Improved strategies for enhanced protein characterization of highly glycosylated molecules

CASSS AT, Lisbon
24th May 2022

Philip Widdowson, Ph. D
Product Specialist
philip.widdowson@genovis.com
Utilize the power of nature for the benefit of patients

Genovis provide reagents and technologies that advance science, enable early diagnostics, and accelerate new biologics therapies for patients in need
On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database

Rolf Appellaar, Jaakko Jormakka, Nathan Stansel

Show more

Add to Mendeley Share Cite

https://doi.org/10.1016/S0006-8993(99)00069-8

Abstract

The SWISS-PROT protein sequence data bank contains at present nearly 75,000 entries, almost two thirds of which include the potential N-glycosylation consensus sequence, or sequon, NXST (where X can be any amino acid but proline) and thus may be glycoproteins. The number of proteins listed as glycoproteins is however considerably smaller, 7942, of which 749 have been characterized with respect to the total number of their carbohydrate units and sites of attachment of the latter to the protein, as well as the nature of the carbohydrate-peptide linking group. Of these well characterized glycoproteins, about 90% carry either N-linked carbohydrate units alone or both N- and O-linked ones, attached at 1297 N-glycosylation sites (1.9 per glycoprotein molecule) and the rest are O-glycosylated only. Since the total number of sequons in the well characterized glycoproteins is 1968, their rate of occupancy is 2/3. Assuming that the same number of N-linked units and rate of sequon occupancy occur in all sequon containing proteins and that the proportion of solely O-glycosylated proteins (ca. 10%) will be the same as among the well characterized ones, we conclude that the majority of sequon containing proteins will be found to be glycosylated and that more than half of all proteins are glycoproteins.

Challenges Associated with Glycans

- Glycans are complex and heterogeneous modifications
- Existence of many isobaric structures
  - Composition: Glucose vs galactose vs mannose → all 162 Da
  - Configuration: α vs β linkage
  - Connectivity: Position of the glycosidic bond (β1-3 vs β1-4 linkage)
- Presence of glycans present challenges in the analysis of other protein modifications
  - Glycan heterogeneity can ‘mask’ other PTMs
  - Removal of glycans can improve characterization of the protein
Tools for characterization of complex glycoproteins
Difficulties with O-glycosylation analysis

- Standard digestion (trypsin, Lys-C) difficult for heavy O-glycosylation
- O-glycans are complex and variable
- Analytical toolbox is limited:
  Few O-glycan specific enzymes
  Chemical methods are laborious
OpeRATOR®
O-glycan-specific protein digestion

- O-glycoprotein specific protease
- Hydrolyzes glycoproteins N-terminally to the O-glycosylated serine or threonine residues
- Significantly reduced activity on sialylated O-glycosylation sites
• All O-glycosylation sites of etanercept could be identified
• No enrichment needed
• Site-specific mapping without ETD fragmentation
Quantitative comparison of O-glycosylation patterns in etanercept

Comparison between originator and biosimilars

- All three peptides stem from the same region and suggest underglycosylation of one residue (216) in the biosimilar
- Quantitative differences mapped site-specifically
Tools for characterization of complex glycoproteins

SialEXO®
SialEXO®
SialEXO®
PNGase F
OglyZOR®
• SialEXO is a broad acting sialidase
• Immobilized SialEXO available for on column desialylation
  • No enzyme in resulting samples
• Improved separation in icIEF
Tools for characterization of complex glycoproteins

SmartEnzymes
• LC-MS of Fc/2 shows hydrolysis of galactose
• Released N-glycan analysis confirms complete removal of galactose
• Detection of underlying modifications - glycation
Tools for characterization of complex glycoproteins

SmartEnzymes

GalactEXO™

SialEXO®

FucosEXO™

GalactEXO™

SialEXO®

GalactEXO™

OglyZOR®

GalNAcEXO™

PNGase F

Not this one!
Etanercept case study

- Fusion protein of TNFR and IgG1 Fc region
- 6 N- and up to 24 O-glycans
- Heterogeneous and difficult to characterize
Subunit analysis of an Fc fusion protein

- Separate TNFR from Fc region by FabRICATOR digestion
- Manageable size and complexity
Total Deglycosylation of Etanercept

- Remove glycan heterogeneity for accurate mass determination and analysis of underlying variants
- Fast and simple one-step sample preparation using immobilized enzymes
Total Deglycosylation of Etanercept

- Complete deglycosylation
- Comparison of originator and biosimilar etanercept
- Highlights the benefit of subunit analysis
Summary

• A variety of Genovis SmartEnzymes available for N- and O-glycan processing
  • Glycan trimming
  • Deglycosylation
  • O-glycan mapping

• Simplified characterization of protein modifications
  • Removal of heterogeneous glycans enhances ability to observe and analyse some PTMs
  • Allows batch-to-batch comparability
  • Originator vs biosimilar

• Versatile and application specific analysis
  • Sample preparation tools to support a range of analyses
  • SmartEnzymes can be used in combination
Acknowledgements

• Helén Nyhlén
• Hanna Toftevall
• Andreas Nägeli
• Andrea Persson
• Erica Andersson
• Olivia Eliasson
• Magdalena Widgren Sandberg

• Rolf Lood
• Maria Nordgren
• Fredrik Leo
• Emil Åberg
• Pearse Hall

• Linda Andersson
• Kristina Nordén
• Therese Lindvall
• Anna Rosengren
• Anna-Karin Carlshaf
• Rawya Antar

• Maria Ekemohn
• Jonathan Sjögren
• Fredrik Olsson
Resources – www.genovis.com

SmartEnzymes Applications

- Isomerization
- Glycation
- Deamidation
- Subunit Analysis
- Fragmentation
- Site-specific Conjugation
- Oxidation
- Aggregation
- Fab-glycosylation
- Pyroglutamation
- ADC Characterization
- O-glycan Analysis
- O-glycan Hydrolysis
- O-glycan Mapping
- Fc-glycan Analysis
- Galactosylation Assay
- Hydrolysis of Gal
- Hydrolase of Gal
- Charge Variant Analysis
- Stalic Acid Analysis
- Sialic Acid Linkage Analysis
- Oxidation
- Sialic Acid Analysis
- Fc-glycan Analysis
- Unmasking Charge Variants
- Tn Antigen
- N-Terminal

SmartStories

- Weiming Yang
  Johns Hopkins University
- Antibody mimetics generation using GingisKHAN
- Hanlieh Khalili
  University of East London
- OpACRATOR changes the game in the field of O-linked glycoproteomics
- Bastiaan Duivelshof
  University of Geneva
- Generating site-specific ADCs using the GlyCLICK technology
- Min Kyung So
  KOBO Osong Medical Innovation Foundation
- FabRICATOR for Intact Analysis of Biologics
- Dan Bach
  Kristensen
  Symphosan A/S
- FabALACTICA in non-reducing study of antibody disulphides
- Vahid Faid
  LFB Biotechnologies

23 SmartEnzymes