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Ovarian Development and Yolk Protein of the Mushroom Fly, *Coboldia fuscipes* (Diptera: Scatopsidae)

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Abstract

Ovarian development and yolk protein (YP) of mushroom fly, *Coboldia fuscipes*, were characterized. *C. fuscipes* has a pair of ovaries, composed of 130~140 ovarioles, respectively. Ovarian development begins at 1 day of pupa, and growth of the ovaries continued to become a matured shape at 1 day after emergence. The YP of *C. fuscipes* identified by SDS-PAGE analysis revealed that the protein is composed of three subunits, designated YP1 (61 kDa), YP2 (50 kDa), and YP3 (47 kDa). These three subunits of YP gradually decreased during embryogenesis. The YP was first detected in the 3 day-old pupal ovary and was continually detected up to 2 day-old adult, but not in the hemolymph. Furthermore, Western blot analysis of male and female adult hemolymph and ovary revealed that the antibodies against YP1, YP2, and YP3 reacted with three YP bands in ovary and egg extract, respectively. However, this reactivity was not observed in the male and female hemolymphs. Therefore, it is assumed that the YP of *C. fuscipes* is synthesized in the ovary at 3 days after pupation.

Key words – *Coboldia fuscipes*, Ovarian development, Yolk protein, Embryogenesis

Introduction

Insect yolk proteins (YPs) have biological importance because they are the most abundant proteins in the eggs, and because they have an important role during embryogenesis [12]. This has probably triggered to study the YP in the several aspects, such as molecular weight, amino acid sequence, and composition of subunits, and so on [2,8,12,18].

Generally speaking, YPs are synthesized as single or multiple precursors, vitellogenin (Vg), in the fat body. This water-soluble percursor is subsquently secreted into hemolymph, and then selectively absorbed into oocytes [15]. In higher Diptera, such as *Dorsophila melanogaster* and *Anastrepha suspensa*, however, the ovaries also synthesize Vg [1,9]. In most insects, the YP is a large protein with the molecular weight ranging from 210~652 kDa and consists of a large subunits (150~190 kDa) and a small subunits (40~65 kDa) [15]. However, the protein of the higher Diptera is known to be composed of 3~5 pieces of small subunits (44~51 kDa) [4,7,14].

Until now, the Vg has been extensively studied in relation to physiology, endocrinology, biochemistry and molecular biology from diverse insect species. Furthermore, the YP has also been extensively characterized in higher Diptera including *D. melanogaster* [1,3,4], *A. suspensa* [9], *Musca domestica* [7], *Ceratitis capitata* [16] and *Phormia regina* [20], but the relevant study of the mushroom fly, *Coboldia fuscipes* is nearly absent. In the present report, we have described the ovarian development and the characteristics of YP in *C. fuscipes*.

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Materials and Methods

Insects

Adults *Coboldia fuscipes* were collected with an aspirator in the oyster mushroom house in Saengbilyang Kyungsangnamdo in October, 1997. Insects were reared under the conditions described previously [6]. The pests collected were reared in a growth chamber at 23±1℃ with 60±5% of relative humidity under 12L : 12D. Spawn of a velvet stem, *Flammulina velutipes*, was grown on PDA (potato dextrose agar) medium (Difco), and were supplied as a diet.

Staged eggs

To examine development of the embryos, female adults were allowed to fertilize and oviposit in the moist petri dish, and eggs were incubated at 23±1℃ with 60±5% of relative humidity. Ten eggs were collected from the dish every 12 hrs over 60 hrs post oviposition. These eggs were punctured by a fine needle in 10 ㎕ phosphate-buffered saline (PBS; 120 mM NaCl, 2 mM KCl, 4.5 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.4) containing 5 mM EDTA and 1 mM PMSF to obtain egg component. After the centrifugation of the component at 13,000 rpm for 20 min at 4℃, the supernatant was used for the SDS-PAGE analysis.

