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Vitellogenesis in the giant tiger prawn, *Penaeus monodon* Fabricius, 1789

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Vitellogenesis can be induced in the ovaries of the penaeid shrimp, *Penaeus monodon*, by eyestalk ablation; 80% of shrimp spawned within 7 days after ablation. Using immunofluorescence, it was observed that vitellin commences to accumulate in the yolk globular stage oocytes. The vitellin of atretic oocytes is reabsorbed and transferred to the newly matured oocytes. Four vitellin-like peptides are synthesized *in vitro* by the ovaries with mol. wt 220, 168, 130 and 74 kDa, respectively. Amongst them, the 168 and 74 kDa peptides are secreted into the culture medium. The results of immunostaining showed that the anti-Ep antisera is able to react with the ovary extracts of *Penaeus japonicus*. Each antiserum may react to the compatible peptide of the *P. monodon* vitellin peptide.

Key words: *Penaeus monodon* Fabricius; Vitellogenesis.

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Introduction

Vitellin (Vt), a major egg yolk protein, is synthesized on a large scale in the yolk production tissues which, in vertebrates are the liver and ovary and, in insects, the fat body and ovary. The primary translation products are precursor molecules, vitellogenins (Vg), which may be cleaved and modified to yield the mature yolk proteins in the course of secretion, transport and deposition in the developing oocyte. In several species of crustaceans, vitellogenin has been isolated and characterized; it has been described as a lipoglycol-carotenopoprotein (Tom *et al.*, 1987; Quinitio *et al.*, 1990; Vazquez-Boucard and Ceccaldi, 1986; Yano and Chinzei, 1987; Rankin *et al.*, 1989; Browdy *et al.*, 1990). In several species of decapod crustaceans, the ovary (OV) (Quackenbush, 1989; Yano and Chinzei, 1987; Browdy *et al.*, 1990; Rankin *et al.*, 1989), subepidermal adipose tissue (SAT) (Aiken and Waddy, 1980; Tom *et al.*, 1987) and hepatopancreas (HP) (Vogt *et al.*, 1989; Paulus and Laufer, 1987) have been implicated in contributing to synthesis of vitellin or its precursor molecule, vitellogenin.

Ovarian development in crustaceans may be promoted by eyestalk ablation in several species of penaeid shrimps. In *Penaeus monodon*, the isolated vitellin possesses a molecular weight of approximately 540 kDa and is composed of four major subunits of mol. wt 74, 83, 104 and 168 kDa (Quinitio *et al.*, 1990). Egg extracts are immunologically identical to hemolymph of maturation stage female shrimp (Chen and Chen, 1993). Previously, we have studied the synthesis of vitellin in ovarian culture *in vitro*. The present study showed that a non-secreted vitellogenin precursor (pre-Vg) is proteolytically cleaved within the ovary into two products with molecular weights of over 200 kDa and secreted into the culture medium.

Materials and Methods

Animals

Broodstock female *P. monodon* were obtained from Tungang, southern Taiwan. Shrimp were maintained in seawater 2.8% at 28 ± 1°C and fed squid and oysters twice daily. Unilateral eyestalk ablation induces ovarian development.

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Table 1. The number of nauplius produced from 25 *Penaeus monodon* females

	Days after ablation								
	3	4	5	6	7	8	9	10	
Spawning no. (Total No. 25)	3	5	6	7	11	6	3	2	Total batch no. 43
Average nauplius no. (×1000)	55	67	58	60	72	83	56	62	Mean of nauplius 6.27/batch

