Glycan Analysis of Murine IgG2a by Enzymatic Digestion with PNGase F and Trypsin, Followed by Mass Spectrometric Analysis

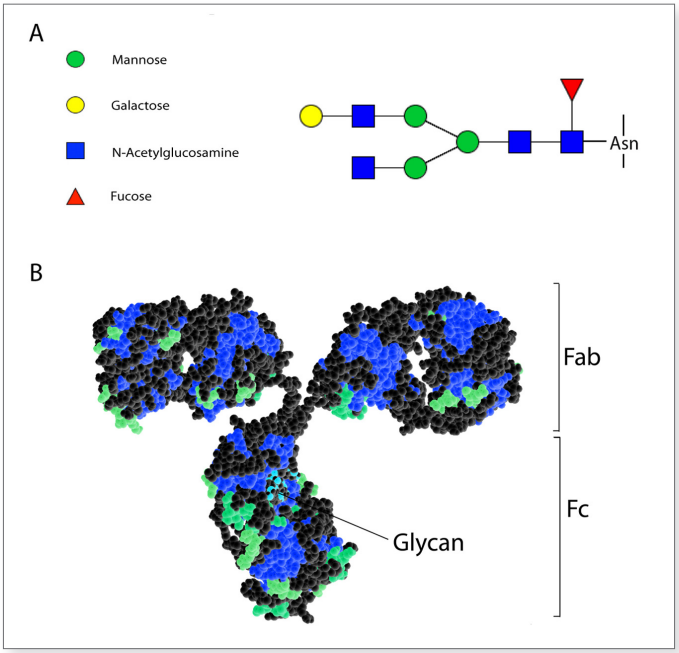
Alicia Bielik, Colleen McClung and Cristen Ruse

Proteomics, the systematic study of proteins in biological systems, has expanded the knowledge of protein expression, modification, interaction and function. However, in eukaryotic cells, the majority of proteins are post-translationally modified (1). A common post-translational modification, essential for cell viability, is the attachment of glycans. Glycosylation defines the adhesive properties of glycoconjugates and it is largely through glycan-protein interactions that cell-cell and cell-pathogen contact occurs.

Glycosylation is also important in the production of therapeutic proteins as it can significantly affect the potency of a biological drug. Producing a homogenously glycosylated protein is very difficult and often impractical. For this reason, development and manufacturing processes are highly monitored to minimize glycosylation variability. Therefore, the ability to determine the presence or absence of a glycan at a particular site is critical to the production of therapeutic proteins. A combination of enzymes (PNGase F and Trypsin) in tandem with mass spectrometry can be used to release the *N*-glycans present on glycoproteins and determine the sites of *N*-glycosylation on the protein.

Immunoglobulin Gs (IgGs) are antibody molecules that are composed of four peptide chains – two heavy chains and two light chains (Figure 1). There are four IgG subclasses (IgG1, 2a, 2b, and 3) in mice. The heavy chains are known to be glycosylated. The glycans present on the heavy chains of IgG are attached to asparagine residues (*N*-linked). *N*-linked glycans are produced by the secretory pathway (ER and Golgi). Synthesis of *N*-glycans begins with the transfer of a common oligosaccharide to a nascent polypeptide in the ER. Some *N*-glycans remain unmodifided ("high mannose"), while others are initially trimmed and then extended as the glycoprotein matures in the Golgi ("complex").

Figure 1: Structure and glycosylation of a murine lgG



(A) Schematic representation of the fully substituted IgG heavy-chain glycan.
(B) Structural model of murine IgG. In the IgG, beta-sheets are colored blue, loops are colored black and the helices are colored green. The brackets indicate the antigen-binding Fab portion and the Fc effector portion of IgG. The arrow indicates the two conserved glycans (aqua) attached to Asn-180 of the heavy chains. The model was generated using JMOL 12.2.23 from a model deposited in the Protein Data Bank by L. Harris (University of California, Riverside).

Materials

- PNGase F (Glycerol-free) (NEB #P0705)
- GlycoBuffer 2 (10X, supplied with enzyme)
- Glycoprotein Denaturing Buffer (10X, supplied with enzyme)
- Trypsin-ultra™, Mass Spectrometry Grade (NEB #P8101)
- Trypsin Buffer (2X, supplied with enzyme)
- Trypsin-digested BSA MS Standard (CAM Modified) (NEB #P8108)
- Anti-MBP Monoclonal Antibody (Murine IgG2a) (NEB #E8032)
- Agilent® 6210 TOF MS with both 1200 Series Capillary and Nano pumps coupled with a HPLC-Chip Cube, with a custom-PLRP-S Chip (75 µm x 150 mm with 40 nl trap) or equivalent TOF or Q-TOF and nanoLC system
- Thermo LTQ Orbitrap XL™ ETD MS with Thermo (Proxeon) EASY-nLC or equivalent nanoLC and high resolution MS/MS system and a 20 cm C18 reverse phase analytical column
- FASP™ Protein Digestion Kit (Expedeon #44250)

