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cDNA ENCODING GROWTH HORMONE FROM HUMPBACK GROUPER (*CROMILEPTES ALTIVELIS*)

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ABSTRACT

Growth hormone (GH) that plays an important role in growth, reproduction, seawater adaptation, and immune function was isolated and sequenced from humpback grouper, *Cromileptes altivelis*. The cDNA was isolated from pituitary using RT-PCR. The 618 bp open reading frame encodes a 205 amino acid (aa) protein, which represents an 18 aa signal peptide followed by a 187 aa mature GH polypeptide. The fragment contained conserved domain of somatotropin–1, somatotropin–2, casein kinase II phosphorylation, protein kinase C phosphorylation, N–myristoylation and N–glycosilation. The similarity of deduced protein of humpback grouper GH was 65.0 - 89.5% with other fishes.

Key words: isolation, cloning, sequencing, growth hormone cDNA, Cromileptes altivelis

INTRODUCTION

Growth hormone (GH) is a 22-kDa protein of pituitary origin that has conserved a pleiotropic action throughout the evolution of vertebrata. There is evidence for the involvement of fish GH in growth, seawater adaptation, reproduction and immune system (Calduch-Giner *et al.* 2000). The secretion of this hormone was regulated by growth hormone releasing hormone (GHRH) and inhibiting hormone (somatostatin) (Anderson *et al.* 2004). Isolation of cDNA encoding growth hormone has been done in Europe such as from rainbow trout (*Oncorhynchus mykiss*) (Yao *et al.* 1991), red sea bream, and salmonid (Voigt and Botta 1990). Growth hormone cDNAs from

the catfishes like *Ictalurus punctatus* (Tang *et al.* 1993), *Pangasius gigas* (Lemaire *et al.* 1994) and *P. pangasius* (Lemaire and Panyim 1993) have been cloned, sequenced and characterized.

Humpback grouper is one of the high economic value seawater fish. In Indonesia, the humpback grouper popularly known as "kerapu bebek" could be found in coral reefs in Banten Bay, Ujung Kulon, Madura, Kalimantan and Nusa Tenggara. It could also be found at Riau, Seribu and Karimunjawa Archipelagoes (Heemstra and Randall 1993). The growth of this fish is slow; therefore to understand the role of GH in regulating the growth of fish, the GH cDNA was isolated, cloned and sequenced.

MATERIALS AND METHODS

RNA Extraction

Pituitary glands were collected from six adult *C. altivelis* of 0.5 kg in weight and quick freezed in liquid nitrogen. Total RNA was extracted following guanidine isothiocyanate (GIT) method (Suharsono et al. 2002). All solutions were prepared from diethylpyrocarbonate (DEPC)-treated autoclaved to avoid the high levels of RNase activity. The visualization of RNA was detected by GelDoc (labquip) transluminator and captured by f1.8 full bright (Olympus) digital camera. RNA pellet was dissolved in DEPC-treated autoclaved, distilled water and stored at –70oC.

cDNA Synthesis

The synthesis of growth hormone cDNA used SuperScript™ Double-Strand cDNA Synthesis Kit from Invitrogen. This kit had an ability of Superscript™ II RNase H- Reverse Transcriptase at the first strand reaction. Reverse Transcriptase-Polymeration Chain Reaction (RT-PCR) of total RNA from *C. altivelis* pituitary used the conserved specific primer that were designed according to 6 GH gene sequences from GenBank database: Epinephelus akaara (accession number: AY326406), *E. awoara* (AF232711), *E. coioides* (AY038606, AY513647, AF376771) and *Sebastes schlegeli* (AY542548). Those conserved sequences were selected by multiple sequence alignment and analyzed by primer 3 software. The primers designed were IKf Forward 5'-cagacctgatcccagacca-3' (19 bp) and IKR Reverse 5'-ctacagggtacagttggcctca-3' (22 bp).

One microgram of total RNA was used as a template for RT–PCR, then added with 2 x 25 µl of reaction buffer, 0.5 µl of each forward and reverse primers (20 pmol), 1 µl of Taq polymerase, 1.2 µl of MgSO4 (2.5 mM) and added DEPC water until 50 µl final volumes. The RT-PCR (PTC-100TM from MJ Research Inc.) protocols was as follows: 45°C for 30 min and 92°C for 2 min-denaturation, 92°C for 15 s, 45°C for 30 s-annealing, 68oC for 90 s-extension for 35 cycles and a final extension at 72°C for 5 min.