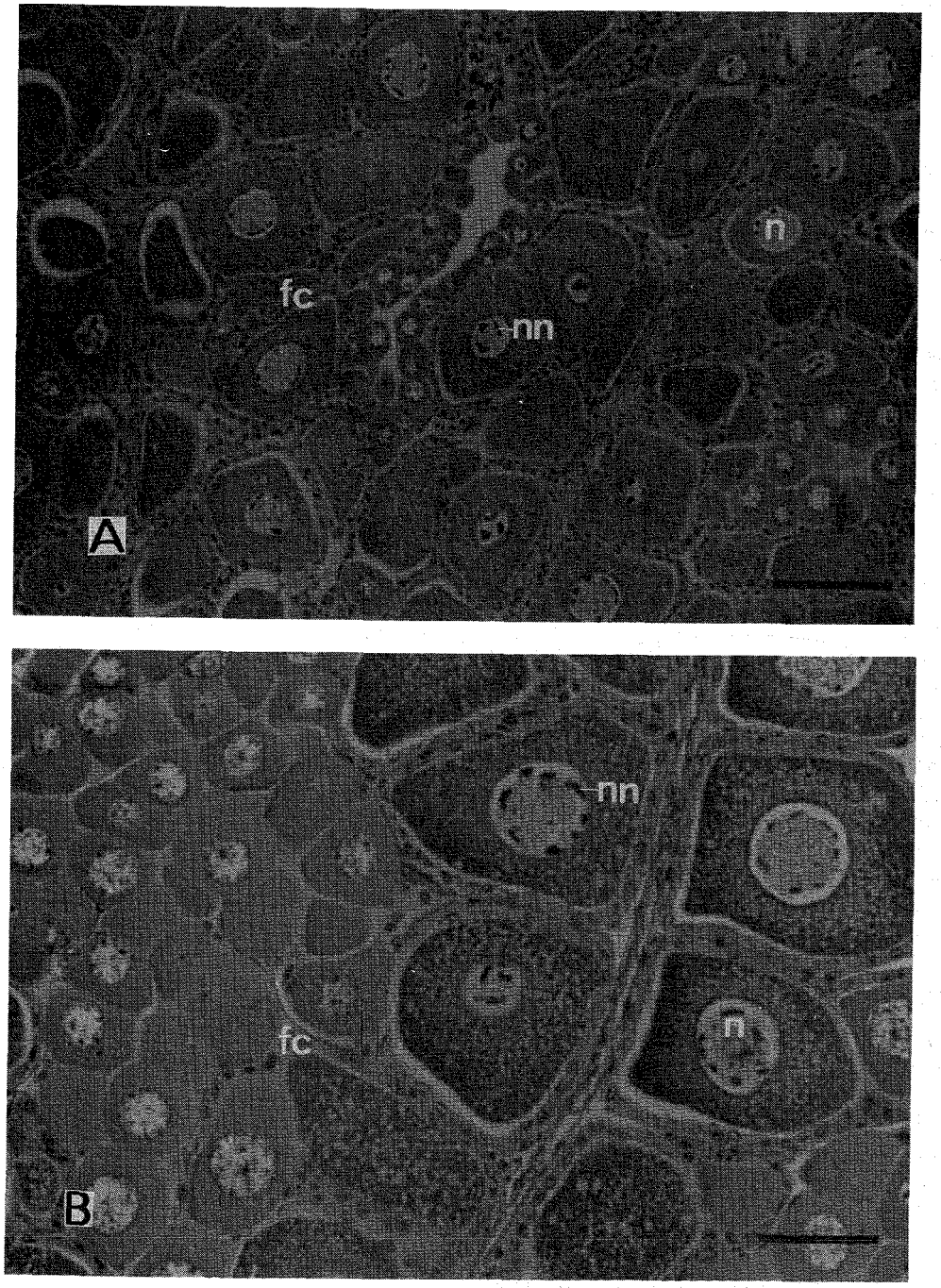

Fig. 1. Histologic section of ovaries of *Penaeus monodon* which were taken for *in vitro* culture (3 days after eyestalk ablation). A, HE stain; B, PAS stain; n, nucleus; nn, nucleoli; fc, follicle cell. Scale: 100 μm (A), 50 μm (B).

The vitellogenesis stage was confirmed visually using the criteria described by Motoh (1981).

