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Distribution of crustacean hyperglycemic hormones (CHH) in the mud crab (*Scylla olivacea*) and their differential expression following serotonin stimulation

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ABSTRACT

Crustacean hyperglycemic hormone (CHH) has gained wide attention for its diverse physiological functions, including in metabolism and reproduction. In this study, cDNA sequence encoding CHH was cloned from the brain and ventral nerve cord (VNC) of the mud crab, *Scylla olivacea*. CHH of *S. olivacea* (*Scyol-CHH*) consists of 642 nucleotides, which encode a 108-amino acid CHH prepropeptide. Based on in situ hybridization, *Scyol-CHH* was expressed in the neurons within the eyestalk, including at neuronal clusters 1-4. *Scyol-CHH* was also detected in the neurons within the neuronal clusters 6 and 8-11 of the brain. In the VNC, its expression was found in the neurons within the subesophageal ganglion as well as the abdominal ganglion. We found that administration of serotonin (5-HT) significantly increased the expression of *Scyol-CHH* in the brain and VNC at 3 and 6 h post-injections, respectively, as compared with the controls. This suggests that 5-HT may play an important role in the regulation of CHH expression in the nervous system. Moreover, injection of 5-HT up-regulated *Scyol-CHH* expression in ovary, suggesting a reproductive-related role for 5-HT and CHH in *S. olivacea*.

**Keywords**: Crustacean hyperglycemic hormone, Serotonin, Central nervous system, Reproduction, *Scylla olivacea*
1. Introduction

Crustacean hyperglycemic hormone (CHH) is a neuropeptide hormone that is synthesized and released from the neurosecretory complex within the eyestalks of decapod crustaceans, called the X-organ-sinus gland complex (XO-SG complex) (Cooke and Sullivan, 1982). Generally, CHH functionally elevates intracellular glucose through the degradation of glycogen by activating the enzyme phosphorylase, lipid metabolism, stress-induced hyperglycemia, and it regulates carbohydrate metabolism (Keller, 1992). CHH is also associated with molting, ion and water balance, and reproduction (Hohnke and Scheer, 1970; de Kleijn et al., 1998; Chung et al., 1999; Santos et al., 1997; Lorenzon et al., 1997; Lee et al., 2014). CHH belongs to the CHH neuropeptide family in which two members are classified based on their precursor and primary structures, including type-I and -II peptides. Type-I peptides include the CHH and ion transport peptide (ITP), while type-II peptides include molt-inhibiting hormone (MIH), gonad-inhibiting hormone/vitellogenesis-inhibiting hormone (GIH/VIH), and mandibular organ-inhibiting hormone (MOIH) (Lacombe et al., 1999; Webster et al., 2012). Regarding biological activities, CHH-superfamily members are multifunctional peptides, which play a role in the control of carbohydrate metabolism, molting, gonad maturation and osmotic regulation. Expression of CHH is not limited to the eyestalk as its expression is also detected in the other organs such as the brain, pericardial organ, reproductive organ, gut, gill, stomach and muscle (Tsai et al., 2008). Based on molecular cloning, multiple isoforms of CHH are likely generated by mRNA alternative splicing while CHH-primary structure has been directly determined by protein sequencing in various species, including crabs, crayfish, lobsters, shrimp, and an isopod (de Kleijn and Herp, 1995; Soyez, 1997; Lacombe et al., 1999; Chang et al., 1999). Likewise, CHH has been identified in the mud crab, Scylla olivacea, in which two isoforms, which are presumably a result from alternative splicing, are present such as Sco-CHH and Sco-CHH-L.
(CHH-like peptide). Full-length of Sco-CHH complementary DNA (cDNA) sequence was cloned from the eyestalk ganglia, whereas Sco-CHH-L cDNA was cloned from extra-eyestalk tissues (i.e., pericardial organ and thoracic ganglia) (Tsai et al. 2008). These two preprohormones possess an identical signal peptide, CHH-precursor-related peptides (CPRP), and the N-terminal 40 amino acid residues of CHH mature peptide, while the remaining C-terminal region is substantially different. Both CHH variants contain six highly conserved cysteine residues that are a characteristic of the CHH family peptides, and share higher sequence identities with other brachyuran CHH sequences than those of other decapods (Tsai et al., 2008). We hypothesize that a different spliced form of CHH in *S. olivacea* may have a different biological activity.

