Studies on Vitellin Accumulation in Freshwater Shrimp, *Palaemon paucidens*

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**Abstract** The vitellin accumulation was studied in freshwater shrimp *Palaemon paucidens* by electrophoretic, immunological, and histological techniques. Egg formation and vitellin accumulation proceeded as follows.

Young oocyte stage was defined as the oocytes less than 1.5 in gonadosomatic index (GSI) and less than about 300 μm in maximum egg diameter (MED). The oocyte had nucleus and cytoplasm which were positive in hematoxylin staining, but did not include components immunologically identical with vitellin.

Early yolk-formation stage corresponded to the ovary ranged 1.5 to 4.5 in GSI and 250 to 550 μm in MED, and was characterized by appearance of eosin-positive cytoplasm. The yolk globules consisted of lipoprotein were accumulated in the cytoplasm from the stage. A hemolymph lipoprotein immunologically identical to vitellin of mature ovary appeared in the ovary of which MED was more than 350 μm.

Late yolk-formation stage was defined as the ovary having more than 4.5 in GSI and 550 μm in MED. The nucleus disappeared and the cytoplasm filled with eosin-positive yolk globules. With the appearance of a vitellogenin in the hemolymph, the yolk constituents were actively accumulated in the ovary.

Vitellogenin synthesized extraoarially was probably incorporated in the oocytes as vitellin after slight conformational changes.

**INTRODUCTION**

In crustacean, the female-specific protein, vitellogenin appears in the hemolymph during vitellogenesis as extraovarian sources of the yolk protein, vitellin. The precursor of vitellin closely correlates to the vitellogenin in chemical and immunological properties. However, incorporation mechanism of the vitellogenin into developing oocyte and mode of vitellin accumulation have been a matter of controversy.

Adiyodi and Adiyodi (1970) suggested that incorporation of hemolymph components into the developing ovary is controlled under hormonal antagonism relating to molting and growth. The vitellin released into hemolymph correlates highly to molting and/or molting hormone (Souty and Picaud, 1981; Picaud and Souty, 1981; Hartnoll, 1982; Meusy and Charniaux-Cotton, 1984; Charniaux-Cotton, 1985; Suzuki, 1987).

In the freshwater shrimp *Palaemon paucidens*, hormonal regulation in vitellin accumula-
tion is not constant throughout egg formation, because the eyestalk removal gives different effect on ovarian growth at various developing stages (Kamiguchi, 1971). Nakagawa et al. (1982) suggested that the female-specific protein was discontinuously released into hemolymph. These phenomena imply that vitellin is accumulated discontinuously under the certain hormonal control.

The present paper dealt with possible mechanism of vitellin accumulation in the oocyte by means of histological and biochemical methods.

**MATERIALS AND METHODS**

**Shrimp**

The freshwater shrimp *Palaemon paucidens* was collected from Umeda Fish-Farming Pond in Hiroshima Prefecture. The hemolymph was withdrawn from the abdomen through the integument with a glass capillary tube and mixed with 50 µl of 0.01 M sodium citrate. The hemolymph solution stored in a freezer at −20ºC was used for electrophoretic analysis. The ovaries were submitted to biological measurements, electrophoresis, and histological observation. For electrophoresis, the ovary was homogenized with 0.9% saline solution and the soluble layer was obtained by centrifugation at 3000 rpm. Oocyte diameter was measured microscopically in histological section.

**Electrophoresis**

The hemolymph and ovary extract were electrophoretically analyzed on polyacrylamide gradient gel (4–30%) in Tris-borate-Na₂EDTA buffer at pH 8.30. The sample solution (5–10 µl) was applied on the top of gel. A constant voltage of 200 V was charged for 6 hr at 10ºC. Protein bands were visualized by the staining with Coomassie brilliant blue R-250. Sudan black B was used for lipoprotein detection. As standard marker, an Electrophoretic Calibration Kit HWM (Pharmacia Fine Chemical Co. Ltd.) was used for molecular weight estimation.

**Immunological Procedure**

The mature ovary was homogenized with 0.9% saline solution and centrifuged. After removing the brownish top layer and insoluble precipitation, the transparent soluble layer was used for the antigen. The rabbit antiserum against the egg protein was prepared by three hypodermic injections of the mature ovary extract with complete ajuvand at the ratio of 1:1. Each antigen included 7-13 mg protein.

The antiserum formation was checked in 1% agarose gels by the Ouchterlony method. Thus obtained rabbit antiserum was kept at −20ºC until analysis. After electrophoresis of the hemolymph, the electrophoretic gradient gel was incubated overnight with 1% agarose gel which contained 10% antiserum.

