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Growth hormone from striped catfish (*Pangasianodon hypophthalmus*): Genomic organization, recombinant expression and biological activity

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ABSTRACT

Growth hormone is an essential polypeptide required for normal growth and development of vertebrates. In this report, striped catfish (*Pangasianodon hypophthalmus*) growth hormone gene and cDNA were isolated by reverse transcriptase-polymerase chain reaction. The striped catfish growth hormone (scGH) encoding gene contains 5 exons and 4 introns. The cDNA sequence of the scGH gene contains a 603 bp open reading frame and encodes for a 200-aa protein consisting of a putative 22-aa signal peptide and the mature 178-aa protein. The recombinant histidine-tagged scGH protein which expressed in *Escherichia coli* as inclusion bodies was unfolded, refolded and purified to near-homogeneity by Ni^{2+}–NTA chromatography. Analysis of the secondary structure content by CD spectroscopy showed that the α-helical content of the refolded scGH is 55%. Elucidation of the folding pathway of scGH by fluorescence spectroscopy showed that denaturation transition of scGH is coincident and cooperative, consistent with the two-state denaturation mechanism. The purified scGH was biologically active and exhibited growth-promoting activity in striped catfish, but not tilapia.
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1. Introduction

Growth hormone (GH) produced by somatotrophs in the anterior portion of the pituitary gland is a single polypeptide of approximately 22 kDa. In addition to being responsible for somatic growth and development of vertebrates, GHs have been shown to be involved in reproduction, osmoregulation, fuel utilization and immune response in fish (Canosa et al., 2007). GHs have been isolated from a wide range of vertebrates including mammals, birds, reptiles, amphibia and fish. Although there is a considerable difference in the amino acid sequences among species, GHs from all species contain approximately 50–60% α-helices and are believed to share a similar three-dimensional structure (Clackson et al., 1998). X-ray diffraction studies of porcine and human GHs show that their structure is a four helix bundle protein in which the first two helices are parallel to each other and antiparallel to the last two helices (Abdel-Meguid et al., 1987; Schiffer et al., 2002). Each molecule of GH had been shown to contain two distinct sites (site 1 and site 2) that bind two identical receptor molecules. The mechanism of signaling involves sequential binding of site 1 to a first receptor followed by binding of site 2 to a second receptor (Walsh et al., 2004). This sequential dimerization activates receptors by bringing the intracellular domains of the receptors into contact, thereby activating cytosolic components. Interest in understanding the structure–function relationships of GH and its receptor interactions has increased recently because of commercial applications of these hormones in the treatment of growth disorders in humans (Wells, 1996). The first salmon GH produced in *Escherichia coli* was in 1985, since then GH from numerous fish have been produced (Sekine et al., 1985).

Giant catfish (*Pangasianodon gigas*) and striped catfish (*P. hypophthalmus*) are two fresh water fish indigenous to the Mekong River in Southeast Asia. Giant catfish is one of the world's largest fresh water fish, which can weigh up to 300 kg and grow more than 3 m in length (Lemaire et al., 1994) and had been listed by IUCN as critically endangered species. Striped catfish is considered as a promising species for aquaculture. With a total cultured production of over 1 million tons, it generated revenues over US $1 billion for the aquaculture industry in 2008 (FAO Fishery Statistic). Although closely related to the giant catfish, striped catfish grow at a much slower rate. Recently, administration of recombinant GH has been considered by the aquaculture industry as a solution to enhance the growth of striped catfish. GH administration has been shown to enhance the growth rate in a number of animals, especially fish (Funkenstein, 2000). The purpose of this research is to clone and express scGH and utilize it for simulation of the growth of stripped catfish. Although cDNA sequence of giant catfish GH had been determined, the degree of sequence similarity between the giant catfish's and striped catfish's GH cDNA remains unknown. This is because of the fact that classification of fish is based primarily on morphology. In addition to cDNAs which are used primarily for cloning and expression of recombinant proteins, characterization of the genomic

Abbreviations: GH, growth hormone; scGH, striped catfish growth hormone; RT-PCR, reverse transcriptase-polymerase chain reaction; UTR, untranslated regions; aa, amino acid; CD, circular dichroism; SDS, sodium dodecyl sulfate.

