Comparative Biochemistry and Physiology, Part B 183 (2015) 49–57

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Molecular characterization of the Chinese alligator follicle-stimulating hormone β subunit (*FSHβ*) and its expression during the female reproductive cycle

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ARTICLE INFO

Article history:
Received 29 July 2014
Received in revised form 17 December 2014
Accepted 16 January 2015
Available online 24 January 2015

Keywords:
Chinese alligator
FSHβ
cDNA cloning
Homology analysis
Gene expression

ABSTRACT

The Chinese alligator *Alligator sinensis* is an endangered species endemic to China, it has a highly specialized reproductive pattern with low fecundity. Up to date, little is known about the regulation of its female reproductive cycle. Follicle-stimulating hormone (FSH), a glycoprotein hormone, plays a key role in stimulating and regulating ovarian follicular development and egg production. In this study, the complete *FSHβ* cDNA from the ovary of the Chinese alligator was obtained for the first time, it consists of 843-bp nucleotides, including 120-bp nucleotides of the 5′-untranslated region (UTR), 396-bp of the open reading frame, and 3′-UTR of 327-bp nucleotides. It encodes a 131-amino acid precursor molecule of *FSHβ* with a signal peptide of 18 amino acids followed by a mature protein of 113 amino acids. Its deduced amino acid sequence shares high identities with the American alligator (100%) and birds (89–92%). Phylogenetic tree analysis of the *FSHβ* amino acid sequence indicated that alligators cluster into the bird branch. Tissue distribution analyses indicated that *FSHβ* mRNA is expressed in ovary, intestine and liver with the highest level in the ovary, while not in stomach, pancreas, heart, thymus and thyroid. Expression of *FSHβ* in ovary increases in May (breeding prophase) and peaks in July (breeding period), it is maintained at high levels through September, then decreases significantly in November (post-reproductive period) and remains relatively low from January to March (hibernating period). These temporal changes of *FSHβ* expression implicated that it might play an important role in promoting ovarian development during the female reproductive cycle.

1. Introduction

The Chinese alligator *Alligator sinensis*, belonging to the Crocodilia Alligatoridae family, is an endangered species and indigenous to China, and is listed by the Chinese government as a first-level state-protected species in 1972 (Yan et al., 2005). Nature reserves and artificial farms of the Chinese alligator were set up in the Anhui and Zhejiang Provinces. Though the issues with artificial incubation and breeding of the Chinese alligators have been successfully resolved, the productivity of the Chinese alligator is considerably affected by inherent problems such as lower and highly annual fluctuating egg-laying rate (Cheng et al., 2003). The Chinese alligators start mating in June every year, laying eggs in July, then hatching until September, March to May is the breeding prophase, June to September is the breeding period, September to November is the post-reproductive period (Cheng et al., 2003). Up to this date, little is known about the regulation of its female reproductive cycle.

Reproduction in vertebrates is mainly under endocrine control of the hypothalamus–pituitary–gonad axis leading to circulating sexual steroids affecting several peripheral target organs. The follicle-stimulating hormone (FSH), a glycoprotein hormone, is the key reproductive hormones involved in gonadal development. FSH belongs to the glycoprotein hormone family along with thyroid-stimulating hormone (TSH), luteinizing hormone (LH) and chorionic gonadotropin (CG) (Liao et al., 2003). They are composed of two different subunits designated as α and β. The α subunit is common among these hormones within a species and is structurally conserved among different species, whereas the β subunit is specific to each hormone and confers biological specificity (Landomiel et al., 2014). *FSHβ* functions together with *LHβ* to promote the growth and development of gonads, to control gametogenesis and regulate gonadal endocrine functions (Chauvigné et al., 2012). In females, *FSHβ* stimulates ovarian follicular development. The follicles receiving insufficient *FSHβ* support are doomed to atretic degeneration during the critical stage of follicle development (Zhao et al., 2010).

The structure, function, and regulation of *FSHβ* molecules have been investigated most extensively in mammals (Noguchi et al., 2006; Scammell et al., 2008) and fish (So et al., 2005; Hellqvist et al., 2006),

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http://dx.doi.org/10.1016/j.cbpb.2015.01.003
1096-4959/

Table 1
Primers used for cloning cDNA of the Chinese alligator *FSHβ*.

Primer names	Primer sequences	Technique
FS1S	5′ TAAGTATCCTCCAGTGTCA 3′	RT-PCR
FS1R	5′ GCTGAGGATAGCAGGAAT 3′	RT-PCR
5′-GSP1	5′ CTCTCACAGTACAGTC 3′	5′RACE
5′-GSP2	5′ GCATTCTGTAGCCACTGGGTAT 3′	5′RACE
5′-GSP3	5′ ACTGTTTCATACACAAGCTCCT 3′	5′RACE
3′-GSP1	5′ CAGTGAAGATCCCTGGATGTGGTGACC 3′	3′RACE
3′-GSP2	5′ TAGGCCCAAGCTACTGCTCCTTCAGT 3′	3′RACE
YFS2S	5′ TAAGTATCCTCCAGTGTCA 3′	qRT-PCR
YFS2R	5′ TCAGTGCTGTCCGTATCA 3′	qRT-PCR
β-act1S	5′ ACCGAAACAAGAACCCAT 3′	qRT-PCR
β-act1R	5′ CCGACACGCTAAGACTGC 3′	qRT-PCR

and relatively less in birds (Shen et al., 2006; Zhao et al., 2010) and amphibians (Urbatzka et al., 2006), wherein conservation of the molecular structure of *FSHβ* was found, while some specific features were also reported in different species. In reptiles, the *FSHβ* cDNA encoding has been reported recently, for Reeves's turtle (Aizawa and Ishii, 2003) and Chinese soft-shell turtle (Chien et al., 2005), no Crocodilian *FSHβ* has yet been characterized.