**Histological Procedure**

The ovary fixed with Bouin’s solution was dehydrated with ethanol and butanol. The paraffin and/or ceroidin section were stained with the hematoxilin-eosin.
RESULTS

Ovarian Maturation

The developing ovary was classified into three stages by histological characterization, as follows: young oocyte stage, early yolk-formation stage, and late yolk-formation stage. The ovary appeared on the mid-gut gland was greyish white in color at the young oocyte stage. The color of the ovary changed into dark green with the progress of maturation.

Histological Observation

Plate 1 shows histological section of the ovary. The young oocyte stage was defined as

Plate 1. Photomicrographs of sections through ovary in various developmental stages in *Palaemon paucidens*

Magnification of all figures 100 (a), 200 (b) times.

Abbreviations

C, cytoplasm; M, mid-gut gland; N, nucleus; V, vacuole; Y, yolk granule

1-a, 1-b, Germ cells in young oocyte stage

2-a, 2-b, Germ cells in early yolk-formation stage

3-a, 3-b, Germ cells in later yolk-formation stage
the ovary which had the eggs of less than about 300 μm in MED and less than 1.5 in GSI. The oocytes had prominent large nucleus which was stained with hematoxylin (Plate 1-1). The nucleus was surrounded by thin hematoxylinphlic cytoplasm of which layer gradually thickened and was followed by change of stainability into eosinphlic. The oocytes ranged 1.5 to 4.5 in GSI and 250 to 550 μm in MED were cagelized in the early yolk formation stage which was characterized by appearance of vacuoles in eosin-positive cytoplasm (Plate 1-2). The incorporation of yolk grabules into the oocyte started in the stage.

The oocytes of the late yolk-formation stage were more than 4.5 in GSI and 550 μm in MED. The cytoplasm filled with the eosinphlic granules (Plate 1-3). The nucleus was no more visible in the oocytes. However, the oocytes having hematoxylinphlic cytoplasm which were characterized by the young oocyte stage were found even in the late yolk-formation stage.

Biochemical Analyses

The hemolymph obtained from various ovarian developing stages were supplied to the polyacrylamide gradient gel electrophoresis (Fig. 1). Coomasie brilliant blue staining visualized totally 29 proteins in the hemolymph. The pattern was not different between both sexes in nonbreeding season. However, with the progress of maturation, the pattern of female hemolymph began to differentiate from that of male. The lipoprotein components with low mobility (>600,000 daltons) were fluctuated

Fig. 1. Schematic electrophoregram of hemolymph on polyacrylamide gradient gel at various ovarian developmental stages in *Palaeon paucidentis*

1. Authentic protein markers
   Human serum albumin (MW 60,000), Lactate dehydrogenase (MW 100,000), Catalase (MW 175,000), Ferritin (MW 300,000), Thyroglobulin (MW 550,000).
2. Young oocyte stage
3. Early yolk-formation stage
4. Late yolk-formation stage
5. Male hemolymph

Protein and lipoprotein were respectively visualized by Coomasie blue and Sudan black B staining.

Fig. 2. Relationship between maximum egg diameter of ovary and appearance of vitellogenin in *Palaeon paucidentis* hemolymph

— negative immunoreactivity
+ positive immunoreactivity
in proportion and mobility. The lipid staining visualized 3–5 components, and two of them (200,000–250,000 daltons) were constantly observed in every developing stages, as well as in the male hemolymph.

In the female hemolymph at the young oocyte stage, the Sudan black B staining revealed 5 bands, in which 3 bands were also found in the male hemolymph. Two lipoproteins with low mobility found at the early yolk-formation stage disappeared with the progress of yolk formation. At the late yolk-formation stage, a broad lipoprotein band of which molecular range was 650,000–1,000,000 (average 860,000) daltons specifically appeared in the female hemolymph. The female-specific component was immuno-reactive against anti-yolk antiserum.

In addition, the immunoelectrophoresis of the hemolymph yielded one main precipitin line with the mature yolk protein. The reaction reveals immunological identity between hemolymph protein and vitellin.

Immunocross reactivity of yolk protein with hemolymph protein was determined. Fig. 2 shows the relation between egg size and immunocross reactivity of the hemolymph to anti-yolk antiserum on the Ouchterlony agarose plate. Whereas a substantial precipitin line and slight lines were found in the female hemolymphs at the early and the late yolk-formation stages, the reaction was negative in the hemolymph at the young oocyte stage or male hemolymph. The hemolymphs at the early yolk-formation stage and the late yolk-formation stage included the immunologically same component, but different in electrophoretic properties.

