STRUCTURAL ANALYSIS OF CARBOHYDRATE CHAINS OF NATIVE AND RECOMBINANT-DNA GLYCOPROTEINS

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ABSTRACT. A review is presented on basic analytical concepts in carbohydrate structural analysis focusing on *N*,*O*-glycoprotein derived oligosaccharide chains. Applications are shown from the field of recombinant-DNA glycoproteins. When carbohydrate is taken into account, comparison of different cell lines for the expression of human glycoproteins of potential therapeutic value have made clear that, although not generally suited, the preferred cell type seems to be the Chinese hamster ovary cell.

Introduction

In recent years the academic and industrial interest in the carbohydrate part of glycoproteins has grown dramatically, mainly because of accumulating evidence concerning the relevance of the glycan chains in the physical and biological behaviour of these biopolymers [1-3]. *N*-Linked glycoprotein glycans confer essential physical properties like proper folding and stabilization of the conformation of glycoproteins, protease resistance, and charge and waterbinding capacity. Furthermore, they are relevant in biological recognition processes like protein targeting and cell-cell interactions. It has been well established that *N*-linked glycoprotein glycans, especially the ensemble of non-reducing terminal structural elements, are species specific, tissue specific, cell-type specific, and protein specific. The terminal sequences are often differentially expressed during early development, provide masking functions to prevent rapid clearance from circulation, activate host immunological systems, act as blood-group determinants, and influence bioactivity. The importance of *O*-linked carbohydrate chains has been mainly discussed in terms of charge, waterbinding properties, and stability. This information has influenced the discussion with respect to genetically engineered proteins prepared in heterologous cell types, with or without a glycosylation machinery [4, 5]. In the context of therapeutic administration of recombinant-DNA glycoproteins, an increasing interest of pharmaceutical industries can be observed to consider the glycosylation patterns of the engineered proteins, with regard to applicability, tolerance and patent position.

In this paper a survey will be presented of general structural features of the two major classes of carbohydrate chains of glycoproteins, of basic analytical concepts for the determination of the primary structure of glycoprotein glycans, and of oligosaccharide analysis data of recombinant-DNA glycoproteins.

General Features of Glycoprotein Glycans

Glycoproteins are biopolymers consisting of a polypeptide backbone with covalently attached carbohydrate side chains [1, 6-8]. Two major classes of chains can be distinguished, namely, glycans *N*-linked to the amide nitrogen of L-asparagine (Asn), and glycans *O*-linked to the hydroxyl func-

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tion of L-serine (Ser) or L-threonine (Thr). Glycoproteins may contain more than one glycosylation site, and *N*- as well as *O*-linked chains can occur in one protein. Moreover, heterogeneity of the oligosaccharide structures attached to one amino acid residue is a known phenomenon.

N-GLYCOPROTEINS

In general, the *N*-linked carbohydrate chains [1, 6, 7, 9] share a common pentasaccharide core element $Man_3GlcNAc_2$ (Man, D-mannose; GlcNAc, *N*-acetyl-D-glucosamine) connected via GlcNAc to Asn, and can be extended in several ways. Four types can be distinguished (Fig. 1), namely, (i) the oligomannose type, consisting of Man, GlcNAc, and sometimes *O*-phosphorylated Man. It includes also structures with non-reducing-end terminal D-glucose (Glc) extensions. Biosynthetically, this type represents the biosynthetic precursor of the other types [7]; (ii) the *N*-acetyllactosamine type, being composed of Man, GlcNAc, D-galactose (Gal), and *N*-acetyl/*N*-glycolyl-D-neuraminic acid (NeuAc/NeuGc). In addition, L-fucose (Fuc), *N*-acetyl-D-galactosamine (GalNAc), D-xylose (Xyl), *O*-acetylated sialic acid, *O*-methylated Gal or Man, and *O*-sulfated Gal, Man, GlcNAc and GalNAc may be present. The *N*-acetyllactosamine type can be divided into di- to penta-antennary structures, with or without βMan-linked GlcNAc units (bisecting GlcNAc), poly(*N*-acetyllactosamine) elements, or blood-group determinants; (iii) the hybrid type, showing the characteristics of both the oligomannose and the *N*-acetyllactosamine type; (iv) the xylose type, in which the tetra- or penta-saccharide core is extended with Xyl, and frequently Fuc occurs.

