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Recombinant expression of soluble gonad inhibiting hormone (GIH) from *Penaeus monodon* in a bacterial expression system

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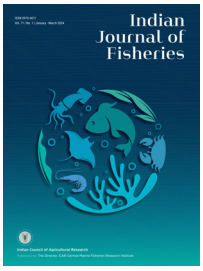

Abstract

Successful reproduction of captive shrimp has been the primary goal of commercial breeding program. Gonad inhibiting hormone (GIH), a member of CMG neuropeptide hormone family present within the neural tissue of the eyestalk of crustaceans, exert profound influence on reproductive maturation in shrimps. Present study reports the recombinant expression and purification of the GIH gene, using an *Escherichia coli* expression system. This is the first report of expressing the recombinant GIH of *P. monodon* using *E. coli* expression system in its native soluble form secreted to the media. The purified recombinant peptide is useful in structure and function analysis, which can be used for identifying its interacting partners and elucidating its mode of action. Production of an antibody/antagonist for the GIH would facilitate the development of an immunological tool for induced maturation in shrimp industry. This procedure could replace the physiologically destructive eyestalk ablation technique.

Introduction

Penaeid shrimps have emerged as the most valuable and globally traded aquaculture commodity. Shrimp aquaculture has experienced tremendous growth during the last few decades; which has grown from around 1.5 million t in 1950 to nearly 5.9 million t by 2024 (Mandal and Singh, 2025). However, development of shrimp farming has not been without problems. Historically, shrimp broodstock caught offshore were used in the commercial hatcheries for seed production (Moss *et al.,* 2012). This reliance on wild-caught spawners has hampered the consistent availability and sustainability of high-quality broodstock. Wild spawners often carry pathogens such as White spot syndrome virus (WSSV), Taura syndrome virus (TSV), Infectious hypodermal and hematopoietic necrosis virus (IHHNV) and the microsporidian parasite causing Enterocytozoon hepatopenaei (EHP), that can be vertically transmitted to seed, resulting in mass mortality and crop loss in the farms. At this context, global shrimp farming industry has increasingly adopted selective breeding programmes using specific pathogen-free (SPF) and specific pathogen-resistant (SPR) stocks to enhance disease resilience and productivity (Bhassu *et al.,* 2024). In India, revival of *Penaeus indicus* as a native SPF-bred species is underway, although hatcheries still depend heavily on wild broodstock, limiting opportunities for genetic improvement and sustainability (Anand *et al.,* 2023).

Complete control over treproduction is the prime requisite for selective breeding and further improvement of production (Taranger *et al.,* 2010). In crustaceans, ovarian maturation is negatively controlled by a neuropeptide, gonad/vitellogenin inhibiting hormone (G/VIH) produced by the X-organ sinus gland complex of eyestalk by inhibiting production of vitellogenin synthesis (Hopkins, 2012). GIH belongs to a peptide family, the crustacean hyperglycemic hormone (CHH) family, which includes CHH (*sensu stricto)*, molt inhibiting hormone (MIH) and mandibular

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organ inhibiting hormone (MOIH) (Treerattrakool *et al.,* 2014). These peptide hormones are synthesised in the *medulla terminalis* of the optic ganglion and transported along the axon and stored in neurohaemal organ, the sinus gland (Lorenzon, 2005). CHH family peptides are classified into two subfamilies based on their primary structure, type 1 peptides that include CHH peptides and type 2 that include GIH, MIH and MOIH peptides. The carboxyl terminus of the type I family is amidated whereas type II sub family lacks amide moiety (Katayama *et al.,* 2002). Structurally type 11 CHH prepropeptide is composed of a signal peptide and hormonal part with 72 to 83 aminoacid and molecular mass of 8 to 9 kDa (Treerattrakool *et al.,* 2014).

GIH is a key hormone or master molecule of reproduction in crustaceans as the inhibitory effect of GIH is more intense than any other hormones controlling reproductive maturation (Vaca and Alfaro, 2000). The existence of gonad-inhibiting hormone (GIH) in crustaceans was first demonstrated when eyestalk ablation was shown to induce precocious vitellogenesis in *Palaemon serratus* (Pasteur, 1959). This classical endocrinological procedure has been the most widely used induced maturation technique in shrimp industry since its inception. Although the eyestalk ablation procedure thought as a stopgap measure until a physiologically compatible procedure developed (Wurts and Stickney, 1984), this procedure could not be replaced so far, due to the unavailability of an effective alternative. A convincing alternative option for the physiologically destructive eyestalk ablation technique is neutralising or blocking the endogenous inhibitory effect of GIH by an antagonist/antibody of this hormone (Okumura, 2004). Although the native neuropeptide purified by RP-HPLC has inherent advantages of having naturally folded peptide that completed all post-translational changes (Mosco *et al.,* 2012), it is extremely difficult to get chromatographically purified GIH in enough quantities; for example, single sinus gland yields 2-4 µg of neuropeptide only (Chung and Webster, 2003). The exquisite sensitivity of molecular biological tools facilitated the production of recombinant protein at reasonable quantities.

Escherichia coli has been the most attractive and widely used expression host for several years because of their ability to grow rapidly at high density on inexpensive substrates, its well characterised genetics and physiological knowledge and availability of large number of cloning vectors (Terpe, 2006). However, most biochemically interesting proteins, particularly protein containing di-sulfide bonds, are extremely difficult to produce as soluble protein in *E. coli* expression system (Esposito and Chatterjee, 2006). These problems in protein production have led to the use of alternate eukaryotic expression systems that can overcome the problems of *E. coli*. However, versatility and economic potential of *E. coli* based protein production make this species still as an excellent choice for the commercial scale production of recombinant proteins (Swartz, 2001).

