Aquaculture 437 (2015) 1–9

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Correlation between gonad-inhibiting hormone and vitellogenin during ovarian maturation in the domesticated *Penaeus monodon*

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ARTICLE INFO

Article history:
Received 6 June 2014
Received in revised form 4 November 2014
Accepted 14 November 2014
Available online 20 November 201

Keywords:
Black tiger shrimp
Eyestalk
Gonad-inhibiting hormone
Vitellogenesis
Ovarian maturation

ABSTRACT

Vitellogenesis in crustaceans is a process by which a major yolk protein, vitellin (Vn) is proteolytically produced from vitellogenin (Vg) and deposited into developing oocytes. Vitellogenesis is regulated by a gonad-inhibiting hormone (GIH) produced in the eyestalk ganglia. In this study, *Vg* and *GIH* mRNA expression and their physiological concentrations at the protein level were examined during ovarian maturation in domesticated broodstock of *Penaeus monodon*. *GIH* mRNA was expressed at the highest level in the eyestalk ganglia of the shrimp with immature ovary while the GIH peptide was actively released into the hemolymph. The release of GIH dropped dramatically at stage I of the ovary onwards conforming to its negative regulatory function on Vg synthesis. *Vg* mRNA expression study confirmed that Vg was synthesized in both the ovary and the hepatopancreas of *P. monodon*. The expression of *Vg* increased as ovarian maturation progressed similarly to that demonstrated in the wild broodstock. Vg protein was found in the hemolymph since stage I of ovarian maturation suggesting a rapid release of Vg into the hemolymph before deposition into oocytes as shown by a significant increase of Vn in the ovary at the following stage. Unlike previous studies in wild *P. monodon* broodstock, Vn was localized to follicle cells of late perinucleolar oocytes and to both follicle cells and ooplasm of the vitellogenic oocytes of domesticated broodstock. We speculate that the incorporation of Vn from follicle cells to the oocytes occurred more slowly in domesticated shrimp; this may account for the retarded reproductive maturation of the domesticated broodstock comparing with the wild broodstock. Our study thus provides insights on vitellogenesis in domesticated *P. monodon* that will be useful for improvement of their reproductive maturation.

1. Introduction

Ovarian maturation in oviparous animals occurs by two phases of vitellogenesis: the synthesis of a yolk precursor, vitellogenin (Vg) in vitellogenic organs and the accumulation of a major yolk protein called vitellin (Vn) into oocyte cells. In penaeid shrimps, Vg is synthesized as a large precursor molecule in both the ovary and the hepatopancreas (Avarre et al., 2003; Phiriyangkul and Utarabhand, 2006; Tsang et al., 2003; Tseng et al., 2001; Tsutsui et al., 2000; Xie et al., 2009). Following its synthesis in the hepatopancreas, Vg is cleaved into two subunits that are subsequently released into the hemolymph. The Vg subunits in the hemolymph are further processed by proteolytic cleavage before deposition into developing oocytes where they undergo biochemical modifications such as glycosylation and conjugation to lipid and carotenoids into vitellin that serves as a nutrient source during embryogenesis (Sappington and Raikhel, 1998; Wilder et al., 2010).

Vitellogenin synthesis in crustaceans is controlled by two antagonistic hormones; one stimulates and another inhibits ovarian development. It was demonstrated that implantation of thoracic ganglion or injection of the ganglion extract could induce vitellogenesis in previtellogenic shrimp (Yano, 1992; Yano et al., 1998), indicating that thoracic ganglion contains the substance that can induce Vg synthesis and/or Vg release into the hemolymph. However, identification of such vitellogenesis-stimulating factor has not been successful so far. On the other hand, vitellogenin expression is inhibited by a well characterized eyestalk's hormone called gonad-inhibiting hormone (GIH) (Nagaraju, 2011; Yano, 1998). GIH is synthesized and secreted by the x-organ-sinus gland neurosecretory system in crustacean eyestalk. Although it is generally known that a decrease in the amount of GIH by eyestalk ablation can induce ovarian maturation in shrimp, the mechanism by which GIH regulates vitellogenesis is still unclear. It

