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Isolation and Characterization of Two Coho Salmon Gonadotropins, GTH I and GTH II[1]

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ABSTRACT

Two gonadotropins, GTH I and GTH II, were isolated from pituitaries of spawning coho salmon (*Oncorhynchus kisutch*) using sequential extractions with ammonium acetate (pH 9.0) and 40% ethanol, precipitation with 80% ethanol, gel filtration chromatography (Sephadex G-100), anion-exchange chromatography (Mono-Q Sepharose), and gel filtration chromatography (Sephadex G-75). Coho salmon GTH I and GTH II stimulated steroidogenesis in vitro in a similar dose-dependent manner when incubated with either ovaries or testes of prepubertal coho salmon. An in vivo bioassay using coho salmon parr demonstrated that coho salmon GTH I and GTH II did not contain thyrotropic activity. Molecular weights were estimated by gel filtration chromatography to be 43 000 and 39 000 for GTH I and GTH II, respectively. Analysis of coho salmon GTH I and GTH II on reversed-phase high-performance liquid chromatography (rpHPLC) revealed that they consist of α and β subunits with N-terminal amino acid residues of Tyr, Gly (α,β of GTH I) and Tyr, Ser (α,β of GTH II). Coho salmon GTH I-β and GTH II-β differed from each other in amino acid composition, N-terminal amino acids (Gly vs. Ser), and molecular weights in SDS-PAGE (19 000 vs. 20 000) and had a high degree of chemical similarity to chum salmon GTH I-β and GTH II-β, respectively. Specific rabbit antisera to the β subunits of coho salmon GTH I and GTH II were generated. The observation of two GTHs with distinctly different chemical characteristics in coho salmon is similar to what has previously been found in chum salmon.

INTRODUCTION

Control of gonadal function by pituitary gonadotropins (GTHs) is a general feature of vertebrate reproduction. In most tetrapods, gonadal function has long been known to be regulated by two GTHs: FSH and LH [1]. LH, FSH, and a third pituitary hormone, thyroid-stimulating hormone (TSH), are chemically related. All three consist of an α and a β subunit that interact noncovalently and are both glycosylated. In mammals it has been found that the α subunits of TSH, LH, and FSH are identical within a species, whereas the β subunits are hormone-specific and structurally conserved between species [2].

Whether fish reproduction is regulated by one or two pituitary GTHs has been controversial for nearly two decades. A single GTH, sometimes referred to as maturational GTH, has been isolated from several teleost species: chinook salmon, *Oncorhynchus tschawytscha* [3]; carp, *Cyprinus carpio* [4]; silver carp, *Hypophthalmichthys molitrix* [5, 6]; pike eel, *Muraenesox cinereus* [7, 8]; tilapia, *Orechromis mossambica* [9]; African catfish, *Clarias gariepinus* [10] and Atlantic croaker, *Micropogonias undulatus* [11]. Maturational GTH has been thought to regulate all aspects of gametogenesis (reviewed by Burzawa-Gerard [12]; Fontaine and Dufour [13]). The single type of GTH consistently isolated from the teleost species so far examined appears to be chemically related to tetrapod FSH and LH because of its glycoproteic and subunit nature. In contrast, Idler and colleagues (reviewed by Idler and Ng [14]) prepared two GTH fractions from pituitaries of four teleost species; one adsorbed to Concanavalin-A Sepharose and stimulated gonadal steroidogenesis (Con-A II) while the other did not adsorb to Concanavalin-A Sepharose and stimulated in vivo vitellogenin uptake by ovarian follicles (Con-A I). Con-A II shows some chemical similarity to classic tetrapod pituitary GTHs, whereas Con-A I does not; Con-A I is low in carbohydrate content, and its subunit nature has not been demonstrated.

More recently, Kawauchi and colleagues [15–21] definitively identified two chum salmon (*O. keta*) pituitary GTHs, GTH I and GTH II, which are distinctly different from each other in chemical characteristics and structurally homologous to tetrapod FSH and LH. Chum salmon GTH I-β and GTH II-β have only about 31% amino acid sequence identity to each other. Amino acid sequence comparisons of chum salmon GTH I-β and GTH II-β to β subunits of bovine FSH (bFSH) and bovine LH(bLH) revealed that GTH I-β has slightly greater sequence identity to bFSH-β (41%) than to bLH-β (35%), whereas GTH II-β has greater identity to bLH-β (42%) than to bFSH-β (38%). Comparisons of the cDNA sequences of chum salmon GTH β subunits to the cDNAs of bovine LH-β and FSH-β demonstrate the same structural

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relatedness [18, 22]. The cDNA of chum salmon GTH I-β has 53% and 45% sequence identity to bFSH-β and bLH-β cDNAs, respectively. The cDNA of GTH II-β has slightly greater sequence identity to the cDNA of bLH-β (51%) than to that of bFSH-β (48%). The amino acid sequences of chinook salmon [23], carp [24], and silver carp [6], and pike eel [8] GTH β subunits, which were either determined directly or deduced from the nucleotide sequence of the cDNA, show about 40% sequence homology to mammalian LH-β and about 75% sequence identity to chum salmon GTH II-β. Therefore, it is apparent that both GTH I and GTH II have structural homology to tetrapod FSH and LH, and the single GTH molecule previously isolated and characterized from several teleost species is structurally similar to chum salmon GTH II. GTH I had not been identified in previous studies.

During the isolation of chum salmon GTH I and GTH II, chemical methods were used to identify GTH I and GTH II [19]. Bioassays used to verify that the purified GTHs were biologically active showed that GTH I and GTH II were equally potent in stimulating gonadal growth in trout after in vivo injections. In another study, Suzuki et al. [25] demonstrated that chum salmon GTH I and GTH II have similar steroidogenic activities when tested in vitro in both vitellogenic ovarian follicles and mature ovarian granulosa and thecal tissue incubations. Therefore, it is likely that the duality of salmon GTHs was not previously detected because the classic GTH bioassays used to monitor fractions during purification studies could not distinguish the two GTHs.

The present study was undertaken to determine whether two pituitary GTHs are present in coho salmon (*O. kisutch*) and to determine their chemical similarity to GTHs of chum salmon. Purified coho salmon GTH I and GTH II were also necessary for future investigations of the physiological roles of GTH I and GTH II in reproductive development of coho salmon. This report describes the isolation and characterization of coho salmon GTH I and GTH II, and generation of β-subunit-specific antisera. During the purification steps, chemical methods were primarily used to identify fractions containing GTH I and GTH II.

MATERIALS AND METHODS

Pituitary Collection and Extraction

Pituitary glands were collected from spawning male and female coho salmon at Domsea Farms, Inc. (Rochester, WA). Glands were frozen on dry ice and stored at −70°C until processing. Pituitaries (40 g wet wt.) were homogenized in 0.2 M ammonium acetate, 1 mM PMSF (pH 9.0), using a VIRTIS (Gardiner, NY) homogenizer (3 000 rpm, 1 min) maintained on ice. The homogenate was stirred 1 h at 4°C and centrifuged at 23 000 × *g* for 30 min. The supernatant was adjusted to 40% ethanol by addition of ice-cold ethanol and stirred overnight at 4°C. After centrifugation, the supernatant was adjusted to 80% ethanol by addition of ice-cold ethanol and was stored 24 h at 4°C without stirring to allow precipitation of proteins. The resulting precipitate was collected after carefully decanting most of the supernatant and centrifuging the remaining fluid. The precipitate was evaporated to near dryness in a vacuum-dessicator. The moist precipitate was dissolved in 0.1 M ammonium bicarbonate (pH 9.0) and lyophylized. In subsequent purification studies, this lyophylization step was omitted.

