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Molecular characterization of Chinese sturgeon gonadotropins and cellular distribution in pituitaries of mature and immature individuals

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ABSTRACT

Chinese sturgeon (*Acipenser sinensis*) is a rare and endangered species, and also an important resource for the sturgeon aquaculture industry. To understand molecular characterization of Chinese sturgeon gonadotropins (GTHs), we cloned the full-length cDNAs of gonadotropin subunits common α (GTH-α), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from a pituitary cDNA library of mature female. Two subtypes of GTH-α were identified. The nucleotide sequences of *A. sinensis* common α I (*As*GTH-α I), common α II (*As*GTH-α II), FSHβ (*As*FSHβ) and LHβ (*As*LHβ) subunit cDNAs are 345, 363, 387 and 414 bp in length, and encode mature peptides of 115, 121, 129 and 138 aa, respectively. Then, three polyclonal antibodies were prepared from the *in vitro* expressed *As*GTH-α I, *As*FSHβ and *As*LHβ mature proteins, respectively. Significant expression differences were revealed between immature and mature sturgeon pituitaries. Western blot detection and immunofluoresence localization revealed the existence of three-gonadotropin subunits (*As*GTH-α, *As*FSHβ and *As*LHβ) in mature sturgeon pituitaries, but only *As*FSHβ was detected in immature individual pituitaries during early stages in the sturgeon life, and obvious difference was observed between males and females. In males, *As*FSHβ was expressed in 4-year-old individuals, whereas in females, *As*FSHβ was just expressed in 5-year-old individuals.

1. Introduction

Chinese sturgeon (*Acipenser sinensis*) is a rare and endangered species. It mainly lives in continental shelf of the Yellow Sea and the East China Sea. It spawns in the upper Yangtze River and is one of the largest fish to enter fresh water. Its reproduction migratory route was blocked in 1981 by the Gezhouba Dam, which caused a drastic decline of the natural population. Nowadays, it was listed as a Grade I protected animal in China and Appendix II species in CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora).

Chinese sturgeon is also an important resource for the sturgeon aquaculture industry, because its growth is the fastest among all species of sturgeons. In comparison with other sturgeons, however, Chinese sturgeon is an extremely late sexual maturation species in Acipenseriformes. For Chinese sturgeon, mature males and females need at least 9 and 14 years, respectively. However, for Russian sturgeon (*A. gueldenstaedtii*), most males mature at the age of 4 years, and females reach first sexual maturity from 6 to 12 years (Hurvitz et al., 2005). In sterlet (*A. ruthenus*), males mature at the age from 3 to 6 years, and most females get sexual maturation before 8 years (Holcik, 1989). In stellate sturgeon (*A. stellatus*), most males and females reach their first sexual maturity from 4 to 8 years and 6 to 11 years, respectively (Holcik, 1989).

To save this species from extinction and develop its aquaculture industry for future, artificial propagation has been attempted since 1983, but mature males and females have not been obtained from the propagated offspring. One of the major reasons is lack of the data regarding the first early years in the life of the sturgeon. Therefore, the understanding of its reproduce regulation should start from the early stages. Previous studies on its reproduction were mainly focused on the gonad ultrastructure and histology at different developmental stages (Yi et al., 1999; Chen et al., 2004), but molecular information is very limited with regards to the reproduction regulation.

Gonadotropins (GTHs), secreted by pituitary, are the key hormones for reproduction control in vertebrates. In other sturgeons, Moberg et al. (1995) showed the existence of two GTHs in white sturgeon (*A. transmontanus*). Doroshov et al. (1997) observed reproductive cycle of cultured white sturgeon. Quérat et al. (2000) cloned two β-subunits of GTHs in Siberian sturgeon (*A. baeri*) and termed them FSH and LH. Hurvitz et al. (2005) reported GTH-α, FSHβ and

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Table 1
Basic information of three wild mature female sturgeons.

Sampling time	Age	Length (cm)	Weight (kg)	Use
Nov. 2005	24	303	192	RNA extracting and cDNA synthesis
Nov. 2006	24	262	223	Immunofluoresence localization
Nov. 2007	17	332	242	Western blot detection

LHβ subunits in Russian sturgeon and revealed their expression pattern in immature individuals.

