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Purification and characteristics of Lipophorin in *Bombyx mori*

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High density lipophorin-L (HDLP-L) was purified from the hemolymph of *Bombyx mori* using KBr density gradient ultracentrifugation and gel permeation chromatography (Sephadex G-200). Lipophorin has native molecular weight of 730 Kd and consists of Apo-Lp I and Apo-Lp II with molecular weights of 250 Kd and 90 Kd, respectively. Lp contains large amounts of glutamine & glutamic acid, threonine, leucine but small amounts of cysteic acid & oxidized cystine, tyrosine, methionine. Lp also contains diacylglycerol, cholesterol, phosphatidylcholine, and phosphatidylethanolamine. Anti-lipophorin showed positive reaction with fat body and ovarial extracts and also revealed immunological identity with lipophorin of Fall webworm, *Hyphantria cunea*. Lipophorin maintains constant level during larval and pupal stages but greatly increases during adult stage in both male and female.

Apo-Lp III was purified from adult hemolymph. Hemolymph was subjected to KBr ultracentrifugation and Lp-free fraction was submitted to cation exchange chromatography after ammonium sulfate precipitation. Apo-Lp III has molecular weight of 17 Kd, and similar amino acid composition as those of other species Lp but contains high amounts of tryptophan which other species are lacking in.

KEY WORDS: *Bombyx mori*, lipophorin, apolipophorin III

Lipophorin (LP) known to be present throughout life cycle as lipoprotein in hemolymph is known to transport lipid in insects. Lp is synthesized by fat body and released into hemolymph during last larval instar stage. Lp carries lipid absorbed from midgut to growing tissues or fat body for storage (Chino *et al.*, 1981). Usually Lp is known to transport diacylglycerol to muscle tissue for energy source (Wheller *et al.*, 1984) and to ovary for maturation (Kawooya and Law, 1988).

Lipophorin is composed of protein and lipid. Proteins are apolipophorin-I (M.W. ≒ 250 Kd), apolipophorin-II (M.W. ≒ 80 Kd) and apolipophorin-III (M.W. ≒ 17-20 Kd), and lipids are combined with small amounts of triacylglycerol, hydrocarbon, and cholesterol (Chino and Kitazawa, 1981).

Density of hemolymph Lp is different depending upon the contents of lipid in Lp (Beenakkert *et al.* 1988). Lp is mostly divided into high density lipophorin (HDLp) and low density lipophorin (LDLp).

HDLp consists of apolipophorin-I (apoLp-I) and apolipophorin-II (apoLp-II) and contains 30-50% lipid and is present in hemolymph of larval and nonactive adult. HDLp is combined with diacylglycerol to become LDLp (density 1.02-1.09 g/ml). Diacylglycerol in fat body is released into hemolymph by the action of adipokinetic hormone (AKH) during flight in several insects

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including *Locusta migratoria* (Chino *et al.*, 1986) and *Manduca sexta* (Shapiro and Law, 1983) using lipid as flight energy source. At this time apoLp-III is combined with apoLp-I and apoLp-II complex to increase loading capacity for lipid (Chino and Yazawa, 1986).

Present work is to purify HDLp-L present in hemolymph of last instar larvae in *Bombyx mori* and also to investigate physicochemical property and characteristics of HDLp-L and also to determine the presence of HDLp-L in various organs and concentration of HDLp-L during developmental stage using immunological analysis. Also, apolipophorin-III was purified from lipophorin-free fraction (Lp-free fraction) in adult hemolymph and their characteristics were investigated.

Materials and Methods

Insects

Bombyx mori were obtained from National Sericultural Experimental Station and reared on Mulberry leaves and artificial diet (Dong Bang Oil Company) at the temperature 27°C and relative humidity of 75% under the photoperiod of 16L: 8D.

Hemolymph collection

Hemolymph was collected from last instar larvae by cutting abdominal legs and from 2 day old male adult by piercing back plate. Small amounts of phenylthiourea were added to hemolymph to prevent darkening. Hemolymph was centrifuged at 10,000 g for 10 min to remove hemocytes and cell debris and supernatant was kept at −70°C until used.

Gel Electrophoresis

Sample was dialyzed against 0.1 M phosphate buffer to remove KBr. Non-SDS PAGE was carried out on 5% polyacrylamide gel at 3mA per gel according to Davis (1964). SDS PAGE was conducted on 10% SDS gel for Lp of last instar larvae and on 12% gel for that of adult at room temperature at the current of 30mA as described by Laemmli (1970). After electrophoresis, gel was stained in Coomassie brilliant blue R 250.

Purification of lipophorin (HDLp-L)

Purification process of apoLp-III was outlined in Table 1. Lipophorin was purified from the hemolymph of last instar larvae by KBr density gradient ultracentrifugation. The pooled hemolymph (approximately 3 ml) was centrifuged at 2,000 g (5°C) for 5 min to remove the hemocytes and 2.64 g of KBr was added, with stirring, to 3 ml of the supernatant to give a final density of 1.13 g/ml. The KBr-hemolymph mixtures (6.5 ml) was put in a 13 ml centrifuge tube overlayered with 6.5 ml of 0.9% NaCl (density, 1.007 g/ml). The tube was placed in ultracentrifuge TST 41.14 rotor and centrifuged at 200,000 g for 16hrs at 4°C. Yellow lipophorin obtained from ultracentrifugation was passed through desalting column (Sephadex G-25) to remove salts. Desalted lipophorin was eluted from column (2 × 60 cm) on Sephadex G-200 (Pharmacia) with 0.05 M phosphate buffer (pH 7. 0) at flow rate of 30 ml/hr with 2 ml per fraction.

