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In-vitro and *in-vivo* biological activity of recombinant yellowtail kingfish (*Seriola lalandi*) follicle stimulating hormone

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ABSTRACT

Biologically active recombinant yellowtail kingfish follicle stimulating hormone (rytkFsh) was produced in yeast *Pichia pastoris* and its biological activity was demonstrated by both *in-vitro* and *in-vivo* bioassays. Incubation of ovarian and testicular fragments with the recombinant hormone stimulated E_2 and 11-KT secretion, respectively. *In-vivo* trial in immature female YTK resulted in a significant increase of plasma E_2 levels and development of oocytes. In males at the early stages of puberty, advancement of spermatogenesis was observed, however plasma 11-KT levels were reduced when administered with rytkFsh.

1. Introduction

The genus Seriola includes highly active pelagic fish belonging to the Carangidae family and are found in the Atlantic, Indian and Pacific Oceans. The culture of Seriola began in Japan over 70 years ago (Nakada, 2008). Yellowtail kingfish (*Seriola lalandi*, YTK) is one of the larger members of the genus and its importance for the aquaculture industry is growing worldwide due to its fast growth, high flesh quality and suitability for farming in both cage and recirculating aquaculture systems (RAS) (Chen et al., 2006; Orellana et al., 2014; Poortenaar et al., 2001). YTK is a gonochoristic species with an asynchronous oocyte development, which provides the capacity for multiple spawning within a reproductive season, between spring and summer (Poortenaar et al., 2001). YTK puberty age can vary between male and female fish, and between geographical locations. According to observations in farm conditions in South Australia, precocious males can start to spermiate from 12 months old onwards, however females take 4–5 years to reach sexual maturity. Currently, a genetic selection program is being undertaken for YTK (Whatmore et al., 2013), and there is interest to shorten its generation time to increase genetic gain. Advancing pubertal development is the first step towards shortening generation time, and as such has been the focus of many studies (Carrillo et al., 2009; Taranger et al., 2010). Strategies to advance the onset of puberty range from the control of environmental parameters, as reported for Atlantic salmon (*Salmo salar*) where the control of salinity and photoperiod modulates pubertal development (Melo et al., 2014), to hormonal therapies such as in female red sea bream (*Pagrus major*), where continuous administration of GnRHa resulted in precocious induction of puberty (Kumakura et al., 2003).

The development of gonads in fish is mainly controlled by two pituitary gonadotropins: follicle stimulating hormone (Fsh) and luteinizing hormone (Lh) (Levavi-Sivan et al., 2010). Evidence suggests that Fsh plays a key role in the onset of puberty and early stages of reproductive development while Lh plays a role at the advanced stages of maturation (Yaron et al., 2003). However, studies in Japanese eel and goldfish reported recombinant Fsh activates the later stages of maturation (Kobayashi et al., 2006; Hayakawa et al., 2008). Gene knockout studies of *fsh* and *lh* in zebrafish have confirmed that the *fsh-fshr* signalling pathway is essential in puberty onset and gonadal growth in male and female fish (Zhang et al., 2015, 2014). Until recently, knowledge gaps still exist in relation to the exact actions of Fsh and Lh (Mazón et al., 2014) in most perciforms. This is partly due to the lack of biologically active pure forms of these hormones (Yu et al., 2010; Zhang et al., 2014). This

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problem has recently been circumvented with the production of recombinant gonadotropins in heterologous expression systems resulting in pure forms of the hormones with biological activity (Levavi-Sivan et al., 2010). To date, piscine recombinant Fsh have been produced successfully for 12 species using different heterologous expression systems (Levavi-Sivan et al., 2010; Yu et al., 2010; Kobayashi et al., 2010; Hayakawa et al., 2009; Chen et al., 2012; Mazón et al., 2014). However, recombinant Fsh has been tested *in-vivo* in six species only. Recombinant goldfish (*Carassius auratus*) Fsh (Hayakawa et al., 2008; Kobayashi et al., 2006) and recombinant Manchurian trout (*Brachymystax lenok*) Fsh (Ko et al., 2007) successfully induced milt production in goldfish. Also, recombinant goldfish Fsh induced ovulation in bitterling (*Rhodeus ocellatus ocellatus*) and promoted the initiation of spermatogenesis in sexually immature male Japanese eel (*Anguilla japonica*) (Hayakawa et al., 2008, 2009). Recombinant Japanese eel Fsh induced spermatogenesis and oocyte maturation in sexually immature Japanese eel (Kobayashi et al., 2010). Recombinant orange-spotted grouper (*Epinephelus coioides*) Fsh administrated to juvenile grouper increased serum sex steroid levels and induced early ovarian development (Chen et al., 2012). In zebrafish (*Danio rerio*) it was found that the native form of the recombinant Fsh had higher activity when compared to its 6His-tagged form (Yu et al., 2010). In prepubertal European sea bass male (*Dicentrarchus labrax*), recombinant Fsh (rFsh) was able to trigger the process of spermatogenesis (Mazón et al., 2014). In the same species, rFsh was tested for its *in-vivo* stability, allowing the comparison of the recombinant hormones produced in two different expression systems (Molés et al., 2011).

In the present study, we have generated a single chain recombinant yellowtail kingfish Fsh (rytkFsh) using the yeast (*Pichia pastoris*) expression system (Hollenberg and Gellissen, 1997). The recombinant hormone was tested both *in-vitro* and *in-vivo* for its efficacy in inducing sex steroid secretion and promoting gonadal development in immature YTK females and YTK males at the onset of sexual maturation.

