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Purification and Characterization of Recombinant Human Follicle Stimulating Hormone Produced by Chinese Hamster Ovary Cells

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Abstract Biologically active recombinant human follicle stimulating hormone (rhFSH) was produced in Chinese hamster ovary cells and purified by a series of chromatographic steps. The chromatographic steps included anion-exchange chromatography (DEAE Sepharose F/F, Q Sepharose F/F), hydrophobic interaction chromatography (Source 15 PHE), and hydroxyapatite chromatography (Macro-Prep ceramic hydroxyapatite type I). A distinctive step of the purification process developed was the use of $ZnCl_2$ for the removal of non-glycosylated or lowly-glycosylated FSH and impurities through co-precipitation with Zn^{2+}. Purified rhFSH was identified and characterized by several physicochemical and biological methods such as gel electrophoresis, high-performance liquid chromatography, amino acid analysis, carbohydrate analysis, and biological activity. The overall yield of the purification was ~30%. The rhFSH preparation obtained showed high purity (>99%) and high *in vivo* potency (>16,000 IU/mg). Carbohydrate analysis suggested that the purified rhFSH contained approximately 40% (w/w) carbohydrate with di- or tri-antennary structure on average, which is somewhat more heavily sialylated than commercially available rhFSH. In conclusion, the results of these analyses established an identity of the purified rhFSH with natural FSH from human pituitary glands, and furthermore, the purified rhFSH preparation showed higher *in vivo* potency and was slightly more heavily sialylated than commercially available rhFSH.

Key words: Follicle stimulating hormone, recombinant, Chinese hamster ovary cells, infertility

Follicle stimulating hormone (FSH) is a 34 kDa glycoprotein hormone consisting of two noncovalently linked subunits, the α subunit and β subunit. The α subunit consists of 92 amino acids and two asparagine-linked glycosylation sites on positions α52 and α78, whereas the β subunit is comprised of 111 amino acids and includes two asparagine-linked glycosylation sites on positions β7 and β24 [1, 2]. FSH is produced and secreted from the anterior pituitary gland and plays an essential role in the regulation and maintenance of reproductive processes such as ovarian follicular development, ovulation, and gametogenesis [5, 10]. FSH is widely used to stimulate follicular development for inducing ovulation in anovulatory women wishing to conceive [21, 29]. It has also now become routine treatment for stimulating multiple follicular developments in ovulatory women undergoing assisted reproduction treatment such as *in vitro* fertilization and embryo transfer treatment [15, 25, 29]. Until recently, FSH could be obtained only from human urinary sources such as human menopausal gonadotropin, containing equal amounts of FSH and luteinizing hormone, or as purified urinary FSH. These preparations imply a number of disadvantages such as low purity, urinary contaminations (including cytokines, growth factors, transferrins, and other proteins that might modulate ovarian activity), limited urine sources, and batch-to-batch inconsistencies [13]. Rapid advances of recombinant DNA technology have made it possible to overcome these limitations. High-level expression of glycoproteins can be achieved in recombinant Chinese hamster ovary (CHO) cells, which have been used for the production of therapeutic proteins [6, 7, 17, 20]. A stable cell line producing recombinant human FSH (rhFSH) has been established by transfection of CHO cells with a plasmid containing the two-subunit genes encoding human FSH [11, 16, 23]. Purified rhFSH from the CHO cell line has a high purity, a high specific activity, and batch-to-batch consistency. Several methods to purify pituitary and urinary FSH have been reported [4, 14, 24, 27]. However, the purification method for pituitary and urinary FSH was not directly applied to the purification of recombinant human FSH. Also, the purification method

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for rhFSH has not been reported in detail. In this article, we describe a novel purification method of rhFSH from culture supernatant of CHO cells by pretreatment and column chromatography. The highly purified rhFSH obtained by the purification processes was extensively characterized for physicochemical, immunological, and biological properties and compared with both natural FSH from human pituitary glands and commercially available rhFSH product.

MATERIALS AND METHODS

Cell Line and Tissue Culture

Development of the CHO cell line, expressing rhFSH used in this study, was described previously by Yang *et al.* (Patent No. KR 2003-68641. Method for mass production of human follicle stimulating hormone) and Koh *et al.* [18]. Briefly, the cell line, DPFC325 (KCLRF-BP-00082, Korean Cell Line Research Foundation, Seoul, Korea), was established by transfection of Rc/CMV-dhfr-TPL-hFSH plasmid, which is able to express FSH-α and -β subunits, into dihydrofolate reductase (DHFR)-deficient CHO cells (CHO/dhfr-) and subsequent methotrexate (MTX)-mediated gene amplification. Culture medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, U.S.A.). The roller bottle for mass culture was purchased from Corning (Corning, NY, U.S.A.). Initially, CHO cells producing recombinant human FSH were cultivated in 175 cm^2 T-flasks with MEM-α supplemented with 10% dialyzed FBS in a humidified incubators at 37°C with 5% CO_2. Exponentially growing cells were inoculated into 850 cm^2 roller bottles with 200 ml of DMEM/F12 media supplemented with 10% FBS and incubated at 37°C on roll-in incubator (Bellco, Vineland, NJ, U.S.A.) at 0.3 rpm. After a proliferation stage for 3 days, the growth medium in roller bottles was replaced with 200 ml of a fresh serum-free medium, CHO-S-SFM II, supplemented with 1 mM methotrexate (Sigma, St. Louis, MO, U.S.A.). The supernatant of cell culture containing rhFSH was harvested and replaced with fresh medium everyday. The amounts of expressed rhFSH were measured by FSH EIA test kit (BioCheck, Foster City, CA, U.S.A.) according to the manufacturer's instruction.

