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Molecular Characterization of Apolipophorin-III in the Fall Webworm, *Hyphantria cunea* Drury

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We isolated and sequenced a cDNA clone corresponding to apolipophorin-III (apoLp-III) from the fall webworm, *Hyphantria cunea*. The cDNA for apoLp-III codes for a 187-residue protein (561 bp) with a predicted molecular mass of 20 kDa. The calculated isoelectric point is 8.76. Multiple alignment analysis of the amino acid sequence revealed that *H. cunea* apoLp-III is most similar to that of *Spodoptera litura* (71.5% identity), followed by that of *Manduca sexta* (69.7% identity). They share five amphipathic α-helices that are proposed to play a critical role in the binding of apoLp-III to lipophorin.

Key words : *Hyphantria cunea*, Fall webworm, Apolipophorin-III, cDNA clone, Amphipathic α-helix, Lipophorin

Introduction

ApoLp-III plays a critical role in the transport of lipids during flight in several species of insects (reviewed by Soulages and Wells, 1994). ApoLp-III assists in the loading of diacylglycerol, generated from triacylglycerol stores in the fat body through the action of adipokinetic hormone, into the hemolymph lipoprotein lipophorin. Such loading is an obligatory part of the pathway that transfers lipid from the fat body to the flight muscles, where the lipid is used to fuel flight.

ApoLp-III has been isolated and characterized from various insect species (Kawooya *et al.*, 1984; Wells *et al.*, 1985; Chino and Yazawa, 1986; Burks *et al.*, 1992) and apoLp-III cDNA sequences have been determined in several species (Cole *et al.*, 1987; Kanost *et al.*, 1988; Smith *et al.*, 1994). Yamauchi *et al.* (2000) compared the amino acid sequence of apoLp-III from distantly related insects and concluded that precise amino acid sequence identity was not as important as the conservation of a common structural feature, namely the presence of five amphipathic helices, for their common function.

It was reported that lipophorin is accumulated in insect oocytes during oogenesis (Kawooya *et al.*, 1988; Telfer *et al.*, 1991). In *H. cunea*, the accumulation of lipophorin and apoLp-III in ovary has been reported (Yun *et al.*, 1994; Yun and Kim, 1996). Recently, the presence of apoLp-III in testis was demonstrated in *H. cunea* (Yun and Kim, 1996).

In this study, we determined the *H. cunea* apoLp-III cDNA sequence and further compared the sequence of *H. cunea* with those of several insects.

Materials and Methods

Animals

Fall webworm, *H. cunea*, was reared on artificial diet at 27°C and 75% relative humidity with a photoperiod of 16L : 8D.

Primer synthesis, PCR, and subcloning of PCR products

Total RNA was isolated from adult whole body by lysis buffer, spin column, and wash buffer according to the protocol recommended by the manufacturer (Qiagen Inc., Chatsworth, USA). All RNA samples were evaluated in agarose gels to ensure that they contained intact rRNA and were free of DNA. Degenerate primers were designed from two internal consensus sequences from 1st and 4th α-helix structure of apoLp-IIIs reported (Cole *et al.*, 1987;

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1    CAT TCA CTG TTG TGT TAC AGG CAG TAA CAC CCG AGC TCT CGT CTC TCC GTA TTG TTC AGT AGT ACA TCA ATC GTC GCA   78
                                                                                  ↓
79   ATG GCA GCC AAG TTT ATC ATC CTC CTC GCT TTA TTC GCT CTC AGC CAA GCC AGT GTT GTG CGC CGT GAT GCG CCG TTG  156
1     M  A  A  A  K  F  I  I  L  L  A  L  F  A  L  S  Q  A  S  V  V  R  R  D  A  P  L                      26

157  GCG AAT TTC CTG CAG GAT TTG GAG AAG CGT GCT GCC GAT ATC CAG AAG ACC TTC AGC GAA CAA TTC CAA GCG ATC TCC  234
27    A  N  F  L  Q  D  L  E  K  R  A  A  D  I  Q  K  T  F  S  E  Q  F  Q  A  I  S                        52

181  AAC TCG AAG AAC GTG CAA GAT GTA AAC AAA GCA GTT AAA GAA AGT TCC GAT GTA GTT CTG AAG CAA CTG TCC ACG TTG  312
53    N  S  K  N  V  Q  D  V  N  K  A  V  K  E  S  S  D  V  V  L  K  Q  L  S  T  L                        78