Considering the role of CHH in reproduction, it has been reported that CHH stimulates oocyte growth *in vitro* in *Homarus americanus* (Tensen et al., 1989), while CHH inhibited the synthesis of vitellin protein and ovarian RNA in the ovarian explants of *Penaeus semisulcatus* (Khayat et al., 1998). The stimulatory effect of CHH on cGMP production in the hepatopancreas obtained from the vitellogenic adult female, has been demonstrated in the blue crab, *Callinectes sapidus* (Zmora et al., 2009). In *C. maenas*, an increase in cGMP level after the incubation of CHH was observed in the gill, midgut, and hindgut glands, and this was correlated with a high glucose concentration in those tissues (Chung et al., 2006). Moreover, CHH expression increased following ovarian developmental stages (from stage I to IV), and decreased at premoult stage as determined by quantitative PCR (qPCR), which suggests that CHH might promote vitellogenesis in the mud crab, *Scylla paramamosain* (Fu et al., 2014). As indicated by *in situ* hybridization, CHH mRNA expression localized at the perikarya of neurosecretory cells of the X-organ (Fu et al., 2014). Recently, several other reports have shown the CHH transcript expression in the reproductive organs, which suggested that CHH might be involved in reproduction (Tsai et al., 2008; Bao et al., 2015).
Serotonin (5-hydroxytryptophan or 5-HT) is a neurotransmitter that regulates many physiological functions in crustaceans, including growth, behavior, and gonad maturation (Sainath and Raddy, 2011). Various studies have revealed the expression and distribution of 5-HT in the CNS and gonads of decapods (Tinikul et al., 2009; Khornchatri et al., 2015). In *S. olivacea*, the role of 5-HT in the stimulation of other neurohormones and reproductive related proteins that are important for reproduction, i.e. red pigment-concentrating hormone (RPCH) and farnesoic acid O-methyltransferase (FAMeT), has been extensively studied (Kornthong et al., 2013, 2014). Although CHH expression in reproductive organs has been studied, the relationship between 5-HT and CHH has never been investigated. In this study, we hence investigate the presence of CHH transcript in our previously generated *S. olivacea* transcriptome (Kornthong et al., 2014), and explore the CHH expression in the *S. olivacea* CNS and ovary. In addition, the effect of 5-HT-primed administration on CHH expression is also investigated.

2. Materials and Methods

2.1. Experimental animals

Mature mud crabs, *S. olivacea* [400 ± 50 g body weight (BW) and 100-120 mm carapace width], were obtained from a commercial farm, Chanthaburi province, Thailand. Animals were kept in the concrete tanks filled with aerated seawater at 26–28 °C, and approximately 50% of seawater was changed every day. The crabs were kept under a photoperiod of 12:12 h light-dark cycle and fed with food pellets twice daily for 10 days for acclimatization before experimental treatment.

2.2. RNA preparation and gene identification

Various organs (gill, gut, brain, thoracic ganglion, heart, hepatopancreas, eyestalk,
ovaries I-V) were collected separately from the mud crabs, and immediately frozen in liquid nitrogen before being stored at -80 °C until used. Total RNA was prepared from each tissue using Trizol reagent (Invitrogen, CA, USA), following the manufacturer’s protocol, and kept at -80 °C until used. The purity and quantity of RNA was measured by using a nanodrop spectrophotometer. Total RNA (1 µg) was used for first-strand cDNA synthesis, using QuantiTect Reverse Transcription Kit (Qiagen, Germany), following the manufacturer’s protocol. For PCR amplification of CHH gene, primers were designed from the CHH transcript derived from S. olivacea transcriptomes (Kornthong et al., 2014). cDNA was subsequently used as a template for PCR, using the forward and reverse primers shown in Table 1. Thermocycling condition used for PCR amplification was set as follows: one cycle at 94 °C for 5 min, followed by 35 cycles of 30 sec at 94°C, 45 sec at 55°C, and 45 sec at 72°C, with a final extension of 10 min at 72 °C.

