Molecular characterization of local isolates of *Bacillus thuringiensis* effective against *Achaea janata*: Identification of Cry toxins, their genes and receptors - cloning of a novel fat body aminopeptidase as alternate target for Cry toxins and its comparison with midgut aminopeptidase

Thesis Submitted for the degree of

DOCTOR OF PHILOSOPHY

by

BUDATHA MADHUSUDHAN

Department of Animal Sciences
School of Life Sciences
University of Hyderabad
Hyderabad, 500 046, INDIA

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Enrollment No. 01LAPH02

Dedicated to

Parents

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DECLARATION

I hereby declare that the work embodied in this thesis entitled "**Molecular characterization of local isolates of *Bacillus thuringiensis* effective against *Achaea janata*: Identification of Cry toxins, their genes and receptors - cloning of a novel fat body aminopeptidase as alternate target for Cry toxins and its comparison with midgut aminopeptidase**" has been carried out by me under the supervision of Prof. Aparna Dutta Gupta and that this has not been submitted for any degree or diploma of any other university earlier.

Budatha Madhusudhan _____
01LAPH02
(Ph.D Student)

Prof. Aparna Dutta Gupta _____
(Research Supervisor)

University of Hyderabad
(A central university established in 1974 by an act of parliament)

Hyderabad- 500 046, INDIA

CERTIFICATE

This is to certify that **Mr. Budatha Madhusudhan** has carried out the research work embodied in this thesis under my supervision and guidance for a full period prescribed under the Ph.D. ordinance of this University. We recommend his thesis **"Molecular characterization of local isolates of *Bacillus thuringiensis* effective against *Achaea janata*: Identification of Cry toxins, their genes and receptors - cloning of a novel fat body aminopeptidase as alternate target for Cry toxins and its comparison with midgut aminopeptidase"** for submission for the degree of Doctor of Philosophy of this University.

Prof. Aparna Dutta Gupta _____
(Research Supervisor)

Head _____
Department of Animal Sciences

Dean _____
School of Life Sciences

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Abbreviations

APN	:	Aminopeptidase N
Bt	:	*Bacillus thuringiensis*
20E	:	20-Hydroxyecdysone
BCIP	:	5-Bromo-4-chloro-3-indolyl phosphate
BSA	:	Bovine serum albumin
BBMV		Brush border membrane vesicles
cDNA	:	Complimentary DNA
CRD	:	Cross reacting deteminant
DEPC	:	Diethyl pyrocarbonate
DNA	:	Deoxyribonucleic acid
DOR	:	Directorate of Oil seeds Research
dNTPs	:	Deoxyribonucleoside triphosphates
DTT	:	1, 4-Dithiothreitol
EDTA	:	Ethylenediaminetetraacetic acid
EGTA	:	Ethylene glycol-bis(2-aminoethylether)-N,N,N`,N`-tetraacetic acid
HEPES	:	N-(2-hydroxyethyl) piperazine-N`-(2-ethanesulfonic acid)
GPI	:	Glycosylphosphotidylinosital
Hex	:	Hexamerin
IgG	:	Immunoglobulin
IPM	:	Integrated pest management
IPTG	:	Isopropyl D-thiogalactoside
kDa	:	Kilo Dalton
LB	:	Luria-Beratani medium
LLI	:	Late-last instar
mg	:	milligram
MOPS	:	3-Morpholinopropanesulfonic acid

GalNAc	:	N –Acetyl galactosamine
GluNAc	:	N-Acetyl glucosamine
SBA	:	Soybean agglutinin
WGA	:	Wheat germ agglutinin
mRNA	:	messenger ribonucleic acid
NBT	:	Nitrotetrazolium blue
ng	:	Nanogram
PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PKC	:	Calcium/phospholipid dependent protein kinase or Protein kinase C
PMSF	:	Phenylmethylsulfonyl fluoride
RNA	:	Ribonucleic acid
RACE	:	Rapid amplification cDNA ends
SDS	:	Sodium dodecyl sulfate
SDS-PAGE	:	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
TAE	:	Tris-Acetate-EDTA buffer
TBS	:	Tris buffered saline
TE	:	Tris-EDTA
TEMED	:	N, N, N`, N`, tetramethylethyleneediamine
Tris	:	Tris (hydroxymethyl) aminomethane
v/v	:	Volume/volume
w/v	:	Weight/volume
X-Gal	:	5-Bromo-4-chloro-3-indolyl D- galactoside
µg	:	microgram
µM	:	micromolar

Introduction and Review of literature

Introduction:

A fundamental shift to a total system approach for crop protection is urgently needed to resolve escalating economic and environmental consequences of combating agricultural pests. Pest management strategies have long been dominated by quests for "silver bullet" products to control pest outbreaks (Lewis et al, 1997). The present approach to agrochemical discovery has shifted focus from traditional *in vivo* screens, to more closely resemble the pharmaceutical model of mechanism, which will address several critical components of the discovery process including target identification and validation. Mainly the research is directed at key invertebrate pests of crops principally lepidopteran insects (Lewis et al, 1997; Trewavas, 2001; Kumar et al, 2004).

In India, the use of the amount of chemical pesticides increased five times from 1965-66 to 1990-91. Extensive usage of pesticides causes additional hazards like insecticide resistance and resurgence of insect pests. So far, over 700 insect pests are reported to have developed resistance world wide to a variety of insecticides, out of which 33 have been reported from India (Rai, 2004). Crop losses due to arthropods, diseases, and weeds, though disputed by some as a valid measure, have increased on a world basis from 34.9% in 1965 to 42.1% in 1988-1990 (Lewis et al, 1997) despite the intensification of pest control methods. Chemical control of insect pests is one of the most costly aspects of crop production, estimated to be $3-5 billion annually world wide. The costs for pesticide application and from economic losses to pests can be staggering. According to recent estimations, each year U.S.A is spending over $400 million just for the control of lepidopteran pests. The therapeutic approach of killing pest organisms with toxic chemicals has been the prevailing pest control strategy for over 50 years (Kumar et al, 2004). Recently in 2006, World Watch Institute released a report mentioning that around the world people are using on an average 3 kg pesticides per hectare and due to this overuse nearly three lakh people are getting affected by cancers, other diseases and birth defects (Pesticide News Letter, 2006). So safety problems and ecological disruptions continue to develop (Wright, 1996), and there are renewed appeals for not only effective, but also safe, and economically acceptable alternatives (Benbrook, 1996). Considerable efforts are being made and trend is shifting towards the use of modern chemistry and molecular biology to replace traditional pesticides with less hazardous chemicals or nontoxic biological agents.

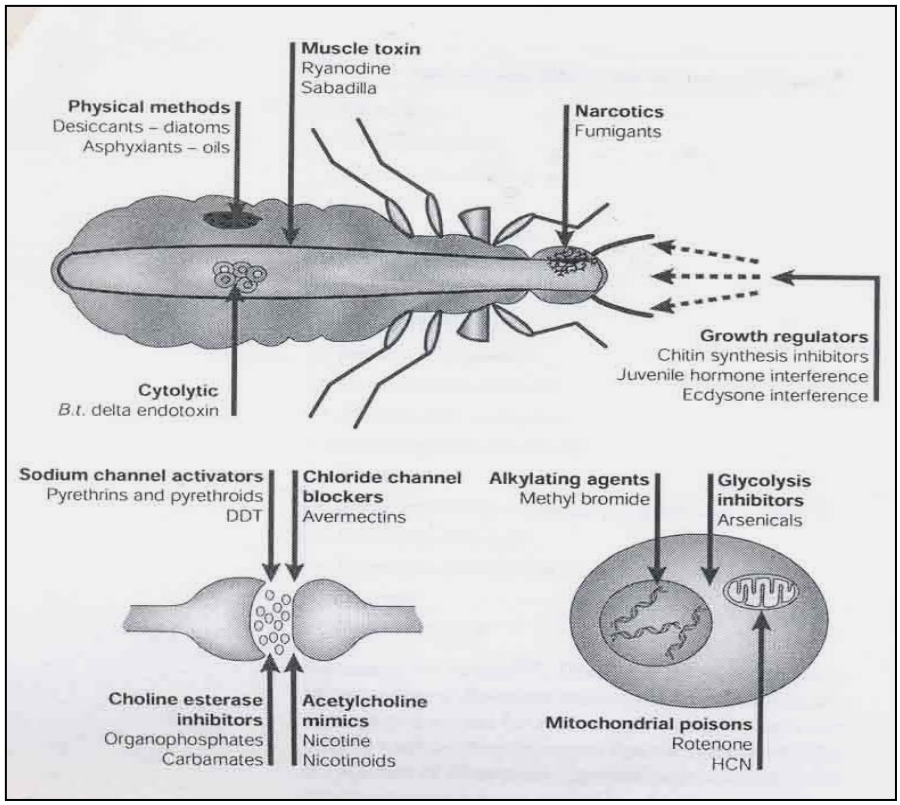


Fig. 1: Different types of pestcidal agents acting on different targets: (Adopted from Schneider, 2000, Nature Reviews) Resistance to wide variety of Chemical insecticides. The process of development of safe eco-friendly pesticides with new/novel target sites, which are less or not hazardous to human, is never ending process. To achieve this, one has to (i) identify important physiological processes, (ii) characterize the molecule involved in, and (iii) clone the corresponding genes

In fact satisfactory solutions to pest problems will also require a shift to understanding and promoting naturally occurring control agents and at present IPM (Integrated Pest Management) refers to the implementation of the principle of incorporating the full array of pest management practices together with production objectives into a total systems approach (Lewis et al, 1997). The primary objective in IPM is not the elimination of a pest organism totally from ecosystem but to bring it into acceptable bounds. The four major problems encountered with conventional pesticides are toxic residues, pest resistance, secondary pests, and pest resurgence. Therefore, a mere switch to nontoxic pesticides, such as microbial or inundate releases of natural enemies, even though helpful in reducing environmental contamination and safety problems, still does not completely address the ecological weakness of the conventional pest control approach. Instead of having an agent which kills pest

quickly, it is desirable to have products, which retard the development of the pest as well as block the reproduction. Furthermore the rapid killing of pests may provide, immediate results but it destroys a resource for parasitoids leading to resurgence of subsequent generations of pests (Lewis et al, 1997). So the approach of slow killing of pests is preferable in larger perspective and it has been already proved beneficial in number of instances. For example *Cotesia marginiventris* is a key parasitoid, for managing the beet armyworm, when the larvae get intoxicated by sub lethal dosages of MVP (a *Bacillus thuringiensis* derived biopesticide), it experiences retarded development and feeding. Further, it is subjected to higher parasitism than non-treated beet armyworm larvae (Soares et al, 1994). Others also made similar report for *B. thuringiensis* and a parasitoid of gypsy moths. In total these kinds of approaches strengthen the parasitoids' effect during subsequent generations.

Review of literature:

Bacillus thuringiensis:

The Japanese bacteriologist Ishiwata first discovered *Bacillus thuringiensis* (Bt) in a diseased silk worm in 1901. It was co-discovered by Berliner from Thuringia in Germany, as a causative agent of a flour moth disease and was named. It was first used as a commercial insecticide in France in 1938, and then in the USA in the 1950's. However, these early products were replaced with more effective ones in the 1960's, when various potent pathogenic strains were discovered with particularly activity against different types of insects.

Bacillus thuringiensis is a gram positive, rod shaped, aerobic and spore forming entomo pathogenic bacterium. Its toxicity is due to crystal toxins (Cry) contained in the parasporal crystal inclusion, produced during the sporulation stage. In addition to Cry proteins other kinds of insecticidal proteins have been described in *B. thuringiensis* like cytolytic (Cyt) toxins and vegetative insecticidal proteins (Vip) proteins. The Vips are secreted during vegetative growth and do not exhibit any similarity with either Cry or Cyt toxins (Schnepf et al, 1998). In susceptible insects, crystals are solubilized in the midgut and the released proteins are activated by midgut proteases. Activated toxins bind to specific receptors in the apical membrane of the midgut cells, producing pores that lead to the lysis of the cell and finally to the death of the insect (Gill et al, 1992). Unlike typical nerve-poison insecticides, Bt acts by producing toxic proteins which interact with the cells of the gut lining

of susceptible insects (Van Rie et al, 1990). These Bt proteins paralyze the digestive system, and the infected insect stops feeding within hours. Bt-affected insects generally die from starvation, which can take several days. Occasionally, the bacteria enter the insect's body cavity or hemocoel and multiply within it (Cerestiaens et al, 2001). However, in most insects it is the interaction of the protein crystal that is lethal to the insect.

Intensive screening programs are being carried out worldwide in order to identify novel or different combination of *cry* gene and Cry proteins with toxic potential against different organisms with specificity for a much broader range of insect pests or to provide alternatives, after the appearance of insect resistance (Schnepf et al, 1998).

B. thuringiensis genome:

B. thuringiensis strains have a genome size of 2.4 to 5.7 million bp (Carlson et al, 1994). Comparison of physical map of Bt with *B. cereus* chromosomal map suggests that all chromosomes of two species have a similar organization with variability in the terminal half (Carlson et al, 1996). Most *B. thuringiensis* isolates have several extrachromosomal elements, some of them circular and others linear (Carlson et al, 1994). Its well known that the Cry toxins are encoded by genes, which are located on plasmids (Gonzalez et al, 1981) of *B. thuringiensis*. There could be 5 or 6 different plasmids in a single Bt strain, and they could encode different toxins. The plasmids can be exchanged between Bt strains by a conjugation-like process, so there could be horizontal transfer of *cry* genes between different strains, which leads to the potentially wide variety of strains with different combinations of Cry toxins. In addition to this, Bt also contains transposon like transposable genetic elements that flank genes and could be excised from one part of the genome and inserted elsewhere (Mahillon et al, 1994). These above features increase the variety of toxins produced naturally by Bt strains, and provide the basis to create genetically engineered strains with novel toxin combinations.

Transcription and translation of Cry toxin genes:

A common characteristic of the *cry* genes is their expression during the stationary phase. Their products generally accumulate in the mother cell compartment to form a crystal inclusion that can account for 20 to 30% of the dry weight of the sporulated cells (Aronson, 1993). The very high level of crystal protein synthesis in *B. thuringiensis* and its coordination

with the stationary phase are controlled by a variety of mechanisms occurring at the transcriptional, post transcriptional, and post translational levels (Baum, 1995). During sporulation, *B. thuringiensis* produces intracellular, crystalline inclusions comprised of a mixture of protoxins. A major class of these protoxin genes, designated as *cry1*, is transcribed from two overlapping promoters (BtI and BtII) utilizing RNA polymerase containing sporulation sigma factors σ^E and σ^K, respectively (Hofte and Whiteley, 1989; Aronson, 1993; Sedlak et al, 2000). RNA polymerase generally transcribes the *cry* genes throughout the sporulation, but there are variations in the types of promoters, as well as in the times of transcription of these genes (Agaisse and Lereclus, 1995; Baum, 1995). Different subspecies of *B. thuringiensis* produce their own array of protoxins, which very often is a mixture of Cry1 types (Hofte and Whiteley, 1989; Aronson, 1993; Cheng et al, 1999). The *cry*1 genes are transcribed differentially (Aronson, 1995) and there is variation in the relative amounts of the protoxins in inclusions (Masson et al, 1998). The differences in the composition of the medium also influences the type and yield of protoxins (Dulmage, 1970). Hence, the overall toxicity profile of an isolate, not only depends on regulation of expression of individual *cry* genes but also on the relative amounts of the various protoxins in inclusion, their solubility (Jaquet et al, 1987; Aronson, 1995) and synergism between certain toxins (Tabashnik, 1992; Lee et al, 1996; Perez et al, 2005). The stability of mRNA is an important contributor to the high level of toxin production in *B. thuringiensis*. The half-life of *cry* mRNA, about 10 min, is at least five fold greater than the reported half-life of an average bacterial mRNA. The Cry proteins generally form crystalline inclusions in the mother cell compartment. Depending on the protoxin composition, the crystals have various forms: bipyramidal (Cry1), cuboidal (Cry2), flat rectangular (Cry3A), irregular (Cry3B), spherical (Cry4A and Cry4B), and rhomboidal (Cry11A). This ability of the protoxins to crystallize determine their susceptibility to proteolytic degradation (Schnepf et al, 1998).

Cry toxin structure-function interpretation:

The structures of three crystal proteins namely Cry3A (Li et al, 1991), Cry1Aa (Grochulski et al, 1995) and Cry4Ba (Boonserm et al, 2005) have been solved by X-ray crystallography. Sequence identity has been demonstrated that Cry3A and Cry1Aa show about 36% amino acid identity and the same similarity was reflected in their three-dimensional structures**.** The tertiary structure of delta endotoxin is comprised of three distinct functional domains connected by a short conserved sequence. Domain 1 is a bundle of seven

alpha–helices, some or all of which can insert into the gut cell membrane, creating a pore through which ions can pass freely (Li et al, 1991).

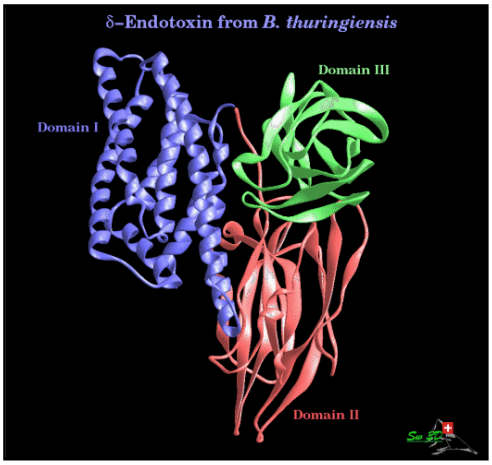


Fig. 2: Three dimensional structure of delta endotoxin (from Prof. Ellars home page)

Domain 2 consists of three anti-parallel beta-sheets, similar to that of antigen-binding regions of immunoglobulins, suggesting that this domain binds receptors in the gut. Domain 3 is a tightly packed beta sandwich, which is thought to protect the exposed end (C-Terminus) of the active toxin preventing further cleavage by gut proteases and also a determinant of insect specificity/receptor binding (Schnepf et al, 1990; Li et al, 1991). The core of the molecule is built from five sequence blocks, which are a highly conserved feature of all the Bt toxins indicating that all the proteins in this Cry family will adopt the same general fold. Phylogenetic analysis of the domains of delta endotoxins revealed that domain I is the most conserved and domain II is hyper variable among all endotoxins (Bravo, 1997). It was further observed that there was a high degree of sequence similarity among the proteins in the N-terminal region, which confers toxicity to the protein, but also there were highly conserved blocks in the C-terminal region (Bravo et al, 2002).

Bt toxin classification:

Cloning of the first *cry* gene, named *cry1A*a, was reported in and since then over 200 *cry* genes encoding a variety of crystal proteins have been cloned and characterized (Schnepf and Whiteley, 1981; Crickmore et al, 1998). The Cry toxins have been classified into Cry1 to Cry 40 families, on the basis of amino acid sequence homology (www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/index.html). The toxins within the Cry1A

subfamily exhibit variation in toxicity and specificity due to minor amino acid substitutions (Wu and Aronson, 1992). Previously Cry toxins were classified based on their crystal morphology as bypyramidal specific to lepidoptera (Cry1Aa, Ab, Ac etc.), cuboidal (Cry 2) to lepidoptera and diptera and flat or irregular (Cry 3) to coleoptera.

Mechanism of action of Cry toxin:

The mechanism of action of the *B. thuringiensis* Cry proteins involves (a) solubilization of the crystal in the insect midgut, (b) proteolytic processing of the protoxin by midgut proteases, (c) binding of the active Cry toxin to midgut receptors and (d) insertion of the toxin into the apical membrane of midgut cell to create ion channels or pores (Schnepf et al, 1998). In the mechanism of action of Cry toxin, these steps vary from insect to insect and each step has its unique role in the toxicity, as well as in the development of resistance (Oltean et al, 1999). So it is extremely important to study the mechanism of action of Cry toxins in various pests.

(a) Solubility: For the toxin to be effective, susceptible insects must feed on crystal proteins. The solubility of particular crystal depends on the compositon of the protoxins and pH in the gut of the susceptible insect. However, the crystals have to be solubilized rapidly and

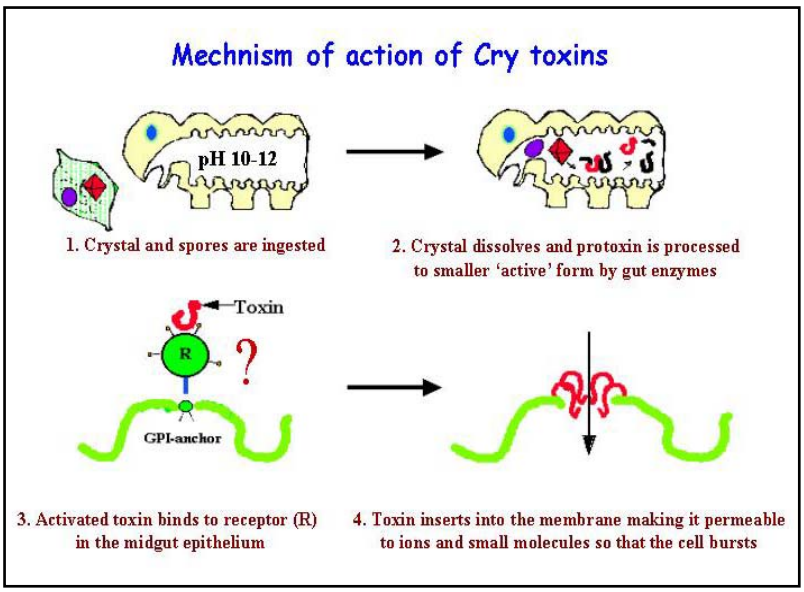


Fig. 3: Mechanism of action of Cry toxins (modified from Prof. Ellar home page)

efficiently in the larval gut of insect to become biologically active. The structure and the solubility characteristics of a crystal presumably depend on factors like the secondary structure of the protoxin, the energy of the disulfide bonds, and the presence of additional *B. thuringiensis*-specific components (Aronson et al, 1991). For most lepidopterans, protoxins are solubilized under the alkaline conditions of the insect midgut. Differences in the extent of solubilization sometimes, explain variations in the degree of toxicity among Cry proteins (Aronson et al, 1991; Du et al, 1994). A reduction in solubility is speculated to be one of the potential mechanism for insect resistance (McGaughey et al, 1998).

(b) Proteolytic processing by midgut proteases: The midgut lumen of lepidopteran larvae is known to contain a variety of alkaline proteases, mainly of serine protease class, includes predominantly trypsin and chymotrypsin like protease activities (Terra and Ferreira, 1994; Broadway, 1997). Such midgut proteases are likely to be responsible for delta endotoxin activation. The different types of protoxins undergo extensive proteolysis at both their N and C termini by trypsin like proteases to produce activated toxin with molecular weight of ≈ 65 kDa. This protease-resistant toxic core is derived from the N-terminal half of the protoxin by removal of 500 to 600 amino acid residues from the C terminus and the first 27 to 29 N-terminal residues (Schnepf et al, 1985; Rukmini et al, 2000). The small fragments which form along with activated toxin, range between 10- to 35-kDa, do not play any role in toxicity and are proteolysed into peptides.

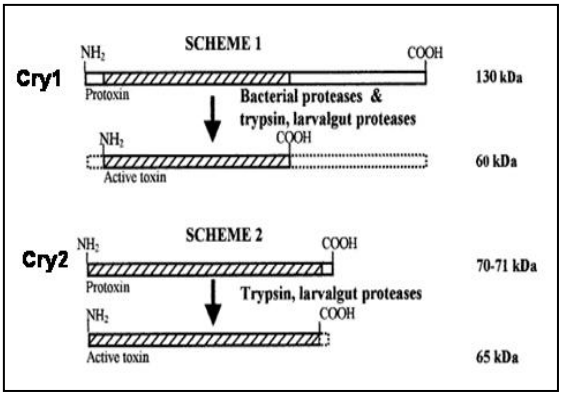


Fig. 4: Schematic representation for the activation of Cry1 and Cry2 toxins (Rukmini et al, Biochimie, 2000).

The proper proteolytic cleavage of *B. thuringiensis* delta endotoxin is the prerequisite for toxicity and insufficient processing or over digestion of a toxin leads to its inactivation

(Lightwood et al, 2000). For example it has been demonstrated that *Plodia interpunctella* (Indian meal moth) which is resistant to the delta endotoxins of ***B. thuringiensis*** subsp. *entomocidus* HD-198 exhibited a lower protoxin activation rate than susceptible insects because of the decrease in the total proteolytic activity of the gut extract (Lightwood et al, 2000). Oppert et al, (1997) and Sayyed et al, (2005) reported that lack of major Bt protoxin activating gut protease resulted in the development of Bt resistance in *P. interpunctella.* Inagaki et al, (1992) demonstrated that in *Spodoptera litura* processing of *B. thuringiensis* subsp. *kurstaki* HD-1 leads to complete degradation of the toxin by proteases is the cause of the lack of potency of HD-1 against this pest. The reduced sensitivity of the Cry1C to fifth instar larvae of S. *litura* was due to the degradation of toxin by proteases (Keller et al, 1996). The battery of midgut proteases that an insect possesses is therefore likely to be a major determinant of toxin potency. Henceforth, it is extremely important to study the gut protease activities of the individual insect in order to understand the proteolytic activation of Bt toxins and its successful usage in pest management.

(c) Binding of activated toxin to receptors: The activated toxin binds to different types of receptors present in the midgut epithelium of susceptible insects. The toxin-binding proteins from several insects have been identified by ligand blot analysis of brush border membrane vesicles (BBMVs) prepared from the midguts of the insects. Different types of molecules, i.e., membrane bound aminopeptidase N, (Sangadala et al, 1994; Gill, 1995; Lee et al, 1996; Denolf et al, 1997; Lorence et al, 1997; Yaoi et al, 1997; Oltean et al, 1999; Gill and Ellar, 2002; Nakanishi et al, 2002; Rajagopal et al, 2002, 2003; Herrero et al, 2005), cadherin-like protein (Vadlamudi et al, 1995; Nagamatsu et al, 1999; Gahan et al, 2001; Hara et al, 2003), anionic glycoconjugate (Valaitis et al, 2001), Glycolipids (Griffitts et al, 2005) and alkaline phosphatase (Jurat-Fuentes and Adang, 2004; Fernandez et al, 2006) in the insect midgut have been found to serve as receptors for the Cry toxins.

Aminopeptidase N (APN): Apparently, APN is the most extensively studied Cry toxin receptor identified and isolated from several lepidopteran insect pests. Membrane bound APNs are well-characterized ectoenzymes that are widely distributed in animal tissues and are implicated in a variety of functions viz. acting as receptors, cell adhesion molecules and signal transduction components (Taylor, 1993). Gene silencing by means of double stranded RNA clearly demonstrated that APN acts as a receptor to Cry1C in *S. litura* (Rajagopal et al, 2002). Further, transgenic expression of APN in *Drosophila* rendered this dipteran

susceptible to Cry1Ac confirming the role of APN as receptor for this toxin (Gill and Ellar, 2002). Also, resistance of *S. exigua* to Cry1Ca was traced to the lack of APN gene expression (Herrero et al, 2005). In another study, the toxin-induced increase in the $86Rb^{+}$ efflux from lipid vesicles reconstituted with APN and the damping of short circuit current (I_{SC}) for Cry1Ac following APN removal from the midgut cell membranes by phosphotidyl inositol phospholipase C treatment provided further support for the role of APN as a receptor (Sangadala et al, 1994; Lee et al, 1996). The 170 kDa APN from *H. virescens* plays a role in pore formation in membrane vesicles (Luo et al, 1997). In *Manduca sexta*, 120 kDa APN mediates channel formation in planar lipid bilayers (Schwartz et al, 1997). Altogether the results from *in vivo* as well as *in vitro* experiments prove that APNs play significant role in establishment of the toxic and lytic effect in the insect gut caused by Cry toxins.

Alkaline phosphatase: Possible involvement of alkaline phosphatase (ALP) in the Cry toxin action has recently been proposed (Jurat-Fuentes and Adang, 2004; Fernandez et al, 2006) however, additional data are needed to acknowledge its significance. Alkaline phosphatase was shown to be a Cry1Ac binding protein in *M. sexta* (McNall and Adang, 2003) and *Heliothis virescens* (Jurat-Fuentes and Adang, 2004). The membrane-anchored form (m-ALP) is tethered to the membrane by a GPI-anchor, whereas the soluble form is localized primarily in the cavity of the goblet cells (Eguchi et al, 1995).

Cadherin: On the other hand, the role of cadherin-like protein as Cry toxin receptor has been confirmed by a number of studies. For example, disruption of a cadherin gene by a retrotransposon-mediated insertion has been linked to the high level of resistance to Cry1Ac toxin in *H. virescens* (Gahan et al, 2001). Expression of a cadherin-like protein from a *Bombyx mori* on the surface of Sf 9 cells made these otherwise resistant cells, sensitive to Cry1Aa (Nagamatsu et al, 1999). Hara et al, (2003) observed inhibition of the binding of Cry1Aa and Cry1Ac to BtR175 after pretreatment with anti-BtR175 antibody, as this suppressed the lytic activity of the toxins on collagenase-dissociated *B. mori* midgut epithelial cells. Above reports suggest that the cadherin-like protein plays an important role in Cry toxin susceptibility and it is the functional Cry toxin receptor in the insect midgut at the genetic and cellular levels. Recently, a model of the sequential participation of cadherin receptor and APN leading to Cry1A toxin insertion into lipid rafts was proposed (Bravo et al, 2004). Competition binding studies demonstrated a correlation between toxin affinity and insecticidal activity (Hoffman et al, 1988; Van Rie et al, 1990). On contrary, Wolfersberger

(1990) showed that Cry1Ab was more active than Cry1Ac against gypsy moth larvae, despite exhibiting a relatively weaker binding affinity. Liang et al, (1995) confirmed that the affinity of toxin was not directly related to toxin activity and they proved a direct correlation between the irreversible binding rate and toxicity.

Several APN isoforms expressed differentially in the insect midgut are known to play an important role in the adaptation of various dietary proteins from different host plants (Telang et al, 2005). In addition studies indicate that different APN isoforms in the midgut can interact differentially with various subtypes of the Cry toxins (Rajagopal et al, 2003). Further occurrence of APNs in other insect organs, apart from midgut has also been well demonstrated biochemically (Hall, 1988). Wang et al, (2005) used enzymatic assays and RT-PCR analysis to demonstrate APNs synthesis by Malphigian tubules, and suggested that its role may be analogous to the APN function of vertebrate kidney. The role of cell surface peptidases in metabolism of neuropeptides has already been demonstrated in *M. domestica* (Lamango et al, 1993). Biochemically characterized APNs from *Drosophila* were shown to have high activities during metamorphosis, and were inducible by 20-hydroxyecdysone (Hall, 1986; Siviter et al, 2002). It has been suggested that APNs along with some other proteases play role in the recycling of amino acids from the proteins of histolysed larval tissues as well as larval hemolymph (Hall, 1988; Haunerland, 1996; Siviter et al, 2002). Although in *L. dispar*, alanyl aminopeptidase activity has been found in larval as well as pupal haemolymph plasma and the enzyme has been extracted and purified but the exact site of enzyme synthesis is not reported (Masler and Kovaleva, 1997). Further the toxicity/mortality obtained after hemocoelic injection of Cry toxins in larvae of *L. dispar* suggests the presence of additional target tissues for Cry toxins in the body cavity of insects (Cerestiaens et al, 2001). However, no information is available till date on the molecular target(s) of Cry toxins in the larval body cavity. Cheon et al, (1997) demonstrated pore forming cytolytic ability of Cry toxins on the *in vitro* cultured fat body and suggested the possibility of existence of binding proteins in fat body. Since the fat body is a pivotal metabolic organ in the body cavity of insects and plays important role during postembryonic as well as adult development, its possible interaction with the Cry toxins deserves attention.

The fat body:

The insect fat body plays a role similar to mammalian liver during the acute-phase immune response. The large size and the position of this tissue in the insect body cavity make the fat body a powerful machine that enables the secretion of peptides into the haemolymph and ensures that they rapidly reach their effective concentrations.

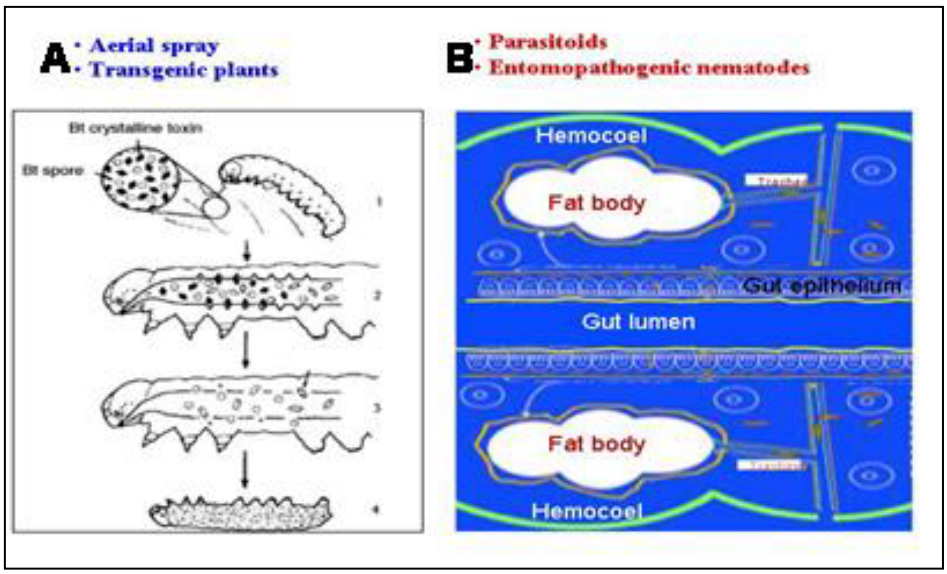


Fig. 5: A. The normal delivery of Cry toxin by ingestion. **B.** The possible delivery of Cry toxins into the body cavity of insects by parasitoids and entomopathogenic nematode (modified from IPCS intex data bank).

The in-depth understanding of how pathogenic mechanisms and host defense responses interact in evolutionarily diverse hosts should contribute to the understanding of key aspects of the pathogenic process that may help in the design of novel preventive and therapeutic approaches (Tzou et al, 2002). In the case of *D. melanogaster*, it has been shown that certain microorganisms are capable of penetrating the exoskeleton or the intestinal epithelium to cause an infection following the so-called "physiological" or "natural" route of infection (Tzou et al, 2002). The physiological infection consists of either (i) larval feeding on the microorganism of interest distributed in the food or (ii) spraying microorganisms directly onto the exoskeleton (body surface).

As fat body is the major multifunctional tissue and plays crucial role in the metabolism and is known to have variety of enzymes including proteases therefore it is most

likely organ, with which Cry toxins could interact. To address this problem, we undertook cloning, biochemical characterization and comparative analysis of APNs from both the larval fat body and the midgut of *A. janata* and evaluated their interaction with different Cry toxins.

Hexamerin and their uptake by fat body:

During the last larval instar, holometabolan insects undergo dramatic physiological changes that prepare it for pupation and metamorphosis. As insect pupae do not feed, they depend on material that has been accumulated during the larval life. In most insects studied, the fat body cells produce large amounts of storage proteins, generally referred to as hexamerins during larval life. A rise in the level of the steroid hormone 20-hydroxyecdysone (20E) at the end of larval life triggers the incorporation of hexamerins into the fat body. Hexamerins are stored into storage granules, and serve as energy and amino acid pool during metamorphosis (Haunerland, 1996; KiranKumar et al, 1997; Burmester and Scheller, 1999). It has been demonstrated that endocytosis, mediated by cell-surface receptor, is essential for the uptake of hexamerins and this receptor is unique and does not belong to the low density lipoprotein (LDL) superfamily (Burmester and Scheller, 1999). Hexamerin receptors have been recognized in dipteran as well as lepidopteran insects (Ueno and Natori, 1984; Wang et al, 1993; Chung et al, 1995; KiranKumar et al, 1997; Arif et al, 2003; Hansen et al, 2003; Persaud and Haunerland, 2004).

Using *Corcyra cepholonica* (rice moth) as a model, our group has already reported that the 120 kDa polypeptide act as a receptor for hexamerin in the fat body (KiranKumar et al, 1997). During larval pupal transformation the receptor protein gets phosphorylated and the phosphorylation is mediated partly by a tyrosine kinase which is activated by ecdysteroid hormone, and the activation is responsible for the hexamerin sequestration (Kiran Kumar et al, 1997; Arif et al, 2003). Although hexamerin receptor cDNAs have been cloned from *Calliphora* as well *Sarcophaga* and the presence of receptor cleavage product has been demonstrated but typical transmembrane domain or endocytosis sorting signals were not reported in these hexamerin receptor sequences (Burmester and Scheller, 1999; Hansen et al, 2003; Persaud and Haunerland, 2004), therefore still its uncertain how the receptors are linked to the fat body cell membrane.

Need for the present study:

India is a major producer of castor in the world with an annual production of about 8.5 lakh tones. The crop is grown mostly in dry land areas of Andhra Pradesh and Gujarat. The major problem encountered with the crop are the attack of insect pests viz. castor semilooper, red hairy caterpillar, capsule borer etc. and diseases like wilt and botrytis, gray rot accounting to an average loss of up to 70%. Castor semilooper, *Achaea janata* is a voracious feeder causing extensive defoliation and at times of severe infestation these larval forms also feed on developing capsules. Out breaks of this pest are common during July to September (Sujatha and Laksminarayana 2005). Under normal conditions, this pest is known to be attacked by two larval parasitoids, viz. *Microplitis maculipennis* and *Euplectrus maternae*. However, the incidence of parasitization by these natural enemies occurs late in the season when the severe damage by the pest has already been done. Eventhough the high-yielding castor varieties can improve productivity, but carry risk of devastating epidemics due to genetic uniformity and are more susceptible to pests. Because of the problem of resistance to chemical insecticides, there is an increased emphasis on developing ecofriendly, safer and more effective methods for control this pest. Of all these biological control measures, the widely used lepidiopteran specific *Bacillus thuringiensis kurstaki* HD-1 is found to be effective against *A. janata* (Devi et al, 1996; Laksminarayana and Sujatha, 2005). Microbial insecticides like *B. thuringiensis* (Bt) are the preferred over chemical insecticides, because of their narrow host range as well as more or less no toxicity to non targets organisms. Reports already show that on long-term use, insects are developing resistance to this biological pesticide also (Tabashnik et al, 2005). It has been suggested that mixture of different Cry toxin could be more effective than a single toxin in delaying the rapid onset of resistance. Lee et al, (1996) demonstrated the existence of synergism between Cry1Aa and Cry1Ac against *Lymantria dispar* and also possible antagonism between Cry1Ab and Cry1Ac in the same insect. Furthermore, if resistance management strategies are to be implemented to preserve the long-term use of *B. thuringiensis* based insecticides, combination of Cry proteins sharing the same binding site is to be discouraged. Hence search and development of more effective Bt strains with new target sites is a never –ending process, in order to overcome the problem of resistance and to sustain the usage of this environmental friendly control agent. Further in future, instead of directly embarking on the programme of genetic engineering for castor transgenics, this type of study will be

advantageous to identify suitable candidate genes and the reaction of castor semilooper to various Cry proteins as there is no information regarding this is available in the data base.

During the last decade the research in agrochemical areas have been focused on the identification of novel molecules as well as novel control targets and their validation. The research is directed at key invertebrate pests of crops principally lepidopteran insects and recent genome sequencing projects are identifying new genes from target tissues of key pests at an accelerating rate and these are useful in genetic transformation to identify and validate those genes that have potential as new targets for pest control. In addition to this scientist are using RNA interference as a means of knocking out activity of specific genes to gain insights into gene function (Rajagopal et al, 2002). This technique has facilitated the efforts in finding genes useful for the control of insect pests (Schneider 2000). To achieve this, a detail understanding of insecticde (in present study Bt Cry toxin) interaction with the host is critical. In addition identification of new target organs and the knowledge of genes and process which are essential for insect survival will be very useful for the development of effective microbial insecticide by genetically modifying organisms. With this back ground four major objectives were formulated for the present thesis work.

Objectives of the present study:

1. Molecular characterization of local isolates of *B. thuringiensis* effective against larval forms of *A. janata*
2. Identification and characterization of larval midgut proteases of *A. janata* and their role in protoxin activation
3. Identification and cloning of novel aminopeptidase from the larval fat body of *A. janata* as a receptor for Cry toxins and its comparison with the midgut aminopeptidase
4. To confirm whether or not the GPI anchored fat body aminopeptidase is a putative hexamerin binding protein

Materials and Methods

Materials and Methods:

GeneRacer ™ kit, Superscript™ first-strand RNA synthesis kit, *platinum pfx*, set of 4 dNTPs and *Taq* DNA polymerases were acquired from Invitrogen (USA). Reverse transcriptase and oligo $d(T)_{18}$ were purchased from Finzyme (Finnland). [α^{32}P] dATP (3000 Ci/mmol) and [γ^{32}P] ATP (3000 Ci/mmol) were procured from BRIT, Trombay, India. Monoclonal anti-phosphotyrosine antibody (PY-99) from Santa Cruz Biotechnology (USA), biotin protein labeling kit and streptavidin detection kit from Bangalore Genei, (India) were used. All the restriction enzymes, modifying enzymes, InsT/A cloning kit, hexalabel random primer labeling kit, IPTG and X-gal were from MBI Fermentas ™ (Germany). Plasmid purification, gel purification kits and TRI reagent™ for RNA isolation were procured from Sigma-Aldrich (USA). All commercial antibodies, adjuvants, affinity matrix for GST protein purification, substrates for immuno-detection and protein expression vector pGEX-5X were purchased from Amersham Biosciences (USA), while the BL21 (DE3) pLysS cells were from Novagen (USA). Alkaline phosphatase conjugated anti-mouse, anti-rabbit secondary antibodies, NBT and BCIP were purchased from Bangalore Genei, (India). Whatman No. 1 and 3 were procured from Whatman (UK). Acrylamide, N-N`-methylene bisacrylamide, 2-mercaptoethanol, glycerol, SDS, glycine, silver nitrate, Tris base and ethylene glycol were purchased from SRL (India). Hybond N+ and Hybond-N nylon membranes were procured from Amersham Biosciences (USA), while PVDF membrane, 3, 10, 30 kDa protein concentrator columns were from Pall-Gelman (Germany). Chromogenic protease substrates like BApNA (N-benzoyl-L–arg p-nitroanilide), BTEE (N-benzoyl–tyr ethyl ester), SAAPPpNA (N-succinyl–ala–ala–pro–Phe p-nitroanilide), SAAPLpNA (N-succinyl–ala–ala–pro–leu p-nitroanilide) and LpNA (Leu p-nitroanilide) and specific inhibitors like TLCK (N-p-tosyl-L–lys chloromethyl ketone), SBTI (soybean trypsin inhibitor), TPCK (N-tosyl-L–phe chloromethyl ketone), elastatinal, bestatin and PMSF (phenylmethanesulfonyl fluoride) were procured from Sigma-Aldrich (USA). All the other molecular biology grade chemicals were acquired from Sigma-Aldrich, Amersham Biosciences and Amresco. Disposable plastic wares were acquired from Axygen and Tarsons, while all the glasswares used were from Schott Duran and Borosil.

Bacterial strains and *cry* genes:

B. thuringiensis subsp.kurstaki HD1 was obtained from Bacillus Genetic Stock Center (BGSC) Ohio State University, USA and used as a standard. *E. coli* JM 103 strains carrying recombinant plasmids over expressing *cry1Aa* (ECE52), *cry1Ac* (ECE53) and *cry1Ab* (ECE54), cloned in the vector pKK223-3 backbone were provided by Dr. Daniel R. Zeigler, BGSC, USA. Other *B. thuringiensis* strains used in this study were isolated from soil samples from different regions of India (kindly provided by Dr. P.S. Vimala Devi, DOR, Hyderabad). *B. thuringiensis* strains were grown in glucose/yeast extract/salts medium at 30°C with agitation at (200 rpm) until sporulation was complete. *E. coli* was grown at 37°C in Lauria-Beretani broth containing ampicillin (100 ug/ml).

