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ORIGINAL ARTICLE

Aquaculture

Development and validation of enzyme-linked immunosorbent assays specific for follicle-stimulating hormone and luteinizing hormone in Japanese eel

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Abstract
Two types of gonadotropins (Gths), follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh), are primary regulators of reproduction in vertebrates, including teleost fish. In this study, recombinant Japanese eel Gths (reGth; reFsh and reLh), and recombinant chimeric Gths (rcGth; rcFsh and rcLh), consisting of an eel β-subunit with rabbit glycoprotein hormone α-subunit, were produced as single-chain proteins, using a mammalian expression system and subsequently highly purified. Antibodies raised against rcGths were affinity purified with the corresponding reGth, and specific enzyme-linked immunosorbent assays (ELISAs) for Japanese eel Fsh and Lh were developed using these antibodies for the first time. The ELISAs were sensitive and parallelism was seen between the standard curve and serial dilutions of Japanese eel serum and pituitary extract (PE). Contents of Fsh and Lh protein in the pituitaries of the female Japanese eel at the pre-vitellogenic stage were comparable and the Lh contents at the migratory nucleus stage were highly induced during artificial induction of maturation by injections of salmon PE. Furthermore, the accumulated Lh was released at a high level by administration of a Lh-releasing hormone analogue and pimozide. Recombinant Gths and homologous ELISAs established for Japanese eel may be useful tools for studying reproductive eel biology.

Keywords Gonadotropin · Reproductive regulation · *Anguilla japonica* · Maturation

Introduction

The Japanese eel *Anguilla japonica* is one of the most important commercial fishes in the Japanese aquaculture industry. Since eel aquaculture entirely depends on the number of glass eels caught in the wild, their recent decrease is of great concern and prices for eel have soared. Thus, the establishment of a method for artificial seed production that can ensure a stable glass eel supply is highly desirable. However, both cultured and wild adult Japanese eels caught

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in coastal areas are immature and cannot undergo further gonadal development under captive conditions (Kagawa et al. 1998). Therefore, great attention has been paid to artificially induced maturation in this species, and there have been many reports on the administration of exogenous gonadotropic hormones (Gths) to elicit this.

It is well known that two Gths [follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh)] play a central role in regulating reproduction in vertebrates (Gharib et al. 1990). However, the differential actions of each Gth in teleosts have not been well established, except for salmonids, where it has been proven that Fsh and Lh primarily regulate early gamete development and final maturation, respectively (Tyler et al. 1991; Planas et al. 2000; Swanson et al. 2003). One of the main reasons for this insufficient knowledge is the lack of large quantities of Gths, especially Fsh, for functional analyses and the establishment of reliable assays for the measurement of Fsh and Lh in non-salmonids (Levavi-Sivan et al. 2010).

Full sexual maturity was achieved experimentally in female Japanese eels, for the first time, by injections of salmon pituitary homogenate or extract (SPE) containing a large amount of Gth (Yamamoto and Yamauchi 1974; Yamauchi and Yamamoto 1982). Thereafter, various improvements have been made to artificial maturation methods (Tachiki et al. 1997; Kagawa 2003; Kagawa et al. 2013). In a currently used method, female eels receive repeated weekly injections of SPE until the ovaries contain oocytes at the migratory nucleus (MN) stage. Furthermore, final oocyte maturation/ovulation is induced by injecting SPE following the administration of a maturation-inducing steroid (17α, 20β-dihydroxy-4-pregene-3-one) or its precursor (17α-hydroxyprogesterone). Owing to these techniques, the acquisition of eggs has become relatively easy; however, the quality of the resultant eggs is unstable and poor in most cases (Kagawa et al. 2012). The cause of this inadequate egg quality is still unclear, but homologous Gths may be strong candidates to provide a solution to this problem. Therefore, further optimization of hormonal treatment for inducing oocyte growth (i.e., vitellogenesis) and final oocyte maturation/ovulation is urgently required. For this, it is essential to establish a technique for the preparation of the two homologous eel Gths, Fsh and Lh, and to gain knowledge of their different functions in ovarian growth/maturation.

