Immunocytochemical Identification of the Site of Vitellogenin Synthesis in the Freshwater Prawn *Macrobrachium nipponense*

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The site of vitellogenin synthesis in the freshwater prawn *Macrobrachium nipponense* was examined immunocytochemically using antibodies raised against lipovitellin purified from mature *M. nipponense* ovaries. The molecular weight of purified lipovitellin was estimated to be 350 kDa by gel filtration. The antiserum cross-reacted with vitellogenin in the hemolymph of the mature female prawn, and could therefore be used to demonstrate the possible site of vitellogenin synthesis immunocytochemically employing the peroxidase-anti-peroxidase (PAP) reaction. Hepatopancreas, sub-epidermal tissue, muscle, and ovary were fixed with periodate-lysine paraformaldehyde solution after which peraffin sections were made. In mature females, oocytes at the yolk globule stage, the hepatopancreas and the sub-epidermal tissue of the lateral somites showed positive PAP reactions. However, the sub-epidermal tissue of the ventral somites, follicular cells of the ovary and muscles did not. No positive reaction was observed in males and immature females. These results suggest that vitellogenin is synthesized in the hepatopancreas and at the sub-epidermal tissue in females at the exogenous vitellogenesis stage.

**Key words:** prawn, vitellogenin synthesis, lipovitellin, hepatopancreas, immunocytochemistry, *Macrobrachium nipponense*; such techniques cannot fully elucidate the site of vitellogenin synthesis but can at least confirm the existence of vitellogenin and in which tissues it is significantly present. We used antiserum raised against purified lipovitellin from mature ovaries for the following immunocytochemical study.

### Materials and Methods

**Prawns**

*M. nipponense* were obtained in May from Lake Kasumigaura in Ibaraki Prefecture, Japan. Prawns were transported to the laboratory and stocked in outdoor culture tanks. Mature ovaries were taken and used for purification of lipovitellin. Hemolymph was collected from mature females and males with micro-hematocrit tubes after cutting the terminal somites of the abdomen. Hemolymph samples were stored at −70°C until analysis.

**Purification of Lipovitellin**

Mature ovaries were removed from the female prawns and rinsed with cold 0.15 M NaCl. The ovaries (1.5 g) were homogenized in 10 ml of 0.02 M Tris-HCl buffer (pH 8.7) containing 0.15 M NaCl with a glass-teflon homogenizer, and centrifuged at 10,000 × g for 30 min at 4°C. The supernatant (5 ml) was applied to a Toyopearl HW60S (TOSOH Co.) column (2.5 cm × 75 cm), and eluted with the same buffer at a flow rate of 20 ml/h. Absorbance of each fraction (5 ml/tube) was monitored at 260, 280, and 460 nm. The protein content of each fraction was measured with a Bio-Rad protein assay kit using bovine serum albumin as the standard. The main protein fractions were pooled, and dialyzed against 0.02 M Tris-HCl buffer (pH 8.7) at 4°C. The dialyzed protein fraction was then applied to a DEAE-Toyopearl 650S column (2.5 cm × 10 cm) previously equilibrated with the same buffer. The applied proteins were first eluted with the buffer, and then eluted with a linear gradient of NaCl (0.01 M NaCl/h) dissolved in the buffer. The lipovitellin peak fractions were pooled and dialyzed against the starting buffer. The fractions were rechromatographed and purified lipovitellin was obtained.

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Fig. 1. Gel filtration chromatography and elution pattern of crude ovarian extract using a Toyopearl HW60S column.

At each purification step, the fractions containing lipovitellin were identified by Ouchterlony's immunodiffusion test and immunoelectrophoresis using antibodies against maturing female and male hemolymphs.

The molecular weight of purified lipovitellin was determined by gel filtration on a Toyopearl HW60S column using thyroglobulin (MW 669 kDa), ferritin (MW 440 kDa), catalase (MW 232 kDa), and aldolase (MW 158 kDa) as standard molecules.

Polyacrylamide Gel Electrophoresis
Polyacrylamide gel electrophoresis (PAGE) was performed on a 7.5% gel according to the method of Davis. The proteins were stained with 0.25% Coomassie brilliant blue R-250 in 45% ethanol and 45% acetic acid.