Preparation of hemolymph and ovary extracts

Hemolymph and ovary were collected from 1 day-old pupa to 2 day-old adult. Hemolymph was collected by puncturing thorax of pupae and pterothorax of adults with a fine needle. This was subsequently diluted into PBS containing 5 mM EDTA and 1 mM PMSF. This hemolymph was centrifuged at 13,000 rpm for 20 min at 4℃ to remove hemocytes and cell debris. The supernatant was stored at -70℃ until use. Ovaries of pupae and adults were dissected in the cold PBS. Ovaries were rinsed three times with cold PBS and then homogenized in PBS containing 5 mM EDTA and 1 mM PMSF using micro-homogenizer. The mixture was then centrifuged at 13,000 rpm for 20 min at 4℃, and the supernatant was stored at -70℃ until use.

Electrophoresis

SDS-PAGE was performed according to Laemmli [13] at 25 mA until the bromophenol blue tracking dye reached the bottom of the gel. After electrophoresis, gels were fixed and stained with 0.1% Coomassie brilliant blue R-250. Molecular weight markers [myosin (205,000), β-galactosidase (116,000), phosphorylase b (97,000), bovine serum albumin (66,000), egg albumin (45,000) and carbonic anhydrase (31,000); Bio-Rad] were used as standards.

Preparation of polyclonal antibody

Ovaries isolated from unfertilized female adults of *C. fuscipes* were homogenized, and then proteins were separated on the SDS-PAGE gel. After staining the gel with Coomassie blue, the major three YP bands were cut off, and were eluted using dialysis tube (Bio-Rad) at 100 V for 60 min, respectively. The eluted YPs were mixed with equal volume of Freund's complete adjuvant (a total of 400 ㎕) and injected into a Balb/c mice, respectively. Three successive injections were performed with one-week intervals beginning a week after the first injection with the antigens mixed with equal volume of Freund's incomplete adjuvant (a total of 400 ㎕). Bloods were collected 3 days after the last injection and centrifuged at 3,000 rpm for 5 min. The supernatant antisera were stored at -70℃ until use.

Western blotting

For Western blot analysis, SDS-PAGE was carried out as described above. Proteins were blotted to a sheet of nitrocellulose membrane (Sigma, 0.45 ㎛ of pore size) [17]. The blotting was performed in transfer buffer (25 mM Tris and 192 mM glycine in 20% methanol) at 30 volt overnight at 4℃. After blotting, the membrane was

blocked by incubation in 1% BSA solution for 2 hrs at room temperature. The blocked membrane was incubated with Vn antiserum solution (1:500 v/v) for 1 hr at room temperature and washed in TBST (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.05% Tween 20). Subsequently, the membrane was incubated with goat anti-mouse IgG alkaline phosphatase conjugate (1 : 5,000 v/v, Sigma) for 1 hr at room temperature. After repeated washings, substrate solution (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$) containing NBT (nitro-blue tetrazolium) and BCIP (5-bromo-4-chloroindolyl phosphate) was added, and the reaction was quenched with distilled water.

Results

The ovary of *C. fuscipes* was composed of a pair, and each ovary has 130~140 ovarioles, respectively (Fig. 1). We could observe that ovarian development begins at 1 day-old female pupa (Fig. 1A), and growth of the ovaries continued to become a matured shape (approximately 1.4 mm in length) at 1 day after emergence. (Fig. 1D).

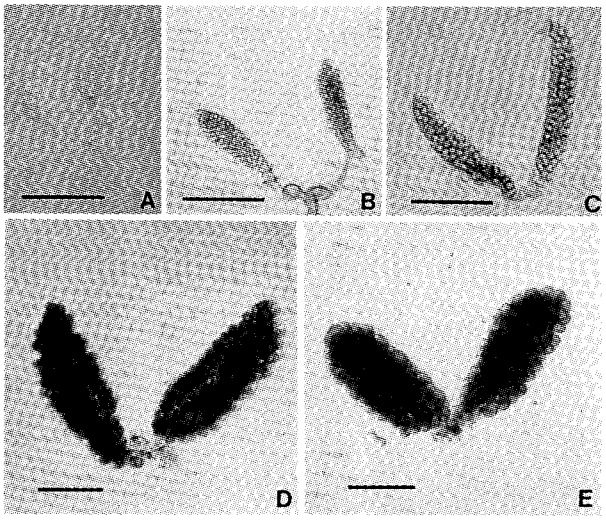

Fig. 1. Ovarian development of mushroom fly, *C. fuscipes*. A, 1 day-old pupal ovary; B, 2 day-old pupal ovary; C, 3 day-old pupal ovary; D, 1 day-old adult ovary; E, 2 day-old adult ovary. Size bars indicate 500 ㎛.