Purification of Recombinant Human FSH

Culture supernatant was harvested and concentrated by using a 10,000 Da cutoff membrane (Millipore, Bedford, MA, U.S.A.) In order to remove lowly-glycosylated or nonglycosylated proteins and peptides, 100 mM $ZnCl_2$ was added to the concentrate and incubated at 4°C for 1 h. The precipitate formed with Zn^{2+} was removed by centrifugation, and the resulting supernatant was collected and dialyzed against 10 mM Tris-HCl buffer, pH 8.0. The dialyzed supernatant was filtered with 0.2 μm filter and loaded onto a DEAE Sepharose F/F (Amersham Biosciences, Uppsala, Sweden), previously equilibrated with 10 mM Tris-HCl buffer, pH 8.0. Proteins were eluted with 80 mM NaCl, and with 0.5 M NaCl in 10 mM Tris-HCl buffer, pH 8.0 at a flow rate of 10 ml/min. The fractions containing FSH immunoreactivity, detected by ELISA, were pooled (IEC-I fraction), and ammonium sulfate was added to the fraction to make a final concentration of 1 M. The fraction was filtered with 0.2 μm filter and applied to a Source 15 PHE column (Amersham Biosciences) equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 1 M $(NH_4)_2SO_4$. Proteins were eluted with increasing concentration of 2-propanol to a final concentration of 20% (v/v). Fractions containing rhFSH immunoreactivity were pooled and dialyzed against 10 mM sodium phosphate buffer, pH 7.0. The FSH-containing fraction from the PHE column was applied onto a Macro-Prep ceramic hydroxyapatite type I column (Bio-Rad, Hercules, CA, U.S.A.) equilibrated in 10 mM sodium phosphate buffer, pH 7.0, at a flow rate of 10 ml/min. The rhFSH was eluted in the runoff fractions. The rhFSH-containing fractions were pooled and dialyzed against 10 mM Tris-HCl buffer, pH 8.0, and the fraction was applied onto a Source 15 Q column (Amersham Biosciences) in 10 mM Tris-HCl buffer, pH 8.0. The elution was carried out with 50–200 mM NaCl linear gradient after 50 mM NaCl step elution. The highly purified rhFSH fractions were pooled and dialyzed against water by ultrafiltration (10,000 Da cutoff membrane). The rhFSH bulk was filtered with 0.2-μm membrane and stored frozen below -20°C before use.

SDS-PAGE and Western Blotting

Analyses of the sample of each purification step and rhFSH preparations were carried out by SDS-PAGE as described by Laemmli [19]. The samples were mixed with Tris-glycine SDS sample buffer (Invitrogen, Carlsbad, CA, U.S.A.) and applied onto a 4–20% Tris-glycine gel (Invitrogen). After electrophoresis, the protein bands were visualized by Coomassie brilliant blue staining, using PhastGel Blue R (Amersham Bioscience). For Western blot, proteins from the SDS-PAGE gels were transferred to nitrocellulose membrane at 250 mA for approximately 1 h. After blocking, the transferred proteins were probed by using mouse anti-hFSH monoclonal antibody (USBiologicals, Swampscott, MA, U.S.A.). Subsequent detection of immobilized primary antibody was performed, using rabbit anti-mouse immunoglobulin conjugated with alkaline phosphatase (Sigma).

Isoelectricfocusing (IEF)

The isohormonal profiles of rhFSH preparations were analyzed by IEF. The samples of rhFSH were mixed with IEF sample buffer pH 3–7 (Invitrogen), applied onto a pH 3–7 IEF gel (Invitrogen) and run at 2 W for 2 h according

to the manufacturer's instructions (Invitrogen). After electrophoresis, the protein bands were visualized by PhastGel Blue R staining. The isoelectric points of the different isomers of rhFSH preparations were determined by comparing with IEF Marker 2.5-6.5 Amersham Biosciences.

Size-Exclusion High-Performance Liquid Chromatography (SE-HPLC)

The purified rhFSH preparation was analyzed, using a TSK-GEL G2000SW XL column (7.8×300 mm, Tosoh, Tokyo, Japan) at 30°C. Elution was carried out with 0.1 M sodium phosphate buffer, pH 6.8, at a flow rate of 0.5 ml/min. The eluted materials were monitored by a UV monitor at 280 nm, and retention time and peak area were compared with those of a reference sample.

