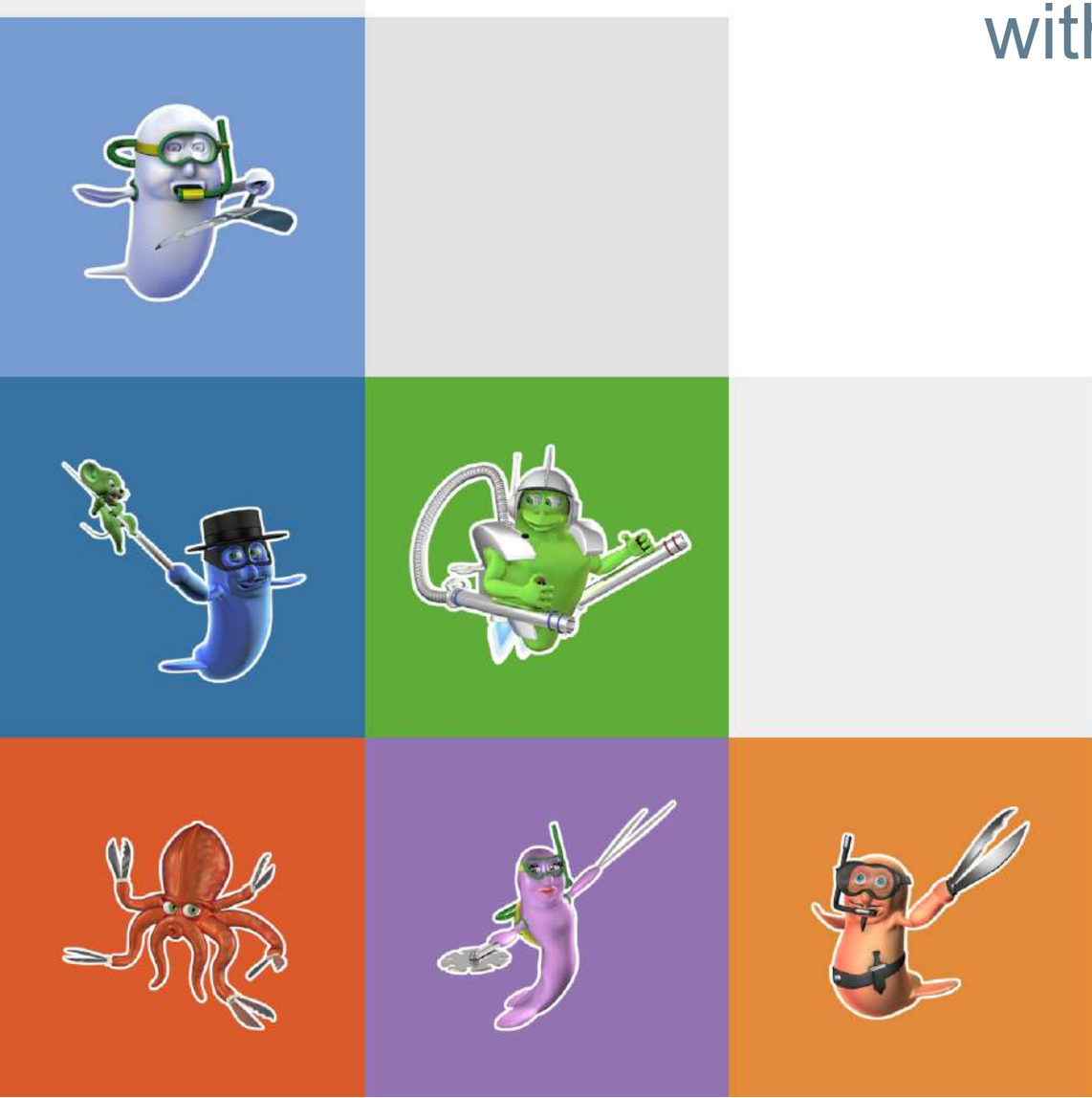


An *O*-glycan Specific Endoprotease with Applications in Glycoprotein Analysis using LC-MS

