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Use of immobilized metal ion affinity chromatography and dye–ligand chromatography for the separation and purification of rainbow trout pituitary gonadotropins, GTH I and GTH II

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Abstract

A new procedure is described for the purification of gonadotropic hormones (GTHs) from the pituitary glands of vitellogenic rainbow trout. The procedure utilizes immobilized metal ion affinity chromatography (IMAC) on a column containing immobilized iminodiacetic acid (Toyopearl AF Chelate) charged with Cu^{2+} ions as a critical step for the efficient separation of GTH I and GTH II. Further purification of both GTH fractions on Cibacron Blue F3GA immobilized on Toyopearl was followed by HPLC size-exclusion for GTH II. The resulting electrophoretically homogeneous preparations possessed a characteristic range of biological activity in the stimulation of steroidogenesis in vitro. N-Terminal sequences of the GTH I and GTH II subunits purified using reversed-phase HPLC revealed a high level of homology with those of GTH subunits found in three other salmonid fish species. In contrast to salmon GTH II, two different α-subunits were observed in trout GTH II. The cross-reactivity between GTH I and GTH II was studied in radioimmunoassay using antibodies against Chinook salmon GTH II β-subunit and rainbow trout GTH I dimer. © 1997 Elsevier Science B.V.

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1. Introduction

Fish pituitary gonadotropins GTH I and GTH II belong to the glycoprotein hormone family. GTH I and GTH II are the two main pituitary hormones involved in the regulation of the fish reproductive cycle, as are tetrapod gonadotropins, the luteinizing hormone (LH) and the follicle-stimulating hormone (FSH). Like all known glycoprotein hormones, GTH I and GTH II consist of two nonidentical subunits; both share a common α-subunit but have different β-subunits. In salmonid species, two versions of an α-subunit, designated α1 and α2, were characterized [1–3]. In our present study, we have purified rainbow trout GTH I and GTH II. The commonly used procedures for the purification of individual glycoprotein hormones from the pituitaries of fish and Tetrapoda may be subdivided into the following three steps: first, group isolation of the total glycoprotein hormone fraction containing the gonadotropins and the thyroid-stimulating hormone (TSH) from the rest of the pituitary proteins (including growth hormone and prolactin); second, separation of the individual gonadotropins (GTH I and

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GTH II in fish or LH and FSH in Tetrapoda) and TSH; third, final purification of each hormone. The most difficult step in such procedures is usually that of separating gonadotropins from each other and from TSH. Historically, ion-exchange chromatography was used for this purpose [2,4–6]. The difficulty of this approach, hence, its generally low efficiency, stems from the extensive microheterogeneity of each individual glycoprotein hormone due to different glycosylation patterns [7]. Thus, in many cases, charge distribution of gonadotropins (GTH I and GTH II in fish and FSH and LH in mammals) overlaps, which accounts for the overlapping of their elution patterns on ion-exchange resins [2,8–10]. In such cases, fractions with extreme isoelectric points (p*I*s) are selected in order to avoid the cross-contamination of gonadotropin preparations. These problems emphasize the need to apply chromatographic approaches other than ion-exchange for glycoprotein hormone separation. Recently, reversed-phase high-performance liquid chromatography (RP-HPLC) at neutral pH [9,11,12] and hydrophobic interaction chromatography [13,14] were successfully applied in an effort to separate out individual glycoprotein hormones. In the present study the separation of rainbow trout GTH I and GTH II was performed using immobilized metal ion affinity chromatography (IMAC). To our knowledge, this is the first application of IMAC for the separation of pituitary glycoprotein hormones. Herein we also describe the use of dye–ligand chromatography so as to further purification of GTH I and GTH II.

2. Experimental

All procedures, except HPLC, were performed at 4°C. All chemicals were obtained from Sigma (L'Isle d'Abeau, France) unless otherwise indicated.

2.1. Pituitary extracts

Pituitary glands were collected between July and September from a commercial fish farm, from two-year-old rainbow trout undergoing exogenous vitellogenesis. The extraction procedure was performed as described by Breton et al. [15]. The acetone-dried pituitary glands (33 g wet mass) were first homogenized in five volumes (m/v) of 50 m*M* Tris HCl–0.5 *M* NaCl (pH 7.8), containing 1 m*M* PMSF, 1 m*M* Ca^{2+}, 1 m*M* Mn^{2+} and 0.009% ascorbic acid (buffer A), and then extracted for 2 h under constant stirring. After ultracentrifugation (40 000 *g*, 40 min) the pellets were reextracted for 2 h in three volumes of the same buffer.

2.2. Affinity chromatography on concanavalin A Sepharose

Affinity chromatography on concanavalin A (Con A) Sepharose was performed according to Breton et al. [15]. In summary, the pooled extract was adsorbed onto a 2.5×16 column packed with Con A Sepharose and equilibrated with ten volumes of buffer A. After being washed with the same buffer, the glycoproteins were eluted with buffer A containing 0.15 *M* methyl-α-D-glucopyranoside.

2.3. Immobilized metal ion affinity chromatography

IMAC was performed on a 1×4 cm column, packed with Toyopearl AF Chelate 650 M (Tosohaas). The column was charged with Cu^{2+}, as described by Hedman et al. [16], and equilibrated with ten volumes of 20 m*M* sodium phosphate–1 *M* NaCl (pH 7) (buffer S). The Con A adsorbed fraction was diluted with two volumes of 30 m*M* sodium phosphate–1.25 *M* NaCl buffer (pH 7) and applied to the column. The column was then washed with buffer S and subsequently developed with a stepwise pH gradient of 0.1 *M* sodium acetate–1 *M* NaCl buffer using the following steps: pH 6, pH 4.8, pH 4. The column was washed next with buffer S and developed with a stepwise concentration gradient of 1-methylimidazole in buffer S with the following steps: 2 m*M*, 10 m*M* and 50 m*M*. The column was irrigated with buffer S. Fractions were then dialyzed against 20 m*M* sodium acetate buffer (pH 6).

