Eur. J. Biochem. *193*, 263–271 (1990)

Isolation and structure determination of the intact sialylated N-linked carbohydrate chains of recombinant human follitropin expressed in Chinese hamster ovary cells

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(Received April 11, 1990) – EJB 90 0408

Biologically active recombinant human follitropin has been expressed in Chinese hamster ovary cells. The carbohydrate chains of the recombinant glycoprotein hormone were enzymatically released by peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F. The oligosaccharides were separated from the *N*-deglycosylated protein by gel-permeation chromatography on Bio-Gel P-100, and fractionated by a combination of FPLC on Mono Q and HPLC on Lichrosorb-NH_2. The structures of the carbohydrate chains were determined by 500- or 600-MHz ^{1}H-NMR spectroscopy. The following types of carbohydrates occur: monosialylated diantennary (10%), disialylated diantennary (43%), disialylated tri-antennary (5%), trisialylated tri-antennary (13%), trisialylated tri′-antennary (8%), and tetrasialylated tetraantennary (12%) *N*-acetyllactosamine type of carbohydrate chains, all bearing exclusively α2-3-linked *N*-acetylneuraminic acid (Neu5Ac). Previously, for pituitary follitropin mono-, di-, tri-, tri′-, and tetra-antennary oligosaccharides containing α2-3- as well as α2-6-linked Neu5Ac residues were reported. The bisecting GlcNAc residues present in native follitropin were not detected in the recombinant glycoprotein. Of the oligosaccharides 29% have an α1-6-linked Fuc residue at the asparagine-bound GlcNAc, whereas this amount is about 50% in pituitary follitropin. In some of the tri-, tri′- and tetra-antennary oligosaccharide fractions small amounts (< 5%) of compounds were detected having one or more additional *N*-acetyllactosamine units.

Follitropin (follicle-stimulating hormone, FSH) belongs to a heterodimeric glycoprotein hormone family in which each hormone is composed of two non-covalently linked subunits, denoted α and β. The amino acid sequences of the α subunits are essentially identical, whereas those of the β subunits are hormone-specific. The structures of the carbohydrate chains differ between the glycoprotein hormones and are believed to confer hormone specific-functions [1–3].

FSH is synthesized by the gonadotropes in the anterior pituitary and is required for normal reproductive functioning in both females and males. There is a need of FSH for clinical purposes since it is applied, either alone or in combination with human LH, for controlled ovarian hyperstimulation in *in vitro* fertilization [4, 5]. Human FSH is also used to stimulate the development of ovarian follicles in anovulatory women suffering from luteal phase deficiency [6], or chronic anovulatory syndrome [7].

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Abbreviations. PNGase-F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F; Neu5Ac, *N*-acetylneuraminic acid; Fuc, L-fucose; hFSH, human follicle-stimulating hormone (follitropin); LH, luteinizing hormone (lutropin); hCG, human chorionic gonadotropin; CHO, Chinese hamster ovary; t-PA, tissue plasminogen activator.

Enzymes. Peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (EC 3.5.1.52); β-galactoside α-2,6-sialyltransferase (EC 2.4.99.1).

Biologically active hFSH has recently been expressed in Chinese hamster ovary cells (CHO cells) [8] (and unpublished results). This cell line is an obvious choice because glycoproteins synthesized by CHO cells can have oligosaccharide chains which are closely related to those found in man [9]. The use of malignant mammalian cell lines may give rise to immunoreactive carbohydrate determinants [10], whereas other eukaryotic species, like yeast, can synthesize carbohydrate structures for which no human counterparts are known [11].

hFSH bears two N-linked carbohydrate chains on each subunit, but no O-linked oligosaccharides. A proper glycosylation, including sialylation, is required for the expression of the biological functions of hFSH [1, 12]. It has been shown that deglycosylated hFSH can still bind to its receptor but can not activate the adenylate cyclase enzyme system [13]. In order to be clinically suitable, the carbohydrate structures of the recombinant product have also to be compatible with the immune system of the recipient. Therefore, a detailed knowledge of the structure of the carbohydrate chains of the recombinant glycoprotein is required. Up to now the structures of the carbohydrate chains of only a few recombinant human glycoproteins have been elucidated (e.g. [9–11, 14–20]). In most of the published reports the fractionation of the oligosaccharides and the structural work has been done on desialylated oligosaccharides [21]. Here, we present the fractionation and structure determination of the enzymatically released intact sialylated oligosaccharides of recombinant hFSH produced by CHO cells, and a comparison of the estab-

lished carbohydrate chains with those reported for pituitary hFSH [22, 23].

MATERIALS AND METHODS

Materials

Recombinant hFSH expressed in a CHO cell line and urinary hFSH were provided by Organon International b.v. (Oss, The Netherlands). The specific *in vitro* bioactivity of the hormone preparations, as determined by the induction of aromatase activity in Sertoli cells [24], were approximately 12000 IU/mg protein for the recombinant product and 8500 IU/mg protein for the urinary product. Peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGase-F) from *Flavobacterium meningosepticum* was obtained from Boehringer Mannheim, FRG.