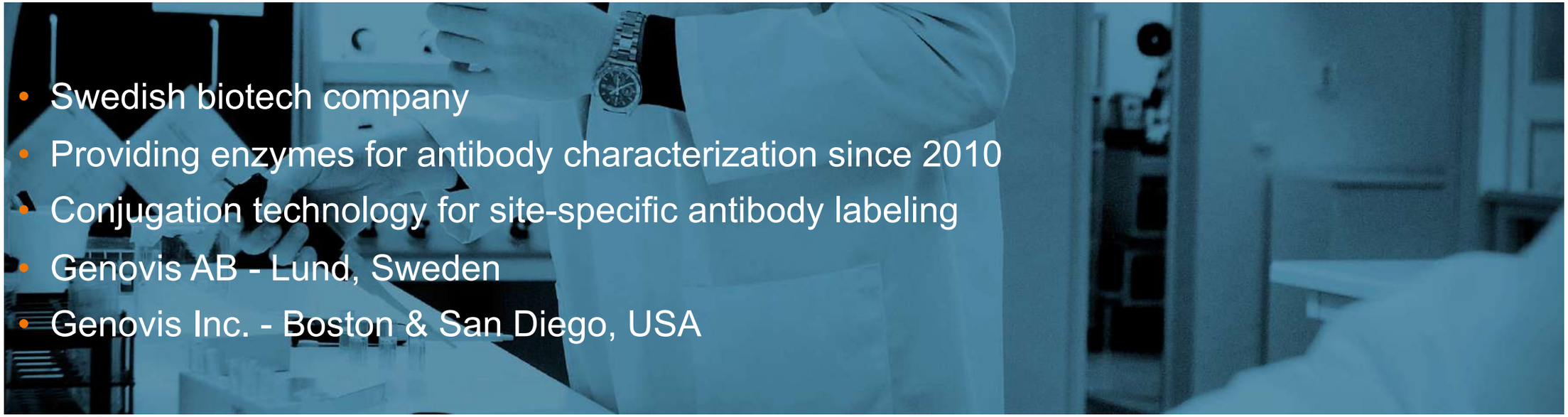
GlycoBioTec 2019

Andreas Nägeli, PhD

Senior Scientist

Genovis AB

Genovis Overview



- Swedish biotech company
- Providing enzymes for antibody characterization since 2010
- Conjugation technology for site-specific antibody labeling
- Genovis AB - Lund, Sweden
- Genovis Inc. - Boston & San Diego, USA

SmartEnzymes™

PROTEASES

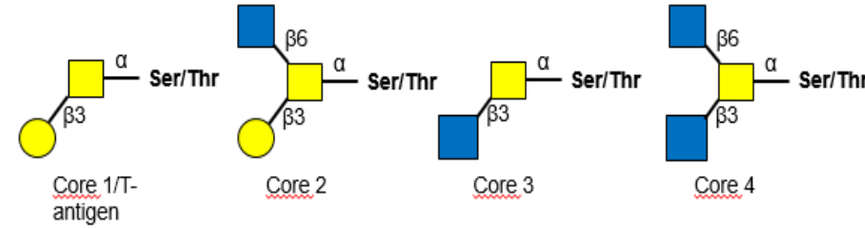
GLYCOSIDASES

ANTIBODY LABELLING

O-GLYCAN ENZYMES

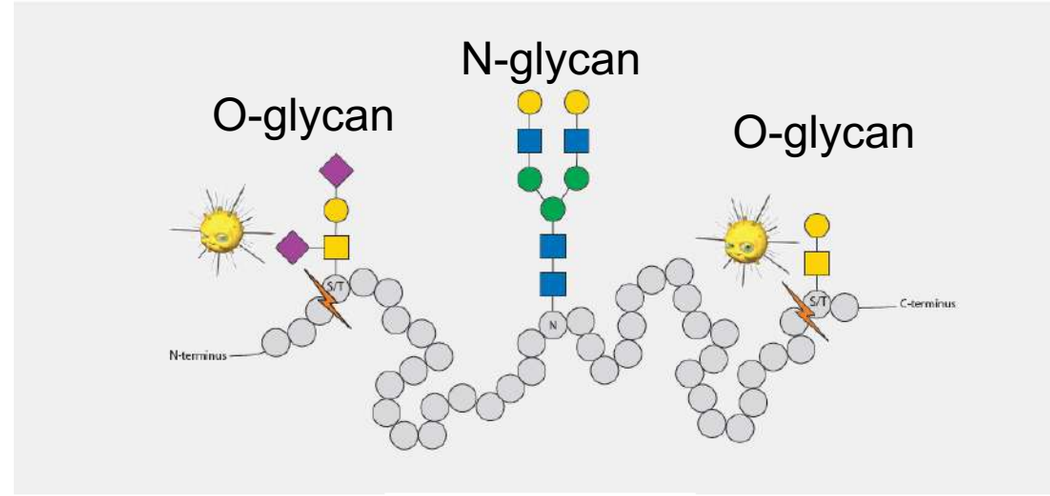
O-glycosylation – a difficult PTM to analyze

- Structurally diverse
- Labile which makes MS analysis difficult
- Analytical toolbox is limited
  - Few O-glycan specific enzymes
  - Chemical methods are laborious
- Standard digestion (trypsin, Lys-c etc) difficult on heavily *O*-glycosylated proteins
- *O*-glycosylated biopharmaceuticals (Etanercept, EPO, hCGβ, C1inh etc.) are difficult to analyse/quality control



OgpA – *O*-glycoprotease from *A. muciniphila*

- *O*-glycoprotein specific protease
- Recognizes mucin type *O*-glycans and hydrolyzes glycoprotein N-terminally to the glycosylated serine or threonine residue
- Cleaves at core 1 and core 2 glycosites
- Higher activity on non-sialylated *O*-glycosylation sites
- No activity on *N*-glycosylation sites