Vitellogenesis-inhibiting hormone (VIH/GIH) was first characterised in *Homarus americanus* (Soyez *et al.,* 1987). Later, expression profile of GIH during the reproductive cycle was studied in few species (DeKleijn *et al.,* 1998; Vijayan *et al.,* 2013). Biological activity, the inhibition of vitellogenin synthesis, of VIH/GIH during reproductive cycle was reported in some crustaceans, for example, *Marsupenaeus japonicus* (Ohira *et al.,* 2006a), *Procambarus bouvieri* (Aguilar *et al.,* 1992) and *Penaeus monodon* (Treerattrakool *et al.,* 2014). As *E. coli* failed to produce sufficient amount of recombinant GIH, Treerattrakool *et al.* (2014) used a eukaryotic expression system, *Pichia pastoris*, to evaluate the possibility of production of recombinant GIH in soluble form. Large scale production of shrimp GIH is the essential prerequisite for providing sufficient quantity of GIH for basic research as well as aquaculture application. The objective of the present study is, therefore, to clone and express GIH protein in native and soluble form using *E. coli* expression system to enable further studies leading to development of alternative strategies to replace eyestalk ablation in shrimps.

Materials and methods

Cloning of GIH gene

The complex nature of the CMG family hormones coupled with the lack of proper sequence information for GIH, made the amplification and isolation of GIH challenging. During our initial experiments, primers designed based on the GIH sequence of *Metapenaeus enesis* (AF294648), *Nephrops norvegicus* (AF163771) and *Homarus americanus* (X87192) failed to amplify GIH from *P. monodon*. Subsequently, GIH was characterised from *Penaeus monodon* collected in Thai waters (Treerattrakool *et al.,* 2008) (DQ643389). Primers (GIH and GIHR) mentioned in the publication has been synthesised and used to amplify GIH gene from eyestalk cDNA of female *P. monodon* broodstock with immature, mature and spent ovaries. However, to our surprise the primers failed to produce amplicon of expected size from the *P. monodon* collected from Indian waters. Hence, we designed a pair of primers manually based on the nucleotide sequence of the Pm-GIH cDNA (DQ643389). The primer PmGIH gene F that starts exactly at the start codon of the GIH and Pm1.1R primer amplified a cDNA fragment with the complete coding sequence (CDS) of the GIH gene.

Eyestalks from wild caught female *P. monodon* at immature stages of ovaries were dissected out and stored in *RNA later* solution at -80°C for total RNA extraction. One micrograms of total RNA isolated from eyestalk using TRI Reagent (Sigma-Aldrich, USA) was used for reverse transcription with First Strand cDNA Kit (Fermentas, CA, USA) using anchored oligod$T_{(24)}$ VN primer. The reaction mixture comprised of 1x RT buffer, 0.5mM dNTP, 1µM anchored oligo dT_{24}VN primer, and 1 U reverse transcriptase. The reaction mix was subjected to PCR amplification with the following temperature profile: 70°C for 5 min, then 25°C for 5 min, 42°C for 60 min and 70°C for 10 min in a S1000 Thermal cycler (BioRad, Hercules, CA, USA).

The first strand cDNA was used as template to amplify the complete CDS of *P. monodon* GIH with the primers, PmGIH Gene F (5`-ATGAAAACATGGCTGCTATTAGCG-3`) and Pm1.1R (5`-TGGGATGCTTTCAGAGAAGG-3`). The reaction mixture comprised 1x PCR buffer with, 2 mM $MgSO_4$, 0.2 mM each of dNTPs, 0.5 µM of each primers and 1.25U *Pfu* DNA polymerase and 1 µg of template cDNA. The PCR reaction conditions were 95°C for 3 min, then 35 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 60 s followed by a final extension at 72°C for 10 min. The amplified PmGIH fragment was column purified using MinElute® PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and cloned into pCR™4-TOPO®TA vector (Invitrogen, USA) and transformed into One Shot® TOP10 Chemically Competent *E. coli* cells (Invitrogen, USA). The transformant was screened and selected on LB agar plates containing ampicillin (100 µg ml^{-1}) for the presence of insert by

colony PCR. Plasmids from selected positive clones were purified using QIAprep kit (Qiagen GmbH, Hilden, Germany) to reconfirm the presence of insert by automated DNA sequencing (SciGenom Labs, India).

Sequence analysis

The nucleotide sequence was edited and aligned using BioEdit (version 7.01), and amino acid sequence was deduced by EMBOSS Transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/). The sequence was analysed for identity and similarity to known sequences by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). GIH sequences of decapods available in the GenBank were retrieved and combined with sequence obtained in the present study and aligned by using CLUSTAL X program. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 11 (Tamura *et al.*, 2021). Neighbor joining tree was constructed using MEGA 11 and to verify the robustness, bootstrap analysis was carried out using 1000 pseudo replications. Signal peptide sequence was analysed using SignalP version 4.1 and protein family signature was identified using ScanProsite tool (http://prosite.expasy.org/scanprosite/).

Preparation of gene construct

Without signal peptide

PrimersGIH_Nco1.nosig_F (5`-ATT ATT CCA TGG CTA ACA TCC TGG ACA GCA AAT GCA GGG G-3`) and GIH_ Not1.Nstp_ R (5`-TAT ATA GCG GCC GCC CAC GGC CGG CCG GCA TTG AGG-3`) were designed specifically to amplify the mature peptide coding region encoding PmGIH to be inserted into expression plasmid pET-28b (Novagen, USA). PCR was conducted with these primers with restriction sites, GIH_ Nco1. nosig_ F contained the Nco1 site and the reverse primer GIH_ Not1. Nstp_ R contained the Not1site using the pCR™4-TOPO®TA vector plasmid (Invitrogen, USA) containing the complete CDS of GIH as the template.

