PhyTip® Normal Phase Columns for Enrichment of Glycans

Introduction

Among the co-translational and post-translational modifications, the cellular process of enzymatic addition of carbohydrates to proteins, called glycosylation, has been a subject of much attention. The resulting glycoprotein is modified with branched or unbranched chains of carbohydrates that form oligosaccharides and polysaccharides, which are simply called glycans. The addition of glycans can occur at asparagine residues, called N-linked, or at the hydroxal oxygens of serine and theronine residues, called O-linked. Glycoproteins are an important subclass of proteins and are involved in a diverse set of cellular functions including signal transduction, inflammation, cell-cell interactions, and development.

There is a high recent interest in the biopharmaceutical industry towards glycoproteins. Glycoproteins are among the high potential targets because of their crucial function in cellular processes. In addition to the interest in this class of proteins as drug targets, researchers are conscious of the fact that glycans are implicated in activity and efficacy of therapeutic peptides and proteins. Any biotherapeutic drug development program takes glycosylation into serious consideration as a means of producing a better product with higher production reproducibility.

As genomics and proteomics have matured, researchers are looking increasingly towards the study of all glycan molecules produced by an organism. These include glycoproteins, glycolipids, lipopolysaccharides, peptidoglycans and proteoglycans. The field of glycomics acknowledges that the complexity of life cannot be explained by genes and protein interactions alone, but that interactions of proteins and nucleic acids take place in the presence of complex sugar moieties and in some cases glycans modulate protein activity. In addition, irregular glycosylation patterns are potentially associated with disease making glycans a promising class of potential biomarkers.

Identification and quantification of glycans is a major challenge, which requires advances in analytical tools and sample preparation. Glycans are made up of a number of different sugars linked together and form branches (Fig.1). The possibility of having a large number of possible isoforms makes glycans especially difficult to identify and requires high performance analytical tools such as capillary electrophoresis or HPLC for analysis. In this technical note, we present recent published works that have utilized fluorescent labeling and cleanup methods for improved detection and quantification of complex carbohydrates[1,2]. The data are courtesy of the laboratory of Dr. András Guttman at the University of Innsbruck, Austria.

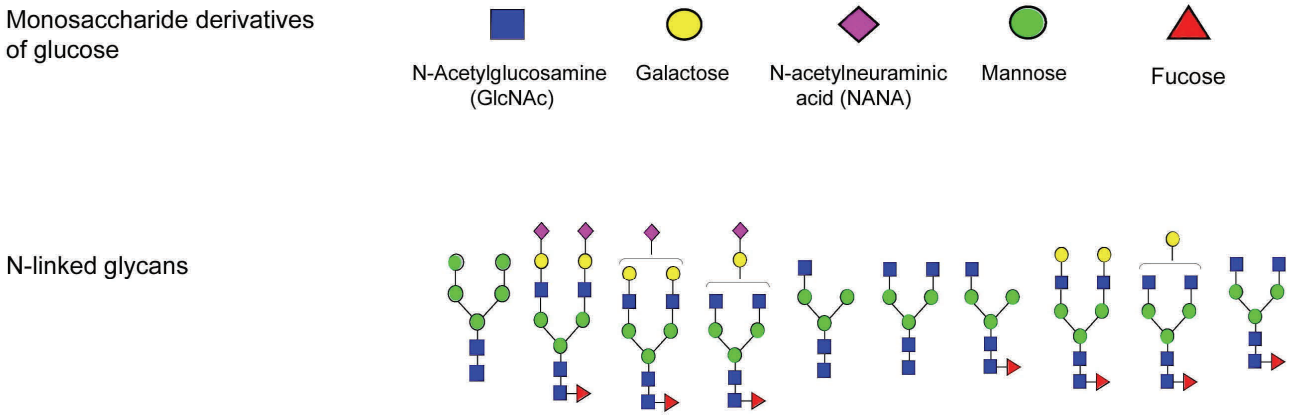


Figure 1: Representation of different carbohydrates and schematic depictions of some common N-linked glycans.

