Molecular characterization and expression analysis of vitellogenin in the marine crab *Portunus trituberculatus*

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Abstract

As a precursor of the major yolk protein vitellin (Vn), vitellogenin (Vg) has been studied in crustacean for decades, mainly in shrimp. However, little is known about the Vg molecule in crabs. In the present study, we report a cDNA encoding Vg in the marine crab *Portunus trituberculatus*, an important commercial species in aquaculture. The cDNA sequence is 7846 bp in length, containing a 7680 bp open reading frame, that encodes 2560 amino acid residues. The deduced amino acid sequence showed 32–75% identity with other known crustacean Vgs. Seven potential cleavage sites (RXXR) were identified in the deduced Vg precursor. SDS-PAGE and Western blotting analysis demonstrated that Vns in the ovary of the mature crab consisted of three major polypeptides (102, 100 and 85 kDa). Northern blotting analysis revealed that Vg was mainly expressed in hepatopancreas in vitellogenesis females. A Vg transcript was also identified in the ovary of the crab by reverse transcription-polymerase chain reaction analysis.

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1. Introduction

As observed in other oviparous animals, vitellin (Vn) is the major yolk protein stored in crustacean oocytes, where it provides free amino acids, lipids, carbohydrates, carotenoids, and minerals (Byrne et al., 1989; Kunkel and Nordin, 1985; Niimi et al., 1993; Khalaila et al., 2004) to the growing and developing of embryo and larva. Vitellogenin (Vg), the precursor of vitellin, is synthesized extraovary or intraovary, transported by the hemolymph, and sequestered into the growing oocytes through receptor-mediated endocytosis. In the oocytes, Vg undergoes proteolytic processing to produce several subunits (Vns) (Meusy, 1980; Adiyodi and Subramoniam, 1983; Meusy and Payen, 1988; Tsukimura, 2001). The synthesis and accumulation of vitellogenin is therefore, crucial for oocyte and embryo development.

Many studies have focused on gene structure and gene expression of decapods vitellogenin (Charniaux-Cotton, 1985; Tsang et al., 2003; Kung et al., 2004). cDNAs encoding Vg have been reported in several shrimp including *Marsupenaeus japonicus*(Tsutsui et al., 2000), *Macrobrachium rosenbergii*(Okuno et al., 2002), *Penaeus semisulatus*(Avarre et al., 2003), *Metapenaeus ensis*(Tsang et al., 2003; Kung et al., 2004), and *Pandalus hyspinotus* (Tsutsui et al., 2004), and in the crayfish *Cherax quadricarinatus*(Abdo et al., 2002). However, Vg has been reported for only one crab, the red crab *Charybdis feriatus* (Mak et al., 2005). These Vg cDNAs are about 7–8 kb in length, which encode more than 2500 amino acid residues, and these amino acid sequences show considerable conservation, especially in their amino terminus. Crustacean Vgs are enzymatically digested soon after their synthesis in extraovarian tissues, and further cleavage of these molecules takes place in the hemolymph or in the ovary (Avarre et al., 2003; Okuno et al., 2002; Mak et al., 2005). In insects, Vgs are cleaved into subunits before being secreted into the hemolymph (Chen et al., 1997). In vertebrates, Vgs are enzymatically processed into smaller yolk proteins, lipovitellins, and phosvitins only after reaching the growing oocytes (Polzonetti-Magni et al., 2004).

In general, Vgs are synthesized by extraovarian tissues such as the liver in vertebrates (Byrne et al., 1989), fat body in insects (Sappington and Raikhel, 1998), and intestine in...
nematodes (Sharrock, 1983). In crustaceans, various Vg synthesis sites have been reported in different species (Kerr, 1968; Meusy and Payen, 1988; Yang et al., 2000; Tsutsui et al., 2000; Tsang et al., 2003; Jasmani et al., 2004; Kung et al., 2004; Mak et al., 2005;). In these investigations, either Northern blotting analysis or immunohistochemical techniques were used to examine the expression and distribution of Vg. The results showed that in crustacean Vg were mainly expressed in the hepatopancreas and the ovary.

