Studies on *Bacillus thuringiensis* proteases involved in the production of insecticidal toxins from protoxins

THESIS SUBMITTED TO THE OSMANIA UNIVERSITY
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN BIOCHEMISTRY

BY

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2001

To
My Parents and Brothers
Whose support and love has made it possible

And to
My Teacher, Prof. G. Venkateswerlu
Who provided invaluable source of strength and inspiration

DEPARTMENT OF BIOCHEMISTRY
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DECLARATION

I hereby declare that this Ph.D thesis entitled ***"Studies on Bacillus thuringiensis proteases involved in the production of insecticidal toxins from protoxins"****, represents the original research work carried out by me in the Department of Biochemistry, Osmania University, Hyderabad under the supervision of* ***Prof. G. Venkateswerlu****, and that I have not submitted this thesis for any other degree of this or any other University or Institute.*

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CERTIFICATE

This is to certify that the thesis entitled ***"Studies on Bacillus thuringiensis proteases involved in the production of insecticidal toxins from protoxins"****, submitted for the degree of Doctor of Philosophy in Biochemistry represents the original research work carried out by Mr. Y. Chandrahasa Reddy in the Department of Biochemistry, Osmania University, Hyderabad, under my supervision during the period of 1997-2001, and that it has not been submitted for any other degree of this or any other University or institute. I hereby recommend the submission of this thesis for the degree of Doctor of Philosophy in Biochemistry.*

Date: 21th April, 2001

Prof. G. Venkateswerlu
Research Supervisor

ACKNOWLEDGEMENTS

It is with great pleasure and respect, I wish to express my deep sense of gratitude and reverence to my research supervisor Prof. G. Venkateswerlu for inspiring me with his constant encouragement, moral support, erudite discussions and guidance throughout my studies. As a matter of fact I do not find words to express my gratitude to him for his contribution to my intellectual growth. I take pride in having associated with such a teacher and he shall remain a guiding force, in all my future endeavours.

I would like to express my profound gratitude and heartfelt thanks to Dr. H.C. Sharma, Principal Scientist, ICRISAT, for his guidance, unstinted encouragement, critical analysis, care and magnanimity during my stay at ICRISAT.

I am grateful to Dr. V. Rukmini who has associated with me in the initial stages of this work and her help during the preparation of the manuscript.

I wish to record my special thanks to Dr. K.K. Sharma, Principal Scientist, Dr. V. Anjaiah, visiting scientist of ICRISAT for conducting PCR and Southern analysis.

I am grateful to Prof. S. Rambhav, Prof. C. Subramanyam, Prof. Vijay Deshpande, former Heads and Prof. P. Maruthi Mohan, present Head for the facilities offered during the course of work, and Dr. B. Sashidhar Rao for his suggestions in immunological studies.

I am very grateful to my friends, well wishers and colleagues, Balaji, Thudi Nagesh, G. Satyanarayana, Dakshayani, Manjula, Suneetha, Dwarak, Soma Singh, Rakesh, Pampapathy, B.V. Singh, Padma Bhavani, Anand, Malli, Raju, Ravi, Sreedhar (CCMB), Satish Reddy and especially appreciative of the travails undergone by Mr. J. Venkata Narayana and Dr. Narasa Raju who accompanied during my vicissitudes.

I acknowledge the generous help from the support staff of ICRISAT and non-teaching staff of this department.

I am grateful to the Department of Biotechnology, Govt. of India, New Delhi, for the financial support during my research work.

It will be incomplete if I do not mention how much my parents, brothers Ramanath and Srikanth, sister Subhashini, and other family members have cooperated with me in scaling these heights.

Chandrahas

CONTENTS

List of Abbreviations and Symbols

A	: Absorbance
ALP	: alkaline phosphatase
BBMV	: brush border membrane vesicle
bp	: base pairs
BSA	: bovine serum albumin
Bt	: *Bacillus thuringiensis*
Bti	: *Bacillus thuringiensis* subspecies *israelensis*
Btk	: *Bacillus thuringiensis* subspecies *kurstaki*
Btt	: *Bacillus thuringiensis* subspecies *tenebrionis*
cm	: centimeter
ddH_2O	: double distilled water
DEAE-cellulose	: diethylaminoethylcellulose
DNA	: deoxyribonucleic acid
DTT	: dithiothreitol
ED_{50}	: median effective dose
EDTA	: ethylenediaminetetraacetic acid (disodium salt)
EGTA	: ethylene glycol tetraacetic acid
ELISA	: Enzyme linked immunosorbent assay
Fig.	: Figure
g	: grams
g	: acceleration due to gravity
h	: hour(s)
IAA	: iodoacetic acid
ICP	: insecticidal crystal protein
kb	: kilobase pairs
kDa	: kilo Dalton
KSCN	: potassium thiocyanate
LC_{50}	: median lethal concentration

LD_{50}	: median lethal dose
lt	: litre(s)
M	: Molar
mA	: milli Amperes
mg	: milligram(s)
min	: minute(s)
ml	: millilitre
mM	: milliMolar
mol. wt.	: molecular weight
MOPS	: 3-N-[Morpholino]propanesulphonic acid
NEM	: N-ethylmaleimide
ng	: nanogram
nm	: nanometer
NTP	: Nucleotide tri-phosphate
PAGE	: polyacrylamide-gel electrophoresis
PBS	: phosphate buffered saline
PCR	: Polymerase Chain Reaction
PMSF	: phenylmethylsulfonylfluoride
rpm	: revolutions per minute
SDS	: sodium dodecyl sulfate
sec	: seconds
sq.	: square
subsp.	: subspecies
Tris	: Tris (hydroxymethyl) amino methane
U	: unit(s)
UV	: ultraviolet
V	: volt
v	: volume
W	: watts

List of Symbols

α	: alpha
β	: beta
δ	: delta
γ	: gamma
µg	: microgram
µl	: microlitre
µM	: micromolar
µmol	: micro moles
^{0}C	: centigrade/ celsius degrees
/	: per
%	: per cent

One letter codes for nucleotides

A	: Adenosine
C	: Cytosine
G	: Guanosine
T	: Thymidine
U	: Uracil

One letter symbols of amino acids

A	: Alanine
R	: Arginine
N	: Asparagine
D	: Aspartic acid
C	: Cysteine
E	: Glutamic acid
H	: Histidine
I	: Isoleucine
L	: Leucine
K	: Lysine
M	: Methionine
F	: Phenylalanine
S	: Serine
T	: Threonine
W	: Tryptophan
Y	: Tyrosine
V	: Valine

Chapter 1

Introduction

INTRODUCTION

Despite significant increases in agricultural production over the last five decades, the challenge of producing sufficient food still remains a daunting task due to increasing population, reduced availability of water, and limiting arable land, etc.

Presently, 800 million people are chronically undernourished in the developing world, and millions more suffer the debilitating diseases related to micronutrient deficiencies and contaminated food and water (Krattiger, 1997). Everyday, one out of five people in the developing world is deprived of sufficient food to meet the daily needs. Paradoxically, over 60 percent of the world's poor largely live in the agrarian countries of South Asia and Sub-Saharan Africa where 65% and 79% of the population, respectively, depends on agriculture for their livelihood (Krattiger, 1997).

There are numerous ways by which agricultural productivity can be raised in a sustainable manner. Different technologies must be deployed concurrently to counteract the negative impact of land degradation and environmental pollution. These include biological fertilizers, soil and water conservation, biodiversity conservation, improved pest control. Of these measures, biotechnology and conventional systems, if properly integrated into crop production systems, offer newer opportunities to increase the production and productivity of crop.

Economic losses in agriculture owing to pests are huge. It is estimated that nearly 15% of these are due to insect attacks on crops (Bravo and Quintero, 1993). Insecticide residue in food and food products, environmental pollution, toxic effects on target and non target organisms, and induced resistance in insects has provoked the use of microorganisms as

an alternative for efficient control of insect pests (Lacey and Harper, 1986). Among the groups of organisms which are being studied for their potential as biocontrol agents the bacteria such as *Bacillus thuringiensis* (*Bt*) and *Bacillus sphaericus* are the most important, because of their entomopathogenic activity. Conventional products based on *Bt* occupy the key position, accounting for nearly 90% of the total biopesticide sales (Bernhard and Utz, 1993; Neale, 1997). It has been used in the field for the past 40 years. It is the most important biological insecticide with an annual sales of nearly US $90 million (Lambert and Peferoen, 1992) and there are 67 registered *Bt* products with more than 450 formulations (Shewry and Gutteridge, 1992). *Bt* subsp. *israelensis* has been used extensively for the control of mosquitoes (Barjac de and Sotherland, 1990).

Two companies, Abbot Laboratories and Novartis, dominate the market with approximately 70% of the total *Bt* production worldwide. The rest is produced by about 30 companies, lead by Phillips Duphar, with over 100 *Bt* product formulations. Most are based on one *Bt* protein, and several contain as many as 5 different *Bt* toxins, with some newer biopesticides containing recomibinant products (e.g. Ecogen's Raven®).

For biopesticide applications, the *Bt* protein is usually used in a formulation containing the spores and crystalline inclusions that are released upon lysis of *Bt* cells during growth. The molecular potency of the toxin is 300 times greater than synthetic pyrethroids, and the toxin breaks down quickly when exposed to ultraviolet light/sunlight (Krattiger, 1997; Koziel *et al.*, 1993).

These *Bt*-based biopesticides also have some disadvantages (McGaughey and Whalon, 1992; Krattiger, 1997). The production of *Bt* based biopesticides is relatively expensive; its application requires the use of agricultural machinery; need to be applied several times during the season;

sunlight breaks down the active ingredient; and water (rain or dew) washes the protein from the plants, thus limiting the time when insects are exposed to it. Biopesticides therefore must be applied where and when the target insects are feeding. Most of these difficulties are circumvented in crops transformed with *Bt* genes encoding insecticidal crystal proteins (ICPs). However, consistent expression of *Bt* toxins in transgenic plants increases the selection pressure in insects. The fact that the resistance development in the open field with conventional *Bt*-preparations is very rare until now, opens a debate on the utility of transgenic insect resistant plants against which the resistance development is rapid or quick. Survival and propagation of resistant pests is a major concern. Transgenic plants with *Bt* toxin gene enhance the foliar activity, but are restricted to a given plant. Besides this, some of the recent reports showed the transgenic corn triggering allergic reactions in humans (Barboza, 2000; - Biotech. Intl.; Taylor and Hefle, 2001; See Fig. 1), and also effecting the larvae of monarch butterflies responsible for cross pollination (Losey *et al.*, 1999). In addition to this, *Bt* corn root exudate releases toxin protein into soils and has effect on earth worms (Saxena *et al.*, 1999).

1.1. General characteristics

The leading biorational pesticide, *Bacillus thuringiensis* (*Bt*) is a ubiquitous gram-positive, spore-forming bacterium which produces parasporal crystals during sporulation (stationary phase of its growth cycle). These crystals are predominantly comprised of δ-endotoxins or insecticidal crystal proteins (ICPs), known to possess insecticidal activity when ingested by certain insects. This observation has led to the development of bioinsecticides based on *Bt* for the control of certain species of insects belonging to the orders of Lepidoptera, Diptera and Coleoptera (Beegle and Yamamoto, 1992). Recent reports of *Bt* isolates have indicated their activity against

Figure 1

Modified Animal Corn in Food Supply Stirs Concerns

In a discovery that may have far-reaching consequences for bioengineered food, genetically modified corn approved only for feeding livestock has been identified in U.S. grocery products. The producer, Aventis CropScience, a subsidiary of French-based Aventis S.A., suspended sales of the corn, called StarLink, and withdrew its registration with the U.S. Environmental Protection Agency (EPA).

Whether the modified corn can harm humans is unknown. The corn was initially approved only for livestock because of concerns that it might cause allergies in humans. However, no evidence of allergies has as yet been found. In the meantime, U.S. regulators are scrambling to determine where and how StarLink got mixed with unmodified corn, while elevator operators worry that their stocks of corn may be contaminated with StarLink.

Contd on page 3

other insect orders (Hymenoptera, Homoptera, Orthoptera and Mallophaga) and nematodes, mites, fungi, and protozoa (Edwards *et al.*, 1988; Feitelson *et al.*, 1992; Feitelson, 1993; Marroquin *et al.*, 2000; Duvick, 2001). *Bt* has been used as a potential alternative to synthetic chemical insecticides in agriculture, forestry, and mosquito control. *Bt* spore formulations are also a key source of genes for transgenic expression to provide effective control of key crop pests. The occurrence, classification, isolation, mechanism of toxin and, its pathogenicity against pests have been previously addressed in several current reviews (Cannon, 1996; Dean *et al.*, 1996; Kumar *et al.*, 1996; Lesieur *et al.*, 1997; Schnepf *et al.*, 1998; Nielsen-LeRoux *et al.*, 1998; Oppert, 1999; Rukmini *et al.*, 2000; Aronson and Shai, 2001). Crickmore *et al.* (1998) proposed a new nomenclature based on ICP gene sequences, which will be used in this study.

1.2. History of *Bacillus thuringiensis* (*Bt*)

Bacillus thuringiensis (*Bt*) was discovered from diseased silkworm (*Bombyx mori L.*) larvae in 1901 by Ishiwata. It was re-isolated in 1911 by Berliner from a diseased Mediterranean flour moth (*Ephestia kuehniella*) population and designated as *Bacillus thuringiensis* (Berliner, 1915). Further research by Steinhaus (1951), on *Bt* led to renewed interest in biopesticides, and as a result, the more potent products such as Thuricide® and Dipel® were introduced. The insecticidal activity of the bacterium is due to crystal protein produced during sporulation (Hannay, 1953; Hannay and Fitz-James, 1955). The crystalline inclusion is made up of protoxin subunits, called δ-endotoxins. Different strains of *Bt* produce more than 25 insecticidal crystal proteins (ICPs), effective against different insect species. Today, a number of *Bt* based products are available for the control of agricultural pests.

The first practical application of *Bt* was reported by Husz (1928), and the earliest commercial production of *Bt* began in France in 1938, under the trade name Sporeine (Luthy *et al.*, 1982). The efficacy of *Bt* formulations for different insect pests is given in Table 1. During 1960s, several formulations of *Bt* were manufactured in the United States, Soviet Union, France, and Germany with various degrees of commercial success. A major step forward in the commercial success of *Bt* was the isolation of *Bt* subsp. *kurstaki* (*Btk*) HD1, a strain proved to be highly potent and still forms the basis of today's commercial insecticides. Initially, it was believed to be active against lepidoptera alone. In 1977, Goldberg and Margalit isolated a *B. thuringiensis* subsp. *israelensis*, from a mosquito-breeding pond. This subspecies is highly toxic not only to the larvae of mosquitoes but also blackfly. In 1983, Krieg and Huger isolated *B. thuringiensis* subsp. *tenebrionis* which is highly effective against elm leaf beetle (*Agelastica alni*) and colorado potato beetle (*Leptinotarsa decemlineata Say*) larvae (Krieg and Huger, 1986). These findings led to the conclusion that *Bt* can be used against other economically important insects.

1.3. Classification of *Bt* toxins

There are two major classes, the cytolytic (Cyt) toxins and the crystal δ-endotoxins. The cytolytic toxins are produced by *Bt* subspecies active on Diptera. The crystal δ-endotoxins are more prevalent, belong to a large and variable family of insecticidal glycoproteins which can be divided on the basis of their activity into five major classes: i) Lepidopteran specific; ii) Lepidopteran and Coleopteran specific; iii) Coleopteran specific; iv) Dipteran specific; and v) Nematode specific and the genes for these crystal proteins comprise of several classes and subclasses (Cannon, 1996). Because of the crystalline nature of these proteins, the term Cry is used in gene (*cry*) and protein (Cry) nomenclature. More than 60 *cry* genes

Table 1. Efficacy of *Bt* formulations for different insect pests.

Variety/Serotype of bacteria	Trade name	Crop	Pest	Source
Bacillus thuringiensis var.*kurstaki* (H 31,3b HD7)	Dipel B1	Cotton Brinjal Tomato Okra	Bollworm Fruit borer Fruit borer Fruit borer	Cheminova India Ltd,159 CST Road, Kalina, Santacruz (E) Mumbai 400 098
Bacillus thuringiensis var. *kurstaki* (H3a, 3b, HD7)	Bio Wit WP	Brinjal Cabbage Cotton Pulses Cabbage	Fruit borer Diamond back moth Boll worms Helicoverpa Diamond back moth	Rallis India Ltd, Agro Chemical Station, Plot No.21 & 22 Phase II, Peenya Indus Arena, P.B.No.5813, Bangalore
Bacillus thuringiensis var. *kurstaki* (H3a, 3b 3c)	Wock Biological (Half-Bt)	Cotton Brinjal	Boll worms Fruit borer	Workhardt Ltd, Ready Money Terrace, 167 Dr. A.B. Road, Mumbai 400 018
Bacillus thuringiensis var. *kurstaki* (H3a,3b, strain Z-52)	BioLep WP	Cotton Tomato	Boll worms Fruit borer	Biotech International Ltd, Vipps Centre, 2nd Block, EFGH, Masji Moth, Greater Kailash-II, New Delhi 110 048
Bacillus thuringiensis var. *kurstaki* (H3a, 3b, strain 7a)	BioAsp WP	Pigeonpea Brinjal Tomato	Helicoverpa Fruit borer Helicoverpa	---do---
Bacillus thuringiensis var. *kurstaki* (H5a,5b)	SPIC Biomass		Houseflies Mosquitoes	Southern Petrochemical Industries (SPIC) Agribusiness Division,SPIC Science Foundation,Guindy Chennai 600 018

Source: Resource Inventory for IPM-I, Compiled by S.N.Puri, K.S. Murthy and O.P.Sharma (NCIPM, New Delhi – pp.19-27 (1997)

(encoding 26 distinct insecticidal crystal proteins) have been sequenced (Adang *et al.*, 1993), and over 90 insecticidal crystal protein genes have been cloned and sequenced (Baum and Malvar, 1995). Thompson *et al.* (1995) compared primary sequences of 50 full length toxins and devised a computer generated dendrogram of possible evolutionary relationships. The *Bt* δ-endotoxins are now known to constitute a family of related proteins for which 140 genes have been described (Crickmore *et al.*, 1998), with specificities for different orders of insect species belonging to Lepidoptera, Coleoptera, and Diptera.

1.4. Structure of *Bt* toxin proteins

1.4.1. Tertiary structure

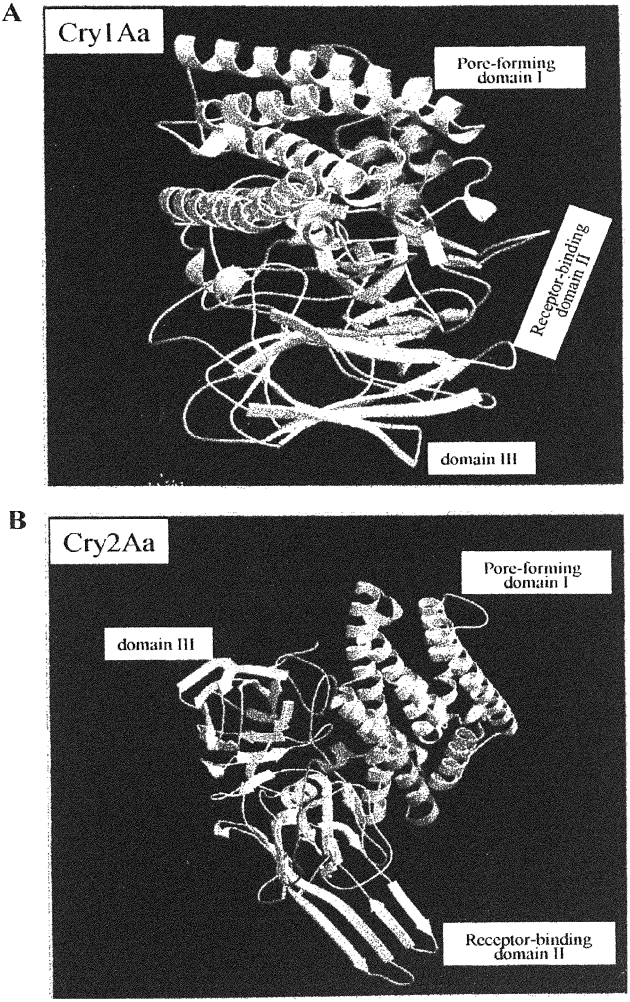
Atomic structures of four crystal proteins - Cry3A (Li *et al.*, 1991; Aronson and Shai, 2001), Cry1Aa (Grochulski *et al.*, 1995), Cry2Aa (Morse *et al.*, 2001), and CytA (Li *et al.*, 1996) have been elucidated by X-ray crystallography. Recently the tridimensional structure of the Cry11Bb toxin was also obtained by homology modelling with the structures of the Cry1Aa and Cry3A toxins (Gutierrez *et al.*, 2001). The structures of Cry1Aa, Cry2Aa, Cry3A, and CytB are given in Figs. 2 and 3. The two Cyt toxins that have been sequenced and characterized, CytA with 249 amino acid residues and CytB with 259, share 39% sequence identity and are very similar in structure (Koni and Ellar, 1993; Chothia and Lesk, 1986). CytA scored <20% amino acid sequence homology with Cry3A and Cry1Aa (Li *et al.*, 1991; Grochulski *et al.*, 1995; Crickmore *et al.*, 1998), and structurally different from these two proteins, with a single domain in which two outer layers of α-helix wrap around a mixed β-sheet. In the protoxin form, CytB is a dimer linked by the intertwined N-terminal strands in a continuous, 12-stranded β-sheet. Proteolytic processing cleaves the intertwined β-strands to release the active CytB as a monomer, as well as removing the C-terminal

tail to uncover the three-layered core. The Cry3A and Cry1Aa proteins show 36% amino acid sequence identity (Crickmore *et al.*, 1998) and contain three domains connected by single linkers. The Cry1Aa, Cry1Ab and Cry1Ac proteins manifest 98% amino acid identity in domain I and II, but differ significantly in domain III (Karim and Dean, 2000). Domain I consists of seven antiparallel α-helices in which α5 is encircled by the remaining helices. The helices in domain I are largely amphipathic, with the α4-loop-α5 region being the most hydrophobic (Grochulski *et al.*, 1995). There are numerous studies, primarily mutagenesis, implicating domain I, especially helices α4 and α5, in the formation and function of the ion channel (Schnepf *et al.*, 1998). The hairpin α4 and α5 inserts into the membrane in an antiparallel manner, while other helices lie on the membrane surfaces (Masson *et al.*, 1999; Nunez-Valdez *et al.*, 2001). Since, helix α7 is located at the interface between the pore-forming domain and receptor-binding domains (Fig. 3), its ability to coassemble with α5 and α6 may assist the insertion of the α4-loop-α5 hairpin into the membrane (Aronson and Shai, 2001). Helix α4 and α5 have a role in establishing the properties of the ion channel and oligomerization, respectively (Gazit *et al.*, 1998; Kumar and Aronson, 1999; Gerber and Shai, 2000). All the δ-endotoxins share a common hydrophobic motif of eight amino acids in helix α7 (Chandra *et al.*, 1999). Domain II forms the most variable part of the toxin and consists of three antiparallel β-sheets, joined in a typical "Greek-key" topology. Domain III consists of two antiparallel β-sheets forming a sandwich with a "jelly roll" topology.

1.4.2. Functions of domains

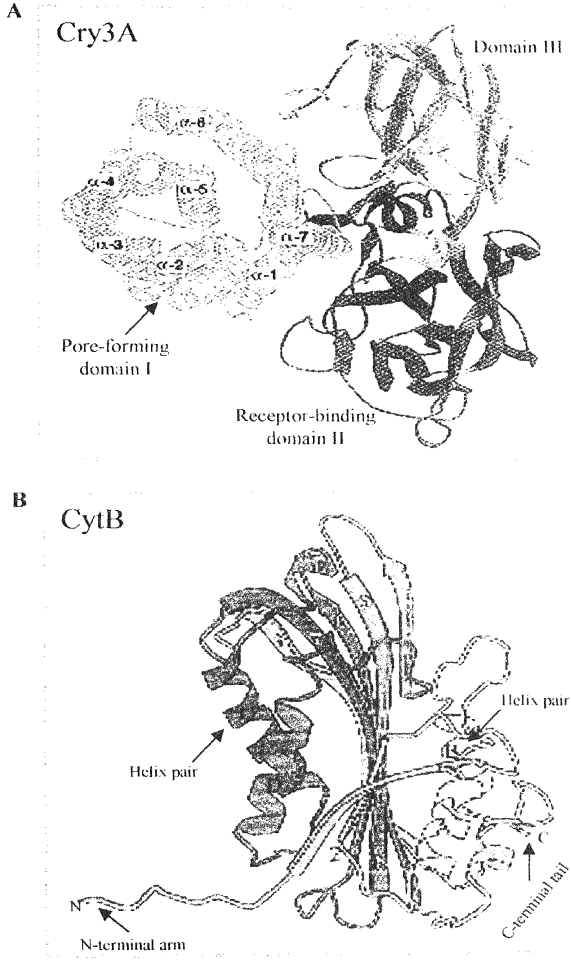
The domain I is the pore-forming segment of the toxin (Li *et al.*, 1991; Gazit and Shai, 1993; Cummings *et al.*, 1994; Gazit and Shai, 1995; Meza *et al.*, 1996; Nunez-Valdez *et al.*, 2001). It has a role in irreversible binding (Chen

Figure 2. Three dimensional structure of Cry1Aa and Cry2Aa



A) Grochulski *et al.*, 1995; B) Morse *et al.*, 2001

Figure 3. Three dimensional structure of Cry3A and CytB



A) Aronson and Shai, 2001; B) Li *et al.*, 1996

et al., 1995; Hussian *et al.*, 1996), membrane insertion, and ion channel function (Dean *et al.*, 1996; Alcantara *et al.*, 2001; Nunez-Valdez *et al.*, 2001). Domain II plays a role in irreversible association (Rajamohan *et al.*, 1995), initial binding (Rajamohan *et al.*, 1996; Abdul-Rauf and Ellar, 1999), membrane insertion (Wu and Dean, 1996), and receptor binding/membrane insertion of toxin (Dean *et al.*, 1996; Smedley and Ellar, 1996). Domain III is involved in receptor binding (Lee *et al.*, 1995, 1999), carbohydrate-mediated receptor recognition (Burton *et al.*, 1999), ion channel function (Chen *et al.*, 1993), receptor binding/membrane insertion (Dean *et al.*, 1996), and membrane permeabilization (Wolfersberger *et al.*, 1996; Schwartz *et al.*, 1997).

Hofte and Whiteley (1989) described five sequence blocks that are highly conserved throughout the δ-endotoxin family. These sequences, as distributed in the active beetle toxin molecule (Cry3A) are described below, starting from the N-terminal (Li *et al.*, 1991). Block I (189-218) and block II (residues 239-305) corresponds to the α5 and α7 of domain I respectively, but includes β1 of domain II. The residues involved in the three salt bridges between domain I and domain II lie within block II; block III (residues 491-538), block 1V (560-569), and block V (633-644) constitute the three buried strands in domain III.

I.4.3. Primary structure

The multigenic nature, complexity of molecular size and sequence, and host specificity of protoxins is listed in Table 2. The crystal (*cry*) genes of class I encode 130-160 kDa protoxins with active toxin embedded in the N-terminal end and conserved C-terminal end involved in the packaging of toxins within the crystalline inclusion (Hofte and Whiteley, 1989). Cry1Aa, 1Ab and 1Ac proteins share more than 80% common amino acid sequence (Hofte and Whiteley, 1989). Similarly, the Cry1Ba and Cry1Ca protoxins show 58%

Table 2. Multigenic nature, host specificity, and molecular sizes of protoxins and toxins

Gene	Subclass	Host specificity	Protoxin (Molecular mass)	Toxin (Molecular mass)
Cry1	1Aa	Lepidoptera	130-160 kDa	Ca. 60 kDa
	1Ab	Lepidoptera/ Diptera	130-160 kDa	Ca. 60 kDa
	1Ac	Lepidoptera	130-160 kDa	Ca. 60 kDa
	1Ba	Lepidoptera	130-160 kDa	Ca. 60 kDa
	1Ca	Lepidoptera	130-160 kDa	Ca. 60 kDa
	1Da	Lepidoptera	130-160 kDa	Ca. 60 kDa
	1Ea	Lepidoptera	130-160 kDa	Ca. 60 kDa
	1Fa	Lepidoptera	130-160 kDa	Ca. 60 kDa
Cry2	2Aa	Lepidoptera/ Diptera	70-71 kDa	65 kDa
	2Ab	Lepidoptera	70-71 kDa	65 kDa
	2Ac	Lepidoptera	70-71 kDa	65 kDa
Cry3	3Aa	Coleoptera	73 kDa	55 kDa
	3Ba	Coleoptera	73 kDa	55 kDa
	7Aa	Coleoptera	73 kDa	55 kDa
	3Ca	Coleoptera	73 kDa	55 kDa
Cry4	4Aa	Diptera	134 kDa	46-48 kDa
	4Ba	Mosquitoes	128 kDa	46-48 kDa
	10Aa	Blackflies	58 kDa	?
	11Aa	Nematode	72 kDa	30 kDa
	Cyt1Aa		27 kDa	?
Cry5	1Ia	Lepidoptera/ Coleoptera	81.2 kDa	?

and 67% amino acid sequence homology, respectively with Cry1Aa protoxin (Hofte and Whiteley, 1989). The toxins of Cry1A exhibited activity against lepidopteran larvae and Cry1Ba for coleopteran larvae. Genes of class *cry*2 encode 70-71 kDa protoxins which are converted into 65 kDa toxins. The Cry2Aa is toxic to both lepidopteran and dipteran larvae, while Cry2Ab is only toxic to lepidopteran insects (Wider and Whiteley, 1990). These two toxins possess nearly 87% of homology, differing mostly between residues 307-822 (Aronson, 1993).

The *cry*3 genes, which occur in *Bt* subsp. *tenebrionis* (*Btt*)and other strains with coleopteran activity, encode Cry3Aa, Cry3Ba, Cry7Aa, Cry3Ca, and Cry8Aa toxins (Sekar and Carlton, 1985; Hernstadt *et al.*, 1987; Hernstadt *et al.*, 1986). The *cry*3 genes encode circa 73 kDa protoxins which are proteolytically activated to circa 67 kDa intermediate size toxins which are further activated to 55 kDa active toxin in the larval gut (Carroll *et al.*, 1989). Cry3Aa and Cry3Ba proteins are 75% identical (Donovan *et al.*, 1992). Cry3Ca protein shares 74%, 61%, 33% homology with Cry3Aa, Cry3Ba, and Cry7Aa proteins respectively (Lambert *et al.*, 1992).

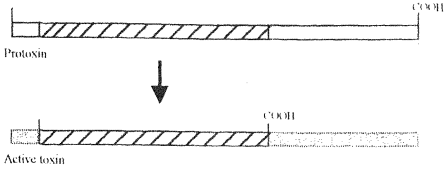
The *cry*4Aa, *cry*4Ba, *cry*10Aa, *cry*11Aa and *cyt*1Aa genes encode proteins with molecular masses in the range 20-140 kDa. The *Bt* subsp. israelensis (*Bti*) crystals are composed of at least four polypeptides of approximately the following molecular masses: 27 kDa (Cyt1Aa); 58 kDa (Cry10Aa); 70 kDa (Cry11Aa); 128 kDa (Cry4Ba), and 134 kDa (Cry4Aa) (Hofte and Whiteley, 1989; Hurley *et al.*, 1985; Lee *et al.*, 1985). Synergistic action between Cyt1Aa and both Cry11Aa and Cry4Ba proteins is known. Cry4Aa is only 54% identical with cry4Ba and 29% identical with cry10Aa. Cry4Ba and cry10Aa are 29% related (Hofte and Whiteley, 1989). The *cry*3 and *cry*4 share only 20% homology with *cry*1A genes (Hofte and Whiteley, 1989).

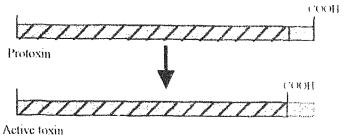
The 81.2 kDa Cry1Ia protoxin produced by *Bt* subsp. *kurstaki* DSIR 732 is larvicidal to both coleopteran (*L. decemlineata*) and lepidopteran (*Ostrinia nubilalis Hubner*) species, but is more active against lepidopteran larvae (Tailor *et al.*, 1992).

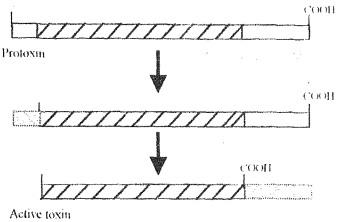
Notwithstanding the complexity of form and size, the protoxins of class 1 to 5 are made as inactive protoxins and are activated by proteolysis into toxins. It is not known how these proteins of different size and amino acid sequence fold to generate common protease processing sites. There is virtually no information on the role of glycosylations in protease activation of toxin. The process of activation appears to resemble that of mammalian gut proteases such as pepsinogen and trypsinogen, in that a relatively small N-terminal peptide is removed. However, in case of protoxin activation, extensive C-terminal processing is involved, but there are no internal cleavages generated within the toxic moiety during activation. It appears that conformational changes occurring during activation are rather subtle, affecting the tertiary structure but not the secondary structure of proteins. The polypeptide of toxic moiety in protoxin when compared to that of active toxin has different thermal unfolding properties (Choma and Kaplan, 1990, 1991).

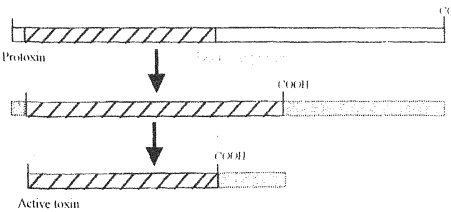
1.5. Processing of protoxins by proteases

Figure 4 illustrates the various schemes for processing of Cry protoxins. The 130 kDa Cry1 protoxins are cleaved quite precisely at both N- and C-terminii giving rise to activated toxins of circa 60 kDa (Hoftee *et al.*, 1986; Hofte and Whiteley, 1989). Approximately 50% of protoxin is mature toxin with a small N-terminal peptide, and a large C-terminal peptide removed during proteolysis (Fig. 4, scheme 1). Amino acid analysis of the activated Cry1Ac toxin shows that N- and C-terminal residues are Ile^{29} and Lys^{623}, respectively (Bietlot *et al.*, 1989). For Cry1Ab, N- and C-terminii of the


COOH
Protoxin
COOH
Active toxin


COOH
Protoxin
COOH
Active toxin


COOH
Protoxin
COOH
67 kDa
COOH
Active toxin


COOH
Protoxin
COOH
72 kDa
COOH
Active toxin

activated toxin appear to be Ile^{29} and Arg^{601}, respectively. Unlike in case of Cry1Ac, a short carboxylic stretch is removed during hydrolysis of Cry2 (Fig. 4, scheme 2), the mature toxin is only slightly smaller than the protoxin (Aronson, 1993). As per scheme 3 (Fig. 4), N-terminal processing of 73 kDa Cry3 protein in *Bt* subsp. *tenebrionis* (*Btt*) gives rise to a major 67 kDa polypeptide which is further processed by trypsin or larval gut proteases into an active polypeptide of 55 kDa with $Aspn^{159}$ of deduced amino acid sequence (Harnstadt *et al.*, 1986; Carroll *et al.*, 1989). Scheme 4 (Fig. 4) is similar to scheme 1, where more than 50% of the protoxin is removed during hydrolysis. However, in scheme 4 the processing takes place in two steps. Processing of the 135 kDa Cry4Aa protoxin results in an active 72 kDa toxin (Paointara *et al.*, 1988; Ward and Ellar, 1988), while the Cry4Ba toxic fragment is between the amino acids 30 and 695 (Yosida *et al.*, 1989) yielding in 76 kDa activated toxin. However, incubation of Cry4Aa and Cry4Ba proteins with mosquito gut extracts results in the formation of 46-48 kDa protease resistant cores (Angasuthanasombat *et al.*, 1988). Cry11Aa proteins are similarly degraded to 32-40 kDa proteins by unidentified proteases associated with parasporal body, trypsin, chymotrypsin or mosquito gut proteases (Cheung *et al.*, 1985; Ibarra and Federici, 1986; Pfannenstiel *et al.*, 1986). The *in vitro* trypsin processed Cry11Aa polypeptides are apparently more active to the mosquito cell lines than the solubilized intact 72 kDa toxin (Chilcott and Ellar, 1988). Dai and Gill (1993) demonstrated that Cry11Aa toxin is first cleaved between Thr^{347} and Phe^{348} and between Phe^{348} and Tyr^{349}, generating a 40 kDa N-terminal fragment, and 32.5 kDa C-terminal fragment. The N-terminal fragment is further processed to an approximately 30 kDa fragment.

1.6. Variations in processing

Variations in protoxin processing by different larval gut proteases may provide *Bt* with selectivity observed in some insect species. The ICP of *Bt* subsp. *aizawai* IC1, is lepidopteran specific when it is activated by lepidopteran proteases or dipteran specific when the lepidopteran active toxin is further processed with dipteran proteases (Haider and Ellar, 1989; Haider *et al.*, 1989). Protease composition within a given strain may also vary as a function of larval age (Keller *et al.*, 1995). The differential effect of protease inhibitors on proteases obtained from different larval instars indicated that gut juice protease profiles change with larval age (Keller *et al.*, 1995). Trypsin and chymotrypsin account for vast majority of the endoprotease activity in midgut luminal fluids (Keller *et al.*, 1995; Chirsteller *et al.*, 1992; Peterson *et al.*, 1994; Lee and Anstee, 1995; Jannston *et al.*, 1995; Novillo *et al.*, 1999; Oppert *et al.*, 1996; Zhu *et al.*, 1997). Slower protoxin processing by trypsin like proteases was observed in resistant strains compared to susceptible ones (Oppert *et al.*, 1996). A three fold variation in chymotrypsin like activity was also observed between resistant and susceptible varieties (Valaitis, 1995). Resistance to *Bt* toxin is associated with lack of protoxin processing in *Plodia interpunctella* (Oppert *et al.*, 1997).