With signal peptide

The complete coding sequence (CDS) of GIH gene including the signal peptide region was amplified using Primers GIH_Nco1F (5`-GGT GGT CCA TGG GCA AAA CAT GGC TGC TAT TAG C-3`) contained the Nco1 site and GIH_ Not1. Nstp_ R (5`-TAT ATA GCG GCC GCC CAC GGC CGG CCG GCA TTG AGG-3`) designed with the Not1 restriction sites specifically to be inserted into expression plasmid pET-28b (Novagen, USA). PCR was conducted with these primers; using the pCR™4-TOPO®TA vector plasmid (Invitrogen, USA) containing the complete CDS of GIH as the template.

Cloning in to expression vector

The amplified product was double digested with NcoI/NotI restriction enzymes to generate the insert for expression vector pET28b. The digested fragments were column purified using MinElute® PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and unidirectionally ligated into the identically treated expression vector pET-28b downstream to T7 promoter and C-terminal hexa-histidine (6xHis) tag using T4 DNA ligase. The ligated product was transformed into One Shot® TOP10 Chemically competent *E. coli* cells (Invitrogen, USA) and transformants were selected on LB agar plates containing kanamycin (50 µg ml^{-1}). The transformants were screened for the presence of insert by colony PCR. Positive clones were selected for plasmid purification using QIAprep kit (Qiagen GmbH, Hilden, Germany) to reconfirm the presence of insert by automated DNA sequencing (SciGenom Labs, India). The recombinant expression plasmids were termed pET28b-*PmGIH_no sig* and pET28b-*PmGIH_with sig*.

Expression of recombinant PmGIH with and without signal peptide

The competent *E. coli* BL21 (DE3) cells (Novagen, USA) were transformed with expression plasmids pET28b-PmGIH_*No sig* and pET28b-PmGIH_*With sig* and transformants, were subsequently selected on LB agar plates containing kanamycin (50 µg ml^{-1}). Bacterial cells from a single colony were grown at 37°C overnight in LB medium containing kanamycin (50 µg ml^{-1}), and then diluted 50-fold with the same medium. The diluted medium was incubated at 37°C for 3 h until the OD_{600} reached 0.6-0.8. The recombinant protein was induced by the addition of isopropyl-b-D-thiogalactoside (IPTG) to a final concentration of 0.4 mM with gentle shaking at 37°C temperature for 5 h. The bacterial cells and media fractions were harvested after induction by centrifugation. The pellet and supernatant were stored at -80°C until analysis. After cell lysis, soluble, insoluble and media fractions were subjected to 15% Tricine SDS Polyacrylamide gel electrophoresis (Tricine-SDS PAGE) (Schägger, 2006) under denaturing conditions.

Affinity purification of rPmGIH without signal peptide

Host cells expressing 6xHis-tagged recombinant protein (r*PmGIH_No sig*) harvested by centrifugation were subjected to lysis and affinity purification using the Ni-NTA Fast Start Kit (Qiagen GmbH, Hilden, Germany) under denaturing conditions. Briefly, pelleted cells from 100 ml of induced culture were centrifuged at 3000 *g* for 10 min. The cell pellet was suspended in 10 ml denaturing lysis buffer and incubated at room temperature for 60 min with intermittent mixing. The lysate was centrifuged at 14000 *g* for 30 min at room temperature to pellet the cellular debris, and the supernatant was loaded into a fast start column containing Ni-NTA resin. The protein-bound resin was washed serially several times with denaturing washing buffer, and the bound 6xHis-tagged recombinant protein was eluted serially three times in 1 ml elution buffer. Fractions were analysed by Tricine-SDS polyacrylamide gel electrophoresis under reducing conditions to detect the presence of recombinant protein.

Affinity purification of rPmGIH with signal peptide

Media fraction from the culture of host bacterial cells expressing 6xHis-tagged recombinant protein (r*PmGIH_with sig*) harvested by centrifugation was purified by a Ni-NTA matrix affinity column under native conditions according to the protocol of the Ni-NTA Fast Start Kit (Qiagen GmbH, Hilden, Germany). Briefly, the supernatant from 100 ml of induced host bacterial culture, cleared by centrifugation at 3000 *g* for 10 min, mixed with equal volume native wash buffer was loaded to a column (10 ml) containing 1 ml of Ni-NTA resin pre-equilibrated with the lysis buffer (10 mM Imidazole, 50 mM NaH_2PO4, 500 mM NaCl, pH 8.0). The protein bound resin was washed six times with native washing buffer (20 mM Imidazole,

50 mM NaH_2PO_4, 500 mM NaCl, pH 8.0). The protein was serially eluted three times in 1 ml elution buffer (500 mM Imidazole, 50 mM NaH_2PO_4, 500 mM NaCl, pH 8.0), and fractions were analysed by Tricine-SDS polyacrylamide gel electrophoresis under reducing conditions to detect the presence of recombinant protein.

Results

Cloning GIH gene

A 316 bp PmGIH cDNA fragment was cloned and characterised (GenBank Accession no: JN836930). Conceptual translation of the CDS sequence using the EMBOSS Transeq showed that it contains a 291 bp open reading frame encoding 96 amino acid residues (Fig 1). A signal peptide at the N-terminus of the deduced peptide by SignalP4.1 program was identified and its cleavage site was located between the amino acid positions Ala17 and Asn18. Based on these predictions, the molecular mass of the mature peptide was estimated as 9.4 kDa. This value does not include other post-translational modification if any, *i.e.*, glycosylations and amidation

Multiple alignment analysis of deduced amino acid sequence of putative PmGIH with other closely related decapod crustacean GIH sequences showed six conserved cysteine residues (Cys24, Cys41, Cys44, Cys57, Cys61, and Cys70) in the mature peptide region in all the sequences, a characteristic of the CMG family hormones (Fig. 2), which is believed to be involved in the formation of internal disulfide bonds. ScanProsite tool detected the presence of CMG neuro hormone family signature ([LIVM]-x(3)-C-[KR]-x-[DENGRH]-C-[FY]-x-[STN]-x(2)-F-x(2)-C.) in the deduced amino acid sequence of PmGIH, a C type lecithin domain (C-[LIVMFYATG]-x(5,12)-[WL]-{T}-[DNSR]-{C}-{LI}-C-x(5,6)-[FYWLIVSTA]-[LIVMSTA]-C), has also been detected.

