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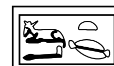

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IDENTIFICATION, ISOLATION AND CLONING OF cDNA ENCODING AMINOPEPTIDASE FROM THE MIDGUT OF THE EGYETIAN COTTON LEAF WORM THAT SERVES AS A RECEPTOR FOR CRY TOXIN

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***B**acillus thuringiensis* (*Bt*) is an aerobic, spore-forming bacterium that produces crystalline inclusions during the sporulation phase of growth that are composed of proteins known as δ-endotoxins (Bravo *et al.,* 2005). The δ-endotoxins comprise two multigenic families, *cry* and *cyt*. Cry proteins are specifically toxic to different insect orders, Lepidoptera, Coleoptera, Hymenoptera, Diptera or nematodes (Go´mez *et al.,* 2007). The crystal inclusions ingested by susceptible larvae, dissolve in the alkaline environment of the gut, and the solubilized inactive protoxins are cleaved by midgut proteases yielding active monomeric 60–70 kDa protease-resistant toxin (Pardo-Lo´ pez *et al.,* 2009; Bravo *et al.,* 2005), then the Cry toxic fragment binds to specific receptors located in the microvilli of the apical membrane of midgut epithelial cells that leads to membrane insertion, pore formation, cell lysis and the eventual death of the host (Bravo *et al.,* 2004 & 2005).

For Cry1A toxins (lepidopteran specific toxins), at least four different binding-proteins have been described as receptors for the Cry toxins; a cadherin-like protein (CADR), a glycosylphosphatidyl –inositol (GPI) - anchored aminopeptidase-N (APN), a GPI-anchored alkaline phosphatase (ALP) and a 270 kDa glycoconjugate (Vadlamudi *et al.,* 1995; Knight *et al.,* 1994; Jurat Fuentes *et al.,* 2004; Valaitis *et al.,* 2001).

APN is the most extensively studied Cry receptor identified and isolated from several lepidopteran insects. APN serves as a binding molecule for Cry1C in *Manduca sexta* (Luo *et al.,* 1996) and *Spodoptera litura* (Agrawal *et al.,* 2002), for Cry1Aa in *Bombyx mori* (Nakanishi *et al.,* 2002; Jenkins and Dean 2001; Yaoi *et al.,* 1999) and for Cry1Ac in *M. sexta* (Garczynski *et al.,* 1995; Knight *et al.,* 1994 & 1995; Sangadala *et al.,* 1994), *Lymantria dispar* (Lee *et al.,* 1996; Valaitis *et al.,* 1995) and *Heliothis virescens* (Gill *et al.,* 1995; Luo *et al.,* 1997; Oltean *et al.,* 1999)

The characterization of receptors in the economically important pests by studying their binding to different Cry toxins will help in evaluating the molecular mechanism of action of Cry toxin and will lead to a better understanding of the mechanism of development of resistance to these proteins in insects.

The Egyptian cotton leaf worm (*Spodoptera littoralis* Boisduval) is an important pest in Mediterranean and Asian countries; it populates fields in relatively close locations (Hamal *et al.,* 1991) and is considered a totally polyphagous pest (Lutfallah *et al*., 1993) affecting various economically important crops in Egypt such as cotton, maize, rice, soybeans and vegetables (Salama and Matter, 1991).

To identify the precise toxin binding targets in insects, midgut cell membranes have been electrophoretically separated and incubated with *Bt* cry toxins in Western blot analysis. We have identified the proteins involved in CryIAc toxicity to *Spodoptera littoralis*. A 109-kDa *B. thuringiensis* toxin-binding protein from the midgut brush-border membrane that binds the Cry1Ac toxin was determined. A cDNA clone encoding this protein was cloned, sequenced and characterized. The analysis of the predicted amino acids sequence reveals that the protein belongs to the aminopeptidase N family of proteins.

MATERIALS AND METHODS

Preparation and solubilization of parasporal crystal toxins

Preparation and solubilization of the pro-toxin containing crystals from *Bt* isolates were performed as described by Luo *et al*. (1999). Cry toxin was prepared from *B. thuringiensis* subsp. *kurstaki* strain HD-1. The bacterial strain was grown on a rotary shaker (200 rpm) for 3 days at 28- 30°C in 250 ml of T3 medium until sporulation and cell lysis. The crystals, spores, and debris were collected by centrifugation at 8000 rpm for 10 min at 4°C, and the pellet was washed with 1 M NaCl containing 0.1% Triton X-100 and

then with distilled water. The crystals were dissolved in 5 ml of 50 mM Na_2CO_3 (pH 9.6) containing 10 mM DTT by stirring for 2 h at room temperature and then centrifuged at 14000 rpm for 15 min to remove the insoluble debris. The supernatant that contains the solubilized crystal protein (pro-toxin protein) was dialyzed overnight at 4°C against 2 liters of 20 mM Na_2CO_3 buffer (pH 9.6) containing 0.2% β-Mercaptoethanol. Pro-toxin concentration was calculated by the following equation: $\mu g/\ \mu l = (1.55 \times \text{value at } A_{280}) - (0.76 \times \text{value at } A_{280})$.

