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Short communication

Differential regulation of hepatopancreatic vitellogenin (VTG) gene expression by two putative molt-inhibiting hormones (MIH1/2) in Pacific white shrimp (*Litopenaeus vannamei*)

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ABSTRACT

Molt-inhibiting hormone (MIH), a peptide member of the crustacean hyperglycemic hormone (CHH) family, is commonly considered as a negative regulator during the molt cycle in crustaceans. Phylogenetic analysis of CHH family peptides in penaeidae shrimps suggested that there is no significant differentiation between MIH and vitellogenesis-inhibiting hormone (VIH, another peptide member of CHH family), by far the most potent negative regulator of crustacean vitellogenesis known. Thus, MIH may also play a role in regulating vitellogenesis. In this study, two previously reported putative MIHs (LivMIH1 and LivMIH2) in the Pacific white shrimp (*Litopenaeus vannamei*) were expressed in *Escherichia coli*, purified by immobilized metal ion affinity chromatography (IMAC) and further confirmed by western blot. Regulation of vitellogenin (VTG) mRNA expression by recombinant LivMIH1 and LivMIH2 challenge was performed by both *in vitro* hepatopancreatic primary cells culture and *in vivo* injection approaches. In *in vitro* primary culture of shrimp hepatopancreatic cells, only LivMIH2 but not LivMIH1 administration could improve the mRNA expression of VTG. In *in vivo* injection experiments, similarly, only LivMIH2 but not LivMIH1 could stimulate hepatopancreatic VTG gene expression and induce ovary maturation. Our study may provide evidence for one isoform of MIH (MIH2 in *L. vannamei*) may serve as one of the mediators of the physiological progress of molting and vitellogenesis. Our study may also give new insight in CHH family peptides regulating reproduction in crustaceans, in particular penaeidae shrimps.

Introduction

In crustaceans, molt-inhibiting hormone (MIH) is a neuropeptide produced by neurosecretory cell soma located in the medulla terminalis X-organ of the eyestalks and then transported to the neurohemal sinus gland, from which MIH is released into hemolymph [12]. Structurally, MIH is considered as a member of the crustacean hyperglycemic hormone (CHH) family. Other peptide hormones in the CHH family include CHH, vitellogenesis-inhibiting hormone (VIH), mandibular organ inhibiting hormone (MOIH) and ion transport peptide (ITP) [5,12]. Traditionally, based on the protein primary sequences and structures, the CHH superfamily is divided into two subfamilies, namely CHH-I (containing CHH and ITP) and CHH-II (containing MIH, VIH, and MOIH) [12]. Hormones from the CHH family are found to often display overlapped biological functions such as: regulation of carbohydrate metabolism, osmostasis, molting and reproduction [29].

Traditionally, the basic function of MIH is found in control of molting rhythm during the molt cycle [12,29]. Molting is an essential and repeated physiological process for somatic growth in arthropods [21]. In 1990, the first peptide with molt-inhibiting activity has been isolated from the sinus gland extracts of lobster,

Abbreviations: MIH, molt-inhibiting hormone; VTG, vitellogenin; CHH, crustacean hyperglycemic hormone; VIH, vitellogenesis-inhibiting hormone; MOIH, mandibular organ inhibiting hormone; ITP, ion transport peptide; IMAC, immobilized metal ion affinity chromatography; ANOVA, analysis of variance; ORFs, open reading frames; GSI, gonadosomatic index; IPTG, isopropyl-β-D-1-thiogalactopyranoside; EDTA, ethylene diamine tetraacetic acid; BCA, bicinchoninic acid; PVDF, polyvinylidene difluoride; TBST, Tris-buffered saline with 0.05% Tween 20; HBSS, Hanks balanced salt solution; DMEM, Dulbecco modified Eagle medium.

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Homarus americanus [2]. Based on *in vitro* co-incubation [6,28] and/or *in vivo* injection experiments [13,16,18,31] performed in different crustacean species, it is found that MIH administration may decrease hemolymph ecdysteroid that synthesized in the Y-organ, and subsequently prolong the duration of the molt cycle. To date, the cDNA and amino acid sequences of MIH have been reported in several crustacean species such as *Litopenaeus vannamei* [3], *Metapenaeus ensis* [6], *Penaeus monodon* [31] and *Marsupenaeus japonicus* [15].

The ovarian development of oviparous animals is characterized by the accumulation of a major yolk protein (vitellin) in the oocytes (namely vitellogenesis) [8]. The precursor of vitellin is called vitellogenin (VTG). In different crustacean species, VTG is synthesized only in the ovary [8,17], or only in the hepatopancreas [10,27], or both in the ovary and hepatopancreas [7,20], and then transported to the oocytes subsequent accumulated in the ooplasm as vitellin after further modification. The process of vitellogenesis in crustaceans is widely assumed to be negatively controlled by VIH [4,23,26]. However, recent studies revealed that other members of CHH family may also play a role in regulating vitellogenesis [5,12,25,29]. MIH is one of the potential reproductive regulators of CHH family. In *M. ensis*, injection of an MIH isoform (MIH-B) can increase the mRNA and protein levels of VTG in ovary and hepatopancreas [22]. In *Callinectes sapidus*, MIH can stimulate vitellogenesis at early ovarian stages in addition to molt-inhibiting [32,33]. However, in kuruma prawn, *M. japonicus*, neither MIH-A nor MIH-B may significant alter VTG gene expression in *in vitro* ovarian fragments incubation [24]. Therefore, it is believed that the regulation of MIH on reproduction in crustaceans is still controversial and need to be further studied.

