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MOLECULAR CLONING AND CHARACTERIZATION OF GIH GENE OF BLACK TIGER PRAWN

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ABSTRACT

The GIH gene of Penaeus monodon has an important role in reproduction. Here we report a novel GIH1 gene of P. monodon (Pem-GIH1). The cloned gene is 827 bp in size and composed of 3 exons and 2 introns. The conceptual peptide of Pem-GIH1 has 103 amino acids and six conserved cysteine residues. Similarity searches using BLAST revealed that the Pem-GIH1 gene is more closely related to SGP C1 of P. monodon. The phylogenetic analysis showed that Pem-GIH1 is clustered under type II hormones. By using SWISS MODEL softwares tertiary structure of the conceptual protein of Pem-GIH1 was constructed.

Key words: *Penaeus monodon,* Phylogeny, neuropeptide, eyestalk ablation, post transcriptional gene silencing

INTRODUCTION

Crustacean hyperglycemic hormone (CHH), moult inhibiting hormone (MIH) and gonad inhibiting hormone (GIH) family (CMG family) include a group of highly homologous neuropeptides which control and co-ordinate many physiological functions in crustaceans. The X-organ-sinus gland complex of the eyestalk forms the major secretory centre for these hormones. All these hormones are structurally related and some exhibit overlapping biological activities. The primary function of CHH is the regulation of carbohydrate metabolism through elevation of sugar in the haemolymph (5, 13). MIH regulate moulting cycle (2, 12) and GIH inhibit gonad development (4). Comparison of amino acid sequences of these hormones revealed significant degrees of similarity (7,1). Members of this family are typically 70-80 amino acid long with six conserved cysteine residues`forming three disulfide bonds (6).

The CMG peptides are grouped into type I and type II (10, 13). Type I hormones comprise all CHHs and type II include MIH and GIH. With regard to their gene structure type I genes are composed of 4 exons and 3 inrons, and type II genes contain 3 exons and two introns. Type I hormones consists of 72-73 amino acids. The amino terminus of type I hormones are blocked by a pyroglutamyl residue and caboxyl termini are blocked by amides, whereas type II hormones consists of 74-83 amino acid residues with a Glycine residue at position 12. The amino and carboxyl termini of type II hormones are free (3).

Unilateral eyestalk ablation is a classical method practiced in shrimp hatcheries to induce growth and maturation. The removal of eyestalk destroys the function of X-organ-sinus gland complex from its negative control over growth and reproduction. The removal of eyestalk is an invasive procedure that results in high mortality. The molecular tools like post-transcriptional gene silencing can be used to disrupt the function of these inhibitory hormones in a non-invasive manner. Only five of type II genes of *P. monodon* are cloned and characterized till to date. In an attempt to clone more genes in the type II peptides of CMG,

we have cloned a novel gene encoding gonad inhibiting hormone (Pem-GIH1) and determined its genetic structure.

MATERIALS AND METHODS

P. monodon were collected from a hatchery near Calicut. The prawns were washed with double distilled water and hepatopancreas dissected out. The genomic DNA was extracted from the hepatopancreas of the black tiger prawn, *P. monodon.* The tissue was homogenized using a glass pestle and mortar. The genomic DNA in the homogenate was extracted using the Nucleic acid Purification Kit (Nucleospin Tissue, Machery-Nagel, Germany) in accordance to the manufacturer's instructions. About 2 nanogram of genomic DNA was amplified for the GIH gene using the foreward primer with DNA sequence 5'-CCGCCCCAGGATACACTT-3' and reverse primer with DNA sequence 5'-TTATTTATGTTCATCCTAAAAATGAGG-3'. The PCR reaction mixture consisted of 2 nanogram of genomic DNA, 1 µl each forward and reverse primers at a concentration of 2.5 µM, 2.5 µl of dNTPs (2mM), 2.5 µl of 10X reaction buffer, 0.20 µl of Taq polymerase (3U/µl) and 16.8 µl H_2O. The PCR profile consisted of an initial denaturation step of 2 minutes at 95ºC, followed by 40 cycles of 5s at 95ºC, 45s at 50ºC and 45s at 72ºC and ending with a final phase of 72ºC for 3 minutes. The PCR products were resolved on a 1% TAE-agarose gel, stained with EtBr. After ascertaining the PCR amplification of the GIH fragment, the remaining portion of the PCR product was column purified using Mo Bio Ultraclean PCR Clean-up kit (Mo Bio Laboratories Inc. California) as per the manufacturer's instructions. The purified PCR product was sequenced from both ends using the forward and reverse primers used for the PCR using Sanger's sequencing method at SciGenom Labs Pvt. Ltd., Cochin. The forward and reverse sequences obtained were trimmed for the primer sequences, assembled by using ClustalW and the consensus was taken for analysis. The nucleotide sequence and peptide sequence were searched for its similarity using BLAST programme of NCBI (www.ncbi.nlm.nih.gov/) and MEGA5 software was used for phylogenetic analysis. Disulphide bond prediction of the conceptual protein was done by DiANNA web server. The tertiary structure of the conceptual protein is constructed by SWISS MODEL software.

