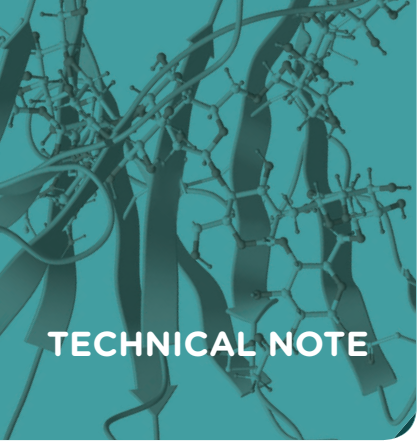


PROzyme

Gly-Q

Gly-*X*

GlykoPrep

Glyko Enzymes

Glyko Standards

InstantPC

InstantAB

InstantQ

2-AB

APTS Express

PhycoLink

PhycoPro

RPE & APC Conjugates

Streptavidins

An Intact Protein Approach to Exoglycosidase Sequencing Followed by Released N-Glycan Analysis from Biotherapeutic Glycoproteins

Andres Guerrero, Anna Fong, John Yan, Tom Rice, Justin Hyche, Aled Jones
ProZyme, Hayward, CA

SUMMARY

- Analysis of N-glycans released from biotherapeutics frequently relies on derivatization with a fluorescent label.
- Exoglycosidases cleave specific terminal monosaccharides allowing for sequential digestion thus aiding in the identification of complex N-glycans.
- Glycoprotein samples can be first treated in parallel by a panel of specific exoglycosidases followed by N-glycan sample preparation using Gly-*X* with InstantPC or InstantQ.

Keywords

Biotherapeutic
Exoglycosidase
N-Glycans
InstantPC
InstantQ
Capillary Electrophoresis
HILIC

INTRODUCTION

The structure of N-linked glycans can play a critical role in the pharmacology of therapeutic proteins, potentially affecting immunogenicity, pharmacokinetics and pharmacodynamics, making the characterization of N-glycans an essential part of the biotherapeutic development process (1). A common analytical approach for the analysis of N-glycans includes the enzymatic release of the glycans with PNGase F, derivatization with a fluorophore (InstantPC, 2-AB, InstantQ) and analysis by liquid

chromatography (LC) with fluorescence detection (FLD) with potential hyphenation to mass spectrometry (MS), or capillary electrophoresis (CE) with LED-induced fluorescence (LEDIF). In addition, the use of exoglycosidase enzymes (Figure 1) offers a specific set of tools for the sequential digestion and release of common monosaccharides (sialic acid, galactose and N-acetylglucosamine) from the non-reducing terminus of the N-glycan, aiding in structural characterization of biotherapeutic proteins containing these important post-translational modifications.

Here we present a workflow for the sequential digestion of terminal monosaccharides from N-glycans found on biotherapeutic proteins. In this unique approach, exoglycosidases are used on the intact glycoprotein prior to PNGase F digestion and labeling of the released N-glycans. This approach allows for a simple workflow that can be applied for different labeling chemistries while providing flexibility in fluorescent dye selection.

METHODS

Materials

MabThera Lot # H0102803, Enbrel Lot # R180844

Sialidase A [GK80040]

β(1-3,4)-Galactosidase [GKX-5013]

β(1-2,3,4,6) N-Acetylhexosaminidase, Sequencing-grade [GKX-5023]

N-Glycan sample preparation kits

Gly-X N-Glycan Rapid Release and Labeling with InstantPC Kit (96-ct) [GX96-IPC]

Gly-X N-Glycan Rapid Release and Labeling with InstantQ Kit (96-ct) [GX96-IQ]

Exoglycosidase digestion and N-glycan sample preparation conditions

Glycoprotein samples were treated with exoglycosidase enzymes under the following conditions prior to release and labeling of N-glycans (Figure 2).

