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GLYCOANALYSIS PROTOCOLS

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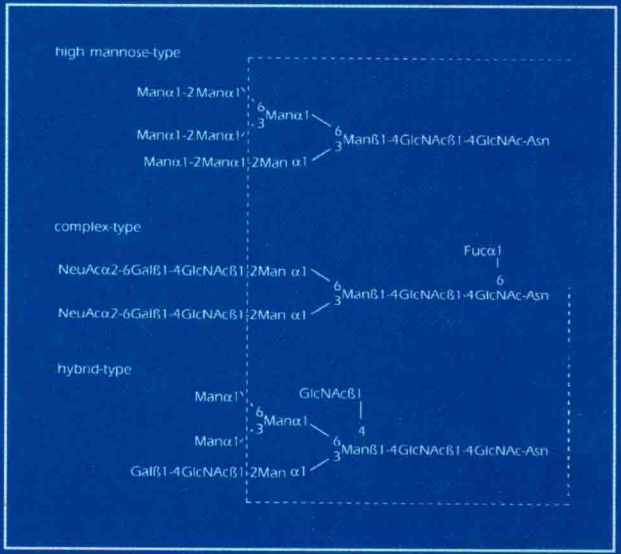
Edited by

Elizabeth F. Hounsell



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METHODS IN MOLECULAR BIOLOGY™

Glycoanalysis Protocols

SECOND EDITION

Edited by

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Preface

Protein glycosylation is now acknowledged as a major posttranslational modification with significant effects on protein folding, conformation distribution, stability, and activity. The added oligosaccharide chains are large and diverse and have specific recognition motifs important in many aspects of cell interactions and regulation. As such, there is a growing need to communicate the analytical methods of the specialist carbohydrate chemist, biochemist, and physicochemist to protein experts and the pharmaceutical industry. Other areas that come under the influence of the glycosciences are DNA interactions with ubiquitous saccharide-containing antibiotics and antitumor drugs; inhibitors of viral infection; bacterial, mycobacterial, and parasite antigens; glycolipids; glycophosphatidylinositol protein membrane anchors; and (glyco)protein–proteoglycan interactions. Compared to the first edition of this book, *Glycoprotein Analysis in Biomedicine,* less emphasis is given to biomedical aspects, but these chapters are still pertinent today. The significant differences in the content relate to advances in analysis relevant to biotechnology; for example, the production of recombinant glycoproteins and other therapeutics. It must also not be forgotten that the methods here described in *Glycoanalysis Protocols* are relevant to exploiting the commercial potential of carbohydrates in fields related to agriculture, food, and the domestic and chemical industries. The emphasis of the book remains in bringing the glycosciences into mainstream biochemistry.

The analytical methods covered in *Glycoanalysis Protocols* are the result of experts translating their life's works into easy-to-follow recipes. I am very grateful to the authors for adhering so well to the format, as well as for their time and effort. The important areas covered start with intracellular glycosylation, *O*-GlcNAc linked to Ser/Thr (of nuclear and cytoplasmic glycoproteins, Chapter 2), with the growing understanding of its relevance to cell regulation and aging processes. Next, proteins destined for transport via intracellular trafficking pathways have oligosaccharide chains linked to Asn (*N*-linked) that have multiple roles in correct folding expression and tissue distribution of the resulting glycoproteins. Several chapters deal with the struc-

