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Method Article

Establishment of specific enzyme-linked immunosorbent assay (ELISA) for measuring Fsh and Lh levels in medaka (*Oryzias latipes*), using recombinant gonadotropins



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A B S T R A C T

The paucity of information on understanding the regulatory mechanisms that are involved in the control of piscine Fsh and Lh synthesis, secretion, and function, prompted the present work. Part of the problem is related to the molecular heterogeneity and the unavailability of Fsh and Lh assays for quantifying gonadotropins, in particular assays regarding the measurement of Fsh, and such assays are available today for only a few teleost species. The present study reports the development and validation of competitive ELISAs for quantitative determination of medaka Fsh and Lh by first producing medaka recombinant (md) gonadotropins mdFsh β , mdLh β , mdFsh $\beta\alpha$, and mdLh $\beta\alpha$ by *Pichia pastoris*, generating specific antibodies against their respective β subunits, and their use within the development of ELISAs.

The advantages of this protocol include:

- The reproducibility of the ELISA demonstrated was relatively high, as shown by reasonably low intra- (Fsh 2.7%, Lh 3%) and interassay CVs (Fsh 5.3%, Lh 5.7%).

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- The high degree of parallelism between serial dilutions of the recombinant and native pituitary-derived Fsh and Lh, may be a sign of similar structures and immunologically similarity.
- Two new competitive ELISAs for the quantification of medaka Fsh and Lh were established for the first time.

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Method details

Background

The gonadotropins follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) play essential roles within the brain-pituitary-gonadal (BPG) axis by controlling gonad development and maturation in all vertebrates [2]. Detailed knowledge of function and regulation of these hormones is still limited. The accepted model in fish suggests that Fsh is important for early stage of gametogenesis, spermatogonial proliferation, and vitellogenesis in female, whereas Lh is mostly involved in processes leading to final gametogenesis, oocyte maturation and ovulation in females and spermiogenesis and spermiation in males [3,4]. When studying gonadotropin regulation and function, an important tool is the quantification of hormone levels in blood and pituitary by enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA). ELISA and RIA assays to determine gonadotropin levels in fish traditionally have been based on native gonadotropins from fish pituitaries and their specific antibodies. The purification of native gonadotropins has been shown challenging, and recombinant gonadotropins are now being used to substitute native hormones. They have various advantages compared to natively purified hormones because they can be continually produced, and potential cross-contamination with other related glycoproteins is minimized [4]. Based on the abovementioned considerations, the objective of this study was to develop and validate homologous competitive ELISAs for medaka (*Oryzias latipes*) Fsh and Lh using recombinant gonadotropins, thus enabling quantification of biologically more relevant protein levels, which will considerably advance the value of future studies of gonadotropin physiology in this important fish model.

Animals

Japanese medaka (*Oryzias latipes*) of the d-rR strain were kept and bred in our fish facility in recirculating systems under a photoperiod of L14:D10 and water temperature of 28 ± 1 °C. Embryos were incubated in embryo culture medium (E3; 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM

MgSO₄ (all Sigma-Aldrich, St. Louis, U.S.A.), and kept at 26 °C until hatching and transfer to system tanks. The fish were fed three meals per day with a combination of dry feed and live brine shrimp nauplii larvae (*Artemia salina*). All fish were raised under the same conditions regarding to temperature, photoperiod, food, tank size, and density. Handling, husbandry and all experimental procedures of fish were in compliance with the guidelines and requirements for the care and welfare of research animals of the Norwegian Animal Health Authority and of the Norwegian University of Life Sciences. In addition, the work described has been carried out in accordance with the EU Directive 2010/63/EU for animal experiments and Uniform Requirements for manuscripts submitted to Biomedical journals, and informed consent was obtained for experimentation with animal subjects.