1. **Denaturation:** 20 µL of 2 mg/mL (40 µg) glycoprotein stock along with 2 µL Gly-*X* denaturant was incubated at 90°C for 3 minutes.
2. **Exoglycosidase digestion:** After denaturation, separate glycoprotein samples were incubated overnight at 42°C with the following sequences of exoglycosidase enzymes (2 µL of each enzyme) after the addition of 6 µL of 0.5 mM Ammonium Acetate pH 5.5.
   a. Sialidase A [GK80040]
   b. Sialidase A [GK80040] + β-Galactosidase [GKX-5013]
   c. Sialidase A [GK80040] + β-Galactosidase [GKX-5013] + β-N-Acetylhexoseaminidase [GKX-5023]
3. **N-Glycan sample preparation:** Following exoglycosidase digestion N-glycans were prepared following standard Gly-*X* (with InstantPC and InstantQ) protocols starting at the 5 minute deglycosylation step.

N-Glycan separation and analysis

HILIC separation conditions

60-minute UHPLC high resolution method

Column: 2.1 x 150 mm, 1.7 µm

Flow rate: 0.4 mL/minute

Gradient: 25–38% 50 mM ammonium formate pH 4.4, between 2.5–50 minutes

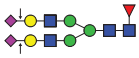
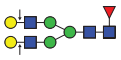
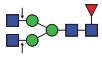
Column temperature: 60°C

Gly-Q CE separation

InstantQ labeled N-glycans were analyzed with Gly-Q, a capillary electrophoresis-based 2-minute separation along with LED induced fluorescence detection (LEDIF)

Instrument method: N-glycans ProZyme

Processing methods: Rituxan_Method/Enbrel_Method

Exoglycosidase [Product Code]	Short Name	Example Glycan	Specificity
Sialidase A (Arthrobacter) [GK80040]	ABS		α (2–3, 6, 8, 9) N-Acetylneuraminic acid linkages
β-Galactosidase (Bovine testis) [GKX-5013]	BTG		β (1–3, 4) Galactose linkages
β-N-Acetylhexoseaminidase (Jack bean) [GKX-5023]	JBH		B (1–2, 3, 4, 6) GlcNAc and GalNAc linkages

◆ N-Acetylneuraminic acid (Neu5Ac) ● Galactose ■ N-Acetylglucosamine (GlcNAc) ● Mannose ▼ Fucose

Figure 1: Exoglycosidase enzymes along with example glycans and substrate specificity.

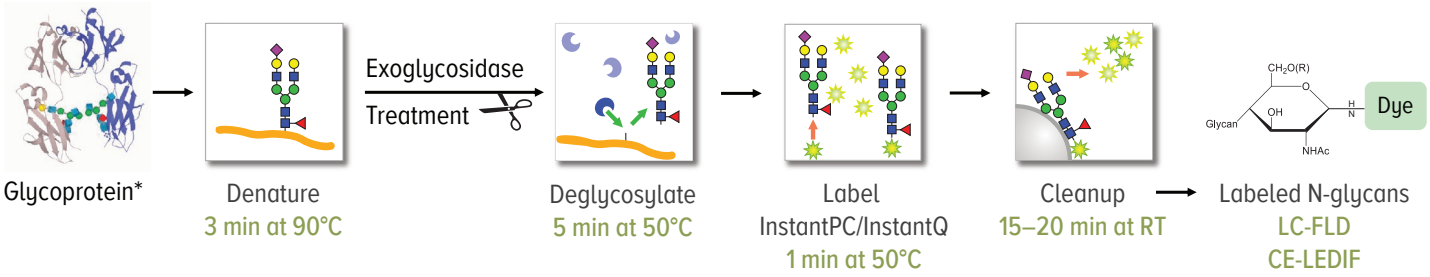


Figure 2: General workflow for exoglycosidase treatment, PNGase F digestion, InstantDye labeling and cleanup prior to LC-FLD and CE-LEDIF analysis.

