韓應昆誌 34(2) : 140~146 (1995)
Korean J. Appl. Entomol.

Protein Contents During Oocyte Development and Some Characteristics of Egg-Specific Protein in *Lucilia illustris*

연두금파리의 난세포성숙에 따른 단백질의 변화와 난특이성단백질의 특성

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ABSTRACT Changes in protein content during oocyte development was measured and egg-specific protein was characterized from the eggs in *Lucilia illustris*. During normal development ovarian protein was rapidly increased at 72 hr and reached maximum at 96 hr after a protein meal, when the eggs were fully matured. Purified protein from the ovaries by gel filtration of DEAE-cellulose and Sephacryl S-200 was loaded on 7.5% native polyacrylamide gel electrophoresis and identified at R_f 0.4 as egg-specific protein, which has a mol. wt of 110,000. A total of 13 amino acids in the egg-specific protein was identified and especially asparagine, glutamic acid, and tyrosine were highly concentrated. Five fatty acids were also identified. It is suggested that there is a specific protein in the eggs of *L. illustris* except yolk protein synthesized and secreted by fat body.

KEY WORDS Oocyte development, Yolk protein, Ovary, Egg-specific protein, *Lucilia illustris*

초 록 연두금파리의 난세포성숙에 따른 단백질의 변화와 난특이성단백질의 특성을 확인하기 위하여 gel filtration, 전기영동 및 분자량측정, 아미노산과 지방산함량을 측정하여 얻은 결과는 다음과 같다. 연두금파리 암컷성충의 난소단백질은 단백질원을 섭식시킨 후 72시간 이후 빠르게 증가하였고, 완전한 성숙이 일어나는 96시간에 최고의 함량을 나타냈다. DEAE-cellulose와 Sephacryl S-200으로 gel filtration하고 7.5% native polyacrylamide gel에서 전기영동한 결과 난소에서 분리된 특이단백질은 R_f 0.4에서 혈림프 및 난소와 다른 밴드가 확인되었으며, 분자량은 110,000 dalton이였다. 분리된 난특이성단백질내 아미노산 조성은 asparagine 외 모두 13종이 검출되었으며, asparagine, glutamic acid와 함께 tyrosine이 특이하게 높게 나타났다. 지방산은 난소와 함께 난특이성단백질에서 palmitic acid 외 4종이 분리되었다. 따라서, 연두금파리의 난에는 지방체에서 합성, 분비된 난황단백질이외에 난소에서만 존재하는 특이단백질이 있음을 알 수 있다.

검색어 난세포성숙, 난황단백질, 난소, 난특이성단백질, 연두금파리

Oogenesis in insects mainly depends on the uptake of extra-ovarian protein, especially vitellogenin which is synthesized in the fat body and released into the haemolymph (Engelmann 1969, Wyatt and Pan 1978, Yin *et al.* 1988). In many insects vitellogenin and vitellin (major yolk protein) have been characterized and provided a good system for the study of hormonal control of egg maturation (Zou *et al* 1989).

On the other hand, a different kind of yolk protein, synthesized in the ovaries themselves and contributed to the protein stores during oogenesis, has been studied in several insect species (Irie and Yamashita 1983, Shirk 1987, Kawooya *et al.* 1988, Kawooya and Law 1988). The ability of an insect ovary to synthesize proteins was first reported in

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Hyalophora cecropia by Melius and Telfer (1969), and Anderson and Telfer(1969) showed the follicular epithelial cells were the site of protein synthesis. These proteins have been referred to as paravitellogenin in *H cecropia* (Telfer *et al.* 1981); yolk polypeptide in *Plodia interpunctella* (Shirk 1987); ovary-specific proteins in mosquitoes (Borovosky and Van Handel 1980); follicle-specific proteins in *Manduca sexta* (Tsuchida *et al.* 1992); and egg-specific protein in *Bombyx mori* (Irie and Yamashita 1983). The protein from *B. mori* was purified and characterized ; it contains 2% carbohydrate and about 4% lipid, with a molecular weight of 125,000 (Irie and Yamashita 1983) and is predominantly phosphorylated in a cAMP-dependent manner in the late developmental stages of the ovary (Takahashi 1987).