Production and purification of recombinant gonadotropins mdFsh β , mdLh β , mdFsh $\beta\alpha$, and mdLh $\beta\alpha$

Using the methylotrophic yeast *P. pastoris* expression system recombinant proteins were produced generally according to Kasuto and Levavi-Sivan [5] and Yom-Din et al. [6]. Synthesis of genes for medaka *fshb* (Accession Number NM_001309017.1), *lhb* (Accession Number AB541982.1), *fshba*, and *lhba* (*gpa*; Accession Number NM_001122906) was outsourced to GenScript, New Jersey, U.S.A. (see sequences in Burow et al. [7]). Gene expression constructs were produced (see Fig. 1 in Burow et al. [7]) and gene expression cassettes were generated with *P. pastoris* codon optimized DNA sequence. The synthetic genes were joined to form a fusion gene that encodes a "tethered" polypeptide (mdFsh $\beta\alpha$, mdLh $\beta\alpha$) in which one of the β chains forms the N-terminal domain and the α chain forms the C-terminal domain. A "linker" sequence containing six amino acids (three Gly-Ser pairs) [8] was positioned between the β and α chains to assist in chimerization of the subunits. The Gly-Ser linker sequence is minimally hydrophobic and can thus maximize the possibility for chimerized subunits folding into their native conformational structure enabling independent interaction with their receptors [5]. A six-His tail was placed at the end of the β subunit enabling purification of the recombinant protein. Synthesized DNA fragments were cloned into pPIC9K vector using EcoRI and NotI restrictions sites and confirmed by sequencing (Weizmann Institute, Rehovot, Israel). The pPIC9K plasmid contained the yeast α mating factor (α MF) secretion signal that directs the recombinant protein into the secretory pathway. The constructs were digested with Sall and used to transform *P. pastoris* strain GS115 (Invitrogen, Carlsbad, U.S.A.) by electroporation (GenePulser, Bio-Rad, Hercules, U.S.A.). This resulted in insertion of the construct at the AOX1 locus of *P. pastoris*, generating a His⁺ Mut^S (slow methanol utilization) phenotype. Transformants were selected for the His⁺ phenotype on 2% agar containing regeneration dextrose-biotin medium (1 M sorbitol, 1.34% yeast nitrogen base without amino acids, 4×10^{-5} % biotin, and 0.005% of L-glutamic acid, L-methionine, and L-leucine (all from Sigma-Aldrich), and 0.005% of L-lysine, and L-isoleucine (all from Biological Industries, Kibbutz Beit-Haemek, Israel)). An additional selection for antibiotic geneticin G418 (Gibco-BRL, Carlsbad, U.S.A.) resistance was performed on 2% agar containing 1% yeast extract (Becton, Dickinson and Company, Franklin Lakes, U.S.A.), 2% peptone (Becton, Dickinson and Company), 2% dextrose medium (Sigma-Aldrich) and the antibiotic G418 (1 mg/ml) (Gibco-BRL). Ten His⁺ Mut^S clones from each construct were cultivated with shaking for 24 h (growth phase) in 1.5 ml of buffered minimal glycerol (BMG) (1.34% yeast nitrogen base without amino acids, 1% glycerol, 4×10^{-5} % biotin, and 100 mM potassium phosphate buffer (all from Sigma-Aldrich), pH 6) at 28 °C, supplemented with methanol 0.5% [v/v] every 24 h. Afterwards, cells were harvested (1500 g, 5 min), resuspended and cultivated for 3 days (induction phase) in 1 ml of buffered minimal methanol (BMM) (BMG, supplemented with 0.5% methanol every 24 h). The protein was expressed in a shaker flask and harvested at 72 h after induction by methanol. Recombinant medaka Fsh β (mdFsh β), medaka Lh β (mdLh β), medaka Fsh $\beta\alpha$ (mdFsh $\beta\alpha$), and medaka Lh $\beta\alpha$ (mdLh $\beta\alpha$) were purified using nickel nitrilotriacetic acid agarose (Ni-NTA) (Qiagen, Hilden, Germany) according to the method described by Kasuto and Levavi-Sivan [5] and Yom-Din et al. [6] with certain modifications. Briefly, the pH of the supernatant was adjusted to 8.0 with 5 N sodium hydroxide (Sigma-Aldrich). Beads (QIAexpressionist, Qiagen) were supplemented to the medium and stirred on magnetic stirrer for 18 h at 4 °C. The purification was performed by batch purification of the his-tagged proteins by washing with 5 mM imidazole (Sigma-Aldrich) that prevents binding of unspecific proteins to the beads. The bound protein was eluted with PBS pH 4.5 containing 250 mM imidazole. The eluted fractions of mdFsh β , mdLh β , mdFsh $\beta\alpha$, and mdLh $\beta\alpha$ were dialyzed with

