# Studies on Follicle-Stimulating Hormone in Japanese Eel, *Anguilla japonica*

（ウナギの卵濾胞刺激ホルモンに関する研究）

A Dissertation Submitted in Partial Fulfillment of the Requirements
for The Degree of Doctor of Philosophy

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# ABBREVIATIONS

| | |
|---|---|
| 11-KT; | 11-ketotestosterone |
| 2-ME; | 2-mercaptoethanol |
| 3-D; | three-dimensional |
| 4-D; | four-dimensional |
| ABC; | avidin-biotin-peroxidase complex |
| ANOVA; | analysis of variance |
| BAS; | bovine serum albumin |
| BCA; | bicinchoninic acid |
| BCIP; | 5-bromo-4-chloro-3-indolyl phosphate |
| BMGY; | buffered glycerol-complex medium |
| BMMY; | buffered methanol-complex medium |
| BSS; | balanced salt solution |
| BW; | body weight |
| CAPS; | 3-cyclohexylamino-1-propanesulfonic acid |
| CBB; | Coomassie brilliant blue |
| CG; | chorionic gonadotropin |
| DAB; | 3, 3'-diaminobenzidine tetrahydrochloride |
| DTT; | dithiothreitol |
| E2; | estradiol-17β |
| FSH; | follicle-stimulating hormone |
| GP; | glycoprotein |
| GSI; | gonadosomatic index |
| GTH; | gonadotropin |
| GnRH; | gonadotropin-releasing hormone |
| HIS; | hepatosomatic index |
| HPG; | hypothalamus-pituitary-gonad |
| HPLC; | high-performance liquid chromatography |
| LH; | luteinizing hormone |
| MAP; | multiple antigen polylinker |
| MEM; | minimum essential medium |

| | |
|---|---|
| NTB; | 4-nitrotetrazolium blue |
| PAGE; | polyacrylamide gel electrophoresis |
| PB; | phosphate buffer |
| PCR; | polymerase chain reaction |
| PFA; | paraformaldehyde |
| PMSF; | phenylmethylsulphonyl fluoride |
| PPD; | proximal pars distalis |
| PVDF; | polyvinylidene difluoride |
| RIA; | radioimmunoassay |
| SDS; | sodium dodecylsulfate |
| SPH; | salmon pituitary homogenate |
| T; | testosterone |
| TFA; | trifluoroacetic acid |
| TSH; | thyrotropin |
| YPD; | yeast extract-peptone-dextrose |
| YPG; | yeast extract-peptone-glycerol |
| cDNA; | complementary deoxyribonucleic acid |

# GENERAL INTRODUCTION

Reproduction is one of the most characteristic features of every organism. The reproduction manner, which is the method for a certain organism to reproduce the same kind, is diversified. It can be roughly classified into sexual reproduction and asexual reproduction. Although advantages and disadvantages exist between the two, the choice of respective manners reflects evolution of the organism. In most cases of asexual reproduction, a certain part of the individual dissociates for a new individual, and thus produced offspring possesses the same genetic information as a parent. Division, sporulation, budding and vegetative reproduction are typically known as manners of asexual reproduction. On the other hand, in most cases of sexual reproduction, sperms as a male gamete and oocytes as a female gamete are produced in the testis and ovary, respectively. In this manner, new individuals are formed by fusion of the specialized distinct cells; that is, sperms and oocytes. Thus, sexual reproduction allows unique recombination of parental genes, a prerequisite for generating a variety in population of a certain species. Only gametes are permitted to run the next generation of the species, and therefore spermatogenesis and oogenesis in respective gonads are critical for successful conservation and evolution of sexually differentiated species, typically seen in vertebrates.

Gametogenesis processes are predominantly controlled by reproductive endocrine systems in most vertebrates; the HPG-axis is mainly involved in the reproductive endocrine system. Internal and external stimuli are integrated within the brain, resulting in an output to the pituitary mediated by the GnRH. In the pituitary, GnRH stimulates secretion of GTH. GTH is transported through the circulation to the gonads, where it stimulates the secretion of sex steroid hormones, and stimulates gamatogenesis. The HPG-axis-dependent reproductive endocrine system is well conserved among vertebrate species, suggesting that conservation of above-mentioned endocrine regulators is essential for successful reproduction of vertebrates. Among these endocrine regulators, GTH, a key substance mediating between the "central" and "terminal" reproductive

signals, has been found in only vertebrates so far, and the chemical properties and physiological roles are relatively diversified and complicated among vertebrate species.

In mammals, FSH and LH secreted from the pituitary gland, as well as CG secreted from the placenta, are categorized as GTH. GTHs form a glycoprotein hormone family together with TSH, and these hormones are heterodimers consisting of two noncovalently associated subunits, a common GPα subunit and a hormone-specific β subunit (FSHβ, LHβ, CGβ or TSHβ) which confers biological specificity to each hormone. Recently, novel glycoprotein hormone subunits, GPα2 and GPβ5, were discovered from the human genome database (Hsu *et al.*, 2002). Thyrostimulin, which consists of GPα2 and GPβ5, is a novel glycoprotein hormone; however, the gonadotropic activity of this novel glycoprotein hormone has not been reported so far (Nakabayashi *et al.*, 2002).

GTHs are chemically complicated ligands with the molecular mass ranging from 28-37 kDa (Ryan *et al.*, 1987). All these hormones possess one or two consensus *N*-glycosylation sites on respective subunits, occupying 15-35% of the total mass. CGβ possesses *O*-linked carbohydrates on its C-terminus region (Hearn *et al.*, 2000). The carbohydrate moieties are critical for preventing from intracellular degradation of the hormones, as well as for subunit folding, heterodimerization and the secretion of the matured hormones (Grossmann *et al.*, 1997; Ulloa-Aguirre *et al.*, 2001; Szkudlinski *et al.*, 2002). In blood circulation, the terminus sialyation or sulfation in the carbohydrate moieties of β subunits is critical for their metabolic clearance (Green *et* al., 1988; Szkudlinski *et al.*, 1993; Baenziger *et al.*, 1992; Perlman *et al.*, 2003). In addition, the specific carbohydrate moiety in α subunit has been reported to be predominantly involved in receptor activation (Sairam *et al.*, 1990; Bishop *et al.*, 1994). Thus, it has been shown that the carbohydrates on respective subunits of GTHs contribute toward maintaining gonadotropic activities in higher vertebrates.

With the recent introduction of molecular biological techniques in endocrinology, such as cDNA cloning, recombinant protein preparation, genetic mutagenesis, gene knock-out/in tequniques and so on (Min *et al.*, 1996; Kumer *et al.,* 1997; Boime and Ben-Menahem, 1999; Saneyoshi *et al*; 2001; Baker *et al.*, 2003; Ma *et al.*, 2004), further studies on more detailed biological functions of GTHs in higher vertebrates are conducted in search of potential application of GTHs, for the purpose of clinical

application and developing more advanced breeding technology (Fares *et al.*, 1992; Lapolt *et* al., 1992; Kanda *et al.*, 1999; Weenen *et al.*, 2004; Min *et al.*, 2004). In the ovary, mammalian FSH predominantly stimulates ovarian follicle development and production of estrogen through aromatization of androgens in granurosa cells, whereas LH stimulates the production of androgen in theca cells (Richards, 1994; Albanese *et al.*, 1996). In the ovary of mature females, FSH-receptor (FSH-R) expression is confined to the granulosa cells. Meanwhile, LH-receptor (LH-R) exists in the theca cells, but also in granulosa cells and luteal cells, depending on the developmental stage of the ovulatory cycles (Camp *et al.*, 1991; Minegishi *et al.*, 1997a and b; Liu *et al.*, 1998). In addition, LH induces ovulation, and maintains the production of progesterone in corpus luteum (Misrahi *et al.*, 1998). In the testis, on the other hand, mammalian FSH and LH influence Sertoli and Leydig cells, respectively, also due to restricted distribution of their specific receptor distributions (Misrahi *et al.*, 1996). LH stimulates androgen production in Leydig cells, whereas FSH enhances Sertoli cell activity to support the germ cells (McLachlan *et al.*, 2002). In testicular development of rodents, FSH also stimulates early Sertoli cell proliferation and differentiation in immature testes (Singh and Handelsman. 1996).

Though elucidation of biological functions of mammalian GTHs is advanced, those in lower vertebrates, like teleosts, are relatively delayed. To date, in spite of request from the fields of basic science and aquaculture industries, abundant homologous GTH is mostly lacking, and thus limited use of homologous GTH partly makes it difficult to progress the functional analysis of teleost GTHs. Indeed, like salmonids, functional analysis of two kinds of GTHs is conducted only in limited species.

In teleosts, two types of GTH have been purified from the pituitaries of chum salmon, *Oncorhynchus keta* (Suzuki *et al.*, 1988a), coho salmon, *Oncorhynchus kisutch* (Swanson *et al.*, 1991), common carp, *Cyprinus carpio* (Van Der Kraak *et al.*, 1992), bonito, *Euthynnus plelamis* (Koide *et al.*, 1993), Atlantic croaker, *Micropogonias undulatus* (Copeland and Thomas, 1993), red seabream, *Pagrus major* (Tanaka *et al.*, 1993), tuna, *Thunnus obesus* (Okada *et al.*, 1994), Mediterranean yellowtail, *Seriola dumerilii* (Garcia-Hernandez *et al.*, 1997), rainbow trout, *Oncorhynchus mykiss* (Govoroun *et al.*, 1997) and mummichog, *Fundulus heteroclitus* (Shimizu and Yamashita, 2002). When firstly isolated from the chum salmon pituitary, the two GTHs

were named GTH-I and GTH-II (Suzuki *et al.*, 1988a). Subsequent attempts to clone cDNAs encoding their specific β subunits revealed the duality of GTHs in fish, as is the case with higher vertebrates (Sekine *et al.*, 1989; Querat *et al.*, 2000; Swanson *et al.*, 2003). Accumulating cDNA sequences and deduced primary structures indicate that teleost GTH-I and II are orthologs of mammalian FSH and LH, respectively. Based on their biochemical properties, teleost GTH-I and II are now renamed FSH and LH, respectively (Li and Ford, 1998).

Since the cDNAs encoding two distinct GTH subunits were cloned, their expression profiles have been investigated in many teleost species. The LHβ gene expression and LH secretion generally increase with the advance of final maturation of gametes (Suzuki et al., 1988b; Breton *et al.*, 1998; Jackson *et al.*, 1999, Melamed *et al.*, 2000; Kajimura *et al.*, 2001a, b; Han *et al.*, 2003; Mateos *et al.*, 2003; Weltzien *et al.*, 2003a). Purified LH virtually stimulates final maturation by inducing progestins (17, 20β-dihydroxy-4-pregnen-3-one; DHP or 17, 20β, 21-trihydroxy-4-pregnen-3-one; 20β-S) secretion in fully matured ovaries (Trant *et al.*, 1986; Suzuki *et al.*, 1988c; Thomas *et al.*, 1989; Koide *et al.*, 1992; Nagahama., 1997; Kagawa *et al.*, 1998). Thus, LH has been established to be essential for the final maturation of gametes in teleosts (Swanson *et al.*, 2003; Yaron *et al.*, 2003). On the other hand, expression patterns of FSHβ genes are diversified among teleost species and between their sexes (Sohn *et al.*, 1999; Gen *et al.*, 2000), suggesting diversed functions of FSH in teleosts.

In red seabream, which spawns daily during the spawnig season, LH stimulates final oocyte maturation, but FSH does not (Kagawa *et al.*, 1998). The gene expression of FSH in red seabream remains low during the spawning period, while that of LH greatly increased (Gen *et al.*, 2000). In goldfish, *Carrasius auratus*, the profile of FSHβ was dissimilar to those of salmonids and red seabream; FSHβ and LHβ mRNAs increase synchronously during its spawning period (Sohn *et al.*, 1999). Accordingly, the profile of FSHβ during ovarian development is diversified among different teleost species with different spawning patterns. Because of the limited species examined so far, it is difficult at present to generalize the physiological functions of teleost FSH in ovarian development. Apparently, more information is required for the comprehensive understanding of teleost FSH functions.

However, biologically active FSH has been successfully isolated in a limited number of

teleosts, due to its small content in the pituitary (Suzuki *et al.*, 1988a; Swanson *et al.*, 1991; Van Der Kraak *et al.*, 1992; Tanaka *et al.*, 1993; Okada *et al.*, 1994; Gracia-Hernandez *et al.*, 1997; Govoroun *et al.*, 1997; Shimizu and Yamashita, 2002; Weltzien *et al.*, 2003b). Inevitably, purified intact FSH is not available for most teleost species, and information on its physiological function is less accumulated than that of LH.

The eel is a highly valued species as a food resource in both Eastern and Western countries. In spite of its economical importance, little is revealed about the reproductive endocrine system and life cycle of this species, partly because the matured eel have not been caught in the wild. Neither maturing silver eel during its spawning migration nor immature cultivated eel undergo further gametogenesis processes in captive conditions. It is well known that eels require the exogenous GTH administration for artificial induction of gonadal maturation, indicating a deficiency in plasma GTHs.

In Japanese eel, *Anguilla japonica*, human CG (hCG) and SPH are often administerd to immature male and female eels, in order to induce the gonadal maturation of the fish (Yamamoto and Yamauchi, 1974; Miura *et al.*, 1991b; Ohta *et al.*, 1996). However, the eggs thus obtained are not always of good quality as fertilization and survival rates are frequently low. It is well known in salmonids that final oocyte maturation is induced by GTH. However, in captive eels, final oocyte maturation is not consistently induced by exogenous GTHs. This makes us consider that the GTHs derived from other species might not be able to execute the essential part of native GTH in this species. However, because isolation of native GTH from eel pituitaries has not been accomplished, it has been difficult to fully elucidate the biological function of the two GTHs in this species.

Today, Japanese eel has been highlighted not only for the importance in aquaculture, but also for its specific biological features as a significant model species. Under culture conditions, as mentioned above, the male Japanese eel stays completely immature. However, the administration of hCG can induce the complete process of spermatogenesis from spermatogonia to sperms (Miura *et al.*, 1991a). Furthermore, spermatogenesis of the eel has also been induced in an *in vitro* organ culture system and a germ-somatic cell co-culture system (Miura *et al.*, 1991b; Miura *et al.*, 1998, Miura *et al.*, 2003). Hence, the fish provides an excellent system for studying the mechanisms of spermatogenesis. Although exogenous GTH (hCG) stimulates spermatogenesis in

immature eel, it is not clear how endogenous GTHs, FSH and LH, contribute toward stimulating spermatogenesis processes in this fish.

The lack of information on the biological activities of eel GTHs delays both improving the technology for artificial maturation and advancing the better understanding of gametogenesis processes of this species. In Japanese eel, cDNAs encoding two GTHs have been cloned, and GTH-Iβ and IIβ subunits were phylogenetically assigned to the FSHβ and LHβ sub-families, respectively (Nagae *et al.*, 1996; Yoshiura *et al.*, 1999). Therefore, in this thesis, GTH-Iβ and IIβ subunits in eel are called FSHβ and LHβ, respectively. However, the biological functions of both eel GTHs have not been elucidated, as it remains difficult to obtain native GTH molecules. Now, production of recombinant Japanese eel GTH is highly desirable.

To date, only exogenous LH or its equivalents have been used for artificial induction of maturation in this species. Consequently, elucidation of the physiological function of the homologous GTHs, especially FSH, is required for the better understanding of gametogenesis processes of eel and for establishment of good techniques for artifitial induction of maturation. Hence, in this thesis, preparation of the homologous eel FSH by genetic engineering and biochemical procedures is conducted for the first time to solve above-mentioned problems.

There are two major aims in this study: one is to shed some light on the functional aspect of eel FSH, and another is to provide a useful tool for improving the artificial maturation of eels. For those purposes, recombinant Japanese eel FSH (rjeFSH) was prepared and its biological activities were investigated *in vitro* and *in vivo* in this thesis. In the first chapter, expression of biologically active rjeFSH using methylotropic yeast, *Pichia pastoris*, was described (Chapter I). Purification of native eel FSH from immature Japanese eel pituitaries was performed and its biochemical properties and steroidogenic activities were investigated in Chapter II. In the course of these studies, it was demonstrated that rjeFSH could express similar steroidogenic activities of native one in immature eel testis. Steroidogenic activities of rjeFSH in different developmental stages of male and female gonads were investigated in Chapter III. Finally, Chapter IV described characterization of specific activities of rjeFSH following purification of biologically active rjeFSH, and the assessment of *in vivo* gonadotropic effect of rjeFSH.

Eel life in nature is so mysterious, as mentioned previously. The preparation of eel FSH is a piece of the study for understanding reproductive endocrine system in eel, and for developing a technique of artificial maturation of this species. It is needless to say that the recombinant hormone, rjeFSH, established in this thesis is an "artificial" tool. I believe, however, that we will be able to induce the eel maturation more "naturally" in future, if rjeFSH would be an aid for elucidating the reproductive systems of this species.

# CHAPTER I

## Expression of a biologically active recombinant follicle-stimulating hormone of Japanese eel, *Anguilla japonica*, using methylotropic yeast, *Pichia pastoris*

### INTRODUCTION

For studying the function of fish GTHs, several recombinant GTHs and GTH subunits have been produced using various expression systems to date (Hew and Yip, 1976; Huang *et al.*, 1991; Elizur *et al.*, 2000; Blaise *et al.*, 2000; Meiri *et al.*, 2000; Choi *et al.*, 2003; Kobayashi *et al.*, 2003; Morita *et al.*, 2003; Vischer *et al.*, 2003; Zmora *et al.*, 2003). The bacterial expression system generally allows high yielding, though the expressed proteins might not have suitable structure (Langley *et al.*, 1987). Since the comparatively high yielding and the suitable forms for expressed proteins are expectable in baculovirus expression system using insect cells, it has been well used for functional expression of recombinant proteins in eukaryote (Lucknow and Summers, 1988). The expression system using mammalian cell line also provides the suitable forms for expressed proteins, however the system costs higher and is generally low yielding. On the other hand, in expression system using yeast, the suitable structure for the expressed proteins and high yielding of recombinant proteins are expectable. Furthermore, the system using yeast can be established more easily than the use of another eukaryotic cells (Sudbery, 1996; Cereghino and Cregg, 2000). Along these lines, in this study, I attempted to produce a recombinant Japanese eel FSH, rjeFSH, in methylotrophic yeast, *Pichia pastoris*, in order to gain better understanding of GTH function in this species.

# MATERIALS & METHODS

### *Animals*

Sexually immature male Japanese eels, *Anguilla japonica*, were purchased from a commercial dealer. Body weight ranged from 200-300 g and GSI was 0.12-0.35%. Immature testes were dissected out from eels anesthetized with 0.05% v/v of 2-phenoxy ethanol (Wako, Japan). Small pieces of each testis were fixed in Bouin's fixative for morphological identification of reproductive stage. Other small portions of about 20 mg were used to estimate *in vitro* steroidogenic activities of hormones.

### *Construction of expression vector*

The cloning of cDNAs encoding Japanese eel GPα and FSHβ subunits (jeGPα, jeFSHβ) was performed as described previously (Nagae *et al.*, 1996; Yoshiura *et al.*, 1999). In this study, we estimated that the N-terminal amino acid residue of jeFSHβ subunit is $Ser^{-3}$ at 3 amino acids upstream from the published $Cys^{1}$ residue, as the probability of signal cleavage between $Ala^{-4}$ and $Ser^{-3}$ is higher than that between $Ser^{-1}$ and $Cys^{1}$ as calculated by the Signal-Scan program (Prestridge, 1991). The four oligonucleotide primers shown in Table 1-1 were designed based on the nucleotide sequences of jeGPα and jeFSHβ subunit cDNA. cDNA fragments encoding mature proteins of the jeGPα and jeFSHβ subunits were amplified by PCR using the primers and inserted into pBluescript II SK(-) (Stratagene, USA) at the *Eco* RI site. PCR was carried out under a cycle protocol of 94 °C for 2min, 15 cycles of 94 °C for 30 sec, 55 °C for 60 sec and 72 °C for 60 sec. Each subunit cDNA was digested by *Eco* RI (TaKaRa, Japan) and ligated into an expression vector pPIC9K (pPIC9K-jeGPα and -jeFSHβ, Fig. 1-1). The DNA ligation was performed according to the handling description using DNA ligation kit (DNA ligation kit, Ver. II; TaKaRa, Japan). The DNA digestion was performed at 37 °C for 1 hr using each restriction enzyme with suitable buffer solution. Subsequently, the jeGTHα subunit expression unit containing a AOX1 promoter, α-factor signal sequence and jeGTHα subunit cDNA was amplified by PCR using the primer set which contained an *Xba* I site (Fig. 1-1). The PCR product was digested with *Xba* I (TaKaRa, Japan) and then the α subunit expression unit was inserted into the pPIC9K-jeFSHβ with the *Xba* I site. This vector obtained was

designated as pPIC9K-jeFSHβ/α (Fig. 1-1). The junction of pPIC9K vector and insertional cDNAs encoding jeFSH subunits are shown in Fig. 1-2. The expression vector, pPIC9K-jeFSHβ/α, contains a histidinol dehydrogenase gene (HIS4) as a marker to isolate *P. pastoris* recombinant strains and a kanamycin registant gene ($Kan^r$) as a marker to select multi-copy inserts by increased resistance to geneticin disulfate. Nucleotide sequences of encoded jeFSHβ and jeGPα subunit cDNAs inserted into the vector and its directions were confirmed by DNA sequencing.

***Expression of recombinant Japanese eel FSH (rjeFSH)***

The expression vector, pPIC9K-jeFSHβ/α, was amplified in bacterial cells (*Escherichia coli* strains) cultured in LB medium containing 1% w/v yeast extract (Difco, USA), 2% w/v Bacto-peptone (Difco, USA), 1% w/v sodium chloride (Wako, Japan) and 0.02 mg/mL of ampicillin sulfate (Wako, Japan) at 37 °C for 18 hrs. The vector was purified using Marligen High Purity Plasmid-Prep Systems (Marligen Biosciences, USA) according to manufucture's instruction. Then, the purified vector was digested with *Bsp* EI (NEB, UK) and utilized for yeast transformation. The methylotrophic yeast *P. pastoris* KM71, which is a histidine-deficient strain (his-), was transformed with the linearized pPIC9K-jeFSHβ/α using a multi-copy *Pichia* expression kit (Invitrogen, USA). Histidine-independent transformants (his+) were selected on a regeneration dextrose medium plate lacking histidine. The plate lacking histidine was composed of 1 M sorbitol (Wako, Japan), 2% w/v dextrose (Wako, Japan), 1.34% w/v yeast nitrogen base with ammonium sulfate and without amino acids (Difco, USA), $4x10^{-5}$% w/v biotin (Wako, Japan) and 0.005% amino acids, L-Glu, L-Met, L-Lys, L-Leu and L-Ile (Difco, USA). Based on the size of colonies corresponding to large number of the transforming DNA on the *Pichia* genome, ten transformants organizing larger colonies were further screened on a YPD plate containing 1% w/v yeast extract (Difco, USA), 2% w/v Bacto-peptone, 0.2% w/v dextrose and 3.0 mg/mL of geneticin disulfate (Sigma, USA). The selected colonies were cultivated in BMGY containing 1% w/v yeast extract, 2% w/v Bacto-peptone, 1.34% w/v, yeast nitrogen base with ammonium sulfate and without amino acids, 1% v/v glycerol, 4x10-5% w/v biotin and 100 mM potassium phosphate under shaking at 29.5 °C for 18 hrs. The cells were harvested by centrifugation at 1,500 x *g* for 10 mins and subsequently resuspended in

1/10 volume of BMMY containing the same components as in BMGY with the substitution of 0.5% v/v methanol instead of 1% v/v glycerol. The incubation was continued at 29.5°C for another 72 hrs. Methanol was added at a concentration of 0.5% every 24 hrs during the period of incubation. The culture supernatant was collected by centrifugation at 1,500 x *g* for 10 mins. The clone that seemed to have the highest expression level was selected by the results obtained from western blotting mentioned thereinafter. As a negative control, *P. pastoris* KM71 transformed with an expression vector not containing the jeFSH subunit cDNAs was cultivated, and fractions were prepared in the same manner.

***Antisera preparation***

Anti-jeGPα, jeFSHβ and jeLHβ subunit antisera were respectively raised in rabbit against the synthetic peptides consisting of 15 amino acid residues corresponding to the amino acid positions 37 to 51 of the jeGPα subunit which was conjugated with MAP-resin (Fig. 1-3), against the synthetic peptide consisting of 18 amino acid residues corresponding to the amino acid positions 32 to 49 of the jeFSHβ subunit which was conjugated with BSA (Fig. 1-3), and against the synthetic peptide consisting of 18 amino acid residues corresponding to the amino acid positions 36 to 53 of the jeLHβ subunit which was conjugated with BSA (Fig. 1-3). These synthetic peptides were designed based on the specific area among subunit molecules. The preparation of synthetic peptides used as antigens and the conjugation of synthetic peptides to each carrier substance (MAP-resin or BSA) were entrusted to the commercial dealer (Sawady Technology, Japan). Each of the synthetic peptide conjugated with carrier substance was injected intradermally in the animal's back. Nine injections were given at 2-week intervals using 300 μg of each antigen dissolved in 1 mL of 0.8% NaCl, emulsified with 1 mL incomplete Freund's adjuvant. The animals were bled 2 weeks after the last injection and respective antisera were prepared. The cross-reaction of anti-jeFSHβ antiserum against jeLHβ antigen or anti-jeLHβ antiserum against jeFSHβ antigen was never observed in the dot blotting. Furthermore, immature and maturing eel pituitary extracts were separated by SDS-PAGE and the immunoreactive tests were performed by western blotting using respective antiserum. The immunoreactive bands were observed over both immature and artificially maturing eel pituitary extracts using anti-jeGPα, and

the immunorective band against anti-FSHβ and anti-jeLHβ antiserum was observed over immature and maturing eel pituitary extract, respectively (data not shown).