tures of these chains and their analyses by high resolution chromatographic methods (HPLC, HPAEC, and HPTLC), which remain the workhorses for purification and characterization (Chapters 1, 5–8). Now the techniques have come of age and they are available in a rational and user-friendly format. There are also powerful shortcuts to detailed analysis, e.g., the use of lectins, gels, electrophoresis and enzymes, that are now alternative techniques for profiling (Chapters 3 and 4). The mucins and proteoglycans, which are mostly secreted, have predominantly other*O*-glycosylation patterns (Chapters 9–11). Glycoproteins at the cell surface and in the serum can have both *N*- and *O*-glycosylation. This diversity shows altered regulation in cancer, normal cells, and differentiation (Chapter 1). Glycoproteins have distinct oligosaccharide–protein core regions that follow certain rules of nature and can be classified by structure. The more distal glycosylation can be highly diverse and shared by both classes of glycoprotein core and glycolipids (Chapters 12 and 13). These sequences have various functions in specific oligosaccharide recognition. The role of another glycan structure linked to both protein and lipid, the glycan of GPI membrane anchors (Chapter 14), is so far not known, but its presence on diverse proteins and highly regulated biosynthesis is being explored, not least because it is an important feature of prions. The effects of glycosylation on glycoprotein conformation and function are being studied by methods such as NMR (Chapter 1), and fluorescent energy transfer mechanisms (Chapter 15) are particularly important in the glycosciences. *Glycoanalysis Protocols* covers all the areas noted above, encompassing glycobiology, glycoimmunology, glycopathology, and glycotherapeutics.

Elizabeth F. Hounsell

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Contents

1

Characterization of Protein Glycosylation

Elizabeth F. Hounsell

1. Introduction

The majority of proteins are posttranslationally modified, the most significant change being glycosylation, i.e., the attachment of one or more oligosaccharide chains. Because of their long history, but also relative neglect until recently, the terminology for saccharides is diverse. Also a major problem in the glycosciences is that many different methods are necessary for oligosaccharide analysis, and this does not at first seem straightforward. I hope this chapter will demystify the structures and the analysis of glycoconjugates (glycoproteins, GPI-anchored proteins, glycolipids, and proteoglycans). The terminology is in fact easy to follow. It has simple beginnings: from glucose comes the generic term glycose, which is used in words such as glycosidic ring, glycoprotein, and so forth; from sucrose (a disaccharide of glucose and fructose) comes the word saccharide and, hence, oligosaccharide chain. In addition to glucose (Glc), there are seven other possible orientations of hydroxyl groups in hexoses of the formula $C_6H_{12}O_6$ (from whence comes the term carbohydrate) in the series allose (All), altrose (Alt), Glc, mannose (Man), gulose (Gul), idose (Ido), galactose (Gal), talose (Tal). However, in addition to hydroxyl groups on the ring carbons, there are also acetamido groups (**Fig. 1**), e.g., at C-2 in *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc), and at C-5 in *N*-acetylneuraminic acid (NeuAc). There may also be present sulfate and phosphate esters. Other commonly occurring monosaccharides are the 6-deoxyhexose fucose (Fuc), the pentose xylose (Xyl), and the C-6 carboxyl uronic acids, glucuronic acid (GlcA), iduronic acid (IdoA), and galacturonic acid (GalA). The monosaccharides are linked together between the hydroxyl groups numbered around the glycosidic ring as shown in **Fig. 1** and with α or β (anomeric) configuration, depending on the ring geometry ($^4C_1$ or $^1C_4$

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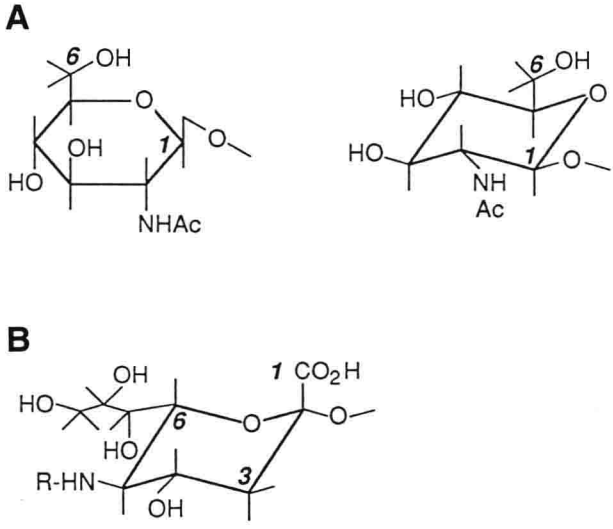