To identify a full-length CHH gene, 5’ RACE and 3’ RACE were performed using the SMARTer RACE 5’ and 3’ cDNA library construction kit (Clontech, CA, USA), following the manufacturer’s protocols. Universal primers and gene-specific primers (5CHHR and 3CHHF) were used to obtain the complete gene sequence (Table 1). Thermo cycling condition was set as follows: one cycle at 94°C for 5min, followed by 35 cycles of 30 sec at 94°C, 30 sec at 52°C, and 1 min at 72°C, with a final extension of 10 min at 72°C.

All amplification products were analyzed by gel electrophoresis using 2% agarose gel. The amplicon with predicted full-length size was purified using a GeneJET gel extraction kit (Thermo Scientific, USA), and inserted into a pGEM®-T Easy vector (Promega, USA). Plasmids with insert sequences were purified using GeneJET Plasmid Mini-prep Kit (Thermo Scientific, USA) before they were sent for sequencing (Macrogen, Korea).

2.3. CHH gene expression in various tissues by in situ hybridization
The *S. olivacea* CHH gene was amplified by PCR using purified plasmid with gene insert as template, and M13 primers (forward primer: 5’-GTAAAACGACGGCCAGT-3’; reverse primer: 5’-AACAGCTATGACCATG-3’). PCR products were extracted using GeneJET gel extraction kit (Thermo Scientific, USA). The CHH riboprobe was synthesized using a DIG-oligonucleotide labeling kit (Roche, Germany). Mud crab eyestalks, brains, and ventral nerve cords (VNCs) were dissected and immediately fixed in the Bouin’s fixative for 12-15h. Tissues were then processed before embedding into paraffin blocks. Consecutive paraffin sections (5-6 μm-thick) were cut and mounted onto silane-coated slides. Sections were deparaffinized by being immersed in fresh xylene twice (10 min each), and subsequently rehydrated by being immersed in a decreasing concentration of ethanol solutions. The sections were then treated with a TE buffer [100 mM Tris-HCl, 50 mM EDTA, pH 8.0) containing RNase-free proteinase-K (5 μg/ml) (Roche, Germany)], at 37 °C for 30 min. Post-fixation was performed by incubating sections with DEPC-treated PBS containing 4% paraformaldehyde, at 4 °C for 5 min. The sections were then incubated with prehybridization buffer (4x SSC containing 50% deionized formamide), at 37 °C for 10 min, to prevent non-specific binding. After prehybridization, the buffer was removed and the sections were incubated with hybridization buffer (40% deionized formamide, 10% dextran sulfate, 1xDenhardt’s solution, 4x SSC 10 mM DTT, 1 mg/ml yeast t-RNA, 1 mg/ml denatured and sheared salmon sperm DNA) containing 10 ng of sense or anti-sense DIG-labeled RNA probe, at 42°C overnight within a humid chamber. Sections were then washed in 2x SSC (twice, 15 min each) and 1x SSC (twice, 5 min each), at 37°C with rocking. After washing, sections were incubated in NTE buffer (500 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0), containing 20 μg/ml RNase A, for 30 min at 37 °C. After being washed twice in buffer-1 [100 mM Tris-HCl (pH 7.5), 150 mM NaCl], sections were blocked by being incubated with blocking solution [0.1% Triton X-100 and 2% normal sheep serum (Sigma) in
buffer-1] for 30 min. Sections were then incubated in blocking solution containing sheep anti-DIG-alkaline phosphatase (Roche, Germany) at dilution 1:500. After being washed in buffer 1 (twice, 10 min each) and buffer 2 [100 mMTris-HCl (pH 9.5), 100 mMNaCl, 50 mM MgCl₂; twice, 10 min each], signals were developed by incubating sections with NBT/BCIP solution (at dilution 1:200 in buffer-2; Roche, Germany) for approximately 1–2 h under the dark. To stop the reaction, sections were immersed in DEPC-treated water, before being mounted in 90% glycerol. Finally, sections were observed and photographed using a Nikon E600 microscope and digital camera (Nikon, Tokyo, Japan).