As a first step to a deeper understanding of the differential functions of the two eel Gths and their potential for producing high-quality eggs, we created recombinant homologous eel Gths (reGths) and recombinant chimeric Gths (rcGths) consisting of Japanese eel β-subunits of Gth (Fshb or Lhb) and rabbit common glycoprotein hormone α-subunit (cga) following an established method for this (Nyuji et al. 2016; Okuzawa et al. 2016). Homologous specific enzyme-linked immunosorbent assays (ELISAs) for eel Fsh and Lh were developed with specific antibodies generated against rcGths. Furthermore, the dynamics of serum Lh levels in female eels at the maturation phase after treatment that contribute to Lh release from the pituitary [administration of luteinizing hormone releasing-hormone analogue (LHRHa) and pimozide] were determined by the developed ELISA.

Materials and methods

Animals and samples

Eighteen immature female eels used in this study at the pre-vitellogenic (PV) stage were produced by treating glass eels with estradiol-17β (Ijiri et al. 1995). Artificial induction of eel ovarian development by treatment with SPE has been previously described (Kazeto et al. 2000; Kazeto et al. 2012). After acclimatization to seawater, five of the female eels served as pre-treatment controls before hormonal treatment and the other 13 female eels were administered with SPE weekly [40 mg/kg body weight (BW)] until they reached the MN stage. Eels at the PV stage ($n = 5$) and the MN stage ($n = 5$) were sampled while deeply anesthetized with 2-phenoxyethanol (Wako, Osaka, Japan), and the pituitary glands, ovaries and sera were collected. Each pituitary was homogenated in Dulbecco's phosphate-buffered saline (PBS) containing the cOomplete Protease Inhibitor Cocktail set (Roche Diagnostics, Tokyo) and centrifuged at 15,000 *g* for 15 min. The resultant supernatant was designated as pituitary extract (PE).

Four eels at the MN stage (L + P group), were administered with a cholesterol pellet containing LHRHa (600 μg/kg BW), des-Gly^{10}—[δ-Ala^{6}] LHRH ethylamide (Lee et al. 1986; Matsuyama et al. 1992) and pimozide (20 mg/kg BW; Sigma, St. Louis, MO) dissolved in dimethylsulfoxide (DMSO) to induce release of endogenous Lh. Four more fish at the MN stage received a LHRHa-free cholesterol pellet and DMSO to serve as the control. Fish of the L + P and control groups were periodically bled prior to and after the last treatment (0, 6, 12, 24, 48 h) and the ovaries were removed at the end of the experiment.

The removed ovaries were weighed to determine the gonadosomatic index (GSI; gonadal weight/body weight × 100). GSI was calculated as (ovulated egg weight + gonadal weight)/body weight × 100 when ovulation had been confirmed. Developmental stages of the ovary were classified as previously described (Kazeto et al. 2000). All PE and sera were frozen and stored at − 30 °C until analysis.

Construction of the expression vectors

Details of the construction of single-chain Gths by In-Fusion reaction have been described in our previous reports (Fig. 1;