RESULTS

A novel GIH gene of *P. monodon* (Pem-GIH1) was cloned. The Pem-GIH1is 827 bp in length and composed of 3 exons and 2 introns. The first intron separates the signal peptide and the second intron separates the mature peptide in the coding region. The conceptual peptide of GIH1 yielded 103 amino acids with six conserved cysteine residues and a glycine residue at position 12 (Figure 1).

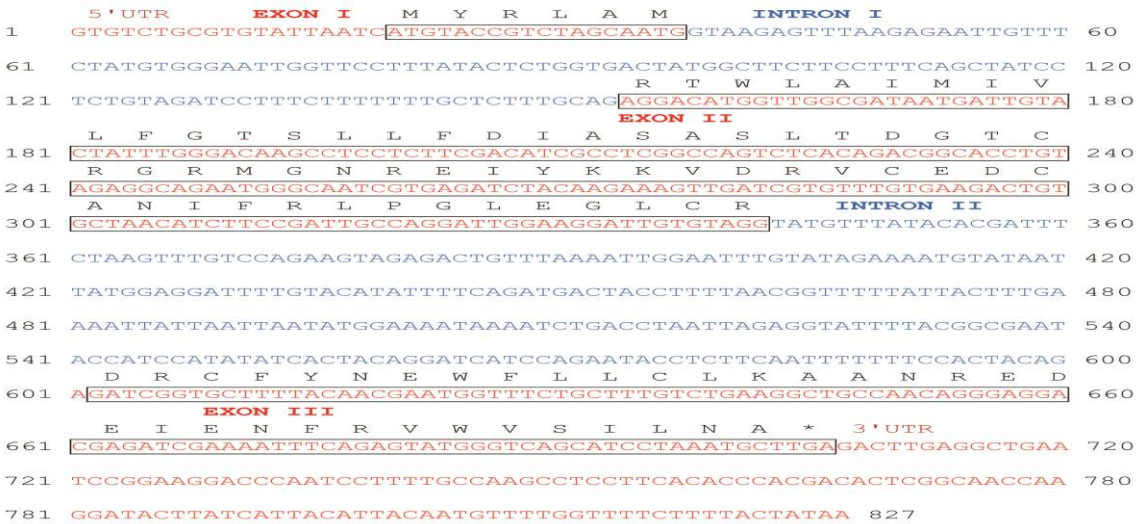

Fig. 1: Nucleotide and deduced amino acid sequences of Pem GIH1. The exons are given in open boxes; the amino acids are presented as one-letter symbols and shown above their codons in each line. An asterisk marks the stop codon.

The numbers on the left and right of the sequences show the co-ordinate of nucleotides in corresponding lines. The GIH1 gene contains three exons and two introns, intron I interrupt the sequence coding for signal peptide and the intron II separates the mature peptide sequence. The N- terminal and C-terminal segments may play important roles in hormone function.

The amino acid sequence of Pem-GIH1 is compared with type II peptide sequences from *P. monodon* and is given in figure 2. It showed that the amino acid sequences are highly conserved towards the C-terminal part of these proteins.

Fig. 2: Comparison of amino acid sequences of GIH1 with type II peptides of *P. monodon* : Pem SGP C1 (BAB70610.1), Pem SGP C2 (AAR89516.1), Pem MIH 2 (AAR89517.1), Pem MIH 1(ACS88073.1), Pem GIH (ABG33898.1). The highly conserved amino acids are shaded in yellow. Note that C terminus has a highly conserved part SILNA, characteristic of type II hormones. The high degree of homology at C-terminus indicates the possibility of forming multimeric proteins and functions in a co-operative manner.

Similarity searches using BLAST revealed that the nucleotide sequence of the mature peptide showed 98% similarity with SGP C1 of *P. monodon*. The peptide sequence showed 99% similarity with SGP C1 of *P. monodon.* The phylogenetic tree constructed with MEGA 5 software revealed that GIH1 of *P. monodon* is clustered under type II peptide hormones of crustaceans (Figure 3).