Insect culture:

Achaea janata:

Achaea janata, castor semilooper, belongs to the order Lepidoptera and family Noctuidae. The larval forms were obtained from the Directorate of Oil Seeds Research (DOR), Hyderabad, India and maintained in the rearing facility of our laboratory. The larvae

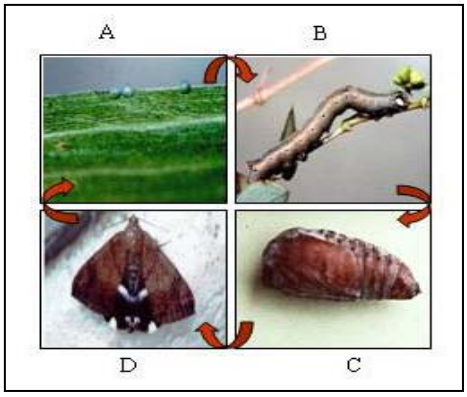


Fig. 6: Simplified representation of the life cycle of castor semilooper, *Achaea janata.* The larva stages were mainly used for the present study. A. Eggs; B. larva; C. pupa; D. adult.

were reared on fresh castor leaves at 25±1°C and 70% RH with a photoperiod of 12 h light and 12 h dark. Based on their age after the fourth ecdysis they were categorized as early-last instar (ELI - 1 to 2 days old), mid-last instar (MLI - 5 to 6 days old) and late-last instar (LLI - 8 to 9 days old). Prepupae were also used. Fat body, haemolymph and other tissues from above stages were collected and used for various studies.

***Spodoptera litura*:**

It is commonly known as tobacco cut worm, belongs to the order Lepidoptera and family Noctuidae. Early larval stages were procured from Directorate of Oil seed Research (DOR), Hyderabad, India and reared on fresh castor leaves. For the present study the 5th (last) instar larvae were used.

Primers:

All the custom primers were designed and procured from Sigma (India) or Bioneer (South Korea), while the sequencing service of Macrogen (South Korea) was used. The list of primers used for the present study is given below:

cry1+:5'MDATYTCTAKRTCTTGACCTA 3'

cry1-: 5'TRACRHTDDBDGTATTAGAT3'

cry1Aa: 5'TTCCCTTTATTTGGGAATGC3'

cry1Ab:5' CGGATGCTCATAGAGGAGAA3'

cry1Ac: 5'GGAAACTTTCTTTTTAATGG 3'

cry1C: 5'ATTTAATTTACGTGGTGTTG 3'

cry1E: 5'ATTTAATTTACGTGGTGTTG 3'

cry2+: 5'TAAAGAAAGTGGGGAGTCTT 3'

*cry2-:*5'AACTCCATCGTTAT TGTAG3'

cry2A: 5'TCTCATAGGGGCGACTAATC3'

cry2B: 5'TGATATAGGTGCATCTCCGT 3'

M13F: 5'GTAAAACGACGGCCAGT3'

M13R: 5'GCGGATAACAATTTCACACAGG 3'

GeneRacer^{TM} 5'forward: 5'CGACTGGAGCACGAGGACACTGA 3'

GeneRacer^{TM} 5'forward nested: 5' GGACACTGACATGGACTGAAGGAGTA3'

GeneRacer^{TM} 3'reverse: 5' GCTGTCAACGATACGCTACGTAACG 3'

GeneRacer^{TM} 3'reverse nested: 5' CGCTACGTAACGGCATGACAGTG

Forward (F1): AFPCYDEP- 5'GCTTTYCCYTGYTAYGAYGAR CC3'

Forward (F2): GAMENWG- 5'GGNGCNATGGARAAYTGGGG 3'

Riverse (R1): HMWFGN -5'TTNCCGAACCACATTG 3'

Riverse (R2): YRVNYD -5'ATCGTAGTTCACGCGGTA3'

AjFBAPNF3 forward: 5' CACTTCTACAGACAACGTGTTGC 3'

AjFBAPNF4 forward nested: 5'AACATTGTTTCTCATGAGATCGCG 3'

AjFBAPNR3 rev: 5'GAAGGTCGGTGATCTGACAATG 3'

AjFBAPNR4 rev nested: 5'TGATATCGAATCGGGCCTTAAATC 3'

AjMgAPNF3 forward: 5'GAAGCTGGAGATTACTGGGTTCTC3'

AjMgAPNF4 forward nested: 5'TTCAACGTTGCTCAGTCTAGTCTG3'

AjMgAPNR3 rev: 5'TTGGTATTATCTGGGTCGTAAAGAAG3'

AjMgAPNR4 rev nested; 5'TACGCCTCCCTGTAGTTAACCATG3'

SpoFBAPNF3 forward: 5'CAGTCCACACCGTACTTCAAATAC 3'

SpoFBAPNF4 forward nested: 5'AGTGGCACAAATCATGGCCCATG 3'

Cry toxin isolation and purification:

All of the *B. thuringiensis* strains were grown for 3 days at 30°C in glucose/yeast extract/salts medium at 30°C with agitation until sporulation and cell lyses was complete. The crystals, spores, and debris were collected by centrifugation, and the pellet was washed with 1 M NaCl containing 0.1% Triton X-100 and then with distilled water. The crystals were purified using sucrose density gradients centrifugation (Thomas and Ellar, 1983), solubilized in 50 mM sodium carbonate buffer (pH 10.5) containing 0.1% 2-mercaptoethanol for 2 h at room temperature and then centrifuged at 27,000 X *g* for 30 min to remove the insoluble debris.

E. coli JM103 strains expressing Cry1Aa, Cry1Ab, Cry1Ac, and DH5α strains expressing Cry1C, Cry1E were grown with ampicillin (50 ug/ml) for 48 h at 37°C (Lee et al,

1992). Cells were harvested (10000 X g, 10 min) and the pellet was resuspended in buffer (50 mM Tris-HCl, 50 mM EDTA, 15% sucrose, pH 8). Cell suspension was sonicated on ice (3X 3 min 30 sec) and centrifuged 15000 X g, for 15 min. The pellet then obtained was washed three times with ice cold 0.5 M NaCl containing 2% Triton X -100, five times with 0.5 M NaCl and finally two times with distilled water. The crystal protein was solubilized in 50 mM sodium carbonate buffer pH 10.5 containing 10 mM DTT at 37°C for 2 hours.

The solubilized crystal proteins from both native as well as recombinant strains were treated with trypsin in a trypsin/protoxin ratio of 1:50 (by mass) at 37°C for 2 h and the profile was checked by SDS-PAGE. Activated toxins were purified according the method of Lee et al, (1992).

Serotyping of local isolates:

For serotyping studies H antigens and corresponding antisera were prepared from the different Bt serotypes. The commercial antisera obtained from the Paster Institute. Agglutnation studies were carried out using local isolates of Bt (Ohba and Aizawa, 1978; Laurent et al, 1996).

Scanning electron microscopy:

Spore crystal suspensions were diluted and placed on aluminum stubs and air-dried. Samples were coated with gold in a Fullam EMS-76M evaporator for 9 min and examined under Geol scanning electron microscope.

Insect bioassay:

Bioassay performed with second-instar larvae of *A. janata*, was a castor leaf surface contamination trial, in which 100 µl aliquots of solubilized suspensions of crystal toxin proteins were pipetted onto the castor leaf surface (surface area, 7 cm^2) in rearing cups and spread with a camel hair brush. The leaf surface was allowed to dry for 15 min, and second-instar larvae were transferred individually to the cups and allowed to feed on the treated leaf for a 3-day period. Subsequently, mortality was assessed daily until 3 days post-inoculation and LC_{50} value was determined by probit analysis (SPSS, version 8).

Injection assay on *A. janata*:

Individual activated Cry toxins were injected into the body cavity of fifth instar larvae following the method described by Cerestiaens et al, (2001). The larvae were maintained individually in plastic petri plates with castor leaves until analyzed.

Determination of protein content (Bradford, 1976):

Protein content in various samples was determined by the method of Bradford (1976). The protein content in the sample was calculated using a standard curve prepared using bovine serum albumin (fraction V).

Purification of GST fused recombinant protein:

GST-fused recombinant proteins were purified using glutathione-CL agarose affinity chromatography (Bangalore-Genei). Briefly, after 2 h of induction, *E. coli* pellet was harvested and the cells were lysed by sonication. The protein was extracted in the 1X PBS containing 2% Triton X-100 and the insoluble proteins were removed by centrifugation. The soluble lysate was then applied to glutathione-agarose column and washed with 5X column volume of 1X PBS. Finally the protein was eluted from the column using elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM reduced glutathione) and the purity was checked by SDS-PAGE.

Generation of polyclonal antibodies against various proteins:

Three-month-old male rabbits (New Zealand variety) were injected with 100 µg of purified protein (emulsified with 500 µl of complete Freund's adjuvant) by subcutaneous injections into various sites on the back. Prior to injection, the lateral ear vein was bled to collect pre-immune serum. After a fortnight, first booster injection was given followed by a second booster injection after seven days. For booster injections, 50 µg protein emulsified with Freund's incomplete adjuvant was used. The blood was collected from the rabbit after a week of second booster injection. The collected blood was left overnight at 4°C for clotting and serum was obtained by centrifugation at 5,000 X g for 20 min (Sambrook et al, 1989).

Purification of IgG:

The IgG fraction was purified by affinity chromatography using protein A-agarose column (Amersham) according to the manufacturer's protocol. The serum protein was diluted (1:1) with the wash or binding buffer (20 mM sodium phosphate pH 7.4 and 150 mM

NaCl) and was loaded on to the protein A-agarose column pre equilibrated with wash or binding buffer. This was followed with the washing of column using the same buffer until the absorbance of elutes at 280 nm approaches the background level. The bound IgG fraction was eluted with 4 column volumes of elution buffer (100 mM glycine-HCl, pH 3.0) in tubes that contained 100 µl of neutralization buffer (1 M Tris-HCl, pH 9.0) for rapid neutralization of the eluted purified IgG fraction. The purified IgG fraction was aliquoted, lyophilized and stored at -20°C till further use.

Preparation of gut extract from *A. janata*:

In brief, larvae were dissected in 0.15 M NaCl at 4°C (Girard et al, 1998), total gut was collected after removal of lumen content and stored frozen (-20°C) until use. The tissue was homogenized in 0.15 M NaCl and centrifuged at 12,500 X g for 20 min at 4°C. The supernatant was collected and frozen (-80°C) and used for enzymatic assays.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE):

SDS-PAGE was carried using discontinuous buffer system as described by Laemmli, (1970). Throughout this study 1.5 mm thick 7.5 % polyacrylamide (30:0.8, acrylamide to bisacrylamide ratio) gel containing 0.1% SDS was used for electrophoretic separation of proteins. Electrophoresis was performed at a constant voltage of 100 or 150 V using 0.025 M Tris and 0.2 M glycine buffer (pH 8.8) containing 0.1% SDS. After the electrophoresis, the gel was either stained with Coomassie brilliant blue or silver otherwise processed for western blotting directly. In most of the cases 10 -15 µg protein per lane was used for SDS-PAGE.

Western blotting and immunostaining:

The electrophoretically separated polypeptides were transferred (electro-blotted) to nitrocellulose membrane using trans-blot apparatus (Bio-Rad) according to the procedure of Towbin et al, (1979). For this, the gel was first equilibrated in Towbin buffer (25 mM Tris, 192 mM glycine and 20% methanol) for 30 min followed by transfer to the membrane for 3 h at 70 V with 250 mA current limit. In case of native gel, methanol was omitted from the Towbin buffer and transfer was carried at 4°C. The transfer of protein to membrane was checked by reversible Ponceau S staining (100 mg Ponceau S in 100 ml of 5% acetic acid). The stain was removed by 3-4 washes with TBST (10 mM Tris-HCl 7.4, 150 mM NaCl and

0.1% Tween-20 (v/v)]. For immunostaining, the protein blot was processed with 3% BSA (w/v) in TBST for 1 h at room temperature to block the non-specific binding sites followed by washing with TBST (10 min X 5 changes). The blot was then incubated with the primary antibody diluted in TBST containing 3% BSA (w/v) for 2 h to overnight. This was again followed by a thorough wash in TBST (10 min X 5 changes). The antibody-bound to proteins was detected using ALP-conjugated anti-rabbit IgG for 1 h. Once again the blot was washed in TBST (10 min X 5 changes). The visualization of the specific cross-reactivity was carried with the BCIP-NBT

Zymographic study with insect gut extract:

Gut homogenate samples were prepared with 5X non-reducing sample buffer (0.4 M Tris pH 6.8, 5% SDS, 20% glycerol, and 0.03% bromophenol blue) without heating, incubated at room temperature for at least 10 min, centrifuged at 15,000 X g for 5 min and supernatant was loaded onto the gel. Gel electrophoresis was carried out according to Lammeli, (1970) using 7.5% SDS-PAGE. Zymograms of protease activities were done according to the method of Garcia-Carreno et al, (1993). Electrophoresis was carried out for 60 min at a constant voltage of 100 V per gel at 5°C. After electrophoresis, the gels were placed in 2.5% (v/v) Triton X-100 for 45 min, then washed and incubated in substrate solution (2% casein (w/v) in 0.1 M glycine-NaOH buffer pH 10.5) for 30 min at 5°C with gentle agitation. Then, gel was kept for 2 h in the same solution at 37°C without agitation. Gels were stained in 30% methanol, 10% acetic acid, 0.5% Coomassie brilliant blue G-250 and destained with 20% ethanol, 7% acetic acid. Irreversible proteinase inhibitors were added to the samples before SDS–PAGE, whereas reversible inhibitors were added to the substrate buffer or incubation buffer after electrophoresis.

Partially purified protoxin substrate solution was obtained by sucrose density gradient centrifugation, from bacteria grown in glucose/yeast extract/salts medium at 30 °C for 2-3 days with agitation until sporulation was complete. In order to identify the proteases which are responsible for the conversion of protoxin to active toxin, zymogram study was carried out in the same way as mentioned above except that instead of casein, protoxin was used as a substrate.

Activity blots:

Proteolytic enzymes capable of hydrolyzing pNA substrates were identified in western blots according to Oppert et al, (1996). After transferring the proteins, nitrocellulose membrane was incubated in substrate solution (0.2 mM pNA in 20 mM Tricine pH 8.0) for 5 minutes at room temperature without agitation. Released nitroanilin was diazotized by subsequent incubations of 5 min each in 0.1% sodium nitrite in 1M HCl, 0.5% ammonium sulphamate in 1M HCl, and 0.05% N-(1-Naphtyl)-ethylenediamine in 47.5% ethanol. Membranes were stored at -20^{o}C. Highlighted red bands show sites of substrate reaction. For certain studies, reversible inhibitors were added to the substrate solution in which the nitrocellulose membrane was incubated (co-incubation).

Collection and preparation of haemolymph sample:

The larval stages of interest were narcotized on ice. The prolegs were cut with a fine sharp scissors and the oozing haemolymph was collected with the help of capillary tube into tubes precoated with 0.025% phenylthiourea in order to prevent tyrosinase activity and malanization. All the haemolymph samples thus obtained were diluted (1: 20) with 10 mM Tris-HCl buffer (pH 7.4), and were centrifuged at 1,000 X g for 3 min at 4°C to sediment the hemocytes. This supernatant *i.e.,* the haemolymph samples were used immediately.

Preparation of homogenate protein sample:

Homogenate protein sample of the desired tissue of appropriate developmental stage was prepared from different insects. The desired tissues were rapidly dissected in cold insect Ringer (130 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2$ and 1 mM PMSF) and were homogenized in 150 µl of homogenization buffer (10 mM Tris-HCl pH 7.4, 0.1% Triton X-100, 1 mM PMSF, 1 mM EDTA and 1 mM DTT). The homogenates were centrifuged at 1,000 X g for 5 min to remove larger debris and the aliquots of the supernatant were used for various studies.

***In vitro* phosphorylation of proteins:**

In vitro phosphorylation of various insect tissue proteins was carried out according to the method of Shanavas et al, (1998) with slight modifications. A 40 µl reaction mixture contained 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, 10 µM ATP, 10 µM sodium orthovanadate and 20 µg of membrane proteins. The reaction mixture was preincubated for 5 min at 30°C and the phosphorylation was initiated by addition of 4 µCi of

[γ^{32}P] ATP. The reaction was terminated after 1 min by addition of 20 µl of SDS sample buffer (0.188 M Tris-HCl pH 6.8, 6% SDS, 30% glycerol, 15% 2-mercaptoethanol and 0.003% bromophenol blue) followed by immersion in boiling water for 3 min. The tubes were centrifuged at 10,000 X g for 5 min and equal amount of supernatant that contained phosphorylated proteins (10 µg) were subjected to 7.5% Tris-glycine SDS-PAGE. The gels were silver stained, vacuum dried and exposed to Kodak X-Omat AR film at -70°C for autoradiography.

Purification of hexamerins:

Hexamerin were partially purified from haemolymph of last instar larvae of *A. janata*. The haemolymph was diluted (1:20) with insect Ringer's solution (130 mM NaCl, 5 mM KCl and 0.1 mM $CaCl_2$) containing 0.01% phenylthiourea, and centrifuged to remove the hemocytes. The supernatant was added with ammonium sulfate to make 40% saturation and was centrifuged at 10 000 X g. The supernatant was collected and ammonium sulfate saturation was raised to 60% and was centrifuged at 10 000 X g**.** Once again ammonium sulfate in supernatant was raised to 70% saturation. It was centrifuged at 10 000 X g**,** the pellet thus obtained was suspended in insect Ringer. The purity was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and the protein was stored at −20°C till use.

Preparation of brush border membrane vesicle (BBMV):

Midgut were dissected from early fifth instar larvae, rinsed in MET buffer (300 mM mannitol, 5 mM EGTA, and 17 mM Tris base, pH, 7.5). BBMV were prepared by the method of Wolfersberger et al, (1987). The tissue was homogenized in 10 volumes of ice-cold buffer (supplemented with the protease inhibitor cocktail) in a glass Dounce homogenizer. An equal volume of 24 mM $MgCl_2$ solution was added and mixed thoroughly with the homogenate. This mixture was allowed to stand for 15 min on ice. Following low-speed centrifugation, the supernatant was removed, transferred to a fresh tube, and centrifuged for 30 min at 30,000 X g. Same steps were repeated second time. The final high-speed pellet was resuspended in same MET buffer (pH 7.5), and flash frozen in liquid nitrogen.

Preparation of fat body membranes:

The fat body tissue from the larvae was dissected in ice cold insect Ringer and homogenized on ice in buffer A [5 mM HEPES (pH 8.5) and 0.1 mM $CaCl_2$]. The membrane fraction from the total fat body homogenate was prepared according to the method of KiranKumar et al, (1997). The fat body homogenate was centrifuged at 1,000 X g for 10 min at 4°C and the resultant supernatant was further centrifuged at 30,000 X g for 30 min at 4°C and the pellet thus obtained was washed once with buffer A followed by resuspension in small volume of buffer B [5 mM HEPES (pH 7.0) and 0.1 mM $CaCl_2$]. This particular fraction was used as membrane preparation and stored at -80°C.

Biotinylation of various proteins:

Cry1Aa, Cry1Ab and Cry1Ac toxins and hexamerins, each 100 µg were biotinylated using the protein biotinylation module kit (Banglore Genei, India) and then purified with Sephadex G25 columns. The biotinylated toxins were detected with ALP conjugated streptavidin.

Ligand and lectin blot analysis of the membrane proteins:

The fat body membrane and BBMV protein preparations were separated by SDS-PAGE and electrotransferred to nitrocellulose membranes. After blocking the membrane for 1 h in 3% BSA in TBST buffer, the blots were incubated in the blocking buffer containing one among the following, primary antibody for western analysis, biotin-labeled Cry toxins for ligand blot analysis or appropriate lectins for lectin blot analysis. After incubation, blots were washed with TBST and then incubated for 2 h in blocking buffer containing either ALP conjugated goat anti-rabbit IgG or ALP conjugated streptavidin. APNs, bound toxins or lectins were detected using the ALP substrate BCIP-NBT.

Immunoprecipitation of binding proteins-

Triton X-100 solubilized fat body membrane and CHAPS solubilized BBMV proteins were incubated with a selected Cry toxin (5 µg) in binding buffer (50 mM sodium phosphate, pH 7.5, 50 mM NaCl and 3 mM $MgCl_2$) for 3 h at 4°C followed by its incubation with 5 µl of Cry1Aa antisera for another 3 h. Subsequently, 100 µl of protein A-Sepharose 4B was added and incubated for 2 h at 4°C on a rotatory shaker. The Sepharose beads were pelleted, washed 6 times with PBS buffer, resuspended in 20 µl of SDS sample buffer containing ß-

mercaptoethanol and heated at 100°C for 5 min. Eluted proteins were subjected to 7.5% SDS-PAGE and electrotransferred to nitrocellulose membrane. The membrane was then incubated with antiAPN antibodies followed by ALP-conjugated goat anti-rabbit antibodies and developed with NBT-BCIP.

GPI anchor detection with anti-CRD antibody-

The larval fat body membrane and the BBMV preparations were digested with 5 units/10 ml of bacterial phosphatidylinositol phospolipase C, (PI-PLC, Molecular Probes) at 30°C for 90 min and subjected to SDS-PAGE. Proteins were electrotransferred to nitrocellulose membrane and the presence of cleaved GPI epitope was detected (Broomfield et al, 1991) using 1: 200 dilution of anti-cross-reacting determinant antibody (anti-CRD).

Binding studies:

Biotinylated toxins were incubated with 15 µg of BBMV in the presence or absence of 300 fold excess unlabelled toxins. Subsequently unbound toxin was removed by centrifugation and pellet was washed twice with the PBS buffer. The pellet was then suspended in sample buffer, boiled for 5 min and subjected to SDS-PAGE. The proteins from SDS-PAGE were transferred to nitrocellulose membrane. The biotinylated proteins that bound to the membrane were visualized by incubating with streptavidin ALP conjugate for 1 h, and finally detected with BCIP-NBT.

Aminopeptidase activity assay:

The APN activity was determined by incubating crude homogenate preparations of different tissues (20 µg) or haemolymph plasma (20 µl) with the substrate leucine *p*-nitroanilide (1.0 mM in 50 mM Tris-HCl, pH 7.3) for different time periods at 30°C. The formation of hydrolysis product (*p*-nitroaniline) was monitored by measuring the increase in absorbance (A_{405}).

Isolation of plasmids from *Bacillus thuringiensis*:

Most strains of *B. thuringiensis* and *Bacillus cereus* contain an array of plasmids, from the small to very large size (Gonzalez et al, 1982; Carlson and Kolsto, 1993; Carlson et al, 1994). Standard alkaline lysis or boiling protocols are efficient for isolation of small size plasmids but give very low yield of the larger ones. Hence, the following method was used for isolation of plasmids. *B. thuringiensis* strain was grown overnight at 30°C in 2 ml LB, to

obtain final OD_{600} of 11-15. Cells were pelleted with a brief spin and resuspended in 100 µl of STEE buffer (15% w/v sucrose, 40 mM Tris-HCl, 2 mM EDTA, pH 7.9). After pipeting them up and down several times, 200 µl lysing solution (3% SDS, 50 mM Tris-HCl, pH 12.5) was added and lysate was incubated at 60°C for 30 min. Further 5 U proteinase K was added and the tube was inverted for several times and incubated at 37°C for 90 min. To this 1 ml phenol-chloroform-isoamyl alcohol mix was added, the tube was inverted for several times and centrifuged at high speed for 15 min. The aqueous layer was collected and analyzed by 0.5% agarose gel electrophoresis.

Triplex PCR methodology:

Identification of the *cry* gene content of *B. thuringiensis* was done by using triplex PCR techniques (Juarez-Perez et al, 1997; Masson et al, 1998). The reaction was carried out using either ordinary family primers (primers directed toward a specific gene class) or type-specific family primers (primers used to identify a specific gene subclass or type). The triplex PCR reaction consisted of 250 ng of total DNA, 1 µM reverse primer I(−), 0.5 µM each forward primer I(+) and/or a type-specific primer, 3 mM $MgCl_2$, 200 nM deoxynucleoside triphosphates, and 2.5 U of *Taq* DNA polymerase (Invitrogen) in a final volume of 50 µl. All reactions were performed in MJ research thermal cycler with an initial 5-min denaturation step at 94°C followed by 25 cycles of amplification consisting of a 1-min denaturation at 94°C, 45 s of annealing at 45°C, and 2 min of extension at 72°C. After 25 cycles, an extra extension step of 10 min at 72°C was added. Family and type-specific primers were selected from the work of Juarez-perez et al, (1997) and Masson et al, (1998). This technique employs the use of both specific and degenerate primers prepared against common regions shared by the different gene classes in order to form a pair of opposing family or class-specific primers. To determine the presence of a gene of a particular subclass or type, a type-specific primer is included with the family primers, creating a triplex PCR. Therefore, the production of a single PCR product confirms the presence of a *cry1* class (family primer), and the appearance of a second band indicates the presence of and identifies a specific *cry1* gene type

RNA isolation from *B. thuringiensis*:

B. thuringiensis culture was pelleted during sporulation stages. Cell pellets were initially preserved in RNA later and were then transferred to a 2 ml screw-cap tube containing 1 ml of Trizol reagent (Sigma). The samples were kept on ice during the entire

procedure to prevent RNA degradation. Samples were sonicated, followed by three cycles of freezing and thawing at –80°C and 65°C, respectively. After disruption, samples were vortexed and incubated on ice for 5 min. Two hundred microliters of chloroform was added to the homogenate, and the tubes were shaken vigorously for 15 sec and then incubated on ice for 3 min. These samples were centrifuged at 14,000 X *g* for 15 min at 4°C. The aqueous phase was transferred to a new tube, and the same volume of isopropanol was added. Samples were incubated for 1 h at room temperature, then centrifuged at 14,000 X *g* for 15 min at 4°C, and washed with 75% ethanol. Total RNA thus obtained was resuspended in diethyl pyrocarbonate (DEPC) treated water. Aliquots of isolated total RNA was treated with the DNase (free of RNase) according to the manufacturer's instructions (Fermentas). RNA samples were run in a formaldehyde gel to check the integrity of the RNA. Samples contaminated with DNA or degraded RNA were excluded from further analysis.

Bacterial RT-PCR:

The cDNA was prepared from the total RNA isolated from sporulating cells of *B.thuringiensis* strains using super script RT kit. Random hexamers were used as primers for synthesizing cDNA. Using cDNA as template triplex PCR was carried out, in the same way as triplex PCR using DNA as template.

Isolation of total RNA from insect tissue:

RNA was isolated using TRI reagent™ following the manufacturer's instructions. In brief, 200-300 mg of tissue was homogenized in 1 ml of TRI reagent™. The homogenates were allowed to stand for 5-10 min at room temperature to ensure complete dissociation of nucleo-protein complexes. The samples were centrifuged at 12,000 X g for 10 min at 4°C to remove the insoluble materials (membranes, polysaccharide, fibers, and high molecular weight DNA). The clear supernatants were transferred to fresh tubes and 0.2 ml of chloroform was added per 1 ml of sample. The tubes were closed tightly and shaken vigorously for 15 sec. and then allowed to stand for 2-15 min at room temperature. The resulting mixtures were centrifuged at 12,000 X g for 15 min at 4°C. The aqueous phase containing RNA was transferred to fresh tubes and 0.5 ml of isopropanol was added. The two phases were mixed properly and the tubes were allowed to stand for 10 min for RNA to precipitate. The tubes were centrifuged at 12,000 X g for 10 min at 4°C. The RNA pellet was washed with 1 ml of 75% ethanol by vortexing. The samples were finally centrifuged at

7,500 X g for 5 min at 4°C, ethanol was discarded, and pellets were dried for a brief period. RNA pellets were dissolved in 30 to 40 µl DEPC-treated milli-Q water.

Gel electrophoresis of RNA under denaturing condition:

Formaldehyde/formamide procedure (Sambrook et al, 1989) was slightly modified and used for denaturing RNA gel electrophoresis. 1.2 g of agarose (for 1.2% gel) was weighed and dissolved by boiling in 72 ml sterile DEPC treated water. The agarose was cooled to 65°C, to this, 10 ml of 10X MOPS buffer (0.2 M MOPS, 0.05 M sodium acetate and 5 mM EDTA, pH 7.0) and 18 ml of formaldehyde (from stock of 40% formaldehyde) were added so that the final concentration of MOPS and formaldehyde are 1X and 0.7 M, respectively. The components were mixed properly, the gel was casted.RNA samples (4-5 µg) were taken in microfuge tubes, and the volume of all the samples was made to 10 µl using DEPC treated water. To this 10 µl of 2X RNA loading dye (95% deionised formamide, 0.025% each of SDS, bromo phenol blue, xylene cyanol FF and ethidium bromide, and 0.5 mM EDTA) from MBI Fermentas was added. Samples were incubated at 55°C for 15 min, cooled for 2 min, loaded on the gel and run initially at 100 volts for 10 min and subsequently at 60 volts for 3 h till the dye front moves by 8 cm. Following electrophoresis, the RNA was visualized with UV light and it l was documented. Integrity of RNA was assessed by absence of smearing and appearance of two strong bands corresponding to 28s and 18s rRNA.

Isolation of total genomic DNA from insect tissues:

The genomic DNA from total body of LLI larvae was isolated as described in Birren et al, (1997). The narcotized larvae (~1.5 g) were thoroughly rinsed in sterile insect Ringer and dried on Whatman-1 filter paper. These larvae were then grounded in a mortar half filled with liquid nitrogen. The pulverized tissue was transferred to a 15 ml conical centrifuge tube and excess of liquid nitrogen was allowed to evaporate away. To this lysis buffer (27% sucrose, 1X SSC, 1 mM EDTA, 1% SDS and 200 µg/ml proteinase K) was added (1 ml/100 mg) and mixed thoroughly. The lysate was incubated at 37°C overnight and it gradually turned extremely viscous due to the release of chromosomal DNA. The lysate was transferred to a fresh tube to which 10 ml of equilibrated phenol, chloroform and iso-amyl alcohol (25:24:1) was added and mixed thoroughly to form an emulsion. The emulsion was centrifuged at 6,000 X g for 10 min at room temperature. The upper aqueous layer was carefully removed into a fresh tube and re-extracted with 5 ml of phenol, chloroform and iso-

amyl alcohol mix. The upper aqueous phase was collected to which equal volume of chloroform was added, mixed thoroughly, centrifuged at 5,000 X g for 5 min at room temperature. The aqueous phase, free from the residual phenol was collected and to this $1/10^{th}$ volume of 3 M sodium acetate was added and mixed thoroughly. The mixture was then added with equal volume of iso-propanol and incubated for 30 min at -20°C. The DNA was collected after centrifugation at 12, 000 X g for 15 min at room temperature. The DNA pellet was washed twice with 70% ethanol followed by drying under vacuum for 5 min. The genomic DNA was dissolved in little volume of TE buffer (10 mM Tris HCl, pH 8.0 with 1 mM EDTA) and stored until use. Quality and quantity of the DNA samples were assessed by spectrophotometry and agarose gel electrophoresis.

Restriction endonuclease digestion of total genomic DNA and agarose gel electrophoresis:

10-15 µg of total genomic DNA was digested with 20-30 units of any of the following enzymes: EcoRI, HindIII, and BamHI as per the requirement, in their respective 1 X buffers, incubating overnight at 37°C in a water-bath. The reaction was stopped by addition of 1 µl of 0.5 M EDTA, pH 8.0. Samples were stored at -20^0C until use. The digested DNA was resolved on 0.8% agarose gel was TAE buffer. As a reference for size marker, λ DNA digested with HindIII/EcoRI was separated along with the samples. The ethidium bromide stained gels were documented to mark the position of the λ-H*ind*III/E*co*RI double digest marker.

Southern blotting:

After destaining the ethidium bromide containing gel for 2 times with double distilled water, the restriction digested DNA was depurinated by soaking the gel in 0.25 N freshly diluted HCl (depurination solution) for 15 min with gentle shaking, till the color of bromophenol blue changes from blue to yellow. The gel was washed twice with double distilled water and soaked in denaturation buffer (87.66 g NaCl and 20 g NaOH in 1L of double distilled water) for 45 min with gentle rocking. After denaturation the yellow color of bromophenol blue returns to blue. Next, the gel was soaked in neutralization buffer (87.66 g NaCl and 60.5 g Tris, pH 7.5 in 1 L of double distilled water) with gentle shaking for 30 min. Finally, the last treatment of the gel was with 20X SSC (88.23 g Trisodium citrate and 175.3 g NaCl in 1 L of double distilled water), pH 7.4 for 30 min and the neutral capillary transfer

was also performed in the same buffer (Sambrook et al, 1989). After the transfer the membrane was briefly washed in 5X SSC to remove any gel pieces adhering to it, air dried and finally cross linked in an Amersham Pharmacia UV cross linker by exposing the membrane to 120 milli joules of UV light and stored in cool and dry place between the folds of Whatmann filter paper until used.

Northern blotting:

Northern blotting was done following the same capillary transfer method explained in Southern blotting section (Sambrook et al, 1989). However, processing the denaturing gel before northern transfer is different from the previous one. The gel was immersed in sufficient volume of DEPC-treated water and kept on a rocker for 30 minutes to remove formaldehyde from the gel. Next, the gel was immersed in 20X SSC, pH 7.0 and kept on the rocker for 30-45 min. The set up for capillary transfer is similar except for the buffer, in this case 10X SSC, pH 7.0 was used.

Preparation of radiolabeled probe:

PCR amplified or restriction digested inserts of the DNA of interest, after eluting from agarose gel were used for preparation of the probe. DNA was radiolabeled using HexaLabel^{TM} DNA labeling kit from MBI Fermentas and [α^{32}P] -dATP following the manufacturer's instructions. The labeled DNA was either used for hybridization reaction directly or stored at –20°C for future use within the period of first half-life for the radiolabel. Before adding the probe in the hybridization buffer, the probe DNA was denatured in a boiling water bath for 5 min and snap cooled on ice.

Nucleic acid hybridization:

Hybridization of both, Southern and northern blots, were performed in bottles in a rotisserie device at 65°C. For hybridization, buffer comprised of 0.5 M phosphate buffer, pH 7.2, 7% sodium dodecyl sulfate, and 10 mM EDTA (Sambrook et al, 1989) was used. Appropriate volume of buffer, at least 70 µl/cm^2 of the blot, was used for hybridization. The blot was prewetted in water in a dish and followed by in 2X SSC; rolled and kept in a clean, dried hybridization bottle with the nucleic acid side upward. Usually 20 ml of buffer was prewarmed and poured in the bottle. 200 µg of sonicated salmon sperm DNA was boiled for 5 min, quickly chilled on ice and added in the prehybridization buffer to reduce background

and nonspecific binding. The blot was prehybridized for 4 h along with rotation at 2-3 rpm. A small volume of prehybridization buffer was taken out in a 15 ml disposable tube; denatured probe was mixed with the buffer and was poured back in the bottle and hybridization was carried out for 12-16 h. Blots were washed in excess of (at least 1-5 ml/cm^2) wash buffers twice, 5 min each in 2X SSC containing 0.1% SDS, followed by 1X SSC, containing 0.1% SDS for 15 min, and finally with 0.1X SSC containing 0.1% SDS for 2 x 10 min, at 65°C. Blots were exposed to Konica X-ray film inside a radioactive cassette with intensifying screen and stored in –70°C for required time period. Cassettes were taken out of –70°C and allowed to come to room temperature before they were developed and fixed, using X-ray film developer and fixer. Autoradiographs were washed thoroughly under running tap water and dried for storage.

Reverse transcription for preparation of cDNA:

For reverse transcription of RNA to form cDNA, the following components were mixed in a sterile 0.2 ml centrifuge tube using disposable RNase-free plastic wares, keeping all the components on ice. 1-5 µl of total RNA (1 µg/µl), 1 µl oligo-d $(T)_{18}$ (500 ng/µl), 1 µl 10 mM dNTP mix and DEPC-treated water to final volume of 12 µl**.** The components were mixed thoroughly by pipetting and it was briefly spun down. The RNA sample was denatured at 65°C for 5 min, snap chilled on ice for 2 min and centrifuged briefly. To this 8 µl of master mix containg 4 µl of 5 X First-Strand buffer, 2 µl 0.1 M DTT, 1 µl RnaseOut (40U/µl), 1 µl MMuLV Reverse Transcriptase (200U/µl) was added. Once again components were mixed well by pipetting up and down and centrifuged briefly. The reaction mixture was incubated at 42°C for 50 min using a thermal cycler, with a heated lid at 100^0C. Subsequently RT reaction was inactivated at 70°C for 15 min, chilled on ice for 2 min and centrifuged briefly. This RT product was used directly for PCR. In general 1-2 µl of RT reaction was used for 50 µl of PCR reaction volume. However, in some cases, like RACE and with SuperScript II RT, another optional step was followed. After inactivation, 1 µl of RNase H (2U/µl) was added to each tube and incubated at 37°C for 20 min. This product was either stored at –20°C or used immediately for PCR reaction.

Polymerase chain reaction (PCR):

The basic protocol, which served as a general guideline and a starting point for the PCR amplification is given below. However, optimal reaction conditions (incubation time

and temperatures, concentration of $MgCl_2$, primers, template DNA and *Taq* DNA polymerase) were altered according to the need. The following components were added to a sterile 0.2 ml microcentrifuge tubes kept in ice. 5 µl, 10 X PCR buffer mix without $Mg,^{++}$ 1.5 µl 50 mM $MgCl_2$, 1 µl 10 mM dNTP mix (each 0.2 mM), 6 µl primer mix (each 5 pico moles), 1-10 µl template DNA (1 pg-5 ng), 0.25 µl, 2.5 units *Taq* DNA polymerase (5U/µl) and sterile water to volume of 50 µl.

For PCR using platinum *pfx* from Invitrogen, $MgSO_4$ was used instead of $MgCl_2$. The 10X assay buffer for *Taq* DNA polymerase contains 200 mM Tris-HCl (pH 8.0) and 500 mM KCl. The PCR was carried out according to the following programme: (i) initial denaturation at 94°C for 2 min, (ii) denaturation at 94°C for 45 sec, (iii) primer annealing at 52-60°C for 30 sec, (iv) primer extension at 72°C (or 68 for *pfx*) for 1 min/kb and (v) final extension at 72°C (or 68°C for *pfx*) for 10 min. The lid temperature was set at 94°C before start of the cycles. After I step of initial denaturation, step ii- iv were repeated for 29 cycles, followed by the step of final extension and maintained at 4°C. The PCR reaction mixtures were either stored at –20°C or visualized upon agarose gel electrophoresis.

5' and 3' Rapid amplification of cDNA ends (RACE):

5' and 3' RACE was carried out following the manufacturer's instruction (Invitrogen). A flow chart for describing the different steps is presented in below**.** The internal primers designed for this purpose are presented in materials. The manufacturer supplied the RACE primers, and the RACE nested primers. 5 µg of a good quality total RNA was taken and sequentially subjected to dephosphorylation, decapping and RNA-oligo ligation. This processed RNA was then subjected to reverse transcription using GeneRacer^{TM} anchored oligodT primer. The first PCR reaction for 5' RACE was performed with gene specific reverse primer and GeneRacer^{TM} 5'primers, while that of 3' RACE was performed with gene specific forward primer and GeneRacer^{TM} 3'primers, using the RT reaction product as template. Next round of nested PCR was performed with gene specific reverse nested primer and GeneRacer^{TM} 5'nested primer for 5' RACE and gene specific forward nested and GeneRacer^{TM} 3' nested primer for 3' RACE, respectively, with the first round of PCR product as template. The RACE products were cloned in pTZ57R/T vector and sequenced. When all the sequences were aligned pair-wise by using BLAST program of

www.ncbi.nlm.nih.gov, full-length cDNA sequence along with the 5' and 3' UTRs was obtained.

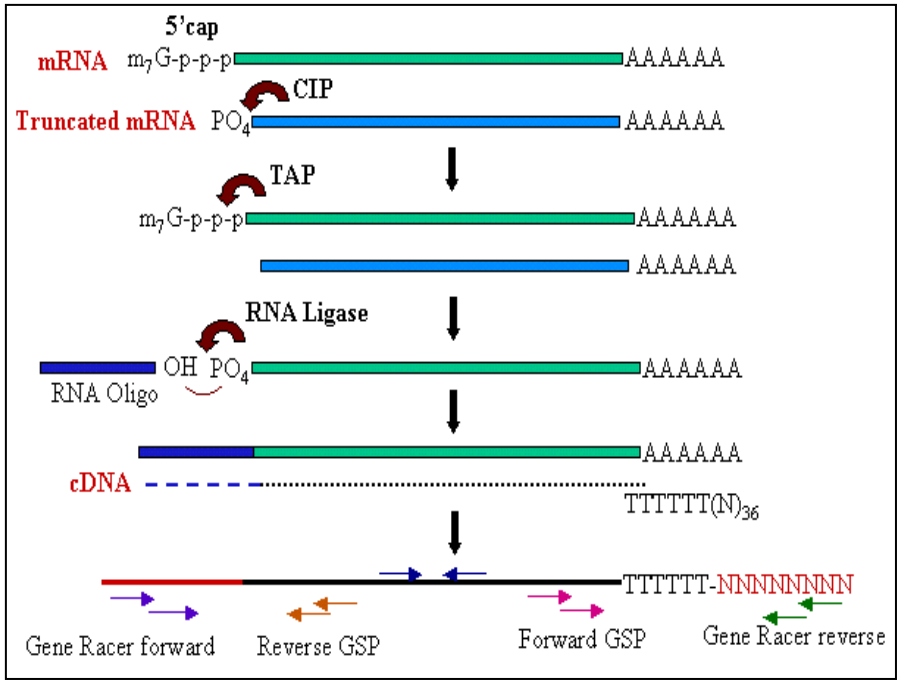


Fig. 7: Diagrammatic representation of the strategy followed for RACE and cloning of RACE products. 5 µg of total RNA was treated sequentially with calf intestinal phosphatase (CIP) for removal of exposed 5'PO_4 groups from the truncated RNAs, followed by Tobacco acid pyrophosphatase (TAP) for removal of CAP structure from the 5' ends of intact RNAs, and finally ligation of an RNA oligomer of known sequence to the newly exposed 5'PO_4 end. Reverse transcription is carried out with GeneRacer oligodT primer and PCR was performed with GeneRacer forward and gene specific reverse primer for 5' RACE, whereas, GeneRacer reverse primer and Gene specific forward primer for 3' RACE.

Gel elution of DNA:

Gel Cleanup kit from Sigma (USA) was used for extracting DNA from agarose gel, exactly following manufacturer's instructions. The DNA was eluted in 20-40 µl (depending on the initial concentration of DNA) of prewarmed sterile water. 2 µl of eluted DNA was run on an agarose gel to check the concentration and quality before using for further work and then stored at –20°C.

Cloning of PCR product into InsT/Aclone™ vector pTZ57R/T:

DNA polymerases (e.g. *Taq*) that are lacking 3'-5' exonuclease activity posses deoxynucleotidyl transferase (TdT) activity in addition to primer extension activity, which frequently results in the addition of extra adenines at 3'-ends of amplified DNA molecules. InsT/AcloneTM PCR product cloning kit was used for one-step cloning of *Taq* amplified, gel purified PCR fragments into a specialized vector pTZ57R/T vector, containing 'T' overhangs at the ends of the linearized vector for easy ligation with PCR products having 'A' overhangs. PCR amplified fragments were either column purified or separated on a 0.8% agarose gel, required fragments were excised and DNA was purified using DNA binding affinity matrices. The products were finally eluted in 20-30 µl of sterile water and used in next step for cloning. About 0.165 mg (0.18 p mol) of pTZ57R/T DNA *i.e.,* 3 µl was taken into a 1.5 ml centrifuge tube. To this, 12 µl (≈ 0.54 pmol) of PCR product was added and mixed thoroughly by pippetting up and down. Then 2 µl of 10X ligation buffer (400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP, pH 7.8 at 25^0C), 2 µl of 50% (w/v) PEG 4000 and 1 µl of T4 DNA ligase enzyme (5 U/µl in storage buffer 20 mM Tris-HCl pH 7.5, 1 mM DTT, 50 mM KCl, 0.1 mM EDTA and 50% glycerol) were added. Contents of the tubes were mixed thoroughly, briefly spun down and incubated at 22°C for 16 h. Ligation products were stored at –20°C until used for DH5α transformation.

'Blunt-end' cloning of PCR product:

PCR products amplified with high fidelity DNA polymerases, e.g. *pfx* (Invitrogen) or KOD hot start polymerase (Novagen) were blunt end products, which were cloned directly at blunt ends of S*ma*I digested vectors (pTZ57R and pGEX-5X-2). The following components were added keeping on ice in a sterile microfuge tube. About 3 µl of S*ma*I digested vector was taken into a 1.5 ml centrifuge tube. To this, 14 µl of PCR product was added and mixed thoroughly by pippetting up and down. Then 2 µl of 10X ligation buffer and 1 µl of T4 DNA ligase enzyme were added. The components were mixed thoroughly by pipetting and incubated in a water bath at 22°C for 16 h. Ligation mixture was either transformed immediately or stored in -20°C.

