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Isolation and Characterization of Gonad Inhibiting Hormone (GIH)-coding Gene in black tiger shrimp (*Penaeusmonodon* Fabricius, 1798)

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Abstract. One of the methods to increase the production in crustaceans is a highly complex hormonal process that requires precise coordination of external and internal factors to be successful. One of the important peptide hormones controlling reproduction in crustaceans is the gonad-inhibiting hormone (GIH). This gene is believed to be capable of encoding important components of the reproduction process, primarily inhibiting gonadal maturation in shrimp. As a key component in understanding the reproduction of *Penaeusmonodon*, we characterised the expression pattern of the GIH gene in the eyestalks. This study aimed to isolate and characterize the gonad inhibiting hormone-encoding gene. The GIH gene was isolated using PCR with GIH-F and GIH-R primers. The PCR products (cDNA fragments) were sequenced. Sequence analysis was conducted in GENETYX version 7 and BLAST-N *(*basic local alignment search tool-nucleotide*)* was used to determine the similarity of sequences generated with existing sequences in GenBank. The cDNA sequences were 316 bp, and had 96%-99% identity with *P. monodon* GIH accessions in GenBank. We conclude that the PCR amplified cDNA fragments of the GIH encoding gene sequences from the shrimp.

1. Introduction

Indonesia is an archipelagic country that has great fisheries sector potential, including being one of the most important shrimp suppliers worldwide, especially to Japan, the United States and the European Union [1]. However, in recent years the volume of shrimp originating from Indonesia has begun to decline and has been replaced by shrimp from Thailand, China and Vietnam. Constraints on shrimp development in Indonesia include low productivity and sub-optimal quality with respect to international requirements. Sustainable export success can only be achieved if efforts to increase production are combined with improvements in quality [2].

The substantial increase in productionrequired due to efforts to meet export market demand has resulted in increasing demand for shrimp seeds, so the production of shrimp seeds must be carried out intensively. Recently, many efforts have been directed at providing shrimpbroodstock by developing, maturing and breeding broodstockin a controlled manner, especially tiger shrimp.The tiger shrimp (*Penaeusmonodon*) is one of the coastal seafood commodities that has been cultivated widely in many

Southeast Asian countries [3,4]. To increase tiger shrimp production, it is necessary to understand the mechanism of controlled gonad maturation in female broodstock.

Gonad maturation and spawning control are major problems in the development of shrimp culture, with a lack of significant development in the reproductive technology applied in shrimp hatcheries. There is a lack of basic knowledge regarding the mechanisms and roles of hormones in shrimp reproduction. The reproduction of female crustaceans is controlled by a complex endocrine system. One common cellular activity during ovarian development is known as vitellogenesis[5]. The main compound produced during this process is vitellin lipoprotein derived from a precursor called vitellogenin (Vg). Vg can be synthesized insideor outside the ovary tissue [6]. Vg synthesis and gonad maturation are regulated by a single eye stem endocrine factor that leads to the production of vitellogenesis inhibiting hormone (VIH) or gonad maturation inhibitor hormone (GIH) [5,7]. GIH suppresses gonad maturation through the inhibition of Vg expression [8]. Apart from playing a role in inhibiting vitellogenin synthesis in female crustaceans, GIH also inhibits the work of the androgenic gland hormone (AGH) in male crustaceans [9]. The gonad maturation inhibiting hormone (GIH) of crustaceans is generally located in the X-organ and sinus glands that are in the eye stalk [10]. To accelerate the process of gonadmaturation in shrimp, it is necessary to inhibit GIH production [11].

Efforts that have been made to inhibit the production of GIH in shrimp involve ablating the eye stalk [9,11]. However, eye stalk ablation can have a negative impact on broodstock [8] and even cause death [7,12]. In addition, ablation of the eye stalk in the broodstock not only suppresses the production of GIH, but also reduces the production of other hormones that play a role in shrimp physiological processes [13] which in turn will affect the quality and quantity of eggs produced [14]. The recent and ongoing development of increasingly advanced molecular techniques provides many opportunities to study the reproductive hormone control mechanism, especially with respect to inhibiting the action of the GIH hormone. This study aimed to isolate and characterize the gene encoding for gonad maturation inhibiting hormone production in tiger shrimp in order to better understand the reproductive mechanism of crustaceans, especially shrimp.

2. Research Methods

2.1. Tiger Shrimp (Penaeusmonodon) Sampling

Three female *P. monodon* tiger prawns were collected from the wild. Thesesamples were transported in live condition to the Biotechnology Laboratory, BarruWindu Shrimp Cultivation Installation in Maros District, South Sulawesi, Indonesia in order to provide eye stalks for total RNA extraction.

