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

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Abstract   The vitellin accumulation was studies 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 extraovarially 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 hormon (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 withdrown 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 $-20°C$ 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-Na$_2$EDTA 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 10°C. Protein bands were visualized by the staining with Coomasie 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 $-20°C$ 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
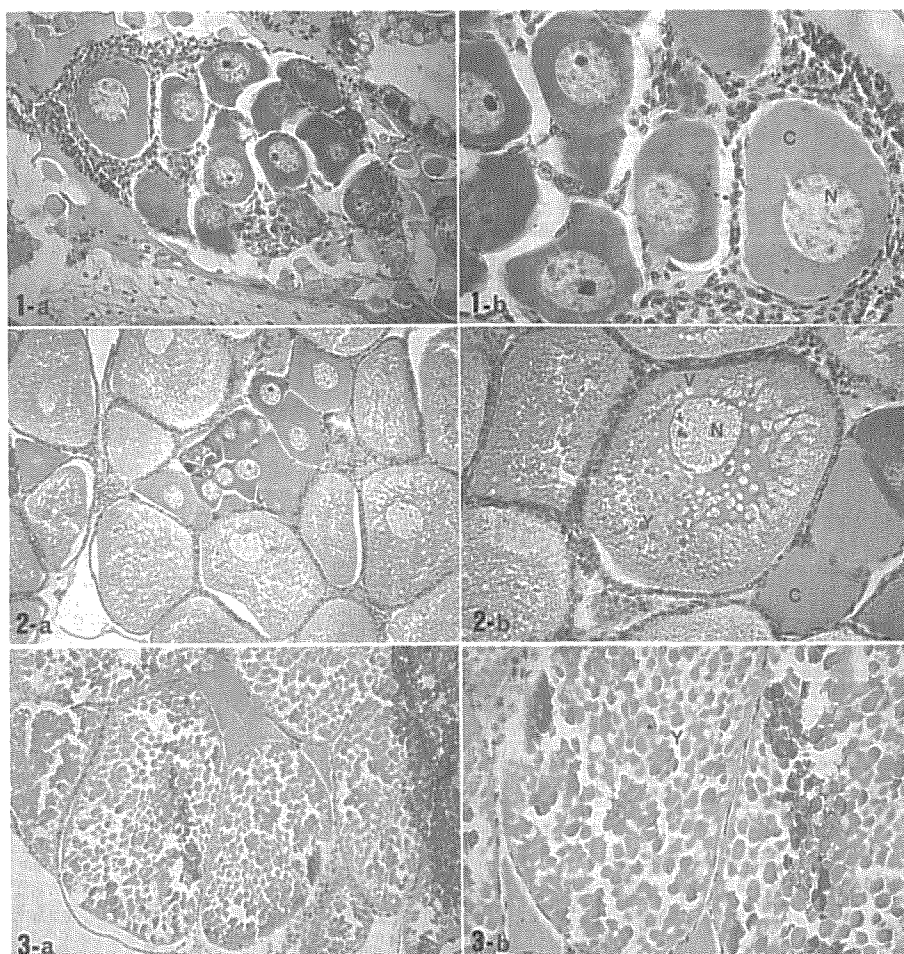

Plate 1. Photomicrographs of sections through ovary in various developmental stages in
Palaemon paucidens
Magnification of all figures 100 (a), 200 (b) times.
Abbreviations
C, cytoplasm; M, mid-gut gland; N, nucleus; V, vacuole; Y, yolk glanule
1-a, 1-b, Germ cells in young oocyte stage
2-a, 2-b, Germ 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 prominant large nucleus which was stained with hematoxylin (Plate 1-1). The nucleus was surrounded by thin hematoxylinpholic cytoplasm of which layer gradually thickened and was followed by change of stainability into eosinpholic. The oocytes ranged 1.5 to 4.5 in GSI and 250 to 550 μm in MED were categolized 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 eosinpholic granules (Plate 1-3). The nucleus was no more visible in the oocytes. However, the oocytes having hematoxylinpholic 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
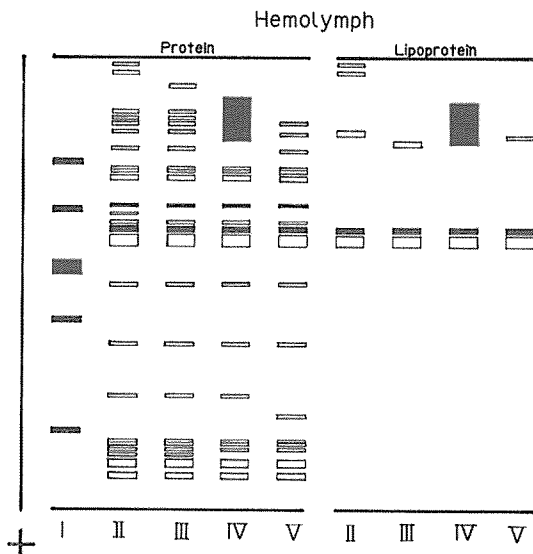