313  TCA TCT AGC CTA CAA AGT GCG TTG ACC GAC GCA AAC GGC AAG GCT AAG GAG GCC CTG GAG CAG ACG CGC CAA AAC CTG  390
79    S  S  S  L  Q  S  A  L  T  D  A  N  G  K  A  K  E  A  L  E  Q  T  R  Q  N  L                       104

391  GAG AAG ACC GCG GAG GAG CTG AGG CGC GCG CAC CCC GAC GTC GAA AAG CAG GCC AAC CAG CTG CGC GAC AAG CTG CAG  468
105   E  K  T  A  E  E  L  R  R  A  H  P  D  V  E  K  Q  A  N  Q  L  R  D  K  L  Q                       130

469  GCC GCC GTG CAG TCC ACC CTA CAG GAG ACC CAG AAG CTC GCT AAG GAA GTG GCC GCT AAC ATG GAG CAG ACC AAC GAG  546
131   A  A  V  Q  S  T  L  Q  E  T  Q  K  L  A  K  E  V  A  A  N  M  E  Q  T  N  E                       156

547  AAA CTG GCT CCT AAG ATT AAG GAG GCG TTC GAA GAC TTC GTG AAG CAG GCC GAG GCC GTG CAG AAG AAG GTG CAC GAC  624
157   K  L  A  P  K  I  K  E  A  F  E  D  F  V  K  Q  A  E  A  V  Q  K  K  V  H  D                       182

625  GCC GCC ACC AAA CAG TGA GCT CAC CCA CAC CAC GCG AAA CGC AAT AAA ACT ATA TTC CAC TGA AAA AAA AAA  702
183   A  A  T  K  Q  *                                                                                  187

703  AAA AAA AAA                                                                                       711
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Fig. 1. Nucleotide and deduced amino acid sequence of the cDNA encoding *H. cunea* apoLp-III. Arrow indicates signal peptide cleavage site. Underlined amino acids were confirmed by N-terminal amino acid sequence analysis of apoLp-III. An asterisk marks the translation stop codon and the polyadenylation signal AATAAA is double underlined.

Kanost *et al.*, 1988; Kim *et al.*, 1998; Yamauchi *et al.*, 2000). The primers are listed below: numbers in parentheses refer to amino acid positions as given in Fig. 1.

5'-AARACNTTYWSNGARCAR-3' (42-47 a. a.)

5'-GCYTCRTGNARYTTYTTYTG-3' (141-147 a. a.)

The thermal cycle profile of PCR involved 35 cycles of 30 sec at 94°C, 1 min at 47°C, and 1 min at 72°C and a further 7 min at 72°C. The resulting 320 bp PCR product was separated on 1% agarose gel. This fragment was excised from the agarose gel, purified, ligated into a pGEM T-easy vector (Promega), and amplified in XL1-Blue competent cells.

Rapid amplification of cDNA end (RACE) PCR

To obtain the beginning region of apoLp-III open reading frame, 5-RACE PCR was performed as described by the manufacturer (Gibco-BRL). Two gene specific primers (A and B) were synthesized as follows: A, 5-TGACAACGTGGACAGTTG-3 (74-79 a.a.) and B, 5-CACGGCCCTGCAGCTTG-3 (128-133 a.a.). Total RNA was extracted from adult whole body using RNeasy mini kit (Qiagen) according to the manufacturers instruction. From 5 μg of total RNA, single-stranded cDNA was synthesized using SuperScript reverse transcriptase (Gibco-BRL) and gene specific primer B.

After cDNA synthesis, the product was purified using Glass MAX Spin Cartridge and tailed with dCTP and TdT. The tailed cDNA was amplified with the Abridged Anchor primer and nested gene specific primer A, and reamplified using the same primers again.

For 3-RACE, 3 μg of total RNA was reversely transcribed according to the protocol recommended by the manufacturer (Korea Bioneer Inc.).

This cDNA was amplified with an adaptor primer and a gene specific primer, 5-GACGCAAACGGCAAGGCT-3 (88-93 a.a.). Reamplified product was separated by agarose gel electrophoresis. The 480 bp product was excised and subcloned into the pGEM-T vector (Promega). Several clones were isolated and sequenced using Sequenase (US Biochemical).

DNA sequencing

The manual nucleotide sequence analysis of both DNA strands was performed by the dideoxy chain termination method (Sanger *et al.*, 1977). 6% acrylamide gels, ^{35}S-radiolabeled nucleotides, and sequenase (US Biochemical) were used according to the manufacturers recommendations.