2.4. Effect of serotonin (5-HT) on CHH gene expression by quantitative real-time PCR

Sixty female mud crabs, which are at late intermolt stage and carry mature ovaries, were divided into five groups: (1) control group in which the animals were administered with vehicle control (100 µl of 0.9% normal saline); (2-5) treatment groups in which the animals were administered with 100 µl of 5-HT (5 µg/g BW dissolved in 0.9% normal saline) and then sacrificed at 3, 6, 12 and 24 h post-injections, respectively. Administration of 5-HT or vehicle control was performed by injection solution into the muscle at the base of the fifth walking leg. At each post-injection time, the brains, VNCs, and ovaries were collected from each group (n=12). The tissues were frozen briefly in liquid nitrogen before being stored at -80 C until used. Trizol reagent (Invitrogen, USA) was used to extract total RNA from the individual brains, VNCs, and ovaries of each individual crab following the manufacturer’s protocol. First-strand cDNA was synthesized using a Quantitect Reverse Transcription Kit (Qiagen, Germany), and then used in real-time PCR. Real-time PCR for amplification of S. olivacea CHH gene was performed in duplicate for each sample (n=12). The β-actin gene was used as a normalization control (Kornthong et al., 2013, 2014). A list of gene-specific primers is provided in Table 1. Amplification was conducted using a KAPA SYBR FAST
qPCR kit master mix for ABI Prism (Kapabiosystems, USA) and a 7500 Real Time PCR System (Applied biosystems, USA). Cycling condition for each reaction was: 95 C for 10 min, followed by 45 cycles of 95 C for 15 sec, 55 C for 10 sec, and 72 C for 10 sec. RotorGene’s software (version 6-0-22) automatically calculated the reaction efficiencies in the reactions. The REST 2009 (Relative Expression Software Tool; Qiagen, Germany) was used to calculate the relative expression of CHH against β-actin and the variance between individual treatments. Statistical significance analyses were performed by SPSS program (Statistical Product and Service Solutions; version 11) using a one-way analysis of variance (ANOVA). A probability value less than 0.05 (p<0.05) indicated a significant difference.

3. Results

3.1. Cloning and sequence analysis of S. olivacea CHH cDNA

S. olivacea CHH transcript was previously identified in the S. Olivacea CNS transcriptome (Kornthong et al., 2014). The partial sequence of S. olivacea CHH was analyzed by using CLC workbench program and it was matched to the sequence of S. olivacea CHH cDNA from previous studies (GenBank accession no. AY372181) (Tsai et al., 2008). The PCR product is 263 nucleotides in length and was predicted to encode 87 amino acids of CHH protein. BLAST searches of S. olivacea CHH against the NCBI GenBank database showed that S. olivacea CHH shares more than 75% of sequence similarity to other known crustacean CHHs (data not shown). This partial sequence was subjected to 3’RACE and 5’RACE in order to obtain a full-length sequence of S. olivacea CHH (Scyol-CHH), which contains 642 nucleotides and encodes a 108-amino acid CHH precursor protein (Fig. 1). A signal peptide of Scyol-CHH prepropeptide was predicted to be 27 amino acids (cleaved between positions A27 and R28). The CHH propeptide was predicted to be cleaved into the CHH and its associated peptide at the K65R dibasic cleavage site (Fig. 1). A
comparison of full-length amino acid sequences of Scyol-CHH with other crustacean CHHs (Fig. 2) showed that *S. olivacea* CHH shares high sequence similarity to *Scylla paramamosain* (89%), *Potamoni bericum* (61%), and *Discoplax celeste* (57%), but less sequence similarity to *Procambarus clarkii* (37%), and *Macrobrachium rosenbergii* (33%).