Preparation of Antibody and Immunological Testing
Antiserum against purified lipovitellin and ovarian extract were prepared by the modified method of Newbould. The emulsified antigen with Freund's complete adjuvant was injected into lymph nodes of rabbit hind limbs. After the first inguinal injection, antigen was subcutaneously injected into the backs of the rabbits four times once a week. The rabbits were bledd from the carotid artery one week after the last injection. Antisera were frozen at -70°C.

Histology and Immunocytochemistry
The gonads, hepatopancreas, sub-epidermal tissue and muscle of both sexes were fixed with periodate-lysine-paraformaldehyde (PLP) solution for six hours and then transferred to 70% ethanol. After dehydration, these samples were embedded in paraffin, and sectioned at 3-5 μm thickness. Sections were stained with Carazzi's hematoxylin-eosin and PAS (periodic acid-Schiff) for histological observations. For immunocytochemistry, the sections were stained using the peroxidase anti-peroxidase (PAP) method of Sternberger et al. modified by Kaneko et al. using rabbit antiserum against lipovitellin. To verify the specificity of the immunocytochemical reaction, normal rabbit serum and antiserum absorbed with purified lipovitellin were used as controls.

Results

Purification of Lipovitellin
The elution pattern for gel filtration of ovarian extract is shown in Fig. 1. Five elution peaks were observed, and the second peak of yellow-brownish color contained the most protein. This peak was analyzed by normal PAGE. Figure 2A shows the normal PAGE patterns of the second peak: one main band and one sub-band were observed (arrows). In order to purify the main band of yolk protein, the second peak was pooled, and applied to ion-exchange chromatography. Two main peaks were obtained (Fig. 3), and the first main peak was pooled and rechromatographed. The single peak thus obtained was collected as purified lipovitellin. This gave a single band on normal PAGE as shown in Fig. 2B. The molecular weight of the lipovitellin was estimated to be 350 kDa by gel filtration.
Site of Vitellogenin Synthesis in Prawn

**Immunological Analysis**

Figure 4 shows the results of Ouchterlony's immunodiffusion test. The antiserum raised against crude ovarian extract formed two precipitin bands in response to mature female hemolymph, and formed a single precipitin band against the purified lipovitellin, while no precipitin band was formed between the antiserum and male hemolymph samples. The precipitin band formed by the purified lipovitellin fused with the main precipitin band formed by mature female hemolymph (Fig. 4A). The antiserum raised against purified lipovitellin formed a single fused precipitin band against mature female hemolymph and lipovitellin, but did not react with male hemolymph (Fig. 4B).

**Identification of the Putative Site of Vitellogenin Synthesis**

Figure 5A and 5B show the results of staining with PAP reaction and hematoxylin-eosin, respectively, using adjacent sections of the ovary containing oocytes during several developmental stages. The oocytes having yolk globules showed distinct positive reactions against anti-lipovitellin serum, whereas the oocytes which had not accumulated yolk globules did not show any positive reaction. Figure 6 shows the results of immunocytochemical reaction in the follicular cells of ovary, hepatopancreas, sub-epidermal tissue and...
The hepatopancreas in mature females showed a positive reaction (Fig. 6A), whereas those of mature males and immature females did not show any positive reaction (Fig. 6B). The follicular cells of vitellogenic and non-vitellogenic oocytes (Fig. 6C) and muscles (Fig. 6D) did not show any positive reaction. The sub-epidermal tissue of lateral somite showed a positive reaction (Fig. 6E), whereas those of dorsal somite did not show any positive response (Fig. 6F).

**Discussion**

From the results of the above immunocytochemical analysis, the hepatopancreas and the sub-epidermal tissue of the lateral somites were considered to be the likely sites of vitellogenin synthesis in the freshwater prawn *M. nipponense*. Since the sub-epidermal tissue does not harbor a significant volume, the main source of vitellogenin in this species may be the hepatopancreas. Vitellogenin synthesis in the hepatopancreas had already been demonstrated in...
Penaeus vannamei, although the type of cells concerning vitellogenesis was not determined in the study, since whole tissue was used for in vitro tracer analysis.

The hepatopancreas is composed of numerous anastomosing fragile tubules that are lined with a single layer of epithelial cells. The cells can be classified into three cell types by electron microscopy: R-cells, B-cells and F-cells. Although it is difficult to distinguish the three cell types on immunocytochemical preparations, R-cells are considered to be vitellogenin synthesizing cells. This is demonstrated by the observations that R-cells are the only cells which show active lipoprotein synthesis in the hepatopancreas of mature females; the cells possess small vacuoles of lipid and numerous electron-dense material within the rough endoplasmic reticulum (in preparation).