Fig. 1. **(A)** There are two alternative forms for portraying monosaccharides as shown here for β-D-*N*-acetylglucosamine (GlcNAc). Different monosaccharides vary by the number and orientation of their functional groups, i.e., OH, NHAc, and the like. Compared to GlcNAc, GalNAc has the C-4 hydroxyl group above the plane of the ring. In addition to linkage to each other via one or more (giving branching) hydroxyl group, monosaccharides and oligosaccharides are also linked to protein and lipid. The main linkages are GalNAcα to the hydroxyl group of Ser or Thr (*O*-linked, mucin type), Xylα to the hydroxyl group of Ser (proteoglycan type), GlcNAcβ to the acetamido nitrogen of Asn (*N*-linked) or to the hydroxyl group of Ser (*see* Chapter 2), and Glcβ to ceramide (glycolipids). **(B)** Sialic acids are a family of monosaccharides where R = CH_3–CO–(*N*-acetylneuraminic acid) or CH_2OH–CO–(*N*-glycolylneuraminic acid); the hydroxyl groups can be substituted with various acyl substituents, and those at C-8 and C-9 by additional sialic acid residues.

for hexopyranoside rings) and linkage above or below the plane of the ring (**Fig. 1**).

The analysis of glycoconjugates follows approximately the progression in this and subsequent chapters of the book, i.e., detection of the presence of glycosylation is achieved by colorimetric analysis or the use of glycosylation-specific enzymes, the glycosyltransferases (e.g., to add radioactively labeled sugars; Chapter 2) and the glycosidases; exoglycosidases to remove monosaccharides sequentially from the end distal to the conjugate linkage (Chapters 4, 14, and 16) or endoglycosidases to cleave within the oligosaccharide chain or at the conjugate–oligosaccharide linkage (Chapters 4–6 and 8). Oligosaccharides or monosaccharides released by enzymatic or chemical methods are sepa-

rated by high-performance liquid chromatography (HPLC), high pH anion-exchange chromatography (HPAEC) or gas–liquid chromatography (GC). These methods are complemented by lectin affinity chromatography (Chapter 3), methylation analysis (Chapter 6), and gel electrophoresis (Chapter 8). Discussed in the present chapter are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy for the detection and characterization of oligosaccharides, glycopeptides, glycoproteins, glycolipids, and so forth.

The molecules classically called glycoproteins comprise mammalian serum and cell membrane glycoproteins of an approximate molecular weight range of 20–200 kDa, having oligosaccharide chains linked to the hydroxyl group of Ser/Thr or the nitrogen of Asn, i.e., *O*- and *N*-linked, respectively, making up from 10–60% by weight. Mucins are traditionally defined as high molecular weight glycoproteins of 10^6 kDa upward having >60% oligosaccharide, which is mainly *O*-linked via GalNAc-containing oligosaccharide cores. Proteoglycans (*see* Chapter 9) also have a high carbohydrate/protein ratio. Their glycosaminoglycan chains are disaccharide repeating units, which, in most cases (i.e., heparin, heparan sulfate, chondroitin sulfate, and dermatan sulfate), have alternating uronic acid and amino sugar residues, and a large degree of sulfation (the exceptions are unsulfated hyaluronic acid and keratan sulfate which is a sulfated Gal-GlcNAc repeat). The distinction between these categories of glycoconjugates is becoming increasingly blurred; they can now be seen as a spectrum of the varying glycosylation patterns occurring on high and low molecular weight, secreted, and cell-surface glycoproteins. As examples of this, classical mucin and proteoglycan sequences can occur on cell-membrane-attached proteins of relatively low molecular weight, and glycoproteins and proteoglycans are found in forms attached to the membrane by lipid-linked glycosylphosphatidylinositol (GPI) anchors. GPI-anchored glycoproteins were first found in trypanosomes, but are now known as a common membrane anchor in mammalian cells as described in Chapter 14. The present book largely restricts its analysis to mammalian glycoconjugates, but the methods are equally applicable to the glycoconjugates of microorganisms, some of which were discussed in the first edition ***(1)***.

