Korean J Biol Sci 2: 367-370, 1998

# Purification and Characterization of Apolipophorin-III in the Hemolymph of the Wax Moth, *Galleria mellonella* L.

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Key Words:
*Galleria mellonella*
Hemolymph
Apolipophorin-III

**Two molecular species of apolipophorin-III (apoLp-III) were purified from the last instar larval hemolymph of *Galleria mellonella* by gel permeation chromatography (Sephadex G-100), ion exchange chromatography (DE-52), heat treatment (90℃ for 30 min) and Mono S FPLC, and were named apoLp-III-a and apoLp-III-b, respectively. They were indistinguishable by SDS-PAGE but could be separated by native PAGE. The molecular mass of apoLp-III determined by SDS-PAGE was approximately 18 kDa. The N-terminal amino acid sequence of apoLp-III-b revealed high similarities with the apoLp-III from *Manduca sexta*.**

Lipophorin (Lp) is composed of two different subunits termed apoLp-I and apoLp-II (Chino et al., 1981; Yun and Kim, 1993; Yun et al., 1994, 1996; Yun and Lee, 1997), and combined with the third subunit known as apoLp-III when the insect is injected with adipokinetic hormone (AKH) or during flight (Kawooya et al., 1984; Wells et al., 1985; Yun et al., 1994; Yun and Kim, 1996). After injection of AKH, large amounts of diacylglycerol are released into the hemolymph from the fat body which combines with high density lipophorin (HDLp) to become low density lipophorin (LDLp) (Van der Horst, 1990). The third subunit, apoLp-III, combines in advance with a complex composed of apoLp-I and apoLp-II to make LDLp stable as a lipid-rich structure (Kawooya et al., 1986; Wells et al., 1987).

ApoLp-III has been studied in *Locusta migratoria, Gastimargus africanus, Thasus acutangulus, Lethocerus medius,* and *Hyphantria cunea* (Kawooya et al., 1984; Wells et al., 1985; Chino and Yazawa, 1986; Haunerland et al., 1986; Kanost et al., 1995; Yun and Kim, 1996) since purification and characteristics of apoLp-III in the adult hemolymph of *Manduca sexta* has been reported by Shapiro and Law (1983). Molecular mass of apoLp-III was reported to be 20 kDa in *Barytettix psolus* (Ziegler et al., 1988), *Locusta migratoria* (Chino and Yazawa, 1986), *Gastimargus africanus* (Haunerland et al., 1986), 19 kDa in *Lethocerus medius* (Kanost et al., 1995), 18 kDa in *Hyphantria cunea* (Yun and Kim, 1996), and 17 kDa in *Manduca sexta* (Shapiro and Law, 1983). However, the two molecular species of apoLp-III were not described so far.

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The present study reports on the purification of apoLp-III from the last instar larval haemolymph by gel filtration (Sephadex G-100), ion exchange chromatography (DE-52), heat treatment, and Mono S FPLC. The biochemical characteristics such as molecular mass, and N-terminal amino acid sequence of purified apoLp-IIIs are described.

## Materials and Methods

### *Insects*

Larvae of *Galleria mellonella* were reared on an artificial diet (The Quaker Oats Co., USA) by the method of Beck (1960) at 30℃ in a rearing room.

### *Collection of hemolymph*

Hemolymph was collected into cold test tubes containing anticoagulation buffer (128 mM NaCl, 1.8 mM $CaCl_2$, 1.3 mM KCl, 30 mM trisodium-citrate, pH 6.4) and the forelegs of larvae were cut with a needle. A few crystals of phenylthiourea were added to the tubes to prevent melanization. Hemolymph was centrifuged at 10,000 g for 10 min to remove hemocytes and other cell debris, and the supernatant was stored at -70℃ until used.

### *Purification of apolipophorin-III*

Gel permeation chromatography: The larval hemolymph (4 ml) was eluted from Sephadex G-100 (Pharmacia, LKB) column (2×60 cm) with 0.05 M phosphate buffer (pH 7.0) at a flow rate of 1.5 ml/min with 2 ml per fraction. Each fraction was collected, dialyzed and concentrated with speed vac, and then electrophoresed. Fractions containing only apoLp-III were used as

samples for ion exchange chromatography.