Liberation of the carbohydrate chains

Prior to the liberation of the carbohydrate chains from recombinant hFSH, gel-permeation chromatography on a Bio-Gel P-4 column (2.5 × 35.0 cm, 200 – 400 mesh, Bio-Rad) was performed in order to remove mannitol, which had been added as a stabilizer. The elution was carried out in 25 mM NH_4HCO_3, adjusted to pH 7 with HCl, at a flow rate of 25 ml/h. The effluent was monitored with a refractive index detector and the void volume fraction, containing the glycoprotein, was collected and lyophilized.

The N-linked carbohydrate chains were enzymatically released from the protein moiety according to a modified version of a previously described protocol [25]. Two batches of approximately 3 mg recombinant hFSH each were dissolved in 0.75 ml 50 mM Tris, adjusted to pH 8.4 with HCl, containing 50 mM EDTA, and 1% (by vol.) 2-mercaptoethanol. The mixture was shaken for 5 min after which SDS was added to a concentration of 2% (mass/vol.), and then boiled for 4 min. After cooling to room temperature the nonionic detergent NP-40 was added to a final concentration of 4% (mass/vol.) and the solution was incubated with 7.5 U PNGase-F for 4 h at room temperature in an end-over-end mixer. Then the mixture was boiled for 2 min, another batch of 7.5 U enzyme was added, and the incubation was continued for 16 h at room temperature. SDS/PAGE and Coomassie brilliant blue staining shows that the material originally present as a diffuse band with an apparent molecular mass between 20.5 kDa and 24.5 kDa appears with slightly reduced molecular mass. After lyophilization, the residue was dissolved in 25 mM NH_4HCO_3, adjusted to pH 7 with HCl, and fractionated in the same buffer on a Bio-Gel P-100 column (1.8 × 47.5 cm, 200 – 400 mesh, Bio-Rad) at a flow rate of 21 ml/h. Fractions of 10 ml were collected and stained for carbohydrate with orcinol/H_2SO_4. Carbohydrate-positive fractions were pooled, lyophilized and desalted in water on a Bio-Gel P-2 column (1.2 × 19 cm, 200 – 400 mesh, Bio-Rad).

FPLC

Fractionation according to charge of the enzymatically released carbohydrate chains was carried out on a Mono Q HR 5/5 anion-exchange column (Pharmacia FPLC system), using an NaCl gradient essentially as described [26]. The fractionation was monitored at 214 nm, and carbohydrate-containing fractions were detected by orcinol/H_2SO_4, lyophilized, desalted on Bio-Gel P-2, and lyophilized again.

HPLC

Subfractionation of the carbohydrate-containing Mono Q fractions was carried out on a Kratos SF 400 system (ABI Analytical, Kratos Division) equipped with a 10-μm Lichrosorb-NH_2 column (0.46 × 25 cm, Chrompack). The column was eluted with 15 mM K_2HPO_4/KH_2PO_4 pH 7.0/acetonitrile (35:65, by vol.), at a flow rate of 1.5 ml/min at room temperature. Runs were monitored at 205 nm. The HPLC fractions were desalted on Bio-Gel P-2 prior to ^{1}H-NMR spectroscopic analysis.

500- and 600-MHz ^{1}H-NMR spectroscopy

The oligosaccharide samples were repeatedly treated with ^2H_2O, finally using 99.96 atom-% ^2H_2O (Merck) at p^{2}H 7 and room temperature. Resolution-enhanced 500- or 600-MHz ^{1}H-NMR spectra were recorded on a Bruker AM-500 spectrometer (Department of Chemistry, Utrecht University) or a Bruker AM-600 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, Nijmegen University), respectively. The probe temperatures were 27 °C and chemical shifts are expressed in ppm relative to internal acetone in ^2H_2O (δ = 2.225 ppm) [27].

Quantification of oligosaccharides

The molar ratio of oligosaccharides present in the FPLC fractions is determined on basis of the number of C = O groups (absorbance at 214 nm) being known after structural identification. The molar ratio of constituent oligosaccharides within each FPLC fraction is determined on the basis of HPLC peak areas (corrected for the number of C = O groups) at 205 nm [25]. For overlapping HPLC peaks the ^{1}H-NMR spectra have also been used for the quantification of relative amounts of oligosaccharides.

Monosaccharide analysis

Monosaccharide analysis was carried out by gas-liquid chromatography on a capillary CP-Sil 5 WCOT fused silica column (0.32 mm × 25 m, Chrompack) using a Varian Aerograph 3700 gas chromatograph. The trimethylsilylated (methyl ester) methyl glycosides were prepared by methanolysis, *N*-(re)acetylation, and trimethylsilylation [28].

