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Characterization of lipophorin from hemolymph of Fall Web-worm, *Hyphantria cunea* Drury

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Lipophorin (LP) was purified from hemolymph of late last instar larvae of *Hyphantria cunea* D. by KBr density gradient ultracentrifugation. Chemical composition of LP was investigated by electrophoresis, thin layer chromatography and gas chromatography. LP consisted of Apo-LP I and Apo-LP II, and M.W. of them were 230 Kd and 80 Kd, respectively. Lipid of LP was mostly composed of neutral lipid including triacylglycerol, diacylglycerol, monoacylglycerol and free cholesterol, and phospholipid rich in phosphatidylethanolamine and phosphatidylcholine. Fatty acids present in these lipids were found to have be 14:0, 16:0, 18:1, and 20:1.

KEY WORDS: *Hyphantria cunea*, Hemolymph, Lipophorin

Lipophorin is present in hemolymph of insects to transport lipids to various target tissues such as fat body, flight muscle, and ovary (Chino *et al.*, 1981). Lipophorin also functions to transport dietary lipids absorbed from gut to storage organ such as fat body during larval stage (Ventatesh and Chippendale, 1987) and phospholipid necessary for vitellogenesis to ovary in vitellogenic female (Gondim *et al.*, 1989). Lipophorin was reported to transport energy source from fat body to flight muscle in Lepidoptera, Hemiptera, and Orthoptera using lipid as flight energy source(Van der Horst *et al.*, 1979) and also juvenile hormone to target tissues (de Kort and Koopmanschap, 1989). Lipophorin was known to transport diacylglycerols, cholesterols, carotenoids, hydrocarbons (Van der Horst *et al.*, 1979; Katase and Chino, 1984; Prasad *et al.*, 1986; Ryan *et al.*, 1986; Tsuchida *et al.*, 1987; Germain *et al.*, 1988), phospholipid (Gondim *et al.*, 1989) and various xenobiotics (Haunerland and Bowers, 1986).

Lipophorin consists of two subunits called Apo-LP I and Apo-LP II (Chino *et al.*, 1981; Shapiro *et al.*, 1984; Robbs *et al.*, 1985; Prasad *et al.*, 1986; Germain *et al.*, 1988), and is known to be combined with another subunits Apo-LP III or C protein as insect is injected with adipokinetic hormone during flight (Kawooya *et al.*, 1984; Van der Horst *et al.*, 1984; Wells *et al.*, 1985; Haunerland *et al.*, 1986).

Also, it was reported that lipophorin contains large amounts of diacylglycerol but small amounts of phospholipid (Chino and Kitazawa, 1981; Prasad *et al.*, 1986; Germain *et al.*, 1988). However, little was known about the lipid analysis of lipophorin from Lepidoptera.

In the present work with *Hyphantria cunea*, lipophorin present in hemolymph of late last instar larvae was purified and their chemical composition was investigated using electrophoresis, thin layer chromatography, and gas chromatography.

Materials and Methods

Insects

Hyphantria cunea were reared on artificial diet at the temperature of 27 ± 1°C and R.H. of 75

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± 5 % under the photoperiod of 16L:8D.

Hemolymph collection

Last instar larvae were injected with 0.5 ml of EDTA-saline (150 mM NaCl, 5 mM EDTA, 50 mM phosphate buffer, pH 6.8) and hemolymph was collected from abdomen by cutting abdominal legs (Chino *et al.*, 1987). A few crystals of PTU (phenylthiourea) were added to hemolymph to prevent melanization. The hemolymph was centrifuged at 10,000 g for 10 min at 4° C and the supernatant was stored at -70° C until used.

Purification of lipophorin by ultracentrifugation

Lipophorin was purified from hemolymph of last instar larvae by KBr density gradient ultracetrifugation. Hemolymph (2.4 ml) and KBr (1.144 g) were put in tube and well mixed on vortex to become final density of 1.31 g/ml. This mixture was overlayed with 2.4 ml of 0.9 % NaCl (density, 1.007 g/ml). This tube was ultracentrifuged at 35,000 rpm for 18 hrs at 4° C. After centrifugation, yellow lipophorin band located in upper half was extracted and dialyzed against phosphate buffer and stored at -70° C.