3.2. *Tissue expression of CHH transcript by in situ hybridization*

The CNS of female mud crab was selected for *in situ* hybridization to determine the spatial expression of Scyol-CHH transcript. A hematoxylin and eosin (H&E) stained median sagittal section of the right eyestalk displays the anatomy of the eyestalk (Fig. 3A). Consecutive sections of the eyestalk with Scyol-CHH mRNA expression at various locations was shown (Fig. 3B), particularly within the neuronal clusters 2-4 (Fig. 3D-K) and the X-organ (Fig. 3L). Scyol-CHH mRNA was also detected in the brain (Fig. 4). A mid horizontal section of the brain stained with H&E was shown at low magnification for reference in Fig. 4A. Consecutive sections of the brain with a strong positive signal of Scyol-CHH transcript within various neuronal clusters were shown (Fig. 4B) and in particular, neuronal cluster 6 (Fig. 4D-E), neuronal clusters 8 and 11 (Fig. 4G–I), and neuronal clusters 9 and10 (Fig. 4J-L). The H&E stained section of the VNC is presented as a reference in Fig. 5A, which includes the subesophageal ganglion (SEG), five fused thoracic ganglia (TG), thoracic artery (TA), and abdominal ganglia (AG). The Scyol-CHH mRNA was observed in various types of neurons, including small-, medium-, and large-sized neurons in VNC (Fig. 5B). Scyol-CHH mRNA was detected in the small-, medium-, and large-sized neurons within the SEG (Fig. 5D-F), TG (Fig.5G-I), and AG (Fig. 5J–L). Negative control sections using a sense-strand DIG-labeled riboprobe showed no positive signal in the cytoplasm of any neuron (Fig. 3C, 4C and 5C).

3.3. *Effect of 5-HT on CHH mRNA expression*
Female mud crabs were injected with 5-HT (5 µg/g BW) and then sampled at different times in order to study whether administration of 5-HT affects the level of Scyol-CHH mRNA expression in the brain, VNC, and ovary. We normalized the Scyol-CHH mRNA expression with β-actin expression and then the differential expression of Scyol-CHH gene between each group was evaluated. After 5-HT administration, Scyol-CHH expression in the brain, VNC, and ovary were significantly increased at 3h post-injection when compared with the controls. In addition, Scyol-CHH expression in VNC was significantly increased at 6 h post-injection when compared with the controls (p<0.05) (Fig. 6).

4. Discussion

In this study, we investigated the presence of CHH gene in the previously generated S. olivacea transcriptome (Kornthong et al., 2014), identified the full-length cDNA sequence of S. olivacea CHH gene (Scyol-CHH), and investigated tissue-specific expression of Scyol-CHH in the CNS and ovary. The expression of Scyol-CHH was restricted to the neural tissue and ovary by in situ hybridization. In addition, administration of 5-HT increased the level of Scyol-CHH expression at 3 and 6 h post-injections as indicated by qRT-PCR.

The common characteristics of neuropeptides in the CHH family include six conserved cysteine residues (which are responsible for the formation of three disulfide bridges) (Keller, 1992; Soyez, 1994; Webster et al., 2012), cyclization of N-terminal glutamine, and C-terminal amidation (Chang et al., 2010). The locations of disulfide bridges are predicted from the results of trypsin cleavage of C. maenas-CHH by Kegel et al. (1989). Based on their results, disulfide bridges in C. maenas CHH are predicted between cysteine residues at position 7 and 43, 23 and 39, and 26 and 52. The C-terminus contains a dibasic cleavage site (Arg67-Lys68) that is potentially cleaved, whereas the N-terminus contains a conserved Val-Glu-Met-Val-NH₂ peptide, which has been previously shown to be necessary for biological
activity (Kegel et al., 1989). The sequence analyses indicated that CHH is N- and C-
terminally blocked, with the C-terminal amide critical for its biological activity (Fanjul-
Moles, 2006). In this study, the alignment of Scyol-CHH with other CHHs revealed six
cysteine residues that are highly conserved, which is the common characteristic of the CHH
family (Chan et al., 2003; Fu et al., 2014).

