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Molecular cloning and characterization of cortical rod protein in the giant freshwater prawn *Macrobrachium rosenbergii,* a species not forming cortical rod structures in the oocytes

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Abstract

The formation of cortical rod structures is a characteristic of fully mature oocytes in penaeid prawns, but such structures are absent from oocytes of giant freshwater prawn *Macrobrachium rosenbergii*. In the present study, we first demonstrated the presence of a 30-kDa protein, which was immunologically related to kuruma prawn cortical rod protein (CRP), in the ovary of giant freshwater prawn, and subsequently purified this protein. Furthermore, a cDNA encoding the CRP-like protein was isolated. Based on the high homology (98%) in the amino acid sequence with kuruma prawn CRP, the 30-kDa protein has been identified as a CRP homologue of giant freshwater prawn, designated *mr*CRP. The RT-PCR analysis revealed that *mr*CRP mRNA was present in the ovary from a prawn with a gonadosomatic index (GSI) of 0.2. Western blot analysis revealed the presence of a CRP-immunoreactive band of 30kDa in the ovary with GSI of 1.6. By immunocytochemistry, CRP-immunopositive signals were detected in the ovary with GSI of 0.9, that had started to accumulate considerable amounts of vitellins and lipids in the peripheral cytoplasm. With progress of vitellogenesis, *mr*CRP was apparently accumulated in the mature oocytes, although it was not detectable, presumably because a relatively small amount of *mr*CRP was masked with large amounts of vitellin and lipids. In giant freshwater prawn without forming cortical rod structures, our findings indicate that the oocytes produce *mr*CRP, a homologue of CRP found in penaeid prawns.

Keywords: Cortical rod protein; Oocytes; *Macrobrachium rosenbergii*

1. Introduction

The giant freshwater prawn *Macrobrachium rosenbergii* and kuruma prawn *Marsupenaeus japonicus* are economically important species for shrimp and prawn farming. However, the physiological mechanisms controlling their reproduction still remain unknown in many respects of hormonal regulation, environmental and nutritive factors, and yolk protein characteristics. For stable and profitable shrimp and prawn farming, it is necessary to supply juveniles stably by means of artificial production of juveniles. Since mother prawns are collected from the wild stock for farming at present, juvenile production is possible only during the natural breeding seasons. To seek effective ways of artificially inducing maturation and producing juveniles, studies on crustacean oogenesis and its regulation have been conducted, but such attempts have not been very successful thus far (Suitoh, 1996; Yamano et al., 2003, 2004).

In the penaeid prawns, biological events leading to ovarian maturation include two important steps, vitellogenesis and cortical rod formation (Avarre et al., 2001; Khayat et al., 2001; Kim et al., 2005; Okumura et al., 2006). As in all ovaiparous animals, vitellogenesis is a process in which oocytes accumulate a large amount of yolk materials via the internalization of a yolk precursor protein, vitellogenin (Vg). This is a female-specific complex carotenolipoprotein synthesized during the reproductive

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phase in crustacean (Meusy and Payen, 1988), which is transformed into vitellin (Vt) on its uptake into the oocytes. Subsequently, Vt undergoes further processing into several subunits. In kuruma prawn, ovarian Vt consists of three polypeptide subunits of 91-kDa, 128-kDa and 186-kDa (Kawazoe et al., 2000), which serves as nutritive materials needed for embryonic growth and development (Tsukimura, 2001). Tsutsui et al. (2000) have shown that, in kuruma prawn, both the ovary and hepatopancreas are the sites of Vg synthesis.

Toward the completion of vitellogenesis, on the other hand, cortical rods first appear in the periphery of the oocytes. Since the appearance of cortical rods is restricted to the fully matured oocytes, this serves as an indicator of upcoming spawning. The cortical rods contain highly-organized and tightly-packed "bottle-brush" or "feathery" structures (Clark et al., 1990). Recently, the components of cortical rods in penaeid prawns have been identified; that is, shrimp ovarian peritrophin-like protein (SOP), cortical rod protein (CRP), and extracellular matrix proteins in a thrombospodin (*mj*TSP), and their amino acid and nucleotide sequences were determined (Khayat et al., 2001; Kim et al., 2004; Yamano et al., 2003, 2004). Our previous study has revealed that the oocytes of kuruma prawn are responsible for CRP synthesis (Kim et al., 2005). When the oocytes are spawned into seawater, the contents of cortical rods are released, forming a jelly layer, a corona, around the egg (Clark et al., 1990). The jelly layer is thought to function as a barrier against polyspermy and external environments until the second embryo cleavage (for review, see Guraya, 1982). After spawning, the surface coat covers the egg beneath the jelly layer, followed by the formation of the hatching envelope a few minutes later.