In vitro *organ culture*

Tissues dissected from early vitellogenic females were incubated in 200 μl (2 ×) Leibovitz's L-15 medium (Hazleton), with osmolarity of 720 ± 10 mmol/kg at 28 ± 1°C as described by Chen *et al.* (1989). New synthetic vitellogenins were labeled in methionine-free medium with 200 μCi/ml ^{35}S-methionine (specific activity 1209.3 Ci/mmol). After incubation, tissue and medium were centrifuged at 15,000 ***g*** for 10 min in an Eppendorf microfuge at 4°C.

Immunoprecipitation with anti-Ep-serum

Crude tissue extracts (100 μl) or culture medium (100 μl) were added to 50 μl anti-Ep-serum and 50 μl protein-A Sepharose CL-6B with incubation at 4°C for 4 hr or overnight. The precipitated pellet, after washing twice with 0.1 M Tris–HCl buffer (pH 7.5), was redissolved in 50 μl SDS sample buffer.

Indirect immunofluorescence microscopy

Paraffin sections were prepared according to standard methods. Tissue sections were incubated with 200× anti-Ep-serum for 1 hr at room temperature, and washed with TBS. FITC-conjugated goat IgG (anti-rabbit-IgG, 1:20 diluted) was applied for 1 hr at room temperature. The sections were mounted in 1 M Tris–HCl (pH 8.1) and glycerol (1:9, vol/vol) then observed and photographed with an Olympus (Model BH2) microscope, with an incident UV attachment and fluorescence optics.