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1    atgaaaacatggctgctattagcgactctggcggtgggagcgagcttagctaacatcctg
      M  K  T  W  L  L  L  A  T  L  A  V  G  A  S  L  A  N  I  L

61   gacagcaaatgcaggggtgcaatgggtaatcgggatatgtacaacaaggtggagcgtgtt
      D  S  K  C  R  G  A  M  G  N  R  D  M  Y  N  K  V  E  R  V

121  tgcgaggactgcaccaatatctaccggttaccacagctggatggcttgtgcagaaatcga
      C  E  D  C  T  N  I  Y  R  L  P  Q  L  D  G  L  C  R  N  R

181  tgcttcaataaccagtggttcctgatgtgcctccactcggccaagcgcgaggccgaactc
      C  F  N  N  Q  W  F  L  M  C  L  H  S  A  K  R  E  A  E  L

241  gagcatttcagactctggatcagcatcctcaatgccggccggccgtggtgatccttcctt
      E  H  F  R  L  W  I  S  I  L  N  A  G  R  P  W  *

301  ctctgaaagcatccca
```

Fig. 1**.** Nucleotide sequence of PmGIH cDNA from *P. monodon* and its deduced amino acid sequence. The start codon is underlined and termination codon is indicated with asterisk (*). The predicted 17 amino acid signal peptide sequence is in bold. Six conserved cysteine residues which form three disulfide bonds, characteristics of CMG family hormones are shaded. A possible amidation site is inside the box.

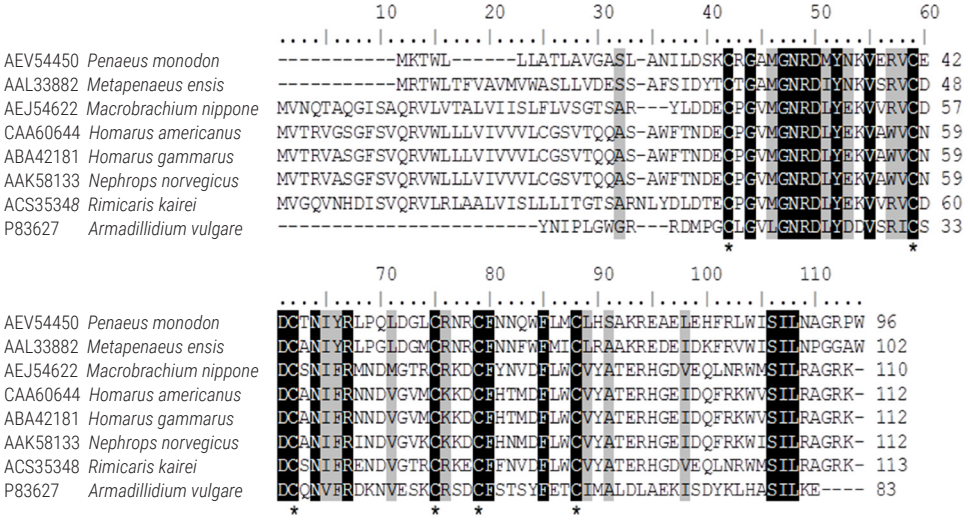

Fig. 2. Multiple alignment of the PmGIH with other members of this family. Amino acid numbers (excluding gap) are shown on the right. Invariant residues are shaded black, conserved residues in >75% of sequences are shaded grey. The position of 6 conserved cysteine residues are marker with asterisks.

Phylogenetic relationships of GIH from tiger shrimp and other selected decapod crustaceans estimated using GIH from *Armadillidium vulgarae* as the out-group. GIH of *P. monodon, Penaeus (Litopenaeus) vannamei* and *M. ensis* formed one distinct cluster, supported by high bootstrap value indicating close genetic relationship, whereas GIH from *H. americanus*, *H. gammarus* and *N. norvegicus*, formed another distinct clade (Fig. 3). In addition, the GIH from *Macrobrachium nipponense* and *Rimicaris kairei* formed a third cluster. The study has revealed a closer relationship among GIH of penaeid shrimps.

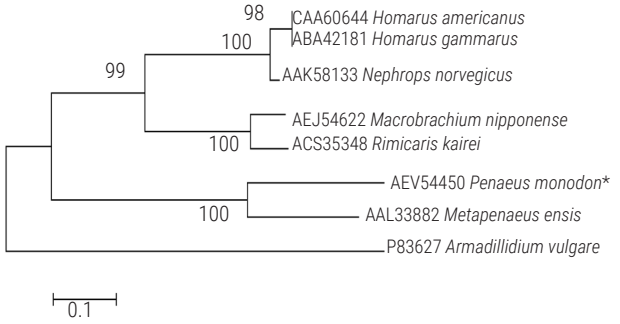

Fig. 3. Phylogenetic relationship of GIH gene from *P. monodon* and other decapod crustaceans based on neighbour joining (NJ) analysis of amino acid dataset. The scale shows genetic distance and GenBank Accession nos. are shown next to each species.

Recombinant PmGIH

As the presence of a secretory signal peptide was detected, two sets of expression plasmids were constructed, one with the complete CDS of PmGIH including native signal peptide and one without the signal peptide *i.e.*, mature peptide region for recombinant expression. Sequencing of the expression plasmids did not show any mutations, the mature GIH peptide coding sequence was in the frame with the C-terminal hexa-histidine site. The sequence information of the pET28b-*PmGIH_No sig* construct predicted that the recombinant protein would contain 92 amino acids: 78 amino acids of the mature GIH peptide with 2 vector amino acids at the N-terminal and 11 vector amino acids, including six histidine residues at the C-terminal. The expected molecular weight of the recombinant product is 10.92 kDa. Whereas the sequence information of the pET28b-*PmGIH_With sig* construct predicted the recombinant protein will contain 108 amino acids: 96 amino acids of the GIH prepro peptide with one vector amino acids at the N-terminal and 11 vector amino acids, including 6 His residues at the C-terminal with an expected molecular weight of 12.51 kDa.

The expression plasmids were transformed into *E. coli* BL21 (DE3) cells and IPTG was added to a final concentration of 0.4 mM to induce protein expression. Production of sufficient quantity of biologically active soluble form of recombinant GIH without signal peptide could not be accomplished by using different approaches in spite of optimisation of protocols using different strategies for enhancing the solubility of recombinant GIH without signal peptide in *E. coli*. Majority of the recombinant GIH without signal peptide was expressing in the form of inclusion bodies. Therefore, we purified it from the inclusion bodies. The recombinant GIH was purified without signal peptide through denaturing Ni-NTA affinity column chromatography. Tricine-SDS-PAGE analysis of the purified rPmGIH_*No sig* (Fig 4) showed single band products that migrate at ~11 kDa, which matched the theoretical molecular weight calculated from its sequence. This protein band was not detected in un-induced culture.

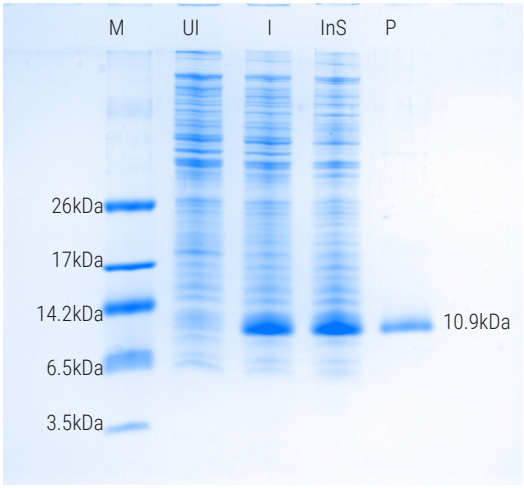

