Biochemical Characterization and Immunohistochemical Identification of Vitellin in the Ovary of Kuruma Prawn (Marsupenaeus japonicus)

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Abstract: Vitellin (Vn) was purified from the mature ovaries of the kuruma prawn (Marsupenaeus japonicus) with gel filtration, polyacrylamide gel electrophoresis (PAGE), and electro-elution. Native- and SDS-PAGE revealed that the purified Vn was a lipo-glyco-caroteno-protein of 530 kDa consisting of four subunits (70, 83, 105, and 190 kDa). Gly, Ala, and Glu were the dominant amino acids of Vn. The antiserum against the purified Vn, which was raised in a rabbit, immunoreacted with vitellogenin (VTG) in the hemolymph and Vn in the ovary by immunodiffusion and Western blotting. Positive immunoreaction with the antiserum was detected in the cytoplasm of oocytes at the late perinucleolus stage. However follicle cells surrounding the oocytes were not immunostained at any developmental stage. No immunoreaction was detected in the cortical rods of mature oocytes. Immunohistochemical observation revealed that the accumulation of Vn in the cytoplasm of the oocytes began at the early vitellogenic stage. These results indicate that the level of in situ cellular VTG in follicle cells is below the limit of immunodetection.

Key words: Kuruma prawn; Vitellin; Follicle cells; Immunohistochemistry

Vitellin (Vn), a lipo-glyco-caroteno-protein, is the major yolk protein in eggs of decapod crustaceans with high molecular weights ranging from 300 to 500 kDa (Meusy et al. 1987; Chang et al. 1993ab). This molecule has 2-11 polypeptide subunits in various penaeid species (Tom et al. 1987; Longyant et al. 1999). Vn accumulates in oocytes during vitellogenesis, and its precursor is vitellogenin (VTG), a female-specific protein found in the hemolymph of crustaceans.

Kuruma prawns are important commercial species in aquaculture. Vn functions as the nutritive material necessary for development of embryos and early larvae (Harrison 1990). Hemolymph VTG and ovarian Vn have been demonstrated to be immunologically identical (Vazquez-Boucard and Ceccaldi 1986; Chang et al. 1994). VTG synthesis in decapod crustaceans was reported to occur in the ovary (Yano and Chinzei 1987; Rankin et al. 1989; Khayat et al. 1994) or the hepatopancreas (Quackenbush 1989; Han et al. 1994; Rani et al. 1997). Recently, Tsutsui et al. (2000) and Avarre et al. (2003) showed the expression of VTG-mRNA in the ovary and hepatopancreas in penaeid shrimps. Tsutsui et al. (2000) detected VTG-mRNA in follicle cells surrounding vitellogenic oocytes in the ovary and parenchyma cells in the hepatopancreas. The temporal sequence between VTG synthesis in follicle cells and Vn accumulation in oocytes is not well understood.

This study was undertaken to examine Vn accumulation processes in oocytes of kuruma prawns. Antiserum was obtained against Vn purified from matured ovaries, and the accumulation of Vn into oocytes was immunohistochemically examined at various stages of ovarian maturation.

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Materials and Methods

Animals

Mature female prawns (Marsupenaeus japonicus) were obtained from culture ponds at the Momoshima Station of the National Center for Stock Enhancement in Hiroshima Prefecture, Japan, and their ovaries were sampled on site.

Dissected ovaries were washed in phosphate buffered saline (PBS: 8.1 mM Na₂HPO₄, 137 mM NaCl, 2.68 mM KCl, 1.47 mM KH₂PO₄, pH 7.2), immediately frozen by dry ice-acetone, and stored at −85°C until analysis. They were homogenized in 0.05 M Tris-HCl (pH 8.5) containing 0.25 M NaCl and 5 mM phenylmethylsulfonyl fluoride using a tissue grinder on ice. After centrifugation at 10,000 × g for 60 min at 4°C, the middle layer was collected and used for the ovarian extract.