Crabs constitute one of the largest crustacean families. To date, investigations of Vg in crabs focused primarily on the purification and characterization of its subunits (Vns). In the sand crab Emerita asiatica, at least six Vn subunits, ranging from 42 to 65 kDa, have been identified (Tirumalai and Subramoniam, 1992). In the land crab Potamon potamios, three major Vn peptides of 115, 105 and 85 kDa have been reported (Pateraki and Stratakis, 2000). Likewise, in the Chinese mitten-handed crab, Eriocheir sinensis, Chen et al. reported that Vg had a native molecular mass of 520 kDa and that denaturing SDS-PAGE revealed two subunits of 97 and 74 kDa (Chen et al., 2004). Up to date, only one complete Vg cDNA has been reported, in the red crab Charybdis feriatus (Mak et al., 2005). It was reported the full-length cDNA of the C. feriatus Vg consists of 8051 nucleotides with a long open reading frame encoding a precursor of 2579 amino acid residues. The C. feriatus Vg is expressed only in the hepatopancreas and the expression level fluctuates in different vitellogenesis stages. However, the biochemical nature of Vg and Vn in crabs remains unclear.

The marine crab Portunus trituberculatus supports a large crab fishery in China, and over-fishing has caused a rapid decline in its population. The annual yield of the crab has declined from 59,000 to 11,000 tons between 1996 and 2001. A need exists for a clearer understanding of reproduction processes in this crab to provide the necessary background knowledge to support P. trituberculatus farming. In the present study, we reported a cDNA encoding Vg in the marine crab P. trituberculatus. Vns in this crab was found to include three major peptides of 80, 100, and 102 kDa. Northern blotting analysis and reverse transcription-PCR demonstrated that Vg was synthesized both in the hepatopancreas and ovary in this crab.

2. Materials and methods

2.1. Animals

Crabs (Portunus trituberculatus) of both sexes were obtained from a commercial farm in NingBo, China, and were reared in a 15 L glass aquarium with artificial seawater until...
sacrificed. They were acclimated for at least two weeks before experimentation and were fed twice daily. Crabs were collected from June to November, and ranged from early developmental stages to vitellogenesis stages (Xue et al., 1997). Crabs were classified based on their body weight, the morphological characteristics of the ovary, the width of the carapace, and the coloration of oocytes. Crabs in early developmental stages weighed from 20 to 100 g and no ovary or only a small milky white ovary was observed. Vitellogenesis crabs had a large orange ovary and weighed over 140 g. A variety of tissues (heart, gill, muscle, hepatopancreas, subepidermal adipose tissue, and ovary) was ablated, snap-frozen in liquid nitrogen, and stored at -80 °C until extraction.

2.2. Total RNA extraction

All tissues were homogenized in Trizol Reagent (Invitrogen), and total RNA was prepared according to the manufacturer’s instructions. Total RNA was quantified on a Genova UV/visible spectrophotometer at 260 nm.

2.3. Reverse transcription-polymerase chain reaction

First-strand cDNA was synthesized from 2 μg of total RNA from hepatopancreas of vitellogenesis crabs, using the SuperScript™/First-strand cDNA Synthesis Kit (Invitrogen), according to the manufacturer’s protocol. The first-strand cDNAs were used as a template for PCR and one cDNA fragment of approximately 1 kb was amplified by two degenerate primers, PF1 and PR (Table 1 and Fig. 1). The PCR program included an initial denaturation at 94 °C for 4 min, then 40 cycles of 30 s at 94 °C, 30 s at 54 °C, 2 min at 72 °C, and a final polymerization step at 72 °C for 10 min. The PCR product was subcloned into the PCR 2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen) for sequencing analysis.

