Module 5
Metabolic Engineering/Glycan Labeling: Release & Detection

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Background:
There are a number of approaches that can be utilized to analyze the glycan component of glycoproteins. Glycans can be analyzed while still attached to proteins, or released from proteins prior to analysis. In many cases the choice of technique depends on the question you wish to answer, the tools and equipment available to you, and to the purity and amount of your glycoprotein of interest.

Analysis of glycans attached to proteins

Glycans can be detected on proteins utilizing mass spectrometry, which will be covered in a lecture later in this course. The other approaches we typically use to detect glycans on proteins can include: 1) labeling proteins with glycosyltransferases, in which either a radiolabeled nucleotide sugar is used or an unnatural sugar which enables "detection" using Click-chemistry; 2) Metabolic labeling, again with radiolabeled or unnatural sugars; 3) Periodate oxidation, followed by colorimetric or fluorescent detection; 4) Separation of purified proteins - followed by release of glycans using PNGase F, beta-elimination, or acid hydrolysis; and 5) Detection of glycans using lectins or antibodies. **Lectins** are carbohydrate-binding proteins that are highly specific for sugar moieties. While there are a number of proteins with lectin domains in mammalian systems, we typically use either bacterial or plant lectins for detection of glycoproteins. Lectins can be found in arrays, but also directly conjugated to Biotin or horse-radish peroxidase. The latter can be used in western blotting applications.

Analysis of released glycoproteins

Enzymatic Release of Glycans:

There are several chemical and enzymatic methods that can be used to release glycans:

1) **β-elimination:** This releases O-linked sugars, but the carbohydrates must be reduced to prevent a peeling reaction. The resulting sugar alditols are not compatible with many labeling techniques, but if acetylated are volatile and easily detected by GC-MS.
    a. β-elimination will cleave the peptide backbone after proline residues.
    b. This method can be performed on blot (PVDF), and can be used as a specificity control during western/lectin blotting.

2) **Hydrazinolysis:** Hydrazine hydrolysis is an effective method for the complete release of unreduced O- and N-linked oligosaccharides. It is not as popular as other techniques as it's toxic and can affect the integrity of the peptide/protein backbone. Notably, under controlled conditions it can be used to selectively release N-linked glycans.
3) **Enzyme hydrolysis:** This technique is more applicable to N-linked glycan analysis, as PNGase F releases most N-linked glycans. O-Glycanase typically only releases βGal-1-3βGalNAc-O-Ser/Thr. A combination of exoglycosidases can be used to release carbohydrates from proteins and probe glycan structure (Figure 47.2, below).

Notably: Glycans can be released from proteins on PVDF sequentially providing an enormous amount of information. Proteins are first treated with PNGase F and then β-elimination. While the latter step results in some damage to the protein backbone, unknown proteins can still be identified by Trypsin-digestion and mass spectrometry.