Chromatography Procedures

The ethanol-precipitated proteins were initially fractionated by gel filtration chromatography on a 3.0 × 85-cm column of Sephadex G-100 superfine (Pharmacia-LKB Biotechnology, Inc., Piscataway, NJ). The extract (0.8 g) was divided such that approximately 100 mg protein was loaded onto the column for each run and the column was run 8 times. The fractions containing proteins with average molecular weights ranging from 25 000 to 50 000 were pooled and lyophylized. In previous studies, this fraction was shown to contain both thyrotropic and gonadotropic activities [26]. The lyophylized gel filtration fraction containing GTHs was subsequently applied to an anion-exchange column (1.0 × 12.5-cm) of Mono-Q Superose (Pharmacia-LKB Biotechnology, Inc.) equilibrated with 0.05 M ammonium bicarbonate (pH 9.0). The sample (30–50 mg) was dissolved in 0.05 M ammonium bicarbonate (pH 9.0), and unadsorbed proteins were eluted with the same buffer. Adsorbed proteins were eluted with a stepwise gradient of 0.1, 0.2, 0.3, and 1.0 M ammonium bicarbonate (pH 9.0). The fractions were lyophylized and subjected to gel filtration chromatography on a 1.6 × 98-cm column of Sephadex G-75 superfine (Pharmacia-LKB Biotechnology, Inc.). The column was equilibrated and proteins were eluted with 0.1 M ammonium bicarbonate (pH 9.0).

Analytical Procedures

During the purification, fractions were examined by both gel electrophoresis and reversed-phase HPLC (rpHPLC). SDS-PAGE was done according to the method of Laemmli [27] with a 5% stacking and 15% separating gel. Samples were examined both with and without reduction by 5.0% β-mercaptoethanol. Proteins were also examined in nondenaturing 7.5% PAGE (pH 8.6) [28]. Gels were stained with 0.2% Coomassie Brilliant Blue R-250 in methanol/acetic acid/water (5:1:5; v/v). rpHPLC was performed on a TSK gel ODS-120T column (4.6 × 250-mm; 5-μm particle size) with a linear gradient of 20–45% acetonitrile, 0.1% trifluoroacetic acid (TFA) for 40 min at a column temperature of 40°C. Protein peaks from rpHPLC were then analyzed for molecular weight by SDS-PAGE. Amino acid composition was determined by the PTC method [29, 30] after proteins were hydrolyzed for 22 h at 110°C with constant-boiling 6 N HCl containing 1.0% phenol. N-terminal amino acid residues were

determined by the Dansyl method [31]. Fractions from rpHPLC were identified as α and β subunits by comparison of amino acid compositions, N-terminal amino acid residues, and molecular weights to those reported for chum salmon GTH I and GTH II [19, 20]. The criteria used for chemical identification and purity of intact GTHs obtained after gel filtration chromatography (Sephadex G-75) were as follows: 1) the protein eluted as "intact" size (30 000–50 000) during gel filtration chromatography on Sephadex G-75; 2) the protein migrated as a single band in nondenaturing PAGE and as two bands in SDS–PAGE; 3) the protein had two N-terminal residues, one for each subunit; 4) both α and GTH-specific β subunits were present when examined by rpHPLC, and each had a single N-terminal residue. GTH I and GTH II were identified according to elution positions on anion-exchange chromatography, amino acid compositions, and N-terminal amino acid residues reported for chum salmon GTH I and GTH II [19–21].

Bioassays

Gonadotropic activity was evaluated by the ability of the proteins to stimulate in vitro estradiol or androgen production by juvenile coho salmon ovarian or testicular tissue, respectively. Thyrotropic activity was monitored with an in vivo bioassay using coho salmon parr. Procedures for these bioassays were as described previously [26, 32]. The major protein peaks from ion-exchange chromatography (Mono-Q Sepharose) and the fractions from gel filtration chromatography (Sephadex G-75) that were identified as GTH I and GTH II by analytical methods were tested for biological activity. Bioassay data were statistically analyzed by analysis of variance and comparisons with treated versus control groups were done by Student's *t*-test.

β-Subunit Isolation and Preparation of Antisera

To obtain highly purified GTH I and GTH II β subunits without utilizing valuable preparations of purified intact GTH I and GTH II, β-subunit preparations obtained by analytical rpHPLC of fractions from ion-exchange chromatography (containing both GTH I and GTH II) were further purified. When mixtures of GTH I and GTH II were subjected to rpHPLC as described above, GTH I-β was frequently contaminated slightly with GTH II-β, whereas highly purified GTH II-β could be obtained. To achieve a complete separation of GTH I-β and GTH II-β, the preparations were fractionated by rpHPLC on a TSK gel ODS-120T column (0.46 × 25-cm) with a linear gradient of 20–45% acetonitrile in 0.01 M ammonium acetate (pH 6.0) in 40 min at a column temperature of 40°C. β subunits of GTH I and GTH II purified by this method were used to immunize New Zealand white male rabbits (two for each subunit). Each rabbit was initially injected intradermally in several sites on the back with 50 μg of β subunit in 0.5 ml 0.9% NaCl emulsified with 0.5 ml Freund's complete adjuvant. Subsequent injections contained 10 μg of β subunit. Each rabbit received 5 injections at 3-wk intervals. Animals were completely bled by carotid cannulation 3 wk after the last injection. Serum was aliquoted, lyophylized, and stored dessicated at −20°C. Specificities of the antisera were determined by immunoblotting and immunocytochemistry [33]. Procedures for immunostaining of Western blots are described in detail elsewhere [34].

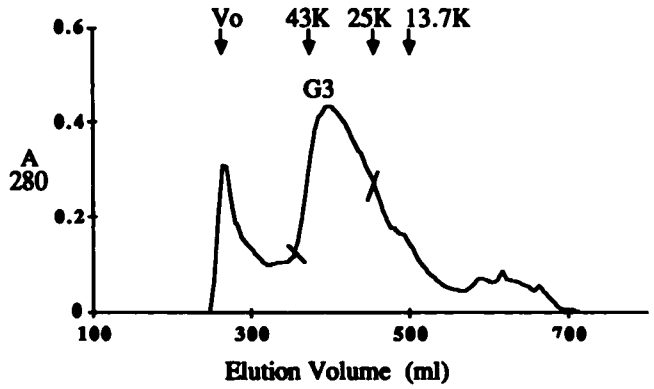

FIG. 1. Gel filtration chromatography of pituitary glycoprotein extract (100 mg) on a column of Sephadex G-100 superfine (3.0 × 85 cm). Column was equilibrated and samples were eluted with 0.1 M ammonium bicarbonate (pH 9.0) at a flow rate of 16 ml/h, 20 min/fraction. TSH and GTH activities coeluted in peak G3. The yield of G3 was 62.3 mg.

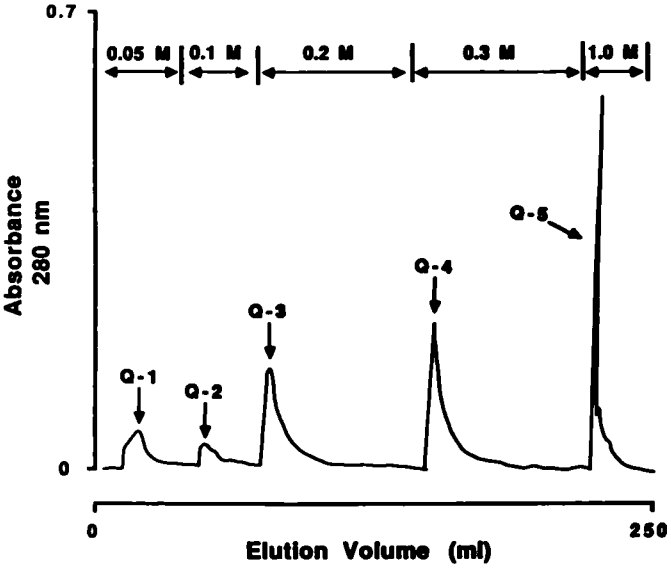

FIG. 2. Anion-exchange chromatography of 20 mg of fraction G3 (*Fig. 1*) on a column on Mono-Q Sepharose (1.0 × 12.5 cm). The sample was dissolved in the 0.05 M ammonium bicarbonate (pH 9.0) and applied to the column with the same buffer. The proteins were eluted with stepwise increases in ammonium bicarbonate concentrations as indicated at a flow rate of 1.0 ml/min, 8 min/fraction. The yields by weight were Q-1, 2.5 mg; Q-2, 1.4 mg; Q-3, 5.3 mg; Q-4, 5.1 mg; Q-5, 3.9 mg.