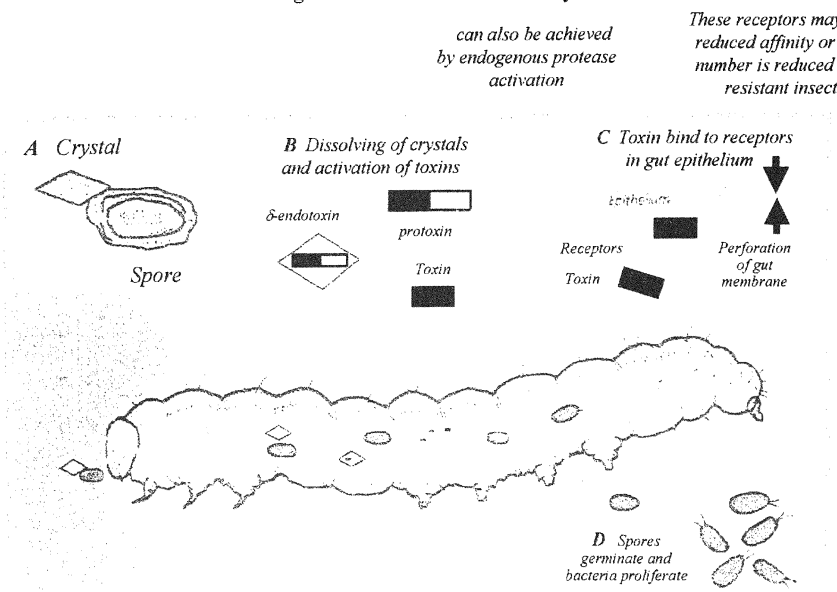
1.7. Mechanism of action

The mode of action of *Bt* has been reviewed by Schnepf *et al.* (1998) and is summarized in the following stages: 1) ingestion of sporulated *Bt* and ICP by an insect larva; 2) solubilization of the crystalline ICP in the midgut; 3) activation of the ICP by midgut proteases; 4) binding of the activated ICP to specific receptors in the midgut cell membrane; 5) insertion of the toxin in the cell membrane and formation of pores and channels in the gut cell membrane, followed by destruction of the epithelial cells (Cooksey, 1971;

Norris, 1971; Fast, 1981; Huber and Luthy, 1981; Luthy and Ebersold, 1981; Smedley and Ellar, 1996); and 6) subsequent *Bt* spore germination and septicemia may enhance mortality (Fig. 5).

When *Bt* crystals are ingested by insects, the crystal proteins are dissolved from the crystal. The pH in the gut of lepidopteran larvae varies between 9 and 12 and lepidopteran-specific crystal bodies can only be solubilized above pH 9.5 (Knowles and Dow, 1993). On getting solubilized in the midgut, the crystalline bodies release the proteins called δ-endotoxins. The toxin portion is derived from the N-terminal half of the protoxin, while the C-terminal portion is involved in the formation of parasporal inclusion bodies and is usually hydrolyzed into small peptides (Choma *et al.*, 1990). The activated toxin can be divided into three structural regions. The region within the N-terminal toxin domain (amino acids 1 to 279) is composed of α-helices, which are considered important in penetrating the peritrophic membrane. At least six α-helices are identified in most Cry toxins. Based on the crystal structure of Cry3A, the other proteins may adopt the same folding scheme with a central hydrophobic helix (helix 5) surrounded by six amphipathic helices (Li *et al.*, 1991). Reducing the hydrophobicity of these regions can reduce the toxicity (Wu and Aronson, 1990; Lee *et al.*, 2000). The C-terminal region (amino acids 461-695) and the highly variable region (amino acids 280-460) are considered important in toxin specificity by coding for open β-sheets that bind to glycoprotein receptors in the midgut. The main target for *Bt* toxins is the insect midgut (Knowles, 1994). The crystalline protoxins are inactive, until they are hydrolysed by the gut proteases (Tojo and Aijawa, 1983; Gill *et al.*, 1992; Milne and Kaplan, 1993), which cleave nearly 500 amino acids from the C-terminus of 130 kDa protoxin and 28 amino acids from the N-terminus leaving 55 to 65 kDa protease resistant active core mostly comprising the N-terminal half of the protoxin (Hofte and Whiteley, 1989). The Cry1A toxin is cleaved at the

Figure 5. Mechanism of toxicity of *Bt*



amino terminal arginine (R2) residue (Nagamatsu *et al.*, 1984), and the carboxyl terminal lysine (K) residue (Bietlot *et al.*, 1989). Recently, it has been reported that a heterogenous DNA fragment of 20 kilobase is associated with the toxin and involved in the proteolytic processing of the protoxin (Bietlot *et al.*, 1993). The 70 kDa Cry2, Cry3 and Cry11Aa proteins are naturally occurring truncated forms.

Brush border membrane vesicles (BBMVs) have been identified as the primary binding site for several insect species (Lee *et al.*, 1992). The active toxins initially bind reversibly to the specific receptors located on the apical brush border membrane of the columnar cells (Schnepf *et al.*, 1998). The initial reversible binding involves certain loops within domain II of the toxin which are in close proximity to helix α7 of domain I (Schnepf *et al.*, 1998) (See Fig. 3). A swinging out domain I with exposure of helix α7 to the membrane surface has been postulated on the basis of disulfide cross-linking of various domain I helices with each other and with loops within domain II (Schwartz *et al.*, 1997; Schwartz and Laprade, 2000). There may be many toxin binding protein receptors, and some have been identified as 12 to 180 kDa glycoproteins (Garczynski *et al.*, 1991; Knowles *et al.*, 1991; Oddou *et al.*, 1991; Yaoi *et al.*, 1999; Schwartz and Laprade, 2000; Jenkins *et al.*, 2000). A 210 kDa membrane protein is the receptor in *M. sexta* for Cry1Ab toxin (Vadlamudi *et al.*, 1995), and a 120 kDa aminopeptidase N is the receptor for Cry1Ac toxin (Knight *et al.*, 1994; Jenkins *et al.*, 1999; Burton *et al.*, 1999). Cry1Ac binding amino peptidase in *Manduca sexta* has a glycosyl phosphatidylinositol anchor (Garczynski and Adang, 1995). After binding to the receptor, the toxin inserts irreversibly into the plasma membrane of the cell leading to lesion formation. There is a positive correlation between toxin activity and ability to bind BBMV (Gill *et al.*, 1992), and the toxicity is correlated with the number than affinity of the receptor (Van Rie *et al.*, 1989).

The toxicity of *Bt* lies in the organization of α-helices derived from domain I. After binding to the midgut epithelial cells, the α-helices penetrate the apical membrane (Knowles and Dow, 1993). Whatever the mechanism for inserting δ-endotoxins into the membrane, most of the toxin molecule is intimately associated with the membrane and forms aggregates therein (Aronson *et al.*, 1999; Kumar and Aronson, 1999; Gerber and Shai, 2000; Aronson, 2000). The formation of toxin induced pores in the columnar cell apical membrane allows rapid fluxes of ions. The pores are K^+ selective (Sacchi *et al.*, 1986), permeable to cations (Wolfersberger, 1989), anions (Hendrickx *et al.*, 1989), and solutes such as sucrose, irrespective of the charge (Schwartz *et al.*, 1991). Carroll and Ellar (1993) observed that midgut permeability in the presence of Cry1Ac was altered for cations, anions, neutral solutes and water. Knowles and Dow (1993) suggested that *Bt* toxins lead to cessation of K^+ pump that leads to swelling of columnar cells and osmotic lysis. The disruption of gut integrity leads to death of the insect through starvation or septicemia. These pores possess both selective (only K^+ passes through) and nonselective (Na^+ and anions pass through) properties depending on the pH (Schwartz *et al.*, 1993). The lepidopteran insect midgut is alkaline and the pores probably permit K^+ leakage. Formation of this cation selective channel destroys the membrane potentials (English and Slatin, 1992) resulting in midgut necrosis, degeneration of peritrophic membrane and epithelium and ultimately bacterial speticaemia (Sneh and Schuster, 1981; Salama and Sharaby, 1985).

Differences in solubilization of different toxins may explain the variations in the toxicity of various proteins (Meenakshisundaram and Gujar, 1998). Decreased solubility could be one potential mechanism for insect resistance to *Bt* proteins (McGaughey and Whalon, 1992). In cotton bollworm (*Helicoverpa zea*), Cry2Aa is less soluble than Cry1Ac and fails to bind to a saturable binding component in the midgut brush border membrane

(English *et al.*, 1994). The unique mode of action of Cry2Aa may provide a useful tool for management of resistance to *Bt* toxins. Although binding of the Cry toxins to the receptors determines the species sensitivity to various toxins, there are distinct exceptions, e.g., Cry1Ac binds to the ligand bands of beet armyworm (*Spodoptera exigua*) brush border membrane proteins, but there is very little toxicity to the insect (Garczynski *et al.*, 1991). Cry1Ab is more toxic to the gypsy moth than Cry1Ac, but does not bind well with the receptors in the brush border membrane (Wolfersberger, 1990).

1.8. Histopathological changes induced by δ-endotoxin

The δ-endotoxin induces histopathological and histochemical changes in the midgut epithelium of insect larvae. Histopathological changes caused by *Bt* subsp. *israelensis* (*Bti*) toxin in silkworm, *Bombyx mori* midgut (Mathavan *et al.*, 1989) included hypertrophy of epithelical cells, vacuolation of cytoplasm and nuclei, disruption of the microvilli, eversion of the plasma membrane, disappearance of basal striations, necrosis of nuclei, disorganization of cytoplasmic organelles, and disintegration of the cell. Histochemical changes observed in the columnar cells were : loss of basophilia due to fall in RNA, decreased protein content, and increased uptake of glycogen. Similar light microscopic observations were made in case of larvae from lepidoptera and coleoptera (Bravo *et al.*, 1992).

1.9. Sporulation phase of *Bacillus thuringiensis*

The growth kinetics of *Bt* is typical of any other aerobic bacterium. The post-log phase is followed by sporulation. Sporulation stages could be followed by recognizable morphological events of spore formation (Ellar, 1978). Axial filament formation (stage I) is followed by an asymmetric division to form forespore compartment (stage II), membrane proliferation to give an immature forespore free within the mother cell (stage III), spore cortex (stage IV) and spore coat formation (stage V and early stage VI) and

finally release of mature spores after cell lysis (stage VI). Crystal protein formation usually begins in the early stages of sporulation, and is synthesized until the end of stage IV while crystals continue to enlarge until stage VI (Lecadet and Dedonder, 1971). The pH changes of the culture medium during the growth cycle of this bacterium could be conveniently used to catch the sporulation stages. The pH of the medium during the exponential growth phase decreases from 7.0 to 5.8 and then increases to about 8.0 during sporulation. The pH remains constant during stationary phase and after completion of sporulation (Lee and Kang, 1989).

1.10. *Bt* toxins and proteases

A good deal of information is known about the initial interaction of proteases and ICPs, relatively little is understood about the involvement of proteases in toxicity once the toxin is formed. In *in vitro*, the toxin can be degraded further with papain, elastase, or trypsin after boiling under denatured conditions (Choma *et al.*, 1990). Some insensitive insects were found with either higher proteolytic activity or higher concentration of proteases in the gut, which resulted in hydrolysis of the core polypeptide (Ogiwara *et al.*, 1992; Shao *et al.*, 1998; Monnerat *et al.*, 1999). This provided evidence that proteases may be involved in toxin specificity and resistance development, as will be discussed in subsequent sections.

Proteases have also been reported as membrane receptors for *Bt* toxins. Aminopeptidase N, and exopeptidase in the brush border membrane, have been documented as a Cry1Ac binding protein in the tobacco hornworm, *Manduca sexta*, tobacco budworm, *Heliothis virescens*, *Plutella xylostella*, the gypsy moth, *Lymantria dispar* (Knight *et al.*, 1994; Sangadala *et al.*, 1994; Gill *et al.*, 1995; Valaitis *et al.*, 1995; Lee *et al.*, 1996; Luo *et al.*, 1997; Jenkins *et al.*, 1999; Burton *et al.*, 1999). However, there is no evidence that aminopeptidase hydrolyzes the toxin. Toxin binding did not

affect aminopeptidase activity, unlike alkaline phosphatase in *H. virescens*, which was inhibited by Cry1Ac (English and Readdy, 1989).

Another Cry1A binding protein in *M. sexta*, a 210 kDa cadherin-like protein is associated with proteases (Vadlamudi *et al.*, 1993; Francis and Bulla, 1997). A trypsin-like protein copurified with the cadherin protein, and the activity of the trypsin-like protein was unaffected by toxin binding. Decreased binding of Cry1Ab was observed when membrane vesicle preparations were held at room temperature, indicating that the receptor was degraded by intrinsic proteases.

Insufficient data is available on the involvement of proteases with the later stages of toxicity, such as protease interactions with toxin-receptor complexes or the membrane pore formation process. However, the ability of toxins to form channels in lipid bilayers was dependent upon the enzyme source used for protoxin activation, suggesting that proteases may influence pore formation (Smedley *et al.*, 1997)

1.11. Protoxin and toxin stability

Studies on proteases and *Bt* proteins have provided information about functional stability. Harsh denaturation and hydrolysis of Cry1 toxins by serine proteinases resulted in two smaller protease-resistant fragments (Chestukhina *et al.*, 1990; Convents *et al.*, 1991). Sequencing of these regions revealed that they are subdomains of the toxin, corresponding to the conserved toxic region, participating in pore formation, and the variable, host recognition region that binds to midgut receptors. Identical subdomain organization was also found in Cry3 toxins (Ort *et al.*, 1995).

A 20 kb DNA fragment was found associated with Cry1Ac crystal protein in *Btk* HD73 and apparently playing a role in an unusual proteolysis observed in the generation of active toxin (Bietlot *et al.*, 1993; Clairmont *et al.*, 1998). The protoxin was associated with a 20 kilobase DNA fragment,

resistant to digestion by DNase. However, removal of the DNA fragment from the protoxin by extensive DNase digestion prevented proteolysis and subsequent activation of the protoxin by trypsin. Therefore, it was proposed that the association with DNA provided the complex with a tertiary structure amenable to proteolysis, and removal of the DNA altered the protoxin structure such that the proteolytic sites were protected or unaccessible.

Research with mutant toxins indicated that toxin stability and/or expression levels are affected by the N-terminal amino acid sequence. Trypsin-hydrolysis of protoxin Cry9Ca1 produced a toxin with an N-terminus of arginine-44 (Lambert *et al.*, 1996). The toxin was further degraded to smaller nontoxin peptides after extensive incubation with trypsin. Elimination of trypsin cleavage sites in mutants resulted in a toxin resistant to further degradation, but toxicity was not enhanced.

A similar report described the alteration of a gene encoding Cry1C to increase expression levels. Purified Cry1C is effective against the *Spodoptera sp.* army worms, yet transgenic plants containing Cry1C toxins have not provided protection from these insects, presumably due to low toxin expression. The trypsin-resistant core of the toxic fragment of Cry 1C ranged from isoleucine-28 to arginine-627 (Strizhov *et al.*, 1996). However, bacteria expressing toxins with truncated N-terminii did not grow, suggesting that the N-terminus is important to cell viability and toxin stability and/or expression.

1.12. Role of proteases in toxin selectivity

In view of the involvement of proteases in the solubilization and activation of *Bt* protoxins, they were thought to control the degree of toxicity at an early step in the mode of action. Haider *et al.* (1986) provided data suggesting that insect proteases could determine *Bt* specificity. This study examined ICPs from *Bt* subsp. *aizawai* (*Bta*), which were toxic to both

lepidopteran and dipteran insects. Protoxins activated by gut extracts from the mosquito, *Aedes aegypti*, were toxic only to dipteran cell lines and larvae, whereas those activated by extracts from *P. brassicae* were toxic only to lepidopteran cells and larvae. Incubation of the protoxin with lepidopteran proteases or mammalian trypsin resulted in conversion to a 55 kDa protein, whereas hydrolysis with dipteran proteases resulted in a 52 kDa protein. Further processing of the lepidopteran-specific toxin by dipteran gut proteases yielded the smaller, dipteran-specific form. Haider and Ellar (1987) also found that trypsin activation of *Bta* protoxin yielded toxins that bound specifically to lepidopteran but not dipteran cells. However, when these trypsin-activated toxins were further hydrolyzed with gut proteases from *A. aegypti*, a protein was obtained that bound to *A. aegypti* but not lepidopteran cells.

The types and/or activity levels of gut proteases may influence toxin sensitivity. A direct correlation was found in *Pieris brassicae*, *Mamestra brassicae*, *S. littoralis*, between the toxicity of *Bt* subsp. *thuringiensis*, gut protein concentration, and protease activity (Bai *et al.*, 1990). Toxin degradation was considered as a mechanism of toxin insensitivity in the cotton bollworm, *Helicoverpa armigera* (Shao *et al.*, 1998). *Btk* ICPs, toxic to *Bombyx mori* but not to *H. armigera*, were hydrolyzed to toxin when incubated with *B. mori* gut proteases. Relatively lower amounts of toxin were produced with *H. armigera* midgut fluid because of excessive toxin degradation by chymotrypsin-like proteases. *In vivo* combination of protoxin and serine protease inhibitors resulted in a synergism of activity in *H. armigera* larvae. These results support the hypothesis that serine proteases were responsible for the lack of toxicity in *H. armigera*, because of a reduction in toxin titer.

Excessive toxin degradation was also implicated in the insensitivity of the Eastern spruce budworm, *Choristoneura fumiferana* to *Bt* (Pang and Gringorten, 1998). Low amounts (1%) of *C. fumiferana* midgut fluid incubated with *Btk* and *Bt* subsp. *sotto* yielded large amounts of toxin and relatively higher toxicity to *B. mori* than incubations containing 50 times more midgut fluid. Increasing volumes of midgut fluid from *B. mori*, (more sensitive to *Btk*), resulted in correspondingly more toxin and enhanced toxicity. Therefore, it was predicted that *C. fumiferana* had a proteinase-mediated mechanism to eliminate toxins, rendering it less susceptible to *Bt* toxins than *B. mori*, lacking such an protective mechanism.

Another possible mechanism for the reduction of *Bt* toxicity is removal of the activated protein by sequestration. Toxin sequestration by gut proteases has been reported in *C. fumiferana*. Precipitation of *Bt* subsp. *sotto* toxins by a gut proteinase of *C. fumiferana* led to limited proteolysis of the toxin and a loss of larval toxicity (Milne *et al.*, 1995). The precipitating proteinase had elastase-like activity and interacted with the C-terminal region of Cry1Aa toxin. These results suggested that some insensitive insects may degrade, precipitate, or eliminate the toxin faster than the susceptible strains.

A suspension of crystalline Cry3A was toxic to the potato aphid. *Macrosiphum euphorbiae*, yet Cry3A which was solubilized and filtered to remove spores or crystalline toxin lacked activity (Walters and English, 1995). It was suggested that the toxin may have been more potent as a suspension due to the need for slow solubilization in the aphid midgut. These results indicated that activation/solubilization processes may occur in insects other than lepidoptera and coleoptera and affect *Bt* toxicity.

Larvae often undergo a decrease in sensitivity to *Bt* toxins as they age (McGaughey, 1978; Sneh *et al.*, 1981; Bai *et al.*, 1993; Johnson and

McGaughey, 1996) and this may also be related to proteinase activity. In *S. littoralis*, an increase in toxin degradation in fifth instar larvae was associated with an increase in the specific activity of gut proteases, which was proposed to account for the loss of Cry1C sensitivity in older larvae (Keller *et al.*, 1996).

Other *in vivo* studies of gut proteases and the specificity of activated toxins have been inconclusive. Toxins were obtained by incubating different strains of *Bt* with proteases from *S. littoralis* and *P. brassicae* (Lecadet and Martouret, 1987). Toxins from either protease source were as active as native crystals in *S. littoralis* larvae. However, *P. brassicae* larvae were much more sensitive to native crystals than to proteolytically activated toxins.

The toxicities of 14 different *Bt* strains were determined for *P. brassicae*, *H. virescens*, *S. littoralis* (Jacquet *et al.*, 1987). The relative toxicities varied greatly, depending on whether insects were fed crystsls, solubilized crystals, or *in vitro*-activated toxins. Similarly, solubility of crystals was a factor for toxicity in some lepidopteran larvae but not others (Aronson *et al.*, 1991). Variable toxicities were reported in lepidopteran forest pests to *Btk* HD1 activated by *B. mori* midgut fluid, although no comparisons were made to crystal toxicity (Van Frankenhuyzen *et al.*, 1991).

1.13. Role of insect proteases in the development of *Bt* resistance

Successful insect control with *Bt* transgenic crops has resulted in their expanded use. However, extensive planting of *Bt* transgenic crops increased the toxin exposure to insects leading to additional selection pressure for the development of resistance. McGaughey and Oppert (1999) reviewed the literature on insect resistance to *Bt* toxins in the laboratory. Resistance has also been reported with *Bt* sprays from field collected *P. xylostella* (Kirsch and Schmutterer, 1988; Tabashnik *et al.*, 1990; Hama *et al.*, 1992; Shelton *et*

al., 1993; Perez and Shelton, 1997). Assessment of the frequency of resistance alleles in field populations of *H. virescens* indicated that resistance in nontarget species could develop in 3 to 4 years (Gould *et al.*, 1997). Because proteases are important at different stages in the mode of action of *Bt*, resistance management may be improved by studying protease interactions with *Bt* toxins in insects that survive on *Bt* transgenic plants.

Studies on physiological adaptations in insects resistant to *Bt* toxins suggest that changes occur in toxin receptors (McGaughey and Oppert, 1999). Receptor-mediated mechanisms may include loss of toxin binding sites, increase in nonspecific binding unrelated to toxicity, and reduction in toxin/receptor aggregation that is associated with pore formation. However, resistance is also caused by changes in ICP solubilization and/or activation reactions that are mediated by proteases (Forcada *et al.*, 1996; Monnerat *et al.*, 1999). These resistance mechanisms may include changes in the pH or modifications of proteases in the insect gut that could lead to changes in solubility, differences in the degree of protoxin activation, and enhanced toxin or receptor-toxin degradation.

Laboratory studies with the Indianmeal moth, *Plodia interpunctella*, found no differences in midgut proteinase activity of *Btk* susceptible and resistant strains (Johnson *et al.*, 1996). However, an another strain *P. interpunctella* resistant to *Bt*, selected with *Bt* subsp. *entomocidus* (*Bte*), had significantly lower soluble gut proteinase activities (Oppert *et al.*, 1994, 1996). Proteinases in gut extracts from the *Bte* resistant insects processed *Bt* protoxin less efficiently than those from the susceptible parent strain or a strain resistant to *Btk*. Comparison of the phenotypic expression of gut proteinases in *P. interpunctella* strains revealed the absence of a major serine proteinase activity in *Bte* and *Bt* subsp. *aizawai* resistant strains (Oppert *et al.*, 1997). This proteinase was shown to hydrolyze *Bt* protoxin; the loss of

the proteinase was genetically linked to *Bt* resistance. Furthermore, the absence of this proteinase was not transient. When insects lacking the serine proteinase placed on untreated diets, the proteinase activity was never recovered, indicating that this genotype was stable. Because the serine proteinases are involved in the activation of *Bt* protoxin in *P. interpunctella*, loss of enzymes involved in protoxin activation could contribute to toxin resistance (Oppert *et al.*, 1996).

Slower Cry11A1 protoxin processing was also reported in *Bt*-resistant strains of *Culex quinquefasciatus* (Dai and Gill, 1993). Similarly, differences in Cry1Ab protoxin processing were described in *Bt* resistant *H. virescens* (Forcada *et al.*, 1996). However, not only did proteases from a *Btk* HD73 resistant strain hydrolyze Cry1Ac protoxin more slowly than those from a susceptible strain, but a subsequent degradation of the activated toxin was also faster with resistant strain proteases (Forcada *et al.*, 1996). Increased toxin degradation results in lower toxicity, but other toxin-eliminating mechanisms, such as toxin sequestration, and precipitation have not yet been reported in resistant insect populations. Any resistance mechanism by toxin elimination would have serious implications for transgenic plants expressing the activated forms of *Bt* toxins.

1.14. Endogenous proteases in *Bt*

During the early sporulation phase, an increase in intracellular protease activity occurs in *Bt* cultures. Proteases endogenous to *Bt* have been described belonging to cysteine, metallo, and serine families of enzymes (Li and Yousten, 1975; Bulla *et al.*, 1977; Chestukhina *et al.*, 1980; Nickerson and Swanson, 1981; Stepanov *et al.*, 1981; Bibilos and Andrews, 1988; Dalhammar and Steiner, 1984; Pfannenstiel *et al.*, 1984; Andrews *et al.*, 1985; Suresh Kumar and Venkateswerlu, 1997; Reddy and Venkateswerlu, 1997; Reddy *et al.*, 1998, 2000; Rukmini *et al.*, 2000). Major proteases in

most *Bt* species are thermostable and many are metalloproteases, with some exceptions. *Btt* which has ICPs with selectivity to coleopteran insects, and *Btk* HD1, towards lepidopteran insects, have proteases of metallo-class. However, serine proteinases constituted approximately 50% of the total activity in *Bti*, which has ICPs selective for dipteran insects (Reddy *et al.*, 1998). In *Btk* HD1, three intracellular proteases with molecular masses of 92, 78 and 69 kDa were characterized by gelatin zymography and azocasein hydrolytic activity (Suresh Kumar and Venkateswerlu, 1998b). The intracellular protease extract of *Btt* showed gelatinolytic activities corresponding to 92, 81 and 69 kDa (Reddy and Venkateswerlu, 1997). Subsequently, it was reported that a 69 kDa protease is specifically involved in the conversion of protoxin (73 kDa) to toxin (67 kDa) (Reddy *et al.*, 2000). *Bti* disclosed a protease pattern consisting of 90, 75, 60 and 30 kDa enzymes (Reddy *et al.*, 1998).

The endogenous proteolytic activities in *Bt* may hydrolyze crystal proteins and generated active toxins (Chestukhina *et al.*, 1980; Bulla *et al.*, 1981; Andrews *et al.*, 1985; Haider *et al.*, 1986; Bibilos and Andrews, 1988; Carroll *et al.*, 1989; Kunitate *et al.*, 1989; Dai and Gill, 1993; Donovan *et al.*, 1997; Suresh Kumar and Venkateswerlu, 1997). A reduction in the size of *Btt* ICPs occurred during sporulation, and proteolysis was prevented by the addition of protease inhibitors (Carroll *et al.*, 1989). Enhanced pesticidal activity was reported when *Bt* crystal proteins mixed with trypsin inhibitors (MacIntosh *et al.*, 1990). Mosquitocidal ICPs were also degraded in the crystal by proteases (Dai and Gill, 1993). Less mosquitocidal proteins were found in the strains of the mosquito-larvicidal bacterium *Bacillus sphaericus* that produced high levels of protease activity (Thanabalu and Porter, 1995). By deleting neutral protease A from *Bt* using genetic engineering, increased the content of crystal protein (Donovan *et al.*, 1997). Extracellular neutral

and alkaline protease deficient mutants are being exploited in enhanced production of toxin by fermentation (Tan and Donovan, 2001).

ICPs from *Btk* crystals, incubated in denaturing and reducing conditions, were hydrolyzed by metalloproteases in the crystal (Suresh Kumar and Venkateswerlu, 1997). Interestingly, the toxin produced under these conditions was highly active against the cotton leafworm, *Spodoptera littoralis*, a species insensitive to native *Btk* crystals or toxins generated by exogenous proteases (Suresh Kumar and Venkateswerlu, 1998a). Although the crystal contained multiple Cry proteins, the toxin was homogenous, as demonstrated by two-dimensional polyacrylamide gel electrophoresis, and lacked the first 29 amino acids of the protoxin with Glu^{30} at the N-terminus (Suresh Kumar and Venkateswerlu, 1998b) and differed from a trypsin generated toxin, lacking an N-terminal peptide of 28 amino acids with Ile^{29} at the N-terminus (Bietlot *et al.*, 1989) both differing in their toxicity.

1.15. *Bacillus thuringiensis* genome and *cry* gene expression

Bt strains have a genome size of 2.4 to 5.7 million bas pairs (Carlson *et al.*, 1994). A complete physical map of the chromosome was constructed for two strains of *Bt* (Carlson and Kolsto; 1993; Carlson *et al.*, 1996). Most *Bt* isolates have extrachromosomal elements, some of them circular and some linear (Carlson *et al.*, 1994). The crystal (*cry*) genes are chromosomal or extrachromosomal or both (Kronstad *et al.*, 1983; Aronson *et al.*, 1986; Debro *et al.*, 1986). The Cry proteins are generally encoded by large transmissible plasmids (Gonzalez *et al.*, 1981), and are characteristically expressed during the stationary phase of the bacterium (Aronson *et al.*, 1986). The parasporal crystal inclusions accumulate through the sporulation phase and account for 20 to 30% of the dry weight of the sporulating cells (Lilley *et al.*, 1980). The high level of crystal protein synthesis in *Bt* and its coordination with the stationary phase are controlled by a variety of

mechanisms occurring at transcriptional, posttranscriptional, and posttranslational levels (Agaisse and Lereclus, 1995; Baum and Malvar, 1995). The *cry* genes are considered to be typical examples of sporulation-specific genes, as in case of *cry*1Aa gene (Wong *et al.*, 1983). However, in case of *Btt*, the *cry*3A gene expression is not directly dependent on the major sporulation-specific factors. The expression begins during the late-exponential phase and not during sporulation (Schnepf *et al.*, 1998). The ICP transcript and ICP antigen were detectable in vegetative cells (Sekar, 1988).

1.16. *Bt* - Transgenic crop plants

Transgenic plants have been constructed with a combination of *Bt* toxin genes and protease inhibitor genes in an effort to increase insecticidal activity, and reduce the potential of resistance development. Trypsin and chymotrypsin inhibitors potentiated the insecticidal activity of *Bt* toxins both in the diet and in tobacco plants with transgenes from *Bt* Cry1Ab and squash (*Curcurbita maxima*, trypsin protease inhibitor) (MacIntosh *et al.*, 1990). With *P. xylostella*, however, soybean trypsin inhibitors had no significant effect on *Bt* toxicity or resistance to *Bt* (Tabashnik *et al.*, 1992). These conflicting results may be due to the choice of inhibitors, differences in bioassay procedures, or tolerance by the insect for the presence of inhibitor, as has been reported in other insects (Oppert, 1999).

The first results concerning the transfer of *Bt* genes in tobacco and tomato were published in 1987 (Barton *et al.*, 1987; Vaeck *et al.*, 1987; Fischhoff *et al.*, 1987), and now extended to many major crop species (Adang *et al.*, 1993; Fujimoto *et al.*, 1993; Koziel *et al.*, 1993; Nayak *et al.*, 1997; Perlak *et al.*, 1990, 1993; Van der Salm *et al.*, 1994). A Belgium biotechnology company, Plant Genetic System, reported the first successful transfer of *Bt* genes into tobacco (Vaeck *et al.*, 1987). These plants

produced endotoxins, which killed the neonates of *M. sexta*. Insects placed on the leaves of the plants displayed the same response as insects placed on leaves sprayed with commercial *Bt* products with feeding suppression after 18 h, and mortality in 3 days. Levels of the endotoxin as low as 30 mg per gram of leaf protein provided complete protection against *M. sexta* neonates, and the production of endotoxin was shown to be inherited as a simple dominant character. Since then, *Bt* genes have been transferred to a number of other crop species such as cotton, rice, and maize with lepidopterous insects as the main targets. Synthetic *cry*3 genes have also been expressed in tobacco and potato plants for the control of *L. decemlineata* by Perlak *et al.*, in 1993, followed by tobacco and tomato plants expressing *cry*1Ab and *cry*1Ac genes to control many lepidopterous pests (Van der Salm *et al.*, 1994).

Considerable progress has been made in developing transgenic crops with resistance to target pests over the past decade (Estruch *et al.*, 1997; Hilder and Boutler, 1999). Successful control of pink bollworm (*Pectinophora gossypiella*) has been achieved through transgenic cotton (Wilson *et al.*, 1992). Field trials of transgenic maize containing Cry toxins have shown to be potential against the European corn borer and can tolerate up to 50 and 300 larvae per plant at the leaf whorl and anthesis stages respectively (Armstrong *et al.*, 1995). Transgenic maize expressing Cry9C from *Bt* subsp. *tolworthi*, effectively controlled both the generations of European corn borer (Jansens *et al.*, 1997). Arencibia *et al.* (1997) used a truncated *cry*1Ab gene in transgenic sugarcane plants under the control of the CaMV35S promoter against neonate larvae of sugarcane borer (*Diatraea saccharalis*). The truncated *cry*1Ab gene has also been introduced into several cultivars of rice (indica and japonica) by microprojectile bombardment and protoplast systems (Datta *et al.*, 1998). Maqbool *et al.* (1998) transformed the rice cultivars Basmati 370 and M7 by using *cry*2A

insecticidal gene against the yellow rice stem borer and the rice leaf folder. Nayak *et al.* (1997) reported that two rice lines transformed with synthetic Cry1Ac were highly toxic to yellow stem borer larvae and reduced the in feeding. *Bt* genes have also been inserted into an elite maintainer line, R68899B with the *cry*1Ab gene (Alam *et al.*, 1999). Transformed brinjal plants have shown to possess higher insecticidal activity (transgenic brinjal fruits) against the larvae of fruit borer (*Leucinodes orbonalis*) (Kumar *et al.*, 1998). A modified gene of *Bt* subsp. *tolworthi*, encoding a coleopteran insect-specific Cry3Ba toxin has been used to transform the female parent of the egg plant commercial hybrid, Rimina (Arpaia *et al.*, 1997). Selvapandian *et al.* (1998) transformed tobacco plants using Cry1Ia5 insecticidal toxin from *Bt* strain from India, which provided complete protection against *H. armigera.* The effectiveness of this toxin was comparable to *cry*1Ab or *cry*1Ac genes.

A commercial formulation of *Bt* (Biolep®) has been found to be effective against the sorghum shoot fly. In addition toxins from *Bt* subsp. *morrisoni* also manisfested appreciable biological activity against the shoot fly larvae. Cry1A, Cry1Ca, Cry1Ea, and Cry2Aa are active against the larvae of *C. partellus*, while Cry1A is most effective against *H. armigera* (Sharma *et al.*, 1999). Although there are no reports of *Bt* transformed sorghum, Van Rensburg (1999) reported that transformed maize was tolerant against the spotted stem borer and the maize stalk borer (*Busseola fusca*), two most important pests of sorghum in Asia and Africa. A codon-modified *cry*1Ac gene has been introduced into groundnut (Singsit *et al.*, 1997). Chickpea cultivars ICCV 1 and ICCV 6 have been transformed with *cry*1Ac gene (Kar *et al.*, 1997).

Adamczyk *et al.* (1998) studied the survival and development of S. frugiperda on the leaves and bolls of normal and Cry1Ac transformed cotton

plants. Hoffmann *et al.* (1992) evaluated the efficacy of transgenic tobacco plants contaning genes encoding *Bt* δ-endotoxin or cowpea trypsin inhibitor (CpTI) against *Helicoverpa zea* under field conditions and reported that the larval mortality was high and the leaf damage was low for the genotypes contaning *Bt* gene as compared to control and CpTI genotype. Transgenic tobacco contaning *Bt* and cowpea trypsin inhibitor genes showed insecticidal activity towards *H. armigera* (Zhao *et al.*, 1997). Mortality of the larvae was low on transgenic tobacco expressing *Bt* alone than the plants expressing both *Bt* and CpTI. It was concluded that gene pyramiding could be a valuable strategy for resistance management and the sustainable use of *Bt* transgenic crops. Santos *et al.* (1997) transformed *Landsberg erecta* plants with either *Bt cry*1Ac gene, or CpTI gene or for both genes and tested them against *S. exigua*, *H. zea*, *Pseudoplusia includens* and *H. virescens*. Both genes reduced growth of the species tested, but *cry*1Ac was more effective in controlling caterpillar growth than the cowpea trypsin inhibitor gene. The resistance of plants with both transgenes was lower than that of plants expressing the *cry*1Ac gene alone, but higher than that of plants expressing only the CpTI gene. Transgenic tobacco plants containing *Bt* or CpTI or both of the genes proved toxic to larvae of *H. armigera*. Plants with both transgenes had enhanced resistance compared to those with single transgenes (Zhao *et al.*, 1996) These results correlated to those of Shao *et al.* (1998), in which a synergism of activity in *H. armigera* was observed with both *Bt* protoxin and serine proteinase inhibitors. *Bt* Cry1Ac protein expressed in transgenic cotton has biological activity specific for lepidopterous insects. (Sims, 1995). Benedict *et al.* (1996) studied the field performance of transgenic cotton plants (*Btk* lines) for resistance to *H. virescens* and *H. zea*. Tobacco and tomato plants expressing *cry*1Ab and *cry*1Ac genes have also been developed (Van der Salm *et al.*, 1994) to control lepidopteran insects.

The expression of *cry*1Ab and *cry*1Ac genes provided protection against *S. exigua*, *M. sexta* and *H. virescens*.