Insect rearing and midgut isolation

The larvae of *S. littoralis* were reared on an artificial diet at 25°C and 70% relative humidity with a photoperiod of 12 h light and 12 h dark. Early-fifth-instar *S. litura* larvae were chilled on ice for 15 min and dissected to isolate the midgut tissue. The midgut was slit longitudinally, the peritrophic membrane was removed, and the residual midgut contents were rinsed away with ice-cold buffer (300 mM mannitol, 5 mM EGTA, 17 mM Tris-HCl, pH 7.5). The midgut tissue used for isolating RNA was rinsed with diethyl pyrocarbonate-treated water. The tissue was either used immediately or snaped frozen in liquid nitrogen and then stored at -80°C.

Preparation of Brush Border Membrane Vesicles (BBMVs)

BBMVs were prepared from fifth-instar larval midguts by the differential $MgCl_2$ precipitation described by Wolfersberger *et al.* (1987) in the presence of protease inhibitors (1 mM PMSF) and stored in buffer (300 mM mannitol, 5 mM EGTA, 17 mM Tris-HCl, pH 7.5) at -80°C until use.

Western blot analysis

The BBMVs protein was subjected to SDS-PAGE on a gel containing 10% polyacrylamide, electroblotted onto a polyvinylidene difluoride (PVDF) membrane for 4 hours by using a semi-dry blotting unit (Bio-Rad), and blocked for 2 h at room temperature with phosphate-buffered saline (TBS; pH 8.0) containing 5% nonfat dry milk powder and 0.5% Tween 20 (freshly prepared). The PVDF membrane was then incubated with solubilized Cry1A (1-10 µg/ml) in PBS blocking buffer overnight at 4°C and subsequently washed three times with PBS blocking buffer to remove the unbound toxin (100 ml per wash for 20 min). The membrane was then incubated with primary antibody (polyclonal rabbit anti-Cry1A; 1µl / 1ml) in PBS blocking buffer overnight at 4°C, washed three times with PBS (100ml per wash for 20 min). The membrane was incubated with goat anti-rabbit immunoglobulin G (IgG) secondary antibody (1:10,000 - Euroimmun, Lübeck, Germany) in TBS blocking buffer at 37°C for 2 h and washed three times with TBS blocking buffer (100 ml per wash for 20 min). After repeated washings with buffer, the immunoreactive proteins were visualized using substrate solution 1-3 min till the appearance of bands in dark room then the membrane was rinsed with water.

Cloning of S. littoralis APN

Total RNA was isolated from the *S. littoralis* midgut by using Trizol. cDNA was synthesized by using an oligo (dT) primer with 5 µg of total RNA as the template and reverse transcriptase (Promega M-MLV) according to the manufacturer's instructions. Primers for screening *APN* (F7:5'CAGAACTTGGTCCGAGAAGG3' & R8 5' ATGTCTCTGTCGT GGGCTCT 3') were designed on the basis of already published sequence of *APN* of *Spodoptera litura* obtained from the National Center for Biotechnology Information (NCBI) protein database (http://www. ncbi. nih.gov, accession number: AF320764). Using these primers, PCR was performed with midgut cDNA as a template. Conditions for the PCR included initial denaturation at 94 0C for 3 min, followed by 35 cycles each of denaturation (94°C, 45 sec), annealing (53°C -1°C in each cycle for 10 cycles then 25 cycles at 43°C, 1 min) and extension (72°C, 3 min). The reaction was ended by incubating the tubes in 72°C for 10 min as a final extension step.

A 2882-bp fragment was amplified and this was cloned into pGEM-T easy vector (Promega) and sequenced by the primer walking method at the AGERI sequencing facility. Both DNA strands were fully sequenced using primers as shown in Table (1). DNA sequences were assembled and analyzed using Vector NTI® Suite software version 11. Upon obtaining positive sequencing results, a BLAST nucleotide search was performed on each sample in order to determine to which genes the fragment was most closely related.

RESULTS AND DISCUSSION

Identification of a Spodoptera littoralis BBMVs protein that binds to Cry toxin

To identify the APN in *S. littoralis* BBMV, an immunoblot analysis was performed using Cry toxin specific antibody that recognized 109 kDa proteins in BBMV (Fig. 1, lane 3). Hence, a 109 kDa protein of the BBMV might serve as a receptor for the Cry type *Bt* toxin in the insect midgut, as shown in Fig. (1) Lane 3.

Also a 108 kDa brush border aminopeptidase-N has been identified as the Cry1C receptor in the polyphagous pest *Spodoptera littora* by ligand blot, immunoflurescence toxin binding studies and immunoprecipitation analysis (Agrawal *et al*., 2002).

The reason of using *Btk*HD1 strain in this study is that the most naturally occurring *B. thuringiensis* strains contain more than one type of ICP within their parasporal crystals; *Btk*HD1 revealed all three *cry1A* genes (Aa, -Ab, -Ac) and two *cry2* genes, *cry2A*, and the cryptic *cry2B* (Bauer, 1995). Some or all of the ICPs present in the crystals of a particular strain may recognize different binding sites in the same insect (van Rie *et al*., 1990). The combination of two or more insecticidal factors that have different target sites is considered to diminish the chance of resistance development (Bauer, 1995).