Preparation and transformation of competent cells:

Competent cells of *E. coli* {DH5α and BL-21 (DE3) pLysS strains} were prepared following Ausubel et al. (1994), with slight modification in the $CaCl_2$ method. Competent

cells of DH5α and BL-21 (DE3) pLysS (200 µl) were transformed using 10 µl of ligation product and transformed cell were selected using appropriate marker following blue white selecton.

Transformation of BL21 (DE3) plys cells with cloned plasmid and over expression of recombinant protein in bacterial system:

Competent cells of BL21 (DE3) pLysS cells were prepared following the similar method as mentioned for DH5α. However, the bacterial culture (without vector) was grown with chloramphenicol (17µg/ml of LB). Following transformation, colonies were observed after 16 h. A scoop of colonies were used for inoculation of 5 ml pilot culture for checking the protein expression. For overexpression of the recombinant protein, 1 ml over night grown starter culture was used for inoculation of 100 ml LB with appropriate antibiotics till OD_{600} reached 0.6-0.8. A stock solution of 1 M IPTG was prepared in sterile water and from this IPTG was added to the grown culture for induction to the final concentration of 1mM. The bacterial culture was induced for 1-12 h, depending on the requirement. The bacterial cells were harvested and then resuspended in PBS (1/20th volume of original culture) and sonicated (30 sec for 3-5 pulses). The presence of recombinant protein was analyzed on SDS-PAGE before further purification.

Computational work, sequence alignment and analysis:

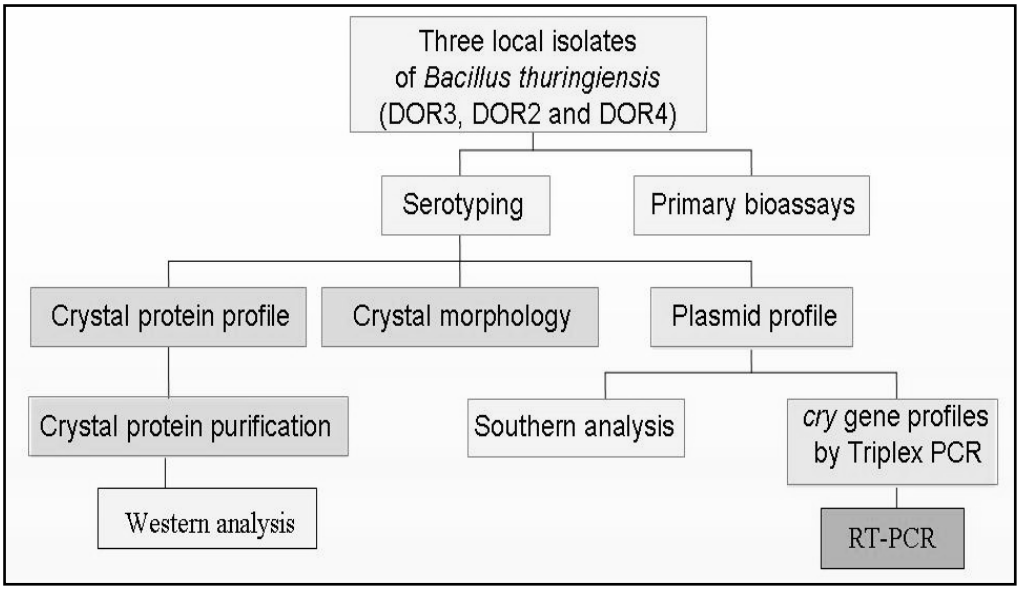
Among the software used for analysis of the data, were NCBI nucleotide and protein databases for BLAST searches, EBI toolbox and Expasy for protein analysis. Adobe systems and Microsoft office tools for rest of the purposes. The complete cDNA sequences and their putative translation products were analyzed using multiple alignments with other reported insect APNs assembled in a dendrogram using CLUSTAL W program. N-terminal and C-terminal GPI anchor, signal sequences were identified with the program PSORT II (http://psort.nibb.ac.jp) and GPI prediction program DGPI (http://129.194.186.123./GPI-anchor/index_en.html) respectively. The sequences were also analyzed for the presence of N- and O-glycosylation sites using NetOGlyc2.0 program (http://www.cbs.dtu.dk./services/netOGlyc).

Chapter I

Molecular characterization of local isolates of B. thuringiensis effective against larval forms of A. janata

Background:

Larval forms of castor semilooper (*A. janata*) cause serious damage and loss of castor crop. Out breaks are common during August-September in Deccan plateau. The crop is grown mostly in dry land areas of Andhra Pradesh and Gujarat in India (Sujatha and Lakshminarayana, 2005). Its control by chemical insecticides is becoming increasingly difficult due to development of resistance. So there is increasing emphasis on developing ecofriendly, safer and more effective methods of pest control (Prabagaran et al, 2002). The safe alternatives from the environmental standpoint are the biopesticides. Of all these biological control measures, exploring *Bacillus thuringiensis* (Bt) is an assured and profitable choice (Devi et al, 1996). In order to overcome the problem of resistance to Bt, there is renewing interest in the search for novel toxins to delay resistance as well as to incorporate the genes of Bt in to castor to develop transgenic lines. So before embarking on the programme of genetic engineering it is advantageous to identify suitable candidate genes and the reaction of castor semilooper to different Cry proteins as there is no information regarding this in the data base. This chapter deals with the morphological, biochemical and molecular characterization of three local isolates of *B. thuringiensis* DOR3, DOR2 and DOR4 potent against castor semilooper. In order to achieve the goal of identification and characterization of local isolates the following experimental strategy was followed:



Results:

Primary feeding bioassays using larval forms of *A. janata*:

In the primary feeding bioassays Bt ssp.*kurstaki* HD-1 served as a positive control. The toxicity of Bt isolates DOR3, DOR2 and DOR4 was found to be higher than that of *kurstaki* HD-1 (Fig. 1.0). Further extensive bioassays were carried out with pure crystal protein along with individual recombinant Cry toxins to understand the higher toxicity of these three isolates in comparison with control and data is included in the later part of this chapter.

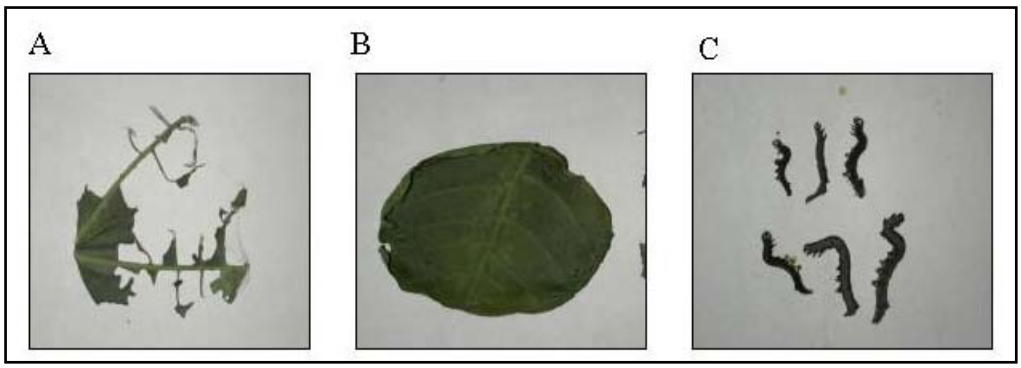


Fig. 1.0: Primary feeding bioassays. A. Control leaves fed by larval forms; B. DOR3 Bt toxin coated leaf surface; C. Larval mortality (please note that the mortality was seen in all the larval stages tested 2nd to 5th instar).

Serotyping of local isolates:

For serotyping studies H antigens and corresponding antisera were prepared from the different Bt serotypes. The results obtained clearly indicated that all three isolates DOR3, DOR2 and DOR4 are belong to serotype *kurstaki* H3a3b3c. Serotyping of three isolates was further confirmed using commercial antisera obtained from Paster Institute (data not presented).

Protein and plasmid profiles of local isolates:

SDS – PAGE analysis (Fig. 1.1A) of alkali solubilized crystal suspension of DOR3, DOR2 and DOR4 revealed similar protein profile to that of *kurstaki* HD1, with major proteins of 130 and 65 kDa (lanes 2-5). Trypsinization of this crystal suspension yielded the release of 65 kDa active toxin in all the isolates (Fig. 1.1B, lanes 1-4).

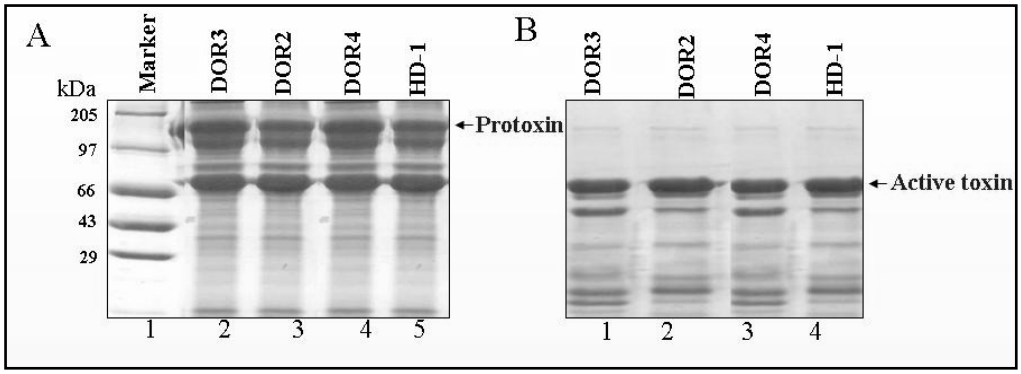


Fig. 1.1: SDS-PAGE analysis of alkali-solubilized parasporal inclusion proteins of Bt isolates **(A)** Two major proteins of 130 kDa and 65 kDa can be seen. Trypsin treatment of alkali-solubilized proteins **(B)**. **A**. Lanes 2, 3, 4, 5 are alkali solubilized crystal proteins from DOR3, DOR2, DOR4 and HD-1 isolates respectively. **B.** Lanes 1, 2, 3 and 4 are active toxins of crystal proteins obtained after trypsinization of DOR3, DOR2, DOR4 and HD-1isolates. Note the presence of 65 kDa active toxin in all the lanes. Lane 1**(A),** molecular weight marker.

Plasmids were isolated from the local isolates and they were found to be of very high molecular size. Plasmid profile of DOR3 and DOR4 was different from that of reference strain *kurstaki* HD1 (Fig. 1.2, lanes 2 and 4), However, DOR2 plasmid profile was quite similar to that of reference strain (lanes 1 and 3).

Morphological structure of parasporal crystals:

Figure 1.3 shows the scanning electron micrographs of the crystals produced by DOR3, DOR2, DOR4 and Bt *kurstaki* HD1. Three isolates under study predominantly showed bipyramidal shaped crystal along with few amorphous type crystals (Fig. 1.3A, B & C). Although there were some morphological differences observed between the local isolates of Bt, over all pattern was similar to those found in Bt *kurstaki* HD1 (Fig. 1.3 D). However, DOR3 morphology closely resembles more with HD-1 than DOR2 and DOR4.

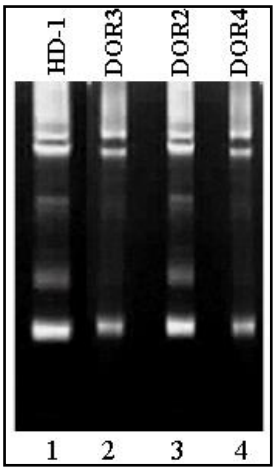


Fig. 1.2: Plasmid DNA pattern of local Bt isolates and its comparison with Bt *kurstaki* HD1. Lane1, Bt ssp *kurstaki* HD-1; lane 2, DOR3; lane 3, DOR2 and lane 4, DOR4.

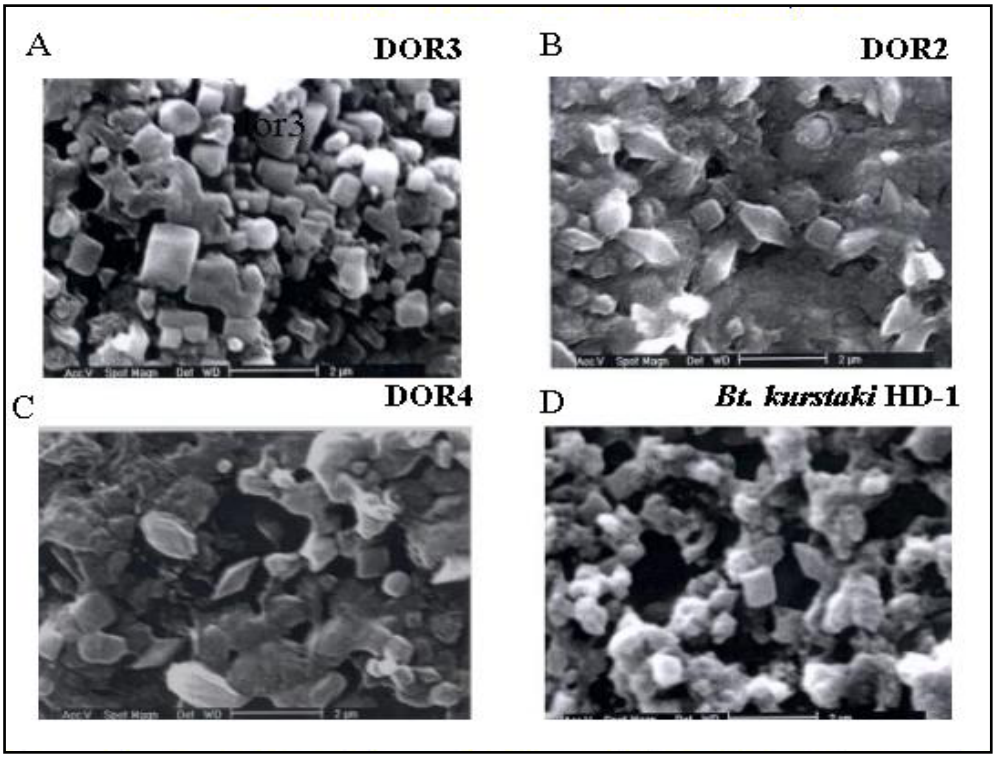


Fig. 1.3: Scanning electron micrographs of parasporal crystal inclusions from different local isolates, under same magnification. **A.** Bt isolate DOR3; **B.** DOR2; **C.** DOR4; D, Bt *kurstaki* HD-1.

Identification of nature of *cry* genes present in local isolates:

To determine the *cry* gene content of all the isolates under study, triplex PCR was carried out using *cry*1 and *cry*2 specific degenerate family primers with the inclusion of type specific primers, wherever appropriate as described in materials and methods.

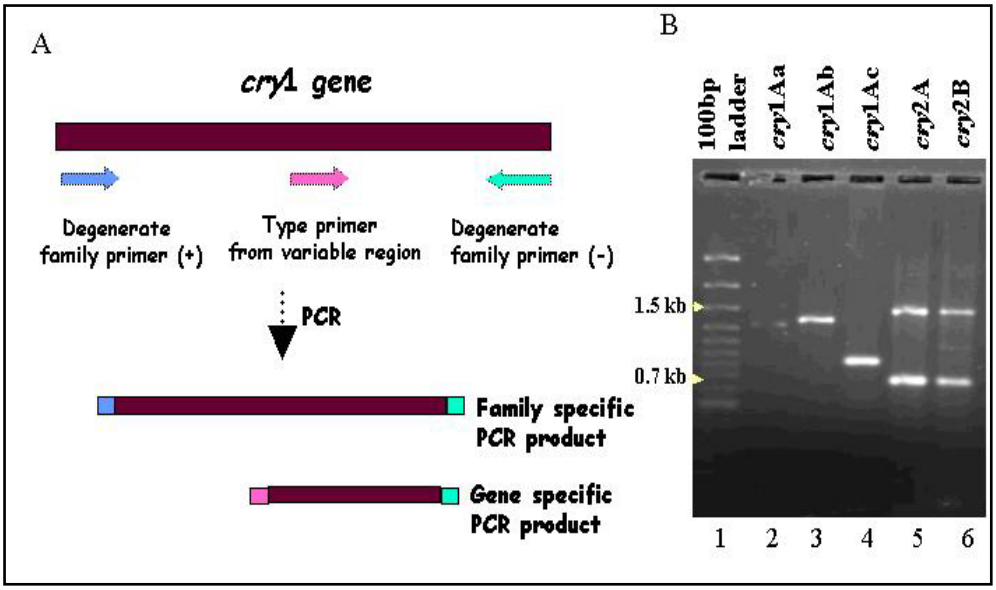


Fig. 1.4: A. Diagrammatic representation of Triplex PCR. B. Agarose gel electrophoresis of the PCR products obtained with control strain Bt *kurstaki* HD-1. Triplets of primers were used for identification of gene product. All lanes, except 1 contain a type primer mixed with degenerate family primers I (+) and I (-). Lane 2, primer 1Aa; lane 3, primer 1Ab; lane 4, primer 1Ac; lane 5, primer 2A; lane 6, primer 2B; lane 1, 100 bp DNA ladder.

The production of single PCR product confirms the presence of specific class, and appearance of second band identifies the specific gene type (Fig. 1.4, A). The consistency and reproducibility of the method, was ascertained by carrying out triplex PCR for *kurstaki* HD1 with *cry*1 and *cry*2 degenerate family and type specific primers. The control strain Bt *kurstaki* HD-1 showed expected PCR products of *cry*1Aa, *cry*1Ab, *cry*1Ac, *cry*2A and *cry*2B (Fig. 1.4B, lanes 2-6). After confirming the fidelity of triplex PCR, the study was extended to identify the *cry* gene content of all the three isolates under study (Fig. 1.5). All of them were positive with the type primer *cry*1Ac (Fig. 1.5A, B & C, lane 4 in each) but interestingly for DOR2 *cry*1Ac PCR product was slightly larger than the expected size (Fig. 1.5B, lane 4)

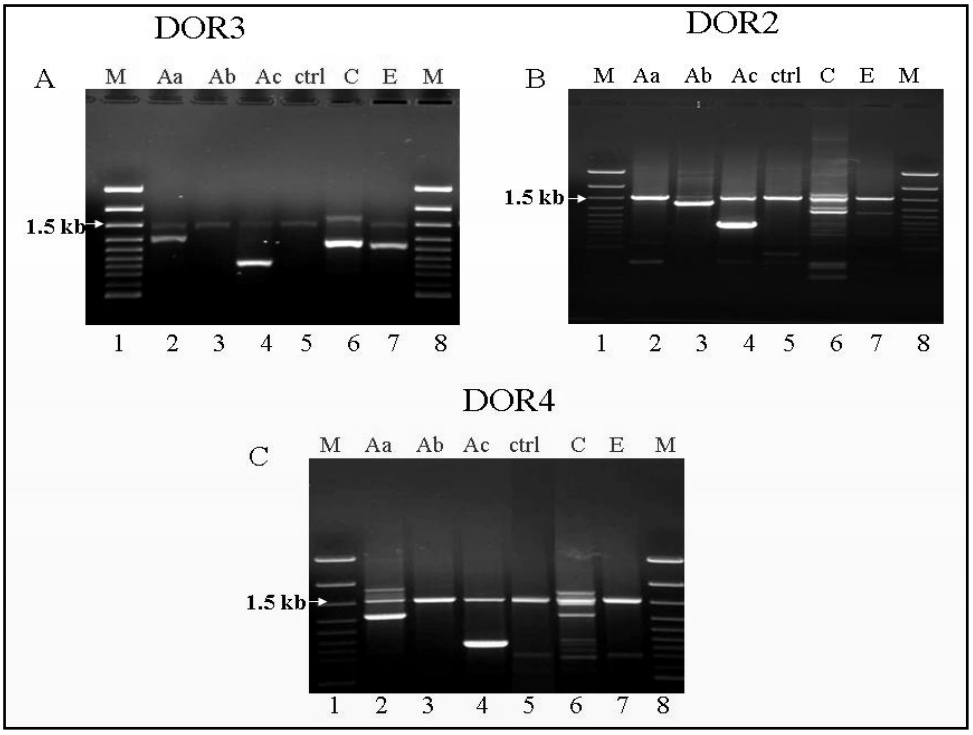


Fig. **1.5:** Identification of the *cry*1 genes present in isolates DOR3, DOR2 and DOR4. A. DOR3; B. DOR2; C. DOR4. Triplets of primers were used to identify *cry* gene content of three isolates. Lanes 2-6, contain a type primer mixed with primers I (+) and I (-). Lane 2, primer 1Aa; lane 3, primer 1Ab; lane 4, primer 1Ac; lane 5, ctrl (only family primers); lane 6, primer1C; lane 7, primer1E; lanes 1 and 8, molecular weight marker-100 bp DNA ladder.

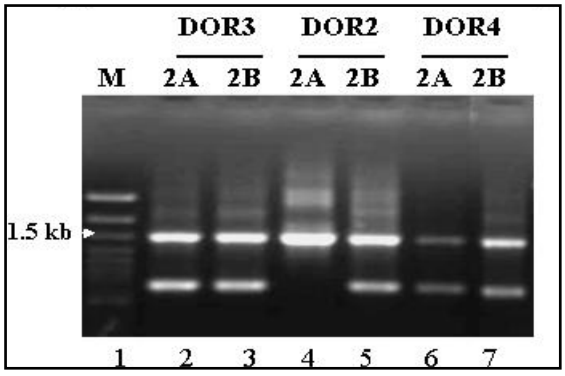


Fig. 1.6: Identification of the *cry*2 genes present in local isolates. Triplets of primers were used to identify *cry* gene content. Lanes 2-7, contain a type primer mixed with primers II (+) and II (-). Lane 2, primer 2A; lane 3, primer 2B; lane 4, primer 2A; lane 5, primer 2B; lane 6, primer 2A; lane 7, primer 2B, lane 1, molecular weight marker.

The *cry*1A gene content of DOR3 and DOR4 (Figs. 1.5A & C) are found to be similar to that of reference strain (Fig. 1.4 B, lanes 2-4) with the exception of *cry*1Ab, which was absent in these local isolates. On the other hand, DOR2 was negative for *cry*1Aa (Fig. 1.5B, lane 2) and positive for *cry*1Ab (1.5B lane 3). Further DOR3 was positive with *cry*1C and *cry*1E type primer (Fig. 1.5A lanes 6 & 7) where as DOR2 and DOR4 (Fig. 1.5 B & C) both positive with *cry*1C type primer (lane 6) but negative with *cry*1E type primer (lane 7). The *cry*2 gene profiles of local isolates found to be similar (Fig.1.6, lanes 2, 3 & 5-7) to that that of reference strain (Fig. 1.4B, lanes 5 and 6) with the exception of DOR2 in which *cry*2A was absent (Fig.1.6, lane 4).

In recent years, a number of secreted insecticidal proteins called vegetative insecticidal proteins (Vip) have been identified during the vegetative growth phase. These Vip proteins have a broad insecticidal spectrum that includes a wide variety of lepidopteran and coleopteran species. One of the most important features of Vip is that it shares no sequence homology with known Bt delta endotoxins. In order to prevent or delay the development of resistance, a multiple toxin approach has been suggested. So there is a need to screen the Bt isolates in order to find out the variants or new *vip* genes. In the present study DOR3, DOR4 and HD-1 were found positive for *vip* genes (Fig. 1.7 lanes 1, 3 & 5) where as DOR2 was negative (lane 2).

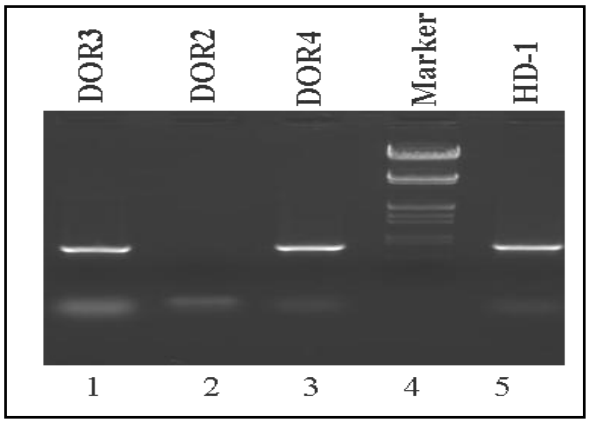


Fig. 1.7: Identification of *vip* genes**.** Lane 1, DOR3; lane 2, DOR2; lane 3, DOR4; lane 4, marker; lane 5, Bt *kurstaki* HD-1.

A comparative account of the gene content present in three isolates is presented in table1. The data clearly shows the presence of different gene profiles in DOR3, DOR2 and DOR4.

Bt isolates	*cry* and *vip* genes							
HD-1 Ctrl	Aa	Ab	Ac	-	-	2A	2B	Vip
DOR3	Aa	-	Ac	C	E	2A	2B	Vip
DOR2	-	Ab	Ac	C	-	-	2B	-
DOR4	Aa	-	Ac	-	E	2A	2B	Vip

Table 1: Summary of the *cry* gene profile in three local isolates and control. The *vip* gene profile is also included for comparison.

So after observing clear variations in the plasmid profile, crystal morphology and *cry* gene content extensive studies were carried out to understand the basis for higher toxicity of local isolates in spite of their similarity in the protein profile because all the *cry*1 genes code for 130-140 kDa protein, so it is very difficult to conclude merely based on PCR profile which *cry* 1 genes are getting translated into proteins.

Location of *cry* genes on plasmids by Southern blotting:

Existing literature clearly suggests that in Bt, *cry* genes are normally located on large plasmids. Hence in the present study Southern analysis was carried out using local strains to find out copy number and location of different *cry* genes. Isolated plasmids were digested with various restriction enzymes and blotted on to nylon membrane. Hybridization was carried out and the blots were probed with radiolabelled *cry*1Ab and *cry*1Ac fragments which were generated by triplex PCR using either DOR2 or DOR3 total DNA as the template.

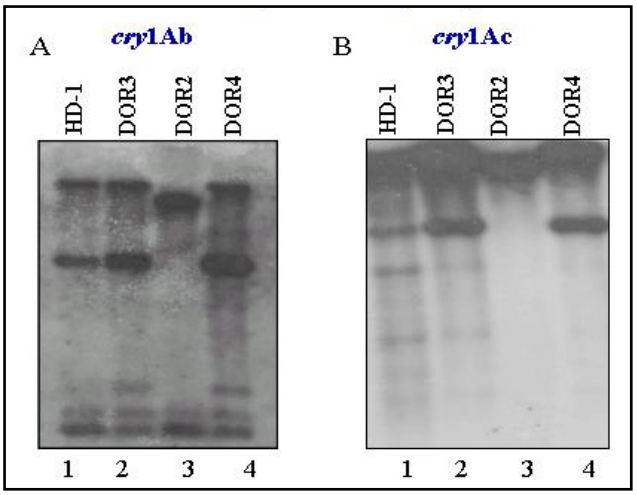


Fig. 1.8: Southern analysis of *cry* genes in three local isolates of Bt and control HD-1. The blot was probed with *cry*1Ab fragment generated from DOR2 (**A**) and *cry*1Ac generated from DOR3 (**B**). Lane 1, HD-1; lane 2, DOR3; lane 3, DOR2; lane 4, DOR4.

Southern blotting with DOR2 *cry*1Ac, as the probe revealed the hybridization in all three isolates (Fig. 1.8A lanes 2-4), but there was difference in the location of band (lane 3). Similar difference in the hybridization pattern of *cry*1Ab was observed between DOR2 and other two DOR strains (DOR3 and DOR4). Further the hybridization pattern of the DOR3 and DOR4 found to be similar to that of HD-1, while DOR2 pattern differed significantly from HD-1 (Fig. 1.8B, lane 3).

Crystal protein purification, antibody generation and immunological study:

Based on the primary bioassay data DOR3 was found to be most toxic followed by DOR4 and DOR2. So in order decipher the reasons for the differential toxicity of three isolates, the crystal proteins were purified (Fig. 1.9A, lanes 2 & 3) from all of them using sucrose density gradient centrifugation (Thomas and Ellar, 1983). The crystal proteins were solubilized in sodium carbonate buffer (pH 10.5). The protoxin was treated with trypsin in 50:1 ratio for generation of activated toxins (Fig. 1.9B, lane 2). After trypsin digestion smaller fragments were removed from the activated toxin using 10 kDa cut off protein concentrators. The activated toxins obtained from three different isolates were used for extensive bioassays. Antisera was generated for the DOR3 activated toxin in order to find out binding proteins for Cry toxins. The specificity of the generated DOR3 antisera was

confirmed by immunoblotting (Fig. 1.9C). Cross reactivity of DOR3 antisera, with crystal proteins of other isolates demonstrated the antigenic similarity of the Cry toxins among these isolates. DOR3 antisera also cross reacted with purified activated toxins as well as 130 kDa protoxin suggesting that activated toxins originated from the protoxin (Fig.1.9C,lanes 2 & 3).

Generation of individual recombinant Cry toxins and corresponding antibodies:

Individual recombinant Cry toxins were over expressed in the form of protoxins (Fig. 1.10A, lanes 1-5) and digested with trypsin to generate activated toxins and purified (Fig. 1.10B, lanes 1-5) according to the method of Lee et al (1992). The purified recombinant toxins were used in bioassays and subsequently individual antisera was generated for Cry1Ac, Cry1C and Cry1E toxins. The specificity of the antisera was confirmed by immunoblotting (Fig. 1.10C, lanes 1-3).

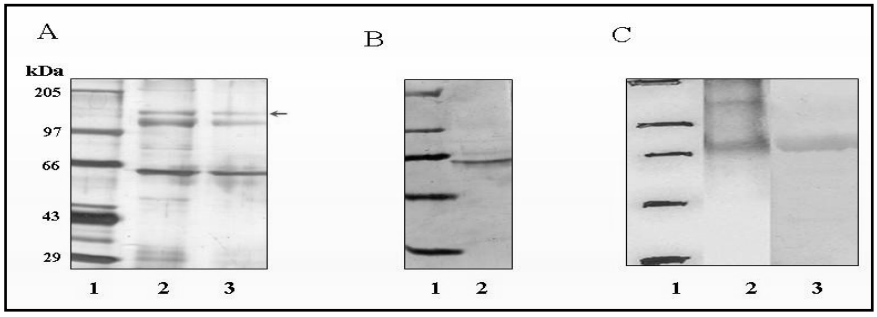


Fig. 1.9: Electrophoretic analysis of local Bt strain DOR3 purified *cry*stal proteins (A) and trypsin-activated toxins (B). A. Lanes 2, crude protoxin; lane 3, density gradient fraction; **B**. Lane 2, purified activated toxin**. C**. Immunoblot analysis of DOR3 crystal protein with DOR3 antisera. Lane 2, purified protoxin; lane 3, activated toxin. Lane 1, in all the figures is molecular weight marker.

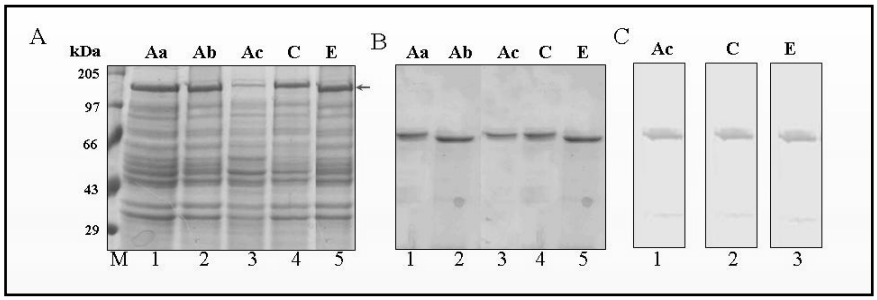


Fig. 1.10: SDS-PAGE analysis showing expression of different recombinant Cry protoxins (**A**) and corresponding trypsinized toxins (**B**). **A**. Lane 1, BGSC ECE52 *cry*1Aa; lane 2 ECE54 *cry*1Ab; lane 3, ECE53 *cry*1Ac; lane 4, *E. coli* DH5α strain ECE125 *cry*1C and lane 5, ECE127 *cry*1E. **B**. Lanes 1- 5 are corresponding activated toxins obtained after protoxin trypsinization. **C.** Immunoblot analysis of the activated toxins. Lane 1, Cry1Ac; lane 2, Cry1C; lane 3, Cry1E toxins probed with respective antisera

Evaluation of insecticidal activity of local Bt isolates and its comparison with Bt *kurstaki* HD-1:

Bt *kurstaki* HD-1 served as a positive control in the bioassay against *A. janata* larvae. The results presented in the table 2 clearly show the toxicity of the local isolates (DOR3, DOR4 and DOR2) was higher than that of control. Furthermore isolate DOR3 is more

Strain	LC_{50} ng/cm^2	Regression equation
HD-1	50.09	Y= -1.70+0.034X
DOR3	18.08	Y= -1.61+0.09X
DOR2	33.40	Y= -1.52+0.045X

Table 2. Bioassays carried out with second instar larval forms of *A. janata* using purified Crystal proteins prepared from DOR3, DOR2 and HD-1

toxic and DOR2 was found to have lowest level of toxicity (Table 2). The toxicity variation among these isolates may be due to the different gene composition and their differential expression. In addition extensive bioassays were carried to find out the degree of toxicity of each of them and data is presented in table 3. The results show that Cry1Ac is more toxic to the larval forms of *A. janata* than Cry1Ab and Cry1Aa was least toxic (Table 3).

Toxin	LC_{50} ng/cm^2	Regression equation
Cry1Aa	61.01	Y= -1.91+0.031X
Cry1Ab	32.03	Y= -1.57+ 0.049X
Cry1Ac	23.70	Y= - 2.18+0.092X

Table. 3. Bioassays carried out with second instar larval forms of *A.janata* using purified recombinant Cry toxins. The recombinant clones were obtained from BGSC.

After carrying out bioassays with individual purified Cry toxins, further studies were carried out, to confirm whether this difference in toxicity of these local isolates is associated with differential expression of *cry* genes leading to production of protein crystals of different composition. Furthermore, since crystals are usually composed of more than one protein type, the relative proportions of the different proteins within the crystal can influence bioassays. Morphological studies (Fig. 1.3) also revealed variations of crystals produced by the local strains (DOR3, DOR2 and DOR4). Additionally, other factors, such as plasmid stability and nutritional requirements as well as mobile genetic elements can also introduce variability in the composition and the potency of crystals within the same strain (Masson et al, 1998). So, RT-PCR analysis was carried out to confirm the corresponding transcript presence in these isolates.

RT-PCR analysis of *cry* gene expression in local isolates of Bt:

To investigate whether the cDNA generated from the local isolates was representative of genes expressed under the conditions used and to test for the presence of these sequences in each cDNA population, PCR was performed using the cDNAs as templates with various primer pairs which were used for direct triplex PCR. DNase-I-treated RNA population isolated during sporulation stage was used for cDNA synthesis. Total RNA also was used as negative control. In most of the cases, expected sizes of amplicons were observed (Fig. 1.11A lanes 1, 3 & 5; Fig. 1.11B lanes 2-4), suggesting that promoter sequences upstream of the open reading frames are functional and full-length messages were produced. Amplification products were neither obtained with *cry1C* nor *cry1E* type primers (data not presented). Further it is interesting to note that transcript size of DOR2 *cry1Ac* (Fig. 1.11B, lane 3) was slightly larger than DOR3 and DOR4 *cry1Ac* (Fig. 1.11B, lanes 2 and 4). In addition, no amplification products were obtained when DNase-treated RNA samples were used as templates, demonstrating the absence of contaminating genomic DNA.

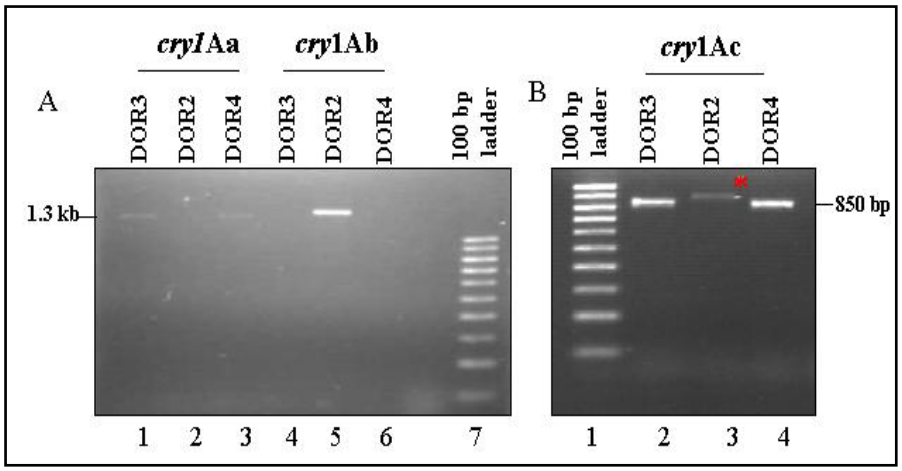


Fig. 1.11: Identification of different *cry* gene transcripts by bacterial RT-PCR. A. *cry1Aa* transcript: Lane 1, DOR3; lane 2, DOR2; lane 3, DOR4. *cry1Ab* transcript: Lane 4, DOR3; lane 5, DOR2; lane 6, DOR4; lane 7, 100 bp DNA ladder. **B.** *cry1Ac* transcript: Lane 1, 100 bp DNA ladder; lane 2, DOR3; lane 3, DOR2; lane 4, DOR4.

Characterizations of new variants of *cry* genes:

After seeing variations in the toxicity of local isolate and combination of *cry* gene expression in them as well as and differences in the sizes of the expected PCR products, the *cry* genes from them were cloned and sequenced. Further clustal W analysis was carried out to analyse variations in the amino acid residues of the cloned genes. The PCR product of DOR2 isolate obtained with *cry*1Ac and *cry*1Ab type primers were sequenced. BLAST analysis indicated that the *cry*1Ac product has homology with *cry*1B (95% identity). It further showed only 84% homology to the reported *cry*1Ac (Fig. 1.12). Even though the DOR2, *cry*1Ac sequenced product showed homology to *cry*1B, when we used *cry*B type specific primer in the PCR, the result was negative. Where as *cry*1Ab PCR product showed 98 % homology to *cry*1Ab reported sequences.

*cry*1Aa and *cry*1Ac PCR products from DOR3 were also cloned and sequenced. The Blast analysis of the DOR3, sequences showed 99% homology with reported sequences with little variations in the amino acid residues.

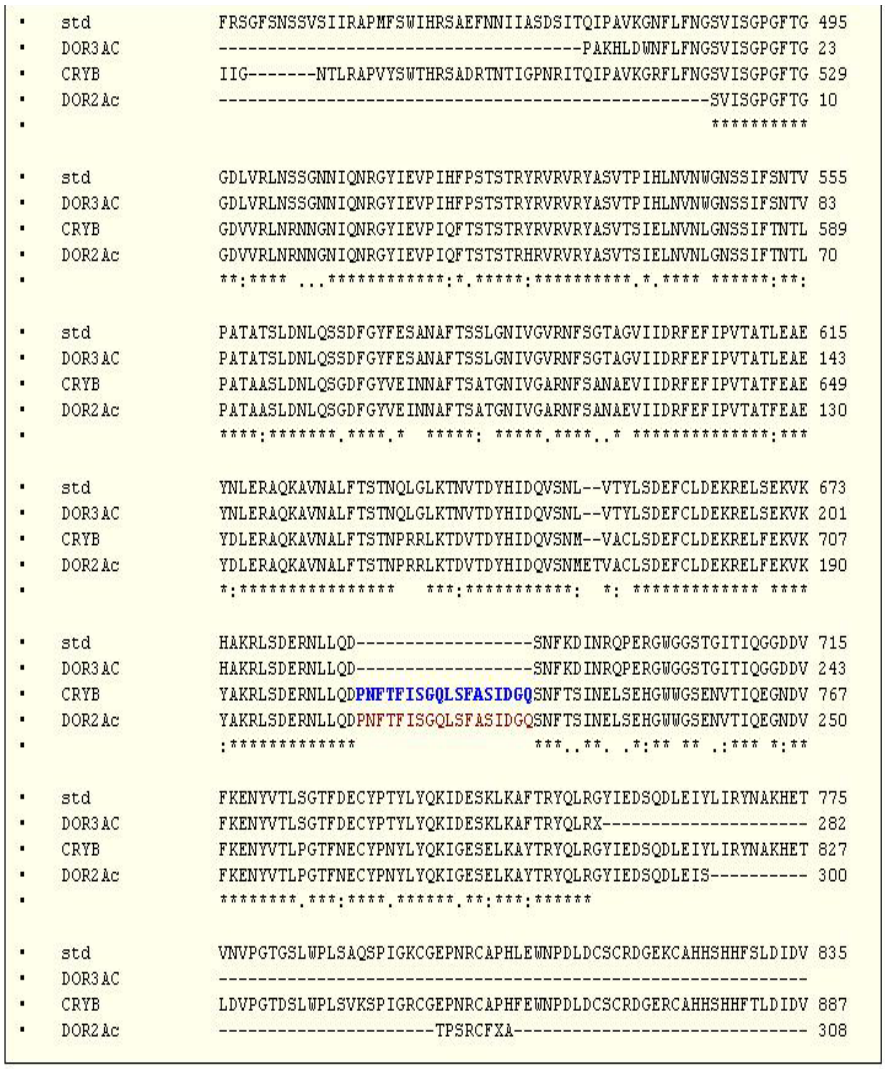


Fig. 1.12: Clustal W analysis carried out with DOR2 Ac, DOR3 Ac, and *Cry*1B and the standard *Cry*1Ac from Bt *kurstaki* HD-1.

Discussion:

The local isolates of Bt that showed higher toxicity than *Bt kurstaki* HD-1 against *A. janata* larval forms were identified primarily by phase contrast microscopy, serological identication and by bioassays. The three isolates under study showed similar protein profiles but exhibited different plasmid profile. Crystal morphology of the three isolates also showed

variations in the shapes of the crystal among them and then with the reference strain Bt *kurstaki* HD-1. Identification of the *cry* genes was carried out by triplex PCR method. Earlier several PCR, PCR-RFLP and hybridization based strategies have been used (Prabagaran et al, 2002) to screen local Bt isolates and to identify *cry* genes having minor amino acid sequence variations (Porcar and Juarez-Perez, 2003). But here we used triplex PCR, in order to identify the existing *cry* genes as well variants of known *cry* genes, called putative novel *cry* genes in the three isolates under study. The triplex PCR method displayed completely different *cry* gene profil in three local DOR isolates when compared with the reference control *kurstaki* HD-1. Two isolates DOR3 and DOR4 showed the combination of *cry*1Aa and *cry*1Ac, while DOR2 alone showed the presence of *cry*1Ab and *cry*1Ac genes.

Present study demonstrates that although *kurstaki* HD-1 expresses all three types of Cry toxin (Aa, Ab and Ac), but toxicity of three local DOR isolates is more than that of reference strain HD-1. Although identification of the *cry* gene content of a strain is important, it formulates only part of our understanding of the behavior of a particular isolate in insect bioassays (Masson et al, 1998). As shown in this study, it is not immediately clear which genes are eventually translated, based solely on information gained from PCR screening. For that after identifying *cry* genes by PCR, RT-PCR was carried out basically to ensure the expression of the identified genes at the RNA level. The RT-PCR studies clearly showed the presence of respective transcripts during the period of sporulation. RT-PCR experiments further demonstrated the expression of the variant *cry*1Ac gene in DOR2 with similar size difference in transcript what was observed in triplex PCR. Comparison of the DOR2 *cry*1Ac deduced protein sequence with reported *cry*1Ac showed that there are variations in the important residue, and these are known to be specifically responsible for the binding of toxin to receptor. However the other genes *cry*1Aa, *cry*1Ac from DOR3 showed almost 98% homology to the reported *cry* genes. In the present study neither the *cry*1C nor *cry*1E transcripts were observed in any of the three isolates. The presence of *cry*1C and *cry*1E is not very unique, because *B. thuringiensis* cells have been known to spontaneously lose plasmids and consequently plasmid-borne *cry* genes, as well as gain new *cry* genes because of homologous recombinations (Masson et al, 1990; Aronson et al, 1991). There are reports of *B. thuringiensis* ssp *kurstaki* isolates containing *cry*1C and *cry*1E crystal proteins.

But till to date, no report is available of Bt *kurstaki* strain containing *cry*1B. The homology of DOR2 *cry*1Ac to cry1B looks unique. Therefore, although these isolates are serotyped as ssp. *kurstaki,* these three isolates DOR3, DOR2 and DOR4 may be new Bt *kurstaki* strains with different gene profile.