Employing real-time quantitative PCR analysis, we have demonstrated in the previous study that the major yolk proteins, vitellin and CRP, show different patterns in gene expression during ovarian development of kuruma prawn (Kim et al., 2005). The CRP mRNA is highly expressed and accumulated in the previtellogenic oocytes, while Vg mRNA in the ovary and hepatopancreas is low during this early developmental stage. The CRP mRNA levels remain high in the following endogenous vitellogenic stage, but decrease rapidly in the more advanced stages. In contrast, Vg mRNA in the ovary showed high levels in the endogenous vitellogenic stage, followed by a decrease in the later stages. As maturation proceeds, the expression site of Vg mRNA shifts from the ovary to hepatopancreas; Vg mRNA levels in the hepatopancreas increase in the exogenous and maturation stages, in which ovarian Vg mRNA levels stay low.

In giant freshwater prawn, vitellogenic processes are apparently different from those in kuruma prawn; Vg is synthesized only in the hepatopancreas, but not in the ovary or ovarian follicles (Yang et al., 2000). In this species, Vg synthesized in the hepatopancreas is proteolytically cleaved into 199-kDa and 90-kDa components before being released into hemolymph. The 199-kDa component undergoes further processing to 90-kDa and 102-kDa components to give rise to three separate vitellin proteins before being incorporated into the oocytes (Okuno et al., 2002).

In spite of increasing knowledge of vitellins in giant freshwater prawn, information is very limited on CRP. Since cortical rods are not formed even in the fully matured oocytes, it is generally believed that this prawn species lacks CRP in the oocytes. In the present study, we first demonstrated the presence of a 30-kDa protein that reacted with an antiserum raised against 28.6-kDa CRP of kuruma prawn in western blot analysis, and subsequently purified this protein. Furthermore, we isolated cDNA encoding the CRP-like, 30-kDa protein in the giant freshwater prawn ovary. Based on the high identity (98%) in the amino acid sequence with kuruma prawn CRP, we identified the 30-kDa protein as a CRP homologue of giant freshwater prawn. We also examined the expression pattern of giant freshwater prawn CRP during ovarian development.

2. Materials and methods

2.1. Samples

Immature and mature female giant freshwater prawns, *Macrobrachium rosenbergii*, obtained from a commercial source in China, were used for this study. For biochemical and molecular cloning, the ovaries were dissected out, frozen immediately in liquid nitrogen, and stored at − 80°C until use. Parts of the ovary were fixed in 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB, pH 7.4) for 24 h for histological observations and immunocytochemistry.

2.2. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

For SDS-PAGE and western blot analysis, we used four prawns of different maturity (body mass (BW), 10.5–46.7g; gonadosomatic index (GSI), 0.5–6.4). The ovaries were homogenized in SDS sample buffer (50mM Tris–HCI buffer, pH 6.8, 2% SDS, 10% Glycerol, and 0.002% Bromophenol blue) using a Handy Sonic homogenizer UR-20P (Tomy Seiko, Tokyo, Japan). The homogenate was centrifuged at 10,000×*g* for 10min at 4°C. This procedure was repeated twice. The combined supernatant was then subjected to SDS-PAGE, which was performed on a 15–25% gradient polyacrylamide gel (Daiichi, Tokyo, Japan) (Laemmli, 1970). The gel was stained with Coomassie Brilliant Blue R-250 (CBB). After SDS-PAGE, western blot analysis was carried out according to the method reported previously (Kim et al., 2004). Briefly, the proteins were transferred onto a polyvinylidene-difluoride (PVDF) membrane, blocked with a block solution, and incubated with an antiserum raised against purified 28.6-kDa CRP from kuruma prawn (Kim et al., 2004) as the primary antibody (1:1,000 dilution). The membrane was incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) and ABC (Vector Laboratories). Finally, immunoreactive bands were visualized by a 0.1M Tris–HCI buffer solution (pH 7.5) containing 0.05% 3, 3′-diaminobenzidine tetrahydrochloride (DAB) and 0.01% H_2O_2.

2.3. Extraction and purification of CRP-like protein

An ovary from a giant freshwater prawn (BW, 30.2g; GSI, 1.6) was homogenized in 20mM phosphate buffered saline (PBS), pH 7.0, containing 2mM phenylmethylsulfonylfluoride