2.4. Dye–ligand chromatography

The Cibacron Blue F3GA (CF3GA) was immobilized on Toyopearl-65 M (Tosohaas) according to Aligman et al. [17]. The blue resin obtained was packed into a 1×8 cm column. The column was

washed with fifteen volumes of distilled water and equilibrated with ten volumes of 20 m*M* sodium acetate buffer (pH 5.5) containing 25 m*M* sodium caprilate. Sodium caprilate was added to a GTH I- or to a GTH II-rich fraction obtained after IMAC at a concentration of 25 m*M*. The fraction was adjusted to pH 5.5 with 1 *M* acetic acid before being applied to the column. After application of the fraction, the column was washed with equilibrating buffer, followed by 20 m*M* sodium acetate buffer (pH 5.5). The column was eluted with 10 m*M* potassium phosphate buffer (pH 11). The fractions which showed the presence of gonadotropin-like molecules using SDS-PAGE, were rechromatographed on blue resin under identical conditions, dialyzed against 10 m*M* ammonium bicarbonate (pH 8) and then lyophilized.

2.5. Gel permeation chromatography

Gel permeation chromatography was performed at room temperature on a 60×2.15 cm TSK-G 2000 SW column (Tosohaas), equilibrated with five volumes of 50 m*M* potassium phosphate buffer (pH 7). Proteins were applied to and eluted from the column in the same buffer. The separation was controlled by a HPLC system (Merck). The GTHs containing fractions were dialyzed against 10 m*M* ammonium bicarbonate (pH 8) and then lyophilized.

2.6. Reversed-phase HPLC

The separation of GTH I and GTH II subunits was performed according to Suzuki et al. [18] with minor modifications. After dissociation in 0.1% trifluoroacetic acid (TFA) for 2 h, the GTH samples were applied to a 25×0.4 cm LiChrocart Lichrosphere RP-18 column (particle size: 5 μm) (Merck), equilibrated with acetonitrile–water mixture (20:80, v/v) in 0.1% TFA at 40°C. Elution was performed at 40°C by a linear gradient with an acetonitrile–water mixture from (20:80, v/v) to (45:55, v/v) in 0.1% TFA for 40 min at a flow-rate of 1 ml/min. Peaks showing insufficient resolution were rechromatographed on the same column using a linear gradient with an acetonitrile–water mixture from (20:80, v/v) to (60:40, v/v) at neutral pH, as described for Chum salmon GTH Iβ [18].

2.7. SDS-PAGE

Disk electrophoresis in SDS-polyacrylamide slab gels (80×50×0.75 mm) was performed in Laemmli buffer system [19] with a 4% stacking gel, cross-linked to 2.7% and a 17% separating gel, cross-linked to 0.5%. After separation for 45 min at 20 mA, the proteins were visualized by silver staining [20]. For M_r determination, an electrophoresis calibration kit from Pharmacia was used.

2.8. Protein sequencing

The derivatisation of cystein residues was performed with 4-vinyl-pyridine according to Henschen [21] after reduction conducted in 100 m*M* Tris HCl buffer (pH 8.4) containing 20 m*M* DTT and 6 *M* guanidinium chloride, for 2 h at room temperature. For N-terminal sequencing, the protein solutions diluted 1:1 with water were adsorbed onto Prosorb (Perkin-Elmer) PVDF membranes previously dampened with 10 μl of pure methanol and washed three times with 200 μl of a methanol–water mixture (20:80, v/v) prior to sequencing. The membrane was placed in the upper blot-cartridge block of a Perkin-Elmer Procise 494 A sequencer. Automated Edman degradation of the whole protein and of peptides was performed with reagents and methods recommended by the manufacturer.

2.9. Biological activity

The biological activities of the gonadotropins were studied by in vitro determination of their steroidogenic potency. Midvitellogenic or postvitellogenic ovarian follicles were incubated at 12°C for 17 or 24 h in 24 or 12 plastic well culture plates (20–25 follicles per well), in an osmotically adapted trout media, reproducing the ion composition of the trout serum [22] and containing increasing concentrations of gonadotropins. After incubation, all media were collected and their steroid concentrations (17α,20β-dihydroxy-4-pregnen-3-one and estradiol-17β) were measured using radioimmunoassay (RIA) [23,24].

2.10. GTH radioimmunoassay

Antibodies directed against GTH I were obtained from a rabbit after four multipoint intradermic injections on the back of the animal at 3-week intervals with 100 μg of GTH I in 0.5 ml of 0.8% NaCl and emulsified with 0.5 ml of complete Freund adjuvant. The rabbit anti-chinook salmon GTH IIβ antibody preparation was reported previously [25]. Iodination of GTH I and GTH II was carried out according to the chloramine T method [26]. 100 μl of serially diluted GTH I and GTH II standards in an assay buffer (50 m*M* Tris–HCl, 5 m*M* $MgCl_2$, 1% bovine serum albumin (BSA) RIA grade, 0.1% Triton X-100, 0.1% sodium azide; pH 7.8) were incubated with 100 μl of anti GTH IIβ or anti GTH I antisera at respective concentrations of 1:30 000 and 1:20 000 in the assay buffer containing 1% normal rabbit serum. After incubation for 24 h at 12°C, 100 μl of iodinated GTH I or GTH II (15 000–20 000 cpm) was added and incubation continued for 24 h at the same temperature. The antibody–hormone complex was precipitated by addition of 200 μl of 5% goat anti-rabbit IgG in an assay buffer containing 7.5% polyethylene-glycol 6000. After precipitation for 12 h at room temperature, 1.5 ml of the assay buffer without BSA was added to each tube. The tubes were then centrifuged for 60 min at 4000 *g*. The supernatants were discarded and the tubes counted in a Packard Cobra gamma counter.