A

B

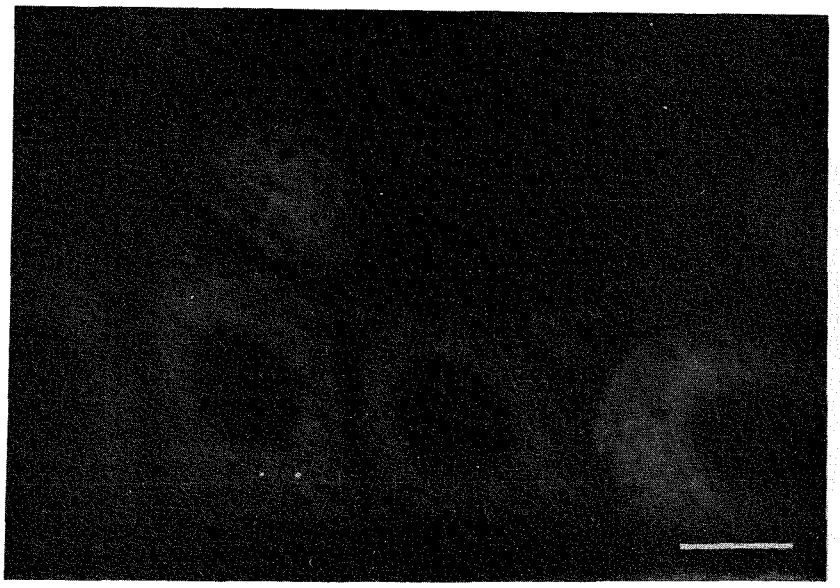

Fig. 2A and B

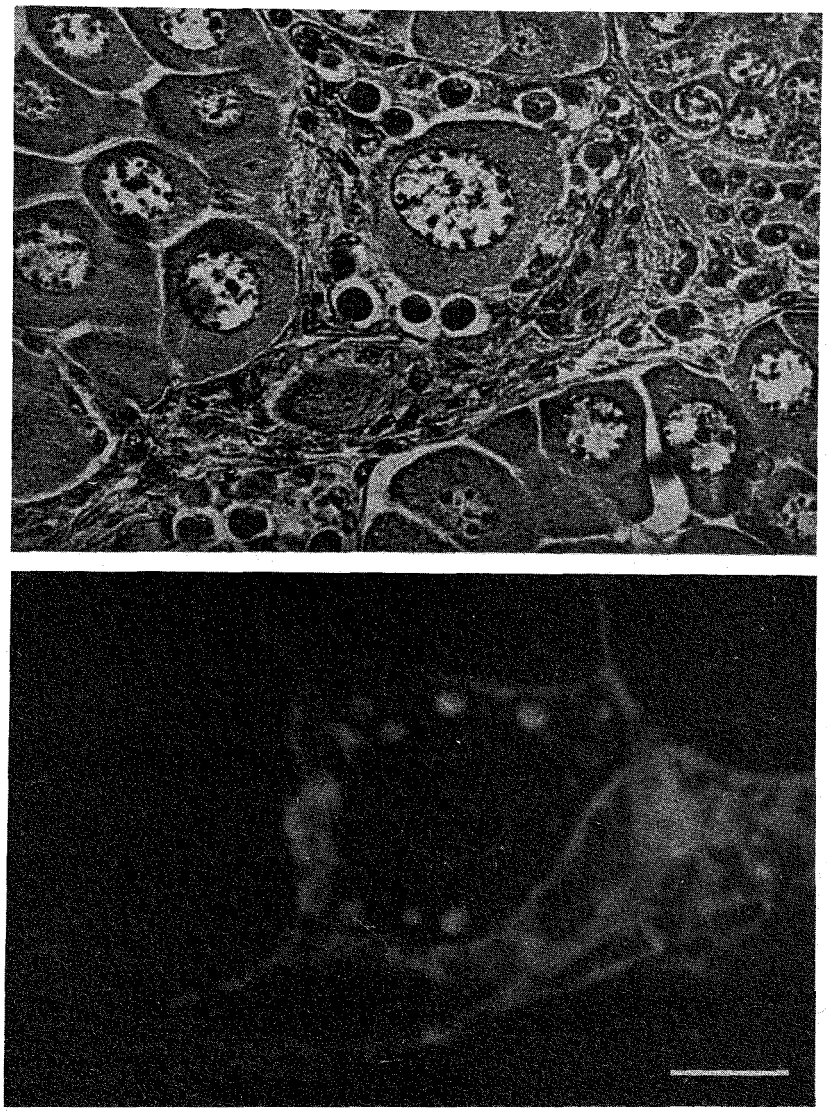

Fig. 2. Immunofluorescence microscopy of developing ovaries of *P. monodon*. A, C, phase contrast; B, D, immunofluorescence. A, B, stage 1; C, D, spent stage. Scale: 50 μm (A, B); 100 μm (C, D).

Digestion of Vg with Endo-H and calf alkaline phosphatase

The culture medium was incubated in 20 mM Tris–HCl buffer plus 20 units of calf alkaline phosphatase (EC 3.1.3.1) (pH 8.0), at 37°C for 2 hr. The reaction was stopped by adding SDS re-educating sample buffered solution and boiling for 3 min. The culture medium was incubated in 20 mM phosphate buffer plus 5 m units endoglycosidase H (EC 3.2.1.96) (pH 5.5) at 25°C for 12 hr. Protease activity was inhibited by 0.5 mM PMSF.

Results

Ovarian development

Eyestalk ablation was used to induce ovarian development; 80% of the eyestalk-ablated animals spawned within 7 days (Table 1). The green color of the ovaries may be derived from carotenoids associated with vitellogenins. Animals in stage I were used. Using hemotoxylin–eosin (HE) stain (Fig. 1A), the ovaries appeared to be in vitellin stage (diameter 40–200 μm). Using PAS stain (Fig. 1B), the vitellinic oocytes (diameter $>100\,\mu$m) showed an accumulation of large numbers of glycoproteins.

