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Purification and Characterization of Apolipophorin-III from Haemolymph of Fall Webworm *Hyphantria cunea* Drury

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Apolipophorin-III (ApoLp-III) was purified from adult haemolymph of *Hyphantria cunea* and their molecular weight and synthetic place were investigated. ApoLp-III purification was performed by KBr-density gradient ultracentrifugation followed by gel permeation chromatography (Sephadex G-100) and ion-exchange chromatography (CM-52) and their purity was confirmed on 10% SDS-PAGE. ApoLp-III has the molecular weight of 18 kDa and is synthesized by fat body.

KEY WORDS: *Hyphantria cunea*, Haemolymph, Apolipophorin-III

Lipophorin (Lp) as lipid carrier is present in haemolymph as high-density lipophorin (HDLp) and low-density lipophorin (LDLp) depending on the lipid content (Chino *et al.*, 1981; Chino and Kitazawa, 1981; Beenakker *et al.*, 1988). It was reported that large amounts of diacylglycerol from fat body were released into haemolymph by the action of adipokinetic hormone (AKH) during flight and bind to HDLp to become LDLp (Van der Horst, 1990) in insects using lipid. At this time, apoLp-III binds to complex of apolipophorin-I (apoLp-I) and apolipophorin-II (apoLp-II) to become stable lipid-rich Lp (LDLp) which transport lipid from fat body to various lipid-using organs (Kawooya *et al.*, 1986; Chino and Yazawa, 1986; Wells *et al.*, 1987; Van der Horst, 1990; Burks *et al.*, 1992). In flightless grasshopper, *Barytettix psolus*, however, HDLp doesn't mobilize lipid in spite of the presence of apoLp-III and AKH (Ziegler *et al.*, 1988). Since apoLp-III had been purified and characterized by injection of AKH into adult of *Manduca sexta* (Shapiro and Law, 1983), apoLp-III was continuously studied in *Locusta migratoria*, *Gastimargus africanus*, and *Thasus acutangulus* (Kawooya *et al.*, 1984; Wells *et al.*, 1985; Chino and Yazawa, 1986; Haunerland *et al.*, 1986). ApoLp-III was also discussed in larval haemolymph in some insects (Kawooya *et al.*, 1984; Kanost *et al.*, 1988). Molecular weight of apoLp-III was determined to be 20 kDa in *Locusta migratoria* (Chino and Yazawa, 1986) and *Gastimargus africanus* (Haunerland *et al.*, 1986) and 17 kDa in *Manduca sexta* (Shapiro and Law, 1983). Lipophorin of *H. cunea* was purified and characterized (Yun and Kim, 1993) and found in testis as well as ovary (Yun *et al.*, 1994). Present work is to purify apoLp-III present in adult haemolymph of *H. cunea* by KBr density gradient ultracentrifugation and determine the property and synthetic place of it.

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Materials and Methods

Insects

Fall webworm, *Hyphantria cunea* were reared on artificial diet at 27±1°C and 75±5% R.H. with a photoperiod of 16L:8D.

Haemolymph collection

Haemolymph was collected from one or two day old adult by cutting heads. A few crystals of PTU (phenylthiourea) were added to the haemolymph to prevent melanization. The haemolymph was centrifuged at 10,000 rpm for 10 min to remove haemocytes and cell debris and the supernatant was stored at -70°C until used.

Purification of apolipophorin-III

Ultracentrifugation

Approximately 1 ml of the pooled adult haemolymph was put into the ultracentrifuge tube and 2.64 g of KBr was added with constant stirring to the haemolymph and filled to 6ml with 0.1M phosphate buffer (pH 7.0) and to 7 ml with 0.9% NaCl (density:1.007 g/ml) to give final volume of 13 ml. The tube was placed in the ultracentrifuge TST 41.14 rotor and centrifuged at 40,000 rpm for 16 hrs at 4°C After centrifugation, 1 ml each was taked out from the top of the tube and thus 12 fractions were dialyzed against 0.05 M phosphate buffer (pH 7.0) and electrophoresed. The lipophorin-free fractions were used as sample for gel filtration.

