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Apolipophorin-III uptake by the adult ovary in the wax moth *Galleria mellonella*

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꿀벌부채명나방의 성충 난소에 의한 아포리포포린-III의 흡수

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Abstract Apolipophorin-III (apoLp-III) was isolated and purified from the last instar larval hemolymph of *Galleria mellonella* by gel chromatography (Sephadex G-100) and ion exchange chromatography (CM-52). In the present study, I wanted to show that apoLp-III is taken up into the adult ovary in *Galleria mellonella*. Adult ovary tissues were incubated at room temperature for 30 min with fluorescein isothiocyanate (FITC)-labeled apoLp-III. Fluorescence microscopy and sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) revealed that adult ovary tissues internalize fluorescence-labeled apoLp-III. The results suggest that apoLp-III is taken up by the adult ovary.

요 약 아포리포포린-III를 꿀벌부채명나방 종령유충 혈림프에서 젤크로마토그래피 (Sephadex G-100)와 이온교환크로마토그래피 (CM-52)방법을 이용하여 분리, 정제하였다. 본 연구에서는 아포리포포린-III가 꿀벌부채명나방의 성충 난소에 의해 흡수되는 지를 조사하였다. 성충난소조직을 형광물질로 표지한 아포리포포린-III와 상온에서 30분간 배양하였다. 배양결과를 형광현미경과 전기영동을 이용하여 확인한 결과 형광물질로 표지된 아포리포포린-III가 성충난소로 흡수된다는 사실을 알았다.

Key Words : Apolipophorin-III, ovary, FITC

1. Introduction

Lipophorin(Lp) has been shown to act as a vehicle for transporting lipid from fat body to organs that can use it such as the ovaries, testes and flight muscle [1]. Most insect Lp contain two apolipoproteins, apolipophorin-I (apoLp-I, Mr=250 kDa) and apolipophorin-II (apoLp-II, Mr=80 kDa)[1,2], A third apolipoprotein (apoLp-III) of molecular weight approximately 18 kDa, is present in the adults of some species [1]. ApoLp-III is a hemolymph protein that associates hydrophobically with lipoprotein surfaces to facilitate lipid transport in aqueous medium, and plays a critical role in the transport of lipids during flight in several species of insects [3]. ApoLp-III assists in the loading of diacylglycerol (DAG), generated from triacylglycerol (TAG) stores in the fat body through the action of adipokinetic hormone, into lipoprotein, the hemolymph lipoprotein. Such loading is an obligatory part of the pathway that transfers lipid from the fat body to the flight muscles, where the lipid is used to fuel flight [1].

It was also reported that low density lipoprotein (LDLp), adults lipoprotein is taken up into ovary where LDLp is cleaved into very high density lipoprotein

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(VHDLp) and apoLp-III, supplying a protein component in yolk in *Manduca sexta* [4]. Insect oocytes synthesize only small amounts of protein. However, most proteins are taken up during oocyte development by receptor mediated-endocytosis [5]. The accumulation of protein reserves in insect oocytes is well studied [6-8]. Recently, Van Hoof et al. (2005)[9] examined lipoprotein and transferrin trafficking in insect cells.

To investigate the uptake of the apoLp-III by the adult ovary, adult ovary tissues of *Galleria mellonella* were incubated at room temperature for 30 min with fluorescein isothiocyanate (FITC)-labeled apoLp-III. Using fluorescence microscopy and sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), we revealed that ovary tissues internalize fluorescence labeled apoLp-III.

2. MATERIALS AND METHODS

2.1 Insects

Larvae of *Galleria mellonella* were reared on an artificial diet mixed with a natural diet (bee wax) as described by Han et al. (2003) [10]. The insects were kept at 32±1℃ and 75±5% relative humidity in darkness.

2.2 Collection and processing of hemolymph and ovaries

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.

Ovaries were dissected from day-1 or -2 adults in cold Ringer's solution and were used for tissue culture and electrophoresis.

