IDENTIFICATION OF GROWTH HORMONE GENE OF *Pangasionodon hypophthalmus*

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ABSTRACT

Identification of growth hormone (GH) gene in a target fish is the first step in the construction of "all fish genes transfer vector" to generate transgenic fish. The research was done to identify and characterize the GH gene of *Pangasionodon hypophthalmus*. There were several activities performed in identifying the GH gene: RNA extraction, cDNA synthesis, PCR amplification, and DNA fragment isolation. The characterizations were done using the nucleotide sequencing engine ABIPRISM 3100. The results were then analyzed using BLASTN/P and GENETYX version 7 program. The full-length GH gene of *P. hypophthalmus* was 1151 bp in length, coding for an open reading frame (ORF) of 603 bp. The 5' and 3' untranslated regions of the GH gene were 22 bp and 526 bp long, respectively. The GH gene *of P. hypophthalmus* had some common characteristics that are owned by GH genes, such as single tryptophan residue (W) on the 104th amino acid, 5 cysteine residues (C) on the amino acid 71, 135, 173, 190, and 198 and a motif of Asn-Xaa-Thr on C terminus which is the potential location for N-linked glycosilation. Polyadenylation signal (aataaa) was on the 14 bp at the upstream of polyadenylation location. Growth hormone *of P. hypophthalmus* consisted of over 200 amino acids from GH cDNA deduction. The highest proportion of amino acid composition was leusin (14%) while the lowest was tryptophan (0.5%).

KEYWORDS: *Pangasionodon hypophthalmus*, GH gene, identification, characterization

INTRODUCTION

Growth hormone (GH) is a polypeptide of fundamental importance for growth regulation in vertebrate (Meier *et al*., 2006). Pituitary growth hormone, also known as somatothropin in fish, is the key protein responsible in the regulation of somatic growth and many aspects of metabolic processes detected in all vertebrate (Ryynanen & Primmer, 2006). In fish, growth hormone is involved in numerous physiological processes including ionic balance, lipid and protein metabolism, growth, reproduction, and immune function as well as various aspects of behavior (reviewed by Perez-Sanchez, 2000). Since GH is commercially important in the areas of medicine, animal husbandry, aquaculture, and animal feed formulation, the gene coding for the hormone has been studied extensively in several mammalian and piscine species (Anathy *et al*., 2001).

In the last twenty years, development of gene transfer was technologically intended to obtain fishery products with certain desired characteristics. Transgenesis or DNA recombinant technology is a genetic engineering that allows the recombination or reunification of

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genes from different sources by *in vitro*, which then will be introduced to an individual. Genes that have undergone this recombination are called recombinant genes or foreign genes. Gene that is introduced or called transgene is a certain encoding protein that controls a desired character.

At this time, the most successful transgene and the first used in the cultivation of fish for human consumption contains construction of GH genes. The reason is that the construction of growth hormone can improve growth substantially on a wide variety of fish species such as common carp and gold fish (*Cyprinus carpio* and *Carrasius auratus* gibelio) (Zhu, 1992), channel catfish (*Ictalurus punctatus*) (Dunham *et al*., 1987), tilapia (*Oreochromis niloticus*) (Martinez *et al*., 1996), Atlantic salmon (*Salmo salar*) (Du *et al*., 1992), coho salmon (*Oncorhynchus kisutch*) (Devlin *et al*., 1994), rainbow trout (*Oncorhynchus mykiss*) (Devlin *et al*., 2001), mud loach (*Misgurnus mizolepis*) (Nam *et al*., 2001), and Arctic charr (*Salvelinus alpinus*) (Pitkanen *et al*., 1999).

Indonesia has many species of fish that have been successfully cultivated. Among various species of potential freshwater fish for commercial cultivation is *Pangasionodon hypophthalmus* which is culturally special for several consumers, especially in Sumatra and Kalimantan (Nasution *et al*., 1997). Development of fish culture technology encourages the spreading of *P. hypophthalmus* products, especially seed to other areas such as Java.

