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Comparative Biochemistry and Physiology, Part B 138 (2004) 305–311

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Purification of vitellin from the ovary of Chinese mitten-handed crab (*Eriocheir sinensis*) and development of an antivitellin ELISA

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Received 16 January 2004; received in revised form 16 April 2004; accepted 18 April 2004

Abstract

Vitellin was purified from ovaries of mature female Chinese mitten-handed crab (*Eriocheir sinensis*) using gel filtration chromatography. Analysis by native PAGE showed the vitellin had a native molecular mass of 520 kDa, while denaturing SDS-PAGE revealed two subunits of 97 and 74 kDa. Purified vitellin was used to raise polyclonal antisera, with which an enzyme-linked immunosorbent assay (ELISA) was developed. The ELISA was sensitive and could effectively detect vitellin in the range of 7.8–500 ng. Furthermore, vitellin levels in various developmental stages of oogenesis were measured with the ELISA assay. The results indicated that levels of vitellin increased significantly from 0.22 mg/ovary at Stage II to 360.31 mg/ovary at Stage IV.

Keywords: Decapoda; Developmental stages; Enzyme-linked immunosorbent assay; *Eriocheir sinensis*; Ovary; Gel filtration chromatography; Purification; Vitellin

1. Introduction

Oogenesis is an energetically expensive reproductive process that can be divided into six phases (Xue et al., 1987; Tsukimura et al., 2002). The latter phases of oogenesis characterized by the accumulation of yolk proteins in the growing oocytes and by significant increases in oocyte diameter, are referred to as primary and secondary vitellogenesis (Meusy and Payen, 1988). The most important sources of nutrients for the embryonic development are yolk proteins (Adiyodi, 1985). Vitellin (Vn) is the common form of yolk stored in oocytes. In many species, vitellogenin (Vg), the precursor molecule to Vn, is transported through the hemolymph to developing oocytes, where it is sequestered and modified with the addition of polysaccharides and lipids into Vn. Thus, the synthesis of yolk proteins is a good indicator of female reproductive activity in many species (Tsukimura, 2001). In crustaceans, Vn is the major yolk protein required for the survival and development of embryos until they can feed independently. Therefore, the quality and quantity of Vn accumulated in eggs are crucial for embryonic survival (Harrison, 1990).

It has been suggested that Vg is synthesized in ovary and hepatopancreas, depending on the species (Yano and Chinzei, 1987; Quackenbush, 1989; Quackenbush and Keeley, 1988; Lee and Watson, 1995; Chen et al., 1999; Tseng et al., 2001). Vg is broken down into several vitellin subunits and accumulated within developing oocytes (Quinitio et al., 1990; Chang et al., 1994; Lee and Watson, 1995; Lubzens et al., 1997; Pateraki and Stratakis, 2000; Okuno et al., 2002). Extensive studies of vitellin have been conducted in a wide range of crustacean species (Fyffe and O'Connor, 1974; Komatsu and Ando, 1992; Tom et al., 1992; Chen and Chen, 1993; Chang et al., 1996; Qiu et al., 1997; Chen and Kuo, 1998; Kawazoe et al., 2000; Garcia-Orozco et al., 2002), but little information was available for the Chinese mitten-handed crab (*Eriocheir sinensis*), one of the most

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1096-4959/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpc.2004.04.012

important species for commercial production in China. Although significant advances have been made in culturing the Chinese mitten-handed crab in the last several decades, the variable quality of eggs spawned by captive broodstock remains a considerable constraint for the production of larvae and postlarvae in large-scale hatcheries. In the present study, we isolated and purified the vitellin, for the first time from ovary of the Chinese mitten-handed crab, and developed an antivitellin enzyme-linked immunosorbent assay (ELISA), in order to assess and control the reproduction process of the crab.