1.1. How Do You Know You Have a Glycoprotein?

Table 1 shows the different types of methods that can be used for the identification of glycosylation. Oxidation with periodate is a classical method for oligosaccharide detection, e.g., the periodate-Schiff reagent (PAS) and Smith degradation, more recently adopted as part of a microsequencing strategy for structural analysis ***(2)*** and as commercial kits for glycoprotein detection in conjunction with lectins or antibodies ***(3)***. The phenol-sulfuric acid assay can be carried out at microscale in a multiwell titer plate and read by an ELISA plate

Table 1
Examples of Analysis Techniques that Detect Carbohydrates

Biological
Release of monosaccharides by exoglycosidases
Release of oligosaccharides by endoglycosidases
Metabolic labeling with ^{35}S or ^{3}H monosaccharides
Addition of monosaccharides by glycosyl transferases
Binding to lectins or anticarbohydrate antibodies
Physicochemical
Characteristic molecular weight by MS
Characteristic chromatographic profile
Characteristic signals in a NMR spectrum
Chemical
Oxidation with sodium metaperiodate, which cleaves specifically between two adjacent hydroxyl groups (as in PAS)
Phenol-sulfuric acid charring of mono- or oligosaccharides having a hydroxyl group at C-2
Reduction of mono- or oligosaccharides having a free reducing end after release from protein or hydrolysis of glycosidic bonds
Addition of a chemical label by reductive amination.
Nitrous acid cleavage of oligosaccharides at non-*N*-acetylated hexosamine residues
Detection of polysulfated oligosaccharides by dimethylmethylene blue staining

reader to detect down to 500 ng of monosaccharides having a C-2 hydroxyl group (e.g., Gal, Man, Glc). Reduction methods (concomitant with oligosaccharide release for *O*-linked chains) can be used to detect oligosaccharides specifically by introduction of a radioactive label and purification on a phenylboronic acid (PBA) column ***(4)***. High-sensitivity analysis can also be achieved by the addition of a fluorescent label by a related technique called reductive amination (*see* Chapters 6–8 and 15). This relies on the fact that a reduced chain can be oxidized by periodate to give a reactive aldehyde for linkage to an amine-containing compound, or that free reducing sugars exist for part of the time in the open-chain aldehyde form. Derivatives chosen include amino-lipids for TLC overlay assays and TLC-MS analysis ***(4,5)***, UV-absorbing groups that also give sensitive MS detection ***(5,6)***, and sulfated aromatic amines for electrophoretic separation ***(7,8)***. These can be detected down to the picomole level.

1.2. What Type of Oligosaccharide Sequences Are Present?

Essential in any analysis strategy is an initial screen for the types of oligosaccharide chain present, e.g., *O*- or *N*-linked chains, and also for the pres-

ence of any labile chemical linkages that might be destroyed by the subsequent analysis techniques used. High-sensitivity analysis by HPLC or HPAEC ***(9,10)*** can be achieved (*see* Chapters 5–7). However, the analysis method described in the present chapter using trimethylsilyl ethers of methyl glycosides is the most widely applicable, being able in one run to identify pentoses (e.g., ribose, xylose, arabinose), deoxyhexoses (e.g., fucose, rhamnose), the hexoses, hexosamines, uronic acids, and sialic acids by gas–liquid chromatography (GC). GC of chiral derivatives ***(11)*** can be additionally used to determine the D and L configurations of monosaccharides. The technique of GC-MS analysis of partially methylated alditol acetates (*see* Chapter 6) is also a very useful technique that can identify the hydroxyl group, through which each monosaccharide is linked, thus establishing their presence in a chain and giving vital structural information. This type of analysis can now be conveniently performed on bench-top GC-MS equipment at the picomole-to-nanomole level.