To improve the efficiency of *P. hypophthalmus* culture in Indonesia, it is necessary to develop a transgenesis technology to produce superior broodstocks with fast growth (supergrowth) character in order to increase the culture efficiency on feed, energy and cost. As a first step to produce *P. hypophthalmus* supergrowth, the research was conducted with the aim to identify gene encoding growth hormone (GH) in *P. hypophthalmus*.

MATERIALS AND METHODS

RNA Extraction

Identification of the GH gene expression was carried out by extracting RNA from organ tissues of pituitary, muscle, brain, liver, testes, and lymph. About 25—50 mg of tissue sample was stored in a sample vial (Eppendorf containing 200 µL isogen). Tissue was destroyed using pestle which was previously sterilized with DEPC 1%. Isogen was added into the Eppendorf to a final volume of 800 µL. Two hundred microliter of chloroform was added and solution was centrifuged with a speed of 12,000 rpm for five minutes at room temperature. Supernatant was then transferred to a new eppendorf containing 400 µL isopropanol. Solvent was then centrifuged with a speed 12,000 rpm for 15 minutes at a temperature of 4°C. Pelleted RNA was kept in the Eppendorf, and the liquid in it was removed. One mL of 70% cold alcohol was added into the Eppendorf and then centrifuged with a speed 12,000 rpm for 15 minutes. Pelleted RNA was dried by removing solution in the eppendorf. Pelleted RNA was dissolved by adding 30 µL 1% DEPC. Concentration of total RNA was measured using a gauge concentration RNA/DNA (GeneQuant). Absorbance was measured at wavelength of 260 and 280.

cDNA Synthesized

The RNA samples were added with DEPC-treated water to reach a volume of 25—30 µL in an RNase-free micro-centrifuge tube. The mixture was heated at 65°C for 10 minutes, and then chilled in ice cubes for 2 minutes. During the chilling process, two beads were visible in the bottom of the tube of first-strand reaction mix beads (white tube). If necessary, the tube can be gently tapped against a hard surface to bring the beads to the bottom. RNA solution was then transferred to the tube of first-strand reaction mix beads (do not mix). Three microliters of 'dT3'RACE-VECT "(5'-gta ata cga ctc act ata ggg cac gcg tgg tcg acg gcc cgg gct ggt ttt ttt ttt ttt ttt ttt-3') with concentration of 1 µg/3 µL was added to reach a final volume of 33 µL. Those samples were kept at room temperature for approximately 1 minute. The contents were mixed by gently vortexing, or by repeatedly pipetting the mixture up and down. To collect the content at the bottom of the tube, the mixture was centrifuged briefly and incubated at 37°C for 60 minutes. The completed first-strand reaction was ready for PCR amplification.

PCR Amplification

One micro liter of cDNA was used as a template for PCR, and then mixed with 1 µL FGHP primer (5'- tca gag aga ttt ggc aaa atg gct-3 '), 1 µL RGHP primer (5'–taa tgc aag aat tag ctt

tat 3 '), 1 µL dNTP, 1 µL Ex Taq buffer, 0.05 µL Ex Taq polymerase (TAKARA) and added with SDW to reach a final volume of 10 µL. PCR was performed with program: 94°C for 3 minutes; 94°C for 30 seconds, 59°C for 30 seconds, 72°C for 1 minute of 35 cycles, 72°C for 3 minutes and 4°C (unlimited). Observation of PCR amplification was carried out with electrophoresis using 0.7% agarose gel.

DNA Fragment Purification

DNA fragment, which was resulted from the PCR analysis was isolated from the gel using Mobio Ultra Clean™ 15 DNA Purification kit (MoBio Laboratories, CA, USA). Weight of agarose gel band slice was determined in a 1.5—2.0 mL tube. A half volume of ultra TBE Melt and 4.5 volume of ultra salt were added and mixed well. The mixture was incubated at 55°C to melt the agarose gel. Ultra bind was resuspended by vortexing at highest speed with ultra bind tube in a horizontal position until homogeneous. Ultra bind was added to the mixture. Incubation was done for 5 minutes at room temperature. The mixture was centrifuged for 5 seconds. Supernatant was removed and set aside. The pellet was resuspended in 1 mL of ultra wash by vortexing for 5—10 seconds. The mixture was then centrifuged for 5 seconds and then the supernatant was discarded. The mixture was centrifuged again for 5 seconds. To remove all traces of ultra wash, as pirating was done with a narrow pipette tip. The pellet was resuspended in water. After that, incubation was done for up to 5 minutes at room temperature followed by spinning the mixture for 1 minute. The supernatant was then removed immediately and transferred to a new tube. DNA was ready to be used.