2. Materials and methods

2.1. Sampling and preparation of ovarian homogenates

Chinese mitten-handed crabs (*E. sinensis*) were obtained from a commercial farm in Shanghai, P.R. China. The different maturation stages of ovaries were determined by the classification of Xue et al. (1987). Ovarian samples were taken from various developmental stages: Stage II (34 mm, milky white), Stage III_1 (40–50 mm, milky white), Stage III_2 (60–90 mm, lightly brown), and Stage IV (92–100 mm, purplish brown). The samples were removed and immediately frozen at −70 °C until purification.

To obtain pure vitellin, ovaries at Stage IV were homogenized in 10 vol. extraction buffer (0.1 M NaCl, 0.01% EDTA, 0.02 M Tris–HCl, pH 7.6, 0.1 mM PMSF in isopropyl alcohol) at 4 °C. The homogenate was centrifuged at 12,000 × *g* for 30 min at 4 °C to make crude egg homogenates. Saturated ammonium sulfate was added to the purple layer of the supernatant to produce a 50% saturated ammonium sulfate solution, and allowed to sit on ice for 10 min before centrifugation. After centrifugation, the pellet was redissolved in extraction buffer and sequentially combined with saturated ammonium sulfate to produce 50% solution and incubated on ice for 10 min before centrifugation. This step was repeated 3× and the final pellet was resuspended in 1 ml of phosphate buffered saline (PBS, 10 mM sodium phosphate, pH 7.7, 0.01% EDTA, 0.1 M NaCl) and was subjected to gel filtration chromatography.

2.2. Chromatography

The egg extract was purple and was further separated on a Sephacryl S-300 HR column (Hiprep, Pharmacia, Sweden; 60 cm, 2.6 cm i.d.), equilibrated in PBS with 0.1 mM PMSF (pH 7.7), at a flow rate of 0.6 ml/min. The effluent was collected in fractions of 1 ml, and the absorbance was measured at 280 and 474 nm. The effluent was concentrated in centrifugal concentrators (Macrosep, Pall Filtron, USA), and each concentrated peak was analyzed on native (7%) PAGE. Proteins were quantified according to Lowry et al. (1951), using bovine serum albumin (BSA) as a standard.

2.3. PAGE and SDS-PAGE

Polyacrylamide gel electrophoresis (PAGE) was carried out under native conditions on 7% polyacrylamide gels with 1.5 M Tris-glycine (pH 8.8). SDS-PAGE separation was conducted on 7.5% polyacrylamide gels in the presence of 0.1% SDS dissolved in 1.5 M Tris-glycine, (pH 8.8). A solution of 0.5 M Tris–HCl (pH 6.8), 1% SDS, 1% 2-mercaptoethanol, 10% glycerol and 0.05% bromophenol blue was used as the sample loading buffer where 1% 2-mercaptoethanol was added. The molecular mass of the identified proteins was determined by comparison with markers of known molecular mass. For native PAGE, markers of 669, 440, 232, 140 and 67 kDa (Amershan Pharmacia, UK) were used; for SDS-PAGE, markers of 200, 130, 97.4, 66.2 and 43 kDa (Dongfeng, Biotech, Shanghai, PRC) were used. The gels were stained with Coomassie Brilliant Blue R-250 for proteins, Sudan Black B for lipids, periodic acid-Schiff's reagent for carbohydrates. Furthermore, the gel with the purified vitellin was stained by "Stains-all" for phosphorus (Campbell et al., 1983). Native PAGE was conducted at 150 V and 4 °C, while SDS-PAGE at 170 V and room temperature (RT).

2.4. Preparation of antiserum

Antisera against the crab vitellin were produced in two New Zealand male rabbits. Briefly, freshly purified vitellin mixed 1:2 (v/v) with Freund's adjuvant was injected in four sites along the backs (1 ml per rabbit) of the animals. Four additional injections using Freund's incomplete adjuvant were given at 2-week intervals. Blood samples were collected every other week for testing the antibody titer. When antibody titer reached its peak, animals were bled. Blood was allowed to clot at 4 °C for 24 h and the antiserum was centrifuged at 3000 × *g*, 10 min, and stored at −70 °C.