3. Results

3.1. Immobilized metal ion affinity chromatography

Since it is impossible to differentiate between GTH I and GTH II on the basis of their biological activities [2,27] and since no specific antibodies against them were available, the presence of gonadotropins in the fractions during the purification procedure was surveyed using SDS-PAGE. This is similar to what had been done during the isolation of salmon and bonito fish gonadotropins [4,9]. In comparison with other proteins, gonadotropins have a characteristic pattern on SDS-PAGE. When samples are loaded onto the gel after incubation at room temperature with a sample buffer lacking a reducing agent, they migrate as native dimers with a M_r of 30 000–45 000. However, if they are incubated at 100°C with a sample buffer containing a reducing agent, the gonadotropins migrate in the form of dissociated subunits, which are revealed as bands with a M_r of 15 000–25 000 on the polyacrylamide gel, while the bands denoting the gonadotropin dimers disappear. Hence, the simultaneous analysis of gonadotropins in their dimeric conformation and that of dissociated subunits in parallel tracks of the same gel allows them to be identified in unknown samples. In preliminary experiments, it was found that some glycoproteins originated from a Con A adsorbed fraction, tend to have a different affinity for IDA-Cu^{2+}-Toyopearl. Among them, two glycoproteins might conceivably be gonadotropins in terms of their characteristic electrophoretic behavior. They were designated as GI-I and GI-II. GI-I was not retained as strongly as GI-II on IDA-Cu^{2+}-Toyopearl and, in contrast to GI-II, it was desorbed at pH 4. However, a linear concentration gradient (1 m*M*–10 m*M*) and a stepwise concentration gradient (with the steps: 2 m*M*, 5 m*M*, 10 m*M*) of 1-methylimidazole were not effective in separating GI-I from GI-II, resulting in the elution of the mixture of both proteins (data not shown). Taking into account these data, in the final protocol we used a combination of a stepwise falling pH gradient with a subsequent 1-methylimidazole stepwise concentration gradient elution. The chromatography of the Con A adsorbed fraction on an IDA-Cu^{2+} column resulted in five peaks (Fig. 1). SDS-PAGE revealed the presence of GI-I in peak I-1 eluted at pH 4.8 and the presence of GI-II in peak I-3 eluted with 10 m*M* 1-methylimidazole (Fig. 2a). GI-I and GI-II were identified on SDS-PAGE in the fractions as dimeric proteins with an M_r of 45 000 and 35 000 respectively, which dissociated into subunits with an M_r of 19 000–23 000 after reduction at 100°C in a sample buffer.

3.2. Dye–ligand affinity chromatography

Dye–ligand affinity chromatography was performed for the subsequent purification of GI-I and GI-II. In preliminary experiments buffer solutions of different pH values and ion concentrations were tested to establish the optimal conditions of the GI-I and GI-II binding and successful elution from

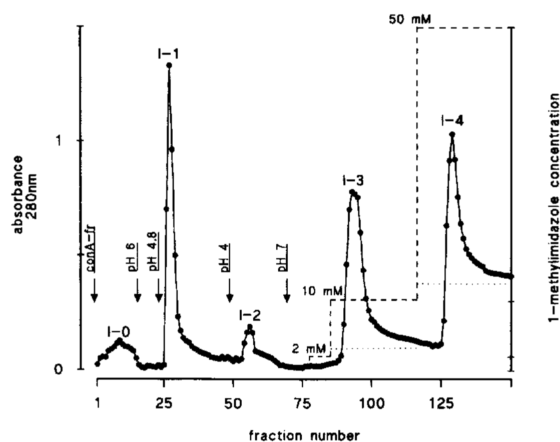

Fig. 1. Metal affinity chromatography on an IDA-Toyopearl 650 M column, shared with Cu^{2+}. A 40-ml volume of a Con A adsorbed fraction (Con A fr) was diluted with 2 volumes of 0.03 *M* sodium phosphate–1.25 *M* NaCl buffer (pH 7). After being adjusted to pH 7 with 1 *M* HCl, the fraction was loaded onto the column, equilibrated with buffer S at a flow-rate of 109 ml/h and fractions of 10 ml were collected. Stepwise elution was performed at a flow-rate of 60 ml/h, fractions of 1.4 ml were collected. (····) absorbance at 280 nm of 1-methylimidazole containing buffer.

CF3GA-Toyopearl. The increasing amount of GI-I and GI-II was observed in the breakthrough fraction when the pH of the loading buffer increased beyond 5.5. The decrease of pH of the loading buffer below 5.5 led to the increase of contaminant protein adsorption. The sodium caprilate added to the starting buffer at a concentration above 25 m*M*, prevented the adsorption of both GI-I and GI-II and contaminant proteins on the blue resin. The selective binding of GI-I and GI-II on CF3GA-Toyopearl was achieved in 20 m*M* sodium acetate buffer (pH 5.5) containing 25 m*M* sodium caprilate, and their optimal elution was obtained with 10 m*M* potassium phosphate buffer (pH 11). The elution profile of fraction I-1 on a blue resin is shown in Fig. 3a. GI-I was recovered in peak B-1, which was eluted at pH 11, whereas most of the contaminant proteins were stayed in nonadsorbed I1-0 fraction (Fig. 2a). In order to remove the traces of contaminants, fraction B-1 was rechromatographed on a Blue-adsorbent using the same conditions. We obtained 7 mg of electrophoretically homogenous GI-I after dialysis and lyophilisation (Fig. 2b). The chromatography of fraction I-3 on a blue column resulted in a similar profile as those obtained with fraction I-1 (Fig. 3b). Nevertheless, a major contaminant migrating in SDS-PAGE as a 65 000 protein was partially retained on the dye–ligand adsorbent (Fig. 2a). It was present in the fraction containing GI-II (fraction B-2-r), obtained after rechromatography of fraction B-2 on the blue column. The removal of the contaminant was performed by means of gel permeation.

3.3. Gel permeation chromatography

In addition to the electrophoretic analysis, gel permeation chromatography on a TSK-SW G-2000 column demonstrated the homogeneity of GI-I preparation, which resulted in one major peak (Fig. 4a). Two other small peaks, which represented only 7% of the total peak area, were eluted but no proteins were identified after their migration on SDS-PAGE and silver staining (data not shown). These small peaks could be attributed to the formation of GI-I aggregates and dissociation into subunits of a part of the GI-I molecules during the HPLC. However, their quantity was insufficient for further analysis. Gel permeation of fraction B-2-r resulted in three peaks (Fig. 4b). Electrophoretically homogenous GI-II was recovered in peak T-3. (Fig. 2b). Following dialysis and lyophilisation, 3 mg of GI-II were obtained.

3.4. Reversed-phase HPLC

The separation of GI-I and GI-II by RP-HPLC after dissociation using 0.1% TFA at 40°C is shown in Fig. 5. The elution of GI-I on RP-HPLC revealed five peaks, while GI-II elution resulted in four peaks. As is shown in Fig. 6 peaks aI-1, aI-2, aII-1, aII-2, bI-3, bII-1, bII-2 migrated as single bands in SDS-PAGE, with and without reduction and temperature treatment, the M_r being 19 000–23 000. Peak bI-1 was electrophoretically identical to GI-I and migrated in SDS-PAGE as a 45 000 dimeric protein, which dissociated into subunits after reduction and heating at 100°C. Such behavior indicates that peak bI-1 represented the stable form of GI-I, which is resistant to acid treatment. Heating at 100°C in a sample buffer without reduction did not result in the dissociation of stable GI-I into subunits, rather in an alteration of its M_r in SDS-PAGE, decreasing from 45 000 to 35 000. The stable form comprised 20% of GI-I preparation, as determined using RP-HPLC.