Analysis of sequence data

The EMBL DataBank was searched with BLAST. Editing

and analysis of the DNA sequence data were performed with DNASTAR software (DNASTAR Inc., Wisconsin). In particular, we used the program "MEGALIGN" to generate pairwise alignment and dendrogram.

Results and Discussion

Sequence of apoLP-III gene and its deduced primary structure

Figure 1 shows the cDNA sequence of apoLp-III from *H. cunea*. Using degenerate primers designed from the internal consensus sequences, a 320 bp fragment of apoLp-III was amplified from RNA of adult whole body by RT-PCR. In an attempt to obtain the 5- and 3- ends of coding sequence, 5- and 3-RACE PCRs were performed using gene specific primers. The resultant products from 5- and 3-RACE PCRs contained initiation and stop codons. The 705 bp sequence of the assembled apoLp-III cDNA contains an open reading frame beginning with an ATG codon at nucleotide 79 and extending to nucleotide 639, followed by a 66 bp 3-untranslated sequence ending with a poly $(A)^+$ tail. The apoLp-III cDNA has a length of 561 bp coding for a 187-residue protein. This open reading frame predicts a molecular mass of 20,744.49 daltons, equivalent to the estimated molecular weight of 18 kDa by SDS-PAGE (Yun and Kim, 1996). The deduced amino acid sequence encoded by the cDNA matching the NH_2-terminal sequence of the apoLp-III is preceded by a sequence encoding 22 amino acids, which might function as a signal peptide. Based on the cDNA sequences reported, the apoLp-III mRNAs encode putative signal peptides of 18-23 amino acids.

The amino acid sequence from apoLp-III cDNA of *H. cunea* lacks putative N-linked glycosylation site (Asn - X - Ser / Thr), although one or two N-linked glycosylation sites are found in apoLp-III of other insect species such as *Spodoptera litura* (Kim *et al.*, 1998) and *Locust migratoria* (Kanost *et al.*, 1988). The biological significance of glycosylation of this protein is not known.

Sequence comparisons and phylogenetic analysis

To retrieve protein sequences similar to that of apoLp-III, relevant databases were searched using the BLAST (Altschul *et al.*, 1990). *H. cunea* apoLp-III showed a relatively high degree of sequence identity with those of other lepidopteran insects such as *S. litura* (71.5% identity), *M. sexta* (69.7% identity), *Galleria mellonella* (69.3% identity), and *Bombyx mori* (56.1% identity). The orthopteran *L. migratoria* showed large difference with lepidopteran insects in the sequence identity of apoLp-III (17.3% identity) (Table 1). One possible biological explanation for this low similarity is that long distance migratory flight, up to 500 km in a single night, is an important component of the life history of locusts (Farrow, 1990). Because a major role of apoLp-III is to assist in lipid transport during flight, there maybe a difference in selection pressure for apoLp-III function in locusts compared with the other species (Smith *et al.*, 1994).

The insect apoLp-III conteins five putative amphipathic α-helices (Fig. 2) with helical contents ranging from 54.3% to 84.5%, depending on the program being used and the sequence being analyzed (Smith *et al*, 1994). Five-helix bundle has been postulated to open at putative hinge domains to expose the hydrophobic interior, thereby facilitating with the lipoprotein surface (Breiter *et al.*, 1991). Amphipathic α-helices in apoLp-III appear to be the fundamental structural motif required for lipid association. Using X-ray diffraction analysis, *L. migratoria* apoLp-III is proven to contain five long amphipathic helices with the hydrophobic residues oriented inward away from the aqueous environment of the hemolymph (Smith *et al.*, 1994).

The model for the association of apoLp-III with lipophorin consists of the protein binding to a nascent lipid patch on the surface of a lipophorin particle via the two

Table 1. Sequence identities among representative insect apoLp-IIIs. Sequences were aligned using the method of Feng and Doolittle (1987). Percent identities (above the diagonal line) and distance score (below the diagonal line) were calculated by computer program MEGALIGN. Abbreviations and sequence sources are described in Fig. 2

	Percent Identity					
	Hc-apoLpIII	Sl-apoLpIII	Ms-apoLpIII	Gm-apoLpIII	Bm-apoLpIII	Lm-apoLpIII
Hc-apoLpIII	-	71.5	69.7	69.3	56.1	17.3
Sl-apoLpIII	33.0	-	71.7	69.3	61.6	18.5
Ms-apoLpIII	36.8	35.5	-	70.6	68.3	17.3
Gm-apoLpIII	37.4	39.4	35.4	-	64.4	17.3
Bm-apoLpIII	56.8	48.0	35.4	44.3	-	17.3
Lm-apoLpIII	291.0	315.0	330.0	309.0	339.0	-