CHH plays regulatory roles not only in carbohydrate metabolism, but also in other
physiological processes, e.g., molting, osmoregulation, and reproduction (Chang et al., 2010).
CHH has been identified in the eyestalk ganglion of the crab, *S. paramamosain*, and it plays a
role in promoting vitellogenesis, a process of yolk formation that is important in animal
reproduction (Fu et al., 2014). Previous studies reported that CHH superfamily peptides were
distributed in both central and peripheral nervous system of crustaceans (Hsu et al., 2006). It
has been suggested that CHH is expressed in the neurosecretory cells of the X-organ, and the
neuroheamal endings in the sinus gland in eyestalk (Webster et al., 2012). In addition, CHH
was reported to be localized in regions other than the eyestalk of crustaceans, such as the
VNC, thoracic ganglia, and SEG of *H. americanus* using immunocytochemistry detection
(Chang et al., 1999). Also, CHH peptides have been found in the brain, thoracic ganglion,
and pericardial organs (PO) of *C. maenas* using immunoreactivity assay (Keller et al., 1985;
Dircksen and Heyn, 1998). As revealed by immunofluorescence detection, the CHH-
containing neurosecretory cells were located in the eyestalks of *C. maenas* (Jaros and Keller,
1979). Based on whole-mount immunohistochemistry, CHH was expressed in the multipolar
neurons of the PO of *C. maenas* (Dircksen et al., 2001). In addition, CHH was detected in the
tapetal cells in retina of the crayfish, *P. clarkii* (Escamilla-Chimal et al., 2001). Furthermore,
CHH mRNA expression in the neuroendocrine cells and ovary of the mud crab, *S.
paramamosian*, has been shown (Fu et al., 2014).
The CNS of *S. olivacea* includes the eyestalk ganglion, brain, and VNC (Kornthong et al., 2014; Khornchatri et al., 2015). We found that *Scyol-CHH* gene was highly expressed in various neuronal clusters of the brain and the X-organ of the eyestalk. This is similar to previous studies in which CHH expression has been shown in the XO-SG system and nerve terminals of *C. productus* using immunoreactivity detection (Hsu et al., 2006). We also showed that *Scyol-CHH* mRNA was detected outside the eyestalk, specifically within neuronal clusters 6, 8, 9, 10 and 11 of the brain. This supports a previous *in situ* hybridization showing that *S. paramamosain* CHH (*Sp-CHH*) is positive in the cell body of neuroendocrine cells belonging to the X-organ of eyestalk ganglion (Fu et al., 2014). They suggested that CHH might play a vital role in the regulation of molting cycle (Fu et al., 2014). The CHH is known to be synthesized in the neurosecretory cells in the eyestalk and subsequently transported to the neurohemal sinus gland before released into the hemolymph (Keller, 1992).

In addition, CHH and CHH-like peptides were co-localized in the eyestalk ganglia and PO of *S. olivacea* (Tsai et al., 2008). In our study, we found the expression of *Scyol-CHH* in small- and giant-sized neurons within the SEG and in the small-, medium- and giant-sized neurons within the TG. CHH-positive neurons located at the dorsal level of the SEG are classified as small-sized neurons (Figure 5D). Based on the neuronal classification of the VNC in our previous study (Kornthong et al., 2014), small- (9-13 µm) and medium-sized (17-34 µm) neurons, corresponded to the CHH-positive neurons in the current study. The small-sized neurons normally serve as coordinating or association neurons, whereas medium-sized neurons are typically neurosecretory cells (TiniKul et al., 2001; Schachtner et al., 2005). Therefore, CHH produced from the medium-sized neurons surrounding the thoracic artery may be released into the hemolymph, functioning as a neurohormone. On the other hand, CHH expression within the small-sized neurons implicates CHH in the coordination of neural circuits. In the AG, *Scyol-CHH* mRNA was also detected in the small- and medium-sized
neurons. This is similar to the finding in *H. americanus*, where CHH was specifically detected within the neuronal somata of the small clusters in the vicinity of the bifurcation of thoracic second roots neurons (Chang et al., 1999). They suggested that CHH released from the extra-eyestalk tissues could serve a neuromodulatory role in addition to an endocrine role (Chang et al., 1999).