Here, we describe the enzymatic removal of *N*-linked glycans using PNGase F from a model glycoprotein, murine monoclonal IgG type 2a, expressed in a mouse hybridoma cell line. We also demonstrate the use of trypsin to identify the site of glycosylation. Two mass spectrometers (MS) are used in this protocol: an Agilent 6210 Time-of-Flight (TOF) MS for analysis of the intact protein and a Thermo LTQ Orbitrap XL MS for analysis of the trypsin-digested murine IgG.

General Protocols

Deglycosylation occurs optimally under denaturing conditions (using SDS and heat). However it can be performed under native conditions without the addition of detergents.

Keep all enzyme solutions on ice.

Denaturation and PNGase F Digestion

1. Add 25 µl glycoprotein at 1 µg/ml (25 µg total) to a 200 µl tube.
2. Add 2.5 µl of 10X GlycoBuffer 2.
3. Add 5 µl of 10X Glycoprotein Denaturing Buffer.
4. Mix and incubate at 95°C for 5 minutes.
5. Cool on ice for 2 minutes.
6. Add 1 µl PNGase F.
7. Mix and incubate at 37°C for 2 hours.
8. Either remove detergent from reaction using the detergent removal protocol below and analyze by LC-MS or continue with Trypsin Digestion using NEB Trypsin-ultra and the FASP Protein Digestion Kit.

Simultaneous PNGase F & Trypsin Digestion

1. Add 25 µl of glycoprotein at 1 µg/µl (25 µg total) to a 1.5 ml tube.
2. Add 25 µl of 2X Trypsin Buffer.
3. Mix and incubate at 95°C for 5 minutes.
4. Cool on ice for 2 minutes.
5. Add 6 µl of PNGase F.
6. Add 250 ng of Trypsin (1:100 enzyme:substrate).
7. Mix and incubate at 37°C for 3 hours.

Detergent Removal by Acetone Precipitation

Generally, a minimum of 1 µg of protein will produce a visible pellet upon precipitation.

1. Add 50 µl of protein solution to a 1.5 ml microcentrifuge tube.
2. Add 450 µl of acetone to the tube and mix.
3. Place on a dry ice/ethanol slurry (-78°C) for 10 minutes.
4. Centrifuge at 14,000 x g for 20 minutes.
5. Carefully remove and discard the supernatant without disturbing the pellet.
6. Wash the pellet with 100 µl of ice cold 9:1 acetone:water.
7. Place on dry ice/ethanol slurry (-78°C) for 10 minutes.
8. Centrifuge at 14,000 x g for 20 minutes.
9. Carefully remove and discard the supernatant without disturbing the pellet.
10. Remove any remaining supernatant and dry by vacuum centrifugation.
11. Resuspend protein pellet in 1X Trypsin Buffer.

Trypsin Digestion using NEB Trypsin-ultra and FASP Protein Digestion Kit

1. Add 200 µl 8 M Urea/10 mM DTT to 10 µg deglycosylated protein solution. Vortex briefly (See Note 1).
2. Rock at room temperature for 30 minutes.
3. Transfer protein-urea mixture to spin filter (provided in FASP Kit). Centrifuge at 14,000 x g for 10 minutes.
4. Add 200 µl fresh urea solution (no DTT) (See Note 2).
5. Centrifuge at 14,000 x g for 10 minutes.
6. Discard flow-through.
7. Add 10 µl prepared iodoacetamide solution and 90 µl urea solution (no DTT). Incubate without mixing for 20 minutes in the dark (See Note 3).
8. Centrifuge at 14,000 x g for 10 minutes.
9. Add 200 µl urea solution (no DTT). Centrifuge at 14,000 x g for 10 minutes.
10. Discard flow-through.
11. Add 200 µl 50 mM ammonium bicarbonate solution (provided with FASP Kit). Centrifuge at 14,000 x g for 10 minutes.
12. Transfer filter to new collection tube.
13. Add digestion solution. Pipette up and down to mix on top of filter. Incubate at 37°C for 2 hours (no rocking) (See Note 4)
14. Add 80 µl 50 mM ammonium bicarbonate solution. Centrifuge at 14,000 x g for 10 minutes.
15. Add 30 µl 50% acetonitrile/0.1% formic acid. Centrifuge at 14,000 x g for 10 minutes.
16. Add 40 µl 0.1% formic acid/water. Centrifuge at 14,000 x g for 10 minutes.
17. Filtrate contains digested peptides. Total filtrate volume = 150 µl.
18. Analyze the peptides by LC-MS/MS.