Amino Acid Composition and *N*-Terminal Amino Acid Sequencing

The rhFSH preparation was lyophilized and subjected to amino acid analysis. Amino acid composition and *N*-terminal amino acid sequencing were carried out with the technical assistance of Korea Basic Science Institute (Daejeon, Korea). Briefly, the amino acid composition of rhFSH was analyzed by the Pico-Tag method (Waters, Milford, MA, U.S.A.) after hydrolysis in constant boiling with 6 N HCl (Sigma) containing 0.5% phenol (w/v) at 110°C for 24 h. For the cysteine content analysis, cysteine residues were oxidized to cysteic acid with a mixture of formic acid and hydrogen peroxide (19:1, v/v). In the case of tryptophan analysis, lyophilized FSH was hydrolyzed in constant boiling with 4 M methane-sulfonic acid at 110°C for 24 h [32]. The two subunits were isolated, using C4 reversed phase HPLC for *N*-terminal amino acid sequencing, and each *N*-terminal amino acid sequences of two subunits of rhFSH were analyzed using the automated Edman degradation method [32] with the Procise 491 Protein sequencing system (Applied Biosystems, Foster City, CA, U.S.A.).

Deglycosylation

The rhFSH samples were reduced and denatured by boiling in the presence of 1% (v/v) β-mercaptoethanol and 1% (w/v) SDS. Release of N-linked or O-linked oligosaccharide from denatured rhFSH (10 μg) was accomplished by using 40 mU of N-glycosidase (Sigma) and/or 0.25 mU of O-glycosidase (Sigma) in reaction buffer containing 0.5% Nonidet P-40 (Sigma). Desialylation of rhFSH (10 μg) was performed with 0.25 mU of sialidase (Sigma) at 37°C for 18 h in the same buffer. The enzymes were added individually or sequentially in order to determine types of oligosaccharides present on the rhFSH.

Monosaccharide Composition

Determination of the monosaccharide composition of the rhFSH preparation was carried out by high performance anion-exchange chromatography (CarboPac PA1, Dionex, Sunnyvale, CA, U.S.A.) and pulsed amperometric detector (HPAEC-PAD, Dionex). Separate sets of hydrolysis conditions were employed to achieve quantitative release of the amino and neutral sugar residues as well as the sialic acid residues. For release of amino sugars, the sample was hydrolyzed with 6 N HCl at 100°C for 4 h. Two M trifluoroacetic acid (at 100°C for 4 h) and 0.1 N HCl (at 80°C for 1 h) were used to release neutral sugars and sialic acids, respectively.

In Vivo Bioassay

The *in vivo* bioactivities of rhFSH were examined by the rat ovarian weight gain method of Steelman and Pohley [31], using NIBSC 92/642 as the international standard. Protein content was determined by measuring UV absorbance at 277 nm (a coefficient of 1.066 mg/ml^{-1}). Briefly, the 21-22-day-old intact Sprague-Dawley female rats were randomized into experimental groups of eight rats. Groups of rats received 1.5 IU, 3 IU, or 6 IU FSH, plus 42 IU human chorionic gonadotropin (hCG) subcutaneously over 3 days. Autopsy was performed on the fourth day (72 h after the first injection). The ovaries were removed, dissected free of surrounding tissue, and weighed on a balance. The *in vivo* bioactivities of the rhFSH samples were evaluated using the statistical model of the 3×3 balanced, paralleled line assay versus the rhFSH international standard IS92/642 [9].

RESULTS AND DISCUSSION

Purification of rhFSH

The purification of rhFSH was accomplished by a pretreatment step and four chromatographic steps. The pretreatment step and first two chromatographic steps were designed to remove most of the impurities, while the remaining two steps further purified the product by eliminating contaminants present in trace amounts. The chromatograms of each purification steps are shown in Fig. 1.

A distinctive feature of the purification method described in this article is the use of $ZnCl_2$ as divalent metal cation for the purpose of removing nonglycosylated or lowly-glycosylated peptides and proteins through precipitation with Zn^{2+}. $ZnCl_2$ pretreatment removed cell culture medium-related proteins as well as degraded FSH in large quantities (Fig. 2, Lane 3). The first two chromatographic steps (ion-exchange and hydrophobic interaction chromatography) removed most of the impurities and yielded highly homogeneous FSH, exclusive of a small amount of impurity with molecular weight of approximately 75 kDa (Fig. 2, Lanes 4 and 5).

Further purifications on hydroxyapatite (Macro-prep ceramic hydroxyapatite type I) removed most of these impurities, resulting in over 95% purity. Final chromatographic step on Source 15 Q removed a small amount of the residual