Immunofluorescence microscopy

The early maturation oocytes (diameter $<100\,\mu$m) neither react to the antiserum nor exhibit fluorescence (Figs 2A,B). The late-stage vitellogenic oocytes had significant fluorescence that was not observed in the early-stage oocyte. The follicular cell around the late-stage oocyte reacted with the anti-Ep antiserum showing strong fluorescence. The reabsorbed oocytes

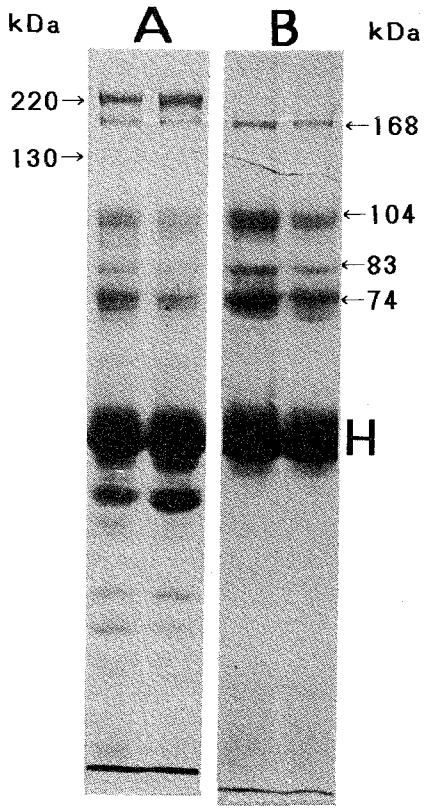

Fig. 3. SDS–PAGE (10% acrylamide) of immunoprecipitates from *in vitro* culture ovary samples. A, ovary extract; B, culture medium.

(Fig. 2D) exhibited vitellin transport to the new maturation oocytes.

Immunoprecipitates of vitellin in ovaries

Vitellin-like proteins of tissues *in vitro* were electrophoretically analysed. Immunoprecipitates by anti-Vn antiserum and protein from the tissues and medium were tested using SDS–PAGE. Figure 3 shows that the ovary extract possesses five major bands of 200, 168, 104, 83 and 74 kDa molecular weight, respectively, and one minor band of 130 kDa. The cultured medium revealed similar patterns but lacked the 220 kDa band.

The *in vitro* culture medium had three new polypeptides, with mol. wt of 70, 78 and 95 kDa, respectively. In the proteolytic map, the 78 kDa polypeptide was observed as Vp3 (83 kDa), and the 95 kDa peptide as Vp2 (104 kDa) (Fig. 4).

Vitellogenin was treated with Endo-H to determine carbohydrates. The 220 kDa peptide to Endo-H showed a reaction of about 20 kDa (Fig. 5A). The other vitellin protein showed no difference when Endo-H-treated. These results may suggest that the 220 kDa peptide was a mass glycolation, and that other peptides contained little or no carbohydrate. In the Sudan Black B stain, the Vp1 and 220 kDa vitellin proteins stained positively, as shown in Fig. 5B. After digestion with alkaline phosphatases, Vg revealed no degradation (Fig. 5C).

In vitro *protein synthesis*

The result obtained from ^{35}S-methionine labeling (Figs 6A,B) showed new synthesis of ovarian polypeptides *in vitro*. After 1 hr of culture, three new synthesized patterns were present in the ovary extract, with mol. wt of 220, 168 and 130 kDa, respectively. Eight hours after culture, the three patterns were more intense, and some smaller patterns were present. In the culture medium, 1 hr after culture, two patterns were found with mol. wt 168 and 74 kDa, respectively. There were no other patterns found in culture medium with incubation periods of up to 8 hr. Autoradiography showed that the ovary may synthesize vitellin-like proteins, with mol. wt of 220, 168, 130 and 74 kDa, plus several smaller polypeptides. In the ovary, there were three patterns of 220, 168 and 130 kDa, respectively. The culture medium contained two secreted polypeptides of 168 and 74 kDa (Fig. 6D), respectively.