Notes:

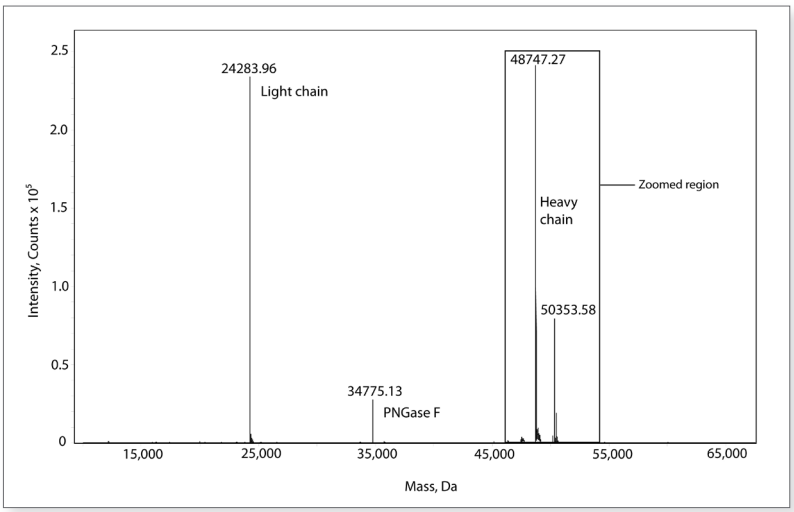
1. To prepare urea solution: Add 1 ml 100 mM Tris-HCl pH 8.5 (provided with FASP Kit) to one tube of urea (provided with FASP Kit). Vortex until all powder dissolves and add DTT to a 10 mM concentration.
2. To prepare urea solution: Add 1 ml 100 mM Tris-HCl pH 8.5 (provided with FASP Kit) to one tube of urea (provided with FASP Kit). Vortex until all powder dissolves.
3. To prepare iodoacetamide solution: Add 100 µl prepared urea solution (no DTT) to 1 tube iodoacetamide (provided with FASP Kit). Pipette up and down 10-15 times to mix well.
4. To prepare digestion solution: Add 1 ml 50 mM ammonium bicarbonate solution (provided with FASP Kit) to 20 µg Trypsin-ultra (NEB #P8101) to make a 20 ng/µl trypsin solution.
5. Add trypsin solution to top of filter at a 1:50 protein:trypsin ratio, and add 50 mM ammonium bicarbonate solution to bring total digestion solution volume to 100 µl.

Results:

Intact Protein MS Data Acquisition

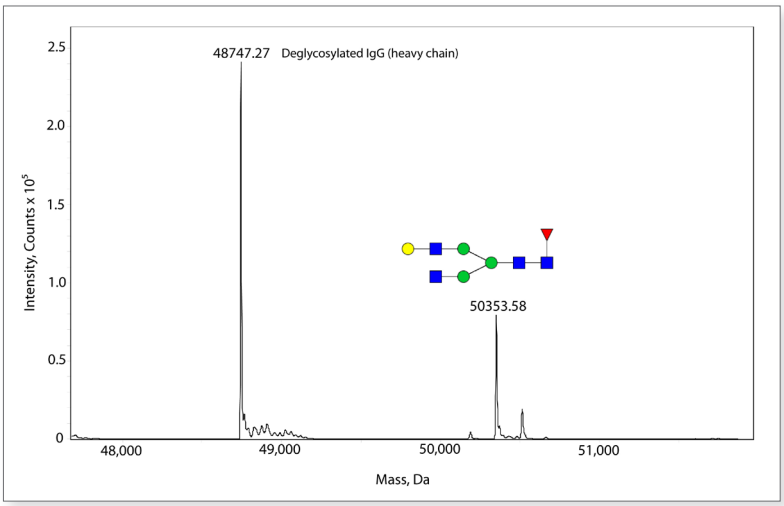
Samples of protein prepared as described above were analyzed by reverse phase liquid chromatography (RPLC) (Figure 2) and electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS) (Figure 3). A custom reverse-phase chip, containing an integrated trapping column (40 nl capacity), separation column and nano-ESI emitter (75 µm x 150 mm both packed with PLRP-S, 5 µm particles, 1000 Å pore size) was used for the separation of proteins (1). The chip trap column was loaded at 4 µl/min and the separation column was run at a flow rate of 500 nl/min using an Agilent 1200 series nano LC connected directly to an Agilent 6210 series ESI-TOF MS. The column was equilibrated with 0.1% formic acid in water containing 5% acetonitrile. One to eight microliters of protein sample was injected onto the column and proteins were separated with a gradient of acetonitrile. The acquired spectra were extracted and the protein spectra were deconvoluted.