For identification of YP in *C. fuscipes*, the staged eggs were analyzed by SDS-PAGE (Fig. 2). The gel, stained with Coomassie blue, revealed three major protein bands with the molecular weights of about 61 kDa, 50 kDa and 47 kDa. These three protein bands clearly represent major yolk proteins (YPs) as they are gradually used up during embryogenesis (Fig. 2). Therefore, the YP of *C. fuscipes* composed of three proteins (YP1, 61 kDa; YP2, 50 kDa; and YP3, 47 kDa).

To study the synthesis of YP in *C. fuscipes*, samples of the ovaries and hemolymphs from 1 day-old pupa to 2 day-old adult were analyzed by SDS-PAGE (Fig. 3, 4). Three protein bands of YP first became detectable in the 3 day-old pupal ovary and were continuously detected up to 2 day-old adult (Fig. 3). However, the YP bands were not detected in the female and male hemolymphs, respectively (Fig. 4). Western blot analyses using the each of polyclonal antiserum against YP1 (61 kDa), YP2 (50 kDa) and YP3 (47 kDa), showed that the three YP bands reacted in the ovary and egg extract, respectively

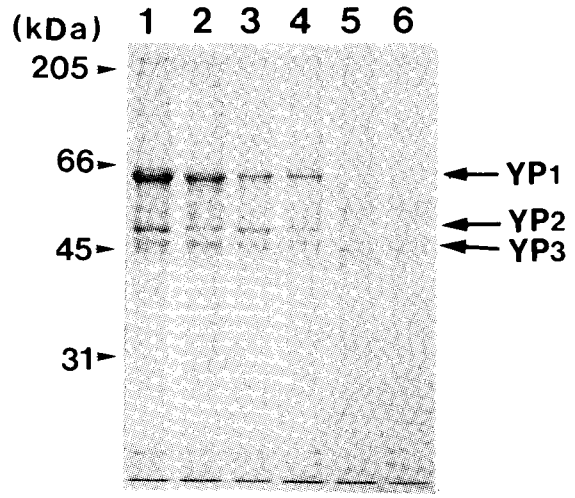

Fig. 2. SDS-PAGE analysis of embryogenesis from *C. fuscipes* eggs.
Lane 1, oviposited egg; Lane 2, 12 hrs after oviposition; Lane 3, 24 hrs after oviposition; Lane 4, 36 hrs after oviposition; Lane 5, 48 hrs after oviposition; Lane 6, 60 hrs after oviposition. Molecular weight markers are indicated at the left.

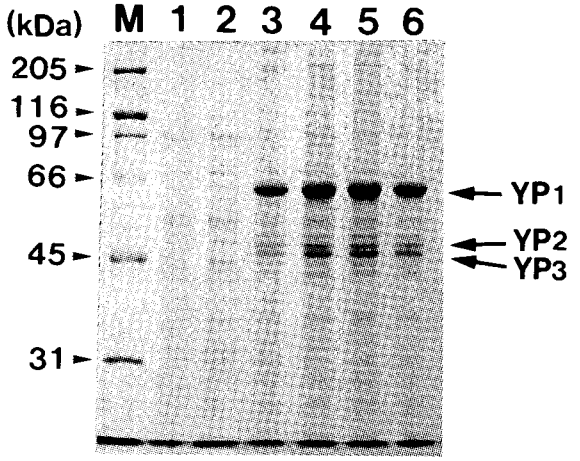

Fig. 3. SDS-PAGE analysis from ovary extracts in *C. fuscipes*.
Lane 1, 1 day-old pupa; Lane 2, 2 day-old pupa; Lane 3, 3 day-old pupa; Lane 4, 1 day-old adult; Lane 5, 2 day-old adult; Lane 6, egg extract. M indicates molecular weight markers.