In the previous reports we have been purified and characterized vitellin (Lee *et al.* 1992), analysed amino acids and fatty acids in the ovaries (Lee *et al.* 1994), and studied on juvenile hormone control of oocyte development (Lee *et al.* 1995) in the blowfly, *Lucilia illustris.* In the present paper, we report on changes in protein content during oocyte development and some properties of egg-specific protein in the egg, not observed yet at all, of *L. illustris.*

MATERIALS AND METHODS

Insects

The blackblow fly, *Lucilia illustris,* was reared by standard methods (Sing and Moore 1985). Adults were maintained on sugar granule and water in 25 ± 2°C chambers with 55± 5% relative humidity and 16h of light daily. To induce synchronization of ovary maturation, flies were fed on fresh beef liver as a protein meal on day 6 after emergence. Following a protein meal eggs were collected every 24h for 5 days and were stored at −20°C immediately until used.

Oocyte Development and Protein Measurements

Oocyte development was determined by measuring the terminal oocyte length which reflects the state of oocyte maturation. Ovaries of non-liver fed and liver-fed flies were dissected out in saline solution and length of terminal oocytes were measured using an ocular micrometer.

Proteins in fat body, haemolymph, and ovaries were determined by Bradford (1976) with bovine serum albumin as the standard. Haemolymph samples were obtained from small surgical incisions at the base of wings and diluted 20-fold with a phosphate buffer saline (PBS, 0.02M KH_2PO_4, 0.3M NaCl, pH 7.0) before protein was assayed. Fat bodies and ovaries were dissected out from females, rinsed in saline solution, homogenized in PBS, and assayed for protein content.

Purification of Crude Extracts

For purification of egg-specific protein, about 1g of the eggs at 96 hr after a protein meal was ground in a glass-Teflon homogenizer in 10ml of 0.05M phosphate buffer, pH 7.5 containing 0.15M KCl and 1 mM phenyl methylsulphonyl fluoride (PMSF). Unless otherwise stated, the same buffer solution was used through all purification steps. The homogenate was centrifuged at 10,000g for 10 min at 4°C and the supernatant was used for the purification listed in the following section.

Chromatography on DEAE-cellulose

Throughout the column chromatography, protein was monitored at 280 nm (Zou *et al.* 1988). The crude egg extract (about 200 mg protein/10 ml) was applied to a column of DEAE-cellulose (DE 23, 2.0 cm i.d.×30 cm) previously equilibrated with the buffer. The materials were eluted first with 200 ml of buffer and then with a linear KCl gradient from 0.15 to 0.5 M. The fractions eluted with 0.35 M KCl were pooled and were concentrated with an immersible CX-10 ultrafilter (Millipore Co., Bedford, U.S.A.). This sample (about 20 mg proteins/ml) was subjected to gel permeation chromatography.

Chromatography on Sephacryl S-200

The preparation (40 mg proteins/2 ml) was applied to a Sephacryl S-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (2.6 cm i.d.×35 cm) equilibrated previously with the buffer (Irie and Yamashita 1983). Proteins were eluted with the

same buffer at a flow rate of 10 ml/hr. Fractions containing egg-specific protein were pooled and concentrated as above. The concentrated samples (0.5 ml) were subjected to a second Sephacryl S-200 column (1.5 cm i.d.×80 cm) which was eluted with 0.5M phosphate buffer, pH 7.5 at a flow rate of 5 ml/hr. Again the fractions containing egg-specific protein were pooled and concentrated as above.