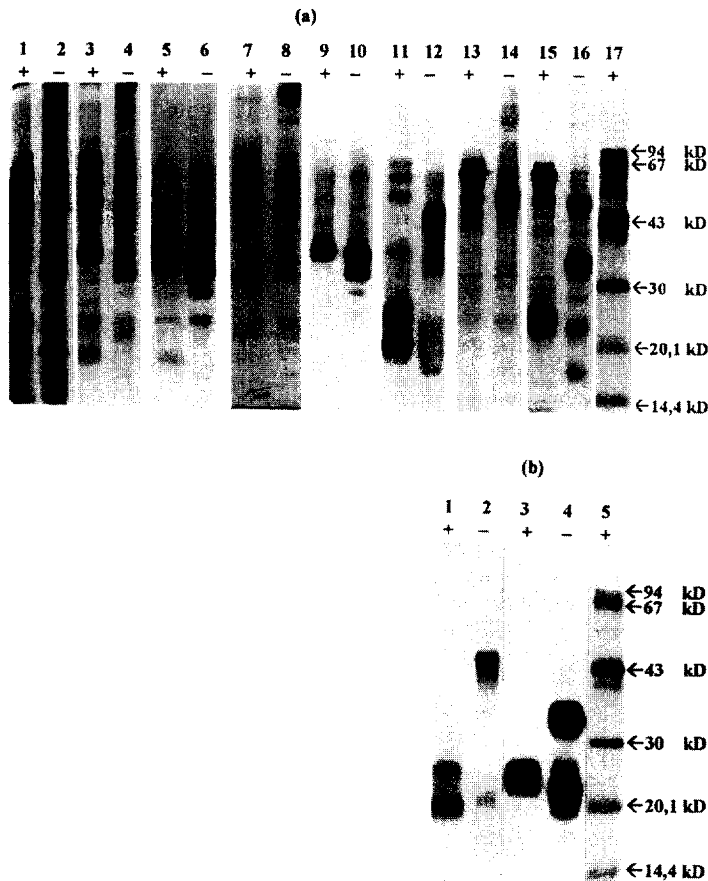

Fig. 2. SDS-PAGE of GI-I and GI-II samples. Each sample (0.7–1 μg) was electrophoresed in two parallel tracks: –, samples were loaded onto the gel after incubation for 10 min at room temperature with a sample buffer lacking a reducing agent; +, samples were loaded onto the gel after incubation for 10 min at 100°C with a sample buffer containing 10 m*M* DTT. (a) Samples from various steps of the GI-I and GI-II purification procedure; lanes 1–2, rainbow trout pituitary extract; lanes 3–4, Con A adsorbed fraction; lanes 5–6, fraction I-1; lanes 7–8, fraction I-3; lanes 9–10, fraction I1-0; lanes 11–12, fraction B-1; lanes 13–14, fraction I3-0; lanes 15–16, fraction B-2; lane 17, M_r standards. (b) Highly purified GI-I, lanes 1–2; GI-II, lanes 3–4; lane 5, M_r standards.

Peak bI-2 was electrophoretically identical to peak bI-3, but it was contaminated with peak bI-1 (data not shown). After rechromatography on RP-HPLC at pH 6, it was combined with peak bI-3 and designated as bI-c.

3.5. N-Terminal amino acid sequence

No difference was found in sequences of twenty N-terminal amino acid residues between aI-1 and aII-1, between aI-2 and aII-2 and between bII-1 and bII-2, respectively. The comparison of N-terminal sequences we obtained with those of GTH subunits from other salmonid fish species allows us to conclude that aI-1 and aII-1 correspond to the rainbow trout GTH 1-subunit, aI-2 and aII-2 to the GTH 2-subunit and bI-c to the GTH I β-subunit, while bII-1 and bII-2 represent the GTH II β-subunit (Fig. 7). These data suggest that GI-I is a trout GTH I and GI-II a trout GTH II. In view of the fact that bII-1 and bII-2 was not deglycosylated and that aI-1, aII-1, aI-2, aII-2 and bI-c were neither reduced nor carboxymethylated nor deglycosylated before sequencing, the absence of any signal at certain positions suggest the presence of cystein (positions 11 and 14 of α1 and α2; positions 4 and 8 of GTH Iβ) or

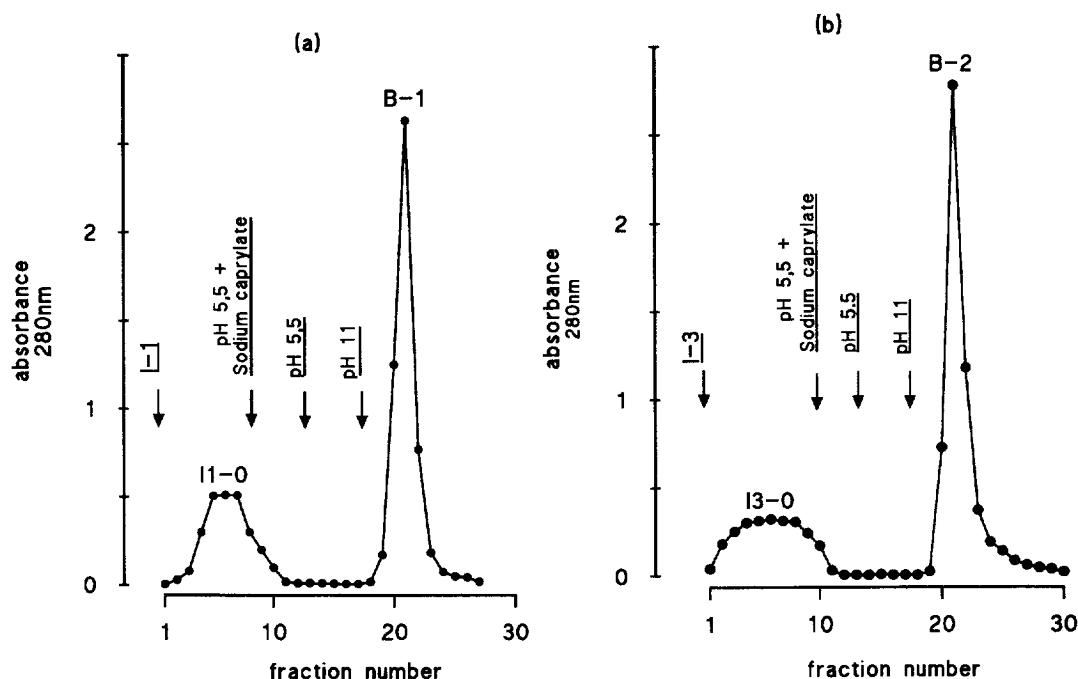

Fig. 3. Dye–ligand chromathography on a CF3GA-Toyopearl 650 M column. Fraction Σ_5I-1 (a) or fraction Σ_5I-3 (b) was applied on the column, equilibrated with 20 m*M* sodium acetate buffer (pH 5.5) cotaining 25 m*M* sodium caprilate, at a flow-rate of 96 ml/h, fractions of 8 ml were collected. Elution was performed at a flow-rate of 51 ml/h, 1.7 ml fractions were collected. Σ_5I-1 and Σ_5I-3 represent the sum of fractions I-1 and I-3, respectively from chromathography of five 40 ml samples of a Con A adsorbed fraction on a Cu^{2+}-IDA column.