RESULTS

The monosaccharide analysis data of recombinant hFSH and urinary hFSH are presented in Table 1. Both glycoproteins show a very similar monosaccharide composition, but small variations in the amounts of GlcNAc and Neu5Ac are observed. For both hormone preparations the absence of GalNAc implies that neither mucin-type of carbohydrate chains nor N-linked chains containing the sulfated GalNAcβ1-4GlcNAc element occur. The carbohydrate content in the recombinant hFSH (42% by mass) is slightly higher than in the urinary hFSH (36% by mass).

Medium-pressure anion-exchange chromatography of the Bio-Gel P-100 oligosaccharide fraction of PNGase-F-digested recombinant hFSH on Mono Q gives rise to four carbohydrate-positive peaks, denoted N1 to N4 (Fig. 1). Fraction N1 has the elution volume of a monosialylated diantennary oligosaccharide, fraction N2 that of a disialylated diantennary oligosaccharide, fraction N3 that of trisialylated tri-/tri′-

Table 1. *Monosaccharide analysis data of recombinant hFSH and urinary hFSH*
The molar carbohydrate compositions are given relative to Man = 3.0. The GlcNAc values are corrected for the amount of Asn-linked GlcNAc that is not cleaved under the conditions of methanolysis

Monosaccharide	Recombinant hFSH	Urinary hFSH
Fuc	0.5	0.5
Gal	2.5	2.5
Man	3.0	3.0
GlcNAc	4.8	5.4
Neu5Ac	2.8	2.2

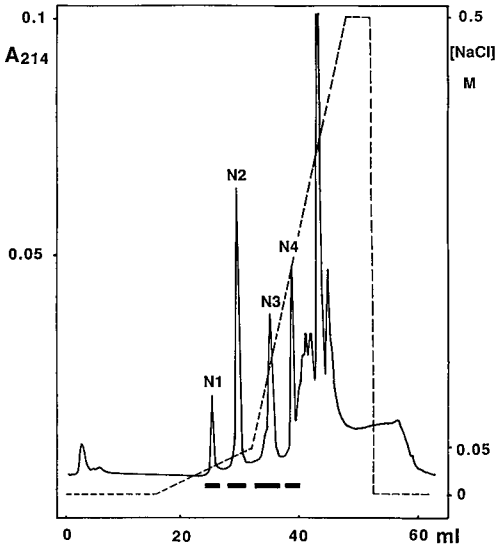


Fig. 1. *Fractionation pattern at 214 nm of the carbohydrate-containing Bio-Gel P-100 fraction, derived from PNGase-F-treated recombinant hFSH on a FPLC HR 5/5 Mono Q column.* The carbohydrate-containing fraction was desalted, lyophilized and dissolved in 0.7 ml H_2O. The column was first eluted isocratically with 16 ml H_2O, followed by a linear concentration gradient (----) of 0 – 50 mM NaCl in 16 ml H_2O, and finally by a steeper gradient of 50 – 500 mM NaCl in 20 ml H_2O at a flow rate of 2 ml/min. The injection volume was 0.1 ml. Fractions were collected as indicated

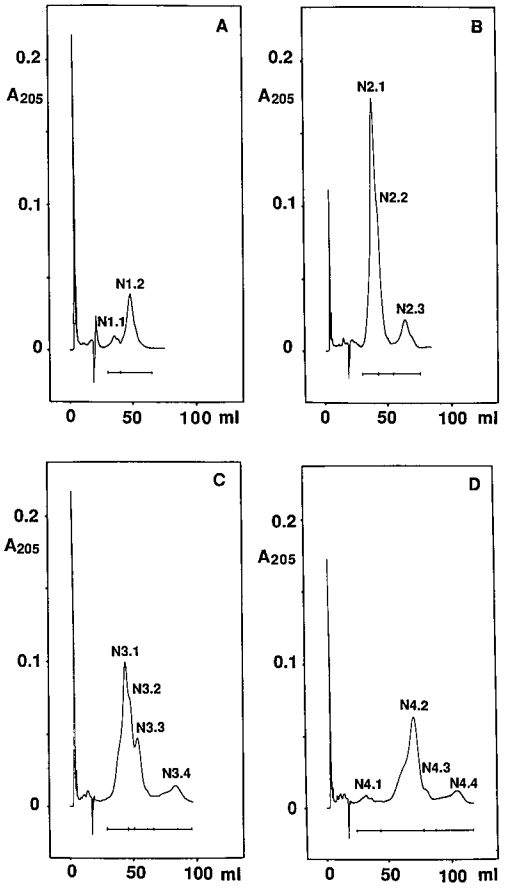
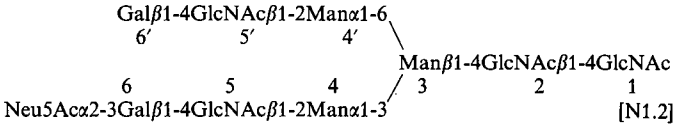