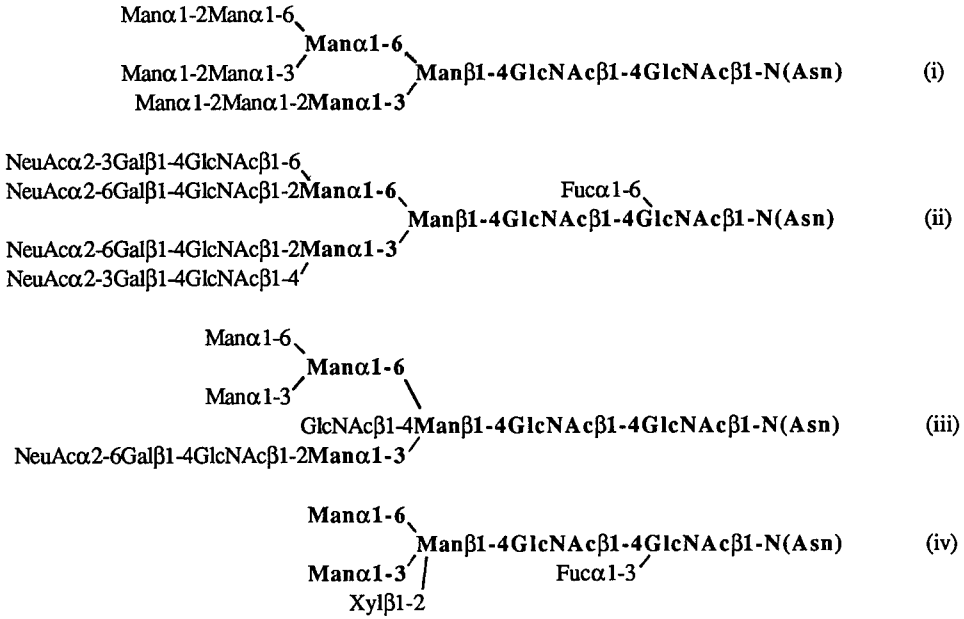


Figure 1. Examples of the four types of *N*-linked carbohydrate chains.

O-GLYCOPROTEINS

For the *O*-linked mucin-type chains [8], in which GalNAc is connected to Ser or Thr, several core structures have been established (Fig. 2). General extensions are built up from *N*-acetyllactosamine (Galβ1-4GlcNAc) and Galβ1-3GlcNAc elements, whereas terminating monosaccharides as Fuc, Gal, GalNAc, and NeuAc are frequently part of blood-group determinants. Moreover, *N*-glycolylated and *O*-acetylated sialic acid, and *O*-sulfated Gal and GlcNAc can be present. *N*- and *O*-linked glycans differ primarily in their core structures, while peripheral sequences exhibit similarities, especially in the larger carbohydrate chains.

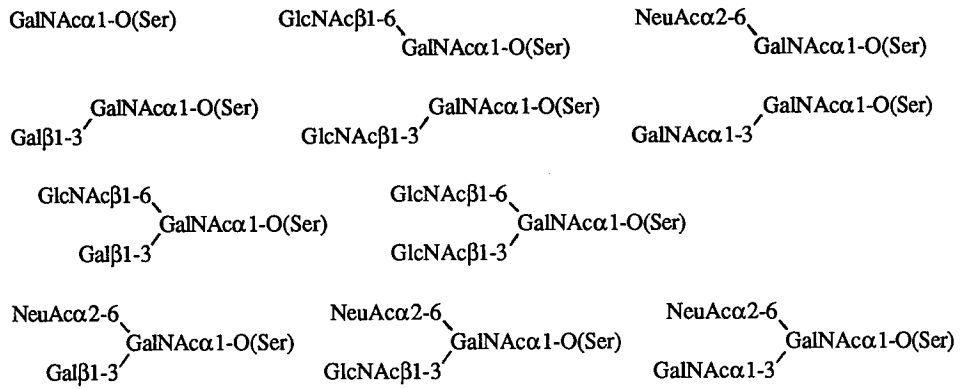


Figure 2. Core structures for *O*-linked carbohydrate chains (GalNAc-Ser/Thr).

In the context of this review also *O*-linked glycans in which the reducing-end monosaccharide Man is condensed with the hydroxyl function in the side chain of Ser or Thr are of importance.