Fig. 1. Schematic electrophoregram of hemolymph on polyacrylamide gradient gel at various ovarian developmental stages in *Palaemon paucidens*
I . Authentic protein markers
Human serum albumin (MW 60,000), Lactate dehydrogenase (MW 100,000), Catalase (MW 175,000), Ferritin (MW 300,000), Thyroglobulin (MW 550,000).
II . Young oocyte stage
III. Early yolk-formation stage
IV. Late yolk-formation stage
V . Male hemolymph
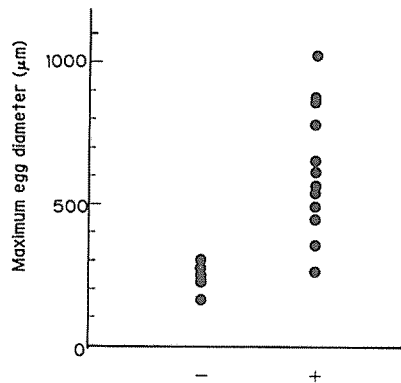Protein and lipoprotein were respectively visualized by Coomasie blue and Sudan black B staining.

Fig. 2. Relationship between maximum egg diameter of ovary and appearance of vitellogenin in *Palaemon paucidens* hemolymph
— negative immunoreactivity
+ positive immunoreactivity
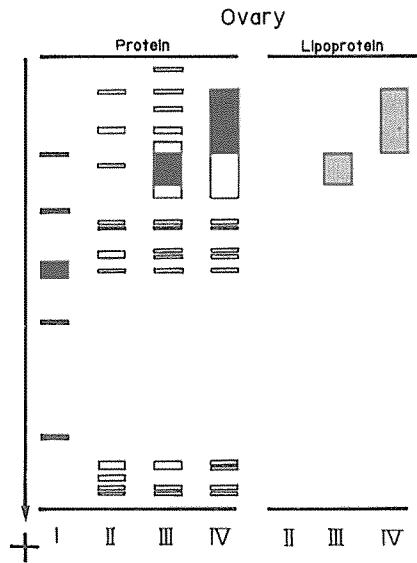
Ovary

Protein   Lipoprotein

Fig. 3. Schematic electrophoregram of ovary extract on polyacrylamide gradient gel at various ovarian developmental stages in *Palaemon paucidens*
  I. Authentic protein markers
  II. Young oocyte stage
  III. Early yolk-formation stage
  IV. Late yolk-formation stage
Staining method was the same as Fig. 1.

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 hemocyanins, 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 clasification 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 begining 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 (Charniaux-Cotton, 1985; Meusy and Charniaux-Cotton, 1984). Two types of the vitellin accumulation have been presented controversically; intraoocytic synthesis of yolk and incorporation of vitellogenin synthesized extraoocytically 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 paucidens,* Kamiguchi (1971) suggested some antagonistic inteaction between molting and ovarian growth.

ACKNOWLEDGEMENTS

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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）成長期の卵黄は卵巣指数 (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。この段階の親の血リンパ中には免疫学的に卵巣ビテリンと同一の成分は検出されなかった。

　2）卵黄形成前期の卵巣は 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, 細胞質は増大してエオシン好染性に変じ, 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パ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エオシ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パ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。