สกว

รายงานวิจัยฉบับสมบูรณ์

โครงการ

การโคลนและศึกษาหน้าที่ของ gonad-inhibiting hormone ในกุ้งกุลาดำ

โดย

นายอภินันท์ อุดมกิจ

สถาบันอณูชีววิทยาและพันธุศาสตร์
มหาวิทยาลัยมหิดล

15 สิงหาคม 2549

สัญญาเลขที่ RSA4680008

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สถาบันอณูชีววิทยาและพันธุศาสตร์
มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
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ชื่อโครงการ: การโคลนและศึกษาหน้าที่ของ gonad-inhibiting hormone ในกุ้งกุลาดำ

ชื่อนักวิจัย: นายอภินันท์ อุดมกิจ
สถาบันอณูชีววิทยาลพันธุศาสตร์
มหาวิทยาลัยมหิดล ศาลายา
อำเภอพุทธมณฑล
นครปฐม 73170

E-mail Address : staud@mahidol.ac.th

ระยะเวลาโครงการ: 15 สิงหาคม 2546 ถึง 14 สิงหาคม 2549

Gonad-inhibiting hormone หรือ GIH เป็นฮอร์โมนสำคัญที่ทำหน้าที่ยับยั้งการพัฒนาของไข่ในกุ้งเพศเมีย แหล่งผลิต GIH ได้แก่ปมประสาทในบริเวณก้านตาที่เรียกว่า X-organ ซึ่งผลิตฮอร์โมนที่สำคัญหลายชนิดเช่น crustacean hyperglycemic hormone (CHH) และ molt-inhibiting hormone (MIH) GIH ยับยั้งการพัฒนาของไข่โดยการยับยั้งการสร้างไวเทลโลจีนิน ซึ่งจะถูกเปลี่ยนไปเป็นไวเทลลิน และสะสมในไข่ที่กำลังเจริญ ด้วยเหตุนี้จึงใช้วิธีตัดตาเพื่อเร่งให้แม่กุ้งวางไข่ในการผลิกลูกกุ้งในฟาร์มเพาะเลี้ยง ซึ่งทำให้มีการใช้กุ้งแม่พันธุ์จำนวนมากในแต่ละปี ปัจจุบันยังไม่ทราบกลไกที่แน่ชัดในกระบวนการสร้างไวเทลโลจีนินและการเปลี่ยนไวเทลโลจีนินเป็นไวเทลลิน รวมทั้งยังมีข้อมูลเกี่ยวกับ GIH ไม่มากนัก โครงการนี้จึงมีวัตถุประสงค์เพื่อศึกษาโครงสร้างและหน้าที่ของ GIH ในกุ้งกุลาดำ เพื่อเป็นแนวทางในการเร่งให้แม่กุ้งวางไข่โดยไม่ต้องตัดตา โดยได้ทำการโคลนชิ้น cDNA จากก้านตากุ้งแม่พันธุ์ ด้วยวิธี 3' Rapid Amplification of cDNA Ends (3' RACE) โดยใช้ไพรเมอร์ที่ออกแบบจาลำดับกรดอะมิโนอนุรักษ์ของฮอร์โมนในกลุ่มนี้จากสิ่งมีชีวิตชนิดอื่นๆ จากการวิเคราะห์ลำดับนิวคลีโอไทด์พบโคลนที่มีลำดับนิวคลีโอไทด์ของชิ้น cDNA insert คล้ายคลึงกับ GIH จำนวน 8 โคลนจากทั้งหมด 213 โคลน โดยทั้ง 8 โคลนมีลำดับนิวคลีโอไทด์เหมือนกันและใช้ในการออกแบบไพร์เมอร์เพื่อโคลน cDNA ชิ้นเต็ม ซึ่งมีความยาว 861 คู่เบส และมี open reading frame ขนาด 288 คู่เบส ที่ถอดรหัสได้เป็นโปรตีนที่ประกอบด้วยส่วนที่เป็น signal peptide ความยาว 17 กรดอะมิโน และส่วนที่เป็น mature peptide ประกอบด้วยกรดอะมิโนจำนวน 79 หน่วย จากการศึกษาการแสดงออกของ GIH ในอวัยวะต่างๆของกุ้ง พบว่า GIH มีการแสดงออกในก้านตา และในปมประสาทบริเวณอื่นๆ ได้แก่ brain, thoracic ganglia และ abdominal nerve-cord การศึกษาหน้าที่ของ cDNA ที่สร้าง GIH ในการยับยั้งการสร้างไวเทลโลจีนิน ทำโดยใช้เทคนิค RNA interference (RNAi) เพื่อยับยั้งการแสดงออกของ GIH และดูผลต่อการสร้างเทลโลจีนินในภาวะที่ไม่มีการสดงออกหรือมีการแสดงออกที่ลดลงของ GIH โดยสร้างอาร์เอ็นเอสายคู่ (dsRNA) จากลำดับนิวคลีโอไทด์ส่วนที่สร้าง mature GIH และพบว่า GIH dsRNA นี้สามารถยับยั้งการแสดงออกของ GIH ใน abdominal nerve-cord ที่เลี้ยงในอาหารเพาะเลี้ยงได้ จากนั้นได้ฉีด GIH dsRNA เข้าไปในกุ้งกุลาดำที่มีขนาดประมาณ 10-15 กรัม ในปริมาณ 5 ไมโครกรัมของ dsRNA ต่อน้ำหนักตัวของกุ้ง 10 กรัม พบว่าสามารถลดระดับการแสดงออกของ GIH ได้ประมาณ 75 % และกุ้งที่มีการแสดงออกของ GIH ลดลงนี้ จะพบการแสดงออกของไวเทลโลจีนินใน hepatopancreas เพิ่มขึ้นประมาณสองเท่า ซึ่งบ่งชี้ว่า GIH มีความเกี่ยวข้องในกระบวนการยับยั้งการสร้างไวเทลโลจีนินซึ่งมีผลต่อการเจริญของไข่ เมื่อทดลองฉีด GIH dsRNA เข้าไปในกุ้งแม่พันธุ์ แล้วติดตามดูการเจริญของรังไข่ พบว่ากุ้งในกลุ่มควบคุมที่ตัดตามีการเจริญของรังไข่และสามารถวางไข่ได้ จำนวน 4 ตัวจากกุ้งทั้งหมด 9 ตัวในกลุ่ม กุ้งในกลุ่มควบคุม

อีกกลุ่มหนึ่งที่ฉีดด้วย buffer อย่างเดียวสามารถวางไข่ได้เพียง 1 ตัวจาก 9 ตัว ส่วนกุ้งในกลุ่มทดลองที่ฉีดด้วย GIH dsRNA มีการเจริญของรังไข่และสามารถวางไข่ได้ 2 ตัวจากกุ้งทั้งหมด 11 ตัวในกลุ่ม ซึ่งมีแนวโน้มที่เห็นว่าการฉีด GIH dsRNA อาจช่วยให้แม่กุ้งวางไข่ได้ดีขึ้น อย่างไรก็ตามการทดลองในส่วนนี้ จะต้องทำการทดลองซ้ำอีกครั้งหนึ่ง เพื่อให้เห็นผลที่ชัดเจนขึ้น นอกจากนี้ยังได้ผลิตโปรตีนลูกผสมของ GIH ใน *E. coli* เพื่อจะนำมาทดสอบหน้าที่ในการยับยั้งการสร้างไวเทลลโลจีนิน และสร้าง GIH แอนติบอดี้ เพื่อนำมาทดลองใช้เร่งการวางไข่ในแม่กุ้งอีกวิธีหนึ่ง

คำหลัก: กุ้งกุลาดำ การเจริญของรังไข่ ไวเทลโลจีนิน RNA interference

Abstract

Project Code : RSA/13/2544

Project Title : Cloning and characterization of gonad-inhibiting hormone of *Penaeus monodon*

Investigator : Mr.Apinunt Udomkit

Institute of Molecular Biology and Genetics

Mahidol University, Salaya Campus

Nakhon Pathom 73170

E-mail Address : staud@mahidol.ac.th

Project Period : 15 August 2003- 14 August 2006

The X-organ in the eyestalk optic ganglia of crustacean produces several peptide hormones that play vital roles in growth and reproduction. Member of these hormones include crustacean hyperglycemic hormone (CHH), molt-inhibiting hormone (MIH) and gonad-inhibiting hormone (GIH). GIH play role in reproduction by inhibiting vitellogenesis, the process that synthesize a yolk protein precursor vitellogenin, in the hepatopancreas. For this reason, female shrimp broodstock are usually induced to ovarian maturation and spawning by eyestalk ablation in the process of shrimp fry production. This leads to the overexploitation of shrimp broodstock because eyestalk ablation normally causes deleterious effect to the shrimp. To date, not much is known about the mechanism that controls vitellogenesis. Moreover, the knowledge about GIH is also limited. This project is therefore aims at studying in both molecular and functional aspects of GIH in *Penaeus monodon*, one of the major cultured shrimp species in Thailand.

A cDNA encoding *P. monodon*'s GIH was cloned by 3'RACE strategy using degenerate primers from the conserved amino acid sequence of GIH among GIH of other species. Eight out of 213 cloned analyzed showed similarity in their deduced amino acid sequences to GIH of *Metapenaeus ensis*. Nucleotide sequence comparison indicated that these eight clones represented a single transcript. Several pairs of specific primers were then designed from this 3' RACE fragment and used in 5'RACE and RT-PCR to obtain a full-length transcript of GIH. The full-length GIH cDNA contains 861 bp with one long open reading frame of 288 bp. The open reading frame encodes 17 amino acid residues of putative signal peptide and a 79 residues mature GIH. The expression study showed that GIH expression is restricted to the nervous tissues such as eyestalk ganglia, brain, thoracic ganglia and abdominal nerve cord. Functional study of GIH cDNA was performed using RNA interference technique. A double-stranded RNA (dsRNA) was synthesized based on the nucleotide sequence of the mature region of GIH. The experiment in nerve cord explants showed that the GIH dsRNA was able to knockdown GIH transcript in the nerve cord. Next the GIH dsRNA was injected into the shrimp (approximately 10-15 g) in the ratio of 5 μg dsRNA per 10 gram of shrimp weight. The expression of GIH in the shrimp injected with GIH dsRNA was reduced by 75% comparing to the control shrimp that did not receive dsRNA. When vitellogenin transcript was determined in the hepatopancreas of the shrimp administered with GIH dsRNA, it showed a two-fold

increase in the transcription level comparing with that in the control shrimp. This resulted suggested a negative involvement of GIH in vitellogeneis that is an index of ovarian maturation. Recently, the GIH dsRNA was injected into female broodstock in order to investigate the effect of GIH knockdown on ovarian maturation. Four out of nine of the control shrimp that were unilateral eyestalk ablated underwent ovarian maturation and successful spawning whereas only one out of nine in the negative control group that was injected with buffer spawned successfully. However, injection of GIH dsRNA induced successful spawning in two out of eleven shrimp in the experimental group. This result, although needs further confirmation, suggested that GIH dsRNA injection may help induce spawning in the broodstock regardless of eyestalk ablation. Moreover, recombinant protein of GIH was produced in *Escherichia coli* and its function will be investigated by inhibition assay of vitellogenin synthesis in the hepatopancreas. The antibody will also be raised against this recombinant GIH and , finally, will be tested for its potential in inducing ovarian maturation in female broodstock.

Keywords : black tiger shrimp, ovarian maturation, vitellogenin, RNA interference

Executive summary

Project Code : RSA/13/2544

Project Title : Cloning and characterization of gonad-inhibiting hormone of *Penaeus monodon*

Investigator : Mr.Apinunt Udomkit
Institute of Molecular Biology and Genetics
Mahidol University, Salaya Campus
Nakhon Pathom 73170

E-mail Address : staud@mahidol.ac.th

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Introduction

Thailand has long been the world's leader in shrimp export with an annual income over two billion US dollars. However, shrimp production is still affected by several problems that lead to dramatic decline in both the production yield and the quality of shrimp. In addition to the influences of viral diseases and growth retardation, the lack of reliable and high quality broodstock is another foremost problem. Ablation of eyestalk is generally used as a method to induce spawning of female black tiger shrimp brookstock. This destroys the source of gonad-inhibiting hormone as well as other hormones that regulates crucial physiological processes such as molting and carbohydrate metabolism, and thus leads to low survival rate of the broodstock due to the disturbance of the hormonal system. As a consequence, several hundred-thousands of broodstock are exploited each year. Although, domesticated broodstock are now successfully developed, spawning of these domesticated shrimp still relies mainly on eyestalk ablation. Therefore the study of gonad-inhibiting hormone and its function in controlling ovarian maturation provides potential alternative for the induction of spawning in female broodstock instead of eyestalk ablation, and will eventually facilitate the efficiency of the domesticate program.

Objectives

1. To clone a cDNA encoding gonad-inhibiting hormone (GIH) of *P. monodon*
2. To characterize the function of GIH cDNA in controlling vitellogenin expression using RNA-interference technique
3. To investigate the effect of double-stranded RNA that is specific to GIH on ovarian maturation of the female broodstock of *P. monodon*.
4. To produce recombinant GIH protein and anti-GIH antibody for the study of its function in the inhibition of vitellogenin synthesis.