In order to obtain satisfactory results in immunocytochemical studies, the quality of the antibody is important; the antibody must have high affinity and high specificity against the antigen. In our experiments, we raised antibodies against lipovitellin from mature ovaries instead of vitellogenin in female hemolymph. This is because contamination by other hemolymph proteins may result in unwanted cross-reactions on application to tissue preparations. Prawns contain many proteins other than vitellogenin in the hemolymph and these proteins are distributed in all the tissues of the body. Therefore, contamination of the antigen by other hemolymph proteins must be minimized. From this objective, purification of lipovitellin from the ovary is much easier and less risky, since the tissue contains extremely large amounts of lipovitellin.

The purified lipovitellin of M. nipponense, used for immunization, has a molecular weight of 350 kDa. This value is nearly equivalent to that of M. rosenbergii, 330 kDa. Similar values have also been reported in other crustaceans: 336 kDa in Homarus americanus, 370 kDa in Pagurus pollicaris, 340 kDa in Uca pugilator, 350 kDa in Sesarma reticulatum, 370 kDa in Libinia emarginata, and 330 kDa in Cancer irroratus. However, values are much higher in some decapods: 540 kDa in Panaeus monodon, 560 kDa in Pandalus kessleri, 500 kDa in Plesionika edwariski, and Procambarus sp. This large difference in lipovitellin molecular weight between the two groups of species may be related to subunit composition.

The antisera raised against this purified M. nipponense lipovitellin was confirmed to be effective for vitellogenin studies as the antibody showed a similar affinity against vitellogenin. The antisera formed only one precipitin line against female hemolymph, which fused with the precipitin line against lipovitellin, while no precipitin line formed with the antiserum raised against lipovitellin was confirmed to be effective for vitellogenin synthesis, this does not necessarily implicate the ovary as the sole origin of vitellen; the existence of vitellogenin in the hemolymph is difficult to explain, if all processes of vitellin synthesis are completed in the ovary. We postulate rather, that the hemolymph vitellogenin, which is the precursor of vitellen, is synthesized exogenously in other organs or tissues. The result of an in vitro tracer experiment in the Isopod Armadillidium vulgare, is suggestive of such, as vitellogenin synthesis was confirmed in the fat body as well as in the ovary. The hepatopancreas is also one of the possible candidate organs for this function, although many studies have not employed the tracer techniques with respect to this organ. This may be because the tissue contains active proteolytic enzyme which degenerate vitellogenin making such techniques difficult. P. vannamei is one decapod species in which vitellogenin synthesis by hepatopancreas has been confirmed by the in vitro tracer techniques. The ovary may participate in the process of vitellin synthesis in many crustaceans.

Immunocytochemical techniques allow clear visualization of the distribution of vitellogenin in various tissues. Vitellogenin has been recognized in the lateral sub-epidermal adipose tissue of the prawns Palaemon serratus, and Parapenaeus longirostris. The adipose tissue of P. serratus showed a positive immunofluorescence reaction, while other organs or tissues showed negative reactions, except for the ovary and hemolymph, which must contain lipovitellin or vitellogenin. Thus, the adipose tissue is one prominent candidate as vitellogenin synthetic organ in Decapoda. Observations in three species of isopods, an amphipod, and in an anostracan support this hypothesis. On the other hand, the root was shown to be the reserve-inclusion cells within connective tissue in the hepatopancreas of crabs. Considering the results of our study, crustaceans may be able to synthesize vitellogenin in multiple organs.

The results reported in this paper indicate that the hepatopancreas and a part of the sub-epidermal tissue are the sites of vitellogenin synthesis with the hepatopancreas appearing to be the main site. However, we can not yet conclude that all of the tissues which showed positive immunoreactions in Crustacea are actually involved in the production of vitellogenin, because immunocytochemical reaction indicates only the existence of vitellogenin in cells. In some crustacean species, vitellogenin synthesis can be induced in male via the loss of androgenic gland functions. The experimental use of males may provide useful information regarding the control of vitellogenin synthesis, as the effects of the female ovary can be eliminated. In addition, direct evidence, such as the occurrence of vitellogenin gene expression, is necessary for final identification.

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