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Differences in Structure of Carbohydrate Chains of Human and Equine Chorionic Gonadotropins

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In recent years the interest in the carbohydrate chains of glycoproteins has grown dramatically, mainly because of accumulating data concerning the importance of these chains in a wide array of biological processes. In particular, this information has influenced the discussion around genetically engineered proteins prepared in cell systems, with and without a glycosylation machinery.

In general, determination of the primary structure of asparagine(Asn)- and serine(Ser)-/threonine(Thr)-bound carbohydrate chains on intact glycoproteins is not possible. Usually these glycoproteins contain more than one glycosylation site, whereby also heterogeneity of the oligosaccharide structures attached to one amino acid residue is a known phenomenon. Therefore, preparation of partial structures of the protein having a single glycosylation site (glycopeptides), or cleavage of the glycan moieties from the glycoprotein, is a prerequisite. Because of microheterogeneity in the carbohydrate chains, proteolytic digestion of the polypeptide has its limitations, even when specific proteases with a relatively narrow specificity are applied. Moreover, when neighboring carbohydrate chains occur, the intervening peptide backbone may resist proteolysis. However, it should be noticed that the protease approach is essential when, after elucidating the occurring oligosaccharide structures,

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information is needed about their native distribution along the polypeptide chain. For the preparation of free glycan chains, mainly two chemical methods are applied (1), namely, hydrazinolysis (2) for the release of Asn-linked oligosaccharides and alkaline borohydride treatment (3) for the release of Ser/Thr-linked oligosaccharides. For the hydrazinolysis extremely dry conditions are needed. Since such conditions are difficult to obtain, currently also cleavage of (N-acetylgalactosamine-)Ser/Thr linkages (1, 2, 4) is observed. In addition, data are accumulating that hydrazinolysis also causes some further degradation/conversion of N-linked chains, introducing still more heterogeneity. Moreover, N- and O-acyl groups are released, whereby the free amino group is always acetylated in the working-up procedure. Probably, sulphate and phosphate groups are partially split off. On the other hand, alkaline borohydride conditions split (N-acetylglucosamine-)Asn linkages to a certain extent (1, 5). Also, in this case O-acyl substituents are cleaved.

To circumvent problems arising from chemical procedures, alternative cleavage methods for carbohydrate chains based on the use of enzymes are worthwhile to apply, in particular when small amounts of material are available and profiling studies are of interest. The use of endo-β-N-acetylglucosaminidases to release N-linked glycans is limited because these enzymes are rather carbohydrate chain specific (6–8). Nowadays, several peptide-N^4-(N-acetyl-β-glucosaminyl) asparagine amidases (PNGases) (7–14) have become available, which seem to be very promising for the analysis of N-linked carbohydrate chains. The enzymes have been shown to release in high percentage oligomannose-, hybrid- and N-acetyllactosamine di-, tri-/tri'- and tetra-antennary types of carbohydrate chains, which makes them generally applicable. The smallest oligosaccharide chain released from a glycoprotein has found to be the N,N'-diacetylchitobiosyl core unit. Concerning the enzymatic release of O-linked chains, the existence of a suitable glycopeptidase has been claimed (15).

During the last few years, we have investigated intensively the value of PNGase-F from Flavobacterium meningosepticum in carbohydrate analysis studies and developed a convenient strategy for the specific release of N- and O-linked carbohydrate chains from N,O-glycoproteins, based on cleavage of the N-linked chains with PNGase-F, followed by alkaline borohydride treatment of the remaining purified O-glycoprotein (10). In this strategy also advanced chromatographic procedures as FPLC on Mono Q and HPLC on Lichrosorb-NH_2 and Carbo Pac play important roles. For identification purposes mainly ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectroscopy is used in combination with monosaccharide analysis,and, if necessary, methylation analysis, exo/endoglycosidases and mass spectrometry (MS), in particular FAB-MS (FAB, fast-atom-bombardment), are used. This approach has turned out to be successful in our recent structural studies on the carbohydrate chains of glycoproteins of different biological sources, including those obtained via recombinant DNA. Examples from the Carbohydrate Research Group in Utrecht on the structural analysis of the carbohydrate chains of recombinant DNA glycoproteins comprise interferon-g (16) and insulin-like growth factor I (17). In this chapter,