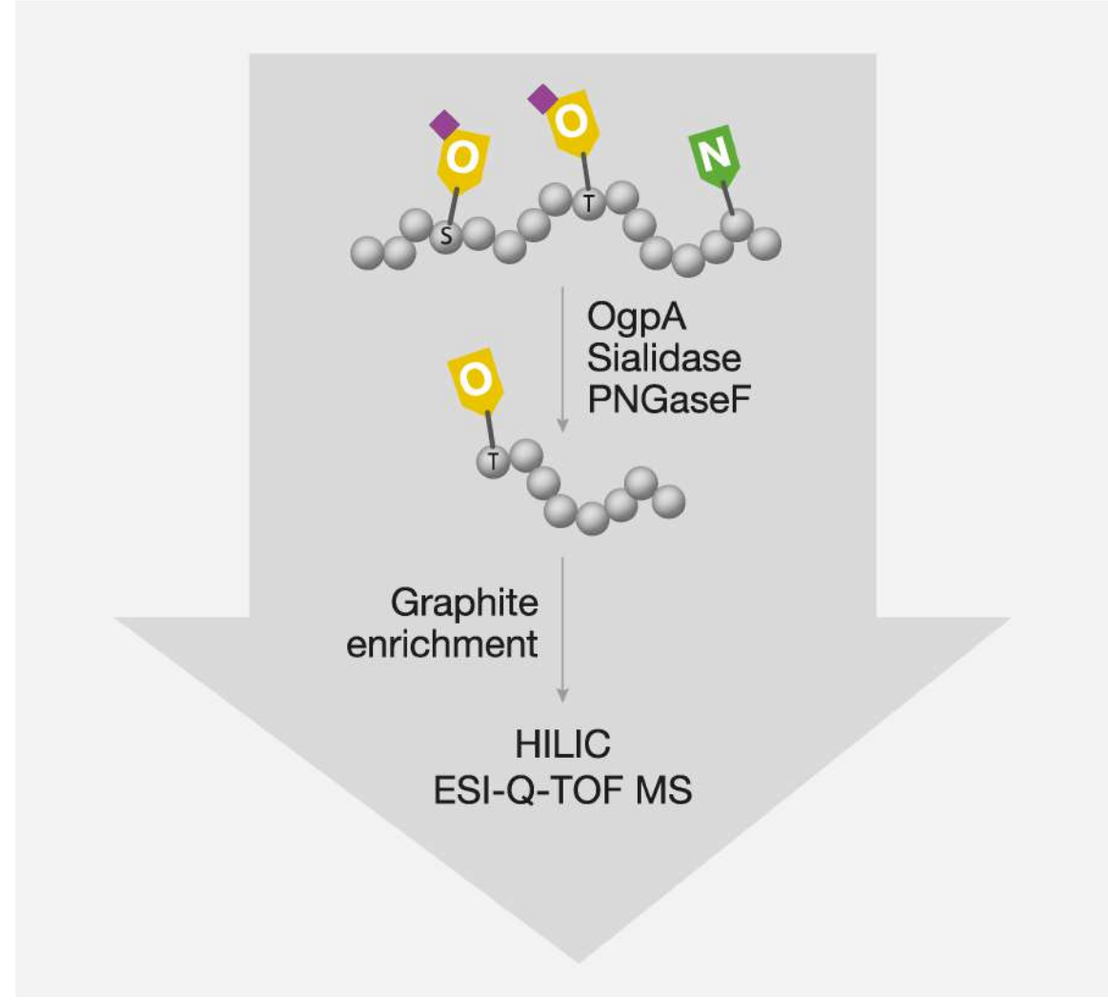


OpeRATOR™

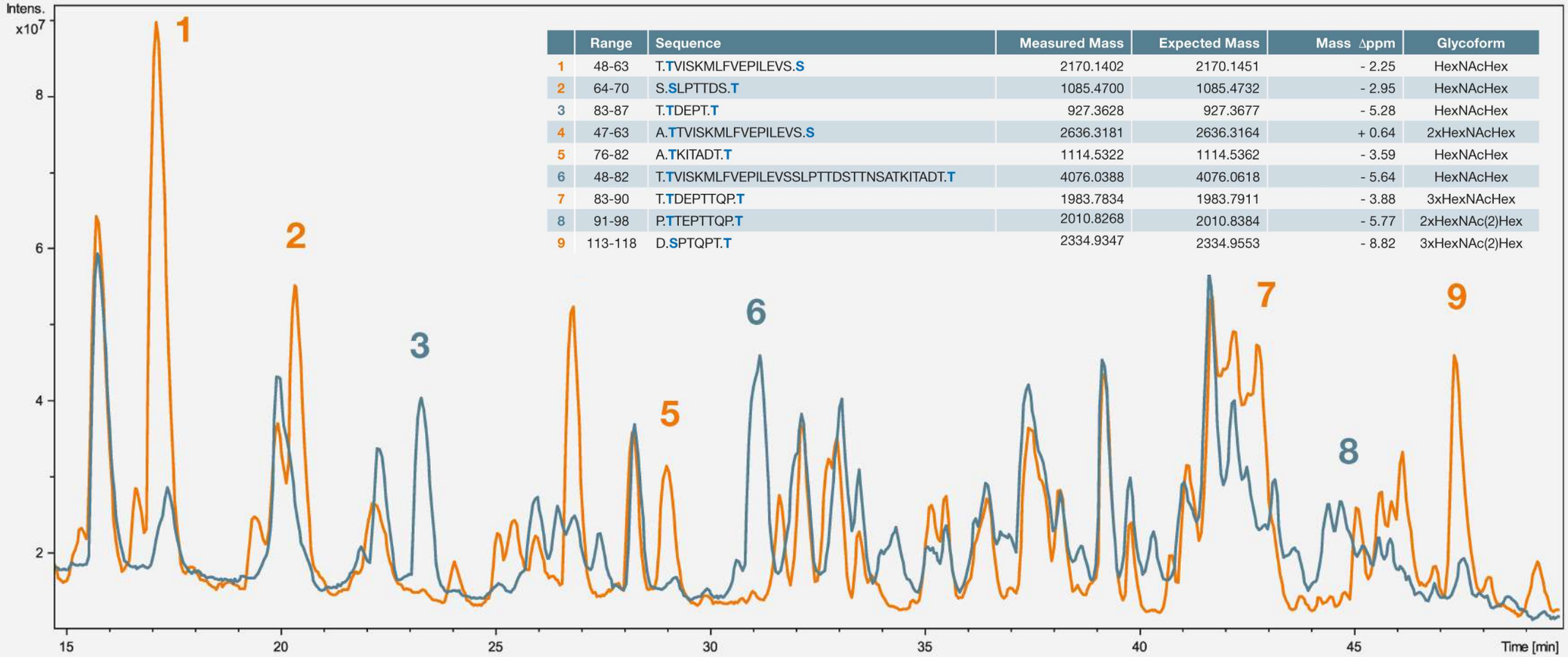
OgpA-based workflow for analysis of *O*-glycoproteins