After identifying the presence of transcripts for *cry*1Aa, *cry*Ab and *cry*Ac in DOR isolates, bioassays with individual recombinant Cry toxins was carried out, basically to find out the basis and to correlate the higher toxicity of these to larval forms of *A. janata* in comparison with the *kurstaki* HD-1. Bioassays with individual Cry toxins proved, that Cry1Ac is more effective followed by Cry1Ab and Cry1Ac. Among the three isolates only DOR2 showed presence of *cry*1Ab gene, Moreover, the 44-MDa plasmid that encodes the *cry1Ab* gene is known to be unstable (Ben-Dov et al, 1997; Bravo et al, 1998). Loss of this plasmid from a *B. thuringiensis* subsp. *kurstaki* isolates would alter the *cry1A* gene PCR profile, resulting in the exclusion of the isolates as a putative *Bt kurstaki* HD1 isolate (Valadares De Amorim et al, 2001). Lee et al, (1996) demonstrated the effects of three combination of *cry* toxin mixture on *L .dispar*, the Cry1Aa -Cry1Ac mixture was about eight times more effective than Cry1Aa -Cry1Ab and four times more effective than Cry1Ab -Cry1Ac and suggested a synergistic effect between *Cry*1Aa -*Cry*1Ac and antagonistic effect between Cry1Aa -Cry1Ab and Cry1Ac -Cry1Ab toxins. This high toxicity of DOR3 and DOR4 in the present study also may be due to the synergistic effect of Cry1Aa - Cry1Ac and less toxicity of DOR2 may due to the antagonism between Cry1Ab – Cry1Ac (Cry1Ab-Cry1B/Cry1Ac). In order to demonstrate the synergistic and antagonistic effect of these three toxins against this particular insect, binding assays were carried out using Cry1Aa, CryAb and Cry1Ac (will be discussed clearly in chapter 3). All these three toxins showed binding to 110-120 kDa protein but binding to ~170 kDa protein was exhibited only by Cry1Aa and Cry1Ac. Heterologous competitive binding assay showed that in presence of *Cry*1Ab, *Cry*1Aa binding was inhibited. This clearly demonstrated that both toxins most likely compete for the same binding site on 110-120 kDa protein. The higher toxicity of DOR3 and DOR4 is most likely due to binding of Cry1Aa and Cry1Ac toxins to different receptors simultaneously. The Cry1Ab alone binds to 110-120 kDa protein. So in *kurstaki* HD-1 synergistic effect of the Cry1Aa - Cry1Ac toxin mixture might be partially masked by the

antagonistic effect of the Cry1Ac-Cry1Ab. The present study clearly shows that because of the absence of Cry1Ab synergistic effect of Cry1Aa - Cry1Ac DOR3 and DOR4 isolates are more toxic than DOR2 and *kurstaki* HD-1 which express *cry1Ab* gene. The higher toxicity of the selected isolates could also be due to other factors: the *cry* genes detected by PCR may represent fragments of genes encoding novel variants of known proteins; the *cry* genes may be identical, but the expression levels of at least some of them may be different; or an undetected factor or protein may be responsible for their higher activity (Masson et al, 1998).

The PCR results obtained with strain DOR2 suggest that it may harbor a putative novel *cry1B* gene and even in the absence of normal *cry* gene content in comparison with *kurstaki* HD-1, DOR2 Cry proteins are fairly toxic to *A. janata* larval forms. So the cloning and expression of the genes and the characterization of its potential insecticidal activity against this pest as well different insect species has to be determined.

Chapter II

Identification and characterization of larval midgut proteases of A. janata and their role in protoxin activation

Background:

The understanding of basic digestive physiology of lepidopteran insect is essential to understand the role of proteases in the activation of Cry toxins (Lecadet and Martouret, 1965; Keller et al, 1996; Lightwood et al, 2000; Mohan and Gujar, 2003). Further it is also useful in the evaluation of toxicity of some protease/proteinase inhibitors (PIs) towards this insect (Broadway, 1997; Pereira et al, 2005; Telang et al, 2005). *B. thuringiensis* delta endotoxins are toxic to a variety of different insect species. Here, we investigated the role of proteolytic processing in determining the potency of the *B. thuringiensis* delta endotoxin towards larval forms of *A. janata*. Larvicidal potency depends on the completion of a number of steps in the mode of action of the toxin. Any alternation in the interaction of gut proteases with Bt toxins leads to the insect survival and development of resistance (Oppert et al, 1997). Henceforth, it is extremely important to study the gut protease activities of the individual insect, in order to understand the proteolytic activation of protoxins and its successful usage in pest management. To the best our knowledge there is no information available on the digestive enzymes of this species, hence the characterization would not only allow the exploitation of novel *B. thuringiensis* isolates but also protease inhibitors like insect control agent, interms of PI transgenics.

In the present chapter complete characterization of midgut protease pattern was carried out in order to identify the proteases which are involved in the protoxin activation in larval forms of A. *janata* and to find out whether midgut homogenate of this insect has the same composition and quantities of proteolytic enzymes, as other lepidopteran insects. Initially, zymogram analysis was carried out either with casein or Bt protoxin as a substrate. Further, activity blots were carried out with the specific substrates and inhibitor, in order to estimate the corresponding molecular masses of proteases.

Results:

Effect of pH on protease activity and composition midgut proteases:

A pH of 9-10.5 and temperature of 30°C was found to be required for optimum midgut protease activity on the basis of the initial studies with buffers of pH range from 6 to 12. The total enzymatic activity of the midgut homogenate was highest at pH 10.5 using azocasein as protein substrate. Keeping this in view zymogram studies were carried out at pH

10.5 during different developmental stages, this also indicated that the major enzymes in the larval midgut of *A. janata* are active at high pH optima.

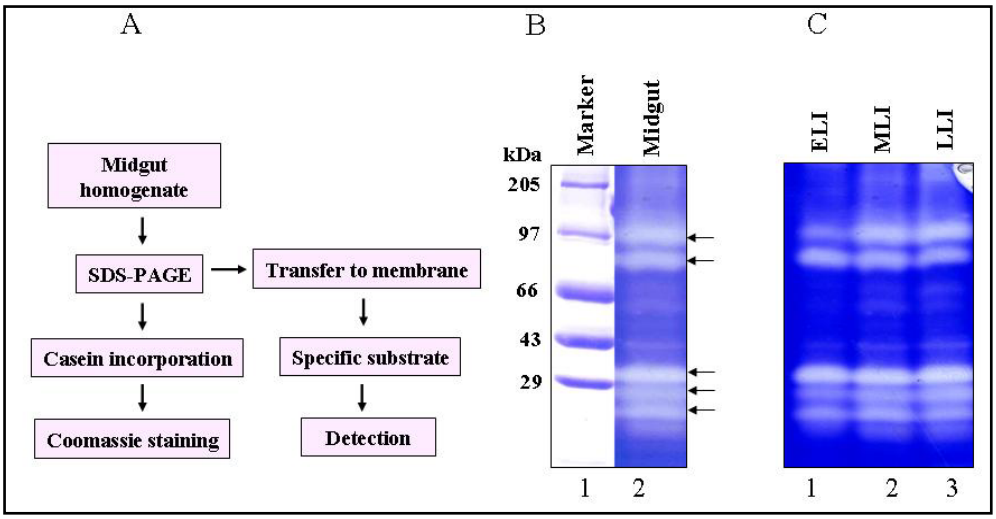


Fig. 2.1: A. Flow diagram for the zymogram and activity blot analysis. **B**. Zymograms of midgut homogenate of *A. janata* subjected to 7.5% SDS–PAGE according to the method of Method of Garcia –Carento et al (1993), for this 2% casein were allowed to penetrate the gel after SDS–PAGE. Lane 1, molecular weight marker; lane 2, midgut proteases. **C.** Developmental profile midgut proteases of *A. janata*. Lane1, early last instars; lane 2, mid-last instars; lane3, late-last instar.

Developmental profile of midgut protease pattern:

Zymograms of midgut homogenate were carried out to determine the composition and molecular weight of the proteases present in it. This was done by subjecting midgut homogenate juice to SDS-PAGE on 7.5 % non-reducing gels and processing it with casein as substrate. The results obtained show the presence of midgut proteases corresponding to approximate sizes of 17, 20, 29 and 80, and 100 kDa (Fig. 2.1B, lane 2). The major protease pattern remained more or less the same through out the last instar development of larvae with slight quantitative variations (Fig. 2.1C, lanes 1-3).

Charcterization of midgut proteases:

The proteases of the midgut homogenate were further characterized using specific substrates and inhibitors (Fig. 2.2). The PMSF, specific inhibitor for serine proteases, at 300 µM concentration inhibited activity of almost all the major proteases (lane 2). The irreversible inhibitor TLCK (lane 4) and reversible inhibitors SBTI (lane 6) and TCI (lane 7)

at the concentration of 300 µM, 100 µg/ml, and 100 µg/ml respectively showed differential inhibitory effect towards different proteases (Fig. 2.2).

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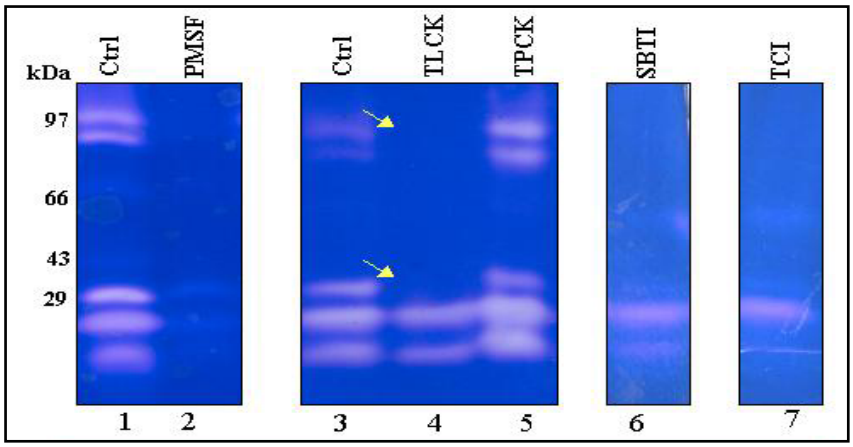

Fig. 2.2: Zymogram of larval midgut homogenate of *A. janata*. Lanes 1 and 3, midgut homogenate; midgut homogenate incubated with PMSF (lane 2); with TLCK (lane 4); with TPCK (lane 5); with SBTI (lane 6); with TCI (lane 7).

The 29, 80 and 100 kDa activities were almost completely inhibited by 300 µM TLCK (lane 4), whereas other activities remained unaffected. The bands inhibited with TLCK were thus identified as trypsin where as bands inhibited with both 100 µg/ml SBTI (lane 6) and 100µg/ml TCI were considered as trypsins or chymotrypsin like serine proteases but interestingly, 300 µM TPCK the chymotrypsin inhibitor did not show any activity when incubated with midgut homogenate (lane 5). Four bands of 17, 29 and 80 and 100 kDa were inhibited by both 100 µg/ml SBTI (lane 6) and 100 µg/ml TCI (lane 7), where as TLCK did not show any effect on 20 kDa protein but slightly inhibited 17 kDa protein (lane 4). The band which was between 17 and 29 kDa proteins was found to be unaffected either by TLCK or reversible inhibitors SBTI and TCI (lanes 4, 6 & 7)

Activity blot analysis:

Activity blot analysis using specific chromogenic substrates BApNA and various inhibitors was carried out to determine the relative sizes of trypsin like enzymes. As observed in the figure 2.3, using PMSF and TLCK inhibitors, major BApNA-hydrolyzing enzymes, one with low molecular weight (29 kDa) and the others with high molecular weight (80 & 100 kDa) were completely inhibited by 150 µM PMSF and 150 µM TLCK respectively.

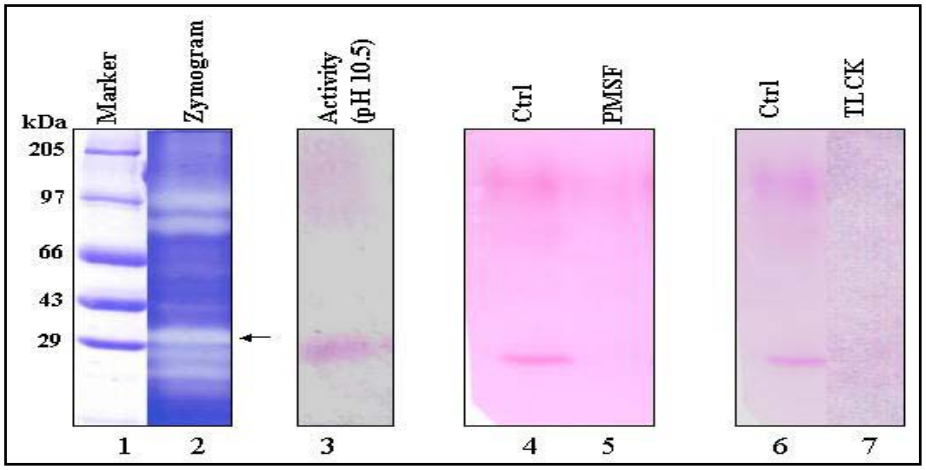

Fig. 2.3: Zymogram of midgut homogenate. Lane 1, molecular weight marker; lane 2, midgut homogenate. Lane 3-7 activity blots with 0.2 mM BApNA as a substrate; midgut homogenate (lane 3); midgut homogenate preincubated without PMSF (lane 4); with 150 µM PMSF (lane 5); without TLCK (lane 6); with 150 µM TLCK (lane 7).

***Bacillus thuringiensis* protoxin activation by bovine trypsin and midgut proteases:**

Trypsin and chymotrypsin like protease activities in the midgut lumina of lepidopteran larvae have been shown to be predominantly responsible for the activation of endotoxins (Choma et al,1990; Milne and Kaplan, 1993; Oppert et al, 1996; Oppert, 1999). We have already described in the chapter 1, that local *B. thuringiensis* strain strain DOR3 was found to be more effective against larval forms of *A. janata* than Bt *kurstak*i HD-1 (a strain which is used in commercial formulations). The following experiments were carried out basically to evaluate and ascertain the role of *A. janata* larval midgut proteases, if any responsible for higher toxicity.

To identify the proteases, which hydrolyze protoxin, as well as to determine the number and sizes of proteases responsible for hydrolysis of protoxin by gut extracts from fifth instar *A. janata* larvae, midgut homogenate was subjected to SDS-PAGE, and the gel was incubated in a solution of partially purified Bt DOR3 protoxin (Fig. 2.4C, lane 3). The presence of clear zones in the zymogram demonstrated activity of enzymes, which are capable of hydrolyzing DOR3 protoxins (Fig. 2.4C, lane 1).

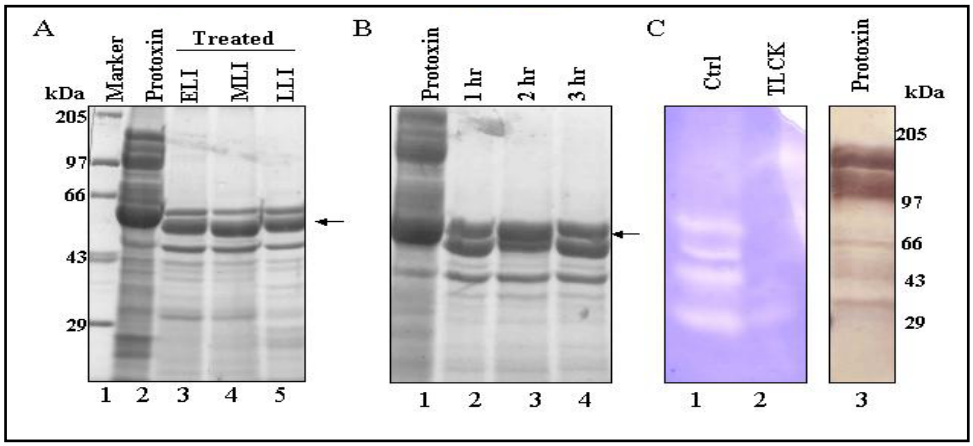

Fig. 2.4: A. Effect of gut proteases from different developmental stages on protoxin digestion. Lane 1, Molecular weight marker; lane 2, Bt protoxin; lane 3-5, protoxin treated with gut proteases; lane 3, ELI; lane 4, MLI; lane 5, LLI; **B.** Effect of LLI gut proteases for different time periods on protoxin digestion. Lane 1, Bt protoxin; lane 2, protoxin treated for 2 h; lane 3, 2 h; lane 4, 3 h. **C.** Zymogram of purified Bt protoxin digestion with LLI proteases. Lane 1, Conttrol in which gut juice is not preincubated with inhibitor; lane 2, gut juice preincubate with TLCK; lane 3, purified protoxin.

Analysis showed the involvement of proteases corresponding to 29 kDa and to a lower extent 43 and 66-70 kDa in activation of protoxin to active toxin. The major pattern of proteases in caseinogram (Fig. 2.1B) was completely different from the pattern obtained in zymogram with protoxin as a substrate (Fig. 2.4C, lane 1). The present study does not show the involvement of higher molecular weight proteases in protoxin hydrolysis. Further the proteases with medium molecular weight were shown to be involved in protoxin hydrolysis (Fig. 2.4C). The trypsin specific inhibitor TLCK inhibited all most all the proteases, which hydrolysed protoxin, suggesting that some of the proteases in the larval midgut of *A. janata*, are very specific for protoxin hydrolysis (Fig. 2.4C, lane 2) and which were not visualized in caseinogram. After incubation of protoxin with midgut homogenate from different developmental stages as well as incubation of protoxin with LLI midgut homogenate for different time periods, the digested products were subjected to SDS-PAGE. With all the treatments the intense active toxin band of 65 kDa was produced (Fig. 2.4A, lanes 3-5 & 2.4B, lanes 2-4). The fragments which were larger than 65 kDa were not observed indicating that activation of the protoxin was complete in all cases.

After identifying the number and size of the trypsin(s) which were responsible for the protoxin activation, further studies were carried out to identify the endoproteases other than trypsins, because PMSF inhibited almost all major midgut protease activities. This was done basically to identify remaining proteases by using other groups of serine protease substrates. Eventhough TPCK did not show any inhibitory effect in the zymogram (Fig. 2.2, lane 5), activity blot analysis were carried out using chromogenic substrates SAAPLpNA and SAAPPpNA, which are well known specific substrates for elastase and chymotrypsin like proteases. Analysis demonstrated that the SAAPLpNA and SAAPPpNA hydrolyzing activities were associated with 17 kDa protein (Fig. 2.5, lanes 3 & 4).

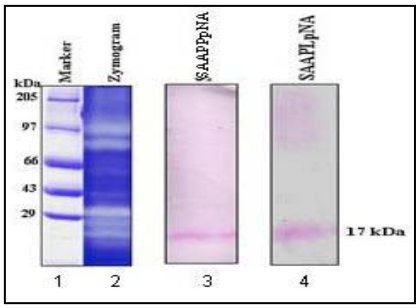

Fig. 2.5: Zymogram of midgut homogenate. Lane 1, molecular weight marker; lane 2, midgut homogenate; lane 3, activity blot with 0.2 mM SAAPPpNA as a substrate; lane 4, activity blot with 0.2 mM SAAPLpNA as a substrate.

Although SAAPPpNA was used as a substrate for chymotrypsin, but in the present study because of the absence of TPCK inhibitory effect and its inhibition by elastinal, the 17 kDa protease is most likely an elastase and not a chymotrypsin.

Detection of aminopeptidase activity:

After identification and characterization of endoproteases studies were extend to exopeptidase with main emphasis on aminopeptidase. Aminopeptidases are known to play crucial role in digestion and responsible for conversion of peptides into amino acids (Billingsley, 1990). Presence of membrane bound aminopeptidases in BBMV, which act as Bt toxin receptors in several lepidopteran insects is well documented (Knight et al, 1994). Results presented in figure 2.6 demonstrate the presence of activity at 65 kDa which was inhibited by bestatin at a final concentration of 500 µM (lane 2). In order to differentiate the cytosolic form of aminopeptidase from membrane bound form, BBMV has been prepared

and subjected to activity blot analysis. The blot analysis once again show the activity at 65 kDa, in addition to which the activity was also seen in the range of 110-120 kDa (Fig. 2.6, lane 3) and the total activity was inhibited by bestatin (lane 4).

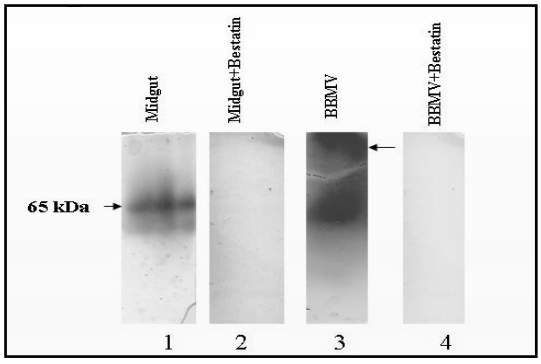

Fig. 2.6: .Activity blots of the midgut homogenate with 0.2 mM LpNA as a substrate. Lane 1, midgut homogenate without inhibitor; lane 2, blot was co-incubated with 500 µM bestatin.lane 3, BBMV without inhibitor; lane 4, blot was co-incubated with 500 µM bestatin.

Discussion:

All classes of proteolytic enzymes known from vertebrates are also present in insects (Reeck et al, 1999). Trypsin and chymotrypsin-like serine protease are usually found in the midgut of lepidopterans and coleopterans (Terra and Ferreira, 1994; Wagner et al, 2002). Elastase like enzymes has been reported in some insects (Wagner et al, 2002). In the present study zymogram analysis revealed that most of the protease activities were inhibited by PMSF, confirming that in *A. janata*, like in other lepidopteran insects serine proteases constitute of major gut proteolytic activity. TLCK specific inhibitor of trypsin completely inhibited the activities of 29 kDa, 80 and 100 kDa proteins. It has been demonstrated that trypsins occur in various isoforms and high molecular weight trypsin like proteases are often aggregates of trypsin of low molecular weights (Novillo et al, 1999; Wagner et al, 2002). The insect proteases frequently do not behave like mammalian trypsins with all inhibitors (Reeck et al, 1999). In the present study the trypsin like activities exhibited by higher molecular weight proteases are most likely not associated with the aggregates of 29 kDa trypsin because these proteases were inhibited differentially by various inhibitors. In the zymograms, chymotrypsin like activity could not be detected using specific inhibitor TPCK, but the

chymotrypsin specific substrate showed reaction on activity blots. The failure of TPCK to detect chymotrypsin like activity may be due to the inability of the chymotrypsin-like protease to recognize phenylalanine, in short chymotrypsin substrates SAAPPpNA (Valaitis, 1995; Elpidina et al, 2005). This kind of differences makes the job of classification of serine proteases of lepidopteran larvae tricky, when compared with classic mammalian trypsins, chymotrypsins and other serine proteases.

Our results show that, protease activity in gut extracts include both tryptic and elastase like activities, while the contribution of trypsin-like enzymes was more evident. The observation of multiple bands of specific activities might be due to the presence of multigene families in the insect's genome (Gatehouse et al, 1997).

Serine proteases viz., trypsin in *Choristoneura fumiferana* (Milne and Kaplan, 1993) and chymotrypsin in *P. interpunctella* (Oppert et al, 1996) were shown to be responsible for *B. thuringiensis* protoxin activation. The zymogram studies using protoxin as a substrate show the role of other proteases along with the trypsins in protoxin activation in *A. janata* larvae. The proteases which showed activity in protoxin digestion, eventhough inhibited by TLCK, the activity blot is not sensitive enough to substantiate the release of azo dye. It is possible that these proteases do not hydrolyze the chromogenic substrate but act exclusively on *B. thuringiensis* protoxin. The inhibition of *B. thuringiensis* protoxin activation with PMSF and TLCK suggest the importance of trypsin-like activity in protoxin activation. The susceptibility of the activated toxins to further proteolysis in the midgut environment could possibly affect toxicity and leads to discrepancies between their in vitro and in vivo activities (Haider et al, 1986). Oppert et al, (1996) reported that lack of major Bt protoxin activating gut protease results in the development of Bt resistance in *P. interpunctella* (Oppert et al, 1997). Eventhough insects seem to possess similar arrays of protease classes; the specificities of the proteases are different (Patankar, 2001). In literature it has been clearly demonstrated that in some insects, proteases degrade protoxin faster (Forcada et al, 1999) while in others they act very slowly or totally ineffective (Haider et al, 1986; Ogiwara et al, 1992; Oppert, 1999). These investigations suggest that changes in the activity or composition of gut proteases are involved in altering the susceptibility of insects to Cry toxins. The 65 kDa activated toxin was stable and it was not proteolysed further on longer incubation when

DOR3 protoxin was treated using midgut homogenate from *A. janata* during different developmental stages as well as for different time periods. So the present study clearly demonstrates the variations in the protease profiles and their differential role in the Bt toxin mode of action. The increasing problems of development of resistance to wide variety insecticidal toxins of *B. thuringiensis* have been reported in several insect (Oppert, 1999). Among these the proteolytic degradation of the toxins has been identified as one of the major reason (Keller et al, 1996). So the information about the proteolytic activities in a complex midgut homogenate of *A. janata* should lead to a better understanding of the Bt toxin proteolysis and provides the basis for designing the strategy for exploration of effective *B. thuringiensis* strains which in turn would be useful in sustaining the long term usage of this environmental friendly microorganism. In addition to this proteinase inhibitors (PIs) have been reported to show toxic effects when ingested by different lepidopterans and insects of other orders (Patankar et al, 2004). Because of the unique digestive physiology of insects, the mode of PI toxicity towards each pest under study will be very important. PIs inhibit the digestive gut proteaes and impair amino acid intake, thus retarding the growth and development of larvae (Telang et al, 2005) and they also decrease the fertility and fecundity of the adult moths (deLeo et al, 2002; Patankar et al, 2004; Zavala et al, 2004; Tamhane et al, 2005; Srinivasan et al, 2005). The success of this control strategy depends on a better identification of PI targets in the insect midgut. The presence multiple proteinase-encoding genes and their variable expression permit the insect to cope with a variety of proteinase inhibitors present in plants and serves as a protection against feeding damages. The demonstration of multiple biological functions of aminopeptidases (Taylor, 1993) has led to successful attempts to control invertebrate pests by direct application of specific aminopeptidase inhibitors (Reed et al, 1999). So aminopeptidases of *A. janata* also could be excellent potential targets for chemical control strategy.

Chapter III

Identification and cloning of novel aminopeptidase from the fat body of A. janata as a receptor for Cry toxins and its comparison with aminopeptidase cloned from the midgut

Background:

The identification and characterization of genes has great promise in agricultural biotechnology, for identifying new targets for pest control involved in processes such as insect-pathogen interactions. It is dependant on the availability of relevant genomic information**.**

Insect larval membrane bound aminopeptidases (APN) have been identified to be the target of *B. thuringiensis* Cry toxins (Sangadala et al, 1994; Gill, 1995; Lee et al, 1996; Denolf et al, 1997; Lorence et al, 1997; Yaoi et al, 1997; Oltean et al, 1999; Gill and Ellar, 2002; Nakanishi et al, 2002; Rajagopal et al, 2002; Rajagopal et al, 2003; Herrero et al, 2005). Several studies showed that crystalline Bt protoxins following proteolytic activation bind to midgut brush border anchored APNs in many lepidopteran species, subsequently the toxin binding leads to several events and to mortality of the insect (Schnepf et al, 1998). APN genes have been isolated from the midgut of variety of insects, and *invitro* as well as *invivo* studies authenticated the role of APN in Bt pathogenicity. Insects in which APN gene was inactivated either by natural mutations (Herrero et al, 2005) or by genetic engineering methods (Rajagopal et al, 2002), become resistant to Bt toxins thus confirming the critical role of APN in Bt toxicity. Bt toxins were also shown to be lethal to lepidopteran larvae when injected into hemocoel (Cerestiaens et al, 2001). However, no information is available on the molecular targets of Bt toxins in the insect larval body cavity.

We had under taken an investigation of the aminopeptidase gene in the lepidopteran *Achaea janata* and *Spodoptera litura*. Firstly we carried out isolation and characterization of two APN genes from *A. janata.* One APN gene designated as *AJFbAPN* is expressed both in fat body and midgut while the other gene named here as *AjMgAPN* is expressed only in the midgut. These APNs manifested variation in their interaction with different Cry1A subtypes and we observed a positive correlation between the binding specificity of the Cry toxins to their APN receptors and their level of toxicity. This is the first report of a fat body specific APN in any insect. Further, in order to substantiate this cloning and characterization of APN was carried out from fat body of another lepidopteran polyphagous pest *S. litura*.

Results:

Identification of DOR3 toxin binding proteins in the BBMV of *A. janata*:

To find out reasons for higher toxicity of DOR3 (reported in chapter 1) at the level of binding proteins (receptors), Cry toxin overlay assay was performed for the identification of putative binding proteins in BBMV for the Cry toxins present in DOR3. Figure 3.1A shows the profile of fat body membrane proteins (lane 2). Ligand blot analysis results presented in figure 3.1B show the intense interaction of DOR3 toxins with two major peptides of molecular weight of 110-120 and ~170 kDa (lane 2) in comparison with the control (lane1). The activity ingel assay with BBMV shows the existence of two forms of aminopeptidases of molecular weights 65 and 110-120 kDa (Fig. 3C).

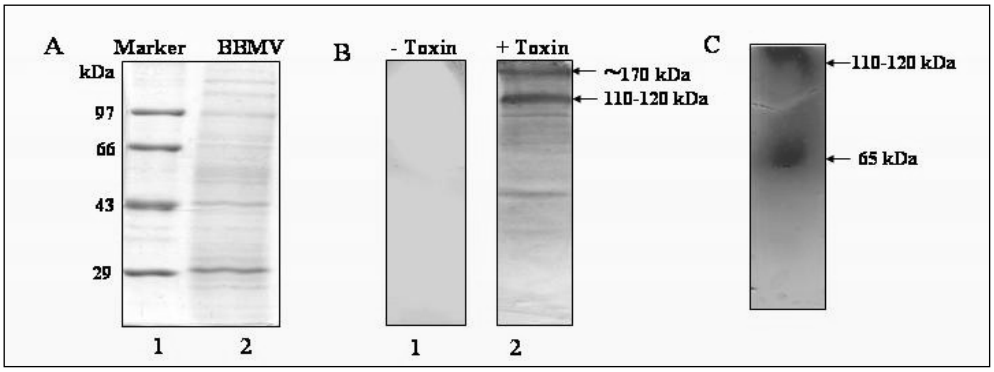

Fig. 3.1: Results obtained from Cry toxin overlay assay with BBMV proteins. BBMV proteins were separated on a 7.5% SDS-PAGE, transferred to nitrocellulose membrane. The blots were incubated either in presence or absence of DOR3 toxins and the interaction was detected with primary antibody raised against Cry toxins followed by secondary antibody conjugated with ALP. **A**. Lane 1, protein molecular weight marker; lane 2, coomassie stained BBMV proteins. **B.** Lane 1, protein blot incubated without DOR3 toxin as control; lane 2, protein blot incubated with DOR3 toxins and subsequently probed with DOR3 antisera. **C**. Aminopeptidse activity ingel assay.

Cloning and characterization of APN cDNAs from larval midgut of *A. janata*:

The midgut specific APN cDNA was partially cloned by RT-PCR using degenerate primers. Primer pair named F1/R1, the details of which are presented in the materials and methods section amplified an 850 bp fragment from midgut (Fig. 3.2, lane 2), which was cloned and sequenced. The sequence proved homologous to APN sequences reported from

other insects. Subsequently, the sequence information was complemented with 5' and 3' RACE, which provided the full-length cDNA sequence.

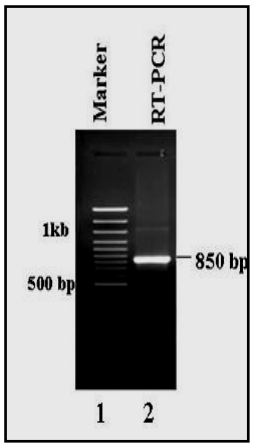

Fig. 3.2: RT-PCR mediated amplification of APN from midgut of *A. janata***:** Total RNA from midgut of the fifth instar larvae was isolated and RT-PCR was performed using degenerate primers, designed based on the conserved regions of the reported insect APNs. Lane 1, 100 bp ladder, lane 2, PCR amplification product (850bp).

The results of RACE reaction are presented in figure 3.3. The first PCR reaction for 5' RACE was performed with AjMgAPNR3 and GeneRacer^{TM} 5'primers (lane 2), while that of 3' RACE was performed with AjMgAPNF3 and GeneRacer^{TM} 3'primers (lane 3), using the RT reaction product as template. Next round of nested PCR was performed with nested primers AjMgAPNR4 and GeneRacer^{TM} 5'nested primer for 5' RACE (lane 4) and AjMgAPNF4 and GeneRacer^{TM} 3'nested primer for 3' RACE (lane 5) respectively, with the first round of PCR product as template. The 5' and 3' RACE of midgut APN cDNA produced 0.6 kb (lane 4) and 1.7 kb (lane 5) fragments, respectively, which were cloned and sequenced. Nucleotide sequence alignment of all these fragments revealed that the full-length midgut APN cDNA is 3056 bp long having 5' and 3' UTRs of 46 and 157 nucleotides, respectively (Fig. 3.4). It has an ORF of 2850 bp, which encodes a putative protein of 950 amino acids with a theoretical molecular mass of 109 kDa and a calculated pI of 4.87. The complete cDNA sequence of midgut APNs was designated as *Achaea janata* midgut aminopeptidase (*AjMgAPN*). This nucleotide sequence has been deposited in the GenBank^{TM}/EBI Data Base under accession number DQ872666 (*AjMgAPN*).

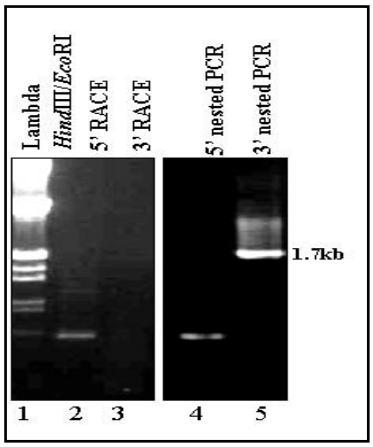

Fig. 3.3: Electrophoretic analysis of RACE products. Lane 1, Lambda HindIII/EcoRI double digested marker; lane 2, 600 bp 5'RACE product; lane 3, no amplification in 3'RACE reaction; lane 4, 600 bp 5'RACE nested PCR product; lane 5, 1.7 kb 3'RACE nested PCR product.

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                                                M  Q  L  L  I
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 I  L  S  I  L  V  G  S  L  V  A  I  P  Q  E  D  F  R  S  N
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 V  E  T  E  I  T  S  T  A  A  R  P  F  K  I  I  S  R  P  G
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```

Fig. 3.4: Complete cDNA sequence and corresponding amino acid sequence of *AjMgAPN* from midgut of *A. janata* (Gen Bank™ Accession No. DQ872666) along with 5' and 3' untranslated regions. The ORF is of 3053 bp long coding for a protein of 950 amino acids shown in black. Where as 5' and 3' UTRs are shown in red. The start and stop codons are shown in blue.

Heterologous expression, purification of truncated APN and generation of anti-APN polyclonal antisera:

The APN-specific PCR fragment obtained from midgut (850 bp), as described in earlier section, was PCR amplified with degenerate primers using High Proof™ *Taq* (Qiagen) to generate blunt end fragments. This was cloned into the *Sma*I site of the pGEX-5X-2 expression vector in the same reading frame as glutathione S-transferase (GST) tag (Amersham Pharmacia) and transformed into *E. coli* BL21 (DE3) PlysS (Novagen).

Expression of recombinant truncated APN-GST proteins was induced with 1 mM IPTG and analyzed by SDS-PAGE. Recombinant MgAPN-GST (58 kDa) protein was detected in the inclusion bodies (Fig. 3.5A, lane 2) , which was solubilized, purified using GST affinity (lanes 4-9) column (Amersham Pharmacia) and used for the generation of antibodies in six months old male New Zealand white rabbit using standard procedures. The antisera was labeled as antiMgAPN antibody, specifically it detected 58 kDa (Fig. 3.5B) protein in bacterial lysates expressing recombinant MgAPN-GST protein.

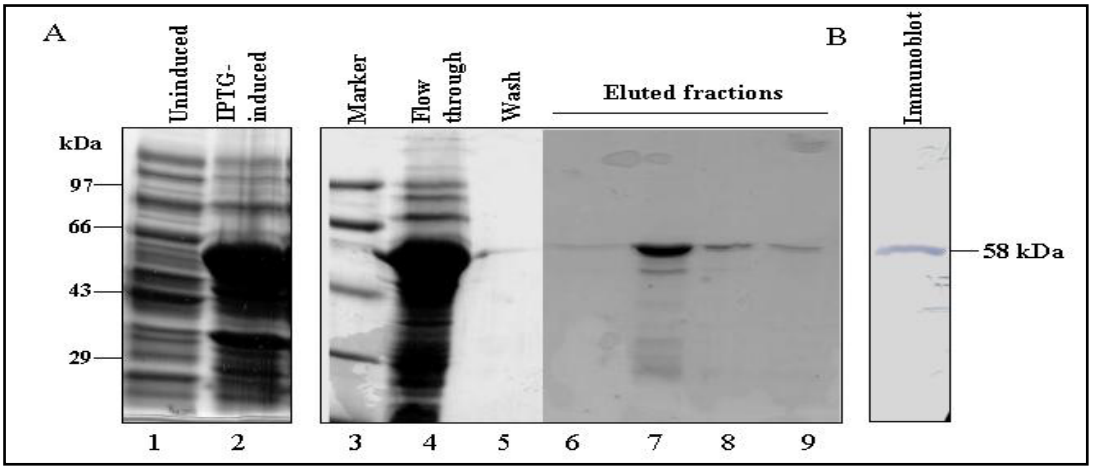

Fig. 3.5: Bacterial overexpression of recombinant MgAPN-GST protein and generation of antiMgAPN antibody in rabbit**. A**. 850 bp cDNA was cloned in the pGEX-5X-2 vector at S*ma*I site, to produce a GST fused APN construct. BL21 cells transformed with vector alone (pGEX-5X-2) or vector with APN were grown up to O.D 600 and induced with 1mM IPTG for 2 h. After harvesting the cells, total protein was extracted with PBS, analyzed by SDS-PAGE and purified using GST affinity columns. Lane 1, total protein from uninduced culture; lane 2, expression of MgAPN-GST fusion protein (58 kDa); lane 3, molecular weight marker; lane 4, flow through; lane 5, wash solution; lanes 6-9 eluted fractions of MgAPN-GST truncated protein. **B.** The specificity of the APN antibody was checked by immunoblot analysis using bacterial lysate expressing MgAPNGST protein (note the presence of 58 kDa cross reacting band).

Immunoblot analysis of APN in larval gut:

In order to determine the regional specificity of the APNs in the gut of *A. janata*, western blot analysis was carried out. The antiMgAPN antiserum recognized the 110-120 kDa protein in different regions of the midgut (Fig. 3.6). The 110-120 protein was found to be located mostly in the anterior (lane 2) and posterior regions (lane 3) of the midgut.

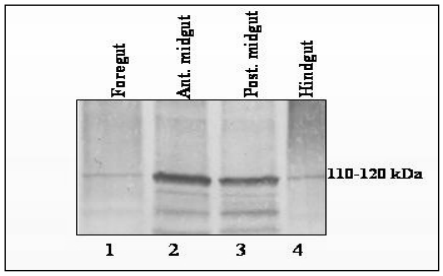

Fig. 3.6: Immunoblot analysis of aminopeptidase in different regions of the larval gut. Homogenates of protein was separated by 7.5% SDS-PAGE and transferred to nitrocellulose membrane. For immunoblot analysis the blots were incubated with antiMgAPN antibodies and detected using standard protocol. Lane 1, foregut; lane 2, anterior midgut; lane 3, posterior midgut; lane 4, hindgut. Note the presence of 110-120 kDa APN.

Identification Cry toxin binding proteins:

The interaction of different Cry toxins with BBMV proteins was studied by ligand blot analysis using biotinylated Cry1Aa, Cry1Ab and Cry1Ac toxins (Fig. 3.7). Differential binding pattern was observed with different Cry toxins. Results obtained show that Cry1Aa and Cry1Ac toxins interact with both 110-120 and ~170 kDa proteins in midgut (Fig. 3.7A & C, lanes 2 and 3 respectively), whereas Cry1Ab interacts only with the110-120 protein (Fig. 3.7B, lanes 2 and 3). Further most of the toxin binding proteins were observed in the anterior and posterior regions of the midgut (Fig. 3.7A, B & C, lanes 2 and 3 respectively) where as in forgut as well as hindgut tissues very faint binding was observed (Fig. 3.7A, B &C, lanes 1 and 4). This result corroborates with the immunoblot analysis (Fig. 3.6), where APN was mostly detected in the anterior and posterior regions of the midgut.

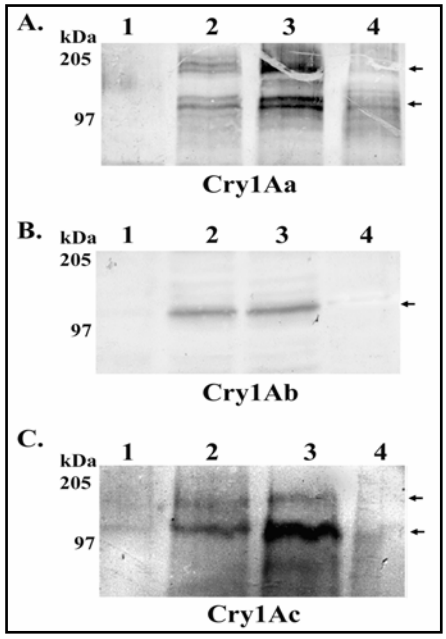

Fig. 3.7: Cry toxin overlay assay with midgut proteins. BBMV proteins were separated on a 7.5% SDS-PAGE, transferred to nitrocellulose membrane. These blots were individually incubated with ~100 ng/ml biotin labeled active Cry1Aa **(A)**, Cry1Ab **(B)** or Cry1Ac **(C)** toxins and detected with streptavidin-conjugated secondary antibody. Lane 1, foregut; lane 2, anterior midgut; lane 3, posterior midgut; lane 4, hindgut.

Binding studies with biotinylated toxins:

The excess amounts of cold Cry1Ac toxin competed with the binding of labeled Cry1Ac toxin to these peptides (MW of peptides 110-120 and ~170 kDa), indicating competitive and specific binding (Fig. 3.8A, lane 2). On the other hand very low competition was obtained with Cry1Aa (Fig. 3.8B, lane 1) and Cry1Ab (lane 2) suggesting that Cry1Aa and Cry1Ab toxins did not recognize Cry1Ac-binding site. Hence they did not displace Cry1Ac toxin from the biding protein.

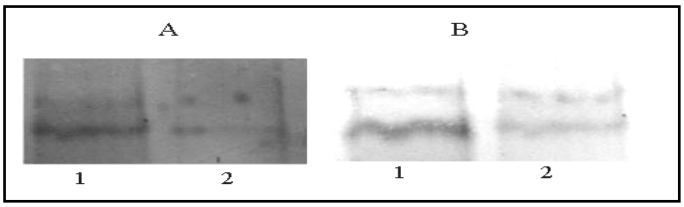

Fig. 3.8: A. BBMV ligand blotting with biotinylated Cry1Ac toxin in the absence (lane 1) and presence (lane 2) of unlabeled Cry1Ac. **B**. BBMV ligand blotting with biotinylated Cry1Ac toxin in the presence of unlabeled Cry1Aa (lane 1) and Cry1Ab (lane 2).

In the Cry1Ab competition assays **(**Fig. 3.9), only homologous Cry1Ab (lane 2) and heterologous Cry1Ac (lane 3) competed with high affinity for Cry1Ab-binding site.

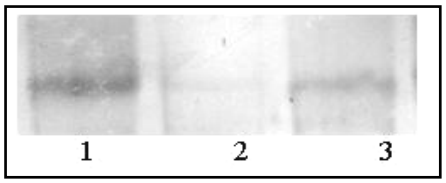

Fig. 3.9: BBMV ligand blotting with biotinylated Cry1Ab toxin (lane 1) in the presence of unlabelled Cry1Ab (lane 2) and in the presence of unlabeled Cry1Ac (lane 3).

As part of the identification of alternate target organs for Cry toxins, Cry toxins were shown to be lethal to lepidopteran larvae when injected into the body cavity of *L. dispar*

(Cerestiaens et al, 2001) and pore forming cytolytic ability of Cry toxins on the *in vitro* cultured fat body cells (Cheon et al, 1997) suggest the presence of Cry binding proteins in the fat body. Since fat body is a pivotal metabolic organ in the body cavity of insects, its possible interaction with the Cry toxins was studied by ligand blot analysis using DOR3 Cry toxin.