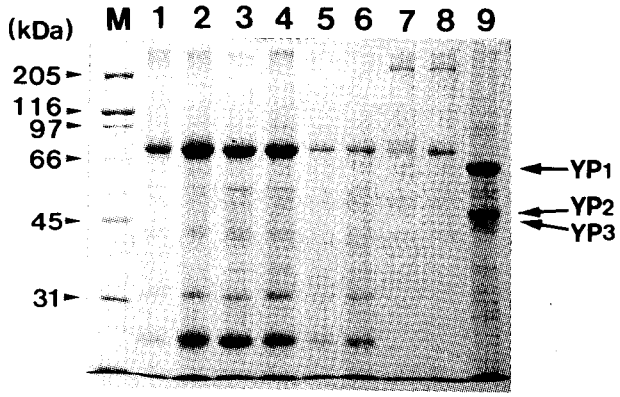

Fig. 4. SDS-PAGE analysis from male and female hemolymphs in *C. fuscipes*.
Lane 1, 1 day-old male pupa; Lane 2, 1 day-old female pupa; Lane 3, 2 day-old male pupa; Lane 4, 2 day-old female pupa; Lane 5, 3 day-old male pupa; Lane 6, 3 day-old female pupa; Lane 7, 1 day-old male adult; Lane 8, 1 day-old female adult; Lane 9, 1 day-old adult ovary extract. M indicates molecular weight markers.

(Fig 5). However, this reactivity was not observed in the male and female adult hemolymphs, respectively (Fig 5). Also, an antiserum against YP1 strongly crossreacted with three YP bands (Fig 5B), and an antiserum against YP2 and YP3 crossreacted with YP1 band (Fig. 5C & D). Therefore, we concluded that the ovary was the major site of synthesis, and the YP of *C. fuscipes* is synthesized at 3 days after pupation.

Discussion

Ovarian development and oogenesis have been extensively studied in the various insects [2,10,11]. In the present study, we have observed that the ovaries of *C. fuscipes* contain about 260~280 ovarioles, and development of the ovary begins at the first day of pupa. During the period between the third day of pupal stage and the first day of adult, the volume of the ovary significantly increased, and almost mature ovarian morphology was identified. Ovarian development of *C. fuscipes* is similar to those of dipteran insects such as *D. melanogaster* [1] and *Lycoriella mali* [11]. In the *D. melanogaster*, the paired ovaries contain 15~20 ovarioles each, and the metamorphosis and vitellogenesis occurs at fifth~sixth day of pupal stage, and then oocytes become mature [1].

Our result indicated that YP of *C. fuscipes* is composed of three subunits with the molecular weights of 61, 50 and 47 kDa. In higher Diptera, the molecule weight of YPs are generally ranged within the 40~54 kDa range, and the number of subunits varies from one to five. For example, *D. Melanogaster* YP is composed of three subunits with the molecule weights of 45, 46 and 47 kDa, respectively [4]. Mediterranean fruit fly, *C. capitata*, is composed of four subunits, two of one kind (46 kDa) and two of another kind (49 kDa), forming a tetramer structure [16]. Also, the house fly, *M. Domestica*, YP is composed of at least five polypeptides with the molecular weights of 46, 48, 51, 52 and 54 kDa [7], and the black blow fly, *P. regina*, has a YP composed of polypeptides with the molecule weight of 42, 43, 44 and 45.5 kDa [20]. In other case, YPs are made up of only one polypeptide: 48 kDa for Caribbean fruit fly, *A. suspensa* [9], and 47 kDa for blow fly, *Lucilia illustris* [14]. Therefore, the