2.2. RNA extraction

Total RNA was extracted using the RNeasy mini kit (Qiagen) according to manufacturer's protocols. For each sample, 20-25 mg of eye stalk material was placed in a 1.5 mL micro-tube and crushed using a frozen mortar, then dissolved in 600 µL of RLT buffer and placed in a container filled with ice. The crushed sample was homogenized with the RLT buffer, and then centrifuged at 14,000 rpm for 3 minutes. The supernatant was transferred to a new centrifuge tube to which 600µL of 70% ethanol was added and then re-suspended with a pipette gently. The sediment formed was placed in a spin column tube which was placed in a 2 ml volume container tube. Samples were centrifuged at 12,000 rpm for 15 seconds to wash the spin membrane column. The spin column was then transferred to a new tube for the next stage. RW buffer (700 µL) was added to the spin column before centrifuging at 14,000 rpm for 15 seconds. After adding500 µL of RPE buffer to the spin column, the extracted RNA was transferred into a new 1.5 mL tube. The extracted RNA was added to 50 µL of Rnase-free water in a new spin column and centrifuged at 14,000 rpm for 1 minute. After centrifuging, the spin column tube was released and the sample was ready for the RNA purity and concentration to be measured.

. Synthesis of cDNA by RT-PCR

Complementary DNA (cDNA) synthesis was carried out using the *Ready-To-Go You-Prime First Strand Beads* (GE Healthcare) kit using the RT-PCR technique. A concentration of 3 µg RNA in 30 µL DEPC 0.1% was homogenized by vortex in a micro-tube, then incubated at 65°C for 10 minutes. Next, the micro tube was placed into ice for 2 minutes, and then the RNA was added to the first strand reaction mix beads tube which already contained 2 white balls. An aliquot of 3 µL prime oligo (dT) 5'-gta atacga or act or cacgcg high tcg high acggcccgggcg high tttttttttttttt t-3 'with a concentration of 1 µg/3 µLwas added to the reaction, then allowed to stand for 1 minute. The micro-tubes were incubated at 37 ° C for 1 hour, and then SDW 50 µL was added to the cDNA formed.

2.4. PCR process

After the RNA had been extracted and cDNA had been formed, PCR was carried out twice. The first PCR was to isolate and amplify the GIH gene, and the second PCR was to isolate the expression of the β-actin gene. GIH gene expression was isolated by using the forward primer specific PmGIH-F 5'-atg aaa aca tgg ctg cta tta gcg-3' and reverse PmGIH-R 5'tgg gat gct ttc aga gaa gg-3' with a target fragmen 316 bp position [4]. The primer used for semi quantitative RT PCR. GIH gene expression can be observed through GIH gene amplification using a PCR Gene Amp 2700 machine (Applied Biosystems). PCR process is run on predenaturation temperature of 95°C for 5 minutes for 35 cycles consisting of 1 minutes denaturation at a temperature of 95°C, 40 seconds annealing at a temperature of 58°C, at a temperature of 72°C extension of 1 minute, and final extension for 10 minutes at 72°C. The second PCR was isolate tiger shrimp β-actin gene as an internal control [15]. The DNA β-actin gene expression fragment targeted was the 400 bp position. Beta-actin gene amplification was performed using the following program: pre-denaturation at 94°C for 2 minutes; 35 cycles consisting of 30 seconds of denaturation at 94°C, 30 seconds of annealing at 55°C, extension at 62°C for 1 minute); and final extension for 5 minutes at 68°C. The successful amplification of target DNA fragments in the PCR product was verified through electrophoresis on 2.0% agarose gel and recorded using the Gel Documentation System (Biometra). The molecular weight of the DNA fragment was determined using the VC 100 bp Plus DNA Ladder (Vivantis) marker.

2.5. Nucleotide Sequences

After the PCR GIH gene amplification process was completed, the PCR product was further purified following the standard procedures in the manual for the kit used. The purifiedproducts from each sample were then sequenced on an ABI PRISM 310 automatic machine at the *First Base* laboratory in Singapore.

2.6. Data Analysis

The nucleotide sequences obtained were analysed in Genetyx Version 7 (Genetyx Corporation) to obtain consensus sequences from the forward and reverse sequences produced. To determine the similarity of the resulting sequences with known sequences, the GIH sequences were aligned with existing GenBank accession sequences using the on-line BLAST-N (basic local alignment search tool-nucleotide) tool. The results were presented and analysed descriptively.