Chromatography

The ovarian extract was dialyzed with an elution buffer (0.02 M Tris-HCl, 0.15 M NaCl, pH 8.3) using a molecular porous dialysis membrane (Spectra/Por cellulose ester; MWCO: 100,000, Spectrum Co.). This extract was subjected to gel filtration on a Sepharose CL-6B column (50 × 2.5 cm i.d., Pharmacia) equilibrated with an elution buffer. Elution was performed at a flow rate of 0.2 ml/min and collected in 3 ml fractions, and the absorbance of each fraction was measured at 280 nm and 474 nm. This chromatographic procedure was performed in a cold room.

Electrophoresis

The purity of Vn was determined by Native polyacrylamide gel electrophoresis (PAGE) in 3–10% gradient sodium dodecyl sulfate (SDS)-PAGE (Lambin et al. 1979). Samples were mixed with an equal volume of sample buffer (0.001% bromophenol blue, 2% SDS, 10% glycerol, 0.0625 M Tris, 5% 2-mercaptoethanol, pH 6.8) and boiled for 10 min at 100°C. Known molecular weight markers were obtained from Amersham Pharmacia Biotech (HMW-SDS marker kit); they included myosin (212 kDa), a2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), and glutamate dehydrogenase (53 kDa).

After electrophoresis, gels were stained with 0.1% Coomassie brilliant blue (CBB) G-250 and silver stain for proteins, and also with periodic acid-Schiff’s reagent and Sudan black B for carbohydrate and lipid moieties, respectively.

Protein elution

Collected fractions containing Vn were subjected to 3–10% Native-PAGE. After electrophoresis, both sides of the gel were cut and stained with CBB as described above. Then the Rf value of the Vn band was calculated. The Vn band estimated by the Rf value was cut off from unstained gel and eluted with an electrophoretic concentrator (Atto) with Tris-glycine buffer, pH 9.2. The elution was carried out under 100 V for 60 min.

Preparation of antiserum and immunodiffusion

A mixture of 0.5 ml of purified Vn dissolved in PBS and an equal volume of Freund’s complete adjuvant was injected subcutaneously into the hind limbs of a rabbit, four times at 14–21 day intervals. Blood was collected from the ear vein two weeks after each booster. Each titer of collected blood was checked by enzyme-linked immunosorbent assay. Immunodiffusion was carried out on a 1% agar gel plate according to the methods of Quinitio et al. (1989, 1990). The antiserum and samples were introduced into different wells at a distance of 3 mm and placed in a humid chamber for immunoreaction over 12 hr at 4°C.

Western blotting

Western blotting was performed for proteins
separated by Native- and SDS-PAGE according to Towbin et al. (1979). Gels were equilibrated in blotting buffer (0.025 M Tris base, 0.192 M glycine, 20% methanol), and separated proteins were electroblotted to a polyvinylidene difluoride membrane (PVDF, Millipore). Electro blotting was performed for 30 min at 100 V under cooling conditions. Then a part of membranes was stained with amido black for proteins. The residual membranes were blocked with 10% skim milk-PBS containing 0.05% Tween 80 at 4°C overnight and then incubated with rabbit antiserum raised against Vn (1:15,000) for 90 min at 4°C. After two washes with 10% skim milk-PBS and one with PBS (10 min each), the membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:1,000, Zymed) for 60 min at 4°C. After washing as mentioned above, Vn was visualized by adding substrate solution (NBT/X-phosphate acid).

**Immunohistochemistry**

Ovarian tissues were fixed in Bouin’s solution, dehydrated through an alcohol series, and embedded in paraffin. Oocyte development was classified into five stages according to the method of Yano (1988). The cytoplasm of oocytes was strongly basophilic at the early perinucleolus stage. In the late perinucleolus stage (oocyte diameter: 65–80 μm), a single layer of follicle cells appeared around the early perinucleolus oocytes. Then, a number of strongly basophilic vesicles arose in the cytoplasm at the yolkless stage (oocyte diameter: 105–150 μm). Yolk granules became for the first time visible at the yolk granule stage (oocyte diameter: 110–224 μm). Finally, cortical rods were found in the periphery of oocytes at the maturation stage (oocyte diameter: 212–272 μm). Follicle cells surrounding oocytes appeared in the ovary from the late perinucleolus stage to the maturation stage. They were rectangular or cuboidal in shape and basophilic.