Ion exchange chromatography: Fractions containing apoLp-III obtained through gel filtration were collected and concentrated to give a final volume of 3 ml. The concentrate was dialyzed against 0.05 M Tris-HCl buffer (0.05 M Tris, 1 mM EDTA, pH 8.3). This sample was used for ion exchange chromatography on a DEAE cellulose (DE-52, Whatman) column (1.2×10 cm). The column was washed with 0.05 M Tris-HCl buffer (pH 8.3) at a flow rate of 3 ml/min with 2 ml per fraction. Bound protein was eluted with a linear gradient (0-0.5 M NaCl). Unbound fractions were collected, heat-treated (90℃ for 30 min), and centrifuged. After centrifugation, the supernatants were collected, dialyzed against 0.02 M sodium acetate buffer (pH 5.0) and then used as a sample for Mono S HR 5/5 (Phamacia) FPLC system. After loading, the column was washed with 0.02 M sodium acetate buffer (starting buffer, solution A, pH 5.0) for 10 min at a rate of 1 ml/min and proteins were eluted with a 25 ml gradient of 20%-70% solution B (0.5 M NaCl in starting buffer).

*Gel electrophoresis and determination of molecular mass*

Non-denaturing SDS-PAGE was conducted on 8% polyacrylamide gel with tris-glycine buffer (pH 8.9) at 100 volts according to Davis (1964). SDS-PAGE was carried out on 12% and 15% SDS gel at room temperature at 15 mA respectively, according to Laemmli (1970). After electrophoresis, gels were stained in Coomassie brilliant blue R250. Molecular mass of apoLp-III was determined as described by Lambin et al. (1976). Molecular mass marker proteins used were phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin

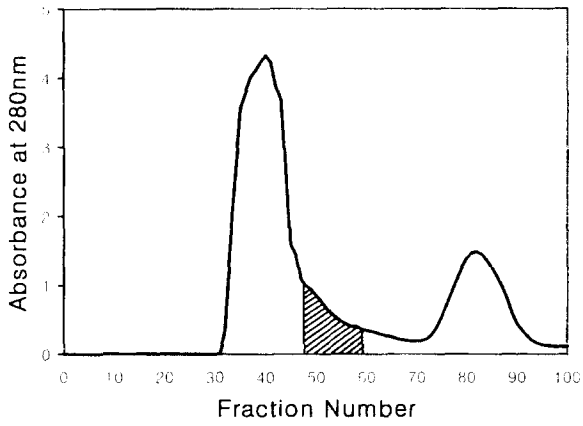


**Fig. 1.** Sephadex G-100 gel filtration profile of larval hemolymph from *Galleria mellonella*. The column was eluted with 0.05 M phosphate buffer at a rate of 30 ml/h and the eluents were collected in 2.0 ml fractions. The fractions represented by oblique lines were subjected subsequently to ion-exchange chromatography.

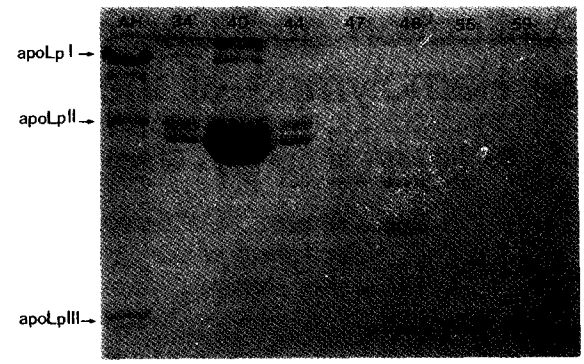


**Fig. 2.** SDS-PAGE of fractions from Fig. 1. AH, adult hemolymph; 34, 40, 44, 47, 48, 55, and 59 fraction number. The arrow indicates apolipophorin-III.

(45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

*N-terminal sequence analysis*

N-terminal amino acid residues were determined by automated Edman degradation on a Miligen 660B sequencer at the Korea Basic Science Center.

## Results

*Isolation and purification of apolipophorin-III*

Apolipophorin-III was isolated from the last instar larval hemolymph by gel permeation chromatography (Sephadex G-100). Most of the protein was eluted in the first major peak (Fig. 1). Each fraction in the first peak was electrophoresed, revealing that apoLp-III is present in fractions 48-59 (Fig. 2). The fractions (48-59) obtained through Sephadex G-100 column chromatography