Identification DOR3 Cry toxin binding proteins in the fat body of *A. janata*:

Cry toxin overlay assays was performed to identify putative DOR3 toxin binding proteins in fat body membrane. Results showed the presence of 110-120 kDa protein as Cry binding proteins (Fig. 3.10B, lane 2). In contrast to this, two proteins of molecular weights of 110-120 and ~170 kDa identified as toxin binding proteins in BBMV (Fig. 3.1B, lane 2) with DOR3 cry toxin overlay assay. In the BBMV of several insect, binding proteins ranging from 110-120 kDa have been identified as APNs. So with the identification of fat body 110-120 kDa protein as DOR3 toxin binding protein, further studies have been carried out to characterize this binding protein.

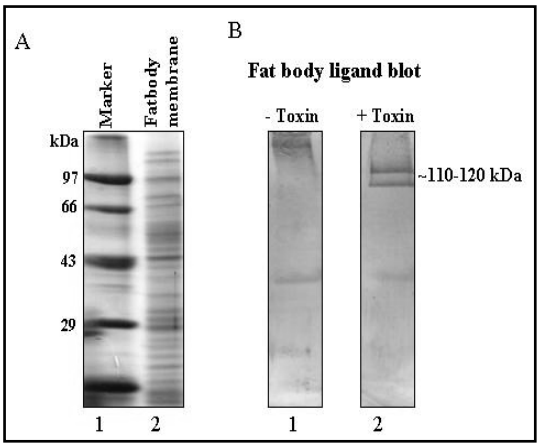

Fig. 3.10: Cry toxin overlay assay with fat body membrane proteins. Fat body membrane proteins were separated on a 7.5% SDS-PAGE, and transferred to nitrocellulose membrane. The blots were either incubated with or without DOR3 toxin and bound toxins were detected with DOR3 antisera followed by ALP-conjugated secondary antibody. **A**. Coomassie stained gel. Lane 1, protein molecular weight marker; lane 2, fat body membrane proteins; **B.** Protein blots**.** Lane1- Control; protein blot incubated without DOR3 toxin; lane 2, protein blot incubated with DOR3 toxin and the blots were subsequently probed with DOR3 antisera.

Cloning and characterization of APN cDNA from fat body of *A. janata*:

The fat body APN cDNA was partially cloned by RT-PCR using degenerate primers (Fig. 3.11). Primer pair named F2/R2 the details of which are given in the materials and methods section, amplified a 550 bp fragment from fat body (lane 2), which was cloned and sequenced. The sequence proved homologous to APN sequences reported from other insects. Subsequently, the sequence information was complemented with 5' and 3' RACE, which provided the full-length cDNA sequence

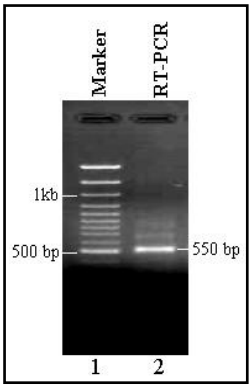

Fig. 3.11: RT-PCR mediated amplification and cloning of *APN* from *A. janata*: Total RNA from fat body of the fifth instar larvae was isolated and RT-PCR was performed using degenerate primers, designed based on the conserved regions of the reported insect APNs. Lanes 1, 100 bp ladder; lane 2, 550 bp PCR amplified product.

Detection of tissue specific expression of the APNs:

The tissue specificity of the identified APNs was determined by northern blot analysis using the fat body (550 bp) and midgut (850 bp) specific APN fragments as probes. As shown in figure 3.12A, the fat body APN fragment could detect expression of a single transcript of ~3.5 kb in the fat body tissue (lane 1), where as it hybridized to two different transcripts of ~ 4.0 and ~5.5 kb in the midgut tissue (lane 2). In contrast, in figure 3.12B, the midgut specific probe recognized both the transcripts detected earlier with fat body probe only in the midgut (lane 2), but failed to recognize any transcript in the fat body (lane 1).

After confirming that fat body APN (AjFbAPN) is different from the midgut APN, by (i) sequencing of the cloned 550 bp insert and (ii) northern analysis, the fat body 550 bp APN fragment was used for generation of tissue specific APN antisera. Further RACE reaction

was carried out for obtaining full length fat body APN by designing primers based on the sequence of the cloned insert, as described for midgut APN in earlier section.

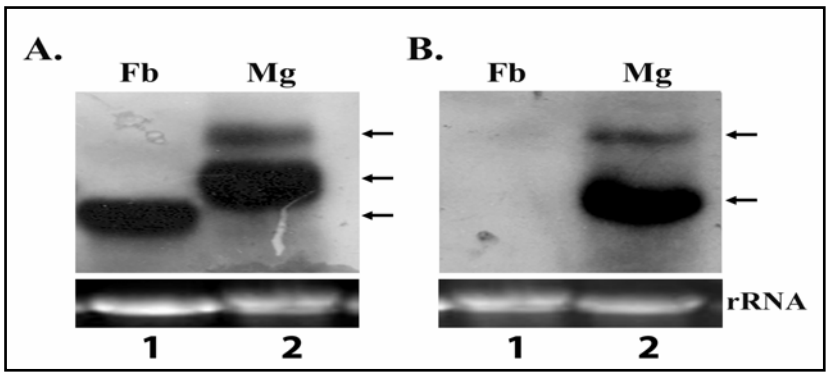

Fig. 3.12: Northern blot analysis of APN mRNA in larval fat body and midgut tissues of *A. janata*. Twenty microgram of RNA from fat body (lane 1) and midgut (lane 2) tissues were denatured and subjected to electrophoresis on a 1% agarose gel containing formaldehyde. The separated RNAs were transferred to Nylon-N+ membrane and hybridized with the [α^{32}P] -labelled probes, which was either fat body 550 bp fragment (**A**) or midgut specific 850 bp PCR fragment (**B**). Equal loading control is shown by ethidium bromide stained RNA in down panel.

Cloning, overexpression and purification of truncated fat body APN and generation of anti-APN polyclonal antisera:

AjFbAPN 550 bp cDNA was PCR amplified with same degenerate primers using Platinum *pfx* to generate blunt end product, subsequently it was cloned into PGEX-5X-2 as described for midgut specific 850 bp fragment. The same has been over expressed as 46 kDa FbAPN-GST fused truncated APN protein (Fig. 3.13A, lane 4), solubilized (lane 5), purified (lanes 6-10) and antisera has been generated in the same way as antiMgAPN (as described earlier). This particular antisera containing antibody has been named as antiFBAPN antisera and it detected specifically 46 kDa protein (Fig. 3.13B) in bacterial lysates expressing recombinant FbAPN-GST protein.

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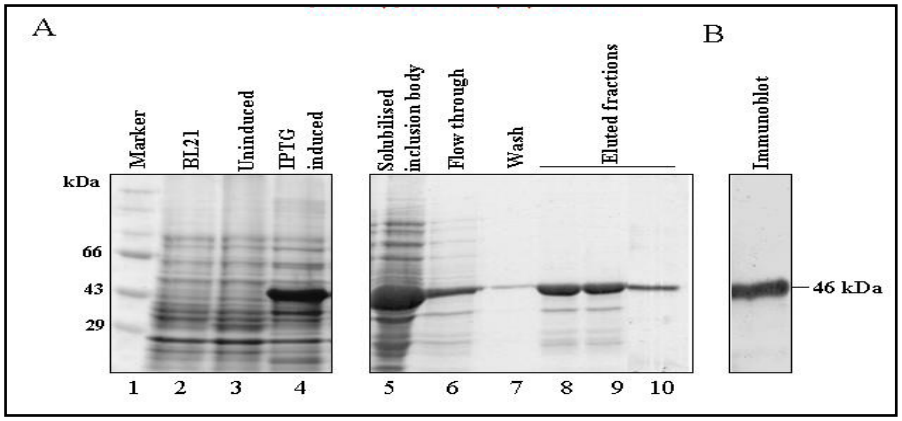

Fig. 3.13: Bacterial overexpression of recombinant FbAPN protein and raising antiFbAPN antibody in rabbit**. A**. 550 bp cDNA was cloned in the pGEX-5X-2 vector at S*ma*I site, to produce a GST fused APN construct. BL21 cells transformed with vector alone (pGEX-5X-2) or vector with indert were grown upto 0.6 O.D and induced with 1mM IPTG for 2 h. After harvesting the cell, total protein was extracted with PBS and analysed by SDS-PAGE and purified using GST affinity columns. Lane 1, molecular weight marker; lane 2, total protein from BL21 culture; lane 3, total protein from uninduced culture; lane 4, expression of FbAPN-GST fusion protein (46 kDa); lane 5, solubilized inclusion proteins; lane 6, flow through; lane 7, wash solution; lanes 8-10, eluted fractions of FbAPN-GST truncated protein. **B.** The specificity of the APN antibody was checked by immunoblot analysis. (Note the presence of 46 kDa cross reacting band in bacterial lysate expressing FbAPN-GST fusion protein.

In order to determine the tissue specificity of the APNs in *A. janata*, western blot analysis was carried out. The antiFbAPN antiserum recognized a 110-120 protein in the fat body (Fig. 3.14, lane 1) and cross-reacted with a ~170 kDa protein in the fore and midgut (lanes 2- 4). On the other hand, the antiMgAPN antiserum recognized only the 110-120 kDa protein, but not the ~170 kDa in different regions of the larval gut (Fig. 3.6). The 110-120 (Fig. 3.6) as well as the ~170 kDa proteins (Fig. 3.14, lanes 3 and 4) were found to be located mostly in the anterior and posterior regions of the midgut.

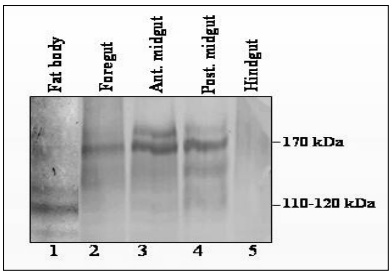

Fig. 3.14: Immunoblot analysis of aminopeptidases in fat body and in different regions of the larval gut. Fat body and gut homogenate proteins were separated by 7.5% SDS-PAGE and transferred to nitrocellulose membrane. For immunoblot analysis the blots were incubated with antiFBAPN antisera. Lane 1, Fat body; lane 2, foregut; lane 3, anterior midgut; lane 4, posterior midgut; lane 5, hindgut (note the presence of 110-120 and ~170 kDa proteins).

Cloning of full length APN from the fat body of *A. janata*:

Based on the nucleotide sequence obtained for the 550-bp APN fragment, primers were designed for 5' and 3' RACE. The RACE reactions (Fig. 3.15) were carried out as described for *AjMgAPN*. The 5'and 3'RACE of fat body APN cDNA yielded a 0.7 kb (lane 4) and a 2.1 kb (lane 5) fragments, respectively. Nucleotide sequence alignment of all these fragments revealed that fat body APN cDNA is 3122 bp long and contains an open reading frame (ORF) of 3015 bp (Fig. 3.16). The cDNA encodes a putative 1004 amino acids protein with a theoretical molecular mass of 113 kDa and a calculated pI of 4.83

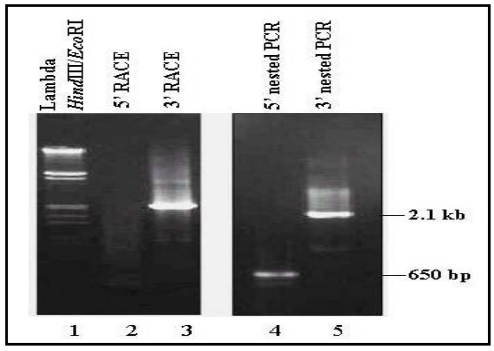

Fig. 3.15: Agarose gel analysis of *AjFbAPN* RACE products: Marker- HindIII/EcoRI double digested Lambda DNA (lane 1); 5' RACE reaction with no amplification (lane 2); 2.1 kb 3'RACE product (lane 3); 650 bp 5'RACE nested PCR product (lane 4); 2.1 kb is 3'RACE nested PCR product (lane 5).

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 X  K  L  L  S  Y  I  Y  D  S  L  -
tattaataaaaaaaaaaaaaaa
```

Fig. 3.16: Complete cDNA sequence and corresponding amino acid sequence of *AjFbAPN* from fat body of *A. janata* (Gen Bank™ Accession No. DQ444715) along with 5' and 3' untranslated regions. The ORF is 3015 bp long coding for a protein of 1004 amino acids shown in black. Where as 5' and 3' UTRs are shown in blue. The start and stop codons are shown in red.

Detailed computational analyses of the deduced amino acid sequences of both the cDNAs were carried out to detect the presence of signature sequences (Fig. 3.17). The SignalP program revealed the presence of a cleavable signal peptide MASRWFNLLLGVLFIQGYLAF (residues 1-21) with a cleavage site between Ala20 and Phe21 at the N-terminal region in *AjFbAPN,* while a peptide MQLLIILSILVGSLVAI (residues 1-17) with a cleavage site between Ala16 and Ile17 was detected in *AjMgAPN* . A GPI anchor signal sequence was detected at the C-terminus of the deduced amino acid sequences of both the APNs. For *AjFbAPN* the predicted sequence consists of three small amino acids DSA (972-974) followed by a stretch of hydrophobic residues SIATLSFVTLFVTLALNLAXKLLSYIYDSL (975-1004), while in the case of *AjMgAPN* it consists of the three residues, SSS (928-930) followed by the hydrophobic stretch of TTFVASTILMLAAMFTHLLR (residues 931-950). Interestingly, we could identify up to twenty-two potential O-glycosylation sites and one potential N-glycosylation site in *AjFbAPN,* while only four of each N- and O-glycosylation sites were detected in *AjMgAPN*. The consensus zinc-binding/gluzincin motif ($HEXXHX_{18}E$) and the gluzincin aminopeptidase motif (GAMEN) are found in all aminopeptidases and their presence in the amino acid sequences of *AjFbAPN* and *AjMgAPN* , along with the other features allowed us to classify both of these *A. janata* APNs as members of the aminopeptidase family. Especially, the gluzincin Zn^{2+}-binding motifs [HEXXH-(18X)-E] that constitute the core of the active site of gluzincin APNs have very less variations in the amino acid residues. However, the hydrophobic N and C terminal sequences of these APNs are significantly different from each other.

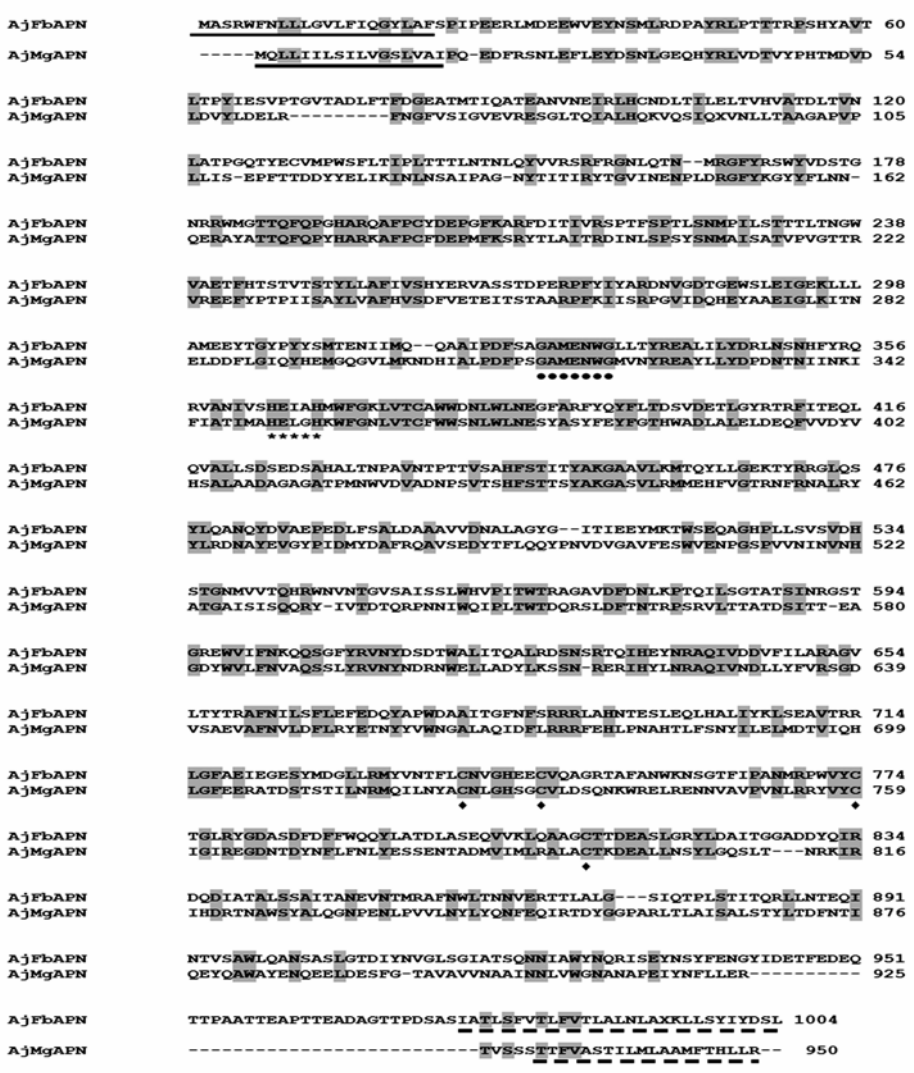

Fig. 3.17: Comparison of the amino acid sequences of fat body (*AjFbAPN*) and midgut (*AjMgAPN*) aminopeptidases. The deduced amino acid sequences of *AjFbAPN* and *AjMgAPN* cDNAs were aligned by Clustal W multiple sequence alignment tool. Gaps are introduced in the sequences for maximizing the alignment. The amino acid residues conserved between the two sequences are highlighted with grey. Solid underlines mark the N-terminal signal peptide sequence and broken underlines denote the C-terminal hydrophobic GPI anchor signal sequence. Below the amino acids stars denote the canonical metal (zinc)-binding motif, dots indicate the *GAMEN* motif. The four cystine residues highly conserved in all the eukaryotic APNs are marked with diamonds at positions 739, 746, 774, and 810, respectively.

Identification of Cry toxin binding proteins:

The interaction of different Cry toxins with the fat body membrane proteins was studied by ligand blot analysis. The protein profile of the fat body membrane preparation was observed by commassie blue staining (Fig. 3.18, lane 2). As seen in Fig 3.18, all the three Cry toxins, Cry1Aa (lane 4), Cry1Ab (lane 5) and Cry1Ac (lane 6) interacted with the 110-120 kDa protein in the fat body membrane. Absence of any signal in the control reaction, where membrane was not incubated with biotin-labeled toxin prior to probing with streptavidin ALP (lane 3) suggests the specificity of the interaction between 110-120 kDa fat body membrane proteins and the Cry toxins.

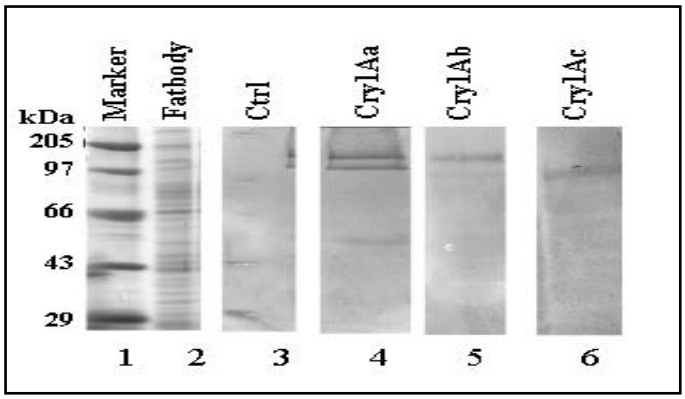

Fig. 3.18: Cry toxin overlay assay with fat body membrane proteins. Fat body membrane proteins were separated on a 7.5% SDS-PAGE, transferred to nitrocellulose membrane. The blots were separately incubated with any one of the biotin labeled active Cry1 toxins (100 ng/ml) and detected with ALP conjugated streptavidin. Lane 1, protein molecular weight marker; coomassie stained fat body membrane proteins (lane 2); protein blot incubated with unlabelled Cry1Aa as control (lane 3); lanes 4-6, protein blots incubated with biotinylated toxins, Cry1Aa (lane 4), Cry1Ab (lane 5), and Cry1Ac (lane 6).

Identification of the glycan moieties present on APNs by lectin blotting-

To investigate the oligosaccharides present on midgut and fat body APNs, lectin-blotting analysis using SBA and WGA was performed (Fig. 3.19). Strong binding of the GalNAc specific SBA lectin to 110-120 kDa protein in the fat body membrane (lane 1) and 110-120 kDa and ~170 kDa proteins in the midgut (lane 2) was observed. On the other hand WGA, specific for GluNAc, cross reacted with the ~170 as well as 110-120 kDa proteins present in the midgut (lane 4), but not in the fat body (lane 3). After lectin blotting, APNs on

blots were detected by antiFbAPN and antiMgAPN antisera in order to confirm that the lectin binding proteins were actually APNs (data not presented).

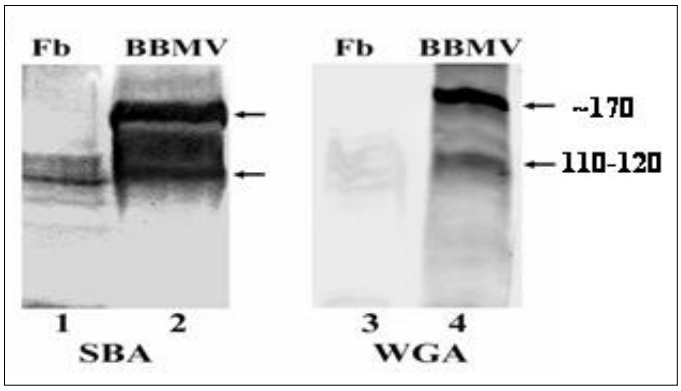

Fig. 3.19: Lectin binding assays. Membrane protein preparations were separated on 7.5% SDS-PAGE and transferred to nitrocellulose membrane. The membranes were incubated either with biotin conjugated soybean agglutinin (SBA) or wheat germ agglutinin (WGA) and lectin bound proteins subsequently detected with ALP conjugated streptavidin. Lanes 1 and 3, fat body membrane proteins (Fb); lanes 2 and 4, brush border membrane vesicles (BBMV).

Results presented in the figure 3.20, clearly show that the presence of GalNAc in the binding assay, selectively reduced binding of Cry1Ac toxin to the ~170 kDa protein in the BBMV (lane 1), but binding of toxin to 110-120 kDa protein in fat body as well as BBMV remained unaffected in the presence of GalNAc (lanes 1 and 2.).

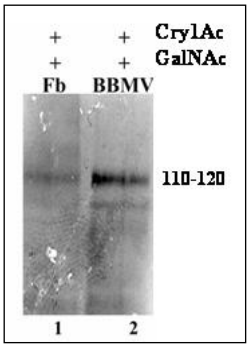

Fig. 3.20: Effect of N-acetylgalactosamine (GalNAc) on the interaction between membrane proteins and Cry1Ac. Membrane protein preparations were separated on 7.5% SDS-PAGE and transferred to nitrocellulose membrane. The blot was incubated with biotin labeled active Cry1Ac (~100 ng/ml) in presence of 100 mM of GalNAc and subsequently the toxin binding

was detected with ALP conjugated streptavidin. Lane 1, fat body membrane protein; lane 2, BBMV.

Immunoprecipitation of Cry toxin binding proteins:

The interaction of the fat body and midgut APNs with the Cry1Aa toxin was further analyzed by co-immunoprecipitation experiments.

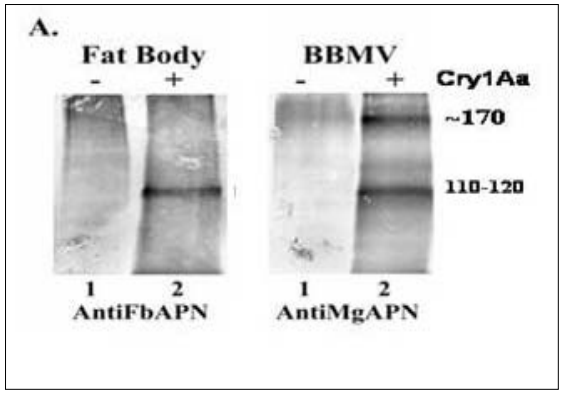

Fig. 3.21: Immunoprecipitation of APN with the Cry toxin. Triton X-100 solubilized fat body membrane proteins and CHAPS-solubilized BBMV proteins from *A. janata* 5th instar larvae were separately incubated with purified activated Cry1Aa toxin. Using corresponding Cry1A antisera, the protein-toxin complex was pulled down with Protein A-Sepharose beads, which were boiled in SDS sample buffer containing ß-mercaptoethanol. The proteins were resolved by SDS-PAGE, and transferred to nitrocellulose membrane. The blot was incubated with corresponding anti-APN antibodies and subsequently detected with ALP-conjugated secondary antibody. **A**: fat body membrane proteins incubated without Cry1Aa (control, lane 1) or with Cry1Aa (lane 2); **B**: BBMV incubated without Cry1Aa (control, lane 1) or with Cry1Aa (lane 2) prior to immunoprecipitation.

Detergent solublized BBMV and fat body membrane preparations were incubated with activated Cry1Aa toxin, and immunoprecipitated using antiCry1Aa antibody. Western analysis of immunoprecipitated proteins using antiAjFbAPN and antiAjMgAPN antisera resulted in the identification of 110-120 kDa APNs in both, fat body (Fig. 3.21A, lane 2) as well as midgut (Fig. 3.21B, lane 2), suggesting that 110-120 kDa APN interacts with Cry toxins. The control immunoprecipitation reaction, in which Cry toxin was not incorporated, did not show any binding proteins (Fig.3.21 A & B, lane 1).

GPI anchor property of the APNs:

Cry proteins, like other pore forming toxins have the ability to bind to receptors, which are anchored to membrane by GPI moiety (Fernandez et al., 2006). As the presence of APN in the fat body was seen for the first time and this APN showed interaction with Cry toxins, it was further examined whether this APN was GPI-anchored protein. Phospholipase C is known to cleave the GPI anchor causing the exposure of the CRD epitope (Broomfield and Hooper, 1993). Upon treatment with phospholipase C, the exposed CRD epitope was detected using anti-CRD antibody (Fig. 3.22, lanes 1-3). As seen in (lane 1) a weak signal of 110-120 kDa in fat body may be because of the cleavage of the GPI anchor during membrane preparation by internal phospholipases. Other than 110-120 kDa fat body showed presence of other lower molecular weight protein which has GPI anchor (lane 1 and 2). The intense thick band seen in midgut (lane 3.) may be the presence of multiple GPI anchor APNs, which after cleavage by phospholipae C, exposed their hydrophobic residues and the signal became intense. The results indicate that the APNs from both, fat body as well as BBMV have a GPI anchor. These results corroborate with the predicted GPI anchor signal in the deduced amino acid sequences of *A. janata* APNs.

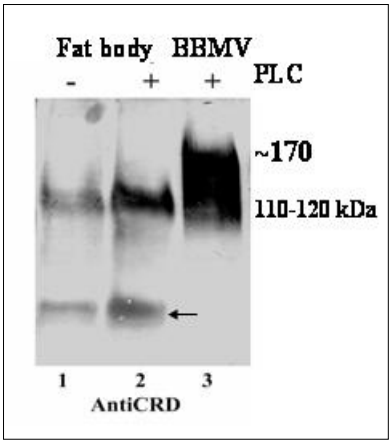

Fig. 3.22: Detection of the cleaved GPI anchors. Fat body membrane proteins and BBMV prepared from 5th instar larvae of *A. janata* were digested with phospholipase C [(PLC), (5 units/ 10ml)] subjected to SDS-PAGE and transferred to nitrocellulose membrane. The blot was incubated with anti-CRD antibodies and cleaved GPI anchors were detected with ALP-conjugated secondary antibody. Lane 1, fat body membrane protein without digestion with PLC; lane 2, fat body membrane protein digested with PLC; lane 3, BBMV digested with PLC.

Southern analysis:

In corroboration with earlier reports, Southern analysis with the partial *AjFbAPN* 550 probe clearly demonstrated that APN gene in *A. janata* exists in multiple copy number, (Fig. 3.23) which clearly supports the notion that APNs in lepidopteran insects like other serine proteases belong to multigene families

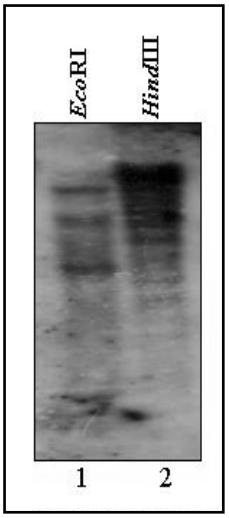

Fig. 3.23: Southern blot analysis of genomic DNA from *A. janata*: Aliquots of genomic DNA were digested with EcoRI and HindIII respectively and were separated on 1% agarose gel. The DNA was transferred to a nylon membrane and then hybridized with [α^{32}P]-labeled 550 bp PCR product from fat body. EcoRI digest (lane 1) and HindIII digest (lane 2)

Activity of aminopeptidases in different tissues

Enzyme activity was assayed using LApNA as the substrate, which demonstrated the leucine aminopeptidase activity in different tissues and the results are presented in table 1. The total midgut homogenate showed a very high specific activity of 21.57 ± 2.5 μ mol min^{-1}mg of protein^{-1} while under similar experimental conditions the fat body homogenate exhibited fairly low specific activity of only 2.6 ± 0.5 pmol min^{-1} mg of protein^{-1} (Table 1). Quite interestingly, a significant increase in haemolymph APN activity was observed from actively feeding last instar larvae to the non feeding prepupae (Table 1). This observation corroborated well with immunoblot analysis of prepupal fat body, which showed considerably higher amount of APN protein as compared to the feeding larval fat body (data not presented).

Table 1:

Aminopeptidase activities in different tissues of *A. janata*:

Sample	APN activity ± S.D.
Larval midgut	**21.57 ± 2.5** μmol min^{-1}mg of $protein^{-1}$
Larval fat body	**2.6 ± 0.5** pmol min^{-1} mg of $protein^{-1}$
Larval hemolymph	**5.4 ± 0.9** pmol min^{-1}μlof $plasma^{-1}$
Prepupal hemolymph	**13.9± 1.51** pmol min^{-1}μlof $plasma^{-1}$

Each value is the mean are ± S.D. of 3 independent assays carried out in duplicates

Variation in the toxic effect of Cry proteins depending on whether ingested by *A. janata* larvae or injected into body cavity:

In order to determine the toxicity effect of Cry1A proteins upon ingestion, force-feeding bioassays were carried out with *A. janata* larvae. As seen in table. 2, Cry1Ac (LC_{50} 23.70 ng/cm^2) was found to be most effective, followed by Cry1Ab (LC_{50} 32.03 ng/cm^2) and Cry1Aa (LC_{50} 61.01 ng/cm^2). Earlier, studies have demonstrated that upon insects feeding on Cry protein expressing transgenic plants, the toxins can penetrate across membranes to be released into haemolymph and thereby interact with different tissues/cell types in the body cavity altering their functions including organs involved in reproduction (Hussein et al, 2006). In order to check the toxic effect of the Cry1A proteins in larval body cavity, the three active toxins, Cry1Aa, Cry1Ab and Cry1Ac, which were already shown to be active in gut, were injected into the hemocoel individually. The three Cry toxins upon injection displayed differential effect on *A. janata* larvae (Table. 2). Consistent with the earlier report by Ceresteins et al, (2001), our studies showed that Cry1Ac had no effect on either feeding behaviour or mortality at a concentration of 0.5 μg/0.2 g of body weight, while Cry1Aa and

Cry1Ab led to lower food consumption associated with reduction in the body weight after 48 h at the same concentration. It is interesting to note that the most potent gut active toxin Cry1Ac terribly failed to show any toxic effect upon intrahemocoelic injection. Furthermore, Cry1Aa and Cry1Ab toxins at the concentration of 0.7 µg/0.2 g of body weight showed complete mortality after 48 h, while Cry1Ac failed to show any effect even at this high concentration (Table 2).

Table 2:

Comparison of the toxicity effects on *A. janata* larvae caused after oral ingestion or intrahemocoelic injection of *B. thuringiensis* Cry toxins

Toxin	**[1]ED inthe hemolymph (ng/ 0.2g body weight)**	**[2]LC_{50} (ng/cm^2)**
Cry1Aa	**>700**	**61.01**
Cry1Ab	**>700**	**32.03**
Cry1Ac	**NS**	**23.70**

[1]ED, the effective dose that caused the mortality of the larvae after 48 h. This assay was carried out using 5th instar larvae

[2]LC_{50} the effective dose that caused 50% mortality in the early second instar larvae following ingestion. NS, not sensitive.

For the first time we demonstrated, cloned and characterized aminopeptidase from the fat body of *Achaea janata*, further we extended our study in order find out whether the presence of aminopeptidase in fat body is unique to *A. janata* or it is also present in other insects of the order lepidoptera. To decipher this question we cloned and characterized the *Spodoptera* fat body aminopeptidase and carried out a comparative analysis.

Cloning of APN cDNAs from the fat body of *Spodoptera litura*:

Cloning of *S. litura* fat body APN was carried out according to the methodology followed for the *A. janata* fat body APN. Using the same degenerate primers, RT-PCR was performed with fat body cDNA as the template. A 550-bp fragment was amplified (Fig. 3.24, lane 2), and this was cloned into the pTZ57R-T vector and sequenced. A set of gene-specific forward primers were designed on the basis of the sequence of the cloned insert.

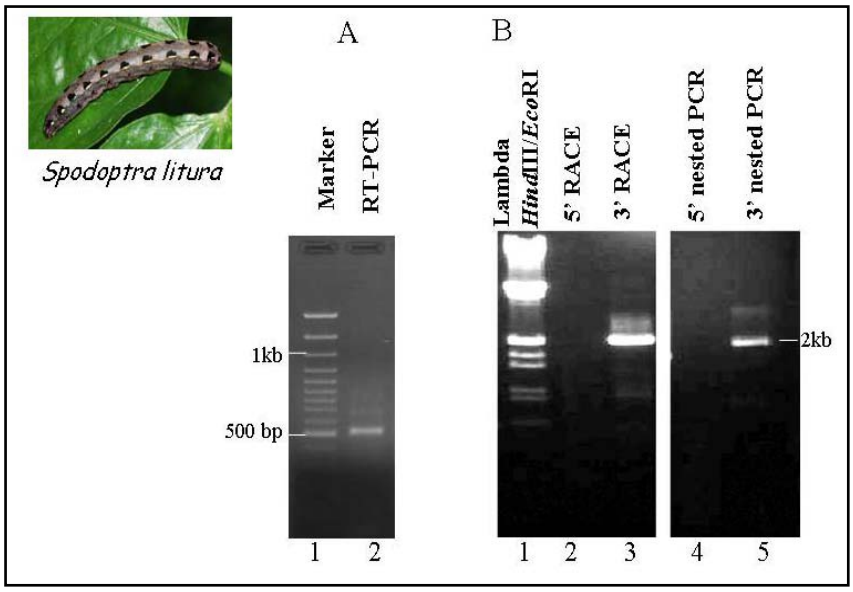

Fig. 3.24: RT-PCR mediated amplification and cloning of APN from *S. litura*: **A**. RT-PCR of fat body APN. Total RNA from from fat body of the fifth instar larvae was isolated and RT-PCR was performed using degenerate primers. Lane 1, 100 bp ladder; lane 2, PCR amplification product (550 bp). **B**. Agarose gel analysis SlFbAPN RACE products: no amplification in 5'RACE reaction (lane 2); 2.0 kb 3'RACE product (lane 3); 5'RACE nested PCR did not yield any product (lane 4); 2.0 kb, 3'RACE nested PCR product (lane 5); and Lambda HindII/EcoRI double digested marker (lane 1).

The RACE reactions were carried out as described earlier. The first 3' RACE was performed with SlFbAPNF3and GeneRacer^{TM} 3'primers, using the RT reaction product as template. Next round of nested PCR was performed with nested primers SlFbAPNF4and GeneRacer^{TM} 3' nested primer for 3' RACE using the first round of PCR product as template. When analyzed by agarose gel electrophoresis, the 3' RACE produced a 2 kb product (Fig 3.25, lane 5). The RACE product was cloned in pTZ57R/T vector and

sequenced. When all the sequences were aligned pair-wise by using BLAST program of www.ncbi.nlm.nih.gov, partial cDNA sequence along with the 3' UTRs was obtained (Fig 3.25). The sequence was submitted to the NCBI Genbank.

```
                Cttttccctgnttatgatgagccggaacttaaagcgacattcgta
                 L  F  P  X  Y  D  E  P  E  L  K  A  T  F  V
attgggattgatcgtcctgccgactaccagccgtctttagccaatactgatattgaacgg
 I  G  I  D  R  P  A  D  Y  Q  P  S  L  A  N  T  D  I  E  R
agagaagttttggccaatggctacatacgagagatattctacccgaccccaagaatgtcg
 R  E  V  L  A  N  G  Y  I  R  E  I  F  Y  P  T  P  R  M  S
acctacttagttgctttcctgatctccgagtttgaagcagctgcttccagccttaatggg
 T  Y  L  V  A  F  L  I  S  E  F  E  A  A  A  S  S  L  N  G
accaatgagtttggaatttatacccgaccagacgcaaagaatcagagtgactatgctttt
 T  N  E  F  G  I  Y  T  R  P  D  A  K  N  Q  S  D  Y  A  F
gacttcggtaggaaagttgtggacgctttgagcagctattttggcataaactactactcc
 D  F  G  R  K  V  V  D  A  L  S  S  Y  F  G  I  N  Y  Y  S
acaaacagtcatttgaggctcgaccatgttgcgttggtcgattttagtgctggtgctatg
 T  N  S  H  L  R  L  D  H  V  A  L  V  D  F  S  A  G  A  M
gagaattggggtctcattaaatatagagaatccctgcttctgtacgttcctggtcagtcc
 E  N  W  G  L  I  K  Y  R  E  S  L  L  L  Y  V  P  G  Q  S
acaccgtacttcaaataccgagtggcacaaatcatggcccatgaaactacacacacgtgg
 T  P  Y  F  K  Y  R  V  A  Q  I  M  A  H  E  T  T  H  T  W
ttcggaagcctggtgacctgtcattggtggagtaacacctggctaaacgagggcttcgct
 F  G  S  L  V  T  C  H  W  W  S  N  T  W  L  N  E  G  F  A
aattatttccaggactacatcacttcatttgtggatccttccgtcggagcagggaaccag
 N  Y  F  Q  D  Y  I  T  S  F  V  D  P  S  V  G  A  G  N  Q
ctggtcataggatctgtctacagtgcatacgacgctgacaacagtccgacttcaccacca
 L  V  I  G  S  V  Y  S  A  Y  D  A  D  N  S  P  T  S  P  P
atcacgaacaacgctgtcaactctccttcggaaatcagcggccattttggaaccatcacc
 I  T  N  N  A  V  N  S  P  S  E  I  S  G  H  F  G  T  I  T
tatcaaaaagctggttctgtaatccgtatgatgcaccatcttatccaggacgaggcattc
 Y  Q  K  A  G  S  V  I  R  M  M  H  H  L  I  Q  D  E  A  F
agatatggacttaattactatttaactcttaactcattcaattctggttacccagataaa
 R  Y  G  L  N  Y  Y  L  T  L  N  S  F  N  S  G  Y  P  D  K
ttgtatgaaggtcttcatcaaggagtgcagcgatataatacattgtcatcttaccctaat
 L  Y  E  G  L  H  Q  G  V  Q  R  Y  N  T  L  S  S  Y  P  N
aataatatttctgatatcatgaattcttggatctcacaggctggtcaccctgtggtgaat
 N  N  I  S  D  I  M  N  S  W  I  S  Q  A  G  H  P  V  V  N
gtgaccattgactacagtacagaaattgttactttgacccaaaaacgctactacgtgaac
V  T  I  D  Y  S  T  E  I  V  T  L  T  Q  K  R  Y  Y  V  N
tcatcaatatcatcgaatgagacgtacaagatccccatcacttacaccacacaaagggca
 S  S  I  S  S  N  E  T  Y  K  I  P  I  T  Y  T  T  Q  R  A
ccagacttcgagaatactagaccggcatttattttggaagaccagacattaacattcatg
 P  D  F  E  N  T  R  P  A  F  I  L  E  D  Q  T  L  T  F  M
atttttaacattagtagggaacatagttgggtcatatttaatctgcaagaaacaggttta
 I  F  N  I  S  R  E  H  S  W  V  I  F  N  L  Q  E  T  G  L
tacagagttaactatgacgaccactcatggagtattataatatcagctttgaaaggaaac
 Y  R  V  N  Y  D  D  H  S  W  S  I  I  I  S  A  L  K  G  N
gacagcgcaatcatacatccactgaatcgtgctaagattattaacgatctcttcgctttg
 D  S  A  I  I  H  P  L  N  R  A  K  I  I  N  D  L  F  A  L
gtttatgctgatgaagttccgttctcaacgctctcaagtgctctagactacctaccatta
 V  Y  A  D  E  V  P  F  S  T  L  S  S  A  L  D  Y  L  P  L
```

```
gaacctgaatacactggctggtttgcggcattgagaggatttagcaaaatatggcatttc
 E  P  E  Y  T  G  W  F  A  A  L  R  G  F  S  K  I  W  H  F
tatttaggagataatgaagtgctaccacatgttgagcattttatactgcagcacttggag
 Y  L  G  D  N  E  V  L  P  H  V  E  H  F  I  L  Q  H  L  E
tctggtatatcaagattaggttacgaagaaaggtccaccgacagtcttgaagaccagagg
 S  G  I  S  R  L  G  Y  E  E  R  S  T  D  S  L  E  D  Q  R
aatcgcatgcagatcctggagtttgcttgcaaattggagcatactggatgtgtagagagg
 N  R  M  Q  I  L  E  F  A  C  K  L  E  H  T  G  C  V  E  R
actgttgagctattcagggctttaagacaaaatggcaccgaggttgcaccaagcttgcgt
 T  V  E  L  F  R  A  L  R  Q  N  G  T  E  V  A  P  S  L  R
cctgtggtatattgcactggtcttcgtcatggatctgccgaagactacgacttcctctgg
 P  V  V  Y  C  T  G  L  R  H  G  S  A  E  D  Y  D  F  L  W
aaccgtatggtgaacaccaatttggcgaatgaggtttgggttattggagatgccttggga
 N  R  M  V  N  T  N  L  A  N  E  V  W  V  I  G  D  A  L  G
tgtacttccgatgaaagcaggataagaagctacctcgtatcaatgacagtagaaaatagt
 C  T  S  D  E  S  R  I  R  S  Y  L  V  S  M  T  V  E  N  S
cctataaggactcaagacttgacagttccattggcgagtgtcctcagggcgtatggcaac
 P  I  R  T  Q  D  L  T  V  P  L  A  S  V  L  R  A  Y  G  N
ctgcacatcgtcatggattctttgaaatctaattatacattgtggagctctatatacccc
 L  H  I  V  M  D  S  L  K  S  N  Y  T  L  W  S  S  I  Y  P
tcaatggacactgttttgaatacagttgcatctgctcttcacactgaagctgattttaat
 S  M  D  T  V  L  N  T  V  A  S  A  L  H  T  E  A  D  F  N
gagttcgaaaccttcctgtctgagtgcaccgtctgcacagaagccacgaagacctctggc
 E  F  E  T  F  L  S  E  C  T  V  C  T  E  A  T  K  T  S  G
cgtaatgctctggtgcaggcaagagccgccacagcttgggcaaacagccacaaagcagac
 R  N  A  L  V  Q  A  R  A  A  T  A  W  A  N  S  H  K  A  D
atattgaaagtgatcagaagtaattccatcatggcggccccatctgtcattgtaatgata
 I  L  K  V  I  R  S  N  S  I  M  A  A  P  S  V  I  V  M  I
atagggctggccatactcatttttaaagagttgtaattatt
 I G  L  A  I  L  I  F  K  E  L
```

Fig. 3.25: Partial cDNA sequence and corresponding amino acid sequence of *SlFbAPN* cloned *from* fat body of *S. litura* along with 3' untranslated regions.

APN mRNA transcript in midgut and fat body of *S. litura*:

The tissue specificity of the identified APNs was determined by northern blot analysis using the fat body *S. litura* fat body 550 bp fragment as probes. As shown in figure 3.26, the fat body specific APN fragment could detect expression of a single transcript of ~3.5 kb in the fat body tissue (lane 1), where as it failed to recognize any transcript in the midgut (lane 2).

Fig. 3.26: Northern blot analysis of APN mRNA in fat body and midgut tissues of *S. litura* Twenty microgram of RNA from fat body (lane 1) and midgut (lane 2) tissues were denatured and subjected to electrophoresis on a 1% agarose gel containing formaldehyde. The separated RNAs were transferred to Nylon-N+ membrane and hybridized with the [α^{32}P]-labelled fat body 550 bp fragment.

The amino acid sequence comparison by multiple sequence alignment, however, it revealed that *SlFbAPN* showed only 33% homology to the reported insect APNs displaying all chacteristic features of APN family (Fig 3.27).