RESULTS

Chromatography

The major peak of protein (G-3; 62.3 mg) obtained after gel filtration chromatography of the pituitary extract (Fig. 1) contained both thyrotropic and gonadotropic activities (data not shown). When this fraction was subjected to anion-exchange chromatography, five major protein peaks (Q-1 through Q-5) were obtained (Fig. 2). Analytical rpHPLC and amino acid composition analysis of the major rpHPLC peaks revealed that the first two Mono-Q Sepharose fractions (Q-1 and Q-2) contained predominantly α subunits (Fig. 3a,b). Fraction Q-2 had thyrotropic activity and no gonadotropic

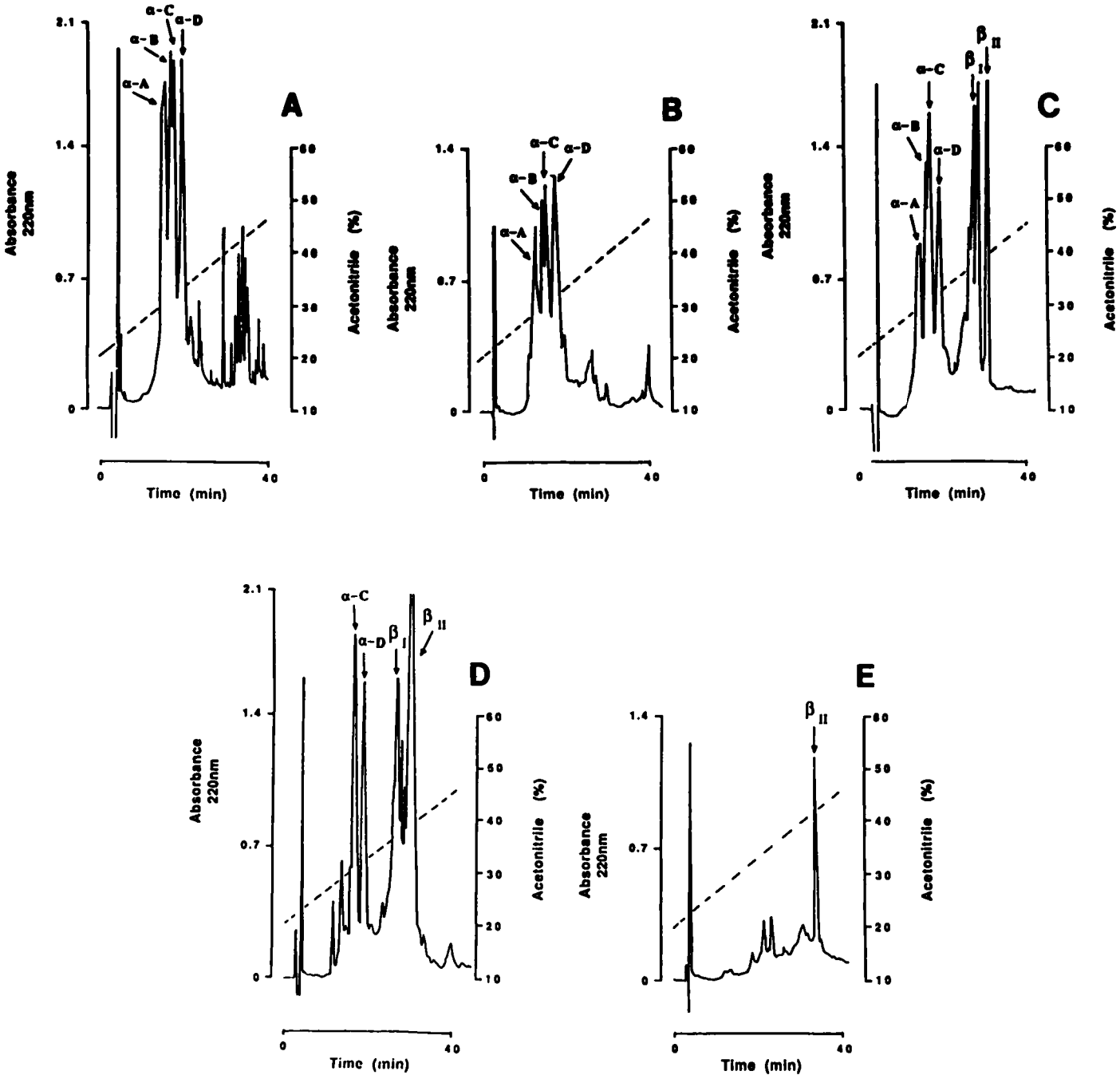

FIG. 3. Reversed-phase high-performance liquid chromatography of fractions from anion-exchange chromatography (*Fig. 2*) on a TSK gel ODS-120T column (4.6 × 250 mm; 5-μm particle size) after samples were dissolved in 0.1% TFA at a column temperature of 40°C and a flow rate of 1.0 ml/min. Dotted line represents a gradient of acetonitrile in 0.1% TFA. A–E are samples Q-1 to Q-5 (*Fig. 2*), respectively. Peaks were identified as α and β after amino acid composition, N-terminal amino acid residue analysis, and gel electrophoresis.

activity (Fig. 4a,b), yet only α subunits could be identified after rpHPLC of this fraction (Fig. 3b). Proteins that eluted after 0.2 M and 0.3 M ammonium bicarbonate were applied to the Mono-Q Sepharose column were designated Q-3 and Q-4 respectively. When analyzed by rpHPLC with subsequent amino acid composition analysis of the major rpHPLC peaks, both Q-3 and Q-4 were found to contain α and β subunits of GTH I and GTH II (Fig. 3c,d). Relative enrichments of GTH I-β in Q-3, and GTH II-β in Q-4 were found. Both Q-3 and Q-4 contained gonadotropic activity (Fig. 4b). The fraction eluting after 1.0 M ammonium bicarbonate (Q-5) contained gonadotropic activity (Fig. 4b). When analyzed by rpHPLC and amino acid composition analysis of the major rpHPLC peak, it was found to contain predominantly GTH II-β (Fig. 3e) with some GTH α subunit. GTH I and GTH II were isolated from fractions Q-3 and Q-4 by gel filtration chromatography on a Sephadex G-75 column (Q-4: Fig. 5; Q-3: data not shown). Fractions 1 and 2 (Fig. 5) were rechromatographed on a Sephadex G-75 column, analyzed by rpHPLC (Fig. 6a,b), and identified as GTH I and GTH II, respectively. Fraction 3 (Fig. 5) contained dissociated α and β subunits.

