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SHORT COMMUNICATION

Expression of clottable protein of tiger shrimp (*Penaeus monodon*) in gonads and its possible role as nutrient source for the embryo

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Summary
We have investigated the expression of clottable protein (CP) in gonad of tiger shrimp (*Penaeus monodon*) and extent of its phosphorylation. Polyclonal antibodies against purified CP were prepared from rabbit serum. Using this anti-CP antiserum, the temporal expression of CP in gonads of tiger shrimp was analyzed. It was found that the CP occurs only in mature ovaries but not in immature ovaries and testes. Results of RT-PCR confirmed that these tissues expressed low levels of CP mRNA transcripts. Upon eyestalk-ablation, the ovaries in female shrimps were induced to develop, and the CP expression levels in ovaries were traced chronically by RT-PCR analyses. The expression level peaked on day 3 with an increase of about 40 folds relative to the basal level and returned to normal level (as the control shrimp) at day 12. The shrimp embryos at different intervals from spawning to 16 h post-spawning were also collected, and it was found that CP contents were gradually decreased in the embryos until the nauplii were hatched. In addition, purified CP was shown to react with specific anti-phosphoserine, anti-phosphothreonine, and anti-phosphotyrosine antibodies suggesting that CP is a phosphoprotein with all types of phosphorylations. Taken together the results suggest that expression of CP in shrimp ovaries is coupled to ovarian development and CP possibly supply nutrition for shrimp embryo.

Introduction

Efficient immune systems and clotting reactions are of vital importance to both vertebrate and invertebrate animals. Coagulation of the hemolymph is part of the innate immune

Abbreviations: CP, clottable protein; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis; VTG, vitellogenin.

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response in Crustacea, it prevents leakage of hemolymph and dissemination of invaders such as bacteria throughout the body. Two coagulation mechanisms have been currently reported in invertebrates [1]. One involves the polymerization of clotting protein molecules by hemocyte transglutaminases [2–10]. The other consists of a proteolytic cascade activated by LPS and β-1, 3-D-glucans in horseshoe crab [11]. Upon activation of its clotting enzyme by a cascade of reactions, two peptidyl bonds in a coagulogen molecule are cleaved, resulting in formation of an insoluble gel and release of a peptide of 28 residues [11].

The hemolymph clottable proteins (CPs) from various crustacean species has been purified and characterized including crayfishes [4,5], shrimps [6–9,12], and lobsters [13,14]. These CPs are homodimeric glycoproteins of about 400 kDa capable of forming oligomers or insoluble clots [14]. Besides playing a clotting role, CP is a very high-density lipoprotein, which was first described to be involved in lipid transport in crayfish [15]. The same phenomena were also described in other shrimp CPs [5,8,9,12]. Four shrimp CPs have been cloned and their deduced amino acid sequences showed limited similarities to those of vitellogenins [16–18]. In addition, haemocyte clotting enzymes (type II transglutaminases) of tiger shrimp [19–21] and other shrimp [22] or crayfish [23] have been cloned and characterized. When activated by physiological level of plasma calcium ion, the hemocyte-released transglutaminases facilitate inter- and intramolecular cross-linking between the Gln and Lys residues in the CP to cause coagulation. Contents of CP in shrimp hemolymph are closely related with the coagulation and hemostasis of the animals [24]. Moreover, recent studies on shrimp immunity using RNA-interference also revealed that transglutaminase and CP are critical molecules for the immune function of shrimp against bacterial and viral infection [25].

Tiger shrimp (*Penaeus monodon*) is an economically important species cultured in Taiwan and southeastern Asia. Previous studies have shown that its plasma CP level was sensitively controlled to maintain hemostasis and could be replenished in traumatic conditions [26]. The gill, central nervous system, and lymphoid organs were found to be the major tissues producing CP in tiger shrimp [26]. The crustacean CP genes are constitutively expressed in both males and females and they are evolutionarily related to vitellogenins (VTGs), which are nevertheless expressed in female animals only [4]. In order to understand the sexual variations in the expression and regulation of shrimp CP, we herein examined the expression of CP in shrimp gonads with Western blot and RT-PCR methods. The CP expression patterns in developing ovaries and hatching eggs were both surveyed. In addition, the phosphorylation status of CP was investigated and possible function role of the CP in shrimp eggs was discussed.

Material and methods

Shrimp culture and management

Immature and mature tiger shrimps, *P. monodon* (60–80 g in body weight) were obtained from an aquaculture farm in Tungkang Marine Laboratory of Taiwan Fisheries Research Institute. The shrimps were reared in plastic tanks supplied with constantly flowing seawater. They were fed on synthetic feed pellets, equivalent to 5% of their body weight twice a day. The molting stage was inferred by examining the partial retraction of the epidermis of the uropods [27]. They are the following: (1) A and B for the postmoult; (2) C for the intermoult; and (3) D_0, D_1, D_2, and D_3 for the premoult. In penaeid shrimp species, VTG synthesis in both ovary and hepatopancreas are under the inhibitory regulation of a neuroendocrine system, the X-organ/sinus gland complex in the paired eyestalks [28,29], and eyestalk ablation (removal of the X-organ/sinus gland complex) is widely used for inducing ovarian development. An eyestalk was ablated at the middle of the optic stalks using fine scissors in the intermolt or early premolt stage (stage C or D_0). The ablated shrimps were kept individually in compartments (20 × 15 × 18 cm) with circulating seawater (flow rate, 250 mL/min) at 28 °C and were sacrificed on 0 (initial sampling), 3, 6, and 12 days after eyestalk ablation (4–5 animals for each sampling) for ovaries collection. Four stages of ovarian maturation, stages I–IV, were visually determined according to that described by King [30] and Yano et al. [31]. The shrimp ovaries, which reached stages III were considered as mature. The spawned eggs were hatched at 28 °C and collected at different intervals of 0, 2, 4, 6, 8, 10, 12, and 16 h post-spawning for CP expression analyses. At 16 h post-spawning, most of the developing eggs were hatched into nauplii under the culturing condition.

Hemolymph collection

Shrimps (20–25 g each) were purchased from local markets and kept in aerated seawater. The hemolymph (0.6–1 ml) was withdrawn from the ventral sinus located in the first abdominal segment, using a 23 G gauged 2.5 ml syringe containing 0.06–0.1 ml anticoagulant (50 mM EGTA, 18 mM Tris–HCl, 0.35 M NaCl, 13 mM KCl, 1.67 mM D-glucose, pH 7.5) [32]. The hemocytes were immediately spun down at 300*g*, 4 °C for 10 min, and the supernatant was pooled for preparing the plasma CP.

Protein purification

The plasma was dialyzed against 50 mM Tris–HCl, 1 mM EDTA, pH 8.0 (TE buffer) for 14 h at 4 °C. It was subjected twice to ion exchange chromatography at 4 °C with a TSK DEAE-650 (S) column (2.5 cm × 9 cm) pre-equilibrated with TE buffer, with or without 0.1 M NaCl. The CP was eluted with a stepwise gradient of NaCl (0.21, 0.24, and 0.3 M) [6]. Protein concentrations were determined by the Bradford method [33], using bovine serum albumin as standard.

The purified CP was subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) [34] with a 5% or 7.5% acrylamide gel, and stained with Coomassie brilliant blue R-250.

Antiserum and immunoblotting

The 190 kDa CP bands obtained in 5% gel after the SDS-PAGE was stained, cut and eluted. After lyophilization, it was used