Cloning of GH cDNA to pGEM T-Easy vector

GH cDNA were ligated to pGEM T-Easy following procedure of Promega (2003). pGEM T-Easy vector and DNA insert control tube were centrifuged briefly to collect contents at the bottom of tube. Ligation reaction follows as 5 µl 2 x of rapid ligation buffer, 1 µl of vector pGEM T-Easy (50 ng), 1 µl of T4 DNA ligase (3 weiss units/ µl) and 3 µl of RT-PCR template. The reactions were mixed by pipetting and incubated overnight at 4°C.

Transformation to *E. coli* DH5±

E. coli DH5± were made competence following the method of Suharsono (2002). A hundred µl of competent cell were added to 10-µl template of GH cDNA (10-50 ng) and incubated on ice for 20-25 min. The mixture was heat-shocked at 42°C for 20-25 min and placed on ice for 5 min, then moved it at room temperature. The medium 2xYT (Yeast extract and Tripton) 100 µl was added to the mixture and incubated in rotary shaker at 250 rpm for 20 min and 37°C. Plasmid-containing GH cDNA are selected by growth on agar containing ampicillin. The bacteria of 100-150 µl were spread in selective medium containing ampicillin (100 µg/ml).

Transforman Identification

Non-transformed cells cannot grow in the presence of ampicillin. The competent cell that contained DNA recombinant grew with ampicillin medium and produced white colonies. The white colony from transformation was replicated and checked by PCR to ensure the plasmid-containing GH cDNA. The reaction of PCR was as follows: a white colony that had been replicated, mixtured with a tube containing ddH2O 7.15 µl, then done the hot start PCR at 95°C for 10 min and 15°C for 5 min. Then, it was added with buffer 1 µl, forward and reverses primer (20 pmol/µl) 0.5 µl, Taq enzyme (5 U/µl) 0.05 µl and dNTP (25 mM) 0.8 µl. The PCR reactions were run at 94°C, 2 min-initial denaturation for 1 cycle, 94°C for 30 s-denaturation, 45°C for 30 s-annealing, 68°C for 90 s-extension for 30 cycles and a final extension at 72°C for 5 min.

Plasmid isolation and sequencing

pGEM T-Easy plasmid that contained GH cDNA in *E. coli* DH5± was isolated by the method described by Suharsono *et al.* (2002). Plasmid cDNA that contained cDNA GH were sequenced following Sanger *et al.* (1977) in automated sequencer ABI PRISM 310.

Sequence analysis

Nucleotide and deduced amino acid sequences were analyzed by Bioedit package and BLAST searches (http://www.ncbi.nlm.nih.gov/blast). The potential domains were analyzed with prosite database program at the ExPASy server http://www.expasy.

ch/cgi-bin/. Restriction map of GH cDNA was analyzed using NEBcutter at web www.tools.neb.com/NEBcutter2/index.php. The similarity between amino acid sequences was analyzed using ClustalW (www.ebi.ac.uk/clustalw/).

RESULTS AND DISCUSSION

The result of sequencing showed that GH cDNA of *C. altivelis* contained 618 bp encoded 205 amino acids, protein, which represents an 18 amino acid signal peptide followed by 187 bp amino acid mature GH polypeptide. GH cDNA sequence of *C. altivelis* can be accessed in GenBank database using accession number EU003991. The four Cys residues in humpback grouper GH are located at conserved position (70, 178, 195, and 203) (Figure 1). The sequence was compared with GH nucleotide of other fishes in Genbank with 50 alignments. The comparison showed 80.5-96.9% similarity with marine and freshwater fishes. The closest similarity is with Epinephelus coioides (96.9%), then *E. awoara* (95.3%), E. akaara (94.8%), *Lepomis cyanellus* (88.9%) and *Acanthopagrus latus* (88.9%). The farthest similarity is *Siniperca kneri* (80.5%), then *Lateolabrax japonicus* (81.5%), *Mugil planatus* (81.7%), *Oreochromis niloticus* (82.3%) and *Monopterus albus* (84.6%),