Physiochemical characteristics and amino acid composition of Lp

Native molecular weight of Lp was measured on 4-6% non-SDS gel according to Hedrick and Smith (1968). Molecular weights of Lp subunits were determined on 10% SDS gel as described by Lambin (1976). Standard marker proteins used were myosin (200,000), β-galactosidase (116, 000), phosphorylase B (97,400), bovine serum albumin (66,200), egg white ovalbumin (45,000).

For amino acid analysis, purified sample was vacuum-dried, hydrolyzed in 6N HCl for 24 hrs, and submitted to HPLC (510 Solvent Delivery Pump, 712 WISP Automated Sample Processor, PicoTag (TM) Column, 990 Photodiode Array Detector ; Millipore). Sample was oxidized with performic acid in advance of acid hydrolysis for cysteine and hydrolyzed with 4M methane sulfonic acid for tryptophan.

Lipid Analysis

Lipid was extracted from purified lipophorin according to Folch *et al.* (1957) with slight modification. Fourteen volumes of chloroform-

methanol (2:1, v/v) were added to purified lipophorin and vortexed and centrifuged at 2,000 rpm for 10 min and half layer containing lipid was concentrated by nitrogen gas. Five to six ml of chloroform-methanol and 1 to 2 ml of benzene were added to concentrated lipid and again concentrated by nitrogen gas. N-hexane is finally to sample and used as sample for Thin Layer Chromatography (TLC). Sample was applied to silica gel F_{254} plate and this developed with petroleum ether-ethyl ether-acetic acid (60:40:1, v/v) for neutral lipid and ethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2, v/v) for cholesterol and chloroform-methanol-water (65:25:4, v/v) for phospholipid. Plates were heated to 120°C and sprayed with iodine vapor for color development.

Preparation of the antiserum and immunological analysis

Purified lipophorin (0.5 ml) and Freund's complete adjuvant (0.5 ml) were mixed and injected into rabbit subcutaneously. Injection was performed every other day for first week and fourth injection was conducted two weeks later. Freund's incomplete adjuvant (0.5 ml) and purified lipophorin (0.5 ml) were thoroughly mixed and used for booster injection two weeks after fourth injection. Blood was collected one week after last injection and centrifuged at 10,000 g for 10 min and the supernatant was used. Immunodiffusion was conducted on 1% agarose gel containing 0.1% sodium azide (w/v) in veronal buffer (pH 8.6) as described by Ouchterlony (1949). The plates were stained in 1% amido black 10B and destained in 2% acetic acid.

Rocket immunoelectrophoresis was performed according to Laurell (1966). Agarose was added to 10 mM veronal buffer (pH 8.6) containing 0.1% sodium azide to give a final concentration of 1% and antibody was added to the 1% agarose gel to give a final concentration of 3% gel. Electrophoresis was conducted in 10 mM veronal buffer (pH 8.6) at 95 V for 4hrs.

Tandem-crossed immunoelectrophoresis was carred out after the procedure of Axelson *et al.* (1973). Agarose, buffer solution, and antibody were the same as described for rocket immunoelectrophoresis. The first dimension was run at 10 v/cm for 3 hrs and the second dimension was run at 5v/cm for 18 hrs.

Identification and purification of apolipophorin-III

Purification process of apoLp-III was outlined in Table 1. Hemolymph was electrophoresed throughout the all stages to confirm the presence of apoLp-III. Adult hemolymph was ultracentrifuged to measure their density. Two yellow bands (HDLp and LDLp) and Lp-free fraction were divided and each electrophoresed and apoL-III was purified from Lp-free fraction. Lp-free fraction was dialyzed against 0.1 M phosphate buffer (pH 7.0) at 4°C for 12 hrs. KBr-free fraction were saturated with constant stirring to 65% with ammonium sulfate at 4°C to induce protein precipitation (Cole and Wells, 1990) and centrifuged at 13,000 g for 30 min. The supernatant was treated one more time as above and second supernatant was concentrated with polyethylene glycol 20,000 and then dialyzed against 0.1M phosphate buffer, 0.01% EDTA, pH 5.5 for 12 hrs for ion exchange chromatography. This supernatant was used for cation exchange chromatography. CM-52 was used as resin and 0.1 M phosphate buffer (pH 5.5) was used as elution buffer. Bound proteins were eluted with 0-0.5 M linear gradient NaCl in elution buffer and fractions containing apoLp-III was confirmed by SDS PAGE and freeze-dried.

Results

Purification of lipophorin

Lipophorin (HDLp-L) was purified from the hemolymph of last instar larvae by KBr density gradient ultracentrifugation. The yellow band was collected with pasteur pipette and density was measured. Density of fraction No. 6 belonging to HDLp-L was 1.14 g/ml (Fig. 1). HDLp-L was purified from this fraction by gel filtration. Purity was confirmed by single band on non-SDS gel (Fig. 2A) and subunits were determined by SDS PAGE (Fig. 2B).

Table 1. Procedure of purification.