Gel filtration

Gel permeation chromatography (Sephadex G-100) was conducted with sample prepared from KBr density gradient ultracentrifugation. Samples were eluted from 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. Absorbance of each fraction was measured at 230 nm and 280 nm, respectively. Each fractions were collected, dialyzed, and concentrated with freeze dryer and then electrophoresed. Fractions containing only apoLp-III were used as sample for ion-exchange chromatography.

Ion-exchange chromatography

Fractions containing apoLp-III obtained through gel permeation chromatography were collected and concentrated to give a final volume of 2 ml. This concentrate was dialyzed against 0.02 M sodium acetate buffer (0.02 M sodium acetate, 0.02 M acetic acid, 0.05 M NaCl, pH 5.0) and subjected to cation ion exchange chromatography

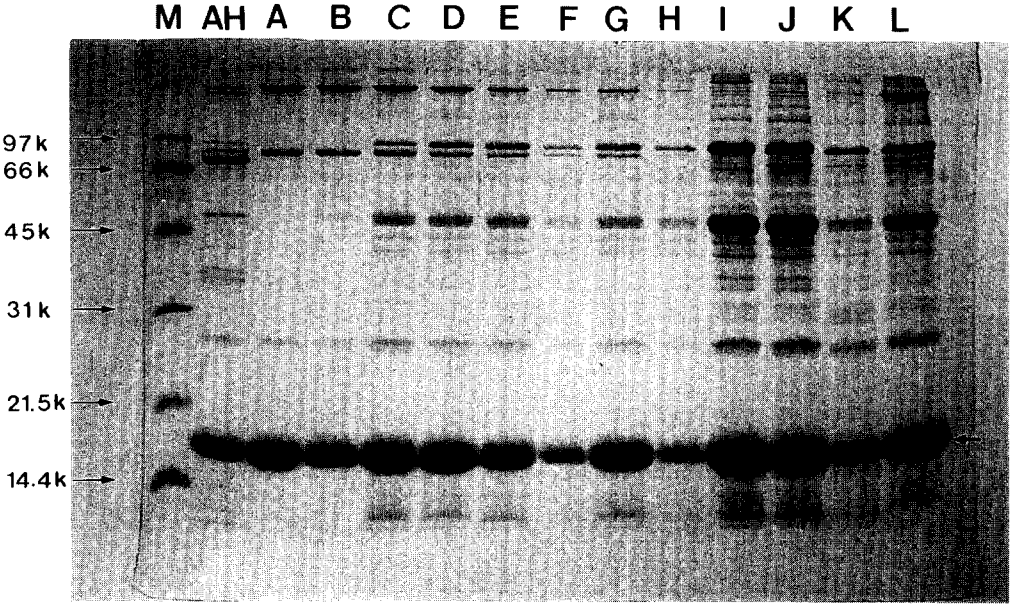

Fig. 1. SDS-PAGE of KBr density gradient ultracentrifugation fractions. M; marker proteins (Phosphorylase b: 97,000, Bovine serum albumin: 66,000, Ovalbumin: 45,000, Carbonic anhydrase: 31,000, Soybean trypsin inhibitor: 21,500, Lysozyme: 14,400), AH; adult haemolymph, A-L; fractions of ultracentrifugation from top of centrifuged tube.

(CM-52). Sample was eluted from CM-52 column (1.2 × 10 cm) with 0.05 M sodium acetate buffer (pH 5.0) at a flow rate of 3 ml/min with 2 ml per fraction. Bound protein was eluted with linear gradient (0 to 1 M NaCl in elution buffer) and absorbance of eluted fractions was measured at