The use of protease inhibitors in combination with *Bt* toxins presents an interesting paradox. Interference with proteases that activate *Bt* protoxins would decrease the production of toxins; this may partially explain the negative results obtained thus far with *Bt* toxins and protease inhibitors. This hypothesis is also supported by protease-mediated resistance in insects that survive when fed *Bt*-treated diets because they lack *Bt*-activating proteases. However, an increase in the expression of and/or activity levels of toxin-degrading proteases could also result in toxin insensitivity in some insects. It was suggested that the increase in *Bt* toxicity observed with protease inhibitors may be due to a reduction in the degradation of toxin in insects with an appropriate adaptive mechanism, such as those with proteases capable of hydrolyzing toxin (Pang and Gringorten, 1998; Zhang *et al.*, 2000). The introduction of proteinase inhibitors may increase the activity spectrum to insects that degrade toxin, either by a species-specific trait or an adaptive mechanism. Regardless, information is lacking on the complexity and regulation of insect digestive proteases. Until we gain more knowledge in this area, responses to the ingestion of proteins that interact with digestive proteases cannot be predicted.

1.17. Resistance to *Bacillus thuringiensis* δ-endotoxin

Evolution of resistance to *Bt* was first observed in a laboratory strain of *P. interpunctella*, a lepidopteran pest of stored grains. Resistance increased to 100-fold on selection over fifteen generations and stabilized when selection was discontinued (McGaughey, 1985). Subsequent receptor binding studies indicated that resistance of *P. interpunctella* was correlated with 50-fold reduction in the affinity of the membrane receptor for Cry1Ab (Van Rie *et al.*, 1990a). Since then, the mechanism of resistance development has been

under scrutiny to identify and evaluate the factors conferring resistance and thereby preserve the performance of *Bt* toxins by delaying and/or preventing the development of resistance in insect populations.

Genetic and biochemical analyses of insect strains with resistance to *Bt* toxins indicated that it was autosomally inherited, as a partially or fully recessive trait and controlled by several genetic factors. The genetic control was unstable and decline over the generations of nonselection (McGaughey, 1985). In case of laboratory strain of tobacco budworm, *H. virescens* resistance was inherited as an additive trait when treated with high doses of Cry1Ac toxin (Gould *et al.*, 1992). Laboratory selection of Egyptian cotton leafworm, *S. littoralis* with *Btk* HD1 (Dipel 2x) for eight successive generations did not establish true resistance, but conversely demonstrated a latent toxicity. This delay in response to the toxin effect was attributed to an increase in tolerance due to intrinsic or extrinsic factors rather than development of true resistance (Schnepf *et al.*, 1998).

1.18. Reistance management

Although *Bt* provides an attractive alternative to chemical insecticides, totally substituting *Bt* for the use of chemicals would be a mistake. Use of the chemical arsenal in combination with *Bt* would probably enable a more judicious use of both, and would also delay the onset of insect resistance (Gill *et al.*, 1992). Resistance management strategies try to prevent or diminish the selection of the rare individuals carrying resistance genes and hence to keep the frequency of resistance genes sufficiently low for insect control. *Bt*-transgenic plants and various management strategies for insect-resistant plants have been reviewed (Estruch *et al.*, 1997; Hendriksen and Hansen, 1998; Jouanin *et al.*, 1998; Hilder and Boulter, 1999; Van Rie, 2000; Shelton *et al.*, 2000)

In transgenic plants, selection pressure could be reduced by restricting the expression of the crystal protein genes to certain tissues of the crop (those most susceptible to pest damage), so that only certain parts of the plant are fully protected, the remainder providing a form of spatial refuge (Shelton *et al.*, 2000). Crystal protein gene expression could be triggered by feeding of the insect itself on a transgenic plant, which resident cry genes controlled by would-inducible promoters (Peferoen *et al.*, 1990). If plants were to express *Bt* toxin only in response to specific damage thresholds, it might provide a mechanism to diminish toxin exposure to insects. Alternatively, toxin expression could be induced by the application of a chemical (Williams *et al.*, 1992).

Another management option is the rotation of plants or sprays of a particular *Bt* toxin with those having another toxin type that binds to a different receptor (Groeters *et al.*, 1993). A very attractive resistance management tactic is the combination of a high-dose strategy with the use of refugia (toxin-free areas). The principle is to express Cry toxins at such a dose that nearly all heterozygotic carriers of resistance alleles will be killed (Tabashnik, 1994).

A specific planting strategy that has been recommended to reduce selection is the use of seed mixtures of toxin-expressing and toxin-free plants to provide prepackaged refugia (Mallet and Porter, 1992; Roush, 1996; Tabashnik, 1994). Another valuable option for resistance management, in combination with the use of refugia, is the expression of multiple Cry proteins in crops or incorporation of multiple proteins in *B. thuringiensis* sprays, provided these toxins have different modes of action (Roush, 1994) with respect to the insect's mechanism of resistance.

Another management strategy is delaying of the evolution of insect populations resistance to the target genes by deploying genes with different

modes of action in the same plant (Gene pyramiding). Activity of *Bt* in transgenic plants can be enhanced by serine protease inhibitors (MacIntosh *et al.*, 1990). Activity of *Bt* can also be increased in combination with tannic acid (Gibson *et al.*, 1995). Cornu *et al.* (1996) reported that transgenic poplars expressing proteinase inhibitor and *cry*3Aa genes exhibited reduced larval growth, altered development and increased mortality as compared to the control. Hoffmann *et al.* (1992) evaluated tobacco plants expressing *Btk* HD73 δ-endotoxin or cowpea trypsin inhibitor (CpTI) for their efficacy against *H. zea* in the field.

1.19. Other insecticidal toxins of *Bacillus thuringiensis*

Apart from the δ-endotoxin, some serotypes of *Bt* produce a second compound designated as β-exotoxin (Cantwell *et al.*, 1964; Mohd-Salleh *et al.*, 1980). It has a broad spectrum but exhibits considerable host specific difference. Species belonging to the order of diptera are the most susceptible hosts. The β-exotoxin (type-I) is a heat stable nucleotide which is secreted into the medium during the vegetative growth phase (Farkas *et al.*, 1977). It is composed of adenine, glucose and allaric acid, inhibits RNA polymerase enzymes by acting competitively with ATP (Faust, 1973; Sebesta and Horska, 1970). The occurrence of type-II β-exotoxin is also reported (Hernandez *et al.*, 2001). The expression of thuringiensin, the heat stable exotoxin is plasmid controlled and it exerts its effect on the host by inhibition of RNA synthesis (Cooper, 1994).

α-exotoxin is a phospholipase-C, which primarily affects the cell membrane phosphlipids (Heimpel, 1954; Bonnefoi and Beguin, 1959). γ-toxin is toxic to sawflies (Tenthredinidae), but the mode of action of this heat-labile toxin has not been determined (Heimpel, 1967).

The so called "water-soluble toxin" paralyses lepidoptera (Fast, 1971), and the "mouse factor exotoxin" is toxic to mice as well as to

lepidoptera (Krieg, 1971). The modes of action of these toxins have not been delineated.

Several enzymes have also been described which may play a role in non-target activity: haemolysin (Baida and Kuzmin, 1995), sphingomyelinase (Gilmore *et al.*, 1989), phospholipases (Damgaard *et al.*, 1996b), protease (Hotha and Banik, 1997), and chitinase (Sampson and Gooday, 1998).

1.20. Vegetative insecticidal proteins (Vips)

Many also produce, during growth, less well characterized insecticidal proteins, the so-called vegetative insecticidal proteins (Estruch *et al.*, 1996) as well as secreting other pathogenic factors (Agaisse *et al.*, 1999). The supernatant of vegetative *Bacillus cereus* culture has two compounds: Vip1 and Vip2, which were shown to possess toxic effects for insects (Estruch *et al.*, 1997). Vip3 has been isolated from *Bt* supernatants is highly toxic to *Agrotis ipsilon* and *Spodoptera littoralis* (Estruch *et al.*, 1996). Vip3A, a 88.5 kDa protein encoded by *vip*3A gene of *Bt* strain AB88 was toxic to a wide range of lepidopterous pests. Vip2A and Vip3A are similar such proteins reported (Warren *et al.*, 1994). The *vip*1A gene encodes a 100 kDa protein and is reported to be toxic to the western corn rootworm larvae in conjunction with the Vip2A protein (Warren *et al.*, 1994). Vip1A shows sequence similarity to the protective antigen of the tripartite *B. anthracis* toxin (Petosa *et al.*, 1997). The activity of these proteins is similar to δ-endotoxins. The acute toxicity of vegetative insecticidal proteins is in the same range as that of the δ-endotoxins from *Bt*. They induce gut paralysis, followed by complete lysis of the gut epithelium cells, resulting in larval mortality.

Aim and scope of the present investigation

Apart from the larval gut enzymes it has been suggested that the endogenous proteases of *Bt* participate in the hydrolysis and activation of protoxin to toxin. Besides, studies on these proteases have also helped in strain improvement. Recently it has been demonstrated that the neutral and alkaline protease deficient mutants of *Bt*, yielded increased toxin by fermentation with greater bioefficacy (Tan and Donovan, 2001).

In view of the role of some of these proteases endogenous to *Bt*, in the generation of active toxin from protoxin, it is worthwhile persuing this problem, by studying the protease deficient strains of this organism, to gain more insight with reference to the production of these proteins. Hence the objectives of the present study are:

- Isolation and characterization of endogenous protease (deficient or hyper producing) mutants of *Bacillus thuringiensis* subsp. *kurstaki* HD1: their role in the activation of protoxin to toxin.
- Elucidation of interrelationship between the production of intracellular proteases of *Bacillus thuringiensis* subsp. *kurstaki* and hydrolysis of δ-endotoxins.
- Evaluation of protoxins and toxins of various strains for a possible endogenous proteolytic activity.

Chapter 2

Isolation and characterization of protease mutants of Btk HD1: their role in activation of protoxin to toxin

Isolation and characterization of protease mutants of *Btk* HD1: their role in activation of protoxin to toxin

2.1. Introduction

Bacillus thuringiensis crystal δ-endotoxin is biosynthesized as an inactive precursor, which is proteolytically converted into an active toxin in the insect gut by exogenous protease(s) and also to a significant extent by endogenous proteases in the producer organism (Suresh Kumar and Venkateswerlu, 1997, 1998a, b; Oppert, 1999; Rukmini *et al.*, 2000). The endogenous proteases involved in the conversion of protoxin to toxin have further been characterized and reported (Suresh Kumar and Venkateswerlu, 1998; Reddy *et al.*, 1998, 2000). However, genetic evidence by way of mutant isolation and characterization to show the role of endogenous proteases in the conversion of protoxin to toxin are lacking. In this regard, Suresh Kumar and Venkateswerlu (1998b) have earlier reported the isolation of endogenous protease deficient mutants of *Btk* HD1. However, a detailed investigation of these mutants was not undertaken by these authors. This study for the first time conclusively proves that endogenous proteases are involved in the conversion of 132 kDa protoxin to 66 kDa toxin. Also this study indicates the role of endogenous proteases in the degradation of toxin, hence enhanced bioactivity by the endogenous protease deficient mutant. The present chapter describes the isolation, characterization and bioassay of protease (endogenous) mutants of *Btk* HD1 and its role in the generation of active toxin.

2.2. Materials and Methods

2.2.1. Bacterial strains

Bacillus thuringiensis subsp. *kurstaki* (*Btk*) HD1 was isolated from DIPEL (TM) (Abbott Laboratories, North Chicago, IL, USA). *Btk* HD73 was obtained from the *Bacillus Genetic Stock Centre* (Ohio State University,

Columbus, OH, USA). Protease mutants of *Btk* HD1 designated as *Btk-q* and *Btk-e* were isolated by chemical mutagenesis of *Btk* HD1 strain.

2.2.2. Chemicals and reagents

Acrylamide, bisacrylamide, β-mercaptoethanol, trypsin, chymotrypsin, coomassie brilliant blue (CBB), dithiothreitol (DTT), sodium dodecyl sulfate (SDS), 3-[N-Morpholino]propanesulfonic acid (MOPS), Tris (hydroxymethyl)-aminomethane (Tris), Triton X-100, azocasein, bovine serum albumin (BSA), and adjuvants were obtained from Sigma Chemicals, USA. Lambda DNA (*Hin*dIII digest) marker, agarose, restriction enzymes, DNA beads, proteinase K, and RNase were obtained from Gibco-BRL, USA. Hybond N^+ nylon membrane was procured from Amersham, UK. Protein markers, goat anti-rabbit IgG conjugated to alkaline phosphatase, 5-bromo-4-chloro-3-indoyl phosphate toluidine (BCIP), and p-nitro blue tetrazolium chloride (NBT) were from Genei, Bangalore, India. Ethyl methanesulfonate was obtained from Spectrochem, Mumbai, India. All other chemicals used were of analytical grade, and obtained locally.

2.2.3. Bacterial growth conditions

Bt was cultured on a liquid medium (1% bacteriological tryptose, 0.3% beef extract, 0.5% NaCl, 5 mM $MgSO_4$, and 20 μM $MnCl_2$; pH 7.2) with shaking (150 rpm; LAB-LINE, IL, USA) at 28^0C to mid-exponential phase (monitored by A_{600} measurements). Sporulation stages were monitored by phase contrast microscopy, and the culture was harvested at early stage VI (Ellar, 1978) of sporulation when more than 90% of the cells were unlysed and carried a mature spore. All cultures were maintained on nutrient agar slants.

2.2.4. Preparation of Milk agar plates

Plates were prepared by using 2% agar and 1.5% of skimmed milk powder. Milk powder (filter sterilized) was added aseptically after autoclaving the other components, and poured in sterile plastic disposable petriplates.

2.2.5. Mutagenesis with Ethyl methanesulfonate (EMS)

The chemical mutagenesis of the bacterium was performed according to Miller (1977). The bacterium was streaked on an agar plate for single colonies. A single colony was chosen and grown overnight (10 ml medium). After 24 h, the culture was aliquoted into 1 ml sterile eppendorfs and spun at 10,000*g* for 10 min at 4^0C. The pellet was washed and resuspended in half the original volume of growth medium containing 0.1 M Tris-HCl (pH 8). To this suspension (2 ml), 30 µl of ethyl methanesulfonate (EMS) was added and mixed vigorously. The tubes were aerated at 28^0C for 2 h followed by washing (twice) the cells with growth medium mentioned above. These cells were resuspended in the same medium and allowed to grow over night.

2.2.6. Screening of intracellular protease mutants

EMS mutagenized cultures were screened for protease deficient mutants on milk agar plates. An overnight grown culture (2 ml) was subcultured into fresh 10 ml medium. After growing for 12 h, it was pelleted and diluted (10^{-2},10^{-4},10^{-6},10^{-8}) with fresh sterile medium in eppendorfs. These dilutions were plated onto milk agar plates. On these plates, the wild type colony producing proteases showed a clearing zone or plaque around. Those colonies which did not show any clearing zone or plaque around them were picked up and further purified to a single cell colony on milk agar plates. These colonies were grown on liquid cultures and retested for plaque forming ability on milk agar plates.

2.2.7. Testing of the mutants

The following methods were followed to test these mutants.

2.2.7.1. Cell lysate preparation

One day old cultures were harvested and the cell sediments were washed twice with 20 mM Tris-HCl (pH 8) containing 1 M NaCl and thrice with the same buffer without NaCl. These sediments were suspended in ddH_2O and sonicated (Virtis Virsonic, USA) for five times (30 sec bursts at 60 watts). After centrifugation (15,000*g* for 15 min at 4^0C; Sigma 3K 30, rotor#12158), the pelleted cell lysate was analysed on SDS-PAGE.

Alternatively where necessary, the organisms were cultured on nutrient agar plates at 28^0C in an incubator. For quick analysis cells scrapped from the nutrient agar plates were suspended in Tris-HCl (0.1 M; pH 8) buffer containing 0.5% Triton X-100. After centrifugation (15,000*g* for 15 min at 4^0C; Sigma 3K 30, rotor#12110), the supernatant was analyzed by X-ray film spot test, for protease activity.

2.2.7.2. X-ray film spot test

This assay was done according to Cheung *et al.* (1991). The gelatin coating on unprocessed X-ray film (20 x 25 cm) of Kodak Chemical Corp. was used as a substrate for a variety of proteolytic enzymes. For this assay, 5 µl of a cell lysate was applied onto a strip of X-ray film followed by incubation for 1 h at 42^0C. After the incubation, the film was washed with running water.

2.2.7.3. Isolation of intracellular proteases

A single colony from overnight grown agar plate was inoculated into 10 ml medium, and incubated for 6 h at 28^0C with shaking. This was subcultured into 50 ml medium and grown to mid-log phase (A_{600} nm 0.4-0.6), and once again inoculated into 250 ml medium. Sporulation stages were monitored by phase contrast microscopy and the culture was harvested at early stage VI

(Ellar, 1978) of sporulation when more than 90% of the cells were intact and carried a mature spore. The culture was pelleted and washed twice with 20 mM Tris-HCl (pH 8) containing 1 M NaCl and thrice without NaCl. The washed pellet was resuspended in 0.1M Tris-HCl, pH 8 and sonicated (6 x 30 sec bursts; Virtis Virsonic, USA). The lysed culture was centrifuged (12,000*g* for 20 min at 4^0C; Sigma 3K 30, rotor#12158) and the supernatant obtained was saturated with ammonium sulfate to 30% at 4^0C and left overnight. The precipitated protein was pelleted down (15,000*g* for 20 min at 4^0C; Sigma 3K 30; rotor#12158) and resuspended in 0.1 M Tris-HCl (pH 8), and dialysed at 4^0C against the same buffer. The protein concentration of the sample was adjusted to 1 mg/ ml by diluting with the same buffer. This preparation was aliquoted to 200 µl volumes and stored at -20^0C, until further use.

2.2.7.4. Gelatin SDS-PAGE

To separate and identify intracellular proteases, gelatin zymography was performed as described by Hummel *et al.* (1996). Samples were mixed with 4x loading buffer (without β-mercaptoethanol or dithiothreitol) and loaded, unheated. Thirty µg protease extract was electrophoresed at 4^0C on a 10% SDS-PAGE containing 1% gelatin. After the run, the gels were rinsed with 0.1 M Tris-HCl (pH 8) containing 1% Triton X-100 for 1 h. Gels were subsequently equilibrated for 30 min (3 x 10 min) in the same buffer without Triton X-100. This was followed by a 5 h incubation at 30^0C in the same buffer. Gels were then stained with coomassie brilliant blue R-250, and destained.

2.2.7.5. Azocasein assay for proteases

Azocasein hydrolysis was carried out as described by Prestidge *et al.* (1971). The reaction mixture .(1 ml) containing 20 µg protease extract, 3 mg azocasein in Tris-HCl (0.1 M; pH 8) buffer, was incubated (JULABO-

SW21, Germany) for 1 h at 30^{0}C. The reaction was stopped with 2 ml ice cold 7% perchloric acid. The protein was allowed to precipitate for 15 min at room temperature and then centrifuged (Remi R4C, Mumbai, India) at 4,000*g* for 10 min at room temperature. To the supernatant, 0.3 ml of 10 N NaOH was added, and the intensity of the color was determined at 440 nm (Beckman DU20, CA, USA). One unit of protease activity is equivalent to the amount of protein that produces an increase of 1.0 unit at A_{440} in 60 min under above mentioned experimental conditions. Protease activity was expressed as units/mg protein.

2.2.7.6. Growth curve

Bacterial cultures (wild type and mutants) were streaked for single colony isolates on agar plates. One such isolate was inoculated into 5 ml medium and incubated for 12 to 16 h at 28^{0}C with shaking. This was again subcultured into 10 ml medium, and the absorbance (A_{600} nm) was measured at every 3 h to monitor the growth.

2.2.7.7. Isolation of toxin

Five day old cultures were centrifuged (12,000*g* for 15 min at 4^{0}C; Sigma 3K 30, rotor#12159) to pellet down crystals, spores and cell debris. The sediment was washed twice with 20 mM Tris-HCl (pH 8) containing 1 M NaCl and thrice with the same buffer without NaCl. The washed sediment (equal to about 2 g of dry technical powder) was extracted with 25 ml of MOPS (0.1 M; pH 7.8, containing 0.5 M DTT and 1 M KSCN) buffer and gently agitated overnight in a shaking water bath (100 rpm; JULABO-SW21, Germany) at 37^{0}C. The resultant slurry was centrifuged (10,000*g* for 20 min at 4^{0}C; Sigma 3K 30, rotor#12159) and the supernatant was dialysed against ddH_2O for 8 h with an hourly change. Most of the times the toxin precipitated at the end of dialysis. However, on occasions, it failed to precipitate. Under such conditions, the toxin was precipitated by saturating

the dialysate by the addition of 17.5 g of ammonium sulfate/100 ml at room temperature. After 3 h, the precipitated protein was recovered by centrifugation (35,000*g* for 20 min at 4^0C; Hitachi 55P-72, Japan, rotor#RP-50-2-768), dialysed once again against ddH_2O until free of ammonium sulfate, lyophilized and stored at -20^0C (Venkateswerlu and Stotzky, 1990).

2.2.7.8. DEAE-cellulose chromatography

Crude preparations obtained as above, was subjected to DEAE-cellulose (fibrous fast flow, Sigma, USA) chromatography. The dialysed sample (20 mg in 5 ml of 20 mM Tris-HCl; pH 8), was applied to a DEAE-cellulose column (24 x 1.5 cm), pre-equilibrated with the starting buffer (20 mM Tris-HCl; pH 8). The unabsorbed material was eliminated by extensively washing with the starting buffer and the bound protein was eluted by discontinuous stepwise-salt gradient. Discontinuous stepwise gradient elution was accomplished in the same buffer using sodium chloride gradient (0.1, 0.3 and 0.6 M) and fractions of 4 ml were collected at a flow rate of 60 ml/h. Appropriate fractions were pooled, dialysed against ddH_2O and lyophilized. The lyophilized protein was stored at -20^0C until further analysis. The protein content of various fractions was monitored by 280 nm absorbance.

2.2.7.9. Isolation of protoxin

The protoxin was isolated using sodium bromide gradient as described by Barbara and Nickerson (1978). Briefly, spores and crystals were removed from the culture medium by centrifugation (10,000*g* for 10 min) and washed three times in ddH_2O. The crystals subsequently were separated from spores and cellular debris by density gradient centrifugation in sodium bromide gradients. Crystals isolated were washed three times in ddH_2O and lyophilized to constant weight. The lyophilized material was solubilized in 5 mM sodium carbonate-bicarbonate buffer (pH 10.5) containing 2 mM EDTA

and the insoluble material was removed by centrifugation. The supernatant was dialysed overnight against Tris-HCl buffer (20 mM; pH 8) containing 10 mM EDTA at 4^0C and concentrated.

2.2.7.10. Protein estimation

Protein content was estimated where necessary by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as the standard. To a suitable aliquot of the sample in 1 ml (containing 10-100 μg protein), 4 ml of alkaline copper reagent (50 ml of 2% Na_2CO_3 in 0.1 N NaOH mixed with 1 ml of 0.5% $CuSO_4$ in 1% sodium potassium tartrate) was added and mixed thoroughly. After 10 min incubation at room temperature, 0.4 ml of 1 N Folin's reagent was added and the mixture vortexed. The blue colour developed after 30 min was measured against a blank at 660 nm.

2.2.7.11. *In vitro* hydrolysis of protoxin with protease extracts

Protoxins from *Btk* HD1, *Btk* HD73 and *Btk-q* were digested with respective protease extracts for 3 h at 37^0C. At the end of digestion, the reaction mixture was centrifuged (6,000*g* for 20 min at 4^0C), and the clear supernatant was extensively dialysed against ddH_2O at 4^0C. The reaction mixture was concentrated by lyophilization and examined by SDS-PAGE.

2.2.7.12. Generation of a 66 kDa toxic polypeptide through proteolytic degradation of the 132 kDa protoxin

Protoxin from *Btk-q* was digested with either trypsin or protease extract of *Btk* HD1. At the end of incubation, the reaction mixture was centrifuged (6,000*g* for 20 min at 4^0C; Sigma 3K 30, rotor#12110), and the clear supernatant was extensively dialysed against ddH_2O at 4^0C, concentrated by lyophilization and analyzed by SDS-PAGE.

2.2.7.13. Screening for protease inhibitor activity by gelatin zymography

Protease extract of mutant (*Btk-q*) was added to the protease extract of *Btk* HD1 and *Btk* HD73 at a concentration of 5% (v/v) and incubated for 3 h at

37^0C. The samples were analyzed by gelatin zymography (Hummel *et al.*, 1996) for proteases.

2.2.7.14. *In vitro* purified toxin processing/degradation

To the purified toxin (1 mg/ml), trypsin/chymotrypsin was added to a concentration of 5% (v/v) and a time course at 37^0C was followed. At the end of incubation, the reaction mixture was centrifuged (6,000*g* for 20 min at 4^0C) and the clear supernatant was extensively dialysed against ddH_2O at 4^0C, and analyzed on SDS-PAGE, followed by immunoblotting.

2.2.7.15. Electrophoresis

SDS-gel electrophoresis of the proteins was performed by the method of Laemmli (1970). The experiment was conducted using 7% gels under reducing (β-mercaptoethanol) conditions, using Tris-glycine buffer (pH 8.3), at a constant current strength of 15 mA, in a Hoefer electrophoresis system. Standard Sigma, USA/Genei, Bangalore (India) molecular weight markers were run along with the protein samples. Electrophoresis was stopped when the tracking dye (bromophenol blue) reached the bottom of the gel and the protein bands were visualized by silver staining method of Blum *et al.* (1987).

2.2.7.16. Silver Staining

All steps were performed at room temperature, with gentle shaking and thorough water wash after each step. The silver staining of the proteins separated on polyacrylamide gels was carried out according to the procedure of Blum *et al.* (1987), with minor modifications. Gels were incubated first with 50% methanol, 12% acetic acid and formaldehyde (50 µl/100 ml) for 1 h followed by three washings in 50% ethanol of 30 min each. Following this the gels were treated with sodium thiosulfate (20 mg/100 ml) for 1 min, rinsed three times (20 sec each) with ddH_2O and impregnated using 0.2%

silver nitrate containing formaldehyde (50 µl/100 ml). After 30 min, they were rinsed with ddH_2O and color was developed by soaking in 6% sodium carbonate (w/v) containing formaldehyde (50 µl/100 ml). The color development was stopped with 1% acetic acid. The stained gels were preserved in 50% methanol after thorough washing with distilled water.

2.2.7.17. Immunological characterization of the mutant

Two male rabbits (New Zealand White) were purchased from National Institute of Nutrition, Hyderabad, and were accilimatized to the laboratory conditions for two weeks. They were maintained on ad libitum diet consisting of leucerne grass and commercial feed.

2.2.7.17.1. Production of polyclonal antibodies

Polyclonal antibodies to the purified toxin was raised in rabbits, according to Smith and Ulrich (1983). A primary dose of 1 mg of toxin in 1 ml of buffer (20 mM Tris-HCl; pH 8) with Freund's complete adjuvant in 1:1 ratio was administered by multiple epidermal injections (25 to 30 sites). After four weeks, three booster doses of 500 µg of toxin with Freund's incomplete adjuvant were administered intramuscularly, at intervals of 15 days. Blood was collected by ear vein puncture in glass tubes and allowed to clot at room temperature. Serum recovered after centrifugation was lyophilized and stored at -20^{0}C until further use.

2.2.7.17.2. Immunological reactivity of antisera

The immunological reactivity of toxin antisera was examined by Ouchterlony double diffusion technique (Ouchterlony, 1953). After coating the plates with 1.2% agar in 10 mM sodium phosphate buffered saline (PBS), pH 7.4, the purified toxin (30 µg/30 µl) was placed in the central well and respective antisera (Neat antiserum upto 1:32 dilution in 10 mM PBS; pH 7.4; 30 µl) in the peripheral wells. The plates were incubated at 37^{0}C for

24 h. Later, they were washed several times with PBS followed by fixing the precipitin line with 1% tannic acid in 1% acetic acid and further destained with 1% acetic acid.

2.2.7.17.3. Western blot ELISA

Immunoblotting of the toxins was performed according to Towbin *et al.* (1979). After SDS-gel electrophoresis, the proteins (40 µg) were electrotransferred (Trans-blot apparatus; Biotech, Salem, India) from the gels to nitrocellulose membrane at a current strength of 200 mA for 5 h. Incubation with each reagent was followed by extensive washing with 25 mM sodium phosphate buffered saline (PBS) and Towbin's phosphate buffered saline (TPBS), containing 0.05% Tween-20, pH 7.6.

The non-specific binding sites were blocked by an overnight incubation of the blots with the blocking buffer (TPBS containing 3% BSA), followed by treatment with primary antibody (1:500 diluted anti-HD73 toxin) overnight at 37^0C. The bands were visualized after incubating with 1:100 diluted secondary antibody i.e., goat anti-rabbit IgG conjugated to alkaline phosphatase followed by treatment of the blot at room temperature for 10 min in the developing solution (0.5 mg p-nitro blue tetrazolium chloride, NBT; and 7.5 mg 5-bromo-4-chloro-3-indoyl phosphate toluidine, BCIP in 50 ml of 0.1 M $NaHCO_3$-NaOH containing 1.0 mM $MgCl_2$; pH 9.8).

2.2.7.18. Electrophoretic analysis of plasmid DNA

Plasmid DNA was extracted using the modified alkaline lysis protocol as described by Jensen *et al.* (1995). Each strain was inoculated into 20 ml nutrient medium and shaken overnight at 28^0C on a rotary shaker (until the A_{600} reached 0.6). A volume of 2 ml culture was pelleted (6,000*g*; Sigma 3K 30, rotor#12110) and suspended in 100 µl TE buffer (40 mM Tris-HCl, 2 mM EDTA; pH 7.9). Cells were then lysed by the addition of 200 µl lysing

solution [3% (w/v) SDS, 15% (w/v) sucrose, 50 mM Tris-hydroxide; pH 12.5]. The preparation was incubated at 60^{0}C for 30 min, followed by the addition of five units of proteinase K (from the stock of 10 mg/ml in 10 mM Tris-HCl; pH 8). Tubes containing the reaction mixture were gently mixed and incubated at 37^{0}C for 90 min. Each preparation was extracted twice with an equal volumes of Tris-saturated phenol (pH 7.8):chloroform:isoamyl alcohol [25:24:1(v/v)] mixture and once with an equal volume of chlorofom:isoamylalcohol (24:1). To the aqueous phase 2.5 volumes of cold absolute ethanol was added and the mixture was stored at –20^{0}C for 30 min. Nucleic acid was pelleted by centrifugation at 13,000*g* for 15 min. The pellet was washed twice with 500 µl of 70% ethanol. DNA pellet was dried in a vacuum drier (Savant Speedvac, medium heat for 5 min) and resuspended in 50 µl of sterile TE (100 mM Tris-HCl and 1 mM EDTA, pH 9.2) buffer, and stored at -20^{0}C until analyzed. The samples were electrophoresed using 0.8% agarose gel (Sambrook *et al.*, 1989) for 2h at 60 V in TAE (40 mM Tris-acetate and 1 mM EDTA; pH 8) buffer. Gels were stained with ethidium bromide and then photographed (UV-transilluminator; Spectroline TR-312 A, Spectronic corporation, Westbury, USA).

2.2.7.19. Restriction enzyme analysis of plasmid DNA

Plasmid DNA was digested with *Eco*RI (G$\downarrow$AATTC), *Bam*HI (G$\downarrow$GATCC), *Hin*dIII (A$\downarrow$AGCTT) and *Pst*I (CTGCA$\downarrow$G). All enzymes were used according to the instructions of the manufacturers. Enzyme digestions in a final volume of 20 µl of 1x reaction mixture (Gibco-BRL) consists of 10-15 µl (200-300 ng) of plasmid DNA, reaction buffer and 1 µl (5-8 U/µl) enzyme. The reaction tubes were incubated at 37^{0}C for 2 h.

Digested products were analysed in a model V16 electrophoresis apparatus (BioRad Laboratories, Maryland, USA) on 1% agarose gel (Sambrook *et al.*, 1989) for 2 h at 60 V in TAE (40 mM Tris-acetate and 1

mM EDTA; pH 8) buffer. Gels were stained with ethidium bromide and viewed on a UV trans-illuminator. Lambda DNA (*Hin*dIII digest; Gibco-BRL)) was used as a molecular weight marker.

2.2.7.20. Plasmid DNA amplification by PCR

PCR program cycle 30 was used for the amplification of plasmid DNA isolated from the strains of *Bt.* Cycle parameters were: Initial denaturation at 94^0C for 3 min (1 cycle), followed by 30 cycles of amplification of denaturation at 92^0C for 1 min, primer annealing at 60^0C for 45 sec and polymerization at 72^0C for 1.5 min, and last cycle at 72^0C for 5 min for extension. The two synthetic oligonucleotide primers (forward primer: 5' AGA GGA TCG AGA CTG GCT ACA 3'; reverse primer: 5' GTG GCT GAG GCG GTG GCT GAA 3'; obtained from ICRISAT), specific to the coding region of the δ-endotoxin gene with 1.2 kb amplification were used. The whole plasmid DNA was used as a template. PCR reaction was performed using Gibco-BRL PCR reagents (Gibco-BRL, Southampton, UK). To obtain dNTP mixture, 25 µl of each of dATP, dCTP, dTTP and dGTP from 100 mM stock were mixed together. The final concentration of each dNTP in this mixture was 10 mM.

PCR reaction mixture:

The following components were added into a sterile 0.5 ml tube:

10x PCR buffer (supplied with the enzyme)	5.0 µl
dNTP mixture 10 mM	0.3 µl
$MgCl_2$ 25 µM	3.0 µl
Forward primer 10 µM	1.0 µl
Reverse primer 10 µM	1.0 µl
Sterile dH_2O	36.25 µl

Taq DNA Polymerase (1.25 U)	0.25 µl
Template DNA	4.0 µl

PCR was performed in a GeneAMP-2400 thermal cycler (PE-Applied Biosystems, USA). Mineral oil was not used as it was fitted with heated covers to prevent evaporation.

Analysis of PCR products

Aliquots (25 µl) of amplified products were mixed with 3 µl of loading dye (0.25% bromophenol blue, and 30% glycerol in distilled water) and electrophoresed on a 1% agarose gel using TAE (40 mM Tris-acetate and 1 mM EDTA; pH 8) buffer system (Sambrook *et al.*, 1989). Gels were stained with ethidium bromide and viewed on a UV trans-illuminator (Spectroline TR-312 A, Spectronic corporation, Westbury, USA).

2.2.7.21. Southern blotting and hybridization

Southern hybridization was performed to confirm the presence of the *cry*1Ab gene in the mutants by using the *cry*1Ab of plasmid pKS$^+$*Bt* as a probe to hybridise with the amplified DNA fragments using protocols described in Molecular Cloning (Sambrook *et al.*, 1989). Further detection of the hybridization signal was performed by using non-radiolabelling (using alkaline phosphatase) methods as per the manufacturers instructions (Amersham, UK).

Plasmid pKS$^+$*Bt* (obtained from ICRISAT) was restricted with *Xho*I and *Xba*I restriction enzymes. Electrophoretic fragment of 1.2 kb was cut and eluted from agarose gel (as per the Qiagen extraction kit; Amersham, UK) and labeled with non-radiolabelling ALKPhos detection system (Amersham, UK).

The agarose gel was rinsed in dd$H_2$0 (3x15 min washes), depurinised with 0.2 N HCl (20 min) and the DNA was denatured (1.5 M NaCl, 0.5 M

NaOH) followed by neutralization (3 M NaCl, 0.5 M Tris/HCl/NaOH). The DNA was transferred on to a Hybond N^+ nylon membrane by capillary blotting with 20x SSC buffer (3 M NaCl, 0.3 M sodium citrate; pH 7.2) for 2 h and treating the membrane with UV light to fix the DNA.

Prehybridization and hybridization was carried out in a Techne Hybridiser IB-1D and the probe was detected by the ALKPhos direct detection system (Amersham, UK) employing alkaline-phosphatase labeled probe and CDP-*Star* chemiluminiscent detection reagent (Amersham, UK). After blotting, the membrane was prehybridised with hybridization buffer (ALKPhos hybridization buffer kit) at 55^0C for 15 min. This was replaced with 10 ml of fresh hybridization buffer containing 10 µl (100-150 ng) of *cry*1Ab gene probe labeled with alkaline phosphatase and hybridization was continued at 55^0C overnight (8 to 14 h). The membrane was then washed (2x 10 min) with agitation at 55^0C with primary wash buffer (2 M urea, 0.1% SDS, 50 mM sodium phosphate, 150 mM NaCl, 1 mM $MgCl_2$, 0.2% blocking reagent; pH 7). It was then washed (2x 5 min) with agitation at room temperature with secondary wash buffer (50 mM Tris base, 100 mM NaCl, 2 mM $MgCl_2$; pH 10). After drained the blot, 1 ml of CDP-*Star* detection reagent was applied and left for 2 to 5 min at room temperature. Membrane was wrapped in saran wrap and exposed to X-ray film. The X-ray films were developed further.

2.2.8. Bioassays

The bioassays were conducted to evaluate the efficacy of *Bt* mutants with respect to wild type *Bt* against cotton bollworm/legume pod borer, *Helicoverpa armigera*, tobacco caterpillar, *Spodoptera litura*, and spotted stem borer, *Chilo partellus*.

2.2.8.1. Insect rearing

Larvae of the pod borer (*H. armigera*) and tobacco caterpillar (*S. litura*) were maintained on a standard artificiel diet (Armes *et al.*, 1992) containing: (per litre of diet gel) agar (Himedia, Mumbai), 17.3 g; ascorbic acid, 4.7 g; sorbic acid, 3 g; methyl-4-hydroxybenzoate, 5 g; vitmix, 10 ml; aureomycin, 11.5 g; chick pea powder, 300 g; yeast, 48 g; and distilled water, 1 lt. Larvae of the spotted stem borer (*C. partellus*) were maintained on the standard artificial diet (used for rearing *H. armigera*) but contained an additional sorghum leaf powder (Sharma *et al.*, 1992).