Fig. 4**.** Tricine SDS-PAGE (15%) analysis of the *r*PmGIH without signal peptide expressed in *E. coli* BL21 (DE3) cells. M: Marker, UI: Uninduced total cellular protein, I: Induced total cellular protein, InS: Insoluble fraction, P: Purified recombinant GIH without signal peptide.

On the other hand, in the case of recombinant GIH with signal peptide, production of recombinant peptide in soluble form was secreted into the media due to the presence of the secretory signal peptide resulted in the complete lysis of the host cells. Prevention of cell lysis was necessary for obtaining recombinant GIH in sufficient quantities, and therefore, different growth conditions were tried to prevent lysis of the host cells by the expressed recombinant protein. Lowering the incubation temperature to 24°C after induction for overnight with gentle shaking helped in reducing the cell lysis and production of soluble recombinant GIH protein in sufficient quantity to carry out affinity purification. The recombinant GIH with signal peptide was purified through native Ni-NTA affinity column chromatography. Tricine-SDS-PAGE analysis of the purified *r*PmGIH_*With sig* showed single band products that migrate at ~12 kDa (Fig. 5), which matched the theoretical molecular weight calculated from its sequence. This protein band was not detected in un-induced culture. The yield of the purified *r*PmGIH_*no sig* was approximately 3 mg l^{-1} and that of *r*PmGIH_*with sig* was approximately 1 mg l^{-1} of culture.

Expression of recombinant GIH with signal peptide in BL21 host showed a negative trend in cell density and extensive host cell lysis was observed suggesting an antibacterial property of the recombinant protein. To verify this, we compared the cell density of un-induced and induced cultures expressing recombinant GIH with signal peptide pre- and post-induction by measuring the cell densities by taking optical density at OD_{600} in different time intervals, which showed (Fig. 6) a drastic reduction in the cell density of the

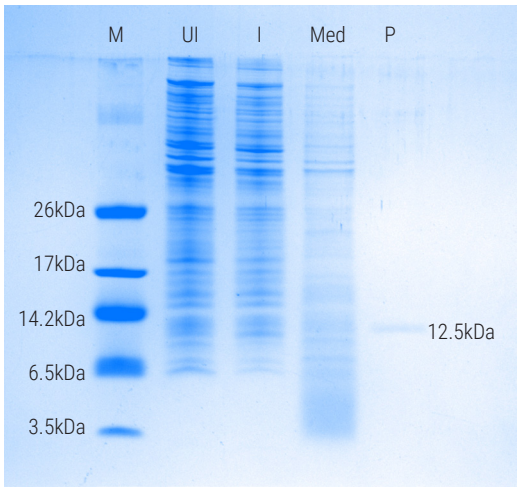

Fig. 5**.** Tricine SDS-PAGE (15%) analysis of the *r*PmGIH with signal peptide expressed in *E. coli* BL21 (DE3) cells. M: Marker, UI: Uninduced total cellular protein, I; Induced total cellular protein, Med: Media fraction, P: Purified recombinant GIH with signal peptide.

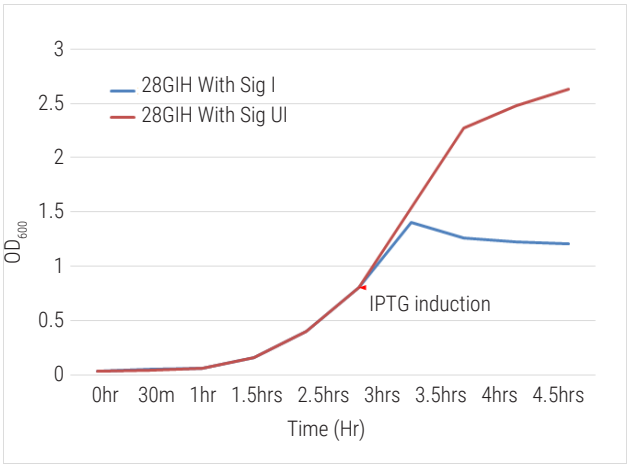

Fig. 6. Comparison of growth pattern of induced and un-induced BL21 host cells expressing recombinant GIH with signal peptide showing drastic reduction in cell density after induction.

induced culture from the third hour onwards. Rapid lysis of the host cells was observed confirming the antibacterial property to the recombinant protein, which can be explained by the presence of C-type lectin domain.

Discussion

In the present study, we report for the first time, the production of recombinant GIH protein in a native, soluble form using an *E. coli* expression system. Further, we have amplified the complete coding sequence (CDS) of GIH cDNA by RT-PCR. This would be a primary step in unfolding the structure and function of GIH in the CMG family as well as developing an antibody/antagonist to GIH. The antibody against GIH could be used as an alternative strategy for inhibiting the biological action of GIH, and it would replace the eyestalk ablation procedure to induce reproductive maturation in penaeid shrimps. Although *E. coli* has been used widely to produce recombinant protein (Jeong and Lee, 2001), it fails to produce substantial yield of more complex eukaryotic protein such as GIH (Treerattrakool *et al.,* 2014). One approach to solve this problem is to have recombinant protein secreted into the periplasm or culture medium (Choi and Lee, 2004). The protein secreted into the periplasm is generally synthesised in the cytoplasm and exported to the periplasm by signal peptide. The production of secretory protein is facilitated by using signal peptides (Jeong and Lee, 2001; Choi and Lee, 2004). An array of signal peptides has been used for the production of secretory recombinant protein (Makrides, 1996). Nevertheless, protein secretion into the periplasm is insufficiently understood, and, therefore, use of these signal peptides would not always be ensured successful production of secretory recombinant protein. The production of secretory recombinant GIH has not been reported so far (Treerattrakool *et al.,* 2014). In this study, we used GIH signal peptide and enabled the production of reasonable amount of recombinant GIH in soluble form.