Immunohistochemistry was performed by the indirect immunostaining method. Paraffin sections (5 μm) were deparaffinized, dehydrated, rinsed in distilled water, and blocked with bovine serum albumin. After removing the excess solution, sections were incubated with the rabbit antiserum against Vn (1:750). After washing, they were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG. All reactions were performed for 10 min at room temperature. After washing, the substrate solution (NBT/X phosphate acid) was added. All sections were counterstained with hematoxylin. Some adjacent sections were treated with anti-Vn antiserum pre-incubated with purified Vn or normal rabbit serum used for a control.

**Amino acid composition**

Purified Vn was hydrolyzed with 6 N HCl for 8 h at 130°C and analyzed for amino acid composition on an automatic amino acid analyzer (Waters).

**Results**

**Purification of Vn**

Two major peaks were obtained from gel filtration on a Sepharose CL-6B column (Fig. 1). Only the second peak showed a distinct orange color, suggesting the presence of Vn, which contains many carotenoids. Native-PAGE showed that the second peak contained a large amount of protein at 530 kDa and two
minor protein bands at about 270 kDa (Fig. 2). The band at 530 kDa was collected for further purification. Purification was performed with electro-elution from Native-PAGE. The molecular weight of native Vn was determined to be approximately 530 kDa by further Native-PAGE (Fig. 3). These results indicate that pure Vn could be isolated from the ovarian extract in the kuruma prawn.

Immunological analysis

Results of Ouchterlony’s immunodiffusion test are shown in Fig. 4. The antiserum raised against purified Vn formed a single fused precipitin line against mature female hemolymph, purified Vn, and the crude extract of the mature ovary, but did not react with male hemolymph, immature female hemolymph, or crude extract from immature ovaries.

Specificity for this anti-Vn antiserum was further investigated by Western blotting. A strong immunoreaction was observed only in the 530 kDa protein, though the ovarian extract included other minor protein bands (Fig. 5). These results indicate that the antiserum against Vn immunoreacted with Vn and VTG specifically.

Characterization of Vn

Vn was separated by SDS-PAGE into four polypeptides, having molecular mass of 70, 83, 105, and 190 kDa (Fig. 6A). Western blotting indicated that these subunit bands strongly reacted with the antiserum against Vn. The antiserum also recognized a weak band of about 130 kDa, which did not appear by Amido black stain, in the ovarian extract and the purified Vn.

Fig. 2. 3–10% linear gradient Native-PAGE of the ovarian extract separated on a Sepharose CL-6B column. Lane 1: molecular weight markers; 2: crude ovarian extract; 3: fraction 23 (the first peak); 4: fraction 40 (the second peak). CBB stain.

Fig. 3. Native PAGE of purified Vn by electro-elution. Silver stain.

Fig. 4. Immunoprecipitin reaction of purified Vn (Vn), the crude ovarian extract of mature (MFO) and immature ovaries (IFO), mature female hemolymph (MFH), immature female hemolymph (IFH), and mature male hemolymph (MMH). Anti-Vn antiserum (A-Vn) was used.
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(Fig. 6B). No immunoreaction was detected on the control membrane incubated with normal rabbit serum (Fig. 6C).

**Amino acid analysis**

The amino acid composition of Vn is presented in Table 1. Gly (13.9%) and Ala (13.4%) are dominant, followed by Glu (10.7%), Asp (9.6%), and Ser (9.5%).

**Immunohistochemistry**

Figs. 7 and 8A show the results of immunohistochemistry at the previtellogenic ovary (the early and late perinucleolus stages). Positive immunoreaction (deep purple color) against the anti-Vn antiserum was observed in the cytoplasm of oocytes at the pre-vitellogenic ovary (Fig. 7A), while no immunoreaction was detected in the sections treated with the control antiserum (Fig. 7B).