General Aspects of the Primary Structural Analysis of Glycoprotein Glycans

In view of the complexity of glycoprotein glycans, determination of the primary structure of Asn- and Ser/Thr-bound carbohydrate chains on intact glycoproteins is not possible. Therefore, preparation of partial structures of the protein, having a single glycosylation site (glycopeptides), or cleavage of the glycan moieties from the glycoprotein (oligosaccharides) is a prerequisite.

GLYCOPEPTIDES

Treatment of the glycoprotein with protease leads to the formation of a mixture of peptides and glycopeptides. With respect to the formed glycopeptides, frequently complex mixtures are obtained, not only heterogeneous in the carbohydrate part, but also in the peptide part. When neighbouring glycans occur, the intervening peptide backbone may resist proteolysis. It may be evident that proteolytic digestion has its limitations, even when specific proteases with a relatively narrow specificity are applied. However, the protease approach is essential when after the elucidation of the oligosaccharide structures present, information is needed about their native distribution.

N-LINKED OLIGOSACCHARIDES

For the release of *N*-linked oligosaccharides chemical as well as enzymatic approaches are widely applied. The chemical cleavage is mainly carried out by using the hydrazinolysis procedure [10]. For the hydrazine treatment extremely dry conditions are needed. Since such conditions are difficult to obtain, currently also cleavage of GalNAc-Ser/Thr linkages is observed. In addition, data are accumulating that hydrazinolysis also causes some further degradation/conversion at the reducing end of *N*-linked oligosaccharides, introducing a further heterogeneity. Moreover, native *N*- and *O*-acyl groups are released, subsequently the generated free amino group is always acetylated in the working-up procedure. Probably, sulfate and phosphate groups are partially removed. For the enzymatic cleavage two classes of enzymes are applied, namely, endo-β-*N*-acetylglucosaminidases splitting the *N*,*N*'-diacetylchitobiose unit, and peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidases (PNGases) cleaving the amide bond of GlcNAc-Asn [11, 12]. The use of endo-enzymes is limited because they are rather carbohydrate chain specific, e.g. Endo-H (*Streptomyces plicatus*) is applied for the release of oligomannose type structures. PNGases A (almond emulsine) and F (*Flavobacterium meningosepticum*) have shown to be generally applicable for the release of *N*-glycans, taking into account restrictions introduced by the lenght of the peptide backbone. Because of differences in sensitivity to different glycoproteins, cleavage conditions have always to be optimized to ensure completeness of the release.

O-LINKED OLIGOSACCHARIDES

Sofar, the release of *O*-linked carbohydrate chains can only be conveyed chemically using the alkaline borohydride treatment [13]. It has to be noted that sometimes peeling reactions are observed. Under the conditions used, also *O*-acetyl substituents are removed, and Asn-GlcNAc linkages are split to a certain extent.

WORKING-UP AND ANALYSIS

Essential steps after enzymatic and/or chemical degradation are the fractionation and purification to homogeneity of the formed mixtures of glycopeptides, oligosaccharides or oligosaccharide-alditols, using combinations of fractionation methods. These procedures can comprise gel-permeation chromatography, ion-exchange chromatography, paper chromatography, high-voltage paper electrophoresis, FPLC, HPLC, and lectin affinity chromatography. When mixtures are not too complicated, sometimes mixture analysis is possible.

In view of the many parameters which define an oligosaccharide, structural analysis of carbohydrate chains is difficult to automate in a similar way as worked out for peptides/proteins or nucleic acids. In general a combination of analytical methods is applied, whereby mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy usually play a main role. To facilitate the structure determination, in several approaches glycan chains are subjected to further chemical degradations such as periodate oxidation, partial solvolysis or hydrazinolysis/nitrous acid deamination, and to enzymatic degradations with exo- or endo-glycosidases.

Generally, the monosaccharide composition of glycoproteins, glycopeptides, oligosaccharides and oligosaccharide-alditols is established by GLC(-MS) following the alditol acetate approach (hydrolysis, reduction, and acetylation) or the methyl glycoside approach (methanolysis, *N*-acetylation, and trimethylsilylation or trifluoroacetylation) [14].

Direct information about the substitution pattern of the constituting monosaccharide residues in

the intact glycan, and thus about the positions of the glycosidic linkages and the ring sizes, is obtained via the methylation analysis procedure, in which GLC-MS of partially methylated alditol acetates plays a crucial role [14].