Physicochemical Properties of Coho Salmon GTH I and GTH II

In gel filtration chromatography (Sephadex G-75) GTH I eluted with an estimated molecular size of 43 000 whereas GTH II eluted as 39 000. In nondenaturing gel electropho-

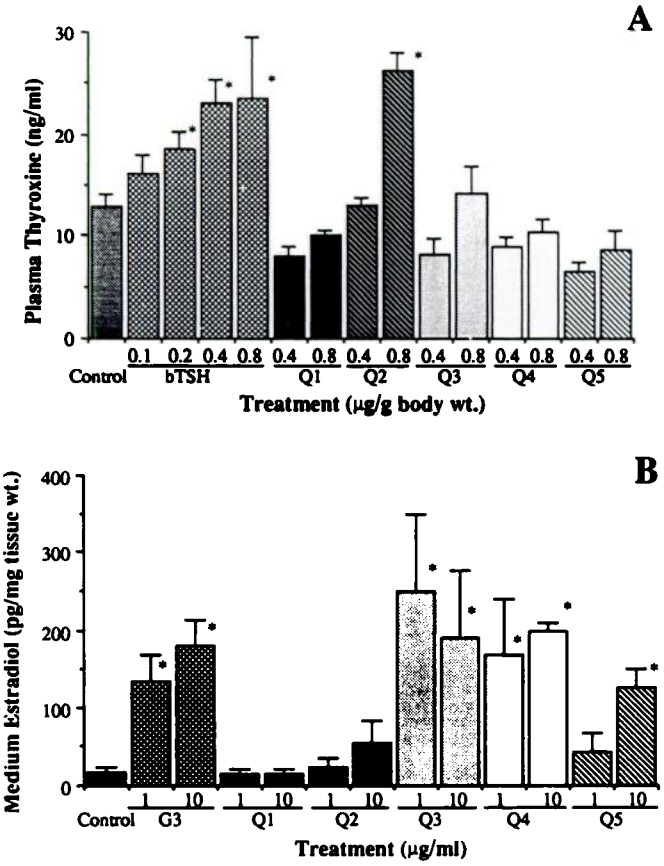

FIG. 4. (A) Thyrotropic activity of bovine TSH (bTSH) and fractions Q-1 to Q-5 (*Fig. 2*) when injected into coho salmon parr. Control fish received i.p. injections of vehicle (0.9% NaCl); treated fish received protein dissolved in vehicle at doses of 0.4 and 0.8 μg/g body wt. Each fish received two injections spaced by 16 h. Blood was collected 8 h after the second injection. Data are expressed as means ± SEM; n = 6/treatment. Asterisk = significant differences from control ($p \leq 0.05$). (B) Steroidogenic activity of fractions Q-1 to Q-5 (*Fig. 2*) in incubations with ovaries from prepubertal coho salmon. After a preincubation for 1 h in control medium, ovaries (1 ovary/ml/well) were incubated for 18 h with fresh control medium or medium containing 1 or 10 μg/ml protein. Data are expressed as means ± SEM; n = 3/treatment. Asterisk = significant differences from control ($p \leq 0.05$).

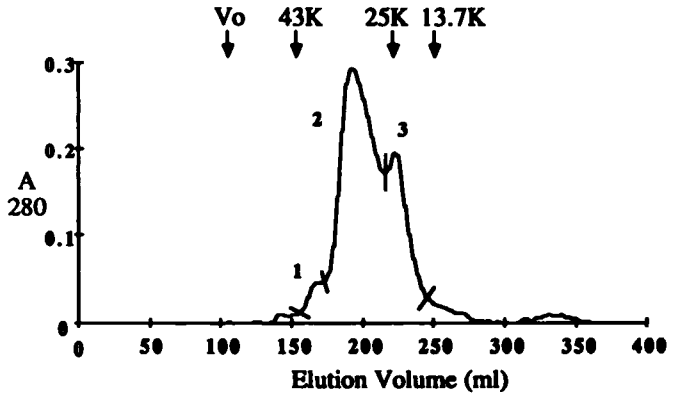

FIG. 5. Gel filtration chromatography of fraction Q-4 (*Fig. 2*) on a column of Sephadex G-75 superfine (1.6 × 96 cm). Sample (5.0 mg) was dissolved in and eluted with 0.1 M ammonium bicarbonate (pH 9.0) at a flow rate of 15 ml/h, 10 min/fraction. Peaks 1 and 2 were rechromatographed on the same column after lyophylization. GTH I (1.7 mg) and GTH II (2.2 mg) were obtained from peaks 1 and 2, respectively.

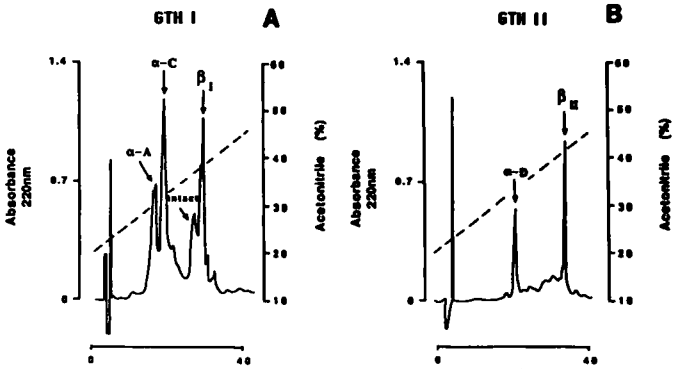

FIG. 6. Reversed-phase high-performance liquid chromatography (rpHPLC) of GTH I (A) and GTH II (B) isolated from fractions 1 and 2 (*Fig. 5*), respectively, after rechromatography on Sephadex G-75. rpHPLC was performed on a TSK gel ODS-120T column (4.6 × 250 mm; 5-μm particle size) after samples were dissolved in 0.1% TFA at a column temperature of 40°C and a flow rate of 1.0 ml/min. Dotted line represents a gradient of acetonitrile in 0.1% TFA. Peaks were identified as α and β after amino acid composition, N-terminal amino acid residue analysis, and gel electrophoresis.

resis, GTH I and GTH II migrated as single bands, whereas in SDS–PAGE, they migrated as three bands under nonreducing conditions (intact, α, and β) and two bands (α and β) under reducing conditions (Fig. 7). When GTH I was analyzed by rpHPLC (Fig. 6a), two α-subunit peaks (α-A,α-C), a β-subunit peak (β_I), and a peak containing intact GTH I with N-terminal amino acid residues of Tyr (α-A,α-C), Gly (β_I), and Tyr,Gly (intact GTH I) were found. In contrast, GTH II (Fig. 6b) contained a single α-subunit peak (α-D) and a single β-subunit peak (β_{II}) with N-terminal amino acid residues of Tyr and Ser, respectively. The molecular weights of the various α and β subunits in SDS-PAGE (Fig. 7) under reducing conditions were as follows: GTH I-α-A: 22 000; GTH I-α-C: 24 000; GTH I-β (Iβ): 19 000; GTH II-α (α-D): 20 000; GTH II-β (IIβ): 22 000. Amino acid compositions of the β subunits of coho salmon GTH I and GTH II (Fig. 8 a,b,c) indicated that coho salmon GTH I-β and GTH II-β are chemically different, yet they are similar to chum salmon GTH I-β and GTH II-β, respectively (data from Itoh et al. [20, 21]). Amino acid composition analysis of the α subunits of GTH I and GTH II indicated that GTH I contained two chemically distinct α subunits, one of which was similar to the single α subunit of GTH II (Fig. 8 d,e,f). A fourth α-subunit fraction (α-B) isolated by rpHPLC from the fraction unadsorbed on Mono-Q Sepharose (Fig. 3a) was not present in the preparations of intact GTH I and GTH II, but had an amino acid composition similar to GTH I-α-A (Fig. 8d), an N-terminal residue of Tyr, and a molecular weight of 15 700 in SDS-PAGE (Fig. 7).

Biological Activities of Coho Salmon GTH I and GTH II

GTH I and GTH II exhibited similar in vitro steroidogenic protencies when incubated with either ovaries or testes of juvenile coho salmon (Fig. 9a, b; see also data reported by Swanson et al. [32]). Neither GTH I nor GTH II (at doses of 0.5 or 1.0 μg/g body wt.) significantly elevated plasma thyroxine levels in coho salmon parr whereas a crude TSH fraction (Q-2, Fig. 2) elevated plasma thyroxine at equivalent doses (Fig. 9c).