2.5. Immunoblotting

Gels were run as described above. After 2 h of electrophoresis, each gel was separated into two. One half was stained with Coomassie Brilliant Blue R-250. The other half was transferred onto a nitrocellulose membrane (NC, Hybond ECL, Amersham, UK.) using a minitransblot with electrophoretic transference cell (Bio-Rad, USA). The nitrocellulose membrane was blocked for 1 h with 5% BSA in PBST (10 mM PBS containing 0.05% Tween-20, pH 7.2), and incubated with a solution of antivitellin antibodies diluted 1:2000 in PBST for 1 h at RT. Membrane was washed with PBST and subsequently incubated for 1 h at 37 °C in a solution containing horseradish peroxidase

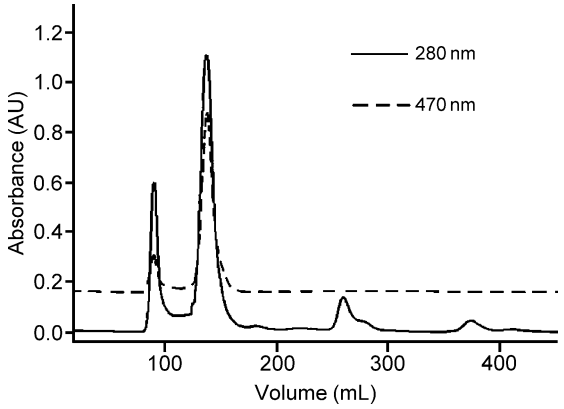

Fig. 1. Vitellin extract of *E. sinensis* fractionated by gel filtration chromatography on a Sphacryl-300 column (60 × 2.6 cm), equilibrated with PBS (10 mM, pH 7.7) and eluted with the same buffer. Flow rate: 0.6 ml/min.

conjugated goat antirabbit IgG (Sigma, USA) diluted 1:1000 in PBST. After washing in PBST, the nitrocellulose membrane was incubated at RT in 10 ml TBS buffer (10 mM, pH 7.5) containing 6 mg of 3,3′ -diaminobenzidine and 10 μl of 30% H_2O_2 until bands were detected. The color reaction was stopped with distilled water.

2.6. Development of enzyme-linked immunosorbent assay (ELISA)

A 96-well polystyrene microtiter plate (Corning, USA) was coated with samples using carbonate buffer (50 mM sodium carbonate, pH 9.6) overnight at 4 °C. Then washed 5 × with PBST (10 mM PBS in 0.05% Tween-20, pH 7.2), and the nonspecific binding sites were blocked by incubating the plate with PBST containing 3% BSA for 2 h at 37 °C. After five successive washes with PBST, antivitellin polyclonal antiserum diluted 1:4000 in PBST was added and the plate was incubated for 1 h at 37 °C. Plates were then washed 5 × with PBST, and incubated for 1 h with goat antirabbit IgG conjugated to horseradish peroxidase (Sigma, USA) diluted in the same buffer. The plate was subsequently washed 5 × with the above buffer. For detection, the OPD enzyme substrate (0.5 mg/ml) was added to each well. After 1 h incubation, 50 ml of 3 M H_2SO_4 was added to each well to stop the reaction. The plate was read at 490 nm on a Universal Microplate Reader (Bio-Tek, USA). The wells for the standard curve were coated with the twofold dilutions of purified vitellin, and the wells coated with BSA served as the nonspecific binding control. Each sample was coated 3 × .

Intraassay variability, measured as a percentage of the coefficient of variation, was assessed by conducting replicated measurements ($n = 7$) in a single ELISA plate. The purified vitellin was first quantified with Lowry method using BSA as a standard and then diluted to three different concentrations, in order to estimate the sensitivity of the ELISA assay. Interassay variability was evaluated in the same manner using the same internal standards analyzed in several different assays ($n = 10$).