cDNA Sequencing and Data Analyzes

Sequencing was done on GH cDNA using automatic ABI Prism machine 3100. Nucleotide and amino acid sequence of *P. hypophthalmus* GH cDNA were compared with the database in the Gene Bank with the BLASTN/P program. Alignment and the percentage of amino acid similarity were analyzed using GENETYX version 7 program.

DISCUSSION

Identification of GH gene of *P. hypophthalmus* was done using tissue samples collected from pituitary, liver, muscle, brain, limph, and testes organs (Figure 1). Based on the results of the

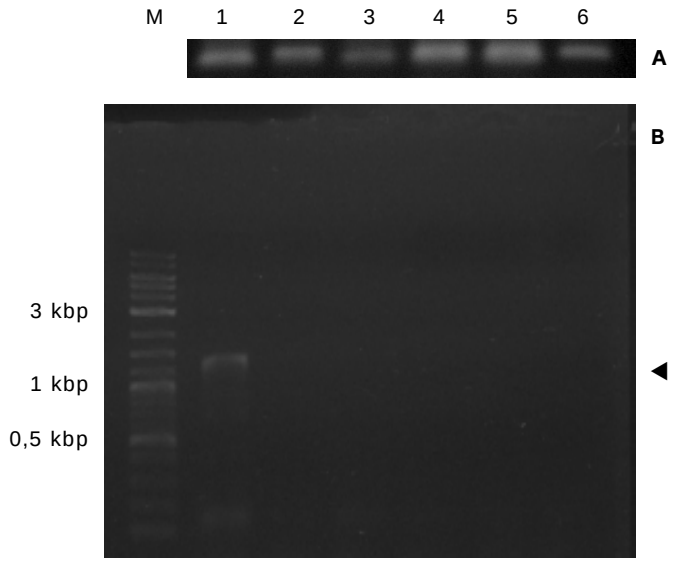

Figure 1. PCR products from some tissues using β-actin gene primer with fragment size of 200 bp (A). Result of PCR analysis from some tissues using FGHP and RGHP primer (B). M is DNA Marker (0.1—10.0 kb, BioLabs Inc., New England). 1= pituitary, 2= liver, 3= muscle, 4= brain, 5= limph, 6= testes. Number in the left image is the size of DNA fragments Marker. Sign of the arrow (◄) on the right shows the DNA target

electrophoresis, GH gene was identified only in pituitary. β-actin gene is used as the internal control. DNA band with a length approximately 1200 bp is a candidate of GH gene fragment. DNA fragments were purified and then used as sample material for sequencing.

The full-length GH gene of *P. hypophthalmus* is 1,151 bp in length, coding for an open reading frame (ORF) of 603 bp. The 5' and 3' untranslated regions of the GH gene are 22 bp and 526 bp long, respectively (Figure 2). Amino acid translation is started with the ATG or sequence known as start codon which is symbolized in the form of methionin (M). Although stop codon is encoded by TAG sequence, it will not be translated into amino acid form, but instead it serves as signal sequence for the end of translation activity.

GH genes between species of fish vary in length. The length of GH gene in *Pangasionodon gigas* is 1,176 bp (accession: L27835; Lemaire *et al*., 1994), *Cyprinus carpio's* length is 1,164 bp (accession: M27000; Koren *et al*., 1989), *Oncorhynchus keta's* length is 1,120 bp (accession: K03050; Sekine *et al*., 1985), *Oncorhynchus mykiss's* length is 1,161 bp (accession: M24683; Rentier-Delrue *et al*., 1989a) and *Oreochromis niloticus's* length is 847 bp (accession: M26816; Rentier-Delrue *et al*., 1989b).