RT–PCR generation of APN-specific primers

In independent PCR experiments, both cDNA from the midgut and F7 and R8 primers were used in order to obtain full-length cDNA clones for *SlAPN*. The previous primers amplified a 2882 bp fragment (Fig. 2). This fragment is called *Sl*fl.

The 2882 bp fragment was cloned into pGEM T-Easy vector. The recombined plasmid pGEM T-Easy/*Sl*fl was transfected to DHl0β competent cells. The positive white clones were selected and inoculated to LB culture including ampicillin. The amplified plasmid DNAs were analyzed by PCR using T7 foreword and SP6 reverse primers. Numbers of clones that contained the right size fragment were used for sequencing.

The miniprep of pGEM T-Easy vector having the 2882 bp fragment was then purified and sequenced using T7 forward and SP6 reverse primers (vector-specific primer). The insert was fully sequenced using internal primers based on already obtained sequences (Sequence-specific primer) (Fig. 3). The sequence results confirmed that the full-length cDNA clones for *SlAPN* were obtained.

cDNA isolation and characterization

The DNA sequence of a maximal-length cDNA clone of *SlAPN* was determined. The sequence was confirmed by repeated sequencing of full length clones. Nucleotide sequence alignment of the full-length fragment revealed that the midgut *SlAPN* cDNA (Fig. 4) is 2882 bp long and contains an open reading frame (ORF) of 2856 bp with the start codon ATG at positions 27 - 29 and the termination codon TAA at positions 2883 - 2885. The predicted start codon is embedded in a consensus Kozak translation initiation sequence (ACCATGG) (Kozak, 1987).

The cDNAs encoded a putative 952 amino acid protein. The predicted protein in its nascent form has a molecular mass of 108.58 kDa. The analysis of the predicted amino acids sequence also reveals an N-terminal cleavable signal peptide for retention in the endoplasmic reticulum (residues 1 to 20), a C-terminal signal sequence for the GPI anchor site stop codon (residues 930 to 952), N-glycosylation sites, and zinc-binding signature sequence characteristic of zinc metallopeptidases (Fig. 5).

A predicted signal sequence, DSA for attachment of a GPI anchor, was present at amino acid 711 (Fig. 5; Italics). *SlAPN* is characterized by a consensus zinc-binding/gluzincin motif ($HEX_2HX_{18}E$, residues 355 to 378) which is part of a typical catalytic active site for the majority of zinc-dependent metallopeptidases and required by APN proteins for enzymatic function (Fig. 5; marked with asterisks). The gluzincin APN motif (GAMENWG, residues 319 to 325) also believed to form part of the active site (Fig. 5; dotted underline).

Potential N-linked glycosylation sites (NXS/T) are shown at residues 103,

377, 430, 574, 711 and 782 (Fig. 5; underlined). The amino acid sequence also contains four Cys residues which are highly conserved among APN molecules of higher vertebrates such as rat, rabbit, pig, and human (Agrawal *et al.,* 2002) (Fig. 5; bold face).

Of the many different *APNs* that have been studied, several common features have emerged. The genes encode proteins of approximately 1,000 amino acids that undergo various forms of post-translational modification to produce mature proteins of between 90 and 170 kDa in size (Pigott and Ellar, 2007); the case under investigation produces about 108 kDa protein. The proteins have a cleavable N-terminal signal peptide that directs nascent polypeptides to the outer surface of the cytoplasmic membrane. There, they are attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor (Takesue, 1992; Knight *et al.,* 1995; Lu and Adang, 1996; Denolf *et al.,* 1997; Agrawal *et al.,* 2002), in contrast to what is shown for vertebrates, where a hydrophobic N-terminal stalk is used for attachment (Pigott and Ellar, 2007). On the other hand, glycosylation is important for some Cry toxin-APN interactions, and in many cases the presence of N- or O-linked carbohydrates has been shown biochemically or predicted by sequence analysis (http://www.cbs.dtu.dk/services/).

In the epithelial cells of mammalian kidney and intestine aminopeptidase-N is a type Π membrane protein, anchored by uncleaved N-terminal signal anchor sequence and with a C-terminal extracellular domain (High, 1992). However, C-terminal extension of the *S. litturalis* sequence, which includes the GPI anchor signal peptide (Fig. 5) reflects the fact that other membrane-bound aminopeptidases are anchored by C-terminal signal anchor sequence. In this case, *S. litturalis* aminopeptidase-N is a type I membrane protein anchored in the membrane by a C-terminal (stop transfer) sequence and with an N-terminal extracellular domain. Such a topology would require an N-terminal cleavable signal peptide to initiate translocation across the endoplasmic reticulum membrane (High, 1992).