Subunit Isolation and Specificites of GTH β-Subunit Antisera

By use of rpHPLC, highly purified GTH I-β and GTH II-β were obtained (Fig. 10). Immunostaining of Western blots of GTH I-β and GTH II-β after SDS-PAGE (Fig. 11) indicated that one of the rabbits immunized with GTH I-β (#8622) produced antiserum that specifically stained GTH I-β, and one of the rabbits immunized with GTH II-β (#8624) produced antiserum that specifically stained GTH II-β. These

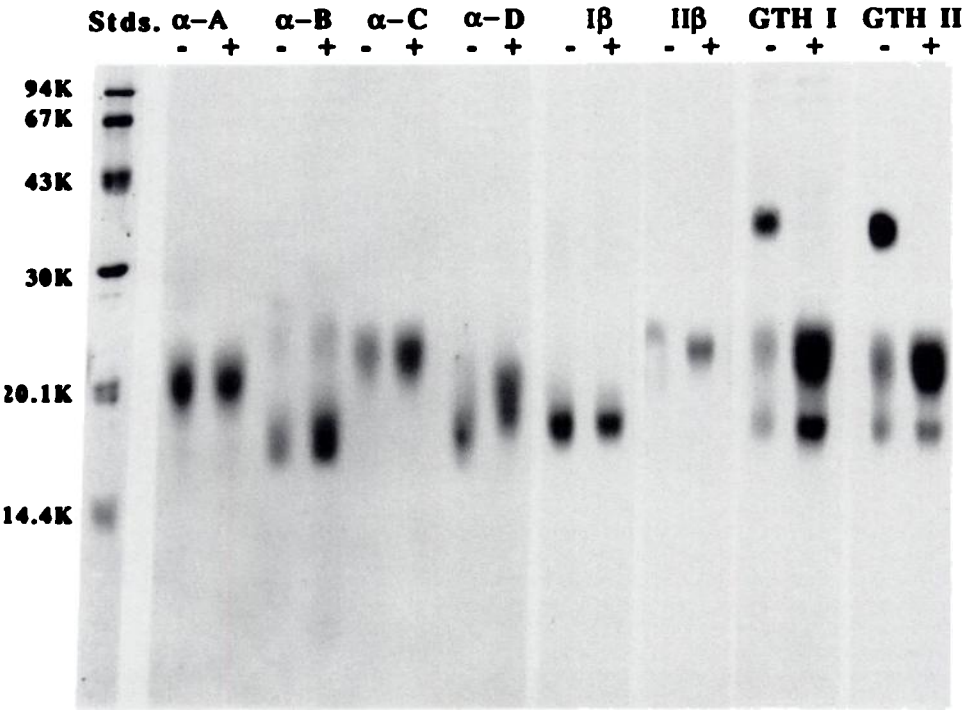

FIG. 7. SDS-PAGE of 10 μg each of intact coho salmon GTH I and GTH II, and their respective subunits with (+) or without (−) reduction by β-mercaptoethanol. α-B was isolated from fractions Q-1 and Q-2 (α-B, *Fig. 3a,b*); α-A, α-C, and β-I were isolated from intact GTH I (α-A,α-C, and βI, Fig. 6A); α-D and β-II were isolated from GTH II (α-D and βII, *Fig. 6B*).

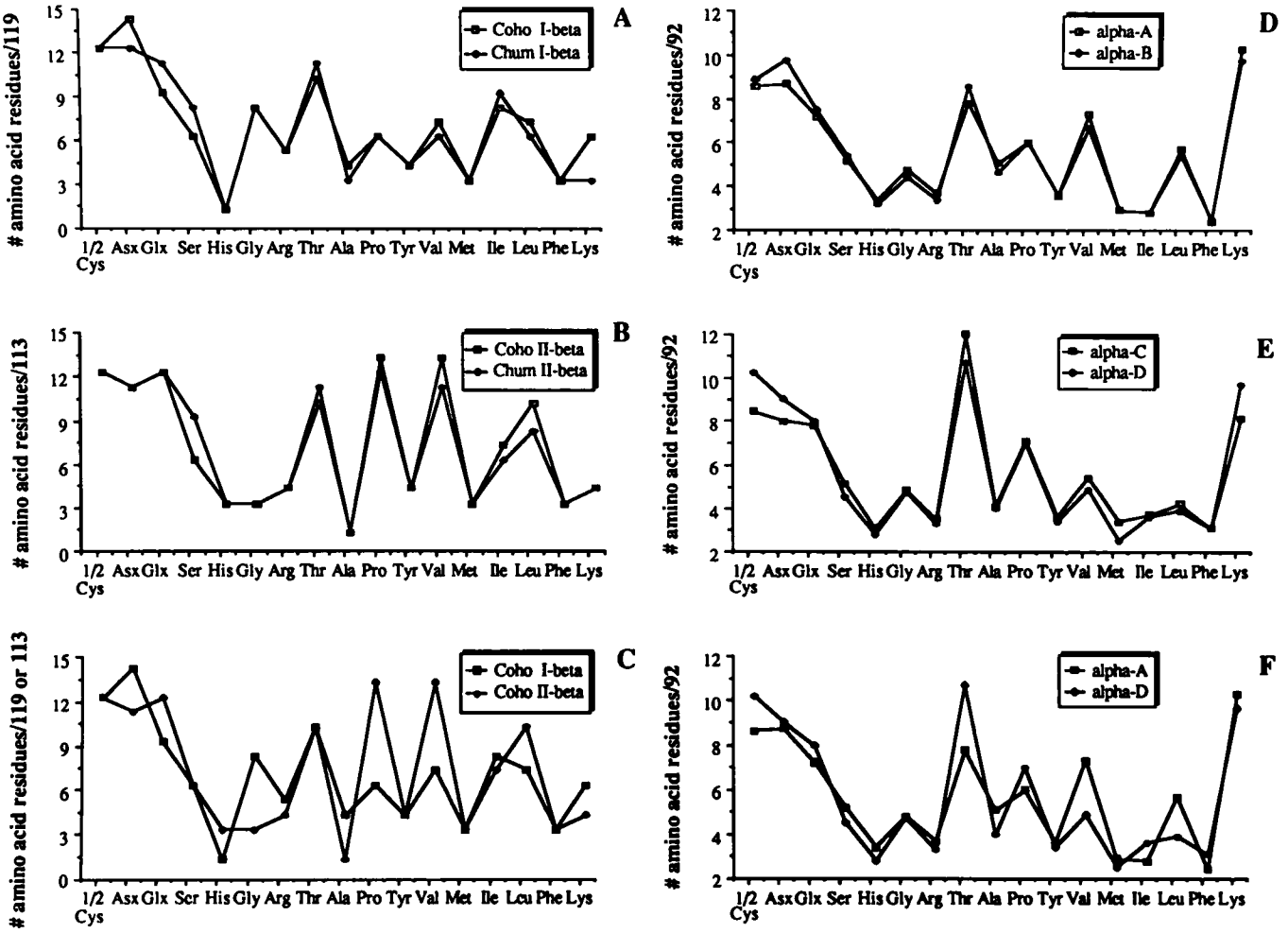

FIG. 8. Comparison of amino acid compositions of coho and chum salmon GTH I-β (A), coho and chum salmon GTH II-β (B), coho salmon GTH I-β and GTH II-β (C), coho salmon α-A and α-B (D), coho salmon α-C and α-D (E), and coho salmon α-A and α-D (F). Data do not include analysis for Trp. Data for chum salmon GTH β subunits is from Itoh et al. [31]. See Figure 7 for source of α subunits and β subunits. Note α-A,α-B,α-C, and α-D correspond to α-A,α-B,α-C, and α-D respectively, from Figure 3.

antisera (#8622 and #8624) also stained two distinctly different pituitary gonadotroph cell types when used for immunocytochemistry in salmonid pituitaries [33].

