

O-glycosylation analysis

Kotsias M, Hendel J, Kozak RP

Ludger Ltd, Culham Science Centre, Oxfordshire, UK

Why is O-glycosylation analysis important?

Protein O-glycosylation is one of the most common types of post-translational modifications. It refers to the covalent attachment of a carbohydrate moiety to the polypeptide backbone. In O-link glycosylation the glycan is attached to the side chain of a Ser or Thr residue.

O-glycosylation is known to have a critical impact on protein secretion and protective immunity against cancer, and maintenance of normal development and physiology. Moreover, a substantial portion of the protein-based pharmaceuticals on the market are glycoproteins where O-glycosylation was found to critically modulate the physicochemical properties of proteins, have a functional impact on their therapeutic potentials, and affect the safety profiles of such drugs. O-glycosylation is prevalent in many classes of therapeutic proteins including Erythropoietin (EPO), Follicle stimulating hormone (FSH), Etanercept, Granulocyte-colony stimulating factor (G-CSF) providing evidence of its critical involvement in drug performance and diseases.

In-depth understanding of the O-glycosylation status of these drug substances will elucidate the structure-function relationship of the O-linked sugars, which may lead to the identification of functionally favorable O-glycan structures to improve drug efficacy and safety profile.

Challenges

O-linked glycans can vary vastly in size, ranging from a single *N*-acetylgalactosamine (GalNAc) monosaccharide at the reducing end terminus to large oligosaccharides exhibiting complex glycan motifs resulting from further modifications by the addition of other sugar types including galactose (Gal), *N*-acetylglucosamine (GlcNAc), GalNAc, fucose (Fuc), and sialic acids (Neu5Ac and/or Neu5Gc). This substantial heterogeneity can generate eight main core structures, each potentially subject to further elongation and substitution (Figure 1).

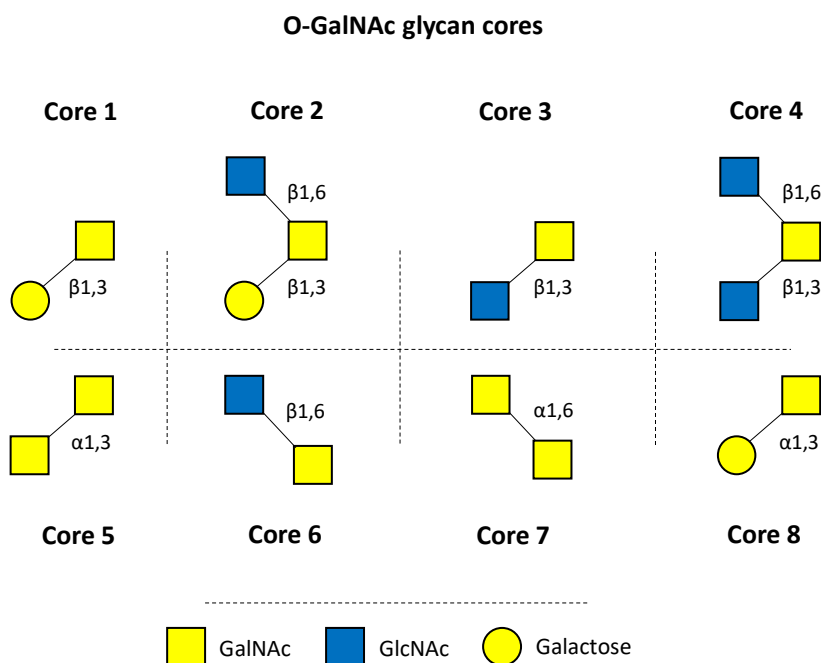


Figure 1. Eight main core O-glycan structures.

The analysis of O-linked glycosylation remains a challenge due to the lack of equivalent enzymes and the inherent structural heterogeneity of O-glycans. The specificities of the reported O-glycanase enzymes are predominantly restricted to T antigen (Gal β 1,3-GalNAc α -Ser/Thr). Despite isolated reports describing O-glycanases of broader specificities, a universal O-glycanase that is capable of liberating O-glycans with more sophisticated branching and elongation is still unknown. Therefore, the release of O-linked oligosaccharides is commonly achieved by chemical approaches where a broad range of samples, including but not limited to glycoprotein therapeutics, mammalian cell lines and bacterial cell components, biological fluids and tissues, can be processed.

How can Ludger help?