Fig. 2. *Fractionation patterns at 205 nm of the recombinant hFSH FPLC fractions N1 – N4 on a 10-µm Lichrosorb-NH_2 column (0.46 × 25 cm).* The FPLC fractions were lyophilized, desalted and dissolved in 50 µl H_2O prior to injection. The column was eluted isocratically with 15 mM K_2HPO_4/KH_2PO_4 pH 7.0/acetonitrile (35:65, by vol.) at a flow rate of 1.5 ml/min at room temperature. The injection volume was 10 µl. Fractions were collected as indicated. (A) FPLC Mono Q fraction N1; (B) fraction N2; (C) fraction N3; (D) fraction N4

antennary oligosaccharides, and fraction N4 that of a tetrasialylated tetraantennary oligosaccharide [29, 30]. The unbound fraction and the material eluting after N4 do not contain carbohydrates.

Further fractionation of Mono Q fraction N1 by HPLC on Lichrosorb-NH_2 gives rise to two subfractions, denoted N1.1 and N1.2 (Fig. 2A). In a similar way Mono Q fraction N2 has been subfractionated into N2.1 – N2.3 (Fig. 2B), Mono Q fraction N3 into N3.1 – N3.4 (Fig. 2C), and Mono Q fraction N4 into N4.1 – N4.4 (Fig. 2D). All HPLC fractions were analyzed by 500- or 600-MHz ^{1}H-NMR spectroscopy. However, the amount of material in fractions N1.1, N4.1, and N4.3 was found to be too low for structure determination by ^{1}H-NMR spectroscopy. In Table 2 the structures, as obtained by high-resolution ^{1}H-NMR spectroscopy, of the oligosaccharides occurring in recombinant hFSH are collected, together with the relative amounts of each oligosaccharide. In the following, the ^{1}H-NMR data of the various compounds will be discussed in order of increasing complexity, and relevant ^{1}H-NMR parameters are compiled in Table 3.

The ^{1}H-NMR spectrum of fraction N1.2 indicates the following monosialylated diantennary structure containing a terminal β-galactosyl residue on the Manα1-6 branch:



In the ^{1}H-NMR spectrum of fraction N1.2 the trimannosyl-*N,N′*-diacetylchitobiose core as part of a diantennary type of carbohydrate chain is reflected by the characteristic set of H-1 and NAc signals of GlcNAc-**1** and GlcNAc-**2**, and the H-1 signals of Man-**4** and Man-**4′**, together with the H-2 signals of Man-**3**, Man-**4**, and Man-**4′** [26, 27]. The 1:1 ratio of the NAc signals of GlcNAc-**1** and Neu5Ac at δ = 2.038 ppm and δ = 2.030 ppm, respectively, confirms the compound to be monosialylated, in agreement with its behaviour on Mono Q. Guided by the NAc signals of GlcNAc-**5** and GlcNAc-**5′**, both resonating at δ = 2.047 ppm, the α2-3-linked Neu5Ac residue (H-3e, δ = 2.756 ppm; H-3a, δ = 1.797 ppm) is located at Gal-**6** (cf. compounds 36 and 37 in [27]). The H-1 signals of

Table 2. *Carbohydrate chains occurring in recombinant hFSH expressed in CHO cells*

Carbohydrate chain	Code	Amount[a]
		mol/100 mol
Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3	N1.2	10
Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3	N2.1	35
Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6 Fucα1-6 Manβ1-4GlcNAcβ1-4GlcNAc Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3	N2.2	8
Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3 Neu5Acα2-3Galβ1-4GlcNAcβ1-4	N2.3	5
Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3 Neu5Acα2-3Galβ1-4GlcNAcβ1-4	N3.1	8
Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6 Fucα1-6 Manβ1-4GlcNAcβ1-4GlcNAc Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3 Neu5Acα2-3Galβ1-4GlcNAcβ1-4	N3.2A	5
Neu5Acα2-3Galβ1-4GlcNAcβ1-6 Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3	N3.2B	2
Neu5Acα2-3Galβ1-4GlcNAcβ1-6 Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6 Fucα1-6 Manβ1-4GlcNAcβ1-4GlcNAc Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3	N3.3	6

Table 2. Continuation

Carbohydrate chain	Code	Amount[a]
		mol/100 mol
Neu5Acα2-3Galβ1-4GlcNAcβ1-6 Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3 Neu5Acα2-3Galβ1-4GlcNAcβ1-4	N4.2A	2
Neu5Acα2-3Galβ1-4GlcNAcβ1-6 Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6 Fucα1-6 Manβ1-4GlcNAcβ1-4GlcNAc Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3 Neu5Acα2-3Galβ1-4GlcNAcβ1-4	N4.2B	10

[a] The structure of the remaining 9%, of which about 4% contain one or more additional *N*-acetyllactosamine units, has not been established

Gal-**6** and Gal-**6′** at δ = 4.545 ppm and δ = 4.473 ppm, respectively, are consistent with the proposed structure.