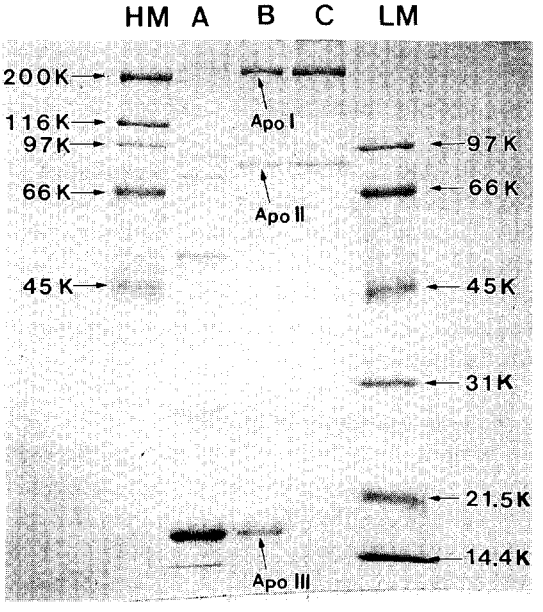

Fig. 2. SDS-PAGE of adult and larval lipophorin. HM, LM; marker proteins (Myosin: 200,000, β-galactosidase: 116,000, Phosphorylase b: 97,000, Bovine serum albumin: 66,000, Ovalbumin: 45,000, Carbonic anhydrase: 31,000, Soybean trypsin inhibitor: 21,500, Lysozyme: 14,400), A; adult haemolymph, B; adult lipophorin, C; larval lipophorin.

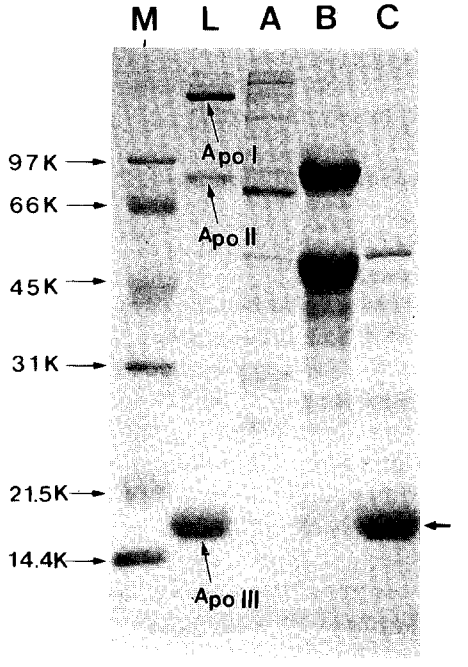

Fig. 4. SDS-PAGE of fractions of each peak in the gel-permeation chremagraphy. M;marker proteins, L; adult lipophorin, A,B and C;elutes of peak a,b,c in the gel-permeation chromatography.

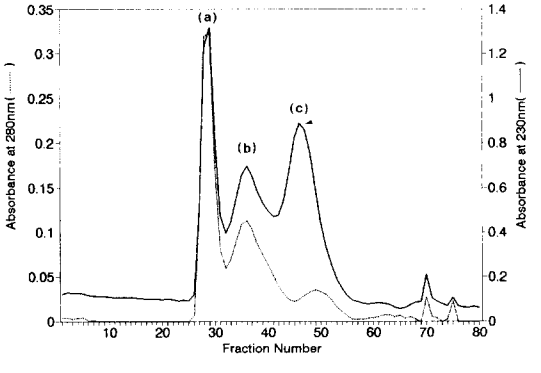

Fig. 3. Gel-permiation chromatography of LP-free fractions of Fig. 1. on a Sephadex G-100. The column was eluted with 0.05M phosphate buffer at the rate of 30 ml/h and the elutes were collected in 2.0 ml fractions.

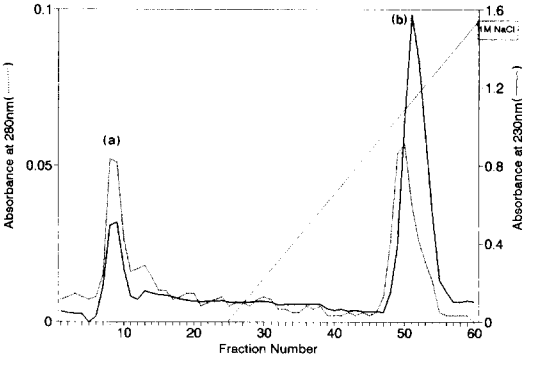

Fig. 5. CM-52 ion exchange chromatography of peak c of gel-permeation chromatography. Equilibration buffer (0.05M acetate buffer) was used as elution buffer. Linear gradient elution was performed from 0M to 1M NaCl in equilibration buffer.

230 nm and 280 nm, respectively. Purity of finally purified apoLp-III was confirmed by SDS-PAGE.

Electrophoresis

SDS-PAGE was carried out on 10% gel at room temperature at 15 mA according to Laemmli (1970). After electrophoresis, gel was stained in coomassie brilliant blue R 250 (0.25%) and destained in 50% methanol including 7% acetic acid and stored at 7.5% acetic acid.