Gonadotropins are well known to be expressed by the pituitary (Saito et al., 2002). However, new studies have revealed that *FSHβ* also showed extrapituitary expression in other tissues. The expression of zebrafish *FSHβ* could be detected in the ovary, testis, brain, kidney, and liver (So et al., 2005), the extrapituitary expression of *FSHβ* in tissues such as gonads, brain, kidney, and liver has also been reported in mammals and other fishes, including humans (Parhar et al., 2003) and African catfish (Vischer et al., 2003b). These results suggested that *FSHβ* may have various physiological functions in different tissues. To pursue the roles of *FSHβ* in the complex reproductive processes of vertebrate species, changes in ovary *FSHβ* expression were monitored in female animals at precise stages of oogenesis during a complete reproductive cycle, including mammals (Parhar et al., 2003), fish (Meiri et al., 2004; Utoh et al., 2005), birds (Shen and Yu, 2002) and amphibians (Saito et al., 2002), whereas no related work has been reported in the Crocodilian order so far.

In this study, the complete cDNA of the Chinese alligator *FSHβ* cDNA was cloned by RT-PCR and RACE methods. The obtained *FSHβ* cDNA and the deduced amino acid sequence of the *FSHβ* were analyzed with web based computer programs. The changes in temporal and spatial expression of *FSHβ* during the reproductive cycle were analyzed by qRT-PCR. The results could expand our knowledge of *FSHβ* gene phylogenetic evolution, provide an insight into the mechanisms that regulate reproduction of the Chinese alligator and could also help towards producing recombinant Chinese alligator *FSHβ*, which may be used in artificial breeding aimed to increase its captive reproductive efficiency.

2. Materials and methods

2.1. Animals and RNA isolation

Sexually mature female Chinese alligators (two animals per season) were obtained from the Xuanzhou Alligator Culturing Centre of Anhui Province. The alligators were anesthetized with an intraperitoneal injection of pentobarbital, the ovary, stomach, intestine, pancreas, liver, heart, thymus and thyroid tissues were excised and immediately kept in RNA-Be-Locker A (Sangon Biotec, Shanghai) and then stored in a −80 °C refrigerator. The above samples were collected in January, March, May, July, September and November during 2013 to 2014. All procedures were approved by the forestry authorities of China.

For the gene cloning and tissue expression experiments, total RNA was extracted from different tissues using a Total RNA Extractor (Sangon Biotec, Shanghai) as in the following steps: taking 100 mg of different tissues and grinding quickly in liquid nitrogen. The rest of the detailed operations was done according to the manufacturer's instructions, RNase-free DNase I (TaKaRa, Dalian, China) was used to remove the genomic DNA contamination. The concentration of total RNA was determined by measuring absorbance at 260 nm, and purity was determined by dividing absorbance at 260 nm by absorbance at 280 nm

Table 2
Species and references of *FSHβ*s used for sequence comparison in this study.

Animal class/species	Scientific name	GenBank ID	Reference
Mammals			
Human	*Homo sapiens*	NM_000510.2	Benson et al. (2013)
Norway rat	*Rattus norvegicus*	NM_001007597.1	Maurer (1987)
Sheep	*Ovis aries*	X15493.1	Mountford et al. (1989)
Chimpanzee	*Pan troglodytes*	NM_001071814.1	Grigorova et al. (2007)
Brush-tailed possum	*Trichosurus vulpecula*	AF008550.1	Lawrence et al. (1997)
Birds			
Quail	*Coturnix japonica*	AB086952.1	Kikuchi et al. (1998)
Chicken	*Gallus gallus*	AB077362.1	Shen and Yu (2002)
Greylag goose	*Anser anser*	KC777370.1	–
Reptiles			
Chinese alligator	*Alligator sinensis*	This study	This study
American alligator	*Alligator mississippiensis*	NM_001287605.1	–
Reeves' s turtle	*Chinemys reevesii*	AB085201.1	Aizawa and Ishii (2003)
Brown tree snake	*Boiga irregularis*	AB575985.1	–
Chinese softshell turtle	*Pelodiscus sinensis*	DQ234263.1	Chien et al. (2005)
Amphibians			
Japanese firebelly newt	*Cynops pyrrhogaster*	AB067752.1	Saito et al. (2002)
Japanese toad	*Bufo japonicus*	AB085668.1	Komoike and Ishii (2003)
African clawed frog	*Xenopus laevis*	AB175888.1	–
Fishes			
Siberian sturgeon	*Acipenser baerii*	AJ251658.1	Quérat et al. (2000)
Australian lungfish	*Neoceratodus forsteri*	AJ578040.1	Quérat et al. (2003)
Grass carp	*Ctenopharyngodon idella*	EF552359	Zhou et al. (2010)
European eel	*Anguilla anguilla*	AY169722.1	Degani et al. (2003)

"–" Only sequence was identified in the GenBank, there was no published report.