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Isolation and Characterization of Lipophorin from *Lymantria dispar* L.

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Lipophorin of *Lymantria dispar* L. has been purified by KBr density gradient ultracentrifugation. The properties and synthetic site of lipophorin and quantitative change of lipophorin during development have been determined using electrophoresis and immunological analysis. Lipophorin is composed of two subunits, apo-LpI (230,000), apo-LpII (49,000), and contains carbohydrates and lipids. Anti-lipophorin showed positive reactions with fat body extract and ovary extract but not with gut extract. The concentration of lipophorin in hemolymph showed gradual decrease during larval and pupal stages. Also, fat body released lipophorin into medium. Immunological test showed some partial identity between lipophorin of *Lymantria dispar* and hemolymph proteins (probably lipophorin) of *Hyphantria cunea* and *Galleria mellonella*.

KEY WORDS : Hemolymph, Fat body, Lipophorin, Immunological analysis, *Lymantria dispar*

Insect hemolymph has special lipid carrier which binds hydrophobic materials such as lipids, carotenoids, cholesterols and hydrocarbons absorbed from the gut and transports them to special storage site and also carries them from storage site to various organs. This carrier is a conjugated protein called lipophorin.

Lipophorin functions to transport the absorbed dietary lipids to storage organ such as fat body during larval stage (Ventatesh and Chippendale, 1987) and provide ovary with phospholipids necessary for vitellogenesis in vitellogenic female (Gondim *et al.*, 1989a, b). Also, lipophorin functions to transport energy source from fat body to flight muscle in adults of Lepidoptera, Hemiptera and Orthoptera using lipids as energy source for flight (Mayer and Candy, 1967; Van der Horst *et al.*, 1978, 1979). Lipophorin was also reported to be JH carrier protein (De Kort and Koopmanschap, 1989). Lipophorin was known to transport diacylglycerols, cholesterols, carotenoids, hydrocarbons (Van der Horst *et al.*, 1979; Katase and Chino, 1984; Parasad *et al.*, 1986; Ryan *et al.*, 1986; Tsuchida *et al.*, 1987; Germain *et al.*, 1988), phospholipids (Gondim *et al.*, 1989) and various xenobiotics (Haunerland and Bowers, 1986). Among these diacylglycerol or cholesterol was bound to lipophorin from fat body while hydrocarbon, at least in the case of *Locusta migratoria*, bound to lipophorin from oenocyte and transported to target cell such as cuticle (Katase and Chino, 1984).

Protein part of lipophorin is composed of two subunits (apo-LpI and apo-LpII) (Chino *et al.*, 1981; Shapiro *et al.*, 1984; Robbs *et al.*, 1985; Prasad *et al.*, 1986; de Bianchi *et al.*, 1987; Germain *et al.*, 1988). It was also known that lipophorin is made up to three subunits (apo-LpI, apo-LpII and apo-LpIII or C protein) in adult of *Manduca sexta*, *Locusta migratoria* and *Schistocerca gregaria* using lipids as energy source for flight (Kawooya *et al.*, 1984; Van der Horst *et al.*, 1984; Wells *et al.*, 1985; Haunerland *et al.*, 1986). Thus, research works on lipophorin have been mostly confined to seperation, purification and

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composition. Present work reports on quantitative change during development and synthetic site of lipophorin, in addition to purification and composition of lipophorin in hemolymph of *Lymantria dispar*. Also, immunological relationship with hemolymph proteins of other insects was tested.

Materials and Methods

Insects

Larvae of *Lymantria dispar* were reared on gypsi moth wheat germ diet, ICN Biochemicals at 27 ± 1°C and 75 ± 5% relative humidity with the photoperiod of 16th light : 8h dark. *Hyphantria cunea* and *Galleria mellonella* were obtained from the colony maintained in Department of Biology, Korea University.

Collection of Hemolymph

Hemolymph was collected in a chilled test tube by cutting the abdominal leg of larvae. A few crystals of phenylthiourea (PTU) were added to the hemolymph to prevent melanization and also a small amount of EDTA added to prevent coagulation of lipophorin. The hemolymph was then centrifuged at 10,000 rpm for 10 min and the supernatant stored at −70°C until used.