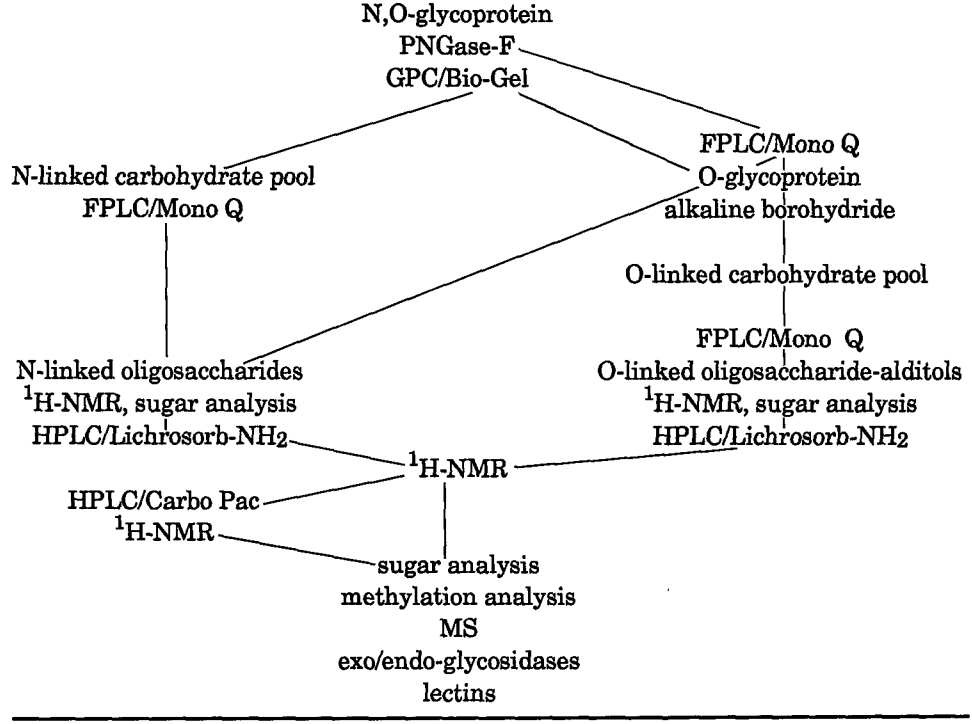
special attention will be paid to differences in the structure of the carbohydrate chains of human and equine chorionic gonadotropins.

ISOLATION PROCEDURES FOR N- AND O-LINKED CARBOHYDRATE CHAINS

In Scheme 1, a survey of our current isolation procedure of N- and O-linked carbohydrate chains is shown. For proteins in their native conformation, the susceptibility to PNGase-F can vary. The rate of deglycosylation is greatly enhanced if the glycoprotein substrate is unfolded prior to enzyme treatment. In our routine approach, the denatured glycoproteins are subjected to enzymatic digestion with free PNGase-F, yielding reducing oligosaccharides. The earlier reported use of Sepharose-4B-immobilized PNGase-F (10, 18) is not advisable in cases where high concentrations of SDS have to be applied, because this leads to a gradual decline of the enzyme activity upon repeated use of the column.

Although several enzymatic deglycosylation conditions have been applied in our laboratory (10, 18, 19), the most suitable approach for a first trial is to use 50 mM Tris/HCl buffer, pH 8.4, containing 50 mM EDTA, 1% (v/v)

Scheme 1. Working-up procedure for the release, fractionation, and analysis of glycoprotein glycans.



