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The site of vitellogenin synthesis in Chinese mitten-handed crab *Eriocheir sinensis*

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Abstract

The objective of this study was to investigate the synthesis site of vitellogenin in the Chinese mitten-handed crab, *Eriocheir sinensis*. Using the RT-PCR techniques, the cDNA fragments isolated from the ovaries of vitellogenic female crab, we found that its deduced amino acid sequence had a high identity with that from other decapods crustacean vitellogenin. This cDNA fragments were used as probes to examine the transcription of mRNAs encoding the Vg. The mRNA expression was observed in vitellogenic female hepatopancreas, which was not detected in any other tissues including muscle, heart, and subepidermal tissues. The positive immunocytological staining with antibody against vitellin were found in ovaries and hepatopancreas of vitellogenic female, which was determined by immunological and immunohistochemical techniques. These results suggest that both ovaries and hepatopancreas are capable of synthesizing vitellogenin. Therefore, it was concluded that hepatopancreas is the extraovarian site of vitellogenin synthesis in *E. sinensis*.

Keywords: Crustacean; *Eriocheir sinensis*; Genomic DNA; Hepatopancreas; Synthesis site; Vitellin; Vitellogenesis; Vitellogenin gene

1. Introduction

Vitellogenesis is a decisive period in the female reproductive cycle of crustacean characterized by the accumulation of yolk and the formation of mature oocytes within the ovary. The main yolk protein, which is called vitellin (Vn), is composed of carbohydrate, phospholipid, carotenoid components and protein (Pateraki and Stratakis, 1997; Chen et al., 2004). It is well established that vitellogenin (Vg) is one of the precursors of Vn in crustaceans. It has been hypothesized that Vg is synthesized by extraovarian tissues, and then transported via hemolymph to developing oocytes where it is incorporated into yolk (Meusy, 1980).

Understanding vitellogenesis processes of crustacean is important because of the growing aquaculture industry and interests in new models for basic research. A large number of methods, including biochemistry, immunology and molecular (Chen and Chen, 1994; Lee and Chang, 1999; Chen et al., 1999, Yang et al., 2000; Tseng et al., 2001; Garcia-Orozco et al., 2002; Okuno et al., 2002), have been used for understanding the vitellogenesis processes. In isopods, the biosynthesis of Vg occurs in the adipose tissue, and is then transported by hemolymph for deposit in oocytes (Picaud, 1980; Suzuki, 1987). The results of several studies on decapod crustaceans indicate that the Vn synthesis is endogenous or/and exogenous. Some species are capable of synthesizing Vg in ovaries (Lui and O'Connor, 1976; Browdy et al., 1990; Fainzilber et al., 1992; Lee and Watson, 1995), and in others, Vn is synthesized by the hepatopancreas (Paulus and Laufer, 1987; Lee and Chang, 1999; Chen et al., 1999; Soroka et al., 2000; Yang et al., 2000). Several crustaceans can produce Vg in both locations (Paulus and Laufer, 1982; Shafir et al., 1992; Sagi et al., 1995; Tsutsui et al., 2000). In *Eriocheir sinensis*, previous histology studies suggested that the oocytes produced endogenous Vg and also absorbed exogenous Vg (Du et al., 1999). However, few studies have been done on the vitellogenesis of *E. sinensis*, especially the site of Vn and Vg synthesis.

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The objective of this study is to locate Vg mRNA in vivo by reverse transcription (RT) and polymerase chain reaction (PCR) and immunohistochemical staining the Vn-immunoreactive proteins in ovary and hepatopancreas to understand the site of Vg synthesis in *E. sinensis*.

2. Materials and methods

Adult Chinese hand-mitten crabs, *E. sinensis,* were collected from pond cultures in aquaculture farm in Shanghai. The vitellogenic females were determined by the ovarian development stages according to Xue et al. (1987). Tissues from both males and females were used for RNA isolation and subsequent verification of the Vg synthesis site in vivo. Hemolymph was collected by withdrawing from the sinuses at the base of the third walking legs with a syringe and mixed (1:2) with anticoagulant containing 1.4% Na_2HPO_4, 1.3% KH_2PO_4, 0.3% EDTA, 2% dextrose, and 0.25% sodium citrate, after centrifuged at 4000 ×*g* for 10 min at 4 °C and stored at −80 °C.

Total RNA was isolated from the ovary, hepatopancreas and other tissues (muscle, heart and subepidermal tissue) of vitellogenic female crab. The tissues were homogenized in Trizol reagent (GibcoBRL, USA) and purified according to the manufacturer' instructions. Purified RNA samples were diluted at about 1 mg/mL for RT-PCR or stored at −80 °C.

Genomic DNA was isolated from 100 mg testis tissue of the male crab by homogenization in 100 mM NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0, 0.5% sodium dodecyl sulfate. The homogenate was then treated with 0.1 mg/mL of proteinase K (Sigma-Aldrich, USA) at 65 °C for 2–4 h then transferred to 4 °C for 12 h. Successive phenol and chloroform–isoamyl alcohol extractions were performed followed by ethanol precipitation, and DNA pellets were resuspended in 100 μL of 10 mM Tris-1 mM EDTA (pH 8.0), and then stored at 4 °C until use for PCR.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out with total RNA isolated from the crabs. First-strand cDNA synthesis was performed using a AMV reverse transcriptase kit (Promega, USA) to transcribe poly A RNA with oligo(d T) as primer. The reaction conditions recommended by the manufacturer were followed. The cDNA was used for the second-strand synthesis and subsequent amplifications. Both genomic DNA and cDNA from vitellogenic female crab were used as templates in the PCR.