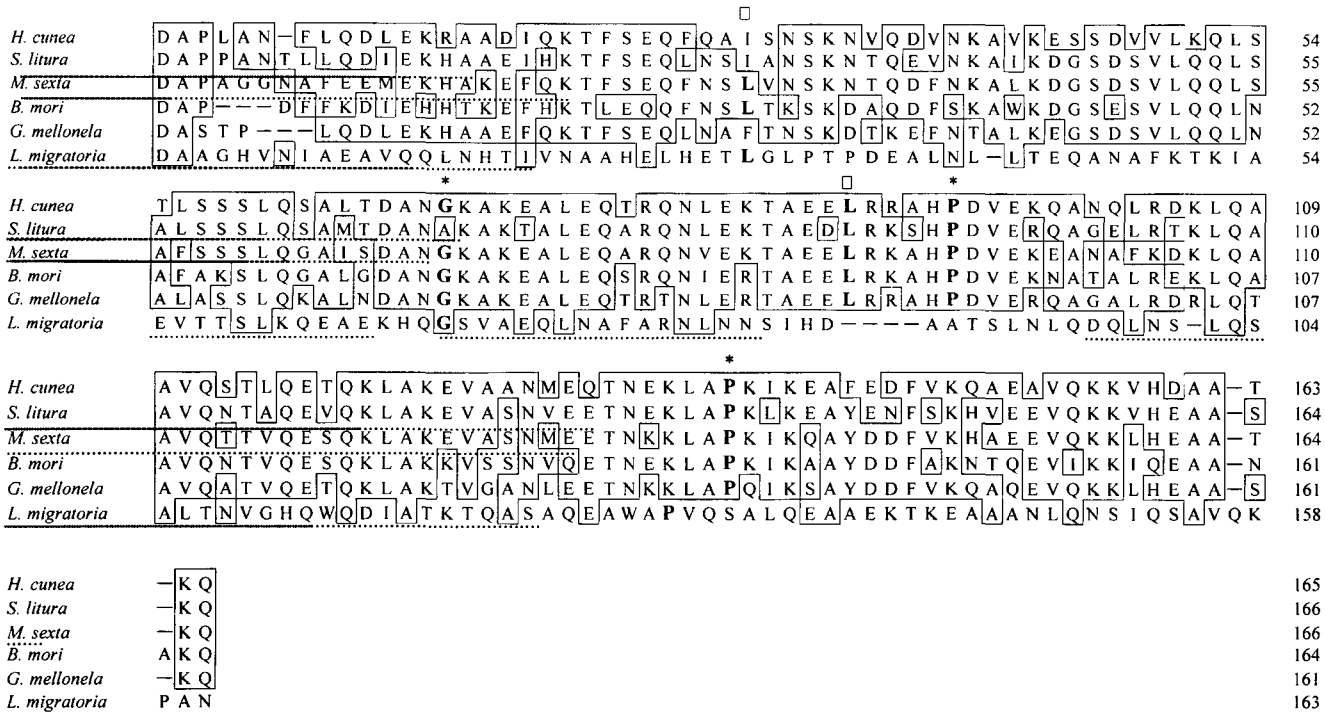

Fig. 2. Alignment of the amino acid sequences of insect apoLp-IIIs. Aligned amino acid sequences for apoLp-IIIs are from *Spodoptera litura* (AF094582), *Manduca sexta* (Cole *et al.*, 1987; M17286), *Bombyx mori* (U59244), *Galleria mellonella* (P80703), and *Locusta migratoria* (J03888). Five amphipathic á-helical domains are indicated by dotted underline. The leucines that are putatively involved in the initial contact with the lipophorin surface are indicated by circle and boldface. Conserved residues that are probably involved in breaks and/or turns between helices are marked by asterisk and boldface. Identical residues and conserved replacements in apoLp-III are in box.

leucines that project into the hemolymph between helices 1 and 2 and helices 3 and 4 (Smith *et al.*, 1994). ApoLp-IIIs from *H. cunea and S. litura* substitute isoleucine (positions 29 and 30, respectively) for first positioned leucine, but second positioned leucine is well conserved (Fig. 2). The substitution from leucine to isoleucine might have no functional difference. It has been proposed that this residue may be important in the binding of apoLp-III to diacylglycerol in lipophorin (Breiter *et al.*, 1991; Soulages and Wells, 1994).