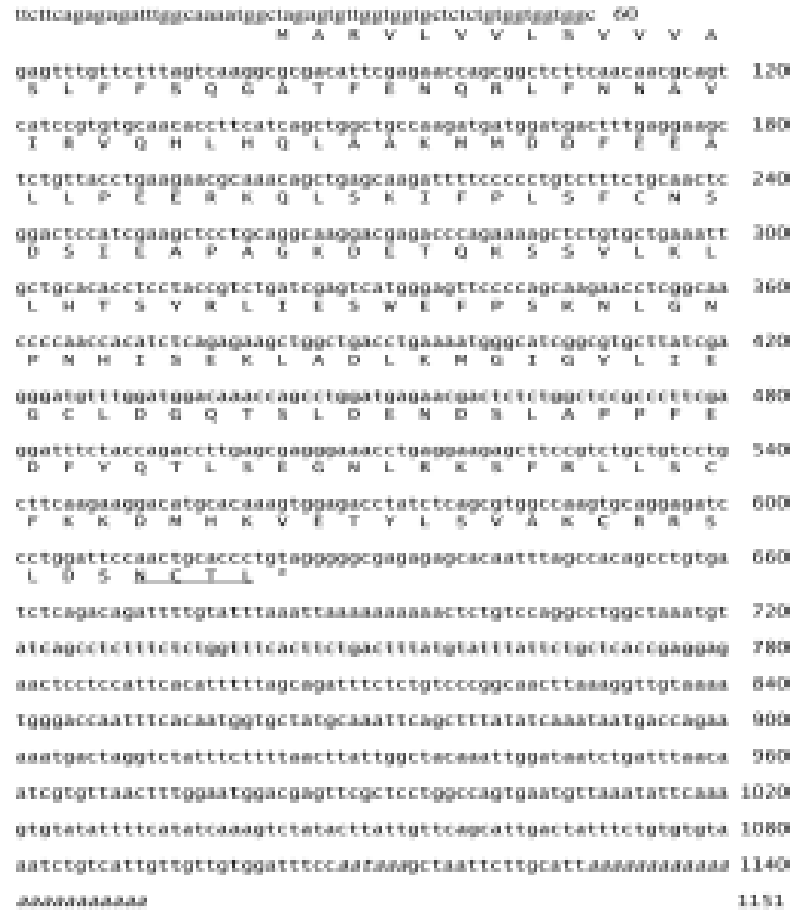

Figure 2. The complete nucleotide sequence of *P. hypophthalmus.* GH cDNA encoding the prepro GH peptide. The cDNA is 1151 long having a signal peptide of 22 residues and the mature peptide of 178 residues. Putative N-glycosilation sites (Asn-Xaa-Thr/Ser) are underlined. The polyadenylation signal (aataaa) and the poly A+ are marked in italic letters

P. hypophthalmus GH gene has some common characteristics that are exclusively owned by GH genes, such as a single tryptophan residue (W) on the 104th amino acid, 5 cysteine residues (C) on the amino acid 71, 135, 173, 190, and 198 and a motif of Asn-Xaa-Thr on C terminus which is the potential location for N-linked glycosilation. Polyadenylation signal (aataaa) was on the 14 bp at the upstream of polyadenylation location.

Growth hormone is a polypeptide of fundamental importance for growth regulation in vertebrates and together with prolactin and somatolactin, constitutes a family of pituitary hormones with similar structure and function which appear to have originated from a common ancestral gene before the evolution of fishes (Kawauchi & Yasuda, 1989 *in* Meier *et al*., 2006). GH has been found in all taxonomic groups of jawed vertebrates (gnathostomes) and, recently, also identified in sea lamprey, an extant representative of a group of the most ancient vertebrates (jawless vertebrates) (Rousseau & Dufour, 2004).

