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Determination site of vitellogenin synthesis in freshwater crayfish *Cherax quadricarinatus* at different maturation stages females

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ABSTRACT. The objective of this study was to determine the site of vitellogenin (Vg) synthesis at different stages of female maturation of freshwater crayfish *Cherax quadricarinatus*. PCR products of 1,100 bp and 900 bp were generated from genomic DNA in the first case, and hepatopancreas and ovary cDNAs in the second case. Results from RT-PCR analyses showed that the mRNA encoding the 3' end of the Vg cDNA was present in the hepatopancreas from secondary-vitellogenesis at first maturation, previously spawner and ovigerous females. The Vg mRNA was present simultaneously in the ovary from secondary-vitellogenesis at first maturation only, but was not detected at previously spawning and ovigerous females. This study provided evidence that the ovary plays a significant role in the production of this major egg yolk protein, but only in some stage of the vitellogenesis cycle.

Key words: *Cherax quadricarinatus*, crayfish, mRNA expression, vitellogenin synthesis, vitellogenesis.

Determinación del lugar de síntesis de la vitelogenina en hembras de la langosta de agua dulce *Cherax quadricarinatus* en diferentes estados de maduración

RESUMEN. El objetivo de este estudio fue determinar el lugar de síntesis de la vitelogenina (Vg) en hembras de la langosta de agua dulce *Cherax quadricarinatus* en diferentes estados de madurez. Un producto de PCR de 1.100 y 900 pb fueron generados a partir de ADN genómico en el primer caso, y de ADNc de tejido de hepatopáncreas y de ovario, en el segundo caso. Resultados del análisis del RT-PCR señalaron que el ARNm que codifica el extremo 3' de ADNc de la Vg estuvo presente en hepatopáncreas de hembras en vitelogénesis secundaria de primera maduración, de hembras con desoves anteriores y de hembras ovígeras. El ARNm de la Vg estuvo presente simultáneamente en el ovario de hembras en vitelogénesis secundaria de primera maduración, pero no estuvo presente en el ovario de hembras con desoves anteriores y de hembras ovígeras. Este estudio da evidencia de que el ovario juega un papel importante en la producción de la principal proteína del vitelo, pero solamente en ciertas etapas del ciclo de la vitelogénesis.

Palabras clave: *Cherax quadricarinatus*, langosta de agua dulce, expresión de RNAm, síntesis de la vitelogenina, vitelogénesis.

INTRODUCTION

Yolk proteins are the most important sources of nutrients for development of oocytes and developing embryo of oviparous animals, including crustaceans. Vitellogenin (Vg) is the precursor of vitellin (Vt) and the main component of yolk proteins (Charniaux-Cotton, 1985). The lipo-glyco-proteinic moiety reserve will be transferred to the eggs and larvae to allow their well development (Serrano-Pinto *et al.*, 2003).

Among researchers concerned with marine invertebrate species, a controversy exists on the site of vitellogenesis. Advance in biomolecular analysis has led to a determination of Vg mRNA expression in crustaceans. In marine species, exogenous Vg

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synthesis in the hepatopancreas of *Penaeus monodon* has been proposed (Tseng *et al*., 2001), of *Metapenaeus ensis* (Kung *et al*., 2004), of *Pandalus hypsinotus* (Tsutsui *et al*., 2004), while endogenous ovarian Vg synthesis has been found in the fiddler crab *Uca pugilator* (Eastman-Reks & Fingerman, 1985); in penaeid shrimps (Yano & Chinzei, 1987; Rankin *et al*., 1989), and in *Callinectes sapidus* (Lee & Watson, 1995). In other penaeid shrimps species the mRNA encoding Vg has been found in both the ovary and the hepatopancreas tissues (Fainzilber *et al*., 1992; Khayat *et al*., 1994; Tsutsui *et al*., 2000, Tsang *et al*., 2003).

In freshwater species the hepatopancreas seems to be the site of synthesis of Vg in *Macrobrachium resenbergii* (Lee & Chang, 1997; Chen *et al*., 1999; Soroka *et al*., 2000; Yang *et al*., 2000; Jayasankar *et al*., 2002; Okuno *et al*., 2002, Jasmani *et al*., 2004); in *M. nipponense* (Han *et al*., 1994) and in freshwater crayfish*Cherax quadricarinatus* (Abdu *et al*., 2002). In this way, the objective of the present study was to determine the site(s) of mRNA expression of Vg in the freshwater crayfish at different stages of female maturation based on RT-PCR analyses.

MATERIALS AND METHODS

Sampling

Freshwater crayfish *Cherax quadricarinatus* were obtained from the laboratory facilities of CIBNOR and maintained at the facilities according to procedures described in Hutchings & Villarreal (1996).