Figure 2: PNGase F treated Murine IgG



Murine IgG-treated with PNGase F, subjected to chromatography, nanoESI and TOF MS. Spectra were deconvoluted and the major peaks were identified.

Figure 3: PNGase F treated Murine IgG Heavy Chain



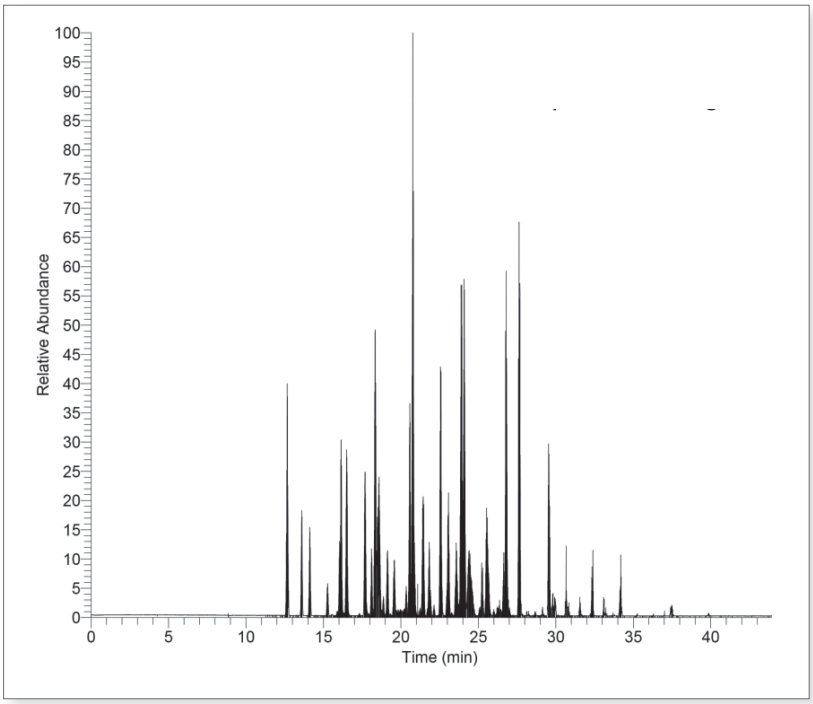
The heavy chain region of the above spectra showing the identity and glycan.

The spectra shown are from a PNGase F digestion done under native conditions. The two heavy chain species observed are the glycan-free heavy chain, 48,747.27 Da, and a small amount of remaining glycosylated species, 50,353.58 Da. The most likely structure of the remaining glycan is also shown.

Trypsin Peptide MS and MS/MS Data Acquisition

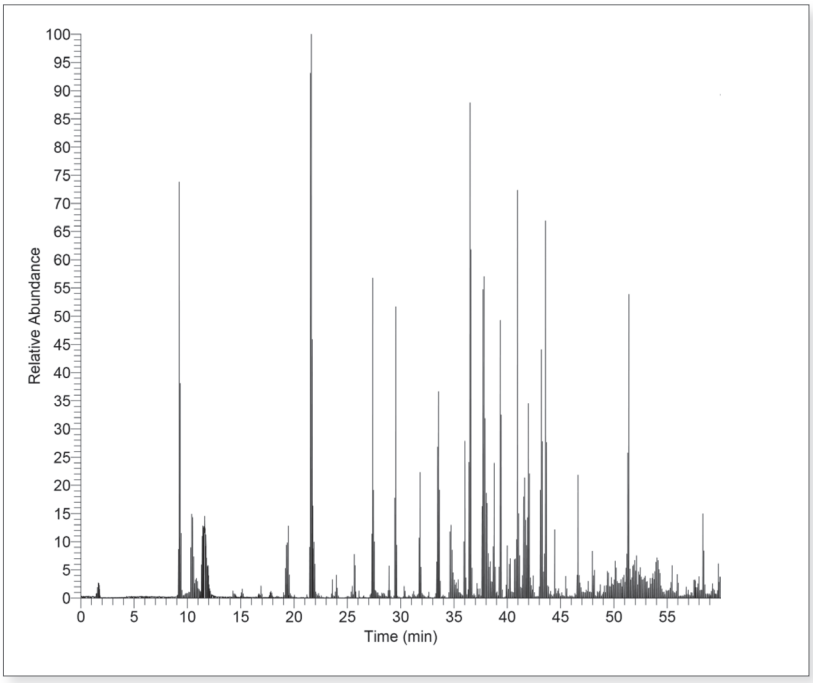
One microliter (400 ng) of digested sample (simultaneous PNGase F/trypsin digestion) was injected onto a self-packed 20 cm 100 ID analytical column (Aqua 3µ C18 packing material) using a Thermo Scientific (Proxeon) EASY-nLC and separated using a 60 min 5-35% FB linear gradient (FA = 0.1% formic acid, FB = CH_3CN, 0.1% formic acid) at a flow rate of 300 nl/min. Multiply charged peptide ions were automatically chosen during a 30,000 amu resolution scan and fragmented by both CID and ETD in a LTQ Orbitrap XL ETD Mass Spectrometer with a nano-electrospray ionization source (Thermo Scientific). A BSA peptide standard online analysis of trypsin digest of BSA (NEB #P8108) was injected (100 fmol) to test the LC and MS system (see Figure 4, 5, 6).