2. Materials and methods

All experiments were conducted with approval from the Animal Ethics Committee of the University of the Sunshine Coast (Queensland, Australia) under approval number AN/A/12/69.

2.1. Isolation of full-length fshb and fsha cDNA sequences

Total RNA was extracted from frozen YTK pituitaries previously stabilised in RNALater (Ambion Life Technologies, Carlsbad, CA, USA) with Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA quality and quantity was established using Agilent's 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). First-strand cDNA was generated from 1 μg total RNA using 5′- and 3′-SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA). Synthesised 5′ and 3′ cDNAs were tested by PCR amplification of the reference gene acidic ribosomal phosphoprotein P0 (ytkARP) (Nocillado et al., 2012).

Gene-specific RACE PCR primers (Table 1) for *fshb* were designed from the previously isolated partial YTK *fshb* sequence (GenBank Accession No. HQ449731). The primers used for the first and semi-nested 3′ RACE PCR were Primer 1 and Primer 3, respectively. The first and semi-nested 5′ RACE PCR reverse primers were Primer 2 and Primer 4. In both reactions, the *fshb* primers were paired with Nested universal primer (NUP). The first round PCR reaction mix contained 1.5 μl 10× PCR buffer, 1.2 μl $MgSO_4$ (50 mM), 0.3 μl dNTPs (10 mM each), 0.3 μl of NUP (10 μM), 0.3 μl of each forward and reverse primer (10 μM), 0.06 μl Platinum high fidelity Taq DNA polymerase (Invitrogen Life Technologies) and 1 μl of the first-strand cDNA. The semi-nested PCR amplification reaction mix contained 2.5 μl 10x PCR buffer, 2 μl $MgSO_4$ (50 mM), 0.5 μl dNTPs (10 mM each), 0.5 μl of NUP (10 μM), 0.3 μl of each forward and reverse primer, 0.1 μl Platinum high fidelity Taq DNA polymerase and 1 μl of the first round PCR product (diluted 20-fold). No template control reactions were performed in order to rule out contamination. The thermal cycling parameters for the first and semi-nested reactions were: initial denaturation at 94 °C (3 min); followed by 35 cycles of denaturation at 94 °C (30 s), annealing at 53 °C (30 s) and extension at 72 °C (1 min); and final extension at 72 °C (5 min). PCR product purification and cloning were as previously described (Nocillado et al., 2012). Plasmid DNA from positive colonies were sequenced by the Australian Genome Research Facility (Brisbane, Australia). Sequences were analysed using NCBI's Basic Local Alignment Search Tool (Altschul et al., 1990) and Sequencher 5.0 (Gene Codes Corporation, (Ann Arbor, MI, USA).

The full length *fsha* cDNA sequence was isolated by 3′RACE PCR. Primers 6 and 7 (Table 1) were designed according to the conserved regions of *Dicentrarchus labrax* (GenBank Accession No. AF269157) and *Scomber japonicus* (GenBank Accession No. JF495131) glycoprotein alpha sequences. The first and semi-nested PCR amplification was performed as for *fshb*. The thermal cycling parameters for both reactions were: initial denaturation at 94 °C (2 min 30 s); followed by 30 cycles of denaturation at 94 °C (30 s), annealing at 60 °C (30 s) and extension at 72 °C (45 s); and final extension at 72 °C (7 min). PCR products were purified, cloned, sequenced and sequence analysed as described for *fshb*. The signal sequences of *fshb* and *fsha* were identified using SignalP 4.1 software (Petersen et al., 2011).

2.2. Synthesis of a single chain fshba and ligation into the pPIC9K expression vector

The design of the rytkFshba was generally according to Kasuto and Levavi-Sivan (2005) and Aizen et al. (2007). The cDNA sequences encoding for YTK *fshb* and YTK *fsha* (excluding the signal sequence) were linked with the nucleotide sequence encoding for GSGSHHHHHHGSGS and were then codon-optimised according to the yeast (*P. pastoris*) coding preference. The codon-optimised sequence was synthesised and ligated into the *EcoR*I-*Not*I sites of pPIC9K (GenScript, Piscataway, NJ, USA). Sufficient quantity of the construct plasmid DNA was generated in JM109 *Escherichia coli*. Plasmid DNA was purified with QIAprep spin midiprep columns (Qiagen, Hilden, Germany). The purified plasmid DNA was linearized with *Sal*I (New England Biolabs, Ipswich, MA, USA). Nine microgram of the linearized construct were transformed into $SuperMan_5His^-$ strain of the yeast (*P. pastoris*) cells (Biogrammatics, Carlsbad, CA, USA) by electroporation (BioRad GenePulser, Hercules, CA, USA).

2.3. Selection of high expressing clones and induction of protein expression

Positive yeast transformants were selected in histidine-deficient media. High copy number colonies were screened based on the resistance to the antibiotic Geneticin (G418 sulphate, Sigma–Aldrich, St. Louis, MO, USA) at concentrations of 0.5–2 mg/ml. Using this protocol, 10 most resistant clones were selected from 800 colonies. From these 10 clones, the highest expressing clones were identified by methanol induction in 125 ml cultures. Protein expression induction by methanol in 1 Litre (L) volume was carried out in 5 L shaker flask at 30 °C. Molecular grade methanol (Sigma–Aldrich) was added every 24 h at 1% final concentration. Yeast was harvested 72 h after induction. The rytkFsh was purified from the culture media using nickel

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