(A) Purification of lipophorin (HDLp-L)

Hemolymph (last larva)
- KBr density gradient ultracentrifugation (200,000 g for 16hrs at 4°C)

Yellow lipophorin
- Desalting column (Sephadex G-25)
- Sephadex G-200 gel filtration (0.05 M phosphate buffer, pH 7.0)

10% SDS-PAGE for confirming HDLp-L

(B) purification of apoLp-III

Hemolymph (adult)
- KBr density gradient ultracentrifugation (200,000 g for 16hrs at 4°C)

Lp-free fraction
- Desalting column (0.1 M phosphate buffer, pH 7.0)
- 65% ammonium sulfate precipitation
- centrifugation (13,000g, 30min)

Supernatant
- CM-52 cation exchange chromatography (Salt gradient:0-0.5M NaCl)

12% SDS-PAGE for confirming apoLP-III

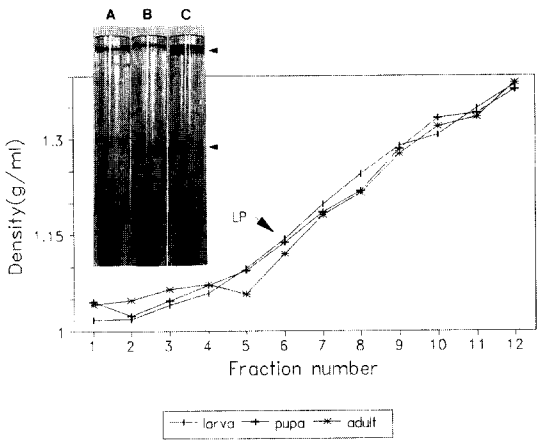

Fig. 1. Density profile obtained by KBr density gradient ultracentrifugation(200,000 g for 16 hrs) of *B. mori* Hemolymph. A, larval; B, pupal; C, adult hemolymph. Fraction 6 was gel permeation chromatographed. Arrows indicate LP.

Molecular weights and amino acid composition of lipophorin

Native molecular weight of Lipophorin was estimated to be 730 Kd and molecular weights ApoLP-I and apoLP-II were determined to be 250 Kd and 90 Kd, respectively (Fig. 3). Lipophorin contains large amounts of GLX (glutamine & glutamic acid), threonine, and leucine but small amounts of CYA (cysteic acid & oxidized cystein), tyrosine, and methionine (Table 2).

Lipid composition

Neutral lipid in HDLp-L was observed using thin layer chromatography. They contain 1,2-diacylglycerol and 1,3-diacylglycerol with small amounts of triacylglycerol and monoacylgycerol. Cholesterol was also found to be present. For phospholipid, phosphatidylethanolamine, phosphatidylcholine, and sphingomyelin were observed (Fig. 4).

Immunological analysis

Antibody against purified HDLp-L was made and used to determine the presence of HDLp-L in various organs, the phylogenetic relationship with other species, and concentration of lipophorin during developmental stages. They showed positive reaction with fat body, ovary, testis, and midgut extracts in immunodiffusion and immunoblotting (Fig. 5). Tandem-crossed immunoelectrophoresis showed continuous precipitine line between peaks from lipophorin, fat body, and ovary, indicating immunological identity