***Western blotting***

The culture supernatant (10 μL) of the yeast transformed with pPIC9K-jeFSHβ/α was separated by 15-25% SDS-PAGE under reducing conditions with 6% v/v 2-ME in sample buffer. Next, the gel was blotted onto PVDF membrane (Immobilon Transfer membrane; Millipore, USA). The membrane was incubated in a blocking solution (Block Ace; Dainippon Pharmaceutical, Japan) at room temperature for 1 hr and then immersed in the 1:5000 antisera diluted with the 0.02 M Tris buffered saline containing 0.05% Tween-20 (TBS-T) at room temperature for 90 mins. The membrane was washed three times at room temperature each with TBS-T for 15 mins, and then incubated with 1:5000 alkaline-phosphatase conjugated goat anti-rabbit IgG (Biosource International, USA) diluted with TBS-T for 2 hrs. The membrane was washed again and equilibrated with developing solution (0.1 M Tris-HCl/0.1 M NaCl/0.05 M $MgCl_2$, pH8.0) for 5 mins. Finally, the membrane was incubated with 0.2 M each of BCIP (Sigma, USA) and NTB (Sigma, USA) diluted with the developing solution for 10 mins. The reaction was stopped by immersion in distilled water.

***De-N-glycosylation of rjeFSH***

Yeast culture supernatant (500 μL) containing rjeFSH were desalted with an ultrafree cartridge (cut off 10 kDa; Millipore, USA) and concentrated to a final volume of 10 μL, which was added to a mixture of 10 μL of denaturing buffer consisting of 0.45% SDS, 1 M Tris-HCl (pH 8.0) and 0.1 M 2-ME. The resulting solution was heated at 100°C for 3 mins. Next, 0.001 U of *N*-glycosidase F (Roche, Switzerland) was added to this solution, which was incubated at 37°C for 15 hrs. The reaction mixture was concentrated and applied to western blotting as described above.

***Preparation of glycoprotein fraction from culture supernatant***

Ethanol was added slowly to the culture supernatant to reach a final concentration of 75%. The resultant suspension was incubated at 4°C overnight and then centrifuged at 10,000 x *g*, 4°C for 30 mins. The supernatant was discarded and the precipitate was

washed with 75% ethanol twice. The washed precipitate was dried up and preserved at -80°C until use for bioassay.

***In vitro bioassay***

To date, many bioassays have been developed for investigating GTH activity in testes (Yamazaki and Dnaldson, 1968; Padmanabhan et al., 1987; Cristin-Maitre *et al.*, 2000). In this study, steroidogenic bioassay was applied to the assessment of GTH activity due to its facilities. Immature eel testes were divided into uniformly sized pieces (about 20 mg/piece), and each piece of testis was pre-incubated using 24 well culture plate (IWAKI, Japan) at 20°C for one hr with 1,000 μL of the eel balanced salt solution (BSS: 140 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 1 mM $NaH_2PO_4$, 2 mM $NaHCO_3$, and 1.5 mM $CaCl_2$) containing 0.88 mg/mL of MEM (Eagle MEM; Nissui, Japan), 0.5 mg/mL of glucose and 1.0% w/v of Anti-Biotics & Micotics (Gibco BRL, USA). The glycoprotein fraction after desalting using a PD-10 column (Amersham Biosciences, USA) was added to the medium and the incubation was continued for another 18 hrs. These incubations were performed using duplicated wells per treatment. The incubation medium was collected and stored at -20°C until used. Concentrations of T and 11-KT released into the culture medium were measured by RIA using the procedures described previously (Aida *et al.*, 1984; Kobayashi *et al.*, 1985).

***Statistics***

Statistical significance between control and experimental groups in each assay using immature or maturing testis was determined using one-way ANOVA followed by the multiple range analysis of Dunnett.

## RESULTS

### *Expression of rjeFSH*

An expression vector (pPIC9K-jeFSHβ/α) was constructed as shown in Fig. 1-1. Subsequently, *P. pastoris* was transformed with the pPIC9K-jeFSHβ/α, and His+ transformants were screened. Transformants having multi-copies of pPIC9K-jeFSHβ/α were selected based on hyperresistance to geneticin disulfate, and 10 large colonies were then selected. These colonies were cultured and induced to produce rjeFSH on a small-scale, and the yields of rjeFSH produced from each clone were determined by western blotting. The clone that produced the highest amount of rjeFSH was selected. Next, we examined the time course expression and secretion of the rjeFSH. The selected clone was cultured and the culture medium was collected every 24 hrs. The collected media were subjected to protein assay with BCA protein assay kit (Pierce, USA) and western blotting, which revealed that total protein contents of medium was decreased gradually; however, the yields of the rjeFSH increased gradually up to 72 hrs after induction, and remained unchanged between 72 to 96 hrs (Fig. 1-4). Therefore, the incubation time was chosen as 72 hrs in further experimentation.

The screened clone was cultured for 72 hrs after induction, and the resultant culture supernatant was collected by centrifugation. The culture supernatant was separated by SDS-PAGE under reducing conditions and subjected to western blotting using antisera raised against jeGPα, jeFSHβ and jeLHβ subunits. A broad immunoreactive band at 26 kDa and multiple bands over 50 kDa were detected using the anti-jeGPα subunit antiserum (Fig. 1-5, A, lane 2), and two immunoreacteve bands were detected at 16.4 and 26.4 kDa (Fig. 1-5, B, lane 2) with the anti-jeFSHβ subunit antiserum. As a negative control, the culture supernatant derived from the yeasts transformed with an expression vector not containing jeFSH subunit cDNAs was subjected to western blotting, which also showed multiple immunoreactive bands over 50 kDa against anti-jeGPα subunit antiserum (Fig. 1-5, A, lane 1), whereas any immunoreactive bands over 50 kDa was not detected with the anti-jeFSHβ subunit antiserum (Fig. 1-5, B, lane 1). Immunoreactive bands were not observed when the antiserum raised against jeLHβ subunit was used (Fig. 1-5, C, lanes 1 and 2). After *N*-glycosidase F digestion, each subunit was decreased in molecular mass (Fig. 1-5, A and B, lanes 3).

In western blotting after separation of the culture supernatant derived from the yeasts transformed with an expression vector containing jeFSH subunit cDNAs by native PAGE under non-reducing conditions, a broad band ranged from 39 kDa to 49 kDa was immunoreacted with both antisera raised against jeGPα and jeFSHβ subunits, similarly (Fig. 1-6, A and B, lanes 2). The culture supernatant derived from the yeasts transformed with an expression vector not containing jeFSH subunit cDNAs did not contain the broad band ranged from 39 kDa to 49 kDa (Fig. 1-6, A, lane 1). In culture supernatant derived from the yeast containing jeFSH cDNAs or not, the bands over 50 kDa were slightly immunoreacted with anti-jeGPα subunit antisera after separation of culture supernatant by native PAGE (Fig. 1-6, A, lanes 1 and 2).

***In vitro Bioassay***

Biological activity of rjeFSH was examined by measuring T and 11-KT released from the immature Japanese eel testes. As a positive control, hCG (Sankyo, Japan) (10 international units; IU) which has already been confirmed to stimulate androgen production and initiate spermatogenesis in immature eel testis (Miura *et al.*, 1991b) was utilized. The glycoprotein fraction containing rjeFSH derived from 2.5 mL of yeast culture supernatant stimulated the release of T and 11-KT from immature testis, as did hCG (Fig. 1-7). In this study, the steroidogenic activity of rjeFSH was compared to that of hCG. The addition of rjeFSH increased the androgen secretion from immature eel testis in a dose dependent manner, similarly to that of hCG. As a result, 11-KT secretion activity of rjeFSH derived from 1 L of yeast culture supernatant was corresponding to that of 1500 IU of hCG (Fig.1-8). The glycoprotein fraction derived from 2.5 mL of the culture supernatant of cells transformed with an expression vector not containing jeFSH cDNAs did not stimulate steroid production.

## DISCUSSION

Recombinant proteins are in general very useful tools for the study of biological function, the production of antibodies and the determination of their three-dimensional structures. Biologically active recombinant mammalian GTHs have been produced using mammalian cell lines (Keene *et al.*, 1989; Mountford *et al.*, 1994; Hakola *et al.*, 1997) and insect cell lines (Narayan *et al.*, 1995; Arey *et al.*, 1997; Kato *et al.*, 1998). However, the production of recombinant GTH by the above system is comparatively costing, harder to manipulate and difficult to enlarge the scale of its culture system. In contrast, the system using methylotrophic yeast, *P. pastoris* does not require any special equipment or harder manipulation, and has enabled large-scale production leading higher yields of recombinant proteins, easily. Moreover, *P. pastoris* has also enabled the addition of *N*-linked carbohydrate to the recombinant proteins, which is required to express the biological activities of GTH (Thotakura and Blithe, 1995). In fact, recombinant mammalian GTHs expressed in *P. pastoris* with biological activity equivalent to that of native GTHs for porcine FSH (Richard *et al.*, 1998), ovine FSH (Fidler *et al.*, 1998) and hCG (Gupta and Dighe, 1999). In this backdrop, we attempted the use of *P. pastoris* as a host organism in the expression of rjeFSH.

In this investigation, expression of rjeFSH was detected by western blotting using antisera raised against jeGPα and jeFSHβ subunits. Western blotting after the separation of culture supernatant by SDS-PAGE revealed that each subunit of rjeFSH molecule was successfully synthesized and secreted into the yeast culture medium. Multiple bands over 50 kDa that immunoreacted with anti-jeGPα subunit antiserum were likely nonspecific bands that originated from the yeast, since these bands were also detected in the culture supernatant of the yeasts transformed with an expression vector not containing jeFSH subunit cDNAs. In addition, it was revealed using *N*-glycosidase F that each subunit is *N*-glycosylated. This is consistent with the previous study in which the deduced amino acid sequences of jeGPα and jeFSHβ subunits have *N*-linked glycosylation site (Nagae *et al.*, 1996; Yoshiura *et al.*, 1999). The multiple immunoreactive bands of rjeFSHβ subunit were detected before and after deglycosylation. These results suggest that the expressed jeFSHβ subunit is glycosylated and some types of carbohydrates which were added to the expressed

jeFSHβ subunit might interrupt deglycosylation, resulting in the multiple immunoreactive bands both before and after *N*-glycosidase F treatment.

Western blotting after the separation of culture supernatant by native PAGE under non-reducing conditions suggested that α and β subunits of rjeFSH associate and form a heterodimeric molecule, as a broad band ranged approximately 39kDa to 49kDa similarly immunoreacted with both antisera against α and β subunits of jeFSH. The moleculer mass of the broad band is in agreement with that of native FSH purified in other teleosts and that of which expressed jeGPα and jeFSHβ met. It is thought that the expressed jeFSHβ subunit is associated with a subunit immunoreacted with anti-jeFSHβ and observed in a broad band. The bands over 50 kDa slightly immunoreacted with anti-jeGPα subunit antiserum after separation by native PAGE and SDS-PAGE would be non-specific immunoreactive bands caused by the same molecules.

*In vitro* bioassay using immature Japanese eel testis was performed in order to investigate the biological activity of rjeFSH. Ten IU of hCG promoted T and 11-KT secretion from immature eel testis, and this demonstrated that the culture system used in this study is suitable for investigating hormone-induced steroidogenic activity in eel testis. The glycoprotein fraction derived from 2.5 mL of the culture supernatant of yeast transformed with an expression vector containing jeFSH cDNAs stimulated the secretion of T and 11-KT, whereas that of the cells transformed with an expression vector not containing jeFSH cDNAs did not. These results clearly indicate that the yeast produced biologically active rjeFSH. It has been reported that 11-KT activates the function of Sertoli cells allowing the completion of spermatogenesis in Japanese eel (Miura *et al.*, 2003). Moreover, T is known as the precursor of not only 11-KT but also E2, which induces spermatogonial renewal in this species (Miura *et al.*, 1999). In this way, T and 11-KT are essential for the induction of spermatogenesis in eel. It has been thought that the increase in T and 11-KT secretion is induced via native eel GTH secreted during the early stage of gametogenesis.

At present, knowledge concerning the functional features of the two types of fish GTH remains limited. A major reason is due to the difficulty in obtaining sufficient quantities of GTH, as many individual animals are needed for the collection of pituitaries and purification of GTH. Moreover, FSH and LH have many similar chemical properties, making it difficult to separate them. Among these lines,

recombinant GTH is a useful tool for elucidating these problems, and the rjeFSH obtained in this study will assist greatly in further investigation of the biological function of fish GTH.

The primary structures of GTH and TSHβ subunits have been elucidated in many teleost species including eel based mainly on cDNA cloning. Phylogenetic analyses of pituitary glycoprotein hormones have revealed that teleost GTH-I and -II belong to FSH and LH sub-families, respectively. In this chapter, it has been confirmed that rjeFSH stimulates T and 11-KT production in immature eel testis, suggesting that native eel FSH, in addition to salmonid FSH, possesses mammalian FSH-like activity that is critical for early gonadal development but not for the final maturation. Therefore, the data obtained in this study supports to rename GTH-I in eel as FSH not only from its phylogenetical aspect but also from its biological function.

In conclusion, producing of the biologically active recombinant Japanese eel FSH using methylotrophic yeast was successful. This is the first arrival of eel GTH with biological activity. This recombinant hormone is expected to contribute to further our understanding of eel reproductive endocrinology.

# CHAPTER II

## Purification of follicle-stimulating hormone from immature Japanese eel, *Anguilla japonica*, pituitaries and its biochemical and physiological properties

## INTRODUCTION

In Chapter I, we produced a recombinant Japanese eel FSH (rjeFSH) and investigated its biological activities in the eel testis. Although rjeFSH was biologically active, there is no means to compare the activities between recombinant and native eel FSHs, because of unavailability of native eel FSH.

Crystallization study and reconstitution of 3-D structure suggest that N-terminal amino acid sequences of GTH subunits are important for the formation of a biologically active heterodimeric GTH molecule (Keutmann, 1992). The N-terminal amino acid of the rjeFSH subunit was designed according to a sequence predicted from cDNA, whereas the chemical structures including the N-terminal amino acid sequences of native eel FSH subunits have not yet been examined. In order to solve the above problems, in this chapter, native eel FSH was purified and its chemical properties and biological activities were examined.

# MATERIALS AND METHODS

### *Collection of Pituitary gland*

For the purification of eel FSH, whole pituitary glands (approximately 10 g) were collected from sexually immature cultivated eel heads, which were kindly provided by a commercial dealer (Sanwa Tansuigyo, Japan). Pituitaries were frozen immediately in liquid nitrogen and stored at -80 °C.

### *Chromatography procedures*

Pituitaries were homogenized in 0.2 M ammonium acetate, pH 6.1, containing 0.02 M PMSF and 0.05 M EDTA, on ice using a Polytron homogenizer (Iuchi, Japan). The homogenate was stirred at 4 °C for 1 hr and centrifuged at 15,000 x *g* for 30 min. The pellet was re-extracted to maximize the recovery. The resulting supernatant was immediately subjected to gel-filtration chromatography on a Sephadex G-100 (Pharmacia biochemicals, USA) column (1.6 x 98 cm) equilibrated with 0.05 M ammonium acetate, pH 9.0, at a flow rate of 3 mL/200 sec/tube at 4 °C. The absorbance was measured at 280 nm and immunopossitive fractions against anti-Japanese eel FSHβ antiserum (aniti-jeFSHβ) were collected. The specific molecular mass was estimated by SDS-PAGE under a non-reducing condition. The gel-filtration fraction containing eel FSH was subsequently applied to anion-exchange chromatography on a DE-52 (Watman, UK) column (1.77 x 30 cm) equilibrated with 0.05 M ammonium acetate, pH 9.0, at a flow rate of 3 mL/200 sec/tube at 4 °C, and unabsorbed proteins were eluted with the same buffer. Absorbed proteins were eluted with stepwise gradients of 0.15, 0.45 and 1.0 M ammonium acetate, pH 9.0. The FSH-containing fractions were lyophilized and dissolved in 0.01 M Tris-HCl, pH 7.0. The fraction was then subjected to further anion-exchange chromatography using HPLC. HPLC was performed on a TSK-gel Super-Q-5PW column (Tosoh, Japan) with a linear gradient of 0-0.5 M NaCl, 0.01 M Tris-HCl, pH 7.0, at a flow rate of 0.5 mL/min at 20 °C for 40 min. Reverse-phase HPLC (rpHPLC) was performed on a TSK-gel ODS-120T column (Tosoh, Japan) with a linear gradient of 15-45% $CH_3CN$ with 0.1% TFA, pH 2.0, at a flow rate of 0.5 mL/min for 60 min at 30 °C. Electrophoresis and western blotting were performed as described in Chapter I.

***De*-N-*glycosylation***

FSH purified from 3 g of immature eel pituitaries was desalted with a 10 kDa cut off ultrafree cartridge (Millipore, USA) and concentrated to a final volume of 50 μL. The denaturing buffer (2.5 μL), consisting of 1% SDS, 0.01 M Tris-HCl, pH 8.6, and 0.001 M 2-ME (Wako, Japan) was added to the concentrated purified FSH solution. The solution was subsequently heated at 100 °C for 3 min. The distilled water (13 μL) and 0.001 U of glycopeptidase F (Takara, Japan) were added to this solution, followed by incubation at 37 °C for 15 hr. The reaction mixture was concentrated and applied to western blotting after separation by SDS-PAGE under a reducing condition.

***N-terminal amino acid sequencing***

Protein samples were separated by SDS-PAGE and blotted onto a PVDF membrane. The membrane was washed with 0.01 M CAPS-buffer, pH 9.0, twice and stained with CBB R-250 (Bio-Rad, CA). The membrane carrying FSH subunit proteins were cut out and applied to a protein sequencer (Applied Biosystems model 491cLc) in a pulsed-liquid mode.

***Heterodimer stability test of intact eel FSH***

To understand the chemical bond of eel FSH subunits, immature eel pituitary homogenate containing intact eel FSH and purified eel FSH were treated with 0.1% TFA or 5% 2-ME at 25 °C for 15 mins. They were then separated by SDS-PAGE under a non-reducing condition, and the associated states of respective FSH subunits were analyzed by subsequent western blotting.

***Pituitaries for western blotting and immunohistochemistry***

For the use of western blotting, maturing female eels were injected with SPH to enhance LHβ mRNA expression in the pituitaries (Suetake *et al.*, 2002). For the use of immunohistochemistry, pituitary of immature cultivated eel was fixed in 4% PFA in 0.1 M PB, pH 7.4, for 18 hr. To examine the distribution of FSH-producing cells, pituitary sections were stained immunohistochemically with the anti-jeFSHβ. After fixed in PFA solution, the pituitaries were dehydrated in ethanol, and embedded in Paraplast. Sagittal

sections were cut at 4 μm thickness and mounted on glass slides (Matsunami, Japan). The sections were immunohistochemically stained by the ABC method (Hsu *et al.*, 1981), using commercial reagents (Vectastain ABC kit; Vector Laboratories, USA). To detect the immunoreactivity, epitope-unmasking treatment was made for the deparaffined section before immunostaining. The sections were immersed in an unmasking solution (12% 2-ME in 0.01 M DTT) for 30 min, and were washed with distilled water. After the unmasking treatment, deparaffined sections were incubated sequentially with: (1) 0.6% $H_2O_2$ for 30 min, (2) 2% normal goat serum for 30 min, (3) anti-jeFSHβ or anti-jeLHβ diluted at 1:1000 at 4 °C, (4) biotinylated anti-rabbit IgG for 30 min, (5) ABC for one hr, and (6) 0.02% DAB containing 0.005% $H_2O_2$ for 5 min. The sections were observed under a microscope (Nikon E800, Japan) equipped with a differential interference contrast device. The specificity of the immunoreaction was confirmed by incubating the sections with normal rabbit serum in place of the specific antibody, or incubating the sections without the epitope-unmasking treatment.

***In vitro bioassay***

Sexually immature male Japanese eel weighing 200 to 300 g, were purchased from a commercial dealer (Sanwa Tansuigyo, Japan). The average GSI was 0.1%. Testes were dissected out from the eel anesthetized in 0.05% v/v of 2-phenoxy ethanol (Wako, Japan). The testes were divided into small pieces, and each piece of the testis was pre-incubated at 20 °C for 1 hr in a well of a 48-well microtiter plate (Iwaki, Japan) with 300 μL of the BSS containing 0.88 mg/mL of MEM (Eagle MEM; Nissui, Japan), 0.5 mg/mL of glucose and 1.0% w/v of Anti-Biotics & Mycotics (Gibco BRL, USA). After preincubation, the medium was changed to fresh one containing various doses of native eel FSH, immature eel pituitary extract, rjeFSH and hCG, and the incubation was continued for another 24 hr. The incubation medium was collected and stored at -20 °C. Content of purified native eel FSH was measured with BCA protein assay kit (Pierce, USA). Concentrations of T and 11-KT released into the culture medium were measured by RIA according to the methods described previously (Aida *et al.*, 1984; Kobayashi *et al.*, 1985).

***Statistics***

Statistical significance between control and experimental groups in the assay was determined by one-way ANOVA, followed by the multiple range analysis of Dunnett.

## RESULTS

### *Purification of intact eel FSH*

Proteins extracted from pituitaries were separated to 6 fractions (G-1~G-6) according to their molecular mass by gel-filtration on a Sephadex G-100 column (Fig. 2-1). In western blotting, only the G-4 fraction with molecular mass of approximately 30-40 kDa was immunoreactive to anti-jeFSHβ. When this fraction was subjected to stepwise elution of anion-exchange chromatography on a DE-52 column, elute was separated into 5 fractions (Fig. 2-2). Among these fractions, western blotting and CBB staining revealed that D-0.15a and b fractions contained FSH (Fig. 2-2). These fractions (D-0.15a and b) were mixed (D-0.15 fraction) and were further separated by anion-exchange chromatography on a TSK-gel Super-Q column using HPLC. The D-0.15 fraction was separated into 10 fractions (Fig. 2-3, Q-1~Q-10). CBB staining and western blotting after separation by SDS-PAGE under a non-reducing condition showed that one major peak, Q-7, was a highly purified protein reacting with both anti-jeGPα and anti-jeFSHβ, but not with anti-jeLHβ (Fig. 2-4, A). The molecular mass was estimated to be approximately 33kDa. These results indicate Q-7 is a highly purified FSH fraction.

### *Chemical properties of eel FSH and its subunits*

When subjected to SDS-PAGE under a reduced condition, the purified FSH decreased its molecular mass, and distinct proteins with the molecular mass of approximately 19 kDa and 21 kDa were detected by CBB staining (Fig. 2-4, A). Anti-jeGPα specifically reacted to the 19 kDa protein (Fig. 2-4, A). Anti-jeFSHβ reacted to the major 21 kDa and minor 17 kDa proteins. Each anti-jeFSHβ-possitive protein would be jeFSHβ subunits having distinct carbohydrate modification.

When the homogenate of maturing female eel pituitaries was subjected to SDS-PAGE under a reducing condition, approximately 18 kDa protein specifically reacted to anti-jeLHβ, whereas no protein reacted to anti- jeFSHβ (Fig. 2-4, B). It was already confirmed that the 18 kDa protein possesses predicted N-terminal amino acid sequence of jeLHβ subunit (data not shown). Anti-jeGPα also reacted to the 17 kDa and 19 kDa proteins (Fig. 2-4, B). The 17 kDa immunopositive protein would be

jeGPα with carbohydrate modification different from that of 19 kDa jeGPα.

N-terminal amino acid sequences of these proteins were revealed as follows: $NH_2$-Tyr-Pro-Asn-Asn-Glu-Met... (anti-jeGPα-positive 19 kDa band) and $NH_2$-Ser-Thr-Ser-X (unidentified) -Gly-...(anti-jeFSHβ-positive 21 kDa band), corresponding to predicted N-terminal amino acid sequences of jeGPα and jeFSHβ mature peptides, respectively (Fig. 2-5).

After de-*N*-glycosylation treatment with *N*-glycopeptidase F, anti-jeGPα and anti-jeFSHβ reacted to 13 kDa and 15 kDa proteins, respectively (Fig. 2-6). These results clearly show the both subunits decreased their molecular mass by de-*N*-glycosylation and were *N*-glycosylated.

In acidic and reductive conditioned solution adjusted with TFA and 2-ME, respectively, intact FSH in the immature eel pituitary homogenate was separated to respective subunits (Fig. 2-7). Purified intact eel FSH was also dissociated to respective subunits in the same test (data not shown). The anti-jeGPα reacted to 13 kDa and 19 kDa proteins in the immature eel pituitary homogenate under non-acidic and non-reductive conditions (Fig. 2-7). Both anti-jeGPα-possitive 13 kDa and 19 kDa proteins might be innate free jeGPα molecules in the immature eel pituitary. Together with the results of de-*N*-glycosylation study, the 13 kDa jeGPα in immature eel pituitary may not be *N*-glycosylated (Fig. 2-7).

***Localization of FSH in eel pituitary***

Immunoreactive FSH cells were detected in PPD, but not in the other part, of the immature eel pituitary section with the epitope-unmasking treatment (Fig. 2-8, A), while FSH cells were hardly detected without the unmasking treatment. (Fig. 2-8, B). On the other hand, immunopositive LH cells were not observed in the immature eel pituitary regardless of the unmasking treatment (Fig. 2-8, C).

***In vitro bioassay of eel FSH in immature testis***

Effects of native eel FSH on T and 11-KT secretion from the immature eel testis are shown in Fig. 2-9. Purified native eel FSH significantly enhanced both T and 11-KT secretion in dose-dependent manners at concentrations between 0.11 to 3.0 μg/mL. Similarly, serially-diluted immature eel pituitary extract, rjeFSH and hCG activated T

and 11-KT secretion from immature eel testes (Fig. 2-9).

## DISCUSSION

Previous studies on the expression profiles of FSHβ and LHβ mRNAs in immature and artificially matured eel pituitaries suggest that a larger amount of FSH would be accumulated in immature eels than in maturing or matured ones, and that little LH would be synthesized in immature fish (Nagae *et al.*, 1996; Yoshiura *et al.*, 1999; Suetake *et al.*, 2002). In this study, we successfully purified eel FSH from pituitaries of cultivated immature eels without contamination of LH and other proteins, as determined by CBB staining and western blotting (Fig. 2-4).