Molecular data from nuclear genes such as the GH gene has been recently used as a source of information in order to evaluate evolutionary relationships of fishes at a variety of taxonomic levels, producing phylogenies with substantial statistical confidence (Meier *et al*., 2006). Analysis of nucleotide sequences is useful in studying relationship between sequences within different orders. The burst of evolution seen in GH evolution could be caused by a number of factors, including (1) altered selection pressure associated with changes in biological function, (2) relaxation of purifying selection due to loss of function, or (3) inadvertent comparison of the products of non-orthologous genes, following gene

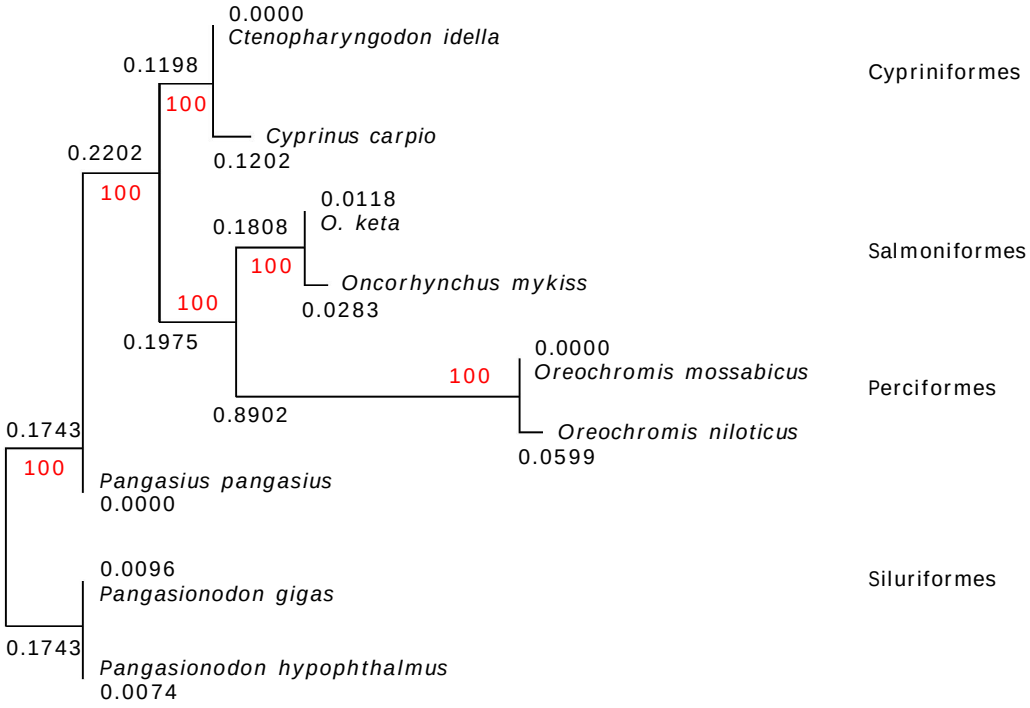

Figure 3. Phylogenetic tree based on nucleotide sequence of GH gene from 4 orders. *Pangasionodon gigas* (accession L27835; Lemaire *et al*., 1994), *Pangasius pangasius* (accession: M63713; Lemaire & Panyim, 1991), *Ctenopharyngodon idella* (accession AY616661; Gong *et al*., 2004), *Cyprinus carpio* (accession M27000; Koren *et al*., 1989), *Oncorhynchus mykiss* (accession M24683; Rentier-Delrue *et al*., 1989a), *Oncorhynchus keta* (accession K03050; Sekine *et al*., 1985), *Oreochromis niloticus* (accession M26916; Rentier-Delrue *et al*., 1989b), *Oreochromis mossambicus* (accession AF033805; Chen *et al*., 1997)

duplication and long periods of divergent evolution (Wallis, 1996).

The consensus phylogenetic tree obtained (Figure 3) showed that *P. hypophthalmus* was in the same order with *P. pangasius* and *P. gigas*. Siluriformes has the highest nucleotide sequence homology with cypriniformes, while the lowest is perciformes.

