Characterization of Glycans from Erbitux®, Rituxan® and Enbrel® using PNGase F (Glycerol-free), Recombinant

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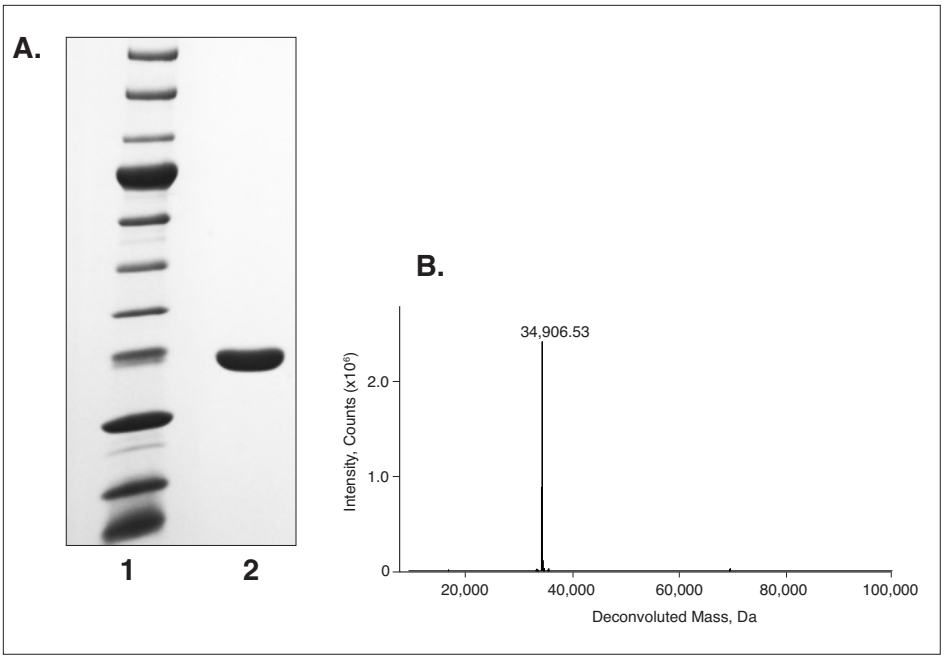
Glycosylation is a post-transcriptional modification that is essential for a wide range of biological processes, including cell attachment to the extracellular matrix and protein-ligand interactions in the cell. Detailed characterization of glycans on therapeutic proteins is critical, as the type and degree of glycosylation can have a profound impact on the stability, activity and effector function of the drug. The microheterogeneity of IgG glycans affects biological functions, such as complement-dependent cytotoxicity (CDC), antibody-dependent cytotoxicity (ADCC), binding to various Fc receptors, and binding to C1q protein (1). Since IgG glycans are typically highly heterogeneous and some glycoforms much less abundant than others, it is critical that the enzymatic reaction and downstream MS analysis be efficient and unbiased so that all species are represented.

PNGase F or Peptide-N-Glycosidase F, is an amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from *N*-linked glycoproteins. PNGase F efficiently removes glycans from a variety of glycoproteins including IgGs, and is used extensively in conjunction with Mass Spectrometry to elucidate the structure of the intact released glycan. Recombinant PNGase F (rPNGase F) is expressed in *E. coli*, highly purified to meet stringent quality control standards, and is produced using no animal products. rPNGase F is used here, in conjunction with downstream LC/MS analysis, to characterize the glycans of three therapeutic glycoproteins: Erbitux, Rituxan and Enbrel.

Figure 1. SDS-PAGE and mass spectrometry show that recombinant PNGase F is highly purified and subjected to stringent quality control assays.



SDS-PAGE Analysis (A) ESI-TOF Analysis (B) of PNGase F (Glycerol-free), Recombinant (MW: 34,906.53 Daltons). SDS PAGE analysis; Lane 1: 15 µl Protein Ladder (NEB #P7703); Lane 2: 5 µl PNGase F (Glycerol-free), recombinant (NEB #P0705). Mass determination by an Agilent 6210 TOF LC/MS.