Obtaining a high-field MS analysis of released oligosaccharide chains in their native form, e.g., by fast-atom bombardment (FAB), liquid secondary ion (LSI), matrix-assisted laser desorption (MALDI), or electrospray (ES) MS, is very useful for discovering any labile groups that would be removed by derivatization. Permethylated oligosaccharides, available as part of the route to partially methylated alditol acetates, can also be analyzed by these techniques to give additional sequence information. Alternative derivatives are peracetylated oligosaccharides, which are readily formed and extracted to give very clean samples for MS analysis ***(12)***. High-sensitivity detection of high molecular weight molecules down to a few picomoles of material can be achieved by the largest mass spectrometers, particularly of oligosaccharides derivatized at the reducing end as discussed above. MS methods for analysis of oligosaccharides, glycopeptides, and glycoproteins are discussed below and in Chapters 2, 13, and 14.

1.3. What Is the Best Strategy for Release of Oligosaccharide Chains?

When initial clues regarding oligosaccharide types have been gained, confirmatory evidence can be obtained by specific chemical or enzymatic release. Both types of methods have been researched extensively over the past decade to achieve a high degree of perfection in minimizing any nonspecific side reactions while maximizing oligosaccharide yield. To obtain typical *N*- and *O*-linked oligosaccharides, chemical release can be best achieved by hydrazinolysis or alkali treatment. Hydrazinolytic cleavage of *N*-linked chains ***(13)*** has been perfected over the last two decades (Chapters 4 and 6–8). At lower temperatures, hydrazinolysis may also be useful for the release of *O*-linked chains (Chapters 6 and 7), but this step is more universally achieved

by mild alkali treatment (β-elimination), e.g., 0.05*M* sodium hydroxide at 50°C for 16 h, which in the presence of 0. 5–1*M* $NaBH_4$ yields intact oligosaccharide alditols (Chapter 11). Alkaline borohydride reduction conditions result in some peptide breakdown, whereas hydrazinolysis for release cleaves the majority of peptide bonds. Enzymatic release leaves the peptide intact and obviates possible chemical breakdown of oligosaccharides. However, occasionally it may be necessary first to protease-digest to achieve complete oligosaccharide release, and the enzymes may not cleave all possible structures (e.g., when working with plants, algae, fungi, insects, viruses, trypanosomes, mycobacteria, and bacteria). The extent of deglycosylation can be readily judged by the detection methods discussed in **Table 1**.

For proteoglycans and GPI anchors, an additional chemical method of release is the use of nitrous acid (Chapters 9 and 14) to cleave at non-*N*-acetylated glucosamine residues. Proteoglycan oligosaccharide sequences are also obtained enzymatically by heparinases and heparatinases (for heparin and heparan sulfate), chondroitinases (for chondrotin and dermatan sulfates), or endo-β galactosidases (for keratan sulfate).

1.4. What Does My Glycoprotein Look Like?

The oligosaccharide chains of glycoproteins are fashioned by a series of enzymes acting in specific sequence in different subcellular compartments. The end product is dependent on a number of factors, including the initial protein message and its processing, availability of enzymes, substrate levels, and so on—factors that can vary between different cell types, different species, and different times in the cell cycle. It is therefore important to address the question of glycoprotein structure to specific glycosylation sites and have profiling methods capable of detecting minor changes in structure, which may be important in function and antigenicity. The following route is discussed in this and subsequent chapters:

1. Initial characterization of type and amount of each monosaccharide and linkage (HPAEC, GC, GC-MS; (picomole-nanomole).
2. Release of *O*-linked chains by alkali, alkaline-borohydride for hydrazinolysis and analysis by labeling and HPLC, PBA, or HPAEC.
3. Protease digestion (Chapter 6) and analysis of the complete digest by high-field MS (peptide in 20-pmol digest identified).
4. HPLC peptide mapping (Chapter 6) and microassay for glycopeptides (*see* **Table 1**) followed by peptide *N*-terminal amino acid sequence analysis of identified glycopeptides.
5. Endoglycosidase release of *N*-linked oligosaccharides and chromatographic profiling as discussed in Chapters 4–7 followed by MS analysis of the separated oligosaccharides and peptides.