2.3 Purification of apoLp-III

The last instar larval hemolymph (5 ml) was eluted from Sephadex G-100 (Phamacia, LKB, Uppsala, Sweden) column (2 x 60 ㎝)with 0.05 M phosphate buffer (pH 7.0) at a flow rate of 0.5 ml/min with 2 ml per fraction. Each fraction was pooled, dialyzed and electrophoresed. Fractions containing only apoLp-III were used as the samples for ion exchange chromatography. These were dialyzed against 0.02 M sodium acetate buffer (0.02 M sodium acetate, 0.02 M acetic acid, 0.05 M NaCl, pH 5.) and subjected to cation exchange chromatography (CM-52, Phamacia, LKB, Uppsala, Sweden). Sample was eluted from CM-52 column (1.2 x 10 ㎝) 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 280 nm. Purity of finally purified apoLp-III was confirmed by SDS-PAGE [11].

2.4 Gel electrophoresis and Western blotting

SDS-PAGE was carried out in a 10% polyacrylamide gel at room temperature at 15 mA according to Laemmli (1970)[13]. After electrophoresis, the gel was stained in coomassie brilliant blue R 250 and destained with a 30% methanol solution containing 3.5% acetic acid. For Western blot analysis, after SDS-PAGE, samples were transferred to a nitrocellulose membrane in Tris-glycine buffer (25 mM Tris, 92 mM glycine, 30% methanol, pH 8.3) at 100 V for 2 h [13]. After transfer, nitrocellulose membrane was equilibrated in TBS solution (20 mM Tris, 500 mM NaCl, pH 7.5) for 10 min and incubated in blocking solution (3% gelatin in TBS) for 30 min. This sheet was then washed twice, for 5 min each, with TTBS solution and incubated for 1 h in a solution containing 300-fold diluted anti-apoLp-III serum. The filtrate was again washed twice with TTBS and then incubated for 1 h in a solution containing 3,000-fold diluted secondary antibody solution (GAR-HRP conjugated IgG)(BioRad). After incubation and two more washes with TTBS, the sheet was submerged in HRP color development solution (60 mg color development reagent, 4-chloro-1-naphthol in 20ml ice-cold methanol, plus 0.015% H_2O_2 in 100ml TBS).

2.5 *In vitro* incubation of ovary tissue with fluorescence labeled apoLp-III

The protein moiety of apoLp-III (1 ㎎/㎖) was labeled

with 20 ㎕/㎖ fluorescein isothiocyanate (FITC) dissolved in 1 ㎍/㎕ dimethyl sulfoxide (DMSO) at room temperature under continuous stiring for 1 h according to Van Hoof et al. (2003) [14]. FITC-labeled apoLp-III was purified with Sephadex G-25 PD-10 column (Amersham Phamacia Biotech, Piscataway, NJ, USA) to separate fluorescence labeled apoLp-III from free fluorescent label. For protein uptake, ovary tissues were incubated with 25 ㎍/㎖ FITC-apoLp-III for 30min at room temperature. After incubation, ovary tissues were washed with in PBS for three times and fixed in 4% paraformaldehyde diluted in PBS for 10-30 min at room temperature.

2.6 Microscopy and image processing

The fluorescence distribution in the ovary was observed using fluorescence Axioskop microscope (Zeiss, Jena, Germany) with a Hg HBO-50 lamp. Using UV and FITC filters, digital images were recorded with a AxioCam HRc digital camera and AxioVision Release 4.5 software (Zeiss).