Purification of lipophorin by ultracentrifugation

Lipophorin was purified from the hemolymph by density gradient ultracentrifugation using Hitachi RPS-50-2-126 rotor. Eleven thousand seventy two mg of KBr and 1 ml of hemolymph were put into ultracentrifuge tube. Phosphate buffered saline (PBS : 0.15 M NaCl/0.10 M Sodium phosphate/0.05% EDTA, pH 7.0) was added to this hemolymph to be final volume of 2.5 ml. KBr was completely dissolved using vortex and then 0.9% NaCl was added to this solution to be final volume of 5 ml. This solution was centrifuged at 38,000 rpm for 10 min at 10°C and then yellow colored lipophorin band was extracted from the tube using peristaltic tube.

Determination of Molecular Weights

Molecular weights of lipophorin subunits were determined as described by Lambin *et al.* (1976). Standard molecular weight markers were carbonic anhydrase (29,000), egg albumin (45,000), bovine plasma albumin (66,000), phosphorylase (97,000), β-galactosidase (116,000) and myosin (205,000).

Composition of Lipophorin

Purified lipophorin was electrophoresed and stained with PAS for carbohydrate (Caldwell and Pigmann, 1965) and Sudan black B for lipid (Chippendale and Beck, 1966).

Preparation of the antiserum and immunological analysis

Purified lipophorin (approximately 600 μg/ml) was mixed with an equal volume of Freund's complete adjuvant (0.5 ml) and injected subcutaneously into rabbits three times every other day with a fourth injection given 1 week later. Booster injections (0.5 ml protein and 0.5 ml Freund's incomplete adjuvant) were given 2 weeks after the fourth injection. Blood was collected 1 week after the fifth injection, allowed to coagulate at 4°C overnight, and centrifuged at 10,000 g for 10 min. The supernatant was used for the immunological tests. Immunodiffusion tests were conducted on 1% agarose gel in 10 mM Veronal buffer (pH 8.6) containing 0.1% sodium azide for 3 days at room temperature as described by Ouchterlony (1949). Gels were stained in 1% Amido black 10B and destained in 2% acetic acid. Rocket immunoelectrophoresis was carried out according to Laurell (1966). One percent agarose in 10 mM Veronal buffer (pH 8.6) containing 0.1% sodium azide was mixed with an appropriate amount of anti-LP serum to yield 5% anti-LP serum. This mixture was coated on a glass plate (6.5 × 6.5 cm). Electrophoresis was conducted in 10 mM Veronal buffer (pH 8.6) at 200 V for 2 h. After electrophoresis, the gel was washed in 0.15 M NaCl for 48 h and stained in Amido black 10B.

Tissue culture

Fat body tissue was dissected and washed two or three times in cold Ringer solution and incubated in Grace's insect culture medium for 4 h. The supernatant was used for immunodiffusion.

Results

Purification of Lipophorin by Ultracentrifugation

Lipophorin was purified from larval hemolymph by KBr density gradient ultracentrifugation. Yellow colored band appeared at upper middle part of tube after centrifugation (Fig. 1). This color is due to the presence of carotenoids bound to lipophorin (Shapiro *et al.*, 1984). This band was regarded to be lipophorin and so collected for sample.

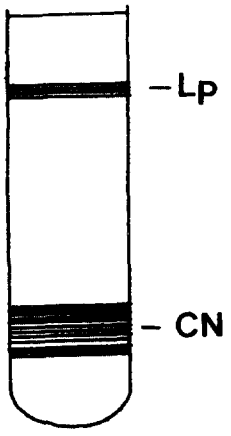

Fig. 1. KBr density gradient ultracentrifugation of *L. dispar* hemolymph. Lp : Lipophorin band (yellow), CN : Cyanoprotein band (blue).

Purity confirmation of purified lipophorin

Hemolymph and purified lipophorin were each electrophoresed under non-denatured condition to confirm lipophorin band on hemolymph electrophorogram as well as purity of purified lipophorin (Fig. 2). As shown in Fig. 2, two lipophorin bands appeared on electrophorogram, among which upper band is considered to be lipophorin.

Properties of lipophorin

Purified lipophorin gave positive reactions with PAS and Sudan black B, indicating that lipophorin contains carbohydrate and lipid (data not shown). Also, purified lipophorin was electrophoresed on 6% SDS-polyacrylamide gel with standard molecular weight markers. Lipophorin is composed of two subunits, apo-LpI (M. W = 230,000) and apo-LpII (49,000)(Figs. 3, 4).