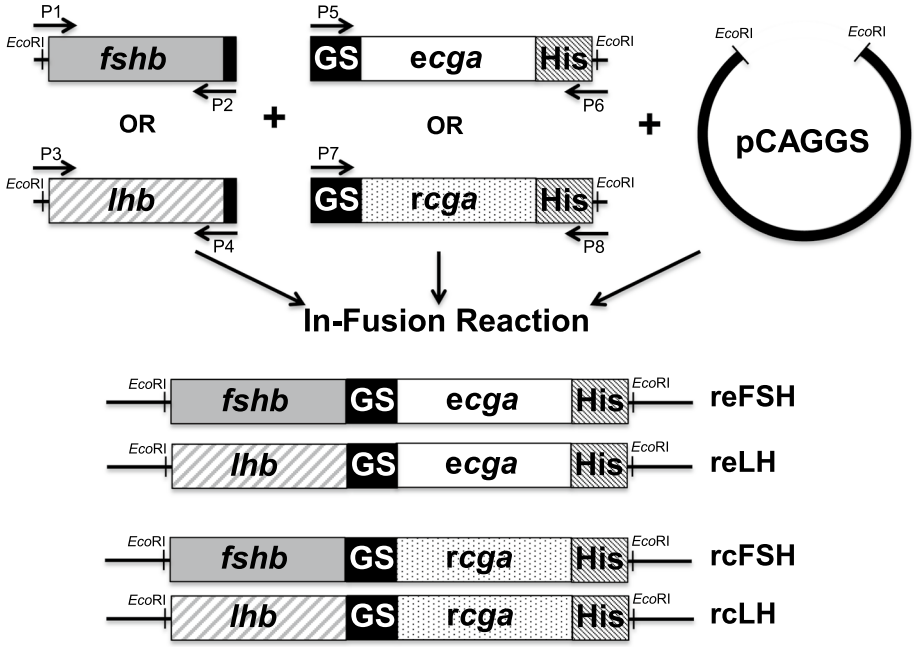

Fig. 1 Schematic diagram of construction of vectors to express recombinant gonadotropins (Gths). *FSH* Follicle-stimulating hormone, *LH* luteinizing hormone, *ecga* Japenese eel common glycoprotein hormone α-subunit, *rcga* rabbit cga, *GS* gonadosomatic, *sse* Sertoli cell enriched, *scc* side chain cleavage

Nyuji et al. 2016; Okuzawa et al. 2016). All primers used for the construction of the expression vectors are listed in Table 1. The complementary DNA (cDNA) of Japanese eel *cga* (e*cga*), *fshb* and *lhb* was amplified by polymerase chain reaction (PCR) with specific primers and a proofreading PrimeSTAR HS DNA polymerase (Takara, Tokyo), using the expression plasmids for *Drosophila* S2 cells (Kazeto et al. 2008) as templates. Stop codons of β subunits were eliminated and a nucleotide sequence encoding a portion of a spacer (GS spacer), GGGSGGGSGGGSGGG, for a tether between β and α subunits was added at the 5′-ends of the reverse primers (P2 and P4) for β subunits and the forward primer (P5) for α subunits, respectively. The 5′-ends of forward primers (P1 and P3) for β subunits and for the reverse primer (P6) for α subunits were designed to possess upstream and downstream sequences from *Eco*RI sites of a mammalian expression vector, pCAGGS (Tokui et al. 1997). In the case of e*cga*, the sequence coding the signal peptide was removed and sequence coding 6xHis was added upstream of the stop codon. The resultant amplicons of either *fshb* or *lhb*, and e*cga* were directionally fused with *Eco*RI-digested pCAGGS plasmid vector by an In-Fusion HD cloning kit (Takara) and designated as single-chain recombinant eel Fsh (reFsh) and Lh (reLh). An expression vector for single-chain recombinant chimeric Gths (rcGth; rcFsh and rcLh) composed of eel β subunit and rabbit *cga* (r*cga*) was also constructed. Total RNA was extracted from a rabbit pituitary, gifted by Professor Yamamoto of Nagoya University, using Trizol (Life Technologies, Carlsbad, CA). One microgram of the total RNA was reverse-transcribed into the cDNA using PowerScript (Takara) after priming with an oligo (dT) primer. The cDNA encoding r*cga* with the same modification at the 5′- and 3′-termini as e*cga* cDNA was amplified by PCR with a set of primers (P7 and

Table 1 Polymerase chain reaction oligonucleotides for the generation of expression vector constructs

Name	Nucleotide sequence	Use and its target DNA
P1	5′-TTTTGGCAAAGAATTCGCTAGCATGGATCTGGCTGTCACAG-3′	Eel *fshb* cDNA
P2	5′-GCCGCTGCCGCCGCCGTGGGTCAGACAGCCTGAC-3′	Eel *fshb* cDNA
P3	5′-TTTTGGCAAAGAATTCGCTAGCATGGCAGTCTACCCAGAATG-3′	Eel *lhb* cDNA
P4	5′-GCCGCTGCCGCCGCCCGCGGGGAGGCTGGCCCG-3′	Eel *lhb* cDNA
P5	5′-GGCGGCGGCAGCGGCGGCGGCAGCGGCGGCGGCAGCGGCGGCGGCTATCCCAACAACGAA ATGGC-3′	Eel *cga* cDNA
P6	5′-CCTGAGGAGTGAATTCTCAATGGTGATGGTGATGATGACCGGTAAATTTGTGGTAGTAGCAGGTGC-3′	Eel *cga* cDNA
P7	5′-GGCGGCGGCAGCGGCGGCGGCAGCGGCGGCGGCTTTCCTGATGGCGAGTTTGCA-3′	Rabbit *cga* cDNA
P8	5′-CCTGAGGAGTGAATTCTCAATGGTGATGGTGATGATGGGATTTGTGATAATAACATGTACTGCAG-3′	Rabbit *cga* cDNA

fshb Follicle stimulating hormone β-subunit, *lhb* luteinizing hormone β-subunit, *cga* common glycoprotein hormone α-subunit

P8). The r*cga* was directionally fused with either eel *fshb* or *lhb*, and pCAGGS. All of the resultant vector constructs were bidirectionally sequenced with an ABI 3130 DNA sequencer to confirm validity.