Materials

- Erbitux (cetuximab) from Imclone, LLC
- Rituxan (rituximab) from Genentech, a member of the Roche Group, and Biogen Idec, Inc.
- Enbrel (enteracept) from Amgen Inc., manufactured by Immunex Corp
- PNGase F (Glycerol-free), Recombinant (NEB #P0709), supplied with 10X Glycoprotein Denaturing Buffer, 10% NP-40 and 10X GlycoBuffer 2
- Supelclean™ ENVI-Carb™ SPE Tube 100 mg, 1 ml (Sigma-Aldrich, cat. #57109-U)
- Acetonitrile (ACN) HLPC/MS grade
- 50 mM NH_4 Formate buffer, pH 4.4 (See Note 1)
- 2-aminobenzamide (2AB, anthranilamide) (Sigma #A89804-5G)
- Sodium cyanoborohydride (Sigma #156159)
- Dimethyl sulfoxide (DMSO)
- Glacial acetic acid
- Spin SPE HILIC columns (Nest Group Inc., SEM-HIL)

General Protocols

Deglycosylation

The protein sample is denatured with DTT and heat (avoiding detergents, which are not compatible with downstream MS analysis). Alternatively, samples are deglycosylated under native conditions (protein is not denatured).

1. To 100 µg of Erbitux, Rituxan or Enbrel, add DTT to a final concentration of 40 mM in a final volume of 100 µl for each sample.
2. Denature at 55°C for 10 minutes, cool on ice.
3. To all samples, add 10 µl of 10X GlycoBuffer 2 and 2 µl of Recombinant PNGase F.
4. Incubate for 24 hours at 37°C.

N-glycan Purification

5. Condition an ENVI-Carb SPE tube with 3 ml of acetonitrile (ACN), followed by 1 ml of 50% ACN (See Note 1). Equilibrate with 3 ml of water.
6. Apply deglycosylated sample from Step 4. Discard flow through, column will retain *N*-glycans. Wash twice with 1 ml of water. Discard flow through.
7. Elute with 300 µl of 40% ACN/60% 50 mM NH_4 Formate, pH 4.4 (See Note 2). Collect *N*-glycans in a 1.5 ml tube.
8. Lyophilize or dry in speedvac (See Note 3).

Fluorescent labeling with 2-aminobenzamide (2AB)

9. To dried sample, add 10 µl of 2AB Labeling Reagent (See Note 4) and 1 µl 50% acetic acid, and mix.
10. Transfer to 0.2 ml PCR tubes. Incubate at 65°C for 2 hours (See Note 5).

Cleanup

11. Condition a HILIC spin column with 350 µl ACN (spin at 1100 rpm for 1 minute, discard flow through), followed by 350 µl of 50 mM NH_4 Formate, pH 4.4 (spin at 3,000 rpm for 1 minute, discard flow through). Add another 50 µl of 50 mM NH_4 Formate, pH 4.4, spin at 1,000 rpm for 5 minutes, discard flow through.
12. Equilibrate the column with 350 µl of 90% ACN/50 mM NH_4 Formate, pH 4.4 (spin at 1,100 rpm for 1 minute, discard flow through). Repeat a second time.
13. Dilute sample from Step 10 to 85% ACN; add 60 µl of ACN and mix (See Note 6). Apply to prepared HILIC column, spin at 700 rpm for 3 minutes, discard flow through.
14. To remove unbound fluorescent label, wash column with 350 µl of 90% ACN/10% 50 mM NH_4 Formate, pH 4.4, spin at 1,100 rpm for 1 minute, discard flow through. Repeat five times.
15. Spin at 3,000 rpm for 2 minutes to dry the column.
16. Elute 2AB-labeled *N*-glycans with 100 µl of 50 mM NH_4 Formate, pH 4.4, spin at 3,000 rpm for 1 minute. Collect in centrifuge tube.

Liquid Chromatography/Mass Spectrometry (LC/MS)

Hydrophylic Interaction Liquid Chromatography (HILIC) in line with mass spectrometry has been successfully used to separate and identify glycoconjugates (2). The fluorescent label at the glycan reducing end (1:1 molar ratio) is crucial for precise quantitation, while it also facilitates electrospray ionization (ESI) for MS. Data is interpreted based on known N-glycan biosynthetic pathways, allowing for the identification of individual glycan species (See Note 7).

17. A sample of labeled *N*-glycans (40 µl) was diluted with 160 µl of ACN in an autosampler vial. The 2AB-labeled *N*-glycans were separated using a XBridge™ BEH Amide column (Waters) on a Dionex UltiMate® LC equipped with fluorescent detection (See Note 8), in line with a LTQ™ Orbitrap Velos™ Spectrometer equipped with a heated electrospray standard source (HESI-II probe) (See Note 9).