Slide-A-Lyzer Dialysis Cassette (Thermo Fisher Scientific, Waltham, U.S.A) according to the manufacturer's protocols against PBS pH 7.5, meaning unwanted compounds were removed by selective and passive diffusion through a semi-permeable membrane. The purified proteins, tagged with a six-His tail, were detected on a Western blot as described below.

Production and validation of specific antibodies for mdFsh β and mdLh β

Polyclonal antisera against recombinant mdFsh β and mdLh β were generated generally following a procedure described by Aizen et al. [9]. Two different rabbits for each protein received three intradermal injections of purified protein (mdFsh β ; 1 mg first injection, 0.5 mg second and third injection; mdLh β ; 0.7 mg first injection, 0.4 mg second and third injection) in 0.9% NaCl and emulsified in an equal volume of complete Freund's adjuvant (Sigma-Aldrich) at 3-week intervals. Two weeks after the final injection, the rabbits were bled, and the serum was aliquoted and lyophilized. To validate the produced antibodies, the recombinant proteins and medaka pituitary extract were visualized using anti-mdFsh β , or anti-mdLh β antisera. To ensure that the plasma of the rabbit before the final injections did not react with mdFsh β and mdLh β , a Western blot using medaka pre-immune serum against medaka pituitary extract, mdFsh β , and mdLh β was conducted as described below.

Western blot analysis

From culture supernatants, reduced and non-reduced samples were resolved by SDS-PAGE for Western blot analysis. Recombinant mdFsh β , mdLh β , mdFsh $\beta\alpha$, and mdLh $\beta\alpha$ were visualized using anti-His (diluted 1:2000) (QIAexpress anti-His antibodies; Qiagen), generally according to Yom-Din et al. [6], and validated by molecular weight. To validate the produced antibodies, the recombinant proteins and medaka pituitary extract were visualized using anti-mdFsh β , or anti-mdLh β (both diluted 1:2000, 1:100,000, 1:600,000) antisera.

To confirm that the plasma of the rabbit before the final injections did not react with mdFsh β and mdLh β , a Western blot using medaka pre-immune serum as a negative control against medaka pituitary extract, mdFsh β , and mdLh β was performed. Precisely, reduced samples from culture supernatants were electrophoresed on 15% SDS-polyacrylamide running gels with a 5% stacking gel. The gels were blotted onto nitrocellulose membranes (Schleicher and Schuell, Dassell, Germany) and blocked with 3% BSA in Tris Buffered Saline (TBS) buffer (20 mM Tris, 150 mM NaCl) to prevent nonspecific binding of the antibodies during subsequent steps. The membranes were incubated in PBS containing 1% nonfat milk with the antisera (dilutions 1:2000, 1:100,000, 1:600,000) for 1 h at room temperature (RT) and afterwards with goat anti rabbit horseradish peroxidase (GAR-HRP) conjugate (dilution 1:5000; Jackson Immuno Research Laboratories) for 1 h at RT. All membranes were washed and treated with enhanced chemiluminescence reagent (Chemiluminescence detection kit for HRP, Biological Industries) to reveal immunoreactive bands. For glycosylation analysis, prior to Western blot analysis, *N*-glycosidase F (PNGase F) was used to produce deglycosylated proteins by hydrolyzing all types of *N*-glycan chains. According to supplier recommendations (Roche Applied Science, Mannheim, Germany), 100 ng of reduced and denatured mdFsh β , mdLh β , mdFsh $\beta\alpha$, mdLh $\beta\alpha$, and medaka pituitary extract were incubated for 2 h at 37 °C in the presence or absence of PNGase F.