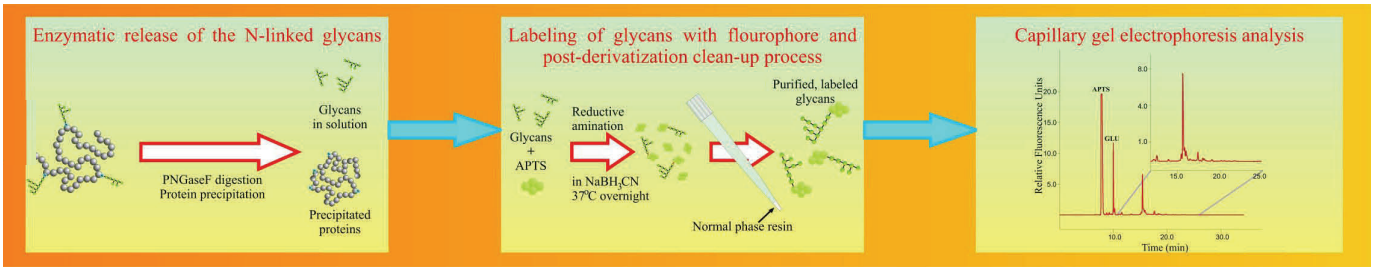


Figure 2: Flow chart of sample preparation for capillary gel electrophoresis profiling of N-glycosylation of standard glycoprotein samples. For the maltooligosaccharide ladder standard the first two steps were not applied, the sample preparation is started with the labeling process.

Materials and Methods

Fluorophore labeling

All reagents are supplied by Sigma Aldrich unless specified. Maltooligosaccharide ladder (Grain Processing Corporation) was labeled with 1 μL 0.2 M 8-amino-1,3,6-pyrenetrisulfonic acid (APTS) in 15% acetic acid and 1 μL 1 M $NaBH_3CN$ in tetrahydrofuran and incubated at 55º for 2 h. The reaction was stopped by addition of 100 μL water. Unreacted, excess APTS was removed by three different methods. First, filter plates (Millipore) were loaded with 100 μL Sephadex G10 gel filtration media and conditioned. 200 μL APTS reaction mixture was loaded and centrifuged at 2500 rpm for 15 minutes. Second, the PhyNexus ME purification system was used to condition PhyTip columns containing 160 μL of Sephadex G10 gel filtration media with 50% acetonitrile (ACN). 200 μL APTS reaction mixture was loaded onto the column and 50% ACN was used to chase and release the labeled maltooligosaccharide. Third, the PhyNexus ME purification system was used to condition PhyTip columns containing 10 μL of normal phase resin with 95% ACN. 200 μL of the APTS reaction mixture was captured with 8 cycles of back-and-forth flow followed by 24 wash cycles in 95% ACN. The labeled maltooligosaccharides were eluted with 350 μL 20% ACN.

Capillary electrophoresis analysis

Maltooligosaccharides were diluted 10-fold in pure water and analyzed on a CarbCE multicapillary electrophoresis system equipped with a blue light-emitting diode excitation source of wavelength 460-470 nm (eGene) using a 12-channel gel cartridge (GCK-CARB). Samples were injected electrokinetically (2 kV, 20 s). Emission was detected by a photodiode using a 520 nm long-pass filter. Separations using 10 cm or 30 cm capillary lengths (20 μm i.d.). The capillaries were filled with CARB Separation Solution (eGene). Separations were carried out at 2-8 kV.

Glycoprotein analysis

For analysis of the glycan composition of glycoproteins, the glycans are first cleaved off of the protein. The proteins analyzed were IgG, RNase B, fetal bovine fetuin (FET) asialofetuin, and α-1-acid-glycoprotein (AGP). 0.1 mg protein in 10 μL water was denatured by addition of Endoglycosidase buffer (New England Biolabs) and incubation at 100º for 10 minutes was followed by digestion with 2 units of PNGaseF at 37º for 2 hours. The deglycosylated protein was removed by cold ethanol precipitation and centrifugation at 11,000 g for 10 minutes. The glycan-containing supernatants were dried by a centrifugal vacuum evaporator. The released glycans were labeled and analyzed in the same manner as the maltooligosaccharide ladder.