On the other hand, Nakanishi *et al.* (2002) reported that the Cry1A toxin-binding regions have many conserved amino acid residues such as RXXFPXXDEP which is conserved in all lepidopteran APNs and was found also in *S. littoralis* (Fig. 5 -highlighted in grey). The authors also reported that Cry1Aa and Cry1Ab toxins may recognize and bind to a common structure in these regions. The binding ability of APN receptor to the *Bt* toxin is needed for toxicity and after binding to a receptor in the insect midgut, the toxin undergoes a conformational change lead to form pores (Sanjay *et al*., 2001).

Aminopeptidase N activity in lepidopteran larvae has been attributed to the APNs associated with the midgut brush border membranes (Terra and Ferreira, 1994; Adang, 2004). As APNs perform the physiological function of protein digestion and may serve as receptors for *Bt*

toxins, studies of insect APN activities and their gene expression have so far been focused on the insect midgut.

SUMMARY

Insecticidal toxins produced by *Bacillus thuringiensis* interact with specific receptors located in the midguts of susceptible larvae, and the interaction is followed by a series of biochemical events that lead to the death of the insect. In order to elucidate the mechanism of action of *Bt* toxins, receptor protein encoding genes from many insect species have been cloned and characterized. In this paper, we report the cloning and characterization of Cry toxin-interacting aminopeptidase N (APN) isolated from the midgut of *Spodoptera littoralis*. The full length *APN* cDNA contains 2882 nucleotides and encodes protein of 952 amino acids. The sequence displays the typical APN features such as N-terminal cleavable signal peptide, several putative N-glycosylation sites, C-terminal signal sequence for the GPI anchor site stop codon, zinc-binding/gluzincin motif ($HEX_2HX_{18}E$) and the gluzincin APN motif (GAMENWG).

REFERENCES

Adang, M. J. (2004). Insect aminopeptidase N. In: Barret, A. J., Rawlings, N. D., Woessner, J. F. (Eds.), Handbook of proteolytic enzymes, Vol 1 Elsevier academic press, New York, 296-299.

Agrawal, N., P. Malhotra, and R. K. Bhatnagar (2002). Interaction of gene-cloned and insect cell-expressed aminopeptidase N of *Spodoptera litura* with insecticidal crystal protein Cry1C. Appl. Environ. Microbiol., 68: 4583-4592.

Bauer, L. S. (1995). Resistance: a threat to the insecticidal crystal proteins of *Bacillus thuringiensis*. Symposium on Pesticide Resistance, Florida Entomologist, 78: 414-443.

Bravo, A., S. S. Gill and M. Sobero´n (2005). *Bacillus thuringiensis* mechanisms and use. In: Comprehensive molecular insect science. Elsevier B.V., 175-206.

Bravo, A., I. Go´mez, J. Conde, C. Munoz-Garay, J. Sa´nchez and M. Zhuang (2004). Oligomerization triggers differential binding of a pore-forming toxin to a different receptor leading to efficient interaction with membrane microdomains. Biochem. Biophys. Acta, 1667: 38-46.

Denolf, P., K. Hendrickx, J. Van Damme, S. Jansens, M. Peferoen, D. Degheele, and J. Van Rie (1997). Cloning and characterization of *Manduca sexta* and *Plutella xylostella* midgut aminopeptidase N enzymes related to *Bacillus thuringiensis* toxin-binding proteins. Eur. J. Biochem., 248: 748-761.

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

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

Go´mez, I., L. Pardo-Lo'pez, C. Mun'oz-Garay, L. E. Fernandez, C. Pe'rez, J. Sa'nchez, M. Sobero'n, and A. Bravo (2007). Role of receptor interaction in the mode of action of insecticidal Cry and Cyt toxins produced by *Bacillus thuringiensis*. Peptides., 28: 169-173.

Hamal, M., M. I. Brownbridge, M. Broza, and B. Sneh (1991). Screening for highly effective isolates of *Bacillus Thuringiensis* against *Spodoptera exempta* and *Spodoptera littoralis*. Phytoparasitica, 19: 9-21.

High, S. (1992). Membrane protein insertion into the endoplasmic reticulum--another channel tunnel? *BioEssays*, 14: 535-540.

Jenkins, J. L. and D. H. Dean (2001). Binding specificity of *Bacillus thuringiensis* Cry1Aa for purified, native *Bombyx mori* aminopeptidase N and cadherin-like receptors. BMC Biochem., 2: 12-19.

Jurat-Fuentes, J. L. and M. J. Adang (2004). Characterization of a Cry1Ac receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae. Eur. J. Biochem., 271: 3127-3135.

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

Knight, P. J., B. H. Knowles and D. J. Ellar (1995). Molecular cloning of an insect aminopeptidase N that serves as a receptor for *Bacillus thuringiensis* CryIA(c) toxin. J. Biol. Chem., 270: 17765-17770.

Kozak, N. (1987). An analysis of 5' noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Research, 15: 8125-8148.

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

Lu, Y. J. and M. J. Adang (1996). Conversion of *Bacillus thuringiensis*

Cry1Ac-binding aminopeptidase to a soluble form by endogenous phosphatidylinositol. Insect Biochem. Mol. Biol., 26: 33-40.

