A novel aminopeptidase in the fat body of the moth *Achaea janata* as a receptor for *Bacillus thuringiensis* Cry toxins and its comparison with midgut aminopeptidase

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Bacillus thuringiensis insecticidal crystal proteins bind to cell-surface receptors which represent a family of aminopeptidases [APN (aminopeptidase N)] present on the brush border membrane of insect midgut cells of susceptible insects leading to pore formation and death of the insect. We report here for the first time the presence of a novel APN in the fat body of the moth *Achaea janata*. Northern blotting detected at least one APN-specific transcript in the fat body, whereas two transcripts of different sizes were detected in the midgut. We have cloned two full-length APN cDNAs of 3015 bp and 2850 bp from fat body and midgut respectively, which encode proteins of 1004 and 950 amino acids. These two APNs share only 33 % amino acid sequence identity, but both display the typical APN features, such as the N-terminal signal peptide, several putative glycosylation sites, C-terminal glycosylphosphatidylinositol anchor signal, the APN-

specific zinc-binding/gluzincin motif HEXXH₁₈E and gluzincin motif GAMENWG. The fat body APN manifested a variation in its expression with respect to tissue and developmental stage. In spite of the abundance of the APN transcript in the fat body, fairly low APN activity was detected in this tissue. The fat-body- and midgut-specific APNs showed differential interaction with various Cry1A toxins. Besides, the level of toxicity of different Cry subtypes varied enormously with mode/site of delivery, such as intrahaemocoelic injections and feeding bioassays. These data indicate that the fat body might be a potential alternative Cry toxin target site in the moth.

Key words: aminopeptidase N (APN), Cry receptor, fat body, glycosylphosphatidylinositol (GPI) anchor, haemolymph.

INTRODUCTION

Development of more effective and environmentally safe pesticides with novel modes of action is a never-ending process. *Bacillus thuringiensis* ssp. *kurstaki* is by far the most successful microbial preparation. An in-depth understanding of its interaction with the host is critical for the development of new *B. thuringiensis* strains and their rational applications. Identification of new targets, characterization of receptors and their genes in the targets, which are known to be involved in insect–pathogen interactions are necessary to overcome the problem of pesticide resistance [1].

Membrane-bound APNs (aminopeptidase Ns) are well-characterized ectoenzymes that are distributed widely in animal tissues and are implicated in a variety of functions, including acting as receptors, cell adhesion molecules and signal transduction components [2].

B. thuringiensis is a Gram-positive, entomopathogenic, sporulating bacterium, which produces several kinds of insecticidal crystalline proteins. Cry proteins are produced as pro-toxins, which upon ingestion are solubilized and proteolytically activated in the midgut of susceptible insects [3]. The activated toxin binds to specific receptors in the brush border of midgut epithelial cells and subsequently creates pores in the cell membrane, which lead to cellular swelling, lysis and eventually insect mortality [4]. Membrane-bound APN [5,6], cadherins [7], glycolipids [8] and alkaline phosphatase [9,10] have been identified as putative Cry

toxin receptors. Previously, a model of the sequential participation of cadherin receptor and APN leading to Cry1A toxin insertion into lipid rafts was proposed [10].

APN is the most extensively studied Cry receptor identified and isolated from several lepidopteran insects. APN genes have been isolated from the midgut of a variety of insects and several *in vitro* as well as *in vivo* studies have authenticated the role of APN in *B. thuringiensis* pathogenesis. Insects in which the APN gene was inactivated either by natural mutations [11] or by genetic engineering methods become resistant to *B. thuringiensis* toxins, thus confirming the critical role of APN in *B. thuringiensis* toxicity [6,12]. Different APN isoforms in the midgut can interact differentially with various subtypes of the Cry toxins [13]. *B. thuringiensis* toxins were also shown to be lethal to lepidopteran larvae when injected into the haemocoel [14]. However, no information is available on the molecular targets of *B. thuringiensis* toxins in the insect larval body cavity. Cheon et al. [15] have demonstrated pore forming cytolytic ability of Cry toxins on the *in vitro* cultured fat body cells indicating the presence of Cry-binding proteins in the fat body. Since the fat body is a pivotal metabolic organ in the body cavity of insects and plays important roles during post-embryonic, as well as adult development stages, its possible interaction with the Cry toxins deserves attention. Another report by Hall [16] demonstrated the occurrence of APNs in other insect organs in addition to the midgut. We undertook a search for APN in the larval fat body of the economically

Abbreviations used: APN, aminopeptidase N; AjFbAPN, *Achaea janata* fat body APN; AjMgAPN, *Achaea janata* midgut APN; ALP, alkaline phosphatase; BCIP, 5-bromo-4-chloroindol-3-yl phosphate; BBMV, brush border membrane vesicles; CRD, cross-reacting determinant; GalNAc, N-acetylgalactosamine; GPI, glycosylphosphatidylinositol; GNA, *Galanthus nivalis* agglutinin; GST, glutathione S-transferase; NBT, Nitro Blue Tetrazolium; ORF, open reading frame; PI-PLC, phosphatidylinositol phospholipase C; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; SBA, soybean agglutinin; TBS, Tris-buffered saline; WGA, wheat germ agglutinin.

The nucleotide sequence reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number DQ444715 for AjFbAPN and DQ872666 for AjMgAPN.

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important pest *Achaea janata* (Castor semilooper), a noctuid moth that causes serious damage and loss of castor crop in large areas of Asia.

In the present study, we have demonstrated the presence of a novel APN from the larval fat body and have cloned the corresponding cDNA, AjFbAPN (*A. janata* fat body APN). The fat body APN showed low homology to the midgut-specific APN cDNA, AjMgAPN (*A. janata* midgut APN). 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.

EXPERIMENTAL

Insect culture

A. janata larvae were obtained from the Directorate of Oil Seeds Research (Hyderabad, India) and maintained in the rearing facility of our laboratory. The larvae were reared on fresh castor leaves at $25 \pm 1^\circ\text{C}$ and 70% relative humidity with a photoperiod of 12 h light and 12 h dark. Early-fifth-instar *A. janata* larvae were chilled on ice for 15 min and dissected to isolate the midgut, fat body and other tissues.

Injection assay on *A. janata*

Individual activated Cry toxins were injected into the body cavity of fifth instar larvae following the method described by Cerestians et al. [14]. The larvae were maintained individually in plastic petri plates with castor leaves until analysed.