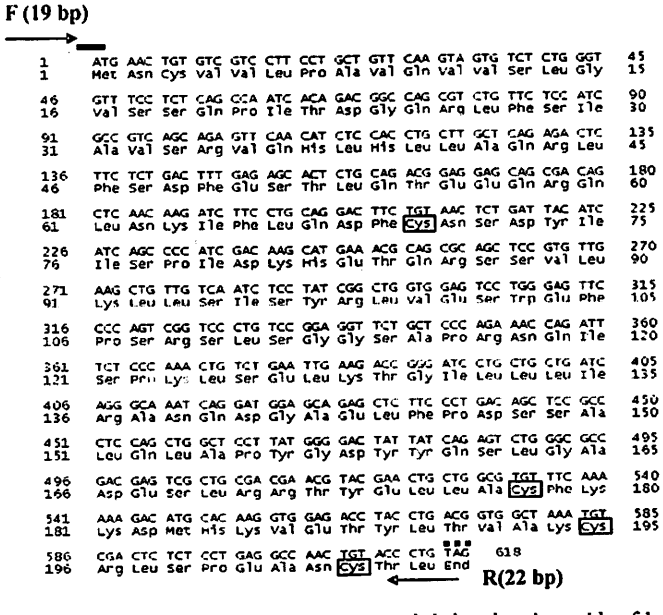
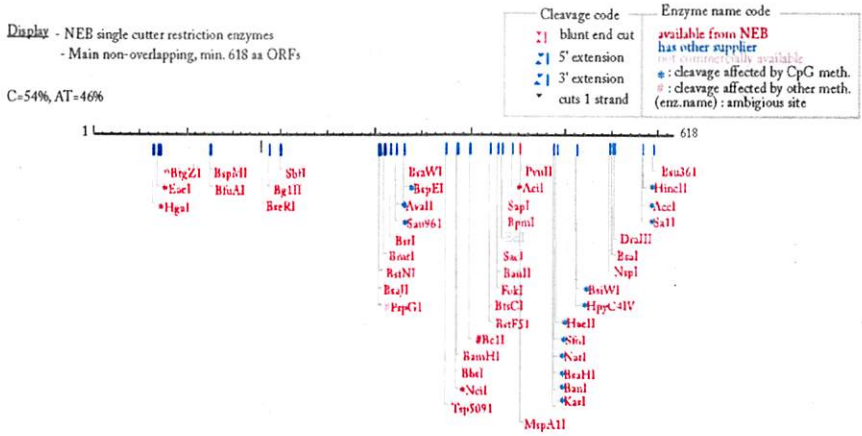


Figure 1. The complete nucleotide sequence and deduced amino acids of humpback grouper (Cromileptes altivelis). Four Cys residues are boxed. Marked (▬▬) start kodon; Marked (▬▬) stop kodon; F (forward primer); R (reverse primer).

Deduced protein of GH cDNA was analyzed based on BLASTP showed high similarity with marine and freshwater fishes (66.0-89.5%). Te highest similarity is with*E. coioides* (89.5%), followed by *E. awoara* (88.6%), *E. akaara* (88.2%), *Siniperca kneri* (87.7%) and *Lepomis cyanellus* (86.8%). Te farthest similarity of GH protein is Limanda yokohamae (65.1%), followed by *Plathicthis bicoloratus* (65.6%), *Hippoglossus hippoglossus* (66.0%), *Fugu rubripes* (70.1%) and *Sciaenops ocellatus* (71.4%).

Proteins or more correctly some of the amino acids they contain are an essential component of the diet for all animals (Houlihan, ***et al.***, 2001). Essential amino acids are those that animals were not able to synthesize, or synthesized insufficient quantity to enable the maintenance of good growth rates, whereas non-essential, or dispensable, amino acids can be synthesized de novo from other compounds.

Deduced protein of GH cDNA encoded 205 amino acids with molecular weight is 23.042 kDa. Te biggest amino acid composition of GH is leusine (14.63%), serine (12.20%) and glutamine (6.83%). Te smallest composition is triptophan (0.49%). Based on amino acids composition, ***C. altivelis*** needed 10 essensial amino acids: leucine (14.63%), valin (5.85%), arginine (5.85%), isoleusine (4.88%), lysine (4.39%), threonine (4.39%), phenylalanine (3.90%), histidine (1.95%), methionine (0.98%) and tryptophan (0.49%). Non-essential amino acids compositions of GH of ***C. altivelis*** are serine (12.20%), glutamine (6.83%), alanine (5.85%), glutamic acid (5.85%), aspartic acid (4.88%), proline (4.39%), glysine (3.90%), tyrosine (3.4 1%), asparagines (2.93%) and cysteine (2.44%). Te restriction site of GH nucleotide of ***C. altivelis*** showed many sites*i.e* : *Bam*HI, *Ban*II, *Sal*I, *Bsa*I, *Nsp*I and *Ban*I (Figure 2).



Restriction site of GH cDNA of humpback grouper ***C. altivelis***
Figure 2.