Electrophoresis

Non-SDS PAGE was carried out on 5% gel at the current of 3 mA/gel as described by Davis (1964). SDS-PAGE was performed on 8-12% concentration gradient gel at the current of 30 mA according to Laemmli (1970). After electrophoresis, gel was stained with Coomassie brilliant blue R 250.

Determination of molecular weights

Molecular weights of subunits were determined on 8-12% concentration gradient gel after the procedure of Lambin *et al.*(1976). Standard markers used were myosin (205,000), β-galactosidase (116,000), phosphorylase B (97, 400), bovine plasma albumin (66,000), egg albumin (45,000), carbonic anhydrase (29,000).

Composition of carbohydrate and lipid

To investigate the presence of carbohydrate and lipid in lipophorin, gel was stained with PAS for carbohydrate as described by Caldwell and Pigman (1965) and with Sudan black B for lipid according to Chippendale and Beck (1966).

Extraction of lipid from lipophorin

Chloroform-methanol (2:1, v/v) was added to 1 ml of purified lipophorin (Nelson, 1975) and mixed on the vortex. Chloroform-methanol (2:1, v/v) was completely evaporated from lipophorin sample at 40° C by rotary vacuum evaporator and also 5-6 ml of chloroform-methanol (2:1, v/v) and 1.5 ml of benzene were added to concentrated sample and again evaporated at 40° C by rotary vacuum evaporator. This process was repeated more than three times and n-hexane is finally added to sample and used for lipid analysis.

Analysis of lipids

For neutral lipid analysis, sample was applied to a plate (silica gel F_{254}, 20x20 cm) which was developed by a solvent system comprising petroleum ether--ethyl ether-acetic acid (60:40:1, v/v) and ethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2, v/v)(Chino *et al.*, 1969). The plate was sprayed with iodine and charred. Standard lipid classes used were tripalmitin, dipalmitin (mixture of 1,2- and 1,3-dipalmitin), monopalmitin and cholesterol.

To analyze phospholipids, plate (silica gel F_{254}, 20x20 cm) was developed by chloroform-methanol-water (65:25:4, v/v) and sprayed with iodine and charred. Standard phospholipids classes used were phosphatidylethanolamine, phsphatidylcholine, and sphingomyelin.

To seperate fatty acids, chloroform-methanol (2: 1, v/v) was completely evaporated from sample with nitrogen gas and 1 ml of chloroform and 0.5 N sodium methoxide (CH_3ONa) each were added to sample at high temperature for 2 or 3 hrs for methylation. One N acetic acid was added to sample and stirred and n-hexane was finally added. The analysis of fatty acids was submitted to gas chromatography (column: 5% Ph Me Silicone, carrier gas : N_2, column temp.: 250° C). Standard fatty acids used were caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, palmitoleic acid, oleic acid, 11-eicosenoic acid, and erucic acid.

Results

Purification of lipophorin by ultracentrifugation

Lipophorin was purified from hemolymph of last instar larvae by KBr density gradient ultracentrifugation. Yellow band appeared at upper half of tube(Fig. 1). Lipophorin was yellow in color because this protein is associated with carotenoids absorbed through food (Shapiro *et al.*, 1984). This yellow protein was extracted from tube using pasteur pipette.

Purity of purified lipophorin

Purified lipophorin and hemolymph were electrophoresed on NON-SDS gel to determine the purity of purified lipophorin and to investigate lipophorin band on gel(Fig. 2).

Number and molecular weights of subunits

Lipophorin was applied to 8-12% concentration gradient polyacrylamide gel to determine number and molecular weights of subunits. Lipophorin was composed of Apo-LP I and Apo-LP II. Molecular weights of them were 230,000 dalton and 80,000 dalton, respectively (Figs. 3, 4).