glycosylated asparagine (positions 12 of GTH Iβ and 10 of GTH IIβ) residues in these positions. This is confirmed by the total identity of the position of these gaps in the sequences obtained with that of cystein or glycosylated asparagine residues in corresponding salmon gonadotropin subunits. The N-terminal amino acid sequence obtained from peaks aI-1 and aII-1 revealed the presence of the additional sequence in low amounts. When twelve amino acid residues were surveyed, this sequence was shown to be identical to an internal salmon GTH α1 sequence started by Ala-50. Also surveyed among twelve amino acid residues, another additional sequence was found in low amounts in peaks aI-2 and aII-2. This sequence was shown to be identical to an internal salmon GTH α2 sequence started by Ser-43. The cleavages occurred after Lys or Arg, which indicates that trypsin activity had taken place during the purification or sequence procedure. It is possible that cleaved GTH α-subunits originated from hypophysis. The existence of cleaved subunits in the pituitary gland was demonstrated for human gonadotropins [31]. Both trout GTH II β-subunits revealed a partial cleavage of the N-terminal amino acid; sequences starting at their second position (Leu-2) were identified.

3.6. Biological activity

The capacity of purified protein to stimulate the ovocyte in vitro steroid production proved their gonadotropic nature. Estradiol-17β production by midvitellogenic follicles was stimulated by both GTH I and GTH II in a dose dependent manner. GTH II was 1.5- to 2-fold more potent than GTH I upon the stimulation of estradiol-17β production (Fig. 8a). Furthermore, as in case of Chum salmon gonadotropins, stimulation of 17α,20β-dihydroxy-4-pregnen-3-one production by postvitellogenic follicles was 5- to 10-fold greater when using GTH II, than when using GTH I, though this was dependent on the dose (Fig. 8b). The stable form of GTH I

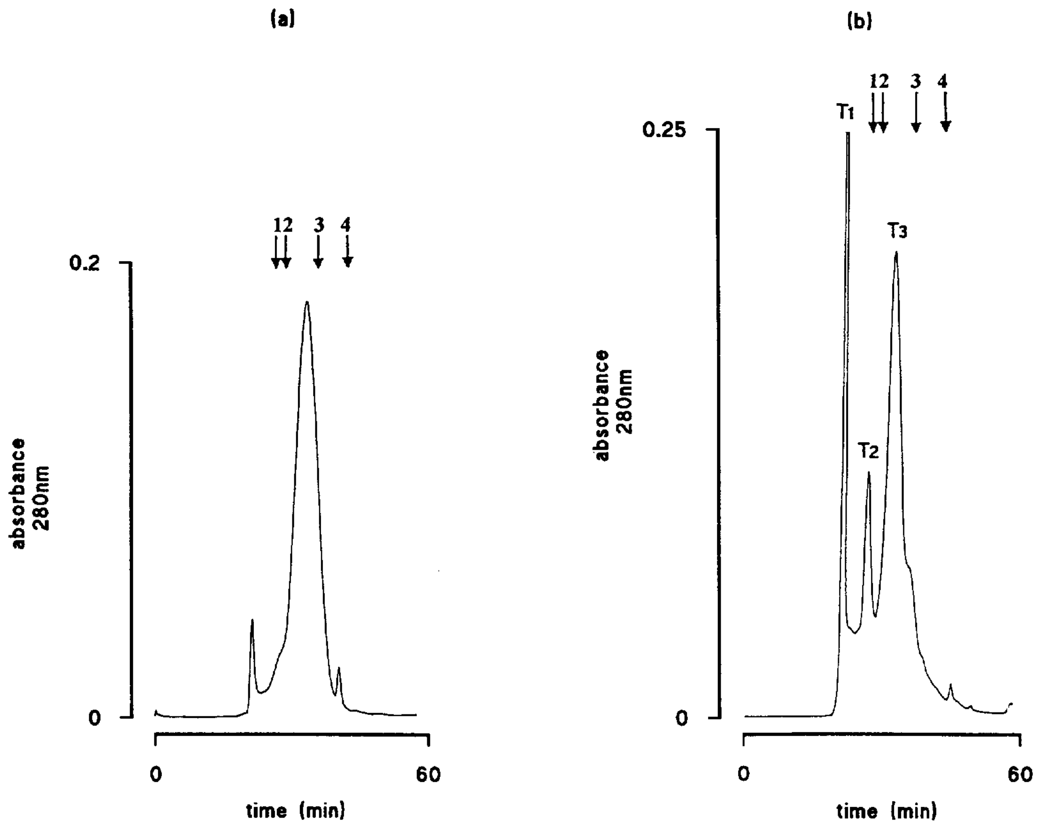

Fig. 4. Gel permeation HPLC on a TSK SW-G2000 column. A 3-mg amount of fraction B1 (a) or 6 mg of fraction B2 (b) were dissolved in 2.5 ml 0.05 *M* potassium phosphate buffer (pH 7) and applied to the column. The column was equilibrated and developed with the same buffer at a flow-rate of 4 ml/min. 1 bovine serum albumin (66 000); 2 ovalbumin (45 000); 3 carbonic anhydrase (29 000); 4 cytochrome *c* (12 000).

possessed similar steroidogenic activity as the native hormone.

3.7. GTH radioimmunoassay

In the GTH II RIA, the competition of GTH II binding by GTH I and of GTH I by GTH II in the GTH I RIA are illustrated in Fig. 9. GTH I never cross-reacted more than 0.0005% with GTH II, when using a specific antibody directed against the Chinook salmon GTH II β-subunit. GTH II cross-reactivity was around 50% in the GTH I RIA, using an antibody directed against the whole GTH I molecule. This high percentage of GTH II cross-reactivity reflected the presence of a common α-subunit in GTH I and GTH II, inducing the loss of specific immunological recognition. The competition curve was parallel to that of the standard curve.