The ^{1}H-NMR spectrum of fraction N2.1 shows that it contains the following disialylated diantennary structure:

Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6
Manβ1-4GlcNAcβ1-4GlcNAc
Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3 [N2.1]

The various structural-reporter-group signals of compound N2.1 match completely those of a disialylated diantennary oligosaccharide obtained from human chorionic gonadotropin (hCG) after incubation with PNGase-F (cf. compound N2B in [26]).

The ^{1}H-NMR spectrum of the partially overlapping fraction N2.2 shows that it contains a mixture of compound N2.1 and its α1-6-fucosylated analogue N2.2 in a molar ratio of 1:4.

Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6 Fucα1-6
Manβ1-4GlcNAcβ1-4GlcNAc
Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3 [N2.2]

Compound N2.2 is identical to the second major disialylated diantennary oligosaccharide obtained from urinary hCG and previously characterized by ^{1}H-NMR spectroscopy (cf. compound N2A in [26]). The effect of the anomerization of the reducing GlcNAc-**1** can be observed on Fuc H-1, resonating at δ = 4.892 ppm (αGlcNAc-**1**) and δ = 4.900 ppm (βGlcNAc-**1**), on Fuc CH_3, resonating at δ = 1.211 ppm (αGlcNAc-**1**) and δ = 1.223 ppm (βGlcNAc-**1**), as well as on the NAc singlets of GlcNAc-**2** at δ = 2.096 ppm (αGlcNAc-**1**) and δ = 2.093 ppm (βGlcNAc-**1**).

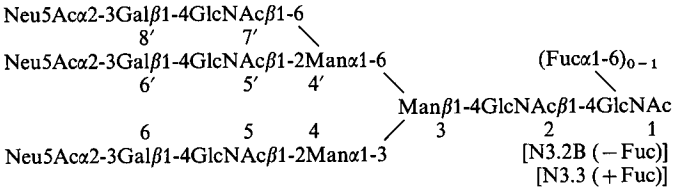
The ^{1}H-NMR spectrum of N2.3 demonstrates the occurrence of the following disialylated tri-antennary carbohydrate chain as the single constituent:

Galβ1-4GlcNAcβ1-2Manα1-6
6′ 5′ 4′
Manβ1-4GlcNAcβ1-4GlcNAc
6 5 4 3 2 1
Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3
8 7
Neu5Acα2-3Galβ1-4GlcNAcβ1-4 [N2.3]

The tri-antennary character of this compound is reflected by the H-1 structural-reporter-group signals of Man-**4** and Man-**4′** at δ = 5.116 ppm and δ = 4.924 ppm, respectively, the H-2 signals of Man-**3**, Man-**4**, and Man-**4′** at δ = 4.21 ppm, δ = 4.21 ppm, and δ = 4.11 ppm, respectively, in combination with the H-1 and NAc signals of GlcNAc-**5**, GlcNAc-**5′**, and GlcNAc-**7**. The presence of two sialic acid residues per molecule is proven by the relative intensities of the NAc, H-3a, and H-3e structural reporters of Neu5Ac in α2-3 linkage. In a tri-antennary oligosaccharide containing an *N*,*N*′-diacetylchitobiose unit the Man-**4′** H-1 signal resonates at δ = 4.92–4.93 ppm if the Manα1-6 branch is terminated by a βGal residue and at δ = 4.90–4.91 ppm if that branch contains a terminal α2-3-linked Neu5Ac residue (cf. compounds 9 in [27] and N3.1 in [30]). Guided by the chemical shift value of Man-**4′** H-1 at δ = 4.924 ppm this branch must have a non-reducing terminal Gal-**6′**. Therefore, the sialic acid residues are unambiguously located at Gal-**6** and Gal-**8**.

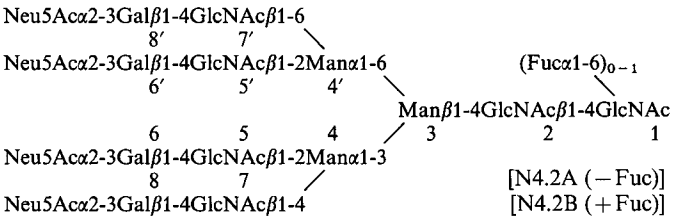
The separation of Mono Q fraction N3 by HPLC on Lichrosorb-NH_2 gives rise to three partially overlapping peaks, denoted N3.1–N3.3, and one well-separated subfraction N3.4. In fractions N3.1–N3.3 four compounds, denoted N3.1, N3.2A, N3.2B, and N3.3, could be identified:

Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6 $(Fuc\alpha1\text{-}6)_{0-1}$
Manβ1-4GlcNAcβ1-4GlcNAc
Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3
[N3.1(−Fuc)]
Neu5Acα2-3Galβ1-4GlcNAcβ1-4 [N3.2A(+Fuc)]