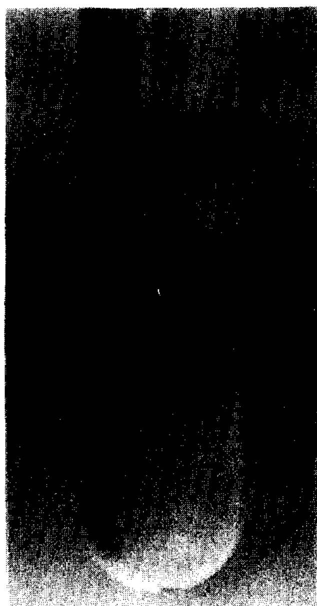

Fig. 1. KBr density ultracentrifugation of *Hyphantria cunea* hemolymph. Lp; Lipophorin band (yellow)

Composition of lipophorin

Lipophorin was positively stained with PAS and Sudan black B, showing that they contain carbohydrate and lipid (Fig. 5).

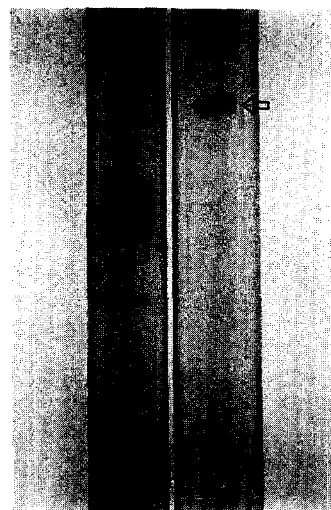

Fig. 2. Electrophoretic patterns of haemolymph and purified lipophorin of *H. cunea*. The open arrow indicates lipophorin band. A; Crude hemolymph, B; Purified lipophorin

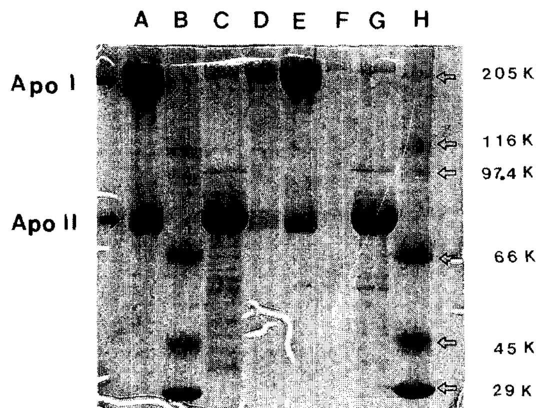

Fig. 3. Purified lipophorin of 8-12% linear gradient SDS-gel. A, D, E, F; Purified lipophorin, B, H; Protein marker, C, G; Crude hemolymph.

Composition of lipids

Neutral lipids were developed by petroleum ether-ethyl ether-acetic acid (60:40:1, v/v) and sprayed with iodine vapour, indicating that they contain triacylglycerol, 1,2- and 1,3-diacylglycerol, monoacylglycerol (Fig. 6). The lipids were also developed by ethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2, v/v) and visualized with iodine vapour, showing the presence of cholesterol in lipophorin (Fig. 7).

Phospholipid was developed by chloroform-methanol-water (65:25:4, v/v) and visualized with iodine vapour, indicating that lipophorin contains phosphatidylethanolamine and phosphatidylcholine with small amounts of unknown materials (Fig. 8).

Also, lipophorin was found to contain fatty acids of 14:0, 16:0, 18:1 and 20:1 (Fig. 9).

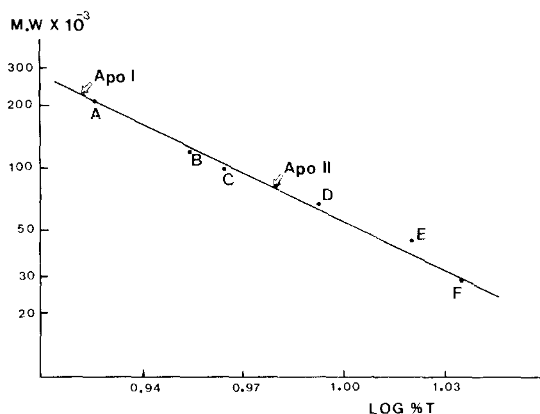

Fig. 4. Determination of M.W. for apolipophorins by electrophoresis on 8-12% linear gradient of polyacrylamide gel in the presence of SDS. Marker proteins used were (A) Myosin (205,000); (B) β-galactosidase (116,000); (C) Phosphorylase B (97,400); (D) bovine plasma albumin (66,000); (E) Egg albumin (435,000); (F) Carbonic anhydrase (29,000)