DISCUSSION

In this study, GTH I and GTH II were isolated from mature coho salmon pituitaries by using predominately chemical procedures to monitor fractions during the purification steps. Bioassays were used to verify the presence of gonadotropic and thyrotropic activities in the major fractions from ion-exchange chromatography and in the purified GTH I and GTH II fractions. A chemical approach to identifying coho salmon pituitary GTH I and GTH II was aided by the detailed chemical information available on chum salmon GTH I and GTH II [19–21]. The work of Suzuki and colleagues [25] indicated that classical bioassays that evaluate steroidogenic activities or the ability of proteins to stimulate germinal vesicle breakdown cannot be used to clearly distinguish salmon GTH I and GTH II. Therefore, chemical identification of GTH I and GTH II are necessary throughout the purification steps until bioassays distinguishing GTH I and GTH II can be developed.

Procedures similar to those described by Suzuki et al. [19] were used to purify coho salmon GTH I and GTH II. However, Mono-Q Sepharose was chosen for anion-exchange chromatography in the present study since preliminary studies indicated it could be used to separate pituitary glycoproteins containing thyrotropic activity from those containing gonadotropic activity. The relative elution positions of thyrotropic and gonadotropic activities in the anion-exchange chromatography used in this study are consistent with those observed in chromatofocusing (i.e. TSH is more basic in pI than GTHs; [26]). The use of chromatofocusing was avoided in the present study since this technique potentially fractionates isoforms of the pituitary gly-

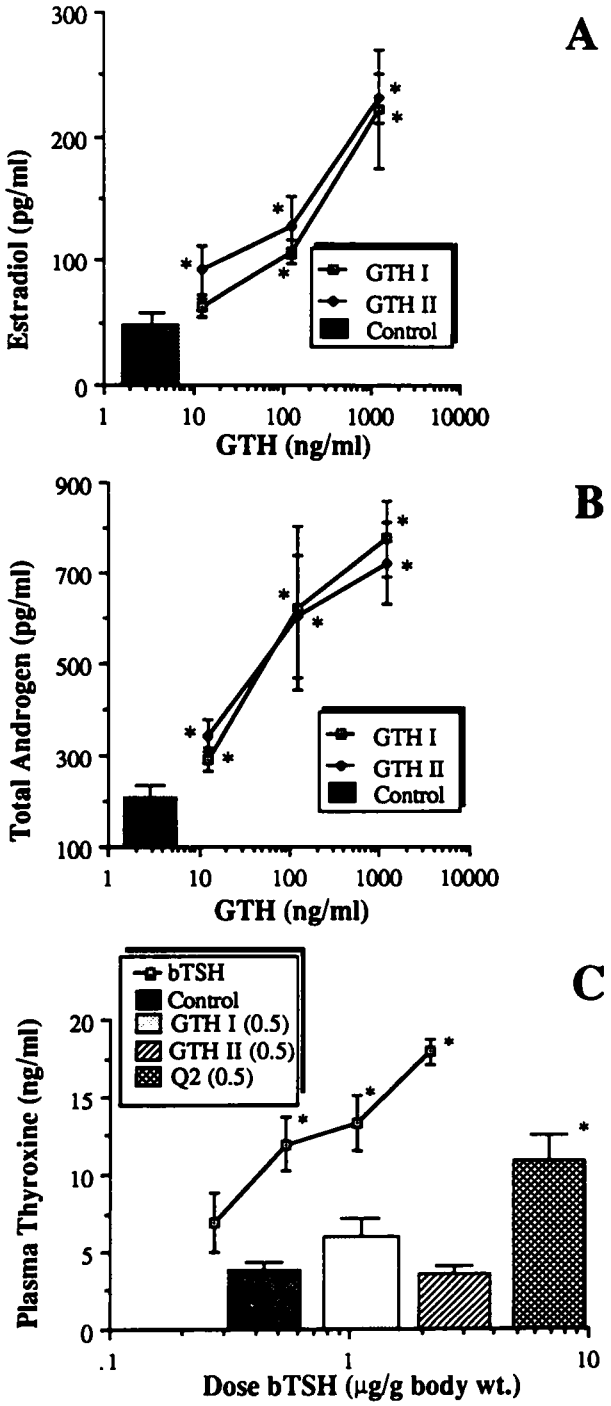

FIG. 9. Steroidogenic activity of coho salmon GTH I and GTH II in incubations with ovaries (A) or testes (B) from prepubertal coho salmon. After a preincubation for 1 h in control medium, gonads (1 ovary or testis/ml/well) were incubated for 18 h with fresh control medium or medium containing 10, 100, or 1 000 ng/ml protein. Data are expressed as means ± SEM; n = 6/treatment. Asterisk = significant differences from control ($p \leq 0.05$). (C) Thyrotropic activity of bone TSH (bTSH), coho salmon GTH I, GTH II, and fraction Q-2 (*Fig. 2*) when injected into coho salmon parr. Control fish received i.p. injections of vehicle (0.9% NaCl); treated fish received coho salmon proteins dissolved in vehicle at a dose of 0.5 μg/g body wt; bTSH was injected at doses of 0.31, 0.62, 1.25, and 2.5 μg/g body wt. Each fish received two injections spaced by 16 h. Blood was collected 8 h after the second injection. Data are expressed as means ± SEM; n = 6/treatment. Asterisk = significant differences from control ($p \leq 0.05$).

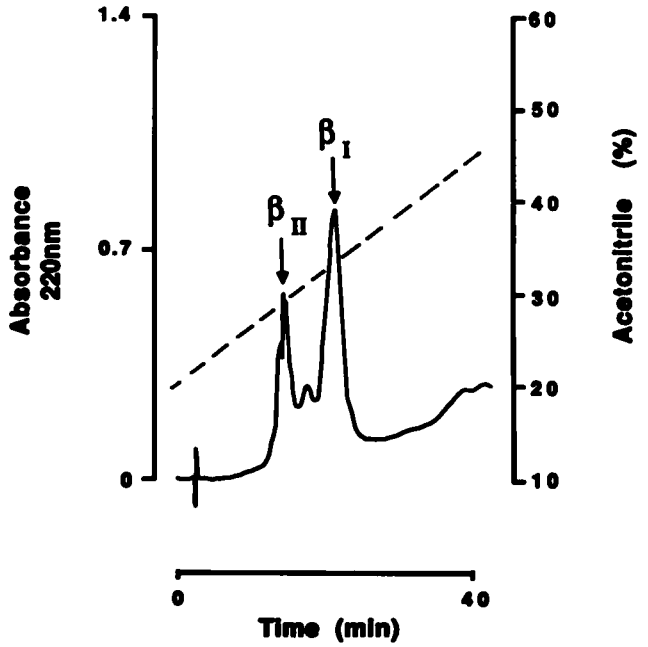

FIG. 10. Reversed-phase high-performance liquid chromatography of a mixture of GTH I-β and GTH II-β on a TSK gel ODS-120T column (4.6 × 250 μm; 5-mm particle size) after samples were dissolved in 0.01 M ammonium acetate (pH 6.0) at a column temperature of 40°C and a flow rate of 1.0 ml/min. Dotted line represents a gradient of 20–45% acetonitrile in 0.1 M ammonium acetate (pH 6.0).