Polyacrylamide Gel Electrophoresis

Haemolymph, fat body, ovary extracts, vitellin and purified egg-specific protein were loaded to polyacrylamide gel electrophoresis in 5~9% gels at pH 8.4, and sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis in 5~10% with a Tris-glycine buffer system, pH 8.4 (Laemmli 1970). Proteins were visualized by staining with Coomassie brilliant blue R (Sigma Chemical Co., St. Louis, U.S.A.) followed by destaining. Molecular weights were determined by native (Hedrick and Smith 1968) and SDS polyacrylamide gel electrophoresis (Laemmli 1970) using protein standards obtained from Sigma Co.

Chemical Analyses

Egg-specific protein sample and ovary at 96 hr after protein meal was hydrolyzed in 6N HCl at 110°C *in vacuo* for 24h and analyzed by Firling (1977) on a JEOL JLC-6AH amino acid analyzer (Japan). Fatty acids were extracted from the ovary (96 hr) and purified sample of egg-specific protein with 1ml of chloroform-methanol (2 : 1 v/v) according to the methods of Fireston and Horwitz (1979). The extracts were esterified by 14% methanolic BF3 and analyzed by gas chromatography (663-50, Hitachi Co., Japan).

RESULTS AND DISCUSSION

Oocyte Maturity and Changes in Protein Content

Results summarized in Fig. 1 showed that when female flies were given a protein meal, oocytes were developed synchronously. Length of terminal oocytes was increased throughout 48 and 72 hr after a protein meal and reached maximum at 120 hr. Between 72 and 120 hr, changes of terminal oocytes in size showed plateau which means that oocytes of *L. illustris* females had been grown up at 96 hr after a protein meal.

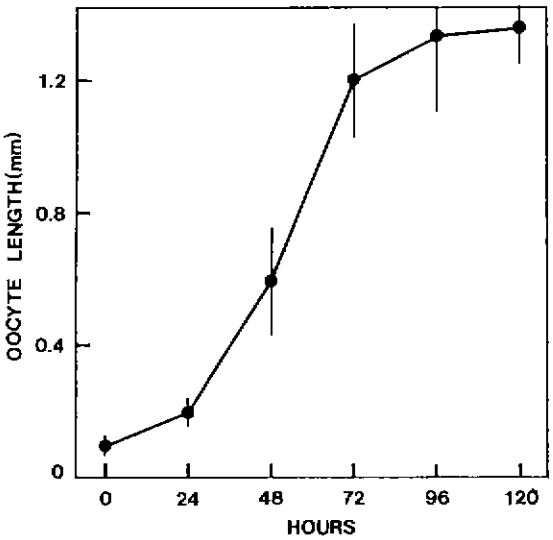

Fig. 1. Length of terminal oocyte in *L. illustris* at various times after a protein meal. Data were the average of three preparations conducted in duplicate. The error bars represent the standard deviation.

Depending upon the above data, changes of protein contents in fat body, haemolymph, and ovaries were measured every 24 hr for 5 days after a protein meal as shown in Table 1. Protein in fat body started increasing at 24 hr after a protein meal and reached maximum at 72 hr, then began to decreased, whereas in haemolymph it built up rapidly at 72 hr, and thereafter it rapidly decreased.

On the other hand, a small amount of ovarian protein was detected by 48 hr after a protein meal, but its content then increased suddenly during the next 24 hr, after which the level remained approximately constant for the next 48 hr. The profiles of protein in *L. illustris* are thus somewhat similar to those found in *Musca domestica* (de Bianchi *et al.* 1988) and *Phormia regina* (Zou *et al.* 1988). Our time course study of the appearance of protein contents implicated that protein was first synthesized in the fat body and secreted into the haemolymph. From there protein was sequestered by developing egg. Growth of the ovaries showed high levels between 72 and 120 hr, at which time oocytes become

Table 1. Protein contents in fat body, haemolymph, and ovary of *L. illustris* female adult after a protein meal

Hours after a protein meal	Fat body (μg/fly)	Haemolymph (μg/ml)	Ovary (μg/ovary)
0	3.50± 0.14	4.30± 0.40	2.10± 0.14
24	37.60± 5.30	8.90± 0.90	6.60± 2.25
48	80.50± 23.00	22.30± 8.70	13.50± 4.80
72	161.60± 31.70	34.70± 4.54	102.30± 19.30
96	94.20± 8.20	19.50± 10.00	168.00± 7.40
120	121.00± 9.30	24.00± 4.80	143.30± 1.80

mature These data(Fig. 1 and Table 1) will basically be used for further work on changes in egg-specific protein content during oogenesis what we are being planned.