Production and purification of recombinant single-chain Gths

For a detailed description of the production and purification of recombinant Gths, see Kazeto et al. (2008) and Nyuji et al. (2016). In brief, the expression vectors were transfected into FreeStyle 293-F cells which were cultured using FreeStyle MAX 293 Expression System (Life Technologies) according to the manufacturer's instructions. After incubation for 5–7 days, the media were harvested by centrifugation and subjected to purification of the recombinant Gths.

The resultant media were concentrated by ultrafiltration or tangential flow filtration, and the recombinant Gths were purified using immobilized metal affinity chromatography (IMAC; Ni–NTA; Qiagen, Valencia, CA), according to the manufacturer's instruction and dialyzed with PBS. Concentration of the protein was determined by a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as a standard.

Generation of antisera

Antisera against rcGths (a-rcFsh and a-rcLh) were raised in rabbits by subcutaneous injections with the corresponding rcGth (1–2 mg) emulsified with an equal volume of Titer-Max Gold (CytRx, Norcross, GA). First, one-quarter of the emulsion was injected into the rabbits. The same amount of emulsion was then used weekly for immunization 2–4 weeks after the first immunization. Bleeding was carried out every 2–3 days 3–6 weeks after the first immunization.

Polyacrylamide gel electrophoresis and western blot

To confirm their purity, the recombinant Gths (reFsh, reLh, rcFsh and rcLh) were analyzed by 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under reducing conditions and stained with Coomassie brilliant blue. Furthermore, reFsh and reLh were also subjected to another SDS-PAGE as above, as well as native-PAGE using 12.5% gel for western blot analysis, and were electrophoretically blotted onto nitrocellulose membranes. The membranes were reacted with either a-rcFsh or a-rcLh (1:2000) diluted in Can Get Signal Immunoreaction Enhancer Solution 1 (Toyobo, Tokyo) after blocking with 5% skimmed milk/TBS-T (0.05% Tween20), followed by the incubation with goat anti-rabbit immunoglobulin G (IgG) conjugated with alkaline phosphatase (Funakoshi, Tokyo) diluted 1:2000 in Can Get Signal Solution 2 (Toyobo). After washing the membrane, immunoreactive bands were retrieved with 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyphosphate p-toluidine salt as chromogen.

Affinity-purification of IgGs specifically bound to reGths and preparation and biotin-labeling of the F(ab')2

IgGs were purified from a-rcFsh (a-rcFsh IgG) and a-rcLh (a-rcFsh IgG) with nProteinA Sepharose (GE Healthcare Biosciences, Piscataway, NJ) from each antisera following the manufacturer's instructions. reFsh and reLh were immobilized on HiTrap NHS-activated HP Columns (GE Healthcare Biosciences) according to the manufacturer's protocol. IgGs (a-rcFsh IgG and a-rcFsh IgG) specifically recognizing either reFsh (apeFsh IgG) or reLh (apeLh IgG) were affinity-purified with the corresponding reGth column. Furthermore, apeFsh IgG and apeLh IgG were digested by pepsin in 0.1 M sodium acetate buffer (pH4.5) and fractioned by a gel-filtration using a Superdex 200 10/300 GL column (GE Healthcare Biosciences). Fractions containing the F(ab')2 (~50 kDa) were pooled and the F(ab')2 labeled with biotin using a Biotin Protein Labeling kit [apeFsh F(ab')2 and apeLh F(ab')2; Roche Diagnostics] according to the manufacturer's protocol.