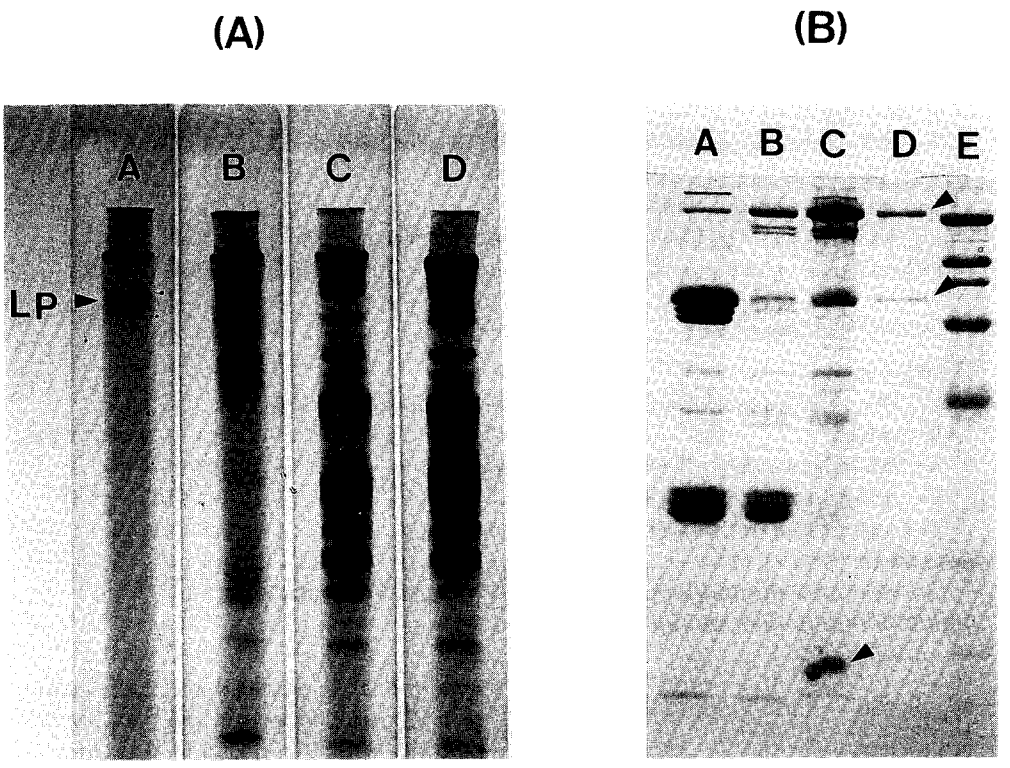

Fig. 2. (A), Native PAGE of hemolymph and purified lipophorin of *B. mori*. A, purified lipophorin(arrow); B, adult hemolymph; C, pupal hemolymph; D, larval hemolymph. (B), SDS-PAGE of hemolymph and purified lipophorin of *B. mori*. A, larval hemolymph; B, pupal hemolymph; C, adult hemolymph (arrow indicates apoLP-III); D, purified lipophorin (arrows indicate apoLP-I, apoLP-II); E, standard marker.

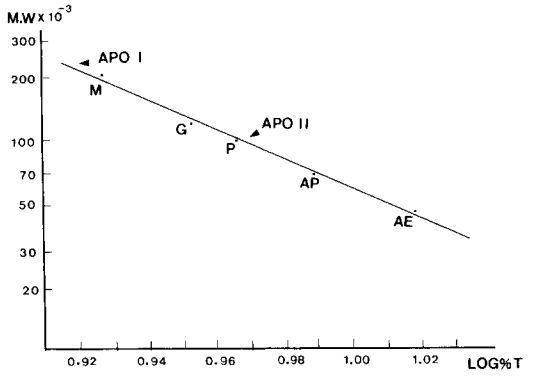

Fig. 3. Determination of M.W. for Apolipophorins by electrophoresis in 8-12% linear gradient of polyacrylamide gel in the presence of SDS. Marker proteins used were M, Myosin (25,000); G, β-galactosidase (116,000); P, Phosphorylase B (97,000); AB, Bovine plasma albumin (66,000); AE, Egg albumin (45,000)

between them (Fig. 6). Concentration of Lipophorin showed constant level during larval and pupal stages but drastic increase during adult stage in both male and female (Fig. 7).

Identification, purification, and amino acid composition of apolipophorin-III

Hemolymph was electrophoresed during developmental stages, indicating that apoLP-III (M. W. = 17 Kd) appears in large amounts in adult stage (Fig. 8A). Adult hemolymph was ultracentrifuged in KBr density gradient tube and divided into HDLp, LDLp, and Lp-free fraction. These fractions each were electrophoresed, indicating that apoLP-III is present in large amounts in Lp-free fraction (Fig. 8B). Also, protein peak was not observed at 280 nm but appeared at 230 nm (Fig. 9). These peaks are concentrated and electrophoresed, showing that

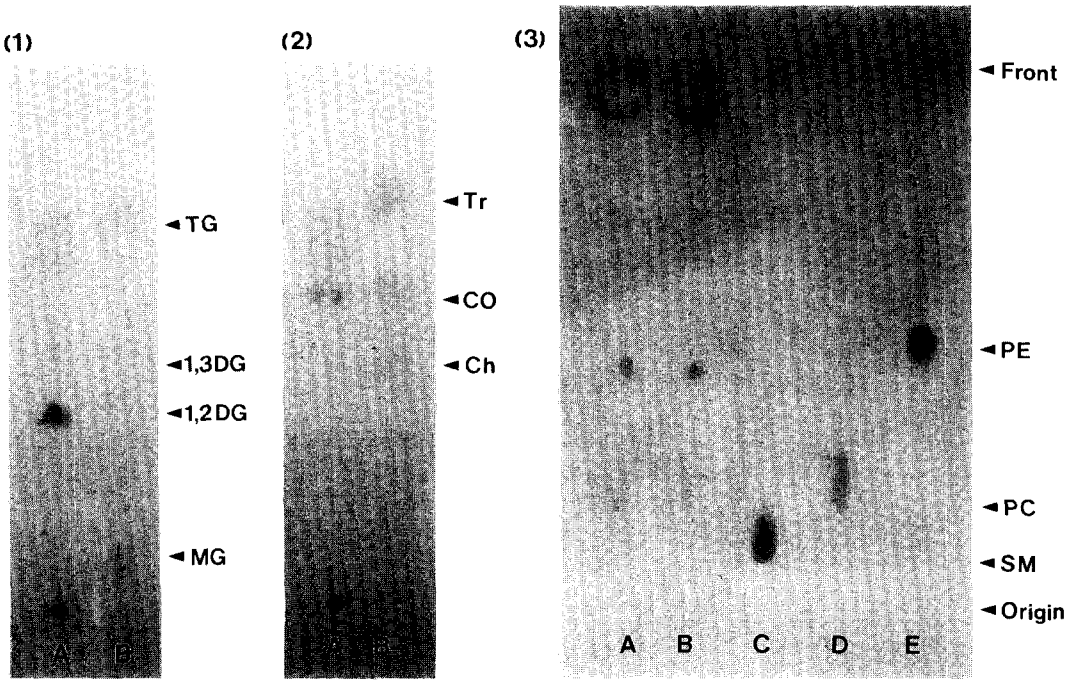

Fig. 4. Thin-layer chromatography of lipid components in Lipophorin. The lipids extracted with chloroform-methanol (2:1, v/v) from purified LP were applied to the plate of silica gel. (1), A, lipophorin (LP); B, monoglyceride (MG), diglyceride (DG), triglyceride (TG) : (2), A, LP; B, cholesterol (Ch), cholesteryl oleate(CO), triolein (Tr) : (3), A,B, LP; C, spingomyelin (SM), phosphatidylcholine (PC), phosphatidylethanolamine (PE).

apoLP-III appears as single band in some fraction (Fig. 10).