Purification of Egg-specific Protein

In the following we have focussed on to clarify if the eggs of *L. illustris* have synthesized a specific protein themselves like *H. cecropia* (Telfer *et al.* 1981), *B. mori* (Irie and Yamashita 1983), and *P. interpunctella* (Shirk 1987). As mentioned in Materials and Methods egg-specific protein from the ovaries was purified using gel permeation chromatography. Separation of protein from the ovaries was achieved by anion exchange chromatography on DEAE-cellulose column. Protein was monitored by the absorbance profile at 280 nm. In 0.15~0.5M KCl at pH 7.5, the proteins in fractions 45~70, presumably containing vitellin, appeared as the second and third sharp peaks of bound protein at the addition of 0.35M KCl (Fig. 2).

The purified protein was subsequently used to gel filtration on Sephacryl S-200. By rechromatography on a Sephacryl S-200 column, egg-specific protein was eluted in a single symmetrical peak as shown in Fig. 3 (Irie and Yamashita 1983).

Polyacrylamide Gel Electrophoresis and Molecular Weight Determination

The proteins extracted from the eggs of *L. illustris* separated into at least eight bands on a 6% polyacylamide gel electrophoresis (Fig. 4C). Egg-specific protein was identified as a band of Rf 0.4 which was absent from male and female haemolymph (Fig. 4A, B). The apparent mol. wt of native egg-specific protein was estimated by SDS-polyacrylamide gel electrophoresis (Fig. 5) that gave a linear relationship between relative motility and the semilogarithm of mol. wts of six protein standards, from which a mol. wt of 110,000 was estimated for egg-specific protein of *L. illustris* (Fig. 5). Irie and Yamashita (1983) reported that in *B. mori* the mol. wt of 130,000 estimated by Sephacryl S-200 column was somewhat higher than that of 120,000 by the native polyacrylamide gel electrophoresis and this difference was possibly due to the asymmetry of the molecule or to the presence of a carbohydrate chain or to both. Takahashi (1987) identified the band 2 protein as egg-specific protein of the eggs in *B. mori*. The ra-

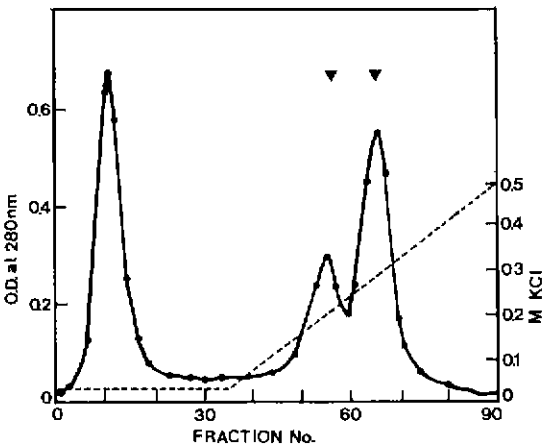

Fig. 2. Elution pattern of anion exchanges chromatography on a DEAE-cellulose column(2.0 cm i.d. by 30 cm). Unbound pretein was eluted with 0M KCl in the phosphate buffer. The bound proteins showing two peaks (arrows) were eluted with linear gradient (0.15~0.5M KCl) at a flow rate 30 ml/hr and were pooled according to Irie and Yamashita (1983)