Determination of molecular weight

Molecular weight of apoLp-III was determined as described by Lambin *et al.* (1976). Standard molecular weight marker proteins used were phosphorylase b (97,000), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

In vitro synthesis of protein

Fat body was dissected from last instar larvae in Ringer's solution (128 mM NaCl, 1.8 mM $CaCl_2$, 1.3 mM KCl ; pH 7.4) and preincubated in Grace's insect culture medium for 10 min. Preincubated fat body was *in vitro* cultured with addition of [^{35}S]-methionine for 4 hrs at 30°C. Cultured tissue was homogenized in 0.05 M phosphate buffer (pH 7.0) and centrifuged at 10, 000 rpm for 20 min. The supernatant was subjected to SDS-PAGE. After electrophoresis, gel was dried and exposed to X-ray film at -70°C for 1 week.

Results

Isolation and purification of apolipophorin-III

Apolipophorin-III (ApoLp-III) was purified from adult haemolymph by KBr density gradient

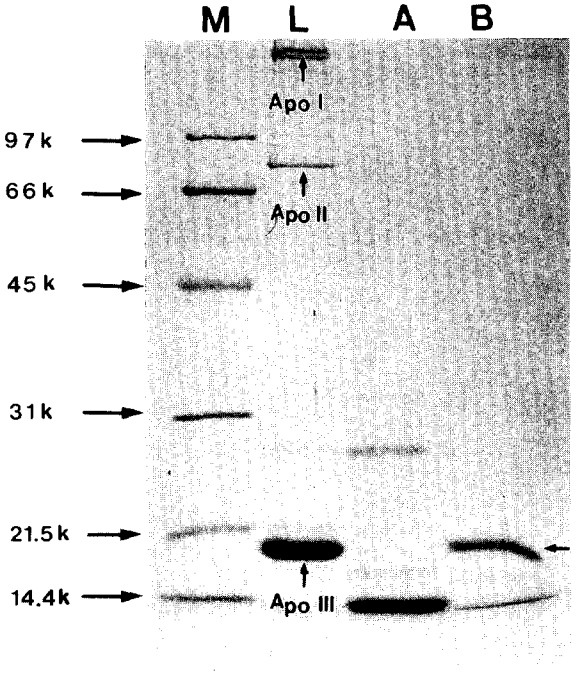

Fig. 6. SDS-PAGE of fractions of each peak in the CM-52 column. M; Marker proteins, L; adult lipophorin, A and B; eluate of peak a,b in the CM-52 column.

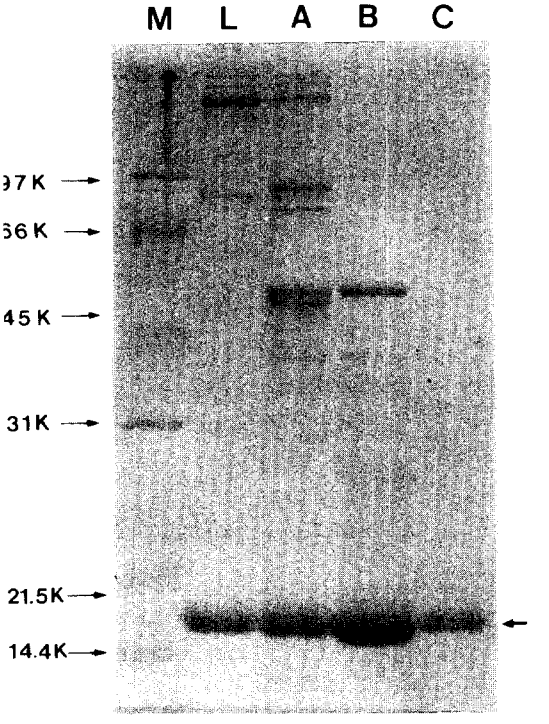

Fig. 7. SDS-PAGE of purification steps of apolipophorin-III from *Hyphantria cunea*. M; marker proteins, L; adult haemolymph, A; fraction after KBr density gradient ultracentrifugation, B; fraction after gel-permeation chromatography on a Sephadex G-100, C; fraction after ion exchange chromatography on CM-52.

ultracentrifugation. One ml each was taken out from the top of the tube and electrophoresed. LDLp was found in first fraction(A) whereas apoLp-III was predominant in remaining fractions (B-L) (Fig. 1). Also, adult Lp and larval Lp each were electrophoresed. Adult Lp consists of three subunits while larval Lp comprises two subunits, indicating that apoLp-III is present in adult Lp (Fig. 2).