ELISA procedure

ELISAs were carried out in 96-well polystyrene plates (Sumitomo Bakelite, Tokyo). Wells were coated with 200 µl of apeGth IgG (500 ng/well) diluted in 100 mM carbonate buffer (pH 9.6) overnight at 4 °C. After washing with phosphate buffered saline–Tween-20 (0.05% Tween20) three times, plates were incubated with 300 µl/well of Universal Casein Diluent/Blocker (UCDB; Stereospecific Detection Technologies, Baesweiler, Germany) for 2-4 h at room temperature (RT). After washing three times with PBS-T, 200-µl samples or standards (reFsh or reLh) diluted with UCDB was added to each well and incubated overnight at RT. After washing as above, each well was incubated with 200 µl of the apeGth F(ab')2 (500 ng/ml in UCDB) for 2 h at RT. After plates had been washed three times, each well received 200 µl of streptavidin-polyHRP80 (400 ng/ml; Stereospecific Detection Technologies) diluted in UCDB and incubated for 1–2 h at RT. After washing, the color was developed with 200 µl of 1-Step Ultra TMB-ELISA solution (Thermo Scientific, Rockford, IL) for 5–15 min at RT in the dark. The reaction was stopped by adding 100 µl of 2 M sulfuric acid. Absorbance at 450 nm was measured using a microplate reader, either an Infinite 200 (Tecan, Grödig, Austria) or an iMark (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

All numerical data are presented as mean ± SEM. Tukey–Kramer's honestly significant difference test was used to determine significant differences between the group mean. Differences were considered significant at $p < 0.05$.

Results

Production and characterization of recombinant Gths and antibodies

Recombinant single-chain eel Gths (reFsh and reLh) and the chimeric Gths (rcFsh and rcLh) secreted into the culture media were purified by IMAC. The purity was assayed by SDS–PAGE under reducing conditions. Blurred bands of about 33 kDa were visualized by total protein staining in reFsh and rcFsh, whereas the molecular masses of reLh and rcLh were slightly lower, at about 31–32 kDa (Fig. 2a). Western blot analyses using a-rcFsh and a-rcLh specifically detected corresponding reGths on both SDS-PAGE (Fig. 2b) and native PAGE (Fig. 2c). The yields of these four recombinant Gths were between 2 and 5 mg/L medium.

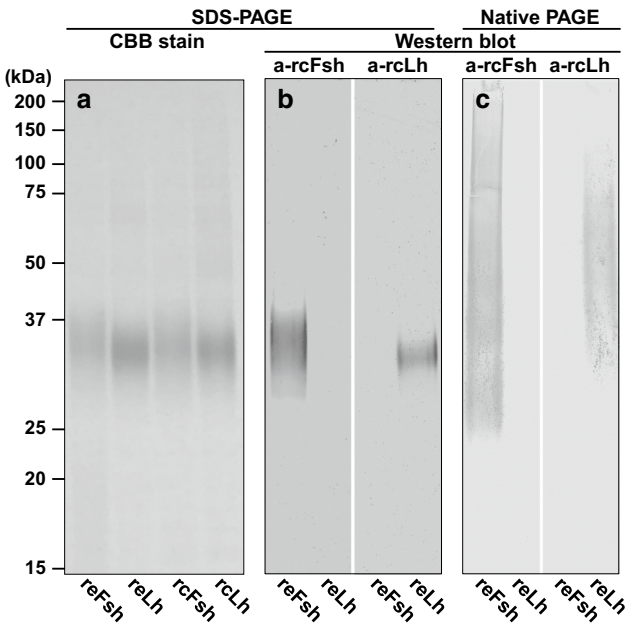

Fig. 2 Polyacrylamide gel electrophoresis (PAGE) and western blot analyses of the purified recombinant Gths produced by FreeStyle 293 cells. **a** Sodium dodecyl sulfate (SDS)-PAGE, total protein staining. **b** SDS-PAGE, western blot with a-rcFsh or a-rcLh. **c** native PAGE, western blot with a-rcFsh or a-rcLh. *PE* Pituitary extract; for other abbreviations, see Fig. 1

Validation of ELISAs for eel Fsh and Lh

The detection range of ELISA for both Fsh and Lh was from a few tens of picograms per milliliter to 50 ng/ml dependent on the duration of the color development reaction. A standard curve with maximum sensitivity to the Fsh ELISA for color development of long duration (15 min) is shown in Fig. 3a. The linear detection range was from 15 to 480 pg/ml. The range could be varied, thus typical sigmoidal curves were obtained with high concentrations (0.39–50 ng/ml) of reFsh standard with color development of short duration (Fig. 3b). Serial dilutions of serum (Fig. 3a, b) and PE (Fig. 3b) showed linearity with parallelism to standard curves. The intra- and interassay CVs were 7.7% ($n = 10$) and 4.4% ($n = 5$), respectively, when 50 ng/ml reFsh in serum from an immature female eel in which both Fsh and Lh were under the level of detection was used.