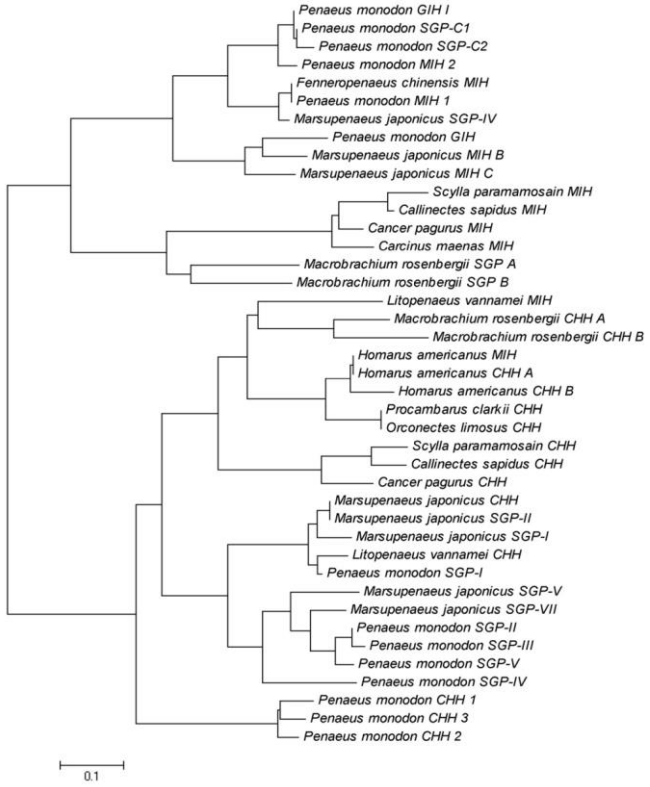

Fig. 3: Phylogenetic tree of amino acid sequences of the CMG peptides from different crustaceans. Type I and type II

peptides of crustaceans form separate clades. Type I clade contained all CHHs from different crustaceans with the CHH2 of *P. monodon* at the base. Type II clade contained all MIHs and GIHs/VIHs from different crustaceans with the SGP B of *M. rosenbergii* at the base. This topology suggested that type I and type II peptides may have originated by duplication of an ancestral gene.

Disulphide bond prediction of Pem-GIH1 and type II hormones are done by DiANNA web server, which showed the presence of 3 disulfide linkages between 6 conserved cysteine residues in all these hormones. The tertiary structure of Pem-GIH1 was determined by homology modeling using SWISS MODEL software and compared with other type II hormones from *P. monodon*. The results showed that Pem-GIH1 is more closely related to SGP C1 (Figure 4).

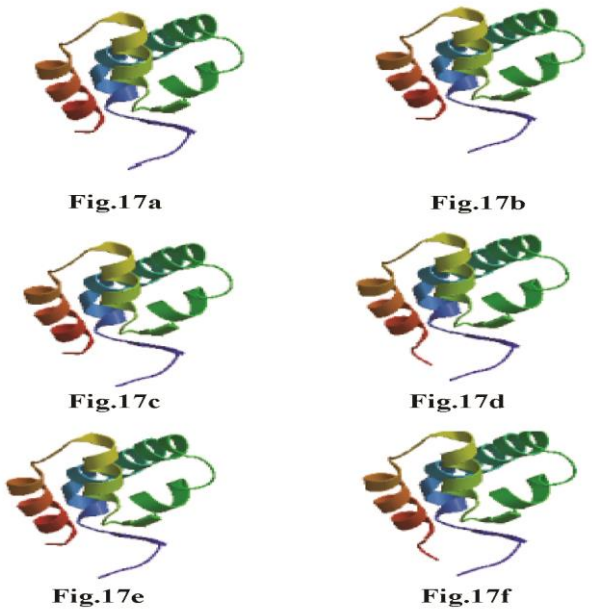

Fig. 4: 3D structure of the mature type II peptides of *P. monodon*.(a: GIH1, b: SGP C1(BAB70610.1), c: SGP C2 (AAR89516.1), d: MIH 1 (ACS88073.1), e: MIH 2 (AAR89517.1), f: GIH (ABG33898.1). Note that all type II proteins are structural homologs. They may have evolutionarily conserved binding sites and shows biologically relevent interactions forming multimeric complexes.

DISCUSSION

Here a novel GIH gene (Pem-GIH1) of *P. monodon* was cloned and characterized. Pem-GIH1 revealed an ORF corresponding to three exons and two introns, which is characteristic of type II gene of crustaceans. Exon-intron organization of type II gene is different from that of the type I gene. Type II gene is composed of three exons, whereas type I gene is composed of four exons (3). The signal peptide of Pem-GIH1 is composed of 28 amino acids and mature peptide consists of 75 amino acids. The presence of six cysteine residues at conserved position and a glycine at position 12 when aligned with other CMG peptides suggested that Pem-GIH1 is a member of type II hormones. The C-terminus has a highly conserved amino acid sequence 'SILNA'.

Phylogenetic analysis showed that Type I hormones and Type II hormones are separately clustered into two distinct sub branches, except the MIH of *H. americanus*. MIH of *H. americanus* is more closely related to CHH type I hormones, showing hyperglycemic effect (Lin *et al*., 1998). The disulphide connectivity pattern of type II peptide is strong evidence that these hormones are homologs. These findings led to the hypothesis that these hormones may act in a co-operative manner by forming interchain disulphide bonds. They form functionally related multimeric complexes, these active units of proteins are structurally and functionally homologs and regulate moulting and reproduction in crustacea. This will provide the groundwork for developing future *in vitro* and *in vivo* studies to understand the cross functional action of these hormones in detail.

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