Results and Discussion

The cDNA encoding gonad-inhibiting hormone (GIH) of *Penaeus monodon* was successfully cloned by RACE strategy using degenerate primers from the conserved region between molt-inhibiting hormone (MIH) and GIH. This cDNA, so-called Pem-GIH, contains 861 bp with one long open reading frame of 288 bp. The open reading frame encodes 17 amino acid residues of putative signal peptide and a 79 residues mature GIH. The expression study showed that GIH expression is restricted to the nervous tissues such as eyestalk ganglia, brain, thoracic ganglia and abdominal nerve cord. This tissue specific expression is similar to GIH of other species. Functional study of GIH cDNA was performed using RNA interference technique. A double-stranded RNA (dsRNA) was synthesized from the nucleotide sequence that codes for the mature region of GIH. The experiment in tissue explants showed that the GIH dsRNA was able to knockdown GIH transcript in the eyestalk ganglia and the abdominal nerve cord. This dsRNA-mediated knockdown of Pem-GIH occurred in sequence specific fashion as unrelated dsRNA of the green fluorescent protein (GFP) failed to knockdown Pem-GIH expression. Next the GIH dsRNA was injected into the shrimp (approximately 10-15 g) in the ratio of 5 μg dsRNA per 10 gram of shrimp weight. The expression of GIH in the shrimp injected with GIH dsRNA was reduced by 75% comparing to the control shrimp that did not receive dsRNA. GIH is known to inhibit vitellogenin synthesis in the hepatopancreas, therefore the consequence of GIH knockdown on the expression of vitellogenin was examined. When vitellogenin transcript was determined by RT-PCR in the hepatopancreas of the shrimp administered with GIH dsRNA, it showed a two-fold increase in the transcription level comparing with that in the control shrimp. This resulted suggested a negative involvement of GIH in vitellogeneis that is an index of ovarian maturation. Recently, the GIH dsRNA was injected into female broodstock in order to investigate the effect of GIH knockdown on ovarian maturation. Four out of nine of the control shrimp that were unilateral eyestalk ablated underwent ovarian maturation and successful spawning whereas only one out of nine in the negative control group that was injected with buffer spawned successfully. However, injection of GIH dsRNA induced successful spawning in two out of eleven shrimp in the experimental group. This result, although needs further confirmation, suggested that GIH dsRNA injection may help induce spawning in the broodstock regardless of eyestalk ablation. Moreover, recombinant protein of GIH was produced in *Escherichia coli* and its function will be investigated by inhibition assay of vitellogenin synthesis in the hepatopancreas. The antibody will also be raised against this recombinant GIH and, finally, will be tested for its potential in inducing ovarian maturation in female broodstock. The utilization of GIH dsRNA or anti-GIH antibody may substitute the eyestalk ablation method to induce ovarian maturation and spawning and will help prevent over-exploitation of wild broodstock as well as increase the efficiency of shrimp domesticated program. This will constantly make Thailand retains its leader position of the shrimp exporter worldwide.

Introduction

Thailand has long been the world's leader in shrimp export with an annual income over two billion US dollars. However, shrimp production is still affected by several problems that lead to dramatic decline in both the production yield and the quality of shrimp. In addition to the influences of viral diseases and growth retardation, the lack of reliable and high quality broodstock is another foremost problem. Ablation of eyestalk is generally used as a method to induce spawning of female black tiger shrimp brookstock. This destroys the source of gonad-inhibiting hormone as well as other hormones that regulates crucial physiological processes such as molting and carbohydrate metabolism, and thus leads to low survival rate of the broodstock due to the disturbance of the hormonal system. As a consequence, several hundred-thousands of broodstock are exploited each year. Although, domesticated broodstock are now successfully developed, spawning of these domesticated shrimp still relies mainly on eyestalk ablation. Therefore the study of gonad-inhibiting hormone and its function in controlling ovarian maturation provides potential alternative for the induction of spawning in female broodstock instead of eyestalk ablation, and will eventually facilitate the efficiency of the domesticate program.

Gonad-inhibiting hormone (GIH) is produced from the x-organ in the medulla terminalis in the eyestalk. GIH is the member of a peptide family called CHH-family, which is composed of three major hormones; crustacean hyperglycemic hormone (CHH), molt-inhibiting hormone (MIH) and GIH. These hormones are structurally related with a typical feature of six cysteines residues that are aligned at conserved positions (Keller, 1992). The members of CHH-family can be divided into two groups, type I and type II. Type I hormone is composed mainly of CHH, whereas MIH and GIH belong to type II. The signal sequence of type I or CHH contains short peptide called CHH precursor related peptide that precedes the dibasic processing site. By contrast, the hormones in type II are preceded directly by the signal peptide. Alignment of amino acid sequences among the hormone in this family revealed the absence of the amino acid glycine at the fifth position after the first cysteine residues in type I (Chen et al., 2005).

The effect of GIH on inhibition of ovarian maturation is exerted through inhibition of vitellogenesis, which is the process whereby the precursor of yolk protein, vitellogenin, is synthesized in the hepatopancreas (Tsukimura, 2001). Additionally, GIH may also block the vitellogenin from being transported into the ovary. GIH was first isolated from the American lobster *Homarus americanus* and its *in vivo* activity was demonstrated (Soyes et al., 1987). The cDNA of GIH was subsequently cloned. Recently, the recombinant protein of *H. americanus* GIH was produced in *E. coli* and this recombinant GIH was able to repress the synthesis of vitellogenin mRNA in the ovary fragment. The same approach was also used to characterize the function of GIH from the kuruma shrimp *Marsupenaeus japonicus* (Tsutsui et al., 2005) and the crayfish *Procambarus bouvieri* (Aquilar et al., 2002). The cDNA encoding GIH-like peptide was also obtained form a few other species, however, whether or not they encode a peptide with GIH activity needs further investigation (Gu et al., 2002; Edomi et al., 2002). In *P. monodon*, no information about GIH is available to date. Only CHH and MIH were both structurally and functionally characterized. Since GIH is a key hormone in controlling vitellogenesis, identification and characterization of this hormone in *P. monodon* will enhance our knowledge on reproduction and thus provide an effective means for the improvement of shrimp breeding in this economically important species.

Objectives

1. To clone a cDNA encoding gonad-inhibiting hormone (GIH) of *P. monodon*
2. To characterize the function of GIH cDNA in controlling vitellogenin expression using RNA-interference technique
3. To investigate the effect of double-stranded RNA that is specific to GIH on ovarian maturation of the female broodstock of *P. monodon*.
4. To produce recombinant GIH protein and anti-GIH antibody for the study of its function in the inhibition of vitellogenin synthesis.

Materials and Methods

Animals

Black tiger shrimp (*P. monodon*), approximately 10-15 g, were purchased from shrimp farms nearby Bangkok. Wild adult female *P. monodon* were caught from the Gulf of Thailand whereas domesticated female broodstock were provided by Prof. Boonsirm Wittayachamnankul and the Bangkok Aquaculture Farm Co. (BAFCO).

RNA Extraction and Reverse Transcription Polymerase Chain Reaction (PCR)

Total RNA was extracted from tissues of *P. monodon* by TRI Reagent (MRC) according to the manufacturer's protocol. The RNA was reverse transcribed into a cDNA by the activity of ImProm-II™ Reverse Transcriptase (Promega) using 500 nM of oligo(dT)$_{16}$ primer (PRT) or GIH-specific primers to prime cDNA synthesis. Approximately 1 μg of total RNA was mixed with primer and heated to 70°C for 5 min. The mixture was chilled on ice before the addition of 3 mM MgCl2, 0.5 mM dNTP mix, 40 units of RNasin ribonuclease inhibitor and 1 μl ImProm-II™ Reverse in 1 x Transcriptase x Improm-II™ reaction buffer. The mixture was incubated at 42°C for 60 min and the reaction was inactivated at 70°C for 15 min.

Rapid amplification of cDNA ends (RACE)

Fourteen degenerate primers used in 3'RACE of a cDNA encoding GIH of *P. monodon* (Pem-GIH) were designed from the conserved amino acid sequences of MIH/GIH from several species of crustacean. Additional five specific primers were also designed from the sequence of *Metapenaeus ensis* GIH. An alignment of amino acid sequences of GIH and MIH and the region used for primer design are shown in figure 1 and table 1, respectively.

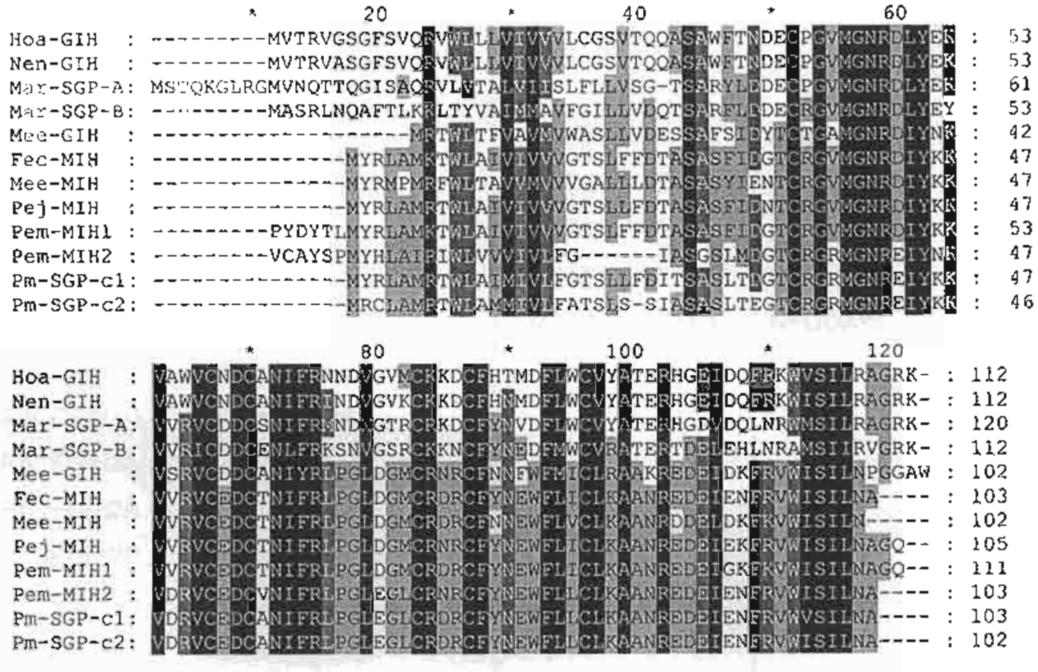

Figure 1 An Alignment of amino acid sequences between molt-inhibiting hormone (MIH) and gonad-inhibiting hormone (GIH) from several crustacean species. Black shading represents identical amino acids. Lower degrees of homology are highlighted in dark and light grey.

GIH: *Homarus americanus* (Hoa-GIH; GenBank accession no. S48747), *Nephrops norvegicus* (Nen-GIH; GenBank accession no. AAK58133), *Macrobraclum rosenbergii* (Mar-SGP-A; GenBank accession no. AAL37948 and Mar-SGP-B; GenBank accession no. AAL37949), *Metapenaeus ensis* (Mee-GIH; GenBank accession no. AAL33882), *Feneropenaeus chinensis* (Fec-MIH; GenBank accession no. AF312977), *Metapenaeus ensis* (Mee-MIH; GenBank accession no. O76534), *Penaeus japonicus* (Pej-MIH; GenBank accession no. P55847), *Penaeus monodon* (Pem-MIH1; GenBank accession no AY496454, Pem-MIH2; GenBank accession no AY496455, Pm-SGP-c1; GenBank accession no AB054187 and Pm-SGP-c/; GenBank accession no AB054188)

Total RNA from adult female at different vitellogenic stages were used in cDNA synthesis. A 1 µl aliquot of cDNA was amplified with GIH-derived primer and PM1 primer (Table 2) in 25 µl of a reaction containing 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton® X-100, 1.5 mM $MgCl_2$, 200 nM each primer, 200 µM each dATP, dCTP, dGTP, dTTP and 1.25 units of *Taq* DNA Polymerase in Storage Buffer B (Promaga). Amplification was performed in a DNA Thermal Cycler (GeneAmp System 2400, PE Applied Biosystems) with 35 cycles of 94°C for 30 sec, 50-55°C for 30 sec and 74°C for 1min following with 7 min incubation at 74°C as a final extension.