In this study, the N-terminal amino acid sequences of α and β subunits of eel FSH were identified, coinciding with those predicted from cDNA sequences. In addition, the de-*N*-glycosylation study revealed that both subunits of eel FSH were *N*-glycosylated. This is consistent with previous findings that the deduced amino acid sequences of jeGPα and jeFSHβ subunits have *N*-linked glycosylation sites (Nagae *et al.*, 1996; Yoshiura *et al.*, 1999). The purified FSH is thus likely to be composed of the subunits previously identified by cDNA cloning. In most vertebrates, GPα and FSHβ contain 10 and 12 Cys residues, respectively (Swanson *et al.*, 2003). This is also confirmed in Japanese eel, suggesting that the 3-D structure of eel FSH would be very similar to that of GTHs in other species reported previously (Lapthorn *et al.*, 1994). However, in some teleosts such as chum salmon, coho salmon and Mediterranean yellowtail, it is accepted that one species has the chemically distinct types of FSHs: one is acid-stable type, and another is acid-unstable type molecule (Suzuki *et al.*, 1988d; Swanson *et al.*, 1991; Gracia-Hernandez *et al.*, 1997). It is suggested that the acid-stable FSH is composed of disulfide-bonded α and β subunits (Suzuki *et al.*, 1988d). Thus, the acid-stable FSH potentially harbors a 3-D structure different from that of conventional non-covalently associated heterodimers. In this study, it is clearly showed that eel FSH is not an acid-stable type molecule (Fig. 7). Interestingly, the Cys positions of FSHβ subunits of the acid-stable type FSH are partially different from those of eel FSHβ subunit.

The cDNA sequences encoding Japanese eel GPα and FSHβ subunits permitted investigating the functional aspects of eel FSH through genetic engineering techniques (Chapter I). When the rjeFSH expression vector was designed, the N-terminal amino acid of the recombinant subunit was determined according to their deduced amino acid

sequences. It is suggested that the construction of heterodimeric form and biological activities of GTH are influenced by the N-terminal amino acid sequence of its β subunit (Keutmann, 1992). In this study, native and recombinant eel FSH expressed similar biological functions in terms of steroidogenic activities. This suggests that rjeFSH can substitute for native one in further experiments. However, the specific activity of native and recombinant ones cannot be compared directly, since rjeFSH has not been fully purified. If the purified rjeFSH can be obtained and compared its specific activity with that of native one, whether or not the N-terminal amino acid sequences of rjeFSH subunits impair FSH activity will be investigated.

Previous studies have demonstrated that exogenous GTH (hCG) stimulates *in vivo* 11-KT secretion in immature eel, and that 11-KT is necessary for promoting spermatogenesis processes of this species (Miura *et al.*, 1991a and b). It is not clear, however, which endogenous GTH, FSH or LH, mainly contributes toward stimulating 11-KT secretion in immature eel testes. Subsequent to the purification of native eel FSH, we investigated its steroidogenic activity, using an *in vitro* tissue incubation system. Purified eel FSH significantly increased T and 11-KT secretion in immature eel testes in dose-dependent manners. As expected, rjeFSH and immature eel pituitary homogenate containing intact eel FSH also enhanced the androgen secretion, and the dose responsiveness was similar to that of purified eel FSH. The activities observed in the immature eel pituitary homogenate would be attributed to FSH, since the content of LH in the pituitary is most likely to be much less than that of FSH (Fig. 2-8). These results suggest that FSH is the GTH, which triggers 11-KT-inducing spermatogenesis in this species.

Both anti-jeFSHβ and anti-jeLHβ used in this study can not recognize respective epitopes without epitope-unmasking treatment of the samples, probably because the antibodies may not approach the epitopes in the intact heterodimeric molecules (Chapter I). In this study, the immunoreactivities were only observed in the sections with the epitope-unmasking treatment. This corresponds well to the results of western blotting in Chapter 1. Immunohistochemistry for eel GTHβ subunits demonstrated that the FSH, but not LH, is mainly synthesized and accumulated in the immature eel pituitary (Fig. 2-8). LH was mainly accumulated in the pituitary of sexually matured eel, as suggested from western blotting (Fig. 2-4, C). All these findings suggest that FSH is the principal

GTH in the immature eel pituitary. This does not oppose the notion that FSH might be more potent in immature gonads than LH (Suzuki *et al.*, 1988c; Suetake *et al.*, 2002).

As mentioned above, there are several lines of evidence that FSH is the predominant GTH molecule in the immature eel, and that FSH induces *in vitro* androgen secretion in the immature testis. Nevertheless, the gonadal development of cultivated eel is completely arrested, suggesting that the mRNA expression and/or protein synthesis of eel FSH might be inactivated or inhibited under cultivated conditions. Establishment of measurement systems for eel GTHs is important to investigate the malfunction of the pituitary-gonad axis in the cultivated eel. Since purified eel FSH and the recombinant one with equivalent bioactivities are now available, an immunoassay system for FSH measurement is ready to be established.

The LH-like GTH in human, hCG, activated the androgen secretion as well as eel FSH. It is likely that hCG may mimic FSH, combining with FSH-R. If eel FSH-R cDNA is cloned and becomes available, expression analysis with the cDNA and the binding assay would elucidate whether or not hCG really combines with eel FSH-R in the immature eel testis. It is widely accepted among vertebrates that androgens are synthesized by Leydig cells in the testis. In the immature eel, FSH may also act on Leydig cells to produce T and 11-KT through FSH-R. However, neither the localization of FSH-R nor its expression pattern has been reported in this species. Purified eel FSH and the recombinant one will be of great significance for characterizing the biological features of eel GTH receptors, as recently reported in some other teleost species, such as amago (Oba *et al.*, 1999a and b), common carp (Basu *et al.*, 2002), chanel catfish (Bogerd *et al.*, 2001; Vischer *et al.*, 2003), and zebrafish (Laan *et al.*, 2002).

# CHAPTER III

## Steroidogenic activities of follicle-stimulating hormone in the testis and ovary of Japanese eel, *Anguilla japonica*

## INTRODUCTION

In the previous chapter (Chapter I and II), the native eel FSH and its recombinant (rjeFSH) were prepared, and their steroidogenic activities in the immature eel testis were examined. The results showed that rjeFSH and native FSH were similarly active in promoting *in vitro* androgen secretion from the immature testis. Thus, rjeFSH could induce the same activities of native eel FSH in terms of steroidogenic activity. This allows us to explore further functional significance of FSH in eel.

The steroidogenic activity of rjeFSH, as mentioned above, has been assessed only in immature eel testis. On the other hand, the physiological roles of eel FSH during the testicular development and oogenesis processes are still uncertain. In this chapter, to further extend our understanding on the physiological functions of eel FSH, the steroidogenic activities of rjeFSH during different reproductive stages of the testis and ovary were examined.

## MATERIALS AND METHODS

### *Animals*

Sexually immature male Japanese eels were purchased from a commercial dealer. Body weight ranged from 200-300 g and GSI was 0.12-0.35%. Sexually maturing male eels, with GSI of 3.8-6.5% were obtained by weekly injection (5 times in total) of human CG (hCG; Sankyo, Japan) (300 IU/fish/week) into immature male eels. Testes were dissected out from eels anesthetized with 0.05% v/v of 2-phenoxy ethanol (Wako, Japan). Small pieces of each testis were fixed in Bouin's fixative for morphological identification of its reproductive stage. Other small portions of about 20 mg were used to estimate *in vitro* steroidogenic activities of hormones.

Maturing female silver eels weighing approximately 240-1,000 g were caught in Mikawa estuary, Aichi, Japan, transported to Tokyo, and reared in a 3-t tank with recirculating seawater at 12-15 °C. To obtain the ovaries with different developmental stages, maturation was induced artificially by injecting the eels with SPH. Weekly intramuscular injections of SPH at a dose of 20 mg/200 mL 0.9% NaCl/individual were repeated 10 times. Fish were sampled before SPH injection and one week after the 1st, 4th, 7th and 10th injection. At the time of each sampling, five fish were used for the following histological observations of the ovary and *in vitro* steroidogenic bioassay. After anesthesia with 0.3% (v/v) 2-phenoxyethanol, eels were weighed, and the ovary was dissected out and weighed for calculating the GSI and HSI. Some pieces of the ovary were fixed in Bouin's fixative for 18 hr for histrological observations. Another portion of the ovary (approximately 150-200 mg) was placed in BSS and immediately used for *in vitro* bioassay, as described below.

### *Tissue sampling for histological observations*

Gonads fixed in Bouin's fixtative were dehydrated through an ethanol series and embedded in Paraplast. The sections were cut at 5 μm thick and mounted on glass slides (Matsunami, Japan). They were stained with Mayer's hematoxylin and eosin to determine the reproductive stages. Then, the diameter of oocytes was measured. For more detailed histological observations, another piece of the fixed ovary was dehydrated through an ethanol series and embedded in Spurr's resin (Polysciences, USA). Sections

were cut at 1 μm thick with glass knives, mounted on glass slides, and stained with 1% toluidine blue. The sections were observed under a light microscope, Nikon E800 (Nikon, Japan).

***Gonadotropins and cyclic AMP***

For *in vitro* bioassay, recombinant Japanese eel FSH (rjeFSH) was prepared as described in Chapter I. Salmon GTH fraction (sGTH) was prepared as previously reported in Sato *et al.* (2000). The sGTH is composed of mostly salmon LH and a smaller amount of FSH. Human chorionic GTH (hCG) was purchased from a commercial dealer (Sankyo, Japan). It is widely known that, in most vertebrates, cAMP functions as an intraceller second messenger of GTH in the target cells. As a positive control for inducing GTH signals in ovarian follicle cells, a membrane permeable cyclic AMP analogue (8-Br-cAMP; Sigma, USA) was added to the incubation medium to a final concentration of 0.1, 1.0 and 10 mM.

***Steroidogenic bioassay***

For the steroidogenic bioassay, the testes and ovaries divided into small pieces were pre-incubated at 15 °C for 1 hr in a well of a 48-well microtiter plate (Iwaki, Japan) with 500 μL of eel BSS containing 0.88 mg/mL of the MEM (Eagle MEM; Nissui, Japan), 0.5 mg/mL of glucose and 1.0% w/v of Anti-Biotics & Mycotics (Gibco BRL, USA). After preincubation, the medium was changed to fresh one containing rjeFSH (1 IU/mL and 10 IU/mL), sGTH (1 μg/mL and 10 μg/mL), hCG (10 IU/mL), or 8-Br-cAMP (0.1, 1.0 and 10 mM), and the incubation was continued for another 24 hr. The incubation medium was then collected and stored at -20 °C. Concentrations of T and 11-KT secreted from the testes, T and E2 secreted from the ovaries into the culture medium were measured by RIA according to the methods described previously (Aida *et al.*, 1984; Kobayashi *et al.*, 1985).

***Statistics***

Statistical significance between control and experimental groups in the assay was determined by one-way ANOVA, followed by the multiple range analysis of Dunnett. Significance was set at $P<0.05$.

## RESULTS

### *Steroidogenesis in immature and maturing eel testes*

rjeFSH was examined in terms of steroidgenic activity using immature and maturing Japanese eel testes. Histological observation showed that immature testis contained only non-proliferated spermatogonia (type A spermatogonia), whereas maturing testis contained proliferated germ cells; spermatocytes and spermatids (Fig. 3-1). rjeFSH significantly stimulated the release of T and 11-KT from immature testis in a dose dependent manner (Fig. 3-2), while maturing testis did not respond (Fig. 3-3). Moreover, 10 IU of hCG, sufficient amount for promoting T and 11-KT secretion from immature eel testis (Fig. 3-2), failed to induce a significant increase in androgen secretion from maturing testis (Fig. 3-3).

### *Classification of maturing ovaries*

Oocyte growth and ovarian follicle development following SPH injections were investigated histologically (Fig. 3-4). The ovaries before the SPH injection possessed pre- or early-vitellogenic oocytes. Around those oocytes, the two cell layers of the ovarian follicle, the theca and granulosa cells, were poorly developed and were hardly distinguishable. The vitellogenesis was promoted by repeated SPH injections, and the oocytes finally reached the mid-vitellogenic stage. In accordance with oocyte growth, the ovarian follicle underwent morphologically distinct developmental processes. During early to mid-vitellogenic stages, the outer theca cells became evident, appearring as flat cells surrounding the oocyte, while the inner granulosa cells were still undeveloped or poorly developed Development of granulosa cells were followed by that of theca cells, as the oocyte developed into the mid-vitellogenic stage. Based on the oocyte growth and follicular development, the ovarian development in the intact and maturation-induced eels was classified into the following three categories (Table 3-1 and Fig. 3-4):

Type A, the ovary possessing poorly developed theca and granulosa cells with pre- or early vitellogenic stage oocytes (150-249 μm in diameter), typically seen in the ovaries before pituitary homogenate injection (n=6, Fig. 3-4, A, B).

Type B, the ovary possessing flat-shaped theca cells and poorly developed granulosa cells around oocytes at the early to mid-vitellogenic stage (250 -349 μm, n=7, Fig. 3-4, C, D).

Type C, the ovary possessing well developed theca and granulosa cells around mid-vitellogenic oocytes (350-450 μm). Granulosa cells of cuboidal shape are typically arranged to form a monolayer epithelium (n=12, Fig. 3-4, E, F).

***Steroidogenic activities of rjeFSH, sGTH, hCG and 8-Br-cAMP in eel ovaries***

Effects of rjeFSH on ovarian steroidogenesis were examined by *in vitro* ovary incubation. The results of T and E2 secretion were expressed as the percentage of the mean value of the saline control in respective treatment to rule out varied individual differences. In the type-A ovary with undeveloped theca and granulosa cell layers, rjeFSH did not induce significant enhancement of both T and E2 secretion. In those ovaries, both T and E2 were not stimulated by 8-Br-cAMP, either (Fig. 3-5). In the type-B ovary with developed theca and poorly developed granulosa cells, rjeFSH significantly stimulated T secretion in a dose-dependent manner (Fig. 3-6, A, rjeSH), but both doses of rjeFSH did not affect E2 secretion (Fig. 3-6, B, rjeFSH). Similarly, T secretion, but not E2, was greatly increased by 8-Br-cAMP in the type-B ovary (Fig. 3-6, 8-Br-cAMP). In the type-C ovary with well-developed theca and granulosa cells, rjeFSH as well as 8-Br-cAMP significantly stimulated both T and E2 secretion (Fig. 3-7).

The sGTH and rjeFSH showed similar steroidogenic activities in any ovarian developmental stage. In type-A and B ovaries, steroidogenic activities of hCG (10 IU/mL) were also similar to those of rjeFSH at a dose of 10 IU/mL; however, hCG failed to stimulate E2 secretion in the type-C ovary (Fig. 3-6, A and B, hCG). There were positive correlation between the oocyte diameter and E2 release from eel ovaries stimulated by rjeFSH ($P<0.0001$, $R^2=0.56$); however, no significant correlation between the oocyte diameter and T release from eel ovaries stimulated by rjeFSH (Fig. 3-8).

## DISCUSSION

*In vitro* bioassays using different reproductive stages of Japanese eel testes showed that rjeFSH stimulated T and 11-KT secretion in immature testis, but not in maturing testis. These results indicate that rjeFSH has higher steroidogenic activity in immature Japanese eel testis than in maturing testis, and the sensitivity to FSH is considered to decrease with the testicular maturation. The mechanism underlying the changes of FSH-inducing steroidogenic activities according to the sexual maturity is interesting. At this point, it can be thought that, at least, three key factors are involved in this mechanism: i) FSH-R in steroidogenic cells, ii) changes of steroidogenic cell density and iii) the functional changes of steroidogenic cells. It is suggested that, after spermatogenesis is completed, DHP is importantly involved in the regulation of sperm maturation (Miura *et al.*, 2003). Specific progestins (DHP and 20β-S) have also been identified as the maturation-inducing hormone of many teleost species (Nagahama and Adachi, 1985; Thomas and Das, 1997) and are converted from progesterone, a precursor of above-mentioned androgens. Thus, it is likely that the testicular steroidogenic cells change its function from androgen synthesis to progestin synthesis when its maturity reaches a certain point.

The changes in FSH-inducing steroidogenic activities in eel testes might be related to the expression profiles of FSH-R molecules. It is probable that rjeFSH binds to the FSH-R in immature eel testis, and stimulates steroidogenesis. However, the cellar localization of eel FSH-R in immature testis is not still fully shown to date. If FSH-targeting cells are identified, we will be able to discuss more detailed mechanisms on changing FSH inducible steroidogenic activities during the testicular maturation of this species. Additionally, in this study, both rjeFSH and hCG which belongs to the LH sub-family exhibited similar activities in immature and maturing eel testis in terms of the steroidogensis. However, it is unclear which receptor hCG binds to, that for FSH or LH in immature eel testis. Therefore, the investigation for cellular localization and profiles of GTH-Rs in eel testis should be conducted in future.

In this chapter, the effects of rjeFSH on *in vitro* steroidogenic activities in the ovaries at different developmental stages were also examined. In teleosts, it is well established that two distinct cells types in the ovarian follicle are involved in E2 synthesis; that is,

the special theca cells in the outer layer and granulosa cells in the inner layer (Nagahama, 1997). In the ovarian follicles, cholesterol is first converted into T through a series of enzyme reactions in the special theca cells, and then T is aromatized into E2 in the granulosa cells. Based on development of the theca and granulosa layers, responsible for production of T and E2, respectively, in the ovarian follicle as well as the oocyte growth, we classified the ovarian developmental stage into three types. It seems reasonable to attempt to correlate those ovarian development stages with *in vitro* T and E2 production in the ovary.

In the type-A ovary with poorly developed theca and granulosa cells, GTHs examined here had no stimulatory effects on T and E2 production. This coincides well with the results that the circulating E2 levels in artificially maturing female eels with pre- and early-vitellogenic ovaries were comparatively low and were increased only slightly by exogenous GTH administration (Matsubara *et al.*, 2002; Adachi *et al.*, 2003). This can be explained by the poor development of the ovarian follicles, which are seemingly insensitive to GTHs at least in terms of steroidogenesis. The lack of steroidogenic activities was further confirmed by cAMP treatment, which was expected to maximize potential steroidogenic activities in the ovary.

In the following developmental stage of the type B, the theca layer started developing in advance of the granulose layer. Such morphological observations imply enhancement of T production in the theca without further conversion into E2 in the granulosa. In fact, GTHs stimulated T secretion, but failed to enhance E2 production. It is thus considered that the secretion of T predominates over that of E2 in the type-B ovary, as also observed in the ovary incubated with cAMP.

When the ovary reached the type C, the granulosa cells became evident to form the two-layer structure of the ovarian follicles, suggesting that the follicles are fully developed for E2 production with enhanced aromatase activity in the granulosa. It is already shown that, during the artificial maturation of eel, the expression of ovarian aromatase is hightest in the mid-vitellogenic stages, and these ovaries are classified as type C in this study. In contrast with type-A and B ovaries, the type C responded well to GTHs, secreting both T and E2 into the culture medium. All these findings are in good agreement with the "two-cell model" that T and E2 are synthesized in the theca and granulosa, respectively (Nagahama *et al.*, 1997). It is notable that steroidogenic

activities of the ovarian follicles are clearly reflected by the histological observations on the oocytes and the surrounding follicle tissues, both developing synchronously.

In this study, we compared *in vitro* ovarian steroidogenic activities between rjeFSH and sGTH. sGTH preparation, which is composed mostly of salmon LH with a much smaller amount of FSH, exhibited similar steroidogenic activities, although effective doses could not be directly compared among them. It is thus speculated that, in Japanese eel, both FSH and LH similarly stimulate ovarian steroidogenesis according to the ovarian developmental stage. This is in accordance with the findings reported in salmonids that both FSH and LH have potentially similar steroidogenic activities, both promoting *in vitro* E2 secretion (Suzuki *et al.*, 1988a and b). In those salmonid species, the gene expression and circulating levels of FSH are reported to be higher than those of LH during the vitellogenic phase, suggesting that FSH, rather than LH, functions as a regulator for ovarian E2 production under the natural conditions. In Japanese eel, however, the profiles of FSH and LH during its native reproductive cycle are unknown; it is reported that the pituitary FSHβ mRNA levels are decreased by exogenous GTH treatment for artificially inducing maturation (Yoshiura *et* al., 1999; Suetake *et al.*, 2002), but it is not clear whether or not those profiles reflect the biological events that actually occur in the wild. Considering that rjeFSH is effective in promoting E2 secretion in mid-vitellogenic ovary rather than in pre- or early-vitellogenic ovary, it is highly likely that, in Japanese eel, FSH is also responsible for E2 production in the ovarian follicles and simulates vitellogenin production.

In spite of limited information on the reproductive endocrinology of Japanese eel in nature, some aspects of FSH and E2 profiles have been investigated in another anguilliform species, common Japanese conger, *Conger myriaster*, caught from the wild (Kajimura *et al.*, 2001b). The plasma E2 in conger increases synchronously with pituitary FSHβ mRNA levels, and both plasma E2 and FSHβ mRNA reach the peak levels during active vitellogenesis. This provides another piece of evidence that FSH is involved in vitelllogenesis though the stimulation of E2 production in Japanese eel.

In addition to sGTH, the ovarian experiments were also conducted using hCG in this chapter. In any developmental stage, hCG affected T secretion in a similar manner to rjeFSH. On the other hand, whereas 10 IU/mL of rjeFSH stimulated E2 secretion from the type-C ovaries, the same unit of hCG failed to stimulate E2 secretion from the same

ovary. These results suggest that effects of hCG to eel ovarian follicle cells are different from those of rjeFSH. The rjeFSH and sGTH showed similar steroidogenic activities in each reproductive stages of the ovary. To date, it is uncertain which type of GTH receptors can bind to exogenous GTHs, such as hCG and sGTH. Considering with the results in eel testes, thus, it will be increasingly important to focus on GTH receptors for the better understanding of the reproductive systems of eels.

It is reported that the spermatogenesis in eel could be induced by 10 ng/mL of 11-KT *in vitro* and *in vivo* (Miura *et al.*, 1991a and b). The immature eel testis already possesses steroidogenic activity and FSH sensitivity, and 11-KT secretion is actually promoted by FSH in the immature eel testis. On the other hand, oogenesis processes in this species seem to be more complicated. In the present study, we focused on short-term effects of GTHs on *in vitro* ovarian steroidogenesis. In the stage-A ovary, although any GTH failed to stimulate remarkable production of ovarian steroids, it does not necessarily indicate that GTHs have no effect on ovarian development. Considering that the pituitary GTH initiates vitellogenesis in oocytes, and that the vitellogenesis is triggered by E2 produced in the ovarian follicles, it is most probable that GTH, presumably FSH, stimulates ovarian development so that the follicles could acquire the steroidogenic ability. I could not address, in the present study, a long-term effect of GTH on ovarian follicle development. Although it is still uncertain in teleosts, FSH has been shown to be involved in ovarian follicle development, as well as in steroidogenesis, in higher vertebrates (Kumar *et al.*, 1997; Burns *et al.*, 2001; Eimerl and Orly, 2002). If the eel ovary can be treated with FSH for extended periods, it could be investigated how the eel FSH affects the ovarian follicle development. The abundant amount of recombinant protein, rjeFSH, will enable us to conduct the long-term *in vivo* examination, instead of scant purified native eel FSH, in future.

# CHAPTER IV

## Purification, specific biological activities and *in vivo* gonadotropic effects of recombinant Japanese eel follicle-stimulating hormone

## INTRODUCTION

In Chapter I, the expression system of recombinant Japanese eel FSH (rjeFSH) was established, and the steroidogenic activities in both eel testes and ovaries *in vitro* have been characterized. We have also revealed that the steroidogenic activity of the recombinant eel FSH is similar to that of the native counterpart. However, the specific activity of rjeFSH remains unclear, because the purification of rjeFSH has not been completed. Additionally, it is still uncertain whether or not rjeFSH is also effective in *in vivo* treatment. To utilize the recombinant protein for studying or controlling the reproduction of this species, information on more detailed biochemical propeties and its *in vivo* bioactivity of rjeFSH is essential.

Thus, in this chapter, purification of rjeFSH was attempted for the characterization of its biochemical properties. Then, the specific activity of purified rjeFSH was investigated and compared with that of native eel FSH. We also characterized *in vivo* effects of rjeFSH by multiple injections to immature male eels.

# MATERIALS AND METHODS

### *Expression of rjeFSH*

Expression of rjeFSH is performed according to the method described in Chapter I with some modifications in the compositions of yeast culture medium and culture scale. The transformed yeast, *P. pastoris*, (pPIC9K-jeFSHβ/α in KM71 strain; see, Chapter I) was grown with shaking at 30 °C for 24 hr in 10 mL of YPD solution which is composed of 1% yeast extract (Difco, USA), 2% polypeptone (Dainippon Seiyaku, Co., LTD, Japan) and 2% dextrose (Wako, Japan) in a sterilized 50 mL tube. Then, the solution was added to the 200 mL of YPG solution which is composed of 1% w/v yeast extract, 2% w/v polypeptone and 1% v/v glycerol (Wako, Japan) in a sterilized 500 mL flask. Shaking was then continued at 30 °C for another 18hr. Next, 100 mL of prior 200 mL YPG solution with well-grown transformed yeast was added to 2 L of newly prepared YPG solution in sterilized 5 L flasks in duplicate, and the yeast was cultivated under shaking at 30 °C for another 18 hr. All the yeast was harvested by centrifugation at 1,500 x *g* for 5 min and was washed by sterilized water. As a pre-induction phase, the yeast was suspended in 500 mL of YP solution composed of 1% w/v yeast extract and 2% w/v polypeptone, and was incubated in 5L flask under shaking at 26 °C for 24 hr. After the pre-induction step, the incubation was continued at 26 °C for another 72 hr. Methanol was added to the medium for expression induction at a final concentration of 0.5% v/v every 24 hr during the period of induction. The culture supernatant containing rjeFSH was collected by centrifugation at 1,500 x *g* for 15 min. The *P. pastoris* KM71 transformed with an expression vector without the jeFSH subunit cDNAs was cultivated in the same manner as rjeFSH producing yeast for preparation of "mock" group.