For the study of intact derivatized (permethylated) and non-derivatized carbohydrate chains by MS several ionization modes are in use, mainly electron impact, chemical ionization and fast atom bombardment (FAB) [14, 15]. FAB-MS in combination with PNGases has shown to be highly attractive in the study of glycosylation sites in glycopeptides.

During the last decade, high-resolution ^{1}H NMR spectroscopy has been developed into an invaluable technique in the structural analysis of glycoprotein glycans [16, 17]. For both the *N*- and *O*-linked carbohydrate chains a concept has been worked out making use of structural-reporter-group signals directly observable at specific positions in the 1D NMR spectrum. Libraries of structural-reporter groups of both *N*- (glycopeptides, oligosaccharides, oligosaccharide-alditols) and *O*- (oligosaccharide-alditols) linked carbohydrate chains have been built up starting from small structures, and the number of NMR data is still rapidly growing. In view of the higher complexity of recently established structures, 2D NMR techniques as COSY, HOHAHA, NOESY, and ROESY are more and more incorporated into the primary structural analysis of glycoprotein glycans.

A Convenient Strategy for Glycoprotein Glycan Analysis

Recently, 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 ($NaOH/NaBH_4$) treatment of the remaining purified *O*-glycoprotein, has been

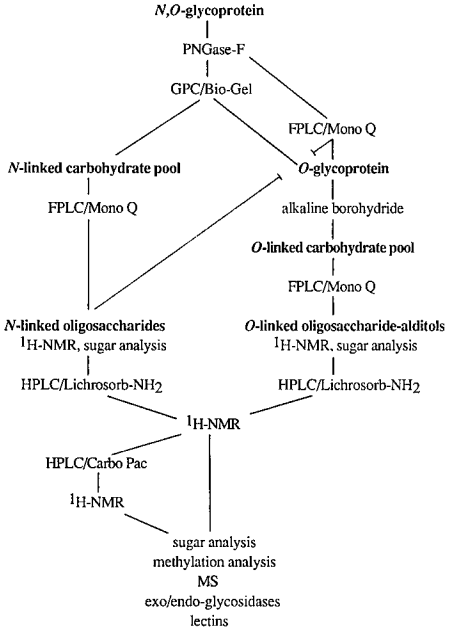


Figure 3. Working-up procedure for the release, fractionation, and analysis of glycoprotein glycans.

developed. A survey of the current set-up is presented in Fig. 3 [18-20]. 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 general, the deglycosylation is carried out between pH 7.2-8.4 in Tris-HCl buffer, in the presence of EDTA, 2-mercaptoethanol and SDS. If necessary, also Nonidet P-40 is added. The extent of enzymatic liberation can be determined in several ways. Routinely, SDS-polyacrylamide gel electrophoresis is applied. As is evident from Fig. 3, in this strategy also advanced chromatographic procedures as FPLC on Mono Q, HPLC on Lichrosorb-NH_2, and MPLC on CarboPac PA1 play important roles. For identification purposes mainly ^{1}H NMR spectroscopy is used in combination with monosaccharide analysis, and, if necessary, methylation analysis, exo/endoglycosidases and MS, in particular FAB-MS, are used. The application of various LC techniques makes the method suitable for microscale oligosaccharide mapping studies. Furthermore, the use of PNGase-F guarantees the presence of native *N*,*O*-acyl groups in the released *N*-linked carbohydrate chains, whereas the conditions are such that desulfation and dephosphorylation will not occur. The establishment of *O*-acyl groups in *O*-linked chains demands for specific, more complicated approaches.

Examples of Recombinant-DNA Glycoproteins

Because of the already mentioned cell-type specificity of glycoprotein glycans, the cellular glycosylation machinery can vary among different cell lines used in recombinant-DNA biotechnology. Furthermore, for a specific cell line holds that also the expressed protein itself influences the final glycosylation pattern. In the following a number of typical examples will be summarized, and for the *N*-linked carbohydrate chains the results will be presented using the symbolic notation system as illustrated in Fig. 4.