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http://dx.doi.org/10.1016/j.aquaculture.2014.11.014
0044-8486/

was shown that GIH level in the hemolymph of an American lobster *Homarus americanus* was high during the immature and previtellogenic stage and decreased in vitellogenic stage (de Kleijn et al., 1998). Correspondingly, *Vg* mRNA expression in both the ovary and hepatopancreas as well as the hemolymph Vg levels of the kuruma shrimp *Marsupenaeus japonicus* were low at previtellogenic stage and drastically increased during vitellogenesis (Okumura et al., 2007). These results, though studied in different crustaceans, evidently suggest the inhibitory role of GIH on vitellogenesis. The investigation of Vg and GIH at both mRNA expression and protein levels during vitellogenesis in a single crustacean species will lead to a more thorough understanding of the regulation of this important reproductive process.

The black tiger shrimp *Penaeus monodon* is one of the important aquatic animals in Thailand and worldwide. The production of *P. monodon* larvae generally relies on eyestalk ablation of the female broodstock (Browdy, 1992). Since removal of the eyestalk not only impedes the production of GIH but also other hormones of physiological importance, other hormonal manipulation approaches have been examined in an attempt to substitute for eyestalk ablation. For instance, the injection of progesterone could stimulate ovarian development in *Metapenaeus ensis* (Yano, 1985). A neurotransmitter serotonin or 5-hydroxytryptamine could also induce ovarian maturation in Penaeid shrimps (Vaca and Alfaro, 2000; Wongprasert et al., 2006). More recently, specific inhibition of GIH expression by dsRNA was demonstrated as a potential alternative to eyestalk ablation to induce vitellogenin expression as well as ovarian maturation in *P. monodon*, especially in the wild broodstock (Treerattrakool et al., 2011). Although these studies provide promising results, an in depth knowledge about the mechanism controlling vitellogenesis process in shrimp is necessary for application of these hormonal manipulation methods to achieve maximum reproductive performance of this shrimp.

In order to understand the control mechanism of vitellogenesis in *P. monodon*, the present study investigates the relationships between GIH and Vg transcripts and protein levels during ovarian maturation in domesticated *P. monodon*. This information will be useful for future development of an alternative method to induce ovarian maturation in female shrimp broodstock.

2. Materials and methods

2.1. Experimental animals and tissue preparation

Domesticated, pond-reared female broodstock of *P. monodon* were kindly provided by the Shrimp Genetic Improvement Center, Suratthani, Thailand. The developmental stages of the ovaries were determined by both the gonado-somatic index (GSI) and histochemical staining with hematoxylin and eosin according to Tan-Fermin and Pudadera (1989), and could be classified into immature (stage 0; GSI = 0.97 ± 0.08%), previtellogenic (stage I; GSI = 1.79 ± 0.06% containing chromatin nucleolar and perinucleolar oocytes), early vitellogenic (stage II; GSI = 2.46 ± 0.09% containing yolkless oocytes as a majority), late vitellogenic (stage III; GSI = 3.88 ± 0.33% mostly composing of yolky oocytes) and mature (stage IV: GSI = 6.24 ± 0.70% as indicated by the massive population of cortical rod oocytes) stages. Tissue samples i.e. eyestalk ganglia, lateral lobe of the ovary and the hepatopancreas were freshly dissected, and immediately frozen in liquid nitrogen and stored at −80 °C. About 2–4 ml of the hemolymph was drawn from dorsal hemolymph sinuses of the shrimp using a syringe and needle containing modified Alsever's solution or anticoagulant-I buffer (27 mM sodium citrate, 450 mM NaCl, 115 mM glucose, 10 mM EDTA, pH is 7.0). The hemolymph samples were centrifuged at 830 ×*g* for 30 min at 4 °C and then transferred to a new tube and stored at −80 °C. All animal experiments were carried out in accordance with animal care and use protocol of the Mahidol University Animal Care and Use Committee.