The Pacific white shrimp (*L. vannamei*) is commercially the most significant shrimp species, development of whose aquaculture has been limited by the artificial ripening of female gonads. In *L. vannamei*, two forms of MIH-like cDNA (LivMIH1 and LivMIH2) have been cloned and characterized previously [3]. The transcripts of LivMIH1 and LivMIH2 have only been detected in the eyestalk ganglia, but not in other tissues. The mRNA levels of LivMIH1 and LivMIH2 are found to be followed a molt-stage related expression [3]. To test whether LivMIH1 and LivMIH2 having some regulatory functions other than molt inhibition in *L. vannamei*, in this study, recombinant protein of LivMIH1 and LivMIH2 was generated in an *Escherichia coli* expression system. The regulation of VTG mRNA expression by recombinant LivMIH1 and LivMIH2 were performed with both *in vivo* injection and *in vitro* hepatopancreatic primary cells culture approaches. Our study may give new insight in CHH family peptides regulating reproduction in crustaceans.

Material and methods

Animals

The Pacific white shrimps (10–15 g in body weight) used for molecular cloning and hepatopancreatic cell culture were purchased in a local market. Previtellogenic female shrimps used in *in vivo* experiments, with a body length of 21 ± 0.5 cm and a body weight of 27.6 ± 0.9 g, were obtained from Dongfang Shrimp Culture Center, Zhanjiang, China. Ovarian developmental stages of shrimp were judged according to criteria established previously [30]. All the shrimps were acclimated in cement tanks containing aerated seawater (salinity 31‰ and pH 7.5) at 28 °C under a 12D:12L photoperiod.

Expression and purification of recombinant LivMIH1/2 in E. coli

To obtain the open reading frames (ORFs) of LivMIH1 and LivMIH2, two pairs of primers were designed (Table 1) based on the previously reported LivMIH1 and LivMIH2 sequences with accession no. AY425615 and AY425616 respectively [3]. The amplification products were then sub-cloned into the expression vector PET28a with *Bam*H I and *Xho* I double digestion. The recombinant plasmids generated were introduced into the host BL 21(DE3) *E. coli* by transformation. Transformed cells were grown in Luria broth containing 25 μg/mL kanamycin at 37 °C. When optic density at 600 nm reached 0.6, isopropyl-β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM was added and bacterial cells were grown for another 10 h at 37 °C for LivMIH1 expression and 8 h at 37 °C for LivMIH2 expression.

Cultured *E. coli* cells were then collected by centrifugation, resuspended in PBS buffer and dissociated by sonication. After separated from soluble fraction by centrifugation at 12,000 × *g* for 10 min at 4 °C, the insoluble fraction was washed twice with inclusion body washing solution I (100 mM Tris–Cl, 10 mM EDTA and 10% Triton X-100, pH 8.0) and II (100 mM Tris–Cl, 10 mM EDTA and 2 M urea, pH 8.0). The recombinant LivMIH1 and LivMIH2 protein that appeared in the inclusion body was dissolved in 8 M urea and purified by using His-Bind Kits (Novagen, Germany) under a denaturing condition. For refolding, purified protein was dialyzed against serially diluted dialysis buffers to remove all urea. After removing precipitated protein by centrifugation at 12,000 × *g* for 10 min at 4 °C, the supernatant (purified and natural protein) was then snap-frozen in liquid nitrogen and stored at −80 °C for further study. The protein content of LivMIH1 and LivMIH2 was determined by bicinchoninic acid (BCA) method (Sangon, China).

Western blotting of recombinant LivMIH1/2

The expressed and purified recombinant LivMIH1 and LivMIH2 were analyzed by western blot. After separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Roche, Germany) by Trans-Blot SD semi-dry electrophoretic transfer cell (Bio-Rad, USA). After that, the membranes were blocked with 5% nonfat dry milk and hybridized with 1:1000 rabbit anti-6 × his polyclonal antibody (Sangon, China) in Tris-buffered saline with 0.05% Tween 20 (TBST). The second antibody used was 1:1000 horseradish peroxidase conjugated goat anti-rabbit IgG (Thermo Scientific, USA). Detection of the recombinant proteins was conducted by X-ray film exposure with SuperSignal West Pico Substrate (Thermo Scientific).

Isolation of shrimp hepatopancreatic cells

Shrimp hepatopancreatic cells were isolated based on the method described previously [4]. Briefly, hepatopancreas from 10 Pacific white shrimps were removed, washed three times in prechilled Ca^{2+}/Mg^{2+}-free Hanks balanced salt solution (HBSS; Sigma; containing 4 mM $NaHCO_3$, 9 mM HEPES, 100 U/mL penicillin, and 100 μg/mL streptomycin, pH 7.63), minced into 0.5-mm thickness and incubated in Ca^{2+}/Mg^{2+}-free HBSS with EDTA (1 mM) at room temperature for 5 min. After that, the hepatopancreatic fragments were digested with collagenase type IV (1 mg/mL, Sigma) and DNase II (0.01 mg/mL, Sigma) in Ca^{2+}/Mg^{2+}-free HBSS at 28 °C for 30 min and dispersed into single cells by gentle pipetting. Dispersed hepatopancreatic cells were then separated from the remaining fragments by filtration through a sterile 30-μm mesh and harvested by centrifugation at 100 × *g* for 5 min at 4 °C. The cells obtained were cultured in Dulbecco modified Eagle medium (DMEM)/F-12 (1:1; Gibco BRL, USA) with 14 mM $NaHCO_3$, 2 g/L bovine serum albumin, 100 U/mL penicillin, and 100 μg/mL streptomycin, pH 7.63. Only those cells showing more than 95% viability evaluated by Trypan blue exclusion assay were used in subsequent experiment. After being diluted to 0.4 × 10^6 cells/mL, the cells were

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