Table 1 Amino acid sequences used for primer design in 3' RACE

Degenerate primers	Amino acid sequence	Specific primers	Amino acid sequence
MG1	RGVMGN	3'RACEGIH-A	FSIDV
MG2	LPGLEGL	3'RACEGIH-B	VTCTGA
3'RACEGIH1	VMGNRD	3'RACEGIH-C	YNKVS
3'RACEGIH1A	C(P/T)GVMGNRD	3'RCAEGIH-D	CLRAAK
3'RACEGIH1B	MGNRD(L/I)YN/EKV	3'RCAEGIH1(Rev)	NPGGAW
3'RACEGIH2	VCDDC		
3'RACEGIH2-1	VC(N/D)DCAN		
3'RACEGIH2A	RVCDDCA		
3'RACEGIH2B	RVCDDCAN		
3'RACEGIH2C	DDCAN(I/L)(Y/F)R		
3'RACEGIH3-1	YATERH		
3'RACEGIH3-2	YATERT		
3'RACEGIH4	RAAKRE		
RevGIH	LIS(V/I/M)W		

For 5'RACE, the first strand cDNA synthesis was performed in a reaction as described for 3'RACE, except that 1 μM of 5'RAEC-GIH1 primer was substituted for PRT primer. The RT reaction was first incubated at 50°C for 60 min. The RNA template was then denatured again at 83°C for 3 min and immediately placed on ice for 5 min. In the second RT step, a 1 μl of ImProm-II™ Reverse Transcriptase was added to the reaction prior to incubation at 50°C for 60 min and then terminated at 70°C for 15 min. The RNA template was degraded with RNase H before proceeding with cDNA purification by QIAquick PCR Purification Kit (QIAGEN). A 20 μl aliquot of purified cDNA was tailed with dATP in 30 μl of 100 mM cacodylate buffer (pH 6.8), 1 mM $CoCl_2$, 0.1 mM DTT, 200 μM dATP and 20 units of terminal deoxynucleotidyl transferase (TdT) (Promaga). The reaction was allowed to occur at 37°C for 20 min and TdT was heat-inactivated at 65°C for 10 min. The first round PCR with 3 μl of the dA-tailed cDNA template was carried out as described for 3'RACE using 200 nM of 5'RAEC-GIH2 and PRT primers, except that annealing was carried out at 55°C for 30 sec. The second round PCR was performed with 200 nM of 5'RAEC-GIH3 and PM1 primers to obtain specific amplified product.

Amplification of full-length Pem-GIH cDNA

Total RNA extracted from one pair of the eyestalk of adult female shrimp in stage IV of vitellogenesis was used to synthesize a cDNA template with PRT primer for the cloning of full-length cDNA of Pem-GIH. A 1 μl aliquot of cDNA was amplified with GIHF and GIHR primers (table 2) in a 25 μl reaction containing 1 x Phusion HF buffer including 1.5 mM $MgCl_2$, 500 nM each primer, 200 μM each dATP, dCTP, dGTP, dTTP and 0.25 units of Phusion DNA Polymerase (Finnzymes). Amplification was performed in a DNA Thermal Cycler (GeneAmp System 2400, PE Applied Biosystems) with 35 cycles of 98°C for 10 sec, 50°C for 30 sec and 72°C for 1min following with 7 min incubation at 72°C as a final extension.

Reverse-transcription polymerase chain reaction (RT-PCR)

Tissue-specific expression of Pem-GIH was investigated in several tissues of *P. monodon* including eyestalks, brain, thoracic nerve cord, abdominal nerve cord, heart, hepatopancreas, ovary, and muscle. Total RNA extracted from these tissues was reverse transcribed into cDNA with PRT primer. The specific transcript of Pem-GIH was amplified with matGIHF and GIHR primers (table 2) to detect the 3' region of the full-length cDNA. The reaction was amplified with 30 cycles of 94°C for 30 sec, 50°C for 30 sec and 74°C for 1 min following with 7 min incubation at 74°C as a final extension. The 5'region of full-length Pem-GIH cDNA was amplified with GIHF and 5'RACEGIH1 (table 2) primers to detect Pem-GIH transcript level in all GIH knockdown experiments. The reaction was amplified with 35 cycles of 94°C for 30 sec, 50°C for 30 sec and 74°C for 1min following with 7 min incubation at 74°C as a final extension. For the detection of vitellogenin transcript in the hepatopancreas, Vg-F (5' CTAAGGCAATTATCACTGCTGCT 3') and Vg-R (5'AAGCTTGGCAATGT ATTCCTTTT 3') primers were used in a reaction with 32 cycles of 94°C for 30 sec, 50°C for 30 sec and 74°C for 1min following with 7 min incubation at 74°C. The actin transcript was amplified with PmActin-F (5'GACTCGTACGTCGGGCGACGAGG 3') and PmActin-R (5' AGCAGCGGTGGTCAT CACCTGCTC 3') primers in a reaction with 21 cycles of 94°C for 30 sec, 55°C for 30 sec and 74°C for 1min followed by further incubation at 74°C for 7 min.

Expression of recombinant Pem-GIH in *Pichia pastoris*

A cDNA encoding mature Pem-GIH was amplified with GIH-X-F and GIH-X-R primers (Table 2) that contain recognition sequences for Sal I and Xba I, respectively at their 5' ends. The mature GIH cDNA was inserted into pPICZaA expression vector as shown in figure 2 for expression in *P. pastoris* system as secreted protein. The recombinant plasmid was integrated into *P. pastoris* genome by electroporation as described previously (Treerattrakool et al., 2003). The recombinant clones that contain integrated Pem-GIH cDNA expression cassette were screened by PCR with specific primers to *P. pastoris* AOX sequences.

A single colony of *P. pastoris* recombinant was cultured in YEPD medium [2% (w/v) peptone, 2% (w/v) glucose and 1% (w/v) yeast extract] at 30(C for 2 days. The culture was then diluted in BMGY medium [1% (w/v) yeast extract, 2% (w/v) peptone, 0.67% (w/v) yeast nitrogen base, 4 μg/ml D-biotin, 100 mM potassium phosphate, pH6.0 and 1% (w/v) glycerol] to an OD_{600} of 0.2 and further incubated at 30°C with shaking until the OD_{600} reached 5-6. The cell pellets were

Table 2. The primers used in this study

Primer	Sequence (5'→ 3')
Reverse transcription and 3'RACE	
PRT	CCGGAATTCAAGCTTCTAGAGGATCCTTTTTTTTTTTTTTTTT
PM1	CCGGAATTCAAGCTTCTAGAGGATCC
5'RACE	
5'RACE-GIH1	CCACGGCCGGCCGGCATTGAG
5'RACE-GIH2	GGCCTCGCGCTTGGCCGAGTG
5'RACE-GIH3	TCGATTTCTGCACAAGCCATCCAGCTG
Full-length Pem-GIH cDNA	
GIHF	GAACGTCTCGTATAAAAGGTCTGCG
GIHR	GGTCGACTTTATTTTAACGGAAAATTAAT
3' region of Pem-GIH cDNA	
matGIHF	AACATCCTGGACAGCAAATGCAGGG
GIHR	GGTCGACTTTATTTTAACGGAAAATTAAT
5' region of Pem-GIH cDNA	
GIHF	GAACGTCTCGTATAAAAGGTCTGCG
5'RACE-GIH1.1	CCGGCATTGAGGATGCTGAT
Amplification for recombinant protein expression in *P. pastoris*	
GIH-X-F	ATGAATTCGTCGACAAAAGAAACATCCTGGACAGC
GIH-X-R	ACTCTAGATCACCACGGCCGGCCGGC
Amplification for recombinant protein expression in *E. coli*	
matGIHx-F	GGAATTCCATATGAACATCCTGGACAGC
matGIHx-R	CGGGATCCTCACCACGGCCGGCCGGC
GIH-dsRNA templates	
T7-matGIHF	TAATACGACTCACTATAGGGAGAAACATCCTGGACAGCAAATGCAGG
T7-matGIHR	TAATACGACTCACTATAGGGAGACCGGCATTGAGGATGCTGAT

* T7 RNA polymerase binding site is underlined

harvested by centrifugation at 6,00 rpm for 5 min at room temperature before resuspended in 1/5 volume of BMMY medium [1% (w/v) yeast extract, 2% (w/v) peptone, 0.67% (w/v) YNB, 4 μg/ml D-biotin, 100 mM potassium phosphate]. The cells were cultured at 30°C with the addition of methanol to a final concentration of 0-4% every 24 h. An aliquot of the culture was collected daily from 0 to 7 days. After the cells were discarded, the culture medium was subjected to further analysis by SDS-PAGE.

Expression of recombinant Pem-GIH in *Escherichia coli* and protein purification

A cDNA encoding Pem-GIH was amplified by PCR with matGIHx-F and matGIHx-R (Table 2) and inserted into pET3a expression vector for recombinant protein expression in *E. coli* system. The recombinant plasmid was transformed into *E. coli* BL21(DE3)pLysS. An overnight culture of recombinant clone was dilute 50 fold in LB broth supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenical, and was further incubated at 37°C with shaking at 250 rpm until the culture reached an OD_{600} of 0.4-0.6. To induce the expression, IPTG was added to the culture to a final concentration of 0.4 mM and thecultutre was incubated at 37°C for another 4 h. The cell pellets were collected by centrifugation and resuspended in 450 μl of STE buffer [25 mM Tris.Cl pH8.0, 150 mM MaCl, 1mM EDTA] with 100 μg/ml lysozyme. The cell suspension was incubated on ice for 15 min prior to the addition of 45 μl of 10% triton-X in STE buffer. The cell lysate was subjected to sonication for 15 sec with 5 sec interval for a total time of 2 min. The cycle was repeated until complete cell lysis was obtained. The cell lysate was centrifuged at 13,000 rpm for 10 min. The pellets were washed three times in distilled water and resuspended in 8 M urea (in PBS pH 7.4). The soluble fraction in 8 M urea was subjected to purification by size exclusion chromatography (Superdex 75 column) and purified protein was analyzed on SDS-PAGE and the fraction containing the recombinant Pem-GIH was dialyzed to refold the protein.

Preparation of GIH-specific double-stranded RNA (GIH-dsRNA)

Two DNA templates for complementary RNA transcripts that were composed of the coding sequence of the mature Pem-GIH, each containing T7 promoter sequence at the 5' end on different strands were synthesized by PCR from the full-length Pem-GIH cDNA. Two separate PCR reactions were set up, one with T7-containing forward primer (T7-matGIHF) and reverse primer (5'RACEGIH1.1) for sense-strand template, the other with forward primer (matGIHF) and T7-containing reverse primer (T7-matGIHR) for antisense-strand template. The reaction was composed of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and extension at 74°C for 1min for 9 cycles with a 1°C decrease in annealing temperature per cycle; then the annealing temperature was remained at 48 °C for another 30 cycles and followed by the final extension at 74°C for 7 min. The expected PCR product were excised and purified with Gel extraction kit (QIAGEN), as described in the manufacturer's protocol. A mixture of 1 μg of each template was used in an *in vitro* transcription reaction with MEGAscript® RNAi Kit (Ambion, USA) according to the manufacturer's protocol with some modifications. Briefly, The two complementary templates were mixed in equal amounts and added to a single transcription reaction to synthesize dsRNA with T7

RNA polymerase at 37° C for 6h. To increase the duplex yield, the transcription reaction was incubated at 75° C for 5 minutes, and allowed to cool to room temperature DNA templates. The remaining DNA template and single-stranded RNA in the solution were digested with DNase I and RNase A at 37° C for 1 h. The proteins, free nucleotides and degraded nucleic acid residues were removed from double-stranded RNA by the filter cartridge as described in manufacturer's instructions. Finally, double-stranded RNA was eluted with 100 µl of 95° C pre-heated 10 mM Tris-HCl pH 7, 1 mM EDTA. The quantity of double-stranded RNA was determined by the UV-spectrophotometry at the absorbancy of A_{260}.

Silencing of Pem-GIH expression in shrimp explant culture by dsRNA

The eyestalk ganglia or abdominal nerve cords of *P. monodon* were dissected from individual shrimp. The eyestalk from a single shrimp was used in each experiment. The XOSG neuron from the left eyestalk was used as a negative control whereas that from the right eyestalk was treated with dsRNA as described below. The nerve cord from the same shrimp was cut into pieces of approximately 0.8-1 cm and used in one set of the experiment. The explant samples were incubated in a well of 24-well plate filled with 1.5 ml of modified M199 culture medium consisting of M199 powder in crab saline (440 mM NaCl, 11 mM KCl, 13.3 mM $CaCl_2$, 26 mM $MgCl_2$, 26 mM Na_2SO_4, and 10 mM HEPES), pH 7.2 supplemented with 100 µg/ml Penicillin-Streptomycin antifungus and 40 µg/ml gentamicin sulphate. The samples were added with 3 µg of GIH-dsRNA and cultured with shaking at 20-24 °C for appropriate time. The samples were then washed with modified M199 medium plus antibiotic before collected for RNA extraction. The level of Pem-GIH transcript was detected by RT-PCR with matGIHF and GIHR primers.

Pem-GIH activity assay by dsRNA-mediated functional knockdown

Adolescent female *P. monodon* (10-15 g each) at the same molting stage (D_0-D_1) were cultured in tanks filled with artificial seawater (approximately 10 ppt of salinity). Shrimp were divided into two groups. The control group was injected through the arthrodial membrane of the second walking leg with 100 µl of PBS and the experimental group was injected with 5 µg of GIH-dsRNA in the same volume. The level of GIH and Vg transcripts in the eyestalk ganglia and hepatopancreas, respectively were detected by RT-PCR at 72 h after administered with dsRNA.

Effect of GIH dsRNA on ovarian maturation

Adult domesticated shrimp (stage 1 of ovarian maturation) were kindly provided by Prof. Boonsirm Withyachumnarnkul. Shrimps were divided into there groups. The shrimp in the positive control group (9 shrimp) were unilaterally eyestalk-ablated whereas the shrimp injected with PBS (9 shrimp) were used as negative control. The experimental group (12 shrimp) was injected with GIH dsRNA at a final concentration of 30 µg/ 10 g shrimp. After eyestalk ablation or injection, the shrimp were cultured in sea water at 30 ppt salinity and the development of the ovary was recorded everyday until spawning.