Bioassays of *Bt* (crystal-spore suspension) were carried out using surface treatment of artificial diet or impregnation of *Bt* toxins into the artificial diet as described by Sharma *et al.* (1992). The choice of bioassay would depend on the insect species, availability of artificial rearing medium, amount of toxins available, and the nature of damage. All bioassays were conducted by exposing neonates (<24 h after hatching) to treated artificial diet. All assays were replicated twice and pooled data were subjected to analysis.

2.2.8.2. Insect studies with Chilo partellus larvae

Glass vials (20 ml capacity) with metal lids without rubber septa (from which the larvae could not escape by making holes) were found to be suitable for experiments with first instar larvae of spotted stem borer, *C. partellus*. Five ml of chickpea based artificial diet (Armes *et al.*, 1992) treated on the surface with 100 µl of the toxin was found to be suitable for bioassay of *Bt* toxins against this insect. Ten larvae were released in each vial, 4 h after treatment.

2.2.8.3. Insect studies with Spodoptera litura larvae

To evaluate the *Bt* toxins against the first instar larvae of *S. litura*, 20 ml plastic vials were used containing 5 ml artificial diet (used for rearing *H.*

armigera). Toxin was sprayed (100 µl) on the diet surface and 10 larvae were released in each vial 4 h after treatment.

2.2.8.4. Insect studies with Helicoverpa armigera larvae

To evaluate the *Bt* toxins against the first instar larvae of *H. armigera*, 7 ml glass vials were used containing 1 ml artificial diet (Armes *et al.*, 1992). Since the larvae being cannibalistic, only one larva was released in each vial. Approximately 1 ml of diet was dispensed into each well and allowed to solidify. Each well was treated with 25 µl of the appropriate toxin concentration prepared in 0.1% Triton X-100 detergent to obtain uniform spreading on the diet surface. Control treatments consisted of diet treated with 0.1% detergent only. Vials were allowed to air dry for 1 h and one neonate was transferred into each well. Vials then were covered with lids, and trays were held at 27^0C, and 80% relative humidity (RH). Mortality and individual larval weights were recorded 5 days after treatment. When mortality was recorded, larvae that had not grown beyond first instar and were <0.1 mg in weight were considered to be dead. As a result, the criterion for "mortality" used in this study accounts for both severe growth inhibition and death.

Bioassays with the crystal toxins were carried out by leaf disc method as described by Albert *et al.* (1989). Tender terminal leaves of cotton were cut into discs (3 cm in diameter) and coated with 50 µl of toxin on each side, air dried and 20 neonates were released per concentration on four discs placed in plastic cups with perforated lids. Five concentrations were tested per assay. Leaves were changed and mortality was recorded daily until the seventh day.

The spore-crystal suspension was obtained by harvesting 3 to 5 day old culture. The cell sediments were washed twice with 20 mM Tris-HCl (pH 8) containing 1 M NaCl and thrice with the same buffer without NaCl.

Nevertheless, a washing procedure was used to eliminate most inter ingredients present in the mixture. This procedure involved centrifugation of 1 ml-batches of crystal-spore suspension at 10,000*g* for 60 sec. The supernatant was discarded and the pellet was suspended in ddH_2O to a 1 ml total volume. This was centrifuged again for 60 sec, the supernatant was removed, and the pellet was resuspended in 1 ml in ddH_2O containing 0.1% Triton X-100. The resulting preparation was used as a stock solution for all bioassays.

All *Bt* purified toxin proteins were produced as described earlier in this chapter. The toxin isolated was further purified on DEAE-cellulose. Proteins were quantified and diluted as 6 to 8 concentrations (10^{-2}, 10^{-4}, 10^{-8}, 10^{-16}.....) in distilled water.

2.2.8.5. Statistical analysis

Bioassays were conducted in duplicate and included at least five *Bt* (or isolated toxins) concentrations that produced significant growth inhibition and mortality as defined previously. Individual larval weights were recorded after 5 days of exposure and transformed to percentage of growth inhibition relative to the controls, and these data were analyzed by non-linear regression fitted to a Probit analysis (Finney, 1971) using the Genstat, 2000 statistical package.

2.3. Results

During sporulation phase of *Bt*, a high protease activity has been observed (Chestukhina *et al.*, 1979). However, no information on the role of these proteases, either in the production of protoxin or toxin or in the physiology of the bacterium. Are there any proteases acting on protoxin and converting it to toxin? or Do they further degrade the protoxin or toxin to smaller fragments? If the enzyme just cleaves the protoxin to toxin, is it beneficial to its insecticidal activity? If the degradation is continued, it is a waste of

protoxin and is a loss of insecticidal activity. In view of this, attempts have made to isolate protease deficient mutants of *Bt*, and characterize them in order to ascertain the role of proteases in the life of the bacterium, answering the above questions to some extent.

2.3.1. Isolation of endogenous protease mutants

Bacillus thuringiensis subsp. *kurstaki* HD1 after treating with EMS were screened on milk agar plates. Figure 1A, shows protease deficient mutant '*Btk-q*' and the wild type strain (*Btk* HD1), where the latter shows zone of clearance but not the former strain on milk agar plate to show the formation of zone of clearance. It can be seen from Fig. 1B, that *Btk* HD1 forms a wide zone of clearance around the colony, whereas *Btk-q* lacking the zone of clearance, with small colony formation, and slow growth.

2.3.2. Assay for protease activity

2.3.2.1. X-ray film spot test

The isolated mutants were also tested for protease activity using X-ray film assay and the results obtained are presented in Fig. 2A. The gelatin clearance by various mutants was compared with the wild type. A trypsin digestion was also carried as an internal marker. Of the seven mutants tested, *Btk-q* (spot 5) did not dissolve the gelatin like the wild type indicating that it is deficient of protease activity. In contrast, the wild type strain *Btk* HD1 exhibited large halo (spot 6). Simultaneously, four other mutants namely, 'c', 'e', 'm' and 'n' were also isolated and tested along with *Btk* HD1 and *Btk-q*. Of these 'c' (spot 3), and 'e' (spot 7), seems to be similar to *Btk* HD1 (could not be distinguished from wild type at this stage), whereas 'm' (spot 2), and 'n' (spot 4) revealed smaller halos.

2.3.2.2. Analysis of proteases by gelatin zymography

The intracellular protease pattern of the above mutants was further examined by gelatin zymographic studies (Fig. 2B). As the extract obtained with 30%

Figure 1A

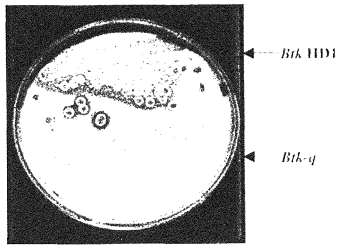
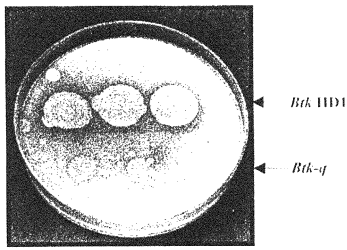
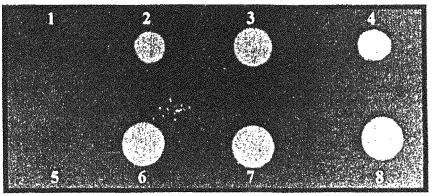


Figure 1B



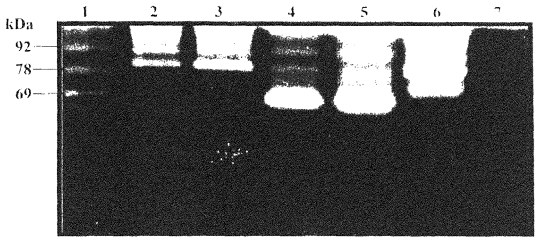
Milk agar plate showing wild type (*Btk* HD1) and protease deficient mutant (*Btk-q*). In the figure the zone of clearance is represented as dark area (In the original plate the halo was a transparent zone against opaque milk agar plate).

Figure 2A



X-ray film spot assay of *Btk* mutants. Spot 1. 0.1% Triton X-100 (-ve control); spot 2. Mutant 'm'; spot 3. Mutant 'e'; spot 4. Mutant 'n'; spot 5. *Btk-q*; spot 6. *Btk* HD1; spot 7. *Btk-e*; spot 8. Trypsin.

Figure 2B



Gelatin zymography of the intracellular proteases of *Btk* mutants. Lane 1. *Btk* HD1; lane 2. Mutant 'm'; lane 3. Mutant 'n'; lane 4. Mutant 'e'; Lane 5. Mutant 'e'; lane 6. Mutant 'e'; lane 7. Mutant 'q'. The amount of protein loaded was 30 μg in all lanes except lane 5, where 50 μg was loaded.

ammonium sulfate fractionation of cell lysate contributed to nearly 95% of the total activity of crude enzyme (12,000*g* supernatant), all experiments were conducted using this fraction. The wild type strain (*Btk* HD1) at the age of 24 h revealed the presence of three intracellular proteases with a molecular mass of 92, 78 and 69 kDa. However, the mutants 'm' and 'n' contained only two proteases of molecular weight of 92 and 78 kDa (and hence a small halo as seen on X-ray film). Another mutant designated as 'c' demonstrated a different pattern (a doublet of 78 kDa) of intracellular proteases. The zymography also reveals that *Btk-q* lacked all the three proteases, and another mutant, *Btk-e* apparently seems to have an elevated activity of 69 kDa protease. Amongst these mutants, one mutant which lacked the protease activity (designated as *Btk-q*) and one with hyperprotease activity (*Btk-e*) were selected for further studies.

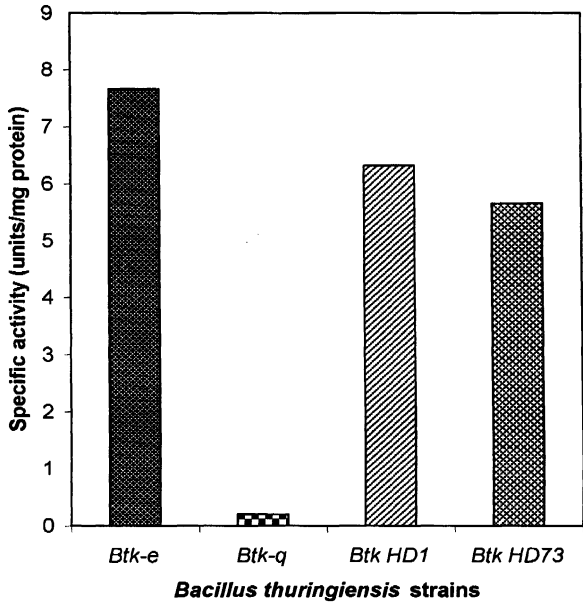
2.3.2.3. Azocasein assay

Further the intracellular protease activity of the above mutants was also quantified using azocasein as a substrate (Fig. 2C). Interestingly, *Btk-q* mutant exhibited only 3.15% activity (only traces, which could not be detected by gelatin zymography) of *Btk* HD1 (100%), whereas *Btk* HD73 and *Btk-e* exhibited nearly 89.50% and 120.25% activity, respectively.

2.3.3. Growth characteristics

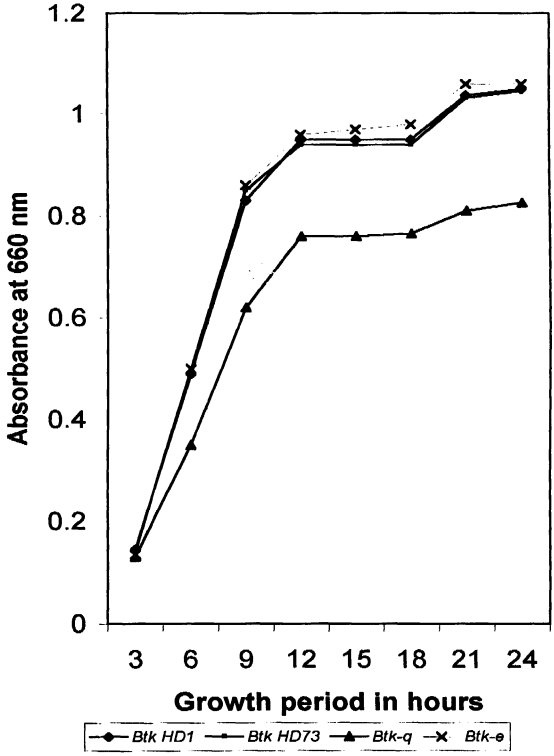
In view of the significant difference in the intracellular protease activity of the mutants *Btk-e*, and *Btk-q*, the growth pattern of these strains was compared to that of the *Btk* HD1, and the data is depicted in Fig. 3. The *Btk-q* mutant seems to be slow growing as compared to the wild type and at the end of 24 h, the growth of this mutant was found to be singnificantly (30%) less than that of wild type (*Btk* HD1).

Figure 2C. Histogram showing intracellular protease activity by azocasein hydrolysis



Protease activity is expressed in terms of specific activity, defined as units/mg protein. One unit of protease activity is equivalent to the amount of enzyme that produces an increase of 1.0 in A_{440} in 60 min under the experimental conditions

Figure 3. Growth curve of wild type and mutant strains of *Btk*



2.3.4. Protoxin and toxin production by mutants

The 'q' mutant was further analyzed for the presence of the protoxin (132 kDa) and toxin (66 kDa) (Fig. 4A). Cell lysate analysis of *Btk* HD1 by SDS-PAGE revealed an intense protein band with a mol. wt. of 66 kDa pertaining to toxin (lane 2). *Btk-q* cell lysate (lane 3) lacked 66 kDa band; instead it showed 132 kDa band. This is probably due to the lack of corresponding protease breaking this protoxin, in *Btk* HD1, converting it into toxin (66 kDa).

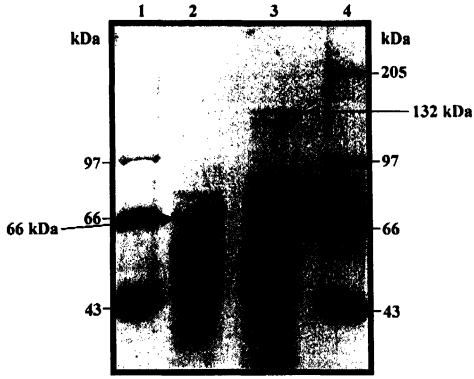
Similarly, toxin isolated by a method described by Venkateswerlu and Stotzky (1990), showed the presence of 66 kDa toxin in *Btk* HD1 (Fig. 4B, lane 2), whereas not in *Btk-q*. The mutant *Btk-q* indicated the presence of 132 kDa (protoxin) but not the toxin (66 kDa) (lane 5). *Btk* HD73 and mutant *Btk-e* also contained the same protein band as that of *Btk* HD1 (lane 3 and 4). This data is in concurrence with the above results presented in Fig. 4A, where it is speculated that *Btk-q* mutant is deficient with respect to a protease converting protoxin to toxin.

2.3.5. *In vitro* hydrolysis of protoxin by endogenous proteases

Isolated protoxins from *Btk* HD1, *Btk* HD73 and *Btk-q* were digested with respective protease extracts (Fig. 5A). Protease extracts from *Btk* HD1 and *Btk* HD73 digested protoxin, producing a 66 kDa toxin along with few low mol. wt. bands. However, the protease extract from *Btk-q* could not digest the 132 kDa protoxin, neither to 66 kDa toxin or the low mol. wt. components. As shown in Fig. 5B, the 132 kDa protoxin in *Btk-q* was shown to be normal in that it was cleaved to 66 kDa toxin by trypsin, and as well by the protease preparation from *Btk* HD1 to 66 kDa toxin along with some low mol. wt. bands.

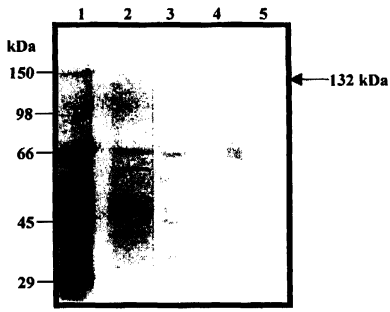
Further as seen from the data in Fig. 5C, the zymographic pattern of proteases from *Btk* HD1 and *Btk* HD73 was not affected, when incubated

Figure 4A



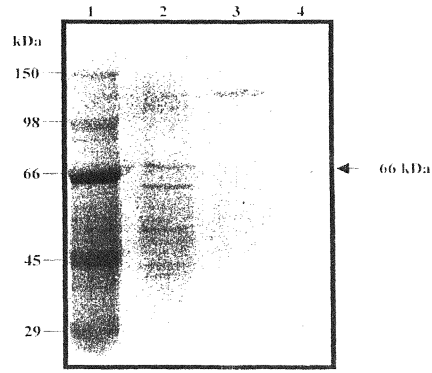
SDS-PAGE of cell lysates prepared from *Btk* HD1and *Btk-q*. Cell lysates were electrophoresed on 7.5% SDS-PAGE. Lane 1. Medium range mol. wt. markers; lane 2. *Btk* HD1 (wild type); lane 3. *Btk-q*; lane 4. High range mol. wt. markers.

Figure 4B



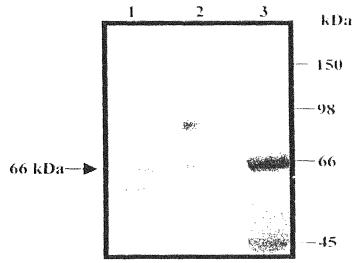
SDS-PAGE of toxin preparations from various *Bt* strains. Toxins were isolated from various *Bt* strains using DTT/MOPS and were electrophoresed on 7.5% SDS-PAGE. Lane 1. Mol. wt. markers; lane 2. *Btk* HD1; lane 3. *Btk* HD73; lane 4. *Btk-e;* lane 5. *Btk-q.*

Figure 5A



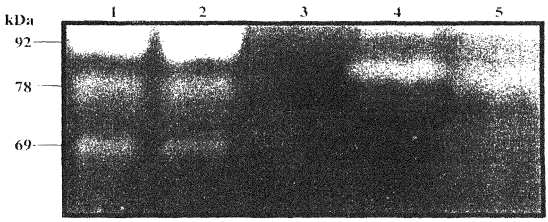
SDS-PAGE analysis of protoxin hydrolysis with protease extracts. Protoxins from various strains were incubated with respective protease extract at 37°C for 3 h. The products of hydrolysis were analyzed after 3 h by SDS-PAGE. Lane 1. Mol. wt. markers; lane 2. *Btk* HD1 protoxin + *Btk* HD1 protease extract; lane 3. *Btk-q* protoxin + *Btk-q* protease extract; lane 4. *Btk* HD73 protoxin + *Btk* HD73 protease extract.

Figure 5B



SDS-PAGE showing hydrolysis of *Btk-q* protoxin with *Btk* HD1 protease extract and trypsin. Protoxin from *Btk-q* was digested with wild type protease extract or trypsin at 37°C for 3 h. Later it was electrophoresed on 7.5% SDS-PAGE gel at 15 mA. Lane 1. *Btk-q* protoxin + *Btk* HD1 protease extract; lane 2. *Btk-q* protoxin + trypsin; lane 3. Mol. wt. markers.

Figure 5C



Gelatin zymographic analysis of *Btk* HD1 and *Btk* HD73 proteases in presence of protease extract from *Btk-q*. The protease extract from wild type strains were mixed with the protease extract of *Btk-q* mutant and incubated at 37°C for 3 h. Later it was electrophoresed on 7.5% SDS-PAGE containing 1% gelatin and stained for protease activity. Lane 1. Protease extract (*Btk* HD1); lane 2. Protease extract (*Btk* HD1) + Protease extract (*Btk-q*); lane 3. *Btk-q* protease extract; lane 4. Protease extract (*Btk* HD73); lane 5. Protease extract (*Btk* HD73) + Protease extract (*Btk-q*).

with the protease extract from *Btk-q*, ruling out that no protease inhibitor present in this mutant.

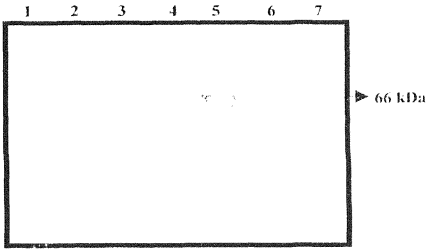
2.3.6. *In vitro* toxin processing/degradation

Most of the studies showed that toxin obtained by exogenous proteases (Trypsin/Chymotrypsin/insect midgut juice) is further resistant to proteolysis. The aim of this experiment is to examine whether endogenous protease activated toxin is resistant (or susceptible) on further proteolysis similar to the toxin obtained by exogenous activation. After 3 or 6 h of incubation of the toxin with trypsin/chymotrypsin, the resulting samples were analyzed by SDS-PAGE (7.5%), followed by immunoblotting. As shown in Fig. 6, the endogenous protease activated toxin is further degraded to few low mol. wt. fragments (lanes 2, 3 and 4), and they appeared to cross react with anti-HD73 antibody like the toxin from *Btk* HD1. Such degradation of purified toxin obtained by exogenous protease activation of protoxin (resistant to further proteolysis) could not be seen (lanes 6 and 7), indicating that the endogenous protease activated toxin is somewhat different (cleavage site) from that of the toxin obtained by exogenous activation.

2.3.7. Immunological characterization of the mutant

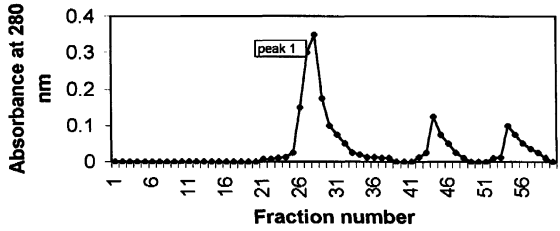
For raising antisera, toxin (66 kDa) was isolated and purified from *Btk* HD73. The toxin protein isolated, revealed on SDS-PAGE/silver staining few other proteins of low molecular weight along with the protein of our interest (Fig. 8A, lane 2) This toxin was hence considered to be crude and further purification of the toxin was carried out. On DEAE-cellulose column chromatography, the bound toxin eluted as a single major peak with 0.3 M NaCl (Fig. 7A) corresponding to the molecular weight of 66 kDa (Fig. 8A, lane 1). This pure toxin was dialysed which on rechromatography eluted at the same place (Fig. 7B)

Figure 6



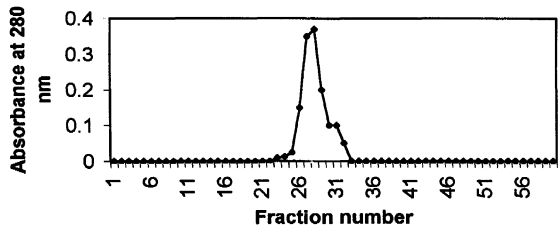
Western blot showing *in vitro* degradation of toxin obtained by an endogenous protease activation of protoxin. Lane 1. Control serum (preimmunized serum); lane 2. Endogenously isolated toxin digested with trypsin (3 h); lane 3. Endogenously isolated toxin digested with trypsin (6 h); lane 4. Endogenously isolated toxin digested with chymotrypsin (3 h); lane 5. •Endogenously isolated toxin (control); lane 6. *Exogenously isolated toxin (control); lane 7. Exogenously isolated toxin digested with trypsin (3 h). *toxin obtained by treating protoxin with trypsin/chymotrypsin. •toxin obtained by treating *Bti* cells with MOPS/DTT/KSCN.

Figure 7A. DEAE-cellulose chromatography of toxin protein



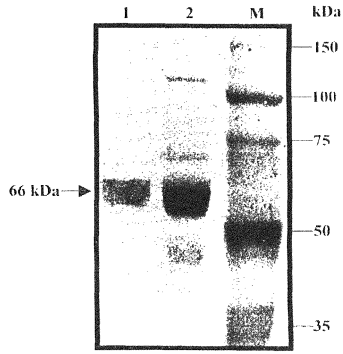
Fractions 1-20 eluted with 0.1 M NaCl. Peak 1 (fractions 25-30), eluted with 0.3 M NaCl. Fractions 40-60, eluted with 0.6 M NaCl.

Figure 7B. Rechromatography of the purified toxin on DEAE-cellulose



Fractions 1-20 eluted with 0.1 M NaCl. Peak (fractions 25-30), eluted with 0.3 M NaCl. Fractions 40-60, eluted with 0.6 M NaCl.

Figure 8A



Separation of *Btk* HD73 crystal toxin (isolated by treating the cells with MOPS/DTT/KSCN) on a 7.5% SDS-PAGE, silver stained. Lane M. Mol. wt. marker proteins (Sigma, USA); lane 1. Purified toxin; lane 2. Crude toxin.

On ouchterlony analysis, antisera to the pure 66 kDa toxin formed a precipitin line with the purified toxin (66 kDa) upto 1:16 dilution (Fig. 8B). This was further confirmed by western blotting, wherein the antisera cross reacted with 66 kDa protein of *Btk* HD73 (Fig. 8C). Few high mol. wt. proteins found as contaminants during the isolation of 66 kDa toxin, also cross reacted with the antisera (lane 1).

2.3.7.1. Dot blot ELISA

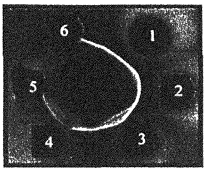
Polyclonal antibodies to 66 kDa toxin protein from *Btk* HD73, were used to study the relationship between the occurrence of toxin among the four strains of *Bt*. When direct culture cells were used as the primary antigen in dot blot ELISA tests, all strains tested positive for the antigen (Fig. 9A). The dot blot ELISA was also performed on MOPS/DTT isolated toxin proteins from all strains (Fig. 9B). Samples were adjusted to 20 μg of toxin per ml and 0.1 ml of each was tested at a dilution of 1:100. All samples reacted with the antibody. This provided evidence for the antigenic relationship of the 66 kDa toxin isolated from each strain.

2.3.7.2. Western blot ELISA

Toxin proteins from *Btk* HD1, *Btk* HD73, *Btk-e* and *Btk-q* analyzed by SDS-PAGE and silver staining (Fig. 4B), were tested further by western blot ELISA (Fig. 9C). The 132 kDa protoxin of *Btk-q* cross reacted with the antibody against 66 kDa protein. *Btk* HD1, *Btk* HD73 and *Btk-e* toxin proteins (66 kDa) also showed reaction with antibody.

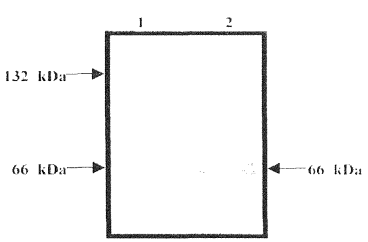
Since the mutant *Btk-q* being a small colony former (Fig. 1A and 1B) and slow growing (Fig. 3), a question may arise whether it is isostrainic and clonal with that of parent. In view of this, *Btk-q* was analysed with respect to its plasmid profile, restriction mapping, PCR amplification and, southern blot hybridization.

Figure 8B



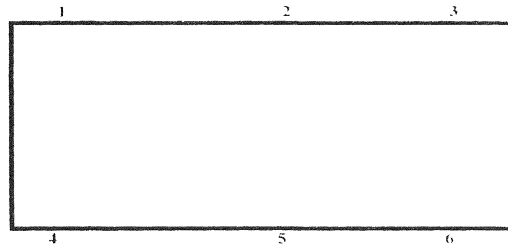
Ouchterlony analysis. 30 μg of pure toxin protein was loaded in the central well. The peripheral wells were loaded with various dilutions of antisera to 66 kDa toxin. Well 1. Undiluted (neat); well 2. 1:2; well 3. 1:4; well 4. 1:8; well 5. 1:16; well 6. 1:32.

Figure 8C



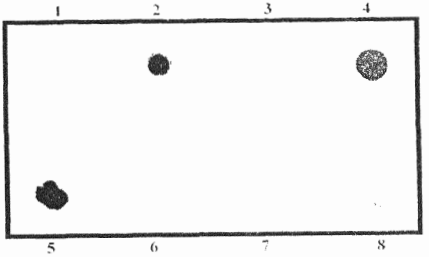
Western blot analysis of *Btk* HD73 crystal toxin using antibodies raised against purified 66 kDa toxin. Lane 1. Crude toxin; lane 2. Purified toxin.

Figure 9A



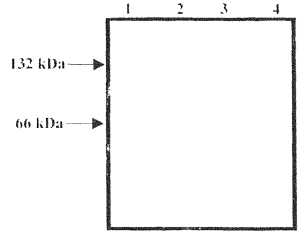
Dot blot analysis of different mutants of *Btk* cells. Spot 1. Mutant 'q'; spot 2. *Btk* HD1; spot 3. *Btk* HD73; spot 4. Mutant 'e'; spot 5. Mutant 'c'; spot 6. Buffer.

Figure 9B



Dot blot analysis of toxins isolated from different *Bt* strains. Spot 1. Control serum; spot 2. *Btk* HD1 toxin; spot 3. *Btk-e*; spot 4. *Btk* HD73 crude toxin; spot 5. *Btk* HD73 purified toxin; spot 6. Mutant 'c'; spot 7. *Btk-q* purified toxin (5 µl); spot 8. *Btk-q* purified toxin (10 µl).

Figure 9C



Western blot analysis of toxins of various *Bt* strains. Toxins isolated (DTT/MOPS) were purified by DEAE-cellulose chromatography and electrophoresed on 7.5% SDS-PAGE. The proteins were transferred to nitrocellulose membrane electrophoretically and probed with antibodies to 66 kDa toxin (*Btk* HD73). Lane 1. *Btk* HD1; lane 2. *Btk-q*; lane 3. *Btk* HD73; lane 4. *Btk-e*.

2.3.8. Plasmid analysis

Plasmid analysis of *Btk* HD1, *Btk* HD73, *Btk-q* and *Btk-e* strains revealed the presence of several plasmids (the reported range is 2-250 kb; Gonzalez *et al.*, 1980; Konstad *et al.*, 1984; Lereclus *et al.*, 1984). All strains were found to have identical plasmid profile. Agarose electrophoresis (Fig. 10A) showed the presence of a >23 kb plasmid in all the strains.

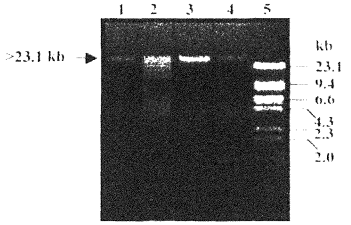
2.3.9. Restriction mapping

The plasmid DNA of each strain had a distinct, *Eco*RI, *Bam*HI, *Hin*dIII and *Pst*I restriction enzyme profile (Fig. 10B and 10C), but showed the pattern as expected of *cry*1A gene. The restriction profile showed that the plasmid has several sites for *Eco*RI, *Bam*HI, *Hin*dIII and *Pst*I enzymes in *Btk* HD1, *Btk-q*, *Btk* HD73 and *Btk-e*. The high mol. wt. plasmids were not able to resolve properly on 0.8% agarose gel. Further results could be ascertained only with PCR analysis.

2.3.10. PCR analysis and Southern hybridization

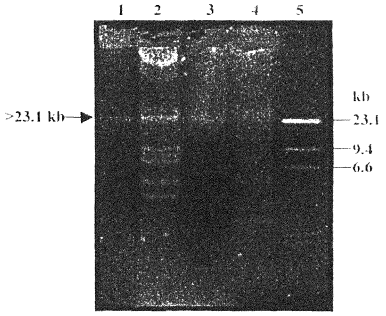
These mutants were further analyzed by PCR amplification and Southern hybridization using two synthetic oligonucleotide primers and enzyme labeled *cry*1Ab gene probe for the determination of presence or absence of the corresponding sequence. We have selected *cry*1Ab primers, since this gene is represented in higher percentage in the crystal. Synthetic oligonucleotide primers specific to regions with in coding sequence of *cry*1Ab gene were used for amplifying the whole isolated plasmid DNA as a template for the presence of *cry*1Ab gene. *Btk* strains HD1 and *Btk* HD73 were used as control strains to test the set of primers. Electrophoretic analysis of the resulting PCR products revealed the presence of a fragement of approximately 1.2 kb, indicating the existence of a *cry*1Ab gene (Fig. 10D).

Figure 10A



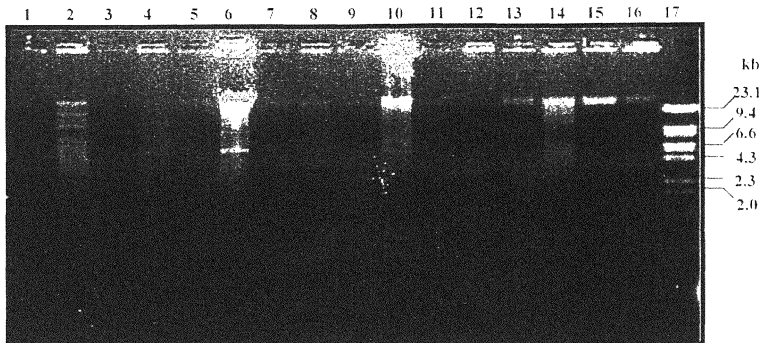
Agarose gel electrophoresis showing plasmid DNA of various *Bt* strains. Lane 1. *Btk* HD73; lane 2. *Btk* HD1; lane 3. *Btk-e*; lane 4. *Btk-q*; lane 5. λ phage *Hin*dIII digest (marker).

Figure 10B



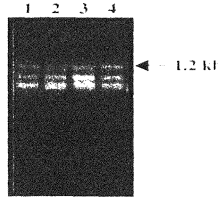
Restriction enzyme analysis of plasmid DNA with *Hin*dIII. Lane 1. *Btk* HD73; lane 2. *Btk* HD1; lane 3. *Btk-e*; lane 4. *Btk-q*; lane 5. λ phage *Hin*dIII digest (marker).

Figure 10C



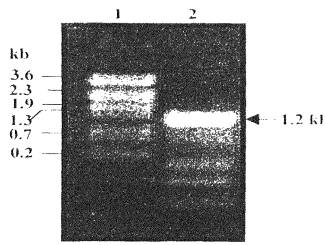
Restriction analysis of plasmid DNA. Lanes (*Eco*RI digest) 1. *Btk* HD73; 2. *Btk* HD1; 3. *Btk-c*; 4. *Btk-q*; lanes (*Bam*HI digest) 5. *Btk* HD73; 6. *Btk* HD1; 7. *Btk-c*; 8. *Btk-q*; lanes (*Pst*I digest) 9. *Btk* HD73; 10. *Btk* HD1; 11. *Btk-c*; 12. *Btk-q*; lane 13. *Btk* HD73; lane 14. *Btk* HD1; lane 15. *Btk-c*; lane 16. *Btk-q*; lane 17. λ phage *Hin*dIII digest (marker).

Figure 10D



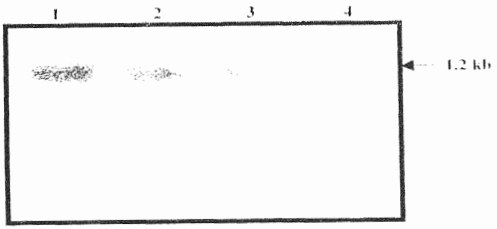
PCR amplification of 1.2 kb fragment of *cry*1Ab gene. DNA isolated from various *Bt* strains were amplified with primers specific for *cry*1Ab gene using *Taq* polymerase. Lane 1. *Btk* HD73; lane 2. *Btk* HD1; lane 3. *Btk-q*; lane 4. *Btk-c*.

Figure 10E



Probe preparation for Southern hybridization. Plasmid pKS$^+$*Bt* was restricted with *Xho*I and *Xba*I and electrophoresed on 0.8% agarose gel. Lane 1. Markers; lane 2. *cry*1Ab gene insert.

Figure 10F



Southern blot hybridization showing *cry*1Ab gene from various *Bt* strains. PCR amplified 1.2 kb fragment of *cry*1Ab gene was probed in a Southern blot with plasmid pKS⁺*Bt* carrying *cry*1Ab gene. Lane 1. *Btk* HD73; lane 2. *Btk* HD1; lane 3. *Btk-q*; lane 4. *Btk-e*.

The amplified product of 1.2 kb *cry*1Ab gene was blotted on the membrane and probed (Fig. 10E) with plasmid sequence of *cry*1Ab gene enzyme labeled with a Gibco-BRL labeling kit and used in Southern hybridization to assess the fedility of amplified fragments of *cry*1Ab of the mutants. And these hybridization experiments confirmed the presence of *cry*1Ab gene in the mutant *Btk-q* and *Btk-e* (Fig. 10F).

2.3.11. Bioassays

The bioassays with three insect species revealed that the larvae became inactivated after 12 to 24 h of exposure to the diet treated with the toxins and they died with in 5 to 10 days, whereas in the untreated diet, the larvae developed normally. Percentage mortality increased with an increase in the concentration of crystal proteins in the diet. Th results of the bioassays are presented in Figs. 11A, 11B, 11C, 12A, 12B, 12C and 13. The data indicated that mutant *Btk-q* was most toxic, followed by *Btk* HD1, *Btk* HD73 and *Btk-e* against *H. armigera*, *S. litura*, *C. partellus*. The *S. litura* and *H. armigera* were significantly more susceptible of all the insects tested where *C. partellus*, was the most tolerant one. The *H. armigera* larvae were the most susceptible than the other two.