* Glycoprotein structure adopted from PDB entry DOI: 10.2210/pdb5VGP/pdb
PDB structure modified and image genrated with Lite Mol: https://webchemdev.ncbr.muni.cz/LiteMol/

RESULTS

- In parallel digestion of glycoprotein samples with Sialidase A, Sialidase A + β-Galactosidase and Sialidase A + β-Galactosidase + β-N-Acetylhexoseaminidase results in the sequential release of terminal sialic acid followed by galactose and N-acetylglucosamine residues.
- Exoglycosidase digestions of MabThera and Enbrel were prepared as described above and N-glycans released and labeled with InstantQ and InstantPC. InstantQ labeled samples from MabThera were analyzed by Gly-Q (Figure 3) and samples from Enbrel were analyzed by both InstantQ and InstantPC (Figures 4 and 5).
- Removal of terminal sialic acid residues results in the shift of sialylated glycans A1, A1F, A2, A2F and G1FS1 to asialylated species G1F, G2 and G2F.
- After the removal of terminal sialic acid, removal of the subsequent terminal galactose residues with β-Galactosidase further shifts the glycan profile towards G0 and G0F.
- Finally, the treatment of glycoprotein samples with all three exoglycosidase enzymes results in the trimming of N-glycans down to the core structures Man3 and Man3F.
- InstantQ labeling and analysis on Gly-Q results in comigrations of Man5 and G0F-N as seen in MabThera (Figure 3). Digestion of MabThera N-glycans with the combination of exoglycosidase enzymes removes the N-Acetylglucosamine from G0F-N resulting in its shift to Man3F.

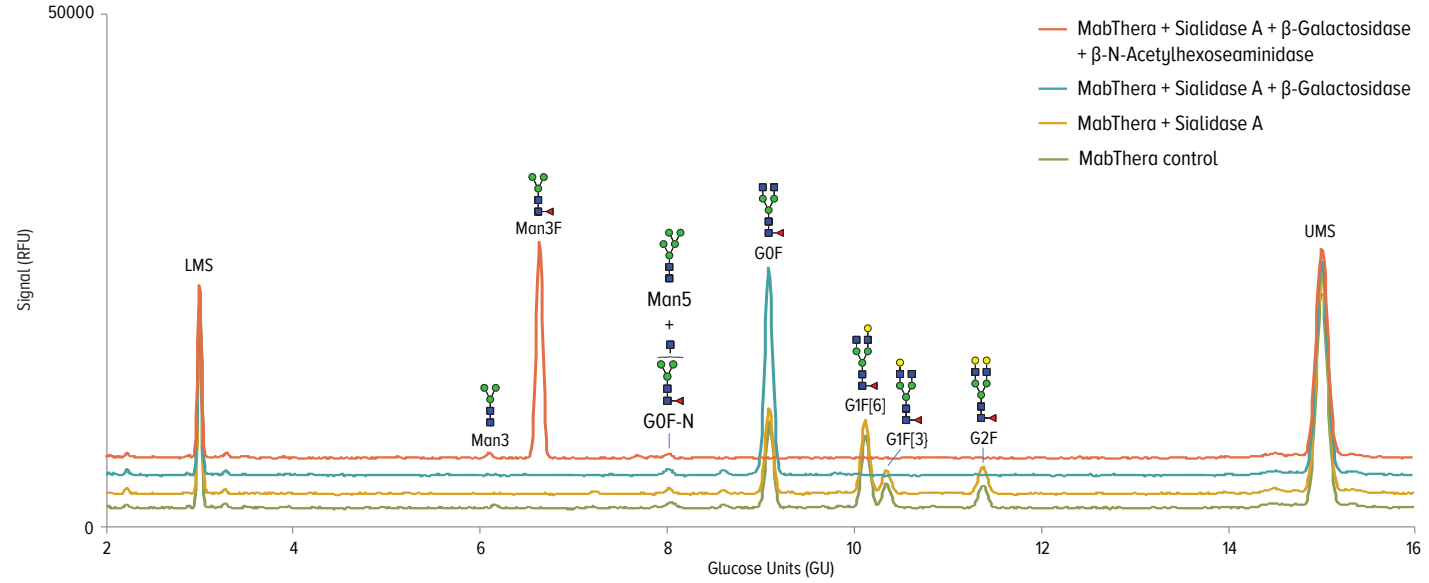


Figure 3: Overlaid Gly-Q electropherograms for sequential digestions of MabThera LMS - lower migration standard (DP3), UMS - upper migration standard (DP15).