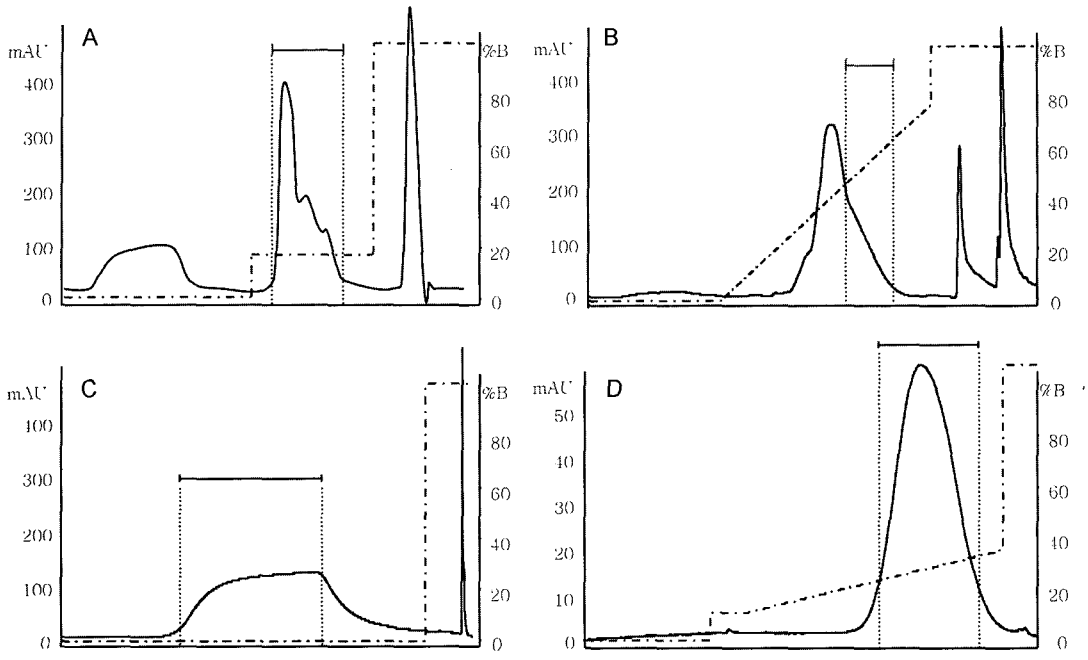

Fig. 1. Chromatograms of each purification step.
(A) DEAE Sepharose F/F column chromatography, (B) Source 15 PHE column chromatography, (C) Hydroxyapatite column chromatography, (D) Source 15 Q column chromatography. —, UV profile at 280 nm (mAU); - -, gradient; ⊢⊣, rhFSH-containing pool.

impurities and yielded rhFSH with purity of >99%. The overall yield was >30%.

Purity and Size

The purity and molecular weight of the rhFSH preparation were determined by several methods, including SDS-PAGE/Western blotting and SE-HPLC. The SE-HPLC profile of rhFSH is shown in Fig. 3.

No other peaks were detectable in the SE-HPLC chromatogram, except for the rhFSH heterodimer. SDS-PAGE and Western blotting further established the purity of the rhFSH preparation. As shown in Fig. 4, the rhFSH preparation contained no detectable amount of free subunits and rhFSH aggregates, and migrated as a heterogeneous, diffuse protein band centered on an approximate mass of 45 kDa. Also, the molecular mass of the rhFSH heterodimer was determined by SE-HPLC by comparing the retention

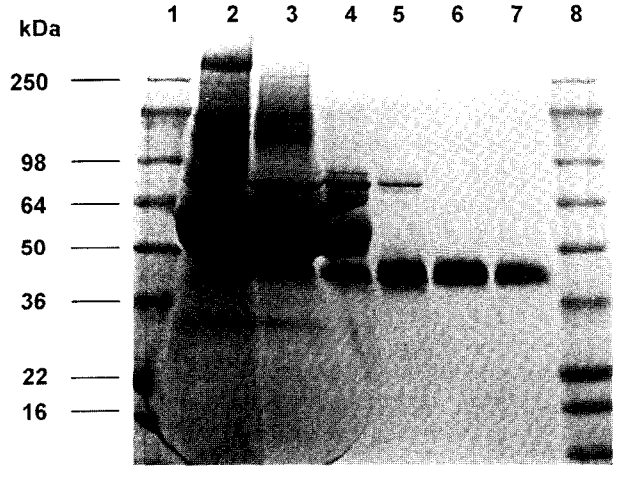

Fig. 2. SDS-PAGE analysis of each samples of rhFSH purification step.
SDS-PAGE was performed in a 4–20% Tris-glycine gel, followed by staining with PhastGel Blue R. Lanes 1 and 8, molecular weight marker; Lane 2, concentrated culture supernatant; Lane 3, sample after pretreatment; Lanes 4–7, samples containing rhFSH after DEAE Sepharose, Source 15 PHE, Hydroxyapatite, and Source 15 Q chromatography, respectively.

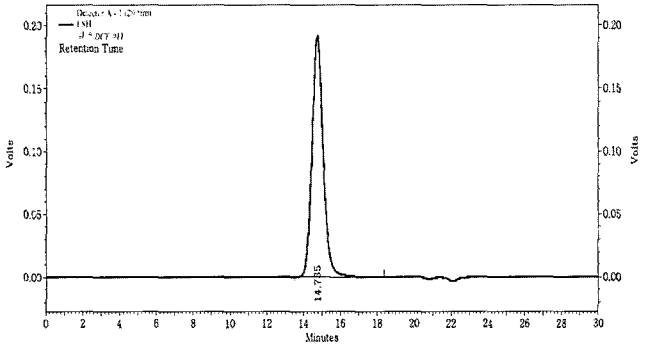

Fig. 3. Size-exclusion high performance liquid chromatography (SE-HPLC) of purified recombinant human follicle stimulating hormone (rhFSH).
The purified rhFSH preparation was analyzed using a G2000SW column. The column was eluted with 0.1 M sodium phosphate buffer, pH 6.8, at a flow rate of 0.5 ml/min. Absorbance was monitored at 280 nm.