The blastp analysis among 4 different orders can be seen in Figure 4. Homology of peptide sequences between *P. hypophthalmus, P. gigas,* and *P. pangasius* is 100%. Based on nucleotide sequence, peptide deduction contains 200 amino acids consisting of 178 "mature peptides" and 22 amino acids of peptide signal. Anathy *et al*. (2001) reported that in fish including silurids, there were two locations of suspected N-glycosylation (Asn-Xaa-Thr or Ser), which normally resided in the amino acid-125 and 175 in "mature peptide". Four cysteine residues were almost entirely in the

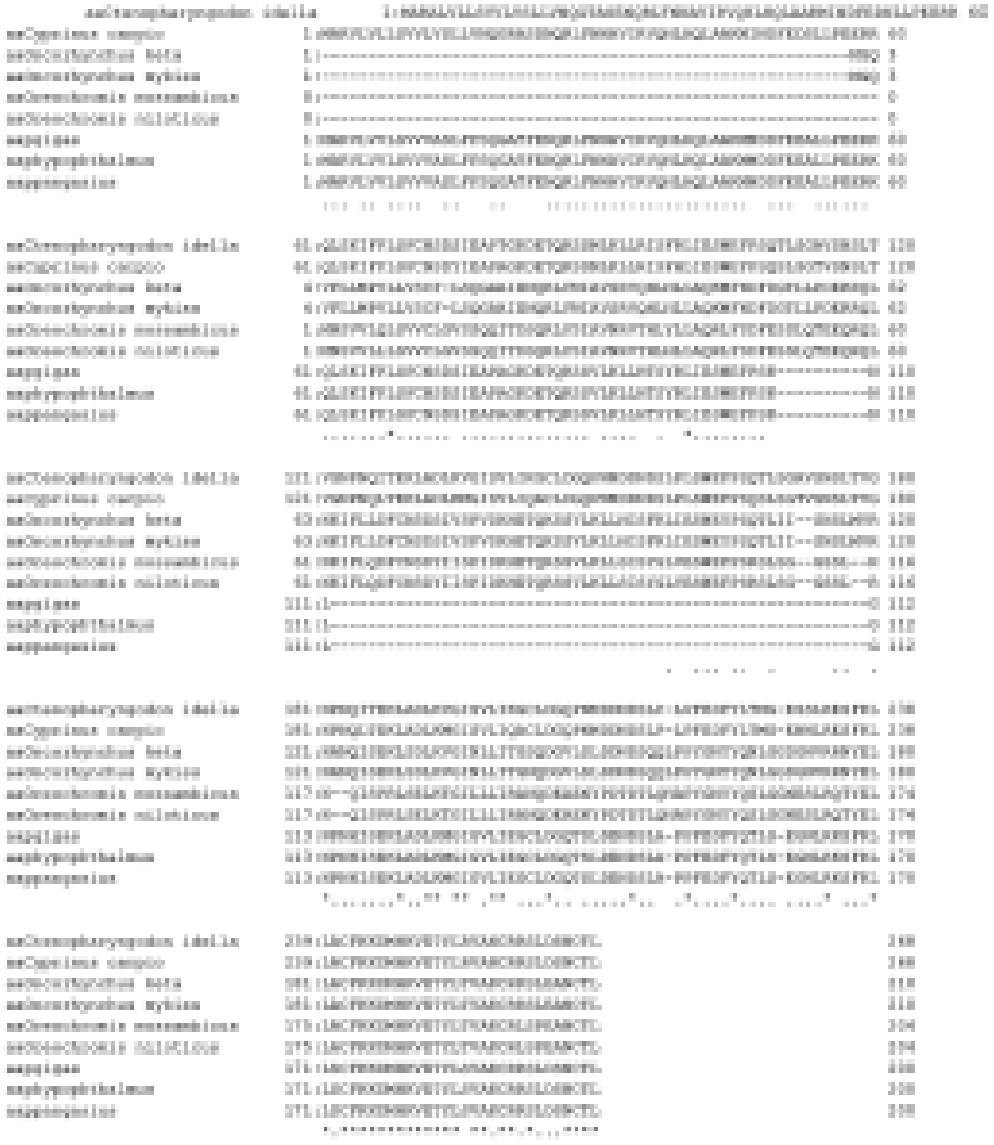

Figure 4. Comparison of GH peptide sequence on 4 different orders

"mature peptide" with a position that was identical to the amino acid-49, 113, 151, and 178.