Feeding bioassay on *A. janata*

Second instar larvae were released into plastic petri plates containing castor leaves coated with a known quantity of activated Cry toxins and allowed to feed for 48 h. Subsequently, mortality was calculated and LC_{50} value was determined by probit analysis (SPSS, version 8).

B. thuringiensis toxin purification

Cry1Aa, Cry1Ab and Cry1Ac protoxins were produced from recombinant *Escherichia coli* ECE52, ECE53 and ECE54, which harbour *cry1Aa*, *cry1Ab* and *cry1Ac* genes respectively, provided by the Bacillus Genetic Stock Center (Ohio State University, OH, U.S.A.). Activated toxins were prepared by proteolytic activation of the protoxins with trypsin and purified according to the method of Lee et al. [17].

Cloning of APN cDNAs from the fat body and midgut of *A. janata*

Total RNA was isolated with the TRI[®] Reagent (Sigma) from the fat body and midgut of fifth instar larvae. The cDNAs were synthesized from 5 μg of total RNA using superscript[™] II RNaseH⁻ RT (reverse transcriptase) and oligo d(T)₁₂₋₁₈ primer (Life Technologies). Degenerate primers used for PCR amplifications were designed from the conserved regions of previously published insect APN genes. The forward primers F1 and F2 were designed based on GAMENWG (5'-GGN GCNATGGA-RAAYTGGGG-3') and AFPCYDEP (5'-GCTTTYCCYTGYTAYGAYGARCC-3') amino acid sequences respectively (the corresponding nucleic acid sequences are in parentheses), while the reverse primers R1 and R2 were designed based on YRVNYD (5'-ATCGTAGTTACGCGGTA-3') and HMWFGN (5'-TTNCCGAACCACATRTG-3') sequences. The respective PCR amplified products were cloned into the pTZ57R-T vector (MBI Fermentas) and sequenced. Based on the nucleotide sequence of these APN fragments, specific primers were designed

further for 5'- and 3'-RACE (rapid amplification of cDNA ends) using Gene Racer[™] kit (Life Technologies). The PCR amplified RACE fragments were cloned, sequenced and aligned to obtain full-length APN cDNAs from fat body and midgut.

Sequence alignment and analysis

The complete cDNA sequences and their putative translation products were analysed for multiple alignments with other reported insect APNs assembled in a dendrogram using the CLUSTAL X program. N- and C-terminal GPI (glycosylphosphatidylinositol) anchor signal sequences were identified with the program PSORT II (<http://psort.nibb.ac.jp>) and GPI prediction program DGPI (http://129.194.185.165/dgpi/index_en.html) respectively. The sequences were also analysed for the presence of N- and O-glycosylation sites using NetOGlyc2.0 program (<http://www.cbs.dtu.dk/services/netOGlyc>).

Expression of truncated APN in *E. coli*, purification of recombinant proteins and generation of polyclonal antibodies

The APN-specific PCR fragments obtained from fat body (550 bp) and midgut (850 bp), as described earlier, were PCR-amplified with degenerate primers using High Proof[™] Taq (Qiagen) to generate blunt-end fragments. These were cloned into the SmaI site of the pGEX-5X-2 expression vector in-frame with the GST (glutathione S-transferase) tag (Amersham Pharmacia) and transformed into *E. coli* BL21 (DE3) PlysS (Novagen). Expression of recombinant truncated APN-GST proteins were induced with 1 mM IPTG (isopropyl β -D-thiogalactoside) and analysed by SDS/PAGE. Recombinant fat body (46 kDa) and midgut (58 kDa) APN proteins were detected in the inclusion bodies, which were solubilized and purified using a GST affinity column (Amersham Pharmacia). Antibodies were generated against GST-tag cleaved APNs in 6-month old male New Zealand white rabbits using standard procedures [18]. The antisera were labelled as anti-MgAPN and anti-FbAPN respectively.

Northern blotting

Total RNA was isolated from the fat body and midgut of the fifth instar larvae and 20 μg of RNA from each sample was denatured and subjected to electrophoresis on a 1% formaldehyde/agarose gel with $1 \times$ Mops buffer (0.2 M Mops, 50 mM sodium acetate, 1 mM EDTA, pH 7.0 with NaOH). The separated RNA was transferred to Hybond N+ membrane (Amersham Pharmacia) by the capillary blotting method. The fat-body- and midgut-specific APN PCR fragments (550 and 850 bp respectively) were labelled with [$\alpha^{32}\text{P}$]ATP using a random-primed hexalabelling kit (MBI Fermentas). Northern hybridization was carried out using standard procedures [18].

Ligand and lectin blot analysis of the membrane proteins

The BBMV (brush border membrane vesicles) were prepared by the method of Wolfersberger et al. [19], whereas the fat body membrane preparation was carried out according to the method of Kirankumar et al. [20]. 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 blocking buffer [3% (w/v) BSA in TBS (Tris-buffered saline)], the blots were incubated in the blocking buffer containing either primary antibodies raised against APNs for Western blotting or biotin-labelled Cry toxins [100 μg of each toxin were biotinylated using the protein biotinylation module kit (Bangalore Genei, India) and then purified with Sephadex G25 columns] for ligand blot analysis or appropriate lectins for

lectin blot analysis. After incubation, blots were washed with TBS containing Tween 20 and then incubated for 2 h in blocking buffer containing either ALP (alkaline phosphatase)-conjugated goat anti-rabbit IgG or streptavidin-ALP conjugate. APNs, bound toxins or lectins were detected using the ALP substrate BCIP (5-bromo-4-chloroindol-3-yl phosphate)-NBT (Nitro Blue Tetrazolium). Dilutions of antibodies used for Western blots were 1:10 000 for anti-FbAPN and 1:8000 for anti-MgAPN, and the lectins, SBA (soybean agglutinin) and WGA (wheat germ agglutinin), were used at 1 µg/ml.

Immunoprecipitation of Cry-binding proteins

Triton X-100 solubilized fat body 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 MgCl₂) for 3 h at 4°C, followed by incubation with 5 µl of Cry1Aa antisera for a further 3 h. Subsequently, 100 µl of Protein A-Sepharose 4B was added and incubated for a further 2 h at 4°C on a rotary shaker. The Sepharose beads were pelleted, washed six times with PBS buffer, resuspended in 20 µl of SDS sample buffer containing 2-mercaptoethanol [125 mM Tris/HCl (pH 6.8), 4 % (w/v) SDS, 20 % (v/v) glycerol, 10 % (v/v) 2-mercaptoethanol and 0.002 % Bromophenol Blue] and heated at 100°C for 5 min. Eluted proteins were subjected to SDS/PAGE (7.5 % gels) and electrotransferred on to nitrocellulose membranes. The membranes were then incubated with anti-APN antibodies followed by ALP-conjugated goat anti-rabbit IgG antibodies and developed with BCIP-NBT.