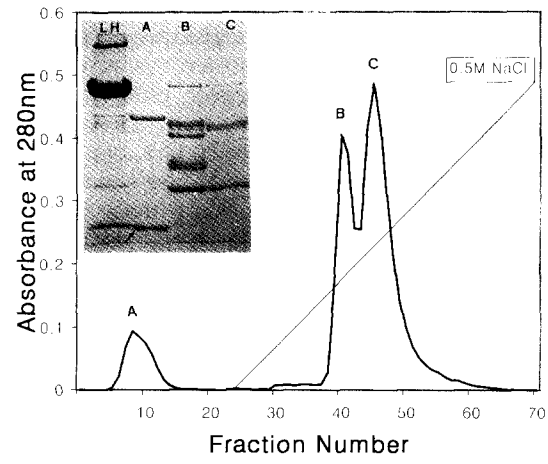


**Fig. 3.** DE-52 ion exchange chromatography of fractions (48-59) of gel filtration (G-100). Equilibration buffer (0.02 M Tris-HCl buffer, pH 8.3) was used as an elution buffer. Linear gradient elution was performed from 0.0 M to 0.5 M NaCl in 0.02 M Tris-HCl buffer. Three peaks from the DE-52 column were resolved on SDS-PAGE. LH, larval hemolymph. The arrow indicates apolipophorin-III.

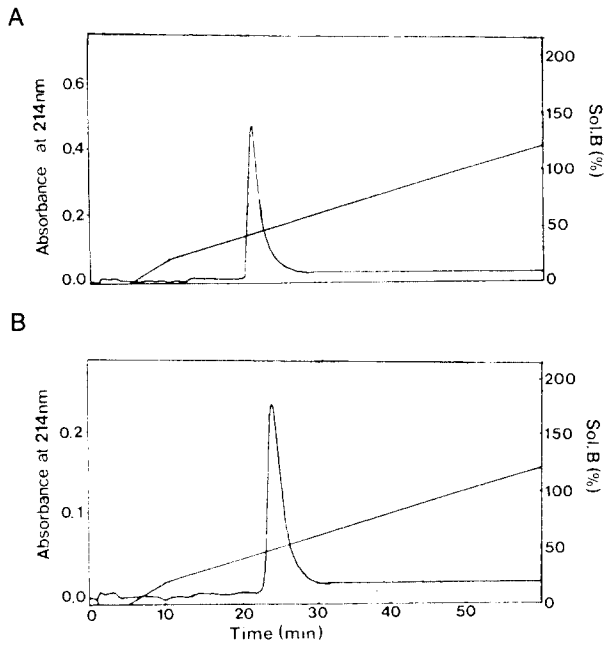


**Fig. 4.** Purification of apoLp-IIIs on Mono S cation exchanger column in FPLC system. After the column was equilibrated with 0.02 M sodium acetate buffer, pH 5.0 (sol.A), proteins were eluted with a gradient of 20-70% sol.B (0.5 M NaCl in equilibration buffer). A, Purified apoLp-III-a. B, Purified apoLp-III-b.

were applied to DE-52 ion exchange chromatography, showing three peaks. The fractions in these peaks were electrophoresed, indicating that apoLp-III appears in the unbound peak (Fig. 3). Fractions in the unbound peak were collected, heat-treated (90℃ for 30 min), centrifuged, and then the supernatant was applied to Mono S HR 5/5 column in a FPLC system, showing two distinct peaks that were designated as apoLp-III-a (42.4%) and apoLp-III-b (48.3%) respectively (Fig. 4). The purity of these two fractions was confirmed by SDS-PAGE. The results showed that both apoLp-III-a

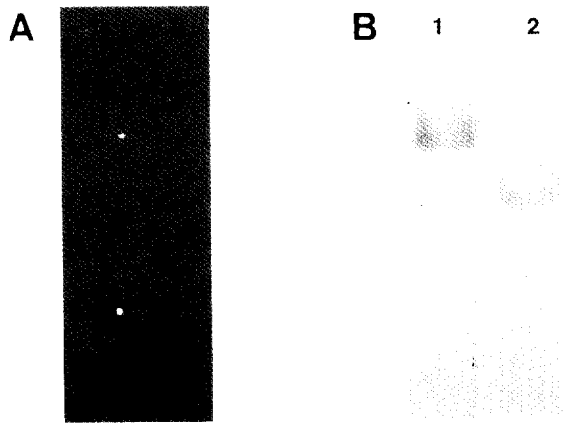


**Fig. 5.** SDS-PAGE (A) and Non-denaturing SDS-PAGE (B) of each peak in Fig. 4. Lane 1, purified apoLp-III-b; lane 2, purified apoLp-III-a.