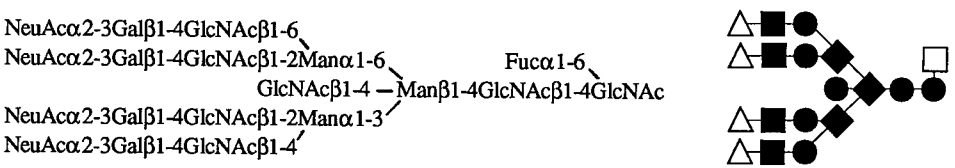


Figure 4. Symbolic notation system for *N*-linked *N*-acetyllactosamine type structures.

CHINESE HAMSTER OVARY CELLS

Analysis of the carbohydrate chains of recombinant human γ-interferon (hIFN-γ) expressed in Chinese hamster ovary (CHO) cells [21] demonstrated the occurrence of a mixture of mono- and disialo diantennary structures partially fucosylated at the Asn-bound GlcNAc residue, with NeuAc in α2-3 linkage only (Fig. 5). Native hIFN-γ contained a similar mixture of oligosaccharides, but with a dominating α2-6 linkage for NeuAc [22].

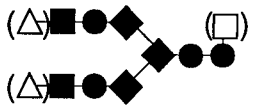
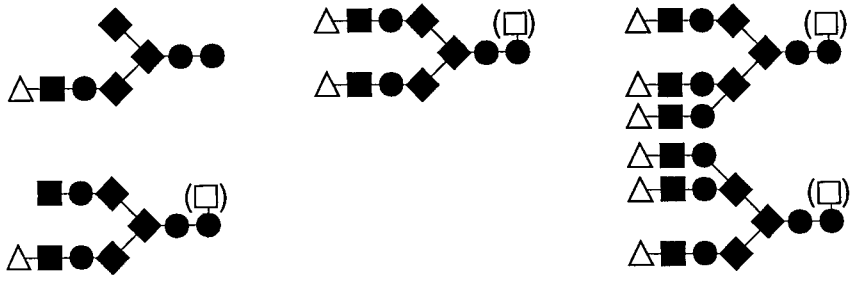


Figure 5. Survey of *N*-linked glycans of recombinant hIFN-γ.

The *N*- and *O*-linked oligosaccharides of human chorionic gonadotropin (hCG) are given in Fig. 6. Comparison of the carbohydrate chains of both urinary hCG [18, 23] and recombinant hCG from CHO cells [24], shows that both products contain the same *N*-linked mono-, di-, tri-, and tri'-antennary glycans, with some variation in the molar amounts. In both cases only α2-3 linked NeuAc is present. For the recombinant product a higher degree of fucosylation is observed. Both native and recombinant hCG contain the *O*-linked tri- and tetrasaccharide. The hexasaccharide is present in low amounts in native hCG, but could not be traced sofar in the recombinant product.



NeuAcα2-3Galβ1-3GalNAc-ol **NeuAcα2-3Galβ1-3(NeuAcα2-6)GalNAc-ol**

NeuAcα2-3Galβ1-3(NeuAcα2-3Galβ1-4GlcNAcβ1-6)GalNAc-ol

Figure 6. Survey of *N*- and *O*-linked glycans of urinary and recombinant hCG.

Structure determination of the intact sialylated *N*-linked carbohydrate chains of recombinant human follitropin (hFSH) expressed in CHO cells, made clear that recombinant hFSH bears α2-3 linked NeuAc only [25] (see Fig. 7), whereas pituitary hFSH carries both α2-3 and α2-6 linked NeuAc [26, 27]. In contrast to native hFSH, recombinant hFSH contains no bisecting GlcNAc residues, whereas the degree of fucosylation of recombinant hFSH is somewhat lower than that of pituitary hFSH [26, 27]. Except for the structures with a bisecting GlcNAc unit, comparison of the desialylated branching pattern of oligosaccharides occurring in recombinant hFSH to that reported in studies on pituitary hFSH, show a closer resemblance to the data in [26] than in [27].