Comparison of the six aligned sequence of apoLp-III revealed a relatively conserved two prolines and a glycine residues, which are involved in breaks or turns between α-helices (Blaber *et al.*, 1993). The conserved pattern of leucine, glycine, and proline between helices in *H. cunea* apoLp-III is most similar to that of *S. litura*. According to the analysis of *S. litura* apoLp-III, this particle is expected to have 89.8% of α-helix content and have five amphipathic α-helices. Because of the high sequence identity, the overall *H. cunea* apoLp-III is almost the same as *S. litura* apoLp-III.

Reconstruction of the phylogenetic relationships based on amino acid-derived distances revealed two subclades

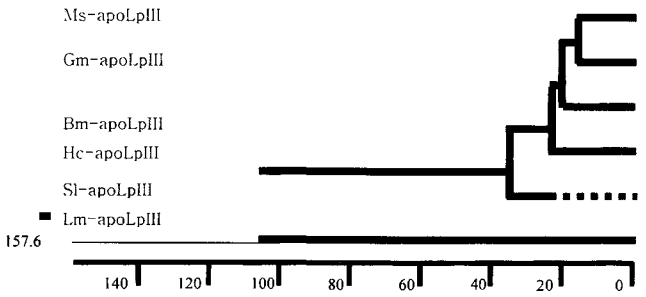

Fig. 3. Distance-based phylogenetic analysis of *H. cunea* apoLp-III and other representative apoLp-IIIs. The phenogram is based on the alignment shown in Fig. 2 and distances are approximate. Lengths of branches along the horizontal axis are proportional to evolutionary distances calculated from the amino acid identity matrix.

corresponding to these two orders of insect, lepidopteran and orthopteran apoLp-IIIs (Fig. 3). This tree shows that the locust apoLp-III is barely related to the lepidopteran apoLp-III. Although apoLp-III from the lepidopteran *G. mellonella* and the orthopteran locust seem to be functionally equivalent in an *in vitro* system (Van der Horst *et*

Table 2. Comparison of amino acid composition of *H. cunea* apolipophorin-III with those of other insects. The names of species and abbreviations are described in Fig. 2

	H. cunea	*S. litura*	*M. sexta*	*G. mellonella*	*B. mori*	*L. migratoria*
Ala	14.55	14.46	14.46	14.72	14.02	18.52
Cys	0.00	0.00	0.00	0.00	0.00	0.00
Asp	6.06	4.22	5.42	5.52	6.10	3.09
Glu	9.70	11.45	11.45	9.82	9.76	8.02
Phe	3.03	1.20	4.82	3.07	4.27	1.23
Gly	0.61	1.20	3.01	2.45	2.44	2.47
His	1.21	3.01	2.41	1.84	2.44	4.32
Ile	1.82	2.41	1.20	0.61	3.05	3.70
Lys	12.12	11.45	13.86	10.43	14.02	4.94
Leu	9.70	9.04	6.63	11.04	7.32	10.49
Met	0.61	0.60	1.20	0.00	0.00	0.00
Asn	5.45	6.63	6.02	5.52	6.10	9.26
Pro	1.82	2.41	1.81	1.84	1.83	2.47
Gln	11.52	9.04	9.04	10.43	9.76	11.11
Arg	3.03	2.41	1.20	4.29	2.44	0.62
Ser	6.67	8.43	7.23	5.52	6.10	6.17
Thr	5.45	5.42	4.22	7.98	4.88	7.41
Val	6.67	6.02	5.42	4.29	4.27	4.94
Trp	0.00	0.00	0.00	0.00	0.61	1.23
Tyr	0.00	0.60	0.60	0.61	0.61	1.00

al., 1988), no relationship between these proteins can be detected on the sequence level (Weise *et al.*, 1998). It is not surprising to show the unexpected values of percent divergence between locust and lepidopteran apoLp-III (Table 1).

Amino acid composition

From deduced amino acid sequence from apoLp-III cDNA of *H. cunea* (Fig. 1), its amino acid composition was determined and compared with those of other insect apoLp-IIIs (Table 2). *H. cunea* apoLp-III lacks cysteine, tryptophan, and tyrosine which make this protein show high A230 / A280 ratio. This protein has each one molecule of methionine and glycine, but contains a considerable amount of alanine, lysine, and glutamine, which is common in insect apoLp-III (Cole *et al.*, 1987; Kanost *et al.*, 1988; Kim *et al.*, 1998; Weise *et al.*, 1998; Yamauchi *et al.*, 2000).

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