GPI anchor detection with anti-CRD (cross-reacting determinant) antibody

The larval fat body and the midgut membrane preparations were digested with 5 units of PI-PLC (phosphatidylinositol phospholipase C; Molecular Probes) per 10 ml at 30°C for 90 min and subjected to SDS/PAGE. Proteins were electrotransferred on to nitrocellulose membranes and the presence of cleaved GPI epitope was detected using 1:200 dilution anti-CRD antibody [21].

APN 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}).

RESULTS

Cloning and characterization of APN cDNAs from fat body and midgut tissues of *A. janata*

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. Primer pair F2/R2 amplified an approx. 550 bp fragment from fat body, whereas primer pair F1/R1 amplified an 850 bp fragment from midgut, both of which were cloned and sequenced. Both of the sequences proved homologous to APN sequences reported from other insects. The 3'- and 5'-RACE of fat body APN cDNA yielded a 2 kb and a 0.6 kb fragment respectively. Nucleotide sequence alignment of all these fragments revealed that the fat body APN cDNA is 3086 bp long and contains an ORF (open reading frame)

of 3015 bp, which encodes a putative 1004 amino acid protein with a theoretical molecular mass of 113 kDa and a calculated pI of 4.83. For midgut APN, the 3'- and 5'-RACE reactions produced 1.7 kb and 0.5 kb fragments respectively, which were cloned and sequenced. The full-length midgut APN is 3053 bp long and contains 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 sequences for fat body and midgut APNs were designated as AjFbAPN and AjMgAPN. The nucleotide sequences have been deposited in the GenBank®/EBI Data Base under accession numbers DQ444715 (AjFbAPN) and DQ872666 (AjMgAPN). Detailed computational analyses of the deduced amino acid sequences of both the cDNAs were carried out to detect the presence of signature sequences (Figure 1). The SignalP program revealed the presence of a cleavable signal peptide, M¹ASRWFNLLL-GVLFIQGYLAF²¹, with a cleavage site between Ala²⁰ and Phe²¹ at the N-terminal region in AjFbAPN, whereas a peptide, M¹QLLIILSILVGSLSVAI¹⁷, with a cleavage site between Ala¹⁶ and Ile¹⁷ was detected in AjMgAPN (Figure 1). 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, D⁹⁷²SA⁹⁷⁴, followed by a stretch of predominantly hydrophobic residues: S⁹⁷⁵IATL-SFVTLFVTLALNLAXKLLSYIYDSL¹⁰⁰⁴. In the case of AjMgAPN, it consists of three serine residues, S⁹²⁸SS⁹³⁰, followed by the hydrophobic stretch: T⁹³¹TFVASTILMLAAMFTHLLR⁹⁵⁰ (Figure 1). Interestingly, we could identify up to 22 potential O-glycosylation sites and one potential N-glycosylation site in AjFbAPN, whereas only four of each N- and O-glycosylation sites were detected in AjMgAPN. The consensus zinc-binding/gluzincin motif (HEXXHX₁₈E) and the gluzincin APN motif (GAMEN) are found in all APNs and their presence in the amino acid sequences of AjFbAPN and AjMgAPN (Figure 1), along with the other features allowed us to classify both of these *A. janata* APNs as members of the APN family. The amino acid sequence surrounding gluzincin Zn²⁺-binding motifs (HEXXHX₁₈E) was fairly well conserved in both sequences. However, the hydrophobic N- and C-termini of the two APN sequences were significantly different from each other.

The amino acid sequence comparison by multiple sequence alignment, however, revealed only 33 % similarity between AjFbAPN and AjMgAPN, implying that they are significantly different from each other (Figure 1). Based on maximum amino acid sequence homology among the reported insect APNs, a phylogenetic tree was constructed. As shown in Figure 2, AjFbAPN and AjMgAPN belong to different classes. AjFbAPN showed a maximum homology of 70 % to *Lymantria dispar* APN4 (GenBank® accession number AF317620) and 64 % to *Bombyx mori* (AF084257), whereas the AjMgAPN manifested highest homology of 73 % to *L. dispar* APN3 (AF317619) followed by 68 % similarity to *Helicoverpa armigera* (AF535165).

Detection of tissue-specific expression of the APNs

The tissue specificity of the identified APNs was determined by Northern blot analysis of total RNA from fat body and midgut tissues using the fat-body- (550 bp) and midgut- (850 bp) specific APN fragments as probes. As shown in Figure 3(A), the fat-body-specific APN probe detects expression of a single transcript of approx. 3.5 kb in the fat body tissue (lane 1), whereas it recognizes two different transcripts of approx. 4.0 and 5.5 kb in the midgut tissue (lane 2). In contrast, as shown in Figure 3(B), the midgut APN-specific probe fails to recognize any transcript in the fat body (lane 1), while it detects the same transcripts of approx. 4.0 and 5.5 kb mentioned earlier in the midgut tissue (lane 2).

AjFbAPN	MASRWENLLLGVLFIQGYLAFSP ⁺ PIPEERLMDEEWVEYNSMLRDPAYRLPTTTREFSHYAVT	60
AjMgAPN	-----MQLLIILSILVGS ⁺ LVAIFQ-EDFRSNLEFLEYDSNLGEQHYRLVDTVYPHTMDVD	54
AjFbAPN	LTPYIESVPTGV ⁺ TADLFTFDGEATMTIQATEANVNEIRLHCNDLTILELTVHVA ⁺ DTLVN	120
AjMgAPN	LDVYLDEL ⁺ R-----FNGFVSIGVEVRESGLTQIALHQKVQSIQXVNL ⁺ LTAGAPVP	105
AjFbAPN	LATPGQTYECVMPWSFLTIPLTTTLNLTNQYVVR ⁺ SRFRGNLQTN--MRGFYRSWYVDSTG	178
AjMgAPN	LLIS-EPFTTDDYYELIKINLSAIPAG-NYITITIRYTGVINENPLDRGFYKGYFLNN-	162
AjFbAPN	NRRWMGT ⁺ TQFQPGHARQAFPCYDEHGFKARFDITIVRSPTFSPTLSNMPILSTTTLTNGW	238
AjMgAPN	QERAYATTQFQPYHARKAFPCFDEHMFKSRYTLAITRDINLSPSYSNMAISATVPVGTR	222
AjFbAPN	VAETFHTSTVTSTYLLAFIVSHYERVASSTDPERPFYIYARDNVGDTGEWSLEIGEKLLL	298
AjMgAPN	VREEFYPTPIISAYLVAHFVSDFVETEITSTAARPFKIISRPGVIDQHEYAAEIGLKITN	282
AjFbAPN	AMEEYTGYPY ⁺ YSMTENIIMQ--QAAIPDFSAGAMENWGLLTYREALILYDRLNSNHFYRQ	356
AjMgAPN	ELDDFLGIQYHEMGQGVLMKNDHIALPDPFSGAMENWGMVNYREAYLLYDPDNTNIINKI	342