Boronic acid lectin affinity chromatography (BLAC)

200 μL PhyTip columns containing 5 μL of BLAC resin was manufactured by mixing 2.5 μL agarose-immobilized concanavalin A and 2.5 μL boronic acid agarose beads. Using the PhyNexus ME instrument, the PhyTip columns were washed with 100 μL water, 100 μL 20mM potassium phosphate pH 2.85 and again with 100 μL water. The PhyTip columns were conditioned in 100 μL binding buffer (50 mM taurine, 1 mM $CaCl_2$, 1mM $MnCl_2$, 20mM $MgCl_2$, 0.05% NaN_3, pH 8.7. 100 μL human serum was diluted in 100 μL binding buffer and the glycoproteins were captured using the PhyTip columns followed by wash in binding buffer and elution with 100 μL 100 mM HCl. The glycoproteins were dried and prepared for PNGaseF digestions and APTS labeling and clean-up as described.

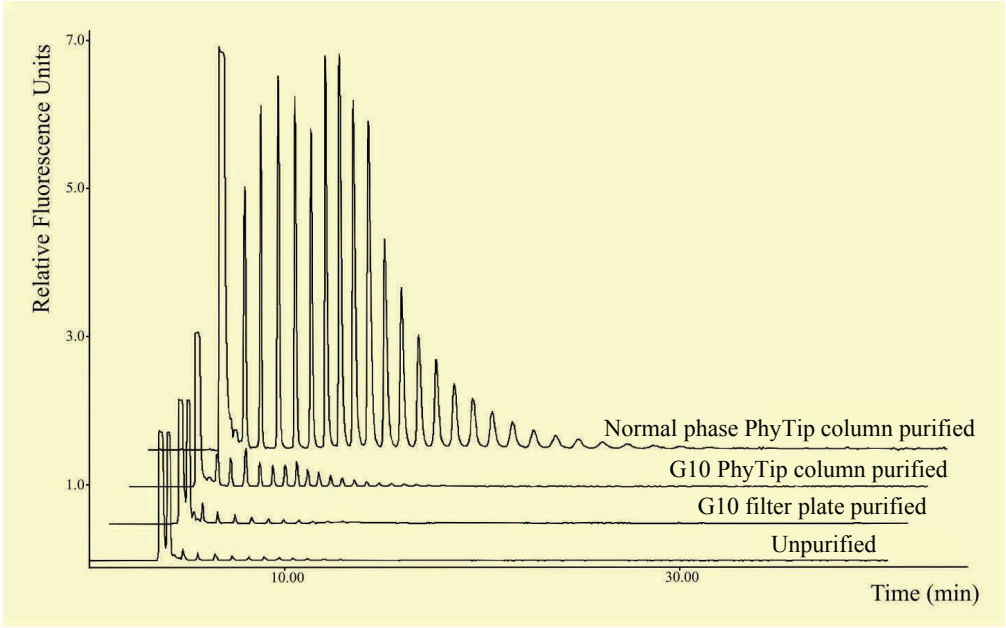


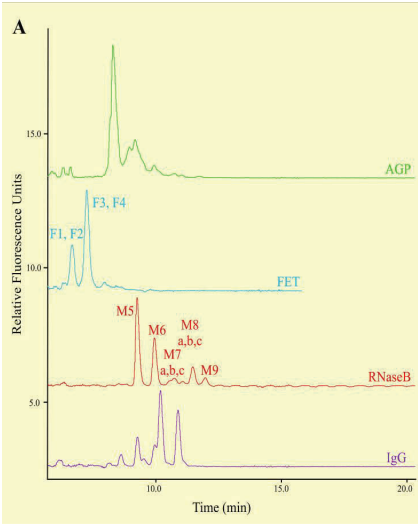
Figure 3: Comparison of methods for removal of unreacted derivatization reagent, APTS, from maltooligosaccharide ladder labeling reactions. CE electropherogram traces of unpurified, Sephadex G10 filter plate purified, Sephadex G10 PhyTip column purified, and normal phase resin PhyTip column purified reaction mixtures are compared for efficiency of dye removal and intensity of recovered maltooligosaccharides peaks. Adapted with permission from Olajos et. al.[1] 2008 American Chemical Society.

Results

Removal of unreacted derivatization reagent

Glycans are composed of monosaccharides. The similarity of the monosaccharide subunits and the ability to form branches makes identification of glycans very difficult (Fig. 1). Advanced analytical methods such as HPLC and capillary electrophoresis (CE) are required to perform the high-resolution separation. However, glycans lack chromophores making the detection of glycans a challenge. Researchers have addressed this problem by labeling glycans with fluorophore tags such as APTS, which provide fluorescence and charge that are quite amenable to a CE separation (Fig. 2).