Fluorescence in situ hybridization (FISH)

To further evaluate the antibody specificity of mdLh β and mdFsh β , fluorescence *in situ* hybridization (FISH) and immunofluorescence (IF) were conducted. FISH was performed on free-floating parasagittal brain-pituitary sections, generally according to Fontaine et al. [10]. Briefly, after being sacrificed with ice water, brain and pituitary from 12 unsexed 6 month-old adult fish were dissected and fixated overnight with 4% PFA at 4 °C. Tissues were then gradually dehydrated with a series of increasing concentrations of ethanol and stored in 100% methanol until used. Tissues were rehydrated, embedded in 3% agarose, para-sagittally sectioned (60 μ m sections) using a vibratome (VT1000S Leica, Wetzlar, Germany), and then treated with proteinase K (1 μ g/ml; P6556, Sigma-Aldrich) for 30 min. *fshb* riboprobe was cloned using AGAGCAGAGGAAGCAACT and

GGGGCACAGTTTCTTTATTTTCAG as primers, and synthesized using PGEM-T vector (Promega, Madison, WI), whereas we used the *lhb* riboprobe previously described [11]. *fshb* and *lhb* sense and antisense riboprobes were conjugated with digoxigenin (DIG; 11277073910; Roche, Basel, Switzerland) using SP6 or T7 RNA polymerase (Promega). Tissues were hybridized with either sense or antisense riboprobes for 18 h at 65 °C and then incubated with sheep anti-DIG conjugated with peroxidase (POD; 1:500; 11207733910; Roche) over night. Signal was revealed using TAMRA-conjugated tyramide constructed in our lab.

Immunofluorescence (IF)

IF staining was conducted on free-floating sections according to Fontaine et al. [10] using anti-Lhb and anti-Fsh β (described earlier). For anti-Lhb, the tissues labelled for *lhb* mRNA by FISH were used (see above). For anti-Fsh β , IF could not be carried out after *in situ* labelling due to an antigen retrieval treatment that was necessary before the IF, destroying the labeling of the FISH. Thus, IF was performed on consecutive parasagittal sections of the one used for the *fshb* FISH. Tissue sections were treated with 2N Hydrochloric acid (HCl) for 1 h at 37 °C and then incubated with primary antibody (anti-Lhb; 1:2000, anti-Fsh β ; 1:1000) overnight at 4 °C. A secondary goat anti-rabbit antibody coupled to AlexaFluor 488 (A-11034, Thermo Fisher Scientific, Waltham, U.S.A.) at a concentration of 1:1000 was used for 4 h incubation. Control experiment without the primary antibody was included. Tissues for anti-Lhb were then treated for nuclei staining with DAPI (4', 6-Diamidino-2-phenylindole dihydrochloride; 32670 Sigma) by incubation at RT for 20 min at a titer of 1:1000 and rinsing.

Imaging

Stained tissues were mounted between slide and coverslip with the mounting medium for fluorescence Vectashield H-1000 (Vector, California, U.S.A.). Images were obtained using a Zeiss LSM710 confocal microscope with 25 \times (LCI Plan-Neofluar 25x/0.8 NA) objective. Channels were acquired sequentially in order to avoid signal crossover between the different filters. Images were processed using the ZEN software (Carl Zeiss AG, Oberkochen, Germany). Z-projections from confocal stacks of images were acquired using Image J software (<http://rsbweb.nih.gov/ij/>). Composites were assembled using Adobe Photoshop and Illustrator CS6 (Adobe Systems, San Francisco, California).