The LC_{50} values for *H. armigera* were 53.8 μg for *Btk* HD1, 50 μg for *Btk* HD73, 21.9 μg for *Btk-q*, and 61.8 μg for *Btk-e* per cm^2 diet (Fig. 11A). ED_{50} values (i.e., concentrations that caused 50% growth inhibition of *H. armigera* larvae) deduced from the data were 24.9, 24.7, 11.2 and 37.7 $\mu g/cm^2$ for *Btk* HD1, *Btk* HD73, *Btk-q* and *Btk-e*, respectively (Fig. 12A). The LC_{50} value for *Btk* HD1, *Btk* HD73, *Btk-q* and *Btk-e* for *S. litura* were 56.6, 62.60, 47.40 and 64 $\mu g/cm^2$ of diet, respectively (Fig. 11B). The LC_{50} for *Btk-q* was the lowest. ED_{50} values for *Btk* HD1, *Btk* HD73, *Btk-q* and *Btk-e* were 22.1, 23.30, 20.80 and 24.4 $\mu g/cm^2$ of diet, respectively (Fig. 12B). The LC_{50} for *Btk* HD1, *Btk* HD73, *Btk-q* and *Btk-e* for *C. partellus*

Figure. 11A. Toxicity of *Bacillus thuringiensis* strains against *Helicoverpa armigera*

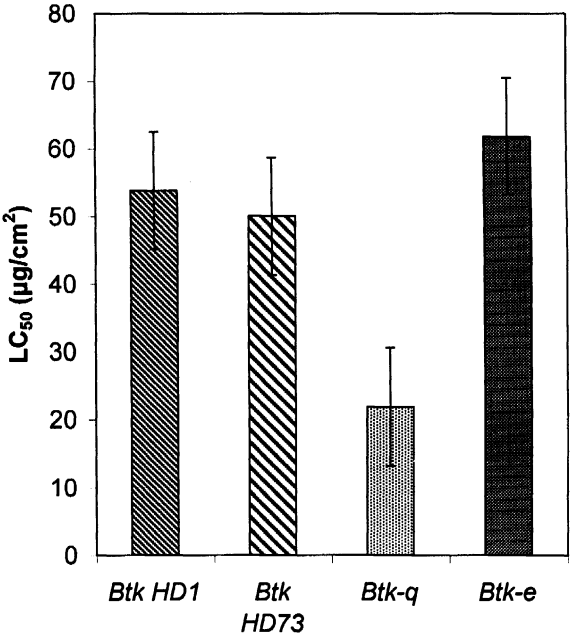


Figure 11B. Toxicity of *Bacillus thuringiensis* strains against *Spodoptera litura*

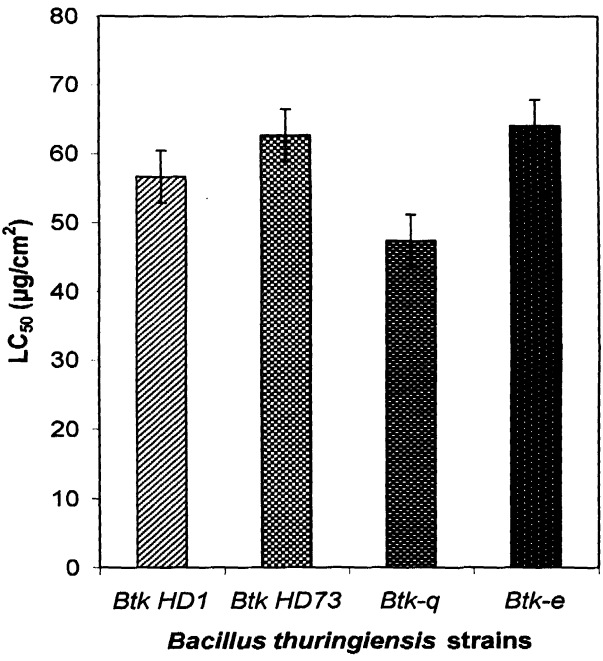
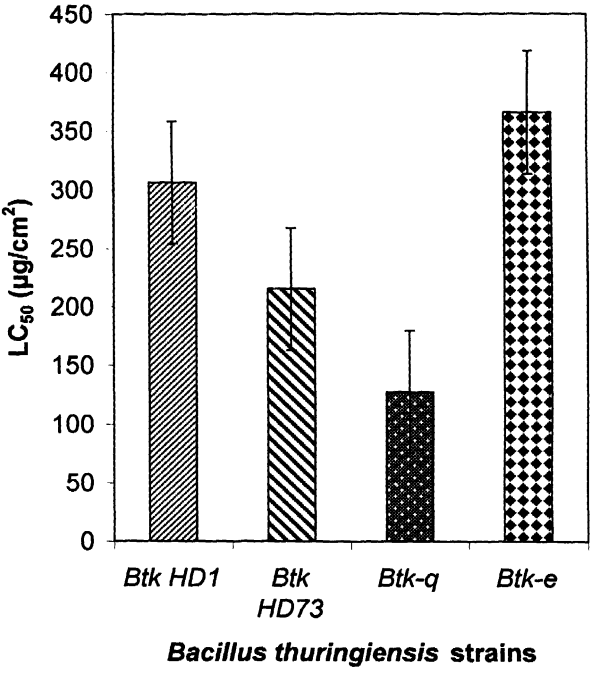


Figure 11C. Toxicity of *Bacillus thuringiensis* strains against *Chilo partellus*



were 306, 215.2, 127.6 and 365.6 μg/cm^2 of diet, respectively (Fig. 11C). ED_{50} values for *Btk* HD1, *Btk* HD73, *Btk-q* and *Btk-e* were 232.8, 183.2, 103.3 and 287.7 μg/cm^2 of diet, respectively (Fig. 12C).

Leaf disc bioassay with purified toxins against neonates of *H. armigera* showed biological activity in the nanogram range. In all cases, increasing toxin concentration significantly decreased the leaf area consumption, which was correlated inversely with larval mortality. *Btk* HD1 exhibited an LC_{50} of 0.412 ng/cm^2, while LC_{50} of *Btk* HD73, *Btk-q* and *Btk-e* were 0.415 ng, 0.330 ng, and 0.479 ng/cm^2, respectively (Fig. 13). The mortality response was 3 days after the exposure of larvae to *Bt* treated leaves, with no further significant increase in mortality between third and seventh day. Larvae that did not exhibit mortality grew satisfactorily on treated leaves up to seven days and till pupation on untreated leaves.

Btk-q showed approximately 4 fold greater activity than controls against *H. armigera*, two fold greater activity against *C. partellus*, and almost same activity against *S. litura* as compared to wild type strains. *Btk-e* (with higher protease activity) showed slightly lower activity against all insects tested when compared to controls *Btk* HD1 or *Btk* HD73.

2.4. Discussion

Intracellular proteases in *Bt* are known to be involved in proteolysis of protoxin in addition to their existence and a possible role in the biochemical events of spore formation (Chestukhina *et al.*, 1982; Andrews *et al.*, 1985; Bibilos and Andrews, 1988; Carroll *et al.*, 1989). Previous work carried out in our laboratory had indicated the involvement of endogenous (bacterial) proteases in the activation of protoxin (Reddy and Venkateswerlu, 1997; Suresh Kumar and Venkateswerlu, 1998b; Reddy *et al*, 1998, 2000; Rukmini *et al.*, 2000). Present studies on protease deficient mutant of *Bt* conclusively proved that endogenous proteases are necessary for the conversion of

Figure 12A. Toxicity of *Bacillus thuringiensis* strains against *Helicoverpa armigera*

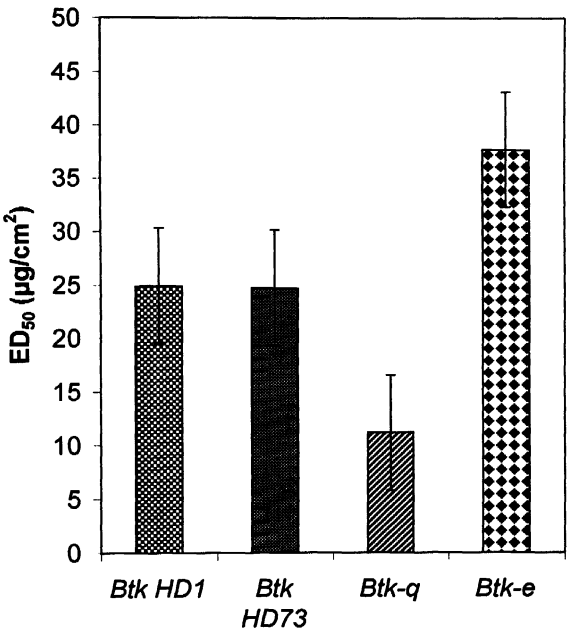


Figure 12B. Toxicity of *Bacillus thuringiensis* strains against *Spodoptera litura*

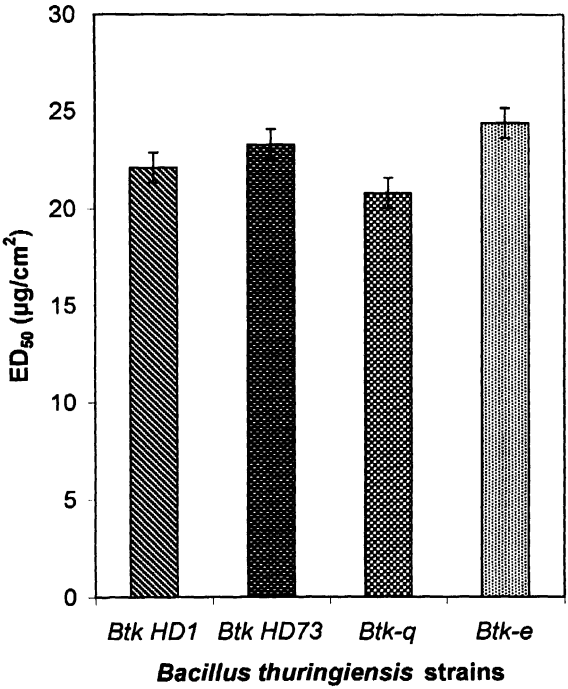


Figure 12C. Toxicity of *Bacillus thuringiensis* strains against *Chilo partellus*

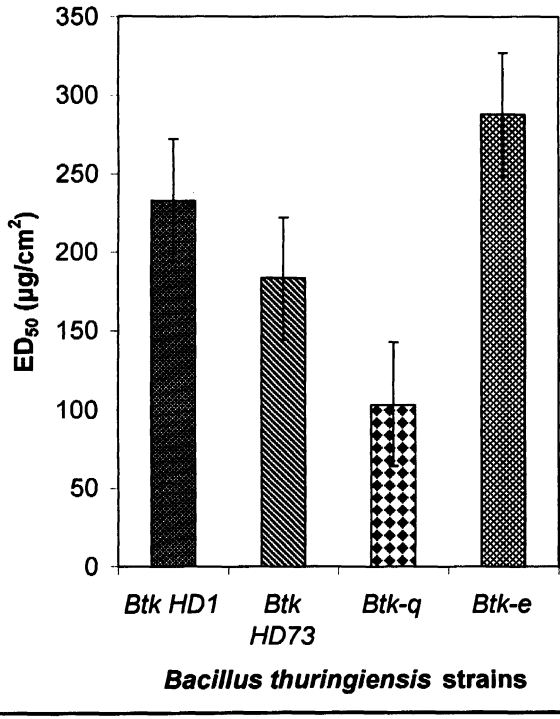
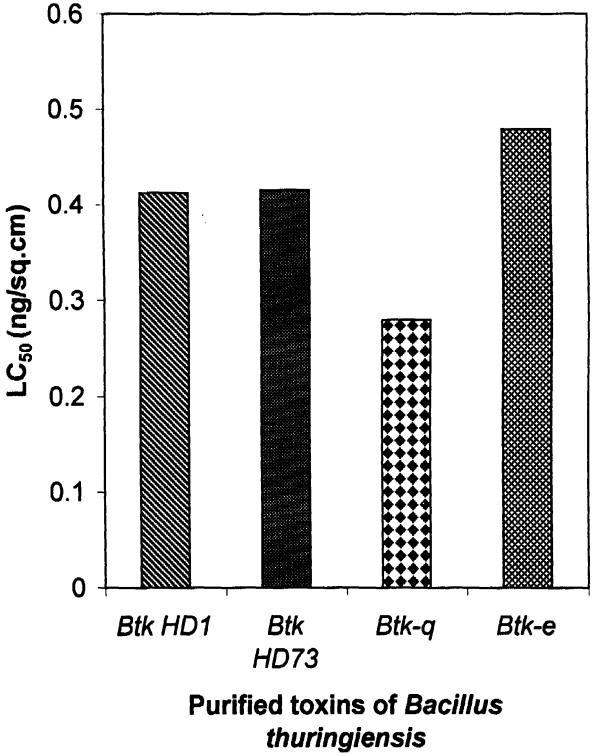


Figure 13. Toxicity of *Bacillus thuringiensis* toxins against *Helicoverpa armigera*



protoxin to toxin. On milk agar plate, protease proficient wild type strain formed a halo around the colony, whereas the deficient mutant lacked this ability. On X-ray film, *Btk-q* mutant did not show any activity as compared to the wild type. In this assay, *Btk-e* could not be distinguished from *Btk* HD1 (wild type), which could be done only by quantitative azocasein hydrolysis and gelatin zymography.

The endogenous protease extract from wild type revealed 92, 72 and 68 kDa bands on gelatin zymography, whereas none of these bands were observed in *Btk-q*. *Btk-e* mutant showed hyperactivity of 69 kDa band. Mutation in *Btk-q* possibly involves regulatory region of the operon, as all the three 92 kDa, 78 kDa and 69 kDa enzymes were lacking in this mutant. Further, the mutants were examined for the δ-endotoxin coding plasmid, and they were found to be intact as indicated by identical plasmid profiles, and the presence of an identical 1.2 kb amplified fragment by PCR analysis of *cry*1Ab gene. Further, southern hybridization with *cry*1Ab probe, the amplified PCR products of *Btk* HD1, *Btk* HD73, *Btk-e,* and *Btk-q* confirmed the presence of a 1.2 kb fragment, which is considered to be responsible for coding protoxin.

The cell lysates isolated from *Btk* HD1 contained a 66 kDa toxin band, which is missing in *Btk-q*. Instead, it showed a 132 kDa protoxin band indicating that the endogenous protease activity is necessary for the conversion of proprotein to toxin (66 kDa). Similarly, the toxin isolated by a specific method, the 66 kDa toxin was observed in the wild type but not in the mutant strain. The immunological cross reactivity of 132 kDa protoxin from *Btk-q,* with the antibody of 66 kDa toxin from *Btk* HD73 (that cross reacts with *Btk* HD1), was confirmed by western blot analysis (In view of the existence of 3 genes for protoxin in *Btk* HD1, we thought it would be better to raise the antibodies against *Btk* HD73, which is coded by a single

gene, which would also cross react with *Btk* HD1). The endogenous protease extracts of *Btk* HD1 and *Btk* HD73 converted 132 kDa protoxin to 66 kDa toxin, while the similar extract from *Btk-q* mutant did not do so. The 132 kDa protoxin from *Btk-q* was normal in that it could be hydrolysed to 66 kDa toxin by trypsin and by the endogenous protease extract from *Btk* HD1 to 66 kDa toxin, along with some other low mol. wt. bands.

The 132 kDa Cry1 protoxins were cleaved quite precisely at both N- and C-terminii giving rise to activated toxins of circa 66 kDa (Hofte *et al.*, 1986; Hofte and Whiteley, 1989). Approximately 50% protoxin is mature toxin with a small N-terminal peptide and a large C-terminal peptide removed during the proteolysis (Hofte and Whiteley, 1989; Rukmini *et al.*, 2000). The toxins generated endogenously (Suresh Kumar and Venkateswerlu, 1998b) lacked the first 29 amino acids of the protoxin with Glu^{30} at the N-terminus and differed from a trypsin-generated toxin, lacking an N-terminal peptide of 28 amino acids with Ile^{29} at the N-terminus (Bietlot *et al.*, 1989), both differing in their toxicity pattern. Haider and Ellar (1989) reported that differential activation of toxin influences the host range of toxin also.

Insecticidal activity of mutants and wild type strains were tested against *H. armigera*, *C. partellus* and *S. litura*. Strains with protease deficiency exhibited a higher pesticidal activity, whereas strains with higher protease activity exhibited lower pesticidal activity. *Btk-q* exhibited 2-3 fold higher activity against all the three insects tested. It is believed that all the 132 kDa protoxin from *Btk-q* mutant is converted in the larval gut to toxin by the exogenous or larval gut proteases as indicated by the *in vitro* experiment. Moreover, absence of endogenous protease in *Btk-q* mutant resulted in 2 to 3 fold greater activity against all the three insects, indicating the role of endogenous proteases in toxin degradation in the insect gut.

Earlier, Suresh Kumar and Venkateswerlu (1997) have also reported that toxin generated by endogenous proteases is active against *Spodoptera litura* a naturally tolerant pest. In other species of *Bt*, particularly in *Btt*, Reddy *et al.* (2000) had reported the presence of a 69 kDa metalloprotease, which is involved in the activation of a 73 kDa protoxin to 68 kDa toxin. Deletion of a specific extracellular neutral protease A from *Bt* using site directed mutagenesis resulted in an increase in the content of crystal protein, indicating that crystal protein stability and yield might have improved the potency of the organism (Donovan *et al.*, 1997). Extracellular neutral and alkaline protease deficient mutants are now being exploited for enhanced production of toxin by fermentation (Tan and Donovan, 2001). MacIntosh *et al.* (1990) found that trypsin inhibitors, when mixed with crystal proteins, enhanced the insecticidal activity of *Bt* against target insects, indicating that a reduction in proteolytic activity could lead to increased toxicity. A similar finding has been reported by Thanabalu and Porter (1995), who showed that strains of the mosquito-larvicidal bacterium *Bacillus sphaericus* that produced higher levels of proteolytic activity, produced less mosquitocidal proteins and the identities of these proteases involved in the loss of the mosquitocidal proteins were not known. The present work provided a further evidence for the involvement of proteases in the generation of an activated 66 kDa toxin in *Btk* HD1 which is active against insects.

Chapter 3

Intracellular proteases in Btk and protease deficient mutant Btk-q

Intracellular proteases in *Bacillus thuringiensis* subsp. *kurstaki* and protease deficient mutant *Btk-q*

3.1. Introduction

Bacillus thuringiensis synthesizes several proteolytic enzymes during early sporulation phase (Doi, 1972). In most *Bt* strains, this increased level of protease activity is associated with both crystal and spore formation (Chestukhina *et al.*, 1979). In *Btk*, *Btt* and *Bti*, the intracellular proteases have recently been suggested to be involved in the activation of protoxins (Chilcott *et al.*, 1983; Carroll *et al.*, 1989; Venkateswerlu and Stotzky, 1990; Reddy *et al.*, 1998). In *Btk*, Andrews *et al.* (1985) have also reported that the proteolytic hydrolysis of protoxin to active toxin was mediated by endogenous proteases. In case of *Btt*, a major polypeptide of activated toxin (68 kDa) along with minor peptides was reported from its precursor of a 73 kDa protoxin (Carroll *et al.*, 1989). Subsequently, it was found that a 69 kDa metalloprotease which appeared at stage II and persisted till stage VI of sporulation evidently involved in the generation of the active toxin (Reddy *et al.*, 2000). Since, proteases play an important role in the production of toxins, and under these conditions there will be a parallel increase of free amino acid pool. With this hypothesis a study has been undertaken to examine the appearance of the intracellular proteases and the concomitant increase of the pool size.

3.2. Materials and Methods

3.2.1. Cell lysate preparation

A single colony from overnight grown agar plate was inoculated into 10 ml medium and incubated for 6 h at 28^{0}C with shaking. This was subcultured into 50 ml medium and grown to mid-log phase (A_{600} nm 0.4-0.6) and once again inoculated into 250 ml medium. The culture was harvested at different stages (3 h to 48 h) and washed twice with 20 mM Tris-HCl (pH 8), containing 1 M NaCl and thrice without NaCl. The washed pellet was resuspended in 0.1M Tris-HCl

(pH 8), and sonicated (Virtis Virsonic, USA) for five times (30 sec bursts at 60 watts).

3.2.2. Intracellular protease extraction

The lysed culture obtained as mentioned above was centrifuged (12,000*g* for 20 min at 4^0C) and the supernatant obtained was saturated with ammonium sulfate to 30% at 4^0C and left overnight at 4^0C. The precipitated protein was pelleted down (15,000*g* for 20 min at 4^0C) and resuspended in 0.1 M Tris-HCl (pH 8), and dialysed at 4^0C against the same buffer. The protein concentration of the extract was adjusted to 1 mg/ml by diluting with the same buffer. This protease extract was aliquoted into 200 µl volumes and stored at -20^0C, until further use. Protein concentration was determined by the method of Lowry *et al.* (1951).

3.2.3. Gelatin SDS-PAGE

To identify intracellular proteases, gelatin zymography was performed as described by Hummel *et al.* (1996). Briefly, 30 µg protease extract was electrophoresed at 4^0C on a 10% SDS-PAGE containing 1% gelatin. Samples were mixed with 4x loading buffer (without β-mercaptoethanol or dithiothreitol) and loaded, unheated. After electrophoresis, the gels were rinsed in 0.1 M Tris-HCl (pH 8), containing 1% Triton X-100 for 1 h. Gels were subsequently equilibrated for 30 min (3 x 10 min) in the same buffer without Triton X-100 at room temperature, followed by a 5 h incubation at 37^0C in the same buffer. Gels were then stained with coomassie brilliant blue R-250 and destained.

3.2.4. Azocasein assay for proteases

Azocasein hydrolysis was performed as described by Prestidge *et al.* (1971). One ml reaction mixture containing 20 µg protease extract, 3 mg azocasein and 0.1 M Tris-HCl (pH 8), was incubated for 1 h at 30^0C. The reaction was stopped with 2 ml ice cold 7% perchloric acid. The protein was allowed to precipitate for 15 min at room temperature and then centrifuged at 4,000*g* (Remi R4C, Mumbai, India) for 10 min at room temperature. To the supernatant, 0.3 ml of 10 N NaOH was added, and the intensity of the color was determined at

440 nm (Beckman DU20, CA, USA). One unit of protease activity is equivalent to the amount of protein that produces an increase of 1.0 unit at A_{440} in 60 min under above mentioned experimental conditions. Specific activity was expressed as units/mg protein.

3.2.5. Ninhydrin assay

Total free amino acid pool in the cell lysates was estimated by the method of Moore and Stein (1954). To 1 ml of the supernatant, 500 µl of cyanide-acetate buffer and 500 µl of 3% ninhydrin reagent was added and kept in a boiling water bath for 15 min and then cooled immediately. To this 5 ml of isopropyl alcohol-water diluent was added rapidly and the color was read at 570 nm (Beckman DU20, CA, USA) against a reagent blank. The amino acid content was expressed as micro moles of L-leucine equivalent per milligram of dry weight of cells.

3.2.6. Paper chromatography

It was performed by the method of Consden *et al.* (1944). Briefly, the lysates were spotted on the Whatman filter paper No.1, and the chromatographs were run for 17 to 18 h by an ascending technique in a chamber thoroughly saturated with the solvent (butanol-acetic acid-water; 25:6:25). The papers were dried and sprayed with a solution of 0.2% ninhydrin prepared in 95% ethanol with the help of an all-glass atomizer and kept in an oven at 105^{0}C for 5 min for color development.

Each extract was run in three replicates and the average R_f values of the ninhydrin positive spots were calculated. The possible amino acids present in different strains of *Bt* were identified with the help of the standard chart prepared under similar controlled conditions.

3.3. Results

When the cultures of *Btk* HD1 at different stages of growth examined by gelatin zymography expressed atleast three intracellular protease activities corresponding to mol. wts. of 92, 78 and 69 kDa (Fig. 1). The first protease to

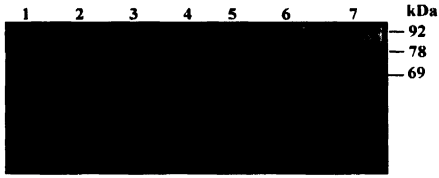
be expressed is a 92 kDa enzyme, at 3 h of sporulation followed by 78 and 69 kDa at 6 h. In contrast the whole protease extract of *Btk* HD73 (similar to the strain *Btk* HD1) revealed the presence of only two major intracellular proteases having mol. wts. of 85 and 78 kDa by gelatin zymography (Fig. 2). However, the perceptible gelatinolytic activity in this case started appearing only from 6 h of sporulation stage. Although the 85 kDa protease persisted till 48 h of growth, the 78 kDa protease continued only up to 9 h of sporulation and then started disappearing from 12 h of sporulation.

These results were further supported by azocasein analysis revealing the increase of protease activity during the growth period of 3 h to 48 h in all strains of *Bt* tested (Figs. 3,4 and 5). In *Btk* HD1 the protease activity was found to be 1.2 U at 3 h of sporulation and gradually increased up to 9 h of growth. The maximum protease activity was detected at 21 h (7 U) of growth and found to be 6 times higher, compared to the activity at 3 h of sporulation. The protease activity reached a plateau at 21 h and continued until 48 h of growth tested. Further as shown in Fig. 3, the protease activity was compared with the production of free amino acids. It also reavealed that the free amino acid pool content increased in a parallel way from 3 h (58.75 μmol) to 24 h of growth (165 μmol) and found to be 3 times higher compared to the production at 3 h of sporulation.

As shown in Fig. 4, the protease activity in *Btk* HD73 was found to be 0.8 U at 3 h of sporulation. This has increased up to 21 h of growth (6.4 U) and found to be 8 times higher when compared to the activity at 3 h of sporulation. It is also evident from the data of Fig. 4, that the protease activity reached a plateau at 21 h of growth. Similarly, the free amino acid pool content has also increased in a parallel way from 3 h (55.5 μmol) to 24 h (155 μmol) of growth and found to be 2.8 times higher compared to the production at 3 h of sporulation.

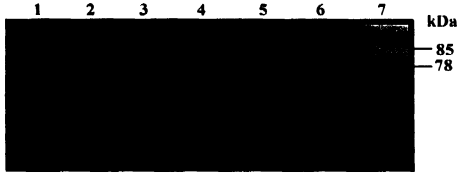
Only traces of protease activity was detected in *Btk-q*, by azocasein hydrolysis, which could not be detected by gelatin zymography (Fig. 5). Further

Figure 1



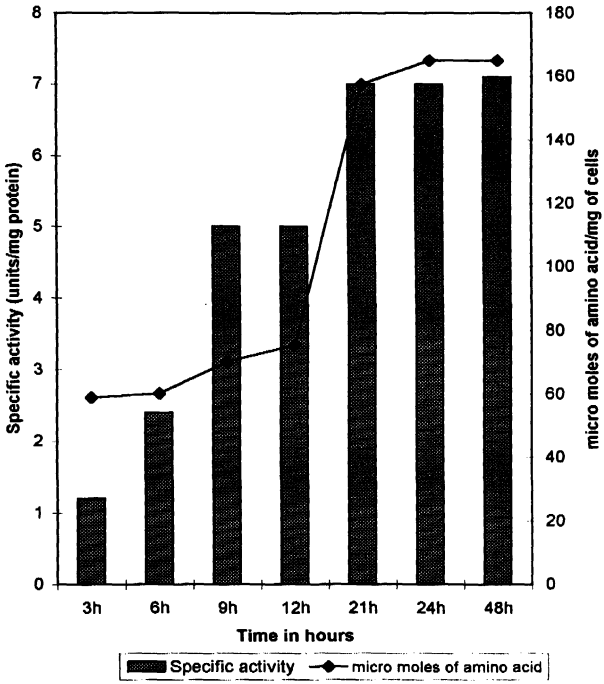
Expression of *Btk* HD1 intracellular proteases during sporulation. Proteases extracted from *Btk* HD1 cells harvested at the following stages of growth were identified on a 7.5% SDS-PAGE containing 1% gelatin. Lane 1. 3 h; lane 2. 6 h; lane 3. 9 h; lane 4. 12 h; lane 5. 21 h; lane 6. 24 h; lane 7. 48 h.

Figure 2



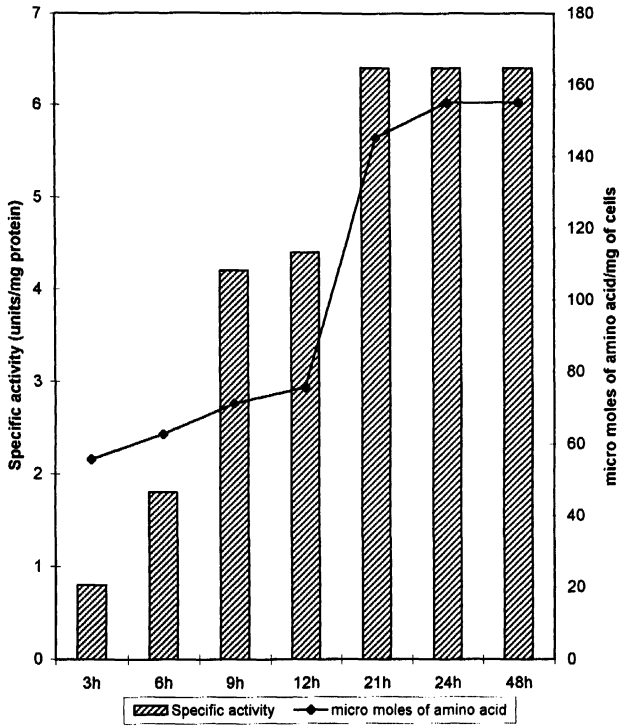
Expression of *Btk* HD73 intracellular proteases during sporulation. Proteases extracted from *Btk* HD73 cells harvested at the following stages of growth were identified on a 7.5% SDS-PAGE containing 1% gelatin. Lane 1. 3 h; lane 2. 6 h; lane 3. 9 h; lane 4. 12 h; lane 5. 21 h; lane 6. 24 h; lane 7. 48 h.

Figure 3. Protease activity and free amino acid pool content of *Btk* HD1 as a function of time



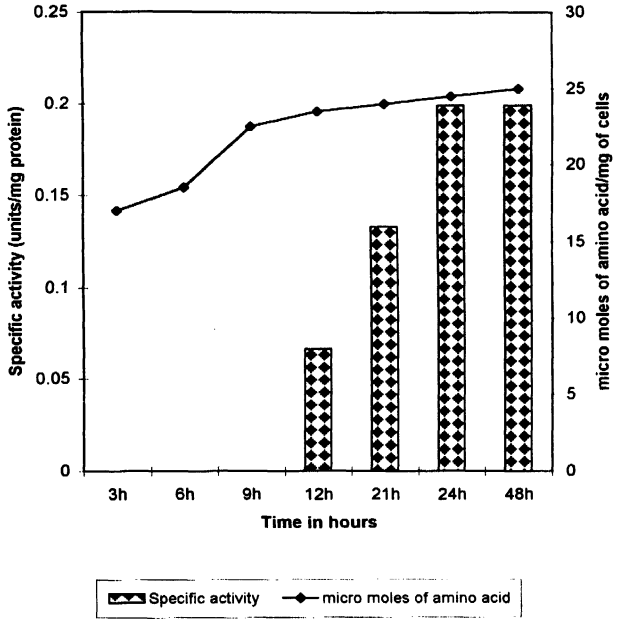
Protease activity is expressed in terms of specific activity, defined as units/mg protein. One unit of protease is equivalent to the amount of enzyme that produces an increase of 1.0 in A_{440} in 60 min under the experimental conditions

Figure 4. Protease activity and free amino acid pool content of *Btk* HD73 as a function of time



Protease activity is expressed in terms of specific activity, defined as units/mg protein. One unit of protease is equivalent to the amount of enzyme that produces an increase of 1.0 in A_{440} in 60 min under the experimental conditions

Figure 5. Protease activity and free amino acid pool content of *Btk-q* as a function of time



Protease activity is expressed in terms of specific activity, defined as units/mg protein. One unit of protease is equivalent to the amount of enzyme that produces an increase of 1.0 in A_{440} in 60 min under the experimental conditions

the results revealed that unlike in parent strains the protease activity could be detected only at 12 h (0.066 U) of growth that has slowly increased till 24 h of growth to a maximum of 0.199 U. It was found to be 3 times higher than the activity at 3 h of sporulation. Maximum protease activity was detected only at 24 h unlike in parent strains at 21 h of growth. The free amino acid content has also increased in a parallel way from 3 h (17 μmol) to 24 h (25 μmol) of growth and was found to be 1.5 times higher compared to the production at 3 h.

Thus qualitative and quantitative variations are noticed at different time periods in these strains (wild type and mutant). Free amino acid content was also found increasing in a parallel way as the protease activity increased in *Btk* HD1 and *Btk* HD73. Whereas in *Btk-q*, this increase either in protease activity (0.2 U as compared to 7 U of *Btk* HD1 at 24 h) or free amino acid pool content (though still in a parallel way) was noticed to be very low (25 μmol out of 165 μmol of *Btk* HD1) when compared to the parent strains (Figs. 3, 4 and 5).

Further analysis of free amino acid pool was carried and the results are presented in Table 1. The amino acids identified were aspartic acid, glutamic acid, tryptophan/methionine/valine, isoleucine/leucine, arginine, alanine, glycine, and tyrosine. The lysates of both *Btk* HD1 and *Btk* HD73 at 21 h, 24 h and 48 h samples manifested the above 8 ninhydrin positive spots, whereas *Btk-q* only 3 spots corresponding to DL-alanine, L-glutamic acid and L-aspartic acid. Besides this decrease in total free amino acid pool content, decrease in the number of free amino acids was also observed in *Btk-q* at 21 h, 24 h and 48 h compared to *Btk* HD1 where the protease production was very high (Table 1).

3.4. Discussion

Bibilos and Andrews (1988) had earlier reported increased intracellular protease activities at the onset of sporulation as measured by activation of protoxin to toixin. They have studied the specificities of proteases extracted from *Btk* 251 and *Btk* LB1 cells at different stages of growth and sporulation. Carroll *et al.* (1989) have demonstrated that the 73 kDa protoxin was first detected at stage II of sporulation and the concentration of this protein was actually reducing from

Table 1. Paper chromatography showing free amino acids

Amino acids	*Btk* HD1 21 h	*Btk* HD1 24 h	*Btk* HD1 48 h	*Btk* HD73 21 h	*Btk* HD73 24 h	*Btk* HD73 48 h	*Btk-q* 21 h	*Btk-q* 24 h	*Btk-q* 48 h
DL-Alanine	•	•	•	•	•	•	•	•	•
L-glutamic acid	•	•	•	•	•	•	•	•	•
L-aspartic acid	•	•	•	•	•	•	•	•	•
Tyrosine	•	•	•	•	•	•			
Tryptophan/Methionine/ Valine	•	•	•	•	•	•			
Arginine	•	•	•	•	•	•			
L-leucine/norleucine/ Isoleucine	•	•	•	•	•	•			
glycine	•	•	•	•	•	•			

stage II to stage VI of growth, while the concentration of 68 kDa active toxin was actually increased during these stages coinciding with the appearance of 69 kDa protease at this stage. Basing on this, Reddy *et al.* (2000) have studied the expression of proteases in *Btt* as a function of growth. A 69 kDa protease appeared first at stage II coinciding with appearance of 68 kDa toxin and continued up to stage VI of sporulation. In our study we found that the protease activity reached plateau at 21 h of growth in *Btk* HD1 and *Btk* HD73 coinciding with the release of free amino acids in a parallel fashion. In *Btk* HD1 and *Btk* HD73, a significant amount of free amino acid pool was found, which could not be observed in *Btk-q*. Only a $1/6^{th}$ amount of free amino acid pool content was noticed as compared to the parent strains. Since the protease activity is low in *Btk-q* mutant the amount and number of free amino acids released is also significantly less compared to the *Btk* HD1 or *Btk* HD73.

Bietlot *et al.* (1989) showed that the trypsin generated toxin lacked an N-terminal peptide of 28 amino acids and the sequence reported prior to Ile-29 is VEVLGGER. Further, they also reported that the amino acid analysis of the trypsin activated toxin of Cry1Ab has C-terminal residue of Arg-601 and the reported sequence following Arg-601 is IEFVPAEVTFEAEYDLLERA (Bernheimer *et al.*, 1986; Rukmini *et al.*, 2000). Suresh Kumar and Venkateswerlu (1998b) reported that the endogenously generated toxin is different from trypsin activated toxin, lacking an N-terminal peptide of 29 amino acids and the N-terminal sequence prior to Glu-30 is VEVLGGERI. But, our results showed that the released free amino acids could be due to the sequential hydrolysis of protoxin either before Ileu-29 or Glu-30 or after Arg-601, exactly coinciding with the previously reported amino acid sequence at the N-and C-terminii. In view of this, we could hypothesize the following possible cleavage sites at N-terminal and C-terminal sequences.

(Met^{1}.....Gly^{25}-Gly^{26}-Glu^{27}-Arg^{28}-$Ileu^{29}$-Glu^{30}.....Phe^{611}-Glu^{612}-Ala^{613}-Glu^{614}-Tyr^{615}-Asp^{616}-Leu^{617}-Glu^{618}-Arg^{619}-Ala^{620}.....)

This might have possibly been achieved by a sequential degradation of protoxin releasing the experimentally observed amino acids. Further our results as shown in the following Table 2, the ratio of protease activity (max/min) and free amino acid pool content (max/min) in wild type strains are very close compared to *Btk-q*, a protease deficient mutant which is very low. This could be due to the more degradation of protoxin in a particular pattern, which is not the case in the mutant strain (The contribution of free amino acids to the pool from general metabolism cannot be ruled out).