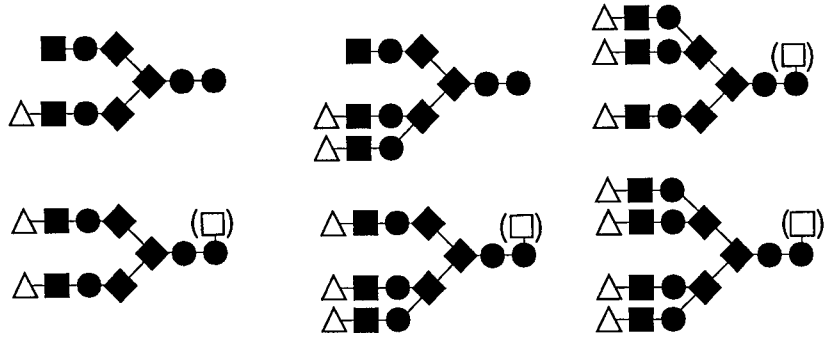


Figure 7. Survey of *N*-linked glycans of recombinant hFSH.

In view of the data presented for recombinant hIFN-γ, hCG, and hFSH, and literature data on the recombinant variants of hIFN-β1 [28], human lutropin [29], human erythropoietin (EPO) [30, 31], human tissue plasminogen activator (tPA) [32, 33], transforming growth factor-β1 precursor [34], and human immunodeficiency virus envelope glycoprotein gp120 [35], it can be concluded that glycoproteins expressed in CHO cells can have (phosphorylated) oligomannose, hybrid, as well as *N*-acetyllactosamine type of structures, depending on the protein under investigation. The latter type comprises mono-, di-, tri-, tri'-, and tetra-antennary glycans, and poly(*N*-acetyllactosamine) sequences can occur. The terminal NeuAc residue is exclusively linked in α2-3 position to Galβ1-4GlcNAc, indicating the absence of β-galactoside α2-6-sialyltransferase activity. It has been shown that the terminal sialylation on *N*-linked glycans in CHO cells can be altered by expression of the latter transferase [36]. Structures with bisecting GlcNAc, the antennary element GalNAcβ1-4GlcNAc, and the antennary element Galα1-3Galβ1-4 are not produced. Small sialylated *O*-linked oligosaccharides (see Fig. 6) are synthesized in a normal way, including α2-3 as well as α2-6 linked NeuAc, as found for the recombinant variants of hCG, hEPO [30], human interleukin-2 [37], and human granulocyte-colony-stimulating factor [38]. This means that the CHO cell has normal *N*-acetylgalactosamine α2-6-sialyltransferase activity.

OTHER CELL LINES

The use of C127 mouse epithelial cells can result in glycans with the NeuAcα2-6Galβ1-4 sequence (see recombinant hIFN-β1 [28] and rtPA [33]) and the immunologically unwanted Galα1-3Gal epitope [4, 28]. Human lung adenocarcinoma PC8 cells used for the expression of hIFN-β1, have the capacity to synthesize NeuAcα2-6Galβ1-4 and Galα1-3Galβ1-4 as terminal sequences, as well as bisecting GlcNAc [28]. Malignant cell lines known to secrete definite glycoproteins as htPA, hIFN-γ and hCG introduce different glycan chains when compared with the native ones.

Saccharomyces cerevisiae cells are capable of introducing in mammalian glycoproteins both *N*- and *O*-linked oligomannose chains, which are typical for yeast glycoproteins. Examples have been reported for recombinant human interleukin-3 [39] with respect to *N*-linked oligomannose chains (polymannans), and for recombinant human insulin-like growth factor I (IGF-I) with respect to *O*-linked manno-oligosaccharides (Manα1-2Man, major; Manα1-3Manα1-2Manα1-2Man, minor) [40]. Native IGF-I does not contain serine-linked manno-oligosacharides. Mammalian glycoproteins produced in plant cell lines may be associated with immunological effects, because of the possible presence of highly immunogenic oligosaccharide chains of the xylose type [4].

FINAL REMARKS

The presented carbohydrate data for a number of native and rDNA glycoproteins give insight into the state of the art. The known importance of the carbohydrate chains in several therapeutically interesting glycoproteins makes it essential that the protein prepared biotechnologically bears carbohydrate chains, that are compatible with the acceptor system. Although in several cases the function of the carbohydrate chains has not been established yet, this does not mean that they have no function. Also in these cases attention should be paid to obtain the right glycosylation pattern. In principle, from a carbohydrate point of view, the preferred cell type seems to be the CHO cell. The observed deviations in carbohydrate structures between native- and CHO-cell-produced materials have to be studied in the near future, in order to find out if corrections are necessary. Finally, it has been shown that batch-to-batch reproducibility is a problem that needs attention. To this end the availability of reliable and fast batch-control procedures are a prerequisite.

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