In the Lh ELISA, the maximum sensitivity of detection ranged from 30 to 960 pg/ml and the sigmoidal curve at the lowest sensitivity ranged from 1.5 to 50 ng/ml. The intra- and interassay CVs were 6.6% ($n = 10$) and 5.7% ($n = 5$), respectively, when 50 ng/ml reLh was used. Serial dilutions of serum and PE indicated linearity with parallelism to standard curves, as shown for Fsh ELISA.

Cross-reactivity of Fsh ELISA with reLh was 8.2% while cross-reactivity of reFsh to Lh ELISA was less than 1%, when 50 ng/ml of antigen in the serum as described above was used. Comparable concentrations of Gth in serially diluted serum samples were detected by both Fsh and Lh ELISA when serum samples were diluted more than ten times.

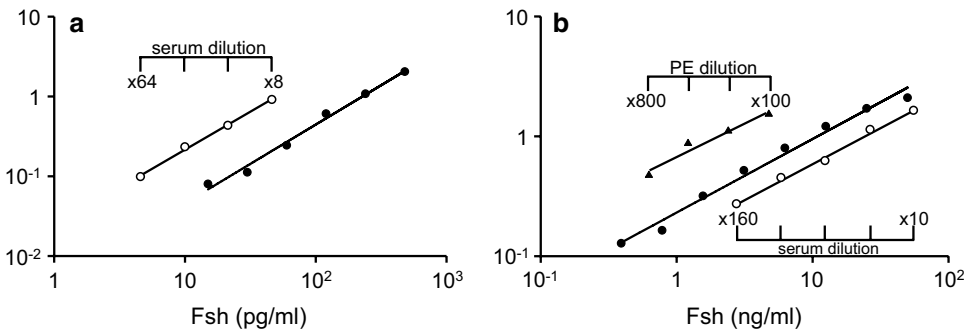

Fig. 3 Typical enzyme-linked immunosorbent assay standard curves for Fsh with color development of long (**a**) and short (**b**) duration. Filled circle Standard curve, open circle Japanese eel serum, filled triangle Japanese eel PE. for abbreviations, see Figs. 1 and 2

Protein contents of Gths in eel pituitary

Pituitary contents of Fsh and Lh were 60.3 ± 12.5 ng/pituitary and 161.7 ± 103.0 ng/pituitary, respectively, in PV eels before the artificial induction of maturation. Lh content in the pituitary of eels at the MN stage after SPE treatment dramatically increased and reached levels more than 1700-fold higher (281 ± 92.4 μg/pituitary) than those in the PV eels (Table 2).

Effects of LHRHa and pimozide on serum Lh level and GSI

Serum levels of Lh in female eels at the MN stage gradually increased until 12 h after administration of LHRHa and pimozide; thereafter, a further increase with statiscal significance was found after 48 h, while there were no significant changes in the control group (Fig. 4). GSI in the L + P group was significantly higher than before the treatment and in the control (Fig. 5). One eel in the L + P group ovulated after 48 h whereas no ovulation was confirmed in the control group.

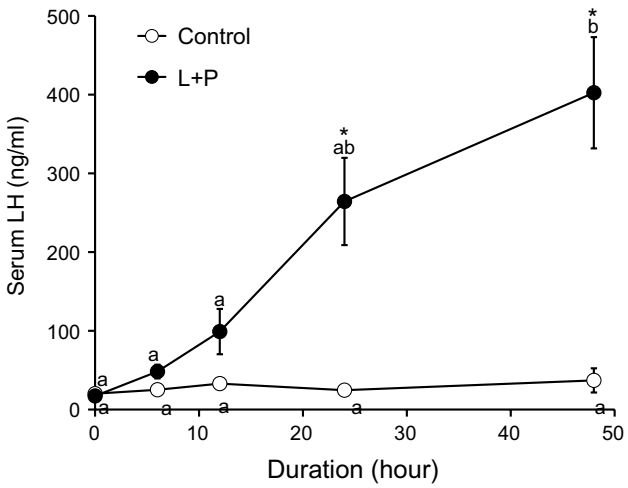

Fig. 4 Serum Lh levels in female Japanese eels at the MN stage after administration of Lh releasing hormone analogue (LHRHa) and pimozide. Different letters indicate significant differences (Tukey–Kramer test, $p < 0.05$) among respective means. Open circle Control, filled circle eels at the MN stage ($L + P$). *$p < 0.05$ (between the levels in the control and L + P group at the same time point; Tukey–Kramer test)