Notes:

1. Apply gentle positive pressure, or use a vaccum manifold.
2. Low pH is needed to elute sialylated glycans.
3. To prevent sialic acid loss, do not overheat.
4. Dissolve 5 mg of 2 AB in 20 µl of DMSO, mix. Add 80 µl of water. Add this solution to 6 mg of $NaCNBH_4$. Use immediately. Discard unused solution following safety regulations.
5. A thermocycler provides excellent temperature control, minimizing evaporation. However, any other suitable incubator can be used for this step.
6. It is critical to maintain a dilution in 85% acetronitrile; higher organic content might cause some glycans to precipitate out of solution, and lower organic content will prevent glycans from binding to the HILIC column.
7. Since various isomers can be present, unequivocal assignment of structure is only possible following extensive analysis, such as MS/MS, which is not described here.
8. The glycans were separated using a gradient of 70%:30% to 62%:38% ACN:50 mM NH_4 Formate, pH 4.4, for 48 minutes at 350 µl/min, 2 AB fluorescence was measured at 350 (ex)/420 nm (em). Injection vol:100 µl
9. Optimized settings for positive mode detection of 2 AB-labeled *N*-glycans: A) ESI: spray voltage, 3.5 kV; capillary temperature, 250°C; sheath gas, 11 psi; Aux gas and sweep gas flow rates, 0; S-lens RF level %, 66. B) Ion Optics settings: Multiple 00 offset, 2.5 V; Lens 0 voltage, 6.5 V; Multiple 0 offset, 7.0 V; Lens 1 voltage, 16 V; Multiple 1 offset, 6.5 V; Multiple RF Amplitude, 600; Front lens, 7.75 V.

Results:

Figure 3. LC/MS Analysis of Erbitux (cetuximab) Glycans released by PNGase F, Recombinant

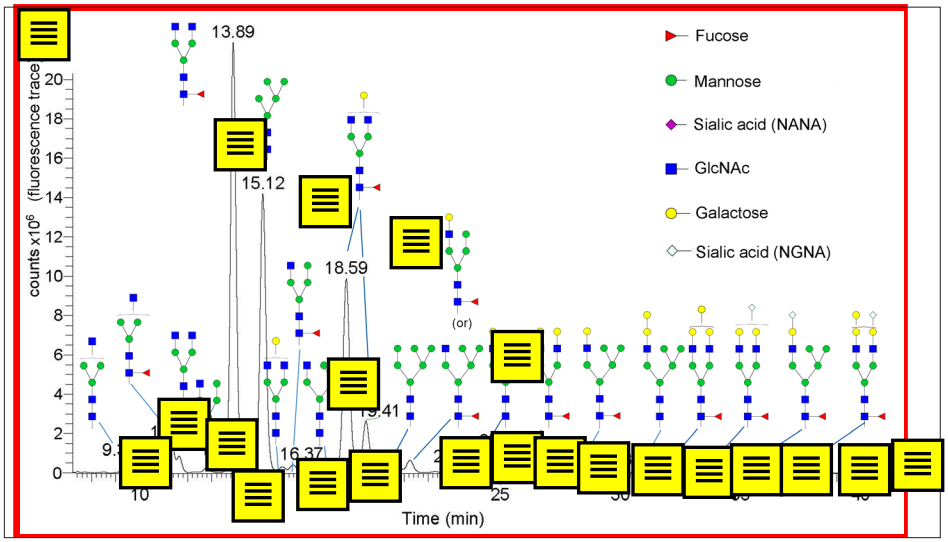
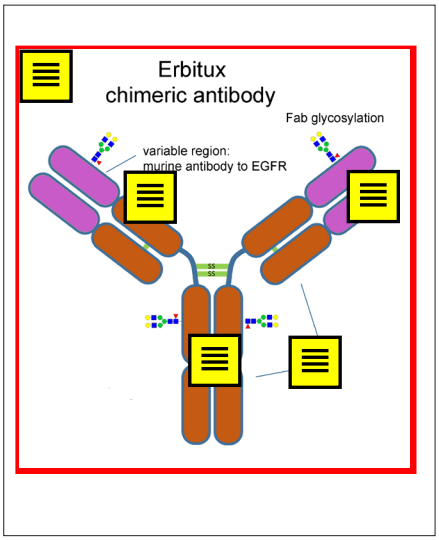


Figure 4. Erbitux functional domain



Erbitux, is a recombinant human-mouse chimeric monoclonal antibody that binds specifically to the extracellular domain of the human epidermal growth factor receptor (EGFR) and is produced in mammalian cell (murine myeloma) culture. Erbitux has two glycosylation sites on each heavy chain, one at Asn 299 in the conserved Fc portion and the other present at Asn 88 in the Fd domain. Masses corresponding to G0F, G1F and G2F glycans are detected as well as high mannose and hybrid structures. Low levels of G2FGal1 are found as well as three glycan species with NGNA sialic acids. The NGNA sialic acids oligosaccharides are likely to have been released from the Fd domain of the antibody, although the majority of the glycoforms observed here are species associated with the conserved glycosylation site in the Fc domain (3).