Results

Cloning of Pem-GIH cDNA from *P. monodon*

The 3'RACE using different primers generated different bands of products. These bands were cloned into pGEM-T Easy vector and characterized by DNA sequencing. A total of 213 clones were analyzed. The results showed that 82 clones harbored the inserts that resembled molt-inhibiting hormone (MIH) whereas only 7 clones contained the inserts that showed high homology to GIH. The result was summarized in table 3.

Table 3 The results of 3' RACE showing the clones that are homology to MIH or GIH

Stage of Broodstock	1st round PCR	2nd round PCR	Approximate product sizes (bp)	No. of clones sequenced	No. of MIH/GIH clones
0-1	3'RACEGIH1 / PM1	3'RACEGIH2 / RevGIH	950, 120	20	-
0-1	3'RACEGIH1 / PM1	MG2 / PM1	950, 650	15	5
0-1	3'RACEGIH1 / PM1	3'RACEGIH1 / PM1	550, 400	5	-
0-1	3'RACEGIH3-1 / PM1	3'RACEGIH3-1 / PM1	200	6	-
0-1	3'RACEGIH3-2 / PM1	3'RACEGIH3-2 / PM1	200, 150	3	-
0-1	3'RACEGIH4 / PM1	3'RACEGIH4 / PM1	250	2	-
0-1	3'RACEGIH2 / PM1	MG2 / PM1	400	3	-
0-1	3'RACEGIH2 / PM1	-	550	2	-
0-1	3'RACEGIH2 / PM1	3'RACEGIH2 / PM1	600	3	-
0-1	MG1 / PM1	3'RACEGIH2 / PM1	500, 400, 350	10	-
0-1	3'RACEGIH2-1 / PM1	-	500-650	5	4
4	3'RACEGIH2-1 / PM1	-	500-650	34	30
4	3'RACEGIH2A / PM1	3'RACEGIH2A / 3'RACEGIH1	150-500	7	-
4	3'RACEGIH2A / PM1	3'RACEGIHD / PM1	250-450	4	-
4	3'RACEGIHA / PM1	3'RACEGIHD / PM1	250-450	6	-
4	3'RACEGIHD / PM1	-	250-450	10	-
2-3	3'RACEGIH1B / PM1	3'RACEGIH2C / PM1	500-600	5	5
2-3	3'RACEGIH2B / PM1	3'RACEGIH2C / PM1	700	2	-
0-1	3'RACEGIH2B / PM1	3'RACEGIH2C / PM1	350-700	21	17
4	3'RACEGIH2B / PM1	3'RACEGIH2C / PM1	200, 550	5	3
0-1	3'RACEGIH2B / PM1	3'RACEGIH2C / PM1	150	2	-
4	3'RACEGIH1A / PM1	3'RACEGIH1B / PM1	700	4	4
4	3'RACEGIH1A / PM1	3'RACEGIH2B / PM1	680	2	2
4	3'RACEGIH1B / PM1	3'RACEGIH2B / PM1	650	4	4
4	3'RACEGIH2C / PM1	-	500, 750, 900	5	-
4	3'RACEGIH1A / PM1	3'RACEGIH2C / PM1	700, 750	3	-
4	3'RACEGIH2B / PM1	3'RACEGIH2C / PM1	700, 750	3	-
2-3	3'RACEGIH1A / PM1	3'RACEGIH2B / PM1	550	1	-
2-3	3'RACEGIH1B / PM1	3'RACEGIH2B / PM1	500-700	16	15

Stage of Broodstock	1st round PCR	2nd round PCR	Approximate product sizes (bp)	No. of clones sequence d	No. of MIH/GIH clones
2-3	3'RACEGIH2C / PM1	-	600, 800	3	-
2-3	3'RACEGIH1A / PM1	3'RACEGIH1B / PM1	750	2	-
		Total		213	89

Nucleotide sequence analysis revealed that MIH-like clones can be divided into two major groups; the first group (18 clones) is identical to the previously identified Pem-MIH1 of *P. monodon* (Yodmuang et al., 2004, see appendices) and the second group (75 clones) is similar to Pem-MIH2. The clones in the Pem-MIH2 group could be divided into four different types that differ mainly in their 3' UTR region as shown in figure 2.

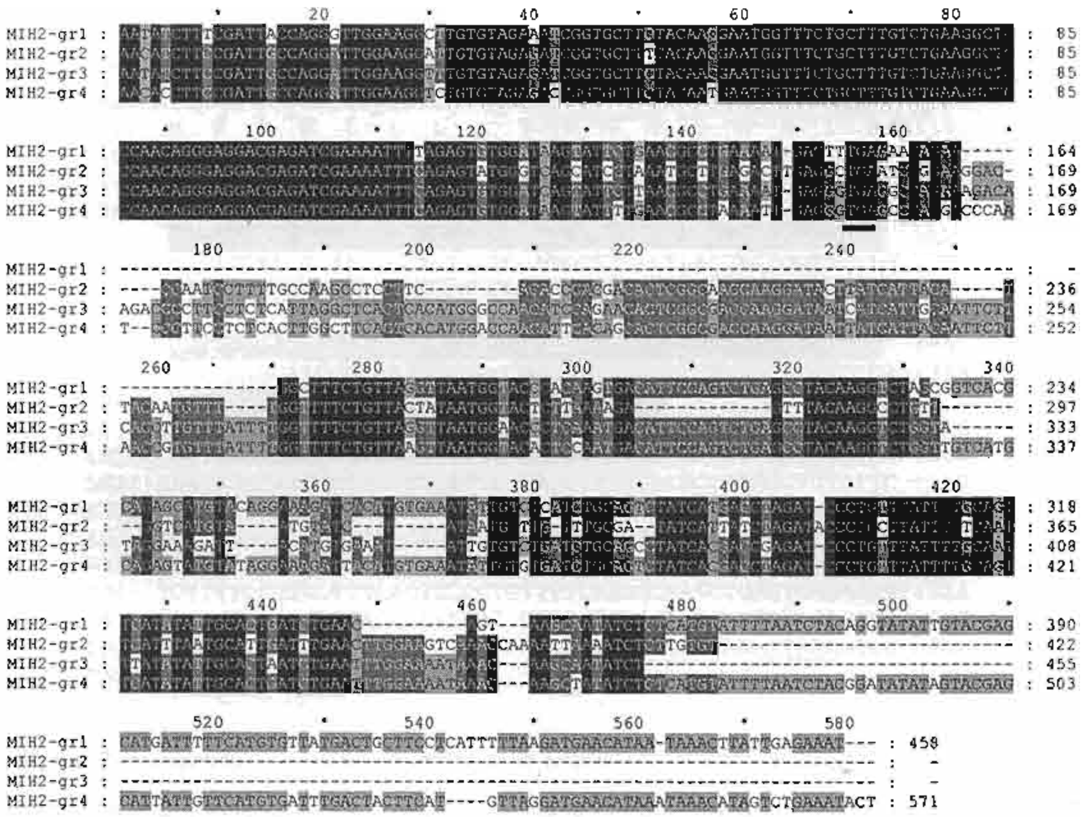

Figure 2. Alignment of nucleotide sequences of the four types of Pem-MIH2. Black shading represents identical nucleotides. Dark and light grey shading represent 75% and 50% homology, respectively. The stop codon is underlined.

The seven clones that contain GIH-like cDNA insert were identical indicating that they were generated form a single type of cDNA. The nucleotide sequence of this 3' GIH fragment was used to design specific primers for 5'RACE to obtain the remaining sequence at the 5' end of

GIH cDNA. Finally, a pair of specific primers was design from the 3' and 5' fragments and was used in the PCR reaction to obtain full-length Pem-GIH cDNA. The full-length cDNA encoding the putative GIH of *P. monodon* was composed of 861 nucleotides containing a 5'-untranslated region (93 bp), an open reading frame (288 bp), a stop codon (TGA), and 3'-untranslated region (476 bp) with a potential polyadenylation signal AATAAA located 7 bp upstream from the poly(A) tail. The open reading frame of Pem-GIH codes for a protein of 96 amino acid residues. The signal peptide, as predicted by SignalP 3.0 Server (WWW Prediction Server' at Center for Biological Sequence analysis, Technical University of Denmark) consisted of 17 amino acids, whereas the rest of the 79 amino acids comprised the mature Pem-GIH peptide (figure 3). The deduced amino acid of putative Pem-GIH showed the conservation of 6 cysteine residues in the mature peptide with a glycine residue at the fifth position after the first cysteine residue. The mature peptide of Pem-GIH showed 68% amino acid identity to the GIH of *M. ensis*, but a lower level of 45 and 48 % amino acid identity to that of *H. americanus* (Hoa-GIH) and *N. norvegicus* (Nen-GIH), respectively (figure 4).

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               AACGTCTCGTATAAAAGGTCTGCGAGCGAGCTACCCCCACAC :42
     AGCTCCACAGGCAGCGGCCCCTGCTACATACTTCAAGCATATCTGCAAGCA :93
 94: ATGAAAACATGGCTGCTATTAGCGACTCTGGTGGTGGGAGCGAGCTTAGCT :144
  1:  M  K  T  W  L  L  L  A  T  L  V  V  G  A  S  L  A  :17
145: AACATCCTGGACAGCAAATGCAGGGGTGCAATGGGTAATCGGGATATGTAC :195
 18:  N  I  L  D  S  K  C  R  G  A  M  G  N  R  D  M  Y  :34
196: AACAAGGTGGAGCGTGTTTGCGAGGACTGCACCAATATCTACCGGTTACCA :246
 35:  N  K  V  E  R  V  C  E  D  C  T  N  I  Y  R  L  P  :51
247: CAGCTGGATGGCTTGTGCAGAAATCGATGCTTCAATAACCAGTGGTTCCTG :297
 52:  Q  L  D  G  L  C  R  N  R  C  F  N  N  Q  W  F  L  :68
298: ATGTGCCTCCACTCGGCCAAGCGCGAGGCCGAACTCGAGCATTTCAGACTC :348
 69:  M  C  L  H  S  A  K  R  E  A  E  L  E  H  F  R  L  :85
349: TGGATCAGCATCCTCAATGCCGGCCGGCCGTGGTGATCTTTCCTTCTCTGA :399
 86:  W  I  S  I  L  N  A  G  R  P  W  *
400: AAGCATCCCACGCGCCCCAGCCTCAGTCCTGATCAGGGACACCTGGCAGGA :450
451: CGCTGGCACAGCTCTCGAGTCTCCCCCTGCGCTATTAAACGTGATCATTGG :501
502: AAATAGACATCTCTAGGCCGATTCGGATGCTTCGACGCCTTGGCTGTATTC :552
553: CTTTATGAATACATTTAGGAGTGGATAGGTCCTCTCTCAGTGTAATTTAAG :603
604: GGCGTATTATTGCATTAGAGAAAGTCTAAGTACTCTAGAATCAAATTTTTC :654
655: TTTCGTTTCACTGCTATCTATTGTTTTCAATTTTGTTTCCCATCTTTTACT :705
706: TGTTTTCCCTGGTCTCAGTGCCCTGTTTTCAAGGGGAACTGATTGGGAAAT :756
757: AAAAATTGAAGATGACCCCATAATGCTTTGTGTTGAAACATTGCGCACTTC :807
808: TTAATGTTTAGCATATCCTGTGTATATTAATTTTCCGTTAAAATAAAGTCG :858
859: ACC(A)n
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Figure 3. Nucleotide and deduced amino acid sequences of Pem-GIH cDNA. The amino acids are presented in one-letter symbol. The mature peptide of Pem-GIH is highlighted in black. The asterisk marks the stop codon, and the polyadenylation signal is underlined.

Tissue-specific expression of Pem-GIH

The expression of Pem-GIH in several tissues of *P. monodon* was examined by RT-PCR to detect specific transcript of GIH. The Pem-GIH transcript at the expected size of 385 bp was found in the eyestalk neural tissues, brain, thoracic ganglia and abdominal nerve cord, whereas no transcript could be detected in other tissues examined. Moreover, tissues from both adolescent and adult male and female *P. monodon* were examined for Pem-GIH expression. The transcript of Pem-

GIH was found to be expressed in the same tissues in both male and female of adolescent and adult shrimp (figure 5).

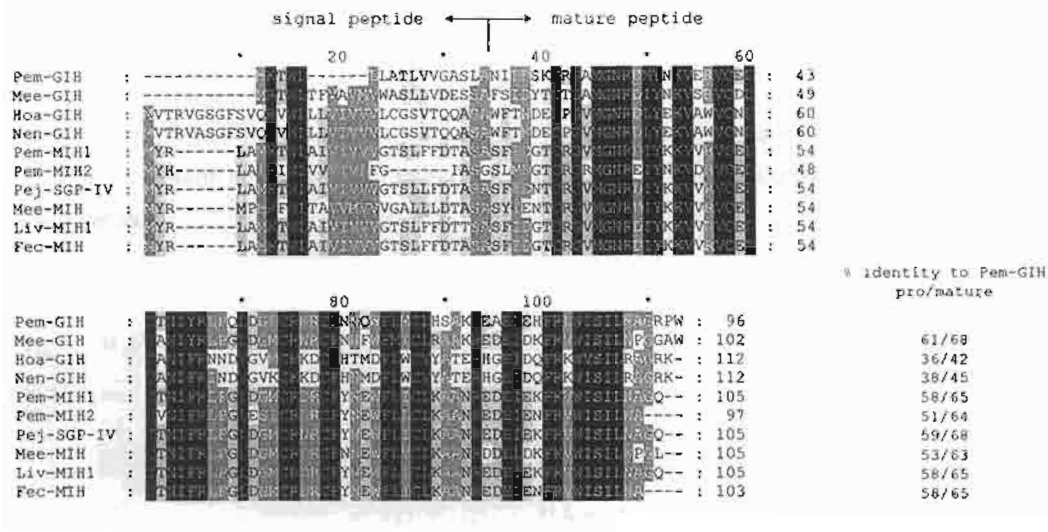

Figure 4. Amino acid sequence comparison GIH and MIH from diverse species. Pem-GIH sequence is shown in the top line. The identity between the Pem-GIH and other hormones in both the pro- and the mature peptides are shown on the right of the alignment.