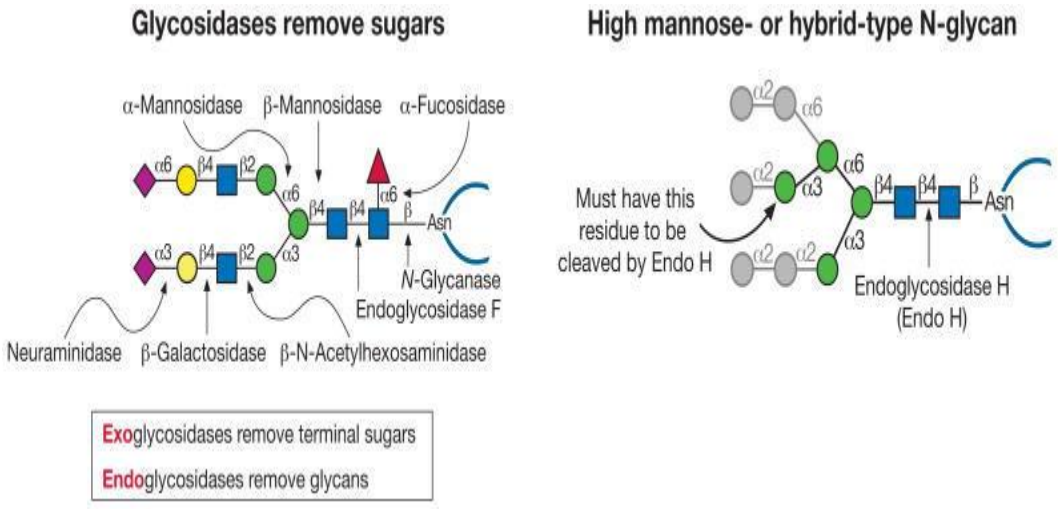


FIGURE 47.2 (Essentials in Glycobiology): A biantennary N-glycan is shown with exoglycosidases that can be used to remove each monosaccharide sequentially. Exoglycosidases act only on terminal sugars. Also shown are endoglycosidases that remove the intact N-glycan. N-Glycanase cleaves the GlcNAc-Asn bond, releasing the N-glycan and converting asparagine into aspartate. Endoglycosidase F cleaves between the core *N*-acetylglucosamine residues and therefore leaves an *N*-acetylglucosamine attached to the protein. (*Right*) Endoglycosidase H (Endo H) cleaves between the core *N*-acetylglucosamine residues of oligomannose or hybrid N-glycans that have at least four mannose residues as shown. Endo H does not act on complex N-glycans.

Labeling of Glycans:

There are numerous methods for labeling glycans, which enhance their detection:

1) **Radiolabels**: Radiolabels are introduced into carbohydrates using either glycosyltransferases or metabolic labeling. The latter is useful, although one has to be careful that the label is not being incorporated into amino acids/proteins. Glycosyltransferases, in combination with tritiated sugar nucleotides, are a powerful way to selectively introduce radiolabels into glycoconjugates. Both of these techniques can also be used to introduce sugars containing chemically reactive groups, for example azido-sugars, into glycoconjugates. Azido-sugars can be selectively reacted with phosphine or alkyne reagents to introduce a fluorescent dye or an affinity probe such as biotin.
2) **Reductive amination:** This technique can be used to label reducing sugars, as such it can be used to label oligosaccharides released by enzymes such as PNGase F or Hydrazinolysis, or monosaccharides released by acid hydrolysis. These techniques are not compatible with the alditols released by β-elimination. Labels used include fluorescent tags such as 2-aminopyridine (2-AP), 2-aminobenzamide (2-AB), 2,6-diaminopyridine (DAP), or biotinylated 2,6-diaminopyridine (BAP).

Desalting Glycans:

Desalting released glycans is essential for the success of many techniques of carbohydrate analysis such as mass spectrometry, capillary electrophoresis, anion exchange chromatography, enzyme degradation, and chemical derivatization. There are only a few robust techniques:

1) **Ion Exchange chromatography:** Cation exchange chromatography is commonly used to desalt released monosaccharides and oligosaccharides. However, there is a risk of losing positively charged sugars.
2) **Size-Exclusion Chromatography:** Released sugars can have a molecular weight as small as ~200 daltons. Thus, even when using a highly cross-linked packing it is difficult to obtain sufficient separation of small oligosaccharides in a timely manner.
3) **Graphitized carbon (solid phase extraction cartridge):** can be used for the purification of oligosaccharides (or their derivatives) from solutions containing one or more of the following contaminants: salts (including salts of hydroxide, acetate, phosphate), monosaccharides, detergents (sodium dodecyl sulfate and Triton X-100), protein (including enzymes) and reagents for the release of oligosaccharides from glycoconjugates (such as hydrazine and sodium borohydride). Notably, appropriate step elutions with acetonitrile are needed to separate the

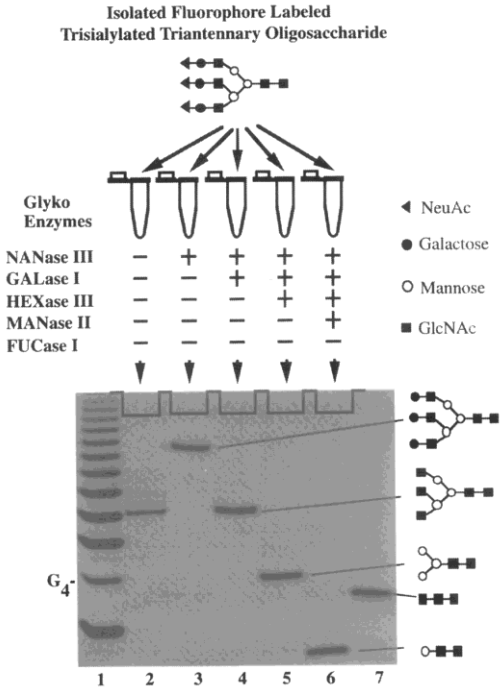
oligosaccharides (elute on low acetonitrile) from hydrophobic compounds such as detergent. The one drawback of this technique is that monosaccharides do not appear to be retained on graphitized carbon.