Table 2. Ratio between the protease activity and production of free amino acids

Strain	Protease activity (Max/min)	Free amino acid pool content (Max/min)
Btk HD1	6	3
Btk HD73	8	2.8
Btk-q	3	1.5

Doi (1972) reported that several proteolytic enzymes are synthesized by *Bacillus* species during early sporulation. Chestukhina *et al.* (1979) have also reported that in most *Bt* strains, serine type proteases appear and steadily increase through different stages of growth. Carroll *et al.* (1989) have found that crystals harvested by sporulated cultures of *Btt* contain a major polypeptide of 68 kDa and several minor polypeptides. Here it was reported that during sporulation 73 kDa polypeptide could be detected at stage I of sporulation. The 68 kDa polypeptide was first detected at stage II and increased in concentration through the later stages of sporulation with a concomitant decrease in 73 kDa polypeptide (its precursor). Further, similar finding using the protease inhibitors was reported by Reddy *et al.* (2000) that 69 kDa protease appeared for the first time at stage II and persisted till stage VI of sporulation coinciding with the appearance of 68 kDa active toxin.

The *npr*A gene encoding *Bacillus thuringiensis* neutral protease A, was cloned by the use of gene-specific oligonucleotides and *npr*A deleted strain has exhibited a higher production of 66 kDa toxin (Donovan *et al.*, 1997). *Npr*A was partially deleted *in vitro* and the deleted allele designated as *npr*A3 was used to construct an *npr*A3 strain (neutral protease deficient of *Bt*). The

extracellular proteolytic activities of *npr*A3 strains was significantly less than *apr*A(+) strain. *Npr*A3 produced insecticidal crystal protein that was more stable than those of isogenic *npr*A(+) strain. Subsequently, it was found that stability and yield of toxin was improved by deletion of a specific protease (Tan and Donovan, 2001).

Chapter 4

Detection of protease activity associated with Btk HD73 toxin

Detection of protease activity associated with *Bacillus thuringiensis* subsp. *kurstaki* HD73 toxin

4.1. Introduction

Bacillus thuringiensis produces δ-endotoxin crystals during sporulation (Aronson *et al.*, 1986). These crystals contain protoxin (130 kDa) which is processed proteolytically to active toxin (60-70 kDa) in the larval midgut by exogenous proteases and also in the producer organism by endogenous proteases (Oppert, 1999; Rukmini *et al.*, 2000). The activated toxin is toxic to several pests (Hofte and Whiteley, 1989) and is associated with several novel properties. In *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) the toxin coding megaplasmid is associated with a phage like particle and with a satellite inclusion (Tam and Fitz-James, 1986). The gene coding for a polypeptide (125 kDa) is also associated with inverted repeat sequences, suggesting that the *Bti* δ-endotoxin gene is located with in a transposable element (Bourgouin *et al.*, 1988). Moreover, the crystal protein production is associated with plasmid (Faust *et al.*, 1983), and with DNA (Yokoyama *et al.*, 1998) in *Bti*. But in *Btk* HD73, a 20 kb DNA fragment is intimately associated with the crystals and represents the unusual proteolysis in the generation of activated toxin (Bietlot *et al.*, 1993; Clairmont *et al.*, 1998). Besides, these δ-endotoxin proteins are also associated with spherical parasporal inclusions of the *Bt* strains (Wasano *et al.*, 1998). But a recent report indicated that noninsecticidal *Bacillus thuringiensis* parasporal inclusion associated with protein parasporin has human leukemic cell-recognizing activity (Mizuki *et al.*, 2000). The function for these associated activities is not known, except in few cases such as 20 kb DNA fragment, required for unusual proteolysis in active toxin generation (Clairmont *et al.*, 1998). In the present study we report that the toxin protein of *Btk* HD73 possessing a metalloprotease activity.

4.2. Materials and Methods

4.2.1. Materials

The enzyme trypsin, enzyme inhibitors like phenylmethyl sulfonyl fluoride (PMSF), iodoacetic acid (IAA), N-ethylmaleimide (NEM), ampholytes, nonidet and ethylenediaminetetraacetic acid (EDTA), benzamidine-HCl were the products of Sigma, St. Louis, USA. Nitrocellulose (NC) membrane was obtained from BioRad, USA. Bacteriological tryptose and beef extract were obtained from Himedia, Mumbai, India. X-ray film was procured from a local diagnostic center (Konica), and 1,10-phenanthroline from Qualigens, India. All other chemicals used were of analytical grade.

4.2.2. Strains

Bacillus thuringiensis strains used were *Bacillus thuringiensis* subsp. *kurstaki* HD1 (DIPEL), *Bacillus thuringiensis* subsp. *kurstaki* HD73 (*BGSC*, USA), *Bacillus thuringiensis* subsp. *tenebrionis* (M-one), *Bacillus thuringiensis* subsp. *israelensis*, *Btk-q* and *Btk-e*. *Btk-q* and *Btk-e* were obtained by chemical mutagenesis of the bacterium *Btk* HD1 (described in chapter 2). *Btk-q* mutant is deficient of protease activity and normally manifests only protoxin (132 kDa) band unlike the parent *Btk* HD1.

4.2.3. Growth conditions

Bt was cultured on liquid growth medium (1% bacteriological tryptose, 0.3% beef extract, 0.5% NaCl, 5 mM $MgSO_4$, and 20 μM $MnCl_2$; pH 7.2) with shaking (150 rpm at 28^0C to mid-exponential phase as monitored by A_{600} measurements). Sporulation stages were monitored by phase contrast microscopy, and the culture was harvested at early stage VI (Ellar, 1978) of sporulation when more than 90% of the cells were unlysed and carried a mature spore. All cultures were maintained on nutrient agar slants.

4.2.4. Isolation and purification of toxin

Toxins from *Bt* strains were isolated by the method as described by Venkateswerlu and Stotzky (1990) and purified on DEAE-cellulose chromatography as described in chapter 2.

4.2.5. Protein estimation

Protein content of the toxin preparations was estimated by the method of Lowry *et al.* (1951) using BSA as the standard.

4.2.6. X-ray film assay

This assay was done according to Cheung *et al.* (1991). The gelatin coating on unprocessed X-ray film (20 x 25 cm) of Kodak Chemical Corp. was used as a substrate for a variety of proteolytic enzymes. For this assay, 5 µl of a sample was applied onto the film followed by incubation for 1 h at 42^0C. After the incubation, the film was washed with running water. Where necessary assay was repeated by the addition of protease inhibitors.

4.2.7. Azocasein assay

Quantitative protease assay was accomplished by using azocasein as substrate as reported by Prestidge *et al.* (1971). A 1 ml reaction mixture containing 20 µg protease extract, 3 mg azocasein and 0.1 M Tris-HCl (pH 8), was incubated for 1 h at 30^0C. The reaction was stopped by adding 2 ml ice cold 7% perchloric acid. The protein was allowed to precipitate for 15 min at room temperature, followed by centrifugation at 4,000*g* for 10 min at room temperature. To the supernatant, 0.3 ml of 10 N NaOH was added. Specific activity was expressed as units/mg protein. Assay was also performed by the addition of protease inhibitors to a final concentration of 10 mM.

4.2.8. *In vitro* digestion of *Btk-q* protoxin with purified *Btk* HD73 toxin

Briefly, 20 µl of toxin of *Btk* HD73 was mixed with *Btk-q* 132 kDa protoxin (1 mg/ml) in Tris-HCl (pH 8) and was incubated at 37^0C for 3 h. Reaction was stopped by incubating in a water bath at 95^0C for 5 min and then cooling it to room temperature. To this, 20 µl of reducing sample buffer was added and

samples were again heated at 95^{0}C for 5 min before they were analyzed on SDS-PAGE.

4.2.9. SDS-PAGE

SDS gel electrophoresis of the toxin proteins was carried by the method of Laemmli (1970) with an acrylamide/N, N-methylene bisacrylamide ratio of 30.2:0.8. The samples were mixed with 4x loading buffer, [40% (v/v) glycerol, 0.05% bromophenol blue, 5% (w/v) SDS, 10% β-mercaptoethanol and 0.125 M Tris-HCl buffer; pH 6.8] boiled for 2 min and loaded. The gels were stained with coomassie blue.

4.2.10. X-ray film band assay

It was done according to the procedure described by Pichare and Kachole (1994). Briefly, 20 μg of the toxin was analyzed on SDS-PAGE. Samples were mixed with 4x loading buffer (without β-mercaptoethanol or dithiothreitol) and loaded, unheated. After electrophoresis, the gel was rinsed in 0.1 M Tris-HCl (pH 8), containing 1% Triton X-100 for 1 h. Gel was subsequently equilibrated for 30 min (3 x 10 min) in the same buffer without Triton X-100. Then it was exposed to undeveloped X-ray film and incubated for 3 h. After the incubation the film was thoroughly washed with distilled water.

4.2.11. Two-dimensional gel electrophoresis

Two dimensional gel electrophoresis was performed according to O'Farrell (1975). The proteins (50 μg) were separated in the first dimension according to their isoelectric points (pH ranging from 3.5-10) on a tube gel containing 8M urea, 2% nonidet P-40 and 2% ampholytes (3.5-10). Electrophoretic separation was carried out in the second dimension by SDS-PAGE. The gels were stained with coomassie brilliant blue R250 and destained with methanol-acetic acid-water (5:1:4).

4.2.12. Western blotting

Both crude and purified preparations of 66 kDa separated on 7.5% gel were transferred electrophoretically to nitrocellulose paper by the method of Towbin

et al. (1979). Non-specific sites were blocked with 3% BSA and the nitrocellulose paper was incubated with the antiserum raised against the 66 kDa toxin (*Btk* HD73). After primary antibody incubation and washing, the blot was incubated in secondary antibody (goat anti-rabbit antisera coupled with horse radish peroxidase; Genei, Bangalore, India). After washing with buffer the bound antibody was detected by adding diaminobenzidine and hydrogen peroxide.

4.3. Results

Toxins from six strains of sporulated *Bt* were isolated and tested for any inherent protease activity.

4.3.1. X-ray film assay

The toxin isolates from *Btk* HD1, *Btk* HD73, *Btt*, *Bti*, *Btk-q* and *Btk-e* strains were tested by X-ray film assay for protease activity and the results are depicted in Fig. 1. Interestingly, purified toxin from *Btk* HD73 was found exhibiting considerable amount of protease activity as compared to trypsin. Other strains tested did not show any significant activity. Trypsin was used as a positive control along with buffer.

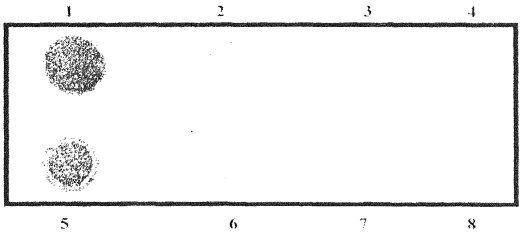
4.3.2. X-ray film assay with inhibitors

Since the purified *Btk* HD73 toxin exhibiting protease activity as evidenced from preliminary X-ray film assay, the class of protease was to be identified. To investigate this, the protease activity was tested by the addition of various inhibitors. As seen in Fig. 2, the protease activity was completely inhibited by EDTA, EGTA and 1,10-phenanthroline, while partial inhibition was detected with PMSF. NEM and IAA did not show any inhibition.

4.3.3. Azocasein assay for detection of protease activity

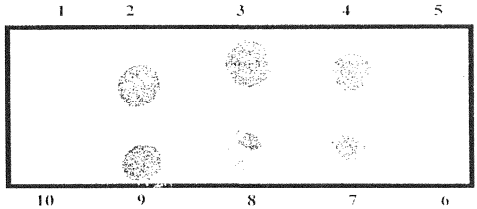
The proteolytic activity of purified toxin of *Btk* HD73 was further quantitatively analyzed by azocasein hydrolysis and the results obtained are shown in Fig. 3. The toxin exhibited nearly 30% activity as compared to trypsin (1 mg/ml) activity (100%), while no activity was found with other toxin proteins.

Figure 1



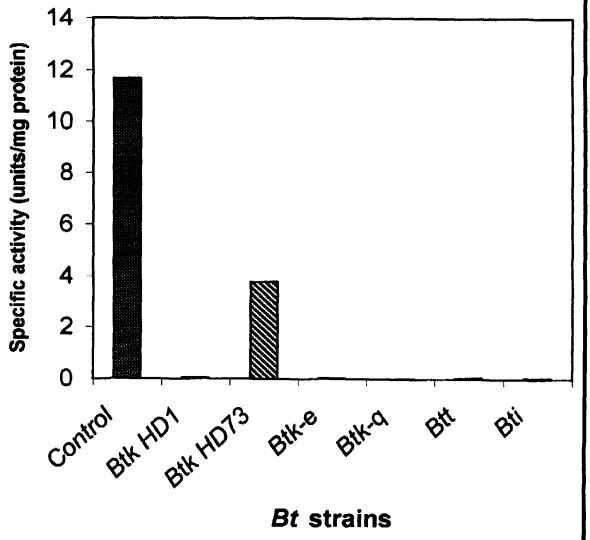
Detection of protease activity of *Btk* toxins by X-ray film. Spot 1. Trypsin; spot 2. *Btk* HD1; spot 3. *Bti*; spot 4. *Bti*; spot 5. *Btk* HD73; spot 6. *Btk-q*; spot 7. *Btk-e*; spot 8. Buffer (-ve control).

Figure 2



Effect of inhibitors on protease activity of *Btk* HD73 toxin. Spot 1. Buffer (-ve control); spot 2. NEM; spot 3. Control (*Btk* HD73, No inhibitor); spot 4. PMSF; spot 5. EDTA; spot 6. EDTA (5 mM); spot 7. 1,10-phe; spot 8. EGTA; spot 9. IAA; spot 10. EDTA control.

Figure 3. Azocasein assay of different *Bt* toxins for protease activity



Protease activity is expressed in terms of specific activity, defined as units/mg protein. One unit of protease is equivalent to the amount of enzyme that produces an increase of 1.0 in A_{440} in 60 min under the experimental conditions

4.3.4. Azocasein assay with inhibitors

To further check the class of protease the enzymatic activity was tested in the presence of various inhibitors. While, EDTA, EGTA and 1,10-phenanthroline inhibited 70 to 80% of the total activity, PMSF showed only 50% inhibition. It was relatively insensitive to other inhibitors and no inhibition was found with IAA, benzamidine-HCl and NEM as observed on X-ray film.

4.3.5. X-ray film band assay

To further identify this toxin protease, we have used undeveloped X-ray film technique as it was found to be more sensitive than gelatin zymography. As shown in Fig. 5, gelatin on the film, corresponding to the toxin protease band was hydrolysed, whereas the places corresponding to other toxin proteins remained unhydrolysed.

4.3.6. *In vitro Btk-q* protoxin (132 kDa) hydrolysis by *Btk* HD73 toxin protease

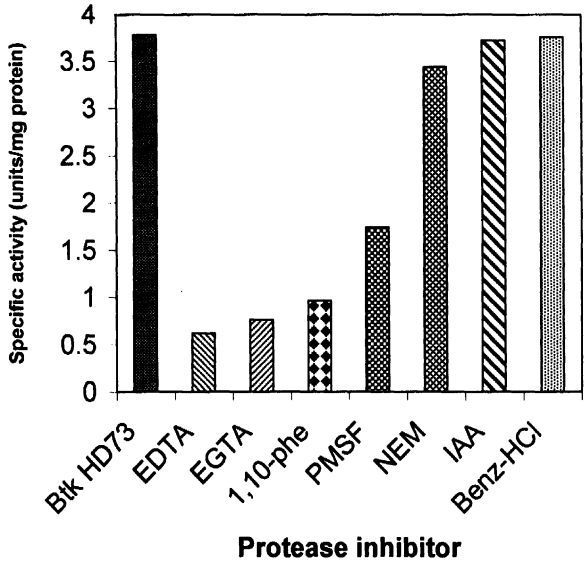
Since the class of protease (*Btk* HD73) was identified, the protease activity of the toxin was subsequently examined by *in vitro* digestion of *Btk-q* protoxin by *Btk* HD73 toxin protease. As seen in Fig. 6, after incubation of protoxin with *Btk* HD73 toxin a conspicuous toxin (66 kDa) protein band and a faint protoxin band was detected (lane 1) indicating that protoxin hydrolysis has occurred. However, this conversion was inhibited by EDTA at a concentration of 10 mM (lane 2).

4.3.7. SDS-PAGE/Western blotting

The antigenic nature of *Btk* HD73 toxin (66 kDa) was confirmed by western blot analysis with known antisera (Fig. 7). It was found to react with anti-HD73-antibody raised against this protein. It also showed that in crude toxin the low mol. wt. fragment(s) appeared to react with antibody (lane 1).

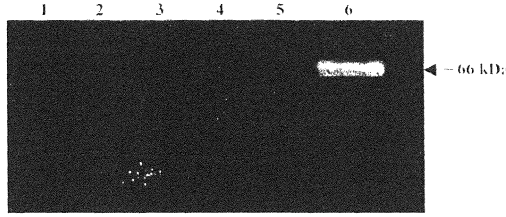
Further purity of the toxin was assessed by two-dimensional gel electrophoresis where it has shown a single spot indicating that the toxin protein is homogenous (Fig. 8).

Figure 4. Effect of protease inhibitors on protease activity of *Btk* HD73 toxin



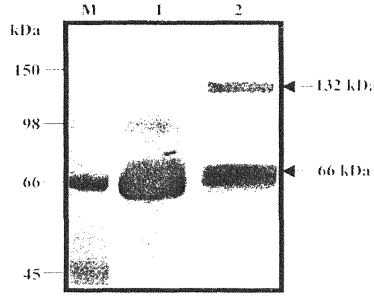
Protease activity is expressed in terms of specific activity, defined as units/mg protein. One unit of protease is equivalent to the amount of enzyme that produces an increase of 1.0 in A_{440} in 60 min under the experimental conditions

Figure 5



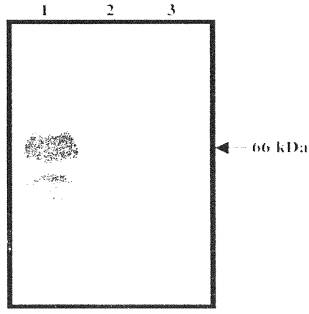
X-ray film band assay showing *Btk* HD73 protease activity. Lane 1. *Btk* HD1; lane 2. *Bt*; lane 3. *Bti*; lane 4. *Btk-q*; lane 5. *Btk-e*; lane 6. *Btk* HD73.

Figure 6



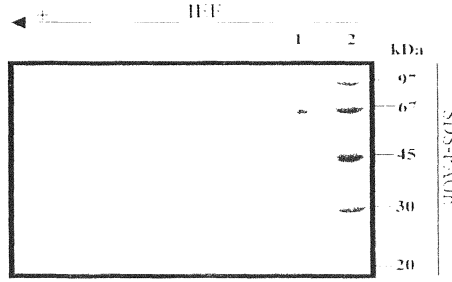
Effect of EDTA on *Btk-q* protoxin activation by *Btk* HD73 toxin protease activity. Lane M. Mol. wt. markers; lane 1. *Btk-q* protoxin + *Btk* HD73 toxin; lane 2. Same as lane 1 + EDTA.

Figure 7



Western blot of crude and purified toxin from *Btk* HD73 (66 kDa). Lane 1. Crude toxin; lane 2. Control serum (preimmunized serum); lane 3. Purified toxin (66 kDa).

Figure 8



Two-dimensional gel electrophoresis of *Btk* HD73 toxin. Lane 1. Purified toxin (66 kDa); lane 2. SDS-PAGE marker proteins comprising of phosphorylase b (97 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and trypsin inhibitor (20 kDa).

4.4. Discussion

The present study reports an unusual activity associated with *Btk* HD73 crystal toxin viz., a metalloprotease activity. *Btk* HD73 contains Cry1Ac toxin and is the most potent among all toxins. The data presented here indicate that it exhibits protease activity by all the methods tested (namely X-ray film spot test, azocasein hydrolysis and X-ray film band test). Whereas the toxins tested from other strains did not show any protease activity.

The inhibitor studies indicated that it is sensitive to EDTA, 1,10-phenanthroline, EGTA completely, while only partilal inhibition with PMSF. NEM, benzimidine-HCl, and IAA did not show any inhibition. This indicates that the protease is a metalloprotease. These results were further supported by azocasein assay.

The X-ray film band assay clearly indicated that the electrophoretically separated 66 kDa toxin exhibits protease activity. The antigenic nature of the toxin was confirmed using antibodies raised against *Btk* HD73 toxin. Further more, the homogeneity of the toxin preparation used in this study was confirmed by a single spot on two-dimensional gel electrophoresis. This indicated the protease activity is intimately associated with *Btk* HD73 toxin.

Earlier investigators also found unusual activity associated with crystal toxin of *Bacillus thuringiensis*. Tam and Fitz-James (1986) reported association of a 75 kDa megaplasmid with crystal inclusion in *Bti*. Other workers also reported such presence of a plasmid associated crystal toxin in *Bti* (Faust *et al.*, 1983). *E. coli* recombinant clones harbouring 125 kDa gene for larvicidal polypeptide was found to be associated with inverted repeat sequences in *Bti* (Bourgouin *et al.*, 1988). Recently evidence was presented for association of 20 kb DNA fragment with Cry1Ac crystal protein in *Btk* HD73. Here it plays a role in unusual proteolysis observed in the generation of active toxin (Bietlot *et al.*, 1993; Clairmont *et al.*, 1998. An unusual property, human leukemikc cell-recognizing activity associated with parasporal inclusions of noninsecticidal *Bacillus thuringiensis* soil isolate was reported and called parasporin (encoded

by a gene 2169 bp long), was a polypeptide of 723 amino acid residues (Mizuki *et al.*, 2000). Very recently it was also reported that lectin activity was associated with *Bt* parasporal inclusion proteins, that could agglutinate specifically sheep erythrocytes (Akao *et al.*, 2001).

Summary and Conclusions

Summary and Conclusions

Two mutants of *Btk* HD1 were isolated, one having elevated protease activity (*Btk-e*, hyper producing) and the other with a deficiency (*Btk-q*). These mutants were characterized by X-ray film spot assay, gelatin zymography, azocasein analysis, western blot analysis, plasmid profiles, PCR, and Southern blot analysis. *Btk-q* mutant displayed 2-3 fold higher activity against *Helicoverpa armigera*, *Spodoptera litura* and *Chilo partellus*, whereas *Btk-e* mutant displayed no significant enhancement in activity, but slightly lower activity against *C. partellus*. Protease deficient mutant *Btk-q* did not reveal the presence of any 66 kDa toxin band as observed with the wild type strain *Btk* HD1. It showed only 132 kda protoxin. The protoxins isolated from *Btk* HD1, *Btk* HD73, and *Btk-q* were treated with respective endogenous protease extracts. In case of *Btk* HD1 and *Btk* HD73 the protease extracts resulted in the conversion of 132 kDa protoxin to 66 kDa toxin, whereas in the case of *Btk-q* no such conversion was noticed. However, the protoxin from *Btk-q* was cleaved to 66 kDa toxin by trypsin. The endogenous protease extract obtained from wild type strain also cleaved the 132 kDa protoxin to 66 kDa toxin from *Btk-q*, simultaneously yielding few other low mol. wt. bands. The data suggests that the organisms possess endogenous proteases that convert protoxin to toxin within the bacterium.

The commencement of intracellular protease synthesis was studied by gelatin zymography in *Btk* HD1, *Btk* HD73 and a protease deficient mutant *Btk-q*. By gelatin zymography, a 92 kDa protease was detected first at 3 h of sporulation which continued till 48 h, where as two other proteases of mol. wt. 78 and 69 kDa were detectable from 6 h onwards and continued till 48 h of growth in *Btk* HD1. Whereas similar studies revealed the presence of two major intracellular proteases in *Btk* HD73 by gelatin zymography, which first appeared at 6 h of sporulation and continued till 48 h of growth. The quantitative azocasein assay confirmed that the total protease activity increases from 3 h to 21 h, thereafter reaching a plateau up to 48 h of growth, both in *Btk*

HD1 and *Btk* HD73. *Btk-q* a protease deficient mutant showed traces of protease activity by azocasein analysis which could not be detected by gelatin zymography. The free amino acid pool content was also increased in a parallel way as the protease activity increased in all three strains. However, this increase was found to be low in *Btk-q* when compared to *Btk* HD1 and *Btk* HD73 strains. Significant amount of ninhydrin positive free amino acids detected at 21 h, 24 h and 48 h in *Btk* HD1 and *Btk* HD73 was not found in *Btk-q*. The following amino acids were detected by paper chromatogram in *Btk* HD1 namely, DL-alanine, L-glutamic acid, L-aspartic acid, tyrosine, tryptophan/methionine/valine, arginine, leucine/norleucine/isoleucine and glycine, whereas in *Btk-q*, DL-alanine, L-glutamic acid, and L-aspartic acid were only detected at 24 h and 48 h where the protease production was maximum.

The toxins isolated from various *Bt* strains were assayed for protease activity and compared by various methods such as X-ray film spot test, azocasein analysis and X-ray film band test. *Btk* HD73, but not other strains exhibited significant protease activity by all the methods tested. The X-ray film band test clearly showed that electrophoretically separated 66 kDa toxin band exhibits protease activity. Toxin purified by DEAE-cellulose chromatography exhibits a single spot on two-dimensional SDS-PAGE indicating homogeneity of the protein. Thus protease activity seems to be intimately associated with 66 kDa toxin *Btk* HD73 only. The protease activity was inhibited by 1,10-phenanthroline, EDTA, EGTA and partially by PMSF, indicating that it could be a metalloprotease.

Thus the following conclusions were drawn from the research work presented in this thesis:

- *Btk-q*, a protease deficient mutant contained only 132 kDa protoxin indicating that endogenous protease activation of this protoxin to toxin is absent.

- This strain has been shown to lack any such enzymes hydrolyzing protoxin to toxin.

- The protoxin of the strain *Btk-q* has been shown to be similar to the wild type strain (*Btk* HD1) as judged by immuloblot experiments.

- The mutation did not involve any alterations in plasmids coding for protoxin as evidenced by their profiles, restriction enzyme analysis, PCR and Southern hybridization.

- It displayed 2-3 fold higher pesticidal activity than the wild type strain, *Btk* HD1.

- The maximum protease activity in *Btk* HD1 was seen at 21 h growth period. The free amino acid pool content also reached a maximum value at this time period in a parallel way. Whereas in *Btk-q* whatever traces of protease activity found at 24 h, the amino acid pool content also reached a maximum value at this time period.

- Only 1/6 of the free amino acid content could be detected in *Btk-q* compared to wild type strain at the stage of maximum protease production. It could be due to the absence of endogenous proteases in *Btk-q* so that no hydrolysis of protoxin to toxin.

- The traces of protease activity observed by azocasein assay in *Btk-q*, a protease deficient mutant, it could not be demonstrated by gelatin zymography.

- Interestingly, it has also been noticed that in *Btk* HD73, one of the wild type strains, the protease activity was observed to be intimately associated with the 66 kDa toxin.

- The protease activity associated with this toxin was inhibited by 1,10-Phe, EDTA, and EGTA indicating that it is a metalloprotease.

REFERENCES

Adang, M.J., Brody, M.S., Cardinau, G., Eagan, N., Roush, R.T., Shewmaker, C.J., Jones, A., Okes, J.V. and McBridge, K.C. (1993). The reconstitution and expression of a *Bacillus thuringiensis cry*3 gene in protoplasts and protoplants. *Plant Mol. Biol.* 21: 1131-1145.

Abdul-Rauf, M. and Ellar, D.J. (1999). Mutations in loop 2 and loop 3 residues in domain II of *Bacillus thuringiensis* Cry1C delta-endotoxin affect insecticidal specificity and initial binding to *Spodoptera littoralis* and *Aedes aegypti* midgut membranes. *Curr. Microbiol.* 39: 94-98.

Adamczyk, J.J.Jr., Church, G.E., Holloway, J.W., Graves, B. and Leonard, B.R. (1998). Larval survival and development of the fall armyworm (Lepidoptera: Noctuidae) on normal and transgenic cotton possessing the *Bacillus thuringiensis* Cry1Ac delta-endotoxin. *J. Econ. Entomol.* 91: 539-545.

Agaisse, H., Gominet, M., Okstad, O.A., Kolsto, A.B. and Lereclus, D. (1999). PlcR is a pleiotropic regulator of extracellular virulence factor gene expression in *Bacillus thuringiensis*. *Mol. Microbiol.* 32: 1043-1053.

Agaisse, H. and Lereclus, D. (1995). How does *Bacillus thuringiensis* produce so much insecticidal crystal protein? *J. Bacteriol.* 177: 6027-6032.

Akao, T., Mizuki, E., Yamashita, S., Kim, H.S., Lee, D.W. and Ohba, M. (2001) Specificity of lectin activity of *Bacillus thuringiensis* parasporal inclusion proteins. *J. Basic Microbiol.* 41: 3-6.

Alam, M.F., Abrigo, E., Datta, K., Datta, S.K., Oliva, N., Tu, J. and Virmani, S.S. (1999). Transgenic insect-resistant maintainer line (IR68899B) for improvement of hybrid rice. *Plant Cell Reports* 18:572-575.

Albert, Z.G., Shivarova, N.I. and Dean, D.H. (1989). Location of the *Bombyx mori* specificity domain on a *Bacillus thuringiensis* δ-endotoxin protein. *Proc. Natl. Acad. Sci.* USA 86: 4037-4041.

Alcantara, E.P., Alzate, O., Lee, M.K., Curtiss, A. and Dean, D.H. (2001). Role of alpha-helix 7 of *Bacillus thuringiensis* Cry1Ab delta-endotoxin in membrane insertion, structural stability, and ion channel activity. *Biochemistry* 40: 2540-2547.

Andrews, R.E.Jr., Bibilos, M.M. and Bulla, L.A.Jr. (1985). Protease activation of the entomocidal protoxin of *Bacillus thuringiensis* subsp. *kurstaki*. *Appl. Environ. Microbiol.* 50: 737-742.

Angasuthanasombat, C., Crickmore, N. and Ellar, D.J. (1988). Comparison of *Bacillus thuringiensis* subsp. *israelensis* CryIVA and CryIVB cloned toxins reveals synergism *in vivo*. *FEMS Microbiol. Lett.* 94: 63-68.

Arencibia, A., Vazquez, R.I., Prieto, D., Tellez, P., Carmona, E.R., Coego, A., Hernandez, L., de la Riva, G.A. and Selman-Housein, G. (1997). Transgenic sugarcane plants resistant to stem borer attack. *Mol. Breed.* 3: 247-255.

Armes, N.J., Bond, G.S. and Cooter, R.J. (1992). The laboratory culture and development of *Helicoverpa armigera*. *Natural Resources Institute Bulletin No.* 57 (Chatham, UK: Natural Resources Institute).

Armstrong, C.L., Parker, G.B., Pershing, J.C., Brown, S.M., Sanders, P.R., Duncan, D.R., Stone, T., Dean, D.A., DeBoer, D.L., Hart, J., Howe, A.R., Morrish, F.M., Pajeau, M.E., Peterse, W.L., Reich, B.J., Rodriguez, R., Santino, C.G., Sato, S.J., Schuler, W., Sims, S.R., Stehling, S., Tarochione, L.J. and Fromm,

M. E. (1995). Field evaluation of European corn borer control in progeny of 173 transgenic corn events expressing an insecticidal protein from *Bacillus thuringiensis*. *Crop Sci.* **35**: 550-557.

Aronson, A.I. and Shai, Y. (2001). Why *Bacillus thuringiensis* insecticidal toxins are so effective: unique features of their mode of action. *FEMS Microbiol. Lett.* **195**: 1-8.

Aronson, A. (2000). Incorporation of protease K in insect larval membrane vesicles does not result in the disruption of the integrity or function of the pore-forming *Bacillus thuringiensis* δ-endotoxin. *Appl. Environ. Microbiol.* **66**: 4568-4570.

Aronson, A.I., Geng, C. and Wu, L. (1999). Aggregation of *Bacillus thuringiensis* Cry1A toxins upon binding to target insect larval midgut vesicles. *Appl. Environ. Microbiol.* **65**: 2503-2507.

Aronson, A.I. (1993). Two faces of *Bacillus thuringiensis* insecticidal proteins and post-exponential survival. *Mol. Microbiol.* 7: 489-496.

Aronson, A.I., Han, E.S., McGaughey, W. and Johnson, D. (1991). The solubility of inclusion proteins from *Bacillus thuringiensis* is dependent upon protoxin composition and is a factor in toxicity to insects. *Appl. Environ. Microbiol.* **57**: 981-986.

Aronson, A.I., Beckman, W. and Dunn, P. (1986). *Bacillus thuringiensis* and related insect pathogens. *Microbiol. Rev.* **50**: 1-24.

Arpaia, S., Mennella, G., Onofaro, V., Perri, E., Sunseri, F. and Rotino, G.L. (1997). Production of transgenic eggplant (*Solanum melongena* L.) resistant to Colorado potato beetle (*Leptinotarsa decemlineata Say*). *Theor. Appl. Genet.* **95**: 329-334.

Bai, C., Degheele, D., Jansens, S. and Lambert, B. (1993). Activity of insecticidal proteins and strains of *Bacillus thuringiensis* against *Spodoptera exempta (Walker). J. Invertebr. Pathol.* **62**: 211-215.

Bai, C., Yi, S.X. and Degheele, D. (1990). Determination of protease activity in regurgitated gut juice from larvae of *Pieris brassicae, Mamestra brassicae, Spodoptera littoralis. Meded. Fac. Landbouwwet. Rijksuniv. Gent.* **55**: 519-525.

Baida, G.E. and Kuzmin, N.P. (1995). Cloning and primary structure of a new hemolysin gene from *Bacillus cereus. Biochim. Biophys. Acta* **1264**: 151-154.

Barbara, J.A. and Nickerson, K.W. (1978). Purification of the protein crystal from Bacillus thuringiensis by zonal gradient centrifugation. *Appl. Environ. Microbiol.* **36**: 625-626.

Barboza, D. (2000). Genetic corn changes dynamics of U.S. grain industry. *The Hindu daily, 14th December.*

Barjac de, H. and Sotherland, D.J. (1990). Bacterial control of mosquitoes and black flies. Rutger University press, New Brunswick, New Jersey, USA, pp.349.

Barton, K.A., Whiteley, H.R. and Yang, N.S. (1987). *Bacillus thuringiensis* δ-endotoxin expressed in transgenic *Nicotiana tobacum* provides resistance to lepidopteran insects. *Plant Physiol.* **85**: 1103-1109.

Baum, J.A. and Malvar, T. (1995). Regulation of insecticidal crystal protein production in *Bacillus thuringiensis. Mol. Microbiol.* **18**: 1-12.

Beegle, C.C. and Yamamoto, T. (1992). History of *Bacillus thuringiensis Berliner* research and development. *Can. J. Entomol.* **124**: 587-616.

Benedict, J.H., Sachs, E.S., Altman, D.W., Deaton, D.R., Kohel, R.J., Ring, D.R. and Berberich, B.A. (1996). Field performance of cotton expressing Cry IA insecticidal crystal protein for resistance to *Heliothis virescens* and *Helicoverpa zea* (Lepidoptera : Noctuidae). *J. Econ. Entomol.* **89**: 230-238.

Berliner, E. (1911). Uber die Schalffsuchi der Mehlmottenraupo (*Ephestia kuehniella*

Zell) und thren Erreger, *Bacillus thuringiensis* n. sp. *Ziets. Angew. Entom.* 2: 29-56.

Bernhard, K. and Utz, R. (1993). Production of *Bacillus thuringiensis* insecticides for experimental and commercial uses. **In: Entwistle, P.F., Cory, J.S., Bailey, M.J. and Higgs, S. (eds.),** *Bacillus thuringiensis an Environmental Biopesticide: Theory and Practice*. John Wiley & Sons, Chichester, England. pp 255-256.

Bernheimer, A.W. and Rudy, B. (1986). Interactions between membranes and cytolytic peptides. *Biochim. Biophys. Acta.* **864**: 123-141.

Bibilos, M., and Andrews, R.E.Jr. (1988). Inhibition of *Bacillus thuringiensis* proteases and their effects on crystal toxin proteins and cell free translations. *Can. J. Microbiol.* **34**: 740-742.

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

Bietlot, H.P., Schernthaner, J.P., Milne, R.E., Clairmont, F.R., Bhella, R.S. and Kaplan, H. (1993). Evidence that the Cry1A crystal protein from *Bacillus thuringiensis* is associated with DNA. *J. Biol. Chem.* **268**: 8240-8245.

Blum, H., Beier, H., and Gross, H.J. (1987). Improved method for silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**: 93-99.

Bonnefoi, A. and Beguin, S. (1959). Recherches sur l'action des cristaux de *Bacillus thuringiensis* souche "anduze". *Entomophaga* 4: 193-199.

Bourgouin, C., Delecluse, A., Ribier, J., Klier, A. and Rapoport, G. (1988). A *Bacillus thuringiensis* subsp. *israelensis* gene encoding a 125-kilodalton larvicidal polypeptide is associated with inverted repeat sequences. *J. Bacteriol.* **170**: 3575-3583.

Bravo, A. and Quintero, R. (1993). *Importancia y potential de Bacillus thuringiensis en el* control de plaga. REDBIO, Santiago, Chile. pp 50.

Bravo, A., Jansens, S. and Peferoen, M. (1992). Immunocytochemical localization of *Bacillus thuringiensis* insecticidal crystal proteins in intoxicated insects. *J. Invert. Pathol.* **60**: 237-246.

Bulla, L.A.Jr., Kramer, K.J. and Davidson, L.I. (1977). Characterization of the entomocidal parasporal crystal of *Bacillus thuringiensis*. *J. Bacteriol.* **130**: 375-383.

Burton, S.L., Ellar, D.J., Li, J. and Derbyshire, D.J. (1999). N-acetylgalactosamine on the putative insect receptor aminopeptidase N is recognized by a site on the domain III lectin-like fold of a *Bacillus thuringiensis* insecticidal toxin. *J. Mol. Biol.* **287**: 1011-1022.