Ludger offers a range of products for analysis of samples containing O-glycans. This includes:

- I. Enzymes (O-glycosidase) for release of unsubstituted Gal β 1,3-GalNAc α disaccharides attached to the Ser or Thr residues of glycoproteins or glycopeptides; (E-G001)
- II. Enzymes (Enzymatic CarboRelease Kit) for protein deglycosylation, for the removal of all N-linked oligosaccharides and many O-linked sugars; (KE-DG01)
- III. Enzymes (Enzymatic DeGlycoMx Kit) containing a premixed cocktail of the enzymes required to remove all N-linked oligosaccharides and most O-linked sugars; (KE-DGMX)
- IV. Kits for complete removal of O-glycans from glycoproteins or glycopeptides:
 - a. Ludger Liberate Hydrazinolysis N- and O-glycan release kit (LL-HYDRAZ-A2)
 - b. Ludger Liberate Orela O-glycan release kit (LL-ORELA-A2)



I. O-Glycosidase

Cleaves only unsubstituted Gal- β (1-3)GalNAc- α disaccharides attached to the serine or threonine residues of glycoproteins or glycopeptides. Released glycans have free reducing termini to allow for fluorescent tagging by reductive amination and analysis by HILIC-(U)HPLC-ESI-MS.



II. Enzymatic CarboRelease Kit

All Ser/Thrlinked (O-linked) Gal-(β 1-3)-GalNAc-(α 1) and all sialic acid substituted Gal-(β 1-3)-GalNAc-(α 1) are removed using the combination of Neuraminidase and O-Glycosidase. The addition of β -Galactosidase and β -N-acetylglucosaminidase assists in the deglycosylation of larger O-link structures. Released glycans have free reducing termini to allow for fluorescent tagging by reductive amination and analysis by HILIC-(U)HPLC-ESI-MS.



III. Enzymatic DeGlycoMx Kit

All Ser/Thrlinked (O-linked) Gal-(β 1-3)-GalNAc-(α 1) and all sialic acid substituted Gal-(β 1-3)-GalNAc-(α 1) are removed using the combination of Neuraminidase and O-Glycosidase. The addition of β -Galactosidase and β -N-acetylglucosaminidase assists in the deglycosylation of larger O-link structures. Released glycans have free reducing termini to allow for fluorescent tagging by reductive amination and analysis by HILIC-(U)HPLC-ESI-MS.

IV. Kits for complete removal of O-glycans from glycoproteins or glycopeptides:



a. Hydrazinolysis

The mechanism by which hydrazine removes O-glycans is not fully understood, it is accepted that it proceeds via an initial β -elimination reaction followed by reaction with hydrazine to form hydrazine derivatives. The glycoprotein or glycopeptide samples are prepared by removing salts, detergent and dyes that could interfere with the release by dialysis against water or 0.1 % TFA. Under the release conditions, the N-acetyl groups of the amino sugars are also removed; therefore, it is necessary to perform a re-N-acetylation step with acetic anhydride. Released O-glycans can be labelled by reductive amination using LudgerTagTM labels such as 2-AB (2-aminobenzamide), 2-AA (2-aminobenzoic acid) or procainamide and analyzed by hydrophilic interaction liquid chromatography (HILIC)-(U)HPLC-ESI-MS with fluorescence detection.

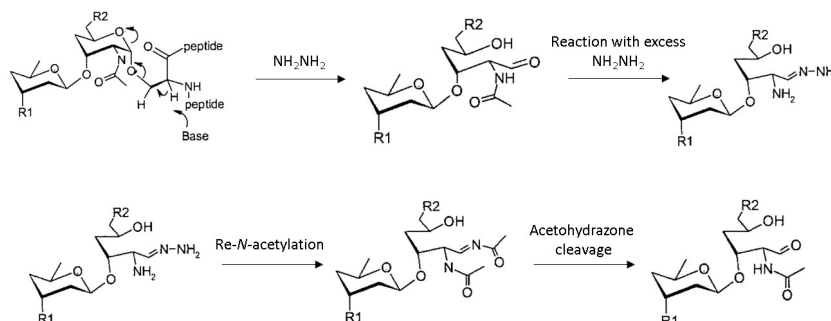


Figure 2. Hydrazinolysis Scheme for O-glycan release.



b. Orela

The Orela reagent reacts at the link between the glycan and the peptide backbone to liberate glycans as glycosylamines. The acid-labile glycosylamine derivative is then hydrolysed to produce the free glycan. As in the hydrazinolysis protocol, the glycoprotein or glycopeptide samples are prepared by removing salts, detergent and dyes that could interfere with the release by dialysis against water or 0.1 % TFA. Released glycans have free reducing termini to allow for fluorescent tagging by reductive amination and analysis by HILIC-(U)HPLC-ESI-MS.