2.7. Statistics

The parallel of the standard curve to sample diluted curve was determined by Student's *t*-test. One-way ANOVA was performed with the content of vitellin and total protein content of ovaries at different developmental stages, and the statistically significant differences were decided using Tukey Test.

3. Results

3.1. Purification of vitellin

Vitellin extract was fractionated by gel filtration chromatography and monitored at a wavelength of 280 nm (Fig. 1). Four distinct peaks were found and vitellin was mainly contained in the fractions from peak 2, as indicated by a peak in absorbance at 474 nm derived from the high content of carotenoids of vitellin. A single protein band was detected with native PAGE (7%) with a molecular mass of 520 kDa (Fig. 2). Vitellin is composed of lipids, carbohydrates and phosphorus as revealed by the respective stain ability of vitellin to Sudan Black B, periodic acid-Schiff's reagent and "Stains-all".

Under denatured conditions on SDS-PAGE, vitellin separates into two subunits with molecular masses of 97 and 74 kDa (Fig. 3). Patterns of subunits were different between the lanes with or without adding 2-mercaptoethanol in SDS-PAGE, indicating secondary structures of vitellin that are disrupted by the addition of 2-mercaptoethanol.

3.2. Specificity of antiserum

The specificity of the antiserum was determined by Western blotting. If the antiserum was highly specific, only

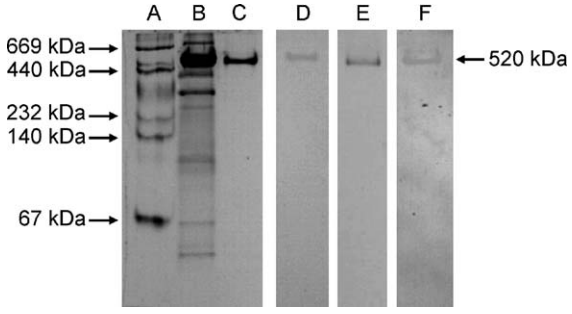

Fig. 2. Native PAGE of *E. sinensis* vitellin ovarian homogenate and vitellin were separated by 7% polyacrylamide. A portion of the gel was stained with Coomassie Brilliant Blue R-250 (A–C); molecular mass markers (A); ovarian homogenate (B) and separated vitellin (C). Vitellin was prestained with Sudan Black B (D); vitellin was stained with periodic acid-Schiff's reagent (E) and "Stains-all" (F).

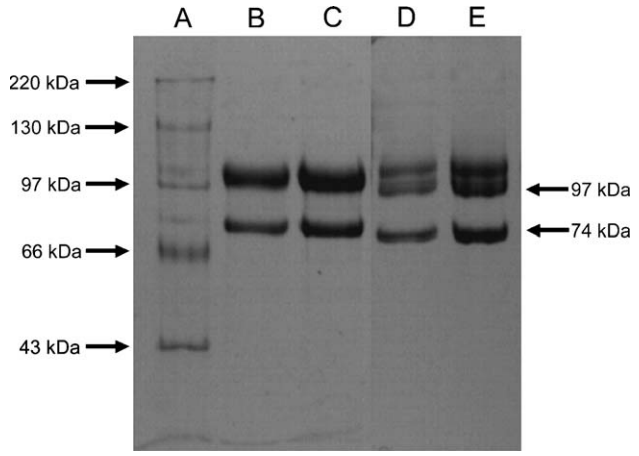

Fig. 3. SDS-PAGE of *E. sinensis* vitellin. The vitellin were separated by 7.5% SDS-polyacrylamide. Molecular mass markers (A); Vitellin was pretreated with 2-mercaptoethanol (B–C) or not (D–E). Note that lane C is a duplication of lane B, with a greater amount of vitellin; and lane E is a duplication of lane D, with a greater amount of vitellin loaded on the gel.

one band representing vitellin was expected. As shown in Fig. 4, when the ovarian homogenates or the purified vitellin were used, only one major band was detected while this band was absent in samples from musk demonstrating that the antiserum was specific for vitellin. Cross-reactivity was negligible.