**Table 1. Amino acid composition of *M. japonicus* vitellin**

<table>
<thead>
<tr>
<th>Amino acids</th>
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<tr>
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<tr>
<td>Glu</td>
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Fig. 5. Native-PAGE and Western blotting of the ovarian extract (lanes 1 and 2), purified Vn (500 ng/lane, lane 3), and mature female hemolymph (lane 4). Lane 1: Amido black stain; lanes 2 – 4: immunostain with anti-Vn antiserum.

Fig. 6. SDS-PAGE and Western blotting of purified Vn (left lane) and ovarian extract (right lane) in each membrane. A: Amido black stain; B: immunostain with anti-Vn antiserum; C: immunostain with normal rabbit serum.
Fig. 7. Immunohistochemical staining of the ovary at the early vitellogenic stage judged by HE stain. Sections were incubated with anti-Vn antiserum (A) and the antiserum absorbed with purified Vn (B). Bars=1 mm.

Fig. 8. Immunocytochemical staining of the ovary at the early vitellogenic (A and D), vitellogenic (B), and mature stages (C). Arrow and asterisk indicate the positive immunoreaction in the cluster of follicle cells and the blood sinus, respectively. BS: blood sinus; CR: cortical rods; EP: early perinucleolus oocytes; FC: follicle cells; LP: late perinucleolus oocytes; M: mature oocytes; YG: yolk granule oocytes. Bars=100 μm.
antiserum absorbed with purified Vn or normal rabbit serum (Fig. 7B). Immunoreaction was strongly detected around the nucleus of ooplasm of oocytes at the late perinucleolar stage, spreading to the periphery gradually (Fig. 8A). These results clearly indicate that the accumulation of Vn in the cytoplasm of oocytes began at the pre-vitellogenic stage. The cytoplasm of oocytes at the yolk granule and maturation stages showed distinct positive reactions against the antiserum (Fig. 8BC). However, follicle cells surrounding the oocytes were immunonegative at all developmental stages (Fig. 8). Clear immunoreaction was also observed in the cluster of expanded follicle cells (Fig. 8B), the blood sinus, and expanded follicle cells with large vesicles in their cytoplasm (Fig. 8D). However, cortical rods in mature oocytes were not immunostained (Fig. 8C).

**Discussion**

Gel filtration and electro-elution from PAGE were utilized to isolate and purify Vn from mature ovaries of kuruma prawns in this study. Two main peaks with an absorbance at 474 nm were obtained from gel filtration on Sepharose CL-6B column. The second peak (fraction 40) contained a protein band of 530 kDa, which was identical to the main band in the ovarian extract. We were able to purify this protein by electro-elution of Native-PAGE. The second peak contained two minor bands, as well.

The native form of Vn with an absorbance at 474 nm was positively stained with PAS and Sudan black (data not shown), suggesting that the Vn contained carotenoid, carbohydrate, and lipid. These results were similar to those of previous studies in *Penaeus monodon* (Quinitio et al. 1990) and *Parapenaeus longirostris* (Tom et al. 1987). The apparent molecular weight for the purified Vn of kuruma prawns was presently determined to be 530 kDa in coincidence with the results of Kawazoe et al. (2000), while Vazquez-Boucard and Ceccaldi (1986) estimated it at 510 kDa by ultracentrifugation and electrophoresis. SDS-PAGE revealed that the Vn appeared four subunits; 70, 83, 105, and 190 kDa. However, Vazquez-Boucard and Ceccaldi (1986) and Kawazoe et al. (2000) reported five polypeptides subunits (76, 86, 92, 105, and 150 kDa) and three subunits (91, 128, and 186 kDa) in kuruma prawns. Tom et al. (1992) reported that smaller peptides in the ovarian extract were increased with ovarian maturation in *Penaeus vanammei*. The difference in the size and number of subunits was considered to be due to the difference in ovarian maturity. Western blotting revealed that the purified Vn and the ovarian extract contained a 130 kDa protein which was weakly immunostained. Avarre et al. (2003) reported similar result in *Penaeus semisulcatus* and suggested that this weakly immunopositive band originated from the resorbing oocytes.