Immunoreactions of P. monodon *vitellin with* P. japonicus *vitellin*

Results of immunoblotting demonstrate the cross-reaction of *P. monodon* vitellin antiserum with ovarian extracts and hemolymph from mature female *P. japonicus* shrimp (Fig. 7). Anti-Ep1 reacted with one 70 kDa vitellin protein only. The anti-Ep2 reacted with the 80 kDa protein, and also showed weak cross-reaction to the 100 kDa protein and the large vitellin

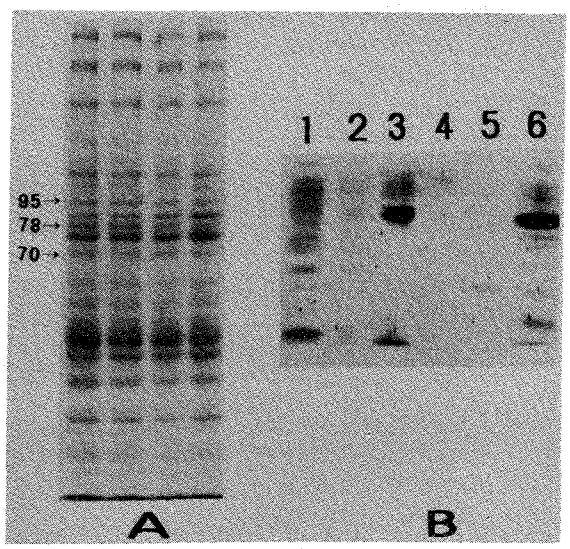

Fig. 4. Immunoblot of peptide maps of *in vitro* culture medium after partial proteolysis. A, 10% SDS–PAGE of culture medium; B, partial proteolysis of the vitellin-like polypeptides. 1, 104 kDa; 2, 95 kDa; 3, 83 kDa; 4, 78 kDa; 5, 74 kDa; 6, culture medium.

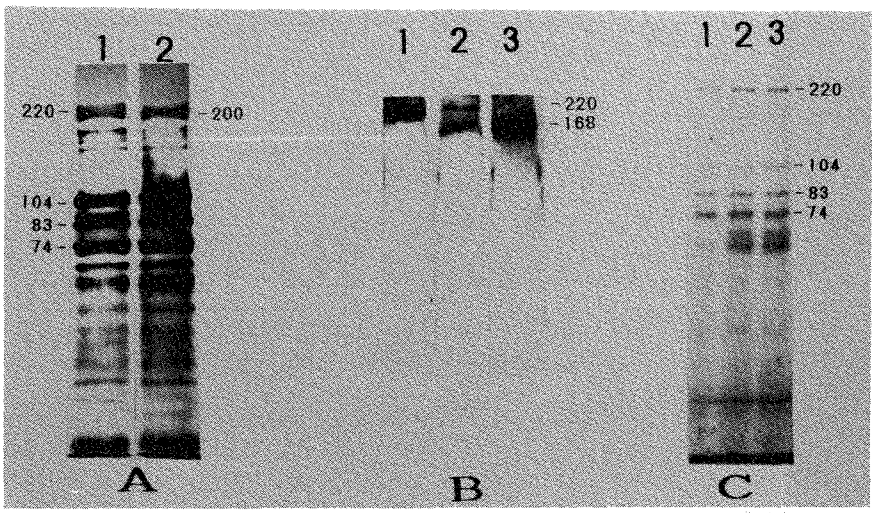

Fig. 5. Effect of Endo-H and calf alkaline phosphatase (CAP) on the *P. monodon* ovary vitellin subunits. A, Endoglycosidase-H treatment: 1, ovary extract; 2, Endo-H digestion, 12 hr. B, Sudan Black B stain: 1, early maturation stage ovary extract; 2, late maturation stage ovary extract; 3, egg extract. C, CAP treatment: 1, ovary extract; 2, CAP digestion, 1 hr; 3, CAP digestion, 2 hr.

protein (160 kDa). The anti-Ep3 reacted to the 100 kDa and some larger proteins with mol. wt ranging from 130 to 170 kDa.

Discussion

The eyestalk-ablated broodstock females entered the vitellogenin stage, which was followed by spawning. The secondary vitellogenin period is less than 4 days; the yolk protein of the spawning eggs is synthesized in this period. Therefore, the vitellin production tissues were found to be at the peptide translation stage.