Figure 5. LC/MS Analysis of Rituxan Glycans released by PNGase F, Recombinant

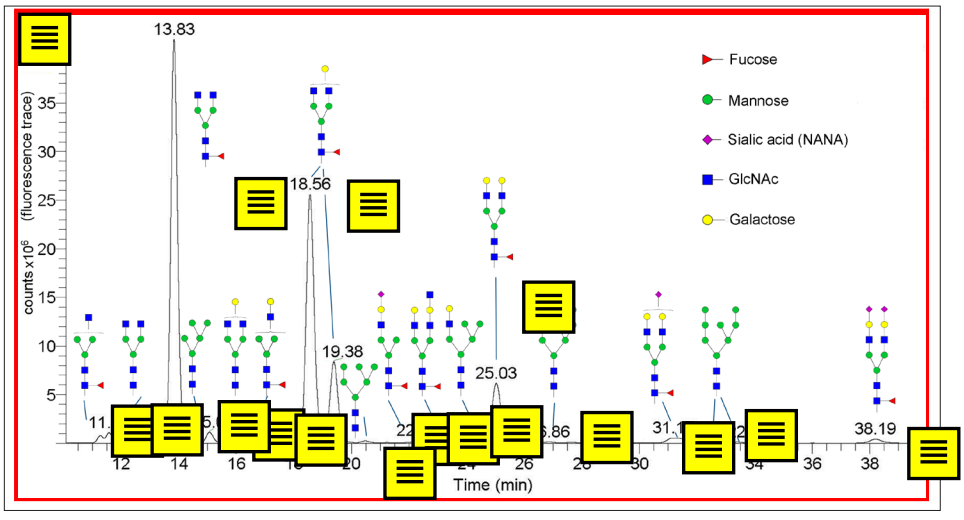
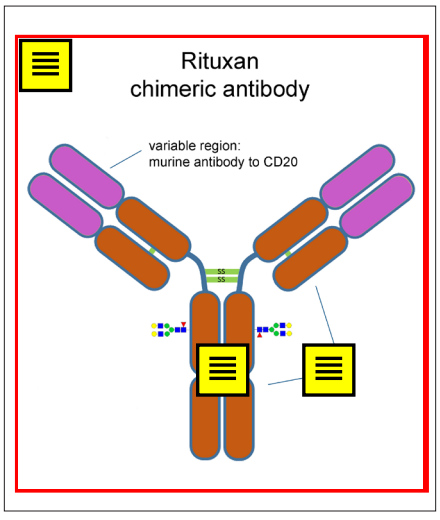


Figure 6. Rituxan functional domain



Rituxan is a genetically engineered chimeric human/mouse monoclonal IgG1 kappa antibody directed against the CD20 antigen which is primarily found on the surface of immune system B cells. Rituxan is produced by mammalian cell (Chinese Hamster Ovary) culture. The most abundant for Rituxan species are G0F, G1F, and G2F. Low levels of high mannose and NANA sialic acid species were detected as well.

Results (continued):