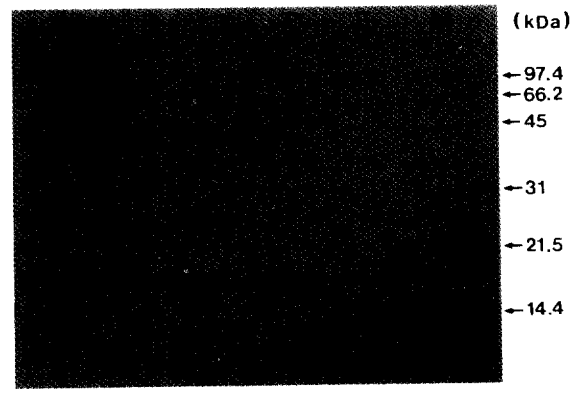


**Fig. 6.** SDS-PAGE analysis of the purification steps. A, larval hemolymph; B, fraction after gel filtration (Sephadex G-100); C, fraction after DE-52 column; D, supernatant of heat treatment; E, purified apoLp-IIIs by cation exchanger column.

and apoLp-III-b were purified and indistinguishable on the basis of their mobility on SDS-PAGE (Fig. 5A). However, the two apoLp-IIIs are separable on native PAGE and migrate rapidly in the order of apoLp-III-b and apoLp-III-a (Fig. 5B). Electropherogram of apoLp-IIIs through a purification process showed that apoLp-IIIs was completely purified in a Mono S HR 5/5 column (Fig. 6).

*Determination of molecular mass*

ApoLp-III was electrophoresed on a 15% SDS gel along with the molecular size markers as described by Lambin et al. (1976) to determine the molecular mass. Molecular mass of apoLp-III was estimated to be approximately 18 kDa (Fig. 6).

*N-terminal amino acid sequence*

N-terminal sequence of purified apoLp-III-b was determined to be the 30 amino acids, which revealed a significant homology with apoLp-III from *Manduca sexta* (Cole et al., 1987). The sequence identity in the analysed portion is 60.5% (18 out of 30) in an optimized alignment as follows;

| | | | |
|---|---|---|---|
| *M. sexta* | DAPAGGNAFE | EMEKHAKEFQ | KTFSEQFNSL VNS |
| *G. mellonella* | DASTPLQDL - | -- EKHAAEFQ | KTFSEQLNAF TNS |

However, because the N-terminal was modified during apoLp-III-a purification, the N-terminal sequence of purified apoLp-III-a was not determined.

## Discussion

The two molecular species of apoLp-III were isolated and purified from the last instar larval hemolymph of *G. mellonella* and its characteristics such as molecular mass, and N-terminal sequence were investigated. In general, insects need to transport large amounts of lipid to the flight muscle from fat bodies during flight or

injection with adipokinetic hormone. Presently, it is known, that apoLp-III binds to Lp to become LDLp which loads large amounts of diacylglycerol. Kawooya et al. (1984) purified apoLp-III from HDLp present in adult hemolymph, while Burks et al. (1992) purified apoLp-III from lipophorin-free fractions in the larval hemolymph. In *Hyphantria cunea,* apoLp-III from the larval hemolymph was associated with lipophorin in the adult hemolymph (Yun et al., 1994; Yun and Kim, 1996).

Molecular mass of apoLp-III from *G. mellonella* was estimated to be 18 kDa according to Lambin et al. (1976). This value was a little lower than 20 kDa of *Barytettix psolus, Locusta migratoria,* and *Gastimargus africanus* but a little higher than 17 kDa of *Manduca sexta* (Kawooya et al., 1984; Chino and Yazawa, 1986; Ryan et al., 1990). In addition, this value corresponded to 8 kDa of *Hyphantria cunea as reported by* Yun and Kim (1996).

The majority of the information about the function and the structure of apoLp-III was gained from studies on adult hemolymph, while the other apoLp-III of larval stage hemolymph has been barely investigated thus far. And so far there was no report on the presence of two molecular species of apoLp-III in the larval hemolymph. In the present work with *G. mellonella,* two molecular species of apoLp-III (apoLp-III-a and apoLp-III-b) were shown to be present in the larval hemolymph. Recently, it was reported that apoLp-III in *G. mellonella* may have immune-regulating function (Wiesner et al., 1997) and the N-terminal sequence of apoLp-III-b seems to be identical with the results described by Wiesner et al. (1997). Also, the sequence for apoLp-III-a was not determined because of the blocking N-terminal.

We will investigate the other functions and relationships of apoLp-III-a and apoLp-III-b more intensively in the future.

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[Received June 2, 1998; accepted June 24, 1998]