Discussion

Recent advancements in recombinant technology have enabled the production of bioactive recombinant Gths in various species of teleosts (Aizen et al. 2007; Kazeto et al. 2008; Molés et al. 2011; Chauvigné et al. 2012). We recently developed methods for the production of recombinant fish homologous and chimeric Gths using a transient expression system of mammalian cells and established reliable homologous immunoassays to quantify the concentration of two Gths in red sea bream *Pagurus major* (Okuzawa et al. 2016) and greater amberjack *Seriola dumerili* (Nyuji et al. 2016). Application of these methods has also been confirmed for Japanese eel in the present study.

reFsh and reLh, consisting of an eel β-subunit and its Cga, were successfully produced and purified, as well as chimeric Gths (rcFsh and rcLh) with rabbit Gphα, instead of eel Gphα, as single-chain proteins. The reGths showed specific immunoreactivity in western blot under both the denatured/reduced condition and in the native condition only to the corresponding antiserum against rcGths. These findings indicate that reGths and rcGth were produced as expected and that the generated antiserum correctly recognized the target reGth. These four recombinant Gths were detected as blurred bands; this characteristic was also found in recombinant amberjack Gth expressed in the same expression system (Nyuji et al. 2016) although sharp bands for each subunit protein were detected when reGths were expressed in *Drosophila* S2 cells (Kazeto et al. 2008). Gths are glycoproteins with N-linked glycans, and it has been reported that eel Fsh (Yoshiura et al. 1999) and Lh (Nagae et al. 1996) potentially contain N-linked glycosylation sites. *N*-glycans

Table 2 Gonadotropin (*Gth*) contents in the pituitaries of Japanese eels

Gth	Ovarian stage	Content (ng/pituitary)
Fsh	PV[a]	60.3 ± 12.5
Lh	PV[a]	161.7 ± 103.0
Lh	MN	$281 \times 10^3 \pm 92.4 \times 10^3$*

For other abbreviations, see Table 1

*$p < 0.05$ [pre-vitellogenic (*PV*) va. migratory nucleus (*MN*) stage; Tukey–Kramer test]

[a]Before the artificial induction of maturation

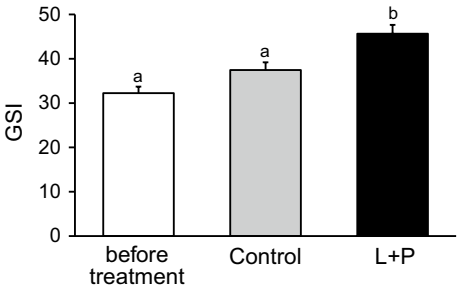

Fig. 5 Gonadosomatic index (*GSI*) in female Japanese eels at the MN stage before and after administration of LHRHa and pimozide. Different letters indicate significant differences (Tukey–Kramer test, $p < 0.05$) among respective means. For other abbreviations, see Fig. 4

of glycoprotein expressed in mammalian cells are complex and composed of a variety of sugars and sialic acids (Betenbaugh et al. 2004; Hossler et al. 2009) while invertebrate cells including S2 cells attached only simple high mannose oligosaccharide to glycoprotein (Kim et al. 2005). Thus, the differences in the patterns and breadth of the recombinant eel Gths may be due to differences in *N*-glycans that depend on the host cells used.