Luo, K., Y. J. Lu and M. J. Adang (1996). A 106-kDa form of aminopeptidase is a receptor for *Bacillus thuringiensis* Cry1C δ-endotoxin in the brush border membrane of *Manduca sexta*. Insect Biochem. Mol. Biol., 26: 783-791.

Luo, K., S. Sangadala, L. Masson, A. Mazza, R. Brousseau and M. J. Adang (1997). The *Heliothis virescens* 170 kDa aminopeptidase functions as "receptor A" by mediating specific *Bacillus thuringiensis* Cry1A δ endotoxin binding and pore formation. Insect Biochem. Mol. Biol., 27**:** 735-743.

Luo, K., J. R. McLachlin, M. R. Brown and M. J. Adang (1999). Expression of a glycosylphosphatidylinositol-linked *Manduca sexta* aminopeptidase N in insect cells. Protein Exp. Purif., 17: 113-122.

Lutfallah, A. F., M. R. Sherif and F. K. El Duweini (1993). Susceptibility of some commercial corn varieties to infestation with certain corn pests in Egypt. Egyptian Journal of Agricultural Research, 71: 717-724.

Nakanishia, K., K. Yaoib, Y. Naginoa, H. Haraa, M. Kitamia, S. Atsumia, N. Miuraa and R. Satoa (2002). Aminopeptidase N isoforms from the midgut of *Bombyx mori* and *Plutella xylostella*, their classification and the factors that determine their binding specificity to *Bacillus thuringiensis* Cry1A toxin. FEBS Lett., 519: 215-220.

Oltean, D. I., A. K. Pullikuth, H. K. Lee and S. S. Gill (1999). Partial purification and characterization of *Bacillus thuringiensis* Cry1A toxin receptor A from *Heliothis virescens* and cloning of the corresponding cDNA. Appl. Environ. Microbiol., 65: 4760-4766.

Pardo-Lo'pez, L., C. Mun'oz-Garay, H. Porta, C. Rodrı´guez-Almaza´n, M. Sobero'n and A. Bravo (2009). Strategies to improve the insecticidal activity of Cry toxins from *Bacillus thuringiensis*. Peptides, 30: 589-595.

Pigott, C. R. and D. J. Ellar (2007). Role of receptors in *Bacillus thuringiensis* crystal toxin activity. Microbiology and Molecular Biology Reviews, 71: 255-281.

Salama, H. S. and M. M. Matter (1991). Tolerance level to *Bacillus thuringiensis* Berliner in the cotton leafworm *Spodoptera littoralis* Biosduval (Lepidoptera: Noctuidae). J. Appl. Entomol., 111: 225-230.

Sangadala, S. S., F. Walters, L. H. English and M. J. Adang (1994). A mixture of *Manduca sexta* aminopeptidase

and phosphatase enhances *Bacillus thuringiensis* insecticidal CryIA(c) toxin binding and $^{86}Rb^{+}$-K^{+} efflux *in vitro*. J. Biol. Chem., 269: 10088-10092.

Sanjay, S., I. N. Trivedi, R. Prasad, J. Kuruvilla, K. K. Rao and H. S. Chhatpar (2001). Aminopeptidase-N from the *Helicoverpa armigera* (Hubner) brush border membrane vesicles as a receptor of *Bacillus thuringiensis* Cry1Ac δ-Endotoxin. Current Microbiol., 43: 255-259.

Takesue, S., K. Yokota, S. Miyajima, R. Taguchi, H. Ikezawa and Y. Takesue (1992). Partial release of aminopeptidase N from larval midgut cell membranes of the silkworm, *Bombyx mori*, by phosphatidylinositol-specific phospholipase C. Comp. Biochem. Physiol., 102: 7-11.

Terra, W. R. and C. Ferreira (1994). Insect digestive enzymes: properties, compartmentalization and function. Comparative Biochemistry and Physiology, 109: 1-62.

Van Rie, J., S. Jansens, H. Hofte, D. Degheele and H. Van Mellaert (1990). Receptors on the brush border membrane of the insect midgut as determinants of the specificity of *Bacillus thuringiensis* δ-endotoxins. Appl. Env. Microbiol., 56: 1378-1385.

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

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

Valaitis, A. P., J. L. Jenkins, M. K. Lee, D. H. Dean and K. J. Garner (2001). Isolation and partial characterization of gypsy moth BTR-270, an anionic brush border membrane glycoconjugate that binds *Bacillus thuringiensis* Cry1A toxins with high affinity. Arch. Insect Biochem. Physiol., 46: 186-200.

Wolfersberger, M., P. Lüthy, A. Maurer, F. Parenti, V. Sacchi, B. Giordana and G. M. Hanozet (1987). Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the cabbate butterfly (*Pieris brassicae*). Comp. Biochem. Physiol., 86: 301-308.

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

Table (1): Sequence of unique oligo-nucleotides used in PCR amplification.