Human C1 inhibitor

- 478 amino acids
- 6 *N*- and up to 26 *O*-glycosylation sites
- Mostly core 1 *O*-glycans with a small amount of core 2
- Two available biopharmaceuticals: recombinant and plasma-derived

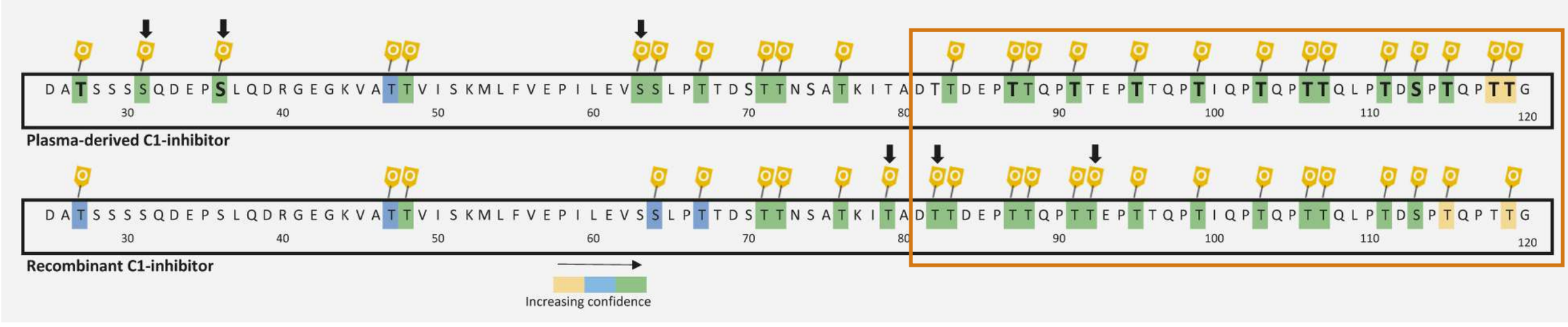


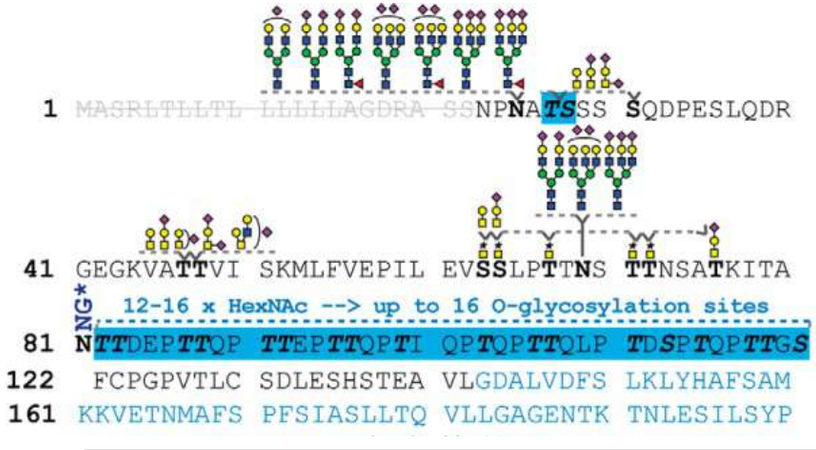
Quantitative comparison of C1 inhibitor *O*-glycosylation



	Range	Sequence	Measured Mass	Expected Mass	Mass Δppm	Glycoform
1	48-63	T.TVISKMLFVEPILEVS.S	2170.1402	2170.1451	- 2.25	HexNAcHex
2	64-70	S.SLPTTDS.T	1085.4700	1085.4732	- 2.95	HexNAcHex
3	83-87	T.TDEPT.T	927.3628	927.3677	- 5.28	HexNAcHex
4	47-63	A.TTVISKMLFVEPILEVS.S	2636.3181	2636.3164	+ 0.64	2xHexNAcHex
5	76-82	A.TKITADT.T	1114.5322	1114.5362	- 3.59	HexNAcHex
6	48-82	T.TVISKMLFVEPILEVSSLPTTDSTTNSATKITADT.T	4076.0388	4076.0618	- 5.64	HexNAcHex
7	83-90	T.TDEPTTQP.T	1983.7834	1983.7911	- 3.88	3xHexNAcHex
8	91-98	P.TTEPTTQP.T	2010.8268	2010.8384	- 5.77	2xHexNAc(2)Hex
9	113-118	D.SPTQPT.T	2334.9347	2334.9553	- 8.82	3xHexNAc(2)Hex

In-depth mapping of *O*-glycosylation sites of human C1 inhibitor





- Two birds with one stone:
  - OgpA generates *O*-glycopeptides specifically
  - *O*-glycosylation sites can be determined from the digestion sites rather than ETD fragmentation

Stavenhagen et al., N- and O-glycosylation Analysis of Human C1-inhibitor Reveals Extensive Mucin-type O-Glycosylation. Mol Cell Proteomics. 2018 Jun; 17(6): 1225–1238.