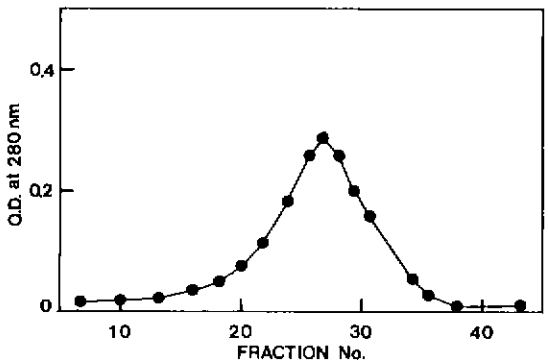

Fig. 3. Elution profile of egg-specific protein on the second Sephacryl S-200 column chromatography. Partially purified samples (20 mg/0.5 ml) after the first Sephacryl S-200 chromatography were applied onto a second Sephacryl S-200 column chromatography. The column was eluted with 0.1 M phosphate buffer, pH 7.5 at a flow rate of 5 ml/hr and fraction size was 2 ml.

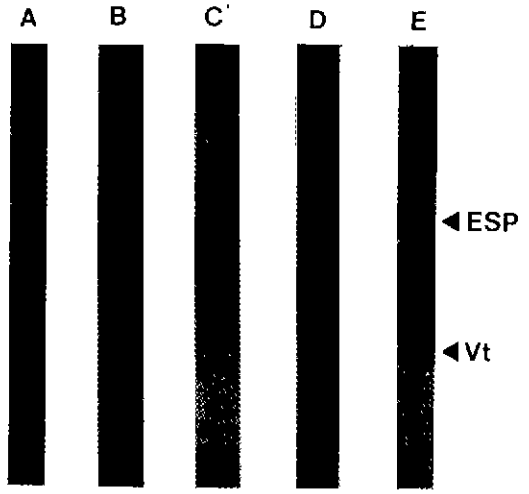

Fig. 4. Bands of proteins prepared from male haemolymph (A), female haemolymph (B), ovary extracts (C), vitellin (D), and purified egg-specific protein (E) using 6% polyacrylamide gel electrophoresis. Each gel contained about 50~100 μg protein was stained with Coomassie brilliant blue. Vt; vitellin, ESP; egg-specific protein.

dioactivity in hydrolysates of ESP appeared to be in the form of serine phosphate(15~25%) and inorganic phosphate(75~85%). This protein was predominantly phosphorylated in a cAMP-dependent manner in the late developmental stages of the ovary.

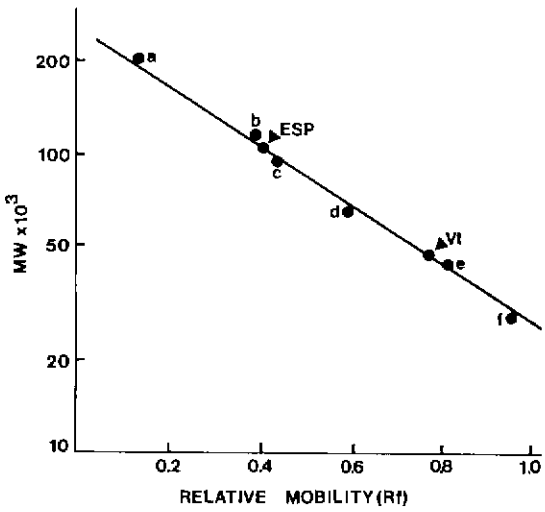

Fig. 5. Molecular weight estimation of egg-specific protein and semilogarismic regression line between log molecular weight of standards and their relative mobility by a SDS polyacrylamide gel electrophoresis. Marker proteins are as follows: a; myosin (205,000), b; β-galactosidase (116,000), c; phosphorylase (97,000), d; bovine albumin (66,000), e; egg albumin (45,000). f; carbonic anhydrase (29,000).

Protein kinase inhibitor protein from rabbit skeletal muscle was shown to inhibit the phosphorylation, indicating the involvement of cAMP-dependent protein kinases in phosphorylation of ESP. Tsuchida *et al.* (1992) have suggested that two proteins were synthesized in developing follicles isolated from the eggs of *M. sexta*. One had a molecular mass of 130 KDa and was composed of two identical 65 KDa subunits. This protein was glycosylated and phosphorylated. The other had a molecular mass of 140 KDa and was composed of two 40 and two 35 KDa subunits. The second one was glycosylated but not phosphorylated. Accordingly, molecular weight of egg-specific protein in the eggs of *L. illustris* was similar to those of *B. mori* and *M. sexta* but not ascertained if it has subunit so that further experiment will be required to clarify this situation.