Remaining fractions (B-L) were collected and subjected to gel permeation chromatography (Sephadex G-100), showing three peaks (Fig. 3). Absorbance of these fractions was measured at 230 nm and 280 nm, respectively. The reason for the measurement at 230 nm is due to the fact that the apoLp-III are lacking in aromatic amino acids. The fractions in these peaks were collected and electrophoresed, indicating that apoLp-III is present in third peak (Fig. 4).

Samples obtained through Sephadex G-100 column chromatography were applied to CM-52 ion exchange chromatography, showing two peaks (Fig. 5). The fractions in these peaks were collected and electrophoresed, indicating that apoLp-III appears in bound peak (Fig. 6). Electropherogram of apoLp-III through purification process showed that apoLp-III was purely purified in CM-52 column (Fig. 7).

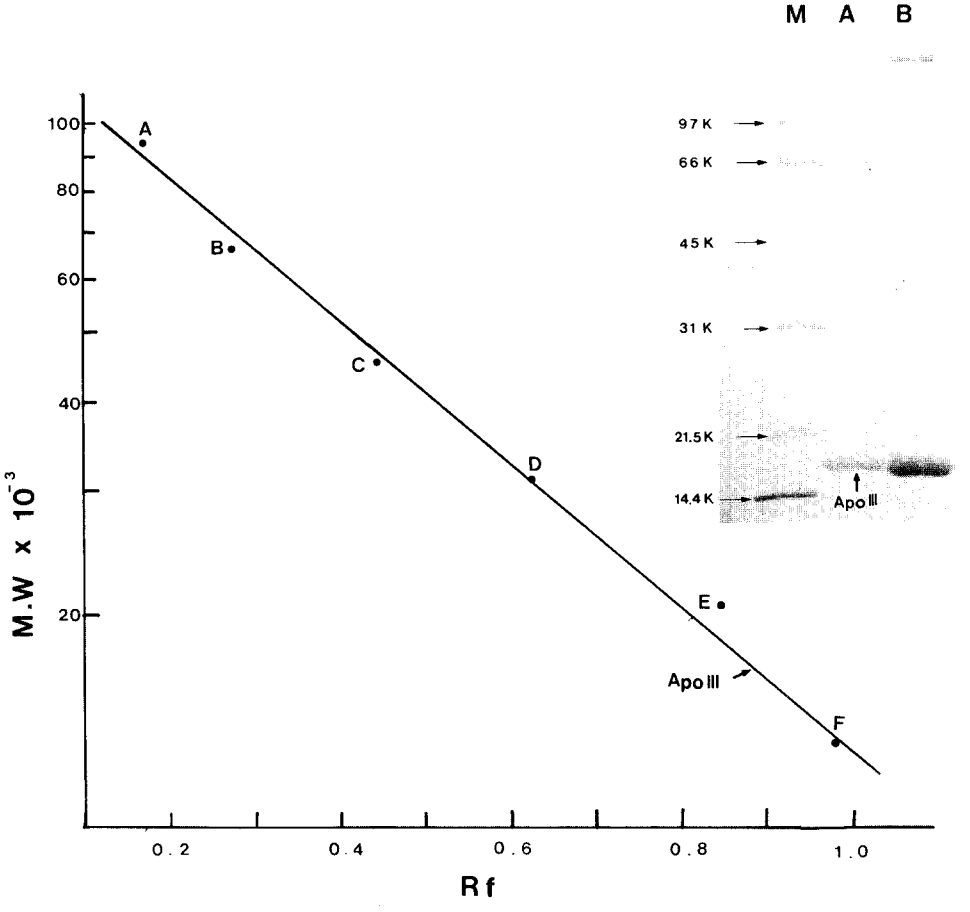

Fig. 8. Determination of molecular weight of apolipophorin-III by 10% SDS-PAGE. A; Phosphorylase b: 97,000, Bovine serum albumin: 66,000, Ovalbumin: 45,000, Carbonic anhydrase: 31,000, Soybean trypsin inhibitor: 21,500, Lysozyme: 14,400). Right upper panel shows electrophoregram of purified apolipophorin-III. M; marker proteins, A; purified apolipophorin-III, B; adult lipophorin.