2-mercaptoethanol, an equivalent amount (w/w) of SDS relative to glycoprotein, and a 2-fold excess (w/w) of NP-40 relative to SDS. The incubation volume applied in our laboratory (10, 18, 19), the most suitable approach for a first trial is to use 50 mM Tris/HCl buffer, pH 8.4, containing 50 mM EDTA, 1% (v/v) should be as small as possible, usually 10 mg glycoprotein/ml buffer. Denaturation of the glycoprotein is accomplished by treating (e.g., boiling) the sample with 2-mercaptoethanol and SDS for 5 min. After cooling the sample, NP-40 is added and the resulting mixture is kept in a shaker for 5 min at ambient temperature. Then, PNGase-F (1 U/5 mg glycoprotein) is added, followed by a second dose (1 U/5 mg) after 4 h, and the incubation is continued for 16 h at ambient temperature. During the incubation, the mixture is gently rotated in an end-over-end mixer. In case a suspension has been formed after 4 h of PNGase-F incubation, the sample is boiled for 4 min prior to the second addition of enzyme.

The extent of enzymatic liberation of the N-linked carbohydrate chains can be determined by a difference assay on an amino acid analyzer, by monosaccharide analysis of the remaining protein fraction after enzyme treatment, or by SDS-polyacrylamide gel electrophoresis. When the glycoprotein as such turns out to be insensitive to the enzyme, a proteolytic digest can be prepared (14). In this respect, it has to be noted that both the α-amino and the carboxyl group of the Asn residue have to occur in peptide linkage. Although for small amounts of material the desalted mixture can directly be separated according to charge on Mono Q (FPLC), it is advisable to remove first SDS and (O-glyco) protein material effectively via precipitation or via conventional chromatography. In case of SDS also extractigel-D can be applied. In the working-up of larger amounts of glycoprotein material (>20 mg), the N-deglycosylated (O-glyco) protein is removed by gel permeation chromatography (GPC) prior to anion-exchange chromatography on Mono Q.

O-Glycoprotein material is subjected to alkaline borohydride treatment (β-elimination) with 0.1 M NaOH, containing 1 M $NaBH_4$ (25 h at 40°C), affording oligosaccharide-alditols. After working-up (10), the released glycans are fractionated according to charge on Mono Q.

N- and O-Glycosidic carbohydrate chains are subfractionated by HPLC procedures and analyzed by 500-MHz ^{1}H-NMR spectroscopy.

^{1}H-NMR SPECTROSCOPY OF GLYCOPROTEIN DERIVED CARBOHYDRATE CHAINS

High-resolution ^{1}H-NMR spectroscopy has been shown to be an extremely powerful method for the structural characterization of carbohydrate chains derived from glycoproteins. The concepts of NMR structural-reporter groups for the analysis of both N- and O-linked carbohydrate chains, established by the Carbohydrate Research Group in Utrecht, have been reviewed extensively (for example, 20, 21).

URINARY HUMAN CHORIONIC GONADOTROPIN

The N,O-glycoprotein hormone human chorionic gonadotropin (hCG) contains about 30% carbohydrate and consists of two dissimilar, noncovalently linked subunits. These subunits are hCGα (15 kDa) and hCGβ (23 kDa) (22). The monosaccharide constituents Fuc/Gal/Man/GlcNAc/GalNAc/Neu5Ac occur in a molar ratio of 0.3/2.4/3.0/4.7/0.9/3.3 for hCG, 0/2.0/3.0/3.4/0/1.1 for hCGα, and 0.9/5.4/3.0/5.4/2.7/7.2 for hCGβ (10). Several studies have been carried out to characterize the primary structures of the N- and O-linked carbohydrate chains of hCG (23–29). It was found that hCGα contains N-linked carbohydrate chains attached to Asn52 and Asn78, and hCGβ N-linked chains to Asn13 and Asn30 and O-linked chains to Ser121, Ser127, Ser132 and Ser138. Concerning the N-linked chains, in previous studies it has been shown that hCGα bears the mono-antennary structure hCG.N1.1 and the nonfucosylated di-antennary structures hCG.N1.2 + N2.1 (Scheme 2) in approximately equal amounts (27). In the hCGβ -subunit, the di-antennary structures hCG.N1.2 + N2.1 and hCG.N1.3 + N2.2 are present in similar amounts (27). The reported O-linked chains comprise the structures hCG0.01, 0.02, and 0.03 (25, 28, 29). Also, partially/completely desialylated forms of the latter structures (29), and Neu5Acα2→6GalNAc-ol (28) have been proposed to occur.