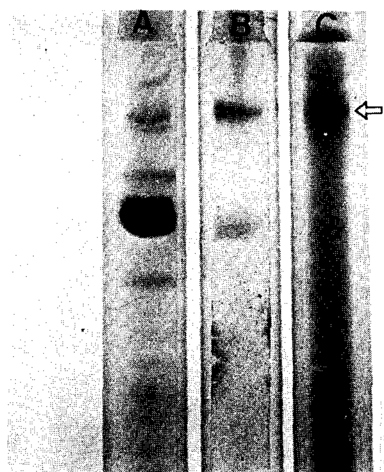

Fig. 5. Electrophoretic patterns of larval hemolymph protein of *Hyphantria cunea*, using different staining methods. A; Coomassie blue staining, B; PAS staining, C; Sudan black B staining.

Discussion

Insect lipophorin functions to transport lipids from storage organ to various organs such as flight

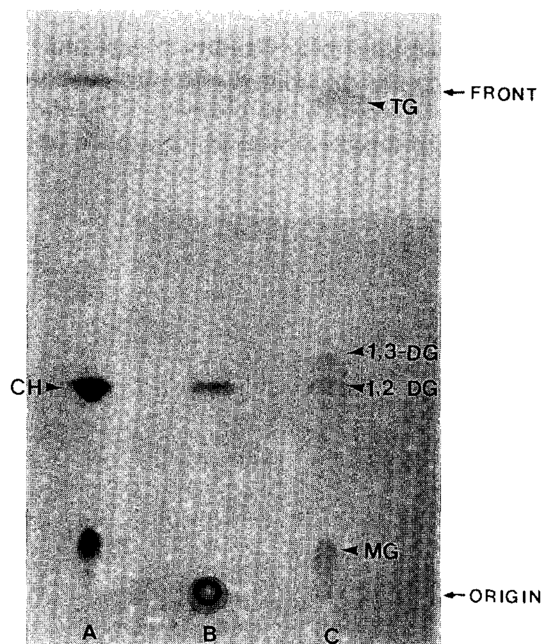

Fig. 6. TLC for neutral lipid components of lipophorin. The lipids extracted with cholroform-methanol (2:1, v/v) from purified LP were applied to the plate of silica gel F_{254}. Solvent system; petroleum ether-ethyl ether-acetic acid (60:40:1, v/v). Detection with I_2 vapour. A; standard cholesterol, B; Lipophorin, C; standard triglyceride (tripalmitin, TG), diglyceride (mixture of 1,3- and 1,2-DG), monoglyceride (monopalmitin, MG)