DNA extraction

Genomic DNA of muscle tissue from tail at secondary vitellogenesis, previous spawned, and ovigerous females was extracted by a phenol/ chloroform procedure, followed by ethanol precipitation, as described by Sambrook *et al*. (1989). The different developmental stages of the ovaries were determined following the classification of Sagi *et al*. (1996).

RNA extraction and cDNA synthesis

Total RNA from the ovary and hepatopancreas at secondary vitellogenesis at first maturation, previously spawned, and ovigerous females were extracted with the Trizol reagent (Gibco BRL, Life Technology, USA) according to the instructions of the manufacturer, followed by treatment with DNAase I. Total RNA (30 g) from each tissue were used. Reverse transcription was performed using an Omniscript RT Random Primer Kit (QIAGEN S.A., France).

PCR amplification

Oligonucleotide primers were designed from the 3' end region of the Vg cDNA, based on the sequence reported recently by Abdu *et al*. (2002) (AF306784). Amplification was primed by a pair of oligonucleotides (VgF-5' GTG CGT CGC CTA CTG GAA CA 3' and VgR-5' CTT GGC GGA ATA CTC GGA CTG 3'). PCR conditions were as follows: denaturing at 94ºC for 2 min, and 35 cycles at 94ºC for 1 min, 45ºC for 1 min, and 72ºC for 4 min. A final elongation step was performed at 72ºC for 10 min. PCR reactions were carried out with *Pfu* DNA polymerase (Promega USA), and using 500 ng of template genomic DNA, 20 nmol dNTP, 25 pmol of each primer, and a buffer supplemented with 5% dimethyl sulphoxide (DMSO). PCR products were resolved by electrophoresis on a 1% agarose gel. A 10,000 bp DNA marker (Eurogentec, EGT Group, France) was simultaneously electrophoresed.

cDNA cloning

PCR fragments were purified and cloned into a pGEM-T Easy Vector System (Promega, USA), to transform *Escherichia coli* (strain XLI-Blue), using standard methods (Sambrook *et al*., 1989). Clones containing the PCR inserts were digested with the appropriate restriction enzyme (*Eco* RI) (Promega, USA) and separated on 0.8% low melting point agarose gel (FMC, USA; Sea Plaque GTG agarose).

RESULTS

A PCR product of 1,100 bp from genomic DNA (Fig. 1) corresponding to the 3' end of the vitellogenin (Vg) gene was amplified. Figure 2 shows the schematic view of the Vg cDNA encoding the 3' end region of *C. quadricarinatus* Vg hepatopancreas cDNA used in this study.

RT-PCR analysis showed that the mRNA encoding the 3' end of the vitellogenin gene was

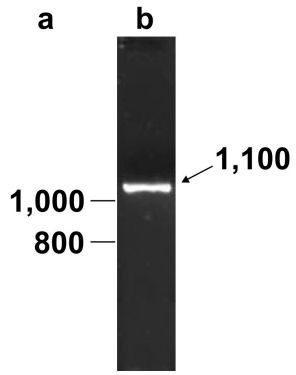

Figure 1. Electrophoregram of the 1,100 bp PCR product from genomic DNA. a) DNA marker (200-10,000 bp) (Eurogentec, EGT Group, France) in a 1% agarose gel in Tris-acetate (TAE) buffer. b) The PCR product corresponds to the 3' end of the full-length vitellogenin cDNA of *Cherax quadricarinatus*.

Figura 1. Electroforegrama de producto de PCR de 1.100 pb a partir de ADN genómico. a) Marcador de ADN (200-10.000S pb) (Eurogentec, EGT Group, France) en gel de agarosa al 1% en amortiguador Tris-acetato (TAE). b) El producto de PCR corresponde al extremo 3' del ADNc del gen de la Vg de *Cherax quadricarinatus*.

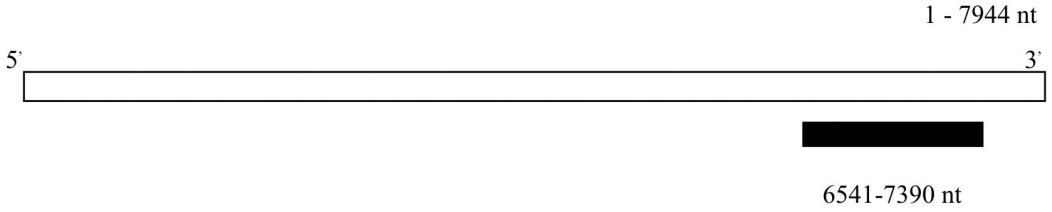

Figure 2. Schematic view of *Cherax quadricarinatus* vitellogenin (Vg) hepatopancreas cDNA (AF306784) (open bar). The solid bar represents the Vg cDNA encoding the 3' end region used in this study.