```
HPAPN2          --MGTNMLLPTVFSILLG-------------------SIAAIPQEDFRSNLEWFD---Y 35
HvAPN110Kd      --MGAKMLLPTVFCILLG-------------------SIAAIPQEDFRSNLEWAD---Y 35
SlAPN           --MGTKMLVPAVLCVLLG-------------------FAAATPLEDFRSNLEFHD---Y 35
TniAPN4         ------MFVPIIFCILLG-------------------AISATPHDDFRSNLEFAD---Y 31
AjMgAPN         ------MQLLIILSILVG-------------------SLVAIPQEDFRSNLEFLE---Y 31
BmAPN4          HEAARYGYNAAAHCTMYPNW-----------------NRPRVPMDELRSNLEWMD---Y 39
PxAPN4          ------MALLLKLAILPA--------------------LLALAWADFPIDADFLSDIVD 33
HAAPN1          --MANRWYTLLLGAALLQ-SALS-----------FGPIE---VTDDEWAEYRNLMR---- 39
HPAPN1          --MANRWYTLLLGAALLQ-SALS-----------FGPIE---VTDDEWAEYRNLLR---- 39
HvAPN170kd      --MASRWFTLLLGVALLQ-SALS-----------FGPIE---VTDDEWIEYRNMMR---- 39
BMAPN1          --MASRWFYFLVGVAFLQ-TSLT-----------LSPIP---VPEDEWVEFARMLR---- 39
MsAPN1          -------FTIFLGVALLQ-GVLT-----------LSPIP---VPEEEWAEFSRMLR---- 34
AjFbAPN         --MASRWFNLLLGVLFIQ-GYLA-----------FSPIPEERLMDEEWVEYNSMLR---- 42
TniAPN1         --MANRFTLLLLGVALAQ-GILA-----------YSPIE---MPEDEWQEYRNLMR---- 39
pxAPNA          --MDSRWFLLVLVLIQAH-DITS-----------LSPIPVDDVAEEDWASFYRMLR---- 42
EPSAPNA         -MAALKLLVFALACYCAS-SFP------QDQPKPRNTIFSDERLQGEIFENLKDMVAFDD 52
LdAPN1          -MARIEFLVLSLACICVQ-GFLNQPAATTSPVTTRNTIFADEKFEGEIFEDLDVFEQLDI 58
BmAPN3          -MANYKVIIFLAACVLAQ-AFPD-----EPIYRTNNTIFLDEKLEGEIFEDIEAFENIDR 53
HAAPN2          -MAAIKLLVLSLACACVI-AHSP------ILQSAG-PSSYTSVKKDLPSRISTPFKNIEL 51
HPAPN3          -MAAIKLLVLSLACACVI-AHSP------IPPVSR-TIFLDERLEGGAFENIDAFNNIEL 51
PxAPN3          ------------------------------------------------------------
TniAPN3         -MVARTTLVLLLACVCTN-AIPR------TPPVLRNTIFADERVEGGVFENVDAFDNIEL 52
PIAPN           -MAAMKWFLLGVLCVSAQ-AFPDR----PTQRSSKTNIFLDEMLEGQIFENLMMEDTSDN 54
SlFbAPN         ------------------------------------------------------------
BmAPN2          --MYLLFITALLG--------------------------------SAYSFPTSTFNNVT 25
MsAPN2          --MYSLIFLALIG--------------------------------AAFGVPLSTNEDST 25
LdAPN2          --MYLLPLLTLLG--------------------------------SAFCVPLNTEIQST 25
TniAPN2         --MYLLALLTVIG--------------------------------SALALPVIENNEKN 25
PxAPN1          --MRLLICLTLLG--------------------------------LVCGNPVQLTDNSI 25
```

```
HPAPN2          STNLDEPAYRLRDVVYPTDVNLDLD--------------VYLNHLNFSGLVQIDVQVRE 80
HvAPN110Kd      STNLDEPAYRLRDVVYPTDVNLDLD--------------VYLDELRFNGLVQIDVEVRE 80
SlAPN           SSNVADPAYRLRPNVYPTDVKVNLEN-------------IDLEGARFTGSVEMIVIVRE 81
TniAPN4         GTNWVNNGYRLNDFVQPTEMIVDLD--------------VYLRQFRFDGVVRINVDVTE 76
AjMgAPN         DSNLGEQHYRLVDTVYPHTMDVDLD--------------VYLDELRFNGFVSIGVEVRE 76
BmAPN4          STNVAESAYRLLDTIQPRTMRVDLD--------------VFLNEARFDGIVSMDIEVLA 84
PxAPN4          TRNDDDVKYRLPESLDPVHCEIEITPHFDAT---------ADRPAFSFDGIVTINVIAKE 84
HAAPN1          -----DPAYRLPTTTKPSNYAINLTP-----YFTG------STLAFTFEGSVAITITATQ 83
HPAPN1          -----DPAYRLPTTTRPSNYVVNLTP-----YFTAT----ATAAAFTFDGTVSITITATQ 85
HvAPN170kd      -----DPAYRLPTTTRPSNYVVNLTP-----YFTG------TTLAFTFDGTVTITFRATQ 83
BMAPN1          -----DPAFRLPTTTRPRHYQVTLTP-----YFDVVPANVN---PFTFDGEVTIYTSPTV 86
MsAPN1          -----DPSYRLPTTTRPRHYAVTLTP-----YFDVVPAGVSGLTTFSFDGEVTIYISPTQ 84
AjFbAPN         -----DPAYRLPTTTRPSHYAVTLTP-----YIESVPTGVT-ADLFTFDGEATMTIQATE 91
TniAPN1         -----DPTYRLVRTTEPETYKVTLTP-----YFDTN-----DAKAFTFDGEVEILIKANQ 84
pxAPNA          -----DPAYRLPTTTKPRLYNVTLTP-----YFENVP---SGITPFTFDGQATIYISATV 89
EPSAPNA         NV-DPA-LYRLPTTTRPIHYDVLWGV-----DFTST--------PQAFSGTVVIQLQATQ 97
LdAPN1          TARNSE-LYRLPNTTKPSHYTVLWTL-----DFSRA--------IPTQSGTVSILLNATQ 104
BmAPN3          SI-AAS-TYRLPTTTRPLHYNVLWAI-----DISR----------LTFSGTVEIQLYATR 96
HAAPN2          SNAAAASPYRLPNTTIPTHYKVLWVI-----NLSEN--------VQSYSGTVDITLQATQ 98
HPAPN3          SN-AAASPYRLPNTTIPTHYKVLWVI-----DIHQT--------VQSYSGNVEITLQATQ 97
PxAPN3          --------YRLPTTTAPSHYKVHWIV-----DPYD----------QRYSGYVAITLSAQQ 37
TniAPN3         SPRNATSPYRLPTTTKPIHYDVVWHI-----DINN----------LQYQGSVDIQIQATK 97
PIAPN           ARNDAVNVYRLPTTTKPYRYNIDWIV-----DTTE----------LTFGGSVAIQLYATQ 99
SlFbAPN         ------------------------------------------------------------
BmAPN2          RNTDLASQYVLPGESFPTFYDVSLFID-------------PANTVSFNGKVSIRIIPRI 71
MsAPN2          RNQNLAALYVLPQTSYPTFYDVRLFID-------------PGYTEAFHGNVSIRIIPNI 71
LdAPN2          TRDDRAQQYVLPPDTIPTFYDVTLFLD-------------PGNPDYFNGSVSIRILPIS 71
TniAPN2         T--VLESLYVLPRETIPLFYDVSLILD-------------PDNEAYFNGTVAIRILPYV 69
PxAPN1          ALQNTYDNYVLPGESFPTFYDVQLFFD-------------PEYEASFNGTVAIRVVPRI 71

HPAPN2          NNLRQIVLHQKVVSIVGVNVVG-PNGP-----VPLQFPYPYTTDDYYEILLINLDEPINI 134
HvAPN110Kd      NDLRQIVLHQKVVSINAVNVVG-PNGP-----VGLQFPYPYTTDDYYEILLINLAEPINI 134
SlAPN           NDLEQISMHQNNLFVTRVNVVNNTNGEN----VQLRSPDPFTYDNYYELLHLHFHLPIVA 137
TniAPN4         NQLNQIVLHQNLVKVEGVNVVNAVTNDP----VPLKITDPFSVNSTFELLVINFDSPINS 132
AjMgAPN         SGLTQIALHQKVQSIQXVNLLT-AAGAP----VPLLISEPFTTDDYYELIKINLNSAIPA 131
BmAPN4          SNIEQIVFHQNVVSIQGVNLVT-ARGDP----VGLKFPDPFTIDRHYELLLINLAQPIAA 139
PxAPN4          DGINSLILQENVREIGAITVTE-ENGRL----IDLNPSSPFERLTEYQFLKINLRSGVTL 139
HAAPN1          ANVNEIVLHCNDLTIESVTVATVASPN-V---NLAASGQTFVCDPVYSFLRIRTAGALAV 139
HPAPN1          ANVDEIVLHCNDLTISSLTVATAANPT-V---NIATPSQTFVCDPTYSFLRIRTTAALAL 141
HvAPN170kd      ANVNEIVLHCNDLTIQTLTVATAASPN-V---NLAASGQTFTCDPVYSFLRIRTAGVLAE 139
BMAPN1          ANVNEVVIHCNDLTIQSLSIGYQSGTNVV---DITATGQTFACEMPFSFLRIRTTEALVL 143
MsAPN1          ANVNEIVLHCNDLTIQSLRVTYVSGNSEV---DITATGQTFTCEMPYSFLRIRTSTPLVM 141
AjFbAPN         ANVNEIRLHCNDLTILELTVHVATDLT-V---NLATPGQTYECVMPWSFLTIPLTTTLNT 147
TniAPN1         A-VSEIVLHCNDLTISKLTVTTETSST-----DLAEAGQTFTCEANTSFLRIKTTSPLEA 138
pxAPNA          ANVSEIVLHCEDLTITKVEVTRNINGEPQ---PVPISNDSPQCEMPYAFLRVAPTQALQL 146
EPSAPNA         AGVNQIVIHSEELNIG-TVSLVQGT---------TAVPVTYVEEPDFQFLRI-SLTTGTL 146
LdAPN1          ANVNEIVIHAHNLTIT-NVRLQLGT---------TEVPVTYTLEPEYHFMRI-RLNEGSL 153
BmAPN3          ANVSEIVIHADDLEIT-SVILRQGT---------VTTPSTYTLQKELQFLRL-RLNTGTL 145
HAAPN2          P-MHEIVIHCDHLTVT-SVVLRQGTAT-----EGTLIPTTPTPQSQYHFLRV-ALNDGVL 150
HPAPN3          PNVNEIVIHCDHLTVT-SVVLRQGTAT-----QGTVIPTTATAQSEYHFLRV-ALNDGVL 150
PxAPN3          ANVSEIVIHCDHVTVT-SVNVTQAN---------TLVPTTASFQREYQFLRV-AVTNGVL 86
TniAPN3         SNVSQIVIHYDHMPSNPSVTLRLGAN---------TIQTSYELEPAYQFLKINLLNNAVL 148
PIAPN           AEVNEIVIQSDDLNIL-NVTLTRNN---------VVVPQTFYLQPEYDFLRV-ELVNGFL 148
SlFbAPN         ------------------------------------------------------------
BmAPN2          A-TNVIVVQAMEMTIRS--ISVFTDR----NSNENLFTSFTLATDDTHFLRISTRTQLLP 124
MsAPN2          N-IDQITIHAMAMRIDS--IRVVSDV----NPNEDLFSDFTLATDDTHLLTIRLTRNITA 124
LdAPN2          I-TNEIVLHAMEMEIEEDAIQVFTDR----EPNVNLFESFTLANNDTHFLRIRLNTQLTV 126
TniAPN2         S-TSQIVIHAMELEIKT--VGVYSDR----TPANDIYLDHEQSTDDTHLLRINLKSPLPV 122
PxAPN1          A-TQEIVLHAMEMEILS--IRAYSDLPSDDNLNENLFSSYTLATDDTHLLKIQFTRVLDA 128

HPAPN2          ---------GNYSITIRYNGQINANPLDRGFYRGYYYL--ND-ELRIYATTQFQPYHARK 182
HvAPN110Kd      ---------GNYSITIRYNGQINDNPIDRGFYKGYYYL--NN-ELRLYATTQFQPYHARK 182
SlAPN           ---------GSYTITIDYRGVINTNPVDRGFYRGYYYY--EN-TRRYYATTQFQPYHARK 185
TniAPN4         ---------GKYAITVRYEGKINENPIDRGFYAGYYFY--NG-AKRYYATTQFQPYHARK 180
AjMgAPN         ---------GNYTITIRYTGVINENPLDRGFYKGYYFL--NN-QERAYATTQFQPYHARK 179
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BmAPN4          ---------GNYTVTVRYRGQINTNPVDRGFYRGYYYV--NN-QLRYYATTQFQPFHARK 187
PxAPN4          SK------SGKYTIRIEYVGHMNETPLSRGMFRGSYVG--KDGKTHWYAATHLQPTHSRQ 191
HAAPN1          DT--------NYVIRSTFR--GNLQTNMRGFYRSWYYD--SSREKRWMATTQFQPGHARQ 187
HPAPN1          NT--------NYIITSSFR--GNLQTNMRGFYRSWYVD--SSGNKRWMATTQFQPGHARQ 189
HvAPN170kd      NT--------DYIIQSTYR--GNLQTNMRGFYRSWYID--SSGTKRWMATTQFQPGHARQ 187
BMAPN1          NR--------EYIIKSTFR--GNLQTNMRGFYRSWYVD--STG-RRWMGTTQFQPGHARQ 190
MsAPN1          NQ--------EYIIRSTFR--GNLQTNMRGFYRSWYVD--RTG-KRWMATTQFQPGHARQ 188
AjFbAPN         NL--------QYVVRSRFR--GNLQTNMRGFYRSWYVD--STGNRRWMGTTQFQPGHARQ 195
TniAPN1         EA--------KYVIKSEFT--GNLQTNMRGFYRSWYVD--SSGNKRWMATTQFQPGHARQ 186
pxAPNA          NQ--------EYTVNVTYR--GNLQTDMRGFYRSWYRD--SSGNKRWMATTQFQPGHARK 194
EPSAPNA         NY--NAQTPVVYTLTISFE--APLRNDMYGIYRSWFRNQPND-PISWMATTQFQATSARK 201
LdAPN1          NY--NPTTPQYYTLTIDFG--ANLRDDMYGIYRTWFRNNPTDTAVSWMASTQFQATAARF 209
BmAPN3          VF--NAASPVIYTLTIDFA--ARLRTDMYGIYRTWFRNSAND-VTRWMASTQFQATSARY 200
HAAPN2          LY--NENVPVQYTLSIAFN--ADMRDDMYGIYRSWYRNLPTDNNIKWMATTQFQATAA-Y 205
HPAPN3          SY--NADVPVQYTLTIEFN--ALMRDDMYGIYRSWYRNLPTDTNIRWMATTQFQATAARY 206
PxAPN3          QY--NAATPVQYVLTIEFN--ANMRDDMYGIYHSWYKNEGSDATISWMATTQFQATAARY 142
TniAPN3         QYTENAQTQTTYLLTVEFGF-TPMRTDMYGIYRSWFKN-AHNQAEQWMASTQFQATAARY 206
PIAPN           DYDAVNATSALYVLTINFE--AELRHDMYGIYRSWFRNDNYNATPNYMATTQFQATSARR 206
SlFbAPN         ------------------------------------------------------------
BmAPN2          DQ----------PYIVNIDYESKYAPNMFGVYVSTYQQ---NGRTVNLVTSQLQPTFARR 171
MsAPN2          LQ----------PHVIHIDYVAQYADDMFGVYVSTYEE---NGRTVNLVTSQLQPTFARR 171
LdAPN2          MQ----------PVTVTISYTAHFAENMFGVYLSTYEE---LGSSVSLITSQLQPTFARR 173
TniAPN2         LQ----------PHTINIKYSGKYAPNMFGIYVSTYET---NNQPQKVITSQLQPTFARR 169
PxAPN1          LQ----------PITVEISYSAQYAPNMFGVYVSRYVE---NGATVSLVTSQLQPTFARR 175

HPAPN2          AFPCFDEPQFKSRYTISITRDTSLSPSYSNM-AIRTSEYIIDN--SRTRETFYTTPIISA 239
HvAPN110Kd      AFPCFDEPQFKSRFTISITRASSLSPSYSNM-AISNTQILG----ARTRETFHPTPIISA 237
SlAPN           AFPCFDEPQFKSRYTISITRPDTLGPSYSNM-AISSTEVIG----NSVRETFYPTPIISA 240
TniAPN4         AFPCFDEPQFKSRFTISITRDNDLTPSFSNM-AISRTEQIG----NRVRETFHPTPIISA 235
AjMgAPN         AFPCFDEPMFKSRYTLAITRDINLSPSYSNM-AISATVPVG-T--TRVREEFYPTPIISA 235
BmAPN4          AFPCFDEPQFKSIYIISITRDRSLSPTYSNM-PISNTETPS-T--NRVKETFFPTPIVSS 243
PxAPN4          LFPSFDEPGFKSTFKIIVNRPANFADTHSNMYAESRSEPIN----GLVKEVFYTTPRMSA 247
HAAPN1          AFPCYDEPGFKATFDITINREADFSPTLSNMPIRTTTNLATG----RVAETFHTTPETST 243
HPAPN1          AFPCYDEPGFKATFDITINRESDFSATLSNMPIRTQTPLASG----RIAETFYTTPVTST 245
HvAPN170kd      AFPCYDEPGFKATFDITINREADFSPSLSNMPIRSTTNVG-A----RVAETFYTTPVMST 242
BMAPN1          AFPCYDEPGFKATFDITMNREESFSPTISNMPIRTTNTLANG----RVSETFWTTPVTST 246
MsAPN1          AFPCYDEPGFKATFDITMNREADFSPTISNMPIRATTTLTNG----RISETFFTTPLTST 244
AjFbAPN         AFPCYDEPGFKARFDITIVRSPTFSPTLSNMPILSTTTLTNG----WVAETFHTSTVTST 251
TniAPN1         AFPCYDEPSFKALFDITIKRLPDFSETLSNMPIKTRGPLTDG----RIAETFHTTPKTST 242
pxAPNA          AFPCYDEPGFKALFNITINREDDFKPSISNMPIRRTISLGNG----RTADSFYTTPLTSS 250
EPSAPNA         AFPCYDEPSFKATFDITIRRPAAYRSWSCTRIASTAPSTTPP---NYEDDIYHRTPIMST 258
LdAPN1          AFPCYDEPSFKATFDVTIRRPVAYQSWFCTVLLRTDTPAAAP---LYRDDVYHTTPVMST 266
BmAPN3          AFPCYDEPSFKATFDITIRRPTTHRSWSCTNIKETRVSTVTG----YQDDIYNRTPLMST 256
HAAPN2          ALPCYDEPGYKAKFDVTIRRPLGYKSWFCTRQRITRPSTTG-----YEEDEYHTTPEMST 260
HPAPN3          AFPCYDEPGFKAKFDVTIRRPTGYKSWFCTRQRVSRVSTVAG----YEEDEYHTTPEMST 262
PxAPN3          AFPCYDEPSFKAKFDVTIIRPANLKSWFCTTRKSTGTSSTPG----FAFDEYNTTPTMST 198
TniAPN3         AFPCYDEPGFKATFKVTIRRPTNLKSWFCTLRESTTPSVVTG----YTDDVYQITPVMST 262
PIAPN           AFPCYDEPSFKATFDISIARRQDVKSWSCTRLAGTAPSDLYGP--EFEVDTFYRTPIMST 264
SlFbAPN         LFPXYDEPELKATFVIGIDRPADYQPSLANTDIERREVLANG----YIREIFYPTPRMST 56
BmAPN2          AFPCYDEPAIKAIFRTTIYAPAAYTVVRHNTPER--AVPLKEDVAGYVKHEFEDTLVMST 229
MsAPN2          AFPCYDEPALKAVFRTTIYAPAAYATVRSNTPER--RDSLKPNEPGYVKHEFEDTLVMST 229
LdAPN2          AFPCYDEPALKAIFRTTIYAPPQYTVVRSNMPLR--EDLLKEPVAGYTKHEFQDTLVMSS 231
TniAPN2         AFPCYDEPALKAKFSTTIYAPPTYPVVRSNMPLKPASENVKEDIAGYTKHEFADTLTMST 229
PxAPN1          AFPCYDEPALKAVFRTTIYAPPAYNVVETNMPLR--TDSLKSDRPGFTKHEFQDTLVMSS 233
                 :* :***  *: :   :           .                   . :  :   *:
HPAPN2          YLVAFHVS-DFVSTEYT-STEAKPFSIISRQGATNQHQ--YAAEIGLKITNELDDYFGIQ 295
HvAPN110Kd      YLVAFHVS-DFVATEYT-STDAKPFSIISRQGVTDQHE--YAAEIGLKITNELDDYLGIQ 293
SlAPN           YLVAFHVS-DFVPTVST-STAPRPFSIISRRGATDQHA--YAAEIGVEITNQLDDYLGIE 296
TniAPN4         YLVAFHVS-DFVSTEYT-STASRPFKIISRQGATAQHA--YAAEIGLKITDALDDYLGIQ 291
AjMgAPN         YLVAFHVS-DFVETEIT-STAARPFKIISRPGVIDQHE--YAAEIGLKITNELDDFLGIQ 291
BmAPN4          YLVAFHVS-DFVETSLT-GTDSRPFGIISRQGVTSQHE--YAAKIGLKITDKLDDYFGIL 299
PxAPN4          YLVTIHISDEFTIIADN-GDAKRPYRILARPDAANQGQ--YALEVGPPLTKWLEEYLGKP 304
HAAPN1          YLIAFIVSHYSQVASN--NNQQRPFHIYARDNVGVHGN--FALEIGVPLLEVMERYTEIP 299
HPAPN1          YLIAFIVSHYKSVATN--NNQLRPFEIYARDNVGVSGN--FALEIGMPLLEVMERYTEIP 301
HvAPN170kd      YLVAFIVSHYTQVATH--SNQQRPFAIYARNNIGNHGN--HALDVGVPLLDVMERYTDIP 298
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BMAPN1          YLLAFIVSHYTVVSTN--NNALRPFDIYARNNVGRTGD--WSLEIGEKLLEAMEAYTQIP 302
MsAPN1          YLLAFIVSHYQVISNN--NNAARPFRIYARNNVGSQGD--WSLEMGEKLLLAMENYTAIP 300
AjFbAPN         YLLAFIVSHYERVASS--TDPERPFYIYARDNVGDTGE--WSLEIGEKLLLAMEEYTGYP 307
TniAPN1         YLLAFIVSHYKEVATG--TDLNRPFKIYARDNAKLTGD--WSLDIGERLLEEMEKITDVP 298
pxAPNA          YLVAFIVSHYEKVESS--NNTLRPFDIYARDNVGVTGQ--WSLEVGEKLLAYMEGHTDYE 306
EPSAPNA         YLLALIVAEYDSLTVNN-AQGQLIYEVIARPNAISTGQGQYALDVGQDLLAEMNDHTNYN 317
LdAPN1          YLLALIVAEYGSITVNN-VNGQLAYEVIARPGAIDAGQGQYALEVGQDLLNQMSLHTNYD 325
BmAPN3          YLIALIVAEYESLEQR--QNGVLRYEVIARPGALSAGQGQYAFDVGMELLATMSRHTAMD 314
HAAPN2          YLLALIVAEYDSLATLD-ADNRVLHEVIARPGAIINGQAAYAQRAGQDLLAEMSDHTDFD 319
HPAPN3          YLLALIVAEYDSLEAVD-DNNDVLHEVIARPGAITNGQAIYAQRVGQELLGNMSEHTGYD 321
PxAPN3          YLLALIVADYDNLPYP--ATGVVRHEVIARPGAIIEGQGDYAQETGQALLEWMSDHTDYD 256
TniAPN3         YLLALIVADYESDSVSN-NNGILQHEVIARPGAMSAGQGKYALETGISLLNMMNNHTAYN 321
PIAPN           YLLAIIVADYKSVEFNN-TQGLLEYEVIARPAAIDNNQYQYAFDVGQELLAEMSDHTAID 323
SlFbAPN         YLVAFLISEFEAAASS--LNGTNEFGIYTRPDAKNQSD--YAFDFGRKVVDALSSYFGIN 112
BmAPN2          YLLAYLVSNFEHVSHEQNPIYRVPFRVYSRPGTQTNAA--FAMDFGQKNMVALEAYNEFP 287
MsAPN2          YLIAYLVSNFNYIENSQNPIYPIPFRVYSRPGTQNTAE--FALEFGQQNMIALEEYTEFP 287
LdAPN2          YLLAYLVSNLGHIEDMTDDLYRIPFKVFSRPGTQDTAA--FALDFGQKNMQALEDYTEFP 289
TniAPN2         YLLAYLVSNFEYIENQQNDIYHIPFRVYSRPGTQTGAE--FALRFGQENMVALEKYVDFA 287
PxAPN1          YLLAYLVSKFDYISNENNPTYDKSMKVFSRPGTQNTAE--FALDFGQKNMVELEKYTEFP 291
                **::  ::                  : :*          :   *      :.

HPAPN2          YHEMGQGALMKNDHIALPDFPSGAMENWGMVNYREAYLLYDANNTNLNNKIFIATIMAHE 355
HvAPN110Kd      YHEMGQGTLMKNDHIALPDFPSGAMENWGMVNYREAYLLYDANNTNLNNKIFIATIMAHE 353
SlAPN           YHDMGQGQIMKNDHIALPDFPSGAMENWGMVNYREAYLLYDPANTNLVNKIFIATIMAHE 356
TniAPN4         YHEMGQGVIMKNDHIALPDFPSGAMENWGMVNYREAYLLYDPEHTNQNNKNFIASIMAHE 351
AjMgAPN         YHEMGQGVLMKNDHIALPDFPSGAMENWGMVNYREAYLLYDPDNTNIINKIFIATIMAHE 351
BmAPN4          YHEMGQGTIMKNDHIALPDFPSGAMENWGMVNYREAYLLYDPQHTNLINKIFIATIMAHE 359
PxAPN4          YYEMAEN--MKNDQIASPFWASGATENWGLVTYRELRLLYEEGETNAVDKMSIGTITAHE 362
HAAPN1          YYGMAQN--MNMKQAAIPDFSAGAMENWGLLTYREALILFDPVNTNNFYRQRIANIISHE 357
HPAPN1          YYNMASN--MNMKQAAIPDFSAGAMENWGLLTYREALILFDPVNTNNFYKQRIANIISHE 359
HvAPN170kd      YYTMATN--MNMKQVAIPDFSAGAMENWGLLTYREALILYDPQNSNSFYRQRIPNIISHE 356
BMAPN1          YYTMAEN--INMKQAAIPDFSAGAMENWGLLTYREALILYDPLNSNHFYKQRVANIVAHE 360
MsAPN1          YYTMAQN--LDMKQAAIPDFSAGAMENWGLLTYREALILYDPLNSNHHYRQRVANIVSHE 358
AjFbAPN         YYSMTEN--IIMQQAAIPDFSAGAMENWGLLTYREALILYDRLNSNHFYRQRVANIVSHE 365
TniAPN1         YYGMALN--MDMKQAAIPDFSAGAMENWGLLTYREALILYDPKHSNHFYKQRVANIVSHE 356
pxAPNA          YYSMAPF--LNMKQAAIPDFSAGAMENWGLLTYREANILYHPENSNHFYKQRVANIVAHE 364
EPSAPNA         FYTMNPN--LKMTQASIPDFSAGAMENWGLLTYREAYIMYDEVHTNSYFKQLIAYILSHE 375
LdAPN1          FYSQHSR--LKMTQASIPDFGAGAMENWGLLTYREAYLMYDEDNTNSHFKQIIAYILSHE 383
BmAPN3          FYSIHPN--LKMTQASIPDFSAGAMENWGLLTYREAYLMYDENHTNGYFKQLIAYILSHE 372
HAAPN2          FYKQDEN--LKMTQAAIPDFGAGAMENWGLLTYREAYILYDEQHTSSNFKQIIAYILSHE 377
HPAPN3          FFSQDVN--LKMTQAAIPDFGAGAMENWGLLTYREAYLLYDEQHTSSNFKQIIAYILSHE 379
PxAPN3          FFSQDPN--LKMTQAAIPDFGAGAMENWGLLTYREAYLLYRPNYTSSYFKQLIAYILSHE 314
TniAPN3         FYDQSPS--LKMTQAAIPDFGAGAMENWGLLTYREAYLMLDDNHTNSYYRQLIAYILSHE 379
PIAPN           YFSVDSN--LKMTQAAIPDFGAGAMENWGLLTYREAYIMYHPNHTNSNYKQLIAYILSHE 381
SlFbAPN         YYSTNSH--LRLDHVALVDFSAGAMENWGLIKYRESLLLYVPGQSTPYFKYRVAQIMAHE 170
BmAPN2          YAFPKLD------KAAVPDFAAGAMENWGLVIYREVALLVTEGVTTTATRQNIARIICHE 341
MsAPN2          YAFPKID------KAAVPDFAAGAMENWGLVIYREVALLVREGVTTTSVKQNIGRIICHE 341
LdAPN2          YLLPKMD------KAAVPDFAAGAMENWGLVIYREVALLVTEGVTTTQTKQNIGRIICHE 343
TniAPN2         YAFPKID------KAAVPDFAAGAMENWGLVVYREVALLVTEGVTTTSVKQNVGRIICHE 341
PxAPN1          YAFPKID------KVAVPDFAAGAMENWGLVIYREIALLVQEGVTTTSTLQGIGRIISHE 345
                :            : :   : :** ****:: ***  ::     :.      :  * .**