Vitellogenesis includes the production of vitellogenin and the accumulation of both organic and inorganic constituents of yolk by the oocytes. In the anti-Ep antisera immunostain (Fig. 2A), vitellin commences to accumulate in the secondary vitellogenesis oocytes (yolk globular stage). However, vitellin is not present in the primary vitellogenesis oocytes (pre-yolk stage, diameter $<100\ \mu l$). According to Tan-Fermin and Pudadera (1991), all the mature oocytes may be spawned out. The atretic oocytes in the secondary vitellogenesis period degenerate and are reabsorbed (Harrison, 1990). In Fig. 2B, the vitellin of degenerating oocytes is transferred to the newly matured oocytes. Vitellin appeared to be used not only for yolk protein formation, but also for nutritious transfer in the vitellogenesis stage.

In the immunoprecipitates of ovary extract and culture, five major vitellin-like peptides occurred. The 220 kDa peptide is only present in

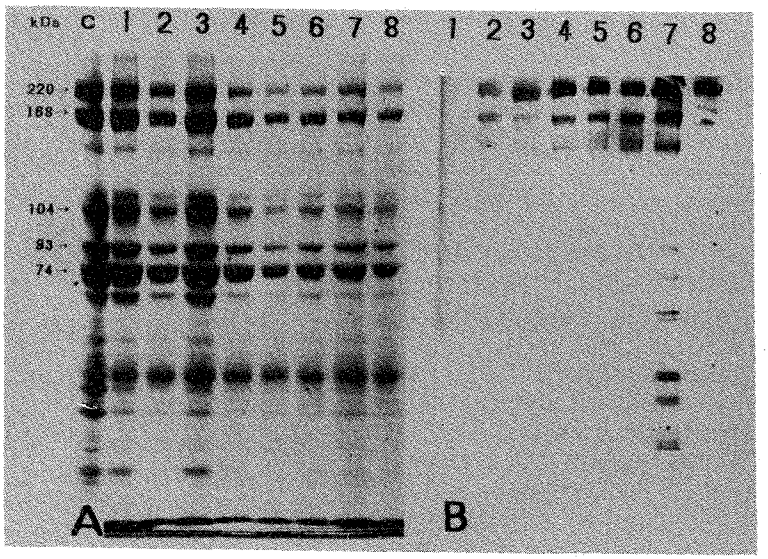

Fig. 6A and B

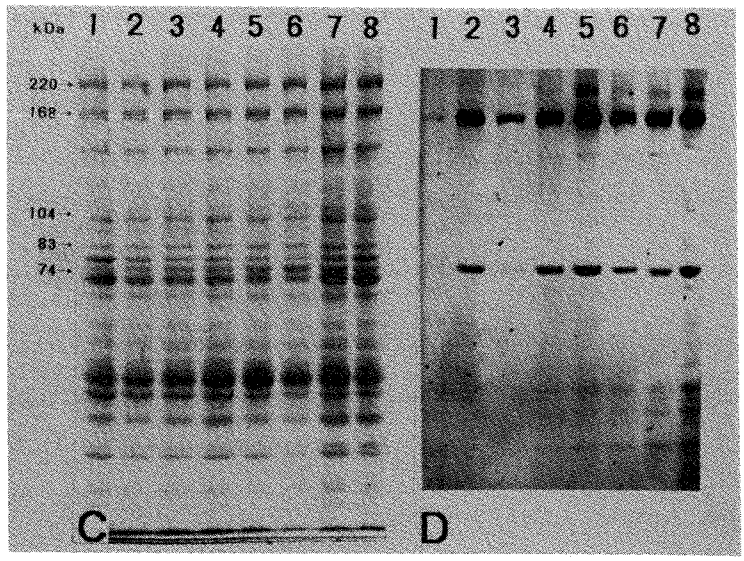

Fig. 6. [^{35}S]methionine labeling of *in vitro* culture ovary proteins. SDS–PAGE fluorography of immunoprecipitates for culture ovary samples. A, C, Coomassie Blue R stain; B, D, autoradiography. A, B, ovary extract; C, D, culture medium. The culture times, C, 0 hr; 1, 0.5 hr; 2, 1 hr; 3, 2 hr; 4, 3 hr; 5, 4 hr; 6, 5 hr; 7, 6 hr; 8, 8 hr.