AjFbAPN	RVANIVSHEIAHMF ⁺ GKLVTCAWWDNLWLN ⁺ EGFARFYQYFLTDSVDET ⁺ LGYRTREITEQL	416
AjMgAPN	FIATIMAH ⁺ ELGHKWF ⁺ GNLVT ⁺ CFWWSNLWLN ⁺ ESYASYFEYFGTHWADLAL ⁺ LEDEQFVVDYV	402

AjFbAPN	QVALLSD ⁺ SEDSAHALTNP ⁺ AVNTPTTVSAHFSTITYAKGA ⁺ AVLKMTQYLLGEKTYRRGLQS	476
AjMgAPN	HSALAADAGAGATPMNV ⁺ VDVADNPSVTS ⁺ SHFSTTSYAKGASVLRMEHFVGT ⁺ TRNFNALRY	462
AjFbAPN	YLQANQYDVAEPEDLFSALDAAAVVDNALAGY--ITIEEYMKTWSEQAGH ⁺ LLSVSDH	534
AjMgAPN	YLRDNAYEVGYPIDMYDAFRQAVSEDTYFLQQYPNVDVGAVFESWVENPGSEV ⁺ VNINVNH	522
AjFbAPN	STGNMVVTQHRWN ⁺ VNTGVSAISSLWHV ⁺ EITWTRAGAVDFDNLKPTQILSGTATSINRGST	594
AjMgAPN	ATGAISISQQRY-IVTD ⁺ TQRFNNIWQIPLTWTDQ ⁺ RS ⁺ LDFTNTRPSRVLT ⁺ TATDSITT-EA	580
AjFbAPN	GREWVIFNKQ ⁺ QSGFYRVNYDS ⁺ DTWALITQALRDSNSRTQIHEY ⁺ NRAQIVDDVFILARAGV	654
AjMgAPN	GDYWVLENVAQSSLYRVNYNDRN ⁺ WELLADYLKSSN-RERIH ⁺ YLNRAQIVNDLLYFVRSGD	639
AjFbAPN	LTYTRAFN ⁺ ILSFLEFEDQYAPWDAAITGFNFSRRRLAHNTESLEQLHALIYKLSEAVTRR	714
AjMgAPN	VSAEVAENVLD ⁺ FLRYETNYVYVNGALAQIDFLRRRFEHL ⁺ PNAHTLFSNYILELMDTVIQH	699
AjFbAPN	LGFAEIEGESYMDGLLRMYVNTFLCNVGH ⁺ EECVQAGR ⁺ TAFANWKN ⁺ SGTFIPANMRPWVYC	774
AjMgAPN	LGFEERATDSTSTILNRMQILNYACNLGHSGCVLD ⁺ SONK ⁺ WRELRENNVAVFVNLRRYVYC	759
	♦ ♦ ♦ ♦	
AjFbAPN	TGLRYGDASDFDFWQQY ⁺ LATDLASEQVVKLQAAGCTIDEASLGRYLD ⁺ AITGGADDYQIR	834
AjMgAPN	IGIREGDN ⁺ TDYNFLNLYESS ⁺ ENTADMVIMLRALACTKDEALLNSYLGQSLT---NRKIR	816
	♦	
AjFbAPN	DQDIATLSSAITANEVNTMRAF ⁺ NWLTNNVERTTALG---SIQTP ⁺ LS ⁺ TITQRLNTEQI	891
AjMgAPN	IHDRTNAWSYALQGNPENLPVVLN ⁺ YLYQNFQIRTDYGGPARLTLAISALSTY ⁺ TD ⁺ FN ⁺ TI	876
AjFbAPN	NTVSAWLQANSASLGTDIYNVGLSGIATSQNNIAWYNQRISEYNSYFENG ⁺ YIDETFEDEQ	951
AjMgAPN	QEYQAWAYENQEELDESFG-TAVAVVNA ⁺ AINNLVWGNANAPEIYN ⁺ FLLER-----	925
AjFbAPN	TPPAATTEAPTTEADAGTTPDSASIA ⁺ TLSFVTLFVTLALN ⁺ LAXKLLSYIYDSL	1004
AjMgAPN	-----TVSSSTTFVASTILMAAMFTHLLR--	950

Figure 1 Comparison of AjFbAPN and AjMgAPN amino acid sequences

The deduced amino acid sequences from the AjFbAPN and AjMgAPN cDNAs were aligned by Clustal W multiple sequence alignment tool. Gaps are introduced in the sequences to maximize the alignment. The amino acid residues conserved between the two sequences are highlighted in 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, asterisks (*) denote the canonical metal (zinc)-binding motif, closed circles (●) indicate the GAMEN motif. The four cysteine residues, which are highly conserved in all the eukaryotic APNs are marked with closed diamonds (♦) at positions 739, 746, 774 and 810. The large boxed area is the region corresponding to the Cry1A toxin binding region of AjFbAPN and AjMgAPN.