Gonadal histology

Histological analysis of testes was performed to assess the maturational stages of fish sampled for developmental series by identifying the germ cell stages (see Table 3 in Burow et al. [7]). Testes from 36 males, grouped according to standard body length (SL), were dissected and transferred to phosphate buffered saline (PBS; Sigma-Aldrich) prior to overnight fixation in ice-cold 4% glutaraldehyde phosphate buffered solution at 4 °C. Note that fish from the smaller size groups had testicular tissue of such low volumes that, in fear of losing it later in the fixation process, it was not dissected out of the body cavity. Rather, all other organs were removed from the abdomen, and body and gonads fixated as a whole. Glutaraldehyde fixative solution was prepared fresh by mixing 28 ml 0.2 M NaH₂PO₄-H₂O with 72 ml 0.2 M Na₂HPO₄-2H₂O (both from Merck Millipore, Billerica, MA, USA), adjusting the mixture to pH 7.2, and then adding 68 ml mq-H₂O and 32 ml of 25% glutaraldehyde (Merck Millipore). Tissues were dehydrated in a series of increasing concentrations of EtOH (70–100%) at RT, each step lasting at least 30 min. The last step (100%) was repeated three and replaced with approx. 5 ml of preparation solution (100 ml Technovit 7100 added 1 g of Hardener I (Heraeus Kulzer, Hanau, Germany)) and kept at slow shaking overnight. All fixation steps were performed in small glass bottles. After infiltration, tissue samples were embedded in cold Histoform S (Heraeus Kulzer) added approx. 1 ml preparation solution w/50 μ l Hardener II (Heraeus Kulzer) and incubated at 37 °C. Cured samples were mounted onto Histoblocs using Technovit 3040 (both from Heraeus Kulzer), before sagittal sections (3 μ m) were prepared using a Leica RM2245 microtome (Leica Biosystems, Wetzlar, Germany). Sections were collected from the periphery until the middle of the testes tissue every

30 μm and placed onto microscope slides. Dried sections were stained with Toluidine Blue O (Sigma-Aldrich) and mounted with Coverquick 4000 (VWR International, Radnor, PA, USA) before histological analysis. Germ cells were determined according to [12], within the five main germ cell stages (spermatogonia type A (SPA), spermatogonia type B (SPB), spermatocytes (SC), spermatids (ST) and spermatozoa (SZ)), no further distinctions were made (see Table 2 in Burow et al. [7]).

Development and validation of specific ELISA for Fsh and Lh, and profile of pituitary levels of Fsh and Lh in male medaka