the early secondary vitellogenesis ovary. The result of Sudan Black B and PAS staining may suggest that the 220 kDa variant is a glycolipoprotein. In *in vitro* culture of the ovary, three newly synthesized vitellin peptides were present in ovary extract with mol. wt 220, 168 and 130 kDa; and two secretory peptides in the culture medium of mol. wt 168 and 74 kDa. The isolated vitellogenin in hemolymph of *P. monodon* is composed of two subunits with mol. wt 170 and 93 kDa (Lee, 1991); that is, it is very similar to the secretory vitellin found in *in vitro* culture. In our present study, *P. monodon* hemolymph contained four egg yolk peptides of 168, 104, 83 and 74 kDa. These may be derived from the reabsorption of vitellin in hemolymph and newly synthesized vitellogenin. In *P. japaneus*, the ovary synthesized two vitellin proteins (Yano and Chinzei, 1987). In *P. semisulcatus*, vitellin protein can be synthesized by the ovary *in vitro* as native vitellin that consists of four subunits (Browdy *et al.*, 1990). Lui and O'Connor (1977) concluded that the ovaries of crab, *Pachygrapsus crassipes*, are capable of synthesizing the proteinaceous yolk found in the mature egg.

Our results did not show Ep2 and Ep3 to be synthesized in ovaries *in vitro*. It may be that the culture time was too short for vitellin to be processed, or that it cannot be processed in *in vitro* culture. From our data, it may be concluded that *P. monodon* ovary synthesizes vitellin *in vitro*. A 220 kDa pre-vitellin and two intermediate vitellins (168 and 130 kDa) were found in the ovary, and 168 and 74 kDa vitellins were secretory to culture medium. *In vivo*, the 168 and 74 kDa peptides may constitute vitellogenin and show absorption by oocytes.

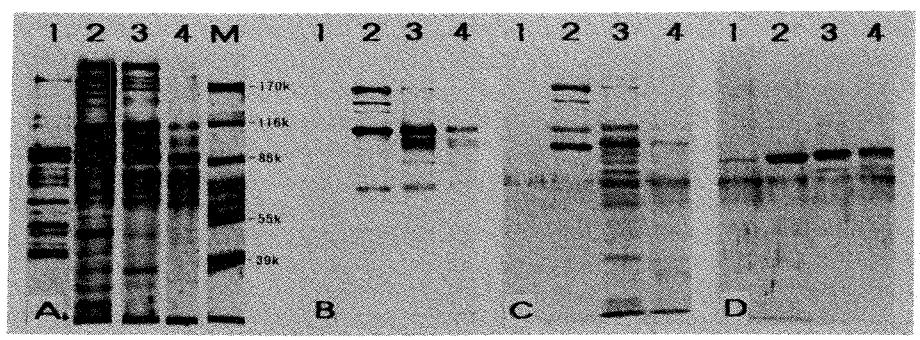

Fig. 7. Immunoblotting showing the cross-reactivity of antiserum to vitellin of *P. monodon* (Pm) with *P. japonicus* (Pj). A, silver stain; B, C, D, immunostain. B, anti-Ep2. B, anti-Ep3. C, anti-Ep2. 1, hemolymph of Pj; 2, ovary extract of Pj; 3, ovary extract of Pm; 4, egg extract.

The vitellin of *P. japonicus* has four subunits that are similar to those of *P. monodon*. When immunostained, they reacted to the anti-Vg antisera of *P. monodon*. This result may suggest that vitellins in *P. monodon* and *P. japonicus* penaeids are very similar immunologically and may mature under the same processes.

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