### *Purification of biologically active rjeFSH*

For the first step of purification, ethanol was added slowly on ice to the culture supernatant containing rjeFSH to reach a final concentration of 80%, and the resultant suspension was settled for 18 hr at 4 °C. The precipitate was collected by centrifugation at 15,000 x *g* for 15 min and dissolved in 0.02 M Tris-HCl, pH 7.0. The solution was desalted using a PD-10 column (Pharmacia Biothech, USA). The desalted solution was separated on a DEAE-FF column (Pharmacia Biothech, USA) by stepwise elution with

0.3 M, 0.6M and 1.0 M NaCl in 20 mM Tris-HCl, pH 7.5. The rjeFSH-containing fraction was confirmed by western blotting, and the fraction was desalted for further anion-exchange HPLC on a TSK-gel DEAE-5PW column (7.5 mm x 7.5 cm; Tosoh, Japan). Separation was performed with a 40-min linear gradient of 0-0.3 M NaCl in 0.02 M Tris-HCl, pH 7.0 at a flow rate of 1.0 mL/min. The elution was monitored by measuring the absorbance at 280 nm. The immunoreactivity against to each jeFSH subunits and the biological activities of each fraction were assessed as described previously. For further purification, the fraction with biological activity was applied to HPLC on the same column. Separation was performed with a 40-min linear gradient of 0.05-0.3 M NaCl in 0.02 M Tris-HCl, pH 8.0. The fraction of purified rjeFSH was desalted using a PD-10 column and then lyophilized immediately. The purified rjeFSH was stored at -80 °C until use.

***In vitro steroidogenic bioassay***

In this chapter, the promotion of 11-KT secretion from immature eel testis was served as an index of the specific biological activity of eel FSH, since its effectiveness was demonstrated in native eel FSH in Chapter II. The steroidogenic bioassay using immature eel testis was performed as described in Chapter I. Serially diluted purified rjeFSH at final concentrations of 0.001, 0.01, 0.1, 1.0, 10 and 100 μg/mL was tested in the steroidogenic bioassay. As a comparison, purified native eel FSH at final concentrations of 0.001, 0.01, 0.1, 1.0 and 10 μg/mL was also tested in the same assay. The concentrations of both purified FSHs were measured by BCA protein assay kit (Pierce, USA).

***Heterodimer stability test of rjeFSH***

To understand the chemical bond of rjeFSH subunits, purified rjeFSH was treated with 0.1% TFA or 5% 2-ME at 25 °C for 15 mins. They were then separated by SDS-PAGE under a non-reducing condition, and the associated states of respective FSH subunits were analyzed by subsequent western blotting using antisera raised against respective jeFSH subunits.

***In vivo rjeFSH administration***

In this study, immature cultivated male eels weighing approximately 150 g were purchased from a commercial dealer (Sanwa tansuigyo, Japan), and acclimated to recirculating seawater in a 1-t tank at 12-15 °C. Partially purified rjeFSH (DEAE-FF 0.3 M fraction) with biological activity, confirmed by *in vitro* bioassay, was utilized for *in vivo* administration to the male eels. Intramuscular injections of rjeFSH at doses of 0.1 and 1.0 IU/g-BW in 300 μL of 0.9% NaCl (saline) were repeated 3 times. The injections were performed at days 0, 3 and 7 and the fish were kept untile day 12. As a comparison, injections with only saline, the "mock" yeast culture meidum similarly processed as rjeFSH containing yeast culture medium, and hCG at a dose of 1.0 IU/g-BW in about 300 μL of 0.9% NaCl were conducted. Fish were sampled before injection and on day 12. At the time of sampling, eels were anesthetized with 0.3% (v/v) 2-phenoxyethanol and weighed, and then testes and livers were dissected out and weighed for calculating GSI and HSI. Plasma samples were also collected to measure the circulating andorogen levels.

***Histological observation***

Testes were fixed in 2% PFA - 2% gltaraldehyde in 0.1 M PB, pH 7.4 for 18 hr. They were then dehydrated through an ethanol series and embedded in Spurr's resin (Polysciences, Inc. PA). Sections were cut at 1 μm thick with glass knives, mounted on glass slides, and stained with 1% toluidine blue. The sections were observed under a light microscope, Nikon E800 (Nikon, Japan).

***Statistics***

Statistical significance between control and experimental groups was determined using ANOVA, followed by the multiple range analysis of Dunnett.

## RESULTS

### *Purification of biologically active rjeFSH*

In respective purification steps, the fraction containing biologically active rjeFSH was confirmed by western blotting and subsequent steroidogenic bioassay. All of expressed rjeFSH was precipitated in 80% ethanol solution. The ethanol precipitated fraction was roughly separated on a DEAE-FF column, and rjeFSH was eluted by 0.3 M NaCl (DF-0.3). No rjeFSH was detected in other fractions, confirmed by western blotting and steroidogenic bioassay (data not shown). DF-0.3 was applied to HPLC on a TSK-gel DEAE-5PW column and separated into 5 fractions (DP7-1-5; Fig. 4-1). The rjeFSH was contained in fraction DP7-2 and DP7-3, and the biological activity was observed only in DP7-2 (Table 4-1). DP7-2 was further separated on the same column under the different elution condition. Finally, highly purified rjeGTHs, DE8-1 and DE8-2, were obtained (Fig. 4-2, -3), and only DE8-1 possessed steroidogenic activiy. DE8-2 was biologically inactive (Fig. 4-4). It is supposed that both DE8-1 and DE8-2 contain purified rjeFSH which possess heterodimeric structure and carbohydrate moiety in respective subunits judging from their molecular mass (Fig. 4-3, -5).

### *Specific activity of rjeFSH*

Although the DE8-2 did not show steroidogenic activity, DE8-1 showed the activity at the concentration of 10 μg/mL *in vitro*. However, purified native eel FSH showed its activity at the concentration of 0.1 μg/mL. The 100 μg/mL of biologically active rjeFSH was shown to be equivalent to 1 μg/mL of native eel FSH (Fig. 4-4). Thus, the specific biological activity of purified rjeFSH (DE8-1) is lower than that of native eel FSH by about two orders of magnitude.

### *Chemical bonds of the subunits of biologically active and inactive rjeFSH*

To consider the chemical structures of the biologically active and inactive rjeFSH, the subunit structures of both rjeFSHs were analyzed by an acid-stability test. Biologically active fraction (DP8-1) possessed both acid-unstable and stable rjeFSH. In acidic conditioned solution (0.1% TFA, pH2.0), acid-stable rjeFSH was dissociated to respective α and β subunits as well as native eel FSH, whereas acid-stable rjeFSH

maintained heterodimeric state (Fig. 4-5, A). On the other hand, the biologically inactive counterpart possessed only acid-stable rjeFSH (Fig. 4-5, B). All rjeFSHs in both biologically active and inactive fractions were dissociated to respective subunits in the solution containing 0.5% 2-ME (Fig. 4-5, B).

***Gonadotropic activity of rjeFSH in vivo***

The biologically active rjeFSH contained in DF-0.3 fraction stimulated the gonadal development of immature male eels. The experimental design of *in vivo* rjeFSH administration is shown in Fig. 4-6. The GSI value of rjeFSH (1.0 IU/g-BW) injected eels significantly increased compared to that before injection (Fig. 4-7, initial controls). As well as rjeFSH, hCG could increase the GSI of immature cultivated male eel. In contrast, other treatments, such as saline and mock, did not (Fig. 4-7). Moreover, plasma T and 11-KT levels were significantly increased by injections of 1.0 IU/g-BW of rjeFSH and hCG, whereas other treatments did not alter the levels (Fig. 4-8). The accumulation of oil droplets in the seminiferous lobules typically increased in the testes of the eels injected with 1.0 IU/g-BW of hCG or rjeFSH (Fig. 4-9). Early type-B spermatogonia (E-GB) were frequently observed in the testes of the eels injected with 1.0 IU/g-BW of hCG or rjeFSH, whereas most of the germ cells are type-A spermatogonia (GA) in the other groups. Moreover, both 1.0 IU/g-BW of hCG and rjeFSH induced the initiation of spermatogenesis of eels, as evidenced by the appearance of late-type B spermatogonia (L-GB) with a denser and heterogeneous nucleus in the testes of these experimental groups. It is in contrast with the absence of L-GB in other groups (Fig. 4-9).

## DISCUSSION

One major aim of this chapter is to obtain information on the detailed chemical and biological properties of rjeFSH produced by the yeast expression system established in Chapter I. Another aim is to investigate whether rjeFSH applicable to the artificial maturation of the eel. In this study, the purification of rjeFSH and *in vivo* administration of rjeFSH were conducted.

The HPLC analysis indicated that rjeFSH was eluted in various retention time on a DEAE-5PW column. Finally, two purified rjeFSH fractions, DP8-1 and DP8-2, were obtained. The specific activities of those fractions and native eel FSH were tested by *in vitro* steroidogenic bioassay using immature eel testis. Only DP8-1 enhanced 11-KT release from immature eel testis; however, the specific biological activity of rjeFSH in DP8-1 was much lower than that of native eel FSH.

Both biologically active and inactive rjeFSH possessed similar molecular weight to native eel FSH, suggesting that these biologically distinct rjeFSHs were similarly glycosylated heterodimers, and other chemical structure, such as the disulfide bond manner in intra-/inter-subunits and 4D-structure, would be varied among them. In order to investigate the disulfide bond manners of both active and inactive rjeFSH, acid stability test was performed, conducted on native eel FSH in Chapter II. As a result, it was shown that biologically active fraction (DP8-1) contained acid-unstable rjeFSH, whereas inactive counterparts (DP8-2) did not. However, the biologically active fraction also contained acid-stable rjeFSH. These results suggest that the acid-stable rjeFSH is inactive and consisted of covalently bridged $\alpha$ and $\beta$ subunits, the 4-D structure being different from native eel FSH probably due to abnormally inter-subunits disulfide bridges. Thus, it is likely that the DP8-1 contains both biologically active and inactive rjeFSHs, and is not fully purified. All of these data show that further purification with another chromatography, such as hydrophobic chromatography, is needed to determine the specific activity of biologically active rjeFSH. The acid-stability test will be an aid for conducting further purification.

It is conceivable that the carbohydrate moiety of native and recombinant eel FSHs is different, and that the carbohydrate moiety is important for the biological activity of GTHs (Sairam *et al.*, 1990). In another report, it is revealed that *P.pastoris* could

modify the carbohydrate moiety in an appropriate site such as *N*- and *O*-glycosylation sites though sialic acid were hardly involved in its glycan structure. The biological significance of sialic acid in teleost GTHs was unveiled though it is reported only in higher vertebrates. If the native eel FSH is treated with sialidase, and the steroidogenic activity before and after the treatment is investigated, biological significance of sialic acid in eel FSH would be shown.

*In vivo* administration of rjeFSH induced significant GSI increases in cultivated immature male eels in a dose-dependent manner. This result clearly demonstrates that rjeFSH is effective for the gonadal development of theeel testis *in vivo*. During the *in vivo* experiment, neither the body weights nor HSI changed (Table 4-2). The saline and mock treatment did not induce testicular development in the experiment. Additionally, no mortality was observed by rjeFSH administration through the experiment. These results support that rjeFSH might not be toxic for eels.

To date, it is reported that hCG strongly induces 11-KT production in eel (Miura *et al.*, 1991a; Ohta *et al.*, 1996). As well as hCG, the 1.0 IU/g-BW of rjeFSH stimulated testicular steroidogenesis and elevated circulating T and 11-KT levels significantly. The remarkable accumulation of oil droplet is well consistent with the activated steroidogenesis in the testes of these experimental groups. In this species, it is well established that 11-KT is essential for the promotion of spermatogenesis processes (Miura *et al.*, 1991b; Miura *et al.*, 2003). T is the important steroid as a precurser of 11-KT. In this study, rjeFSH increased plasma T and 11-KT levels similar to hCG; however, it is uncertain whether or not eel FSH and hCG stimulates the same cells in the eel testis. Further binding assay with testicular membrane preparation will provide the answer to the question, and the purified rjeFSH obtained in this study will be useful for conducting the binding assay.

Intact cultivated male eel possess immature testis mostly consisting of type-A and early type-B spermatogonia that are spermatogonial stem cells. In this experiment, the meiotic germ cells were not observed due to a small amount of injected hormones and the short term of the experiment; however, 1.0 IU/g-BW of rjeFSH induced both spermatogonial renewal and spermatogenesis processes, indicated by the increase in the freaquency of early- and late type-B spermatogonia in these testes. This is consistent with the results that both GSI and androgen secretions were significantly induced by

rjeFSH in a dose-dependent manner. Taken together, rjeFSH is effective for the gonadal development of immature male eel and is now available for both *in vivo* experiments and artificial induction of immature eel.

In this study, rjeFSH was injected to only male eels. Further study must be conducted using immature previtellogenic female eels for the elucidation of the reproductive systems in this species in more detail. Previous studies revealed that pre-vitellogenic eel oocytes possess immature follicle layers. If the rjeFSH could enhance effectively the development of eel follicular cells, the novel method of the artificial induction of eel maturation will be established utilizing the homologous eel FSH, rjeFSH.

# GENERAL DISCUSSION

In Japanese eel, *Anguilla japonica*, the administration of exogenous GTH is necessary for the artificial induction and completion of gonadal maturation due to its GTH deficiency under captive conditions. The isolation of native eel GTH has not been accomplished, which has made it difficult to fully elucidate biological functions of the two GTHs (FSH and LH) in eel. To date, it has been repeatedly pointed out that only the use of exogenous LH-like GTHs for the artificial induction of eel maturation should be reconsidered for the better induction of maturation. However, through the vertebrates, it was extremely hard to purify large amounts of native FSH without contamination of LH. As stated previously, the analyses of FSH functions in higher vertebrates have been proceeded by introducing newly developed experimental procedures (see, general introduction). However, some of these procedures, such as gene knock-out techniques (Kumer *et al.*, 1997; Baker *et al.*, 2003; Ma *et al.*, 2004), can not be applied to the study in teleost species. Moreover, there is no data on the biological activity of eel FSH so far. Under the circumstances, it seemed to be essential to prepare the eel FSH and to assess its biochemical features for the analyses of FSH functions in this spieces. Therefore, in this study, I tried to prepare homologous eel FSH with biological activities using genetic engineering and biochemical procedures.

***Expression of recombinant eel FSH (rjeFSH)***

Initially, in order to provide an abundant experimental tool for studying the physiological functions of eel FSH, the recombinant Japanese eel FSH, named rjeFSH, was produced with biological activity using methylotrophic yeast, *Pichia pastoris*. For this, an expression vector in which jeFSHβ and jeGPα subunit cDNAs were tandemly connected was constructed. This vector permitted the characteristic dual expression of both subunits under the regulation of identical promoter within a single vector, and this makes it possible for the equal production of respective subunits. The construction of similar expression vector was previously reported in producing mammalian FSHs

(Fidler *et al.*, 1998). Successfully, both glycosylated subunits were expressed and were heterodimerized with each other, and thus produced rjeFSH was secreted into the yeast culture medium. In *in vitro* assay, rjeFSH stimulated the release of T and 11-KT from immature eel testes. This is the first observation that how the eel gonad was stimulated by homologous GTH.

Establishment of this expression system enables us to obtain a large amount of biologically active homologous eel FSH (approximately 1,500 IU in 1 L of yeast culture supernatant) within a week without enormous efforts for collecting huge numbers of pituitaries. If the biologicall activity of rjeFSH is identical to the native eel FSH, produced recombinant protein will be a powerful tool for studying reproductive endocrinology of eel. The expression system using *P. pastoris* is expected to produce biologically active recombinant glycoprotein hormones (Cereghino and Cregg, 2000; Fidler *et al.*, 1998; Gupta *et al.*, 1999), and, virtually bioactive rjeFSH was successfully expressed in this study. Although the expression profiles of FSH subunit cDNAs were already studied during artificial maturation (Yoshiura *et al.*, 1999; Suetake *et al.*, 2002), it was uncertain that whether or not native eel FSH showed the same biological effect as that of rjeFSH, because the native eel FSH had not been isolated and its biological activities are not investigated so far.

***Purification of native eel FSH and characterization of its biochemical properties***

Hence, to obtain further information on the biochemical and biological features of eel FSH, intact eel FSH was purified from immature Japanese eel pituitaries by gel-filtration and ion-exchange chromatography. Purified eel FSH was about 33 kDa heterodimeric molecule consisting of distinct glycoprotein subunits, GPα and FSHβ, whose N-terminal amino acid sequences coincided with those of predicted jeGPα and jeFSHβ mature peptides. This provides direct evidence that intact eel FSH is produced and accumulated in immature eel pituitaries. Cells reacting with anti-jeFSHβ antiserum were observed in PPD of an immature eel pituitary, while jeLHβ-immunoreactive cells were not detected, suggesting eel FSH, not LH, is a predominant GTH in immature eel. It should be noticed that all of native eel FSH was dissociated into distinct α and β subunits in the acidic solutions (0.1% TFA, pH 2.0). This suggest that native eel FSH possesses similar 4-D structure, which is formed by non-covalently associated distinct α and β subunits, to that

of generally known as GTH structures through the vertebrates. Thus, it is likely that eel, unlike another teleost species such as salmonids, has no acid-stable type FSH which is probably formed by covalently bonded α and β subunits.

Gonadotropic activities of eel FSH were demonstrated *in vitro* by stimulating T and 11-KT secretions in immature eel testes. Purified eel FSH stimulated the secretion of both androgens from the immature eel testis in dose-dependent manners, similar to immature eel pituitary homogenate and recombinant eel FSH produced by yeast. The 11-KT is a spermatogenesis-inducing steroid in this species (Miura *et al.*, 1991b). Both native and recombinant eel FSH clearly stimulated 11-KT secretion in immature eel testis. These results demonstrated that endogenous FSH in this species and homologous recombinant one possess similar activities, presumably stimulating the gametogenesis through the sex steroid secretion during the early stages of gonadal development.

***Biological significance of eel FSH on its steroidogenesis and spermatogenessis***

Recently, Miura *et al.* (2002) discovered "spermatogenesis-preventing substance (SPS)" in immature eels testis. They clearly demonstrated that 11-KT suppresses SPS mRNA expression and inducees spermatogenesis processes in this species. SPS is thought to be a mammalian "anti-Müllerian hormone (AMH)" homolog in eel owing to their primary structure similarities. In mammals, it is known that AMH is synthesized in Sertoli cells and induces the regression of the anlagen of the uterus and oviduct in the male fetus (Josso *et al.*, 1993; Teixeira *et al.*, 2001). In addition, a growing amount of evidence indicates that AMH also acts as a negative modulator of Leydig cell differentiation and andorogen secretion from the cell (Teixeira *et al.*, 2001; Josso *et al.*, 2001). Recently, it was also reported that AMH production is increased by FSH stimulation through Sertoli cell proliferation and an enhancement of AMH gene transcription (Lukas-Croisier *et al.* 2003). It is uncertain whether or not eel FSH directly increases SPS gene transcripts; however, it is likely that eel FSH suppresses SPS gene expression through 11- KT secretion. Thus, it is highly possible that FSH in eel and mammals differently acts on the spermatogenesis.

In mammalian testes, it is widely known that steroidogenesis is one of the major functions of Leydig cells and LH stimulates this function (Saez, 1994). This strongly suggests that eel FSH stimulates steroidogenesis of immature eel testes through FSH-R

probably existing on Leydig cells in immature eel testis. In testes of mammals, however, the FSH-Rs were observed only in Sertoli cells (Misrahi *et* al., 1996; McLachlan *et al.*, 2002; Allan *et al.*, 2004). Previous studies indicated the presence of two types of GTH-Rs in teleosts; one that interacts with both GTHs and another that interacts with either GTH (Miwa *et al.*, 1994; Oba *et al.*, 1999a and b). However, in teleost species, the expression and localization of GTH-Rs are not generalized to date. Further studies on GTH-Rs in eel will provide information not only for investigating FSH functions in this species but also for understanding the differences in FSH function between teleosts and mammals.

***Characterization of in vitro steroidogenic activities of rjeFSH in eel testis and ovary***

To investigate the further physiological significance of eel FSH in the processes of gametogenesis, steroidogenic activities of rjeFSH were assessed by *in vitro* bioassays using different reproductive stages of Japanese eel testes. It was clearly shown that rjeFSH stimulates T and 11-KT secretion in immature testes but not in maturing ones. This suggests that rjeFSH is highly potent in immature testes, while the potency is reduced in maturing testis. To date, little is known on how the dual GTHs are involved in the testicular development of teleost species. Eel is now an excellent model for investigating spermatogenesis, since the whole processes of eel spermatogenesis, from completely immature spermatogonia to sperms, can be induced by 11-KT treatment *in vitro* (Miura *et al.*, 2003). While, eel FSH significantly induces 11-KT secretion from immature eel testis, it is uncertain whether or not eel FSH possesses biological activities other than steroidogenesis. Thus, in future, it seems worthwhile searching factors involved in such non-steroidogenic FSH activities by utilizing the cDNA subtraction methods, *i.e.*, subtracting the cDNAs expressed in 11-KT treated testis from those of FSH stimulating testis.

The ovaries used in Chapter III could be classified into three stages (type A, B and C) by the difference in developmental stages of ovarian follicular cells. Type-A ovary possessed just poorly developed follicular cells, and rjeFSH did not induce significant sex steroid secretion. Considering that cAMP could not enhance both T and E2 secretion in type-A ovaries, it is suggested that the steroidogenic activity of poorly developed follicle cells is still weak or the population of the steroidogenic cells is smaller. Meanwhile, T

secretion was stimulated by rjeFSH in the type-B ovary with well-developed theca cells, but E2 secretion was not. This suggests that developed steroidogenic theca cells were stimulated by rjeFSH. In the type-C ovary with fully developed theca and granulosa cells, rjeFSH stimulated both T and E2 secretion in a dose-dependent manner. These results suggest that rjeFSH stimulates both theca and granulosa cells, and that aromatase activity is accelerated by eel FSH in the granulosa cell, which develop following the theca cell development in this species. These data also suggest that eel FSH is more potent in maturing ovary than in less developed one.

In terms of steroidogenesis, the eel testes and ovaries in different reproductive stages showed unique responses to rjeFSH; rjeFSH remarkably stimulated immature testes, not maturing ones, whereas maturing ovaries showed higher response to rjeFSH than immature ovaries. Thus, sexually different responses to FSH are a highly interesting phenomenon, suggesting that the physiological roles of eel FSH are differentiated between males and females. It is supposed that these differences are partly due to differences in developmental manners and physiological roles of the FSH-targeting cells between male and female eel gonads. Further investigation on FSH-targeting cells will lead to better understanding of physiological roles of FSHs not only in eel but also in teleosts in general.

***Purification of biologically active rjeFSH***

For the more detailed biochemical characterization, rjeFSH was purified, and the specific biological activity was investigated by *in vitro* steroidogenic bioassay. Anion-exchange chromatography finally separated rjeFSH into biologically distinct two components; one is biologically active and, another is inactive. The *in vitro* bioassay showed that the specific activity of the bioactive rjeFSH fraction is lower than that of purified native eel FSH by about two orders of magnitudes. In this study, the acid-stability test showed that the biological active fraction contains at least two types of molecules, one is acid-unstable rjeFSH similar to native eel FSH, and another is acid-stable one. On the other hand, the biologically inactive fraction did not conatined acid-unstable rjeFSH. These results suggest that the biological active fraction is not completely purified. Actually, it can not be simply compared the specific activity of native eel FSH with that of rjeFSH. Thus, further purification will elucidate the specific

activity of biologically active rjeFSH. These results also suggest that the expression system utilizing in this study can produce a mixture of structurally different FSH molecules. Further determination of 3-D and 4-D structures of these biologically distinct rjeFSHs and their subunits will provides some insight for elucidating structure-function relationships of teleost FSHs. In future, the effect of *N*-linked carbohydrate moieties in rjeFSH should be also investigated for the characterization of its specific bioactivities.

***In vivo administration of rjeFSH***

In *in vivo* bioassay, rjeFSH significantly increased GSI values and plasma T and 11-KT levels of cultivated male eels. Furthermore, the spermatogenesis process of the eels treated with rjeFSH was advanced in a dose-dependent manner probably due to the induced circulating 11-KT. These results clearly demonstrate the rjeFSH is effective in promoting eel gonadal development and its gamategenesis *in vivo*, meaning that rjeFSH is applicable to *in vivo* administration. It is noteworthy that the accumulation of oil droplet was characteristically observed in the seminiferous lobules of the rjeFSH-administrated eel testes. It is unclear that the effect is induced by rjeFSH directly or through some other mediating factors such as androgens. Further *in vivo* experiments will provide the more detailed information on FSH functions in this species.

As described in general introduction, the terminal residues of the carbohydrates on their β subunits play important roles for maintaining its *in vivo* biological activities by preventing them from the metabolic clearance (Matzuk *et al.*, 1990; Szkudlinski *et al.*, 1993). It is probable that rjeFSH and native eel FSH have distinct carbohydrate moieties, because it is reported that *P.pastoris* can hardly attach sialic acid to the terminus of its carbohydrate moieties (Cereghino and Cregg, 2000). Actually, it is unclear whether or not the carbohydrate moieties of rjeFSH spoil the FSH activities *in vivo*. One of human native GTHs, hCG, is considered to be sialyated on its β subunit, and similar results were obtained in rjeFSH. In eel, thus, the stablity of GTHs in blood is seems to be unaffected by sialyation. However, more studies must be conducted to characterize the physiological function of the carbohydrate moieties of eel FSH.

*Future perspectives*

In the present study, physiological roles of eel FSH was, at least in part, elucidated using prepared recombinant and native eel FSHs. The analyses performed in this thesis are mainly focused on the sex steroid production induced by eel FSHs. As mentioned above, other functional aspects of eel FSH, *i.e.*, the infuluence of eel FSH on ovarian follicular development, should be investigated in future. Additionally, the established expression system for biologically active recombinant eel FSH will allow the utilization of a sufficient amount of eel FSH in the fields of basic and application studies. Finally, I do hope that further studies with rjeFSH bring some new insights into the regulatory mechanisms of eel reproduction, together with the results obtained in this research.