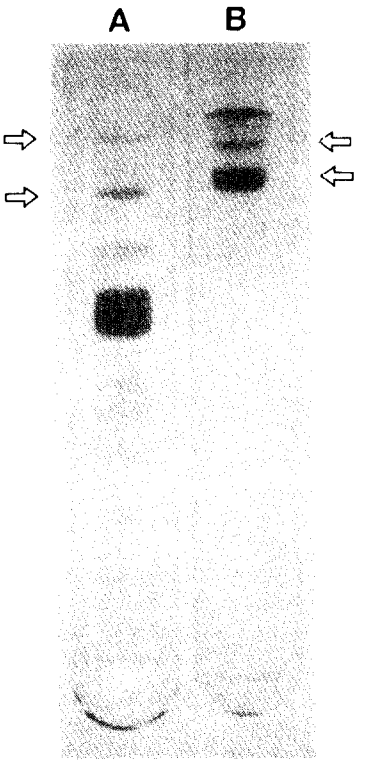

Fig. 2. Electrophoretic patterns of hemolymph and purified lipophorin of *L. dispar*. The open arrows indicate lipophorin band. lane A : hemolymph, lane B : purified lipophorin.

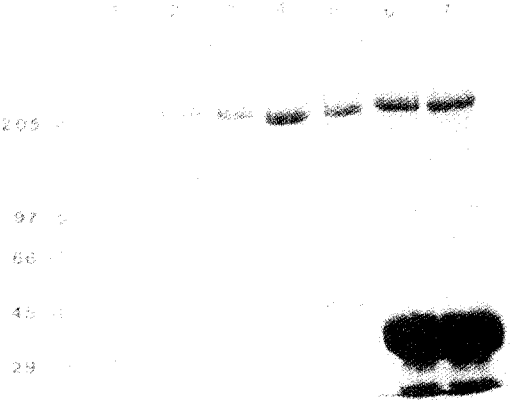

Fig. 3. SDS-PAGE (6%) of hemolymph and purified lipophorin. lane 1 : Standard molecular weight markers, lanes 2-5 : purified lipophorin, lanes 6-7 : hemolymph.

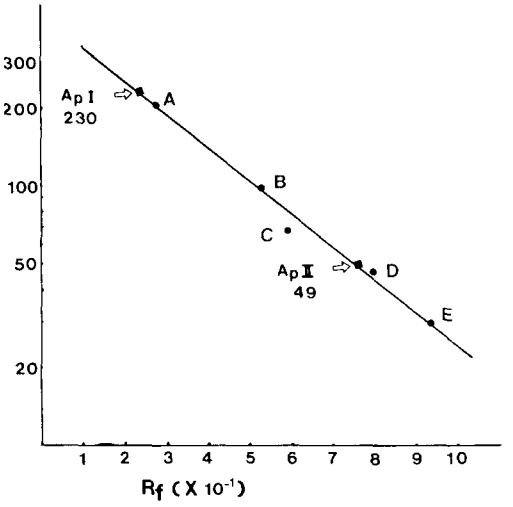

Fig. 4. Determination of M. W. of Lipophorin subunits (Apolipophorin) by 6% SDS-PAGE. Marker proteins used were (A) Myosin (205,000), (B) Phosphorylase (97,400), (C) Bovine plasma albumin (66,000), (D) Egg albumin (45,000), (E) Carbonic anhydrase (29,000).

Distribution of lipophorin in tissues

Immunodiffusion was conducted using antibody against lipophorin to determine the distribution of lipophorin in tissues. Anti-lipophorin showed positive reaction with larval fat body and pupal ovary but not with larval midgut (Figs. 5, 6).

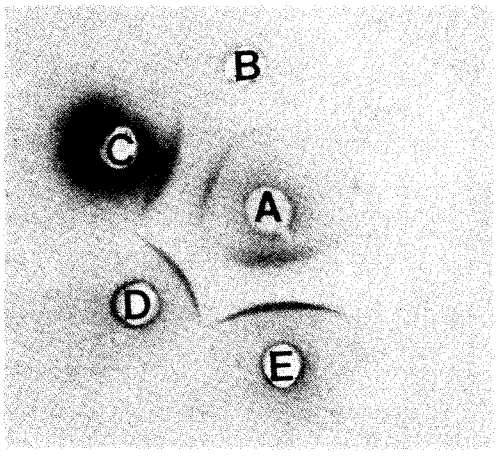

Fig. 5. Double diffusion precipitation on Ouchterlony plate. A : anti-Lp, B : gut extract, C : fat body extract, D : purified lipophorin, E : hemolymph.