The general strategy discussed above has been applied to hCG, hCGα and hCGβ (10, 18). The PNGase-F digestion products of each glycoprotein were directly fractionated on Mono Q. In Figure 1, a typical 214 nm UV fractionation pattern of digested hCGβ on a FPLC HR 5/5 Mono Q column is presented, showing one neutral noncarbohydrate fraction hCG.NN and four charged fractions hCG.N1, N2, N3, and N4, of which hCG.N1, N2, and N3 represent mono-, di-, and trisialo-N-glycans, respectively. Fraction hCG.N4 corresponds with the N-deglycosylated protein-SDS complex. HPLC on Lichro-sorb-NH_2 at pH 7.0 afforded a further subfractionation of each carbohydrate fraction (e.g., the fractionation pattern for hCG.N3 of hCGβ in Fig. 2). In Figure 3, the fractionation pattern of the β-elimination products derived from N-deglycosylated hCG on a FPLC HR 5/5 Mono Q column is shown, evidencing the occurrence of three oligosaccharide-alditol fractions, denoted hCG0.01, 0.02, and 0.03. The various fractions were analyzed by 500-MHz ^{1}H-NMR spectroscopy. Concerning the mono- and di-sialo N-oligosaccharides, the earlier reported structures were confirmed. The hCGα-subunit contains the mono-antennary structure hCG.N1.1 and the nonfucosylated di-antennary structures hCG.N1.2 + N2.1 in the molar ratio 1:1, whereby the percentage of hCG.N1.2 can nearly be neglected. The hCGβ-subunit contains the mono-antennary structure hCG.N1.1, the nonflucosylated di-antennary structures hCG.N1.2 + N2.1, and the fucosylated di-antennary structures hCG.N1.3 + N2.2 in the molar ratio 1:2.5:2.5. Compounds hCG.N1.2 and N1.3 are only present in small amounts. In earlier studies, the small amount of hCG.N1.1 was not detected for the hCGβ-subunit; however, recently it has been established for choriocarcinoma hCGβ (30). Additionally, analysis of hCG.N3 (Fig. 1), after subfractionation on

Scheme 2. N- and O-linked carbohydrate chains from normal hCG (10, 18). (Key: Man, D-mannose; Gal, D-galactose; GlcNAc, N-acetyl-D-glucosamine; GalNAc-ol; N-acetyl-D-galactosaminitol; Fuc, L-fucose; Neu5Ac, N-acetyl-D-neuraminic acid; Neu4,5Ac$_2$, N-acetyl-4-O-acetyl-D-neuraminic acid.)