# SUMMARY IN JAPANESE

## 要旨

脊椎動物の下垂体から分泌される2種類の生殖腺刺激ホルモン（gonadotropin; GTH）、すなわち卵濾胞刺激ホルモン（follicle-stimulating hormone; FSH）および黄体形成ホルモン（luteinizing hormone; LH）は、個体の性成熟を制御する生殖内分泌系の主要因子である。多くの魚類でLHは単離されその生理作用が詳細に調べられているのに対し、FSHは単離が困難でありその性状・作用ともに不明な点が多い。

ウナギ（*Anguilla japonica*）は我が国における重要な水産資源のひとつであるが、現在その種苗は全て天然の稚魚に依存している。天然の成熟個体は採捕されていないことに加え、近年、ウナギ天然種苗の捕獲量は減少しつつあり、その人工種苗生産技術の確立が切望されている。ウナギは飼育環境下において自発的に性成熟することはないため、親魚作出には調製が容易なヒトやサケ等のLHを主成分とするGTH画分の投与が行われている。しかし、このような方法を用いても必ずしも良質の受精卵が安定的に得られるわけではく、未だウナギ種苗生産技術の確立には至っていないのが現状である。その理由のひとつとして、投与した他動物種のLHがウナギ自身のGTH、特にFSHの生理作用を補完し得ていないという可能性が繰り返し指摘されてきた。しかし、これまでウナギFSHは物質として得られておらず、その生理作用の詳細は不明である。このようにウナギの生殖内分泌系を理解するための基礎的知見を欠くことが、その種苗生産技術を確立する上で大きな障害のひとつとなっている。

上記の問題を解決する端緒として、本研究では遺伝子工学的手法ならびに生化学的手法を用い、これまで入手困難とされてきたウナギFSHの調製およびその生理活性の解析を試みた。本研究の成果はウナギの性成熟機構の理解のみならず、新たなウナギの人為催熟法開発に向けての有用な知見を提供することが期待される。

第1章　酵母発現系を用いた組換えウナギFSHの作製

研究を進める上でウナギFSHを調製することが不可欠であるが、天然物の精製からでは今後の実験を行うのに十分量のウナギFSHを得ることが見込めないと判断した。そこで本研究では、まず組換えウナギFSHの作製系の確立を行った。GTHは各ホルモン間で共通のα鎖およびホルモン特異的なβ鎖（FSHβ, LHβ）の2つのサブユニットからなる

ヘテロ二量体の糖タンパク質ホルモンである。糖鎖の付加が可能で収量の多い発現系であること、さらに哺乳類の GTH を活性型で発現させた例が報告されていることから、本研究では酵母発現系を採用した。

既にクローン化されているウナギ FSH の各サブユニットをコードする cDNA を PCR で加工し、同一ベクターでαおよび FSHβ の両サブユニットを発現するベクターを構築した。構築した発現ベクターを用い、メタノール資化性酵母 *Pichia pastoris* を遺伝子相同組換えにより形質転換した。得られた形質転換体をメタノールで発現誘導することで、組換え体を発現させ、酵母培養液中へ分泌させた。培養後の上清を SDS-PAGE により分離後、cDNA より演繹されたアミノ酸配列をもとに作製した抗血清を用いた western blot 解析に供し、組換え FSH の各サブユニットが発現していることを確認した。その後、上清をエタノール沈殿、ゲル濾過に供し組換え体の回収を行った。ウナギ未熟精巣を用いて *in vitro* で生殖腺刺激活性を調べた結果、組換えウナギ FSH はヒト絨毛性 GTH (hCG) と同様に、未成熟精巣からのテストステロン (T) および 11-ケトテストステロン (11-KT) 分泌を有意に促進した。組換えウナギ FSH の活性量を国際単位として定められている hCG の活性単位 (IU) により標準化したところ、本発現系において発現誘導後の酵母培養液 1L に含まれる活性は約 1,500 IU であった。

第 2 章　未成熟ウナギ下垂体からの天然型ウナギ FSH の単離・精製

ウナギ FSH はその遺伝子発現の動態から、主に未成熟個体の下垂体に存在すると考えられる。しかし、未だその化学構造および生理活性について解析された例はない。また、組換え体が真に天然物ウナギ FSH の生理活性を反映しているかを確認する必要性もある。そこで、得られる天然物は微量であることが予想されたが、その化学構造および生理作用の解析を目的に、未成熟な飼育ウナギの下垂体から天然ウナギ FSH の単離を試みた。

ウナギ FSHβ 抗血清に対する免疫反応性を指標に、未成熟ウナギの下垂体抽出物をゲル濾過クロマトグラフィーおよび数段階の陰イオン交換クロマトグラフィーに付し、ウナギ FSH を精製した。精製した FSH は約 33 kDa のタンパク質で、各サブユニットはそれぞれ Asn 結合型糖鎖によって修飾されていた。さらにウナギ FSH は酸性溶液中において各サブユニットが解離する酸不耐性構造を有しており、酸耐性構造をもつサケ科等の FSH とは異なる化学構造であることが示唆された。また未成熟ウナギ下垂体の免疫組織化学により、魚類の GTH 産生部位として知られる前葉主部に抗 FSHβ 血清に対する免疫陽性反応が確認された。精製したウナギ FSH を活性試験に供した結果、未成熟ウナギの下垂体抽出物、hCG、および酵母で作製した組換えウナギ FSH と同様に、精製ウナギ FSH

は用量依存的にTおよび11-KTの分泌を促進した。これらの結果より、酵母で作製した組換えウナギFSHは天然型ウナギFSHと同様の生殖腺刺激作用を有することが示された。

第3章　組換えウナギFSHの雌雄生殖腺における性ステロイド産生作用の解析

ウナギ未成熟精巣に対するFSHの生理活性は示されたが、成熟の進んだ精巣あるいは卵巣の発達にどのようにウナギFSHが関与するかは不明である。そこで、人為的に成熟を進行させたウナギ精巣および卵巣を用意し、異なる成熟段階の生殖腺におけるウナギFSHの性ステロイド産生に与える影響を調べた。作製した組換え体が天然型と同様の生理作用を有することが前章で判明したため、本章では組換えウナギFSHを用いて実験を行った。

組換えウナギFSHは未熟期の精巣におけるTおよび11-KTの分泌を顕著に促進したが、成熟の進行した精巣ではTおよび11-KTの分泌を促進することはなかった。そのためウナギFSHは成熟が進んだ状態よりも、むしろ未成熟な状態の精巣において重要な働きをもつことが考えられた。一方、莢膜・顆粒膜細胞共に未発達な卵濾胞組織をもつ卵巣では組換えウナギFSHによりTおよびE2の分泌は促進されなかった。この様な卵巣にみられた卵には卵黄物質がほとんど蓄積していなかった。T産生を担う莢膜細胞が発達しているが、E2産生に重要な顆粒膜細胞が未発達な卵巣では、組換えウナギFSHはTの分泌を用量依存的に促進したが、E2分泌には影響しなかった。この様な卵巣では卵黄蓄積初期から卵黄蓄積中期にかけての卵が多く観察された。また、莢膜・顆粒膜の二細胞系共よく発達した卵濾胞組織を有していた卵巣では、組換えウナギFSHは用量依存的にTおよびE2の分泌を促進した。この様な卵巣は主に卵黄蓄積中期の卵を有していた。従って、ウナギFSHの卵巣における性ステロイド産生能は、卵濾胞組織が十分に発達する卵黄蓄積期の間に上昇することが示された。従って、ウナギFSHの卵巣における性ステロイド産生能は卵濾胞が十分に発達する卵黄蓄積期の間に上昇することが示された。

第4章　組換えウナギFSHの化学構造、比活性および生体内投与の検討

今後、組換えウナギFSHの有効利用を目指す上で、その詳細な化学構造、比活性および*in vivo*における生理活性についての知見は必須である。そこで、組換えウナギFSHの精製を行い、その構造および活性をより詳細に解析し天然型との比較を行った。また、組換えウナギFSHを未成熟ウナギに投与し、生体内での組換えウナギFSHの生理作用を検討した。

組換えウナギFSHを含む酵母培養上清をゲル濾過および数段階の陰イオン交換クロマ

トグラフィーに付し、抗ウナギ FSHβ 血清に対する免疫反応性および未成熟ウナギ精巣における 11-KT 産生促進を指標に、生理活性型組換えウナギ FSH を精製した。酸耐性試験において、精製した生理活性型組換えウナギ FSH 画分中には天然型と同様の酸不耐性分子が含まれていた。生理活性型組換えウナギ FSH 画分の活性は天然型と比較して 1/100 程度であったが、天然型と組換え体の化学構造の差異がその比活性に影響を与えているものと考えられた。

海水条件化に馴致した飼育雄ウナギに 0.1 IU/g および 1.0 IU/g の濃度で 12 日間に 3 回の組換えウナギ FSH の投与を行ったところ、生理食塩水投与群と比べ 1.0 IU/g の組換えウナギ FSH 投与群において生殖腺体重比の有為な増加が認められた。また、投与した組換えウナギ FSH は用量依存的に血中 11-KT 量に顕著な増加が認められた。以上の結果より、本研究で作製した組換えウナギ FSH は、生体内においても生理活性を発揮しうることが示された。

以上のウナギ FSH に関する研究より、以下の知見を得ることができた。

1) 酵母発現系を用いて生理活性を有する組換えウナギ FSH 発現系を構築した。
2) 未成熟ウナギ下垂体中には天然型ウナギ FSH が蓄積されており、精製した天然型ウナギ FSH は組換えウナギ FSH と同様の生理活性を有していた。
3) 組換えウナギ FSH は、未成熟期の精巣において顕著な性ステロイド分泌活性を示した。一方、卵巣では卵黄蓄積開始時と比べて成熟が進んだ卵黄蓄積中期の卵巣においてより効果的に性ステロイド分泌を促進した。
4) 活性型組換えウナギ FSH 画分の比活性は天然型の 1/100 程度であった。
5) 組換えウナギ FSH は生体内投与により未成熟雄ウナギの血中性ステロイド量を増加させ、生殖腺発達を誘導した。

本研究により天然型および組換え体ウナギ FSH が物質として調製可能となり、それらの生理作用の一端を明らかにすることができた。また、組換えウナギ FSH は生体内においても活性を有しており、今後の人為催熟にも応用可能であることが示された。これらの成果をもとに、ウナギの生殖内分泌系の更なる理解とともに、ウナギ自身の FSH を用いた新たな催熟技術の開発が期待される。

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# Tables and Figures

**Table 1-1** Oligonucleotides used for amplification of jeGPα and jeFSHβ subunits when constructing expression vector, pPIC9K-jeFSHβ/α

| Name | Sequences |
|---|---|
| jeGPα-forward-*EcoR* I primer | 5'-**CG***GAATTC*TATCCCAACAACGAAATGGCA-3' |
| jeGPα-reverse-*EcoR* I primer | 5'-**CG***GAATTC*TTAAAATTTGTGGTAGTAGCA-3' |
| jeGPα-forward-*Xba* I primer | 5'-**CG***TCTAGA*TATCCCAACAACGAAATGGCA-3' |
| jeFSHβ-forward-*EcoR* I primer | 5'-**CG***GAATTC*TCCACCAGTTGTGGTCTCGCC-3' |
| jeFSHβ-reverse-*EcoR* I primer | 5'-**CG***GAATTC*CTAGTGGGTCTGACAGCCTGA-3' |

Note: The 5'-terminal **C** and **G** residues (bold) in respective primers are attached to prevent the removal of the terminus nucleotide by the exonuclease activity of high-fidelity Taq DNA polymerase. An *Eco*R I sites (5'-GAATTC-3'), *Xba* I sites (5'-TCTAGA-3') and the stop codons (5'-TAA-3' or 5'-CTA-3') are shown in italics and underlined characters, respectively.

**Table 3-1** Ovarian developmental stages based on oocyte growth and development of ovarian follicular cells

| | Ovary | | |
|---|---|---|---|
| **Parameters** | **Type A** | **Type B** | **Type C** |
| Oocyte diameter (μm) | $219.2 \pm 15.8^{a}$ | $254.0 \pm 12.2^{a}$ | $349.2 \pm 10.5^{b}$ |
| GSI (%) | $3.1 \pm 0.3^{a}$ | $3.7 \pm 0.2^{a}$ | $7.7 \pm 0.7^{b}$ |
| HSI (%) | $1.5 \pm 0.2^{a}$ | $1.4 \pm 0.1^{a}$ | $1.9 \pm 0.1^{b}$ |
| Number of fish | 6 | 7 | 12 |

Values are means ± S.E.M. (n=6-12). Different letters (a, b) indicate significant difference at $P<0.05$.

**Table 4-1** 11-Ketotestosterone secretion from immature eel testis

| | 11-KT (pg / 100mg testis) | | |
|---|---|---|---|
| Saline | $87.5 \pm 17.8^{a}$ | | |
| | yeast culture supernatant equivarent (mL) / mL incubation medium | | |
| | 3.3 | 10 | 30 |
| DP7-2 | $106.3 \pm 34.1^{a}$ | $477.2 \pm 128.9^{b}$ | $2497.3 \pm 968.1^{c}$ |
| DP7-3 | $101.5 \pm 14.8^{a}$ | $102.1 \pm 18.7^{a}$ | $85.7 \pm 15.3^{a}$ |

Values are means ± S.E.M. (n=3). Different letters (a, b, c) indicate significant difference at $P<0.05$.

**Table 4-2** Changes in body weight during *in vivo* experiment

| Groups | Body weight (g) | | | |
|---|---|---|---|---|
| | Days after injection | | | |
| | 0 | 3 | 7 | 12 |
| Saline | 159.3±3.5[a] | 160.4±3.8[a] | 161.7±4.0[a] | 162.4±3.5[a] |
| hCG (1.0 IU/g-BW) | 155.3±1.5[a] | 155.8±3.2[a] | 154.7±4.3[a] | 155.9±8.0[a] |
| Mock | 156.9±4.9[a] | 157.6±4.8[a] | 161.2±5.3[a] | 161.2±4.2[a] |
| rjeFSH (0.1 IU/g-BW) | 154.8±4.3[a] | 156.5±4.5[a] | 158.6±4.8[a] | 158.7±4.8[a] |
| rjeFSH (1.0 IU/g-BW) | 160.9±3.9[a] | 158.5±3.8[a] | 165.8±5.8[a] | 163.8±4.6[a] |

Values are means±S.E.M. (n=3-5). The same letter (a) indicates statistically equal in respective groups ($P>0.05$)

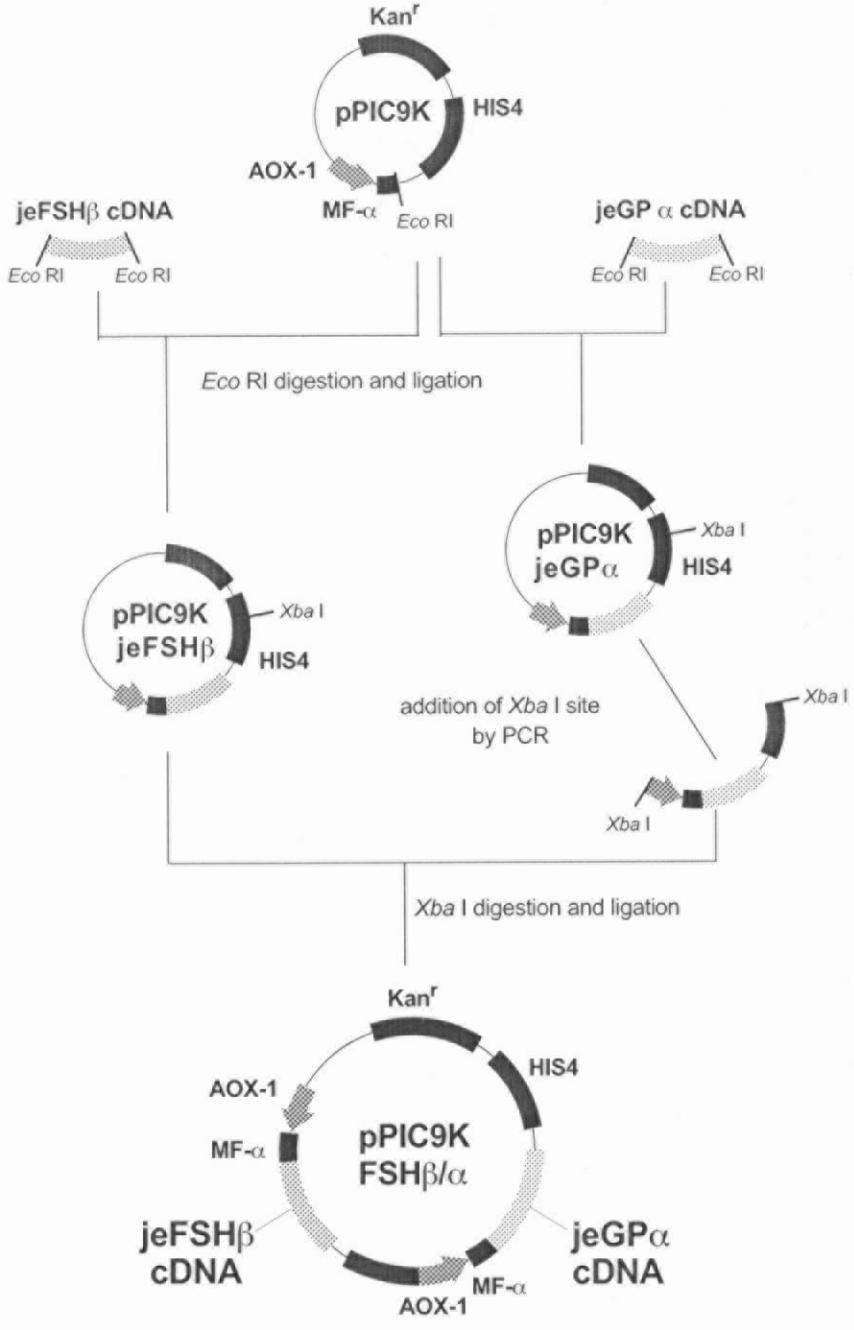
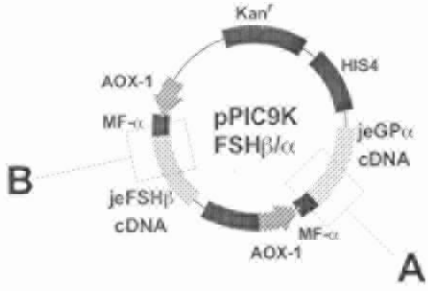
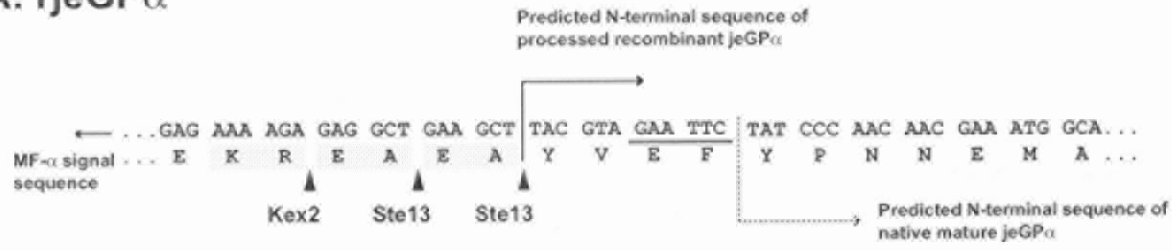



**Fig. 1-1.** Construction of expression vector (pPIC9K-jeFSHβ/α).
MF-α, an α-factor secretion-signal sequence; 5'-AOX, an alcohol oxidase I promoter; HIS4, a histidinol dehydrogenase gene; Kan$^r$, a kanamycin resistant gene.




## A: rjeGPα




## B: rjeFSHβ

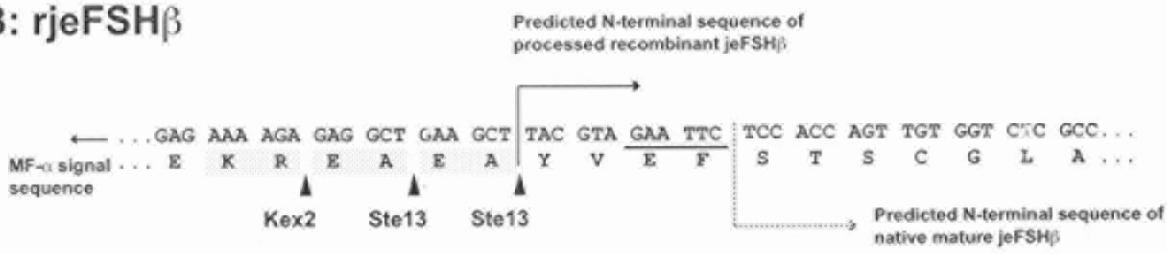



**Fig. 1-2** The cDNA sequences encoding predicted N-terminal region of both rjeFSH subunits in the expression vector (pPIC9K-jeFSHβ/α). The preliminaly cleavage of the signal sequence by Kex2 and Ste13 proteases. The cleavages were occuring where the site of arrowheads. Shaded amino acid sequences, K-R and E-A, are the recognition sites of Kex2 and Ste13 protease, respectively. Underlined cDNA sequence (GAATTCC) indicates the *Eco*R I site utilized when the insertion of cDNAs encoding jeFSH subunits into the pPIC9K vector.

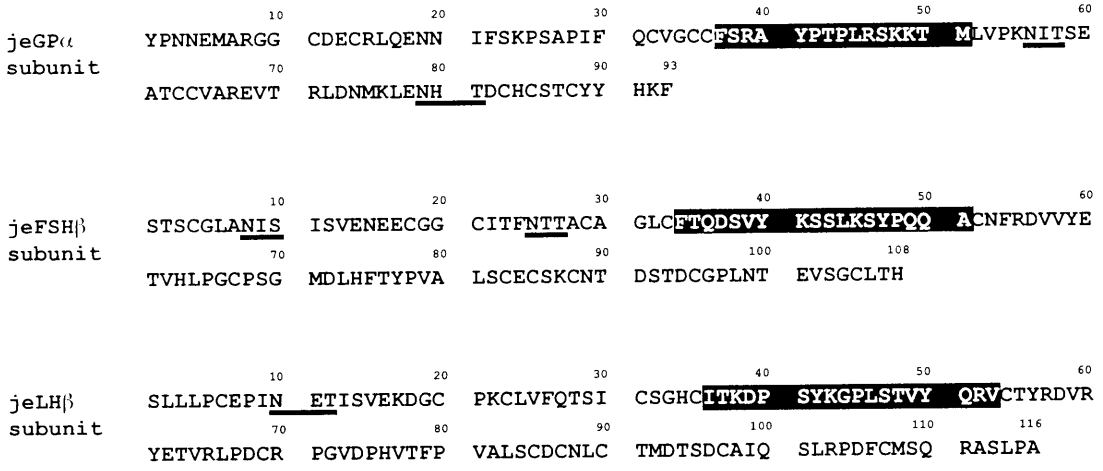



**Fig. 1-3** Deduced amino acid sequences of GPα, FSHβ and LHβ subunits in Japanese eel. The sequences represented in solid boxes are chemically synthesized for antigens to prepare the antibadies of respective subunits. Underlined sequences indicate predicted *N*-linked glycosylation sites. The amino acid identity between jeFSHβ and jeLHβ is 42% (Yoshiura *et al.*, 1999).

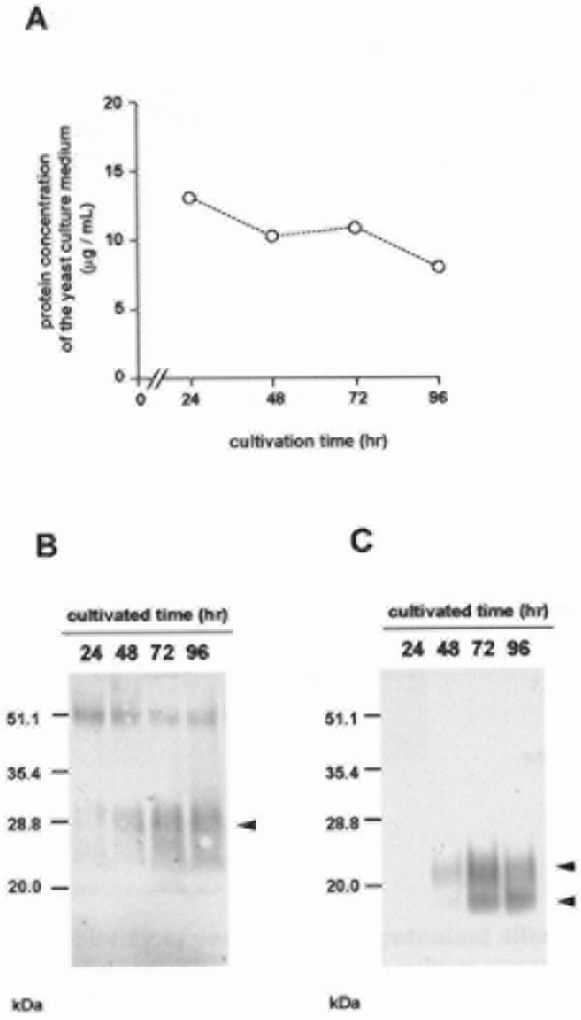



**Fig. 1-4.** The time-course of secretion of each subunit of rjeFSH.
The time-dependent changes of total protain concentrations of the culture supernatant of yeast transformed with an expression vector containing the jeFSH subunit cDNAs after 24, 48, 72 and 96 hours induction (A). Western blotting of yeast culture supernatant after separation by 15-25% SDS-PAGE under reducing conditions. The 10 μL of yeast culture supernatant was applied to each lane. The results of western blotting using antisera against jeGPα subunit (B), against and jeFSHβ subunit (C) are shown in each panels. Arrowheads show specific immunoreactive bands.

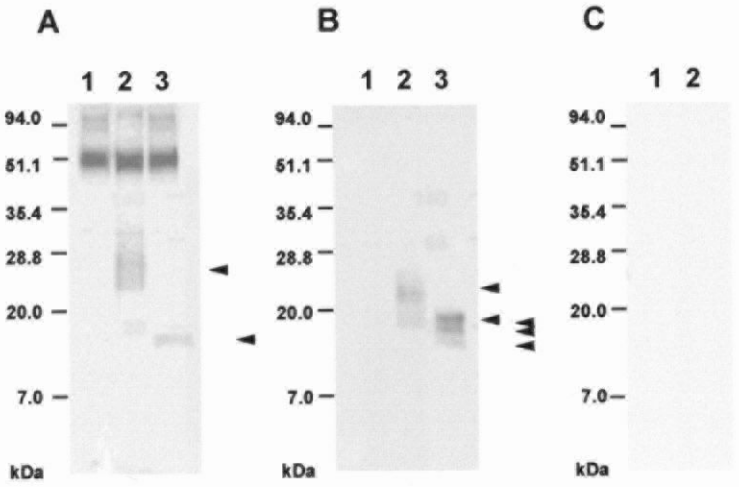



**Fig. 1-5.** Western bloting of yeast culture supernatant after separation by 15-25% SDS-PAGE under reducing conditions. The 10 μL of yeast culture supernatant was applied to each lane. The results of western blotting using antisera raised against jeGTHα subunit (A), against jeFSHβ subunit (B) and against jeLHβ subunit (C) are shown in each panels. Lanes 1, culture supernatant of yeast transformed with an expression vector not containing the jeFSH subunit cDNAs; Lanes 2, culture supernatant of yeast transformed with an expression vector containing the jeFSH subunit cDNAs. Lanes 3, deglycosylated samples of those shown in lanes 2. Arrowheads show specific immunoreactive bands.