3. Results and Discussion

Gonad Inhibiting Hormone (GIH) Coding Genes in tiger prawns were successfully amplified using PCR technique. Visualization of cDNA fragments from the GIH gene on the electrophoresis gelshowed an amplification product cDNA band between 300-400 bp. However, only two of the three samples expressed the GIH gene (Figure 1).

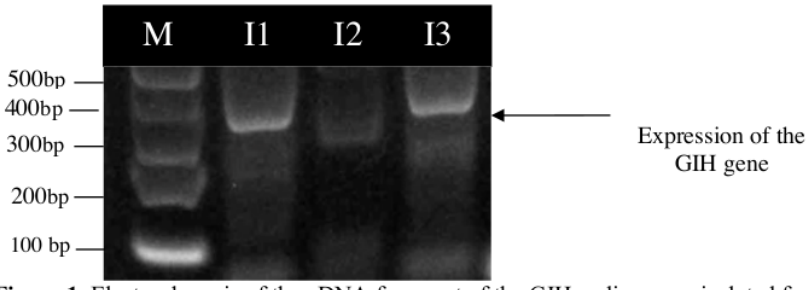

Figure 1. Electrophoresis of the cDNA fragment of the GIH coding gene isolated from three tiger prawn (*Penaeusmonodon*) samples (1, I2 and I3). M = 100 bp Ladder. The black arrow indicates the GIH gene fragment at around 300-400 bp.

Based on the results of shrimp gonad dissection, the first shrimp sampled had not yet entered the mature stage of gonad development (Figure 2), so that it was unable to express the GIH gene. This is consistent with the results of research by Vijayan *et al*. (2013) who found that the GIH mRNA level was low before entering the mature gonad stage of the reproductive cycle because at that stage it was not only GIH that was responsible for inhibiting gonad maturity.

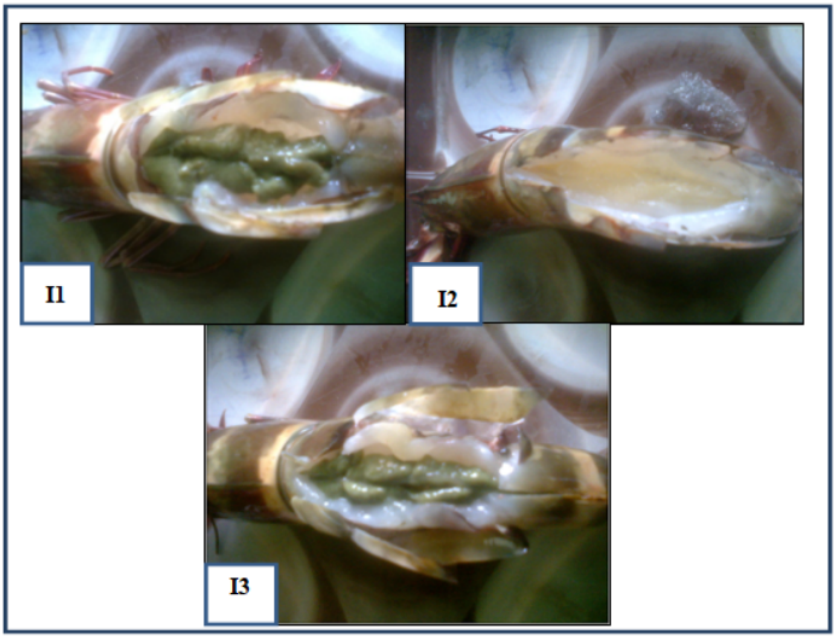

Figure 2. Dissection of sampled tiger shrimp gonads. Samples I1 and I3 show the presence of mature gonads, whereas I2 has immature gonads

The GIH expression profile in tiger shrimp is the inverse of the ovarian development profile, where the highest GIH expression level appears at gonad maturity stage (GMS) I (to maintain the immature state of the ovary) and the expression level decreases significantly with GMS, reaching the lowest level when the ovaries develop to maturity [11]. Shi *et al.*, (2020) also found that the level of expression of GIH in the eye stalk of *Litopenaeusvittata* increases at GMS II and III, but will decrease dramatically at GMS IV. In GMS II, the ovary begins to develop and expand, increasing in GMS III and reaching maturity at GMS IV. This shows that GMS II and GMS III are still immature (albeit maturing) phases, while GMS IV is the mature phase. Based on this, sample I2 is thought to have entered the transitional phase of vitellogenesis (immature phase), while the other two samples (I1 and I3) had entered the mature phase. This is different from the GIH-related processes associated with vitellogenesis (gonad maturation process) in *Homarusamericanus* lobsters described by de Kleijn *et al.* (1998). In the lobster *H. americanus*, GIH in the haemolymphremains relatively high and stable until the previtellogenesis phase. When approaching the vitellogenesis phase, GIH begins to decline to a low but stable level as it enters the vitellogenesis phase, even to the mature phase. Upon re-entering the immature phase (after spawning), GIH increases and becomes relatively stable at a higher level again. This shows that the control of GIH varies in different species [13].