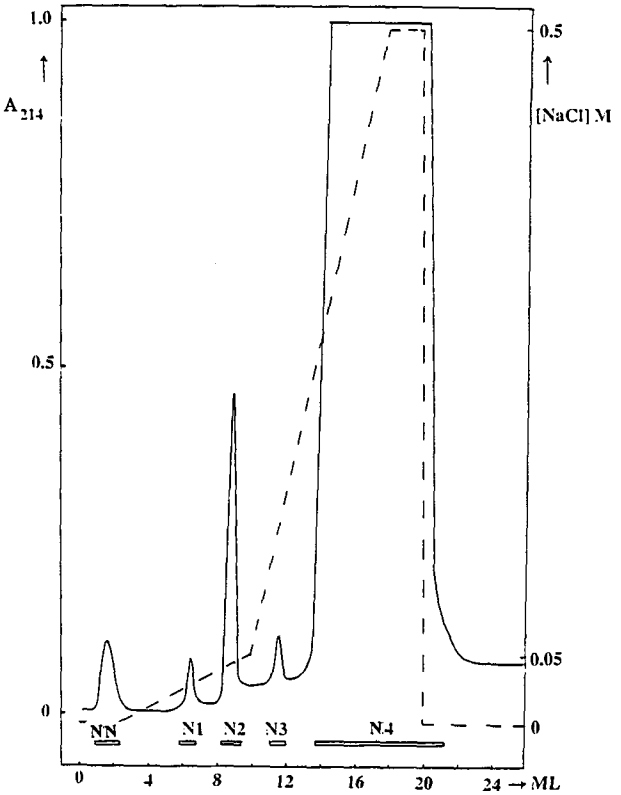


Fig. 1. Fractionation pattern at 214 nm of the PNGase-F digest of hCGβ on a FPLC HR 5/5 Mono Q column. The elution was carried out with a linear concentration gradient (– – – –) from 0–50 mM NaCl in 8 ml H_2O, followed by a steeper gradient from 50–500 mM NaCl in 8 ml H_2O at a flow rate of 60 ml/h.

Lichrosorb-NH_2 (Fig. 2), made clear that hCG also contains small amounts of trisialo tri- and tri'-antennary structures, fucosylated and nonfucosylated. A quantitative survey for the hCGβ-subunit is presented in Scheme 3. Finally there are indications that hCG also contain very small amounts of α2→6-linked Neu5Ac. The O-linked carbohydrate structures hCG0.01, 0.02, and 0.03 in hCGβ were found to occur in the molar ratio 3:1:3.

The finding of tri-antennary N-linked carbohydrate chains in normal hCG is of special interest in view of the data on the claimed significance of these structures for hCGs of patients with specific types of trophoblastic diseases(30–34). Our results strongly suggest that the N-acetylglucosaminyltrans-ferase IV activity is already present in normal trophoblasts and that the occurrence of larger amounts of tri-antennary oligosaccharides associated with invasive mole is merely due to an increase of N-acetylglucosaminyltransferase IV activity. The occurrence of tri'-antennary carbohydrate chains has not been reported so far

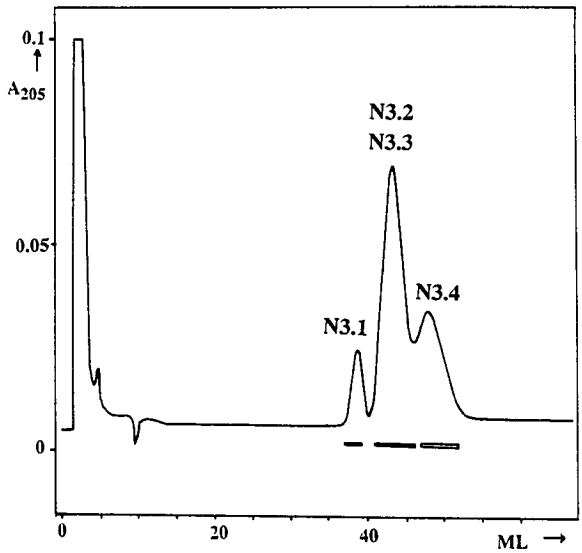


Fig. 2. Fractionation pattern at 205 nm of the Mono Q fraction hCG.N3 of hCGβ on a HPLC Lichrosorb-NH_2 10μ column. The elution was carried out with 30 mM K_2HPO_4-KH_2PO_4, pH 7.0/acetonitrile (35/65, v/v) at a flow rate of 120 ml/h.

for hCG. This study indicates that besides the already mentioned N-acetylglucosaminyltransferase IV activity also N-acetylglucosaminyl-transferase V activity is present in trophoblasts.

SERUM EQUINE CHORIONIC GONADOTROPIN

The N,O-glycoprotein hormone equine chorionic gonadotropin (eCG) contains about 40% carbohydrate and consists of two dissimilar, noncovalently linked subunits: eCGα (17 kDa) and eCGβ (44 kDa) (35–37). Only preliminary information is available about the structure of the carbohydrate chains (38, 39). The

Scheme 3. Molar ratios of N-linked carbohydrate chains from hCGβ.