2.5. Molecular cloning of cDNA encoding Vg

Long-range PCR was performed using ZTaq (Takara) and two gene specific primers, GSP2 and GSP3 (Table 1 and Fig. 1). The PCR program included 35 cycles of 5 s at 98 °C and 50 s at 68 °C. The PCR product was subcloned into the PCR 2.1–TOPO vector using the TOPO TA cloning kit (Invitrogen) for sequencing analysis. The full-length cDNA sequence of vitellogenin from _P. trituberculatus_ was re-constructed from the overlapping cDNA clones. The sequence data was submitted to GenBank under accession number DQ000638.
2.6. Northern blotting analysis

Total RNA was extracted from various tissues using Trizol, as described above. Total RNA was quantified on a Genova UV/visible spectrophotometer. Aliquots containing 10 μg of total RNA corresponding to each tissue were mixed with 4 μl of 6M glyoxal and 12 μl DMSO and heated at 50 °C for 30 min, then placed on ice for over 1 min. The treated RNAs were then separated through a 1.0% agarose gel. After electrophoresis, the gel was stained with ethidium bromide and photographed. The RNAs were then transferred to a nylon membrane (Millipore Immobilon-NY®) and UV cross-linked. The probe for Northern blotting was a non-radioactive partial cDNA fragment corresponding to bases 421–1493 of crab Vg cDNA (Fig. 2), synthesized by the DIG High Prime Labeling Kit (Roche). Prehybridization was performed at 42 °C for 1.5 h and hybridization was performed at 45 °C overnight. The membrane was washed at room temperature twice at low stringency (2 × SSC, 0.1% SDS) for 5 min and then at 65 °C twice at high stringency (0.1 × SSC, 0.1% SDS) for 15 min. After washing, hybridized probes were detected by alkaline phosphatase-conjugated anti-DIG antibody and the CSPD chemiluminescent detection system (Roche).

2.7. SDS-PAGE and Western blotting analysis

Proteins from ovary and hepatopancreas of vitellogenesis crabs were extracted with an extraction buffer (0.1 M Tris-HCl, 1 M NaCl, 6 M urea, pH 7.5). Samples were analyzed on a discontinuous SDS-PAGE with a 4% stacking gel and 7.5% separation gel. After electrophoresis, the gel was stained in Coomassie brilliant blue R-250 until bands appeared. For Western blot analysis, proteins were transferred onto a PVDF membrane (Millipore Immobilon-P) after electrophoresis. Transfer was conducted at 30 V overnight in a transfer buffer (16 mM Tris, 120 mM glycine, 10% methanol, pH 8.3). The membrane was then washed three times for 5 min in T-TBS and incubated with a rabbit anti-vitellin (Macrobrachium nipponense) serum (1:4000 in T-TBS) as the primary antibody for 1 h at room temperature. The membrane was washed an additional four times for 5 min and incubated with goat anti-rabbit IgG (Fc) AP conjugate (Promega) (1:5000 in T-TBS) as the secondary antibody for 1 h at room temperature. Then, the membrane was washed five times for 5 min and soaked in detection buffer for 5 min. The antigen-antibody complexes were revealed by the addition of color-development solution (5-bromo-4-chloro-3-indolyl phosphate/nitrate blue tetrazolium NBT/BCIP) (Promega), according to the manufacturer’s instructions.

2.8. Reverse transcription-polymerase chain reaction analysis of P. trituberculatus Vg in hepatopancreas and ovary

RNAs from hepatopancreas and ovary of vitellogenesis crabs were extracted and first-strand cDNAs were synthesized as above. According to the Vg sequence obtained (Fig. 2), three pairs of gene specific primers (GSP4, GSP5; GSP6, GSP7; GSP8, GSP3) (Table 1) were used to study Vg expression in hepatopancreas and ovary by RT-PCR. The PCR program included denaturation at 94 °C for 4 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 58 °C, 1 min at 72 °C, and a final polymerization step at 72 °C for 10 min. PCR products were analyzed on a 1.5% agarose gel.

Genomic DNA from hepatopancreas of female crabs was extracted with the DNeasy® Tissue Kit (Qiagen), according to the manufacturer’s instructions. PCR was performed using three pairs of gene specific primers (GSP4, GSP5; GSP6, GSP7; GSP8, GSP3) (Table 1). The PCR program included initial denaturation at 94 °C for 4 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 58 °C, 2 min at 72 °C, and a final polymerization step at 72 °C for 10 min. PCR products were then analyzed on a 1.5% agarose gel.