Immunoblot analyses of tissue homogenates

As described in the Experimental section, anti-FbAPN and anti-MgAPN antisera were generated using the recombinant proteins encoded by the fat-body-specific 550 bp and midgut-specific 850 bp cDNA fragments respectively. Initially the specificity of the antibodies was checked by immunoblot analysis using

E. coli-expressed respective recombinant proteins. In order to determine the tissue specificity of the APNs in *A. janata*, Western blot analysis was carried out. The anti-FbAPN antiserum recognized a 113 kDa protein in the fat body (Figure 4A, lane 1) and cross-reacted with a 170 kDa protein in the midgut, (Figure 4A,

Figure 2 Phylogenetic relationships of AjFbAPN, and AjMgAPN with other insect APNs

The amino acid sequences of the two APNs were aligned with those of other APN family members in order to deduce their phylogenetic relationship. The tree was calculated with the ClustalX 1.83 program. AjFbAPN (DQ444715); AjMgAPN, (DQ872666); PxAPN1, *P. xylostella* APN1 (X97878); PxAPNA, *P. xylostella* APNA (AF020389); PxAPN3, *P. xylostella* APN3 (AJ222699); LdAPN1, *L. dispar* APN1 (AF126442); Ld APN2, *L. dispar* APN2 (AF126443); Ld APN3, *L. dispar* APN3 (AF317619); LdAPN4, *L. dispar* APN4 (AF317620); MsAPN1, *M. sexta* APN1 (X89081); MsAPN2, *M. sexta* APN2 (X97877); BmAPN1, *Bo. mori* APN1 (AF084257); BmAPN2, *Bo. mori* APN2 (AB011497); HvAPN170, *H. virescens* 170kDa APN (AF173552); HvAPN120, *H. virescens* 120kDa APN (U35096); HpAPN1, *H. punctigera* APN1 (AF217248); HpAPN2, *H. punctigera* APN2 (AF217249); HpAPN3, *H. punctigera* APN3 (AF217250); PiAPN1, *P. interpunctella* APN1 (AF034483); HaAPN1, *He. armigera* APN1 (AF521659); HaAPN2, *He. armigera* APN2 (AF521660); SIAPN1, *S. litura* APN1 (AF320764); EpAPN1, *E. postvittana* APN1 (AJ222699); BmAPN3, *Bo. mori* APN3 (AAL83943); BmAPN4, *Bo. mori* APN4 (BAA33715); TniAPN1, *T. ni* APN1 (AY836579); TniAPN2, *T. ni* APN2 (AY836580); TniAPN3, *T. ni* APN3 (AY836581); TniAPN4, *T. ni* APN4 (AY836582). *sequences reported in this study.

Figure 3 Northern blot analysis of APN mRNA in fat body and midgut tissues of *A. janata*

RNA (20 μ g) from fat body (lane 1) and midgut (lane 2) tissues were denatured and subjected to electrophoresis on a 1% (w/v) agarose gel containing formaldehyde. The separated RNAs were transferred on to Nylon-N+ membrane and hybridized with the 32 P-labelled probes: (A) the fat-body-specific 550 bp fragment; and (B) the midgut-specific 850 bp PCR fragment. Equal loading control is shown by ethidium bromide stained RNA in the bottom panel.

Figure 4 Immunoblot analysis of APNs in different tissues of *A. janata*

Tissue homogenates (15 μ g) were separated by SDS/PAGE (7.5% gels) and transferred on to nitrocellulose membranes. For immunoblot analysis the blots were either incubated with anti-FbAPN (A) or anti-MgAPN (B) antibodies. Lane 1, fat body; lane 2, foregut; lane 3, anterior midgut; lane 4, posterior midgut; lane 5, hindgut; and lane 6, salivary gland. Positions of protein molecular mass markers are indicated to the left-hand side of the Figure.

lanes 2–5), this could be due to epitope similarity. The presence of slow and fast mobility forms (doublet) of approx. 113 and 170 kDa APNs on immunoblots probably results from the cleavage of GPI anchor by endogenous phospholipases [22]. Lu and Adang [23] reported a similar phenomenon for GPI anchored 120 kDa toxin-binding APN in *Manduca sexta*. On the other hand, the anti-MgAPN antiserum recognized only the 113 kDa protein, but not the 170 kDa protein in different regions of the midgut (Figure 4B, lanes 2–5), whereas it revealed a weak cross-reactivity for the 113 kDa protein of the fat body (Figure 4B, lane 1). The variations in the size of the detected APN proteins may be due to differential glycosylation patterns. The 113 kDa, as well as the 170 kDa proteins, were found to be located mostly in the anterior and posterior regions of the midgut (Figures 4A and 4B; lanes 3 and 4), but not in the foregut and hindgut (Figures 4A and 4B, lanes 2 and 5). None of these antibodies, however, could detect any APN protein in the salivary gland (Figures 4A and 4B, lanes 6).

Cry toxin overlay assay

The interaction of different Cry toxins with the fat body membrane and BBMV proteins was studied by ligand blot analysis. Differential binding patterns were observed with different subgroups of Cry1A toxins. The protein profile of the fat body membrane preparation was observed by Coomassie Blue staining (Figure 5, lane 2). As shown in Figure 5, all the three Cry toxins, Cry1Aa (lane 4), Cry1Ab (lane 5) and Cry1Ac (lane 6) interacted with the 113 kDa protein in the fat body membrane. The absence of any signal in the control reaction, where membrane was not incubated with biotin-labelled toxin prior to probing with streptavidin ALP (Figure 5, lane 3) suggests a specific interaction between 113 kDa fat body membrane protein and the Cry toxins. On the other hand, analysis of BBMV protein interaction with Cry toxins, as shown

Figure 5 Cry toxin overlay assay with fat body membrane proteins

Aliquots of 15 μ g of fat body membrane proteins per lane were separated by SDS/PAGE (7.5 % gels) and transferred on to nitrocellulose membranes. The blots were separately incubated with any one of the biotin labelled activated Cry1 toxins (100 ng/ml) and detected with streptavidin-ALP conjugate. Lane 1, protein molecular mass markers; lane 2, Coomassie Blue stained fat body membrane proteins; lane 3, protein blot incubated with unlabelled Cry1Aa as control; lanes 4–6, protein blots incubated with biotinylated toxins, Cry1Aa (lane 4), Cry1Ab (lane 5), and Cry1Ac (lane 6).

in Figure 6 reveals that while Cry1Aa (Figure 6A) and Cry1Ac (Figure 6C) interact with both 113 and 170 kDa membrane proteins, Cry1Ab interacts only with the 113 kDa protein (Figure 6B). As shown in Figure 6, most of the toxin-binding proteins are present in the anterior and posterior regions of the midgut (lanes 2 and 3), while foregut and hindgut tissues barely show any toxin binding (lanes 1 and 4). This observation corroborates the immunoblot analysis described in the previous section (Figures 4A and 4B, lanes 3 and 4), where APNs were mostly detected in the anterior and posterior regions of the midgut.