The electrophoretic diagram of ovary components was fairly variable throughout vitellogenesis (Fig. 3). Low molecular components located in the gel front might be hemo-cyanins, because they disappeared after hatching. Lipoprotein was not found in the ovary at the young yolk-formation stage. A lipoprotein appeared in the ovary of the early yolk-formation stage was between 400,000 and 550,000 (average 480,000) daltons, and was electrophoretically different from the vitellin of the mature egg. As developed vitellogenesis, the lipoprotein quantitatively increased and the mobility gradually decreased. At the late yolk-formation stage, a broad band of which molecular weight ranged 560,000–1,000,000 (average 800,000) daltons was found to an appreciable amount in the ovary.
DISCUSSION

The morphological classification of the ovarian development seemed to include differences in phase of vitellin accumulation in the oocytes. In the young oocyte stage, no component positive to lipid staining was found on the electrophoretic diagram. The ovary at the early yolk-formation stage included a component immunologically identical with that at the late yolk-formation stage, but they were different each other in electrophoretic properties. The appearance of yolk globule in egg might mean the beginning of vitellin accumulation. The molecular weights of vitellin and vitellogenin estimated by electrophoresis were 800,000 and 860,000 daltons, respectively. However, these values were higher than those of other species reported previously (DURLIAT, 1984; MEUSY, 1980; WALLACE et al., 1967). The differences in molecular weight might be due to the phenomenon that dissociation and aggregation of crustacean proteins are pH-dependent (NAKAGAWA, unpublished data).

In the oocytes with more than 350 μm in MED, the Ouchterlony precipitin reaction and immunoelectrophoresis revealed that the mature ovary and the female hemolymph included common subunits. There has long been discussion for the precursors and the site of synthesis of the vitellin in many crustacean species (CHARNAUX-COTTON, 1985; MEUSY and CHARNIAUX-COTTON, 1984). Two types of the vitellin accumulation have been presented controversially; intraovocytic synthesis of yolk and incorporation of vitellogenin synthesized extracellularly by the micropinocytosis. Vitellogenin of Parapenaeus longirostris found in a subepidermal adipose tissue and in the hemolymph was accumulated at later vitellogenic stage (Tom et al., 1987). In many crustaceans, the vitellogenin was identical with vitellin in electrophoretic and immunological properties (DURLIAT, 1984; KERR, 1969; MEUSY et al., 1983; SUZUKI, 1987; Tom et al., 1987; WOLIN et al., 1973). They have been discussed whether hemolymph proteins enter into the oocytes with or without structural changes. The present study provided that the vitellogenin was released into the hemolymph at the early yolk-formation stage and was incorporated by the ovary through micropinocytotic uptake with partial structural changes.

Release of vitellogenin into the hemolymph seemed to accompany by histological change in the ovary. The histological observation indicated that vitellin deposition and enlargement of ovary were unlikely continuous. Furthermore, growth of GSI and MED did not parallel (NAKAGAWA et al., 1982). Three reposing periods were observed throughout ovarian development, where enlargement of egg diameter intermittently ceased, assuming that GSI increased constantly. The phenomena were difficult to interpret, but would be derived from the participation of molt cycle controlled under certain hormonal regulation in vitellin synthesis, as reviewed by MEUSY (1980). In Palaemon paucidentis, KAMIGUCHI (1971) suggested some antagonistic interaction between molting and ovarian growth.

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REFERENCES


スジエビの卵黄蓄積に関する研究

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スジエビの卵黄形成過程を成長期、卵黄形成前期、卵黄形成後期に分類し、ポリアクリルアミド濃度勾配ゲル電気泳動法、免疫学的方法で卵黄物質の蓄積機構を調べた。

1）成長期の卵黄は卵黄指数（GSI）約1.5以下、最大卵径（MED）300 μm 前後以下で、卵母細胞はヘマトキシリンで染色される核と細胞質を有する。この段階の卵の血リンバ中には免疫学的に卵黄ビテリンと同一の成分は検出されなかった。

2）卵黄形成前期の卵黄は GSI 1.5 から 4.5、MED 250 から 550 μm に相当し、卵細胞内に核を有し、細胞質は増大してオオシリン好染性に変し、卵黄顆粒が出現し始めた。MED 350 μm 以上の卵細胞を持つ親の血リンバには免疫学的にビテリンと同一のリポタンパク質が出現した。

3）GSI 4.5 以上、MED 550 μm 以上の卵細胞は卵黄形成後期に相当し、細胞内には核は消失してオオシリン好染性の卵黄顆粒が充満した。免疫学的に卵黄ビテリン（分子量800,000）と同一の成分（分子量860,000）ビテロジェニンが血リンバ中に活発に放出された。

4）GSI 5、MED 500 μm は組織学的、電気泳動的挙動からみて卵黄形成前期から後期への移行時期と考えられ、この段階以降、卵黄顆粒の蓄積が旺盛となった。