3.3. ELISA of vitellin

A typical calibration curve is shown in Fig. 5 using vitellin as the standard. The standard curve showed linearity between 7.8 and 500 ng with a correlation coefficient of $r = 0.9967$. Vitellin levels as low as 4 ng were found to differ significantly from the background. Specificity of the ELISA

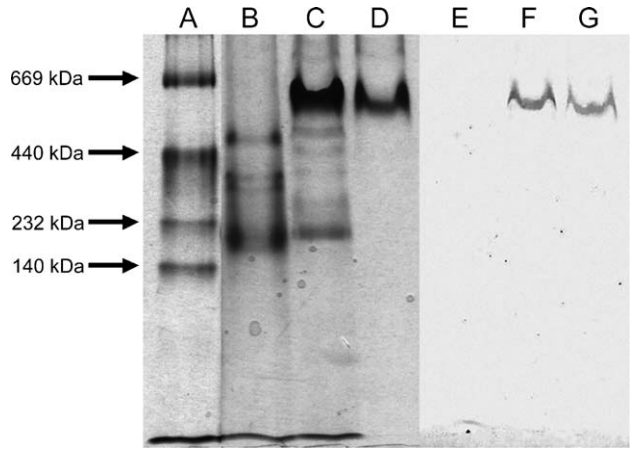

Fig. 4. Western blot analysis of *E. sinesis* antivitellin antiserum. Homogenates of muscle (B and E) and ovary (C and F) and vitellin (D and G) were separated by 7% PAGE. The left portion of the gel was stained with Coomassie Brilliant Blue R-250 and the right portion of the gel was electroblotted to nitrocellulose membrane. Molecular mass markers (A).

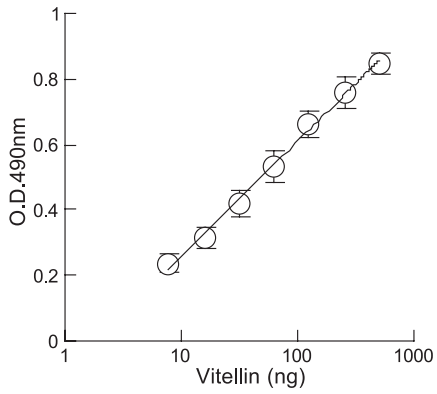

Fig. 5. A typical ELISA standard curve for *E. sinensis* vitellin. Each point represents a mean ± S.D. ($n = 6$).

was tested using serially diluted female muscle homogenates as a negative control. Additionally, the parallelism of the standard curve to serial dilutions of ovarian extracts from mature female over the working range of the ELISA was compared and the results indicated that there was no significant difference in the slopes of the two curves ($P > 0.05$) (Fig. 6). The precision was measured using low, medium and high internal standards. The intraassay and interassay coefficients of variance were 8.2% and 9.6%, respectively.

In order to determine vitellin levels during oogenesis, vitellin levels were measured using the ELISA in ovaries from female in different stages (Table 1). The level of vitellin was 0.22 ± 0.02 (mean ± S.D.) mg/ovary in Stage II and significantly increased during the processes of oocyte maturation ($P < 0.05$). The highest level of the vitellin (360.31 ± 40.15) was detected in Stage IV, which is about 1600-fold over that of Stage II. Levels of total protein in

