Purification and Biological Activities of Isoforms of Human FSH

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It is known that pituitary FSH, LH, and TSH are heterogeneous, existing as families of isoforms with respect to isoelectric point (pI), circulating half-life, in vitro and in vivo activities (for recent review, see [1]). To date, however, no systematic study has been undertaken to purify and characterize these isoforms with a view to establishing the biochemical basis for these differences. To this end, we have utilized a novel method exploiting the differences in charge-based protein separation between isoelectrofocusing and ion exchange chromatography for the purification to homogeneity of the isoforms of human pituitary FSH.

Materials and Methods

The purification procedure was based on a previously described method (2). Frozen human pituitaries supplied by the Human Pituitary Advisory Committee (HPAC, Canberra, Australia) were minced and homogenized in 50-mM phosphate buffer (pH 7.0), and a high-speed supernatant was prepared (100,000 g × 1 h at 4°C). The supernatant was applied to a gel filtration column (Sephacryl S200, Pharmacia, Uppsala, Sweden, 100 × 5 cm) in 50-mM ammonium acetate (pH 7.0), and the FSH radioreceptor active fractions were pooled and lyophilized.

Preparative Isoelectrofocusing (IEF)

The sample (400 mg dissolved in water containing 300-μM EDTA, 1.1-μM pepstatin, 1.3-μM leupeptin, and 0.2-μM phenylmethylsulphonylfluoride) was electrofocused in a 440-mL sucrose density gradient column (LKB, Bromma, Sweden) using carrier ampholytes (1% Ampholines, LKB) in the pH range from 3.5 to 10 for 17–18 h at 2000 V or 30 W at 4°C. The gradient
was eluted at 100 mL/h and 1-mL fractions were collected. The pH was determined in every 5th tube. Phosphate buffer (pH 7.0) (0.5 mL, 0.3 M) was added to neutralize the pH.

**High-Performance Liquid Chromatography**

An FPLC (Pharmacia) system was used with a Mono Q anion exchange (HPIEX) column (HR5/5, 5 × 0.5 cm). The sample (50–60 mL) was sequentially fractionated with (a) a 57-min 0- to 342-mM NaCl gradient in 20-mM piperazine (pH 9.60) and 0.015% Brij-35 and (b) a 34-min 0- to 300-mM NaCl gradient in piperazine buffer (pH 6.0) at 1 mL/min at 20°C–22°C.

A TSK G3000SW column (60 × 0.75 cm, Toya Soda Co.) in 20-mM Tris buffer, 150-mM NaCl (pH 7.5), and 0.015% Brij-35 was employed at a flow rate of 0.4 mL/min. For SDS-PAGE, samples were reduced in dithiothreitol, electrophoresed in 16% gels using the method of Laemmli (3), and silver stained. Samples for amino acid analysis were hydrolyzed in vacuo in 6-M HCl, 0.2% phenol (22 h, 110°C) and analyzed by the Picotag method (Millipore/Waters, MA, USA).

**FSH Radioreceptor Assay (RRA) and FSH In Vitro Bioassays**

The FSH-RRA method of Cheng (4) was employed using calf testis membranes as the receptor source, the first IS for FSH (83/575) as standard, and iodinated human FSH isoform (pl 4.25) as tracer. The binding of tracer to an excess of membrane was 65%. A membrane dilution giving 25% binding with nonspecific binding of 1.5%–2% was used. Parallelism of logit log-dose-transformed dose-response lines was observed between preparations and standard. The between-assay variation was 7.8% (n = 6). The within-assay variation, based on the mean index of precision, was 0.048.

The FSH in vitro bioassay method of Van Damme (5) was used based on the FSH-induced aromatization of 19-hydroxyandrostenedione by immature rat Sertoli cells (Sc) in culture. All samples were assayed in the one assay. The within-assay variation, based on the mean index of precision, was 0.090.

**Results**

Human pituitary high-speed supernatant preparations were sequentially purified by gel filtration on Sephacryl S200, preparative isoelectrofocusing, 2 anion exchange chromatographic steps, and a final gel filtration HPLC chromatographic step (Figs. 32.1a–32.1d). Two of the FSH radioreceptor active regions (region A, pH 3.76–3.97; and region B, pH 4.07–4.34) from the preparative IEF were chosen for further processing. Following HPIEX at pH 9.6 and pH 6.0, regions A and B were further resolved into 3 isoforms