The recombinant gonadotropins were used to develop specific and homologous competitive ELISAs for determination of mdFsh and mdLh in the pituitary, generally according to Mañanós et al. [1]. Competitive ELISAs were developed using specific β -subunit polyclonal rabbit primary antibodies against mdFsh β or mdLh β (described earlier), recombinant β -subunits mdFsh β or mdLh β (described previously) to coat the ELISA microplates, and recombinant mdFsh $\beta\alpha$ or mdLh $\beta\alpha$ (described earlier) for the standard curves. Briefly, ELISA microtiter plates (Nunc-Immuno™ Plates; Nunc, Denmark) were coated with 100 μl /well of 10 ng/ml (1 ng/well) mdFsh β or 5 ng/ml (0.5 ng/well) mdLh β . The following day, plates were washed with 200 μl /well phosphate buffered saline with Tween 20 (PBST) buffer (10 mM Na_2PO_4 , 2 mM KH_2PO_4 (pH 7.4), 140 mM NaCl, 3 mM KCl, and 0.05% Tween 20 (all from Sigma-Aldrich)). To reduce background by blocking the unspecific binding sites, plates were blocked for 1 h with 200 μl /well of PBST buffer containing 1% bovine serum albumin (BSA, Sigma). Single pituitaries were dissected and kept on ice until homogenization (6 m/s, 3×30 s using ceramic beads, cat.no 116933050, MP Biomedicals, California, U.S.A.) using FastPrep-24 (MP Biomedicals) diluted 1:2.7 with 0.1% BSA in PBST, in sufficient volume to allow technical duplicates (125 μl /well). The homogenate was centrifuged at 10,000g for 5 min. Samples and standards were first pre-incubated overnight at RT with the primary antibodies (125 μl /well) (final dilution 1:10,000 for mdFsh β and 1:50,000 for mdLh β in 0.1% BSA in PBST) in 96-well microtest plates (Sarstedt, Nümbrecht, Germany). After pre-incubation, each sample was distributed into the wells (100 μl /well) of the coated microtiter plates and incubated for 3 h at RT. After incubation, the plates were washed with PBST. Formed antigen-antibody complexes were detected by addition of 100 μl /well of GAR-HRP (Bio-Rad) diluted 1:5000 in PBST-0.1% BSA buffer for 2 h at RT. The plates were washed again with PBST. The visualization of the presence of enzyme complexes was performed by addition of 100 μl /well of 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate (KPL, Zotal, Israel) diluted 1:4. The reaction was carried out in complete darkness at RT and stopped after approximately 15 min with TMB stop solution 1 M H_2SO_4 (50 μl /well). Absorbance was read at 450 nm, using a Microplate Spectrophotometer (Epoch 2, Biotek, Winooski, U.S.A.).

The ELISA was validated for medaka Lh and Fsh determinations in pituitary extract of medaka. Displacement curves for pituitary samples were achieved by serial dilutions of the sample in 0.1% BSA in PBST and comparison with the standard curve using recombinant mdFsh $\beta\alpha$ or mdLh $\beta\alpha$. For the parallelism analysis, pituitaries were collected from sexually mature male fish and homogenized using different conditions (10 ceramic beads, 6 m/s; 20 ceramic beads, 4 m/s; 20 ceramic beads, 6 m/s; all 3×30 s). The homogenate was centrifuged at 10,000g for 5 min and the resulting supernatant was used as the pituitary extract. The sensitivity of the assay is defined as the lowest dose of Fsh or Lh capable of reducing the optical density more than the mean plus two standard deviations of the zero dose of Fsh or Lh [$B_0 - 2SD$]; it was calculated by adding the mean of the blank to two times the standard deviation of the blank. Intra-assay coefficient of variation (CV) was determined by assaying six replicates of one of the standard concentrations (1.56 ng/ml) on the same assay plate. Inter-assay CV was calculated by assaying the same sample five times in different plates.

Profile of pituitary levels of Fsh and Lh in male medaka comparing juveniles versus adults

A profile of Fsh and Lh in male medaka pituitaries was conducted using the ELISA method described above. To achieve a profile for Fsh, pituitaries from 24 juvenile males with SL between 12 mm and 16.5 mm, and of 24 adult males between 21 mm and 25.5 mm were dissected. For the profile of Lh pituitaries from 12 juvenile males with SL between 12 mm and 16 mm, and of 12 adult males between 22.5 mm and 26.5 mm were dissected (for both Fsh and Lh 1 pituitary in 40 μl 0.1% BSA in PBST per

biological replicate was used). The distinction between juvenile and adult was based on unpublished results relating medaka testicular maturation stage to SL [7], and showing that males with SL below 16 mm were completely immature and males with SL above 20 mm to be fully mature.

Statistical analysis

Data are presented as mean \pm SEM. All data were tested for normal distribution (Shapiro–Wilk normality test). For sample groups, which did not follow a normal distribution, the data were first log-transformed. For ELISA data calculations, sigmoid curves were linearized using logit transformation. Correlations were calculated by Graph-Pad Prism software (version 7; GraphPad, San Diego, U.S.A.). Significance level was set to 0.05.

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