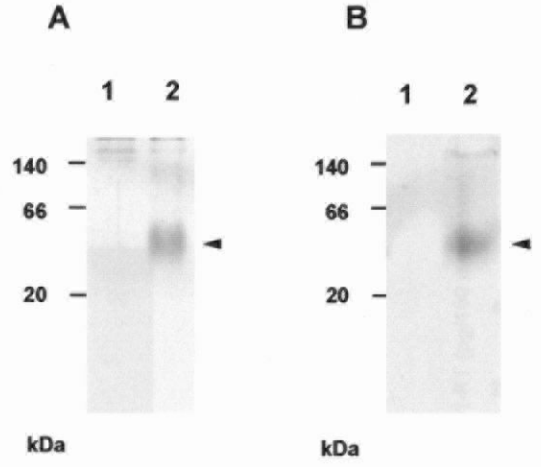



**Fig. 1-6.** Western bloting of yeast culture supernatant after separation by 20% native-PAGE under non-reducing conditions. The 10 μL of yeast culture supernatant was applied to each lane. The results of western blotting using antisera against jeGTHα subunit (A) and against jeFSHβ subunit (B) are shown in each panels. Lanes 1, culture supernatant of yeast transformed with an expression vector not containing the jeFSH subunit cDNA; Lanes 2, culture supernatant of yeast transformed with an expression vector containing the jeFSH subunit cDNAs. Arrowheads show specific immunoreactivities.

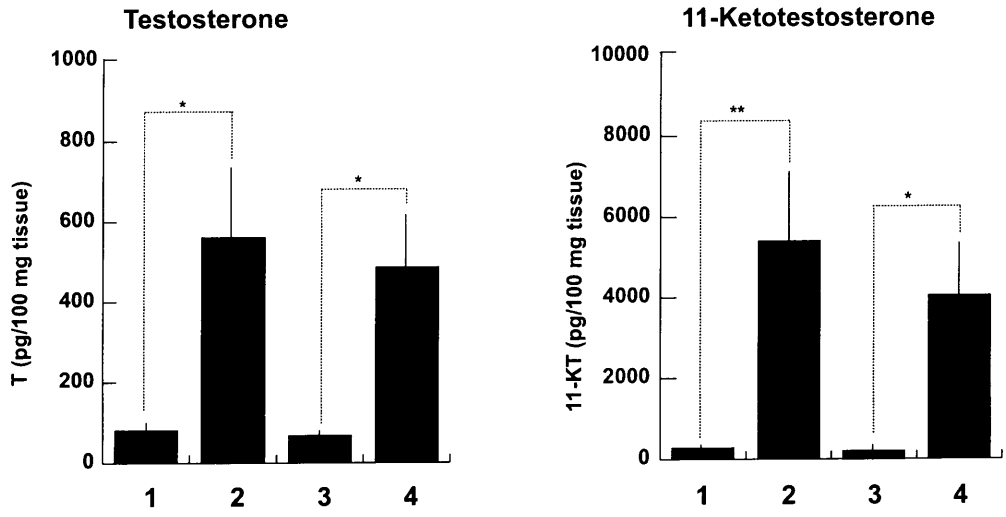



**Fig. 1-7.** Testosterone and 11-ketotestosterone secreting activity of rjeFSH in immature Japanese eel testis. Saline solution (1), treatment with 10 IU of hCG (2), treatment with glycoprotein fraction derived from 2.5 mL of culture supernatant of yeast transformed with an expression vector not containing jeFSH subunit cDNA (3), treatment with glycoprotein fraction derived from 2.5 mL of culture supernatant of yeast transformed with an expression vector containing jeFSH subunit cDNAs (4). Results are expressed as the mean ± S.E.M. of five independent bioassays. * and ** indicate significant differences at $P<0.05$ and $P<0.001$, respectively.

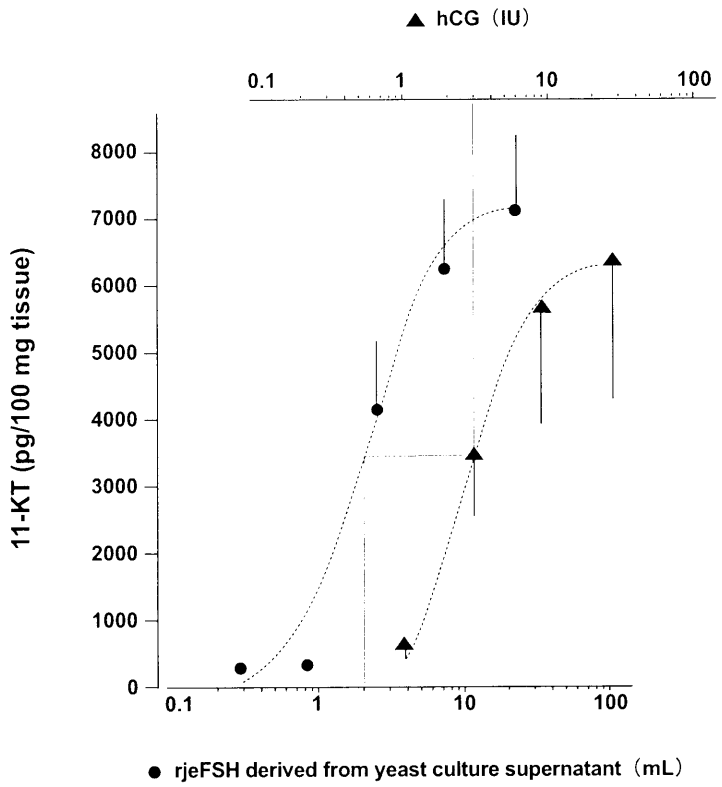



**Fig. 1-8.** Dose-dependent 11-ketotestosterone secreting activity of rjeFSH and hCG in immature Japanese eel testis. Solid circles and triangles show the 11-ketotestosterone release from immature eel testis stimulated by rjeFSH derived from yeast culture supernatant and hCG solution, respectively.

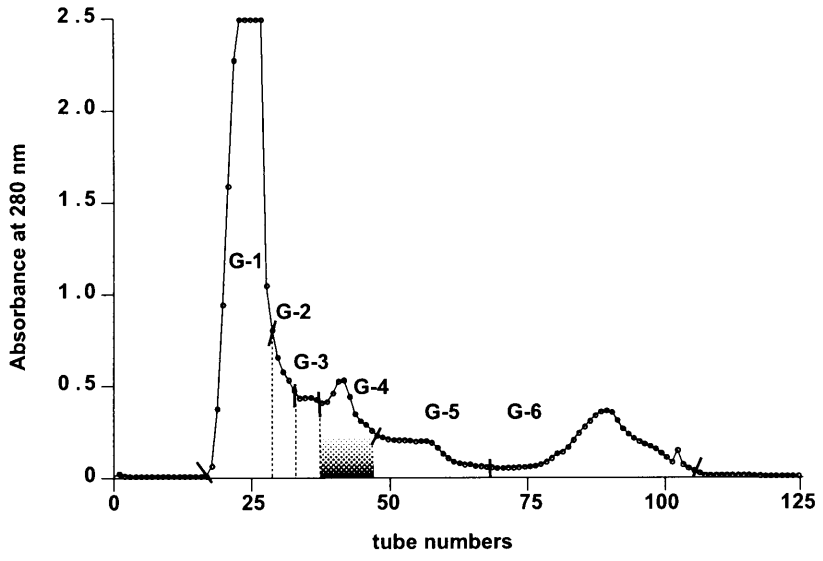



**Fig. 2-1.** Gel-filtration of the immature eel pituitary extract on a Sephadex G-100 (Superfine) column (1.6 x 98 cm). The column was equilibrated with 0.05 M ammonium acetate, pH 9.0. The sample was eluted with the same buffer at a flow rate of 3 mL/200 sec/tube. Immunoreactive FSH examined by western blotting was found only in the fraction G-4, which was subjected to further fractionation.

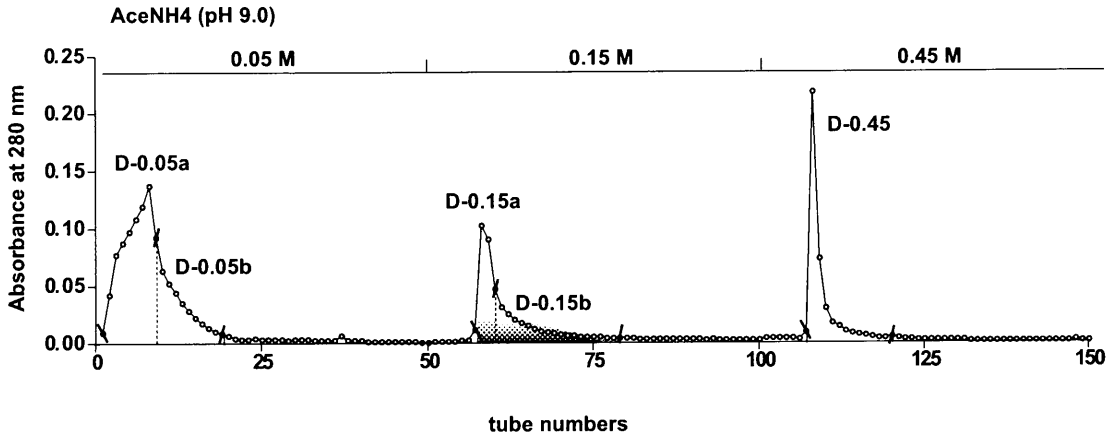



**Fig. 2-2.** Anion-exchange chromatography of the fraction G-4 on a DE-52 column (1.77 x 30 cm). The column was equilibrated with 0.05 M ammonium acetate, pH 9.0. The protein samples were eluted with a stepwise increase in ammonium sulfate, at a flow rate of 3 mL/200 sec/tube. The fractions containing intact eel FSH (D-0.15a and b) were subjected to further fractionation.

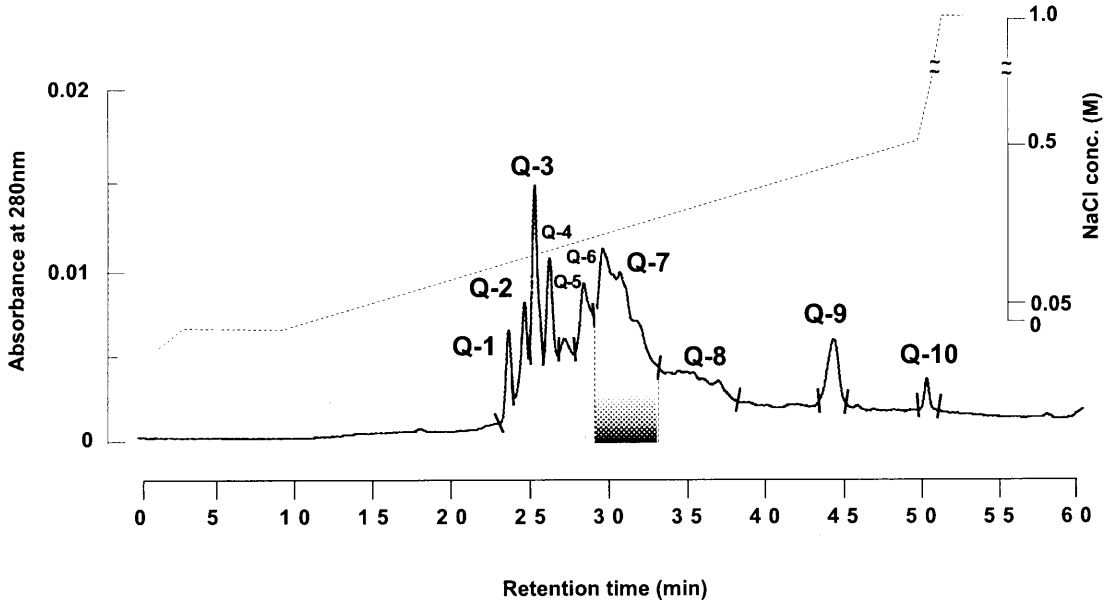



**Fig. 2-3.** Anion-exchange HPLC of the mixed D-0.15 fraction on a TSK-gel Super-Q column. The sample was dissolved in 0.01 M Tris-HCl, pH 7.0, and applied to a column equilibrated with the same solvent. The proteins were eluted with a linear gradient increase in sodium chloride at a flow rate of 0.5 mL/min.

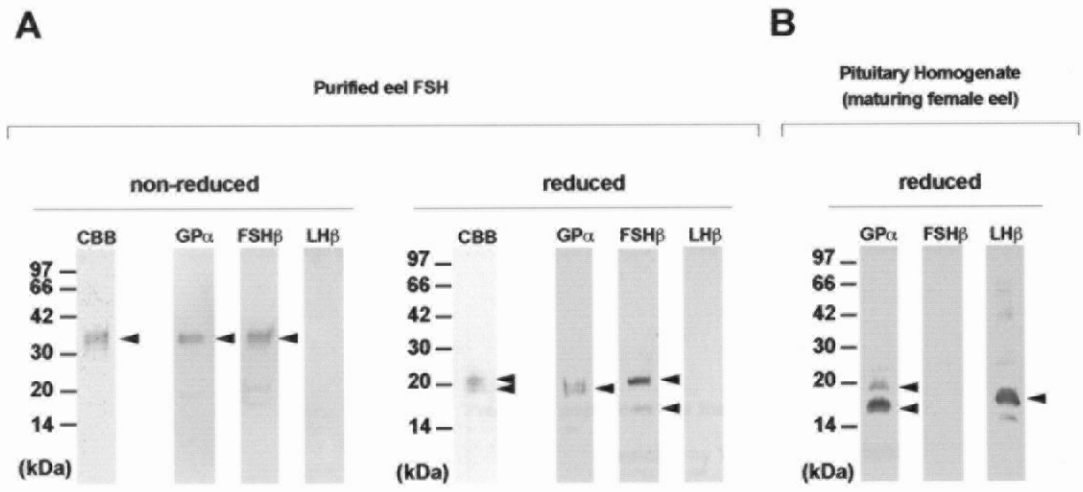



Fig. 2-4. SDS-PAGE and western blotting of purified eel FSH (A, B) in maturing eel pituitary homogenate (C). Samples are separated on 15-25% SDS-PAGE under a non-reduced or reduced condition. Purified eel FSH (1$\mu$g) was separated on SDS-PAGE and stained with CBB. After separation on SDS-PAGE, purified eel FSH was stained with anti-jeGP$\alpha$, anti-jeFSH$\beta$ and anti-jeLH$\beta$. B: Homogenate of a maturing female eel pituitary was separated on SDS-PAGE and was stained with anti-jeGP$\alpha$, anti-jeFSH$\beta$ and anti-jeLH$\beta$. Arrowheads show specific signals.

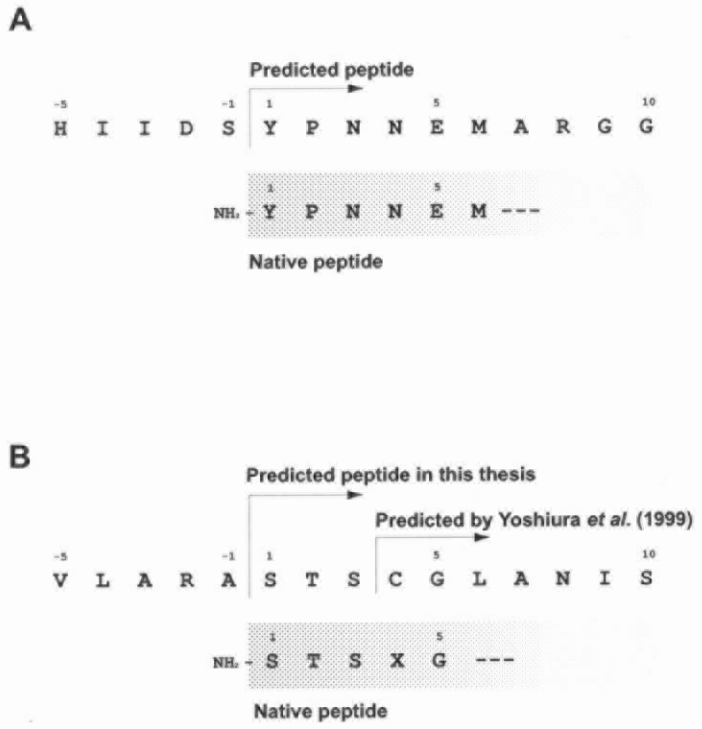



**Fig. 2-5.** The N-terminal amino acid arrangements of native jeGPα (A) and jeFSHβ (B) polypeptides, and those predicted from cDNA sequences.

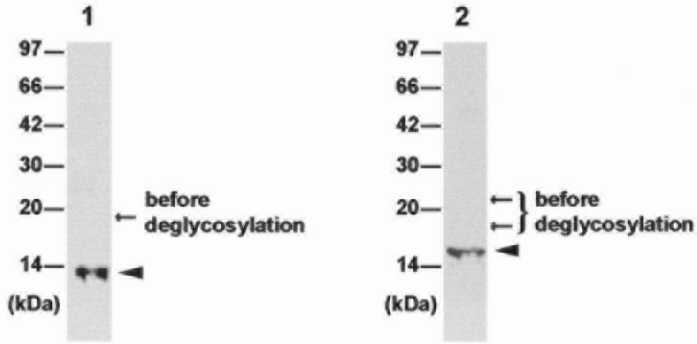



**Fig. 2-6.** De-*N*-glycosylation of eel FSH subunits. De-*N*-glycosylated eel FSH was separated on 15-25% SDS-PAGE under a reduced condition, and stained with anti-jeGPα (1) and anti-jeFSHβ (2). Arrowheads show specific signals.

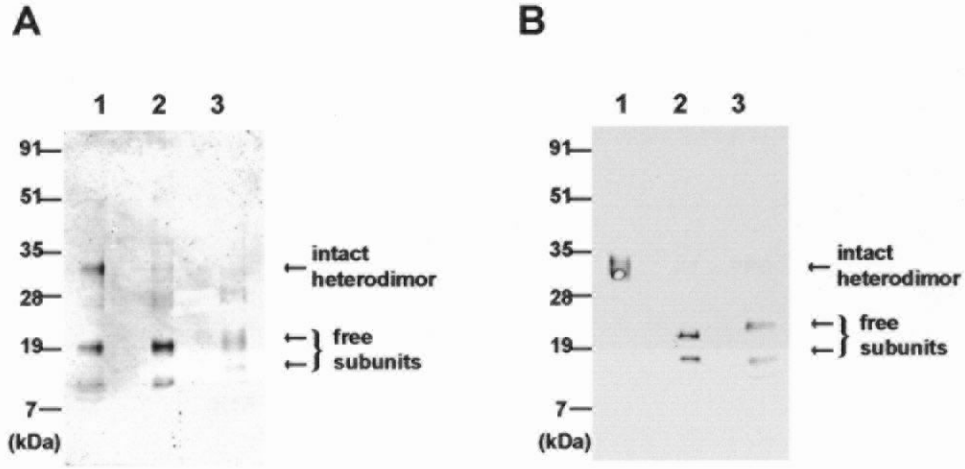



Fig. 2-7. Separation of immature eel pituitary homogenate by SDS-PAGE under a non-reducing condition, and that (1) treated with 0.1 % TFA (2) or 5 % 2-ME (3). Stained with anti-jeGPα (A) and anti-jeFSHβ (B). Arrows show specific signals.

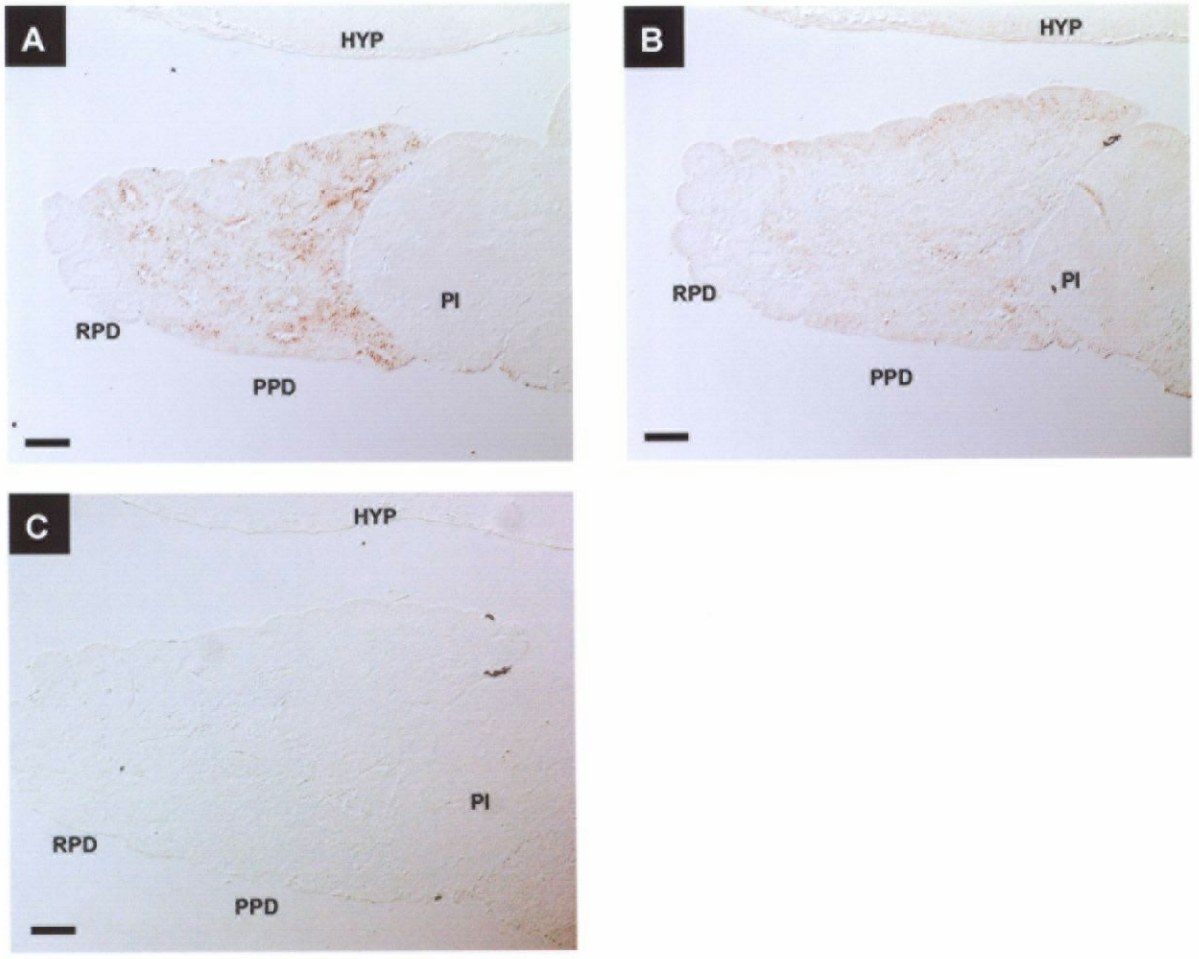



**Fig. 2-8.** Immunohistochemistry of an immature male eel pituitary. Adjacent sagittal sections of the pituitary with (A, C) or without unmasking treatment (B) were stained using anti-jeFSHβ (1:1000) (A, B) and anti-jeLHβ (1:1000) (C). RPD, rostral pars distalis; PPD, proximal pars distalis; PI, pars intermedia; HYP, hypothalamus. Scale bars, 100 $\mu$m.

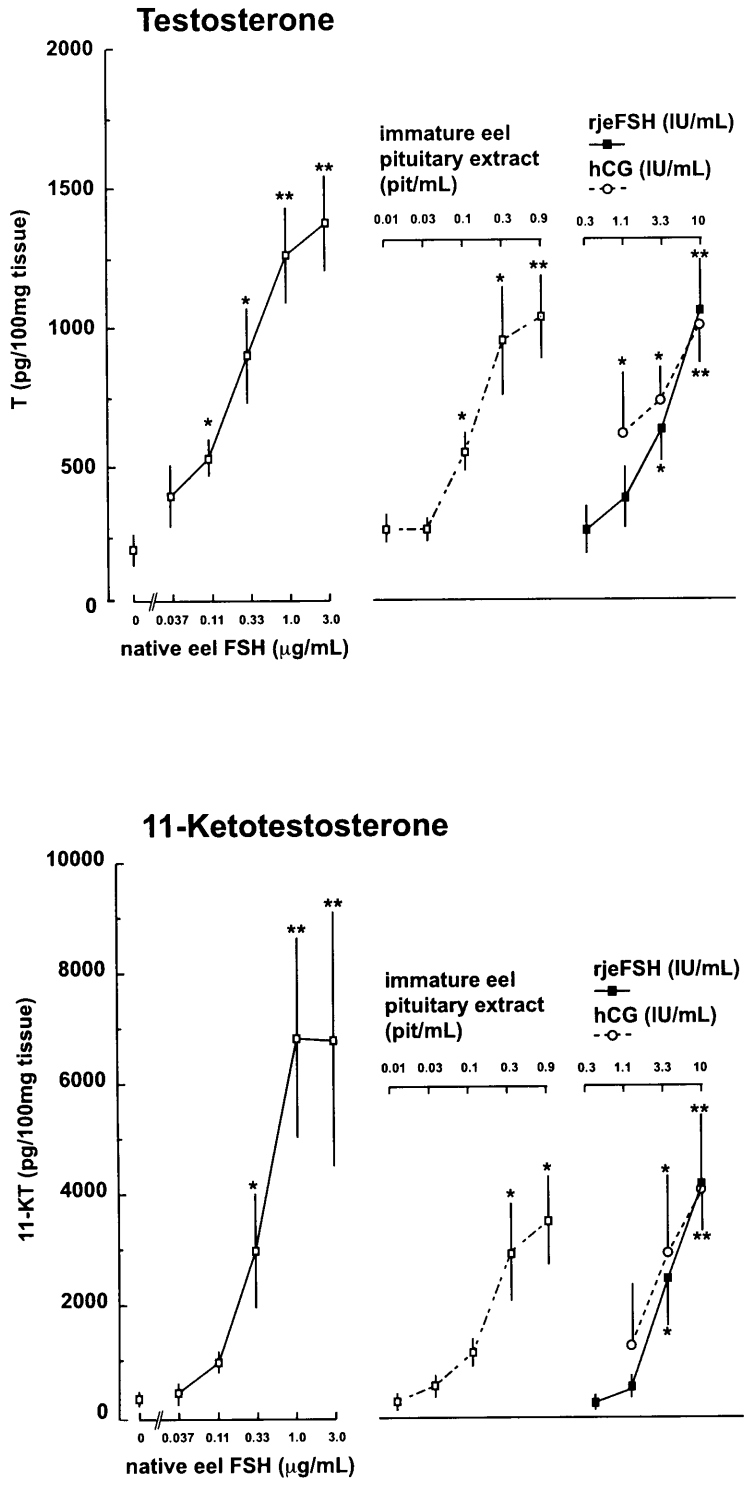



**Fig. 2-9.** Testosterone and 11-ketotestosterone secreting activity of purified eel FSH, immature eel pituitary extract, rjeFSH and hCG. Results are expressed as the mean ± S.E.M. * and ** indicate significant differences at $P<0.05$ and $P<0.001$, respectively.