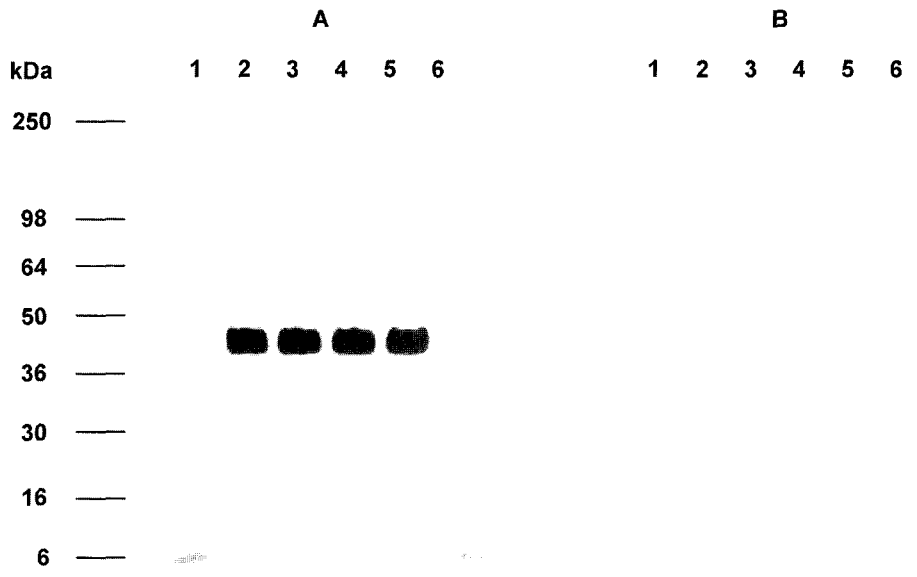

Fig. 4. SDS-PAGE and Western blotting of purified rhFSH.
(A) SDS-PAGE was performed in a 4–20% Tris-glycine gel, followed by staining with PhastGel Blue R. (B) For Western blot, proteins from the SDS-PAGE gel were transferred to nitrocellulose membrane and, after blocking, the transferred proteins were probed, using mouse anti-FSH monoclonal antibody, followed by subsequent detection of immobilized primary antibody with rabbit anti-mouse immunoglobulin conjugated with alkaline phosphatase. Lanes 1 and 6, molecular weight marker; Lanes 2–5, purified rhFSH.

time of rhFSH with those of a number of calibration proteins with known molecular weight. The apparent molecular mass of rhFSH by SE-HPLC is ~45 kDa (data not shown).

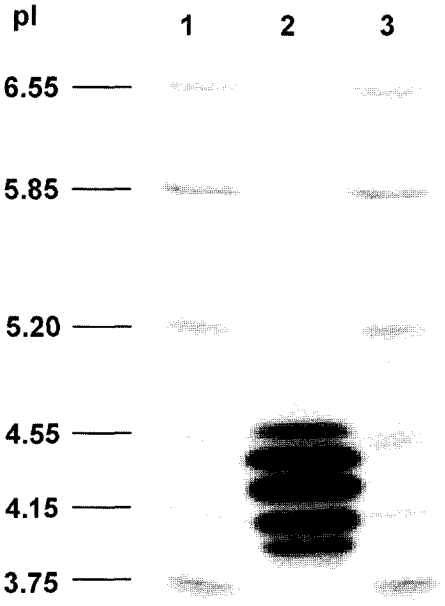

Fig. 5. Isohormone profile of purified rhFSH.
The IEF was performed in a pH 3–7 IEF gel, and the protein bands were then visualized by PhastGel Blue R staining. Lanes 1 and 3, pI marker; Lane 2, purified rhFSH.

Isohormone Profile

Natural follicle stimulating hormone shows microheterogeneity due to differences in carbohydrate chain structure, especially in the degree of sialylation [12]. Isohormones of the rhFSH preparation were separated by isoelectric focusing techniques on the basis of differences in isoelectric points. It has been described that human pituitary FSH could be separated into at least 20 isohormone fractions, which displayed 7 discrete levels of FSH receptor-binding activities [30]. Also, it has been known that the rhFSH isoforms, made in the Chinese hamster ovary cell line, ranged from pI 3.0–5.5 with a modal value of pI 4.2 [3]. Figure 5 shows the isohormone profile of the highly purified preparation. The purified rhFSH showed 7 detectable bands distributed over an isoelectric point range of 3.8–4.8, which is slightly more acidic than two rhFSH preparations that showed 7–9 bands at pI 3.9–5.5 [23] and 6–7 bands at pI 4.0–5.2 [22], respectively.

Amino Acid Composition and *N*-Terminal Sequence

Amino acid composition data showed that the experimental composition of rhFSH was in agreement with the theoretical composition (data not shown). *N*-terminal amino acid sequences of the first 15 residues were established for both α and β subunit polypeptides by Edman degradation as described in Materials and Methods. As can be seen in Table 1, the *N*-terminal sequence of both subunits was in agreement with the natural FSH. Significant amino terminal heterogeneity of the β-subunit was observed. About 50%

Table 1. *N*-terminal amino acid sequence analysis[a] of purified hFSH by automated Edman degradation.