4. Discussion

We now propose a new strategy for gonadotropin separation by means of copper ion affinity chromatography, followed by further purification on a Cibacron F3GA-Toyopearl. Protein affinity for the chelated metal resin strongly depends on surface density and accessibility of histidyl residues, where the imidazole side-chain is considered as the principal electron donor for coordination in IMAC [16,32,33]. The quantity and distribution of histidyl residues between salmon GTH I and GTH II β-subunits are different and they are identical among

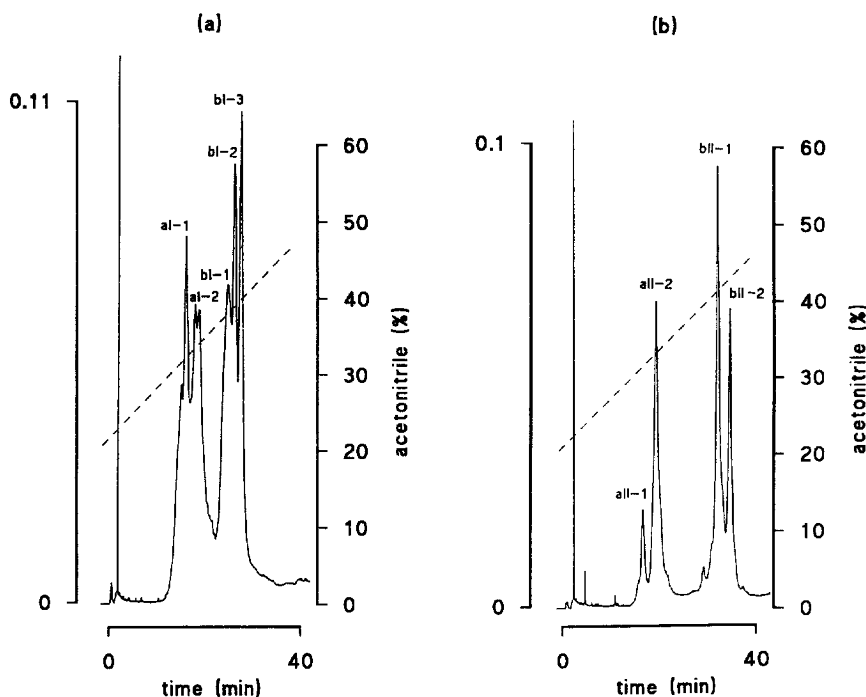

Fig. 5. RP-HPLC of GTH I (a) and GTH II (b). Hormones (500 μg of each) were incubated in 250 μl of 0.1% of trifluoroacetic acid at 40°C for 2 h and loaded onto the RP-18 column, equilibrated with an acetonitrile–water mixture (20:80, v/v) in 0.1% TFA at 40°C. Elution was performed by a linear gradient with an acetonitrile–water mixture from (20:80, v/v) to (45:55, v/v) in 0.1% TFA for 40 min at a flow-rate of 1 ml/min.

Chum salmon [28], Masu salmon [29] and Chinook salmon [30], which are species closely related to rainbow trout. These facts allowed us to anticipate a different affinity of rainbow trout GTH I and GTH II for chelating resin, that was confirmed in our study. The combination of two elution mechanisms was used for the trout GTH I and GTH II separation on this adsorbent: stepwise pH gradient elution, based on the protonation of the imidazole side chain of the protein, and affinity elution with 1-methylimidazole, based on the coordination of the free 1-methylimidazole, contained in the eluent with IDA-Me^{2+}. This approach is very useful if the affinity elution is not effective for the protein separation and on the other hand the pH gradient elution can not be continued at low pH because of the instability of the protein in the very acidic conditions, as is the case of fish GTH II [2,8,9,19].

Cibacron F3GA is widely used as an immobilized ligand for the purification of nucleotide-dependent enzymes [34,35] and some other proteins, especially those of plasma [35–39]. However few applications of dye affinity chromatography using Cibacron F3GA or other dyes as immobilized ligand is known for the fractionation of glycoprotein hormones [40,41]. This study gives an additional example of successful use of dye–affinity chromatography for the purification of this class of proteins. However the optimization of chromatographic conditions is needed in each given case. The structure of the dyes allows multiple interactions, among which are electrostatic, hydrogen-bond or hydrophobic. This explain the great variety of possible adsorption and desorption protocols, depending on the protein. Our purification procedure was based only on the difference in the affinity of the proteins from trout pituitary extract to the immobilized Cibacron F3GA at different pH. In comparison with described techniques for the isolation of gonadotropins on the dye–adsorbents we did not used currently applied elution with increasing salt gradient. We also ascertained a preventive sodium caprylate effect on the

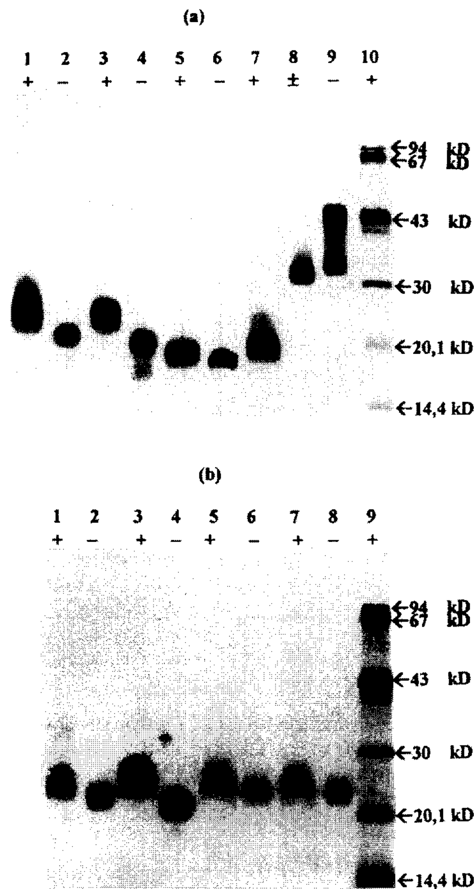

Fig. 6. SDS-PAGE electrophoresis of samples obtained after RP-HPLC of GI-I (a) and GI-II (b). Each sample (0.7–1 μg) was electrophoresed in two or three parallel tracks: −, samples were loaded onto the gel after incubation for 10 min at room temperature with a sample buffer lacking a reducing agent; ±, samples were loaded onto the gel after incubation for 10 min at 100°C with a sample buffer lacking a reducing agent; +, samples were loaded onto the gel after incubation for 10 min at 100°C with a sample buffer, containing 10 m*M* DTT. (a) lanes 1–2, aI-1; lanes 3–4, aI-2; lanes 5–6, bI-3; lanes 7–9, bI-1; lane 10, M_r standards. (b) Lanes 1–2, aII-1; lanes 3–4, aII-2; lanes 5–6, bII-1; lanes 7–8, bII-2; lane 9, M_r standards.

binding of contaminant proteins from rainbow trout pituitary extract on the CF3GA-Toyopearl similar, as was described, for human serum albumin [36,37].