3. Results

3.1. Characterization of P. trituberculatus Vg cDNA

One pair of degenerate primers, PF1 and PR (Table 1 and Fig. 1), was derived from two consensus regions identified by an alignment of amino acid sequences from known crustacean Vgs. A cDNA fragment was amplified, sequenced (Fig. 1), and conceptually translated into a 357 amino acid residue sequence, which shared 46–87% identity with other known crustacean Vgs (Charybdis feriatus 87%, Cherax quadricarinatus 53%, Metapenaeus ensis 46%). Then, a gene specific primer, GSP1 (Table 1 and Fig. 1), was designed for 5’ RACE. A 808 bp 5’ end cDNA fragment was amplified by 5’ RACE (Fig. 1). Because the remaining 3’ end fragment was too long for a single PCR amplification, another degenerate primer, PF2 (Table 1 and Fig. 1), was designed and used for 3’RACE. The sequenced 3’ end cDNA fragment was 841 bp long (Fig. 1). Based on the sequences obtained above, two gene specific primers, GSP2 and GSP3 (Table 1 and Fig. 1), were synthesized. A long-range PCR was performed and a cDNA fragment of about 7 kb (Fig. 1) was amplified, subcloned, and sequenced.

The full-length cDNA sequence of crab vitellogenin was determined by overlapping the three fragments (Fig. 1). This Vg cDNA contains 7846 nucleotides and an open reading frame encoding a precursor of 2560 amino acid residues (Fig. 2). The 5’ and 3’ untranslated regions consist of 27 and 139 nucleotides, respectively. A polyadenylation signal, AATAAA, was also identified upstream of the poly (A) tail (Fig. 2). The predicted molecular mass of this Vg is about 287 kDa with a theoretical pl of 6.81. The first 18 amino acid residues are most likely a signal peptide of this Vg molecule (Fig. 2). Like most other known crustacean Vgs, the deduced amino acid sequence contains several consensus cleavage sites (RXXR) recognized by an endopeptidase belonging to the subtilisin family of serine protease (Fig. 2). A phosvitin/ polyseryl domain was not identified in the deduced Vg sequence. When aligned with other known decapods Vg amino acid sequences, the P. trituberculatusVg exhibited highest identity with Charybdis feriatus Vg (75% identity),
followed by *Cherax quadricarinatus* Vg (37% identity), *Pandalus hypsinotus* Vg (35% identity), *Marsupenaeus japonicus* Vg (34% identity), and *Macrobrachium rosenbergii* Vg (32% identity).

### 3.2. Characterization of Vn in *P. trituberculatus*

SDS-PAGE analysis found that the ovarian protein was composed of three major polypeptides (102, 100, and 85 kDa) and several minor polypeptides (Fig. 3a). Western blotting analysis confirmed that these polypeptides were immunopositive for the Vn antibody (Fig. 3b). In the hepatopancreas, very low levels of protein could be detected by Coomassie blue staining, and no immunoreactivity was observed to the antibody against Vn (Fig. 3).

### 3.3. Expression of Vg in *P. trituberculatus*

Northern blotting indicated a strong hybridization signal of approximately 8 kb only in hepatopancreas of vitellogenesis crabs, but not in any other female tissue (heart, gill, muscle, subepidermal adipose tissue, or ovary) or the male hepatopancreas (Fig. 4). Vg mRNA was only detected in the vitellogenic stage of female crabs, but not in the early developmental stages (Fig. 5).

Using two pairs of gene specific primers (GSP4 and GSP5, GSP6 and GSP7) (Table 1), 400 and 954 bp cDNA fragments, respectively, were amplified by RT-PCR from both hepatopancreas and ovary (Fig. 6a, b). Only one cDNA fragment of 425 bp was obtained from hepatopancreas, but not from ovary, using gene specific primers GSP8 and GSP3 (Table 1, Fig. 6c). A control PCR reaction using genomic DNA showed that the RT-PCR did not amplify the genomic sequence (Fig. 6).