Figura 2. Vista esquemática del ADNc de hepatopáncreas del gen de la vitelogenina (Vg) de *Cherax quadricarinatus* (AF306784) (barra clara). La barra obscura representa al ADNc del extremo 3' utilizado en este estudio.

present in the hepatopancreas from secondary-vitellogenesis at first maturation, previously spawned, and ovigerous females. Vg mRNA was present simultaneously in the ovary from secondary-vitellogenesis in first maturation females, but was not detected in previously spawned and ovigerous females (Fig. 3). The bands were aproximately 900 bp.

DISCUSSION

mRNA represents the site of expression of the yolk precursor gene, and it is the most reliable criterion for defining the site of Vg synthesis. In crustaceans, Vg is synthesized in multiple organs, depending on species and stage of molting or vitellogenesis (Tsutsui *et al*., 2000; Abdu *et al*., 2002; Avarre *et al*., 2003; Tsang *et al*., 2003).

We detected acumulation of Vg mRNA simultaneously in hepatopancreas and ovary tissues during secondary vitellogenesis of first maturation females, based on RT-PCR analyses. The hepatopancreas Vg mRNA was expressed in secondary vitellogenesis of first maturation, previously spawned, and ovigerous females. The vitellogenin was expressed in ovary tissue in secondary vitellogenesis of first maturation females, but it was not detected in previously spawned and ovigerous females. These results are consistent with previous research showing a complete immunological identity of the hepatopancreas and hemolymph vitellogenin and the egg and ovarian vitellins (Serrano-Pinto *et al*., 2003).

Other investigators reported different levels of mRNA encoding vitellogenin expression in both tissues. Tsutsui *et al*. (2000) found Vg mRNA synthesized simultaneously in hepatopancreas and ovarian tissues of vitellogenic *Penaeus japonicus*

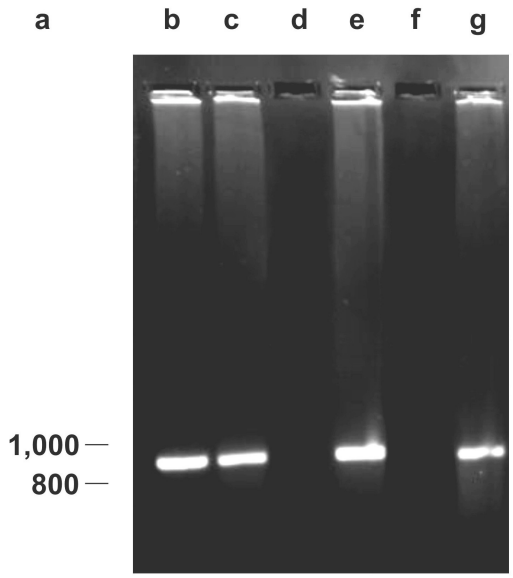

Figure 3. RT-PCR analysis showing the 900 bp mRNA encoding the 3' end of *Cherax quadricarinatus* Vg cDNA: a) DNA marker (200-10,000 bp) (Eurogentec, EGT Group, France; b, c) first maturation female; d, e) previous spawner female; f, g) ovigerous female; b, d, f) ovary; c, e, g) hepatopancreas. Elecrophoresis was performed in a 1% agarose gel in Tris-acetate (TAE) buffer.

Figura 3. El análisis de RT-PCR muestra un ARNm de 900 pb que codifica el extremo 3' del ADNc del gen de la vitelogenina (Vg) de *Cherax quadricarinatus*: a) Marcador de ADN (200-10.000 pb) (Eurogentec, EGT Group, France; b, c) hembras en primera maduración; d, e) hembras con anteriores desoves; f, g) hembras ovígeras; b, d, f) ovario; c, e, g) hepatopáncreas. Elecroforesis en gel de agarosa al 1% en amortiguador Tris-acetato (TAE).

females related to ovarian maturation. They found high mRNA levels in the hepatopancreas during the early and late exogenous vitellogenic stages, but the highest mRNA levels during the early exogenous vitellogenic stage occurred in the ovary. Thereafter, levels rapidly declined. Avarre *et al*. (2003) showed that hepatopancreas and ovarian tissues are involved in the expression of Vg mRNA in *P. semisulcatus*. The Vg from the hepatopancreas is released into the hemolymph and remains in this form, but the Vg in ovary undergoes second cleavage, which probably occurs with a certain delay during ovarian maturation.

The discrepancies between the results obtained in this study and the results obtained by Abdu *et al*. (2002) working with the same species, are probably related to the female maturation stages.

CONCLUSIONS

This study demostrated that mRNA encoding the 3' end of the Vg cDNA was present in the hepatopancreas from secondary vitellogenesis in females at first maturation, previously spawned, and ovigerous. Vg mRNA was present simultaneously in the ovary from females at secondary vitellogenesis at first maturation, but it was not detected at previously spawned and ovigerous females. With these result, we provide evidence that the ovary plays a significant role in the production of this major egg yolk protein, but only in some stages of the vitellogenesis cycle.

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