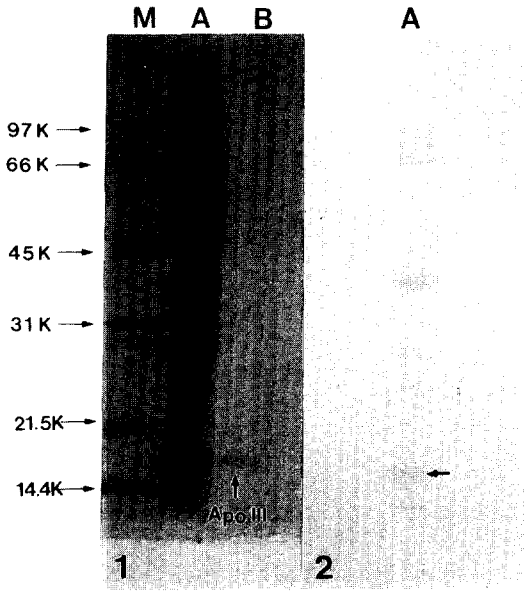

Fig. 9. SDS-PAGE (1) and autoradiogram (2) of [^{35}S]-methionine labelled proteins. M; marker proteins, A; extracts of cultured last instar larval fat body, B; purified apolipophorin-III.

Determination of apoLp-III molecular weight

Molecular weight of apoLp-III was estimated to be 18 kDa as determined on 10% gel according to Lambin *et al.* (1976)(Fig.8).

Biosynthesis of apoLp-III

To detect synthetic place of purified apoLp-III, last instar larval fat body was cultured with [^{35}S]-methionine and electrophoresed. Autoradiogram showed that apoLp-III was synthesized in fat body (Fig. 9).

Discussion

ApoLp-III was purified from the adult haemolymph of *H. cunea* and their molecular weight and synthetic place were investigated. In general, insects have to transport large amounts of lipid to flight muscle during flight. At this time, AKH (Adipokinetic hormone) was released into haemolymph and then 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 haemolymph while Burks *et al.* (1992) purified apoLp-III from lipophorin-free fractions in larval haemolymph. In *H. cunea*, both bound apoLp-III and free apoLp-III are present in adult haemolymph. Therefore, apoLp-III was purified from lipophorin-free fractions in adult haemolymph by KBr density gradient ultracentrifugation and column chromatography. ApoLp-III was measured at 280 nm, measuring range for protein and 230 nm for peptide because apoLp-III was reported to lack aromatic amino acids (Kawooya *et al.*, 1984).

Molecular weight of apoLp-III 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; Van der Horst, 1990).

Also, apoLp-III of locust was synthesized from adult fat body and released into haemolymph (Izumi *et al.*, 1987) and apoLp-III of *Diatraea grandiosella* was also predominantly synthesized in larval fat body (Shelby and Chippendale, 1990). ApoLp-III of *H. cunea* was synthesized in larval fat body.

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미국흰불나방(***Hyphantria cunea*** Drury) 혈림프부터 apolipophorin-III의 순수정제 및 특성

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미국흰불나방(*Hyphantria cunea* D.)의 adult haemolymph에서 apolipophorin-Ⅲ(apoLp-Ⅲ)를 순수분리하여 이들의 분자량 및 합성장소에 관해 조사하였다. ApoLp-Ⅲ의 정제는 KBr-density gradient ultracentrifugation을 행한 다음 gel permeation chromatography(Sephadex G-100)와 ion-exchange chromatography(CM-52)로 분리한 후, 이들의 purity를 10% SDS-PAGE로 확인한 결과, 단일밴드로 나타났다. 순수분리된 apoLp-Ⅲ의 분자량은 약 18kDa로 측정되었으며, apoLp-Ⅲ의 합성장소를 알아보기 위하여 지방체를 tissue culture하여 autoradiography를 한 결과, apoLp-Ⅲ가 지방체에서 합성됨을 확인하였다.