The amount of CMG family peptides obtained from sinus gland extract using analytical route is extremely low, and therefore a great deal of effort is required to obtain these neuropeptides from natural sources, the X-organ complex of the eyestalk (Grève *et al.,* 1999). Considerable amount of recombinant protein is essential to elucidate the precise physiological role of these peptides (Morera *et al.,* 2013). Recombinant CHH of several crustaceans have been produced: *P. japonicus* (Katayama *et al.,* 2002), *N. norvegicus* (Mettulio *et al.,* 2004), *M. rosenbergii* (Ohira *et al.,* 2006a), *Astacus leptodactylus* (Mosco *et al.,* 2012) and *Litopenaeus setiferus* (Morera *et al.,* 2013). On the other hand, to date, only three recombinant GIH: *N.norvegicis* (Edomi *et al.,* 2002), *H. americanus* (Ohira *et al.,* 2006b) and *P. (L.) vannamei* (Tsutsui *et al.,* 2013) were reported, all as insoluble fusion proteins.

However, knowledge of GIH was extremely limited because of the difficulties in preparing a suitable bioassay for evaluating the biological function of this hormone, vitellogenin-inhibiting activities (Wilder *et al.,* 2010). Recently, the gene silencing effect of double stranded RNA (ds RNA) has been used by several researchers to study the biological function of various hormones including crustacean neuropeptides. It was established that administration of GIH dsRNA increased vitellogenin gene transcript levels in female *P. monodon* that confirms the biological role of GIH (Treerattrakool *et al.,* 2011). Although the biological activity not evaluated, we anticipate that the recombinant GIH produced in this study, will have biological activity comparable to that of native GIH.

Recombinant CMG family peptide is generally expressed in the insoluble fraction when using *E. coli* as expression system, and therefore, in order to confer the biological activity it should be subjected to refolding reaction (Tsutsui *et al.,* 2013). This has been one of the major challenges in producing recombinant proteins using *E. coli* expression system. Traditionally, the expression of secretory protein has been largely associated with eukaryote expression systems, which require more demanding efforts, higher operational costs and specialised facilities. Moreover, associated post-translational modifications and heterologous sample preparations can sometimes interfere in downstream applications. To allow for an easy, straightforward and highly cost-effective approach, it is desirable to establish *E. coli* based methods for this purpose (Kotzsch

et al., 2011). Therefore, secretary expression of target proteins into the culture media combines the advantages of easy recovery and the possibility of tailoring the growth conditions for preservation of activity and stability of recombinant proteins. In the present study, we successfully produced recombinant GIH of *P. monodon* for the first time using *E. coli* expression system, yielding a reasonable quantity of *r*PmGIH. Moreover, recombinant PmGIH_with a signal peptide was detected in the culture medium; thereby eliminating the need for refolding steps.

To conclude, the *E. coli* expression system reported here was able to produce sufficient quantities of recombinant PmGIH to produce specific antibodies, which upon administration could act as antagonist by blocking the inhibitory action of GIH hormone *in vivo*. The recombinant PmGIH can also be utilised to develop specific immunoassay assay systems, enabling elucidation of GIH-mediated neuroendocrine mechanisms through *in vivo* bioassays. It can also find application in characterisation of GIH binding partners and associated signaling pathways. The recombinant molecules developed, offer significant potential for advancing our understanding of the molecular mechanisms regulating reproduction in penaeid shrimps, and may contribute to the development of alternative strategies for induced gonadal maturation, reducing reliance on eyestalk ablation in shrimp aquaculture.

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References

Aguilar, M. B., Quackenbush, L. S., Hunt, D. T., Shabanowitz, J. and Huberman, A. 1992. Identification, purification and initial characterization of the vitellogenesis-inhibiting hormone from the Mexican crayfish *Procambarus bouvieri* (Ortmann). *Comp. Biochem. Physiol., B: Com. Biochem.,* 102(3): 491-498. https://doi.org/10.1016/0305-0491(92)90039-T.

Anand, P. S. S., Balasubramanian, C. P., Aravind, R., Biju, I. F., Rajan, R.V., Vinay, T. N., Panigrahi, A., Sudheer, N.S., Rajamanickam, S., Kumar, S., Antony, J., Ambasankar, K., Jithendran, K. P. and Vijayan, K. K. 2023. Reproductive performance of captive-reared Indian white shrimp, *Penaeus indicus*, broodstocks over two generations. *Front. Mar. Sci.,* 10. https://doi.org/10.3389/fmars.2023.1101806.