α-subunit			β-subunit		
No.	Urinary hFSH	Purified rhFSH	No.	Urinary hFSH	Purified rhFSH
1	Ala	Ala	1	Asn (Cys)	Asn (X^b)
2	Pro	Pro	2	Ser (Glu)	Ser (Glu)
3	Asp	Asp	3	Cys (Leu)	X^b (Leu)
4	Val	Val	4	Glu (Thr)	Glu (Thr)
5	Gln	Gln	5	Leu (Asn)	Leu (Y^c)
6	Asp	Asp	6	Thr (Ile)	Thr (Ile)
7	Cys	X^b	7	Asn (Thr)	Y^c (Thr)
8	Pro	Pro	8	Ile (Ile)	Ile (Ile)
9	Glu	Glu	9	Thr (Ala)	Thr (Ala)
10	Cys	X^b	10	Ile (Ile)	Ile (Ile)
11	Thr	Thr	11	Ala (Glu)	Ala (Glu)
12	Leu	Leu	12	Ile (Lys)	Ile (Lys)
13	Gln	Gln	13	Glu (Glu)	Glu (Glu)
14	Glu	Glu	14	Lys (Glu)	Lys (Glu)
15	Asn	Asn	15	Glu (Cys)	Glu (X^b)

[a]Data are shown for the first 15 cycles. Amino terminal heterogeneity of the β-subunit is observed. Amino acids in parentheses show the sequence of *N*-terminal-truncated species of uhFSH or rhFSH (~50%).
[b]X (Cysteine) and [c]Y (Asparagine) were not recovered in this analysis.

of the protein starts at the expected amino acid residue 1 (Asn), whereas the other half of the sequence starts at amino acid residue 3 (Cys). The *N*-terminal truncated species of the sequence of the β-subunit (~50%) were shown to be identical to that of pituitary, urinary, and recombinant human FSH [22, 23, 26, 28].

Deglycosylation Profile and Carbohydrate Content

Following enzymatic release of glycans from denatured rhFSH, the deglycosylation profile was analyzed by electric mobility on SDS-PAGE. As shown in Fig. 6, purified FSH, which has 40–48 kDa molecular weight, was dissociated into two subunits with 24–28 kDa by boiling under reduced condition. Release of sialic acid from denatured FSH by sialidase showed ~2 kDa decrease of molecular weight. In addition, rhFSH, whose N-linked glycans were released by treatment of N-glycosidase, was found to have molecular weight of 14–16 kDa, whereas no decrease of molecular

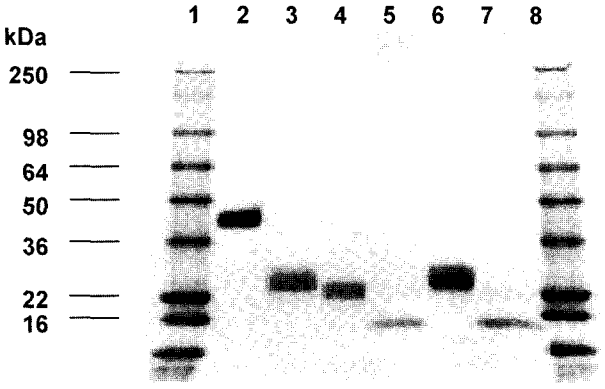

Fig. 6. Deglycosylation profile of reduced rhFSH by enzymatic digestion.
Following enzymatic release of glycans from denatured rhFSH, the deglycosylation profile was analyzed by electric mobility on SDS-PAGE. Lanes 1 and 8, molecular weight marker; Lane 2, rhFSH sample; Lane 3, rhFSH denatured by boiling in the presence of β-mercaptoethanol and SDS; Lanes 4–7, denatured rhFSH digested with sialidase (Lane 4), N-glycosidase (Lane 5), O-glycosidase (Lane 6), and 3 enzymes at a time (Lane 7).

weight by O-glycosidase was observed, implying that purified FSH contains only N-linked glycans, but not O-linked glycans.

The deglycosylation analyses indicated the carbohydrate content of purified FSH to be approximately 40% of the total weight of the glycoprotein.

Monosaccharide Composition

The monosaccharide composition was investigated by high-performance anion-exchange chromatography after the sugars were released from the intact molecule by chemical hydrolysis. Five monosaccharides were identified: mannose, fucose, N-acetylglucosamine, galactose, and sialic acid (Table 2). These results show that half of the oligosaccharides of rhFSH are fucosylated. The content of N-acetylglucosamine and galactose indicates that an oligosaccharide contains over 2.5 antennas, ~90% of which is sialylated.

The overall monosaccharide composition of purified rhFSH was compared with those of pituitary and urinary hFSH as well as recombinant hFSH described previously by Loumaye *et al.* [22]. The monosaccharide content of purified rhFSH was found to be similar, but more heavily

Table 2. Comparison of the monosaccharide compositions of recombinant and natural human FSH normalized for mannose=3.

Monosaccharide	Purified rhFSH	Reference[a]		
		Follitropin-α	Pituitary hFSH	Urinary hFSH
Fucose	0.5	0.5	0.7	0.6
Galactose	2.87	2.8	2.8	3.0
Mannose	3.0	3.0	3.0	3.0
N-Acetylglucosamine	4.56	4.6	4.4	5.2
Sialic acid	2.56	2.2	2.8	2.9

[a]Data from Loumaye *et al.* [22].