HPAPN2          LGHKWFGNLVTCFWWSNLWLNESFASFFEYFGAHWADPSLELDDQFVVDYVHSALNSDAS 415
HvAPN110Kd      LGHKWFGNLVTCFWWSNLWLNESFASFFEYLGAHWADPALELDDQFVVDYVHSALNSDAS 413
SlAPN           LGHKWFGNLVTCFWWSNLWLNESFASYFEYFAAHWADPKLELADQFIVDYVHSALNADAS 416
TniAPN4         LGHKWFGNLVTCFWWSNLWLNESFASFFEYFAAHWADPALELEDQFVVDYVHSALTSDAS 411
AjMgAPN         LGHKWFGNLVTCFWWSNLWLNESYASYFEYFGTHWADLALELDEQFVVDYVHSALAADAG 411
BmAPN4          LAHKWFGNLVTCFWWSNLWLNESFASFYEYFGAHYADPSLELDDQFVVDYVHSALTWDAG 419
PxAPN4          LGHKWFGNLVTARWWDNVWINEGYASYFEYFAMDAVDKSMDLADQFNIMYTQSALATDSS 422
HAAPN1          IAHMWFGNLVTCAWWDNLWLNEGFDTILPVLLDWVVAPEMGFETRFIVEQLHVSMLSDSL 417
HPAPN1          IAHMWFGNLVTCAWWDNLWLNEGFARFYQYYLTGVVAPEMGFETRFIVEQLHVSMLSDSI 419
HvAPN170kd      IAHMWFGNLVTCAWWDNLWLNEGFARFYQYYLTGVVSPEMGYDTRFIVEQVHTSLLSDSI 416
BMAPN1          IAHMWFGNLVTCAWWDNLWLNEGFARFYQYYLTASVAPELGYETRFIVEQVQMAMFSDSV 420
MsAPN1          IAHMWFGNLVTCAWWDNLWLNEGFARFSQYYLTATVDPELGYEIRFIPEQLQVAMFSDSV 418
AjFbAPN         IAHMWFGKLVTCAWWDNLWLNEGFARFYQYFLTDSVDETLGYRTRFITEQLQVALLSDSE 425
TniAPN1         IAHMWFGNYVTCAWWDNLWLNEGFARFYQYYLTDRVDKNLGFDTRFIVEQLHTSLLSDSG 416
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pxAPNA          IAHMWFGNLVTCAWWDNLWLNEGFARYYQYFLTGPVLPDLGYETRFIVEQVHTAMFSDSL 424
EPSAPNA         IAHMWFGNLVTCDWWDVLWLNEGFARYYQYFLTHWVETDMGLETRFITEQVHTALLSDSS 435
LdAPN1          IAHMWFGNLVTCEWWDVLWLNEGFARYYQYYLTYWVE-DMGFGTRFITEQVHTALLSDSA 442
BmAPN3          IAHMWYGNLVTCDWWDVLWLNEGFARYYQYFLTDWVEDYMGLGTRFIVEQIHTSLLSDSA 432
HAAPN2          IAHMWFGNLVTNAWWDVLWLNEGFARYYQYFLTAWVGD-MGLATRFINEQVHASLLSDSS 436
HPAPN3          IAHMWFGNLVTNAWWDVLWLNEGFARYYQYFLTAWVED-MGLATRFINEQVHASLLSDSS 438
PxAPN3          IAHMWFGNLVTNDWWDVLWLNEGFARYYQYFLTDAVEDYMGLGTRFINEQVHTTLLSDSA 374
TniAPN3         IAHMWFGNLVTNQWWDVLWLNEGFARYYQYFLTEWTVG-MGLGTRFITEQVHTSLLSDSS 438
PIAPN           IAHMWFGNLVTCDWWDVLWLNEGFAKYYQYFLTHWVEDHMGFETRFITEQVHTALLSDSA 441
SlFbAPN         TTHTWFGSLVTCHWWSNTWLNEGFANYFQDYITSFVDPSVGAGNQLVIGSVYSAYDADNS 230
BmAPN2          NVHMWYGNEVGPLSWTYTWLNEGFATFFESFATDLVLPEWRMMEQFVVTMQN-VFQSDAV 400
MsAPN2          NTHMWFGNEVGPMSWTYTWLNEGFANFFENYATDFVRPQWRMMDQFVIAMQN-VFQSDAV 400
LdAPN2          NVHQWFGNEVGPQSWTFTWLNEGFANFFENYATDLVLPEWRMMDQFVVALQN-VFQSDAV 402
TniAPN2         NVHMWFGNEVGPLSWTYTWLNEGFANFFENFATDLVRPDWRMMDQFVLMMQN-VFQSDAV 400
PxAPN1          NTHQWFGNEVGPDSWTYTWLNEGFANFFESFATDLVLPEWRMMDQFVINMQN-VFQSDAV 404
                  * *:*. *    *   *:**.:          .        ::          *
HPAPN2          QFATPMNHTDVVDNDSITSHFSVTSYAKGASVLKMMEHFVGWRTFRNALRYYLRNNEYDI 475
HvAPN110Kd      QFATPMNHVDVVDNDSITAHFSVTSYAKGASVLRMMEHFVGSRTFRNALRYYLRNNEYSI 473
SlAPN           PSATPMNWEEVADNPTITQHFSTTSYAKGASVLRMMEHFVGPRTFRNALRHYLRDNAYGI 476
TniAPN4         SGATPMNWDTVEDNLSISAHFSTTSYAKGASVLRMMEHFLGQRNFRNGLRYYLRDNAYKI 471
AjMgAPN         AGATPMNWVDVADNPSVTSHFSTTSYAKGASVLRMMEHFVGTRNFRNALRYYLRDNAYEV 471
BmAPN4          TGATPMNWTEVSNNSSISSHFSTTSYAKGFGSQ-DDGAFPRFQNFQERPEILPQRQCLRH 478
PxAPN4          ASTRALQHT-VNTPTQVSGHFSGISYSKGAALLNMLKHFLGENTFKKSLNYYLDEMKYEY 481
HAAPN1          DSAHALTNPNVNDPTTVSAHFSTITYAKGASIIRMTQHLLGNNTFVKGLRTYLKDNAYGV 477
HPAPN1          DSAHALTNPSVNDPTTVSAHFSTITYAKGASIIRMTQHLLGNDTFVKGLRTYLKDNAYGV 479
HvAPN170kd      DSAHALTDPSVNDPAAVSAHFSTITYAKGASVLRMTQHLLGNDTYVKGLRSYLKDNAYGV 476
BMAPN1          DTAHALTDLNVNDPTTVSAHFSTITYARGAAILRMTQHLLGVETFVKGLRNYLRERQFNV 480
MsAPN1          DSAHALTDTSVNDPVAVSAHFSTITYARGAAILRMTQHLLSYDTFVKGLRQYLRARQFDV 478
AjFbAPN         DSAHALTNPAVNTPTTVSAHFSTITYAKGAAVLKMTQYLLGEKTYRRGLQSYLQANQYDV 485
TniAPN1         VNAHPLTDENVSSPTTVSAHFSTITYAKGASVLRMTQHLLGNSTFEKGLRSYLKARRYDV 476
pxAPNA          DSAHALTNPSVNDPAAVSNHFSTITYARGAAVLRMTQQLLGENTYLTGLRNYLKDRAFDV 484
EPSAPNA         NNPHALTNPGVGSPRAVSAMFSTLSYNKGAAVIRMTEHLLGSEVHRAGLRNYLVANEFDM 495
LdAPN1          NNPQPLTNPGVGSPSSVSAMFSTISYNKGAAVIRMTEHLLGFTVAEQGLRNYLRGKEFQT 502
BmAPN3          NSPQPLTNPGVGSPASVSAMFSTISYNKGAAVIRMTEHFLGFEVHRQGLNNYLIERSFDT 492
HAAPN2          IDAHPLTNPGVGSPAAVSAMFSTITYNKGASVIRMTEHLLGFEVHRAGLRKYLEDKKFKT 496
HPAPN3          ISAHPLTNPGVGSPASVSAMFSTITYNKGAAVIRMTEHLLGFEVHRAGLRTYLQNMQFKT 498
PxAPN3          NNPHPLTNPGVGSPASVSAMFSTITYNKGAAVIRMTEHLLGYETHRKGLREYLKELHFKT 434
TniAPN3         NNPQPLTNPGVGSPAAVSAMFSTITYNKGAAVIRMTEHLLGSVVHTTGLRNYLKKHHFGL 498
PIAPN           ITAHPLSTSGIGSPSQVSSMFSTLSYNKGAAIIRQTEHLLGFEVHRQGLRNYLAQKSFDT 501
SlFbAPN         PTSPPITNNAVNSPSEISGHFGTITYQKAGSVIRMMHHLIQDEAFRYGLNYYLTLNSFNS 290
BmAPN2          LTINPMTHA-VYTPSQIMGQFNAIAYQKSGSVIRMLHHFLTPEIFRRGLVIYIINNSRRA 459
MsAPN2          LSVNPMTHP-VYTPSQIIGTFNAVAYQKSGSVIRMLQHFMTPEIFRRGLVIYIKANSRAA 459
LdAPN2          LSINPMTHP-VYTPAEILSTFNAVAYQKSGSVIRMMQHFLTPEVFRQGLVYYIQTMSRDA 461
TniAPN2         ISVNPMTHP-VYTPSQILGTFNAVAYQKSGSVIRMMQHFLTPEVFRQGLILYIKDKSRSA 459
PxAPN1          LSVNPITFE-VRTPSQILGTFNSVAYQKSGSVIRMMQHFLTPEIFRKSLALYISRMSRKA 463
                     .:    :     :   *.  :* :. .       :
HPAPN2          GFPVDMYTAFKQAVAEDVNYQRDFPNVDVGAVFDTWVQNRGSPVINVSRNNSTGVITVRQ 535
HvAPN110Kd      GFPVDMYAAFKQAVSEDFTFERDFPGIDVGAVFDTWVQNRGSPVLNVARNSNTGVISVSQ 533
SlAPN           GNPSLMYQAFNKAIAEDHTFLSDFPNINFGNVFDSWVQNRGSPVVEVTRNPETGVIVVEQ 536
TniAPN4         GTPENLYDALRQAASEDFTFARDFPNVNVGAVLDSWVQNPGAPVLKVDVNMETGLITVTQ 531
AjMgAPN         GYPIDMYDAFRQAVSEDYTFLQQYPNVDVGAVFESWVENPGSPVVNINVNHATGAISISQ 531
BmAPN4          WYSCGLVRRFERAASEDHVFARDFPNVDVGEILDSWVQNPGSPVINVEFNTNNGVITLTQ 538
PxAPN4          ANPDDVFRGFARAVQEDGALT-QFTNVNITDFLSDWVYEPGYPVINVDINMNTGNIYIEQ 540
HAAPN1          AEPRHLFTALDAAATADNRLANYG-GMTIDRYFRSWSEKAGHPLLTVSIDHSSGRMTIIQ 536
HPAPN1          AEPRNLFTALDAAATADNSLTNYG-GMTIDRYFRSWSEKAGHPLLTVSIDHSSGRMTVIQ 538
HvAPN170kd      AEPADLFRALDAAAAADNALANYG-GITIDRYLRSWSEKAGHPLLTVSVDHSTGRMTIIQ 535
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MsAPN1          AEPYHLFSALDAAAAEDNALAAYR-GITIDAYFRTWSEKAGHPLLSVTVDHESGRMTLVQ 537
AjFbAPN         AEPEDLFSALDAAAVVDNALAGYG--ITIEEYMKTWSEQAGHPLLSVSVDHSTGNMVVTQ 543
TniAPN1         ATPDDLFDALQEAATLDGALTQYP-GATVKAYFETWTSKAGHPLLTVTVGND-GTMKVTQ 534
pxAPNA          SEPRHLFSALDTVAAADSALATYG-GVSIESYLKSWSEQAGHPLLTVVVNHETGLMQVTQ 543
EPSAPNA         VLPINLFEALQDAGTAAGALAEYGPDFSLIDYYKSWTEQRGHPVLEVSVNHQTGDMTIHQ 555
LdAPN1          AEPIDLFEYQEAAGIAAGALNYN--DFSFIEYYKSWTEQPGHPVLNVHVNHQTGDMTIYQ 560
BmAPN3          ALPIHLFQTLEVSARAAGALSAYGPDFSFVDYYKSWTEQSGHPVLNVQVNHQTGDMTIYQ 552
HAAPN2          VQPIDLFTALETAGNDAGALDAYGDHFDFVKYYESWTEQPGHPVLNVHINHQTGHMTIYQ 556
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HPAPN3          VQPIDLFTALQTAGHNAGALDAYGVEFNFVKYYESWTEQPGHPVLNVYINHRTGQMTIYQ 558
PxAPN3          AKPIDLFDALDLAGRADGAFNAYGSDFNFVEYYKSWTEQPGHPVLQVQINHRTGDMTIYQ 494
TniAPN3         ASPQDLFDALDDAGTNAGAFTAYGNDFSLSEYYRSWTEQPGHPVLFVYVDHASGEMTITQ 558
PIAPN           ALPVDLFQHLHDAGVSAGAISEYGPGFSVVDYYRTWTEQGGHPVLNVQVDQQTGRLTISQ 561
SlFbAPN         GYPDKLYEGLHQGVQRYNTLSSYP-NNNISDIMNSWISQAGHPVVNVTIDYSTEIVTLTQ 349
BmAPN2          AGPSDLYCALQQALDA----SDHSIPYSISNVMNRWVNQGGFPVLNVRKSAPNANSVFIS 515
MsAPN2          AAPSDLYVALQQALDE----SSHRIPKPISTIMTEWSTQGGFPVLTVRRTAPNADSVFVA 515
LdAPN2          AAPIHLYAALQRALTE----SNHSIPYALNTVMDRWVNQGGFPVLTVTRSAATAESLVVE 517
TniAPN2         AHPSDLYTSLQKALDA----STHSIPFPLATVMTRWTTQGGFPVLTVTRSAAAAQSIVVA 515
PxAPN1          AKPTDLFEAIQEVVDA----SDHSIRWRLSIIMNRWTQQGGFPVVTVRRSAPSAQSFVIT 519
                  .  :                .         *  : * *:: :          .
HPAPN2          QRY--VLSG-AVAPALWHIPLTWTQHGSLNFNSTRPSTVLTTEYT---NINAA-SGENFV 588
HvAPN110Kd      ERY--VLSG-AVAPALWQIPLTLTQNGSLNFENTRPSLVLTTQSQ---NINGA-SGDNFV 586
SlAPN           KRY--QLSG-EPPTQTWEIPLSWTEQKHLDFSSTKPRQLLNVTST---ALLSE-PGDNFV 589
TniAPN4         ERY--VVTGNQPSNPTWQIPLTWTHAGEMNFTNLRPKTVLSSPST---TIQGA-PGHNWV 585
AjMgAPN         QRY--IVTDTQRPNNIWQIPLTWTDQRSLDFTNTRPSRVLTTATD---SITTE-AGDYWV 585
BmAPN4          ERF--LLTG--SRDQLWRIPITWTDATTRNFSNTRPSLIMNTRTV---NIQGN-AGQHWV 590
PxAPN4          ERF--FTTT-GSSNQVWPLPLTYTSASSPDWSNTKASHVMTGKSY---NITKT-PAHEWT 593
HAAPN1          TRFERNSGVSTATDSLWDIPITWTRAGSIDFDNLKPTQFISGVLT---IIDRGTTGREWV 593
HPAPN1          TRFERNTGVSTATNSLWDIPITWTRAGAIDFNNLKPTQFITGVLT---IIDRGTTGREWV 595
HvAPN170kd      TRFERNSGVS-SSNSLWDIPITWTRARAIDFENLKPTQFVTGVLT---VIERRTTGREWV 591
BMAPN1          ERWERNTGVS-QFPSLWHIPITWTRAGAPEFEDLKPSQFISQQVT---SINRGTTGLEWV 595
MsAPN1          ARWERNTGVS-RFPGLWHIPITWTRAGAPDFENLKPSQVMTGQSL---VIDRGTRGQEWV 593
AjFbAPN         HRWNVNTGVS-AISSLWHVPITWTRAGAVDFDNLKPTQILSGTAT---SINRGSTGREWV 599
TniAPN1         ERFGLTPVTT--FEGTWQIPITWTSQGNVDFYDLKPSRILTGTST---TIDVGTNQRGWL 589
pxAPNA          ARWERNTGVS-GVPTLYQIPITWTRGGAPNFINLKPSQVLTQTTT---TINRESTGYEWV 599
EPSAPNA         RRFNINSGYS-DVTTNWYIPITFASASNIEFANTKPTHIISKAIT---IINRGSIGDEWV 611
LdAPN1          RRFNINTGYS-NINTNYIVPITFATASNPDFVNTKPTHIISKAIT---VINRGSVGDQWV 616
BmAPN3          RRFNINTGYS-NVNTNYIVPITFATARNPNFANTKPTHVLTKAVT---VINRGSVGDEWV 608
HAAPN2          RRFDIDTGYS-VQNRNYIVPITFTTGADPDFDNTKPSHVISKAVT---VIDRGVVGDVWT 612
HPAPN3          RRFDIDTGYS-VQNKNYIVPITFTTGANPNFDNTKPTHIISKAVT---VIDRGVVGDVWT 614
PxAPN3          RRFNINTGYS-SANTRYVIPITFTSRPNPNFENTKPSHILTKPVT---VINRDAYGDHWT 550
TniAPN3         RRFNINTGIS-SRNTNYVVPITFVTGANPDFSNPKPTHIMRKSSI---KINRQVTGDHWV 614
PIAPN           RRFDITNGYA-TPVTNWIVPINFATASNPNFNNTKATHIMTDGTM---FIDTN-ISNEWV 616
SlFbAPN         KRYYVNSSIS--SNETYKIPITYTTQRAPDFENTRPAFILEDQTLTF-MIFNISREHSWV 406
BmAPN2          QERYLTDRSL-TSTDRWHVPVNWVLSSNIDFSDTKPQGWIPPSFPATSIDIPGLANAEWF 574
MsAPN2          QERYLTDRSL-TSTDRWHVPVNWVISSNVNFSDTSPQAWILPTFPATAVDVPGLSNADWY 574
LdAPN2          QVRFLTDNTL-VSSDRWHVPINWVLSTDPDFSDTQPMEWIPPTFPARSIDIPGLSQADWY 576
TniAPN2         QERYLTDRSN-PSNDRWHVPINWVLSTDPDFSDTSPQAWVPPTFPARSFDIPGLSNAEWY 574
PxAPN1          QRRFLTDSTQ-ESNTVWNVPLNWVLSTDVNFNDTRPMAWLPPQLAAEAVQVPGLQNAEWF 578
                         : :*:. .     :: .  .   :                  :
HPAPN2          IFNIAQSGLYRVNYDTNNWQLLASYLKSNN-RQNIHKLNRAQIVNDILYFVRSNNINRTL 647
HvAPN110Kd      IFNNAQSGLYRVNYDTNNWQLLASYLKSNN-RENIHKLNRAQIVNDVLNFVRSNSINRTL 645
SlAPN           IFNIQQSGLYRVKYDENNWRALSSYLNSNN-RERIHKLNRAQIVNDVLHFIRSGDIDRTI 648
TniAPN4         IFNVAQSGLYRVNYDSHNWEMLGSYLKSTN-RQNIHKLNRAQIVNDLLFFIRSNDISKAL 644
AjMgAPN         LFNVAQSSLYRVNYNDRNWELLADYLKSSN-RERIHYLNRAQIVNDLLYFVRSGDVSAEV 644
BmAPN4          MLNIAQSGLYRVNYDDSTWQRIAAFLR-TN-REAVHKLNRAQIVNDVLFFIRAGKITTSR 648
PxAPN4          IFNVKQNGYYRVNYDTHNWELIAEALH-KD-VNAIHYLNRAQIVDDVFALMRSGRMTHAL 651
HAAPN1          IFNKQQTGFYRVNYDQITWGLITQALR-SNVRLSIHEYNRAQIVDDVMLLARAGIMTYSR 652
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BMAPN1          IFNKQEAGFYRVNYDDTNWALLTRALR-SSSRTAIHQLNRAQIVDDIFQLARANVMKYNR 654
MsAPN1          IFNKQVSGFYRVNYDNTTWGLITRALR-SANRTVIHELSRSQIVDDVFQLARSGVMSYQR 652
AjFbAPN         IFNKQQSGFYRVNYDSDTWALITQALRDSNSRTQIHEYNRAQIVDDVFILARAGVLTYTR 659
TniAPN1         IFNKQQTGFYRVDYDPITWAHNTMALRNAEVRKDIHVYNRAQIVDDVFLLARSERMTYRQ 649
pxAPNA          IFNKQESGFYRVNYDDTTWDLITAALK-SNERRVIHDLNRAQIVDDVFALARAGVMSYTK 658
EPSAPNA         IFNKQQTGYYRVNYDDYTWDLITAALRGPQ-RTQIHPYNRAQIVNDVFQFARAGVMSYTK 670
LdAPN1          IFNKQQTGYYRVNYDDYTWDLIAIALRGAD-RTVIHEYNRAQIVNDVFQFARSGLMSYSR 675
BmAPN3          IFNKQQTGFYRVNYDDYTWNLIVIALRGPQ-RTQIHEYNRAQIVNDVFQFARSGLMTYNR 667
HAAPN2          IFNIQQTGFYRVNYDDYTWDLIILALRGAD-REKIHEYNRAQIVNDVFQFARSGLMTYER 671
HPAPN3          IFNIQQTGFYRVNYDDYTWDLIIIALRGAD-RERIHEYNRAQIVNDVFQFARAGLMKYDR 673
PxAPN3          IFNIQQTGFYRVNYDDFTWDLITEALRGND-RAYIHEHNRAQIVNDVFQFARSGIMSYQR 609
TniAPN3         LFNTQQTGFYRVNYDDYTWDLIIQALRGPD-RTKIHEYNKAQIVNDVFQFARSGLMSYER 673
PIAPN           ILNIQQTAFYRVNYDDYTWNLIALALQSNESRAVIHEYNKAQIVNDIFQFARSGLMSYTR 676
SlFbAPN         IFNLQETGLYRVNYDDHSWSIIISALKGND-SAIIHPLNRAKIINDLFALVYADEVPFST 465
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BmAPN2          IFNKQQTGYYRVNYDPENWAALAKILQTNH--AVIHLLNRAQILDDSFNMARNGRLNYNL 632
MsAPN2          IFNKQQTGYYRVNYDVENWVALARVLNNSH--EIIHVLNRAQIVDDAFNLARNGRLHYKN 632
LdAPN2          IINKQQTGYYRVNYDVRNWEALTKALEEDH--EVIQVLNRGQILDDAFNLARNGRLNYEY 634
TniAPN2         IINKQQTGYYRVNYDLQNWIALGRVFNSNH--SLINVLNRAQVIDDAFNLARNGRLNYEH 632
PxAPN1          IVNKQQTGYYRVNYDPENWRALAKVLNDTH--EIIHLLNRAQLIDDSFNLARNGRLDYSL 636
                :.*    . ***.*:  .       :.       ::  .:.::::* : :     :
HPAPN2          GFEVLDFLKDETDYYVWNGALTQIDWILRRLEHLP--PAHAAFSEYILDLMNTVINHLGY 705
HvAPN110Kd      AFEVLDFLRDETDYYVWNGALTQIDWILRRLEHLP--AAHAAFSEYILDLMSTVINHLGY 703
SlAPN           GFEVIDFLRSETDYYVWNGALTQLDWIRRRLEHMP--RAHEAFTSYLRGLMNNVINHLGY 706
TniAPN4         GFGVLDFLGSETDYYVWAGALTQLDWIRRRLEHIP--QAHEAFTNYLLSLMNAVINHLGY 702
AjMgAPN         AFNVLDFLRYETNYYVWNGALAQIDFLRRRFEHLP--NAHTLFSNYILELMDTVIQHLGF 702
BmAPN4          AFDVLSFLENERDYYVWGGAITQLEWIRRRLEHLP--QAHEAFTAYTLDLLRNVINHLGY 706
PxAPN4          GFQILDFLKKDVSYYSWYPAISGFNWLRNRFLHLP--DVLAEFDEILFKYLDAVVTDLGY 709
HAAPN1          ALNILSFLKFEDQYAPWGARITGFNFALRRLAHDA--TALQKLRNEILDLSTAIVNRLGF 710
HPAPN1          ALNILSFLKFEDQYGPWAAVITGFNFALRRLAHDA--TALQKLKAEILDLSTAIVARLGY 712
HvAPN170kd      ALNILSFLKFEDQYAPWIAAITGFNFALRRLAHDT--VAHQNLRNEIIDLSSAIVARLGT 708
BMAPN1          AFNILSFLQFEDEYAPWLAAISGFNFLIRRLAHDS--TNAALLQKLILELSPAVVAKLGY 712
MsAPN1          ALNILSYLRFEDAYAPWLSAISGFNWVIRRFAHDA--ANLQTLQNQIIGLSEAVVARLGF 710
AjFbAPN         AFNILSFLEFEDQYAPWDAAITGFNFSRRRLAHNT--ESLEQLHALIYKLSEAVTRRLGF 717
TniAPN1         AFNILSFLEFEDEYAPWIAAIAGFNFAVRRLAHDE--AALAKLQAHIHSTAAAVVNRLGY 707
pxAPNA          AMDILSFLEFEEQYAPWVAAISGFNWLLRRLAHDP--QTLQSFQQEIIKLSSAVTARLGF 716
EPSAPNA         AFNILSFLEFETEYAPWVAAMTGFTWLRNRLTGT---QFLNPLEGLIADWATVVMADLTY 727
LdAPN1          ALNILSFLQYETDYAPWLAALTGFSWLRNRLASESTVTQLNQVNERLELWSRAVITSLTY 735
BmAPN3          AFNILSFLENETEYAPWVAAITGFNWIRNRLVGT---AHLTTLNNLIARWSSNLMNQLTY 724
HAAPN2          ALNILSYLENETDYAPWVAAITGFNWLRNRLVGKP---QLAELNAKIVQWSSKVMSELTY 728
HPAPN3          ALNILSYLENETDYAPWVAAMTGFNWLRNRLVGKP---QLAELNARIVQWASKVMSELTY 730
PxAPN3          AMNILSFLEFETDYAPWVAAITGFNWVRARLHGTP---ELAYLDAQIVRWAQAAINKLTY 666
TniAPN3         AFNILSFLQFETEYAPWVAALTGFSWIRNRLANNPT--ELAQINTRIQQWSAEIMGQLGY 731
PIAPN           ALSLLSFLQYETDYAPWVAAITGFNWLRNRFAGTS---LQETFETLIATWATTVMADVTY 733
SlFbAPN         LSSALDYLPLEPEYTGWFAALRGFSKIWHFYLGDN--EVLPHVEHFILQHLESGISRLGY 523
BmAPN2          PFEISRYLINEKDYIPWAAINPAFNYLDIVLTGSS---VYNLFREYLLTLTAPLYDEIGW 689
MsAPN2          AFEISRYLEMEKDYIPWAAANPAFNYLDIVLSGAN---SYNLYRYYLLNLTAPMFEDLGF 689
LdAPN2          AFNLSSYLVQEKDYIPWASVNPAFNYLAMVLSESE---VFEEYQYYLLNLTAPLYEELGF 691
TniAPN2         AFEISRYLEKEKNYIPWGAANPAFTYLDGVLSGTG---VYNLFQQYLLALSAPLFEELGF 689
PxAPN1          AFDLSRYLVQERDYIPWAAANAAFNYLNSVLSGSS---VHPLFQEYLLFLTAPLYQRLGF 693
                      :*  :  *  *      :                                 :
HPAPN2          NER-STDSTSTILNRMQIMNYACNLGHSGCVSDSLDKWRQHRANVSNLVPVNLRRHVYCV 764
HvAPN110Kd      NEQ-STDSTSTILNRMQIMNYACNLGHSGCIADSLDKWRQHRENPNNLVPVNLRRYVYCV 762
SlAPN           NEG-PNDSTSTILNRIQILNYACNIGHSGCVSDSLQKWNDYKEN-NEPVPVNLRRHVYCT 764
TniAPN4         NEL-ATDSTSTILNRMQIMNFACNLGHAGCIADSLNKWNSFKNN-NETVPKNLRRYVYCT 760
AjMgAPN         EER-ATDSTSTILNRMQILNYACNLGHSGCVLDSQNKWRELREN-NVAVPVNLRRYVYCI 760
BmAPN4          NER-ATDSTSTILNRMQILNLACNLGHSGCISDSLQKWRQFRNNPTNLVPVNSRRYVYCV 765
PxAPN4          EAVDSNEPLTRTLNRFYVMSFACNIGHEGCVQHATQKYTEMVNG--GKVDPNIRRHVYCS 767
HAAPN1          SEPAVS-NFMDDLLRMNVMTFLCDIGHQGCITAARTSFATWKN--GGVVPPNMRPWVYCN 767
HPAPN1          NEPTVS-NFMDDLLRMNVMTFLCDVGHQGCINAATTSFTTWKN--GGVVPPNMRQWVFCN 769
HvAPN170kd      ASQLQG-TFMDNLLRMYVMTFLCDIGHQGCINAAVTNFAAWKN--GGTVPANMRPWVFCS 765
BMAPN1          LEPENG-SYMTDLQRMYVMEFLCNVGHEECNNFGTQAFRRWST--GTFIPANMRPWVYCA 769
MsAPN1          TEVSGG-TYMTDLQRLHVMQFLCNVGHQQCIDAGRQNFLNWRN--GSFIPANMRPWVYCT 767
AjFbAPN         AEIEGE-SYMDGLLRMYVNTFLCNVGHEECVQAGRTAFANWKNS-GTFIPANMRPWVYCT 775
TniAPN1         EDKGGDDNFMDDLLRMNLMQFLCNVNHEKCIEEGVKSFQSWKVNEAFHIPANHRPWVYCA 767
pxAPNA          AEVPGE-PFMDGLLRMYVLDFLCNVGHEQCVAQAVTNFRNLRN--GTFLPANMRPWVYCA 773
EPSAPNA         YPTANPEDFMRSYLRYQLAPIMCQLNRDNCRAEAVAQFNALRDN-NVEVPADSRNWVYCN 786
LdAPN1          RPITG-EHFLRAHLRSQLAPVLCNIGVAGCLEEARNQLNDLRVS-NIAVPVDNRNWVYCS 793
BmAPN3          SPIPN-ESFMRSYLRYQLAPLLCNINVAACRTAATTQFQALRVN-GQEVPVDNRNWVYCN 782
HAAPN2          MPIEG-EPFMRSYLRWQLAPVMCNLNVPACRAGAKVIFDNLRLY-QHEVPVDSRSWVYCN 786
HPAPN3          APIEG-EDFMRSYLRWQLAPVMCNLNVPACRAGASAIFNDLRVF-GHEVPVDSRNWVYCN 788
PxAPN3          YPLSD-DDFMRSYLRYQLAPMLCNLRRSRLPPMLPTHSLMPESH-GSEVPPDSRNWVYCN 724
TniAPN3         TPIAG-EEFMRSYLRYQMAPVMCNIGVSACLTAANTQFNNLRQN-NVEVPVDNRNWVYCN 789
PIAPN           YPTEG-ESFMQSYKRMQLAPTMCAIGVPECIEAAEIQFNTLMNG-VAEVPVDSRSWVYCN 791
SlFbAPN         EER-STDSLEDQRNRMQILEFACKLEHTGCVERTVELFRALRQN-GTEVAPSLRPVVYCT 581
BmAPN2          EATAN-EEHVMAYHRNIILDINCRLGNQRCVTRAQELLEQFRNNPTQRLNPDLQNTVYCS 748
MsAPN2          DVKSG-EEFVTPYHRNIILDINCRFGNQRCISRAQEILQAFKNNPNQRPNPDIQTLVYCS 748
LdAPN2          NAAAG-EEHVTPYHRNIILDINCRHGNPACINTAQQLLENFRNNPSQRLNPDIQTLVYCS 750
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TniAPN2          LAGD--EEFVTAYHRNIILDLNCRHGNPNCINRAQELLEGFREG-TTQLNADIQTLVFCS 746
PxAPN1           NAATG-EEHVTPFHRNIILNINCLHGNEDCVSTAETLLQNFRDNPTQTLNPDIQTTVFCS 752
                             *  :    *                          . :  *:*
HPAPN2           GLREGNETDYNYLYSVYNSSQNTADMVVILR--ALACTKHQPSLEHYLQQSMYNDKVR-- 820
HvAPN110Kd       GLREGNETDYSYLFSVYNSSENTADMVVILR--ALACTKHQPSLEHYLQESMYNDKIR-- 818
SlAPN            GLREGDRSDYDFLFNAYNSSENAADMVIMLR--ALACTKDLDALGHYLQESMYNDKIR-- 820
TniAPN4          GLREGNATDYEFLLEKYNSGENTADMVVMLR--ALACSRDQTRIVHYLQESMNNDKIR-- 816
AjMgAPN          GIREGDNTDYNFLFNLYESSENTADMVIMLR--ALACTKDEALLNSYLGQSLTNRKIR-- 816
BmAPN4           GVRQGNSSDYNFLFERYNASQNTADMVVMLR--ALACTRDTNSLQHYMFQSMHNDRIR-- 821
PxAPN4           GLRQGSLAQWKVLQVQYLASNNQADEVVKLR--GLGCTSDDQAVKEYLEMVLT-DAVK-- 822
HAAPN1           GVRYGDQSDFTHLWTSTNNLTLLTIKLVHAVRLCLGCTLKPGQLEIFLNDIVSGG-M--T 824
HPAPN1           GLRYGDQSDFTYLWSRYITSDVANDKLVMLS--AAGCTRNQASLNIFLNAIVSGSDD--I 825
HvAPN170kd       GLRYGDASDFTYLWSRYTSSNVANDQLVMLS--AAGCTLNQASLNLFLNAIVSGSDD--I 821
BMAPN1           GLRHGTAEDFNFFWNRYLQEDLSSEKVVMLN--VAGCTTDQASLNRFLDAIVSGNDD--I 825
MsAPN1           GLRYGSAEDFNYFWNRYIVEDLSNEKVVMLE--AAGCTRDQASLEKFLNAIVSGNDD--V 823
AjFbAPN          GLRYGDASDFDFFWQQYLATDLASEQVVKLQ--AAGCTTDEASLGRYLDAITGGADDYQI 833
TniAPN1          GLRAGDASDFDVFWSRYLKEDLASEKVVMVT--AAGCTGDEASLRKFLNAIVDDKED--I 823
pxAPNA           GLRAGTPEDFRFLWSRFESEDLANEIVVLLE--KLGCTKDAASLNVLLNSVVEDNEL--V 829
EPSAPNA          ALREGTADVFTYLRNRFINHNVYTEKILLLQ--VLGCTPHQTALYQFLNDIVLENEV--V 842
LdAPN1           GLRQGGVDEFNYLWNRFLTHNVYTEKILLLQ--NLGCTANAASLNTFLTNIVSENFV--V 849
BmAPN3           ALRDGTEADFNFLYQRFQSHDVYTEKIQILW--VLGCTPHANSLNTLLNAIVQDNFI--I 838
HAAPN2           ALRDGGADEFNHLYNRFKGHNVYTEKILILQ--TLGCTSHAVSLTTLLNDIVTPNNI--I 842
HPAPN3           ALRDGGAQEFDFLYNRFKGHNVYTEKILILQ--TLGCTSHAASLTTLLNDIVTPNNI--I 844
PxAPN3           ALRRGDAEDFNFLWKRFLTDNVYTEKILLLQ--TLGCTSHDASLATLLYAIVLPNNI--I 780
TniAPN3          ALRNGNQEDYNFLRTRFFQHNVYTEKILILG--ILGCTTHEASLNSFMNEIVSDNFI--I 845
PIAPN            ALRRGDDSHFNFLWQRFQSHHVYTEKILLLS--VLGCTNDVASLTVFLDAILEENYL--I 847
SlFbAPN          GLRHGSAEDYDFLWNRMVNTNLANEVWVIGD--ALGCTSDESRIRSYLVSMTVENSP--I 637
BmAPN2           GLRGGDRDNFNFLWEQYLATSDSSGQNILRN--ALGCSSNPELRPFYMNQVFDAHSP--V 804
MsAPN2           SLRAGNVENFNFLWNMYLGTSDSSEQSILLS--ALGCTSNAERRNFYLNQIIDDNSA--V 804
LdAPN2           GLRGGDADNFNFLWDMYRSTSDPSEQSILLN--ALGCTSNEELRSFYLNQVIAEDSQ--V 806
TniAPN2          GLRGGSVENFNFLWNRYVDSSDSSEQSILLN--ALGCTSNVERRAFYLGQVIDDNSP--V 802
PxAPN1           GLRGGDVDNFNFLWARYTATQDSSEQSILLN--ALGCTSNADRRDFLFSQVIASDSQ--V 808
                 .:* *    :  :                    .*: .       :
HPAPN2           -IHDRTNAFSFALQGNPENLPIVLNFLYNNFAAIRETYGGVARLNLCINAIPAFLTDYQI 879
HvAPN110Kd       -IHDRTNAFSFALQGNPENLPIVLNFLYNNFAAIRETYGGVARLNLCINAIPAFLTDYQT 877
SlAPN            -IHDRTNAFSFALQGNLENVQFVSLFLQNNYDTIRTTYGGEARLTLCVNAVAAFLNTFPA 879
TniAPN4          -IHDRTNAWAYALQGNPENLPTVLNFFYNNFAEIRTKYGGEIRLNTCISSFTAHLTSHER 875
AjMgAPN          -IHDRTNAWSYALQGNPENLPVVLNYLYQNFEQIRTDYGGPARLTLAISALSTYLTDFNT 875
BmAPN4           -IHDRTNAFSYALQGNRENLPIVLNFLYQNFAAIRTSYGGEARLVTAVNAISGFLTDFAI 880
PxAPN4           -AQDRVNAFTYLYMGDRGNAQKALQFIKTNHNQIREAVVGSVRFNTVLANLAAY-TDEEG 880
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HPAPN1           RPQDHSAAIASAVRSNEENTMRVFTWLQANVQQTIATLGS---VSPILNEITVRLLNEAQ 882
HvAPN170kd       RPQDYSSAIASAVRSNEENTMRVFTWLQSNVQQTTTTLGS---VSPILNEITARLLNEAQ 878
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AjFbAPN          RDQDIATALSSAITANEVNTMRAFNWLTNNVERTTLALGS---IQTPLSTITQRLLNTEQ 890
TniAPN1          RPQDYSVALNSAIASNEVNTLRAFEWLKTNVDQTVKTLGS---INSPLSTISSRLLNDAQ 880
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EPSAPNA          RPQDYTVAFNSAVTGNEANTQLVFTWVQNNYALVANAFSD---VAVPLSYISSRLRTEPE 899
LdAPN1           RPQDYTTALNSAVSGNEGNTNIVFTYIRTYPDLVASAFGDS--LSTPLLYVASRLRSNQR 907
BmAPN3           RPQDYTNAFNNAVSGNEGNTQIAFRFIQNNLAAVTAAFQS---VATPLSYVSSRLRTEAE 895
HAAPN2           RPQDYTTAFSTAVSGNEENTLFVLNYIQNNLETVLKAFSS---PRTPLSYIAARLRTVED 899
HPAPN3           RPQDYTTAFSTAVSGNEENTLFVFNYVQNNLQAVLNAFAN---PRTPLSYIAARLRTVEE 901
PxAPN3           RRQDYTTAFNTAVSGNEENTQKAFQFIQQNLPAVSLAFGS---PVTPLSYVSARLRTEAE 837
TniAPN3          RPQDYNSAFNSAVTGNEINTQRAFRFVQDNLPRIISAFGS---PVTPLSYISSRLRTNAE 902
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SlFbAPN          RTQDLTVPLASVLRAYG-NLHIVMDSLKSNYTLWSSIYPS---MDTVLNTVASALHTEAD 693
BmAPN2           GGQDRHTILVSVINSSPENMDAALEFVIENFHRIQPRVQGLTGTTNILNAFARRLTTETH 864
MsAPN2           REQDRHSIAVSVINSSPEGMNVALDFVVENFHRIQPRVQALTGTTNILNTFARRLTTSAH 864
LdAPN2           REQDRHTIVVSVTNASPENMNVALDFVIENFHLIQPRVQGLTGTTNILNAFARRLTSQAH 866
TniAPN2          REQDRHTVIVSVTNASPESTEAALDFVVANFAEIQPRVQGLTGTTNILNAFARRLTTEAH 862
PxAPN1           REQDRHSVLVSAINSGPDNMNAALDFVLENFANIQPNVQGLTGTTNILNAFARTLTTQEH 868
                   :*          .   .   .   .                    :   .
HPAPN2           IAQFRTWVESNQL--ELAGSVNAGNNVVSTALANLDWGNDAAVEIVNFLNAR-------- 929
HvAPN110Kd       ITQFQSWVYANQL--ALVGSFNNGVSVVNTALDNLTWGNGAAVEIVNFLNYK-------- 927
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SlAPN          ITQFQTWAYGSQV--GLAGSFNAAVNVVNSAMANLDWGSNNALEVFNFVSVR-------- 929
TniAPN4        IEEFHSFVYQNQI--ALEGSFSVGDSVARSANANMLWGNANAPEIYEFLNTK-------- 925
AjMgAPN        IQEYQAWAYENQE--ELDESFGTAVAVVNAAINNLVWGNANAPEIYNFLLERT------- 926
BmAPN4         IREFQSWVYANQL--ALGTAFSTGVSVINSAISNLEWGNAEATDIYNFLLAR-------- 930
PxAPN4         LQDMESWLEENKD--SIP-EYSVGVSAVASARANMAWGTQNADSILKAARGS-------- 929
HAAPN1         IT----------------AAHTSATNGIATSRSNIQWYTQRVAEFNVYFETGYVEENFAD 925
HPAPN1         ITQVQNWLDA--S-VIGNAAYTSAINGIATSRANIQWYTQRVPEFNAYFETGYVEENFSD 939
HvAPN170kd     ITQVQTWVNANQN-AIGSAAHASAISGIAGARANIEWYNRRVPEFNAYFETGYVEENFFE 937
BMAPN1         INEVSNWLTANQN-TLG-ATYSTALRAIETTRSNLVWSQQRISEFTNYFESGYVEDVIEE 940
MsAPN1         MTQVQTWLDANRN-TIG-AAYNTGVNGIATSRANLQWSANRMSEFLRFFETGFVDDVPSE 938
AjFbAPN        INTVSAWLQANSA-SLGTDIYNVGLSGIATSQNNIAWYNQRISEYNSYFENGYIDETFED 949
TniAPN1        INTVETWLNENAE-IIGASAVAAGRSGIATSKSNIEWLTKRKVEFEDYFETGFEDPLAPP 939
pxAPNA         IVEFETWLNANQN-QIGAASYATGMSGARAARDNLAWSARRLPEIQEYLEYGYTPPPSPS 945
EPSAPNA        VNAYLQWVNSNAA--VLGEAYEDVVDGAEDTRQSIQWTTTIVADVNDYLENGFQEIEPST 957
LdAPN1         IDEFISWAESNQE--SLGNAYTTAINEANSARNSIIWAESVQADITNYIETNGDPITTST 965
BmAPN3         IVSFQNWATQNQV--ALGDAYQAVFRGAETSRESIAWASLVQNDMNSYFVTGDTVYEAST 953
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PxAPN3         VNAFQKWADDNKS--LLEGSYSAVYNGAELTRESLKLAPIIANDLDDFFETG--VLPITT 893
TniAPN3        VQAFQTWATANQA--TLGTHYNSVLQGVQNSVESIQWVSEVQEDLNSYFVNGDAPISTTT 960
PIAPN          IEEFQLWARENRN--ALGNAYNSVYNGAESARASIKWTNEILDDVLNFIENGGDEIEVTT 965
SlFbAPN        FNEFETFLSECTV--CTEATKTSGRNALVQARAATAWANSHKADILKVIRSN-------- 743
BmAPN2         AERINQLISRHQA-ILTAGEQASISAIREHIAASIAWGKDNAAVVEDWLEDNYGEPKPEE 923
MsAPN2         NEKIDELVRRHES-IFSAGERASIAAIRENIAASIAWSNSNAGIVENWLKENYGPP---- 919
LdAPN2         SDKVDELYSRHES-IFTAGELASIAGIRENIAASITWSNENANTVEDWLFENYG------ 919
TniAPN2        LEKVRDFEKRYED-IFSAGEIAAIAGINENIAASVTWTKDNAAVVEAWLRSNYGS----- 916
PxAPN1         ANKIDEFSNKYAN-VFTAGEMASVAAIKENIAASITWNSQNAATVEAWLRKNFGT----- 922

HPAPN2         ----------------------------------------------------SGSTTIL 936
HvAPN110Kd     ----------------------------------------------------SASPSIL 934
SlAPN          ----------------------------------------------------SSSPTIF 936
TniAPN4        ----------------------------------------------------SGAATIV 932
AjMgAPN        ----------------------------------------------------VSSSTTFV 934
BmAPN4         ----------------------------------------------------SSSTTVT 937
PxAPN4         ----------------------------------------------------AAMVL 934
HAAPN1         PTTTSTTTTTTTTTTTTAAPTTTTTTEAPTTTTTTTTEAPTTTTTEATTTPVPGSANIAT 985
HPAPN1         TTTTTTTTTTTTTTTPAPTTTTTTTTEASTTPSTTP---TTTTTTEPTTTPATDSANIAT 996
HvAPN170kd     TTTTTTTTTTTTTTTTTTEAPTTTTTEAPTTTSTTTTT--TPAPTTEATTTPVPDSANIAT 995
BMAPN1         ITEAPP-----TAPPTAPP----------------------TEAPAVTPAPDSANVAA 971
MsAPN1         ATTVAPPAETTVTPSTFPP----------------------TVAPATTPAPGSGNIAA 974
AjFbAPN        EQTTPA------ATTEAPT----------------------TEADAGT-TPDSASIAT 978
TniAPN1        VTETEA-------STSSP----------------------TAAPSTTEAPASASTAA 967
pxAPNA         P-----------PPTDAPP--------------------VETTPDMTPPQPDAASITT 972
EPSAPNA        PAPVTPEVPPVTE--PRPDL--------------------TRPDTP-ELPGSAVTSF 991
LdAPN1         EAPVSPTTVSTTQQVPIVEP--------------------VTPSLP-EPPSSASTAF 1001
BmAPN3         AANVITSPSTTVTPPNLVEP--------------------ATPSLP-VPDAAPVSTF 989
HAAPN2         STTTTTVAPTTVTQPPITEP--------------------STPSLPVPVTDGAMTSF 996
HPAPN3         STTTTTPAPTTISLPAISEP--------------------STPSLP-EVTDGAMTSF 997
PxAPN3         STPEPSTTVPTSARPDLQQP--------------------TTPELP----ASAVSAM 926
TniAPN3        STQAPVTVQVTSTRVPFVNP--------------------GTPTLP--VADSAATSL 995
PIAPN          ATPGTETPVDTETPPDIDEP--------------------TTPDIP--EPDSAVTSA 1000
SlFbAPN        ----------------------------------------------------SIMAA 748
BmAPN2         P---------------------------------------------------SAAHSTT 931
MsAPN2         ----------------------------------------------------SGAKSLT 926
LdAPN2         ----------------------------------------------------SGVAALT 926
TniAPN2        ----------------------------------------------------SGAPMLV 923
PxAPN1         ----------------------------------------------------DGASTVS 929

HPAPN2         ASSILILAAMLLQMFH---------- 952
HvAPN110Kd     ASSILILAAMLVQMFR---------- 950
SlAPN          ASSLLILAAMLIQLYR---------- 952
TniAPN4        ASSILLLAASLVQMLR---------- 948
AjMgAPN        ASTILMLAAMFTHLLR---------- 950
BmAPN4         SSFILMITALVVKMFH---------- 953
PxAPN4         PTALLLIASLFALLM----------- 949
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HAAPN1          LSIVTMIVTLVVNMA---------- 1000
HPAPN1          LSIVTLIVTLVVNMA---------- 1011
HvAPN170kd      LSIVTLIVTLVVNMA---------- 1010
BMAPN1          LSFITLIITLAVNLA---------- 986
MsAPN1          LSVVSLLVTLAINMVA--------- 990
AjFbAPN         LSFVTLFVTLALNLAXKLLSYIYDSL 1004
TniAPN1         LSVVAMLVTLAVNMV---------- 982
pxAPNA          LSAITLALTFAINFVV--------- 988
EPSAPNA         LSMTLLGLAALFNFVL--------- 1007
LdAPN1          ISVVVMVLAVIGNIVF--------- 1017
BmAPN3          LSVAVVALVAVVNLIM--------- 1005
HAAPN2          ASLFIISLGAILHLIL--------- 1012
HPAPN3          ASLFIISLGAILHLIL--------- 1013
PxAPN3          ISIFLLALAAFAHIIL--------- 942
TniAPN3         VSICLLAVAAIANIIL--------- 1011
PIAPN           LSVVAIAVAAIVNLAL--------- 1016
SlFbAPN         PSVIVMIIGLAILIFKEL-------- 766
BmAPN2          AGFIVLLSAFVAFFNIH--------- 948
MsAPN2          AGLLVLISLFVAIFNH---------- 942
LdAPN2          SSLIILISVFVTIFNH---------- 942
TniAPN2         SSLIVLISAFVTIFNY---------- 939
PxAPN1          ASITIIISAMVAIYNIL--------- 946
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Fig. 3.27: Comparison of *A. janata* and *S. litura* APN deduced protein sequence with APN from other inect species by multiple sequence alignment with CLUSTAL W (1.82).

Based on maximum amino acid sequence homology among the reported insect aminopeptidases, a phylogenetic tree was constructed. As seen in figure 3.28 *AjFbAPN* and *AjMgAPN* belong to different classes. *AjFbAPN* is located in a separate class along with *P. xylostella* APNA. This result is in contrast with the report by Nakanishi et al, (2002) that insect APNs reported till date belong to four major classes. *AjFbAPN* showed a maximum homology of 70% to *L. dispar* APN N4 (GenBank® accession number AF317620) and 64% to *B. mori* (AF084257), where as the *AjMgAPN* manifested highest homology of 73% to *L. dispar* N3 (AF317619) followed by 68% similarity to *H. armigera* (AF535165), which belong to entirely different classes of aminopeptidases.

Phylogenetic analysis:

A detailed phylogenetic analysis was carried out using APN sequences obtained from *A. janata*. The amino acid sequence comparison by multiple sequence alignment, revealed only 33% homology between *AjFbAPN* and *AjMgAPN* implicating that they are significantly different from each other (Fig. 3.28). The *SlFbAPN* showed only 33% homology to *A. janata* APNs as well as to other insets. Because of its partial sequence it was not included in the phylogram.

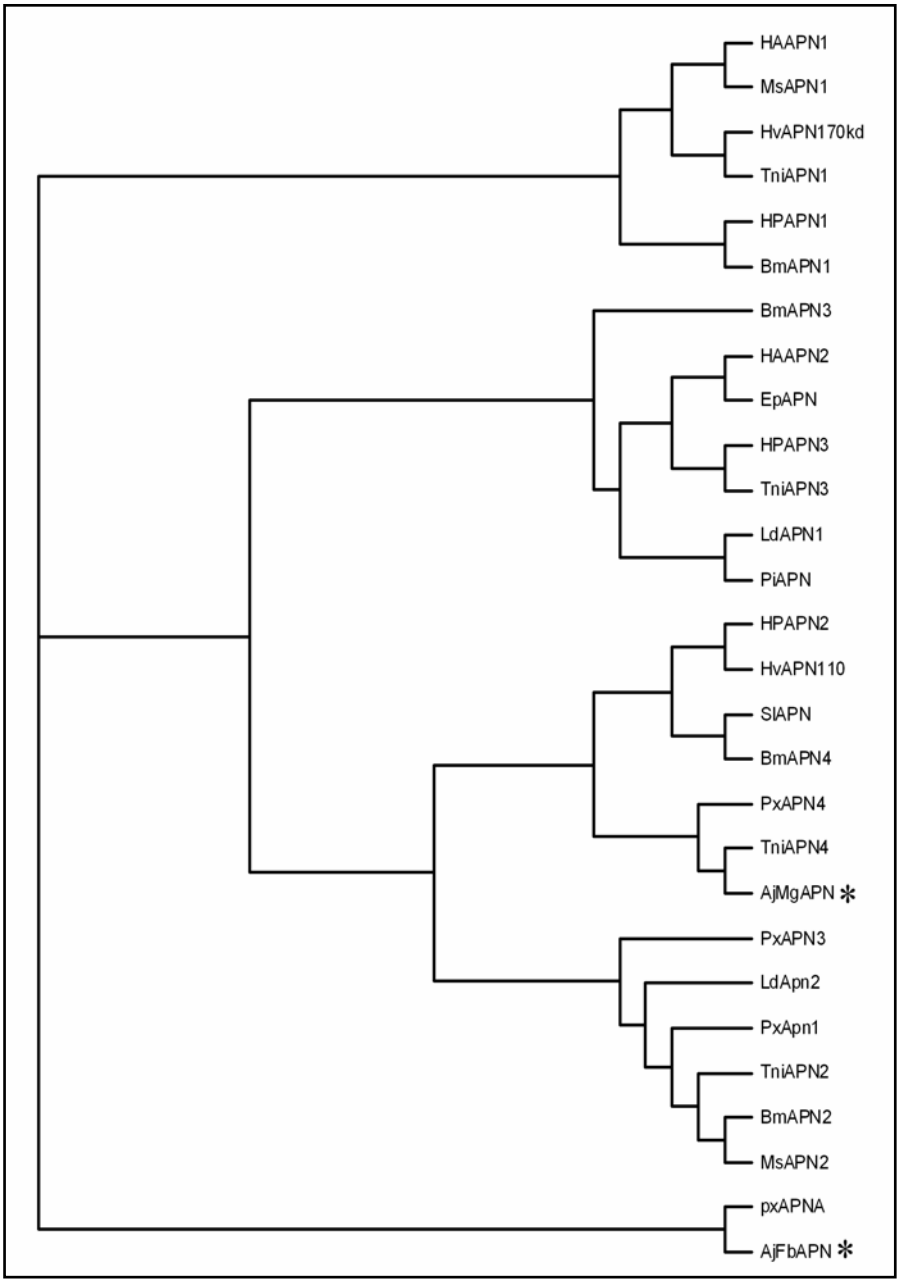

Fig. 3.28: Phylogenetic relationships of *AjFbAPN,* and *AjMgAPN* with other insect aminopeptidase**s**. The amino acid sequences of these two APNs were aligned to those of other APN family members in order to deduce their phylogenetic relationship. The tree was calculated with the clustalX 1.83 program. A*jFbAPN, A. janata* fat body APN; *AjMgAPN, A. janata* midgut APN; PxAPN1, *P xylostella*APN1; PxAPNA, *P. xylostella* APNA; z PxAPN3, P. *xylostella* APN 3; Ld APN1, *L. dispar* APN1; Ld APN2 *L. dispar* APN2; MsAPN1, *M. sexta* APN1; MsAPN2, *M. sexta* APN2 ; BmAPN1, *B. mori* APN1; BmAPN2, *B. mori* APN2; HvAPN170, *H. virescens* 170kDa APN; HvAPN120, *H. virescens* 120kDa APN;

HpAPN1, *H. punctigera* APN1; HpAPN2, *H. punctigera* APN2; HpAPN3, *H. punctigera* APN3; PiAPN1, *P. interpunctella* APN1; HaAPN1, *H. armigera* APN1; HaAPN2, *H. armigera* APN2; SlAPN1, *S. litura* APN1; EpAPN1, *E. postvittana* APN1; BmAPN3, *B. mori* APN3; BmAPN4, *B. mor*i APN4, TniAPN1, *Trichoplusia ni* APN1; TniAPN2, *Trichoplusia ni* APN2; TniAPN3, *Trichoplusia ni* APN3; TniAPN4, *Trichoplusia ni* APN4.

Discussion:

Fat body is a major insect metabolic organ performing wide variety of functions including protein metabolism and storage (Burmester and Scheller, 1999). APNs, along with a battery of other proteases present in the fat body are responsible for the catabolism of larval storage proteins, hexamerins that are sequestered in the fat body at the onset of metamorphosis (Hall, 1988; Haunerland, 1996; Siviter et al, 2002). Earlier, studies by Cerestiaens et al, (2001) demonstrated considerable amount of toxic effect of Cry toxins when they were injected into the hemocoel of *L. dispar* larvae. Furthermore, the findings of Cry binding and pore-forming ability of Cry toxins on *in vitro* cultured fat body cells strongly suggest the presence of Cry binding proteins in the plasma membrane of the fat body cells (Cheon et al, 1997). Our present investigation provides sufficient experimental proof to support this assumption and elucidates the role of midgut/fat body APNs in pathogenesis of the Cry toxins.

To the best of our knowledge, all the insect aminopeptidases registered till date in the database were cloned from the midgut. For the first time we have reported an APN from the larval fat body of *A. janata*, which is significantly different from the midgut APN of the same species. We have cloned and carried out molecular characterization of these two APN cDNAs. The derived amino acid sequences of the fat body (*AjFbAPN*) and midgut (*AjMgAPN*) specific APN cDNAs contain all the canonical features of the M_1 family of zinc-dependent ectopeptidases. Their comparison with other members of the APN super family revealed strong homology to the reported insect APNs. Dendrogram analysis of APN isoforms from the insect midgut categorized them as four distinct classes and isoforms of all four classes are been reported from the midgut of *B. mori, P. xylostella* (Nakanishi et al., 2002) and *T. ni* (Achberger et al, 1982; Nakanishi et al, 2002; Wang et al, 2005). Herrero et al, (2005) proposed the presence five classes of APNs in lepidopteran midgut. In the present study, the phylogenetic analysis revealed that *AjFbAPN* and *AjMgAPN* belong to different classes of APNs in the phylogram and *AjFbAPN* is being from a different tissue it is in a

class five. Phylogenetic analysis results strongly suggest the diversion of *A. janata* APN genes from a single ancestral gene. The multiple APN isoforms existing in insects are believed to have arisen as a consequence of gene duplication events (Chang et al, 1999). In corroboration with earlier reports, our Southern analysis with the partial *AjFbAPN* 550 probe clearly demonstrated that APN gene exists in multiple copy number, which also supports the notion that APNs in lepidopteran insects, like other serine proteases, belong to multigene families. Detection of a single APN transcript in the fat body and two different sized transcripts in the midgut of *A. janata* indicates the expression of a unique APN in the fat body, which is smaller in size from the midgut APN transcripts. The sizes of detected APN mRNAs in both of the tissues were however, slightly larger than their corresponding cDNAs cloned. The likely explanation for this observation could be that less abundant, but post transcriptionally modified shorter mRNAs have given rise to the shorter oligo-d(T) primed cDNAs. However the fat body APN cDNA cloned from *S. litura* did not pickup transcript from the larval midgut and this might be due to very low homology (33%), it exhibited with other reported midgut APNs.