Identification of the glycan moieties present on APN by lectin blotting

In order to investigate the biochemical properties of the newly identified midgut and fat body APNs, we studied the oligosaccharides present on them by lectin blot analysis using SBA and WGA. Strong binding of the GalNAc (*N*-acetylgalactosamine)-specific SBA lectin to the 113 kDa protein in the fat body membrane (Figure 7A, lane 1), as well as to the 113 and 170 kDa proteins in the midgut (Figure 7A, lane 2) was observed. On the other hand *N*-acetylglucosamine-specific WGA cross-reacted with the 113 and 170 kDa proteins present in the midgut (lane 4), but did not bind to the 113 kDa protein in the fat body (lane 3). Following lectin blotting, these membranes were incubated with the anti-FbAPN and anti-MgAPN antisera to confirm that the lectin-binding proteins were actually the APNs being investigated (results not shown). Further, we examined if the oligosaccharide moieties present on the APNs had a role in their interaction with Cry1Ac toxins.

Immunoprecipitation of Cry toxin binding proteins

The interactions of the fat body and midgut APNs with the Cry1Aa toxin were further confirmed by co-immunoprecipitation experiments. Detergent-solubilized fat body membrane preparation and BBMV were incubated with Cry1Aa toxin, and the toxin protein complexes were subsequently precipitated by anti-Cry1Aa

Figure 6 Cry toxin overlay assay with midgut proteins

Aliquots of 15 μ g of BBMV proteins per lane were separated by SDS/PAGE (7.5 % gels) and transferred on to nitrocellulose membranes. These blots were separately incubated with 100 ng/ml biotin labelled activated Cry1Aa (A) and Cry1Ab and Cry1Ac (B) toxins and detected with streptavidin-ALP conjugate. Lane 1, foregut; lane 2, anterior midgut; lane 3, posterior midgut; and lane 4, hindgut.

antibody using Protein A-Sepharose beads. Western blot analysis of immunoprecipitated proteins by anti-FbAPN and anti-MgAPN antisera identified the 113 kDa immunoprecipitated APNs in both fat body (Figure 8A, lane 2) as well as midgut (Figure 8B, lane 2), suggesting that this 113 kDa APN interacts with Cry toxins. As shown in Figure 8(B), lane 2, Cry1Aa toxin was able to interact with multiple APNs, for example, both 113 and 170 kDa proteins, as the midgut is known to have multiple APN isoforms. The control immunoprecipitation reactions were carried out by eliminating the prior incubation of membrane proteins with Cry toxins, which showed the absence of any immunoprecipitated binding proteins, therefore confirming the specificity of Cry toxin interaction with the immunoprecipitated membrane proteins (Figures 8A and 8B, lanes 1).

GPI anchor properties of APNs

Cry proteins have the ability to bind to receptors which are anchored to the membrane via a GPI moiety. As this is the first report showing the presence of an APN in the fat body, and as this APN interacts with Cry toxins, we examined further whether this APN was GPI-anchored. Bacterial PLC is known to cleave the GPI anchor causing the exposure of the CRD epitope [24]. The CRD epitope is exposed when the protein is converted into a hydrophilic form by PI-PLC, and its recognition by an anti-CRD antibody is the strongest evidence for the presence of a GPI anchor.

Figure 7 Lectin binding assays

(A) Lectin binding assays. Membrane protein preparations were separated by SDS/PAGE (7.5 % gels) and transferred on to nitrocellulose membranes. The membranes were incubated either with biotin conjugated SBA or WGA and lectin bound proteins were subsequently detected with streptavidin–ALP conjugate. Lanes 1 and 3, fat body membrane proteins (Fb); lanes 2 and 4, BBMVs. (B) Effect of GalNAc on the interaction between membrane proteins and Cry1Ac. Membrane protein preparations were separated by SDS/PAGE (7.5 % gels) and transferred on to nitrocellulose membranes. The blot was incubated with biotin labelled activated Cry1Ac (100 ng/ml) in presence of 100 mM of GalNAc and subsequently detected with streptavidin–ALP conjugate. Lane 1, fat body membrane proteins; lane 2, BBMVs.

Upon treatment of fat body membrane proteins and BBMVs with PLC, we detected the exposed CRD epitope using anti-CRD antibody (Figure 8C). As seen in Figure 8(C), lanes 1 and 2, there was an increase in intensity of the signal of 113 kDa fat body membrane protein following PLC treatment, because of the cleavage of the GPI anchor and exposure of the CRD epitope. The control lane without PLC treatment (Figure 8C, lane 1) also shows some signal, this could be accounted for by the activity of endogenous phospholipases. The intense signal for the CRD epitope in the BBMVs following PLC treatment (Figure 8C, lane 3) may be due to the presence of multiple GPI anchored APNs.

Activity of APNs in different tissues

Enzyme activity was assayed using leucine *p*-nitroanilide as the substrate, which demonstrated the leucine APN activity in different tissues. The total midgut homogenate showed specific activity of 21.6 ± 2.5 $\mu\text{mol/min per mg}$ of protein, whereas, under similar experimental conditions, the fat body homogenate exhibited specific activity of only 2.6 ± 0.5 pmol/min per mg of protein (Table 1). The specific activity was not altered significantly when aromatic substrates were used with fat body homogenate (results not shown).

Figure 8 Immunoprecipitation of APN with the Cry toxin

Triton X-100 solubilized fat body membrane proteins and CHAPS-solubilized midgut membrane proteins from *A. janata* fifth instar larvae were separately incubated with purified Cry1Aa toxin and subsequently with anti-Cry1Aa antisera. The protein–toxin complex was pulled down with Protein A–Sepharose beads, and resolved by SDS/PAGE, and transferred on to a 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) BBMVs incubated without Cry1Aa (control, lane 1) or with Cry1Aa (lane 2) prior to immunoprecipitation. (C) Detection of the cleaved GPI anchors. Fat body membrane proteins and BBMVs from *A. janata* fifth instar larvae were digested with 5 units of PLC/10 ml, subjected to SDS/PAGE and the proteins transferred on to a 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 PLC digestion; lane 2, fat body membrane protein digested with PLC; and lane 3, BBMVs digested with PLC.

Table 1 APN activities in different tissues of *A. janata* larvae

APN activity is expressed as $\mu\text{mol/min per mg}$ of protein for midgut, pmol/min per mg of protein for fat body, and as pmol/min per μl of haemolymph plasma for larval and prepupal haemolymph. The results are shown as means $\pm$ S.D.