High GIH expression on the eye stalk and haemolymph when the ovary is immature indicates that GIH production is continuous and is released into shrimp haemolymph[13]. The decrease in GIH concentration at ovarian maturation explains the function of GIH which inhibits vitellogenin expression and oocyte growth during ovarian development [16]. In addition, the expression value of GIH has been found to be lower in the eye stalk compared to the ovary, which is thought to be because the GIH in the ovary originated from the primordial cell[11]. The indicator used in this study to determine whether the GIH gene was expressed or not was the presence of the β-actin gene expression fragment, because the β-actin gene is always expressed all the time and in all organs so that its expression can be used as an internal control on the expression of other genes [15]. Figure 3 shows that the expression of the β-actin gene has a similar pattern of fragment length and thickness in the three samples. This result can be considered a validation of the PCR amplification of GIH gene expression.

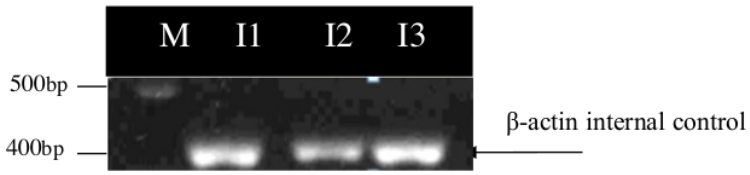

Figure 3. Internal control: β-actin electrophoresis of tiger shrimp samples I1, I2 and I3. M = 100 bpLadder.The arrow shows the β-actin control fragment at the position around 400 bp

To ensure that the isolated cDNA fragment was the GIH gene, the two samples with cDNA fragments capable of expressing the GIH gene were purified from the agarose gel and sequenced. The length of the GIH gene sample fragments was 316 bp. The alignment of the two GIH gene samples in GENETYX 7 is shown in Figure 4.

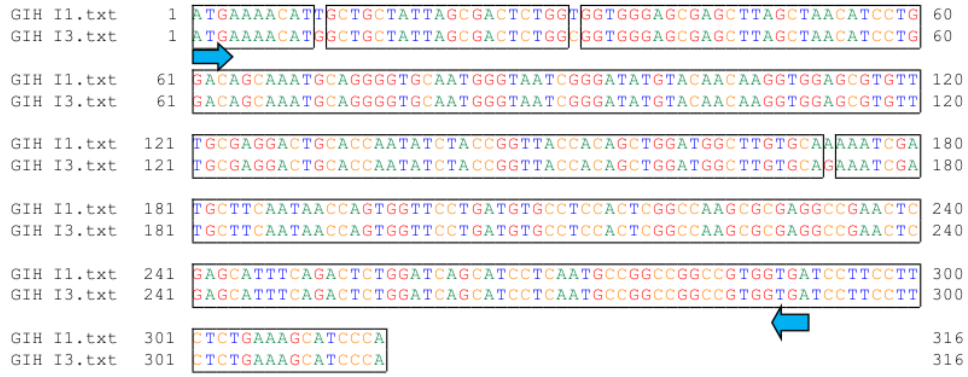

Figure 4. Alignment of the GIH coding gene sequences isolated from tiger shrimp samples I1 and I3

The position of key elements in the sequences reinforces the notion that the fragments isolated by the PCR are genes coding for tiger shrimp GIH. The initial codon (initiation) is in nucleotides 1-3 (the first arrow in the alignment sequence). The final (stop) codon is located at the nucleotide 289-291 bp (second arrow in the sequence alignment). According to Jackson *et al.* (2012) the initial codon (genetic code) or translation initiation codon is a codon for the amino acid methionine (ATG) which initiates the structure of a polypeptide (protein), while the final or stop codon is a translation termination codon in a gene with sequences triplets TAA, TAG or TGA.

From the length of the GIH (316 base pairs), this fragment translates 103 amino acids. GIH is part of the type II Crustacean Hyperglycemic Hormone (CHH) family (Li *et al.*, 2015; Shi *et al.*, 2020). The nucleotide sequences obtained from each sample have a high similarity (99%). The results of the BLAST-N can be seen in the form of a phylogenetic tree in Figure 5.