Ryynanen & Primmer (2006) reported the putative signal peptide region was predicted to include the first 22 amino acids in all teleosts (salmoniformes, siluriformes and cypriniformes) pre-GH proteins, with the exception of those in perciformes, which consisted of the first 17 or 18 residues depending on the species. The C-terminal region is highly conserved among all teleost fishes containing 3 out of 4 cysteine residues known to be important for disulfide bonds formation. The existence of bonds is important for structural integrity and hormone biological activity. The fourth conserved cysteine was in position 71 (72 in perciformes). Putative functionally important sites of teleost GH were also relatively conserved when they were compared to other mammalian GH sequences (Liu *et al*., 2001). Over 30% of the reported sites in mammals were identical to fish amino acids and 54% of the sites in teleost were similar to at least one mammalian species.

P. hypophthalmus growth hormone is arranged by over 200 amino acids which are encoded by 20 types of amino acids (Figure 5). The highest amino acid composition is leusin (14%) while the lowest is tryptophan (0.5%). Syaifudin (2006) reported that the GH gene from *Cromileptes altivelis* cDNA was arranged by 205 amino acids among which the highest composition was leusin (14.63%) and Serin (12.20%) and the lowest was tryptophan (0.49%). These 20 amino acids of this protein were joined by peptide bond (NRC, 1993).

Protein requirement of fish must contain sufficient quantity of 10 essential amino acids (fish can not afford synthetics). These amino acids are important for tissues protein synthesis. GH amino acid composition can be used as a reference in the preparation of feed formulation for *P. hypophthalmus*.

CONCLUSION

The full-length GH gene of *P. hypophthalmus* is 1,151 bp in length, coding for an open reading frame (ORF) of 603 bp. The 5' and 3' untranslated regions of the GH gene are 22 bp and 526 bp long, respectively. *P. hypophthalmus* GH gene has some common characteristics that are exclusively owned by GH genes, such as single tryptophan residue (W) on the 104th amino acid, 5 cysteine residues (C) on the amino acids of 71, 135, 173, 190, and 198 and a motif of Asn-Xaa-Thr on C terminus which is the potential location for N-linked glycosilation. Polyadenylation signal (aataaa) was on the 14 bp at the upstream of polyadenylation location. Growth hormone *P. hypophthalmus* was arranged by over 200 amino acids from GH cDNA deduction. The highest proportion of amino acid composition was leusin (14%) while the lowest was tryptophan (0.5%).

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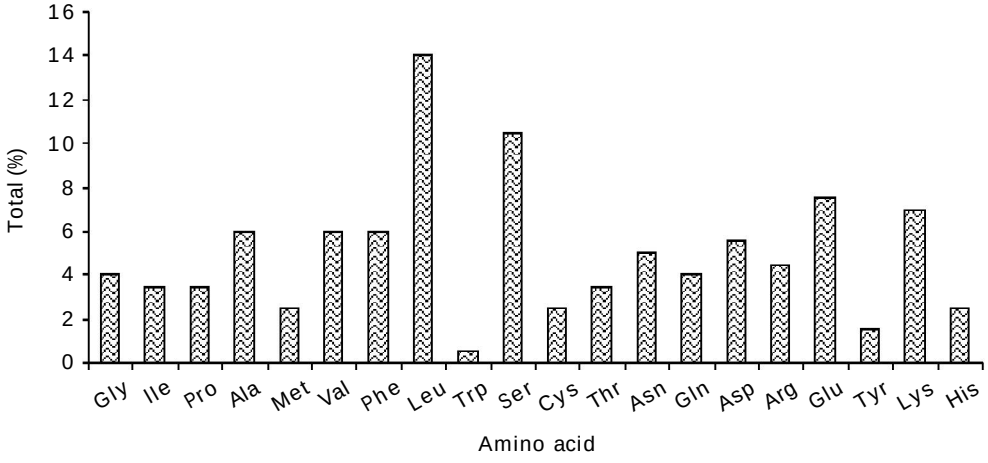

Figure 5. Amino acid composition of *P. hypophthalmus* growth hormone

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