Sample	APN activity
Midgut	21.6 ± 2.5
Fat body	2.6 ± 0.5
Larval haemolymph	5.4 ± 0.9
Prepupal haemolymph	13.9 ± 1.5

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

In order to determine the toxic effect of Cry1A proteins upon ingestion, feeding bioassays were carried out with *A. janata* larvae. As shown in Table 2, Cry1Ac ($\text{LC}_{50} = 23.70$ ng/cm²) was

Table 2 Comparison of the toxicity effects on *A. janata* larvae caused after oral ingestion or intrahaemocoelic injection of *B. thuringiensis* Cry toxins

ED is the effective dose that caused mortality of the larvae and the results are shown as means $\pm$ S.D. The values in parentheses for the ED column indicate the range of values at which mortality was observed during different time periods. NS, not sensitive in comparison with Cry1Aa and Cry1Ab. LC₅₀ is the effective dose that caused 50% mortality among the early second instar larvae following ingestion and the values in parentheses indicate the 95% confidence limits.

Toxin	ED in the haemolymph (ng/0.2 g body weight)	LC ₅₀ (ng/cm ²) (fiducial limits)
Cry1Aa	700 $\pm$ 136 (500–900)	60.11 (47.71–122.31)
Cry1Ab	700 $\pm$ 79 (600–800)	32.03 (14.57–42.52)
Cry1Ac	NS	23.70 (19.73–30.56)

found to be most effective, followed by Cry1Ab (LC₅₀ = 32.03 ng/cm²) and Cry1Aa (LC₅₀ = 60.11 ng/cm²). Earlier studies have demonstrated that, upon insects feeding on Cry protein expressing transgenic plants, the toxins can penetrate across membranes and are released into the haemolymph where they can interact with different tissues/cell types in the body cavity and alter their functions, including those of organs involved in reproduction; however, the mechanism of entry was not established [25]. Further, several large proteins and fusion peptides/proteins have been reported to cross the insect midgut epithelium and reach the haemolymph when fed in the diet [26–29]. So, in order to check the toxic effect of the Cry1A proteins in the larval body cavity, the three active toxins, Cry1Aa, Cry1Ab and Cry1Ac, which were already shown to be active in the gut, were injected into the haemocoel individually. The three Cry toxins, upon injection, displayed differential effects on *A. janata* larvae (Table 2). Our studies showed that at a concentration of > 700 ng/0.2 g of body weight, Cry1Aa and Cry1Ab led to lower food consumption associated with a subsequent reduction in the body weight leading to mortality. Mortality (approx. 40%) was observed after 72 h for lower doses of toxins (500–600 ng) and after 48 h for higher doses (700–900 ng). However, Cry1Ac-injected larvae survived and showed little reduction in body weight in comparison with control larvae.

DISCUSSION

The fat body is a major insect metabolic organ performing a wide variety of functions including protein metabolism and storage [30]. 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 [31–33]. Earlier studies by Cerestiaens et al. [14] demonstrated a considerable toxic effect of Cry toxins when they were injected into the haemocoel of *L. dispar* larvae. Furthermore, the findings of Cry binding and of 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 [15]. The present study shows the presence of a Cry toxin binding protein in the fat body membrane of *A. janata*.

To the best of our knowledge, all of the insect APNs registered to date in the databases (EBI, Swiss-Prot and GenBank®) 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 M1 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 have been reported from the midgut of *Bo. mori*, *Plutella xylostella* and *Trichoplusia ni* [5,34]. Herrero et al. [11] 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. The multiple APN isoforms existing in insects are believed to have arisen as a consequence of gene duplication events [35]. Detection of a single APN transcript in the fat body and two different sized transcripts in the midgut indicates the expression of a unique APN in the fat body, which is smaller in size than the midgut APN transcripts. The sizes of the detected APN mRNAs in both of the tissues were, however, slightly larger than their corresponding cloned cDNAs. The probable 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.

We identified the presence of a fat-body-specific membrane protein of approx. 113 kDa using anti-FbAPN antisera, which we assume could be the fat body APN encoded by the 3086 bp cDNA we cloned from fat body; the APN cDNA has an ORF of 3015 bp, which encodes a putative 1004 amino acid protein with a theoretical molecular mass of 113 kDa and is in agreement with the size of the fat body membrane protein. Interestingly in the midgut, anti-FbAPN antisera recognized an approx. 170 kDa protein, which was, however, not detected by anti-MgAPN antisera. Rather, anti-MgAPN antisera detected an approx. 113 kDa protein in the midgut. Earlier, Oltean et al. [36] reported N-terminal sequencing-based cloning of a 170 kDa Cry1Ac receptor from *Heliothis virescens* larval midgut. This clone encoded a protein with a deduced molecular mass of only 112.21 kDa, but possessed a stretch of poly(threonine) residues at the C-terminus, which they assumed was most likely to undergo glycosylation to give rise to the 170 kDa protein. However, the AjFbAPN cDNA did not show the presence of any poly(threonine) 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 [30], the presence of APNs in the fat body indicates a significant physiological role. Biochemically characterized APNs from *Drosophila* were shown to have high activities during metamorphosis and were inducible by insect steroid 20-hydroxyecdysone [31,33]. The soluble and membrane bound APNs are also known to play a role in neuropeptide inactivation, which are present in insect haemolymph [37]. Although APNs perform multiple physiological activities, they were mostly reported from the midgut, with few exceptions. Recently, Wang et al. [34] 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 successful isolation of an APN from the fat body of *A. janata* primarily relied on the use of highly selective degenerate primer pairs.

The APNs are the most widely characterized *B. thuringiensis* toxin receptors in the larval midgut and as we report here their presence in a vital metabolic organ like the fat body may have far reaching implications. In the present study, the lethal effect of Cry1A toxins on *A. janata* larvae upon intrahaemocoelic injection was manifested as lower food intake leading eventually