Separation of Oligosaccharides:

There are numerous methods that can be used to separate oligosaccharides. Each technique has different capacities, sensitivities, degrees of separation, advantages and disadvantages. In many cases your choice of method will depend on the available equipment, your goals, and the downstream application.

1. **Thin Layer Chromatography (TLC):** Polyhydroxyl compounds such as sugars can be complexed to metals, resulting in a charged complex that can be separated by TLC. In this method, SILICA TLC plates are exchanged with metal containing solutions such as 5% Cu(AcO) – H_2O. Plates are washed in water and dried before the application of the carbohydrates. Separation proceeds in water. It is essential that carbohydrates be desalted. Detection is via autoradiography /fluorography or colorimetric assays. This technique can separate anomers and isomers.
2. **High-voltage Borate Paper electrophoresis:** An excellent method for separating small (<2500 Daltons) molecules that are hydrophilic and typically uncharged, such as neutral sugars. Sugars are complexed with borate (1.9% Borax, pH9.4) or molybdate (2%, pH3-5), giving them a charge. Run times are between 30-60minutes, and 10-20 samples can be run at once. Samples can be detected by chemical methods (destructive) or by autoradiography/fluorography.
3. **Size exclusion/Gel Permeation Chromatography**: Carbohydrates can be separated by size using gel permeation chromatography. Sugars are detected by the incorporation of a radiolabel (such as ^{3}H-Gal using galactosyltransferase) or a fluorescent tag. For O-linked sugars it is possible to introduce this tag during reductive β-elimination, by using tritiated sodium borohydride. It is possible to reduce N-linked glycans with tritiated sodium borohydride after release by PNGaseF. The carbohydrates can also be detected using colorimetric assays, though these are generally less sensitive. Typically Biogel P-2, P-4 and P-6 (<400 mesh resin) are used. Biogel P-4 is best for the separation of 3-24 units of glucose. Typically, the column is run at 55^{o}C to reduce interactions between the carbohydrates and the resin. Columns are calibrated and retention time expressed in units of glucose.

4. **<u>Graphitized Carbon:</u>** Graphitized carbon undergoes both hydrophobic and polar interactions with oligosaccharides, and can separate oligosaccharides of the same size but different shapes. This technique is compatible with online mass spectrometry. Detection is dependent on mass spectrometry or labeling with fluorescent compounds.



5. **<u>Reversed Phase:</u>** An example of the columns and conditions: C_{18} octadecylsilyl reversed-phase column (3μm, 4 × 250 mm) with a mobile of 0.1% acetic acid in water (A) and 0.1% acetic acid in 10:90 acetonitrile:water (B). Requires labeling with *2-aminobenzamide* for detection and this can introduce some variability.

6. **<u>HILIC</u>** <u>(hydrophilic interaction liquid chromatography, Right)</u>**<u>:</u>** The chromatography of carbohydrates conducted on HILIC stationary phases has been shown to involve a partitioning mechanism in contrast to the traditional adsorption chromatography on normal phase materials. Typical equilibration conditions used for oligosaccharides and glycopeptides are 10–25% water in acetonitrile with a low concentration of acid or salt (mostly below 100 mM). Common columns include: TSK Gel-Amide 80 and ZIC-HILIC-SPE. This technique is compatible with mass spectrometry. Detection on typical HPLCs requires derivatization of the carbohydrate.

7. **<u>FACE</u>** <u>(fluorophore-assisted carbohydrate electrophoresis, right):</u> Addition of a tag (by reductive amination) to the reducing end provides a means of detection and a charge to the sugar allowing separation in a polyacrylamide gel. Separation may vary if sugars are sulfated, acetylated of sialylated as this will change the size:charge ratio.