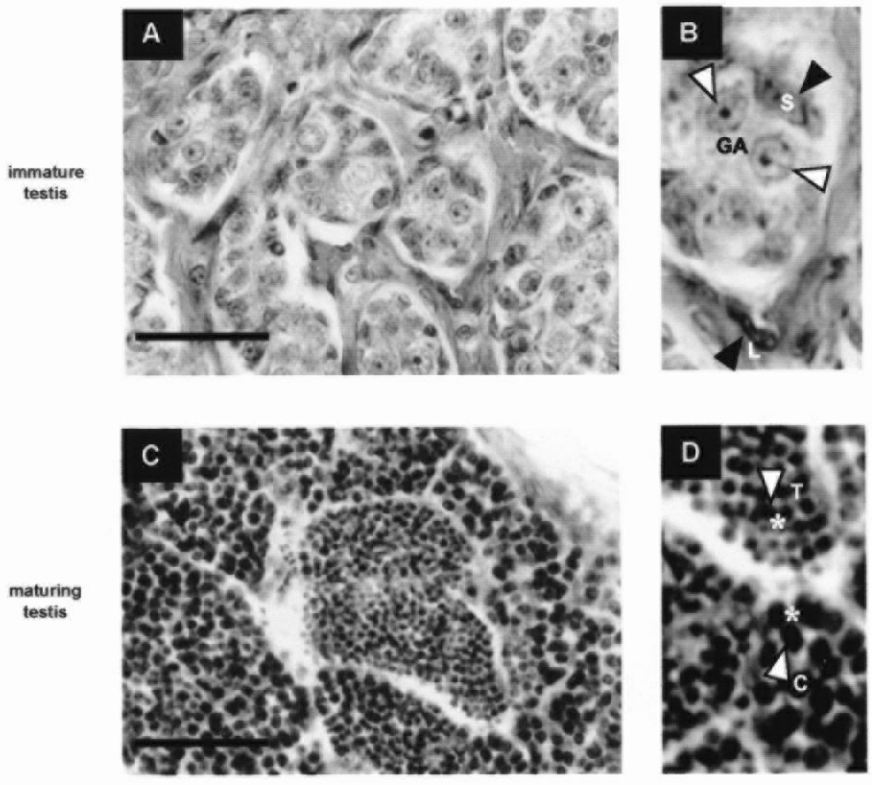



**Fig. 3-1.** Light micrographs of the testes at different reproductive stages. Each panel shows immature testis (A, B) and maturing testis (C, D). Unproliferated early-type A spermatogonia (GA); Sertoli cells (S); Leydig cells (L); spermatocyte (C); spermatid (T) . Panels B and D are magnified views of panel A and B, respectively. Asterisks (*) are marked in the germ cells in meiotic division. Scale bars: 50 μm.

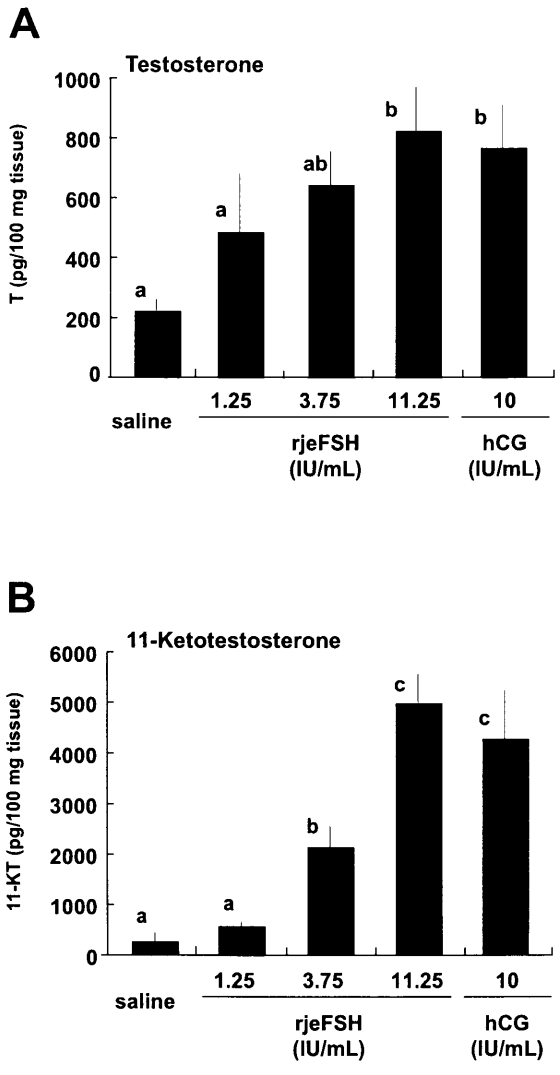



**Fig. 3-2.** Testosterone and 11-ketotestosterone secreting activity of rjeFSH in immature Japanese eel testes. Columns represent secreted androgens in saline solution , and in the 1.25 IU, 3.75 IU and 11.25 IU of rjeFSH. Results are expressed as the mean ± S.E.M. of three independent bioassays. Values with the same letter(s) are not significantly different ($P<0.05$).

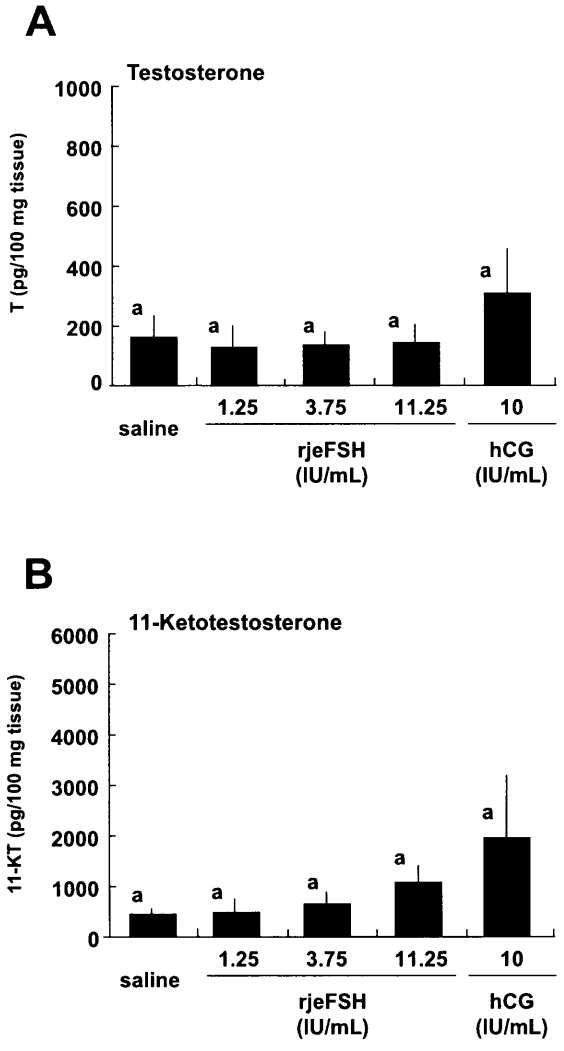



**Fig. 3-3.** Testosterone and 11-ketotestosterone secreting activity of rjeFSH in maturring Japanese eel testes. Columns represent secreted androgens in saline solution , and in the 1.25 IU, 3.75 IU and 11.25 IU of rjeFSH. Results are expressed as the mean ± S.E.M. of three independent bioassays. Values with the same letter is not significantly different ($P<0.05$).

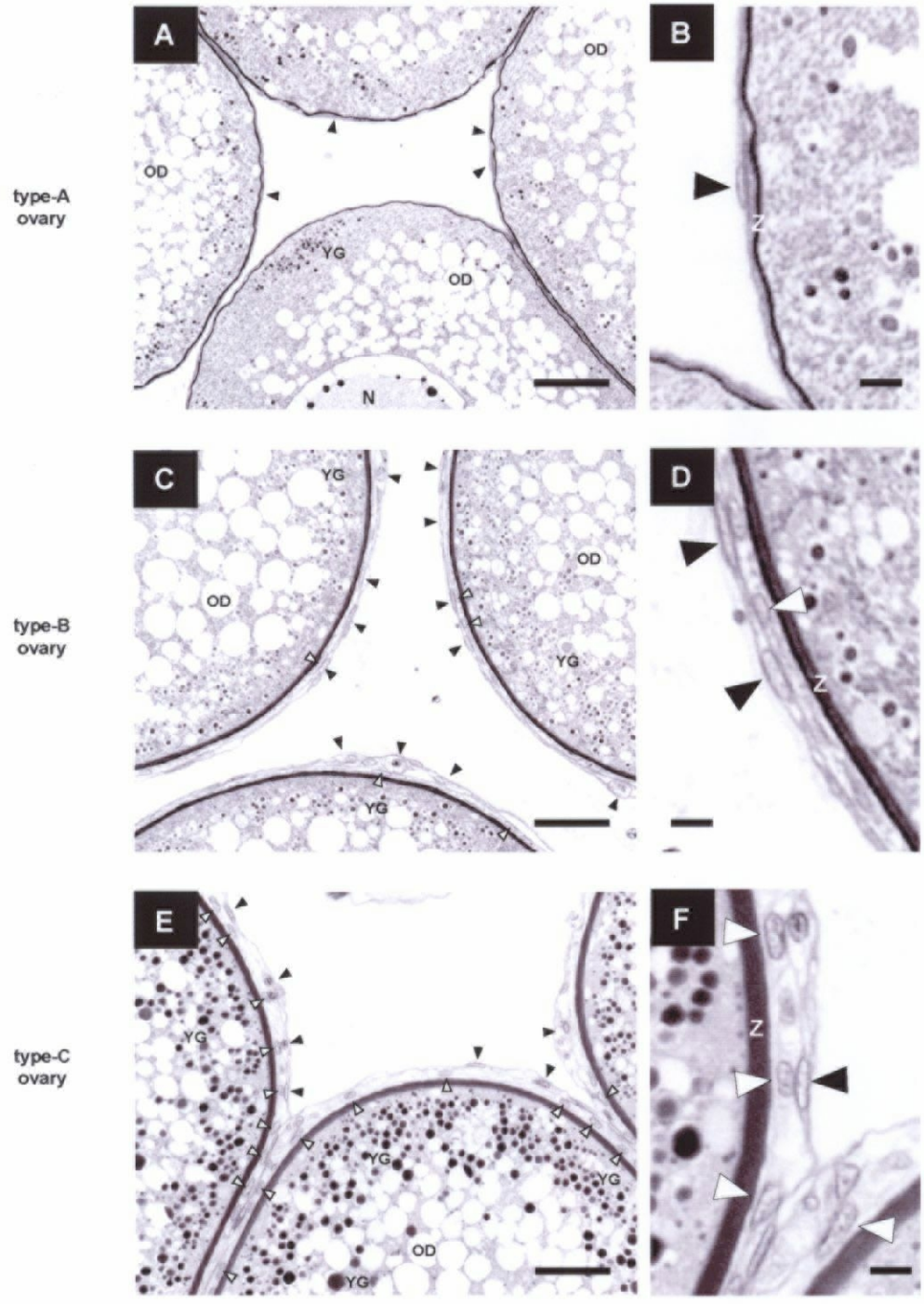



**Fig. 3-4.** Light-micrographs of the ovaries at differential developmental states. Ovarian developmental stages were classified into types A, B and C, based on the oocyte growth and development of ovarian follicular cells. Type A, pre- to early vitellogenic ovaries, possessing poorly developed theca (▲) and granulosa (△) cells (A, B); type B, early to mid-vitellogenic ovaries possessing oocytes which are surrounded by outer flat-shaped theca cells and inner poorly developed granulosa cells (C, D); type C, mid-vitellogenic ovaries with well-developed theca and granulosa cells (E, F). B, D and F, magnified views of ovarian follicular layers. N, germinal vesicle; OD, oil droplet; YG, yolk globule; Z, zona radiata. Scale bars: A, C, E, 50 μm ; B, D, F, 5 μm.

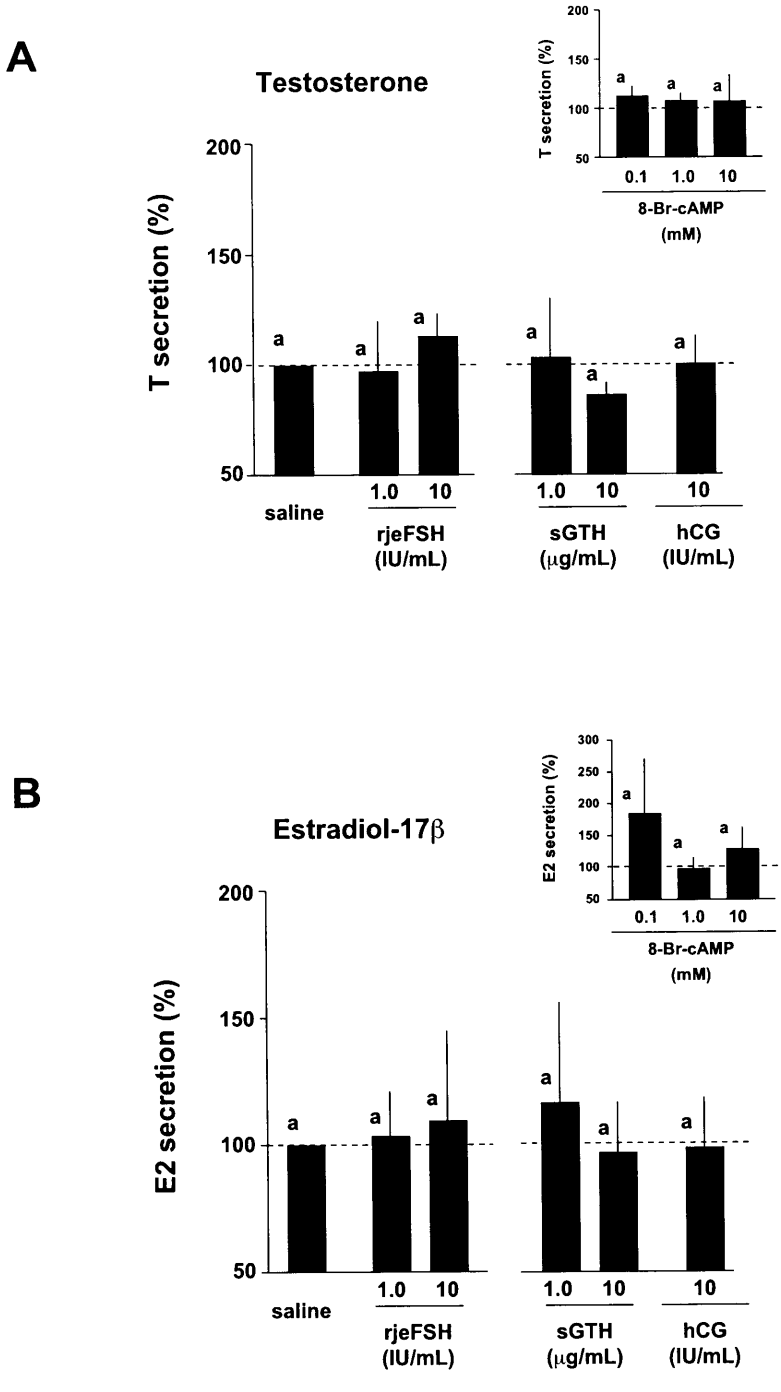



**Fig. 3-5.** Testosterone (A) and estradiol-17β (B) secretion from type-A ovaries incubated for 24 hr in media containing saline (controls), rjeFSH (0.1 and 1.0 IU/mL), sGTH (1.0 and 10 μg/mL), hCG (10 IU/mL), or 8-Br-cAMP (0.1, 1.0 and 10 mM). Data are expressed as the mean±S.E.M (n=6) of the % release of control values. Values marked with the same letter (a) are not significantly different from each other ($P>0.05$).

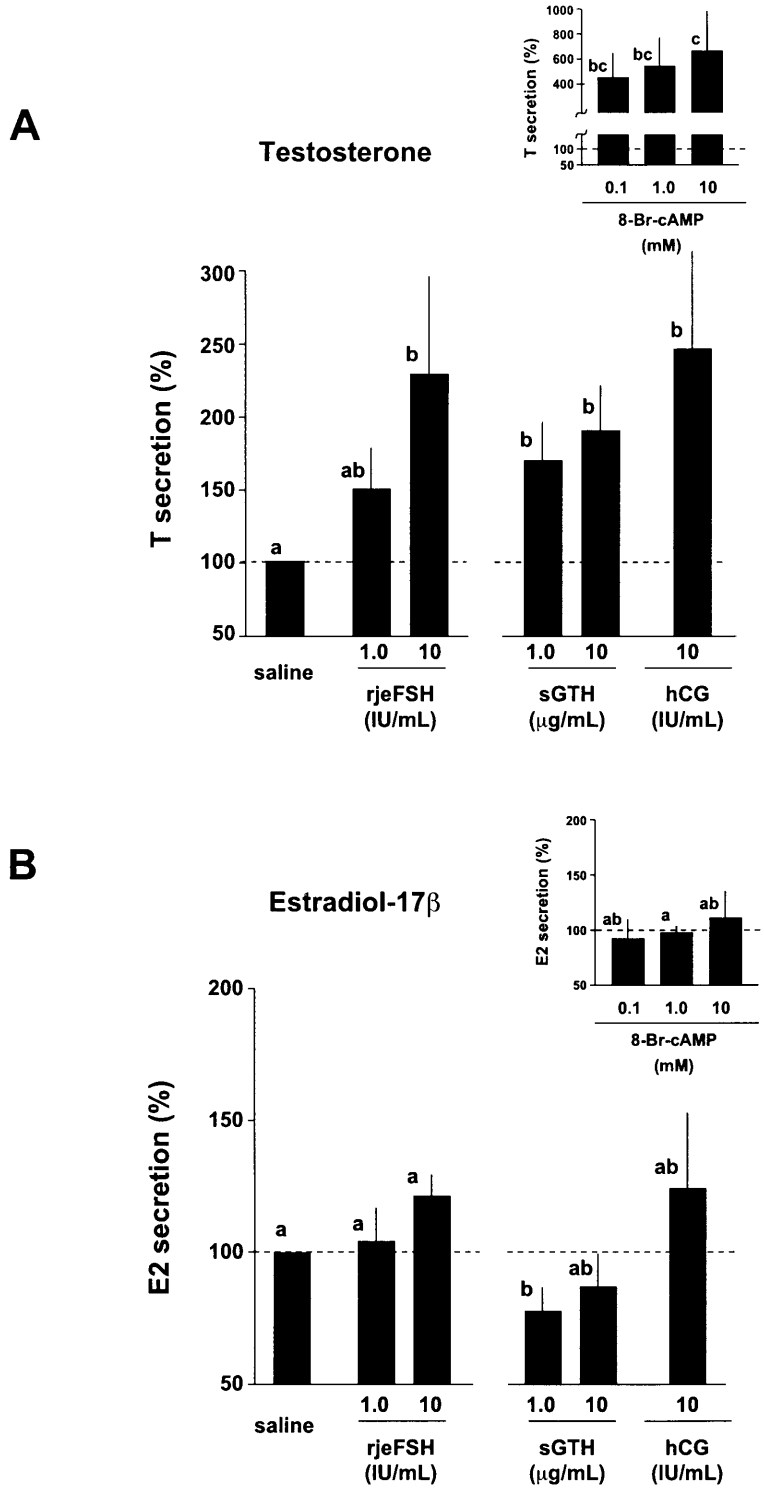



**Fig. 3-6.** Testosterone (A) and estradiol-17β (B) secretion from type-B ovaries incubated for 24 hr in media containing saline (controls), rjeFSH (0.1 and 1.0 IU/mL), sGTH (1.0 and 10 μg/mL), hCG (10 IU/mL), or 8-Br-cAMP (0.1, 1.0 and 10 mM). Data are expressed as the mean±S.E.M (n=7) of the % release of control values. Values marked with the different letters (a, b) are significantly different from each other ($P$>0.05).

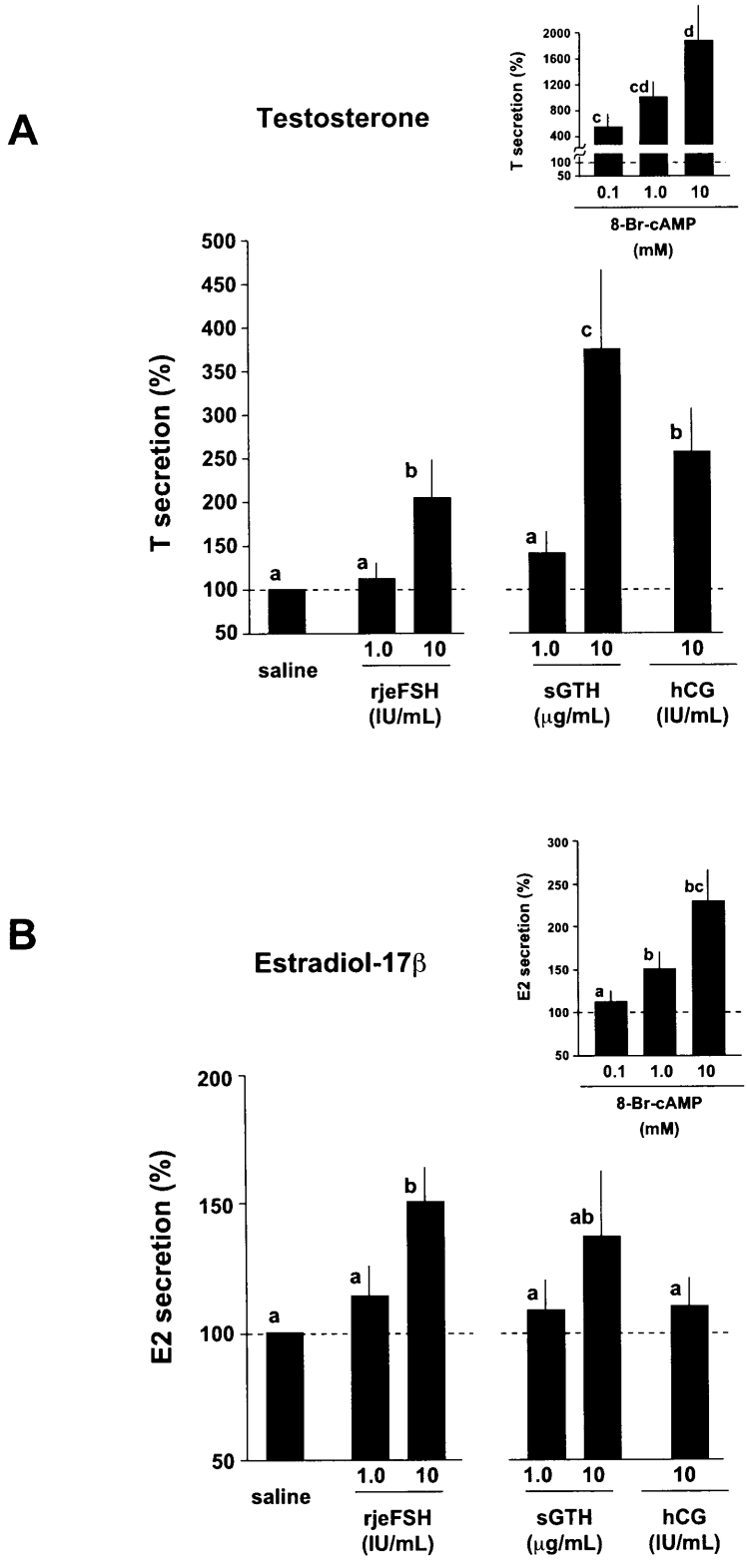



**Fig. 3-7.** Testosterone (A) and estradiol-17β (B) secretion from type-B ovaries incubated for 24 hr in media containing saline (controls), rjeFSH (0.1 and 1.0 IU/mL), sGTH (1.0 and 10 μg/mL), hCG (10 IU/mL), or 8-Br-cAMP (0.1, 1.0 and 10 mM). Data are expressed as the mean±S.E.M (n=12) of the % release of control values. Values marked with the different letters (a, b, c) are significantly different from each other ($P$>0.05).