Composition of Amino Acids and Fatty Acids

The amino acid composition of egg-specific protein was shown in Table 2 along with that of ovary for comparison. A total of 13 amino acids was dec-

Table 2. Amino acid composition (mol%) of egg-specific protein and ovary in *L. illustris*

Amino acid	Egg-specific protein	Ovary*
Asparagine	15.4	13.8
Threonine	7.3	5.6
Serine	6.9	5.8
Glutamic acid	16.3	18.4
Glycine	8.2	6.4
Alanine	9.4	7.3
Valine	6.7	7.0
Isoleucine	5.9	5.6
Tyrosine	7.0	10.5
Phenylalanine	5.5	4.8
Histidine	4.0	3.1
Cysteine	2.2	4.0
Arginine	5.2	8.2

*The ovary sample was hydrolyzed at 96 hr after a protein meal.

Table 3. Fatty acid composition of egg-specific protein and ovary in *L. illustris*

Fatty acid	Egg-specific protein (μg/mg)	Ovary* (μg/ovary)
Palmitic acid	5.67	92.65
Palmitoleic acid	8.42	117.15
Stearic acid	2.11	10.75
Oleic acid	7.25	79.90
Linoleic acid	–	18.80

The values represent the average of two replications.
*96 hr after a protein meal (Lee *et al.* 1994)

tected and egg-specific protein was rich in asparagine, glycine, alanine and glutamic acid. It is apparent that there is some similarity between egg-specific protein and ovary.

In *B. mori* (Irie and Yamashita 1983), 17 amino acids of egg-specific protein were isolated and among them glutamate/glutamine, aspartate/saparagine, glycine and serine were rich. In case of follicle-specific protein in *M. sexta*, 16 amino acid composition was reported (Tsuchida *et al.* 1992). Comparing with *B. mori* and *M. sexta*, a small number of amino acids in egg-specific protein in *L. illustris* was detected and it is thought to be due to technical difficulties. More precise analysis must be needed though it was a comparison between the different orders with one another.

Extracted fatty acids were subjected to gas chromatography to separate the components. As shown in Table 3, palmitic acid, palmitoleic acid, stearic acid, and oleic acid in the egg-specific protein were the main components of fatty acids and linoleic acid was also found at a trace level, but its quantity was not measured.

Egg-specific proteins in several species of holometabolous insects have now been shown, however, it is noteworthy that the proteins have little in common either in terms of the site of synthesis, molecular weight and subunit composition or chemical composition. The *B. mori* egg-specific protein, localized in the follicle cells and yolk sphere, has a 125,000 dalton protein containing one subunit with mol. wt of 55,000, 2% carbohydrate and 4% lipid, which are consumed during early embryogenesis (Irie and Yamashita 1983, Yamashita and Indrasith 1988) through the action of a specific protease (Indrasith *et al.* 1988). Egg-specific proteins may be an important source of amino acids for early embryogenesis, since *B. mori* larvae can hatch from vitellin-deficient eggs (Yamashita and Irie 1980) Based on this observations, we tentatively conclude that the ovary of *L. illustris* has egg-specific protein which is a 110,000 dalton protein containing 13 amino acids and 5 fatty acids besides yolk protein. However, the exact properties of egg-specific protein in *L. illustris* will have to await further experiments such as western blot, immunodiffusion and immunohistochemistry.

Aknowledgement.

This work was supported by Korea Science and Engineering Foundation grants (No. 921-0400-036-2). We wish to thank Dr. Tae-Heung KIM at Department of Agricultural Biology, Chonbuk National University for his critical correction of the manuscript and Miss Kyung LEE for insect care.

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(Received April 4, 1995)