We have found some important differences in the structure of trout GTHs in comparison with that of Chum and Coho salmon. First of all both trout GTH I and GTH II contained two α-subunits α1 and α2 in contrast to the salmon gonadotropins, in which only

			1 10 20
peak aI-1 / peak aII-1	Rainbow trout	*α1*	YPNSDMPNVGXEEXKLKENK
	Chum salmon	*α1*	-Q----T---C--C------
	Masu salmon	*α1*	------T---C--C------
peak aI-2 / peak aII-2	Rainbow trout	*α2*	-----KT-M-X--XT--P-T
	Cum salmon	*α2*	-----KT-M-C--CT--P-T
	Masu salmon	*α2*	-----KT-M-C--CT--P-T
			1 10 20
peak bI-c	Rainbow trout	*GTH Iβ*	GTDXRYGXRLNXMTIIVERE
	Chum salmon	*GTH Iβ*	--EC---C---N--------
	Masu salmon	*GTH Iβ*	---C---C---N---T----
			1 10 20
peak bII-1 / peak bII-2	Rainbow trout	*GTH IIβ*	SLMQPCQPIXQTVSLEKEGC
	Chum salmon	*GTH IIβ*	---------N----------
	Masu salmon	*GTH IIβ*	---------N----------
	Chinook salmon	*GTH IIβ*	---------N----------

Fig. 7. N-Terminal amino acid sequence of samples, obtained after RP-HPLC of GI-I and GI-II in comparison with that of Chum salmon, Masu salmon and Chinook salmon GTH subunits. Amino acids identical to those of the GI-I and GI-II subunits are indicated by dashes. X marks the positions in which no amino acid was identified. The sequences are taken from: Chum salmon GTHα1 and GTHα2 [1], Chum salmon GTH Iβ and GTH IIβ [28], Masu salmon GTHα1 and GTHα2 [3], Masu salmon GTH Iβ and GTH IIβ [29], Chinook salmon GTH IIβ [30].

GTH I shared both α1 and α2, whereas GTH II shared α2 [1,2]. The content of stable form in trout GTH I was 2-fold greater than in the salmon GTH I [42]. Finally two different peaks were detected by RP-HPLC for trout GTH II β-subunit in contrast to the characterized fish GTH IIβ, for which only one peak was identified on RP-HPLC [2,8,9,19]. The complete amino acid sequence of these peaks is needed so as to find out if there are any differences in their primary structure.

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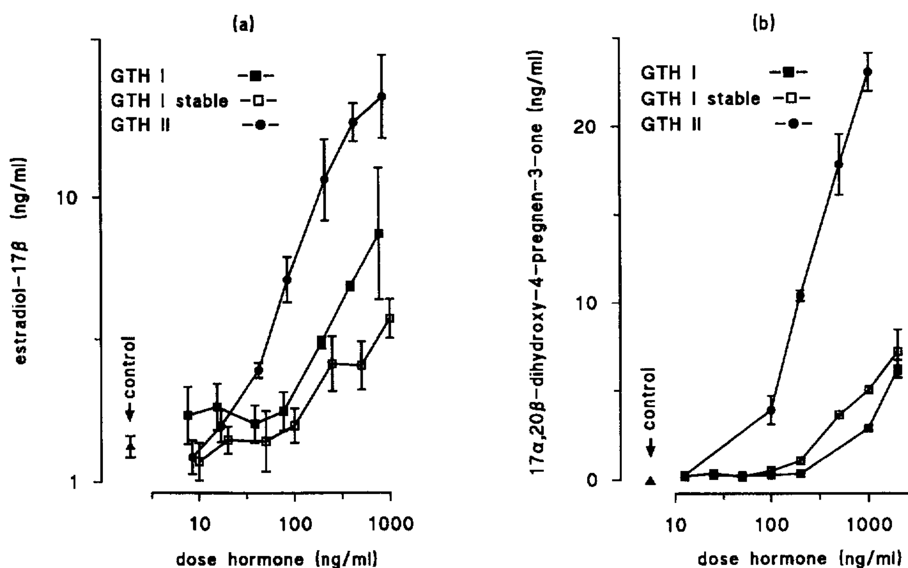

Fig. 8. Effect of GTH I and GTH II on steroid production by rainbow trout ovarian follicles. Each point with a bar represents the mean±S.E.M. of three replicates. (a) The effect of GTH I and GTH II on estradiol-17β production by rainbow trout ovarian follicles. Ovarian follicles (diameter: 2.2 mm) were incubated with GTHs for 17 h in 24 well plastic culture plates, 20 specimen/1.5 ml/well. (b) Effect of GTH I and GTH II on 17α,20β-dihydroxy-4-pregnen-3-one production by rainbow trout ovarian follicles. Ovarian follicles (diameter: 4.5 mm) were incubated with GTHs for 24 h in 12 well plastic culture plates, 25 specimen/3 ml/well.