In European eel *Anguilla anguilla*, Lh has been measured by a heterologous radioimmunoassay (RIA) using an antiserum against the β-subunit of carp *Cyprinus carpio* (Dufour et al. 1983; Dufour et al. 1988; Vidal et al. 2004). It should be possible to determine Japanese eel Lh by this RIA because the Lhb of European eel (Quérat et al. 1990) and Japanese eel (Nagae et al. 1996) show over 98% homology. However, RIA is not a preferable method simply because special equipment and facilities are required for the use of radioisotopes. Furthermore, an assay for Fsh has not been reported for Japanese eel. Therefore, ELISAs for Fsh and Lh in Japanese eel were established in the present study. The detection limit of the assays was a few tens of picograms per milliliter, which is comparable to that of the Lh RIA (Dufour et al. 1983). Furthermore, the inter- and intraassay CVs (4.4–7.7%) were within the range of assay systems established for Gths of other fish species (Aizen et al. 2007; Mateos et al. 2006; Molés et al. 2011). Specificity of the Lh ELISA with Fsh was very high (cross-reactivity < 1%), while the cross-reactivity of the Fsh ELISA with Lh was not particularly low (8.2%) and comparable to that of other assay systems for teleost Gths. Furthermore, parallelism was found between the standard and serially diluted samples of eel serum and PE, therefore these ELISAs should be useful in measuring both Japanese eel Fsh and Lh.

Contents of Fsh and Lh protein in the pituitaries of female Japanese eels at the PV stage were comparable, and the Lh contents at the MN stage drastically statistically significantly increased. Transcript abundances of *lhb* in this species have been shown to be low at the PV stage and rapidly increase during artificial induction of maturation by SPE treatment, peaking at the MN stage (Nagae et al. 1996; Saito et al. 2003), which coincides with changes in protein contents, as shown in this report. The cross-reactivity of Fsh ELISA to Lh was 8.2%, and the content of Lh at the MN stage was approximately 280 µg/pituitary, thus predicted Fsh contents at the MN stage due to cross-reactivity should be about 23 µg/pituitary. Therefore, it is thought that pituitary Fsh contents at the MN stage cannot be determined precisely when the Fsh assay shows a few tens of micrograms per pituitary (data not shown).

LHRHa is widely used in teleost fish and has the advantage of inducing final oocyte maturation/ovulation since it stimulates Lh release from the pituitary gland (Zohar and Mylonas 2001). In contrast, it has been reported that dopamine inhibits Lh release in fish, especially in primitive species; the combined use of LHRHa and dopamine antagonists, e.g. pimozide or domperidone, were found to effect distinctive Lh release in some fish species (Sokolowska et al. 1985; Kim et al. 2011). In addition, the synergic effects of LHRHa and pimozide or L-α-methyl-3, 4-dihydroxyphenylalanine (an inhibitor of catecholamine synthesis) on Lh release have been demonstrated in female silver European eel pretreated with estradiol-17β in vivo (Dufour et al. 1988) and in vitro (Montero et al. 1996). For this reason, these chemicals were administered to Japanese eel at the maturational phase to examine the in vivo effects of LHRHa and pimozide on Lh release and the induction of final oocyte maturation/ovulation. The serum Lh level and GSI increased until 48 h after LHRHa plus pimozide treatment in a time-dependent manner; the Lh level reached over 300 ng/ml, whereas that in the vehicle control did not change. The serum level of Lh was much higher than that reported in estradiol-treated European eel (10–20 ng/ml) (Dufour et al. 1988). This difference may be due to a difference in the pituitary contents of Lh in the different eel species, since the pituitaries of the European eels and the Japanese eels contained 20–60 µg/pituitary and 280 µg/pituitary, respectively. Therefore, artificially induced Japanese eels should be a more sensitive model when the aim is to understand the mechanism of Lh release. Furthermore, ovulation was induced in an eel, which strongly suggests that these chemicals may be applicable in inducing ovulation, in turn making it possible to obtain fertilizable eggs. However, further optimization of the treatment, e.g., timing, doses etc., is essential for increasing the success rate of the induction of ovulation.

In summary, recombinant Gths were produced by utilizing a mammalian expression system and ELISAs for Japanese eel. The protein expression of Gths was determined and a high amount of Lh was found to have accumulated in the pituitaries of mature female eels, indicating that Japanese eel is a suitable model for the investigation of the mechanism of Lh release. Recombinant Gths and the ELISAs established in this report may be powerful tools that can be used for a deeper understanding of the differential roles of the two Japanese eel Gths, Fsh and Lh, in gametogenesis, and to optimize methods that can be used to induce oocyte growth/maturation/ovulation for the production of high-quality eggs of this species.

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