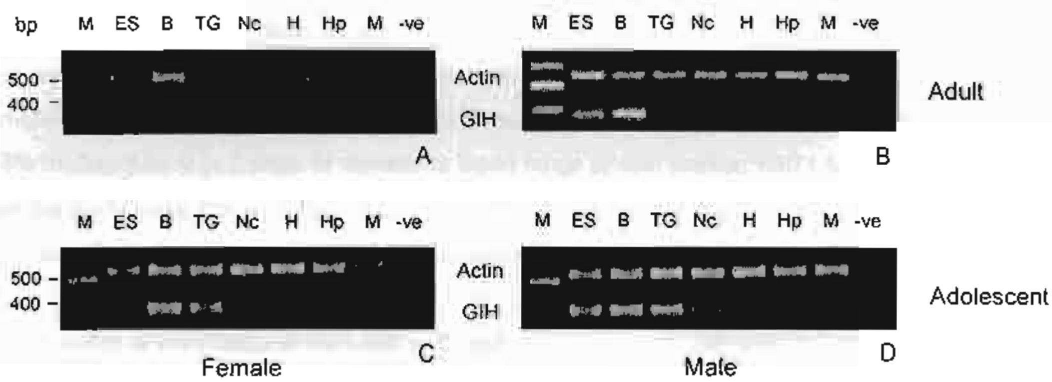

Figure 5. Specific expression of Pem-GIH in *P.monodon*'s tissues. The transcript of Pem-GIH was detected by RT-PCR in muscle (M), eyestalk neurons (ES), brain (B), thoracic ganglia (TG), abdominal nerve cord (Nc), heart (H) and hepatopancreas (Hp). The expression of Pem-GIH in the tissues of both female and male of adolescent and adult *P. monodon* is presented in indicated panels.

Expression of recombinant Pem-GIH in *P. pastoris*

The recombinant clones containing Pem-GIH cassette integrant were screen for expression in 1 ml culture. The clone that gave the highest expression level of recombinant Pem-GIH was selected to determine the optimal expression condition by varying the concentration of methanol from 0 to 4 % and the induction period from 0 to 7 days. The result in figure 6 showed that the highest expression was obtained with the induction by 3% methanol for 2 days. However, the level of recombinant Pem-GIH expression was several folds lower than that of recombinant CHH. To obtain higher amount of recombinant Pem-GIH, the expression was conducted in E. coli expression system instead.

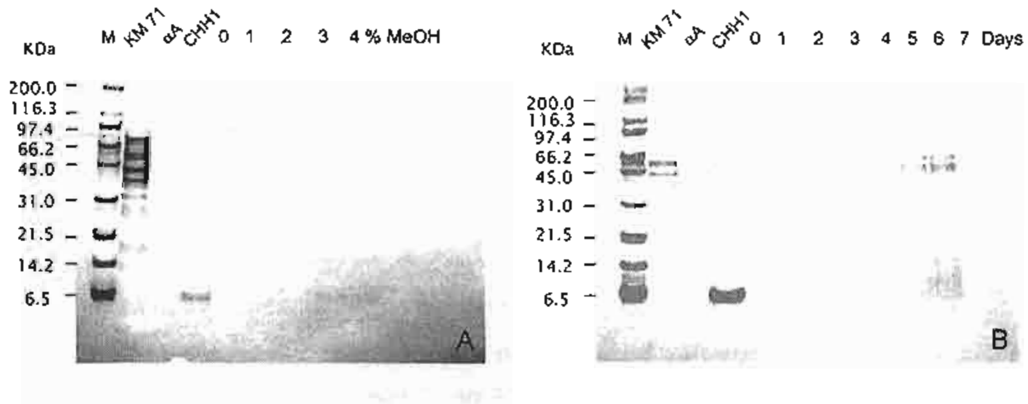

Figure 6. Expression of recombinant Pem-GIH in *P. pastoris*. The figure shows SDS-PAGE analysis of recombinant Pem-GIH in the culture medium of recombinant clones. (A) The concentration of methanol was varied from 1 to 4 % for the period of 2 days. (B) The expression was induced with 3% methanol for 0 to 7 days. M represents broad range protein marker; KM71 is the culture medium of the Km71 host; αA is culture medium of KM71 containing pPICZαA vector and CHH represents the culture medium of recombinant KM71 containing Pem-CHH.

Expression of recombinant Pem-GIH in *E. coli*

The expression of recombinant Pem-GIH in E. coli was induced by 0.4% IPTG at 0.5 OD_{600} of the culture for 4 h. The expression temperature was varied at 30°C and 37°C. Figure 7 showed the expression level of recombinant Pem-GIH of different recombinant clones at both temperatures in the condition either with or without IPTG induction. The result revealed that the expression level from each clones are similar but the expression at 30°C gave a little bit higher level of the recombinant Pem-GIH than expression at 37°C. Clone number 10-1 was selected for further study.

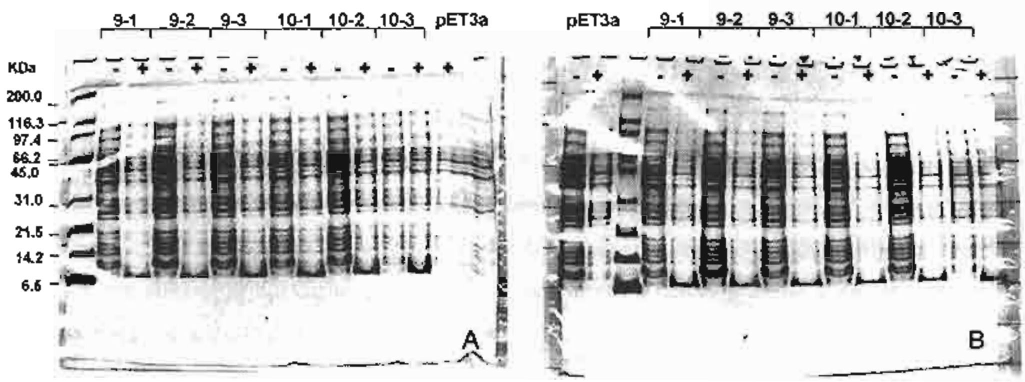

Figure 7. Expression of recombinant Pem-GIH in *E. coli*. SDS-PAGE analysis shows the expression from different recombinant clones. The expression was expressed at 30°C (A) and 37°C (B) either without (-) or with (+) the induction of 0.4 mM IPTG for 4 h. pET3a represents the protein expressed from recombinant clone containing pET3a vector alone.

Purification of recombinant Pem-GIH

The inclusion of the expressed product from clone 10-1 was solubilized in 8 M urea and purified by size exclusion chromatography. The eluted fractions were analyzed by SDS-PAGE. The result in figure 8 revealed that the recombinant Pem-GIH could be purified as a single peak from other proteins in fractions 11 to 14.

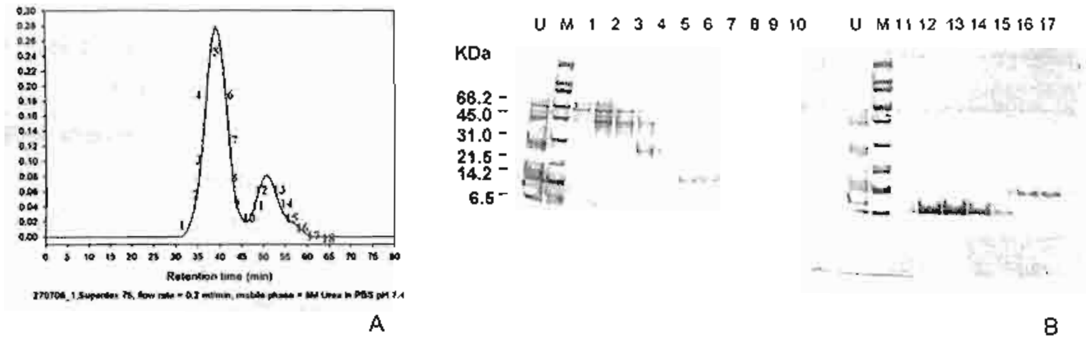

Figure 8. Purification of recombinant Pem-GIH by size exclusion chromatography. The inclusion was solubilized in 8 M urea and purified by Superdex 75 column. The elution profile is shown in (A) the numbers indicate the collected fractions. The proteins in each fraction were analyzed on SDS-PAGE (B). Lane U represents the unpurified soluble proteins. M is broad range standard protein marker. Each lane is corresponded to the fraction indicated in (A).

Production of double-stranded RNA of Pem-GIH

The DNA templates for the synthesis of two complementary RNA strands of GIH were amplified by PCR using T7-containing forward primer (T7-matGIHF) with reverse primer (matGIHR) for sense strand template, and forward primer (matGIHF) with T7-containing reverse primer (T7-matGIHR) for antisense strand template (figure 9A). These two templates were subjected to *in vitro* transcription, and the transcribed sense and antisense RNA of mature Pem-GIH were annealed to yield double-stranded RNA (Figure 9B). The GIH double-stranded RNA (dsRNA) was completely digested with RNase III but resisted to RNase A digestion indicating that the annealing product was really in the form of dsRNA.

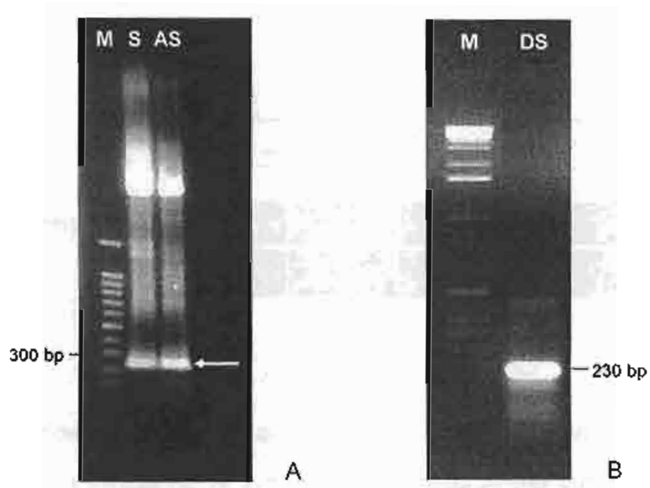

Figure 9. The synthesis of GIH dsRNA

A. Amplification of DNA template for in vitro transcription of sense (S) and antisense (AS) RNA strands of Pem-GIH.

B. Annealling product (DS) between sense and antisense strand transcribed from the template in (A) to produce GIH dsRNA

dsRNA-mediated Pem-GIH knockdown in shrimp explant culture

The GIH-dsRNA was first determined for its efficacy to knockdown GIH expression in GIH expressing tissues ie. eyestalk neurons and abdominal nerve cord. The eyestalk XOSG neurons and abdominal nerve cord explants were cultured in a culture medium that contained GIH-dsRNA. RT-PCR result showed the barely detectable level of GIH transcript in the GIH-dsRNA treated eyestalk XOSG culture from adult female shrimp after 3 h (Fig. 10A) indicating that GIH-dsRNA could efficiently inhibit GIH expression. Similar results were observed when the abdominal nerve cord from either adult or adolescent female shrimp was incubated with GIH-dsRNA at 3 and 6h (Figs. 10B and 10C). However, the irrelevant dsRNA, GFP-dsRNA, failed to knockdown Pem-GIH mRNA expression as the abdominal nerve cord incubated with GFP-dsRNA expressed similar level of Pem-GIH transcript to that of the control sample into which no dsRNA was added. These results indicated that GIH-dsRNA was capable of triggering sequence-specific knockdown of Pem-GIH expression in shrimp explant culture, and thus was a potent tool for functional study of Pem-GIH in the shrimp.