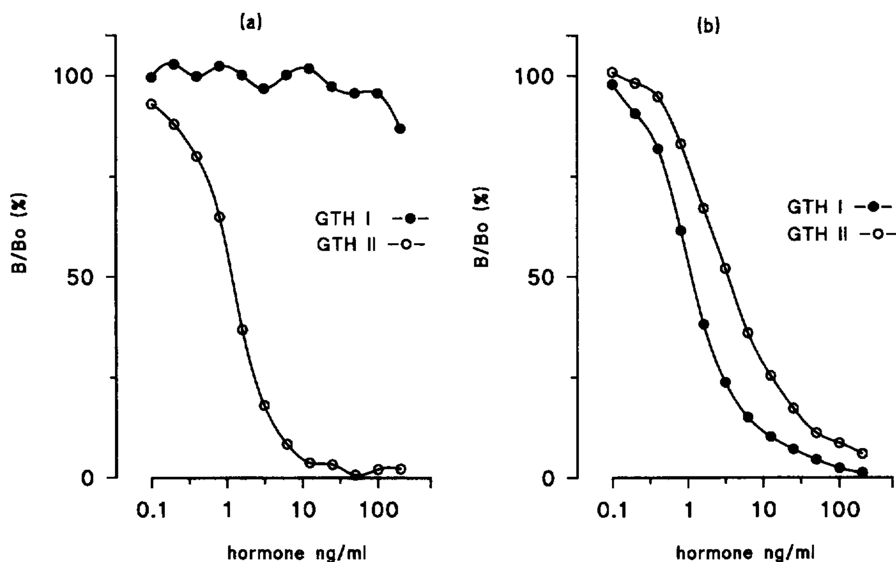

Fig. 9. Competition curves for GTH I in the GTH II RIA using ^{125}I-GTH II and rabbit antibody against GTH IIβ (a) and for GTH II in the GTH I RIA using ^{125}I-GTH I and rabbit antibody against GTH I (b).

References

[1] H. Itoh, K. Suzuki, H. Kawauchi, Gen. Comp. Endocrinol. 78 (1990) 56.
[2] P. Swanson, K. Suzuki, H. Kawauchi, W.W. Dickhoff, Biol. Reprod. 44 (1991) 29.
[3] K. Gen, O. Maruyama, T. Kato, K. Tomizawa, T. Wakabayachi, Y. Kato, J. Mol. Endocrinol. 11 (1993) 265.
[4] K. Suzuki, H. Kawauchi, Y. Nagahama, Gen. Comp. Endocrinol. 71 (1988) 292.
[5] P. Licht, H. Papkoff, S.W. Farmer, C.H. Muller, H.W. Tsui, D. Crews, Rec. Progr. Horm. Res. 33 (1977) 169.
[6] G.R. Bousfield, D.N. Ward, J. Biol. Chem. 18 (1984) 1911.
[7] A. Stockel Hartree, A.G.C. Renwick, Biochem. J. 287 (1992) 665.
[8] G. Van Der Kraak, K. Suzuki, R.E. Peter, H. Itoh, H. Kawauchi, Gen. Comp. Endocrinol. 85 (1992) 217.
[9] Y. Koide, H. Itoh, H. Kawauchi, Int. J. Pept. Protein Res. 41 (1993) 52.
[10] F. Apparaily, V. Laurent-Cadoret, F. Lecompte, M. Chopineau, M.-C. Maurel, F. Guillou, Y. Combarnous, Reprod. Fertil. Dev. 6 (1994) 157.
[11] J. Hiyama, A.G.C. Renwick, J. Chromatogr. 529 (1990) 33.
[12] M.A. Chlenov, E.I. Kandyba, L.V. Nagornaya, I.L. Orlova, Y.V. Volgin, J. Chromatogr. 631 (1993) 261.
[13] J. Hiyama, A. Surus, A.G.C. Renwick, J. Endocrinol. 25 (1990) 493.
[14] S. Hofferer, F. Lecompte, T. Magallon, E. Palmer, Y. Combarnus, J. Reprod. Fertil. 98 (1993) 597.
[15] B. Breton, P. Prunet, P. Reinaud, Biol. Anim. Biochem. Biophys. 18 (1978) 759.
[16] E. Hedman, Y.-J. Zhao, E. Sulkowski, J. Porath, Biochemistry 86 (1989) 1811.
[17] E. Aligman, Y. Kroviarski, S. Cochet, Y.L. Kong Sing, D. Muller, D. Dhermy, O. Bertrand, J. Chromatogr. 510 (1990) 165.
[18] K. Suzuki, H. Kawauchi, Y. Nagahama, Gen. Comp. Endocrinol. 71 (1988) 302.
[19] U.K. Laemmli, Nature 227 (1970) 680.
[20] H. Blum, H. Beier, H.J. Gross, Electrophoresis 8 (1987) 93.
[21] A. Henschen, Protein Microsequence Analysis, Springer-Verlag, Berlin, 1986.
[22] B. Jalabert, A. Fostier, Reprod. Nutr. Dev. 24 (1984) 127.
[23] A. Fostier, B. Jalabert, Fish. Physiol. Biochem. 2 (1986) 87.
[24] A. Fostier, C. Weil, M. Terqui, B. Breton, B. Jalabert, Ann. Biol. Anim. Bioch.. Biophys. 18 (1978) 929.
[25] B. Breton, Y. Zohar, E. Sambroni, Aquaculture 40 (1984) 307.
[26] W.M. Hunter, F.S. Greenwood, Nature 194 (1962) 495.
[27] K. Suzuki, Y. Nagahama, H. Kawauchi, Gen. Comp. Endocrinol. 71 (1988) 452.
[28] H. Itoh, K. Suzuki, H. Kawauchi, Gen. Comp. Endocrinol. 71 (1988) 438.
[29] K. Kato, K. Gen, O. Maruyama, K. Tomizawa, T. Kato, J. Mol. Endocrinol. 11 (1993) 275.
[30] K.Y. Trinh, N.C. Wang, C.L. Hew, L.W. Crim, Eur. J. Biochem. 159 (1986) 619.
[31] D.N. Ward, S.D. Glenn, H.S. Nahm, T. Wen, J. Peptide Prot. Res. 27 (1986) 70.
[32] J. Porath, J. Carisson, I. Olsson, G. Belfrage, Nature (London) 258 (1975) 598.
[33] J. Porath, J. Chromatogr. 443 (1988) 3.
[34] Y.D. Clonis, Crit. Rev. Biotechnol. 7 (1988) 263.
[35] R.K. Scopes, J. Chromatogr. 376 (1986) 131.
[36] D.J. Gisch, T.S. Reide, J. High Resolut. Chromatogr. 11 (1988) 258.
[37] R.J. Leatherbarrow, P.D.G. Dean, Biochemistry 189 (1980) 27.
[38] S.C. Williams, R.B. Sim, J. Immunol. Meth. 157 (1993) 25.
[39] Y. Yamato, M. Eguchi, Comp. Biochem. Physiol. 95 (1990) 347.
[40] J.B. Wu, P.G. Stanton, D.M. Robertson, M.T.W. Hearn, J. Endocrinol. 137 (1993) 59.
[41] J.R. McFarlane, N.M. Czekala, H. Papkoff, Biol. Reprod. 44 (1991) 827.
[42] L. Yan, P. Swanson, W.W. Dickhoff, J. Exp. Zool. 258 (1991) 221.