We identified presence of a fat body specific 110-120 kDa membrane protein using antiFbAPN antisera, which we assume could be the fat body APN encoded by the 3122 bp cDNA we cloned from fat body; the APN cDNA has an open reading frame (ORF) of 3015 bp, which encodes a putative 1004 amino acids protein with a theoretical molecular mass of 110-120 kDa and is in agreement with the size of the fat body membrane protein. Interestingly in the midgut, antiFbAPN antisera recognized an ~170 kDa protein, which was however, not detected by antiMgAPN antisera. Rather, antiMgAPN antisera detected an 110-120 kDa protein in the midgut. Earlier, Oltean et al, (1999) reported N-terminal sequencing based cloning of a ~170 kDa Cry1Ac receptor from *H. virescence* larval midgut. This clone encoded a protein with a deduced molecular mass of only 112.21 kDa, but possessed a stretch of polythreonine residues at the C-terminus, which they assumed to most likely undergo glycosylation to give rise to ~170 kDa protein. However, the *AjFbAPN* cDNA did not show the presence of any polythreonine stretch and therefore suggests that *AjFbAPN* might be different from the ~170 kDa APNs cloned from the midgut of other insects.

As larval fat body is known to be homologous in function to the vertebrate liver and is an essential organ for protein metabolism and storage (Burmester and Scheller, 1999), presence of APNs in the fat body indicates significant physiological role. Biochemically

characterized APNs from *Drosophila* were shown to have high activities during metamorphosis and were inducible by insect steroid 20-hydroxyecdysone (Hall, 1988; Siviter et al, 2002). The soluble and membrane bound APNs are also known to play a role in neuropeptide inactivation, which are present in insect hemolymph. For example, APN in the hemolymph of *D. punctata* participate in the inactivation of allatostatin (Garside et al, 1997; Masler and Kovaleva, 1997). Although APNs perform multiple physiological activities, they were mostly reported from the midgut, with a few exceptions. Recently, Wang et al, (2005) demonstrated APN synthesis in the Malpighian tubules of *T. ni,* but failed to detect any APN in the fat body by using gene specific primers designed based on midgut APN isoforms. It appears that our success to isolate an APN from the fat body of *A. janata* primarily relied on the usage of highly selective degenerate primer pairs.

The APNs are the most widely characterized Bt toxin receptors in the larval midgut and as we report here of their presence in a vital metabolic organ like fat body, it may have far fetched implications. In our present investigation, the lethal effect of Cry1A toxins on *A. janata* larvae upon intrahemocoelic injection was manifested as lower food intake eventually leading to reduction in the body weight of larvae, while higher toxin concentration led to larval mortality. Previously, the toxicity/mortality effect after a hemocoelic Bt injection was observed and reported on the larvae of *L. dispar* (Cerestiaens et al, 2001). The toxicity obtained from hemocoelic delivery opens up the possibility of toxin interaction with various organs present in the body cavity, which could be additional targets for toxins. Cry toxins, which are not effective against *Lygus hesperus* when fed through artificial diet, small amount of these toxins were detected in the cellular outlines within haemolymph and fat body (Brandt et al, 2004). Further, Cheon et al, (1997) demonstrated the pore forming ability of Cry toxins on *in vitro* cultured fat body cells using transmission electron microscopy. Colorado potato beetles, which fed on *cry3A* potato transgenic plants not only laid less number of eggs but also showed dramatically reduced fat body structure and ovaries when compared to beetles fed on non transgenic plants (Perlak et al, 1993). Lepidopteran insect *Spodoptera littoralis*, when fed on coleopteran specific *Cry3A* expressing transgenic potato plants reached smaller pupal weight and laid virtually no eggs Hussain et al, 2006; Kannan and uthamasamy, 2006). This observation insisted the authors to suggest that the sterility was due to low vittelogenin level in hemolymph. They assumed that Cry toxins, by some means penetrated into the hemolymph and were sequestered, which then interacted with different

types of cells like follicular cells, trophocytes and fat body cells that support egg maturation (Hussein et al, 2006). Several others have reported sub lethal effect of Cry toxin on fecundity and female egg laying. All these studies illustrated the effect of Cry toxin, which is not only limited to the midgut but is extended to other important organs those are associated with reproduction, like fat body and ovaries. In order to correlate these pathophysiological effects of Cry toxins with the actual biochemical events, we analyzed the Cry1 toxin interaction with fat body membrane preparations using toxin overlay assay. The results obtained showed strong interaction of all the three, Cry1Aa, Cry1Ab and Cry1Ac toxins, with the 110-120 kDa protein in fat body membrane preparation. The specificity of this interaction was further examined by co-immunoprecipitation experiments carried out using Cry1Aa toxin and its antisera, and subsequently identifying the binding protein of 110-120 kDa as APN by using antiFbAPN antisera. The midgut specific 110-120 kDa protein also showed strong interaction with Cry1Aa, Cry1Ab and Cry1Ac. Interestingly, the midgut specific ~170 kDa protein was found to bind only to Cry1Aa and Cry1Ac, but not to Cry1Ab. The specificity of the interaction of midgut specific 110-120 kDa binding protein with Cry1Aa toxin and confirmation of this protein to be an APN was carried out by co-immunoprecipitation using antiMgAPN antisera.

The insect APNs identified as Bt toxin receptors are known to possess GPI anchor. Targeting of GPI-anchored proteins is a general strategy for most of the pore-forming toxins during interaction with their target cells. The GPI anchored proteins increase the lateral mobility of the toxins, which could result in the oligomerization and toxin insertion in to target cells. The interaction of pore-forming toxins with lipid rafts is responsible for additional cellular events of toxin internalization, faulty signal transduction and cellular response (Fernandez et al, 2006). In the present study, the detection of GPI anchor by using anti CRD antibody further substantiates the potential role of fat body APN as a Cry toxin receptor.

Of the different Cry1A toxins screened by feeding bioassay in our laboratory, Cry1Ac was found to be most effective against *A. janata* larvae followed by Cry1Ab and Cry1Aa. With reference to the results from Cry toxin overlay analysis, which showed differential interaction of midgut APNs with the three types of Cry1A toxins, it may be inferred that higher toxicity of Cry1Ac towards *A. janata* could be because of its simultaneous recognition by different receptors (110-120 and ~170 kDa proteins). In fact, the Cry1Ac was reported to

be the most effective Cry toxin even for *H. virescens*, *H. zea* and *H. armigera* larvae among other noctuid pests (Banks et al, 2001: Rajagopal et al, 2003). On the contrary, the intrahemocoelic injection reveals toxicity mostly for Cry1Aa and Cry1Ab, while Cry1Ac, in spite of its binding to the fat body APNs, terribly failed to show any toxicity. Therefore, it appears that the nature of Cry toxicity in the body cavity is quite different from that in the insect gut and this observation is consistent with the reports on *L. dispar* (Cerestiaens et al, 2001). As carbohydrate moieties have been known to play a crucial role in the interaction of Cry1Ac with its receptors, in order to investigate the basis of differential toxicity of Cry1Ac, we have studied the role of glycan moieties in its interaction with both, fat body as well as midgut APNs. Cry1Ac toxin binding to the 110-120 kDa protein in fat body as well as midgut was not inhibited in the presence of the sugar GalNAc, suggesting the presence of two binding sites for Cry1Ac toxin on 110-120 kDa APN (Burton et al, 1999). Most likely the GalNAc recognition is not essential for Cry1Ac toxins to interact with the 110-120 kDa receptors in the fat body and midgut, although it might be important for the ~170 kDa receptor in the midgut. It appears that all the three Cry1A toxins have alternative binding sites on the APNs and the interactions are independent of the GalNAc moiety (Estela et al, 2004). The variations in the toxicities of three different toxin through force feeding as well as in intrahemocoelic injection could be associated with presence of multiple receptors in migut whereas presence of single receptor in the fat body.

Nontoxic effect of Cry1Ac in the body cavity, in spite of its binding to 110-120 kDa APNs might be due to its interaction with highly glycosylated proteins like hexamerins and other procoagulants in the hemolymph. We observed strong binding of hexamerins to Cry1Ac on ligand blots. Such interaction of Cry1Ac with the hexamerins was also reported in *H. armigera* (Ma et al., 2005). But in case of Cry toxins, Peyronnet et al, (1997) showed that neither Cry1Aa nor Cry1C had any depolarizing effect when applied on the basolateral side of the midgut membrane of *L. dispar*. This might explain the nontoxic effect of Cry1Ac on the basolateral side of midgut, where the toxins were deposited by intrahemocoelic injections in the present study. Yet, another important report shows persistence of a bacteria, *Photorhabdus luminescence* in the gut of entamopathogenic nematode, which upon infection are released into the body cavity of insects, where it not only affects the basolateral side of the midgut, but also the fat body and shows binding of its Tca toxin (Silva et al, 2002). In spite of the contrasting nature of Tca and Cry toxins with respect to infection of basolateral

side of midgut, this report strongly suggests the possibility of interaction of Cry toxin with receptors present on fat body, which most likely appears to be the 110-120 kDa APN. Present study clearly demonstrates the presence and involvement of a fat body receptor in the pathogenesis of Cry toxins that is different from the Cry receptors in the gut. The requirement of higher toxin concentrations to be effective on intrahemocoelic injection could be due to different factors like, the size of the larvae used, presence of basal lamina surrounding fat body, which retards the toxin access to the plasma membrane of fat body cell, the kind of receptors and the type and purity of the toxin. Although, the route of Cry toxin entry into the body cavity of insects is not a general phenomenon, yet this widens the possibility to develop appropriately engineered chimeric Cry toxins, which can be directed to the fat body by using alternate delivery methods, such as entamopathogenic nematodes (Tounsi et al., 2006). By suitable use of insect immune system modifiers for enhancing the activity of bioinsecticide (Schneider, 2000), the present study will be of great significance in order to overcome the problem of resistance in future.

The abundance of APN transcript and protein in the larval fat body is associated with almost negligible level of APN enzymatic activity in the fat body membrane, while a significantly high APN activity detected in the haemolymph during developmental transition stage indicates towards enormous physiological significance of the fat body APN. Although APN activity was reported earlier in the larval haemolymph of *L. dispar* and *Diploptera punctata*, the site of enzyme synthesis was not made clear (Garside et al., 1997; Masler and Kovaleva, 1997). Based on the results from enzymatic studies, it appears that the fat body might be one of the sites for synthesis and release of APN into the haemolymph during postembryonic development. In fact, detection of a considerable amount of signal for cleaved GPI anchor in the fat body membrane preparation, even in absence of phospholipase C treatment supports this assumption. Our results are consistent with the paradigm that most of the haemolymph proteases are synthesized and released either by fat body or hemocytes (Jiang et al, 2005). The increase in the haemolymph plasma APN activity from late larval instar to prepupal stage suggests that during prepupal stage there may be an induction of enzymatic activity, which is regulated by raising ecdysteroid titre (Hall, 1988), which also suggests a possible role of fat body APN in larval to pupal transformation and metamorphosis (Haunerland, 1996). However, much detailed studies are needed to decipher the physiological functions of this newly identified fat body APN as that would particularly

enhance the significance of its being a Cry toxin receptor in the lepidopteran insect pests. With the identification of aminopeptidase in the fat body it emerges as a potential new target site for Cry toxin action, which widens the possibility of development of successful alternate delivery methods for Cry proteins

Chapter IV

Identification of GPI anchored aminopeptidase in the fat body as putative hexamerin binding protein

Background:

During final instar development, holometabolan insect larvae undergo dramatic physiological changes to prepare themselves for pupation and metamorphosis. As pupae do not feed, they depend on material that has been accumulated during the larval life. Hexamerins are synthesized and accumulated in high concentrations and appear to be the central source of a storage pool of amino acid resources for metamorphic development (Haunerland, 1996). A rise in the level of the steroid hormone 20-hydroxyecdysone (20E) at the end of larval life triggers the incorporation of hexamerins from hemolymph into the fat body (Burmester and Scheller, 1997; Kiran Kumar et al, 1997). Even though the endocytosis of vitellogenis by oocytes is well established, the regulation of hexamerin uptake and their delivery to protein storage granules of fat body remains elusive in spite of a extensive studies which intened to analyse whether the uptake process a `classical' endocytosis or different (Burmester and Scheller, 1999).

Although cloning and molecular characterization of hexamerin binding protein (putative hexamerin receptor) has been carried out by some groups, still it is not clear whether these are transmembrane or membrane anchored proteins. Further none of the reported receptor show the features classical endocytic sorting signals (Burmester and Scheller, 1995; Hansen et al, 2003; Persaud and Haunerland, 2004). On the other hand GPI-anchored proteins are cell surface proteins, anchored in the membrane through a glycosylphosphatidylinositol. In recent literature GPI-anchored proteins mediating signaling phenomena have been well documented (Vander Geer et al, 1994). Binding of ligands to GPI-anchored molecules is required for the delivery of certain activation signals to trigger the sequestration of ligands into specialized glycolipid-based domains (Solomon et al, 1998). On the basis of these observations and the fact that tyrosine phosphorylation of cellular substrates has been used as an indication of GPI-anchored protein mediated signaling, we thought to analyse whether binding of hexamerins to 110-120 kDa fat body membrane protein initiates a signaling event which is responsible for sequestration and also investigated its regulation by tyrosine kinase dependent phosphorylation.

Our group works on the process of uptake of hexamerin/arylphorin by the fat body of lepidopteran insects and is trying to decipher the mechanism of this selective uptake during postembryonic development, using *C. cepholonica* larvae. It was demonstrated that this

process is regulated by 20E, involving the participation of a specific binding protein for arylphorin with a molecular mass of 120 kDa (putative arylphorin/hexamerin receptor). This 120 kDa protein was detected in the membrane fraction of fat body (KiranKumar et al, 1997; Arif et al, 2003). The present chapter reports that the hexamerin binding protein is attached to the fat body cell membrane and role of glycan in the uptake of hexamerins. We also report that the 110-120 kDa hexamerin receptor of *A. janata* gets phosphorylated and the phosphorylation is mediated partly by a tyrosine kinase which is activated by ecdysteroid hormone like in *C. cepholonica*. Ligand blotting of phosphorylated fat body proteins with bio-labeled hexamerin and subsequent autoradiographic analysis once again suggests that the phosphorylated 110-120 kDa protein is putative hexamerin binding protein.

Results:

Purification of hexamerins and immunoblotting studies:

Using ammonium sulfate fractionation hexamerins were partially purified from haemolymph, of last instar larvae of *A. janata*, as mentioned materials and method and polyclonal antibodies against them were generated in rabbits. Results presented in figure 4.1 show the profile of proteins obtained after different steps of purification (Fig 4.1A, lanes 2-5). Hexamerins with molecular weight of 82-86 kDa, present in fairly pure form were used for antibody generation (Fig 4.1A, lane 6). The antisera showed selective cross reactivity with hexamerins alone present in the haemolymph (Fig 4.1D).

Developmental profile, stage and tissue specific uptake of hexamerins by the fat body cells:

Results presented in figure 4.1, shows the profile of hexamerins in the prepenultimate, penultimate and final larval instar larval hemolymph (Fig 4.1B, lanes 1-3). The glycoprotein nature of hexamerin was demonstrated by PAS staining (Fig 4.1C). The presence of hexamerins in the fat body was also detected by western blotting (Fig 4.1D). It was found to be fairly high in prepupal fat body (Fig 4.1E, lane 3) when compared with ELI and LLI (Fig 4.1E, lanes 1 and 2). It is well known that the fat body cells do not synthesize hexamerins and proteins present at this stage represent the hexamerins that have been incorporated from the hemolymph.

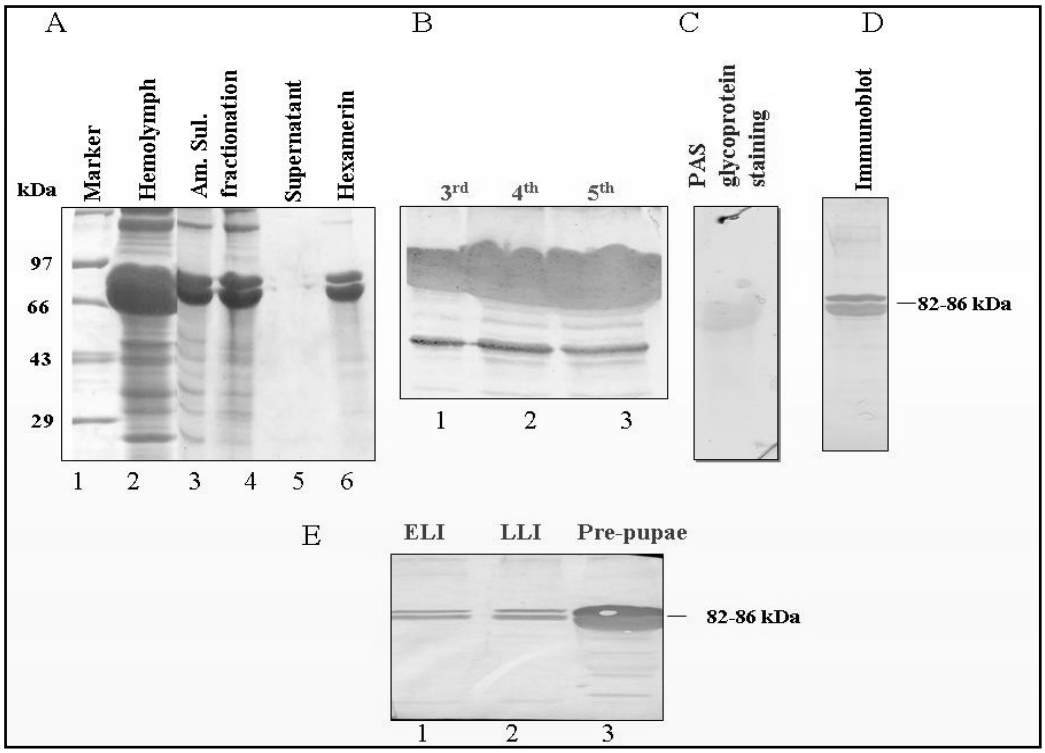

Fig. 4.1: A. SDS–PAGE of haemolymph fractions obtained at various steps of purification. Lane 1, molecular weight marker; lane 2, crude haemolymph; lane 3, 40% ammonium sulfate precipitate; lane 4, 60% ammonium sulfate precipitate; lane5, 70% ammonium sulfate precipitate, lane 6, pure hexamerin. **B**. Hexamerin profiles in hemolymph during different developmental stages present in hemolymph of larvae. Lane 1, pre penultimate; lane 2, penultimate and lane 3, lat instar larva. **C**. Identification of glycoprotein status of hexamerin by PAS staining. **D**. Immunoblot analysis of hexamerins. **E**. Hexamerins profile in the fat body of late larval instars.

Identification of Hexamerin binding protein in the fat body membranes:

The presence of hexamerin binding protein/receptor of 110-120 kDa has been already documented in few lepidopteran insect (Chung et al, 1995; Kiran Kumar et al, 1997; Arif et al, 2003). Hence it was tempting to check whether or not the larval fat body of *A. janata* also has a similar binding protein. Membrane fraction of the fat body cells from late-last instar larvae of *A. janata* were subjected to SDS–PAGE (Fig 4.2, lane 1) and followed by ligand

blotting. The biotinylated hexamerin bound to a major protein of 110-120 kDa molecular weight (lane 2). Here we show that indeed 110-120 protein in larval fat body of *A. janata* is able to bind to hexamerin *in vitro*. Therefore, it can be considered as the hexamerin binding protein of *A. janata*.

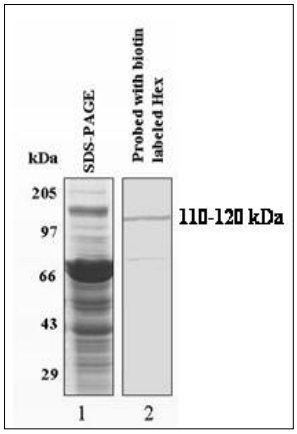

Fig. 4.2: Hexamerin overlay assay with fat body membrane protein. The fat body membrane proteins were separated on a 7.5% SDS-PAGE (lane 1), transferred to nitrocellulose membrane. These blots were incubated with the biotin labeled hexamerins (100 ng/ml) and detected with ALP conjugated streptavidin (lane 2).

20E-induced phosphorylation of the 110-120-kDa protein is tyrosine kinase dependent:

The *in vitro* phosphorylation of *A. janata* larval fat body membrane proteins in the presence of 20E led to the phosphorylation of few proteins, among them 110-120 kDa was the major protein (Fig 4.3A, lane 2). Earlier studies from our laboratory demonstrated that the 20E-induced phosphorylation of the 120 kDa protein was tyrosine kinase mediated, and was calcium as well as protein kinase C independent. (Arif et al, 2003). Hence, in the present study also the possibility of tyrosine kinase mediation was checked. Immunoblot analysis of the phosphorylated protein with anti-phosphotyrosine antibody showed cross reactivity of the antibody with the 110-120 kDa protein (Fig 4.3B, lane 1). The immunoblot clearly shows the strong cross reactivity of P-Tyr antibody with 20E induced phosphorylated 110-120 kDa protein (lane 2) in comparison to the protein phosphorylated in the absence of 20E (lane 1).

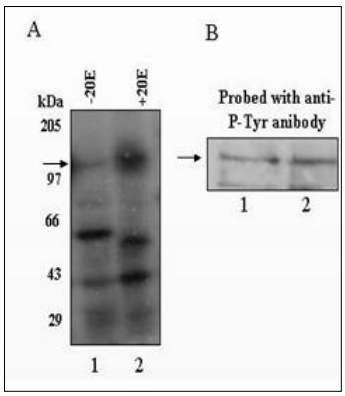

Fig. 4.3: **A**. Phosphorylation of fat body proteins. Lane 1, in the absence of 20E; lane 2, in the presence of 20E. **B**. Western blot analysis of corresponding autoradiograph with phosphotyrosine antibody.

Phosphorylated 110-120 kDa is hexamerin binding protein:

For this we co-immunoprecipitated phosphorylated 110-120 kDa protein from freshly isolated fat body membrane preparation and assessed its binding with hexamerins using hexamerin overlay assay and subsequently subjected to autoradiography. In this experiment 110-120 kDa protein showed exclusive binding to hexamerin (Fig. 4.4)

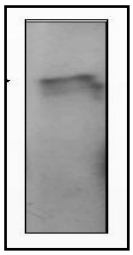

Fig. 4.4: Ligand blot analysis of phosphorylated binding protein by Hex and Hex antisera.

GPI anchor property of the hexamerin binding protein:

Cry proteins, like other pore forming toxins have the ability to bind to receptors, which are anchored to membrane by GPI moiety. Targeting of GPI-anchored proteins is a general strategy for most of the pore-forming toxins during interaction with their target cells (Broomfield and Hooper, 1993). In the present study it was demonstrated by ligand blotting of fat body membrane protein with Cry1Aa toxin. The toxin showed the binding to 110-120 kDa protein (Fig. 4.4, lane 2) where as in control there is no binding (lane 1).

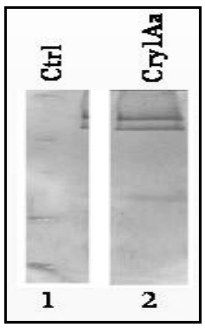

Fig. 4.5: The fat body membrane proteins were separated on a 7.5% SDS-PAGE, transferred to nitrocellulose membrane. The strip was separately incubated with activated Cry1Aa toxins and detected with respective antisera. Lane 1, blot directly probed with antisera (control), lane 2, blot probed with toxin followed by antisera.

Identification GPI moiety on hexamerin binding protein:

PIPLC digestion was used to determine whether the 110-120 kDa protein was anchored to fat body membrane through GPI moiety in *A. janata*. As shown in figure 4.6, after PIPLC digestion, anti-CRD sera recognized the110-120 kDa protein in the fat body membrane (lane 1), suggesting that this protein is anchored to the fat body membrane through GPI moiety, the faint crossreactivity in the absence of PI-PLC (lane 2) may be due to cleavage by internal phospholipases.

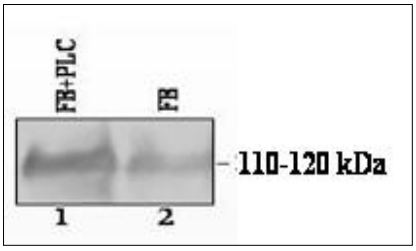

Fig. 4.6: Detection of the cleaved GPI anchors. The blot was incubated with anti-CRD antibodies and cleaved GPI anchors were detected with ALP-conjugated secondary antibody. Lane 1, fat body membrane protein after PLC digestion; lane 2, fat body membrane protein without PLC digestion.

Identification of glycan moieties in hexamerin binding protein:

To investigate the oligosaccharides present, lectin blotting analysis was performed using selected lectins (Fig 4.7). After lectin blotting, Hex binding protein was recognized by SBA (lane 1) but not by WGA (lane 2). To further test the existence of terminal GalNAc on

Hex binding protein, mild periodate deglycosylation studies were carried out, after transferring the fat body membrane proteins from SDS-PAGE to PVDF membrane. The binding of hexamerin to the same protein was not eliminated (lane 3) after mild chemical deglycosylation. This result provides further support for the conclusion that hexamerin binding to the 110-120 kDa protein is not primarily GalNAc mediated.

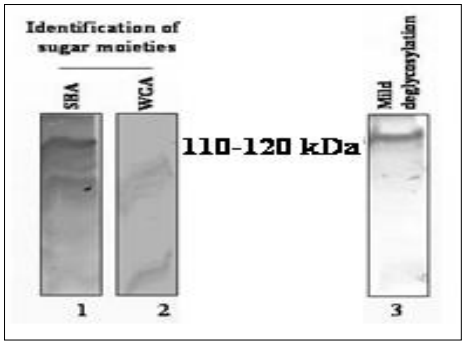

Fig. 4.7: The membranes were incubated either with biotin conjugated soybean agglutinin (lane 1); wheat germ agglutinin (lane 2) and lectin bound proteins were subsequently detected with streptavidin-conjugated secondary antibody. Effect of mild deglycosylation on the binding of hexamerin to its receptor (lane 3).

Identifications of Hexamerin binding protein as Aminopeptidase:

As we described in chapter 3, we identified for the first time an aminopeptidase from fat body of lepidopteran insect and that particular protein also had approximately similar molecular weight and shown to be as GPI anchored protein using different strategies. In the earlier sections of this chapter the Hex binding protein was identified as GPI anchored protein and has the ability to undergo phosphorylation. These findings were further substantiated by co-immunoprecipitation of the phosphorylated Hex binding protein and its subsequent identification by immunoblot analysis using APN antisera (Fig. 4.8, lane 2). In the control co-immunoprecipitation reaction hexamerin was not include and hence there is no binding (lane 1).

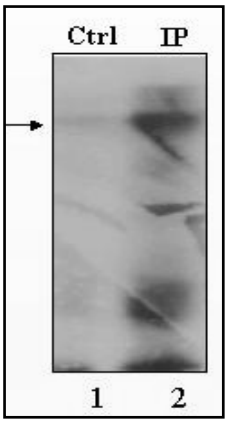

Fig. 4.8: Immunoprecipitation of phosphorylated Hex binding protein and probed with anti-FB APN antisera; lane 1, control without ligand, hexamerin; lane 2, with hexamerin.

Discussion:

In the present study we have demonstrated that the binding of hexamerin to the receptor leads to tyrosine phosphorylation of a 110-120-kDa protein. Recently in the rice moth, *C. cephalonica,* we projected that the hexamerin receptor is activated through phosphorylation by a tyrosine kinase. The extent of phosphorylation was enhanced in the presence of 20HE, which in turn responsible for up take of hexamerins (Arif et al, 2003). The human GPI-linked CD59, CD55, CD48, CD24, and CD14 as well as the mouse Thy-1 and Ly-6 were found to be associate with protein tyrosine kinases related to Src, the key regulators of cell activation and signal transduction. The cross-linking of GPI-anchored proteins triggers the sequestration of ligands into specialized glycolipid based microdomains (Stefanova et al, 1991; Freedman et al, 1999; Togo and Morisawa, 2004). Glycosylphosphatidylinositol **(**GPI**)-**anchored proteins have neither transmembrane nor cytoplasmic amino acid sequences (Solomon et al, 1998). The interaction of GPI-linked molecules with protein tyrosine kinases suggests a potential mechanism of signal transduction in cells. Although hexamerin receptor cDNAs have been cloned from *Calliphora* as well as *Sarcophaga* and the presence of receptor cleavage product has been demonstrated but typical transmembrane domain or endocytosis sorting signals were not reported in these hexamerin receptor sequences (Burmester, 1997), therefore still its uncertain how the receptors are linked to the fat body cell membrane. Lipid-linked signal transduction molecules such as Src family non receptor tyrosine kinases and heterotrimeric G proteins are found to co-immunoprecipitate with GPI-anchored proteins (Schlessinger, 2000;

Haugh, 2002). Arif et al, (2004) demonstrated that phosphorylation of 120 kDa protein in *C. cepholonica* was neither dependent on diacyl glycerol nor on protein kinase C. This further suggests the possibility of involvement of Src kinases which are attached to GPI anchor proteins may be responsible for phosphorylation. Based on this**,** we speculated that this protein may be a GPI anchored, and the signaling is regulated by Src tyrosine kinases. So the tyrosine kinase mediated phosphorylation of Hex binding protein lead us to check its status on the membrane.

Cry proteins, like other pore forming toxins have the ability to bind to receptors which are anchored to the membrane by a GPI moiety. As the Cry toxin showed the binding to 110-120 kDa protein, it was further substantiated by identifying the CRD epitope with anti CRD antibody. Furthermore, most of the reported GPI anchored proteins are known to be glycoproteins. The carbohydrate moieties of hexamerins were not involved in the uptake of hexamerins by fat body and integument (Marinotti et al, 1988). So further we checked the role of glycan moieties which are present on the 110-120 kDa Hex binding proteins. Present study clearly rules out is either glycan involvement of hex binding protein to its ligand. So most probably this interaction through GPI moieties or because of lipid based interactions. Finally the co-immunoprecipitation experiments of Hex binding protein and its subsequent identification by immunoblotting using AntiFbAPN, prompts us to suggest that lead us to assume that GPI anchored fat body aminopeptidase may be responsible for the uptake of hexamerins. The main function of the binding protein may be to anchor arylphorin in the protein granules of pupal fat body, and it may be a different protein from the arylphorin receptor, if any, needed for incorporation of arylphorin into the fat body. However, it is possible that the pupal protein granules containing the 120 kDa protein participate directly in the uptake of arylphorin from the hemolymph into the fat body because this 120 kDa protein is the only protein so far shown to have affinity to arylphorin.

Summary

The work starts with the morphological, biochemical and molecular characterization of three local isolates of *B. thuringiensis* DOR3, DOR2 and DOR4 potent against castor semilooper. In order to achieve the goal, first we carried out primary bioassays to identify the potent strains, then serotyping, purification Cry proteins, PCR and RT-PCR to decipher the reasons for higher toxicity of these local Bt isolate to *A. janata*. The local isolates of Bt that showed higher toxicity than *Bt kurstaki* HD-1 against *A. janata* larval forms were identified primarily by phase contrast microscopy, serological identification and by bioassays. The three isolates under study showed similar protein profiles but exhibited different plasmid profile. Crystal morphology of the three isolates also showed variations in the shapes of the crystal among them and then with the reference strain Bt *kurstaki* HD-1. Identification of the *cry* genes was carried out by triplex PCR method. (Juarez-Perez et al, 1996). Earlier several PCR, PCR-RFLP and hybridization based strategies have been used to screen local Bt isolates and to identify *cry* genes having minor amino acid sequence variations (Porcar and Juarez-Perez, 2003). But here we used triplex PCR, in order to identify the existing *cry* genes as well variants of known *cry* genes, called putative novel *cry* genes in the three isolates under study. The reproducibility of method was validated by using Bt *kurstaki* HD-1 as control. The triplex PCR method displayed completely different *cry* gene profile in three local DOR isolates when compared with the reference control *kurstaki* HD-1. Two isolates DOR3 and DOR4 showed the combination of *cry*1Aa and *cry*1Ac while DOR2 alone showed the presence of *cry*1Ab and *cry*1Ac genes.

Although identification of the *cry* gene content of a strain is important, it formulates only part of our understanding of the behavior of a particular isolate in insect bioassays (Masson et al, 1998). Based on PCR study, it is not immediately clear which genes are eventually translated. Further in order to know which *cry* genes are transcribed, RT-PCR experiments were carried out. RT-PCR results showed expression of the variant *cry*1Ac gene in DOR2 with similar size difference in transcript what was observed in triplex PCR with genomic DNA as template. Comparison of the DOR2 *cry*1Ac deduced protein sequence with reported *cry*1Ac showed that there are variations in the important aminoacid residue, and these are known to be specifically responsible for the binding of toxin to receptor. The homology of DOR2 *cry*1Ac to *cry*1B looks unique because *cry*1B is not reported in *kurstaki* serotype. The *cry*1Aa, *cry*1Ac from DOR3 showed almost 98% homology to the reported *cry* genes. Bioassays with individual Cry toxins proved, that Cry1Ac is more effective followed

by Cry1Ab and Cry1Ac. Among the three isolates only DOR2 showed presence of *cry*1Ab gene, moreover, the 44-MDa plasmid that encodes the *cry1Ab* gene is known to be unstable (Ben-Dov et al, 1997; Bravo et al, 1998). Loss of this plasmid from a ***B. thuringiensis*** subsp. *kurstaki* isolates would alter the *cry1A* gene PCR profile, resulting in the exclusion of the isolates as a putative *Bt kurstaki* HD1 (Valadares De Amorim et al, 2001). Lee et al, (1996) proved the synergistic effect between Cry1Aa - Cry1Ac and antagonistic effect between Cry1Aa -Cry1Ab and Cry1Ac - Cry1Ab against *L. dispar*. This suggests that the higher toxicity of DOR3 and DOR4 also may be due to the synergistic effect of Cry1Aa - Cry1Ac and less toxicity of DOR2 may due to the antagonism between Cry1Ab – Cry1Ac (Cry1B - Cry1Ab). Therefore, although these three isolates DOR3, DOR2 and DOR4, are serotyped as ssp.*kurstaki* these may be new Bt *kurstaki* strains with different gene profile.

The total enzymatic activity of the larval midgut juice was highest at pH 10.5 when azocasein was used as substrate. Zymogram studies (Garcia-carreno et al, 1993) carried out with *A. janata* larvae, revealed the presence of midgut proteases corresponding to approximate sizes of 17, 20, 29 and 80, and 100 kDa. The major protease pattern remained more or less the same through out the larval periods with slight quantitative variation. The 29, 80 and 100 kDa activities were almost completely inhibited by 300 µM TLCK (N-tosyl-L–lys chloromethyl ketone), where as other activities were remain unaffected. The bands inhibited with TLCK were thus identified as trypsin where as bands inhibited with both 100 µg/ml SBTI (soybean trypsin Inhibitor) and 100 µg/ml TCI (trypsin chymotrypsin inhibitor) were considered as trypsins or chymotrypsin like serine proteases but interestingly, 300 µM TPCK (N-tosyl-L–phe chloromethyl ketone) the chymotrypsin inhibitor did not show any activity when incubated with midgut juice. Activity blot analysis using specific chromogenic substrates BApNA (N-benzoyl-L–arg p-nitroanilide) and the respective inhibitors revealed that PMSF (phenylmethanesulfonyl fluoride) and TLCK inhibited, major BApNA-hydrolyzing enzymes, one with low molecular weight (29 kDa) and the others with high molecular weight (80 & 100 kDa). It has been earlier reported that trypsins occur in various isoforms, high molecular weight trypsin like proteases may be the aggregates of low molecular weight trypsins (Novillo et al, 1999; Wagner et al, 2002)

The susceptibility of the activated toxins to further proteolysis in the midgut environment could possibly affect toxicity and leads to discrepancies between their *in vitro* and *in vivo* activities (Haider et al, 1986). The zymogram with DOR3 Bt protoxin as

substrate revealed involvement of proteases corresponding to 29 kDa and to a lower extent 43 and 66-70 kDa in activation of protoxin to active toxin. The incubation of protoxin with midgut juice from different developmental stages as well as for different time periods, revealed the production of intense active toxin band of 65 kDa with all the treatments. Even though insects seem to possess similar arrays of protease classes; the specificities of the proteases are different (Patankar, 2004). In literature it has been clearly demonstrated that in some insects, proteases degrade protoxin faster (Forcada et al, 1999) and in other instances they act very slowly or totally ineffective (Ogiwara et al, 1992; Oppert, 1999). The present study suggests that changes in the activity or composition of gut proteases may be responsible for the more susceptibility of this insect to Cry toxins.

Activity blot analysis with SAAPLpNA (N-succinyl–ala–ala–pro–leu p-nitroanilide) and SAAPPpNA (N-succinyl–ala–ala–pro–phe p-nitroanilide), the specific substrates for elastase and chymotrypsin showed that the SAAPLpNA and SAAPPpNA hydrolyzing activities were associated with 17 kDa protein. Two types of aminopeptidase with molecular weights 65 and 110-120 kDa were observed with LpNa (Leu p-nitroanilide) as a substrate in midgut and brush border membrane vesicles respectively.

We demonstrated for the first time the presence of a novel aminopeptidase (APN) in the fat body of the moth *A. janata*. Northern blotting detected at least one APN transcript in the fat body, while two transcripts of different sizes were detected in the midgut. The fat body and midgut specific APN cDNAs were partially cloned by RT-PCR using degenerate primers and subsequently sequence information was complemented with 5’ and 3’ RACE, which provided the full-length cDNA sequences. The complete cDNA sequences for fat body and midgut APNs were designated as *Achaea janata* fat body aminopeptidase (*AjFbAPN*) and *A. janata* midgut aminopeptidase (*AjMgAPN*). The full-length *AjMgAPN* is 3053 bp long and contains an open reading frame (ORF) of 2850 bp, which encodes a putative protein of 950 amino acids with a theoretical molecular mass of 109 kDa and a calculated pI of 4.87, while *AjFbAPN* cDNA is 3086 bp long and contains an ORF of 3015 bp, encodes a putative 1004 amino acid protein with a theoretical molecular mass of 113 kDa and a calculated pI of 4.83. These two APNs share only 33% amino acid sequence homology among themselves, but both display the typical APN-features, like the N-terminal signal peptide, several putative glycosylation sites, C-terminal glycosylphosphatidylinositol (GPI) anchor signal, the APN-specific zinc binding/gluzincin motif $HEXXHX_{18}E$ and gluzincin motif GAMENWG.

Dendrogram analysis of APN isoforms from the insect midgut categorized them as four distinct classes and isoforms of all four classes have been reported from the midgut of various insects (Wang et al, 2005). Herrero et al, (2005) proposed the presence of five classes of APNs in lepidopteran midgut. In the present study, the phylogenetic analysis revealed that *AjFbAPN* and *AjMgAPN* belong to different classes of APNs in the phylogram and *AjFbAPN* being from a different tissue is in class five. Phylogenetic analysis strongly suggests the diversion of *A. janata* APN genes from a single ancestral gene and it was further substantiated by Southern analysis. Previously, the toxicity/mortality effect after a hemocoelic Bt injection was reported in the larvae of *Lymantria dispar* (Ceresteins et al, 2001). The toxicity obtained from hemocoelic delivery opens up the possibility of toxin interaction with various organs present in the body cavity, which could be additional targets for toxins. Of the different Cry1A toxins screened by feeding bioassay in present study, Cry1Ac was found to be most effective against *A. janata* larvae followed by Cry1Ab and Cry1Aa. On the contrary, the intrahemocoelic injection reveals toxicity mostly for Cry1Aa and Cry1Ab, while Cry1Ac, in spite of its binding to the fat body APNs, failed to show any toxicity. With reference to the results from Cry toxin overlay analysis, which showed differential interaction of midgut APNs with the three types of Cry1A toxins, it may be inferred that higher toxicity of Cry1Ac towards *A. janata* could be because of its simultaneous recognition by different receptors (110-120 and ~170 kDa proteins).

Several others also have reported sub-lethal effect of Cry toxin on fecundity and egg laying in female insects (Perlak et al, 1993, Hussein et al, 2006). All these studies illustrated the effect of Cry toxin, which is not only limited to the midgut but is extended to other important organs that are associated with reproduction, like fat body and ovaries. In order to correlate these pathophysiological effects of Cry toxins with the actual biochemical events, we analyzed the Cry1 toxin interaction with fat body membrane preparations using a toxin overlay assay. The results obtained showed strong interaction of all three Cry1Aa, Cry1Ab and Cry1Ac toxins, with the 110-120 kDa protein in fat body membrane preparation. The specificity of this interaction was further examined by co-immunoprecipitation experiments carried out using Cry1Aa toxin and its antisera, and subsequently identifying the binding protein of 110-120 kDa as APN by using antiFbAPN antisera. The midgut specific 110-120 kDa protein also showed strong interaction with Cry1Aa, Cry1Ab and Cry1Ac. Interestingly, the midgut specific ~170 kDa protein was found to bind only to Cry1Aa and Cry1Ac, but not

to Cry1Ab. The specificity of the interaction of midgut and fat body 110-120 kDa binding protein with Cry1Aa toxin and confirmation of this protein to be an APN was carried out by co-immunoprecipitation using antiMgAPN and antiFbAPN antisera respectively. The detection of the GPI anchor by using anti-CRD antibody (Broomfield et al, 1991) further substantiates the potential role of fat body APN as a Cry toxin receptor because the targeting of GPI-anchored proteins is a general strategy for most of the pore-forming toxins during interaction with their target cells. Based on the results obtained from enzymatic studies, it appears that the fat body might be one of the sites for synthesis and release of APN into the hemolymph during postembryonic development. For the first time we cloned and characterized aminopeptidase from the fat body of *Achaea janata*, further we extended our study in order find out whether the presence of aminopeptidase in fat body is unique to this insect or its presence is conserved throughtout the order lepidoptera. To decipher this question we cloned and characterized the *Spodoptera litura* fat body aminopeptidase and carried out comparative analysis with APN cloned from *A. janata*.

Although the route of Cry toxin entry into the body cavity of insects is not a general phenomenon, this widens the possibility to develop appropriately engineered chimeric Cry toxins, which can be directed to the fat body by using alternate delivery methods, such as entomopathogenic nematodes. By suitable use of insect immune system modifiers for enhancing the activity of bioinsecticide (Schneider, 2000), the present study will be of great significance in order to overcome the problem of resistance in the future.

Further we have demonstrated that the binding of hexamerin (Hex) to the receptor leads to tyrosine phosphorylation of a 110-120-kDa protein. Recently in the rice moth, *Corcyra cephalonica* our group projected that the hexamerin receptor is activated through phosphorylation by a tyrosine kinase. The extent of phosphorylation was enhanced in the presence of 20E, which in turn responsible for the uptake of hexamerins (Arif et al, 2003). However, it is uncertain how these receptors are linked to the fat body cell membrane. The GPI anchored proteins like CD59, CD55, Thy-1 and Ly-6 are reported to be associated with protein tyrosine kinases related to Src, the key regulators of cell activation and signal transduction. The cross-linking of GPI-anchored proteins triggers the sequestration of ligands into specialized glycolipid based microdomains (Freedman et al, 1999; Togo and Morisawa 2004) and these proteins have neither transmembrane nor cytoplasmic amino acid sequences (Solomon et al, 1998).

Based on this**,** we speculated that this protein may be a GPI anchored, and the signaling is regulated by Src tyrosine kinases. So the tyrosine kinase mediated phosphorylation of Hex binding protein lead us to check its status on the membrane. Cry proteins, like other pore forming toxins have the ability to bind to receptors which are anchored to the membrane by a GPI moiety. As the Cry toxin showed the binding to 110-120 kDa protein, it was further substantiated by identifying the CRD epitope with anti CRD antibody. Furthermore, most of the reported GPI anchored proteins are known to be glycoproteins. Earlier studies show that the carbohydrate moieties of hexamerins were not involved in the uptake of hexamerins by fat body and integument (Marinotti et al, 1988). In the present study we checked the role of glycan moieties, present on the 110-120 kDa Hex binding proteins, and results rule out involvement of glycan of hex binding protein to its ligand hexamerin. So most probably the interaction is either through GPI moiety or it is lipid based interaction. Finally the co-immunoprecipitation experiments of Hex binding protein and its subsequent identification by immunoblotting using AntiFbAPN lead us to assume that GPI anchored fat body aminopeptidase may be a putative Hex binding protein responsible for the uptake of hexamerins. The main function of the binding protein may be to anchor hexamerin /arylphorin in the protein granules of pupal fat body, and it may be a different protein from the arylphorin receptor, if any, needed for incorporation of arylphorin into the fat body.

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