Serotonin is a neurotransmitter which is found in the central nervous system and ovary of crayfish (*P. clarkii*) and freshwater prawn (*M. rosenbergii*), and in the hemolymph of various crustacean species (Kulkarni et al., 1992). The effect of 5-HT has been investigated, showing that administration of 5-HT could stimulate ovarian maturation and embryonic development in *M. rosenbergii* (Tinikul et al., 2009). In addition, 5-HT induced ovarian development in *L. vannamei* (Vaca and Alfaro, 2000). In *S. olivacea*, 5-HT stimulated RPCH and FAMeT expressions (Kornthong et al., 2013, 2014). It has been suggested that 5-HT could regulate behavior and a variety of systemic physiological functions (Spitzer et al., 2008). In this study, we have shown the stimulatory effect of 5-HT on *Scyol-CHH* mRNA expression in the brain, VNC and ovary using qRT-PCR analysis. This result suggests that 5-HT play an important role in CHH expression level in *S. olivacea*. Furthermore, the injection of 5-HT stimulated CHH expression and increased glucose level in the hemolymph of *Fenneropenaeus indicus* (Sathyanandam et al., 2008). Hence, it is possible that 5-HT could regulate hyperglycemia in crustaceans. Similarly, injection of 5-HT into the crayfish, *Orconectes limosus*, significantly increased CHH level in the circulation (Santos et al., 2001). Likewise, injection of 5-HT into the crayfish, *A. leptodactylus*, could increase the glucose level in the hemolymph and increase the exocytosis of CHH granules in the sinus gland (Stroelenbarg and Herp, 1977). In addition, *Squilla mantis* and *Astacus leptodactylus* injected with 5-HT showed a significant increase in hemolymph glucose level (Lorenzon et al., 2004). These observations suggest that serotonin plays a major role in controlling glucose mobilization. Furthermore, they suggested that
stimulatory effect of 5-HT on ovarian maturation in crustaceans might be mediated through neuro-regulation system by the CHH release (Sathyanandam et al., 2008). However, the role of Scyol-CHH in reproduction in S. olivacea needs to be verified further.

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References


Table

Table 1
CHH and actin primers used for PCR, RACE PCR, and real-time quantitative RT-PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Direction</th>
<th>Nucleotide Sequence</th>
</tr>
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<tr>
<td>CHH-F</td>
<td>Forward</td>
<td>5’- TGGGCGAATGGGTCGACTGTTGG -3’</td>
</tr>
<tr>
<td>CHH-R</td>
<td>Reverse</td>
<td>5’- TAAGCAGTACCTCCTCCGGATAG -3’</td>
</tr>
<tr>
<td>5CHH-R</td>
<td>Reverse</td>
<td>5’- CGCCCTTGACGAAGATCG -3’</td>
</tr>
<tr>
<td>3CHH-F</td>
<td>Forward</td>
<td>5’- TATTCAGGTATTTAAATAGTC -3’</td>
</tr>
<tr>
<td>Actin-SO-F</td>
<td>Forward</td>
<td>5’- GAGCGAGAAATCGTTGACAT-3’</td>
</tr>
<tr>
<td>Actin-SO-R</td>
<td>Reverse</td>
<td>5’- CCGATGATGATGACCTGCGCGT-3’</td>
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</table>

Figure legends

Fig. 1. CHH nucleotide and deduced amino acid sequences derived from *S. olivacea* cDNA. The amino acid sequence is shown as single letters underneath the encoding nucleotide sequence. The italic and underlined letters represent the signal peptide. The black bold letters represent the CPRP (crustacean hyperglycemic hormone precursor-related peptide), consisting of 37 amino acids, followed by the dibasic cleavage site (KR; red bold italic letters). The italics black sequence represents the mature CHH. An asterisk indicates the stop codon. The numbers on the right indicate nucleotide and amino acid numbers.
Fig. 2. Amino acid alignment of crustacean hyperglycemic hormone (CHH) preprohormone of S. olivacea with other known crustacean CHHs. Gaps (-) was included to allow alignment. ‘*’, identical amino acids; ‘:’, conserved substitutions (same group); ‘.’, semi-conserved substitution (similar shapes). Scyol, Scylla olivacea (orange mud crab); Scypa, Scylla paramamosain (green mud crab; GenBank accession no: AFD28272); Potib, Potamon ibericum (freshwater crab; GenBank accession no: ABA70561); Disce, Discoplax celeste (land crab; GenBank accession no: AEM45615); Proc, Procambarus clarkii (crayfish; GenBank accession no: AFV95078); Macro, Macrobrachium rosenbergii (freshwater prawn; GenBank accession no: AAL40915).