Figure 4: 87% sequence coverage of Trypsin-digested BSA MS Standard



BSA digest solution diluted to 100 fmol/µl with 0.1% formic acid. 1 µl digest solution was injected via a Proxeon Easy n1000 LC System (Thermo Fisher) onto a self-packed C18 column (100 ID x 20 cm, Aqua 3µ C18 packing material). Peptides were separated using a 30 min 5-40% B linear gradient (A = 0.1% formic acid, B = Acetonitrile, 0.1% formic acid) at a flow rate of 400 nl/min and analyzed online by a Q Exactive mass spectrometer (Thermo Fisher) with a nano-electrospray ionization source. Acquisition range was from 400 to 1600 m/z and a source voltage of 2.5 kV was used. 87% sequence coverage was obtained.

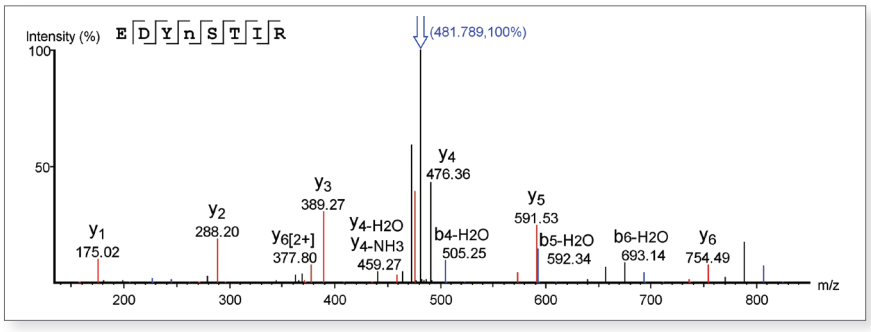
Figure 5: Online analysis of PNGase F/Trypsin-digested Murine IgG



LTQ Orbitrap XL base peak chromatogram of 400 ng Murine IgG simultaneously digested with PNGase F & Trypsin.

The MS and MS/MS fragmentation data were analyzed with both Proteome Discoverer™ 1.4 (Thermo Scientific) and PEAKS7 software. Data was searched using a SwissProt FASTA database. For these analyses, theoretical peptides generated by a tryptic digest with a maximum of two missed cleavages were considered, and the precursor and product mass tolerances were set to ± 10 ppm and ± 0.01 Da, respectively. Variable modifications of asparagine were allowed for (the conversion of asparagine to aspartic acid that occurs when PNGase F removes the glycan). Data was validated using a reverse database decoy search to a false discovery rate of 1%.

Figure 6: MS/MS spectrum of PNGase F-treated peptide



Conclusion

A search of the data collected from the simultaneous PNGase F/Trypsin digested sample identified a peptide with the characteristic N-X-S/T, with an N to D modification (a mass change of +0.98 amu). The peptide identified was EDYNSTLR from the heavy chain of the murine IgG, and is consistent with a previously observed glycosylation site of murine IgG.

References:

1. Spiro, R.G. (2002) *Glycobiology* 12, 43R-56R.
2. Perkins, D.N., et al. (1999) *Electrophoresis* 20, 3551-3567.

www.neb.com
NEW ENGLAND BioLabs® Inc.
New England Biolabs, Inc., 240 County Road, Ipswich, MA 01938-2723 Telephone: (978) 927-5054
Toll Free: (USA Orders) 1-800-632-5227 (USA Tech) 1-800-632-7799 Fax: (978) 921-1350 e-mail: info@neb.com