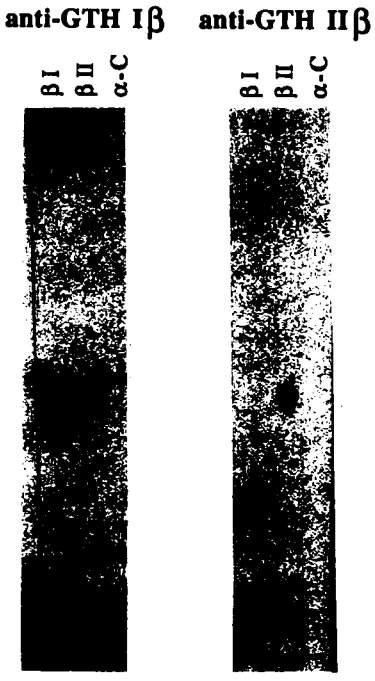

FIG. 11. Immunoblot of coho salmon GTH I-β, GTH II-β, and α (α-C, *Fig. 6a* and *Fig. 7*) stained with anti-GTH I-β (#8622; diluted 1/5 000) and anti-GTH II-β (#8624; diluted 1/5 000). Samples (100 ng each) were first separated by SDS-PAGE and then electroblotted to a nitrocellulose sheet. The immunostaining procedure is described in detail elsewhere [34].

coproteins that have been shown to be microheterogeneous [26]. Since GTH I eluted with greater molecular size than GTH II in gel filtration chromatography, a final gel filtration step was used to completely separate intact GTH I and GTH II from anion-exchange fractions.

The chemical characteristics of coho salmon GTH I and GTH II are similar to those of chum salmon GTHs. The relative elution positions in anion-exchange chromatography of coho salmon GTH I and GTH II were similar to those of chum salmon GTHs; GTH I was relatively more basic than GTH II. In addition, coho salmon GTH I (43 000) eluted in gel filtration chromatography with an average molecular size greater than GTH II (39 000) as was found for chum salmon GTHs. The N-terminal amino acid residues of coho salmon GTH I-β (Gly), GTH II-β (Ser), and α subunits (Tyr) are identical to those of the chum salmon GTH subunits. Molecular weight estimates of the coho salmon GTH subunits in SDS-PAGE were 22 000 and 24 000 for α subunits GTH I; 19 000 for GTH I-β; 20 000 for GTH II-α; and 22 000 for GTH II-β. Suzuki et al. [20] reported similar values for chum salmon GTH α subunits and GTH II-β; however chum salmon GTH I-β was slightly smaller (17 000) than coho salmon GTH I-β (19 000). Since amino acid compositions of the GTH I β subunits of coho and chum salmon were similar, it is possible that they differ in carbohydrate composition, which would result in a difference in apparent molecular weight in SDS-PAGE.

The finding of two coho salmon α subunits that differed in amino acid composition is similar to what has been found for chum salmon α subunits. Sequence analysis of two chum salmon α subunits indicate that they have 72% sequence identity to each other and 65% sequence identity to bovine α subunit [35]. Whether the duality of α subunit has any functional significance is not known.

Coho salmon GTH I and GTH II were distinctly different in their chemical characteristics. However, when the steroidogenic activities of coho salmon GTH I and GTH II were compared by use of an in vitro bioassay, no significant differences in their activities could be found. Coho salmon GTH I and GTH II stimulated estradiol-17β and total androgen production by juvenile ovarian and testicular tissue, respectively, in a similar, dose-dependent manner. These results are similar to those we reported previously [32]. Suzuki et al. [25] also found that chum salmon GTH I and GTH II were equally potent in stimulating in vitro estradiol-17β production by vitellogenic amago salmon (*O. rhodurus*) ovarian follicles. However, chum salmon GTH II was found to be more potent than chum salmon GTH I in stimulating 17α-hydroxyprogesterone production by ovarian thecal layers and 17α,20β-dihydroxy-4-pregnen-3-one production by granulosa layers in the presence of 17α-hydroxyprogesterone [25]. The apparent lack of hormone specificity of the gonadotropin-induced steroid production in the salmonid ovary is reminiscent of previous studies in teleosts using mammalian FSH and LH. Kagawa et al. [36] found that ovine LH was considerably more potent than ovine FSH in stimulating in vitro estradiol secretion in the amago salmon ovary. In contrast, Bona-Gallo and Licht [37] found that ovine FSH was more potent than ovine LH in stimulating testicular testosterone production in the rainbow trout (*O. mykiss*). Extensive studies of several teleost species by Bona-Gallo and Licht [37, 38] have shown that both the testis and the ovary of teleosts have a high degree of interspecific variability in the steroidogenic response to tetrapod FSH and LH and that there is a general lack of hormonal specificity of the response. Further studies are obviously necessary to determine the biological significance of the two salmon GTH molecules and their functional relationships to tetrapod LH and FSH.

Although the salmon GTH I and GTH II appear to have similar steroidogenic activities when tested in vitro, blood and pituitary levels of these two GTHs vary significantly during reproductive development. GTH I was the predominate GTH in the plasma and pituitary of vitellogenic/spermatogenic rainbow trout, whereas GTH II was the predominant GTH at the time of final reproductive maturation [39]. Swanson et al. [32] found that in prespermatogenic and previtellogenic (prepubertal) coho salmon, GTH I was the only GTH detectable in the plasma. Therefore, the physiological relevance of the steroidogenic activity of GTH II in prepubertal fish in questionable.

Coho salmon GTH I and GTH II were not thyrotropic when tested in an in vivo bioassay. However, a potent TSH fraction that did not contain significant gonadotropic activity was found after anion-exchange chromatography. When this fraction (Q-2) was further analyzed by rpHPLC, it appeared to contain four major protein peaks with amino acid compositions similar to α subunit. It is likely that TSH β is present in small quantities relative to α subunits in Q-2, and was not resolved from the α subunits in this rpHPLC system. So far, neither the TSH β subunit nor the chemical nature of the intact TSH have been identified in this fraction. Current studies are being directed toward identification and isolation of TSH β from Q-2.

Highly purified coho salmon GTH I-β and GTH II-β were used to generate specific rabbit antisera. The specificities of these antisera have been verified by immunoblotting and immunocytochemistry [33]. These antisera stained β subunits specifically in immunoblotting, and did not stain α subunits. Cross-reactivities of the anti-GTH I-β or anti-GTH II-β to salmon TSH β could not be done since salmon TSH β is not currently available. However, in immunoblotting, no cross-reactivity to the anti-GTH I-β or GTH II-β antisera was found in the bioactive TSH fraction (Q-2) prepared in this study (data not shown). In addition, Nozaki et al. [33] have demonstrated that these antisera stain two distinctly different gonadotroph cell types in the pars distalis of salmonids and do not stain thyrotrophs, sommatotrophs, lactotrophs, corticotrophs, or melanotrophs. Moreover, in an ontogenetic study of rainbow trout pituitary gonadotrophs,

Nozaki et al. [40] have shown that GTH I-producing cells are present prior to puberty and increase in number during vitellogenesis; GTH II-producing cells do not appear until after the onset of spermatogenesis/vitellogenesis and are greater in number than GTH I-producing cells at the time of final reproductive maturation. These data, in addition to data on blood levels of GTH I and GTH II [39], suggest relationships of GTH I with gonadal growth and GTH II with final maturation of the gonads similar to those of FSH and LH in mammals. More detailed investigations of the specific roles of GTH I and GTH II in salmonid reproductive development are necessary and possible with the availability of purified coho salmon GTH I and GTH II, and β-subunit-specific antisera.