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organization of GH gene has recently captivated interest from researchers. For instance, the intron patterns of GH genes had been shown to be a natural marker used in analysis of the evolutionary relationships of various teleost groups (Venkatesh and Brenner, 1997). Furthermore, although the GH cDNA from numerous fish had been determined, only a selected few of the genomic sequence of the GH gene, including that of giant catfish, are known.

Therefore, this article describes (i) determination of the genomic and cDNA sequences of the scGH gene, (ii) recombinant expression and purification of scGH from inclusion bodies, (iii) analysis of the secondary structure content of refolded scGH by CD spectroscopy, (iv) equilibrium denaturation studies on the folding pathway of recombinant scGH by fluorescence spectroscopy and (v) determination of biological activity of the recombinant scGH on juvenile striped catfish and tilapia.

2. Materials and methods

2.1. Isolation of genomic DNA from striped catfish

One gram of striped catfish liver (*P. hypophthalmus*) from one fish was cut into small pieces and suspended in two volumes of 100 mM Tris–HCl (pH 9.0), 100 mM NaCl, 250 mM EDTA, 200 mM sucrose and 0.5% SDS. The mixture was homogenized and incubated at 65 °C for 30 min. Genomic DNA was prepared by standard protocol (Sambrook et al., 1989). The concentration of genomic DNA was quantified by measuring the absorbance at 260 nm and the purity was determined from the ratio of absorbance at 260/280 nm.

2.2. Amplification of the genomic sequence of scGH gene

Degenerate primers, GH-F1 (5′-GATCTGASAAGTTTCTTCWG-3′) and GH-R1 (5′-ARATCR CAGGCTGWGGCTAA-3′ where R is A/G, S is G/C and W is A/T), were used for amplification of the scGH gene. These primers were designed from consensus sequences, one at the 5′ and the other at the 3′ UTR, of fish GH cDNAs identified after multiple sequence alignments. Database homology search was performed using BlastN and BlastX programs. Multiple alignment was performed using ClustalW program. The cDNAs of the growth hormone gene used in multiple alignments were *Pangasianodon gigas* (GeneBank accession number L27835), *Silurus meridionalis meridionalis* (AF530481) *and Heteropneustes fossilis* (AF147792); these fish are closely related to striped catfish and are in the same Ostariophysi superorder. Amplification of the scGH gene was performed in a 20 μl reaction mixture containing 100 ng of genomic DNA, 200 nM primers, 200 μM dNTPs, 0.2 U Taq DNA polymerase (Takara) and 1× PCR buffer. PCR was performed as follows: initial denaturation at 94 °C for 2 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min and an additional extension at 72 °C for 7 min. PCR product was gel purified with a Qiaquick gel extraction kit, cloned into pGEM-T easy vector (Promega) and sequenced using DNA sequencing services at Macrogen (Korea).

2.3. Isolation of striped catfish total RNA and amplification of scGH cDNA using RT-PCR

Total RNA was extracted from pituitary glands of juvenile striped catfish (5–10 g body weight) with Trizol reagent (GibcoBRL) according to the manufacturer's instructions. The isolated total RNA containing scGH mRNA was used as template for synthesis of the first-strand cDNA with the Improm II reverse transcription system (Promega) as follow. Reverse transcription was carried out in a 20 μl reaction mixture containing 5 μg of total RNA, oligo-dT_{15}, 200 μM dNTPs, 20 U RNAsin, 200 U reverse transcriptase, 3.75 mM $MgCl_2$ and 1× buffer for 1 h at 42 °C. The first-strand cDNA obtained was then used as a template for PCR amplification of the full-length scGH cDNA. PCR was carried out in a 20-μl reaction mixture containing first-strand cDNA (1 μl aliquot), 200 nM GH-F1 and GH-R1 primers, 200 μM dNTPs, 0.2 U *Taq* polymerase (Takara) and 1× PCR buffer. PCR conditions were as described above. The amplified PCR product was purified from agarose gels, ligated into pGEM-T vector and transformed into *E. coli* DH5α. The recombinant pGEM-scGH was purified and sequenced, using T_7 universal primer.