to reduction in the body weight of larvae, whereas higher toxin concentration led to larval mortality. The toxicity obtained from haemocoelic delivery opens up the possibility of toxin interaction with various organs present in the body cavity. In addition to this, large foreign proteins like BSA and anti-BSA antibody have been reported to cross the insect midgut epithelium and reach the haemolymph when fed orally [27]. Roe and Brandt [38] developed a polymer conjugated protein technology for delivering haemocoelic-active foreign proteins (like *B. thuringiensis* Cry toxins) into haemolymph, where the polymer facilitates the crossing of toxic protein across the insect gut into the haemolymph in an amount sufficient to cause toxicity. Furthermore, small amounts of Cry toxins, which are not effective against *Lygus hesperus* when orally ingested, were absorbed into the haemolymph as holoproteins [26]. Gatehouse and colleagues [29] reported that the Snowdrop lectin GNA (*Galanthus nivalis* agglutinin) binds to the gut epithelium and is transported to the haemolymph of the insect after oral ingestion, which can be used as basis for novel insecticides. Subsequently, the authors demonstrated the transport of various recombinant fusion proteins (like GNA linked either to neuropeptides, spider venom toxin or scorpion toxin) across the insect gut into haemolymph and showed toxicity upon injection as well as ingestion. Further, Cry1Ac–Ricin B chain lectin fusion protein conferred resistance to a broader spectrum of insect pest including *Spodoptera* species, which is normally not susceptible to Cry1Ac alone, but the basis for the insecticidal activity was not established [39]. Thus, in order to correlate the pathophysiological effects of Cry toxins with the actual biochemical events, we analysed 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 113 kDa protein in fat body membrane preparation. The specificity of this interaction was examined further by co-immunoprecipitation experiments, which were performed using Cry1Aa toxin and its antisera, and subsequently identified the 113 kDa binding protein as APN using the anti-FbAPN antisera. The midgut-specific 113 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 113 kDa binding protein with Cry1Aa toxin and confirmation that this protein is an APN was carried out by co-immunoprecipitation using anti-MgAPN antisera. Further, the binding of Cry1A toxins to AjFbAPN and AjMgAPN isoforms was substantiated by the presence of Cry1A toxin binding region identified by Nakanishi et al. [5], consisting of 64 amino acids, including 27 residues (Figure 1) which are known to play an important role in the recognition of Cry1Aa toxin [40]. Cry1Ac toxin binds at two distinct sites on insect APNs, of which one is GalNAc dependent other is independent [41,42]. 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 and midgut APNs. Cry1Ac toxin binding to the 113 kDa protein in fat body as well as midgut was not inhibited in the presence of the sugar GalNAc (Figure 7B, lanes 1 and 2), suggesting the presence of two binding sites for Cry1Ac toxin on 113 kDa APN [43]. It is most probable that GalNAc recognition is not essential for Cry1Ac toxins to interact with the 113 kDa receptors in the fat body and midgut, although it might be important for the 170 kDa receptor in the midgut (Figure 7B, lane 2). It appears that all the three Cry1A toxins have alternative binding sites on the APNs and the interactions are independent of the GalNAc moiety [44]. In the present study, the detection of the

GPI anchor using the 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 (113 and 170 kDa proteins). In fact, the Cry1Ac was reported to be the most effective Cry toxin even for *H. virescens*, *He. zea* and *He. armigera* larvae among other noctuid pests [13,45]. On the contrary, the intrahaemocoelic injection reveals toxicity mostly for Cry1Aa and Cry1Ab, whereas Cry1Ac, in spite of its binding to the fat body in Cry toxin overlay analysis, did not show any significant effect in comparison to other two Cry toxins. The Cry1Ac toxin contains a lectin-like domain that recognizes a part of the GalNAc moiety on APNs [43]. The amino acid residues forming the GalNAc binding pocket in Cry1Ac are not conserved in Cry1Aa and Cry1Ab. Furthermore, GalNAc is reported to inhibit the binding of Cry1Ac to APN, but not the binding of Cry1Aa and Cry1Ab [41]. This suggests that Cry1Ac domain III has a sugar-dependent mechanism of binding unique to Cry1A toxins [46]. In addition to APN, the lectin-like domain of Cry1Ac facilitates the GalNAc-mediated binding of toxin to peritrophic membrane proteins and hexamerins [43,47,48]. In *Bo. mori*, the binding of Cry1Aa and Cry1Ac to BBMV APN was demonstrated using ligand blot and microscopic studies, but toxicity was reported only for Cry1Aa [47,49]. The authors revealed that the non-toxic effect of the Cry1Ac is due to GalNAc-mediated binding of Cry1Ac to peritrophic membrane proteins, which delays the passage of toxin to the receptors on brush border epithelium [47,49]. In the present study, the intrahaemocoelic injection of Cry toxins also opens up a number of scenarios for the Cry toxin interaction. Mainly the Cry1Ac toxin, before it reaches the receptor (FbAPN), first may interact with (be exposed to) abundant haemolymph proteins, such as hexamerins, which are known to contain GalNAc moieties (M. Budatha, G. Meur and A. Dutta-Gupta, unpublished work) and form insoluble aggregates. Ceresteins et al. [14] also suggested the possible formation of non-functional Cry1Ac aggregate in the haemolymph of *L. dispar*. The different insecticidal activities of various Cry1A toxins to insects result from the different APN recognition mechanisms of the Cry1 toxins [40]. Furthermore, the variations in the toxicities of three different toxins, through feeding as well as in intrahaemocoelic injection, could be associated with the presence of multiple receptors in the midgut, whereas there is only a single receptor in the fat body. The requirement of higher toxin concentrations to be effective on intrahaemocoelic injection could be due to different factors, such as the size of the larvae used, the presence of the basal lamina surrounding the fat body, which retards the toxin access to the plasma membrane of the fat body cell, the kind of receptors and the type and purity of the toxin. In order to overcome the problem of resistance, to sustain the long-term use of environmentally friendly *B. thuringiensis*-based pesticides and to increase the insecticidal spectrum, engineering of Cry toxins with novel specificity and identification of new targets with an appropriate delivery method are required [27,39,50]. However, further detailed studies are needed in order to substantiate the role of fat body APN in the pathogenesis of Cry toxins.

The abundance of APN transcript and protein in the larval fat body is associated with an almost negligible level of APN enzymatic activity in the fat body membrane, whereas a significantly high APN activity detected in the haemolymph during the developmental transition stage indicates 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 clear [37]. Based on the results from enzymatic studies, it appears that the fat body or haemocytes might be one of the sites for synthesis and release of APN into the haemolymph during post-embryonic development [51]. The increase in the haemolymph plasma APN activity from late larval instar to prepupal stage (Table 1) suggests that, during the prepupal stage, there may be an induction of enzymatic activity which is regulated by rising ecdysteroid titre [31], which also suggests a possible role of fat body APN in larval to pupal transformation and metamorphosis [32]. However, further detailed studies are needed to decipher the physiological functions of this newly identified fat body APN in order to enhance the significance of it being a Cry toxin receptor in the lepidopteran insect pests.

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