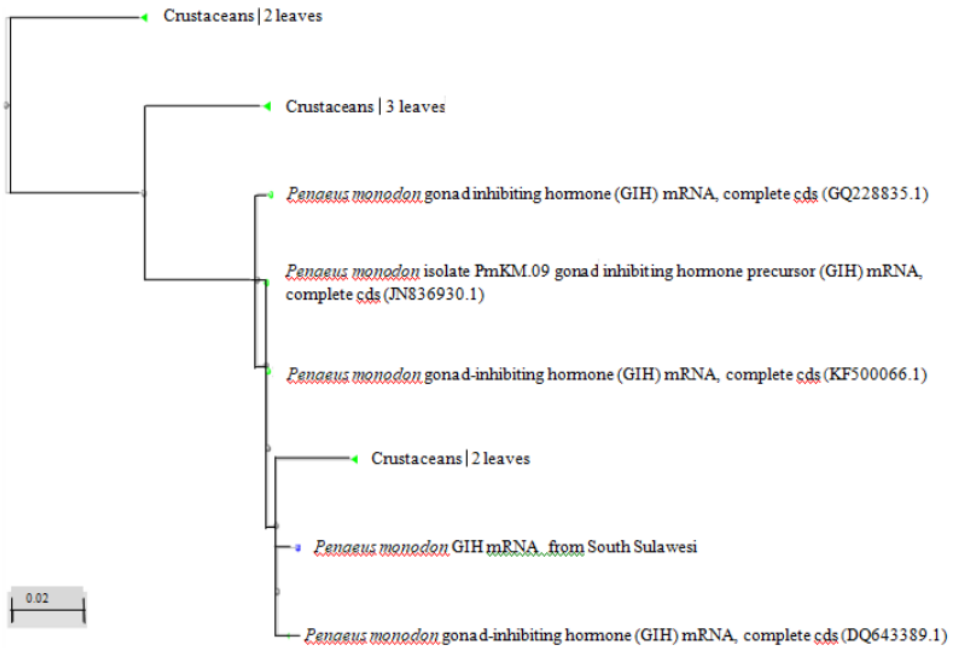

Figure 5. GIH gene phylogenetic tree from isolated samples of tiger shrimp from South Sulawesi.

CHH generally has a length of 72-87 amino acids[16]. GIH peptides consist of 77-83 amino acids with molecular masses between 7-11 kDa[9], while Treerattrakool *et al.* (2008) shows the Open Reading Frame (ORF) of *P. monodon* GIH (Pem-GIH) is 288 bp which produces protein from 96 amino acid triplets. However, the signal peptide is only 17 amino acids, while the other 79 amino acids are mature Pem-GIH peptides. Of the 79 mature Pem-GIH amino acids, there were six amino acids coding for cysteine followed by glycine. Six cysteine amino acid triplets are characteristic of CHH [16]. The 103 amino acids obtained in this study comprised 6 amino acid triplets coding for cysteine residues with glycine after the first cysteine.

Furthermore, the amino acid sequence of tiger prawn GIH obtained in this study showed a high sequence identity (96-99%) with GIH from *P. monodon* GenBank accessions identified using the BLAST-N tool. This high similarity indicates that this geneis relatively unlikely to experience mutations. It is well known that mutations are changes in genetic material that can be inherited and give rise to other forms of genes [19]. Figure 5 shows the phylogenetic analysis of GIH genes that have been registered in the GenBank database. Phylogenetic analysis is inseparable from biological evolution. Evolution is a gradual process, and can allow simple species to become more complex or diversify through the accumulation of changes over several generations. Offspring will have some differences from their ancestors because they will experience evolutionary changes[20].

Phylogenetic analyses or cladistics identify clades, where a clade is a group of descendants from one common ancestor. Phylogenetic analyses are usually represented as a branching system, such as a tree diagram known as a phylogenetic tree. This phylogenetic tree provides information about the classification of populations based on their evolutionary relationships. In the reconstruction of phylogenetic trees, molecular data are increasingly widely used because they are considered more stable in the evolutionary process than morphological data [21]. The results of the analysis of the similarity of the GIH gene to the data in GenBankshown in Figure 5confirm that the gene fragments isolated from the tiger shrimp samples were GIH genes.

4. Conclusion

From this research we conclude that the gonad maturity inhibiting hormone (GIH) gene of tiger shrimp (*Penaeusmonodon*) has a sequence length of 316 bp. The homology between the two samples and other GIH sequences from *P. monodon* deposited in GenBank ranged from 96% to 99%. The GIH gene obtained has a close relationship with the GIH gene of other crustacea that have been registered in the Gen Bank database.

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