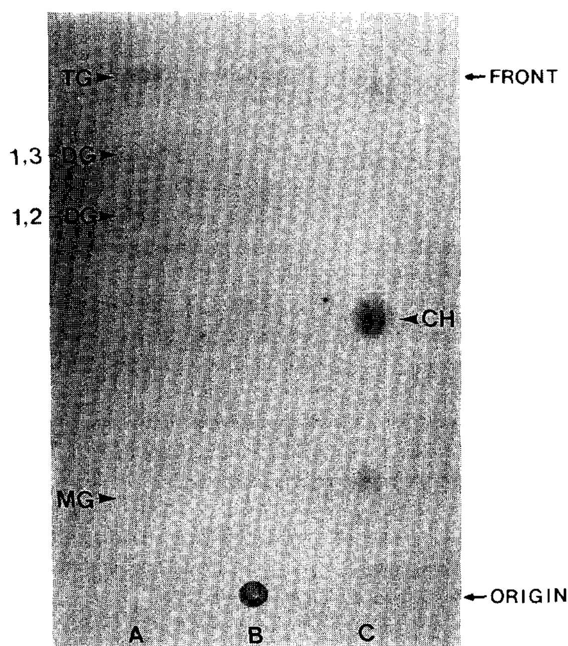

Fig. 7. TLC showing the presence of free cholesterol in lipophorin. The lipids extracted with chloroform-methanol (2:1, v/v) from purfied LP were applied to the plate of silica gel F_{254}. Solvent system; ethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2, v/v). Detection with I_2 vapour. A; standard tryglyceride (tripalmitin, TG), diglyceride (mixture of 1,3- and 1,2-dipalmitin, 1,3- and 1,2-DG), monoglyceride (monopalmitin, MG). B; Lipophorin, C; standard

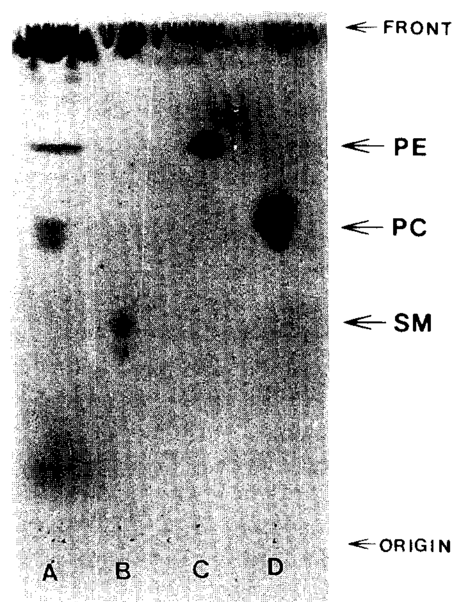

Fig. 8. TLC for phospholipids in lipophorin. The lipids extracted from LP were applied to the plate of silica gel F_{254}. Solvent system: chloroform-methanol-water (65: 25:4, v/v). Detection with I_2 vapour. A; Lipophorin, B; standard sphingomyelin (SM), C; standard phosphatidylethanolamine (PE), D; standard phosphatidylcholine (PC)

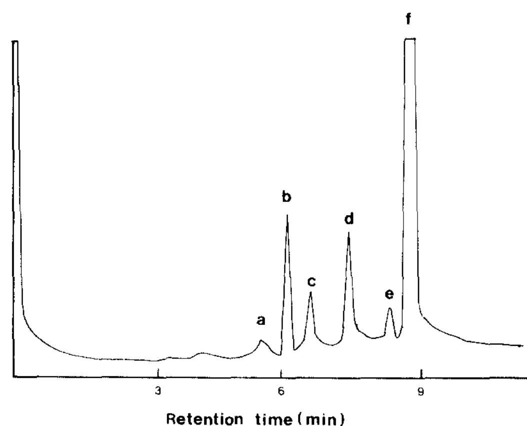

Fig. 9. Gas-chromatogram of fatty acid in lipophorin. a; myristic acid (14:0), b, f; unknown, c; palmitic acid (16: 0), d; oleic acid (18:1), e; 11-eicosenoic acid (20:1).

muscle or ovary (Chino *et al.*, 1981). In general, lipophorin consists of Apo-Lp I and Apo-Lp II and their molecular weights were known to be 250, 000 dalton and 78,000 dalton, respectively (Ryan *et al.*, 1984). In *Manduca sexta* and *Locusta migratoria*, however, since lipid is drastically required during long distance flight of adult stage, lipophorin is combined with Apo-Lp III or C protein which has molecular weight of 20,000 dalton to form low density lipophoin (LDLP) containing greater amounts of diacylglycerol (Van der Horst *et al.*, 1984 ; Wells *et al.*, 1985; Haunerland and Bower, 1986). Adipokinetic hormone (AKH) was reported to stimulate formation of LDLP (Van der Horst *et al.*, 1979, 1984). In the present work with *H.cunea*, lipophorin is composed of Apo-Lp I and Apo-Lp II, molecular weights of which are 230,000 dalton and 80,000 dalton, respectively. These values are usually in agreement with those reported