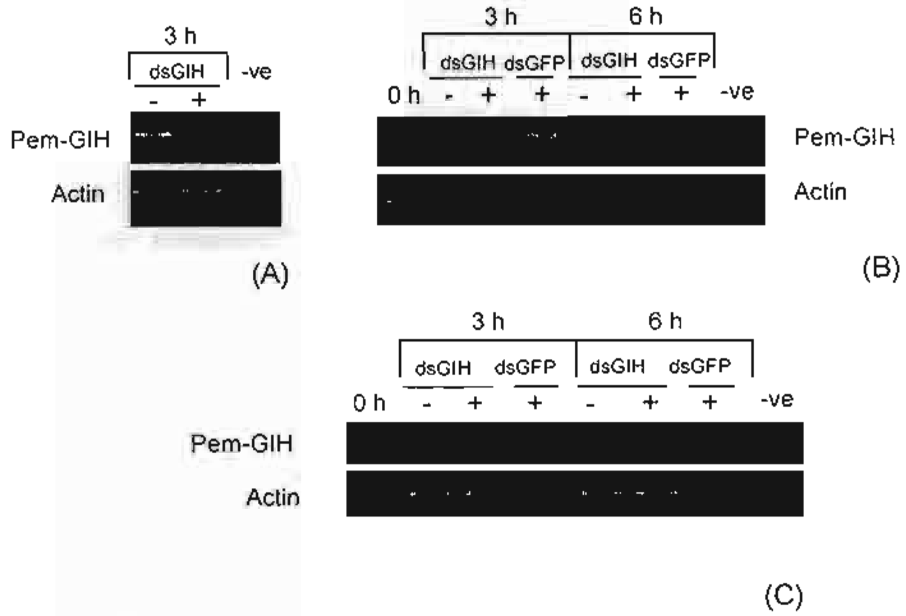

Figure 10. Specific knockdown of Pem-GIH expression by GIH-dsRNA in shrimp explant culture. The eyestalk neurons and abdominal nerve cord were incubated in the culture medium with (+) or without (-) GIH-dsRNA. dsRNA of green fluorescent protein (dsGFP) was used as the control. The Pem-GIH transcript in each sample was detected by RT-PCR after 3 or 6 hour of incubation. The experiments were performed with eyestalk from adult female *P. monodon* (A) and abdominal nerve cord of adult (B) and adolescent (C) female.

Biological assay for vitellogenesis-inhibting activity of Pem-GIH by dsRNA

To test whether Pem-GIH knockdown by dsRNA would interfere with the process of Vg synthesis in the hepatopancreas, female *P. monodon* was injected with GIH-dsRNA and the level of Pem-GIH expression as well as the expression of Vg transcript in the shrimp was determined by RT-PCR. First, the expression of GIH in eyestalk ganglia and Vg in the hepatopancreas was investigated in shrimp at different stages. The RT-PCR result in figure 11 illustrated that GIH and Vg were expressed throughout the life cycle of the shrimp from juvenile to adult. Due to the unavailable of adult female, the effect of GIH-dsRNA on GIH and Vg mRNA level was investigated in adolescent shrimp instead. The result in figure 12 showed that Pem-GIH transcript level in the eyestalk decreased more than 75% in the shrimp administered with GIH-dsRNA at 72 h following dsRNA injection comparing with the control shrimp that was injected with PBS only. The consequence of the depletion in GIH transcript on Vg synthesis was next investigated. RT-PCR analysis revealed that Vg transcription levels were exerted about twofold in the hepatopancreas of GIH-knockdown shrimp

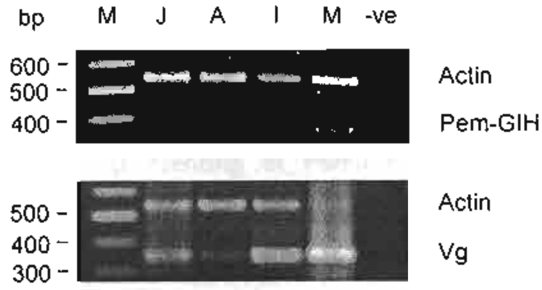

Figure 11. Expression of Pem-AGO and vitellogenin (Vg) at different stages of *P. monodon*. The Pem-AGO and Vg transcripts were detected by RT-PCR in juvenile (J), adolescent (A), immature female (I) and mature female (M) of *P. monodon*.

when compared with that in the control shrimp (figure 12). The increase in the ratio of Vg to actin transcripts in GIH-depleted background suggested that knockdown of Pem-GIH led to the induced expression of Vg in the hepatopancreas.

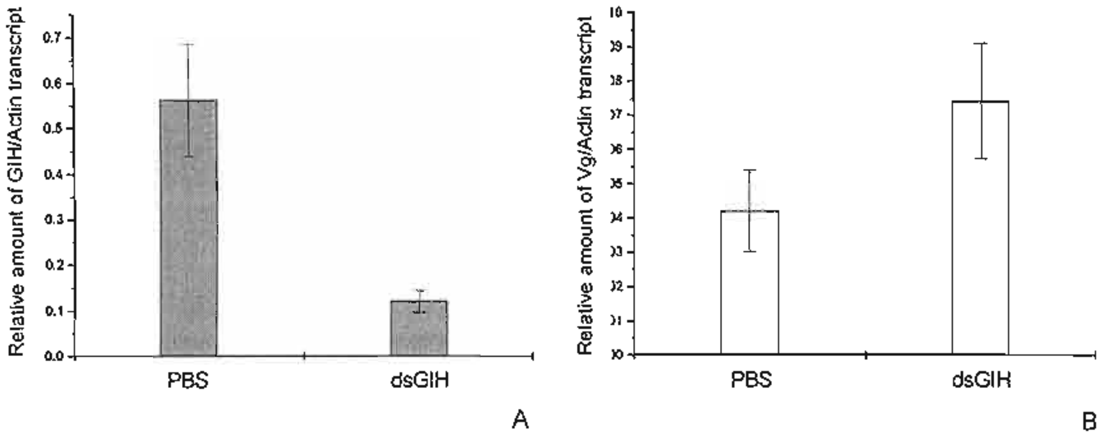

Figure 12. Effect of GIH dsRNA on the expression of GIH and vitellogenin (Vg) in shrimp. Adolescent *P. monodon* were divided into two groups. The control group (n = 14) was injected with PBS whereas the experimental group (n = 8) was injected with dsGIH (5 µg/shrimp. The transcripts of GIH (A) and Vg (B) were detected by RT-PCR and normalized with the level of actin transcript.

Effect of GIH-dsRNA on ovarian maturation in female *P. monodon*

To investigate the consequence of Pem-GIH knockdown on ovarian maturation, GIH-dsRNA was injected into adult female shrimp at stage 0 of ovarian development. After injection the shrimp were returned to the culture pond and the development of the ovary was followed everyday. The control shrimp were subjected to eyestalk ablation in stead of dsRNA injection and the negative control group was injected with PBS. The result in table 4 showed that eyestalk ablation could induce ovarian maturation and successful spawning in four out of nine shrimp in the control group. By contrast, only one from nine shrimp of the negative control group could spawn. The shrimp in the experimental group that were injected with GIH-dsRNA showed moderate percentage of successful spawning (2 out of 11 shrimp) comparing to both the eyestalk-ablated and the PBS-injected groups. This preliminary result suggests that silencing of Pem-GIH expression by dsRNA-mediated knockdown may have positive influence in induction of ovarian maturation that led to successful spawning in adult female shrimp.

Table 4. Ovarian maturation and spawning in female broodstock of *P. monodon*. Female broodstock at stage 0 of ovarian development were unilaterally eyestalk-ablated (A), injected with PBS (B) or injected with GIH dsRNA (C). The development of ovaries and spawning was followed and recorded for 12 days (D0-D11).

A

ES ablation group (right eye) . Total = 9 adult female shrimp (S0)													
		time (Day)											
No.	body weight (g) :length (inch)	D0	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11
110	115g:8.6"	S0	S0	S0	S0	S0	S0	S1	S2	S3	..	S0	S0
111	103g:8.0"	S0	S0	S0	S0	S0	S0	S0	S0	S0	S0	S0	S0
112	97g:8.1"	S0	S0	S0	S0	S0	S0	Molt+AI	S0	S0	S0	S0	S0
113	102g:8.0"	S0	S0	S0	S0	S0	S0	S0	S0	S0	S0	S0	S0
114	92g:8.2"	S0	S0	S0	S0	S0	S0	S0	Molt+AI	S0	S0	S0	S0
116	120g:8.4"	S0	S0	S0	S1	S1	S2	S3	[illegible]	S0	S0	Molt+AI	S0
117	95g:8.1"	S0	S0	S0	S0	S0	S0	S0	Molt+AI	S0	S0	S0	S0
120	107g:8.4"	S0	S1	S2	S3		S0	S1	S0	Molt+AI	S0	S0	S0
121	126g:8.4"	S0	S0	S0	S0	S1	S2	S3/Spawn	Molt+AI	S1	S2	S3	[illegible]

B

dsGIH injected group (10mM NaCl, 10mM TrisCl pH7.0) : Total = 11 adult female shrimp (S0)													
		time (Day)											
No.	body weight (g) :length (inch)	D0	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11
125	133g:8.5"	S0	S0	S0	S0	S0	S0	S0	S0	S0	S0	S0	Molt+AI
133	109g:8.3"	S0	S0	S0	S0	S0	S0	S0	S0	Molt+AI	S0	S0	S0
134	87g:7.7"	S0	S0	S0	S0	S0	S0	Molt+AI	S0	S0	S0	S0	S1
137	108g:8.4"	S0	S0	S1	S1	S2	S3	S3	[illegible]	Molt+AI	S0	S0	S0
138	103g:8.1"	S0	S0	S0	S0	S0	S0	Molt+AI	S0	S0	S0	S0	Dead
140	87g:8.2"	S0	S0	S0	Molt+AI	S0	S0	S0	S0	S0	S0	S0	S1
141	150g:9.2"	S0	S0	S0	S0	S0	S0	S0	S0	Molt+AI	S0	S0	S0
142	114g:8.5"	S0	S0	S0	S0	S0	S0	S0	S0	Molt+AI	S0	S0	S0
144	9.5g:8.1"	S0	S0	S0	S0	S0	S0	S0	Molt+AI	S0	S0	S0	S0
146	83g:8.0"	S0	S0	S0	S0	S0	S0	S0	S0	Molt+AI	S0	S0	S0
147	121g:8.4"	S0	S0	S0	S0	S0	S0	Molt+AI	S1	S2	[illegible]	S0	S0

C

Buffer group (10mM NaCl, 10mM TrisCl pH7.0) : Total = 9 adult female shrimp (S0)													
		time (Day)											
number	body weight (g) :length (inch)	D0	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11
122	115g:8.2"	S0	S0	S0	S0	S0	S0	S0	S0	S0	S0	S0	S0
123	122g:8.0"	S0	S0	S0	S0	S0	S0	S0	S0	S0	S0	S0	S0
126	101g:8.2"	S0	S0	S0	S0	S0	S0	S0	S0	S0	S0	Molt+AI	S0
127	130g:8.5"	S0	S0	S0	S0	S0	S0	S0	Molt+AI	S0	S0	S0	S0
128	122g:8.3"	S0	S0	S0	S0	S0	S0	S0	S0	S0	S0	S0	S0
130	130g:8.5"	S0	S0	S0	S1	S1	S1	S2	S0	S0	S0	S0	S0
131	128g:8.4"	S0	S0	S0	S0	S0	S0	S0	S1	S2	[illegible]	Molt+AI	S0
132	102g:8.4"	S0	S0	S0	S0	S0	S0	Molt+AI	S0	S0	S0	S0	S0
135	102g:8.2"	S0	S0	S0	S0	S1	S1	S1	S0	Molt+AI	S0	S0	S0

S0 to S4 represent stage 0 to stage 4 of ovarian development. AI = artificial insemination

Discussion

This study has identified and characterized a cDNA encoding gonad-inhibiting hormone (GIH) of the black tiger shrimp *P. monodon*. Due to the close similarity in the primary structure of molt-inhibiting hormone (MIH) and GIH, the two hormones that comprise type II of the CHH hormone family of crustacean (Chen et al., 2005), the degenerate primers for cloning of GIH cDNA were designed from the conserved regions of these two hormones. The RNA sample for cDNA cloning was extracted from adult female shrimp with developing ovaries at different stages. Generally, the reproductive stages in shrimp are induced at the intermolt, the stage during which high level of MIH is needed to prevent molting. Therefore, it is not surprising that the majority of cDNA fragments obtained by 3'RACE using degenerate primers from MIH/GIH conserved regions belonged to MIH sequences. Only 7 out of 89 clones showed homology to GIH sequences. These Pem-GIH clones were identical. The deduced amino acid sequence possesses all characteristics that are in agreement with the type II hormone of the CHH family. The C-terminus of Pem-GIH had an extension of two amino acid residues when compared with that of MIH, a typical feature that is shared by other GIH sequences. Moreover, the high expression of Pem-GIH in thoracic ganglia rather than the eyestalk neurons also agreed well with GIH of *M. ensis* (Gu et al., 2002) and the tentative GIH of *Marsupenaeus japonicus* (Ohira et al., 2006). This Pem-GIH cDNA was therefore further examined for its role in inhibition of vitellogenesis , the key process during ovarian maturation.

RNA interference (RNAi) is a process whereby double-stranded RNA (dsRNA) is cleaved into short RNA fragments about 21-23 nucleotides called small interfering RNA (siRNA) which is then triggers the cleavage of cognate mRNA (Hannon, 2002) This specific cleavage requires perfect complementary between the siRNA and the mRNA target. RNAi has been widely used as a powerful technique for functional study of an interesting gene (Volz et al., 2005; Martin et al., 2006). In this study RNAi was employed as a tool to study the function of Pem-GIH cDNA in vitellogenesis inhibition. Double-stranded RNA was synthesized from the coding sequence of mature Pem-GIH. This GIH-dsRNA does not contain any regions in which longer than 21 consecutive identical nucleotides were found between GIH and MIH or CHH of *P. monodon*. Thus, this GIH dsRNA would specifically target the cleavage of Pem-GIH mRNA only.