Figure 7. LC/MS Analyis of Enbrel Glycans released by PNGase F, Recombinant

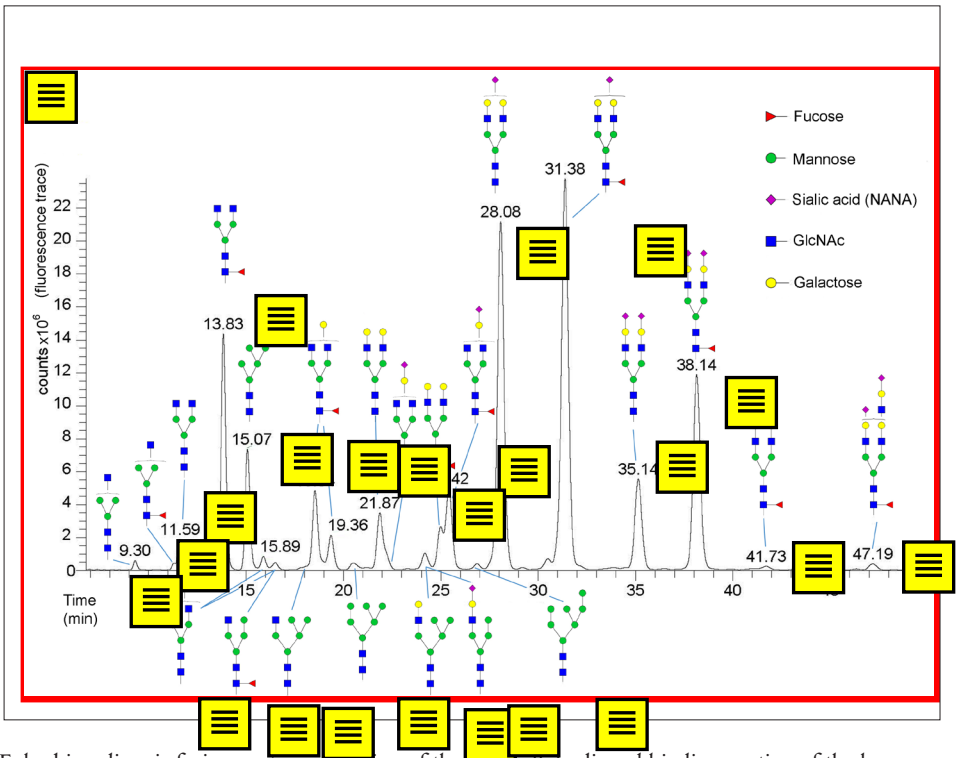
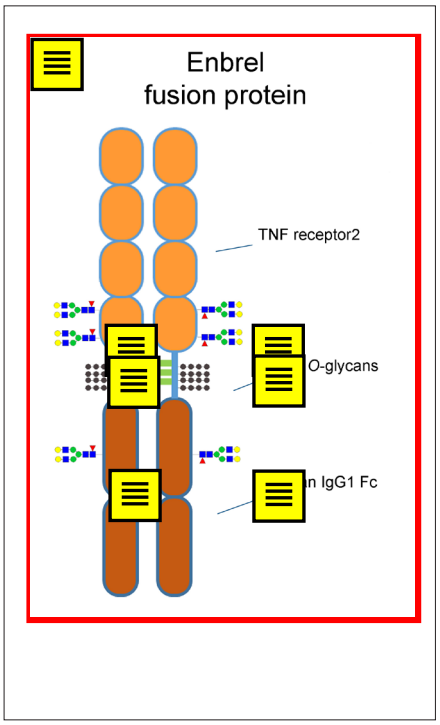


Figure 8. Enbrel Functional Domains



Enbrel is a dimeric fusion protein consisting of the extracellular ligand binding portion of the human tumor necrosis factor receptor (TNFR) linked to the Fc portion of human IgG1. The Fc component of Enbrel contains the CH2 domain, the CH3 domain and hinge region, but not the CH1 domain of IgG1. Enbrel is produced by recombinant DNA technology in a Chinese hamster ovary (CHO) mammalian cell expression system.

Glycoforms identified by LC/MS analyis of intact Enbrel (etanercept). Enbrel contains many NANA-sialylated species, the most abundant being G2 and G2F monosialylated. Low levels of high mannose and hybrid species are detected as well.

In each chromatogramThe identity of each N-glycan peak was annotated manually according to the peak retention time in comparison to known standards, and the corresponding m/z value as determined by ESI-MS, in agreement with the known metabolic pathways of antibody producing expression systems. The elucidated N-glycan structures are shown above each peak using the conventional glycan nomenclature of the Consortium for Functional Glycomics.

Conclusion

Endoglycosidases are critical tools to investigate the nature of protein glycosylation. PNGase F, Recombinant is used here with LC/MS analysis to assign glycans released from three well-characterized therapeutic glycoproteins, Erbitux, Rituxan and Enbrel. A wide range of glycan structures is easily detected using this enzyme including high mannose and sialylated species. PNGase F, Recombinant efficiently deglycosylates these biotherapeutic proteins with results that are reproducible and unbiased with regard to glycan species released. These glycosylation profiles are in agreement with published glycan studies of Erbitux, Rituxan and Enbrel. Finally, it should be noted that protein samples treated with enzymes remain intact, and therefore are ready for downstream proteomic analysis.

References:

1. Wright, A. and Morrison, S. L. (1997) *Trends Biotechnol.* 15(1): 26–32.
2. Wuhrer, M., de Boer, A. R. and Deelder, A. M. (2009) *Mass Spectrom Rev.* 28, 192–206.
3. Ayoub, et al. (2013). Correct primary structure assessment and extensive glyco-profiling of cetuximabby a combination of intact, middle-up, middle down and bottom-up ESI and MALDI mass spectrometry techniques. mAbs 5:5, 699–710.

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