previously (Ryan *et al.,* 1984). Also, lipophorin was positively stanined with PAS reagents, revealing conjugated protein containing carbohydrate. Most of lipophorins were known to contain carbohydrate (Chino and Kitazawa, 1981). In insects including Locust, Cockroach, *Cecropia* silkworm, *Manduca,* and *Drosophila,* lipophorin contains triacylglycerol, diacylglycerol, and cholesterol but no monoacylglycerol (Chino and Kitazawa, 1981; Prasad *et al.,* 1986; Germain *et al.,* 1988). In *H.cunea,* however, monoacylglycerol is present in lipophorin which was different from those reported previously. In the case of phosphlipid, lipophorin contains phosphatidylethanolamine (PE) and phosphatidylcholine (PC) in locust and cockroach but sphingomyelin in addition to PE and PC in *Cecropia* silkworm (Chino and Kitazawa, 1981). Lipophorin of *H.cunea* was found to contain PE and PC. Also, fatty acids in lipophorin was reported to be 12:0,14:0,16:0 and 18:0 (Chino *et al.,* 1969; Prasad *et al.,* 1986). Lipophorin fatty acids of *H.cunea* were found to be 14:0, 16:0, 18:1, and 20:1.

References

Caldwell, R.C., and W. Pigman, 1965. Disc electrophoresis of human saliva in polyacrylamide gel. *Archs. Biochem. Biophys.,* **110:** 19-96.

Chino, H., R.G.H. Downer, G.R. Wyatt and L.I. Gilbert, 1981. Lipophorins, a major class of lipoproteins of insect haemolymph. *Insect Biochem.,* **11:** 491.

Chino, H., and K. Kitazawa, 1981. Diacylglycerol-carring lipoprotein of hemolymph of the locust and some insects. *J. Lipid Res.,* **33:** 1042-1052.

Chino, H., S. Murakami, and K. Harashima, 1969. Diglyceride-carring Lipophorins in insect hemolymph isolation, purification and properties. *Biochem. Biophys. Acta.,* **176:** 1-26.

Chino, H., Y. Hirayama, Y. Kiyomoto, R.G.H. Downer, and K. Takahashi, 1987. Spontaneous aggregation of locust lipophorin during hemolymph collection. *Insect Biochem.,* **17:** 89-97.

Chippendale, G.M., and S.D. Beck, 1966. Haemolymph proteins of *Ostrinia nubilalis* during diapause and prepupal differentiation. *J. Insect Physiol.,* **12:** 1629-1638.

Davis, B.J., 1964. Disc electrophoresis. II. Methods and applications to human serum proteins. *Ann. N.Y. Acad. Sci.,* **121:** 404-427.

de Kort S., and B. Koopmanschap, 1989. Binding of juvenile hormone III to lipophorin from the American cockroach *Periplaneta americana. Arch. Insect Biochem. Physiol.,* **11:** 159-172.

Germain, J.P., Fernando-Warnakulasuriya, and M.A. Wells, 1988. Isolation and characterization of lipoprotein from *Drosophila melanogaster* Larvae. *Archs. Biochem. Biophys.,* **8:** 243-248.

Gondim, K.C., P.L. Olivera, and H. Masuda, 1989. Lipophorin and Oogenesis in *Rhodnius prolixus*: Transfer of Phospholipids. *J. Insect physiol,* **35:** 19-27.

Haunerland, N.H., and W.S. Bowers, 1986. Binding of insecticides to lipophorin and arylphorin. Two hemolymph proteins of *Heliothis zea. Arch Insect Biochem. Physiol.,* **3:** 87-96.

Katase, H., and H. Chino, 1984. Transport of hydrocarbons by haemolymph lipophorin in *Locusta migratoria. Insect Biochem.,* **14:** 1-6.

Kawooya, J.K., P.S. Keim, R.O. Ryan, J.P. Shapiro, P. Samarawera, and J.H. Law, 1984. Insect apolipoprotein III. Purification and properties. *J. Biol. Chem.,* **259:** 10733-10737.