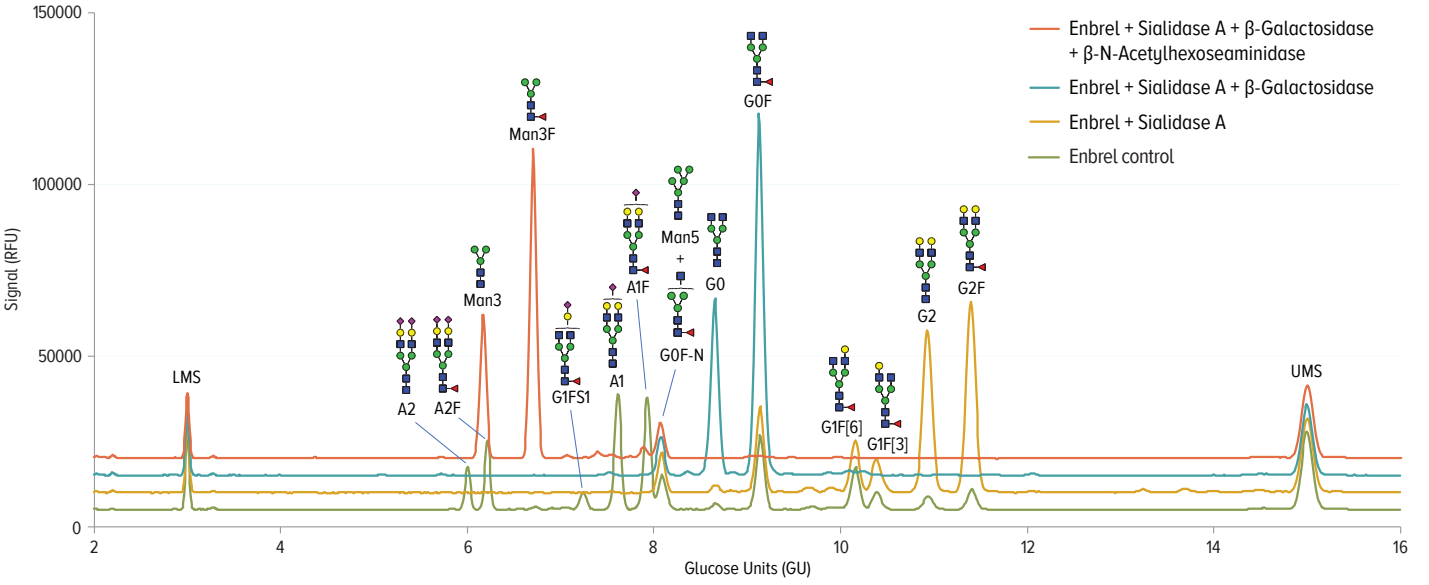


Figure 4: Overlaid Gly-Q electropherograms for in parallel digestions of Enbrel.