Mono-antennary	hCG.N1.1	15.0%	15.0%
Di-antennary	hCG.N1.2	2.0%	78.0%
	hCG.N1.3	2.0%	
	hCG.N2.1	37.0%	
	hCG.N2.2	37.0%	
Tri-antennary	hCG.N3.1	0.9%	2.0%
	hCG.N3.2	1.3%	
Tri'-antennary	hCG.N3.3	3.4%	5.0%
	hCG.N3.4	1.4%	

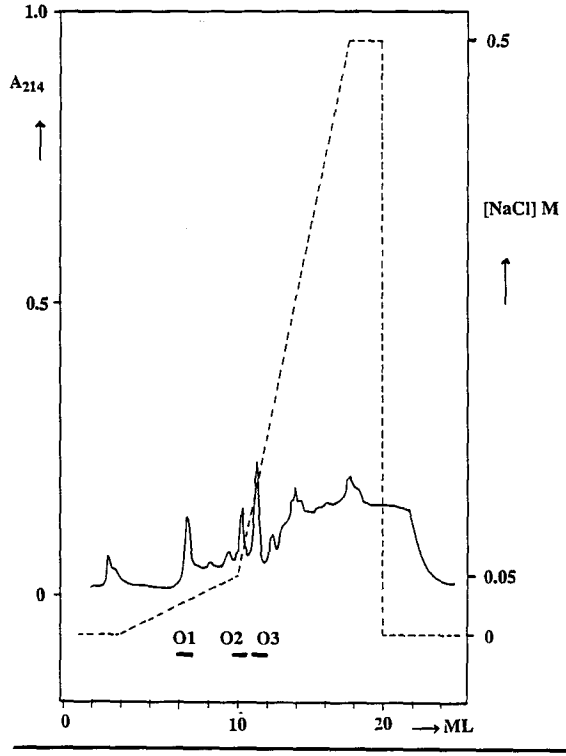


Fig. 3. Fractionation pattern at 214 nm of the β-elimination products released from N-deglycosylated hCG on a FPLC HR 5/5 Mono Q column. For additional details, see Figure 1.

eCGα-subunit has attachment positions for two N-linked chains at Asn56 and Asn82 (36), whereas eCGβ carries one N-linked glycan chain at Asn13 (37, 39) and six O-linked carbohydrate chains. The N-linked chains are characterized as having multi-antennary, bisected, and poly-N-acetyllactosa-mine elements. Also, some of the O-linked chains seem to have large poly-N-acetyllactosamine units.

The monosaccharide analysis of the hormone eCG demonstrated that Fuc/Gal/Man/GlcNAc/GalNAc/Sialic acid occurs in the molar ratio of 0.7/13.5/3.0/13.8/5.2/8.3. In the native glycoprotein, sialic acid comprises a mixture of Neu5Ac and N-acetyl-4-O-acetylneuraminic acid (Neu4,5Ac_2) (40). In the following, structural information will be presented for the major N- and O-linked oligosaccharide chains of eCG, whereby the α- and β-subunits have been studied separately. The subunits were subjected to PNGase-F digestion and the released N-carbohydrate pools were fractionated on Mono Q. Both patterns show the presence of mono- (N1), di- (N2), and tri- (N3) sialo compounds (see Fig. 4, eCGβ), the disialo fraction being the major one. HPLC subfractionation of eCGβ fraction N2 yields three major peaks, designated eCG.N2.1, N2.2,