RNAi knockdown of Pem-GIH expression was first examined in explant culture of eyestalk ganglia and abdominal nerve cord. The result clearly demonstrated that Pem-GIH dsRNA was able to silence expression of GIH efficiently (Figure 11). By contrast, irrelevant dsRNA of green fluorescent protein (GFP) failed to knockdown Pem-GIH transcript suggesting that the silencing of Pem-GIH by dsRNA occurred in a sequence specific manner. The potency of GIH dsRNA to silence Pem-GIH expression was also demonstrated in shrimp (Figure 12A). The consequence of GIH silencing by dsRNA on vitellogenin expression was investigated in GIH knockdown shrimp in order to establish the role of Pem-GIH on vitellogenesis. Although the mode of action of GIH on vitellogenesis is still unclear, it has been demonstrated that recombinant GIH of *Homarus americanus* could inhibit expression of vitellogenin in the ovary of heterologous species, *M. japonicus* (Ohira et al., 2006). In addition to the ovary, hepatopancreas has also been shown to be another site for vitellogenin synthesis (Tseng et al., 2001). Since the adolescent shrimp were used in our study, the consequence on vitellogenin synthesis was only examined in the hepatopancreas. An increase in the level of vitellogenin expression in the hepatopancreas of GIH-knockdown shrimp indicating that Pem-GIH plays inhibitory role in vitellogenesis by inhibiting the expression of vitellogenin. We also achieved high-level expression of recombinant Pem-GIH in *E. coli*. The biological function of Pem-GIH in vitellogenesis could be further confirmed by an assay that utilizes this recombinant Pem-GIH to inhibit the expression of vitellogenin in the ovary or the hepatopancreas.

It has been shown that vitellogenin expression in the hepatopancreas is correlated to ovarian maturation (Jayassankar et al., 2002). Therefore, we also investigated the consequence on ovarian maturation by silencing GIH expression in female broodstock of *P. monodon* by GIH dsRNA and tracking the development of the ovary as well as spawning. Although no conclusive result was obtained, it still provided promising information that silencing of GIH expression by dsRNA could induce ovarian maturation in the female broodstock to certain extent. This result is in concurrence with the precocious ovarian development after the main source of GIH synthesis was removed by eyestalk extirpation (Browdy and Samocha, 1985). Our studies on functional knockdown of Pem-GIH in both the explant culture and the shrimp also revealed the relationship between vitellogenin synthesis in the hepatopancreas and the ovarian maturation. Additionally, it could be postulated that Pem-GIH plays role at the early step in vitellogenesis.

The expression of Pem-GIH was also detectable in male shrimp, which is similar to GIH of *H. americanus* and the Norway lobster *Nephrops norvegicus* (De Kleijn et al., 1992; Edomi et al., 2002). The role of GIH in male crustacean is not well established. However, eyestalk ablation in the crayfish *Cherax quadricarinatus* resulted in an over-expression of androgenic gland (AG) polypeptides, which caused direct effect on the male reproductive system. It is therefore possible that GIH may play a more versatile role in male crustacean as well.

Finally, the GIH dsRNA that was synthesized in this study has been demonstrated to have potency in the induction of ovarian maturation via inhibition of vitellogenesis. Hence, this dsRNA could be applied as an alternative method to induce spawning of *P. monodon*'s female broodstock. This strategy can substitute the eyestalk ablation method and will help prevent over-exploitation of wild broodstock as well as increase the efficiency of shrimp domesticated program. This will constantly make Thailand retains its leader position of the shrimp exporter worldwide.

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Yodmuang S., **Udomkit A.**, Treerattrakool S. and Panyim S. (2004) Molecular and biological characterization of molt-inhibiting hormone of *Penaeus monodon*. *J Exp Mar Biol Ecol* **312**, 101-114.

Outputs

Publications

1. Yodmuang S, **Udomkit A**, Treerattrakool S and Panyim S (2004) Molecular and biological characterization of molt-inhibiting hormone of *Penaeus monodon*. *J Exp Mar Biol Ecol* 312, 101-114.
2. Treerattrakool S, **Udomkit A** and Pamyim S (2006) Anti-CHH antibody causes impaired hyperglycemia in *Penaeus monodon*. *J Biochem Mol Biol* 39, 371-376.

Submitted manuscript

Treerattrakool S, Udomkit A, Chan SM and Panyim S. Molecular characterization of gonad-inhibiting hormone of *Penaeus monodon* and elucidation of its inhibitory role in vitellogenin expression by RNA interference. (submitted to FEBS Journal)

International conferences

1. Treerattrakool S, **Udomkit A**, Chan S-M and Panyim S. cDNA cloning and functional study of gonad-inhibiting hormone (GIH) from the eyestalk of *Penaeus monodon*. World Aquaculture 2005. May 9-13, 2005. Bali International Convention Center. Nusa Dua, Bali, Indonesia.
2. Treerattrakool S, Panyim S and **Udomkit A.** cDNA cloning and functional study of gonad-inhibiting hormone (GIH) from the eyestalk of *Penaeus monodon*. The International Conference on Shrimp Biotechnology: New Challenges through Thai Shrimp Industry. Navember 4-5, 2005. Queen Sirikit National Convention Center, Bangkok.
3. Treerattrakool S. and **Udomkit A.** Characterization of multiple molt-inhibiting hormone genes in the genome of *Penaeus monodon*. The International Conference on Shrimp Biotechnology: New Challenges through Thai Shrimp Industry. Navember 4-5, 2005. Queen Sirikit National Convention Center, Bangkok.

Appendices

Reprints

1. Yodmuang S, **Udomkit A**, Treerattrakool S and Panyim S (2004) Molecular and biological characterization of molt-inhibiting hormone of *Penaeus monodon*. *J Exp Mar Biol Ecol* 312, 101-114.
2. Treerattrakool S, **Udomkit A** and Pamyim S (2006) Anti-CHH antibody causes impaired hyperglycemia in *Penaeus monodon*. *J Biochem Mol Biol* 39, 371-376.

Submitted manuscript

Treerattrakool S, Udomkit A, Chan SM and Panyim S. Molecular characterization of gonad-inhibiting hormone of *Penaeus monodon* and elucidation of its inhibitory role in vitellogenin expression by RNA interference. (submitted to FEBS Journal)

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Molecular and biological characterization of molt-inhibiting hormone of *Penaeus monodon*

Supansa Yodmuang, Apinunt Udomkit*,
Supattra Treerattrakool, Sakol Panyim

Institute of Molecular Biology and Genetics, Mahidol University, Salaya Campus, Nakhon Pathom 73170, Thailand

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Abstract

The action of molt-inhibiting hormone (MIH) on the inhibition of ecdysone release from the Y-organ of decapod crustacean keeps the animal in the intermolt stage that dominates its molting cycle. MIH is thus one of the major keys in mediating growth and reproduction. This study has isolated cDNA encoding two types of MIH, *Pem-MIH1* and *Pem-MIH2*, from the black tiger shrimp, *Penaeus monodon* on the basis of sequence homology to MIH from two other shrimp species. The full-length cDNA of Pem-MIH1 was characterized. *Pem-MIH1* cDNA harbored 318 bp open reading frame that coded for a translated product containing 28 amino acids of the signal peptide and a putative mature Pem-MIH of 77 amino acids. The recombinant Pem-MIH1 was expressed in *Pichia pastoris* as a secreted protein. After purification by gel filtration, the purified Pem-MIH1 exhibited the ability to extend molting duration of *P. monodon* from 11.8 days to 16.3 days suggesting that Pem-MIH1 be responsible for molt-inhibiting function in the shrimp. The attempt to clone *Pem-MIH1* and *Pem-MIH2* genes was achieved by direct PCR amplification and PCR-based genome walking strategy, respectively. The structure of both *Pem-MIH* genes, containing three exons interrupted by two introns, was similar to each other and also to that of MIH genes of other crustaceans reported so far. Expression study of *Pem-MIH1* and *Pem-MIH2* in various tissues of *P. monodon* revealed the difference in expression patterns. *Pem-MIH1* expressed in both the eyestalk and the thoracic ganglia whilst *Pem-MIH2* expression was limited

* Corresponding author. Tel.: +66 2 800 3624x1237; fax: +66 2 441 9906.
E-mail address: staud@mahidol.ac.th (A. Udomkit).

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to the eyestalk. The expression of MIH in non-eyestalk tissue may suggest additional role of this hormone.

Keywords: Eyestalk; MIH; Molting; *Penaeus monodon*

1. Introduction

Molting is an important physiological process that is essential for growth and development in crustacean. The molting process is regulated by two antagonistic hormones. Ecdysteroids, released from the Y-organ, function as a molting hormone to trigger the onset of this process (Skinner, 1985). The synthesis of ecdysteroids is presumed to be negatively regulated by molt-inhibiting hormone or MIH (Mattson and Spaziani, 1985; Skinner, 1985). Molt-inhibiting hormone is synthesized by a cluster of neuro-secretory cells called the X-organ sinus gland complex located in the medulla terminalis of the eyestalk (Keller, 1992).

Owing to amino acid sequence similarity, MIH is classified as a member of the crustacean hyperglycemic hormone (CHH) family (Keller, 1992; de Kleijn and Van Herp, 1995). The hormones in this family share similar sizes and amino acid sequences (approximately 30–80% identity). A distinctive feature of the CHH neuropeptide family is the presence of six cysteine residues that are aligned at the same positions in all members (Keller, 1992). The CHH family is classified into two types, type I and type II based upon their primary structure (Lacombe et al., 1999). All CHHs identified so far fall into type I consisting of 72 amino acids with amidated carboxyl terminus (Chang, 1997). The hormones in type II vary in size from 73 to 81 amino acids and contain an amino acid glycine at position 12, which is not present in type I hormones (Lacombe et al., 1999). Type II of the CHH family includes MIH and the other two hormones, gonad-inhibiting hormone (GIH) and mandibular organ inhibiting hormone (MO-IH).

Although MIH has been shown to inhibit the synthesis of the molting hormone, ecdysone, it is plausible that CHH family peptides other than MIH also play integrated roles to control molting process. For instance, CHH of the crab *Carcinus maenas* showed inhibitory effect on ecdysteroid secretion by the Y-organ in vitro (Webster and Keller, 1986). Likewise, MIH itself may influence other processes. In the shrimp *Metapenaeus ensis*, the level of MIH-B transcript increased as the gonad maturation proceeded, and thus the hormone might have important function in the regulation of female reproduction (Gu et al., 2002).

MIH has been isolated from the sinus gland of the lobsters (Chang et al., 1990; Marco et al., 2000), the crabs (Webster, 1991; Chung et al., 1996), the crayfish (Aguilar et al., 1996; Nagasawa et al., 1996) and the prawn (Yang et al., 1996). Based on the amino acid sequences of these peptides, the cDNA encoding MIH has been obtained from several species by the use of recombinant DNA technology (Klein et al., 1993; Chan et al., 1998; Umphrey et al., 1998; Gu and Chan, 1998a; Lu et al., 2001; Gu et al., 2002). This has enabled further studies on regulation as well as functional aspects of this hormone. Recombinant MIHs have been produced and demonstrated to be biologically active either by the inhibition of ecdysone release from the Y-organ (Ohira et al., 1999) or by extending the molting cycle duration (Gu et al., 2001, 2002). In addition, several putative binding sites for transcription factors have been identified that may play roles in the regulation of MIH gene expression (Chan et al., 1998; Lu et al., 2000).

In this study, the gene and the cDNA encoding molt-inhibiting hormone (Pem-MIH) of the black tiger shrimp, *Penaeus monodon*, one of the most important cultured species in the world were cloned and characterized. The biological activity of the recombinant Pem-MIH was demonstrated. Finally, the expression of Pem-MIH in several types of shrimp tissues was examined.

2. Materials and methods

2.1. RNA isolation and cDNA synthesis

P. monodon were purchased from local farms nearby Bangkok, Thailand. Neural tissues were dissected from the eyestalks of live shrimps and subjected to RNA isolation using TRI Reagent® (Molecular Research Center). Total RNA was used in cDNA synthesis primed with PRT primers in the reaction containing 5 μg of total RNA, 20 mM Tris–HCl pH8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 500 nM PRT primer, 500 μM each dATP, dCTP, dGTP, dTTP and 200 units of SuperScript II Reverse transcriptase (GIBCO BRL). The reaction was incubated at 50 °C for 50 min before terminated by heating at 70 °C for 15 min. The RNA template was degraded by incubation with 2.5 μl of 5N NaOH at 55 °C for 30 min. The reaction was neutralized with 72 μl of 1% acetic acid, and the cDNA was recovered by purification using QIAGEN PCR purification kit (QIAGEN).

2.2. Rapid amplification of cDNA ends

The degenerate MG1 primer used in 3′ RACE of Pem-MIH cDNA was designed from an amino acid block RGVMGN that is conserved between the MIH of *Marsupenaeus japonicus* and *M. ensis*. An 8 μl aliquot of the cDNA was amplified with MG1 and PM1 primers in a reaction containing 20 mM Tris–HCl pH8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 nM each primer, 200 μM each dATP, dCTP, dGTP, dTTP. The reaction mixture was heated to 94 °C for 3 min before the addition of 2.5 units of *rTth* DNA polymerase (Biotools). Amplification was carried out in a DNA Thermal Cycler (GeneAmp 2400, PE Applied Biosystems) with 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min followed by 7 min incubation at 72 °C.