ApoLP-III contains comparatively small amounts of cysteine, tyrosine, and methionine but large amounts of glutamine, alanine, and lysine (Table 2).

Discussion

Native molecular weight of lipophorin was estimated to be 730 Kd which was a little higher than those of Locust (580 Kd), Cockroach (600 Kd), and Silkworm (700 Kd) (Pattnaik *et al.*, 1979). Amino acid composition of HDLp-L in Philosamia silkworm, *Periplaneta americana*, and locust was reported to have a high amount of aspartic acid, glutamic acid and leucine but low amount of methionine (Chino and Kitazawa, 1981). *Bombyx mori* showed similar data with a little lower in ASX (asparagine & aspartic acid) and a little higher in threonine.

Lipophorin was reported to comprise 40-50% lipid (Gellissen and Emmerich, 1980). In neutral fat diacylglycerol (DG) predominates while triacylglycerol (TG) is relatively small. Also, lipophorin contains a large amount of cholesterol, and phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin in phospholipid. Lipophorin of *Bombyx mori* contains large amonuts of 1.2-DG and 1.3-DG but small amounts of TG. Also, Lp contains small amounts of cholesterol, phosphatidylethanolamine, and phosphatidylcholine. Concentration of lipophorin during developmental stages shows increase during larval stage, constant level during pupal stage but decrease during adult stage in *Musca domestica* (de Bianchi *et al.*, 1987) and gradual decrease during larval and pupal stages in *Lymantria dispar* (Ryu and Kim, 1987). Gonzalez *et al.* (1991) reported that lipophorin shows gradual increase during larval stage but increase