OgpA in the literature

analytical chemistry

Article

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Deciphering Protein *O*-Glycosylation: Solid-Phase Chemoenzymatic Cleavage and Enrichment

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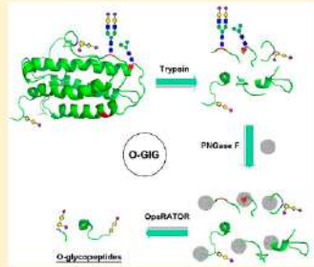
Supporting Information

ABSTRACT: Glycosylation plays a critical role in the biosynthetic-secretory pathway in the endoplasmic reticulum (ER) and Golgi apparatus. Over 50% of mammalian cellular proteins are typically glycosylated; this modification is involved in a wide range of biological functions such as barrier formation against intestinal microbes and serves as signaling molecules for selectins and galectins in the innate immune system. *N*-linked glycosylation analysis has been greatly facilitated owing to a range of specific enzymes available for their release. However, system-wide analysis on *O*-linked glycosylation remains a challenge due to the lack of equivalent enzymes and the inherent structural heterogeneity of *O*-glycans. Although *O*-glycosidase can catalyze the removal of core 1 and core 3 *O*-linked disaccharides from glycoproteins, analysis of other types of *O*-glycans remains difficult, particularly when residing on glycopeptides. Here, we describe a novel chemoenzymatic approach driven by a newly available *O*-protease and solid phase platform. This method enables the assignment of *O*-glycosylated peptides, *N*-glycan profile, sialyl *O*-glycopeptides linkage, and mapping of heterogeneous *O*-glycosylation. For the first time, we can analyze intact *O*-glycopeptides generated by *O*-protease and enriched using a solid-phase platform. We establish the method on standard glycoproteins, confirming known *O*-glycosites with high accuracy and confidence, and reveal up to 8-fold more glycosites than previously reported with concomitant increased heterogeneity. This technique is further applied for analysis of Zika virus recombinant glycoproteins, revealing their dominant *O*-glycosites and setting a basis set of *O*-glycosylation tracts in these important viral antigens. Our approach can serve as a benchmark for the investigation of protein *O*-glycosylation in diseases and other biomedical contexts. This method should become an indispensable tool for investigations where *O*-glycosylation is central.



Published online: November 20, 2018

Method

TRANSPARENT PROCESS OPEN ACCESS

molecular systems biology

Mapping the O-glycoproteome using site-specific extraction of O-linked glycopeptides (EXoO)

Weiming Yang, Minghui Ao, Yingwei Hu, Qing Kay Li & Hui Zhang*

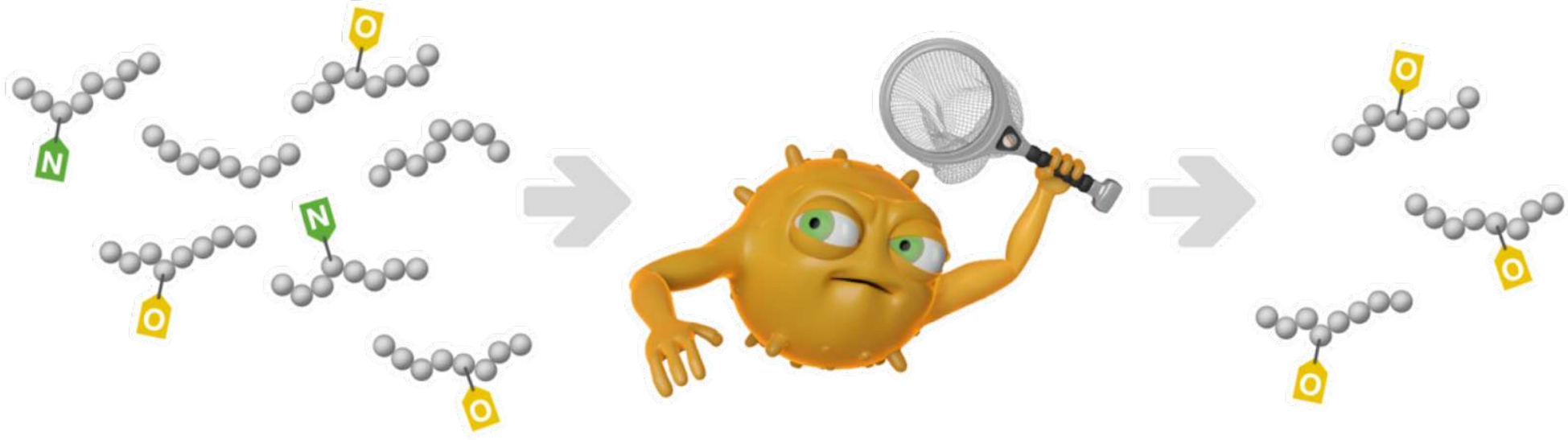
Abstract

Protein glycosylation is one of the most abundant post-translational modifications. However, detailed analysis of O-linked glycosylation, a major type of protein glycosylation, has been severely impeded by the scarcity of suitable methodologies. Here, a chemoenzymatic method is introduced for the site-specific extraction of O-linked glycopeptides (EXoO), which enabled the mapping of over 3,000 O-linked glycosylation sites and definition of their glycans on over 1,000 proteins in human kidney tissues, T cells, and serum. This large-scale localization of O-linked glycosylation sites demonstrated that EXoO is an effective method for defining the site-specific O-linked glycoproteome in different types of sample. Detailed structural analysis of the sites identified revealed conserved motifs and topological orientations facing extracellular space, the cell surface, the lumen of the Golgi, and the endoplasmic reticulum (ER). EXoO was also able to reveal significant differences in the O-linked glycoproteome of tumor and normal kidney tissues pointing to its broader use in clinical diagnostics and therapeutics.