Primer	Primer sequence (5' to 3')	5' Position
F7	CAGAACTTGGTCCGAGAAGG	1
F_{ATG}	ATGGGTACCAAAATGTTGGTTCC	27
R7	AATATTATGCCAAATTGACAGCTAA	1597
F8	TCTTCTATTGCTGTGCGTGT	1528
R8	ATGTCTCTGTCGTGGGCTCT	2885
F9	ACCTTCATTTCCACCTACCC	410
R9	GATCAGCTAATTCAAGTTTTGG	1207
R10	TCACCATATCAGCAGCATTTTC	2380
T7	ATTATGCTGAGTGATATCCC	vector
SP6	ATTTAGGTGACACTATAGAA	vector

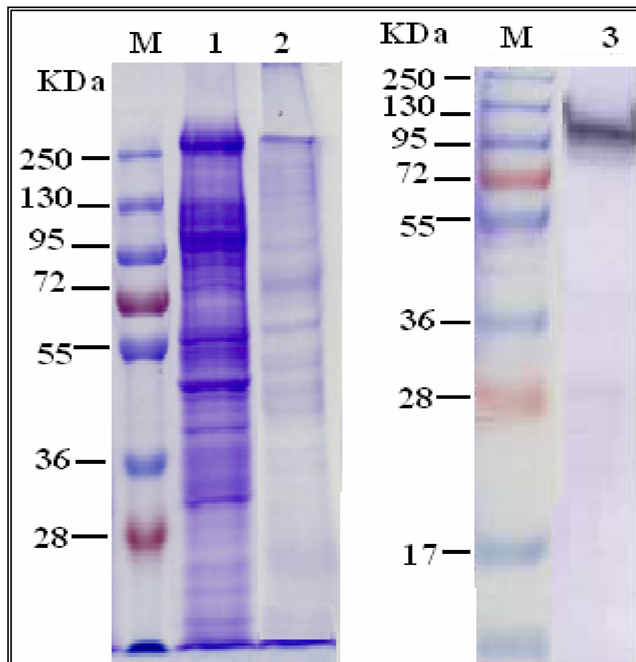
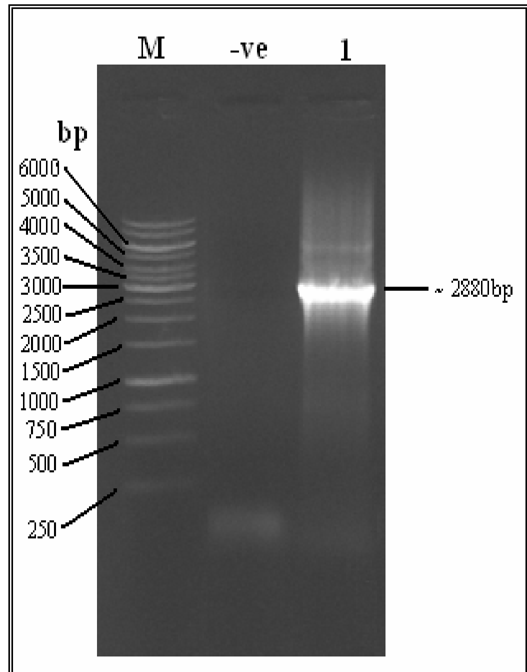


Fig. (1): SDS-PAGE profile of *Spodoptera littoralis* total protein midgut isolates (lane 1), brush border membrane vesicles preparation (lane 2) and immunoblot (lane 3). Lane M contained prestained molecular mass standards (numbers on the left are kilodaltons).

Fig. (2): RT-PCR with *S. litura apn* primers (lane1) on total RNA isolated from: midguts of 5th instar larvae beside a negative control (without RNA) (lane -ve). Lane M contained molecular mass standards 1kb (numbers on the left are bp).



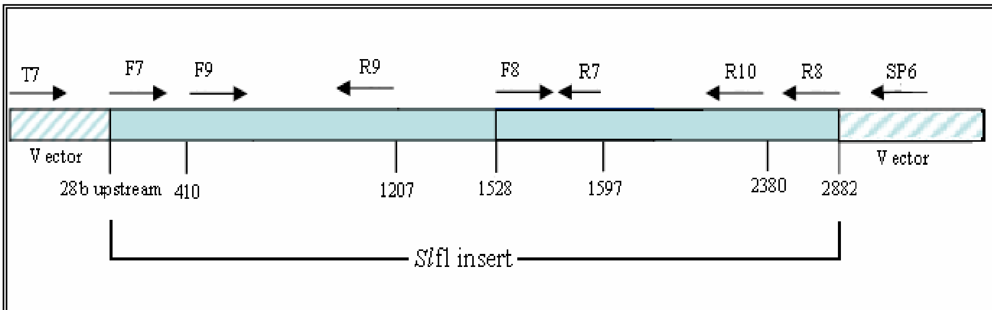


Fig. (3): Schematic for primers used in sequencing of full-length *APN* in the recombined plasmid pGEM T-Easy vector/*Sl*fl. Primer positions are indicated by arrows.