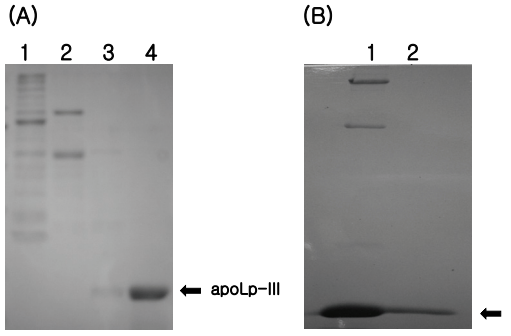
3. RESULTS

3.1 Isolation and purification of apoLp-III

ApoLp-IIIwas isolated from the last instar larval hemolymph by gel filtration (Sephadex G-100) and ion exchange chromatography (CM-52). Electrophero -gram of apoLp-III through a purification process showed that apoLp-III was completely purified in CM-52 column [Fig.1].

3.2 Presence of apoLp-III in the adult ovary

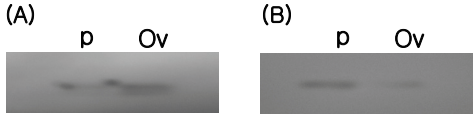
The presence of apoLp-III was examined in the adult ovary by electrophoretic analysis and Western blotting. The results showed that apoLp-III is present in the adult ovary [Fig. 2, A].



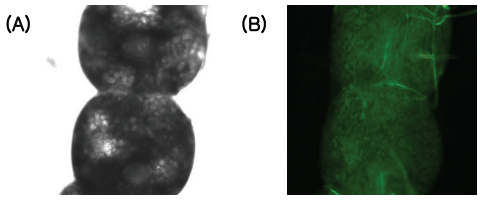
[Fig.1] (A) SDS-PAGE of purification steps of apolipophorin-III from *Galleria mellonella* L. 1; larval hemolymph, 2; fraction after gel chromatography on a Sephadex G-100, 3; fraction after ion exchange chromatography on CM-52, 4; purified apoLp-III. (B) SDS-PAGE of adult lipophorin(1) and purified apoLp-III(2). Arrows are apoLp-III.

3.3 Uptake of fluorescence labeled apoLp-III by the adult ovary

To investigate the uptake of the FITC labeled apoLp-III by the adult ovary, ovary tissues were incubated at the room temperature for 30 min. The results revealed that fluorescence labeled apoLp-III was present in the adult ovary [Fig.2, B]. Also, it was examined the distribution of apoLp-III in oocyte during the transition from early to middle/or late vitellogenic stage of oogenesis. As shown in [Fig.3], FITC labeled apoLp-III was distributed throughout the oocyte in early vitellogenic stage.



[Fig.2] (A) Western blotting of purified apoLp-III(p) and adult ovary extracts (Ov). (B) SDS-PAGE of fluorescein isothiocyanate(FITC)-labeled apoLp-III(p) and adult ovary extracts(Ov). FITC-labeled apoLp-III was present in the adult ovary extracts.



[Fig.3] Fluorescence microscopic images of adult ovary tissues(B) obtained with fluorescein iosthiocyanate (FITC). (A) shows differential interference contrast (DIC) images of the same frame represented in (B).

4. DISCUSSION

ApoLp-III was isolated and purified from the last instar larval hemolymph of *G. mellonella*, its function was investigated by using fluorescence microscopy and SDS-PAGE. In general, insects have to transport large amounts of lipid to flight or in response to injection with AKH (Adipokinetic hormone). At this time, high-density lipoprotein (HDLp) is combined with apoLp-III and converted it into LDLp which has a higher diacylglycerol (DAG) load. ApoLp-III appears in bound- and free-state in adult hemolymph in *Manduca sexta*. Free-state apoLp-III in hemolymph binds to HDLp to mask increased hydrophobic surface of this particle that result from DAG uptake, thus, Lp can remain stable in the aqueous environment of hemolymph despite this increased lipid-carrying capacity [3]. In *Hyphantria cunea*, apoLp-III from larval hemolymph was associated with lipophorin in the adult hemolymph [11]. It was reported that apoLp-III is synthesized by the fat body and secreted to the hemolymph. The protein was taken up by the ovary in the bound form [15]. However, so far there was no report on the presence of free-state apoLp-III in ovary. In the present work with *G. mellonella*, the presence of apoLp-III in the ovary was confirmed by western blotting [Fig.2].To investigate the uptake of the FITC labeled apoLp-III by the adult ovary, ovary tissues were incubated at the room temperature. The results clearly showed that fluorescence labeled apoLp-III was taken up by the adult ovary [Fig.3]. This result suggests that large amounts of proteins can serve as building block and as a source of energy.