Degenerate primers for the RT-PCR were designed according to two highly conserved domain sequences of crustacean Vg: forward, 5′-AAG GC (T/A) CT (G/T) GG (T/C) AAC ATG GG-3′ (VF) and reverse, 5′-GGA GC (A/G) TAG ATG ATG TT (G/C) GA-3′ (VR). The specific primers, sVP (5′-GAT GTG GCT GAG GTG CTG AT-3′) and sVR (5′-AAA GGT CAA TGT TGC GGG AG-3′), which were designed on the basis of the nucleotide sequences of putative Vg fragment. PCR reactions contained 10 mM Tris–HCl buffer (pH 9.0), containing 10 mM KCl, 8 mM $(NH_4)_2SO_4$ and 0.5% NP-40, 2.0 mM $MgCl_2$, 1 U Taq polymerase (Bioasia, China), 0.25 mM dNTPs, and 1 μM primers. PCR conditions used for cDNA amplification with degenerate primers (VF and VR) were: denaturation at 95 °C for 5 min; 30 cycles of denaturation at 94 °C for 1 min; annealing at 42 °C for 1 min; elongation at 72 °C for 2 min; and then followed by a 10 min extension at 72 °C and cooling to 4 °C. The PCR with specific primers (sVF and sVR) was accomplished by the following program: denaturation at 95 °C for 5 min; 30 cycles of denaturation at 94 °C for 45 s; annealing at 60 °C for 30 s; and elongation at 72 °C for 30 s; followed by 72 °C extension for 10 min and cooling to 4 °C.

The housekeeping gene beta actin of crab was used as an internal standard. The primers for RT-PCR in the present study, which were designed according to a highly conserved domain sequences of crustacean beta actin (Genbank accession no: AY910691), were as follows: actF 5′-CCT CCG GTC GTA CCA CTG GTA T-3′ and actR 5′-CCA CGG AAG GTC TCA TTG CCG ATC GTG-3′.

PCR products were excised from the 1.0% agarose gels, followed by purification with a DNA gel extraction kit (Beyotime, China). The purified fragments were sequenced in both directions on a DNA sequencer. Sequence confirmation and amino acid translations were performed using the Vector NTI suite 8.0.

Vn purified from mature ovarian extract of *E. sinensis* by salt precipitation and further with Sephacryl S-300 HR column, was used to prepare rabbit antiserum (Chen et al., 2004). The tissues were homogenized in phosphate buffer saline (PBS, 10 mM sodium phosphate, pH 7.7, 0.01% EDTA, 0.1 M NaCl, 0.1 mM PMSF), and centrifuged at 12,000 ×*g* for 30 min at 4 °C. The supernatants were used for immunodiffusion in a 1% agar gel plate (2 mm thickness) developed according to the methods of Ouchterlony and Nilsson (1978).

Ovary and hepatopancreas were fixed with Bouin's fixative, embedded in paraffin, and sectioned at 6 μm. The sections were stained with hematoxylin and eosin. For immunohistochemical examination, sections were rehydrated through xylene, absolute ethanol, 70% ethanol, 30% ethanol and distilled water. After inactivation of endogenous peroxidase activity with 3% H_2O_2 solution and then blocked in 3% BSA dissolved in PBS, tissue sections were incubated first with antibody to vitellin (1:2000 dilution) for 60 min, while negative control with PBS and then washed with PBS containing 0.5% Tween-20 (PBST) and PBS alone. Horseradish peroxidase conjugated goat anti-rabbit IgG (Sigma-Aldrich) and substrate solution containing 0.5 mg/mL 3,3′-diaminobenzidine (DAB), 0.3% hydrogen peroxide in

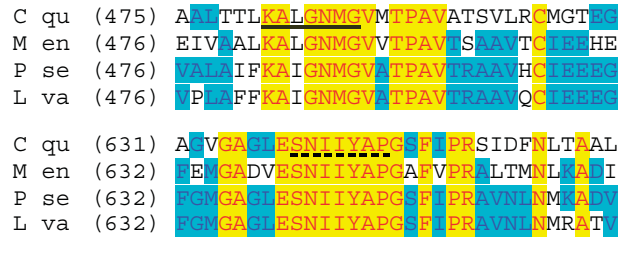

Fig. 1. Amino acid sequence alignments of two vitellogenin fragments from various animal species: *Cherax quadricarinatus* (C qu) (AAG17936.1, Abdu et al., 2002), *Metapenaeus ensis* (M en) (AAM48287.1, Chan, S.M. and Tseng, W. Z.), green tiger shrimp *Penaeus semisulcatus* (P se) (AAL12620.3, Avarre et al., 2003), Pacific white shrimp *Litopenaeus vannamei* (L va)(AAP76571.2, Parnes et al., 2004). Forward and reverse primers employed were designed, based on the sequences indicated by the solid and broken lines.