Fig. 3. In situ hybridization of Scyol-CHH transcripts in the eyestalk of S. olivacea. (A) A median sagittal section of the right eyestalk stained with H&E showing the location of neuronal clusters (numbers) and neuropils. (B) Detection of Scyol-CHH transcripts in eyestalk (purple), including neuronal clusters 1-4. (C) Negative control shows no positive signal in the cytoplasm of the neurons. (D–O) High magnification micrographs shows positive signals in the cluster 2 (D–F), clusters 3 and 4 (G–K), and the XO (L). Scale bars: A :500 μm; B and C: 250 μm; D, G: 100 μm; E and H: 50 μm; F, I, J, K and L : 25 μm. Abbreviations: LG, lamina ganglionaris; ME, medulla externa; MI, medulla interna; MT, medulla terminalis; XO, X-organ; HB, hemielipsoid body; A, Anterior side; L, Lateral side.

Fig. 4. In situ hybridization of Scyol-CHH transcripts in the brain of S. olivacea. (A) A mid horizontal section of the brain stained with H&E shows the locations of neuronal clusters (numbers) and neuropils. (B) Low power micrograph shows positive signals in various neuronal clusters of the brain. (C) Negative control micrograph shows no positive signal in
the cytoplasm of the neurons. (D–L) High magnification micrographs show positive staining in the cluster 6 (D–F), clusters 8 and 11 (G–I), and clusters 9 and 10 (J–L). Scale bars: A, B, C: 250 μm; D, J: 100 μm; E, G, H: 50 μm; F, I, K, L: 25 μm. Abbreviations: PT, protocerebral tract; AMPN, anterior medial protocerebral neuropils; ON, olfactory neuropil; A, Anterior side; L, Lateral side.

**Fig. 5.** *In situ* hybridization of *Scyol-CHH* transcripts in the ventral nerve cord (VNC) of *S. olivacea*. (A) H&E staining of VNC shows the locations of neuronal clusters (arrows) and neuropils. (B) Low power of VNC micrograph shows positive signals in various neuronal clusters (arrows) of the VNC. (C) Negative control micrograph shows no positive signal in the cytoplasm of the neurons. Low (D), medium (E), and high (F) magnification micrographs show positive signals in the small-, medium-, and large-sized neurons within the SEG (asterisks). Arrows in (D) indicate positive signals at different neuronal clusters. Low (G), medium (H), and high (I) magnification micrographs show positive signals in various sizes of neurons in the thoracic ganglia (TG). Low (J), medium (K), and high (L) magnification micrographs show positive signals in the small-, medium-, and large-sized neurons in the AG. Scale bars: A, B, C: 500 μm; D, G: 250 μm; E, H, J: 100 μm; I, K, L: 50 μm. Abbreviations: SEG, subesophageal ganglion; TG, thoracic ganglion; AG, abdominal ganglion; TA, thoracic artery; A, Anterior side; L, Lateral side.

**Fig. 6.** Effect of 5-HT priming on the levels of CHH expression using quantitative RT-PCR. CHH mRNA expression levels were determined at 3, 6, 12, and 24 h post 5-HT injections (N=12). Asterisk indicates significant differences (at p < 0.05) when compared with the control [animals injected with vehicle control (VC)] group.
Figure 3
Figure 4
Highlights

- The distribution of crustacean hyperglycemic hormone (CHH) in central nervous system of mud crab.

- The relationship of serotonin stimulation on CHH expression.

- The proposed reproductive function of CHH in mud crab.