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   1 cactcatttg gtaccgttgg gcaaccATGG GTACCAAAAT GTTGGTTCCC
     GCTGTGCTTT GCGTTCTTCT GGGATTTGCT GCTGCCACTC CCTTAGAAGA
 101 CTTTCGGTCA AATTTGGAGT TCCTCGAGTA TTCCTCAAAT GTAGCTGATC
     CAGCTTACCG TCTCCGTTCA ATTGTGTACC CTACTGATGT CAAAGTAAAT
 201 CTAGAGAATA TAGACCTTGA AGGAGCTCGC TTCACAGGAT CAGTGGAGAT
     GATCGTTATT GTAAGAGAAA ACGATTTAGA GCAAATCTCC ATGCACCAGA
 301 ACAATCTCTT TGTCACCAGA GTCAATGTTG TTAACAACAC AAATGGTGAA
     AATGTTCAAC TGAGGTCCCC TGATCCCTTC ACCTATGACA ACTATTATGA
 401 ACTCCTTCAC CTTCATTTCC ACCTACCCAT CGTTGCCGGC TCCTACACCA
     TTACTATCGA CTACAGAGGC GTCATCAACA CAAACCCTCT CGACAGAGGC
 501 TTCTACAGAG GTTACTATTA CTACGAAAAT ACTCGCAGGT ACTACGCTAC
     CACACAGTTC CAACCCTACC ATGCCAGGAA AGCCTTCCCT TGCTTCGACG
 601 AGCCCCAGTT CAAGTCACGT TACACAATTT CCATCACCCG CCCCGACACT
     CTTGGCCCGT CTTACTCCAA CATGGCCATC AGCTCTACTG AAATTATCGG
 701 CAATAATGTT CGCGAAACAT TCTACCCAAC TCCCATCATT TCTGCCTACC
     TAGTGGCTTT CCATGTGAGT GATTTCGTGC CCACTGCTTC TACATCCACC
 801 GCTCGTAGAC CATTCAGTAT TATCTCTCGC CGAGGAGCGA CAGACCAACA
     CGCATATGCT GCCGAAATCG GTGTGGAAAT AACCAACCAA TTAGATGACT
 901 ACCTTGGCAT TGAGTACCAT GACATGGGAC AAGGGCCAAT TATGAAGAAC
     GATCATATTG CTCTGCCTGA CTTCCCGTCT GGTGCTATGG AAAACTGGGG
1001 AATGGTTAAC TACAGAGAGG CTTACCTTTT ATACGACCCT GCTAACACCA
     ACTTGGTCAA CAAGATATTC ATTGCAACCA TCATGGCTCA TGAACTGGGA
1101 CACAAATGGT TCGGTAACCT GGTCACCTGT TTCTGGTGGA GCAACCTTTG
     GCTAAACGAA TCTTTTGCTA GCTACTTCGA ATACTTTGTT GCGCACTGGG
1201 CTGATCCAAA ACTTGAATTA GCTGATCAAT TCATCGTTGA CTACGTGCAC
     AGTGCCCTCA ATGCAGACGC GAGTCCCTCG GCTACTCCTA TGGACTGGGA
1301 TGAGGTTGCA AATAATCCCA CAATAACGCA ACACTTTAGT ACTACCAGCT
     ATGCCAAGGG AGCTTCTGTT CTTAGAATGA TGGAGCATTT TGTTGGACCA
1401 AGAACCTTCC GTAATGCCCT CAGACATTAT TTGCGGGACA ACGCCTACGG
     CATCGGTACC CCCTCCCTGA TGTATCAAGC ATTTGATAAG GCCATCGCTG
1501 AAGATCATAC ATTTTTAAGT GATTTCCCGA ATATCAACTT TGGGAATGTA
     TTCGACAGTT GGGTTCAAAA CCGTGGCTCT CCAGTCGTGG AAGTAACTCG
1601 TGACCCAGAA AGTGGTGTTG TTGTTGTTGA ACAAAAACGT TACCAACTTT
     CTGGAGAACC TCCCACCCAA ACTTGGGAGA TCCCGCTCAG TTGGACCGAG
1701 CAGAAACATT TAGACTTCAG CTCGACCAAA CCCAGGCAAT TGTTAAATAT
     AACGTCTACG GCTCTTCTAA GTGAGGCAGG AGACAACTTT GTCATATTTA
1801 ACATTCAACA GTCTGGACTG TACCGTGTCA GATACGATGA AAACAACTGG
     AAAGCACTTG CATCGTACCT GAGCAGCAAC AACAGGGAAC GGATTCACAA
1901 ATTGAACAGA GCTCAAATTG TCAATGATGT GCTGCACTTC ATTCGCTCCG
     GGCATATCGA CAGGACTCTT GGCTTCGAAG TTATTGATTT CTTAAGAAGC
2001 GAAACTGACT ACTACGTATG GAACGGTGCT CTTACTCAGC TTGACTGGAT
     CCGACGTCGG TTGGAGCACA TGCCCAGAGC TCATGAAGCG TTCACTAGCT
2101 GCTTACATGG TCTTATGAAC AATGTTATCA ACCACCTCGG ATACAACGAA
     GGCCCCAACG ACTCTGCTTC CACAATCCTG AACAGAATTC AGATTTTGAA
2201 CTATGCCTGC AACATCGGGC ACAGCGGTTG CGTTTCTGAC AGTTTGCAGA
     AATGGAACGA TTACCAAGAA AACAACGAAC CGGTACCTGT GAGCCTTCGT
2301 CGCCATGTAT ATTGCACTGG TCTTCGTGAG GGTGATAGAT CTGACTATGA
     TTTCCTGTTC AATGCATACA ACGCTTCAGA AAATGCTGCT GATATGGTGA
2401 TCATGCTTCG AGCTCTTGCT TGCACCAAAG ATCTTGATGC ACTTGGACAT
     TACTTGCAAG AGAGCATGTA CAACGATAAG ATTCGTATCC ACGACCGTAC
2501 AAACGCCTTC AGTTTCGCTT TGCAAGGAAA CCTGGAAAAT GTACAATTCG
     TTAGCCGCTT CCTTCAAAGT AACTTCGATA CTATCAGAAC TACATATGGT
2601 GGTGAAGCTC GTCTAACCCT CTGCATCAAC GCCGTCGCCG CCTTCCTGAA
     CACGTTCCCA GCAATAACTG AATTCCAGAC GTGGGCCTAT GACAACCAAA
2701 TCGACTTAGC CGGGTCGTTC AACGCCGCTG TGAATGTAGT CAATAGTGCC
     ATGACCAATG TAGAATGGGG TAGCAATAAT GCGCTTGAAG TCTTCAATTT
2801 CGTTTCTGTT AGAAGCAACT CCCCTACCAT CTTCGCTTCA TCATTCCTCA
     TCCTTGCAGC TATGCTCATT CAGCTGTACC GTtaa
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Fig. (4): Complete cDNA nucleotide sequences of *Spodoptera littoralis* aminopeptidases N receptor (*SlAPN*) for the *B. thuringiensis* toxin. The open reading frame is in upper case letters. The ATG translation initiation codon (start codon) and TAA translation stop codon are bold and highlighted in grey.