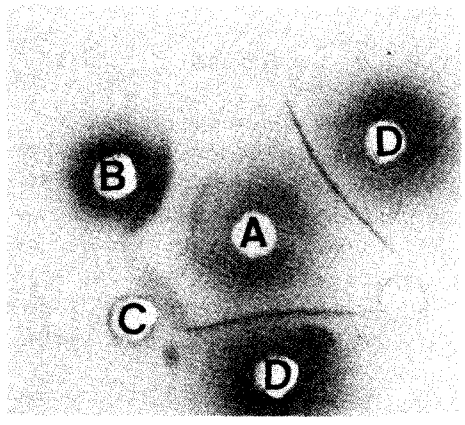

Fig. 6. Double diffusion precipitation on Ouchterlony plate. A : anti-Lp, B : ovary extract, C : fat body culture media, D : hemolymph.

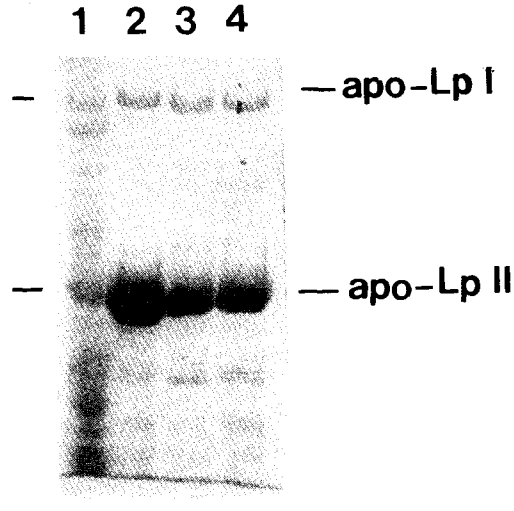

Fig. 7. SDS-PAGE (6%) of hemolymph and fat body extract. lane 1 : fat body extract, lanes 2-4 : hemolymph.

Synthesis of lipophorin by fat body

Fat body tissue was incubated in Grace's insect medium to determine the synthetic site of lipophorin. The supernatant showed the positive reaction with anti-lipophorin, indicating that fat body produces lipophorin into medium (Fig. 6). Also, electrophorogram showed that apo-LpI and apo-LpII bands appeared on SDS gel (Fig. 7).

Quantitative change of lipophorin during development

Hemolymph was collected from 3rd, 4th, 5th

Fig. 8. Rocket immunoelectrophoretic patterns of hemolymph reacted with anti-Lp during developmental stages. L3 : 3rd instar larvae, L4 : 4th instar larvae, L5 : 5th instar larvae, LL : the last instar larvae, P1 : 1-day old pupae, P3 : 3-day old pupae, P5 : 5-day old pupae.

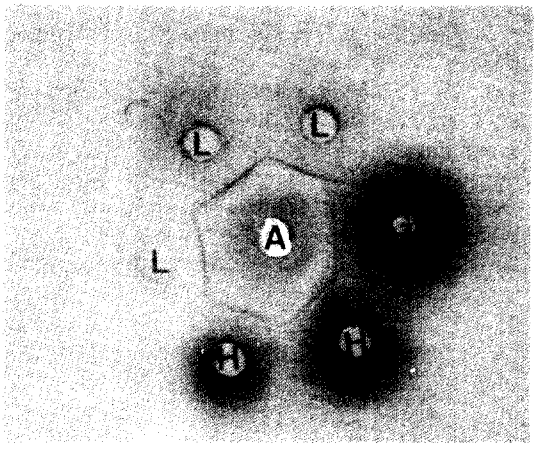

Fig. 9. Double diffusion precipitation on Ouchterlony plate. A : anti-Lp of *L. dispar*, L : hemolymph of *L. dispar*, G : hemolymph of *G. mellonella*, H : hemolymph of *H. cunea*.

and last instar larvae and 1, 3, 5 day old pupae and each hemolymph used for rocket immunoelectrophoresis. As shown in Fig. 8, lipophorin showed a gradual decrease during larval and pupal stages.