Above researches had examined the relationship between gonadotropins and reproduction regulation in other sturgeons, but the relative information was almost unknown in the extremely late sexual maturation Chinese sturgeon. The aim of this study was to clone and characterize Chinese sturgeon GTH-α, FSHβ and LHβ, and observe their localization and cellular distribution in pituitaries of mature and immature individuals. This work will provide useful information for artificial propagation and reproduction regulation of Chinese sturgeon.

2. Materials and methods

2.1. Fish sampling

Since Chinese sturgeon was listed as a Grade I protected animal, we got wild samples under permission of the government for research for one fish per year. All of the three wild females were sex mature, and got in November (the reproduction season) 2005, 2006, and 2007, respectively. The sampling was performed after just spawning. The ages were evaluated by the method described previously (Deng et al., 1985). All their information was provided in Table 1. Immature sturgeons from 1 to 5 years were from the aquaculture offspring propagated from the wild sturgeons, and sampled in December 2007 and October 2008 from the hatchery of Yangtze River Fisheries Research Institute.

2.2. RNA extraction and SMART cDNA synthesis

Pituitary was collected from a 24-year-old female (captured in 2005). Total RNAs were extracted using SV total RNA isolation system (Promega, USA). The RNA quantity was measured spectrophotometrically at A260 nm and the ratio of A260:A280 nm by biophotometer (Eppendorf). The RNA quality was assessed by gel-electrophoresis to ensure the integrality. The cDNAs were synthesized from 50 ng of total RNAs according to previous reports (Zhou et al., 2006) using the Switching Mechanism at 5′-end of RNA Transcript (SMART) cDNA Library Construction Kit (Clontech). Briefly, 50 ng of total RNA was reversely transcribed at 42 °C for 1 h at the presence of both 3′ SMART cDNA synthesis (CDS) primer II A (5′-AAGCAGTGGTATCAACGCAGAGTACTVN-3′) (N = A, C, G, or T; V = A, G, or C), and SMART II A oligonucleotide (5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′). Then 2 μl of first-strand reaction product was added into each 100-μl long-distance PCR system containing 0.2 μM PCR primer (5′-AAGCAG TGGTATCAACGCAGAGT-3′). The LD PCR parameters were 95 °C for 15 s and 65 °C for 30 s and 68 °C for 6 min on Perkin-Elmer PCR System 2400 for 20 cycles. Five microliters of the amplified products were separated by electrophoresis on 1% agarose gels.

2.3. Cloning and sequencing

In order to identify new genes and to profile gene expression in pituitary of wild mature female Chinese sturgeon, we constructed its SMART cDNA library. Briefly, the 3′-end of cDNAs was added dATP-tails by incubating with dATP and Taq polymerase at 72 °C for 20 min. Then, the modified cDNAs were ligated to pMD-18T vector (Promega) and the plasmids were used to transform *Escherichia coli* DH5α super competent cells. The plasmid cDNA library was plated to appropriate density to pick individual colonies. DNA sequencing was performed using dRhodamine terminator cycle sequencing Kit and ABI PRISMTM 310 Genetic Analyzer (Perkin Elmer). The randomly sequenced 2025 clones revealed the full-length cDNAs of *As*GTH-α I and *As*GTH-α II subunits but not *As*FSHβ and *As*LHβ subunits.

Table 2
Primers used for RACE PCR of *As*FSHβ and *As*LHβ.

Primers	Subunits	Positions	Primer sequences
P1	*As*FSHβ	368–388	5′-ACTGACTGTGGCACCCTAAGC-3′
P2	*As*FSHβ	447–463	5′-CCAGCAGGGTACTAATT-3′
P3	*As*LHβ	408–425	5′-CCTCGGACTGTACCATTC-3′
P4	*As*LHβ	470–487	5′-GGAGTGTCAGTAGTTCTG-3′