The main component (90%) in fraction N3.1 represents the non-fucosylated trisialylated tri-antennary compound N3.1. The remaining 10% of the material is due to overlap with fraction N3.2. The structural-reporter-group signals of compound N3.1 are essentially the same as those previously obtained for a trisialylated tri-antennary compound isolated after PNGase-F digestion of the β subunit of hCG (cf. compound N3.1 in [30]). In fraction N3.2 the main component (N3.2A, 60%) is an α1-6-fucosylated trisialylated tri-antennary compound, characterized by the same structural-reporter groups as N3.1, except for the effects of the fucosylation [26]. For oligosaccharides isolated from the β subunit of hCG, it has been established that on Lichrosorb-NH_2 a fucosylated trisialylated tri-antennary compound co-elutes with a non-fucosylated trisialylated tri′-antennary compound [30]. The minor component (N3.2B, 30%) in fraction N3.2 is a non-fucosylated trisialylated tri′-antennary compound, of which the branching pattern of the trimannosyl core is deduced from the Man-**4** and Man-**4′** H-1 signals at δ = 5.121 ppm and δ = 4.874 ppm, respectively, and the Man-**3**, Man-**4**, and Man-**4′** H-2 signals at δ = 4.25 ppm, δ = 4.21 ppm, and δ = 4.11 ppm, respectively. All structural-reporter-group signals of compound N3.2B match those previously obtained for a non-fucosylated trisialylated tri′-antennary oligosaccharide isolated from the β subunit of hCG (cf. N3.2A in [30]). The remaining 10% of the material in fraction N3.2 is due to overlap with fractions N3.1 and N3.3. The main component (75%) in fraction N3.3 is the α1-6-fucosylated form of N3.2B, recognized by the same structural-reporter groups as N3.2B except for the typical effects observed for the fucosylation [26]. The rest of the material in fraction N3.3 stems from the preceding fraction N3.2. It should be noted that the proton assignments for the crowded region δ = 4.53 – 4.60 ppm in the ^{1}H-NMR spectrum of N3.3 differ from those published by Spellmann et al. [20] for the same structure occurring in a mixture of oligosaccharides obtained from recombinant human tissue plasminogen activator (t-PA). The assignments given in the present report for the H-1 atoms of GlcNAc-**5** and GlcNAc-**5′** are based on the observed cross-peaks with the H-1 atoms of Man-**4** and Man-**4′**, respectively, in a rotating frame nuclear Overhauser enhancement spectroscopy experiment. In addition the tentative assignments given in a previous report [29] for the H-1 signals of Gal-**8′** and GlcNAc-**7′** were proven. Fraction N3.4, constituting about 2% of the total amount of oligosaccharides in recombinant hFSH, is more retarded on Lichrosorb-NH_2 than the fucosylated trisialylated tri′-antennary compound N3.3. From its behaviour on Mono Q, fraction N3.4 is suggested to contain three sialic acid residues. The ^{1}H-NMR spectrum of N3.4 shows a mixture of tri- and tri′-antennary compounds, as deduced from the H-1 and H-2 signals of Man-**4** and Man-**4′**. As compared to the ^{1}H-NMR data of N3.3, the ^{1}H-NMR spectrum of N3.4 shows an additional (Gal H-4)-like signal at δ = 4.16 ppm, indicative of a β1-3-linked GlcNAc in an extra *N*-acetyllactosamine unit (cf. compound 60 in [27]).

The ^{1}H-NMR spectrum of fraction N4.2 has been shown to comprise a mixture of a non-fucosylated (N4.2A, 15%) and a fucosylated (N4.2B, 85%) tetrasialylated tetraantennary carbohydrate chain.



The essential structural-reporter groups of N4.2A/B match those previously obtained for a tetrasialylated tetra-antennary carbohydrate chain terminating in GlcNAc-**2**, isolated from sialidosis placenta (cf. compound 241 in [29]), except for the differences observed at the reducing ends.

Fraction N4.4 represents about 2% of the total amount of oligosaccharides in recombinant hFSH. As deduced from the behaviour on Mono Q, this fraction contains a tetrasialylated compound. However, on Lichrosorb-NH_2, N4.4 is more retarded than the fucosylated tetrasialylated tetra-antennary compound N4.2B. Although the ^{1}H-NMR spectrum of N4.4 contains all features of non-fucosylated (10%) and fucosylated (90%) tetrasialylated tetra-antennary carbohydrate chains, the additional Gal H-4 signal at δ = 4.159 ppm suggests that one or more of the antennae is extended by an extra *N*-acetyllactosamine unit (cf. compound 60 in [27]).