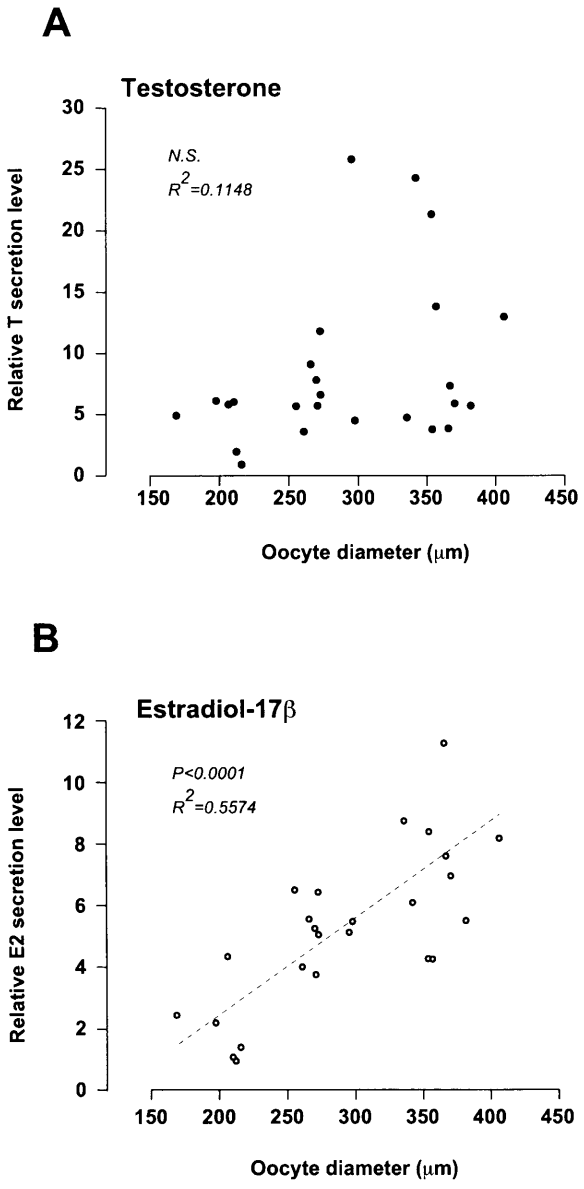



**Fig. 3-8.** Correlation analysis between relative testosterone secretion induced by 10 IU/mL of rjeFSH and oocyte diameter (A) and, between relative estradiol-17β secretion induced by 10 IU/mL of rjeFSH and oocyte diameter (B). Data are expressed in the ratio to minimum values, given 1.0.

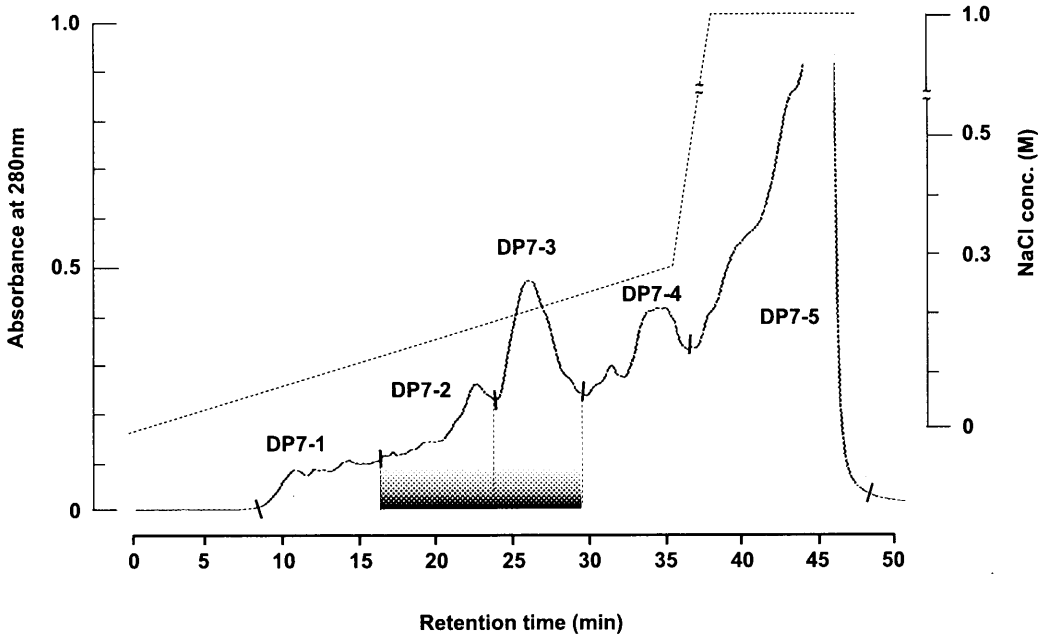



**Fig. 4-1.** Anion-exchange HPLC of the DF-0.3 fraction on a TSK-gel DEAE-5PW column. The sample was dissolved in 0.02 M Tris-HCl, pH 7.0, and applied to the column equilibrated with the same solvent. Proteins were eluted with a linear gradient increase in sodium chloride at a flow rate of 1.0 mL/min. The fractions containing rjeFSH are shaded.

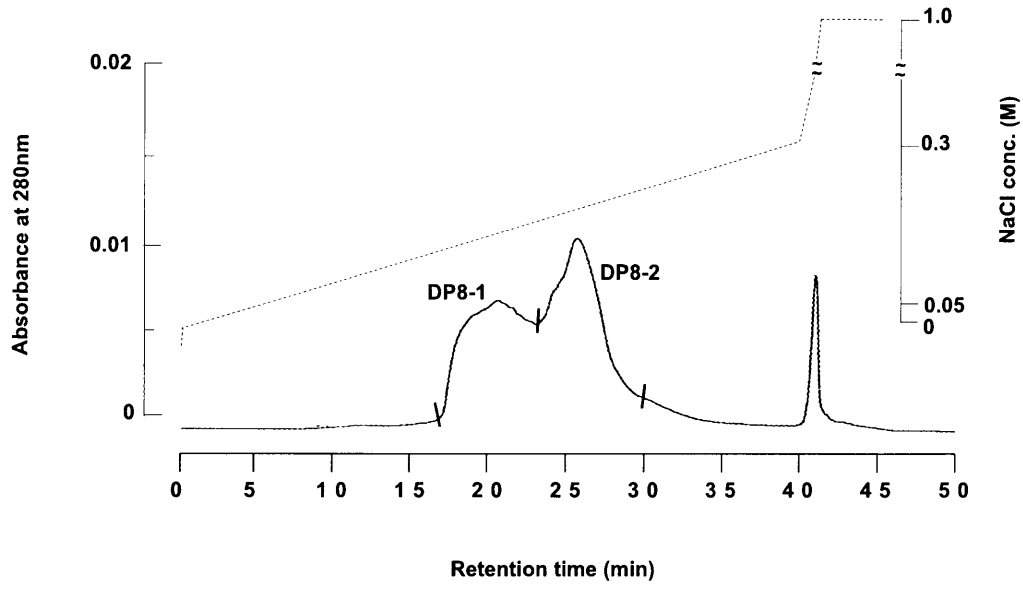



**Fig. 4-2.** Anion-exchange HPLC of the DP7-2 fraction on a TSK-gel DEAE-5PW column. The sample was dissolved in 0.02 M Tris-HCl, pH 8.0, and applied to the column equilibrated with the same solvent. Proteins were eluted with a linear gradient increase in sodium chloride at a flow rate of 0.5 mL/min.

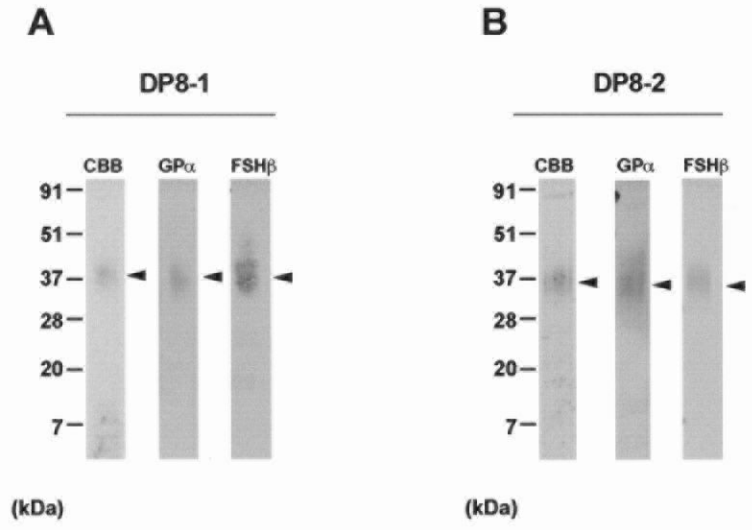



**Fig. 4-3.** SDS-PAGE and western blotting of purified rjeFSH fraction, DP8-1 (A) and DP8-2 (B). Samples are separated on 15-25% SDS-PAGE under a non-reduced condition. Purified protein (0.5 μg) was separated on SDS-PAGE and stained with CBB. After separation on SDS-PAGE, purified eel FSH was stained with anti-jeGPα and anti-jeFSHβ. Arrowheads show specific signals.

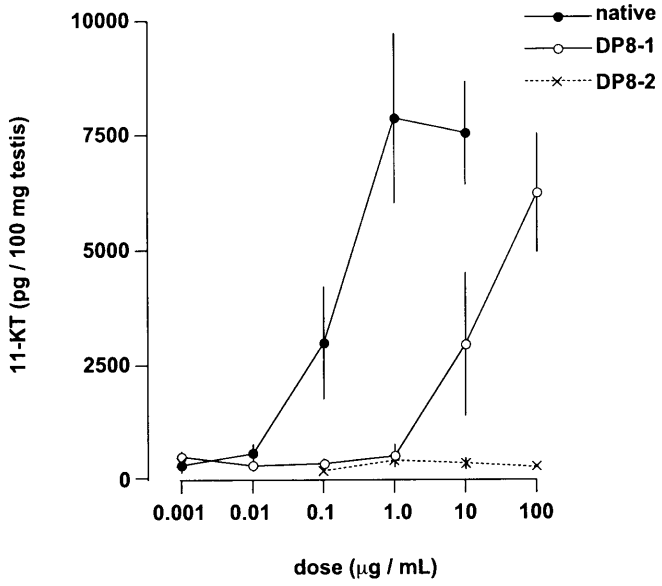



**Fig. 4-4.** Dose-dependent 11-ketotestosterone secreting activity of native and recombinant FSH in immature Japanese eel testis. Closed and open circles, and crosses show the 11-ketotestosterone release from immature eel testis stimulated by native eel FSH and rjeFSH (DP8-1 and DP8-2).

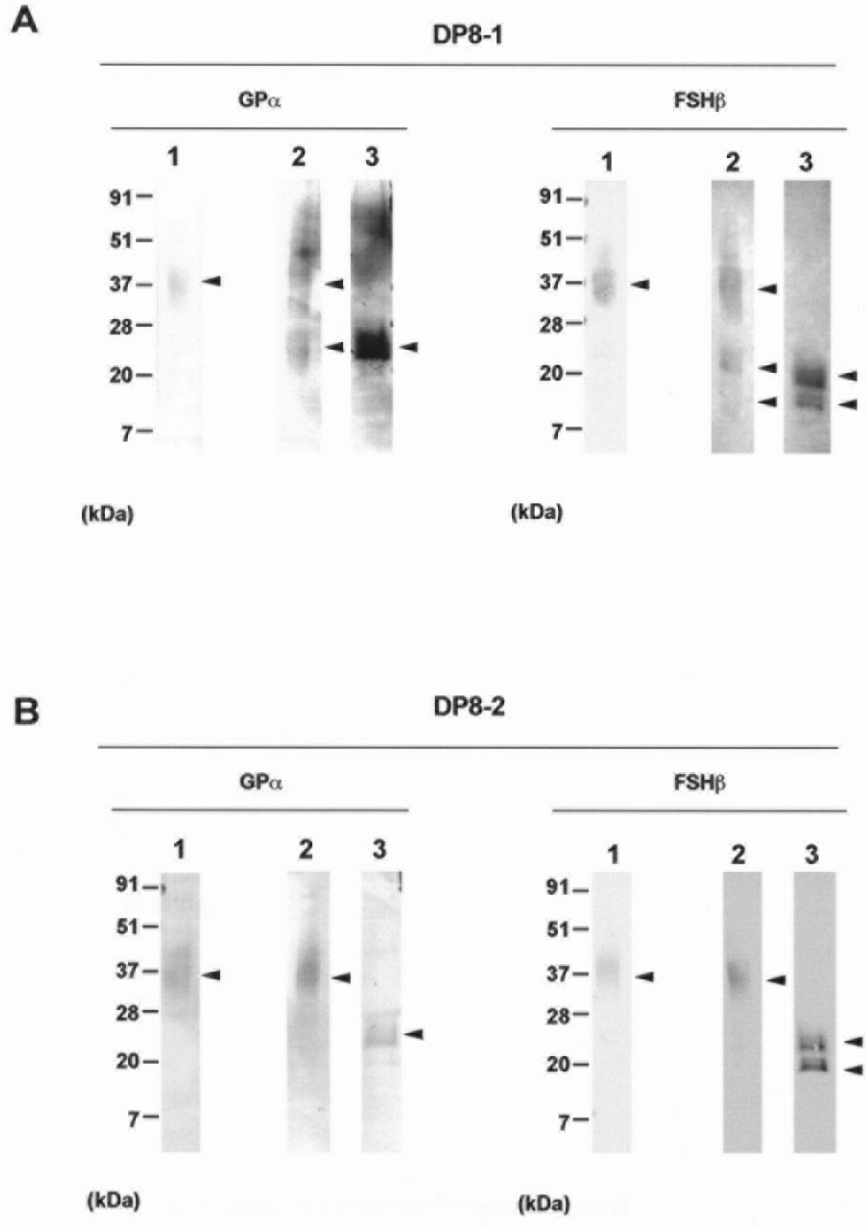



Fig. 4-5. Acid-stability tests of purified rjeFSH fractions, DP8-1 (A) and DP8-2 (B). Samples are separated on 15-25% SDS-PAGE under a non-reduced condition. Purified protein (0.5 μg)( 1) and that treated with 0.1 % TFA (2) or 5 % 2-ME (3). After separation, purified eel FSH was stained with anti-jeGPα and anti-jeFSHβ. Arrowheads show specific signals.

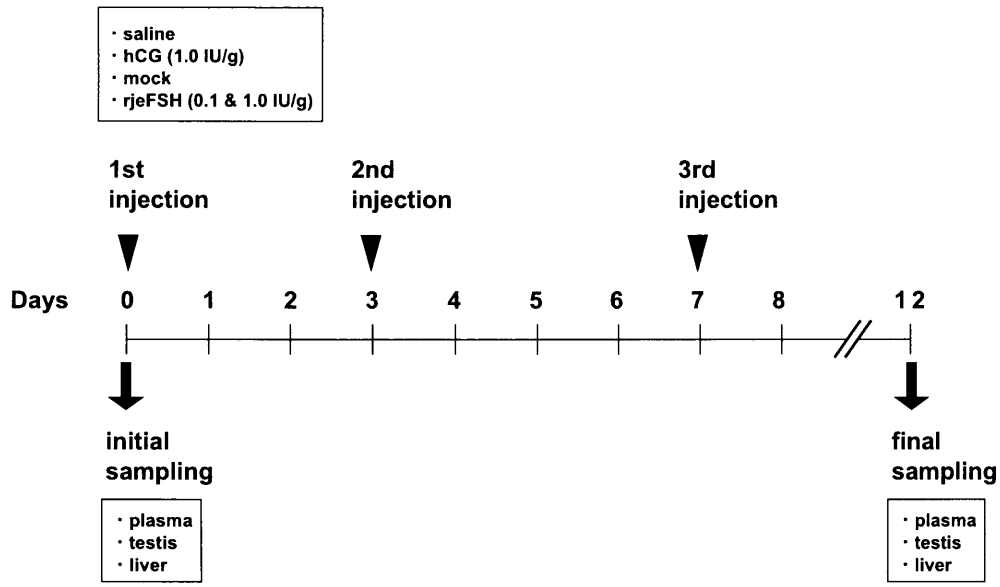



**Fig. 4-6.** Schematic illustration of experimental design of *in vivo* rjeFSH administration.

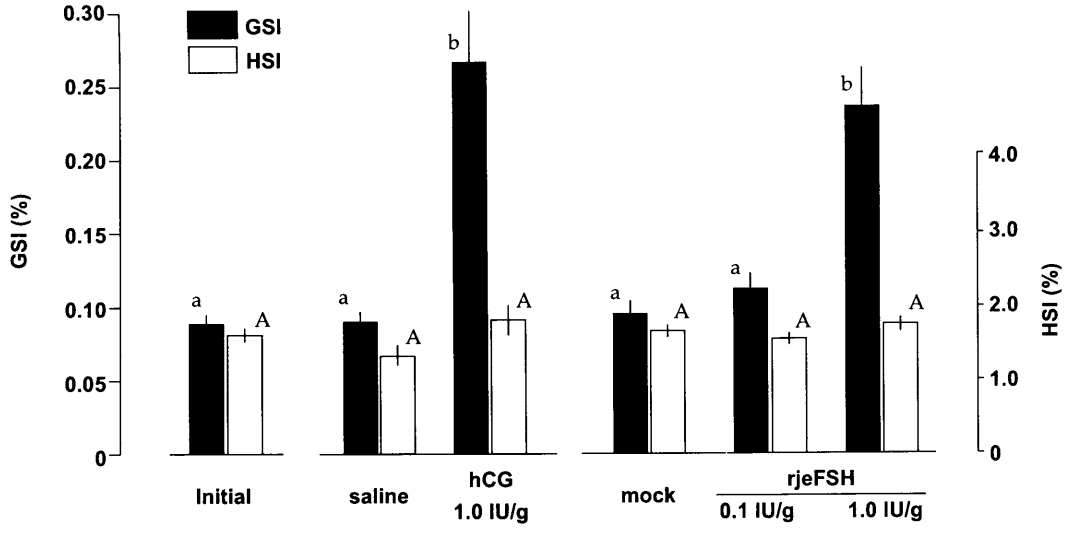



**Fig. 4-7.** Changes in GSI and HSI of cultivated male eels before and after *in vivo* experiment. Values are means±S.E.M. (n=3-5). Different letters (a, b) indicate significant difference at $P<0.05$.

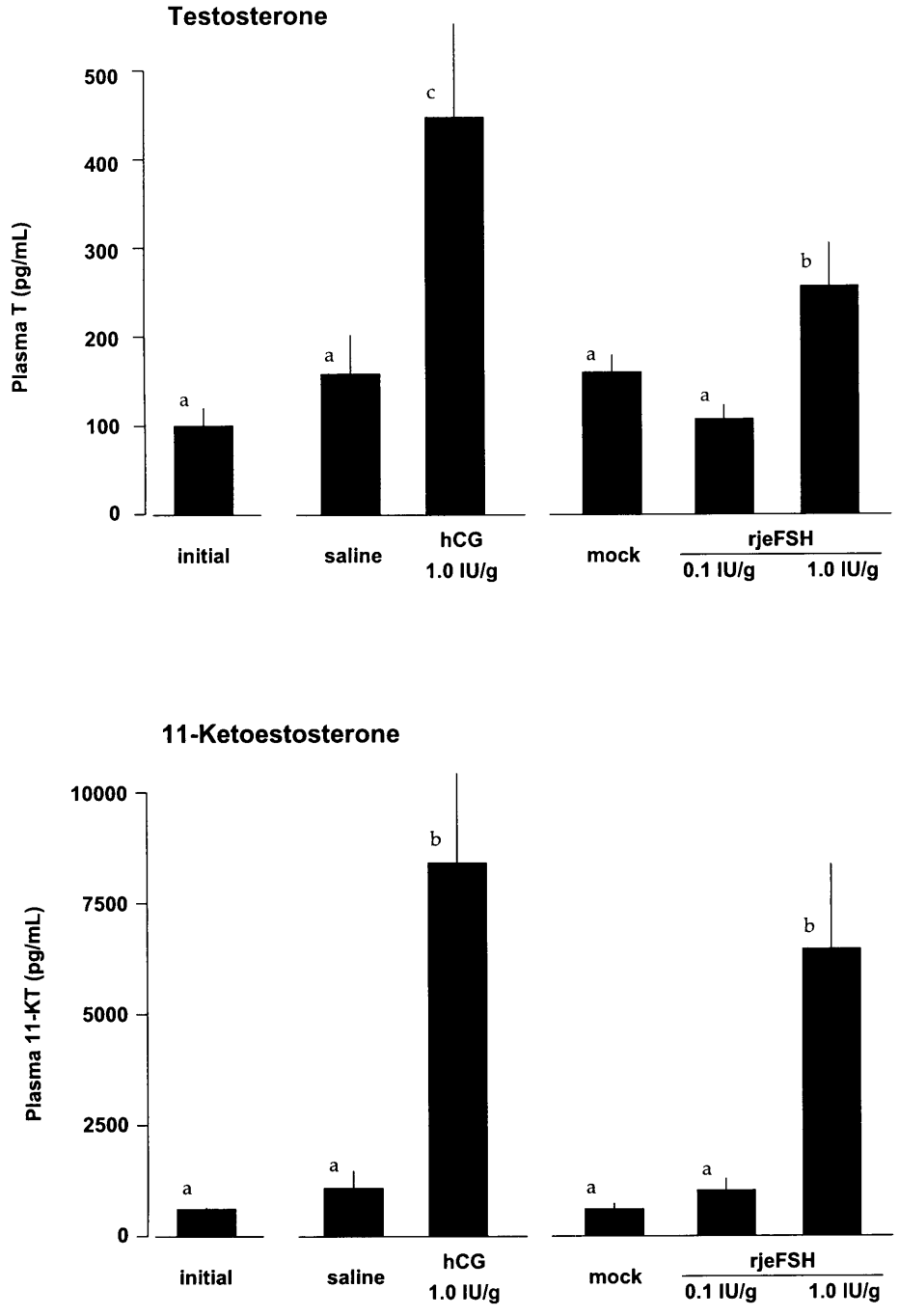



**Fig. 4-8.** Plasma testosteron (T) and 11-ketotestosterone (11-KT) levels of cultivated male eels before and after *in vivo* experiment. Values are means±S.E.M. (n=3-5). Different letters (a, b, c) indicate significant difference at $P<0.05$.

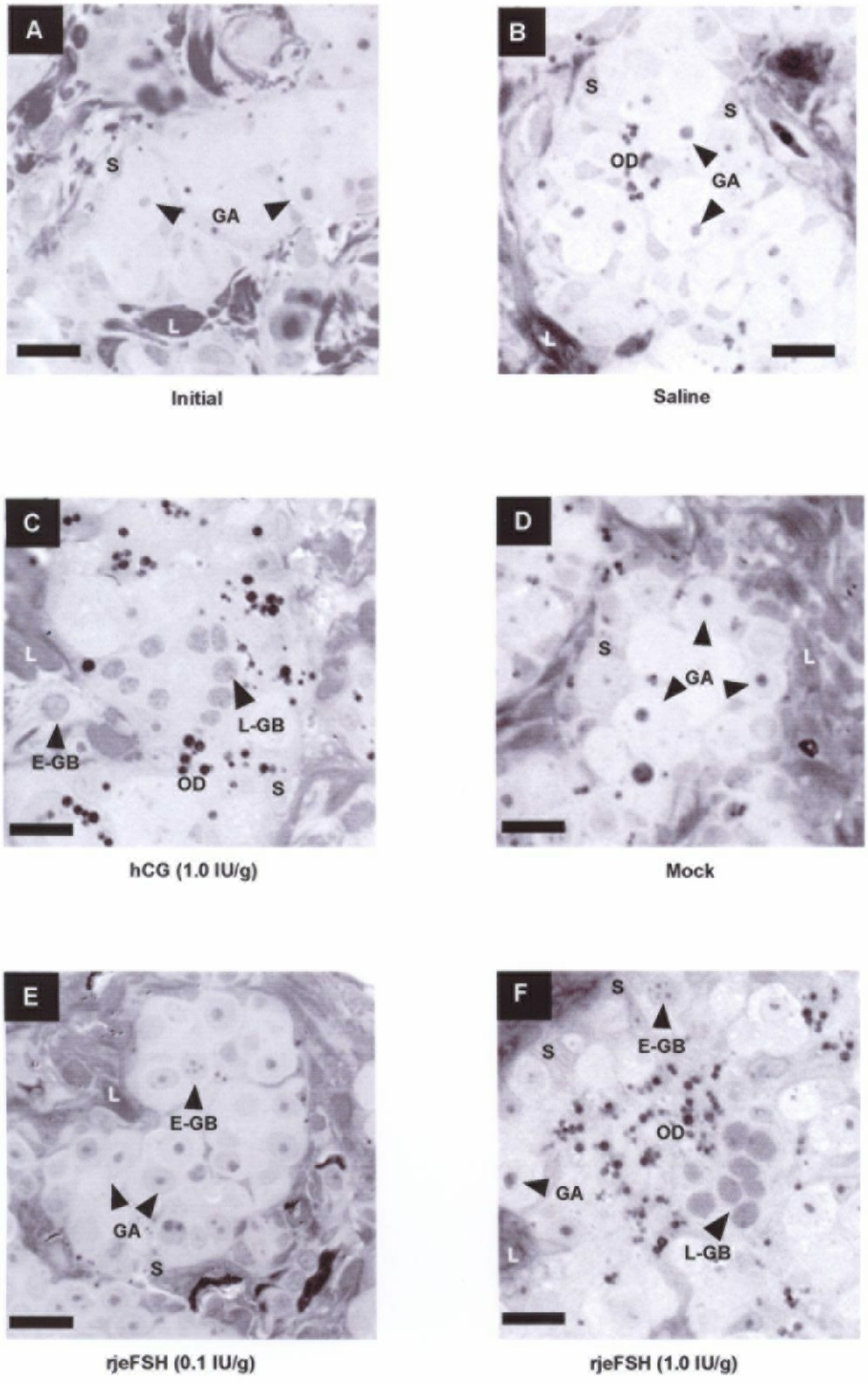



**Fig. 4-9.** Light micrographs of the testes befor (A) and after (B,C,D,E,F) *in vivo* experiment. Unproliferated type A spermatogonia (GA); early-type B spermatogonia (E-GB); prolifelated late-type B spermatogonia (L-GB); Sertoli cells (S) ; Leydig cells (L). Allowheads indicate germ cells. Well-accumulated oil droplet (OD) and L-GB are typically observed in the group of hCG (1.0 IU/g-BW; C) and rjeFSH (1.0 IU/g-BW; F). Scale bars: 10 μm.