2.2. RNA isolation and quantitative reverse transcription real-time polymerase chain reaction

About 50 mg of shrimp tissues (ovary and hepatopancreas) or an optic lobe from eyestalk were homogenized in 500 μl of TRI-REAGENT® (Molecular Research Center, Inc.), and the RNA was extracted following the manufacturer's protocol. RNA concentration was estimated by spectrophotometry and the quality of the RNA was determined by 1.2% TAE agarose gel electrophoresis.

One microgram of RNA was used as a template in first-strand cDNA synthesis reaction with an oligo$(dT)_{16}$ primer using ImProm-II™ reverse transcriptase enzyme (Promega, USA) according to the manufacturer's protocol. Then 1 μl of 1:10 dilution of the cDNA was used in quantitative real-time PCR to detect *GIH* and *Vg* transcript levels. Quantitative real time PCR was performed using KAPA SYBR® FAST qPCR kit with specific primers for *GIH* (GIH-F: 5′-AACATC CTGGACAGCAAATGCAGGG-3′ and GIH-R: 5′-GGCCTCGCGCT TGGCCG AGTG-3′) and *Vg* (Vg-F: 5′-TCCATCTGCAGCACCAATCTTCGC-3′ and Vg-R: 5′-GCAACAGCCTTCATTCTGATGCCA-3′) and following KAPA SYBR® FAST qPCR protocol (KAPABIOSYSTEMS, USA). The reaction composing of 1 × KAPA SYBR® FAST qPCR Master Mix, 200 nM each of forward and reverse primers was carried out in a real-time PCR machine ABI7500-prism model. The expression levels of *GIH* and *Vg* were normalized with, and expressed as a relative level to that of *EF-1α* mRNA determined by EF-1α F (5′-GAACTGCTG ACCAAGATCG ACAGG-3′) and EF-1α R (5′-GAGCATACTGTTGGAAGGT CTCCA-3′) primers. The real-time PCR data was analyzed by $2^{-\Delta CT}$ method.

2.3. Immunohistochemistry

The dissected tissues were fixed in Davidson's fixative (31% Ethanol, 22% Formalin, and 11.5% Glacial acetic acid) for 48 h before processed by paraffin sectioning procedure into 5 μm-thick tissue sections, then histological features of the tissues were determined by hematoxylin and eosin staining according to Bell and Lightner (1988). For localization of Vg/Vn in shrimp ovary and hepatopancreas, the tissue sections were immersed in 0.1% H_2O_2 in PBS to eliminate endogenous peroxidase. Non-specific signal was then blocked by incubating the sections in PBS containing 10% fetal bovine serum prior to incubating with monoclonal antibody raised against *P. monodon* Vn protein (anti-Vn mAb) that reacts to the 140 kDa subunits of both Vg and Vn (Longyant et al., 2000) (kindly provided by Prof. Paisarn Sithigorngul and Asst. Prof. Siwaporn Longyant, Srinakharinwirot University, Thailand) for 16 h at 4 °C and washed in PBS containing 0.1% tween 20. The sections were incubated with 1:1000 dilution of horseradish peroxidase (HRP)-conjugated anti-mouse IgG for 90 min at 37 °C. The signal was developed by diaminobenzidine and the reaction was terminated in PBS. The sections were mounted with permount and visualized under the light microscope.

2.4. Immunofluorescence staining

The eyestalks were fixed in Davidson's fixative and processed by paraffin sectioning procedure as described earlier. For immunofluorescence staining, the sections were rehydrated with decreasing percentages of ethanol from 100% to 50% followed by PBS. The tissues were permeabilized in 0.1% Triton X-100 in PBS for 5 min and washed in PBS. Non-specific signal was then blocked by incubating the sections in PBS containing 10% fetal bovine serum prior to incubating with 1:50 dilution of monoclonal antibody specific to *P. monodon* GIH protein (anti-GIH mAb) in PBS containing 1% fetal bovine serum (Treerattrakool et al., 2014) for 16 h at 4 °C, and washed in PBS containing 0.05% tween 20. Subsequently, the sections were incubated with 1:100 dilution in PBS of the Alexa flour 488-conjugated anti-mouse IgG for 2 h at room temperature. The sections were mounted with ProLong® Gold Antifade reagent and visualized under the Olympus FluoView™ FV1000 confocal microscope.