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REFERENCES

1. Licht P, Papkoff H, Farmer SW, Muller CH, Tsui HK, Crews D. Evolution of gonadotropins structure and function. Rec Prog Horm Res 1977; 33:169–248.
2. Pierce JG, Parsons TF. Glycoprotein hormones: structure and function. Ann Rev Biochem 1981; 50:465–495.
3. Breton B, Purnet P, Reinaud P. Sexual differences in salmon gonadotropin. Ann Biol Anim Biochim Biophys 1978; 18:739–765.
4. Burzawa-Gerard E. Purification d'une hormone gonadotrope hypophysaire de poisson téléostéen, la Carpe (*Cyprinus carpio L.*). Biochimie 1971; 53:545–552.
5. Chang YS, Huang FL, Chen CT, Lo TB. Isolation and properties of the pituitary gonadotropin from silver carp (*Hypophthalmichthys molitrix*). Int J Pept Prot Res 1988; 31:150–156.
6. Chang YS, Huang CJ, Huang FL, Liu CS, Lo TB. Purification, characterization, and molecular cloning of gonadotropin subunits of silver carp (*Hypophthalmichthys molitrix*). Gen Comp Endocrinol 1990; 78:23–33.
7. Huang FL, Huang CJ, Lin SH, Lo TB, Papkoff H. Isolation and characterization of gonadotropin isohormones from the pituitary gland of pike eel (*Muraenesox cinereus*). Int J Pept Pro Res 1981; 18:69–78.
8. Liu C-S, Huang F-L, Chang Y-S, Lo T-B. Pike eel (*Muraenesox cinereus*) gonadotropin. Amino acid sequences of both α and β subunits. Eur J Biochem 1989; 186:105–114.
9. Farmer SW, Papkoff H. A teleost (*Tilapia mossambica*) gonadotropin that resembles luteinizing hormone. Life Sci 1977; 20:1227–1232.
10. Goos HJT, De Leeuw R, Burzawa-Gerard E, Terlou M, Richter CJJ. Purification of gonadotropic hormone from the pituitary of the African catfish, *Clarias gariepinus* (Burchell), and development of a homologous radioimmunoassay. Gen Comp Endocrinol 1986; 63:162–170.
11. Copeland PA, Thomas P. Purification of maturational gonadotropin from Atlantic croaker (*Micropogonias undulatus*) and development of a homologous radioimmunoassay. Gen Comp Endocrinol 1989; 73:425–441.
12. Burzawa-Gerard E. Chemical data on the pituitary gonadotropins and their implication to evolution. Canad J Fish Aquat Sci 1982; 39:80–91.
13. Fontaine YA, Dufour S. Current status of LH-FSH-like gonadotropin in fish. In: Idler DR, Crim LW, Walsh JW (eds.), Proceedings of the Third International Symposium on Reproductive Physiology of Fish. Memorial University of Newfoundland, St. John's Newfoundland, Canada; 1987: 48–56.
14. Idler DR, Ng TB. Teleost gonadotropins: isolation. biochemistry and function. In: Hoar WS, Randall DJ, Donaldson EM (eds.), Fish Physiology, Vol. 9A. New York: Academic Press; 1983: 187–221.
15. Kawauchi H, Suzuki K, Nagahama Y, Adachi S, Naito N, Nakai Y. Occurrence of two distinct gonadotropins in chum salmon pituitary. In: Yoshimura F. Gorbman A. (eds.), Pars Distalis of the Pituitary Gland: Structure, Function and Regulation. Amsterdam: Elsevier; 1986; 383–390.
16. Kawauchi H, Suzuki K, Itoh H, Swanson P and Nagahama Y. Duality of salmon pituitary gonadotropins: In: Ohnishi E, Nagahama Y, Ishizaki H (eds.), Proceedings of the 1st Congress of Asia Oceania Society of Comparative Endocrinology, Nagoya; 1987: 15–18.
17. Kawauchi H, Suzuki K, Itoh H, Swanson P. Dual gonadotropin system in fish. In: Imur H (ed), Progress in Endocrinology 1988. Amsterdam: Elsevier, 1988: 227–232.
18. Kawauchi H, Suzuki K, Itoh H, Swanson P, Nozaki M, Naito N, Nagahama Y. Duality of salmon pituitary gonadotropins. Fish Physiol Biochem 1989; 7: 29–38.
19. Suzuki K, Kawauchi H, Nagahama Y. Isolation and characterization of two distinct gonadotropins from chum salmon pituitary glands. Gen Comp Endocrinol 1988; 71:292–301.
20. Suzuki K, Kawauchi H, Nagahama Y. Isolation and characterization of subunits of two distinct gonadotropins from chum salmon pituitary glands. Gen Comp Endocrinol 1988; 71:302–306.
21. Itoh H, Suzuki K, Kawauchi H. The complete amino acid sequences of β subunits of two distinct chum salmon GTHs. Gen Comp Endocrinol 1988; 71:438–451.
22. Sekine S, Saito A, Itoh H, Kawauchi H, Itoh S. Molecular cloning and sequence analysis of chum salmon gonadotropin cDNAs. Proc Nat Acad Sci 1989; 86:8645–8649.
23. Trinh K-Y, Wang NC, Hew CL, Crim LW. Molecular cloning and sequencing of salmon gonadotropin beta subunit. Eur J Biochem 1986; 159:619–624.
24. Chang YS, Huang CJ, Huang FL, Lo TB. The primary structures of carp gonadotropin subunits deduced from cDNA nucleotide sequences. Int J Pept Prot Res 1988; 32:556–564.
25. Suzuki K, Kawauchi H, Nagahama Y. Steroidogenic activities of two distinct salmon gonadotropins. Gen Comp Endocrinol 1988; 71:452–458.
26. Swanson P, Dickhoff WW, Gorbman A. Pituitary thyrotropin and gonadotropin of coho salmon (*Oncorhynchus kisutch*): separation by chromatofocusing. Gen Comp Endocrinol 1987; 65:269–287.
27. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 1970; 227:680–685.
28. Ornstein L. Disc electrophoresis. I. Background and theory. Ann NY Acad Sci 1964; 121:321–349.
29. Bidlingmeyer BA, Cohen SA, Tarvin TL. Rapid analysis of amino acids using precolumn derivitization. J Chromatogr 1984; 336:93–104.
30. Heinrikson RL, Meredith SC. Amino acid analysis by reverse-phase high-performance liquid chromatography: precolumn derivitization with phenylisothiocyanate. Anal Biochem 1984; 136:65–74.
31. Gray WR, Sequential degradation and dansylation. In: Hirs CHW (ed.), Methods in Enzymology, Vol. 11. New York: Academic Press; 1967: 469–475.
32. Swanson P, Bernard MG, Nozaki M, Suzuki K, Kawauchi H, Dickhoff WW. Gonadotropins I and II in juvenile coho salmon. Fish Physiol Biochem 1989; 7:169–176.
33. Nozaki M, Naito N, Swanson P, Miyata K, Nakai Y, Oota Y, Suzuki K, Kawauchi H. Salmonid pituitary gonadotrophs. I. Distinct cellular distributions of two gonadotropins, GTH I and GTH II. Gen Comp Endocrinol 1990; 77:348–357.
34. Miyazima K, Kawauchi H, Swanson P, Cook H, Kaneko T, Peter RE, Suzuki R, Hirano T. Isolation and characterization of carp prolactin. Gen Comp Endocrinol 1988; 70:407–417.
35. Itoh H, Suzuki K, Kawauchi H. The complete amino acid sequences of α subunits of chum gonadotropins. Gen Comp Endocrinol 1990; 78:56–65.
36. Kagawa H, Young G, Nagahama Y. Estradiol-17β production in isolated Amago salmon (*Oncorhynchus rhodurus*) ovarian follicles and its stimulation by gonadotropins. Gen Comp Endoncrinol 1982; 47:361–365.
37. Bona-Gallo A, Licht P. Gonadotropin specificity of in vitro testosterone secretion by fish testes. Gen Comp Endoncrinol 1981; 43:467–478.
38. Bona-Gallo A, Licht P. Specificity of gonadotropins in the response of in vitro estrogen secretion by fish ovaries. Gen Comp Endoncrinol 1983; 51:316–319.
39. Suzuki K, Kanamori A, Kawauchi H, Nagahama Y. Development of salmon GTH I and GTH II radioimmunoassays. Gen Comp Endocrinol 1988; 71:459–467.
40. Nozaki M, Naito N, Swanson P, Dickhoff WW, Nakai Y, Suzuki K, Kawauchi H. Salmonid pituitary gonadotrophs. II. Ontogeny of GTH I and GTH II cells in the rainbow trout (*Salmo gairdneri irideus*). Gen Comp Endocrinol 1990; 77:358–367.