For 5′ RACE, the cDNA was synthesized with the MIH1 primer that was designed from the nucleotide sequence of the 3′ RACE fragment using the same condition as described in Section 2.1. A 10-μl aliquot of the cDNA was tailed with dATP at 37 °C for 10 min in 25 μl of the following reaction; 10 mM Tris–HCl pH 8.4, 25 mM KCl, 1.5 mM $MgCl_2$, 200 μM dATP and 1 μl of terminal deoxynucleotidyl transferase (TdT) (Promega). After heat-inactivation of TdT at 65 °C for 10 min, the PCR amplification was performed with 5 μl of the dA-tailed cDNA. The reaction was performed as described for the 3′ RACE using 200 nM of MIH2 and PM1 primers. Subsequently, nested amplification was performed with 200 nM of MIH3 and PM1 primers to obtain specific product. The nucleotide sequences of the primers used in all amplifications are shown in Table 1.

2.3. Construction of expression vector and transformation of Pichia pastoris

A cDNA fragment encoding the mature region of Pem-MIH1 was amplified with MIH1-F and MIH1-R primers (Table 1) that contain recognition site for *Sal* I and *Xba* I at the 5′ end, respectively. This *Pem-MIH1* cDNA fragment was digested with *Sal* I and *Xba* I before ligated to the expression vector, pPICZαA that had been previously digested with *Xho* I and *Xba* I resulting in a recombinant expression plasmid, αMIH-EX (Fig. 1). The in-frame insertion of the cDNA was verified by DNA sequencing.

The recombinant expression vector was introduced into *P. pastoris* cells by electroporation at 1.5 kV, 25 μF, 200Ω using a Gene Pulser (Bio-Rad). The transformants were selected on YEPD plate [2% (w/v) peptone, 2% (w/v) glucose, 1% (w/v) yeast extract, 2% agar] containing 100 mg/ml Zeocin™ (Cayla).

2.4. Expression of recombinant Pem-MIH1

An overnight culture of *P. pastoris* transformants was transferred into 100 ml of fresh BMGY medium [1% (w/v) yeast extract, 2% (w/v) peptone, 0.67% (w/v) YNB, 4 μg/ml D-biotin, 100 mM potassium phosphate, pH 6.0 and 1% (v/v) glycerol]. After growing at 30 °C with shaking until the OD_{600} reached 5–6, the cell pellet was collected and resuspended in 1/5 volume of BMMY medium [1% (w/v) yeast extract, 2% (w/v) peptone,

Table 1
Primers used in this study and their nucleotide sequences

Primer	Nucleotide sequence (5′ -->3′)
3′ RACE	
PRT	CCGGAATTCAAGCTTCTAGAGGATCCTTTTTTTTTTTTTTTT
PM1	CCGGAATTCAAGCTTCTAGAGGATCC
MG1	(AC)(GC)NGGNGTNATGGGNAA
5′ RACE	
MIH1	GATCTCGTCCTCCCTGTT
MIH2	TAGACAAATCAGGAACCA
MIH3	CGGAATTCGTTGTAGAAGCACCG
Mature Pem-MIH1 cDNA	
MIH1-F	CCTCGAATTCGTCGACAAAAGAAGTTTCATAGACGGCAC
MIH1-R	GCGTAGATCTTCACTGACCGGCGTTCAGGATG
Amplification of Pem-MIH1 gene	
5′MIH1	ACAGCATCCACGCCGTCT
3′MIH1	AAGCATTCCAGTAACTTT
Genome walking of Pem-MIH2 gene	
5′gMIH-O	CGGAATTCGTTGTAGAAGCACCG
5′gMIH2-I	TACAGGTGCCGTCCATGAGACTGC
RT-PCR	
Pem-MIH2: MIH2-F	TGGAATTCGTCGACAAAAGAAGTCTCATGGACGGCAC
MIH2-R	ACTCTAGATCAGGCGTTCAGAATACT

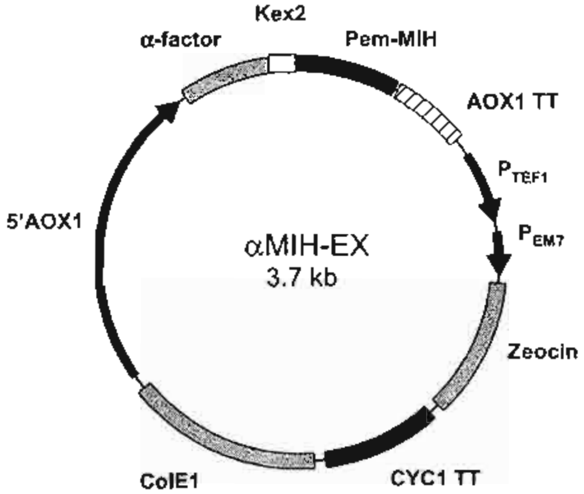

Fig. 1. Physical map of αMIH-Ex. A cDNA encoding the mature Pem-MIH1 was inserted into pPICZαA expression vector downstream of and in-frame with the α-factor secretion signal and the cleavage sites for Kex2 enzyme.

0.67% (w/v) YNB, 4 μg/ml D-biotin, 100 mM potassium phosphate]. Methanol was added to a final concentration of 3% (v/v), and the incubation was continued at the same condition. An aliquot of the culture was collected on day 5 after the induction. The culture medium was subjected to further analysis by 16.5% Tricine-SDS-PAGE.

2.5. *Protein purification*

The culture medium of *P. pastoris* transformants was collected and the recombinant protein was recovered by precipitation with ammonium sulfate at 50–55% saturation. The proteins in the precipitate were resuspended in PBS pH 7.4 before subjected to further purification step by size exclusion chromatography. The protein solution was loaded onto the Superdex 75 PC 3.2/30 column (Amersham Pharmacia Biotech) and subsequently eluted with PBS pH 7.4 at the flow rate of 0.4 ml/min.

2.6. *Biological assay for MIH activity*

Individual juvenile *P. monodon* (13–15 g) was cultured in separate compartment ($15\times15\times15$ cm^3) containing artificial sea water at 10 ppt salinity with aeration. Forty-five shrimps were divided into three groups, each contained fifteen shrimps. The shrimps were allowed to molt once. On the third day after the first molt, individual shrimp was injected as follows: the shrimps in the control group were injected with 100 μl of PBS buffer; the shrimps in the positive control group were injected with one-pair equivalent of the eyestalk's sinus gland extract and the experimental group was injected with 5 μg of purified recombinant Pem-MIH1. Molting cycle duration of the shrimps in each group was monitored. Molting was judged by the pull-off of the newly molted shrimps from their old exoskeleton.

2.7. PCR amplification of Pem-MIH1 gene

Genomic DNA was prepared from the abdominal muscle of *P. monodon* by QIAGEN Genomic-Tip and the Genomic DNA buffer set (QIAGEN). The primers used for PCR amplification, 5′ MIH1 and 3′ MIH1, were designed from the 5′ and 3′ ends of *Pem-MIH1* cDNA, and their nucleotide sequences are shown in Table 1. The reaction contained 150 ng of genomic DNA, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 200 nM each primer and 200 μM each dATP, dCTP, dGTP, dTTP in a total volume of 50 μl. The reaction was heated to 94 °C for 3 minutes then, 2.5 units of *Taq* DNA polymerase (Promega) were added. The amplification was carried out with 35 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 2 min followed by the final extension at 72 °C for 7 min.

2.8. Cloning of genomic fragment by PCR-based genome walking strategy

The 5′ region of *Pem-MIH2* gene was obtained by the use of PCR-based genome walking. GenomeWalker library was constructed with *Dra* I enzyme as described in the Universal GenomeWalker Kit user manual (CLONTECH). The library was first amplified with outer adaptor primer AP1 and outer gene specific primer, 5′ gMIH-O derived from the conserved block DRCFYNE of Pem-MIH sequences. The nested amplification was performed with inner adaptor primer AP2 and inner gene specific primer, 5′ gMIH2-I that were designed to be specific to *Pem-MIH2*. The PCR reactions were carried out according to the manufacturer's instruction.

2.9. Reverse transcription polymerase chain reaction (RT-PCR) of Pem-MIH transcripts

Total RNA extracted from various tissues of *P. monodon*, i.e. eyestalk, gill, heart, muscle and thoracic ganglia was used as a template for reverse transcription with PRT primer as described in Section 2.1. The amplification of specific transcript was performed with specific primers to each *Pem-MIH* cDNA: MIH1-F and MIH1-R for *Pem-MIH1* transcript; MIH2-F and MIH2-R for *Pem-MIH2* transcript.

2.10. Homology modeling of Pem-MIH

The structure of recombinant Pem-MIH1 was determined from the soluble structure of the MIH of *M. japonicus* (Pej-MIH) (Katayama et al., 2003) using SWISS-PDB Viewer (v 3.7) software (http://www.expasy.ch/spdb/ mainpage.html).

3. Results

3.1. Cloning and characterization of Pem-MIH1 cDNA

Two types of cDNA were obtained form 3′ RACE, designated as *Pem-MIH1* and *Pem-MIH2*. These two cDNA fragments shared 60% identity in their nucleotide

Pem-MIH1

```
                                          5′ cagcatccacgccgtctcag
ggtagaggtccttcgagtcgcgtctccttgggttcattccgtccctacgattatacactc
atgtatcggctggcgatgaagacatggctggcgatagtgattgtagtagttgggacaagc
 M  Y  R  L  A  M  K  T  W  L  A  I  V  I  V  V  V  G  T  S
ctcttctttgacaccgcctcggccagtttcatagacggcacttgtcgaggcgtaatgggc
 L  F  F  D  T  A  S  A  S  F  I  D  G  T  C  R  G  V  M  G
aatcgtgacatttacaagaaggtagtgcgtgtgtgtgaggattgcaccaatatcttccga
 N  R  D  I  Y  K  K  V  V  R  V  C  E  D  C  T  N  I  F  R
cttccaggcttggacggcatgtgcagagatcggtgcttctacaacgaatggttcctgatt
 L  P  G  L  D  G  M  C  R  D  R  C  F  Y  N  E  W  F  L  I
tgtctaaaggctgccaacagggaggacgagatcgaaaaattcaaagtttggatcagcatc
 C  L  K  A  A  N  R  E  D  E  I  E  K  F  K  V  W  I  S  I
ctgaacgccggtcagtgaggtcgaacgcgacaggactccttccacttgcaaggcctcgct
 L  N  A  G  Q  -
cccgcaggccagtaccgacgacacttggcgactaatgaaccttatcattagttttttatta
Caattactaactattatttggttttccccgttagtttactggtaccagtaaatgatatgc
cagtctgagcctacaaggcctggcggtcatgttgagtatttagacgaaaaagtacgtgtg
aaatactccctctgatttgctatcatttgttgtaacggaatccggatacctgtttatttt
aaacttcaataggtgtactgattttaatttggaagttcaactaaagaaatatcacgtgtt
ttaatatttatgtatattctccgaggatactttttatgtgatttaattgcttcctcattg
ttatgatggacataaataaaagttactggaatgcttaaag  3′
```

Fig. 2. Nucleotide and deduced amino acid sequences of Pem-MIH1. The amino acids are present in one-letter symbols. The highlighted amino acid sequence represents the putative mature peptide region.

sequences (data not shown). A set of specific primers were designed from the sequences of these 3′ MIH fragment in order to obtain the 5′ portion of each *Pem-MIH* cDNA. Unfortunately, only the product corresponding to *Pem-MIH1* cDNA was

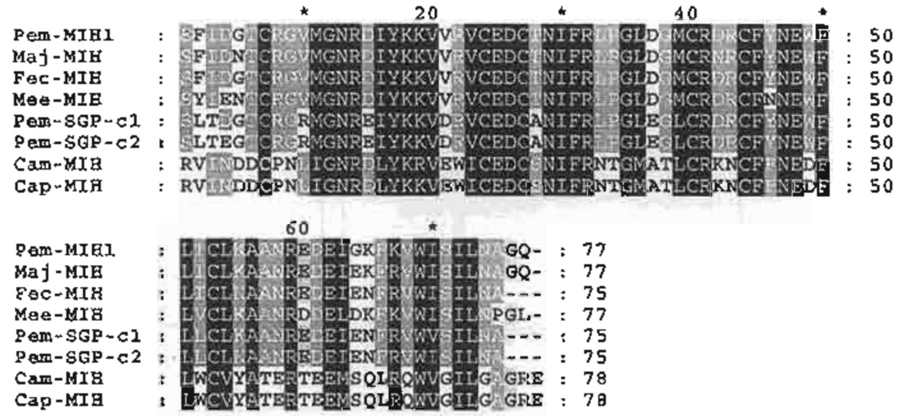

Fig. 3. An alignment of amino acid sequences of MIH from several crustaceans: *P. monodon* (Pem-MIH1; this study, Pem-SGP-C1; GenBank accession no. AB054187 and Pem-SGP-C2; GenBank accession no. AB054188), *M. japonicus* (Maj-MIH; GenBank accession no. P55847), *Feneropenaeus chinensis* (Fec-MIH; GenBank accession no. AF312977), *Metapenaeus ensis* (Mee-MIH; GenBank accession no. O76534), *Carcinus maenas* (Cam-MIH; GenBank accession no. Q27225) and *Cancer pagurus* (Cap-MIH; GenBank accession no. CAC05346). Amino acids that are conserved among all sequences are highlighted in black. Dark and light grey colors represent amino acids with lesser degree of conservation.