Cannon, R.J.C. (1996). *Bacillus thuringiensis* use in agriculture: A molecular perspective. *Biol. Rev.* **71**: 561-636.

Cantwell, G.E., Heimpel, A.M. and Thompson, M.J. (1964). The production of an exotoxin by various crystal-forming bacteria related to *Bacillus thuringiensis* var. *thuringiensis Berliner*. *J. Insect. Pathol.* 6: 466-480.

Carlson, C.R., Johansen, T., Lecadet, M.M. and Kolsto, A.B. (1996). Genomic organization of the entomopathogenic bacterium *Bacillus thuringiensis* subsp. *berliner* 1715. *Microbiol.* **142**: 1625-1634.

Carlson, C.R., Caugant, D.A. and Kolsto, A.B. (1994). Genotypic diversity among *Bacillus cereus* and *Bacillus thuringiensis* strains. *Appl. Environ. Microbiol.* **60**: 1719-1725.

Carlson, C.R. and Kolsto, A.B. (1993). A complete physical map of a *Bacillus thuringiensis* chromosome. *J. Bacteriol.* 175: 1053-1060.

Carroll, J. and Ellar, D.J. (1993). Proteolytic processing of a coleopteran specific δ-endotoxin by *Bacillus thuringiensis* var. *tenebrionis*. *Eur. J. Biochem.* **214**: 771-778.

Carroll, J., Li, J. and Ellar, D.J. (1989). Proteolytic processing of a coleopteran specific delta-endotoxin produced by *Bacillus thuringiensis* var. *tenebrionis*. *Biochem. J.* **261**: 99-105.

Chandra, A., Ghosh, P., Mandaokar, A.D., Bera, A.K., Sharma, R.P., Das, S. and Kumar, P.A. (1999). Amino acid substitution in alpha-helix 7 of Cry1Ac delta-endotoxin of *Bacillus thuringiensis* leads to enhanced toxicity to *Helicoverpa armigera Hubner*. *FEBS Lett.* **458**: 175-179.

Chen, X.J., Curtiss, A., Alcantara, E. and Dean, D.H. (1995). Mutations in domain I of *Bacillus thuringiensis* δ-endotoxin CrylAb reduce the irreversible binding of toxin to *Manduca sexta* brush border membrane vesicles. *J. Biol. Chem.* **270**: 6412-6419.

Chen, X.J., Lee, M.K. and Dean, D.H. (1993). Site-directed mutations in a highly conserved region of *Bacillus thuringiensis* δ-endotoxin affect inhibition of short circuit current across *Bombyx mori* midguts. *Proc. Natl. Acad. Sci.* (USA) **90**: 9041-9045.

Chestukhina, G.G., Zalunin, I.A., Kostina, L.I., Kotova, T.S., Kattrukha, S.P. and Stepanov, V.M. (1980). Crystal-forming proteins of *Bacillus thuringiensis*. *Biochem. J.* **187**: 457-465.

Chestukhina, G.G., Tyurin, S.A., Kostina, L.I., Osterman, A.L., Zalunin, I.A., Khodova, O.A. and Stepanov, V.M. (1990). Sub-domain organization of *Bacillus thuringiensis* entomocidal proteins N-terminal domains. *J. Protein Chem.* **9**: 501-507.

Chestukhina, G.G., Kostina, L.I., Mikhailova, A.L., Tyurin, S.A., Klepikova, F.S. and Stepanov, V.M. (1982). The main features of *Bacillus thuringiensis*: δ-endotoxin molecular structure. *Arch. Microbiol.* **132**: 159-162.

Chestukhina, G.G., Kotova, T.S., Zalunin, I.A. and Stepanov, V.M. (1979). Proteinases in growth and spore formation of *Bacillus thuringiensis*. *Biokhimiya* (Russian) **44**: 796-802.

Cheung, A.L., Ying, P. and Fischetti, V.A. (1991). A method to detect proteinase activity using unprocessed X-ray films. *Anal. Biochem.* **193**: 20-23.

Cheung, P.Y.K. and Hammock, B.D. (1985). Separation of three biologically distinct activities from the parasporal crystal of *Bacillus thuringiensis* var. *israelensis*. *Curr. Microbiol.* **12**: 121-126.

Chilcott, C.N. and Ellar, D.J. (1988). Comparative toxicity of *Bacillus thuringiensis* var. *israelensis* crystal proteins *in vivo* and *in vitro*. *J. Gen. Microbiol.* **134**: 2551-2558.

Chilcott, C.N., Kalmakoff, J. and Pillai, J.S. (1983). Characterization of proteolytic activity associated with *Bacillus thuringiensis* var. *israelensis* crystals. *FEMS Microbiology Letters* **18**: 37-41.

Chirsteller, J.T., Liang, W.A., Markwick, N.P. and Burgess, E.P.J. (1992). Midgut proteinase activities in 12 phytophagous lepidopteran larvae: dietery and protease inhibitor interactions. *Insect Biochem. Mol. Biol.* **22**: 735-746.

Choma, C.T., Surewicz, W.K., Carey, P.R., Pozsgay, M., and Raynor, T. (1990). Unusual proteolysis of the protoxin and toxin from *Bacillus thuringiensis* : Structural implications. *Eur. J. Biochem.* **189**: 523-527.

Choma, C.T. and Kaplan, H. (1991). The toxic moiety of the *Bacillus thuringiensis* protein undergoes a conformational change upon activation. *Biochem. Biophys. Res. Commun.* **179**: 1934-1938.

Choma, C.T. and Kaplan, H. (1990). Folding and unfolding of the protoxin from

Bacillus thuringiensis: Evidence that the toxic moiety is present in an active conformation. *Biochemistry* **29**: 10971-10977.

Chothia, C. and Lesk, A.M. (1986). The relation between the divergence of sequence and structure in proteins. *EMBO J.* **5**: 823-826.

Clairmont, F.R., Milne, R.E., Pham, V.T., Carriere, M.B. and Kaplan, H. (1998). Role of DNA in the activation of the Cry1A insecticidal crystal protein from *Bacillus thuringiensis*. *J. Biol. Chem.* **15**: 9292-9296.

Consden, R., Gordon, A.A. and Martin, J.P. (1944). Qualitative analysis of protein: a partition of chromatographic methods using paper. *Biochem. J.* **38**: 224-232.

Convents, D., Cherlet, M., Van Damme, J., Lasters, I. and Lauwereys, M. (1991). Two structural domains as a general fold of the toxic fragment of the *Bacillus thuringiensis* δ-endotoxin. *Eur. J. Biochem.* **195**: 631-635.

Cooksey, K.E. (1971). The protein crystal toxin of *Bacillus thuringiensis*: Biochemistry and mode of action. *In: Burges HD & Hussey NW ed. Microbial control of insects and mites.* New York, London, Academic Press Inc., pp 247-274.

Cooper, D. (1994). *Bacillus thuringiensis* toxins and mode of action. *Agric. Ecosyst. Environ.* **49**: 21-26.

Cornu, D., Leple, J.C., Bonade-Bottino, M., Ross, A., Augustin, S., Deplanque, A., Jouanin, L., Pilate, G. and Ahuja, M.R. (1996). Expression of a proteinase inhibitor and a *Bacillus thuringiensis* delta-endotoxin in transgenic poplars. In: *Somatic Cell Genetics and Molecular Genetics of Trees*, W. Boerjan and D.B. Neale (eds.), pp.131-136. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Crickmore, N., Zeigler, D.R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J. and Dean, D.H. (1998). Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* **62**: 807-813.

Cummings, C.E., Armstrong, G., Hodgman, T.C. and Ellar, D.J. (1994). Structural and functional studies of a synthetic peptide mimicking a proposed membrane inserting region of a *Bacillus thuringiensis* δ-endotoxin. *Mol. Membr. Biol.* **11**: 87-92.

Dai, S.M. and Gill, S.S. (1993). *In vitro* and *in vivo* proteolysis of the *Bacillus thuringiensis* subsp. *israelensis* Cry11Aa proteins by *Culex quinquefasciatus* larval midgut proteases. *Insect Biochem. Mol. Biol.* **23**: 273-283.

Dalhammar, G. and Steiner, H. (1984). Characterization of inhibitor A. a protease from *Bacillus thuringiensis* which degrades attacins and cecropins, two classes of antibacterial proteins in insects. *Eur. J. Biochem.* **139**: 247-252.

Damgaard, P.H., Jacobsen, C.S. and Sorensen, J. (1996b). Development and application of a primer set for specific detection of *Bacillus thuringiensis* and *Bacillus cereus* in soil using magnetic capture-hybridization and PCR amplification. *System Appl. Microbiol.* **19**: 436-441.

Datta, K., Vasquez, A., Tu, J., Torrizo, L., Alam, M.F., Oliva, N., Abrigo, E., Khush, G.S. and Datta, S.K. (1998). Constitutive and tissue-specific differential expression of the *cry*1A(b) gene in transgenic rice plants conferring resistance to rice insect pests. *Theore. and Appl. Genetics* **97**: 20-30.

Dean, D.H., Rajamohan, F., Lee, M.K., Wu, S.J., Chen, X.J., Alcantara, E. and Hussain, S.R. (1996). Probing the mechanism of action of *Bacillus thuringiensis* insecticidal proteins by site-directed mutagenesis-a minireview. *Gene* **179**: 111-117.

Debro, L., Fitz-James, P.C. and Aronson, A. (1986). Two different parasporal inclusions are produced by *Bacillus thuringiensis* subsp. *finitimus*. *J. Bacteriol.* **165**: 258-268.

Doi, R.H. (1972). Role of proteases in sporulation. *Curr. Top. Cell. Regul.* **6**: 1-20.

Donovan, W.P., Rupar, M.J., Slaney, K.A.C., Malvar, T., Gowranburke, M.C. and Johnson, T.B.C. (1992). Characterization of two genes encoding *Bacillus thuringiensis* insecticidal crystal proteins toxic to coleopteran species. *Appl. Environ. Microbiol.* **58**: 3921-3927.

Donovan, W.P., Tan, Y. and Slaney, A.C. (1997). Cloning of the *nprA* gene for neutral protease A of *Bacillus thuringiensis* and effect of *in vivo* deletion of *nprA* on insecticidal crystal protein. *Appl. Environ. Microbiol.* **63**: 2311-2317.

Duvick, J. (2001). Prospects for reducing fumonisin contamination of maize through genetic modification. *Environ. Health Perspect* **109**: 337-342.

Edwards, D.L., Payne, J. and Soares, G.G. (1988). Novel isolates of *Bacillus thuringiensis* having activity against nematodes. *European Patent Application*, EP 0 303 426 A2.

Ellar, D.J. (1978). Spore specific structures and their function. *Symp. Soc. Gen. Microbiol.* **29**: 295-325.

English, L., Robbins, H.L., Von Tersch, M.A., Kulesza, C.A., Ave, D., Coyle, D., Jany, C.S. and Slatin, S.L. (1994). Mode of action of Cry2Aa: a *Bacillus thuringiensis* delta-endotoxin. *Insect Biochem. Mol. Biol.* **24**: 1025-1035.

English, L. and Slatin, S.L. (1992). Mode of action of δ-endotoxins from *Bacillus thuringiensis*: A comparison with other bacterial toxins. *Insect Biochem. Mol. Biol.* **22**: 1-7.

English, L.H. and Readdy, T.L. (1989). Delta endotoxin inhibits a phosphate in midgut epithelial membranes of *Heliothis virescens*. *Insect Biochem.* **19**: 145-152.

Estruch, J.J., Warren, G.W., Mullins, M.A., Nye, G.J., Craig, J.A. and Koziel, M.G. (1996). Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. *Proc. Natl. Acad. Sci.* (USA) **93**: 5389-5394.

Estruch, J.J., Carozzi, N.B., Desai, N., Duck, N.B., Warren, G.W. and Koziel, M.G. (1997). Transgenic plants: an emerging approach to pest control. *Nat. Biotechnol.* **15**: 137-141.

Farkas, J., Sebesta, K., Horska, K., Samek, Z., Dolejs, L. and Sorm, F. (1977). Structure of thuringiensis, the thermostable exotoxin from *Bacillus thuringiensis*. *Coll. Czech. Chem. Commun.* **42**: 909-929.

Fast, P.G. (1981). The crystal toxin of *Bacillus thuringiensis*. *In: Burges HD ed. Microbial control of pests and plant diseases* 1970-1980. New York, London, Academic Press Inc., pp 223-248.

Fast, P.G. (1971). Isolation of a water-soluble toxin from a commercial microbial insecticide based on *Bacillus thuringiensis*. *J. Invertebr. Pathol.* **17**: 301.

Faust, R.M., Abe, K., Held, G.A., Iizuka, T., Bulla, L.A. and Meyers, C.L. (1983). Evidence for plasmid-associated crystal toxin production in *Bacillus thuringiensis* subsp. *israelensis*. *Plasmid* **9**: 98-103.

Faust, R.M. (1973). The *Bacillus thuringiensis* β-exotoxin: Current status. *Bull. Entomol. Soc. Am.* **19**: 153-156.

Feitelson, J.S. (1993). The *Bacillus thuringiensis* family tree, p 63-71. In L. Kim (ed.), *Advanced engineered pesticides*. Marcel Dekker, Inc., New York, NY.

Feitelson, J.S., Payne, J. and Kim, L. (1992). *Bacillus thuringiensis*: insects and beyond. *Bio/Techn.* **10**: 271-275.

Finney, D.J. (1971). *Probit analysis*. Cambridge University Press, Cambridge, UK.

Fischhoff, D.A., Bowdish, K.S., Perlak, F.J., Marrone, P.G., McCormick, S.M.,

Niedermeyer, J.G., Dean, D.A., Kusano-Kretzmer, K., Mayer, E.J., Rochester, D.E., Rogers, S.G. and Fraley, R.T. (1987). Insect tolerant transgenic tomato plants. *Bio/Techn.* **5**: 807-813.

Forcada, C., Alcacer, R.E., Garcera, M.D. and Martinez, R. (1996). Differences in the midgut proteolytic activity of two *Heliothis virescens* strains, one susceptible and one resistant to *Bacillus thuringiensis* toxins. *Arch. Insect Biochem. Physiol.* **31**: 257-272.

Francis, B.R. and Bulla, L.A.Jr. (1997). Further characterization of BT-R1 the cadherin-like receptor for Cry1Ab toxin in tobacco hornworm (*Manduca sexta*) midguts. *Insect Biochem. Mol. Biol.* **27**: 541-550.

Fujimoto, H., Itoh, J., Yamamoto, M., Jyozuka, J. and Shimamoto, K. (1993). Insect resistant rice generated by introduction of a modified δ-endotoxin gene of *Bacillus thuringiensis*. *Bio/Techn.* **11**: 1151-1155.

Garczynski, S.F. and Adang, M.J. (1995). *Bacillus thuringiensis* Cry1A(c) δ-endotoxin binding aminopeptidase in the *Manduca sexta* midgut has a glycosyl-phosphatidylinositol anchor. *Insect Biochem. Mol. Biol.* **25**: 409-415.

Garczynski, S.F., Crim, J.W. and Adang, M.J. (1991). Identification of a putative brush border membrane-binding molecules specific to *Bacillus thuringiensis* δ-endotoxin by protein blot analysis. *Appl. Environ. Microbiol.* **57**: 2816-2820.

Gazit, E., La Rocca, P., Sansom, M.S.P. and Shai, Y. (1998). The structure and organization within the membrane of the helices composing the pore-forming domain of *Bacillus thuringiensis* δ-endotoxin are consistent with an 'umbrella-like' structure of the pore. *Proc. Natl. Acad. Sci.* (USA) **95**: 12289-12294.

Gazit, E. and Shai, Y. (1995). The assembly and organization of the α5 and α7 helices from the pore-forming domain of *Bacillus thuringiensis* δ-endotoxin. *J. Biol. Chem.* **270**: 2571-2578.

Gazit, E. and Shai, Y. (1993). Structural and functional characterization of the α5 segment of *Bacillus thuringiensis* δ-endotoxin. *Biochemistry* **32**: 3429-3436.

Gerber, D. and Shai, Y. (2000). Insertion and organization within membranes of the δ-endotoxin pore-forming domain, helix 4-loop-helix 5 and inhibition of its activity by a mutant helix 4 peptide. *J. Biol. Chem.* **275**: 23602-23607.

Gibson, D.M., Gallo, L.G., Krasnoff, S.B. and Ketchum, R.E.B. (1995). Increased efficiency of *Bacillus thuringiensis* subsp. *kurstaki* in combination with tannic acid. *J. Econ. Entomol.* **88**: 270-277.

Gill, S.S., Cowles, E.A. and Francis, V. (1995). Identification, isolation, cloning of a *Bacillus thuringiensis* Cry1Ac toxin binding protein from the midgut of the lepidopteran insect *Heliothis virescens*. *J. Biol. Chem.* **270**: 27277-27282.

Gill, S.S., Cowles, E.A. and Pietrantonio, P.V. (1992). The mode of action of *Bacillus thuringiensis* endotoxins. *Annu. Rev. Entomol.* **37**: 615-636.

Gilmore, M.S., Cruz-Rodz, A.L., Leimeister-Wachter, M., Kreft, J. and Goebel, W. (1989). A *Bacillus cereus* cytolytic determinant, cereolysin AB, which comprises the phospholipases C and sphingomyelinase genes: Nucleotide sequence and genetic linkage. *J. Bacteriol.* **171**: 744-753.

Goldberg, L.J. and Margalit, J. (1977). *Mosquito News* **37**: 355-358 (cited in Kumar *et al.*, 1996. The insecticidal proteins of *Bacillus thuringiensis*. *Adv. Appl. Microbiol.* **42**: 1-43).

Gonzalez, J., Jr, M., Dulmage, H.T. and Carlton, B.C. (1981). Correlation between specific plasmids and δ-endotoxin production in *Bacillus thuringiensis*. *Plasmid* **5**: 351-365.

Gonzalez, J., Jr, M. and Carlton, B.C. (1980). Patterns of plasmid DNA in

crystalliferous and acrystalliferous strains of *Bacillus thuringiensis*. *Plasmid* 3: 92-98.

Gould, F., Martinez-Ramirez, A., Anderson, A., Ferre, J., Silva, J. and Moar, W.J. (1992). Broad-spectrum resistance to *Bacillus thuringiensis* toxins in *Heliothis virescens*. *Proc. Natl. Acad. Sci.* (USA) **89**: 7986-7990.

Gould, F., Anderson, A., Jones, A., Sumerford, D., Heckel, D.G., Lopez, J., Micinski, S., Leonard, R. and Laster, M. (1997). Initial frequency of alleles for resistance to *Bacillus thuringiensis* toxins in field populations of *Heliothis virescens*. *Proc. Natl. Acad. Sci.* USA **94**: 3519-3523.

Grochulski, P., Masson, L., Borisova, S., Pusztai-Carey, M., Schwartz, J.L., Brousseau, R. and Cygler, M. (1995). *Bacillus thuringiensis* Cry1Aa insecticidal toxin: crystal structure and channel formation. *J. Mol. Biol.* **254**: 447-464.

Groeters, F.R., Tabashnik, B.E., Finson, N. and Johnson, M.W. (1993). Resistance to *Bacillus thuringiensis* affects mating success of the diamondback moth (Lepidoptera: Plutellidae). *J. Econ. Entomol.* **86**: 1035-1039.

Gutierrez, P., Alzate, O. and Orduz, S. (2001). A theoretical model of the tridimensional structure of *Bacillus thuringiensis* subsp. *medellin* Cry11Bb toxin deduced by homology modelling. *Mem. Inst. Oswaldo. Cruz.* **96**: 357-364.

Haider, M.Z. and Ellar, D.J. (1989). Functional mapping of an entomocidal δ-endotoxin. Single aminoacid changes produced by site directed mutagenesis influence toxicity and specificity of the protein. *J. Mol. Biol.* **208**: 183-194.

Haider, M.Z., Smith, G.P. and Ellar, D.J. (1989). Detection of the toxic coding fragments and an insect specificity region of a dual toxicity *Bacillus thuringiensis* crystal protein gene. *FEMS Microbiol. Lett.* **58**: 157-164.

Haider, M.Z. and Ellar, D.J. (1987). Analysis of the molecular basis of insecticidal specificity of *Bacillus thuringiensis* crystal δ-endotoxin. *Biochem. J.* **248**: 197-201.

Haider, M.Z., Knowles, B.H. and Ellar, D.J. (1986). Specificity of *Bacillus thuringiensis* var. *colmeri* insecticidal δ-endotoxin is determined by differential proteolytic processing of the protoxin by larval gut proteases. *Eur. J. Biochem.* **156**: 531-540.

Hama, H., Suzuki, K. and Tanaka, H. (1992). Inheritance and stability of resistance to *Bacillus thuringiensis* formulations of the diamondback moth, *Plutella xylostella* (LINNAEUS) (Lepidoptera: Yponomeutidae). *Appl. Entomol. Zool.* **27**: 355-362.

Hannay, C.L. and Fitz-James, P. (1955). The protein crystals of *Bacillus thuringiensis* berliner. *Can. J. Microbiol.* 1: 674-710.

Hannay, C. L. (1953). Crystalline inclusions in aerobic spore-forming bacteria. *Nature* **172**: 1004.

Heimpel, A.M. (1967). A critical review of *Bacillus thuringiensis* var. *thuringiensis Berliner* and other crystalliferous bacteria. *Annu. Rev. Entomol.* **12**: 287-322.

Heimpel, A.M. (1954). Investigations of the mode of action of strains of *Bacillus cereus* Frankland and Frankland pathogenic for the larch sawfly, *Pristiphora chrichsonii* (Htg.). Kingston, Canada, Queen's University, pp 155 (Doctoral dissertation).

Hendrickx, K., DeLoof, A. and Van Mellaert, H. (1989). Effects of *Bacillus thuringiensis* δ-endotoxins on the permeability of brush border membrane vesicles from tobacco hornworm (*Manduca sexta*) midgut. *Comp. Biochem. Physiol.* **95**: 241-245.

Hendriksen, N.B. and Hansen, B.M. (1998). Phylogenetic relations of *Bacillus thuringiensis*: implications for risks associated to its use as a microbiological pest control agent. *In insect pathogens and insect parasitic nematodes. Proceedings of the 6th European Meeting "Microbial control of pests in sustainable Agriculture".*

Copenhagen, Denmark, 10-15 August, 1997 (edited by Smits, P.H).Bulletin OILB/SROP (1998) 21 (4) 5-8 ISBN 92-9067-098-3.

Hernandez, C.S., Ferre, J. and Larget-Thiery, I. (2001). Update on the detection of beta-exotoxin in *Bacillus thuringiensis* strains by HPLC analysis. *J. Appl. Microbiol.* **90**: 643-647.

Hernstadt, C., Giltroy, T.E., Solesk, D.A., Bennet, B.D. and Gautner, F.H. (1987). Nucleotide sequence and deduced amino acid sequence of a coleopteran active delta-endotoxin gene from *Bacillus thuringiensis* subsp. *sandiego*. Gene **57**: 81-116.

Hernstadt, C., Soares, G.C., Wilcox, E.R., and Edwards, D.L. (1986). A new strain of *Bacillus thuringiensis* with activity against coleopteran insects. *Bio/Techn.* 4: 305-308.

Hilder, V.A. and Boulter, D. (1999). Genetic engineering of crop plants for insect resistance - a critical review. *Crop Protection* **18**: 177-191.

Hoffman, M.P., Zalom, F.G., Wilson, L.T., Smilanick, J.M., Malyj, L.D., Kiser, J., Hilder, V.A. and Barnes, W.M. (1992). Field evaluation of transgenic tobacco containing genes encoding *Bacillus thruingiensis* δ-endotoxin or cowpea trypsin inhibitor: Efficacy against *Helicoverpa zea* (Lepidoptera: Noctuidae). *J. Econ. Entomol.* **85**: 2516-2522.

Hofte, H., de Greve, H., Seurink, J., Jansens, S., Mahillon, J., Amphe, C., Dekerchore, J., Vanderbruggen, H., Van Matagu, M., Zabeau, M. and Vaeck, M. (1986). Structural and functional analysis of cloned delta-endotoxin of *Bacillus thuringiensis berliner* 1715. *Eur. J. Biochem.* **161**: 273-280.

Hoftee, H., deGreve, H., Seurink, J., Jansens, S., Mahillon, J., Amphe, C., Dekerchore, J., Vanderbruggen, H., VanMatagu, M., Zabeau, M. and Vaeck, M. (1986). Structural and functional analysis of cloned delta-endotoxin of *Bacillus thuringiensis berliner* 1715. *Eur. J. Biochem.* **161**: 273-280.

Hofte, H. and Whiteley, H.R. (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* **53**: 242-255.

Hotha, S. and Banik, R.M. (1997). Production of alkaline protease by *Bacillus thuringiensis* H 14 in aqueous two-phase systems. *J. Chem. Technol. Biotechnol.* **69**: 5-10.

Huber, H.E. and Luthy, P. (1981). *Bacillus thuringiensis* delta-endotoxin: Composition and activation. *In: Davidson EW ed. Pathogenesis of invertebrate microbial diseases.* Totowa, New Jersey, Allanheld-Osmum Publishers, pp 209-234.

Hummel, K.M., Penheiter, A.R., Gathman, A.C. and Lilly, W.W. (1996). Anamalous estimation of protease molecular weights using gelatin-containing SDS-PAGE. *Anal. Biochem.* **233**: 140-142.

Hurley, J.M., Lee, S.G.L., Andrews, R.E.Jr., Khowder, M.J. and Bulla, L.A.Jr. (1985). Separation of the cytolytic and mosquitocidal proteins of *Bacillus thuringiensis* subsp. *israelensis*. *Biochem. Biophys. Res. Commun.* **126**: 961-965.

Husz, B. (1928). *Int. Corn Borer Invest. Sci. Rep.* 1: 191-193 (cited in Kumar *et al.*,1996. The insecticidal proteins of *Bacillus thuringiensis*. *Adv. Appl. Microbiol.* **42**: 1-43).

Ibarra, J.E. and Federici, B.A. (1986). Isolation of a relatively nontoxic 65 kDa protein inclusion from the parasporal body of *Bacillus thuringiensis* subsp. *israelensis*. *J. Bacteriol.* **168**: 527-533.

Ishiwata, S. (1901). On a kind of severe flasherie (Sotto disease). *Dainihan Sanbshi Kaiho* **9**: 1-5.

Jacquet, F., Hutter, R. and Luthy, P. (1987). Specificity of *Bacillus thuringiensis* delta-endotoxin.. *Appl. Environ Microbiol.* **53**: 500-504.

Jannston, K.A., Lee, M.S., Brough, L., Ailder, V.A., Gatehouse, A.M.R. and Gatehouse, J.A. (1995). Protease activities in the larval midgut of *Heliothis virescens*: Evidence for trypsin and chymotrypsin like enzymes. *Insect Biochem. Mol. Biol.* **25**: 373-383.

Jansens, S., Van Vliet, A., Dickburt, C., Buysse, L., Piens, C., Saey, B., DeWulf, A., Gossele, V., Paez, A., Goebel, E. and Peferoen, M. (1997). Transgenic corn expressing a Cry9C insecticidal protein from *Bacillus thuringiensis* protected from European corn borer damage. *Crop Sci.* 37: 1616-1624.

Jenkins, J.L., Lee, M.K., Sangadala, S., Adang, M.J. and Dean, D.H. (1999). Binding of *Bacillus thuringiensis* Cry1Ac toxin to *Manduca sexta* aminopeptidase-N receptor is not directly related to toxicity. *FEBS Lett.* **462**: 373-376.

Jenkins, J.L., Lee, M.K., Valaitis, A.P., Curtiss, A. and Dean, D.H. (2000). Bivalent sequential binding of a *Bacillus thuringiensis* toxin to gypsy moth aminopeptidase N receptor. *J. Biol. Chem.* **275**: 14423-14431.

Jensen, G.B., Andrea, W., Susanne S. P., Jesper, D., James, A.B. and Lars, A. (1995). The Genetics basis of the aggregation system in *Bacillus thuringiensis* subsp. *israelensis* is located on the large conjugative plasmid pXO16. *J. Bacteriol.* 177: 2914-2917.

Johnson, D.E. and McGaughey, W.H. (1996). Natural mortality among Indianmeal moth larvae with resistance to *Bacillus thuringiensis*. *J. Invertbr. Pathol.* **68**: 170-172.

Jouanin, L., Bonade-Bottino, M., Girard, C., Morrot, G. and Giband, M. (1998). Transgenic plants for insect resistance. *Plant Science* (limerick) **131**: 1-11.

Kar, S., Basu, D., Das, S., Ramkrishnan, N.A., Mukherjee, P., Nayak, P. and Sen, S.K. (1997). Expression of *cry*1Ac gene of *Bacillus thuringiensis* in transgenic chickpea plants inhibits development of podborer (*Heliothis armigera*) larvae. *Transgen. Res.* **6**: 177-185.

Karim, S. and Dean, D.H. (2000). Pesticidal and receptor binding properties of *Bacillus thuringiensis* Cry1Ab and Cry1Ac delta-endotoxin mutants to *Pectinophora gossypiella* and *Helicoverpa zea*. *Curr. Microbiol.* **41**: 430-440.

Keller, M., Sneh, B., Strizhov, N., Prudovsky, E., Regev, A., Koncz, C., Schell, J. and Zilberstein, A. (1995). Digestion of δ-endotoxin by gut proteases may explain reduced sensitivity of advanced instar larvae of *Spodoptera littoralis* to Cry1C. *Insect Biochem. Mol. Biol.* **26**: 365-373.

Keller, M., Sneh, B., Strizhov, N., Prudovsky, E., Regev, A., Koncz, C., Schell, J. and Zilberstein, A. (1996). Digestion of δ-endotoxin by gut proteases may explain reduced sensitivity of advanced instar larvae of *Spodoptera littoralis* to Cry1C. *Insect Biochem. Mol. Biol.* **26**: 365-373.

Kirsch, K. and Schmutterer, H. (1988). Low efficacy of a *Bacillus thuringiensis (Berliner)* formulation in controlling the diamondback moth, *Plutella xylostella (L.)*, in the Philippines. *J. Appl. Entomol.* **105**: 249-255.

Knight, P.J.K., Crickmore, N. and Ellar, D.J. (1994). The receptor for *Bacillus thuringiensis* Cry1Ac δ-endotoxin in the bursh border membrane of the lepidopteran *Manduca sexta* is aminopaptidase-N. *Mol. Microbiol.* **11**: 429-436.

Knowles, B.H. (1994). Mechanism of action of *Bacillus thuringiensis* insecticidal δ-endotoxins. *Advan. Insect Physiol.* **24**: 275-308.

Knowles, B.H. and Dow, J.A.T. (1993). The crystal δ-endotoxin of *Bacillus thuringiensis*: Models for their mechanism of action on the insect gut. *Bioassays* **15**: 469-476.

Knowles, B.H., Knight, P.J.K. and Ellar, D. J. (1991). N-acetyl galactosamine is part of receptor in insect midgut epithelium that recognises an insecticidal protein from *Bacillus thuringiensis*. *Proc. Roy. Soc.* (London) Ser. B **245**: 31-35.

Koni, P.A. and Ellar, D.J. (1993). Cloning and characterization of a novel *Bacillus thuringiensis* cytolytic delta-endotoxin. *J. Mol. Biol.* **229**: 319-327.

Koziel, M.G., Beland, G.L., Bowman, C., Carozzi, N.B., Crenshaw, R., Crossland, L., Dawson, J., Desai, N., Hill, M., Kadwell, S., Launis, K., Lewis, K., Maddox, D., McPherson, K., Meghji, M.R., Merlin, E., Rhodes, R., Warren, G.W., Wright, M. and Evola. S.V. (1993). Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. *Bio/Techn.* **11**: 194-200.

Krattiger, A.F. (1997). Insect resistance in crops: A case study of *Bacillus thuringiensis* (*Bt*) and its transfer to developing countries. *ISAAA Briefs* No.2. ISAAA: Ithaca, NY. pp 42.

Krieg, A. and Huger, A.M. (1986). Symposium in memorium Dr. Ernst Berliner, Darmstadt 25.08.1986. Mitteilungen aus der Biologischen Bundesanstalt fur Land- und Forstwirtschaft, Berlin-Dahlem, Heft 233.

Krieg, A. (1971). Concerning α-exotoxin produced by vegetative cells of *Bacillus thuringiensis* and *Bacillus cereus*. *J. Invertebr. Pathol.* **17**: 134-135.

Kronstad, J.W., Schnepf, H.E. and Whiteley, H.R. (1983). Diversity of locations for *Bacillus thuringiensis* crystal protein genes. *J. Bacteriol.* **154**: 419-428.

Kronstad, J.W. and Whiteley, H.R. (1984). Inverted repeat sequences flank a *Bacillus thuringiensis* crystal protein gene. *J. Bacteriol.* **160**: 95-102.

Kumar, P.A., Mandaokar, A., Sreenivasu, K., Chakrabarti, S.K., Bisaria, S., Sharma, S.R., Kaur, S. and Sharma, R.P. (1998). Insect-resistant transgenic brinjal plants. *Mol. Breed.* **4**: 33-37.

Kumar, A.S. and Aronson, A.I. (1999). Analysis of mutations in the pore-forming region essential for insecticidal activity of a *Bacillus thuringiensis* delta-endotoxin. *J. Bacteriol.* **181**: 6103-6107.

Kumar, P.A., Sharma, R.P. and Mallik, V.S. (1996). The insecticidal proteins of *Bacillus thuringiensis*. *Adv. Appl. Microbiol.* **42**: 1-43.

Kunitate, A., Okamoto, M. and Ohmori, I. (1989). Purification and characterization of a thermostable serine protease from *Bacillus thuringiensis*. *Agric. Biol. Chem.* **53**: 3251-3256.

Lacey, L.A. and Harper, J.D. (1986). Microbial control and integrated pest management. *J. Entomol. Sci.* **21**: 206-213.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lambert, B., Buysse, L., DeCock, C., Jansens, S., Piens, C., Saey, B., Seurinck, J., Van Audenhove, K., Van Rie, J., Van Vliet, A. and Peferoen, M. (1996). A *Bacillus thuringiensis* insecticidal crystal protein with a high activity against members of the Family Noctuidae. *Appl. Environ. Microbiol.* **62**: 80-86.

Lambert, B., Theunis, W., Ageda, K.R., VanAudenhove, K., Dewell, K.C., Janssens, S., Seurin, K.J. and Peferoen, M. (1992). Nucleotide sequence of gene *cry*3Ca encoding a novel coleopteran-active crystal protein from strain BT109p of *Bacillus thuringiensis* subsp. *kurstaki*. *Gene* **110**:131-132.

Lambert, B. and Peferoen, M. (1992). Insecticidal promise of *Bacillus thuringiensis*. *Bioscience* **42**: 112-121.

Lecadet, M.M. and Martouret, D. (1987). Host specificity of the *Bacillus thuringiensis* δ-endotoxin toward lepidopteran species: *Spodoptera littoralis* Bdv, *Pieris brassicae* L. *J. Invertebr. Pathol.* **49**: 37-48.

Lecadet, M.M. and Dedonder, R. (1971). Biogenesis of the crystalline inclusion of *Bacillus thuringiensis* during sporulation. *Eur. J. Biochem.* **23**: 282-294.

Lee, M.K., You, T.H., Young, B.A., Cotrill, J.A., Valaitis, A.P. and Dean, D.H. (1996). Aminopeptidase N purified from gypsy moth brush border membrane vesicles is a specific receptor for *Bacillus thuringiensis* Cry1Ac toxin. *Appl. Environ. Microbiol.* **62**: 2845-2849.

Lee, M.K., Rajamohan, F., Jenkins, J.L., Curtiss, A.S. and Dean, D.H. (2000). Role of two arginine residues in domain II, loop 2 of Cry1Ab and Cry1Ac *Bacillus thuringiensis* delta-endotoxin in toxicity and binding to *Manduca sexta* and *Lymantria dispar* aminopeptidase N. *Mol. Microbiol.* **38**: 289-298.

Lee, M.K., You, T.H., Gould, F.L. and Dean, D.H. (1999). Identification of residues in domain III of *Bacillus thuringiensis* Cry1Ac toxin that affect binding and toxicity. *Appl. Environ. Microbiol.* **65**: 4513-4520.

Lee, M.J. and Anstee, J.H. (1995). Endoproteinases from the midgut of larval *Spodoptera littoralis* include a chymotrypsin-like enzyme with external binding site. *Insect Biochem. Mol. Biol.* **25**: 49-61.

Lee, M.K., Young, B.A. and Dean, D.H. (1995). Domain III exchanges of *Bacillus thuringiensis* Cry1A toxins affect binding to different gypsy moth midgut receptors. *Biochem. Biophys. Res. Commun.* **216**: 306-312.

Lee, M.K., Milne, R.E., Ge, A.Z. and Dean, D. H. (1992). Location of *Bombyx mori* binding receptor on *Bacillus thuringiensis* δ-endotoxin. *J. Biol. Chem.* **267**: 3115-3121.

Lee, Y.K. and Kang, S.K. (1989). *Seoul Natl. Univ. J. Agric. Sci.* **14**:187-198 (cited in Ph. D. dissertation, Reddy, S.T. (1999). Intracellular proteases in *Bacillus thuringiensis* subsp. *tenebrionis* and their role in the proteolytic processing of the delta-endotoxin.

Lee, S.G., Eckbald, W. and Bulla, L.A. (1985). Diversity of protein inclusion bodies and identification of mosquitocidal protein in *Bacillus thuringiensis* subsp. *israelensis*. *Biochem. Biophys. Res. Commun.* **126**: 953-960.

Lereclus, D., Ribier, J., Klier, A., Menou, G. and Lecadet, M.M. (1984). A transposon-like structure related to the δ-endotoxin gene of *Bacillus thuringiensis*. *EMBO J.* 3: 2561-2567.