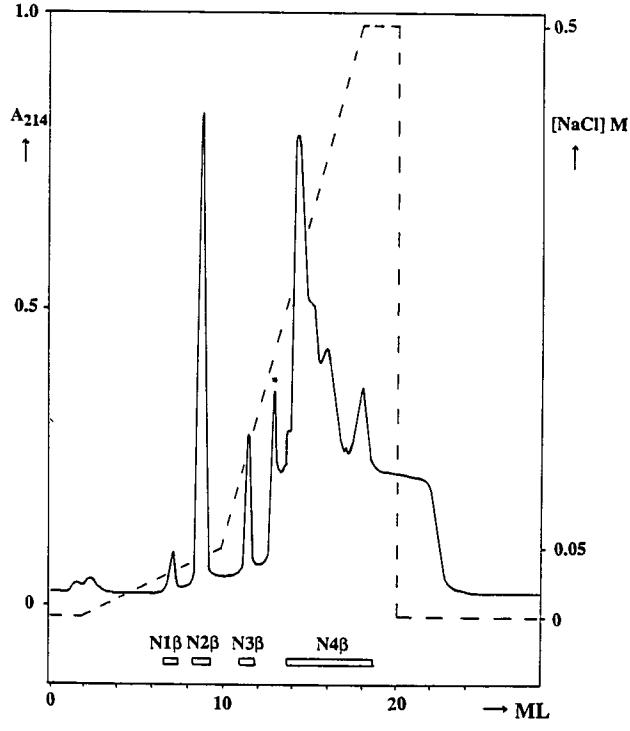


Fig. 4. Fractionation pattern at 214 nm of the PNGase-F digest of eCGβ on a FPLC HR 5/5 Mono Q column. For additional details, see Figure 1.

and N2.3, which correspond with the structures as summarized in Scheme 4. The interpretation of the ^{1}H-NMR spectra was highly facilitated by the data available from a model study carried out on equine fibrinogen (19). In addition, ^{1}H-NMR analysis of the total N2 fraction demonstrated the occurrence of small amounts of α1→6-linked Fuc at the Asn-bound GlcNAc residue. In contrast to hCG, in eCGβ α2→3 sialylation hardly occurs. The use of PNGase-F for the release of N-linked chains, which keeps N- and O-acylation patterns intact, has resulted in the finding of highly interesting carbohydrate chains, terminated with Neu5Ac and/or Neu4,5Ac$_2$. Because of the well-known sialidase-resistance of α-linked Neu4,5Ac$_2$ it may be obvious that the biological lifetime of eCG is strongly influenced, especially when used in animal systems, wherein no natural 4-O-acetylation of sialic acid occurs. The information now available for the α-subunit fraction N2 shows that similar structures occur, but the degree of fucosylation is much higher, the degree of O-acetylation lower, and the degree of α2→3 sialylation much higher.

The O-linked chains of eCGβ have been released by alkaline borohydride treatment. This means that the native O-acetylation pattern (if present) of the O-glycans is lost. The major structures found after b-elimination are identical to those of hCG (hCG.01, 02, 03; Scheme 2).

Scheme 4. Major N-linked carbohydrate chains from normal eCGβ.

Neu5Acα2→6Galβ1→4GlcNAcβ1→2Manα1→6
Manβ1→4GlcNAcβ1→4GlcNAc
Neu5Acα2→6Galβ1→4GlcNAcβ1→2Manα1→3
[eCG.N2.3]

Neu5Acα2→6Galβ1→4GlcNAcβ1→2Manα1→6
Manβ1→4GlcNAcβ1→4GlcNAc
Neu4,5Ac2α2→6Galβ1→4GlcNAcβ1→2Manα1→3
[eCG.N2.2A]

Neu4,5Ac2α2→6Galβ1→4GlcNAcβ1→2Manα1→6
Manβ1→4GlcNAcβ1→4GlcNAc
Neu5Acα2→6Galβ1→4GlcNAcβ1→2Manα1→3
[eCG.N2.2B]

Neu4,5Ac2α2→6Galβ1→4GlcNAcβ1→2Manα1→6
Manβ1→4GlcNAcβ1→4GlcNAc
Neu4,5Ac2α2→6Galβ1→4GlcNAcβ1→2Manα1→3
[eCG.N2.1]

CONCLUDING REMARKS

The adapted approach for the structural analysis of N,O-glycoproteins, making use of PNGase-F and alkaline borohydride treatment, together with FPLC/HPLC separation techniques, has shown to be a highly efficient procedure. By using the enzymatic method of cleavage, attention can also be paid to the native N,O-acylation patterns. In this context it is worthwhile to refer to a recent study on bovine fibrinogen, containing carbohydrate chains terminated with N-acetylneuraminic acid and/or N-glycolylneuraminic acid (41). It is expected that the presented methodology will be of great value for future routine profiling techniques in batch control of rDNA glycoproteins.

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