(O-GalNAc) addition is a major type (Jensen *et al*, 2010; Chia *et al*, 2016; Darula & Medzihradszky, 2018). Definitive characterization of O-linked glycoproteins requires quantitative analysis of O-linked glycosylation sites and their corresponding glycans. In contrast to N-linked glycosylation, where a consensus glycosylation motif has been identified, there is no consensus O-linked glycosylation motif for the amino acid residues surrounding the glycosylated Ser or Thr (Nishikawa *et al*, 2010; Darula & Medzihradszky, 2018). The cellular machinery for O-linked glycosylation, located primarily in the Golgi apparatus, is believed to operate stochastically in response to changes in a wide range of both intrinsic and extrinsic factors (Chia *et al*, 2014; Bard & Chia, 2016). The presence of up to 20 GalNAc-transferases (GalNAc-Ts) for adding the initial sugar to amino acid residues in different sequence regions further complicates the dynamic regulation of O-linked glycosylation (Bennett *et al*, 2012). As a consequence, O-linked glycosylation can exhibit high heterogeneity in different cells, tissues, and diseases (Steentoft *et al*, 2013; Medzihradszky *et al*, 2015).

The enrichment of O-linked glycopeptides from complex biological samples is an essential prerequisite to definitive identification of

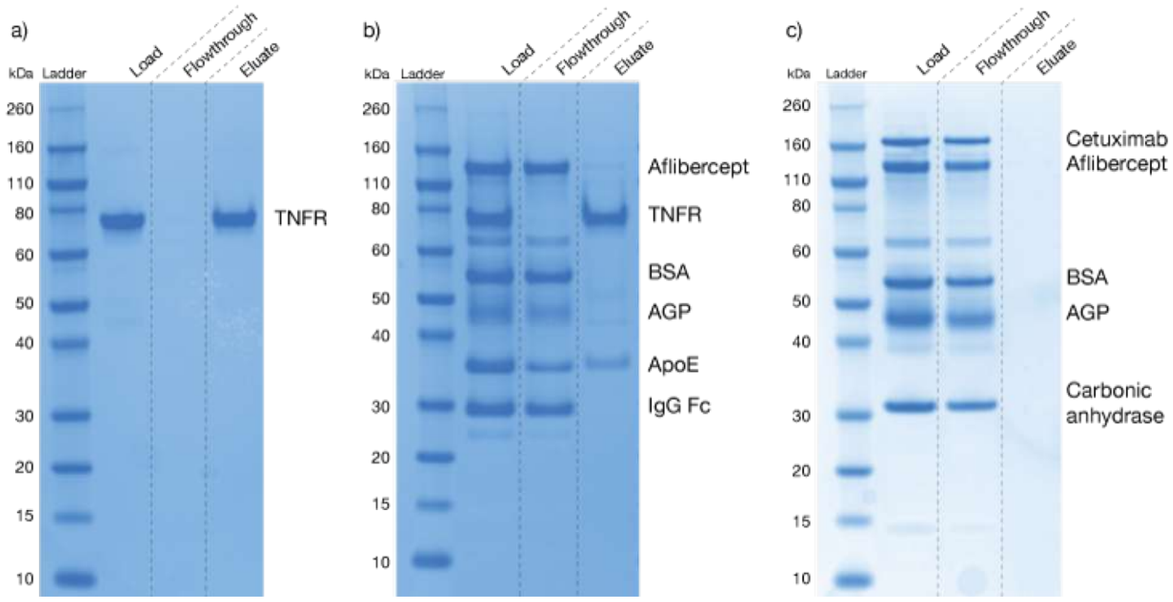
GlycOCATCH™



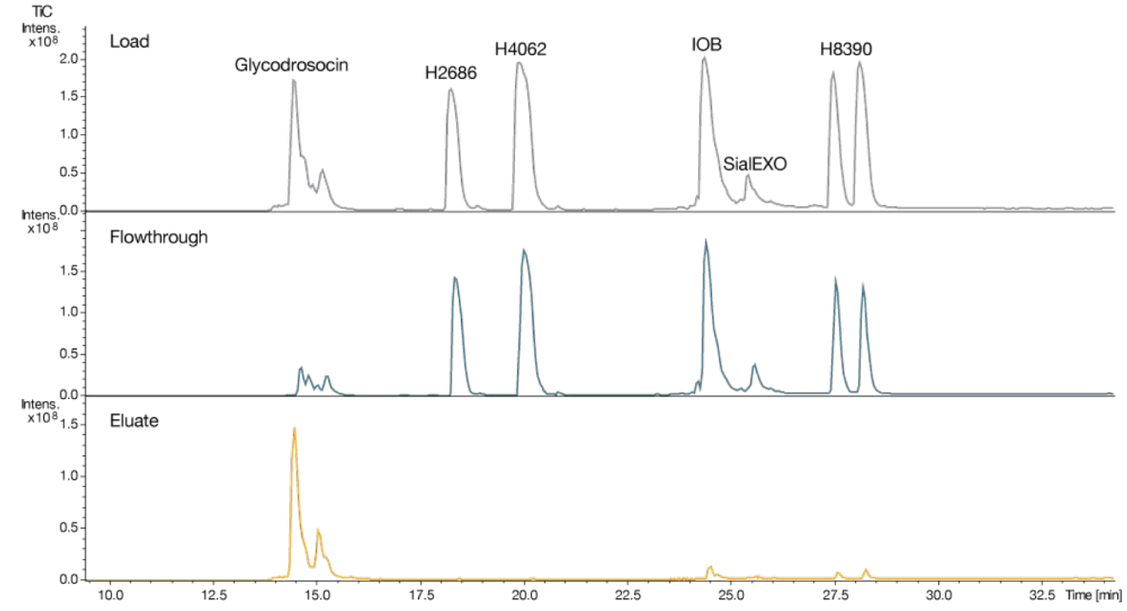
Enrichment of *O*-glycosylated Proteins and Peptides