The antiserum used formed only a single precipitin line against purified Vn, mature female hemolymph, and mature ovarian extract, while no precipitin line was formed with immature female or male hemolymph, or immature ovary extract. Western blotting demonstrated that the anti-Vn antiserum recognized only the 530 kDa band in the ovarian extract and the mature female hemolymph. Immunoreaction with the antiserum was also detected in the cytoplasm of oocytes at the vitellogenic stages, but not in the cortical rods of mature oocytes.

Positive immunoreaction was first found around the nucleus of oocytes and then spread toward the periphery in late perinucleolar oocytes, which were surrounded by a layer of follicle cells. Several researchers (Yano 1988; Tan-Fermin and Pudadera 1989) observed by hematoxylin and eosin stain that yolk granules first accumulated in basophilic vesicles near the nucleus in the secondary vitellogenic oocytes and then spread to the oocyte periphery. However, our immunohistochemical results strongly support the description by Kawazoe et al. (2000), who suggested that the accumulation of Vn commenced as early as the primary vitellogenic stage in the form of small yolk granules, which may be difficult to be identified by light microscopy. These results indicate that the accumulation of Vn into the cytoplasm of oocytes begins at the late perinucleolar stage in
kuruma prawns.

Tsutsui et al. (2000) and Avarre et al. (2003) detected the expression of VTG-mRNA in the ovary and the hepatopancreas at the vitellogenic stage by Northern blotting and real time PCR, respectively. Tsutsui et al. (2000) detected VTG-mRNA expression in follicle cells surrounding vitellogenic oocytes and in parenchyma cells in the hepatopancreas by in situ hybridization. In the present study, however, positive immunoreaction against VTG was not observed in rectangular or cuboidal follicle cells, even though a strong positive reaction was clearly detected in the cytoplasm of developing and mature oocytes. Yano et al. (1996) and Kawazoe et al. (2000) also reported that follicle cells surrounding oocytes were not immunostained at the yolk granule stage in kuruma prawns. These results strongly support the hypothesis of Thurn and Hall (1999) that the synthesis and release of VTG in follicle cells occur at such a rapid rate that the level of in situ cellular VTG is below the limit of immunodetection.

In summary, the native form of Vn (530 kDa) was purified from the vitellogenic ovary of kuruma prawns with gel filtration and electroelution. The purified Vn was a caroteno-lipo-carbohydrate protein and appeared 70, 83, 105, and 190 kDa subunits. Antiserum against the purified Vn specifically reacted with VTG in the hemolymph and Vn in the ovary. Immunohistochemical observation indicated that the accumulation of Vn in the cytoplasm of oocytes begins at the late perinucleolus stage in kuruma prawns. No immunopositive reaction was detected in the follicle cells surrounding oocytes at any development stage.

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References


クルマエビにおける卵巣内ビテリンの生化学的特徴および免疫組織化学的観察

田原大輔・中野幹太・宮台俊明・矢野徹

ゲル濾過クロマトグラフィー、電気泳動およびゲル電気泳出法を用いて、クルマエビの卵巣からビテリン（Vn）を精製し、そのアミノ酸組成を決定した。精製したVnは、脂質・糖・カロテノイドを含み、分子量530kDaで4つのサブユニットバンド（70, 83, 105および190kDa）が観察された。更に、抗Vn血清を作製し特異性を免疫拡散およびウェスタンブロッティング法で検討した結果、この抗血清は体液中のビテロゲン（VTG）とVnを同等に認識した。卵巣の免疫組織化学的観察の結果、周辺仁後期以降の卵母細胞の細胞質に免疫反応は見られなかったが、何れの成熟段階の卵巣細胞にも反応は見られなかった。これらの結果は、卵巣細胞内のVTG濃度は免疫学的検出限界よりも低いことを示している。