*As*FSHβ and *As*LHβ subunits cDNA were amplified by 3′- and 5′-RACE (rapid amplification of cDNA ends) as described previously (Hurvitz et al., 2005). Briefly, gene-specific primers P1 for the *As*FSHβ subunit and P3 for the *As*LHβ subunit (Table 2) were designed according to FSHβ sequence (GenBank Accession No. AJ251658) and LHβ sequence (GenBank Accession No. AJ251656) of *A. baeri*, and were used for 3′-RACE along with the primer provided in the kit. PCR was carried out in a volume of 50 μl containing 2.5 U of Taq polymerase (Promega, Madison, WI), 5× buffer (Promega), 1.5 mM $MgCl_2$, dNTPs (0.2 mM final concentration of each nucleotide), 100 pmol of each primer, and 4 μl of cDNA. PCR conditions were as follows: 94 °C for 3 min, 94 °C for 1 min and 52 °C for 1 min, and 72 °C for 1 min for 40 cycles, followed by 72 °C for 7 min for *As*FSHβ, or 94 °C for 3 min, 94 °C for 1 min and 53 °C for 1 min, and 72 °C for 1 min for 40 cycles, followed by 72 °C for 7 min for *As*LHβ. For the 5′-RACE, the gene-specific reverse primers were designed according to the sequences of the cDNA cloned in the 3′-RACE (P2 for *As*FSHβ and P4 for *As*LHβ, respectively, Table 2). The PCR cycling parameters were 3 min denaturation at 94 °C, followed by 40 cycles of 1 min denaturation at 94 °C, 1 min annealing at 53 °C and 1 min extension at 72 °C for both *As*FSHβ and *As*LHβ. All PCRs were terminated with an additional extension at 72 °C for 7 min. The amplified DNAs were visualized by ethidium bromide staining on the electrophoresed agarose gel, cloned into pMD18-T vector (Takara), and sequenced.

2.4. Database and sequence analysis

Nucleotide sequence identity analysis was performed using the BLAST program (GenBank, NCBI). The signal peptide and putative cleavage sites were predicted using software at ExPASy Molecular Biology Server (http://www.cbs.dtu.dk/services/SignalP/). Amino acid sequence alignment and similarity analysis of *As*GTH-α I, *As*GTH-α II, *As*FSHβ and *As*LHβ subunits with other fish gonadotropins (Table 3) were carried out by Clustal multiple sequence alignment programs. Potential N-glycosylation sites were predicted by searching the motif Asn-Xaa-Ser/Thr.

2.5. Expression of fusion proteins and preparation of polyclonal antibodies

The *As*GTH-α I cDNA coding mature protein was amplified using the primers in Table 4 and digested with EcoRI and XhoI. The digested fragment was inserted in frame to the EcoRI and XhoI double-digested expression vector pET32a (+) vector (Novagen). The coding regions of *As*FSHβ and *As*LHβ cDNAs were amplified using the primers in Table 4, and digested with EcoRI and HindIII. The digested fragment was subcloned into the pET32a (+) vector. After the recombinant constructs were confirmed by DNA sequencing, they were, respectively, transformed into *E. coli* BL21 (DE3). Protein expression was induced with IPTG (final concentration 1 mM), and

Table 3
Amino acid identities between Chinese sturgeon GTH subunits and other fish GTH subunits.

Class/order	Species	*As*GTHα I (%)	*As*GTHα II (%)	*As*FSHβ (%)	*As*LHβ (%)	Accession numbers in GenBank
Chondrichthyes/Carcharhiniformes	*Scyliorhinus canicula*	60	63	50	58	GTHα:AJ310343, FSH:AJ310344, LH:AJ310345
Chondrostei/Acipenseriformes	*Acipenser baerii*	98	84	99	98	GTHα:AJ310342, FSH:AJ251658, LH:CAB93502
Chondrostei/Acipenseriformes	*Acipenser gueldenstaedtii*	91	92	99	100	GTHα:AY519658, FSH:AY519657, LH:AY333426
Teleostei/Anguilliformes	*Anguilla anguilla*	71	66	54	70	GTHα:X61038, FSH:AAN73407, LH:CAA43374
Cypriniformes	*Danio rerio*	66	67	45	65	GTHα:AAR84285, FSH:AAR84282, LH:AAR84284
Siluriformes	*Ictalurus punctatus*	64	65	52	63	GTHα:AAD18004, FSH:AAG32155, LH:Q9DG80
Salmoniformes	*Oncorhynchus kisutch*	65	64	42	65	GTHα:AAO72301, FSH:AAO72299, LH:AAO72300
Cyprinodontiformes	*Fundulus heteroclitus*	53	47	35	50	GTHα:P47744, FSH:P30971, LH:P30972
Perciformes	*Epinephelus coioides*	55	51	32	56	GTHα:P47745, FSH:AAT79786, LH:AY129311
Pleuronectiformes	*Hippoglossus hippoglossus*	56	50	37	51	GTHα:P47746, FSH:CAD10501, LH:CAD10502