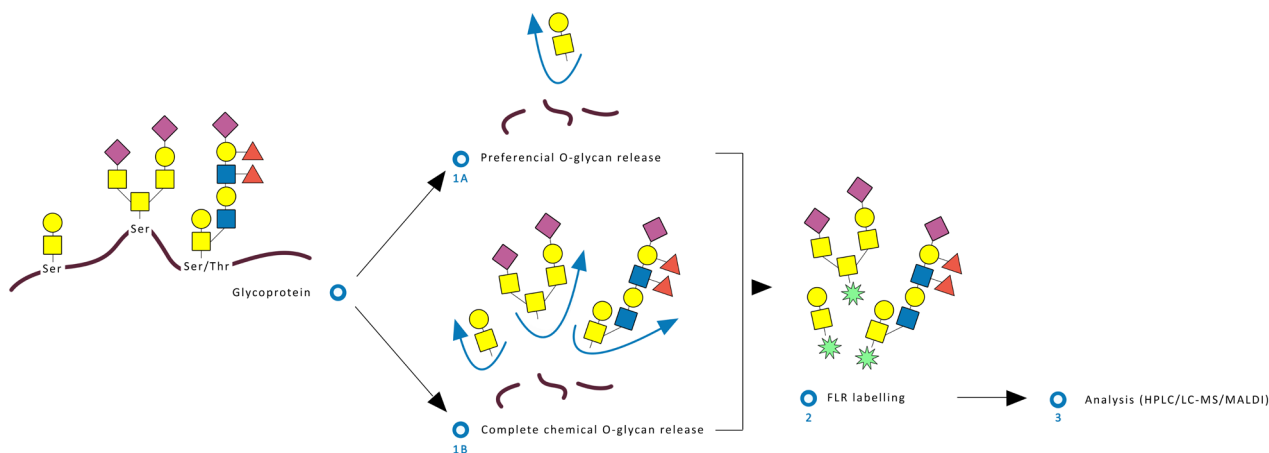


Figure 3. O-glycosylation analysis workflow.

What information is obtained by using Ludger kits and Standards?

The released O-glycans can be fluorescently labelled and analysed by HILIC-HPLC or LC-ESI-MS/MS.

The HILIC-HPLC data provides:

- I. Glucose unit (GU) values for profile comparison between batches where glycan separation is roughly based on glycan size.
- II. Relative quantitation of glycan peaks generated (relative % areas) from fluorescence LC data.
- III. Preliminary structural assignments by comparison against Ludger standards.

The ESI-MS and MS/MS data and provides:

- IV. m/z values
- V. mass composition data matched to LC-separated glycan peaks
- VI. possible glycan structures from matching m/z values from MS/MS with database of glycans

Results

Figure 4 shows HILIC-UPLC profiles for hydrazine released O-glycans from fetuin standard. The data is shown in Table 1.

The fetuin sample contains the following O-glycans at the expected GU values: core 1, Gal α 1-3GalNAc at GU 1.48; mono-sialylated and di-sialylated core 1, Neu5Ac α 2-3Gal β 1-3GalNAc at GU 2.32; Neu5Ac α 2-6(Gal β 1-3)GalNAc at GU 2.97; di-sialylated core 1 O-glycan Neu5Ac α 2-3Gal β 1-3(Neu5Ac α 2-6)GalNAc at GU 3.55; along with some peeled product*, Neu5Ac α 2-3Gal β 1-3Gal at GU 1.69. Fetuin also contains a small amount of the di-sialylated core 2 structure, Neu5Ac α 2-3Gal β 1-3(Neu5Ac α 2-3Gal β 1-4GlcNAc β 1-6)GalNAc at GU 4.72.

*peeling = site reaction that may be defined as stepwise degradation of the polysaccharide starting at the reducing end and removing one sugar residue at a time.