Bhassu, S., Shama, M., Tiruvayipati, S., Soo, T. C. C., Ahmed, N. and Yusoff, K. 2024. Microbes and pathogens associated with shrimps - implications and review of possible control strategies. *Front. Mar. Sci*., 11: 1397708. https://doi.org/10.3389/fmars.2024.1397708.

Choi, J. H. and Lee, S. Y. 2004. Secretory and extracellular production of recombinant proteins using *Escherichia coli. Appl. Microbiol. Biotechnol.,* 64(5): 625–635. https://doi.org/10.1007/s00253-004-1559-9.

Chung, J. S. and Webster, S. G. 2003. Moult cycle-related changes in biological activity of moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) in the crab, *Carcinus maenas*: From target to transcript. *Eur. J. Biochem.,* 270(15): 3280–3288. https://doi.org/10.1046/j.1432-1033.2003.03720.x.

DeKleijn, D. P. V., Janssen, K. P. C., Waddy, S. L., Hegeman, R., Lai, W. Y., Martens, G. J. M. and Van Herp, F. 1998. Expression of the crustacean hyperglycaemic hormones and the gonad- inhibiting hormone during the reproductive cycle of the female American lobster *Homarus americanus*. *J. Endocrinol.,* 156(2): 291–298. https://doi.org/10.1677/joe.0.1560291.

Edomi, P., Azzoni, E., Mettulio, R., Pandolfelli, N., Ferrero, E. A. and Giulianini, P. G. 2002. Gonad-inhibiting hormone of the Norway lobster (*Nephrops norvegicus*): cDNA cloning, expression, recombinant protein production, and immunolocalization. *Gene*, 284(1–2): 93–102. https://doi.org/10.1016/S0378-1119(02)00389-X.

Esposito, D. and Chatterjee, D. K. 2006. Enhancement of soluble protein expression through the use of fusion tags. *Curr. Opin. Biotechnol*., 17(4): 353–358. https://doi.org/10.1016/j.copbio.2006.06.003.

Grève, P., Sorokine, O., Berges, T., Lacombe, C., Van Dorsselaer, A. and Martin, G. 1999. Isolation and amino acid sequence of a peptide with vitellogenesis inhibiting activity from the terrestrial isopod *Armadillidium vulgare* (Crustacea). *Gen. Comp. Endocrinol.*, 115(3): 406–414. https://doi.org/10.1006/gcen.1999.7330.

Hopkins, P. M. 2012. The eyes have it: A brief history of crustacean neuroendocrinology. *Gen. Comp. Endocrinol.*, 175(3): 357–366. https://doi.org/10.1016/j.ygcen.2011.12.002.

Jeong, K. J. and Lee, S. Y. 2001. Secretory production of human granulocyte colony-stimulating factor in *Escherichia coli. Protein Expr. Purif.,* 23(2): 311–318. https://doi.org/10.1006/prep.2001.1508.

Katayama, H., Ohira, T., Aida, K. and Nagasawa, H. 2002. Significance of a carboxyl-terminal amide moiety in the folding and biological activity of crustacean hyperglycemic hormone. *Peptides,* 23(9): 1537–1546. https://doi.org/10.1016/S0196-9781(02)00094-3.

Kotzsch, A., Vernet, E., Hammarström, M., Berthelsen, J., Weigelt, J., Gräslund, S. and Sundström, M. 2011. A secretory system for bacterial production of high-profile protein targets. *Protein Sci.,* 20(3): 597–609. https://doi.org/10.1002/pro.593.

Lorenzon, S. 2005. Hyperglycemic stress response in Crustacea. *Invertebrate Survival Journal,* 2(2): 132–141.

Makrides, S. C. 1996. Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiol. Rev.,* 60(3): 512–538. https://doi.org/10.1128/mr.60.3.512-538.1996.

Mandal, A. and Singh, P. 2025. Global Scenario of Shrimp Industry: Present status and future prospects. In: *Shrimp culture technology*, Springer Nature, Singapore, pp.1–23. https://doi.org/10.1007/978-981-97-8549-0_1.

Mettulio, R., Giulianini, P. G., Ferrero, E. A., Lorenzon, S. and Edomi, P. 2004. Functional analysis of crustacean Hyperglycemic Hormone by *in vivo* assay with wild-type and mutant recombinant proteins. *Regulatory Peptides,* 119(3): 189–197. https://doi.org/10.1016/j.regpep.2004.02.002.

Morera, Y., Lugo, J. M., Ramos, L., Rodríguez-Ramos, T., Huberman, A. and Estrada, M. P. 2013. A chimeric recombinant crustacean hyperglycemic hormone from *Litopenaeus schmitti* (Burkenroad) obtained as C-terminus fusion protein boost hemolymph glucose concentration in *Litopenaeus vannamei* (Boone). *Aquac. Res.,* 44(7): 1066–1075. https://doi.org/10.1111/j.1365-2109.2012.03110.x.

Mosco, A., Zlatev, V., Guarnaccia, C., Pongor, S., Campanella, A., Zahariev, S. and Giulianini, P. G. 2012. Novel protocol for the chemical synthesis of crustacean hyperglycemic hormone analogues - An efficient experimental tool for studying their functions. *PLoS ONE,* 7(1): e30052. https://doi.org/10.1371/JOURNAL.PONE.0030052.

Moss, S. M., Moss, D. R., Arce, S. M., Lightner, D. V. and Lotz, J. M. 2012. The role of selective breeding and biosecurity in the prevention of disease in penaeid shrimp aquaculture. *J. Invertebr. Pathol.,* 110(2): 247–250. https://doi.org/10.1016/j.jip.2012.01.013.