Lesieur, C., Vecsey-Semjen, B., Abrami, L., Fivaz, M. and Gisou van der Goot, F. (1997). Membrane insertion: The strategies of toxins. *Mol. Membr. Biol.* **14**: 45-64.

Li, J., Koni, P.A. and Ellar, D.J. (1996). Structure of the mosquitocidal δ-endotoxin CytB from *Bacillus thuringiensis* sp. *Kyushuensis* and implications for membrane pore formation. *J. Mol. Biol.* **257**: 129-152.

Li, J., Carroll, J. and Ellar, D.J. (1991). Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 A^0 resolution. *Nature* **353**: 815-821.

Li, E. and Yousten, A.A. (1975). Metalloprotease from *Bacillus thuringiensis*. *Appl. Microbiol.* **30**: 354-361.

Lilley, M., Ruffel, R.N. and Somerville, H.J. (1980). Purification of the insecticidal toxin in crystals of *Bacillus thuringiensis*. *J. Gen. Microbiol.* **118**: 1-11.

Losey, J.E., Rayor, L.S. and Carter, M.E. (1999). Transgenic pollen harms monarch larvae. *Nature* **399**: 214.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.

Luo, K., Tabashnik, B.E. and Adang, M.J. (1997). Binding of *Bacillus thuringiensis* Cry1Ac toxin to aminopeptidase in susceptible and resistant diamondback moths (*Plutella xylostella*). *Appl. Environ. Microbiol.* **63**: 1024-1027.

Luthy, P., Cordier, J. and Fischer, H. (1982). *In "Microbial and Viral Pesticides"* (E. Kurtak, ed.), pp 35-74. Dekker, New York (cited in Kumar *et al.*, 1996. The insecticidal proteins of *Bacillus thuringiensis*. *Adv. Appl. Microbiol.* **42**: 1-43).

Luthy, P. and Ebersold, H.R. (1981). The entomocidal toxins of *Bacillus thuringiensis*. *Pharmacol. Ther.* **13**: 257-283.

MacIntosh, S.C., Kishore, G.M., Perlak, F.J., Marrone, P.G., Stone, T.B., Sims, S.R. and Fuchs, R.L. (1990). Potentitation of *Bacillus thuringiensis* insecticidal activity by serine protease inhibitors. *J. Agric. Food. Chem.* **38**: 1145-1152.

Mallet, J. and Porter, P. (1992). Preventing insect adaptation to insect resistant crops: are seed mixtures or refugia the best strategy? *Proc. Roy. Soc.* (Lonon). *Ser.* B **250**: 165-169.

Maqbool, S.B., Husnain, T., Raizuddin, S. and Christou, P. (1998). Effective control of yellow rice stem borer and rice leaf folder in transgenic rice *indica* varieties *Basmati* 370 and M 7 using novel δ-endotoxin *cry*2A *Bacillus thuringiensis* gene. *Mol. Breed.* **4**: 501-507.

Marroquin, L.D., Elyassnia, D., Griffitts, J.S., Feitelson, J.S. and Aroian, R.V. (2000). *Bacillus thuringiensis* (*Bt*) toxin susceptibility and isolation of resistance mutants in the nematode *Caenorhabditis elegans*. *Genetics* **155**: 1693-99.

Masson, L., Tabashnik, B.E., Liu, Y.B., Brousseau, R. and Schwartz, J.L. (1999). Helix 4 of the *Bacillus thuringiensis* Cry1Aa toxin lines the lumen of the ion channel. *J. Biol. Chem.* **274**: 31996-32000.

Mathavan, S., Sudha, P.M. and Pechimuthu, S.M. (1989). Effect of *Bacillus thuringiensis israelensis* on the midgut cells of *Bombyx mori* larvae: a histopathological and histochemical study. *J. Invert. Pathol.* **53**: 217-227.

McGaughey, W.H. and Oppert, B. (1999). Mechanisms of insect resistance to *Bacillus thuringiensis* toxins. *Israel J. Entomol.* **32**: 1-14.

McGaughey, W.H. and Whalon, M.E. (1992). Managing insect resistance to *Bacillus thuringiensis*. *Science* **258**: 1451-1455.

McGaughey, W.B. (1985). Insect resistance to the biological insecticide *Bacillus thuringiensis*, *Science* **228**: 193-195.

McGaughey, W.H. (1978). Effects of larval age on the susceptibility of almond moths and Indianmeal moths to *Bacillus thuringiensis*. *J. Econ. Entomol.* **71**: 923-925.

Meenakshisundaram, K.S. and Gujar, G.T. (1988). Proteolysis of *Bacillus thuringiensis* subsp. *kurstaki* endotoxin with midgut proteases of some important lepidopterous species. *Indian J. Exptl. Biol.* **36**: 593-598.

Meza, R., Nunez-Valdez, M.E., Sanchez, J. and Bravo, A. (1996). Isolation of Cry1Ab protein mutants of *Bacillus thuringiensis* by a highly efficient PCR site-directed mutagenesis system. *FEMS Microbiol. Lett.* **145**: 333-339.

Miller, J.H. (1977). *Experiments in Molecular Genetics*. Cold Spring Harbour Laboratory, Cold Spring Harbour, NY.

Milne, R.E., Pang, A.S.D. and Kaplan, H. (1995). A protein complex from *Choristoneura fumiferana* gut-juice involved in the precipitation of δ-endotoxin from *Bacillus thuringiensis* subsp. *sotto*. *Insect Biochem. Molec. Biol.* **25**: 1101-1114.

Milne, R. and Kaplan, H. (1993). Purification and characterization of a trypsin like digestive enzyme from spruce budworm (*Christoneura fumiferana*) responsible for the activation of δ-endotoxin from *Bacillus thuringiensis*. *Insect Biochem. Mol. Biol.* **23**: 663-673.

Mizuki, E., Park, Y.S., Saitoh, H., Yamashita, S., Akao, T., Higuchi, K. and Ohba, M. (2000). Parasporin, a human leukemic cell-recognizing parasporal protein of

Bacillus thuringiensis. 7: 625-634.

Modified animal corn in food supply stirs concerns (article). **(2000).** *Biotech Journal, Intl.* 5: 11-12.

Mohd-Salleh, M.B., Beegle, C.C. and Lewis, L.C. (1980). Fermentation media and production of exotoxin by three varieties of *Bacillus thuringiensis*. *J. Invertebr. Pathol.* 35: 75-83.

Monnerat, R., Masson, L., Brousseau, R., Pusztai-Carey, M., Bordat, D. and Frutos, R. (1999). Differential activity and activation of *Bacillus thuringiensis* insecticidal proteins in diamondback moth, *Plutella xylostella*. *Curr. Microbiol.* 39: 159-162.

Moore, S. and Stein, W.H. (1954). A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. *J. Biol. Chem.* 211: 907-913.

Morse, R.J., Stroud, R.M. and Yamamoto, T. (2001). Structure of Cry2Aa suggests an unexpected receptor-binding epitope structure. *Medline* 9: pp 409.

Nagamatsu, Y., Itai, Y., Hatanaka, C., Funatsu, G. and Hayashi, K. (1984). A toxic fragment from the entomocidal crystal protein of *Bacillus thuringiensis*. *Agric. Biol. Chem.* 48: 611-619.

Nayak, P., Basu, D., Das, S., Basu, A., Ghosh, D., Ramakrishnan, N.A., Ghosh, M. and Sen, S.K. (1997). Transgenic elite *indica* rice plants expression Cry1Ac delta-endotoxin of *Bacillus thuringiensis* are resistant against yellow stem borer (*Scirpophaga incertulas*). *Proc. Natl. Acad. Sci.* (USA) 94: 2111-2116.

Neale, M.C. (1997). Bio-pesticides-harmonisation of registration requirements with in EU directive. 91-414. An industry view. *Bulletin of European and Mediterranean Plant Protection Organization* 27: 89-93.

Nickerson, K.W. and Swanson, J.D. (1981). Removal of contaminating proteases from *Bacillus thuringiensis* parasporal crystals by density gradient centrifugation in NaBr. *Eur. J. Appl. Microbiol. Biotechnol.* 13: 213-215.

Nielsen-LeRoux, C., Hansen, B.M. and Henriksen, N.B. (1998). Safety of *Bacillus thuringiensis*. *IOBC Bull.* 21: 269-272.

Norris, J.R. (1971). The protein crystal toxin of *Bacillus thuringiensis*: biosynthesis and physical structure. *In: Burges HD & Mussey NW ed. Microbial control of insects and mites.* New York, London, Academic Press Inc., pp 229-246.

Novillo, C., Castanera, P. and Ortego, F. (1999). Isolation and characterization of two digestive trypsin-like proteinases from larvae of stalk corn borer, *Sesamia nonagriodes*. *Insect Biochem. Mol. Biol.* 29: 177-184.

Nunez-Valdez, M., Sanchez, J., Lina, L., Guereca, L. and Bravo, A. (2001). Structural and functional studies of alpha-helix 5 region from *Bacillus thuringiensis* Cry1Ab delta-endotoxin. *Biochim. Biophys. Acta* 1546: 122-131.

O'Farrell, H.P. (1975). High resolution two dimensional electrophoresis of proteins. *J. Biol. Chem.* 250: 4007-4021.

Oddou, P., Hartmann, H. and Geiser, M. (1991). Identification and characterisation of *Heliothis virescens* midgut membrane proteins binding *Bacillus thuringiensis* δ-endotoxins. *Eur. J. Biochem.* 202: 673-680.

Ogiwara, K., Indrasith, L.S., Assano, S. and Hori, H. (1992). Processing of δ-endotoxin from *Bacillus thuringiensis* subsp. *kurstaki* HD-1 and HD-73 by gut juices of various insect larvae. *J. Invert. Pathol.* 60: 121-126.

Oppert, B., Kramer, K.I., Beeman, R.W., Johnson, D. and McGaughey, W.H. (1997). Protease mediated insect resistance to *Bacillus thuringiensis* toxins. *J. Biol. Chem.* 272: 23473-23476.

Oppert, B., Krammer, K.J., Johnson, D., Upton, S.J. and McGaughey, W.M. (1996).

Luminal proteinases from *Plodia interpunctella* and the hydrolysis of *Bacillus thuringiensis* Cry1Ac protoxin. *Insect Biochem. Mol. Biol.* **26**: 571-583.

Oppert, B., Kramer, K.J., Johnson, D.E., MacIntosh, S.C. and McGaughey, W.H. (1994). Altered protoxin activation by midgut enzymes from a *Bacillus thuringiensis* resistant strain of *Plodia interpunctella*. *Biochem. Biophys. Res. Commun.* **198**: 940-947.

Oppert, B. (1999). Transgenic plants expressing enzyme inhibitors and the prospects for biopesticide development. In: Koul, O. and Dhaliwal, G.S. editors. *Advances in Biopesticide Research*. Vol I. Harwood Academic, The Netherlands: Harwood Academic. (In press).

Oppert, B. (1999). Protease interactions with *Bacillus thuringiensis* insecticidal toxins. *Arch. Insect Biochem. Physiol.* **31**: 257-272.

Ort, P., Zalunin, I.A., Gasparov, V.S., Chestukhina, G.G. and Stepanov, V.M. (1995). Domain organization of *Bacillus thuringiensis* Cry3Aa δ-endotoxin studied by denaturation in guanidine hydrochloride solutions and limited proteolysis. *J. Protein Chem.* **14**: 241-249.

Ouchterlony, O. (1953). Antigen-antibody reaction in gel types of reactions in coordinated systems of diffusion. *Acta. Pathol. Microbiol. Scandinavica* **32**: 231-240.

Pang, A.S.D. and Gringorten, J.L. (1998). Degradation of *Bacillus thuringiensis* δ-endotoxin in host insect gut juice. *FEMS Microbiol. Lett.* **167**: 281-285.

Paointara, M., Angusthasombat, C. and Panyim, S. (1988). The mosquito larvicidal activity of 130 kDa delta-endotoxin of *Bacillus thuringiensis* var. *israelensis* residues in the 72 kDa amino terminal fragment. *Biochem. Biophys. Res. Commun.* **153**: 294-300.

Peferoen, M., Jansens, S., Reynaerts, A. and Leemans, J. (1990). In M.E. Vayda and W.C. Park (ed.), *Molecular and Cellular Biology of the Potato*, pp193. *CAB International*, Wallingford, United Kingdom.

Perez, C.J. and Shelton, A.M. (1997). Resistance of *Plutella xylostella* (Lepidoptera: Plutellidae) to *Bacillus thuringiensis Berliner* in Central America. *J. Econ. Entomol.* **90**: 87-93.

Perlak, F.J., Deaton, R.W., Armstrong, T.A., Fuchs, R.L., Sims, S.R., Greenplate, J.T. and Fischhoff., D.A. (1990). Insect resistant cotton plants. *Bio/Techn.* **8**: 939-943.

Perlak, F.J., Stone, T.B., Muskopf, Y.M., Petersen, L.J., Parker, G.B., McPherson, S.A., Wyman, J., Love, S., Reed, G., Biever, D. and Fischhoff, D.A. (1993). Genetically improved potatoes: Protection from damage by Colarado potato beetles. *Plant Mol. Biol.* **22**: 313-321.

Peterson, A.M., Carolina, V., Barillas-Mary, V. and Wells, M.A. (1994). Sequence of three cDNAs encoding an alkaline midgut trypsin from *Manduca sexta*. *Insect Biochem. Mol. Biol.* **25**: 49-61.

Petosa, C., Collier, R.J., Klimpel, K.R., Leppla, S.H. and Liddington, R.C. (1997). Crystal structure of the anthrax toxin protective antigen. *Nature* **385**: 833-838.

Pfannenstiel, M.A., Ross, E.J., Kramer, V.C. and Nickerson, K.W. (1984). Toxicity and composition of protease-inhibited *Bacillus thuringiensis* var. *israelensis* crystals. *FEMS Microbiol. Lett.* **21**: 39-42.

Pfannenstiel, M.A., Couche, G.A., Ross, E.J. and Nickerson, K.W. (1986). Immunological relationships among proteins making up the *Bacillus thuringiensis* subsp. *israelensis* crystalline toxin. *Appl. Environ. Microbiol.* **52**: 644-649.

Pichare, M.M. and Kachole, M.S. (1994). Detection of electrophoretically separated

protease inhibitors using X-ray film. *J. Biochem. Biophys. Methods* **28**: 215-224.

Prestidge, L., Gage, V. and Spizizen, J. (1971). Protease activities during the course of sporulation in *Bacillus subtilis*. *J. Bacteriol.* **107**: 815-823.

Rajamohan, F., Alcantara, E., Lee, M.K., Chen, X.J., Curtiss, A. and Dean, D.H. (1995). Single amino acid changes in domain II of a *Bacillus thuringiensis* Cry1Ab δ-endotoxin affect irreversible binding to *Manduca sexta* midgut membrane vesicles. *J. Bacteriol.* **177**: 2276-2282.

Rajamohan, F., Hussain, S.R.A., Cotrill, J.A., Gould, F. and Dean, D.H. (1996). Mutations in domain II, loop 3 of *Bacillus thuringiensis* Cry1Aa and Cry1Ab δ-endotoxins suggest loop 3 is involved in initial binding to lepidopteran midguts. *J. Biol. Chem.* **271**: 25220-25226.

Reddy, S.T., Suresh Kumar, N. and Venkateswerlu, G. (2000). Identification and purification of the 69-kDa intracellular protease involved in the proteolytic processing of the crystal δ-endotoxin of *Bacillus thuringiensis* subsp. *tenebrionis*. *FEMS Microbiol. Lett.* **183**: 63-66.

Reddy, S.T., Suresh Kumar, N. and Venkateswerlu, G. (1998). Comparative analysis of intracellular proteases in sporulated *Bacillus thuringiensis* strains. *Biotechnol. Lett.* **20**: 279-281.

Reddy, S.T. and Venkateswerlu, G. (1997). Intracellular proteases in sporulated *Bacillus thuringiensis* subsp. *tenebrionis*: Detection and analysis by gelatin zymography. *Biochem. Mol. Biol. Intl.* **43**: 935-943.

Roush, R.T. (1996). Can we slow adaptation by pests to insect transgenic crops?, pp 242-263. In G.J. Persley (ed.), *Biotechnology and Integrated Pest Management. CAB International*, Wallingford, United Kingdom.

Roush, R.T. (1994). Managing pests and their resistance to *Bacillus thuringiensis*: can transgenic crops be better than sprays? *Biocontrol Sci. Technol.* **4**: 501-516.

Rukmini V., Chandrahasa Reddy, Y. and Venkateswerlu, G. (2000). *Bacillus thuringiensis* crystal δ-endotoxin: Role of proteases in the conversion of protoxin to toxin. *Biochimie* **82**: 109-116.

Sacchi, V.F., Parenti, P., Hanozet, G.M., Giordana, B., Luthy, P. and Wolfersberger, M.G. (1986). *Bacillus thuringiensis* toxin inhibits K^+-gradient-dependent amino acid transport across the brush border membrane of *Pieris brassicae* midgut cells. *FEBS Lett.* **204**: 213-218.

Salama, H.S. and Sharaby, A. (1985). Histopathological changes in *Heliothis armigera* infected with *Bacillus thuringiensis* as detected by electron microscopy. *Insect Sci. Appl.* **6**: 503-511.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). *Molecular Cloning: A laboratory Manual 2nd ed.* Cold Spring Harbour Laboratory. Cold Spring Harbour, New York.

Sampson, M.N. and Gooday, G.W. (1998). Involvement of chitinases of *Bacillus thuringiensis* during pathogenesis in insects. *Microbiology* **144**: 2189-2194.

Sangadala, S., Walters, F.S., English, L.H. and Adang, M.J. (1994). A mixture of *Manduca sexta* aminopeptidase and phosphatase enhances *Bacillus thuringiensis* insecticidal Cry1Ac toxin binding and $^{86}Rb^+$-K^+ efflux *in vitro*. *J. Biol. Chem.* **269**: 10088-10092.

Santos, M.O., Adang, M.J., All, J.N., Boerma, H.R. and Parrott, W.A. (1997). Testing transgenes for insect resistance using *Arabidopsis*. *Mol. Breed.* **3**: 183-194.

Saxena, D., Flores, S. and Stotzky, G. (1999). Insecticidal toxin in root exudates from *Bt* corn. *Nature* **402**: 480.

Schnepf, E., Crickmore, N., van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R. and Dean, D.H. (1998). *Bacillus thuringiensis* and its pesticidal crystal

proteins. *Microbiol. Molec. Biol. Rev.* 62: 775-806.

Schwartz, J.L., Garneau, L., Savaria, D., Masson, L., Brousseau, R. and Rousseau, E. (1993). Lepidopteran-specific crystal toxins from *Bacillus thuringiensis* form cation and anion-selective channels in planar lipid bilayer. *J. Membrane Biol.* 132: 53-62.

Schwartz, J.L., Potvin, L., Chen, X.J., Brousseau, R., Laprade, R. and Dean, D.H. (1997). Single-site mutations in the conserved alternating-arginine region affect ionic channels formed by Cry1Aa, a *Bacillus thuringiensis* toxin. *Appl. Environ. Microbiol.* 63: 3978-3984.

Schwartz, J.L., Juteau, M., Grochulski, P., Cygler, M., Prefontaine, G., Brousseau, R. and Masson, L. (1997). Restriction of intramolecular movements within the Cry1Aa toxin molecule of *Bacillus thuringiensis* through disulfide bond engineering. *FEBS Lett.* 410: 397-402.

Schwartz, J.L. and Laprade, R. (2000). Membrane permeabilization by *Bacillus thuringiensis* toxins: protein insertion and pore formation. *In: Entomopathogenic Bacteria: from Laboratory to Field Application* (Charles, A., Delecluse, A. and Nielson-Leroux, C. Eds.) pp 1-19. Kluwer Associate Publishing, Norwell, MA.

Schwartz, J.L., Garneau, L., Masson, L. and Brousseau, R. (1991). Early response of cultured lepidopteran cells to exposure to δ-endotoxin from *Bacillus thuringiensis* improvement of calcium and anionic channels. *Biochim. Biophys. Acta.* 1065: 250-260.

Sebesta, K. and Horska, K. (1970). Mechanism of inhibition of DNA-dependent RNA polymerase by exotoxin of *Bacillus thuringiensis*. *Biochim. Biophys. Acta* 209: 357-367.

Sekar, V. (1988). The insecticidal crystal protein gene is expressed in vegetative cells of *Bacillus thuringiensis* var. *tenebrionis*. *Curr. Microbiol.* 17: 347-349.

Sekar, I. and Carlton, B.C. (1985). Molecular cloning of the delta-endotoxin gene of *Bacillus thuringiensis*. *Gene* 33: 151-159.

Selvapandian, A., Reddy, V.S., Ananda Kumar, P., Tiwari, K.K. and Bhatnagar, R.K. (1998). Transformation of *Nicotiana tobaccum* with a native *cry*11a5 gene confers complete protection against *Heliothis armigera*. *Mol. Breed.* 4: 473-478.

Shao, Z., Cui, Y., Liu, X., Yi, H., Ji, J. and Yu, Z. (1998). Processing of delta-endotoxin of *Bacillus thuringiensis* subsp. *kurstaki* HD1 in *Heliothis armigera* midgut juice and the effects of protease inhibitors. *J. Invertebr. Pathol.* 72: 73-81.

Sharma, H.C., Ananda Kumar, P. Seetharama, N., Hari Prasad, K.V. and Singh, B.U. (1999). Role of transgenic plants in pest management in sorghum. In International Symposium on tissue culture and genetic transformation of sorghum, 23-28 Feb 1999, ICRISAT Center, Patancheru, Andhra Pradesh, India.

Sharma, H.C., Taneja, S.L., Leuschner, K., and Nwanze, K.F. (1992). Techniques to screen sorghums for resistance to insect pests. *Information Bulletin No.* 32, ICRISAT, Patancheru.

Shelton, A.M., Tang, J.D., Roush, R.T., Metz, T.D. and Earle, E.D. (2000). Field tests on managing resistance to *Bt*-engineered plants. *Nat. Biotechnol.* 18: 339-342.

Shelton, A.M., Robertson, J.L., Tang, J.D., Perez, C., Eigenbrode, S.D., Preisler, H.K., Wilsey, W.T. and Cooley, R.J. (1993). Resistance of diamondback moth (Lepidoptera: Plutellidae) to *Bacillus thuringiensis* subspecies in the field. *J. Econ. Entomol.* 86: 697-705.

Shewry, P.R. and Gutteridge, S. (1992). Plant protein engineering, Cambridge University Press, Cambridge, UK, pp.346.

Sims, S.R. (1995). *Bacillus thuringiensis* var. *kurstaki* Cry1Ac protein expressed in

transgenic cotton: effects on beneficial and other non-target insects. *Southwest. Entom.* **20**: 493-500.

Singsit, C., Adang, M.J., Lynch, R.E., Anderson, W.F., Wang, A., Cardineau, G. and Ozias-Akins, P. (1997). Expression of a *Bacillus thuringiensis cry*1Ac gene in transgenic peanut plants and its efficacy against lesser cornstalk borer. *Transgen. Res.* **6**: 169-176.

Smedley, D.P., Armstrong, G. and Ellar, D.J. (1997). Channel activity caused by a *Bacillus thuringiensis* endotoxin preparation depends on the method of activation. *Mol. Membr. Biol.* **14**: 13-18.

Smedley, D.P. and Ellar, D.J. (1996). Mutagenesis of three surface-exposed loops of a *Bacillus thuringiensis* insecticidal toxin reveals residues important for toxicity, receptor recognitition and possibly membrane insertion. *Microbiol.* **142**: 1617-1624.

Smith, R.A. and Ulrich, J.T. (1983). Enzyme linked immunosorbent assay for quantitative detection of *Bacillus thuringiensis* crystal protein. *Appl. Environ. Microbiol.* **45**, 586-590.

Sneh, B. and Schuster, S. (1981). Recovery of *Bacillus thuringiensis* and other bacteria from larvae of *Spodoptera littoralis* Boisd. Previously fed on *B. thuringiensis*-treated leaves. *J. Invert. Pathol.* 37: 295-303.

Sneh, B., Schuster, S. and Broza, M. (1981). Insecticidal activity of *Bacillus thuringiensis* strains against the Egyptian cotton leaf worm *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Entomophaga* **26**: 179-190.

Steinhaus, E.A. (1951). Possible use of *Bacillus thuringiensis* subsp. *berliner* as an aid in the biological control of the alfalfa caterpillar. *Hilgardia* **20**: 350-381.

Stepanov, V.M., Chestukhina, G.G., Rudenskaya, G.N., Epremyan, A.S., Osterman, A.L., Khodova, O.M. and Belyanova, L.P. (1981). A new subfamily of microbial serine proteinases? Structural similarities of *Bacillus thuringiensis* and *Thermoactinomyces vulgarisis* extracellular serine proteinases. *Biochem. Biophys. Res. Commun.* **100**: 1680-1687.

Strizhov, N., Keller, M., Konez-kalman, Z., Regev, A., Sneh, B., Schell, J., Koncz, C. and Zilberstein, A. (1996). Mapping of the entomocidal fragment of *Spodoptera*-specific *Bacillus thuringiensis* toxin Cry1C. *Mol. Gen. Genet.* **253**: 11-19.

Strizhov, N., Keller, M. and Mathur, J. (1996). A synthetic *cry*IC gene, encoding a *Bacillus thuringiensis* δ-endotoxin, confers *Spodoptera* resistance in alfalfa and tobacco. *Proc. Natl. Acad. Sci.* (USA) **93**: 15012-15017.

Suresh Kumar, N. and Venkateswerlu, G. (1997). Involvement of endogenous metalloprotease in the activation of protoxin in *Bacillus thuringiensis* subsp. *kurstaki. Biochem. Mol. Biol. Intl.* **42**: 901-908.

Suresh Kumar, N. and Venkateswerlu, G. (1998a). Endogenous protease-activated 66-kDa toxin from *Bacillus thuringiensis* subsp. *kurstaki* active against *Spodoptera littoralis. FEMS Microbiol. Lett.* **159**: 113-120.

Suresh Kumar, N. and Venkateswerlu, G. (1998b). Intracellular proteases in sporulated *Bacillus thuringiensis* subsp. *kurstaki* and their role in protoxin activation. *FEMS Microbiol. Lett.* **166**: 377-382.

Tabashnik, B.E., Cushing, N.L., Finson, N. and Johnson, M.W. (1990). Field development of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). *J. Econ. Entomol.* **83**: 1671-1676.

Tabashnik, B.E. (1994). Evolution of resistance to *Bacillus thuringiensis. Ann. Rev. Entomol.* **39**: 47-79.

Tabashnik, B.E., Finson, N. and Johnson, M.W. (1992). Two protease inhibitors fail to

synergize *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). *J. Econ. Entomol.* **85**: 2082-2087.

Tailor, L.R., Tippett, J., Gibb, G., Ells, S., Pike, D., Jordan, L. and Ely, S. (1992). Identification and characterization of a novel *Bacillus thuringiensis* delta-endotoxin entomocidal to coleopteran and lepidopteran larvae. *Mol. Microbiol.* **6**: 1211-1217.

Tam, A. and Fitz-James, P. (1986). Plasmids associated with a phagelike particle and with a satellite inclusion in *Bacillus thuringiensis* subsp. *israelensis*. *Can. J. Microbiol.* **32**: 382-388.

Tan, Y. and Donovan W.P. (2001). Deletion of aprA and nprA genes for alkaline protease A and neutral protease A from *Bacillus thuringiensis*: effect on insecticidal crystal proteins. *J. Biotechnol.* **84**: 67-72.

Taylor, S.L. and Hefle, S.L. (2001). Will genetically modified foods be allergenic? *J. Allergy. Clin. Immunol.* **107**: 765-771.

Thanabalu, T. and Porter, A.G. (1995). Efficient expression of a 100-kilodalton mosquitocidal toxin in protease-deficient recombinant *Bacillus sphaericus*. *Appl. Environ. Microbiol.* **61**: 4031-4036.

Thompson, A.T., Schnepf, H.E. and Feitelson, J.S. (1995). Structure, function and engineering of *Bacillus thuringiensis* toxins, in: Setlow, J.K. (Ed.), *Genetic Engineering of Bacillus thuringiensis toxins*, Vol. 17, Plenum Press, New York. pp 99-117.

Tojo, A. and Aijawa, K. (1983). Dissolution and degradation of δ-endotoxin by gut juice protease of silkworm, *Bombyx mori*. *Appl. Environ. Microbiol.* **45**: 576-580.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.

Vadlamudi, R.K., Weber, E., Ji, I., Ji, Y.H. and Bulla, L.A.Jr. (1995). Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. *J. Biol. Chem.* **270**: 5490-5494.

Vadlamudi, R.K., Ji, T.H. and Bulla, L.A.Jr. (1993). A specific binding protein from *Manduca sexta* for the insecticidal toxin of *Bacillus thuringiensis* subsp. *berliner*. *J. Biol. Chem.* **17**: 12334-12340.

Vaeck, M., Reynaerts, A., Hoftey, H., Jansens, S., DeBeuckleer, M., Dean, C., Zabeau, M., Van Montagu, M., and Leemans, J. (1987). Transgenic plants proteacted from insect attack. *Nature* **327**: 33-37.

Valaitis, A.P. (1995). Gypsy moth midgut proteinases: purification and characterization of luminal trypsin, elastase and the brush border membrane leucine aminopeptidases. *Insect Biochem. Mol. Biol.* **25**: 139-149.

Valaitis, A.P., Lee, M.K., Rajamohan, F. and Dean, D.H. (1995). Brush border membrane aminopeptidase-N in the midgut of the gypsy moth serves as the receptor for the Cry1Ac δ-endotoxin of *Bacillus thuringiensis*. *Insect Biochem. Mol. Biol.* **25**: 1143-1151.

Van der Salm, T.I, Bosch, D., Honee, G., Feng, L., Munsterman, E., Bakker, P., Stiekema, W.J. and Visser, B. (1994). Insect resistance of transgenic plants that express modified *Bacillus thuringiensis cry*1Ab and *cry*1C genes: a resistance management strategy. *Plant Mol. Biol.* **26**: 51-59.

Van Frankenhuyzen, K., Gringorten, J.L., Milne, R.E., Gauthier, D., Pusztai, M., Brousseau, R. and Masson, L. (1991). Specificity of activated Cry1A proteins from *Bacillus thuringiensis* subsp. *kurstaki* HD1 for defoliating forest Lepidoptera. *Appl. Environ. Microbiol.* **57**: 1650-1655.

Van Rensburg, J.B.J. (1999). Evaluation of *Bt*-transgenic maize for resistance to the stem borers *Busseola fusca (Fuller)* and *Chilo partellus (Swinhoe)* in South Africa. *South-African Journal of Plant and Soil* 16: 38-43.

Van Rie, J., Jansens, S., Hoftey, H., Degheele, D. and Van Mellaert, H. (1989). Specificity of *Bacillus thuringiensis* - endotoxins. Importance of specific receptors on the brush border membrane of the midgut of target insects. *Eur. J. Biochem.* 186: 239-247.

Van Rie, J., McGaughey, W.H., Johnson, D.E., Barnett, B.D. and Van Mellaert, H. (1990a). A mechanism of insect resistance to the microbial insecticide *Bacillus thuringiensis*. *Science* 247: 72-74.

Van Rie, J. (2000). *Bacillus thuringiensis* and its use in transgenic insect control technologies. *Int. J. Med. Microbiol.* 290: 463-469.

Venkateswerlu, G. and Stotzky, G. (1990). Simple method for the isolation of the antilepidopteran toxin from *Bacillus thuringiensis* subsp. *kurstaki*. *Biotechnol. Appl. Biochem.* 12: 245-251.

Walters, F.S. and English, L.H. (1995). Toxicity of *Bacillus thuringiensis* δ-endotoxins toward the potato aphid in an artificial diet bioassay. *Entomol. Exp. Applicata* 77: 211-216.

Ward, S.E. and Ellar, D.J. (1988). Cloning and expression of two homologous genes of *Bacillus thuringiensis* subsp. *israelensis* which encode 130-kilodalton mosquitocidal proteins. *J. Bacteriol.* 170: 727-735.

Warren, G.W., Koziel, M.G., Mullins, M.A., Nye, G.J., Desai, N., Carr, B. and Kostichka, N.K. (September, 1994). World Intellectual Property Organization patent WO 94/21795.

Wasano, N., Kim, K.H. and Ohba, M. (1998). Delta-endotoxin proteins associated with spherical parasporal inclusions of the four Lepidoptera-specific *Bacillus* strains. *J. Appl. Microbiol.* 84: 501-508.

Wider, W.R. and Whiteley, K.H.R. (1990). Two highly related insecticidal crystal proteins of *Bacillus thuringiensis* subsp. *kurstaki* possess different host range specificities. *J. Bacteriol.* 72: 965-974.

Williams, S., Friedrich, L., Dincher, S., Carozzi, N., Kessmann, H., Ward, E. and Ryals, J. (1992). Chemical regulation of *Bacillus thuringiensis* δ-endotoxin toxin expression in transgenic plants. *Bio/Techn.* 10: 540-543.

Wilson, F.D., Flint, H.M., Deaton, R.W., Fischhoff, D.A., Perlak, F.J., Armstrong, T.A., Fuchs, R.L., Berberich, S.A., Parks, N.J. and Stapp, B.R. (1992). Resistance of cotton lines containing a *Bacillus thuringiensis* toxin to pink bollworm (Lepidoptera: Gelechiidae) and other insects. *J. Econ. Entomol.* 85: 1516-1521.

Wolfersberger, M.G., Chen, X.J. and Dean, D.H. (1996). Site-directed mutations in the third domain of *Bacillus thuringiensis* δ-endotoxin Cry1Aa affect its ability to increase the permeability of *Bombyx mori* midgut brush border membrane vesicles. *Appl. Environ. Microbiol.* 62: 279-282.

Wolfersberger, M.G. (1990). The toxicity of two *Bacillus thuringiensis* δ-endotoxins to gypsy moth larvae is inversely related to the affinity of binding sites on the midgut brush border membrane for the toxins. *Experientia* 46: 475-477.

Wolfersberger, M. G. (1989). Neither barium nor calcium prevents the inhibition by *Bacillus thuringiensis* - endotoxin of sodium or potassium gradient dependent amino acid accumulation by tobacco hornworm midgut brush border membrane. *Arch. Insect Biochem. Biophys.* 12: 267-277.

Wong, H.C., Schnepf, H.E. and Whiteley, H.R. (1983). Transcriptional and translational start sites for the *Bacillus thuringiensis* crystal protein gene. *J. Biol. Chem.* 258:

1960-1967.

Wu, S.J. and Dean, D.H. (1996). Functional significance of loops in the receptor binding domain of *Bacillus thuringiensis* Cry3Aa δ-endotoxin. J. *Mol. Biol.* **255**: 628-640.

Wu, D. and Aronson, A.I. (1990). Use of mutagenic oligonucleotides for defining regions of a *Bacillus thuringiensis* δ-endotoxin involved in toxicity. In: *Proceedings of 5th International Coloquim on Invertebrate Pathology and Microbial Control*, 20-24 August 1990, Adelaide, Australia. *Society of Invertebrate Pathology*, Adelaide, Australia. pp 272-277.

Yaoi, K., Nakanishi, K., Kadotani, T., Imamura, M., Koizumi, N., Iwahana, H. and Stato, R. (1999). *Bacillus thuringiensis* Cry1Aa toxin-binding region of *Bombyx mori* aminopeptidase N. *FEBS Lett.* **463**: 221-224.

Yokoyama, Y., Kohda, K. and Okamoto, M. (1998). CytA protein, a delta-endotoxin of *Bacillus thuringiensis* subsp. *israelensis* is associated with DNA. *Biol. Pharm. Bull.* 21: 1263-1266.

Yosida, K.I., Matsushima, Y., Sen, K., Sakai, H. and Kaino, J. (1989). Insecticidal activity of a peptide containing the 30th and 695th amino acid residues of the 130 kDa proteins of *Bacillus thuringiensis* var. *israelensis*. *Agric. Biol. Chem.* **53**: 2121-2227.

Zhang, J.H., Wang, C.Z. and Qin, J.D. (2000). The interactions between soybean trypsin inhibitor and delta-endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera* larva. *J. Invertebr. Pathol.* 75: 259-266.

Zhao, J.Z., Fan, X.L., Shi, X.P., Zhao, R.M. and Fan, Y.L. (1997). Gene pyramiding: an effective strategy of resistance management for *Helicoverpa armigera* and *Bacillus thuringiensis*. *Resist. Pest Manage.* **9**: 19-21.

Zhao, R.M., Shi, X.P., Wang, J.H. and Fan, Y.L. (1996). Transgenic tobacco plants expressed both B.t and CpTI genes and their homozygotes. *Rice Biotechn. Quart.* **25**: 35-36.

Zhu, Y.C., Oppert, B., Krammer, K.J., McGaughey, W.M. and Dowdy, A.K. (1997). cDNAs for a chymotrypsin like protein from two strains of *Plodia interpunctella*. *Insect Biochem. Mol. Biol.* 27: 1027-1037.

Publications

V. Rukmini, **Y. Chandrahasa Reddy** and G. Venkateswerlu. (2000). *Bacillus thuringiensis* crystal δ-endotoxin: Role of proteases in the conversion of protoxin to toxin. *Biochimie* **82**: 109-116.

Y. Chandrahasa Reddy, V. Rukmini, H.C. Sharma and G. Venkateswerlu. (2001). Isolation and Characterization of Endogenous Protease Mutants of *Bacillus thuringiensis* subsp. *kurstaki* HD1: Their role in Activation of Protoxin to Toxin (Submitted to *Journal of Invertebrate Pathology*).