For efficient derivatization of glycans with tags, an excess of APTS is required. Unreacted APTS must be removed because co-migration of free dye with low molecular weight glycans interferes with quantification and identification. A number of strategies were tested for the ability to remove the excess dye and enrich for labeled glycans. A maltooligosaccharide ladder was labeled with APTS and split into several aliquots. The first reaction aliquot was not purified and run on a CE. The second was purified by a spin plate containing Sephadex G10 gel filtration media. The third and fourth aliquots were purified by the PhyNexus ME semi-automated purification system using pipette tip columns containing different separation media. The PhyTip columns contained either Sephadex G10 or a normal phase resin (Fig. 3). The unpurified sample and filter plate purification method shows a large dye front at that co-migrates with the low molecular weight oligosaccharides. The PhyTip columns containing G10 resin were able to remove unreacted APTS, but the enrichment of labeled



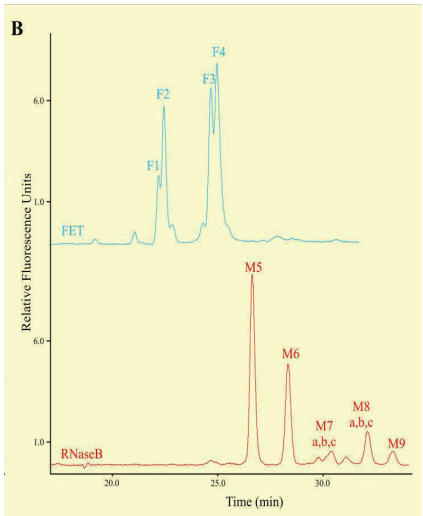


Figure 4: CE electropherograms of normal phase PhyTip column purification of APTS-labeled glycans from IgG, RNaseB, FET and AGP using an effective capillary length of 10 cm (A) or 30 cm (B). Adapted with permission from Olajos et. al.[1] 2008 American Chemical Society.

Results cont'd

oligosaccharides was only slightly better than the filter plate method. The PhyTip columns containing normal phase resin achieved both effective purification and high enrichment.

Glycan composition analysis of glycoproteins

A number of glycoproteins, immunoglobulin G (IgG), ribonuclease B (RNaseB), fetal bovine fetuin (FET) and α-1-acid-glycoprotein (AGP) were analyzed for their respective glycan content. The glycans were first cleaved off of the protein using peptide-N-glycosidase F (PNGaseF) followed by labeling with APTS and purification by PhyTip columns containing normal phase resin. The purified glycans were separated by CE of either 10 or 30 cm capillary lengths (Fig. 4).

Boronic acid lectin affinity chromatography (BLAC)

Glycoproteins present in human serum represent an attractive target for diagnostics discovery. Disease has frequently been correlated with changes in glycosylation of proteins including extra branching, truncation and excessive amounts of certain monosaccharides. Researchers at the University of Innsbruck in Austria have developed a method for glycoproteomics work by performing an affinity enrichment of glycoproteins from serum using a combination of a lectin, concanavalin A and a pseudo-lectin boronic acid[3,4]. This method, termed BLAC was used to purify glycoproteins from serum samples of a healthy pool of individuals and from stage-four prostate cancer patients. The glycoproteins were subject to PGNaseF digestion, APTS labeling, sample clean-up and CE analysis to compare the glycan patterns. Researchers discovered a number of peaks that are specific to prostate cancer samples and set the groundwork for further study[4].

Discussion

Advances in high-resolution separation methods such as HPLC and CE are a good solution for identification, quantification and profiling of glycans. Though these analytical tools are excellent separation methods. Detection of glycans poses an analytical challenge because of the lack of a natural chromophores/charged groups. Labeling strategies circumvent this problem by providing a simple and efficient tagging procedure. Recent advances in sample cleanup using PhyTip columns containing normal phase resin allow users to reproducibly process samples in a high-throughput fashion. Furthermore, this purification strategy can be advanced for diagnostics discovery using the BLAC method. These new tools are essential for the advance of the glycomics and glycoproteomics fields.

References

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