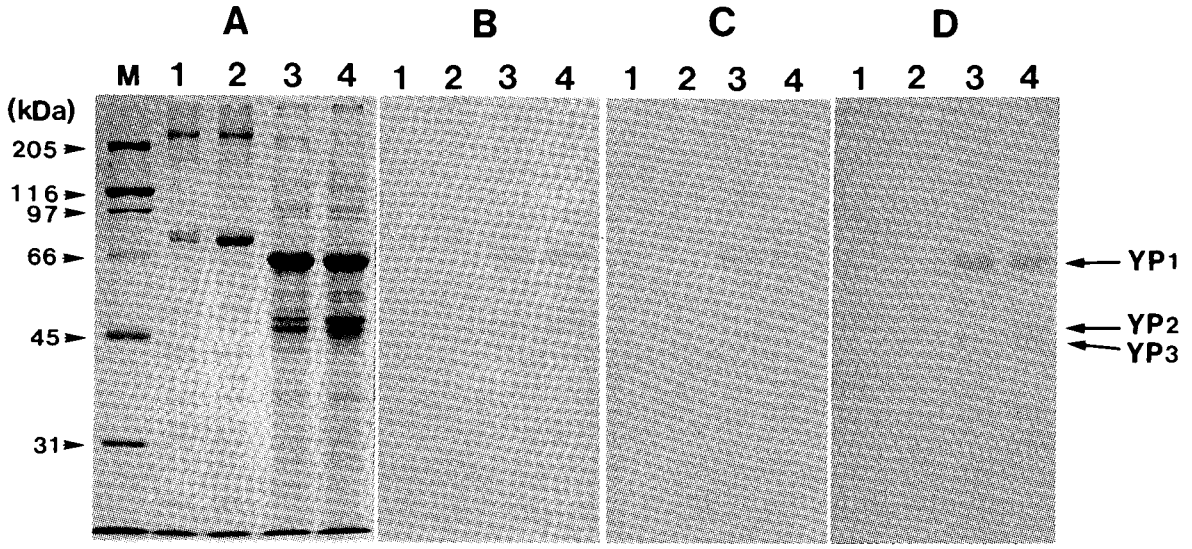

Fig. 5. SDS-PAGE (A) and Western blot (B, C, and D) analyses from adult hemolymph and ovary in *C. fuscipes*. Western blot analysis was used antiserum against YP1 (B), YP2 (C), and YP3 (D), respectively. Lane 1, 1 day- old male adult hemolymph; Lane 2, 1 day-old female adult hemolymph; Lane 3, 1 day-old adult ovary extract; Lane 4, egg extract. M indicates molecular weight markers.

molecular weight and subunits composition of YP in *C. fuscipes* are similar to those of higher dipteran insects.

As the results of SDS-PAGE and Western blot analyses, the ovary was the major site of synthesis, and the YP of *C. fuscipes* is synthesized at 3 days after pupation. Brennan *et al.* [3] first indicated that insect ovaries were also capable of YP synthesis on the basis that *D. melanogaster* YP genes were transcribed in the ovarian follicular epithelium, as well as in the fat body. Since then, YP synthesis has been reported in the ovaries of other Diptera including *C. capitata* [16] and *Stomoxys calcitrans* [5]. Also, the initiation of YP synthesis occurs nearly at the time of eclosion in the higher Diptera [2,9]. Handler and Shirk [9] reported that YP (48 kDa) synthesis in *A. suspensa* became first detectable by 4~5 days after eclosion, and the major site of YP synthesis was in the ovary itself.

Acknowledgements

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초록 : 버섯파리 (*Coboldia fuscipes*)의 난소발육 및 난황단백질에 관한 연구

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버섯파리 (*Coboldia fuscipes*)의 실내사육에 의한 난소발육 및 난황단백질의 특성에 관해서 조사하였다. *C. fuscipes* 성충의 난소는 한 쌍으로서 각각 130~140개의 난소소관으로 구성되어 있으며, 번데기 1일째부터 난소발육이 시작되어 성충 1일째에 성숙한 난소 형태를 갖추었다. *C. fuscipes*의 난황단백질 (yolk protein : YP)을 확인하기 위해서 산란된 알을 12시간 간격으로 SDS-PAGE를 실시한 결과, 분자량 61 kDa (YP1), 50 kDa (YP2), 47 kDa (YP3)인 3개의 주요 난황단백질 밴드를 확인하였다. 이들 난황단백질들은 배자 발생 (embryogenesis)이 진행되는 동안 점진적으로 감소하였다. *C. fuscipes*의 난황단백질은 번데기 3일째부터 난소에서 확인되기 시작하여 성충시기까지 존재하는 것을 SDS-PAGE 통하여 확인하였다. 그러나 혈림프에서는 이들 주요 난황단백질이 확인되지 않았다. 또한 *C. fuscipes*의 난황단백질에 대한 항체를 이용한 Western blot에서도 혈림프에서는 면역학적 반응이 나타나지 않았으나, 난소 및 알 추출물에서는 3개의 주요 난황단백질 밴드에서 면역학적 양성반응이 일어났다. 따라서 *C. fuscipes*의 주요 난황단백질은 번데기 3일째 시기에 대부분 난소에서 합성이 일어나는 것으로 사료된다.