Deduced protein of GH cDNA contained potential domains: Somatotropin-1, Somatotropin-2, Casein kinase II phosporylation, Protein kinase C phosporylation, N-myristoylation and N-glycosilation (Figure 3). Potential domains analysis of deduced protein GH showed that *C. altivelis* had similar conserved domain with epinepheline (*E. coioides, E. akaara* and *E. awoara*), but different with *P. gigas, Salmo salar, Anguilla anguilla, P. pangasius* and *Cyrpinus carpio.* N-glycosilation is the most conserved domain with 100% similarity. N-glycosilation contained 4 amino acids: asparagines, cysteine, threonine and leucine.

Casein kinase II (CK-2) is a protein serine/threonine kinase whose activity is independent of cyclic nucleotides and calcium. CK-2 phosphorylates many different proteins (Pinna 1990). CK-2 is a multy function of protein kinase because of its role in function and cellular process, included mitosis and cellular transformation (Promega 2001). Casein kinase II of GH from *C. altivelis* contained 4 amino acids: serine, aspartic acid, phenylalanine and glutamic acid.

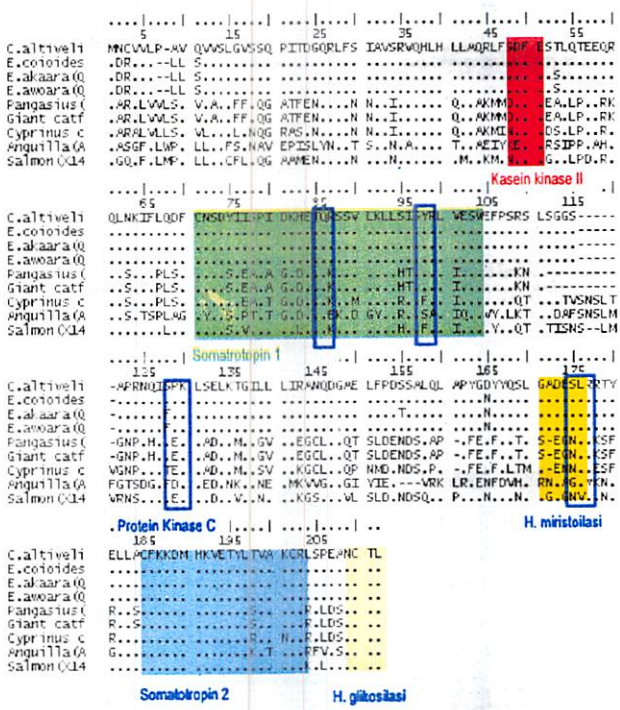


Figure 3. Conserved domain of deduced protein of GH cDNA from humpback grouper (*C. altivelis*) based on prosite database analysis. *Pangasius pangasius* (Genbank accession number: M63713), *Pangasionodon gigas* (L27835), *Cyprinus carpio* (X13670), *Anguilla anguilla* (AY148493)), *Salmo salar* (X14305).

The hormone somatotropin (growth hormone, GH) plays an important role in growth control. Somatotropin-1 is a variety domain (50%-100%). In this domain, *C. altivelis* contained 8 essential amino acid (Ile, Lys, Thr, Val, Leu, Trp, Arg, His). Somatotropin-2 had more conservative domain than somatotropin-1. The similarity was 89.47-100%. The most varied amino acid was at 198th. At this number, threonine in *C. altivelis* was replaced by serine in *P. pangasius* and *Pangasionodon gigas*, lysine in *A. anguilla* and arginine in *C. carpio*. Another difference of amino acid was at 200th and 201st. GH of *C. altivelis* did not contain arginine and serine that may cause the difference in growth characters. Somatotropin-2 of *C. altivelis* contained 7 essential amino acid (Lys, Met, Val, Thr, Leu, Trp, Arg).

N-terminal N-myristoylation is a lipid anchor modification of eukaryotic and viral proteins targeting them to membrane locations, thus changing the cellular function of modified proteins. Protein myristoylation is critical in many pathways; e.g. in signal transduction, apoptosis, or alternative extracellular protein export (Maurer-Stroh *et al.* 2002). There was less conservative amino acid (16.66-33.33%) in this domain, except in *grouper* (epinepheline). The more variation in this domain was supposed to be connected with the function as protein modification to membrane function. Modification in this domain was supposed to increase the growth in *C. altivelis*. Serine and aspartic acid were the possible amino acid to be modified with asparagines and glutamate, respectively.

CONCLUSIONS

The cDNA GH of *C. altivelis* contained 618 bp that encoded 205 amino acids with the conserved domain are: Somatotropin-1, Somatotropin-2, Casein kinase II phosporylation, Protein kinase C phosporylation, N-myristoylation and N-glycosilation. The similarity of deduced protein GH was 65.0-89.5% with other fishes.

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