DISCUSSION

This study presents the determination of structures of the intact sialylated N-linked oligosaccharides of recombinant hFSH expressed in CHO cells (Table 2). The negative charge on the carbohydrate chains of recombinant hFSH can be attributed to α2-3-linked sialic acid only. Pituitary hFSH carries both α2-3- and α2-6-linked sialic acid, the former one being more preponderant [22, 23, 31]. Therefore a comparison of the structures of the asialo-carbohydrate chains of recombinant hFSH with those occurring in pituitary hFSH is more meaningful. This shows that the oligosaccharides occurring in recombinant hFSH resemble more the data reported by Renwick et al. [22] than those by Green and Baenziger [23, 31] (Table 4). It has to be noted that the latter authors have found neither tri′- nor tetra-antennary oligosaccharides. In contrast to pituitary hFSH, recombinant hFSH contains no bisecting GlcNAc residues. Furthermore, the recombinant hFSH contains small amounts (< 5%) of extra *N*-acetyllactosaminyl units as part of tri-, tri′-, and tetra-antennary compounds. The core fucosylation, i.e. fucose α1-6-linked to GlcNAc-**1**, is about 50% in pituitary hFSH [22] and 29% in recombinant hFSH.

The occurrence of Neu5Ac exclusively α2-3-linked, rather than α2-6-linked, is a general feature of glycoproteins expressed in CHO cells. However, it has recently been shown that the terminal glycosylation sequences on N-linked oligosaccharides of CHO cells can be altered by expression of β-galactoside α-2,6-sialyltransferase [32]. In recombinant hFSH from CHO cells no neutral carbohydrate moieties have been detected, whereas pituitary hFSH has between 5% [22] and 10% [31] of such chains. The total amount of partially sialylated oligosaccharides is significantly lower in the recombinant than in the pituitary glycoprotein. It can be suggested that a higher degree of sialylation may lead to a longer half-life in the circulation for the recombinant product.

Table 3. *^{1}H-chemical shifts of structural-reporter-group protons of the constituent monosaccharides of the N-linked oligosaccharides derived from recombinant hFSH*

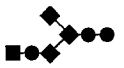
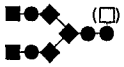
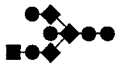
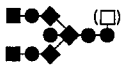
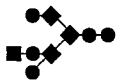
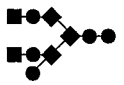
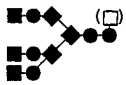
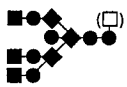
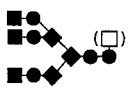
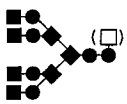
Chemical shifts are given at 300 K and were measured in ^2H_2O relative to internal acetone (δ = 2.225 ppm) [26]. Compounds are represented by short-hand symbolic notation: □, Fuc; ■, Gal; ●, GlcNAc; ◆, Man; △, Neu5Acα2-3. For numbering of the monosaccharide residues, see text

Reporter group	Residue	Chemical shift in								
		N1.2	N2.1	N2.2	N2.3	N3.1	N3.2A	N3.2B	N3.3	N4.2B
		ppm								
H1	GlcNAc-**1**α	5.188	5.190	5.181	5.189	5.189	5.184	5.184	5.182	5.182
	β	n.d.[a]	4.697	n.d.	n.d.	n.d.	4.69[b]	4.69	4.691	4.687
	GlcNAc-**2**α[c]	4.612	4.612	4.663	4.611	4.610	4.662	n.d.	4.663	4.656
	β	4.604	4.604	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Man-**4**	5.118	5.117	5.116	5.116	5.114	5.114	5.121	5.123	5.129
	Man-**4′**	4.928	4.924	4.922	4.924	4.908	4.904	4.874	4.872	4.857
	GlcNAc-**5**	4.573	4.573	4.573	4.562	4.559	4.560	4.576	4.577	4.559
	GlcNAc-**5′**	4.581	4.573	4.573	4.579	4.570	4.574	4.588	4.587	4.591
	Gal-**6**	4.545	4.544	4.544	4.544	4.544	4.545	4.545	4.545	4.543
	Gal-**6′**	4.473	4.550	4.550	4.473	4.544	4.545	4.545	4.545	4.543
	GlcNAc-**7**	–	–	–	4.544	4.544	4.545	–	–	4.543
	GlcNAc-**7′**	–	–	–	–	–	–	4.545	4.545	4.543
	Gal-**8**	–	–	–	4.544	4.544	4.545	–	–	4.543
	Gal-**8′**	–	–	–	–	–	–	4.560	4.561	4.559
H-2	Man-**3**	4.246	4.247	4.248	4.21	4.21	4.21	4.25	4.250	4.203
	Man-**4**	4.190	4.193	4.189	4.21	4.21	4.21	4.21	4.21	4.221
	Man-**4′**	4.11	4.11	4.11	4.11	4.11	4.11	4.11	4.11	4.11
H-3	Gal-**6**	4.113	4.113	4.113	4.116	4.117	4.117	4.117	4.117	4.116
	Gal-**6′**	n.d.	4.119	4.119	–	4.117	4.117	4.117	4.117	4.116
	Gal-**8**	–	–	–	4.116	4.117	4.117	–	–	4.116
	Gal-**8′**	–	–	–	–	–	–	4.117	4.117	4.116
H-3a	Neu5Ac	1.797	1.798[d]/1.801[e]	1.797[d]/1.800[e]	1.800[f]	1.802[g]	1.802[g]	1.802[g]	1.802[g]	1.804[h]
H-3e	Neu5Ac	2.756	2.758[f]	2.758[f]	2.756[f]	2.757[g]	2.757[g]	2.757[g]	2.757[g]	2.756[h]
NAc	GlcNAc-**1**	2.038	2.039	2.039	2.038	2.038	2.038	2.038	2.039	2.038
	GlcNAc-**2**α[c]	2.081	2.082	2.096	2.080	2.081	2.094	2.079	2.094	2.094
	β	2.081	2.082	2.093	2.080	2.081	2.094	2.079	2.092	2.091
	GlcNAc-**5**	2.047	2.048	2.048	2.045	2.043	2.042	2.051	2.052	2.048
	GlcNAc-**5′**	2.047	2.043	2.043	2.045	2.043	2.042	2.038	2.039	2.038
	GlcNAc-**7**	–	–	–	2.072	2.072	2.073	–	–	2.075
	GlcNAc-**7′**	–	–	–	–	–	–	2.038	2.039	2.038
	Neu5Ac	2.030	2.031[i]	2.031[i]	2.031[i]	2.031[j]	2.031[j]	2.031[j]	2.031[j]	2.031[k]
H-1	Fucα[c]	–	–	4.892	–	–	4.90	–	4.899	4.901
	β	–	–	4.900	–	–	n.d.	–	4.905	4.908
H-5	Fuc	–	–	4.10	–	–	n.d.	–	4.10	n.d.
CH_3	Fucα[c]	–	–	1.211	–	–	1.211	–	1.211	1.211
	β	–	–	1.223	–	–	1.223	–	1.223	1.223