Laemmli, U.K., 1970. Cleavage of structure proteins during the assembly of the head of bactriophage T_4, *Nature,* Lond., **227:** 680-685.

Lambin, P., D. Rochu, and J.M. Fine, 1976. A new method for determination of molecular weights of proteins by electrophoresis across a sodium dodecyl sulfate (SDS)-polyacrylamide gradient gel. *Analyt. Biochem.,* **74:** 567-575.

Nelson, G.J., 1975. Analysis of lipids and lipoproteins (ed.E.G. Perkins). Am. Oil Chem. Soc., Champaign, **III:** 9-12.

Prasad, S.V., R.O. Ryan, J.H. Law, and M.A. Wells, 1986. Changes in lipoprotein composition during larval-pupal metamorphosis of an Insect, *Manduca sexta. J. Biol. Chem.,* **261:** 558-562

Robbs, S.L.,m R.O. Ryan, J.O. Schmitt, P.S. Keim, and J.H. Law, 1985. Lipophorin of the larval honeybee, *Apis mellifera. J. Lipid Res.,* **26:** 241-247.

Ryan, R.O., J.O. Schmidt, and J.H. Law, 1984. Chemical and Immunological properties of lipophorin from seven insect orders. *Arch. Insect Biochem. Physiol.,* **1:** 375.

Ryan, R.O., S.V. Prasad, E.J. Henriksen, M.A. Wells, and J.H. Law, 1986. Lipoprotein interconversion in an insect, *Manduca sexta. J. Biol. Chem.,* **261:** 563-568.

Shapiro, J.P., P.S. Keim, and J.H. Law, 1984.

Structural studies on lipophorin, an insect lipoprotein. *J. Biol. Chem.*, **259:** 3680-3685.

Tsuchida, K., S.V. Prasad, and M.A. Wells, 1987. Changes in lipophorin composition during the fourth to fifth instar molt of *Manduca sexta*. *Insect Biochem.*, **17:** 1139-1141.

Van der Horst, D.J., J.M. Van Doorn, and A.M. Beenakkers, 1979. Effects of the adipokinetic hormone on the release and turnover of haemolymph diacylglycerides and on the formation of the diacylglycerides-transporting lipophorin system during locust flight. *Insect Biochem.*, **9:** 627-635.

Van der Horst, D.J., J.M. Van Doorn, and A.M. Beenakkers, 1984. Hormone-induced rearrangement of locust haemolymph lipoproteins. *Insect Biochem.*, **14:** 495-504.

Ventatesh, O.J., and G.M. Chippendale, 1987. Major plasma proteins of larvae of the southwestern corn borer, *Diatraea grandiosella. Arch. Insect Biochem. Physiol.*, **5:** 271-284.

Wells, M.A., R.O. Ryan, S.V. Prasad, and J.H. Law, 1985. A novel procedure for the purification of apolipophorin III. *Insect Biochem.*, **15:** 565-571.

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미국흰불나방 (***Hyphantria cunea*** D.)의 lipophorin의 물리화학적 성질

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미국흰불나방 (*Hyphantria cunea* D.)의 종령유충말기의 혈림프에 존재하는 lipophorin을 KBr 밀도구배 초원심분리법으로 분리, 정제한 다음, 이들의 물리화학적 성질, 지질, 지방산의 종류를 전기영동법, 얇은 막 및 기체 크로마토그래피법을 이용하여 조사하였다. Lipophorin은 Apo-Lp I, Apo-Lp II 2종류의 apolipoprotein으로 구성되어 있는 데, 이들의 분자량은 각각 230 Kd와 80 Kd로 측정되었고, 화학적 구성성분을 조사한 결과 탄수화물, 지질 등이 결합되어 있음을 확인하였다. Lipophorin은 triacylglycerol, diacylglycerol, monoacylglycerol, free cholesterol 등의 중성지질과 phosphatidylethanolamine, phosphatidylcholine 등의 인지질이 존재하고 있는 것으로 나타났다. 이들 지질에 존재하는 지방산들은 14:0, 16:0, 18:1, 20:1인 것으로 밝혀졌다.