REFERENCES

[1] H.K. Yun, W.K. Kim, and H.R. Kim, "Immunological analysis of lipophorin in the hemolymph, ovaries, and testes of the fall webworm, *Hyphantria cunea* (Drury)", Arch. Insect Biochem. Physiol. pp.153-167, 1994.

[2] H.K. Yun, and S.G. Lee, "Purification and characterization of Lipophorin in wax moth, *Galleria mellonella*", Kor. J. Entomol. pp. 257-263. 1997.

[3] M.A. Wells, R.O. Ryan, J.K. Kawooya, and J.H.Law, "The role of apolipophorin-III in *in vivo* lipoprotein interconversion in adult *Manduca sexta*", J. Biochem., pp,4172-4176, 1987.

[4] M.C. Van Heusden, D.J. Van der Horst, J. Voshol, and A.M.Th. Beenakkers, "The recycling of protein components of the flight-specific Lipophorin in *Locusta migratoria*", Insect Biochem., pp,771-776, 1987.

[5] H.M. Cheon, S.J. Seo, J. Sun, T.W. Sappington, and A.S. Raikhel, "Molecular characterization of VLDL receptor homolog mediating binding of lipophorin in oocyte of the mosquito *Aedes aegypti* ", Insect Biochem. Mol. Biol. pp, 754-760, 2001.

[6] A.S. Raikhel, and T.S. Dhadialla, "Accumulation of yolk proteins in insect oocytes", Annu. Rev. Entomol., pp, 217-551, 1992.

[7] E.S. Jung, and H.K. Yun, "Lipophorin uptake by the larval fat body and adult ovary in the wax moth *Galleria mellonella*", Entomological Research, pp,167-171, 2006.

[8] R. Ziegler, and R.V. Antwerpen, "Lipid uptake by insect oocytes", Insect Biochem. Mol. Biol., pp, 264-272, 2006.

[9] D. Van Hoof, K.W. Rodenburg, and D.J. Van der Horst, "Receptor-mediated endocytosis and intracellular trafficking of lipoproteins and transferrin in insect cells", Insect Biochem. Mol. Biol., pp,117-128, 2005.

[10] J. Han, C.S. Lee, C.Y. Yun, B.H. Lee, Y.G. Ko, C.S. Kang, S.D. Lee, J.S. Hwang, S.W. Kang, and H.R. Kim, "Cloning and expression of male-specific protein (MSP)

from the hemolymph of greater wax moth, *Galleria mellonella* L.", Arch. Insect Biochem. Physiol. pp, 110-120, 2003.

[11] H.K. Yun, and H.R. Kim, "Immunological analysis of apolipophorin-III in the hemolymph, ovaries, and testes of the Fall webworm, *Hyphantria cunea* (Drury)", Arch. Insect Biochem. Physiol., pp, 413-426, 1996.

[12] U.K. Laemmli, "Cleavage of structural proteins during assembly of the head of bacteriophage T4", Nature, pp, 680-685, 1970.

[13] H. Towbin, T. Staehelin, and J. Gordon, "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some application", Proc. Natl. Acad. Sci. USA, pp, 4350-4354, 1979.

[14] D. Van Hoof, K.W. Rodenburg, and D.J. Van der Horst, "Lipophorin receptor-mediated lipoprotein endocytosis in insect fat body cells", J. Lipid. Res., pp, 1431-1440, 2003.

[15] W.H. Telfer, M.L. Pan, and J.H. Law, "Lipophorin in developing adults of *Hyalophora cecropia*: Support of yolk formation and preparation for flight", Insect Biochem., pp, 653-663, 1991.

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