Selective enrichment of *O*-glycosylated proteins and peptides

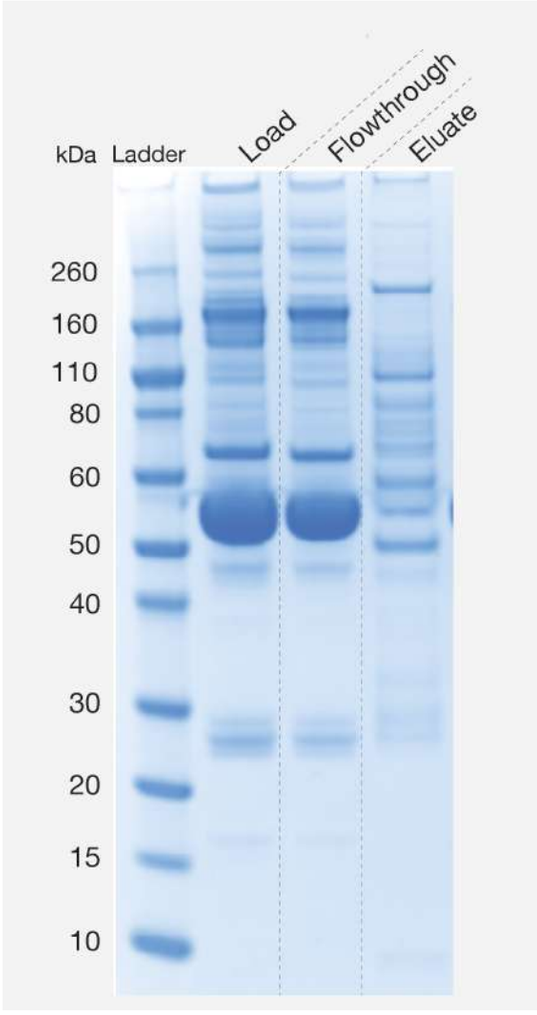
Proteins



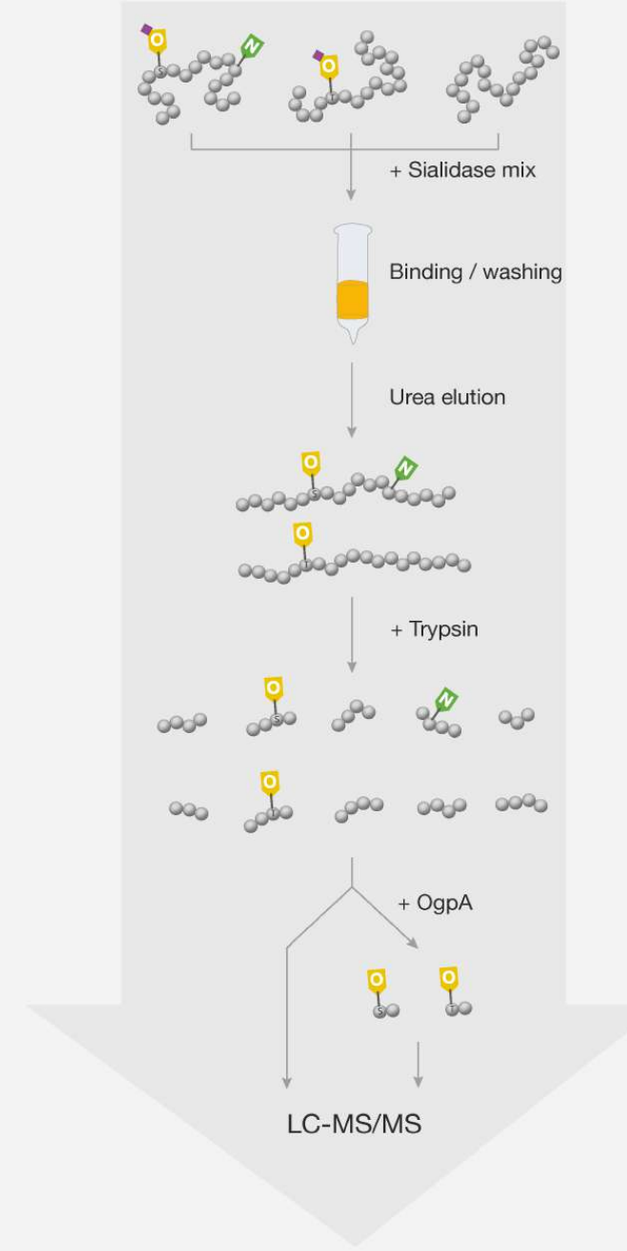
Peptides



Enrichment of *O*-glycosylated proteins from serum



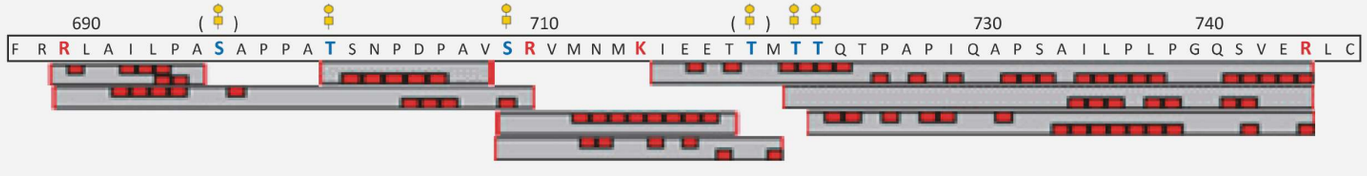
- *O*-glycosylated proteins are specifically enriched using the GlycOCATCH resin