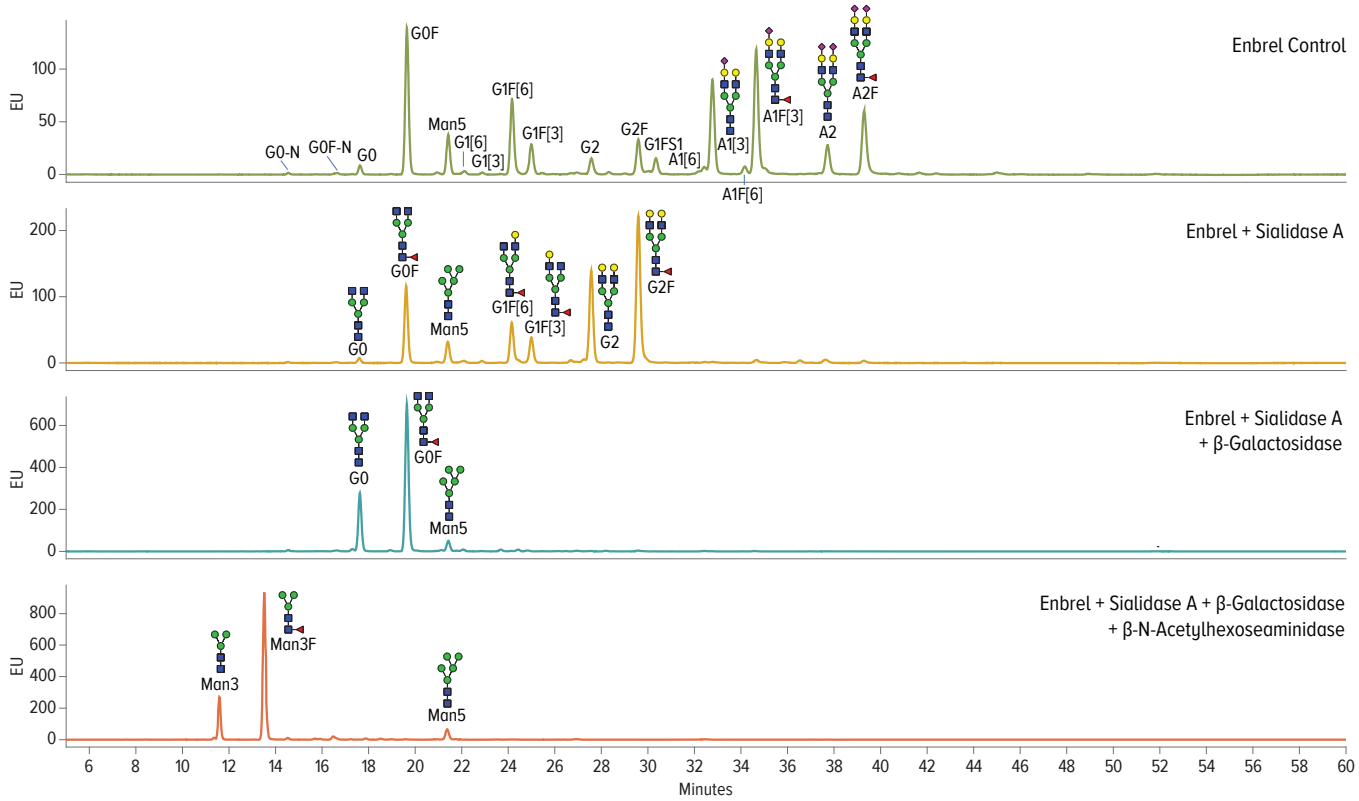


Figure 5: Stacked UHPLC chromatograms for Enbrel N-glycans. A) Total N-glycan profile, B) treatment with Sialidase A, C) Sialidase A + β-Galactosidase, D) Sialidase A + β-Galactosidase + β-N-Acetylhexoseaminidase.

CONCLUSIONS

1. Exoglycosidases may be used on intact and denatured glycoprotein samples prior to the release and labeling of N-glycans.
2. The use of exoglycosidases on intact glycoprotein samples allows for a standard digestion protocol prior to release and labeling allowing for flexibility in fluorescent dye selection.
3. Exoglycosidase digestion can aid in resolving peaks which would otherwise coelute or comigrate.

References

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Toll Free +1 (800) 457-9444 **Phone** +1 (510) 638-6900 **Fax** +1 (510) 638-6919 **Website** www.prozyme.com
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