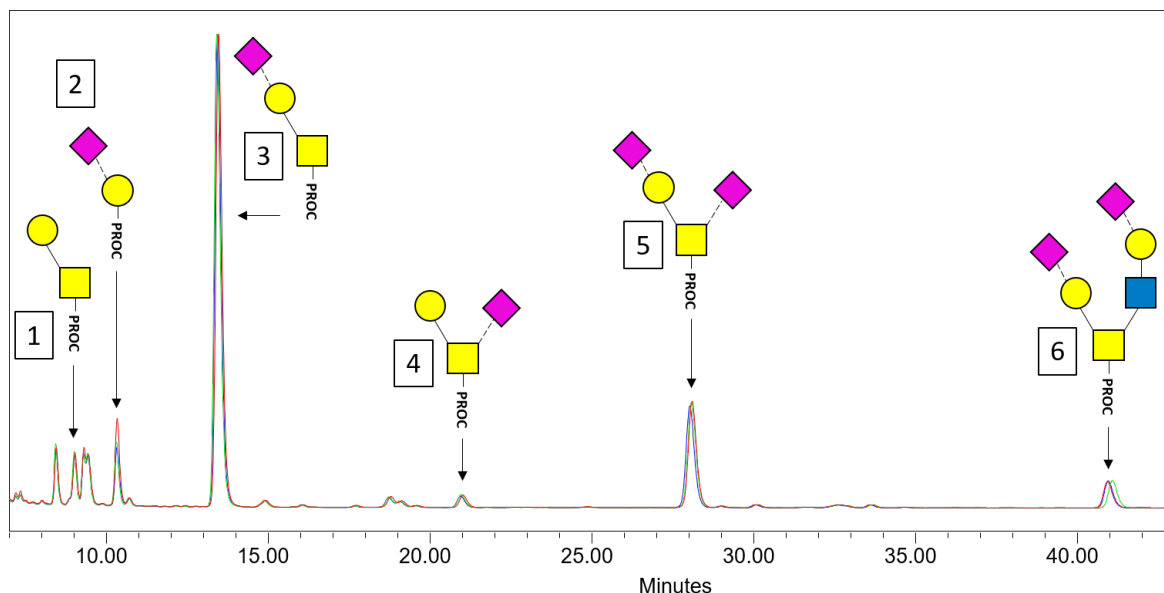


Figure 4. HILIC-UPLC profiles for hydrazine released fetuin O-glycans.

Peak id	Structure	Fetuin-PROC-O-Hy										
		A		B		C		Average	STDev	Average	STDev	CV
		GU	% Area	GU	% Area	GU	% Area	GU			% Area	
1		1.52	4.85	1.51	5.02	1.52	4.79	1.52	0.01	4.89	0.12	2.47
2		1.85	8.35	1.84	6.12	1.85	5.89	1.85	0.01	6.79	1.36	20.01
3		2.51	65.57	2.50	67.06	2.51	68.08	2.51	0.01	66.90	1.27	1.89
4		3.17	2.12	3.16	2.22	3.16	2.06	3.16	0.01	2.13	0.08	3.75
5		3.90	19.11	3.90	19.58	3.89	19.18	3.90	0.01	19.29	0.26	1.32
6		5.13	5.13	5.15	5.43	5.14	5.41	5.14	0.01	5.32	0.17	3.18

Table 1. HILIC-UPLC data for hydrazine released and procainamide labelled O-glycans from Fetuin replicates (A, B, C).

Product/Ordering Information

Glycan Release

E-G001

O-glycosidase for removal of unsubstituted Gal β 1,3-GalNAc α disaccharides attached to the Ser or Thr residues

LL-HYDRAZ-A2

Ludger Liberate Hydrazinolysis N- and O-glycan release and cleanup kit

LL-ORELA-A2

Ludger Liberate Orela O-glycan release and cleanup kit

Glycan Labelling

LT-KAB-A2

LudgerTag 2-AB (2-aminobenzamide) Glycan Labeling Kit

LT-KAB-VP24

LudgerTag 2-AB (2-aminobenzamide) Glycan Labeling Kit containing 2-picoline borane

LT-KAB-VP96

LudgerTag 2-AB (2-aminobenzamide) Glycan High Throughput Labeling Kit containing 2-picoline borane

LT-KAA-A2

LudgerTag 2-AA (2-aminobenzoic acid) Glycan Labeling Kit

LT-KAA-VP24

LudgerTag 2-AA (2-aminobenzoic acid) Glycan Labeling Kit containing 2-picoline borane

LT-KPROC-VP24

LudgerTag Procainamide Glycan Labeling Kit with 2-picoline borane

LT-KPROC-24

LudgerTag Procainamide Glycan Labeling Kit with sodium cyanoborohydride

LT-KPROC-96

LudgerTag Procainamide Glycan Labeling Kit with sodium cyanoborohydride

Glycan Clean Up

LC-S-A6

LudgerClean S Glycan Cleanup Cartridges

Process Controls

GCP-FET-250U

Fetuin Glycoprotein Standard

2-AB Labelled O-glycan Standards

CAB-C1-01

2AB labelled core 1 O- glycan, 100pmol

CAB-C1S(3)1-01

2AB labelled sialylated core 1 O glycan, 100pmol

CAB-C1S(3)1-02

2AB labelled sialylated core 1 O glycan, 50pmol

CAB-C1S(3,6)2-01

2AB labelled di-sialylated core 1 O glycan, 100pmol

CAB-C2S(3,3)2-01

2AB labelled di-sialylated core 2 O glycan, 100pmol

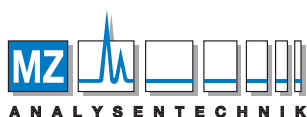
Fetuin O-glycan library

CLIBO-FETUIN-01

A library of O-glycans released from 30 μ g fetuin glycoprotein



LL-ORELA-A2 - Ludger Liberate Orela O-glycan release and cleanup kit



AUTHORIZED DISTRIBUTOR

MZ-Analysentechnik GmbH, Barcelona-Allee 17 • D-55129 Mainz

Tel +49 6131 880 96-0, Fax +49 6131 880 96-20

e-mail: info@mz-at.de, www.mz-at.de