Immunological relationship with hemolymph proteins of other species

Immunodiffusion was conducted to determine the immunological relationship between lipophorin of *Lymantria dispar* and hemolymph proteins of *Hyphantria cunea* and *Galleria mellonella*. Anti-lipophorin showed positive reactions with the hemolymph proteins of *Hyphantria cunea* and *Galleria mellonella*, indicating that these hemolymph have protein immunologically related to the lipophorin of *Lymantria dispar* (Fig. 9).

Discussion

Insect lipophorin is a conjugated protein containing lipids, carotenoids, sterols and hydrocarbon, and also functions to carry orgainc materials between storage organ and other organs which need them.

In most cases lipophorin is composed of two subunits, apo-LpI (M.W = 230,000) and apo-L-pII (M. W. = 80,000) but in several insects when lipid requirement is drastically increased during long distance flight, lipophorin is transformed into low density lipophorin containing high amount of diacylglycerol. In *Locusta migratoria*, lipophorin is transformed into low density conjugated protein by attachment of small protein molecule called protein C (Van der Horst *et al.*, 1984, 1987) whereas in *Manduca sexta* apo-LpIII attached (Wells *et al.*, 1985; Haunerland and Bower, 1986). This process was reported to be controlled by AKH (adipo-kinetic hormone)(Van der Horst *et al.*, 1979, 1984, 1987; Orchard and Lange, 1983; Van Heusden *et al.*, 1984).

In the present work with *Lymantria dispar*, protein part of lipophorin is made up of two subunits, apo-LpI (230,000) and apo-LpII (49,000). Molecular weight of apo-LpI is similar to those of other species but molecular weight of apo-LpII is different from those of other species. In most cases apo-proteins of lipophorin was reported to contain oligosaccharide (Nagao *et al.*, 1987). Based on the positive reaction with PAS, lipophorin of *Lymantria dispar* contains oligosaccharide. Also, electrophoretic pattern of purified lipophorin showed two bands on the gel. When either band was sliced, elutioned and electrophoresed, they showed identical electrophoretic pattern. This fact indicates that upper band is product of aggregation formed through lipophorin purification. Similar result was obtained from Locust lipophorin (Chino *et al.*, 1987).

Quantitative change of lipophorin during development was not much investigated but in *Musca domestica* lipophorin showed gradual increase during larval and pupal stages and then decrease during adult stage (de Bianchi *et al.*, 1987). This pattern was quite different from that of *Lymantria dispar* in which lipophorin showed gradual decrease during larval and pupal stages.

Also, immunological relationship of lipophorins among different species has been reported but some general principle was not attained. In the present work with *Lymantria dispar*, anti-lipophorin of *Lymantria dispar* showed partially positive reactions with hemolymph of *Hyphantria cunea* and *Galleria mellonella*. This result indicates that some partial identity in structure is present between lipophorin of *Lymantria dispar* and hemolymph proteins (probably lipophorin) of *Hyphantria cunea* and *Galleria mellonella*.

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매미나방(*Lymantria dispar* L.)의 Lipophorin의 정제 및 특성

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KBr 밀도구배 초원심 분리법을 이용하여 매미나방의 Lipophorin을 분리, 정제한 후 전기영동과 면역학적 방법으로 Lipophorin의 성질, 암수 상소 및 발생단계에 따른 양적변화를 조사하였다. Lipophorin은 두개의 소단위(apo-LpI : MW = 230 Kd와 apo-LpII : MW = 49 Kd)로 구성되어 있으며 탄수화물과 지질을 함유하고 있다. Lipophorin에 대한 항체는 지방체 추출물과 난소 추출물과는 양성반응을 보였지만, 장 추출물과는 반응을 나타내지 않았다. 혈림프내의 Lipophorin의 농도는 유충과 용시기에 걸쳐서 점진적인 감소현상을 나타내었다. 그리고, 세포배양과정에서 지방체는 lipophorin을 배양액 속으로 방출하였다. 면역학적 실험에서, 매미나방의 Lipophorin과 미국흰불나방의 Lipophorin 그리고, 꼴벌부채명나방의 Lipophorin사이에 부분적 동질성이 확인되었다.