Table 3. *In vivo* bioactivity of purified recombinant human follicle stimulating hormone.

Tests	*In vivo* activity (IU/mg)	Relative potency (%) compared to NIBSC 92/642[a]
#1	16,177	117.2
#2	17,216	124.8
#3	16,940	122.8
Mean[b]	16,778	121.6

[a]First international standard for recombinant human follicle stimulating hormone for bioassay. The specific activity of this preparation has been described as 13,800 IU/mg in http://www.nibsc.ac.uk.
[b]Average of three separate tests.

sialylated, than the rhFSH product described by Loumaye *et al.* [22] and slightly less sialylated than urinary- or pituitary-hFSH. This observation is in good agreement with data obtained from the isoelectric profile of rhFSH product described by Loumaye *et al.* [22], which was slightly less acidic.

In Vivo Bioactivity of rhFSH

This bioassay is based on the fact that intact immature female rats treated with hCG are sensitive to exogenous FSH, and that the relationship between administered FSH and ovarian weight is linearly proportional. The rhFSH preparation showed more than 16,000 IU/mg of protein biopotencies, which is higher than the 13,800 IU/mg of international standard for recombinant hFSH (Table 3). These values are also higher than those of commercially available rhFSH preparations with specific activities of mean ~10,000 IU/mg [2, 8, 22], even if the error inherent in the bioassay is taken into consideration.

In summary, we described a novel purification method of rhFSH produced by Chinese hamster ovary cells. Also, we established the identity of rhFSH by several physicochemical and biological methods. The purification method described in this article provided an efficient removal of impurities and high yield of rhFSH in large-scale production. The highly purified rhFSH has a molecular weight of 45 kDa and isohormonal profile of 7 detectable bands at pI 3.8-4.8. Amino acid sequence and composition analyses established the identity of purified rhFSH with natural FSH. Carbohydrate analysis suggested that the purified rhFSH contained sialylated di- and tri-antennary structure on average and somewhat more heavily sialylated carbohydrate than commercially available rhFSH. Furthermore, the purified rhFSH showed higher *in vivo* potency than commercially used rhFSH preparation [2, 8, 22]. Our previous studies on androgen-sterilized mouse model showed that the purified rhFSH was effective in stimulating follicular development and subsequent induction of ovulation, and also increased the pregnancy and fertilization rates of mice [18]. These results demonstrate that the protein may be useful for treatment of infertile patients in need of both ovulation induction and assisted reproductive techniques.

REFERENCES

1. Baenziger, J. U. and E. D. Green. 1988. Pituitary glycoprotein hormone oligosaccharides: Structure, synthesis and function of asparagines-linked oligosaccharides on lutropin, follitropin and thyrotropin. *Biochim. Biophys. Acta* **947:** 287-306.
2. Bagatti, G., C. Crisci, A. Datola, G. Gostoli, M. Mascia, P. Polletta, G. Roncalli, and P. Simoni. 2001. Characterisation and comparison of recombinant human follicle-stimulating hormones. *J. Clin. Res.* **4:** 91-104.
3. Cerpa-Poljak, A., L. A. Bishop, Y. J. Hort, C. K. H. Chin, R. de Kroon, S. M. Mahler, G. M. Smith, M. C. Stuart, and P. R. Schofield. 1993. Isoelectric charge of recombinant human follicle-stimulating hormone isoforms determines receptor affinity and *in vitro* bioactivity. *Endocrinology* **132:** 351-356.
4. Chapman, G. E., A. G. C. Renwick, and J. H. Livesey. 1981. The isolation of human pituitary hormones from frozen glands. *J. Clin. Endocrinol. Metab.* **53:** 1008-1013.
5. Chappel, S. C., A. Ulloa-Aguirre, and C. Coutifaris. 1983. Biosynthesis and secretion of follicle-stimulating hormone. *Endocr. Rev.* **4:** 179-211.
6. Chun, G.-T., J. B. Lee, S. U. Nam, S. W. Lee, Y. H. Jeong, E. Y. Choi, I. H. Kim, Y.-S. Jeong, and P.-H. Kim. 2002. Screening of high-productivity cell lines and investigation of their physiology in Chinese hamster ovary (CHO) cell cultures for transforming growth factor-β1 production. *J. Microbiol. Biotechnol.* **12:** 121-129.
7. Chung, J. Y., H. K. Ahn, S. W. Lim, Y. H. Sung, Y. W. Koh, S. K. Park, and G. M. Lee. 2003. Development of recombinant Chinese hamster ovary cell lines producing human thrombopoietin or its analog. *J. Microbiol. Biotechnol.* **13:** 759-766.
8. De Leeuw, R., J. Mulders, G. Voortman, F. Rombout, J. Damm, and L. Kloosterboer. 1996. Structure-function relationship of recombinant follicle stimulating hormone (Puregon®). *Mol. Hum. Reprod.* **2:** 361-369.
9. Finney, D. J. 1989. *Statistical Method in Biological Assay*, 3rd Ed. Charles Griffen & Company, London, U.K.
10. Gharib, S. D., M. E. Wierman, M. A. Shupnik, and W. W. Chin. 1990. Molecular biology of the pituitary gonadotropins. *Endocr. Rev.* **11:** 177-199.
11. Howles, C. M. 1996. Genetic engineering of human FSH (Gonal-F®). *Hum. Reprod. Update* **2:** 172-191.
12. Jackson, J. H. and P. J. Russell. 1984. Characteristics of the isoelectric focusing procedure: Importance of column size, pH, and protein-protein interactions. *Anal. Biochem.* **137:** 41-49.
13. Jennings, C. J., K. Moreland, and C. M. Peterson. 1996. *In vitro* fertilization. *Drugs* **52:** 313-343.
14. Jiang, N. S. and A. Albert. 1968. Purification of urinary follicle stimulating hormone by gel filtration in the presence of sodium dodecyl sulfate. *J. Clin. Endocrinol.* **28:** 1295-1299.