Ohira, T., Okumura, T., Suzuki, M., Yajima, Y., Tsutsui, N., Wilder, M. N. and Nagasawa, H. 2006a. Production and characterization of recombinant vitellogenesis-inhibiting hormone from the American lobster *Homarus*

americanus. *Peptides*, 27(6): 1251–1258. https://doi.org/10.1016/j.peptides.2005.10.019.

Ohira, T., Tsutsui, N., Nagasawa, H. and Wilder, M. N. 2006b. Preparation of two recombinant crustacean hyperglycemic hormones from the giant freshwater prawn, *Macrobrachium rosenbergii,* and their hyperglycemic activities. *Zool. Sci.*, 23(4): 383–391. https://doi.org/10.2108/zsj.23.383.

Okumura, T. 2004. Perspectives on hormonal manipulation of shrimp reproduction. *Japan Agri. Res. Quart., JARQ,* 38(1):49–54. https://doi.org/10.6090/jarq.38.49.

Pasteur, C. 1959. Preliminary note on the role of the x organ in the control of molting in the shrimp *Palaemon (=Leander) serratus* (Pennant). *Fisheries Research Board of Canada Translation Series No. 2798. Reports of the monthly sessions of the Society of Natural and Physical Sciences of Morocco*, 25:.123–124. https://waves-vagues.dfo-mpo.gc.ca/library-bibliotheque/20877.pdf (In French) (Accessed 28 August 2025)

Schägger, H. 2006. Tricine-SDS-PAGE. *Nature Protocols,* 1(1): 16–22. https://doi.org/10.1038/nprot.2006.4.

Soyez, D., Van Deijnen, J. E. and Martin, M. 1987. Isolation and characterization of a vitellogenesis-inhibiting factor from sinus glands of the lobster, *Homarus americanus. J. Exp. Zool.,* 244(3): 479–484. https://doi.org/10.1002/jez.1402440314.

Swartz, J. R. 2001. Advances in *Escherichia coli* production of therapeutic proteins. *Curr. Opin. Biotechnol.,* 12(2): 195–201. https://doi.org/10.1016/S0958-1669(00)00199-3.

Tamura, K., Stecher, G. and Kumar, S. 2021. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Mol. Biol. Evol.,* 38(7): 3022–3027. https://doi.org/10.1093/molbev/msab120.

Taranger, G. L., Carrillo, M., Schulz, R. W., Fontaine, P., Zanuy, S., Felip, A., Weltzien, F. A., Dufour, S., Karlsen, Ø., Norberg, B., Andersson, E. and Hansen, T. 2010. Control of puberty in farmed fish. *Gen. Comp. Endocrinol.,* 165(3), pp.483–515. https://doi.org/10.1016/j.ygcen.2009.05.004.

Terpe, K. 2006. Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. *Appl. Microbiol. Biotechnol.,* 72(2): 211–222. https://doi.org/10.1007/s00253-006-0465-8.

Treerattrakool, S., Boonchoy, C., Urtgam, S., Panyim, S. and Udomkit, A. 2014. Functional characterization of recombinant gonad-inhibiting hormone (GIH) and implication of antibody neutralization on induction of ovarian maturation in marine shrimp. *Aquaculture,* 428–429: 166–173. https://doi.org/10.1016/ j.aquaculture.2014.03.009.

Treerattrakool, S., Panyim, S., Chan, S. M., Withyachumnarnkul, B. and Udomkit, A. 2008. Molecular characterization of gonad-inhibiting hormone of *Penaeus monodon* and elucidation of its inhibitory role in vitellogenin expression by RNA interference. *FEBS Journal,* 275(5): 970–980. https://doi.org/10.1111/j.1742-4658.2008.06266.x.

Treerattrakool, S., Panyim, S. and Udomkit, A. 2011. Induction of ovarian maturation and spawning in *Penaeus monodon* broodstock by double-stranded RNA. *Mar. Biotechnol.,* 13(2): 163–169. https://doi.org/10.1007/ s10126-010-9276-0.

Tsutsui, N., Ohira, T., Okutsu, T., Shinji, J., Bae, S. H., Kang, B. J. and Wilder, M. N. 2013. Molecular cloning of a cDNA encoding vitellogenesis-inhibiting hormone in the whiteleg shrimp *Litopenaeus vannamei* and preparation of its recombinant peptide using an *E. coli* expression system. *Fish. Sci.,* 79(3): 357–365. https://doi.org/10.1007/s12562-013-0603-z.

Vaca, A. A. and Alfaro, J. 2000. Ovarian maturation and spawning in the white shrimp, *Penaeus vannamei,* by serotonin injection. *Aquaculture,* 182(3–4): 373–385. https://doi.org/10.1016/S0044-8486(99)00267-7.

Vijayan, K. K., Reynold, P., Mohanlal, D. L. and Balasubramanian, C. P. 2013. Preliminary characterisation of gonad inhibiting hormone (GIH) gene and its expression pattern during vitellogenesis in giant tiger shrimp, *Penaeus monodon* Fabricius, 1798. *Indian J. Fish.,* 60(2): 103-107.

Wilder, M., Okumura, T. and Tsutsui, N. 2010. Reproductive mechanisms in crustacea focusing on selected prawn species: Vitellogenin structure, processing and synthetic control. *Aqua-BioScience Monographs,* 3(3): 73–110. https://doi.org/10.5047/absm.2010.00303.0073.

Wurts, W. A. and Stickney, R. R. 1984. An hypothesis on the light requirements for spawning penaeid shrimp, with emphasis on *Penaeus setiferus*. *Aquaculture,* 41(2): 93–98. https://doi.org/10.1016/0044-8486(84)90086-3.