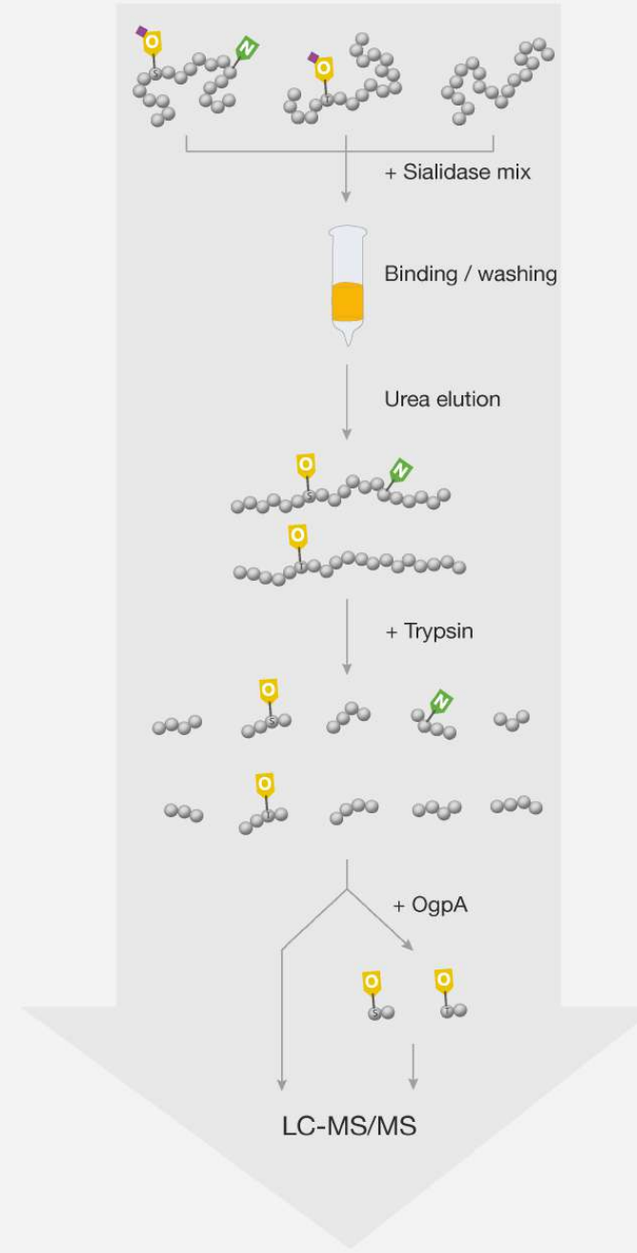
Enrichment of *O*-glycosylated proteins from serum

- Mapping of *O*-glycosylation of very low abundant serum proteins

Inter-alpha-trypsin inhibitor heavy chain H4 (5ng/ml)



Range	Sequence	Measured Mass	Z	Expected Mass	Mass Δppm	Glycosylation site	Indicated Glycosylation site	Glycoform
689 - 695	R.RLAILPA.S	377.2493	2	752.4909	-8.97		S696, T701, S702, S709	
690 - 710	R.LAILPASAPPATSNPDPAVSR.V	925.7851	3	2774.3597	-9.45			2 x HexNAc Hex
701 - 708	A.TSNPDPAV.S	583.2566	2	1164.5034	-4.06	T 701		HexNAc Hex
709 - 719	V.SRVMNMKIEET.T	568.2657	3	1701.7801	-2.82	S 709	T 720	HexNAc Hex
709 - 721	V.SRVMNMKIEETTM.T	645.6276	3	1933.8683	-3.73	S 709	T 722	HexNAc Hex
716 - 745	K.IEETTMTTQTPAPIQAPSAILPLPGQSVER.L	1181.2581	3	3540.7757	-6.54		T720, T 722, T723	HexNAc Hex
722 - 745	M.TTQTPAPIQAPSAILPLPGQSVER.L	801.4025	4	3201.6028	-6.86	T 722		2 x HexNAc Hex
723 - 745	T.TQTPAPIQAPSAILPLPGQSVER.L	912.8074	3	2735.4229	-8.27	T 723		HexNAc Hex



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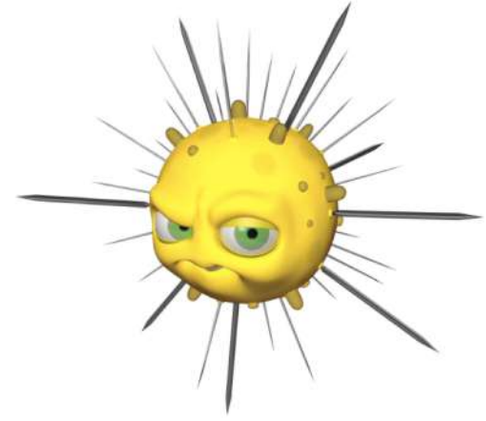

OpeRATOR™

… an O-glycoprotease for in-depth analysis of *O*-glycosylated proteins

GlycOCATCH™

… an affinity resin for selective enrichment of *O*-glycoproteins