15. Johns, H. W. J., G. S. Johns, M. C. Andrew, A. Acosta, C. Bundren, J. Garcia, B. Sandow, and G. Wright. 1982. The program for *in vitro* fertilization at Norfolk. *Fertil. Steril.* **38:** 14-21.
16. Keene, J. L., M. M. Matzuk, T. Otani, B. C. J. M. Fauser, A. B. Galway, A. J. W. Hsueh, and I. Boime. 1989. Expression of biologically active human follitropin in Chinese hamster ovary cells. *J. Biol. Chem.* **264:** 4769-4775.
17. Kim, N. S., K. H. Chang, B. S. Chung, S. H. Kim, J. H. Kim, and G. M. Lee. 2003. Characterization of humanized antibody produced by apoptosis-resistant CHO cells under sodium butyrate-induced condition. *J. Microbiol. Biotechnol.* **13:** 926-936.
18. Koh, S. B., K. S. Seo, S. C. Kim, B. O. Ahn, W. B. Kim, and S. H. Lee. 2002. Effect of recombinant human FSH on ovulation, pregnancy and *in vitro* fertilization in androgen-sterilized mice. *Arch. Pharm. Res.* **25:** 357-363.
19. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.
20. Lee, S.-H., H.-S. Lee, M.-K. Lee, J.-H. Lee, J.-D. Kim, Y.-S. Park, S.-Y. Lee, and H.-Y. Lee. 2002. Enhancement of tissue type plasminogen activator (tPA) production from recombinant CHO cells by low electromagnetic fields. *J. Microbiol. Biotechnol.* **12:** 457-462.
21. Loumaye, E., R. Campbell, and J. Salat-Baroux. 1995. Human follicle-stimulating hormone produced by recombinant DNA technology: A review for clinicians. *Hum. Reprod. Update* **1:** 188-199.
22. Loumaye, E., M. Dreano, A. Galazka, C. Howles, L. Ham, A. Munafo, and A. Eshkol. 1998. Recombinant follicle stimulating hormone: Development of the first biotechnology product for the treatment of infertility. *Hum. Reprod. Update* **4:** 862-881.
23. Olijve, W., W. de Boer, J. W. M. Mulders, and P. M. G. G. van Wezenbeek. 1996. Molecular biology and biochemistry of human recombinant follicle stimulating hormone (Puregon®). *Mol. Hum. Reprod.* **2:** 371-382.
24. Peckham, W. D. and A. F. Parlow. 1969. On the isolation of human pituitary follicle-stimulating hormone. *Endocrinology* **84:** 953-957.
25. Prevost, R. R. 1998. Recombinant follicle-stimulating hormone: New biotechnology for infertility. *Pharmacotherapy* **18:** 1001-1010.
26. Rathnam, P. and B. B. Saxena. 1975. Primary amino acid sequence of follicle-stimulating hormone from human pituitary glands. *J. Biol. Chem.* **250:** 6735-6746.
27. Saxena, B. B. and P. Rathnam. 1967. Purification of follicle-stimulating hormone from human pituitary glands. *J. Biol. Chem.* **17:** 3769-3775.
28. Saxena, B. B. and P. Rathnam. 1976. Amino acid sequence of the b-subunit of follicle-stimulating hormone from human pituitary glands. *J. Biol. Chem.* **251:** 993-1005.
29. Speroff, L., R. H. Glass, and N. G. Kase. 1989. *Clinical Gynecologic Endocrinology and Infertility*, pp. 583-609. 3rd Ed. Williams & Wikins, Baltimore, U.S.A.
30. Stanton, P. G., D. M. Robertson, P. G. Burgon, B. Schmauk-White, and M. T. W. Hearn. 1992. Isolation and physicochemical characterization of human follicle-stimulating hormone isoforms. *Endocrinology* **130:** 2820-2832.
31. Steelman, S. L. and F. M. Pohley. 1953. Assay of the follicle-stimulating hormone based on the augmentation with human chorionic gonadotropin. *Endocrinology* **53:** 604-616.
32. Tarr, G. E. 1986. Manual Edman sequencing system, pp. 155-194. *In* J. E. Shively (ed.), *Methods of Protein Microcharacterization*. Humana Press, Clifton, New Jersey, U.S.A.