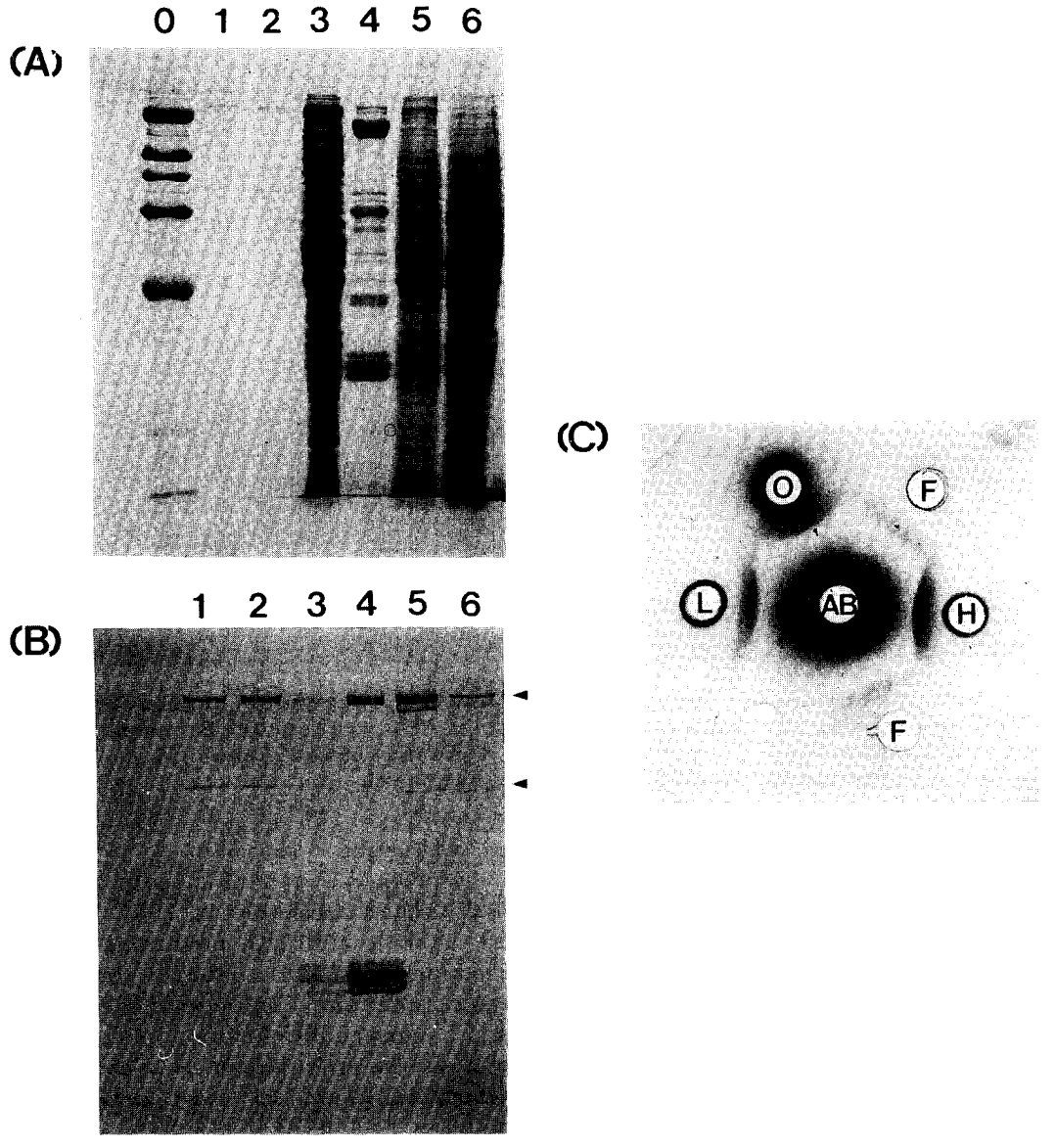

Fig. 5. Immunological relationships of each organ with anti-LP. (A), Each organ was separated by SDS-PAGE; (B), Immunoblotted nitrocellulose. lane 0, standard marker; 1-2, purified LP; 3, fat body extracts; 4, ovary extracts; 5, testis extracts; 6, midgut extracts. (C), Ouchterlony's double diffusion test. AB, anti-LP; L, LP; O, ovary extracts; F, fat body extracts; H, *B. mori* hemolymph.

during adult stage with slight decrease in early phase. *Bombyx mori* LP shows constant level during larval and pupal stages but great increase during adult stage.

Lipophorin shows positive reaction with fat body, ovary, testis, and midgut extracts. LP was reported to be present in fat body (Gellissen and Wyatt, 1981) and in ovary (Kawooya and Law, 1988). Also, storage protein was reported to be synthesized and transported within testis (Miller *et al.*, 1990) but lipophorin was not reported in testis. *Bombyx mori* showed positive reaction with

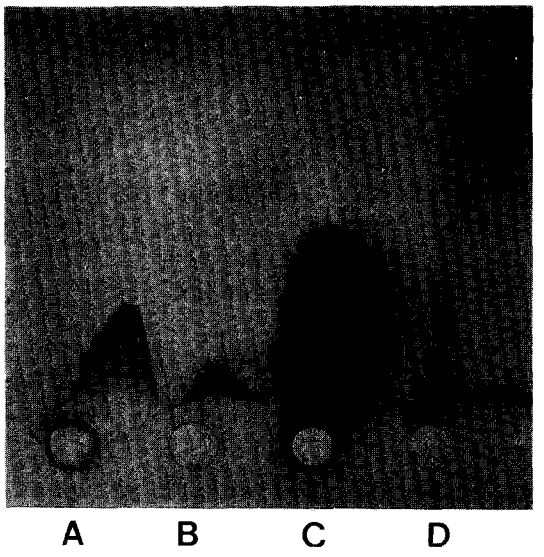

Fig. 6. Tandem-Crossed Immunoelectrophoresis with anti-LP. A, purified LP from larval hemolymph; B, ovary extracts; C, larval hemolymph; D, fat body extracts.

testis, indicating that lipophorin is present in testis.

Lipophorin is at least composed of apoLP-I and apoLP-II and appears in larval and adult stages. Also, HDLp only appears in larval and pupal stages but LDLp as well as HDLp are present in adult stage. Thus, apoLp-III is present in very small amounts in larval and pupal stages and independent of lipophorin but becomes important component of lipophorin in adult. Lipophorin is present as HDLp state in resting stage of adult but adult *Bombyx mori* showed large amounts of HDLp as well as LDLp (Miura and Shimizu, 1989). In the present work with *Bombyx mori*, apoLP-III was combined with LDLp but not with HDLp and also was present in large amounts in Lp-free fraction (Fig. 8).

ApoLP-III was purified from LP present in adult hemolymph (Kawooya *et al.*, 1984) and Lp-free fraction of larval hemolymph (Burks *et al.*, 1992). In the present work with *Bombyx mori*, apoLP-IIIwas purified from Lp-free fraction of adult hemolymph which contains large amounts of apoLP-III. Absorbance was measured at 230 nm because LP is deficient in tyrosine. Amino acid composition of apoLp-III is similar to those of other insect species. They contain small amounts of cysteine, tyrosine, and methionine but large amounts of tryptophan which is scarcely present in other insects. As amino acid composition of apoLP-III and HDLp-L were compared, they both contain large amounts of glutamine, alanine, and leucine but small amounts of cysteine, tyrosine, and methionine.