### 4. Discussion

In the present study, one cDNA encoding Vg in the marine crab *P. trituberculatus* was cloned and characterized. The identity of this cDNA was confirmed by comparison to amino acid sequences of other known crustacean Vgs. In crustaceans, it is now well established that Vg is initially synthesized as a precursor that undergoes further processing into several subunits (Vns) after synthesis. The cleavage sites, which...
content of Vgs in *M. japonicus*, *M. rosenbergii*, and *C. quadricarinatus* is 5%, 8%, and 10%, respectively. In *P. trituberculatus*, serine residues accounted for only 4.96% of the amino acid residues. The absence of phosvitin and polyserine domains in most crustacean Vgs suggests that crustaceans may utilize a different mechanism for Vg-receptor binding during endocytosis of Vg. A Vg-specific receptor has been found in the crab *S. serrata* (Warrier and Subramoniam, 2002) and several other crustacean species (Laverdure and Soyez, 1988; Jogan and Van Herp, 1989).

Several candidate tissues have been identified in crustacean as Vg synthesis sites: subpidermal adipose tissue, hemocytes, hepatopancreas, and ovary (Meusy and Payen, 1988; Kerr, 1968). In *P. semisulatus* (Avarre et al., 2003), *M. japonicus* (Tsutsui et al., 2000), and *C. quadricarinatus* (Abdu et al., 2002; Serrano-Pinto et al., 2004), Vg transcripts have been detected in both hepatopancreas and ovary. In the freshwater prawn, *M. rosenbergii*, and in the red crab, *C. feriatus*, molecular approaches have recently revealed that Vg is exclusively produced by hepatopancreas (Mak et al., 2005; Jasmani et al., 2004). In our study, by Northern blotting analysis, it was observed that Vg was expressed only in the hepatopancreas in vitellogenesis crabs. The difference in synthesis locations in crustacean may be due to the samples in different maturation stages, variation in analytical methodologies and species-specific adaptations.

In the present study, a predominant 8 kb band, with a smear across lower molecular weights, was detected only in the hepatopancreas by Northern blotting analysis. This smear was not an artifact of mRNA degradation as indicated by the intact 18S and 28S ribosomal RNA in the sample (Fig. 4). Similar smears have been reported in *M. ensis* (Kung et al., 2004), *P. semisulatus* (Avarre et al., 2003), and *P. hypsinotus* (Tsutsui et al., 2004). In *M. ensis*, Kung et al. (2004) found that several major small RNA transcripts were labeled during shorter Northern blot exposures, and the relative abundance of these transcripts varied during the reproductive cycle. They suggested these small transcripts originated from alternative expression/splicing of the Vg gene in the *M. ensis* or from multiple members of the Vg gene family. We were unable to identify any obvious small transcripts in these smears during shorter Northern blot exposures.

Although Northern blotting analysis revealed Vg expression only in the hepatopancreas (Fig. 4), RT-PCR analysis detected Vg in both ovary and hepatopancreas (Fig. 6). These results suggest that trace amounts of Vg transcripts are expressed in the ovary during vitellogenesis. Using electron microscopy to study the mud crab *S. serrata*, Cheng et al. observed both intraocytic and extra-ocytic yolk formation during primary vitellogenesis, whereas during secondary vitellogenesis, extra-ocytic Vg played a dominant role in yolk accumulation in oocytes (Cheng et al., 2002). Based on the results presented here, the synthesis of Vg in the marine crab *P. trituberculatus* is proposed to occur as follows. Vg is synthesized both in ovary and hepatopancreas in the female crab. Trace transcripts are produced in the ovary when the crab reaches primary vitellogenesis. However, as the crab reaches secondary
vitellogenesis, a greater need for Vg exists in the maturing oocytes, and therefore, the hepatopancreas starts to synthesize Vg. This extravarian Vg is transported by the hemolymph and is sequestered into the oocytes. Mechanistic details of Vg synthesis in the marine crab *P. trituberculatus* are now under investigation. 

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**References**