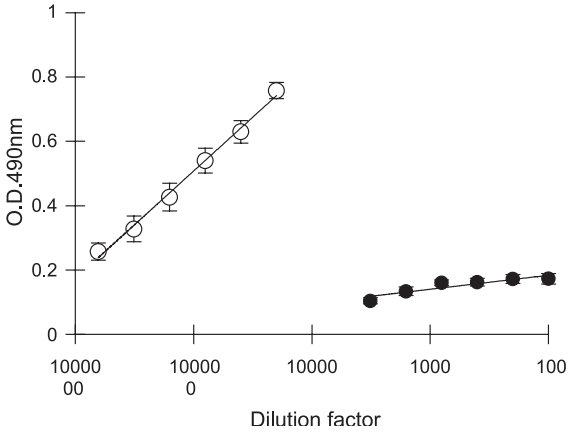

Fig. 6. Linear relationship of vitellin and absorbance at 490 nm as a reflection of specificity of the ELISA assay. The absorption at 490 nm was inversely proportional to the dilution factor with the *E. sinensis* ovarian extracts (○), while very low absorption was observed with the negative control of the muscle extracts (●). Each point represents a mean ± S.D. ($n = 4$).

Table 1
Levels of vitellin and protein in ovaries at different stages of development determined by ELISA

Ovarian stage	Vitellin (mg/ovary)	Ovarian protein (mg/ovary)
II	0.22 ± 0.02^{a}	47.12 ± 4.74^{a}
III_1	103.49 ± 22.8^{b}	180.43 ± 19.41^{b}
III_2	203.42 ± 11.21^{c}	269.81 ± 36.75^{b}
IV	360.31 ± 40.15^{d}	446.86 ± 38.52^{c}

Mean ± S.D. ($n=5$) followed by the same letter are not significantly different (one-way ANOVA, $P>0.05$).

ovaries increased remarkably during ovarian development with a similar pattern to that of vitellin.

4. Discussion

Crustacean vitellin from a number of species has been recently characterized. Vitellin in many species had a native molecular mass ranging from 300–600 kDa (Tom et al., 1992; Pateraki and Stratakis, 1997; Garcia-Orozco et al., 2002; Vazquez-Boucard et al., 2002; Serrano-Pinto et al., 2003). In this study, vitellin has been purified from ovaries of the mature *E. sinensis* by gel filtration chromatography (Oberdorster et al., 2000; Tsukimura et al., 2000). The molecular mass of vitellin in the mature *E. sinensis* has been determined by PAGE to be approximately 520 kDa which is within the range found in the other species (Chang et al., 1996; Pateraki and Stratakis, 1997; Vazquez-Boucard et al., 2002). Only one form of vitellin has been commonly identified in most crustacean species (Lee and Puppione, 1988; Komatsu and Ando, 1992; Pateraki and Stratakis, 1997; Tsutsui et al., 2000; Vazquez-Boucard et al., 2002). However, two forms of vitellin (Vn1 and Vn2) were obtained from secondary vitellogenic *Cherax quadricarinatus* with native molecular masses of 470 and 440 kDa (Serrano-Pinto et al., 2003). Similarly, Chang et al. (1996) reported two forms of vitellin (Vn1 and Vn2) in *Penaeus chinensis* with molecular masses of 500 and 380 kDa. Here only one kind of vitellin was found in the mature Chinese mitten-handed crab.

The difference in size of crustacean vitellin may arise from the different number and size of the subunits, as well as the different ovarian stages and the different methods for purification of vitellin (Quinitio et al., 1990). For instance, two subunits were obtained from *E. japonica* (Komatsu and Ando, 1992), *Uca pugilator* (Quackenbush and Keeley, 1988) and *Potamon potamios* (Pateraki and Stratakis, 1997). Lubzens et al. (1997) reported three subunits of vitellin in *Penaeus semisulcatus* with molecular masses of 200, 120 and 80 kDa. The vitellin from *Callinectes sapidus* contained four subunits (Lee and Watson, 1994). In the present study, under denaturing SDS-PAGE, two subunits were detected in purified vitellin from the ovaries of mature *E. sinensis* with molecular mass of 97 and 74 kDa. As in *P. chinensis* (Chang et al., 1996), the disulfide bond may bind the two subunits of the vitellin together, as demonstrated by the different band patterns of subunits in reducing and nonreducing SDS-PAGE.