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  1MGTKMLVPAV LCVLLGFAAA TPLEDFRSNL EFLEYSSNVA DPAYRLRSIV
 51YPTDVKVNLE NIDLEGARFT GSVEMIVIVR ENDLEQISMH QNNLFVTRVN
101VVNNTNGENV QLRSPDPFTY DNYYELLHLH FHLPIVAGSY TITIDYRGVI
151NTNPLDRGFY RGYYYYENTR RYYATTQFQP YHARKAFPCF DEPQFKSRYT
201ISITRPDTLG PSYSNMAISS TEIIGNNVRE TFYPTPIISA YLVAFHVSDF
251VPTASTSTAR RPFSIISRRG ATDQHAYAAE IGVEITNQLD DYLGIEYHDM
301GQGPIMKNDH IALPDFPSGA MENWGMVNYR EAYLLYDPAN TNLVNKIFIA
351TIMAH*E*LGH*K WFGNLVTCFW WSNLWLNE*SF ASYFEYFVAH WADPKLELAD
401QFIVDYVHSA LNADASPSAT PMDWDEVANN PTITQHFSTT SYAKGASVLR
451MMEHFVGPRT FRNALRHYLR DNAYGIGTPS LMYQAFDKAI AEDHTFLSDF
501PNINFGNVFD SWVQNRGSPV VEVTRDPESG VVVVEQKRYQ LSGEPPTQTW
551EIPLSWTEQK HLDFSSTKPR QLLNITSTAL LSEAGDNFVI FNIQQSGLYR
601VRYDENNWKA LASYLSSNNR ERIHKLNRAQ IVNDVLHFIR SGHIDRTLGF
651EVIDFLRSET DYYVWNGALT QLDWIRRRLE HMPRAHEAFT SCLHGLMNNV
701INHLGYNEGP NDSASTILNR IQILNYACNI GHSGCVSDSL QKWNDYQENN
751EPVPVSLRRH VYCTGLREGD RSDYDFLFNA YNASENAADM VIMLRALACT
801KDLDALGHYL QESMYNDKIR IHDRTNAFSF ALQGNLENVQ FVSRFLQSNF
851DTIRTTYGGE ARLTLCINAV AAFLNTFPAI TEFQTWAYDN QIDLAGSFNA
901AVNVVNSAMT NVEWGSNNAL EVFNFVSVRS NSPTIFASSF LILAAMLIQL
951YR
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Fig. (5): Translated sequence of the full length of *APN* gene amplified with primers F7 and R8 from *S. lituralis*. The 2,856-bp cDNA clone encodes a 952-amino-acid polypeptide. The start methionine is in bold. The putative NH2-terminal cleavable peptide and the GPI signal peptide at the COOH terminus are double underlined. A conserved gluzincin residue is indicated with a dotted line while zinc-binding/gluzincin motif is marked with asterisks. The putative N-glycosylation sites are underlined. The four Cys residues that are conserved among eukaryotic aminopeptidases are in boldface. The conserved amino acid residue for Cry1A toxin-binding regions is highlighted in grey.