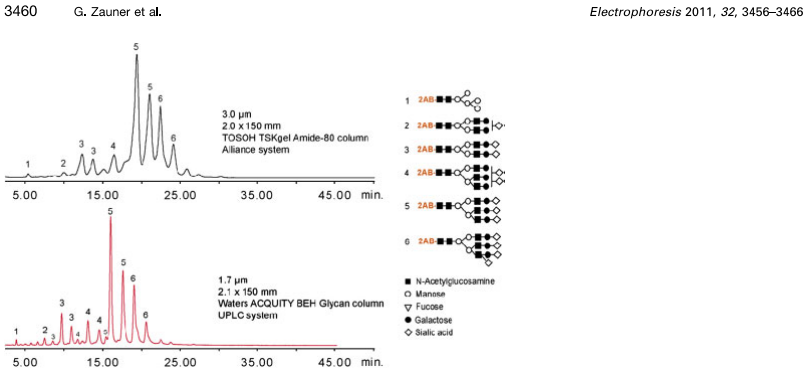
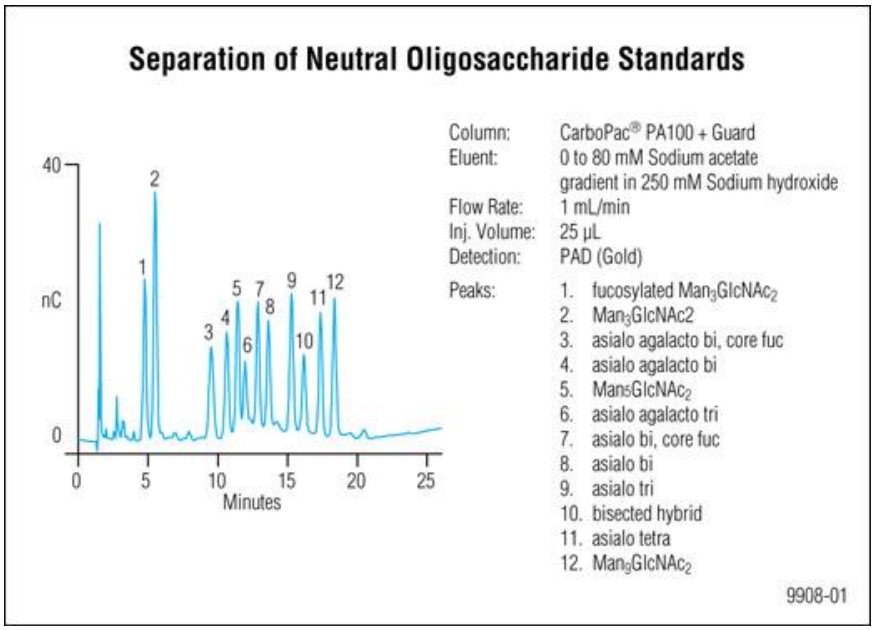


Figure 1. Glycan separation comparison of 3.0 and 1.7 μm sorbent HPLC columns. Ten picomoles of fetuin 2-AB-labeled glycans was separated using 3.0 μm TSK gel Amide-80 column in Alliance 2695 (top) and 1.7 μm BEH Glycan column in UPLC system (bottom). Sialylated biantennary and triantennary glycans including positional isomers were baseline resolved using 1.7 μm column in UPLC system in 45-min gradient time. The UPLC separation was done in gradient 65–55% B in 45 min at 0.5 mL/min using 2.1 mm × 150 mm, and the HPLC separation was done in 65–55% B in 50 min at 0.45 mL/min using 2.0 mm × 150 mm. The column temperature was at 40°C on both runs. Peak assignment was performed on the basis of HILIC-ESI-MS experiments. Notably, to our knowledge the Man5 glycan reported here has hitherto not been reported for fetuin and might represent a contamination. The peaks labeled with same numbers indicate the presence of isomers. Taken from [19] with permission.

8. **GC/MS:** Derivatized sugars are separated by GC, and fragmented in the mass spectrometer. Thus, structures are identified by retention time and fragmentation patterns. GC analysis is limited to derivatized sugars of low molecular weight (up to 12 monosaccharide units). Sugars are typically peracetylated or methylated to make them volatile, however other derivatization methods exist. Some information, such as acetylation patterns, may be lost.



9. **Capillary Electrophoresis (CE):** CE allows the separation and quantification of sugars by retention time. This technique can be coupled to MS allowing for more rigorous characterization of oligosaccharides. For detection of the sugars, typically reductive amination is used to incorporate a label.
10. **ION Exchange:** Charged oligosaccharides can be separated by ion-exchange chromatography on DEAE Sepharose. Detection requires a radiolabel of the incorporation of a fluorescent tag.
11. **HPAEC (DIONEX):** Oligosaccharides released from proteins and desalted can be separated by High Performance Anion Exchange Chromatography (HPAEC) on a PA100 column equilibrated in 250mM Sodium Hydroxide and a gradient against Sodium Acetate. One advantage of this method is that is does not require labeling of the oligosaccharides. Sensitivity will be in the 10-100pmol range. It is possible to separate charged oligosaccharides, however, higher concentrations of sodium acetate are required to elute these sugars. One disadvantage of this technique is subsequent analysis, as the sugars have to be desalted. Sample will often require additional analysis to determine the actual size and composition. This technique requires a specialize HPLC which has no internal metal (degraded by the hydroxide).