A polyclonal antibody was raised against a purified ovarian vitellin of *E. sinensis*, which specifically recognized vitellin in ovarian homogenate. Cross-reactions were not a problem with the other proteins from ovaries and muscles. The observed high specificity indicated that the polyclonal antibody may be useful for various immunological studies of vitellin. This antiserum allowed us to develop an ELISA and to quantify the concentrations of vitellin. The ELISA was sensitive for *E. sinensis* vitellin with a working range of 8–500 ng. Lee and Watson (1994) developed an ELISA for blue crab (*C. sapidus*) based on polyclonal antibodies that had a sensitivity of 148 ng/ml, which is comparable to the present ELISA. The ELISA for *E. sinensis* has an acceptable intraassay coefficient of variation across its working range. The interassay coefficient of variation was comparable to those reported for ELISA for vitellin from other crustacean species (Sagi et al., 1999; Vazquez-Boucard et al., 2002). The precision of the assay is sufficient to allow direct comparison of samples on the same plate and from different plates assayed at different times. In order to validate the applicability of the ELISA for different concentrations of vitellin, we established a standard curve using diluted samples. The dilution curve for ovarian homogenate was similar to the nondiluted ELISA standard curve. Thus, the ELISA is suitable for quantification of vitellin in ovaries where vitellin is expected to be at similar concentrations.

In crustaceans, vitellogenesis is associated with the formation of mature oocytes within the ovary. Vitellogenesis occurs in two stages, the primary vitellogenesis extends for several months and results in slow increase in the size of the oocyte; the secondary vitellogenesis results in rapid increase in oocyte size leading to oviposition (Aiken and Waddy, 1980). Vitellin is generally low in the early developing ovaries (Stage II), but becomes the most abundant protein in the ovaries of developed oocytes. In *E. sinensis*, Stage III_1 and Stage III_2 are active growth phases of oocytes and at Stage IV, full oocytes are ripe (Xue et al., 1987; Du et al., 1995). Corresponding to the development of oocytes, the vitellin levels in ovaries increase remarkably in Stages III_1 and III_2 (Table 1), indicating that the accumulation of vitellin results in the growth of ovarian weight. In *Macrobrachium rosenbergii*, substantial quantities of vitellin accumulate within the developing oocytes from Stage III to Stage V when oocytes grow actively (Lee and Chang, 1997).

It is now widely accepted that vitellin is processed from the precursor protein vitellogenin in ovary. The synthetic site of vitellogenin can differ in various species. In *C. sapidus*, vitellogenin is produced primarily in the developing oocyes (Lee and Watson, 1995). Similarly, various other species are capable of synthesizing vitellogenin in ovaries (Lui and O'Connor, 1977; Rankin et al.,

1989; Browdy et al., 1990). Convincing evidence is available for the synthesis of vitellin by the hepatopancreas and its transportation to the ovary of several decapod crustaceans (Paulus and Laufer, 1987; Quackenbush, 1989; Lee and Chang, 1999; Soroka et al., 2000; Yang et al., 2000). Several crustaceans can produce vitellogenin in both locations (Paulus and Laufer, 1982; Shafir et al., 1992; Sagi et al., 1999; Tsutsui et al., 2000). Although the vitellogenesis in *E. sinensis* has not been investigated in the present study due to the loss of samples, previous histological results suggested that the oocytes produce endogenous vitellogenin by autosynthesis and also absorb exogenous vitellogenin of heterosynthesis directly or indirectly (Du et al., 1999). Further studies are needed to determine the origin of exogenous vitellogenesis in the Chinese mitten-handed crab.

Acknowledgements

This study was supported by grants 30271012, 30130040 and 3028018 from the National Nature Science Foundation of China.

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