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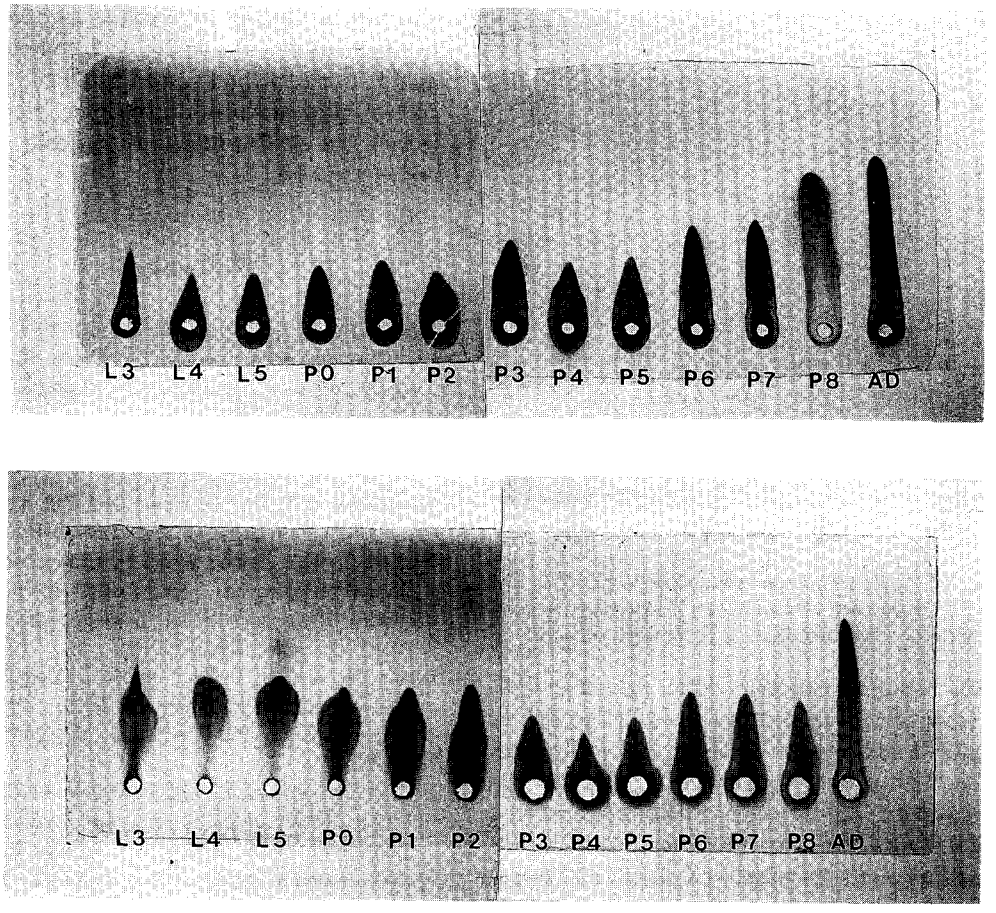

Fig. 7. Rocket immunoelectrophoretic patterns of male (1) and female (2) hemolymph reacted with anti-LP during developmental stages. L3, 3rd instar larvae; L4, 4th instar larvae; L5, 5th instar larvae; P0-P8, 0 to 8-day old pupae; AD, adult.

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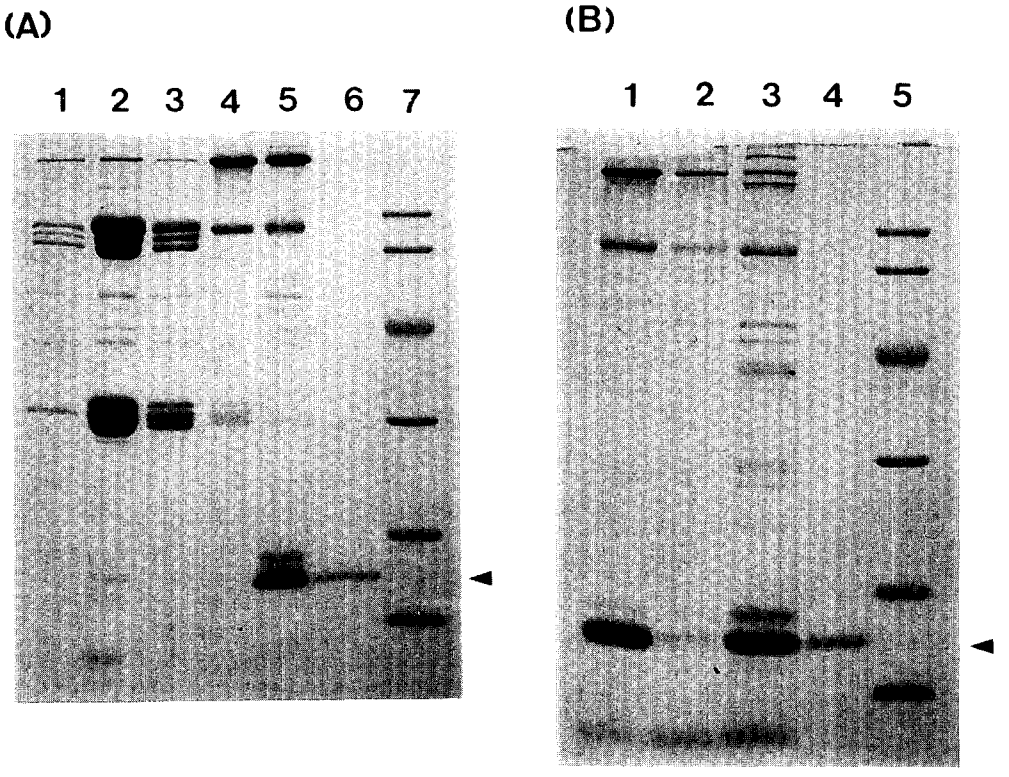

Fig. 8. (A), SDS-PAGE of hemolymph from different stages. 1, 4th instar larvae; 2, 5th instar larvae; 3, prepupae; 4, 5-day old pupae; 5, adult; 6, purified apoLP-III; 7, standard marker. (B), SDS-PAGE (12%) of selected fractions after KBr density gradient ultracentrifugation. 1, LDLp; 2, HDLp; 3, Lp-free fraction; 4, purified apoLP-III; 5, standard marker.