2.4. Construction of scGH expression vector

Once the complete nucleotide sequence of scGH cDNA had been determined, cDNA coding for mature GH protein (excluding signal peptide) was amplified using scGH cDNA as template and another two specific primers, GH-F2 (5′-CATATGTCGAGAACCAGCGGCTCTT CAAC-3′ where underlining indicates the added *Nde*I linker) and GH-R2 (5′-GAGCTCCTACAGGGTGCAGTTGGAATTCCAG-3′ where underlining indicates the added *Xho*I linker). The nucleotide sequence of GH-F2 primer corresponds to position FENQRLF of mature scGH followed by an added *Nde*I linker whereas that of GH-R2 corresponds to position SNCTL and the stop codon followed by an added *Xho*I linker. PCR was performed as described above except that the annealing temperature was 60 °C instead of 56 °C. The amplified product was gel-purified and ligated into pGEM-T vector (Promega). After transformation into competent *E. coli* DH5α, the recombinant plasmid, designated pGEM-scGH, was purified from cell culture by the alkali lysis methods and sequenced to confirm that no mutations were introduced by the PCR amplification step. For protein expression, the 543 bp insert corresponding to the mature scGH cDNA was removed from pGEM-scGH by double digestion with *Nde*I and *Xho*I, gel purified and ligated into pET-28b (Novagen). The ligation mixture was transformed into *E. coli* DH5α and selected on LB containing 50 μg/ml kanamycin. The resultant plasmid, pET-scGH, was purified from cell culture by the alkali lysis method and transformed into *E. coli* BL21.

2.5. Over-expression, refolding and purification of recombinant scGH

For purification of recombinant scGH, a 20 ml culture of *E. coli* BL21 (DE3) carrying pET-scGH grown overnight in LB plus 50 μg/ml kanamycin was inoculated into 1 l of the same medium and shaken at 37 °C. When $OD_{600\ nm}$ reached 0.5, lactose was added at a final concentration of 1 mM and the cells were allowed to grow for an additional 5 h before harvest by centrifugation at 12,000 *g* for 20 min. The cell pellet (4.4 g of wet weight) was resuspended in 20 ml of lysis buffer [50 mM Tris–HCl (pH 8.0), 0.5% Triton X-100, 1 mM EDTA and 0.5 mM PMSF], disrupted by sonication (5 s pulses and a 10 s pause for a total period of 10 min), and centrifuged at 12,000 *g* for 20 min. The pellet containing scGH in the form of inclusion bodies was washed twice with 20 ml of wash buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 10% glycerol and 1% Triton X-100] and centrifuged at 12,000 *g* for 20 min. The washed inclusion bodies were solubilized with 10 ml of solubilization buffer [50 mM potassium phosphate (pH 11), 2 M urea and 1% Triton X-100] and then stirred gently for 2 h on ice. The solubilized scGH was separated from the remained insoluble protein by centrifugation at 10,000 *g* for 30 min. The supernatant containing solubilized scGH (~10 ml) was diluted by addition of an equal volume of 50 mM potassium phosphate (pH 8.0) and dialyzed against 50 mM potassium phosphate (pH 10), 0.5 M urea, and 50 mM glucose to gradually remove urea from the proteins. After 1 h of dialysis, one volume of 50 mM potassium phosphate buffer (pH 7.5) was added to the dialysis buffer. This procedure was repeated five times. The dialyzed scGH solution was centrifuged for 30 min at 12,000 *g* to remove aggregates and then loaded onto a Ni^{2+}–NTA affinity column (Qiagen) which had been pre-equilibrated with the same buffer. After washing with 40 ml of buffer A [50 mM potassium phosphate buffer (pH 8.0) and 300 mM NaCl] containing 20 mM imidazole, protein bound to the column was eluted with 4 ml of buffer A

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