[a] n.d., not determined
[b] Some values are given with only two decimals because of spectral overlap
[c] α and β stand for the α and β anomers of GlcNAc-**1**
[d] Signal stemming from Neu5Ac attached to Gal-**6**
[e] Signal stemming from Neu5Ac attached to Gal-**6′**
[f] Signal stemming from two protons
[g] Signal stemming from three protons
[h] Signal stemming from four protons
[i] Signal stemming from two NAc groups
[j] Signal stemming from three NAc groups
[k] Signal stemming from four NAc groups

Typical examples of human glycoproteins expressed in CHO cells are interferon-γ [9], interferon-β [10, 14], erythropoietin [15, 16], and tissue plasminogen activator (t-PA) [19, 20]. Furthermore the carbohydrate chains of the human-immunodeficiency-virus (HIV) envelope glycoprotein gp120 produced in CHO cells have been investigated [18]. In recombinant human interferon-γ and human interferon-β almost all of the carbohydrate chains are of the diantennary

Table 4. *Comparison of the structure of the asialo-oligosaccharides of recombinant hFSH with corresponding data reported for pituitary hFSH*
For an explanation of the short-hand symbolic notation, see Table 3

Structure	Amount in hFSH		
	recombinant	pituitary	
		[22]	[31]
	%		
	0	0	4
	53	32	20
	0	0	9
	0	14	16
	0	0	4
	0	0	7
	18	23	31
	0	7	18
	8	17	0
	12	5	0

N-acetyllactosamine type, whereas recombinant human erythropoietin carries a heterogeneous mixture of N-linked oligosaccharides yarying in branching between di- and tetra-antennary. t-PA, on the other hand, contains in addition to sialylated *N*-acetyllactosamine type, large amounts of oligomannose type of carbohydrate chains [19, 20]. In some recombinant glycoproteins extra *N*-acetyllactosamine units have been detected. Thus it is obvious that the glycosylation of a recombinant glycoprotein can not easily be predicted, since the protein itself plays an important role in determining its final set of oligosaccharides. So far, from a carbohydrate point of view, the CHO cell line seems to be a proper choice for the expression of therapeutically interesting glycoproteins. In those cases where the natural glycoprotein has also been studied, a close resemblance is found between the recombinant and the natural glycoprotein glycans. Carbohydrate structures with bisecting GlcNAc or the antennary elements GalNAcβ1-4GlcNAc and Galα1-3Galβ1-4 have not been found in normal CHO cells. One of the major open questions is still how different oligosaccharide structures modulate the biological properties of a (recombinant) glycoprotein.

This investigation was supported by the Netherlands Program for Innovation Oriented Carbohydrate Research (IOP-k) with financial aid from the Ministry of Economic Affairs and the Ministry of Agriculture, Nature Management and Fisheries, and by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO). K.H. was supported in part by the Finnish Cultural Foundation and by the Magnus Ehrnrooth Foundation.

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