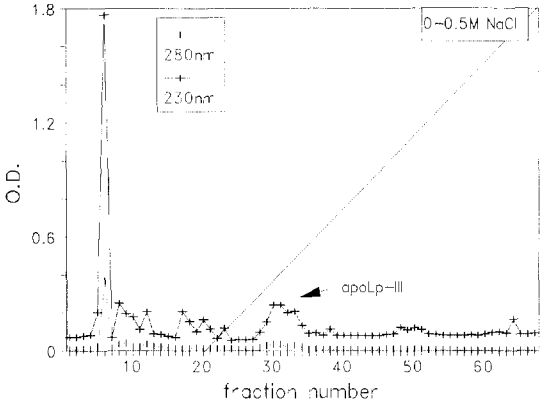

Fig. 9. Cation-exchange chromatography (CM-52) of ammonium sulfate supernatant of Lp-free fraction. After eluted with 0.1 M phosphate buffer (pH 5.5), apoLP-III were eluted with a gradient from 0 to 0.5 M NaCl in the same buffer.

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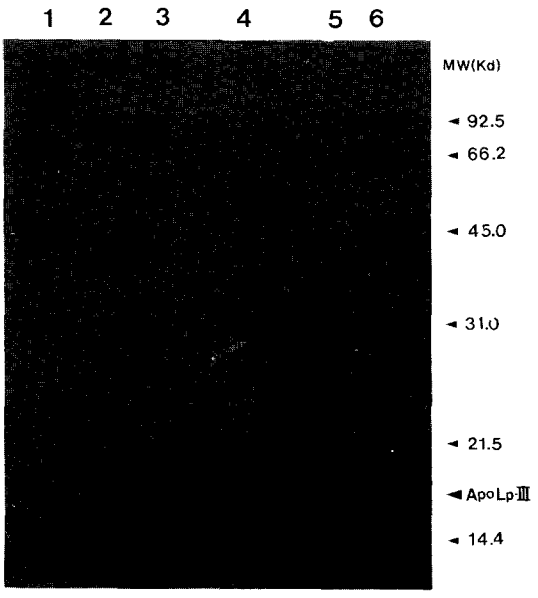

Fig.10. SDS-PAGE of apoLP-III at different stages of purification. 1 and 6, protein standard; 2, crude hemolymph; 3, Lp-free fraction after isolation by KBr density gradient ultracentrifugation; 4, supernatant after 65% ammonium sulfate precipitaion; 5, apoLP-III fractions; 6, cation-exchange chromatography.

Table 2. Amino acid composition of purified Lipophorin and apolipophorin-III from *Bombyx mori*. Data are in residues Mol %.

amino acid	Lipophorin(HDLp-L)	Apolipophorin-III
ASX**	7.53	5.86
THR	10.03	4.94
SER	4.28	3.38
GLX**	10.69	15.67
PRO	7.51	6.58
GLY	6.21	1.82
ALA	8.00	15.06
VAL	7.19	4.96
MET	0.60	0.23
ILE	6.11	3.62
LEU	9.62	9.10
TYR	0.62	0.42
PHE	7.86	5.08
HIS	2.01	2.23
LYS	6.72	13.00
ARG	4.49	2.81
TRP	0.14	5.05
CYA*	0.77	0.16

CYA* means the sum of cysteic acid & oxidized cystine. ASX**, GLX** mean the sum of Asparagine & Aspartic acid and Glutamine & Glutamic acid, respectively.

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누에나방(***Bombyx mori***)의 Lipophorin의 정제 및 특성

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누에나방(*Bombyx mori*)의 HDLp-L을 KBr밀도구배 초원심분리법과 gel permeation chromatography(Sephadex G-200)으로 순수 분리하였다. 리포포린의 native molecular weight는 730kd였고 분자량이 250kd와 90kd인 Apo-Lp Ⅰ Apo-Lp Ⅱ로 나타났으며, 아미노산의 조성은 GLX(glutamine & glutamic acid), threonine, leucine에서 높은 비율로 조사되었고 CYA(cysteic acid & oxidized cystine), tyrosine, methionine은 낮은 비율로 존재하였다. 지질에 대한 조성은 중성지질은 diacylglycerol이 주로 존재하였고 cholesterol과 phosphatidylcholine, phosphatidylethanolamine의 인지질이 존재하였다. 면역학적 실험에서는 지방체와 난소추출물에서 면역반응이 나타났으며, 타종과의 관계에서는 같은목에 속하는 미국흰불나방(*Hyphantria cunea*)의 리포포린과 면역학적 동질성을 보였다. 발생단계에 따른 리포포린의 농도변화는 암수 모두 유충시기와 용시기에는 일정하다가 성충때 크게 증가함을 보였다.

apoLp-Ⅲ에 대한 정제는 성충나방의 혈림프에서 KBr초원심분리를 한후 Lp-free fraction으로 부터 ammonium sulfate precipitation과 cation exchange chromatography를 사용하여 정제하였다. apoLp-Ⅲ의 분자량은 17 Kd였고 아미노산 조성은 타종과 비교했을때 거의 비슷하였으나 다른종에는 없는 tryptophan이 상당량 존재하였다.