8130 Exam—Curved 5pts for Mean 82 / Median 81

Glyco-challenged

GLYCO-LITERATE

# of Grades in Bin

Bin Endpoint

55 60 65 70 75 80 85 90 95 100

Glyco-literacy

Complex Carbohydrate Research Center,
Department of Biochemistry & Molecular Biology
University of Georgia
Requirements for Structural Determination of a Carbohydrate

- Identification of sugars.
- Stereochemistry of each sugar.
- Types of linkages.
- Types of ring structures.
- Anomeric configuration of each sugar.
- Sequence of the different sugar residues.

Determining a Carbohydrate Sequence

Glycoconjugate
- Glycosyl Composition
- Glycosyl Linkages
- NMR Spectroscopy

Enzymatic
Chemical

Oligosaccharides
- Separation
- Glycosyl Composition
- Glycosyl Linkages
- NMR Spectroscopy
- Mass Spectrometry

Sequence of Oligosaccharides
Compositional Analysis Using Derivitized Glycans
often done by GLC Separation
—Azadi Summer Course

Preparation of Alditol Acetates

Preparation of Trimethylsilyl (TMS) Methyl Glycosides

HPAE-PAD Detection of Monosaccharides

Figure 2: Non-extended O-Man on Proteins from WT and POMGnT1 Knock-out mice. Monosaccharides were released from protein, separated, and detected from POMGnT1 +/- and +/- mouse brains. From the two data sets, it appears that proteins carrying non-extended O-Man structures are enriched ~2.4-fold in the POMGnT1 +/- animals.
**Glycosyl Linkages by Preparing Partially Methylated Alditol Acetates (PMAAs)**

GLC/GC Analysis
--Also taught in Azadi’s Summer Courses

**Tandem Mass Spectrometry-Based Approaches For the Characterization of Glycopeptides**
Site Mapping and Characterization
-Glycans, N-linked Sites, O-linked Sites

Current Efforts: Glycopeptides

Block diagram of a mass spectrometer

All MS do one thing: Measure m/z
Glycan Release/Permethylation of Glycans

- Trypsin digestion of protein
- Enzymatic release of N-glycans
- β-elimination for O-linked- additon of NaOH
  - resulting in release of glycan structure from hydroxyl of Ser or Thr
  - Reduction with NaBH₄ prevents re-attaching of glycan
- Permethylation of glycan- OH→OMe
  - Addition of MeI
- Analyzed permethylated glycans by applying MSn fragmentation as needed to completely determine the structure

![Glycan Structure](image1)

![Per-O-Methylated Carbohydrate](image2)

Glycan Analysis

Starting Material → Homogenization and organic extraction → Precipitate proteins by centrifugation

Reduction/amination → Trypsin digestion → Glycoproteins + Peptides

Glycosphingolipids → Cerebroside glycanase digestion → Glycosphingolipid Glycans + N-linked Glycans

Pyridinylation and TSK-amide Chromatography → Permethylated for MALDI-TOF/MS and NSI-LTQ/MS²

N-linked Glycans + Peptides → BEMAD with Thiol enrichment Chromatography

OMe

Permethylated Glycans from wildtype Drosophila embryos

POMGnT1 appears to be essential for O-Man Extension

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<tr>
<th>MW</th>
<th>Structure</th>
<th>-/-</th>
<th>% total</th>
<th>+/+</th>
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Site Mapping and Characterization
-Glycans, N-linked Sites, O-linked Sites

Current Efforts: Glycopeptides

PNGase F Treatment and N-linked Glycosylation Site-mapping

Secreted Proteins from Adipocytes
(insulin responsive vs insulin resistant)

- Sulfated glycoprotein 1 precursor (SGP-1)
- Follistatin-related protein 1 precursor
- Haptoglobin
- Decorin

- Ipprotein lipase
- Follistatin-related protein 1 precursor
- Haptoglobin
- Decorin

56 proteins with 83 N-linked sites
N-linked Site Mapping from ConA-enriched glycopeptides from Drosophila heads--272 sites mapped from 197 Proteins

Pileup of the 272 N-linked Sites to Determine Consensus beyond N-X-S/T

Concerns: PNGase/F/A, Deamidation, C-terminal O-18

PGP N-linked Site Mapping with PNGase F/A

PNGase F  (red = coverage)  3 sites identified

PNGase A (red=coverage)  7 sites identified

High Stringency Filter
Differential isotopic tagging of both cysteine and post-translationally modified ser/thr through β-elimination/Michael addition with light (d0) and heavy (d6) DTT.

Concerns: Specificity, efficiency, recovery

Other Promising Approaches: ECD/ETD
Site Mapping and Characterization
-Where we want to be!

(75%)

(25%)

Alpha-Dystroglycan/Dystrophin Complex
Figure 2. Released O-Man and O-GalNAc linked glycans

Identification of Glycans using TIM-MS

<table>
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<tr>
<th>Structure</th>
<th>Theoretical</th>
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<th>Δ(Th - Obs)</th>
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100.0
The Glycan Problem??: Neutral Loss
Fragmentation of GlcNAc-CTD by CID-MS/MS
Parent ion 535 ([M+2H+] + GlcNAc)

Parent ion 535 (M+2H+) + GlcNAc

CID

HPLC

LTQ ion trap

Mass spectrometer

Nsi

Automated MS/MS

Database

Protein
Nucleotide
ESTs

TurboSEQUEST
Cross-Correlation
Comparison

Peptides sequenced,
Proteins Identified

Peptide mixtures

Capillary Column

Buffer A

Buffer B

Predicted MS/MS

MS

MS/MS

Relative Abundance

GlcNAc

263.8

y6

616.2

b2

259.9

419.3

616.2

y6

819.2

b8

848.8

866.3

848.4

Y--S--P--T--S--P--S--K

b ions - 164 251 348 449 536 633 720 848

866 763 616 519 418 331 234 147 - y ions

O-GlcNAc (204)

NL 7.62e6

Poor Fragmentation

No Site Information
Fragmentation of GlcNAc-CTD by CID-MS/MS/MS

Parent ions 535/866

NL 2.88e6

Y--S--P--T--S--P--S--K

b ions - 164 251 348 449 536 633 720 848

y ions - 866 703 616 519 418 331 234 147

m/z

Relative Abundance

Pseudo Neutral Loss Activated Data

Dependant MS³ for Glycopeptide Mapping

MS Survey Scan

MS/MS scan

Neutral loss?

Top N peaks?

If we have purified protein and glycomics data

http://www.thermo.com
Where we are going next.....

- NL triggered MSn
- “non-NL” triggered MSn
- Further exploring ECD/ETD capabilities
- Enrichment Chromatography and HILIC
- Quantification strategies
Proteomic / Glycomics Quantification

- **Non-isotope**
  (spectral counts, etc.)

- **Total Ion Monitoring¹**
  (normalized peak height/area)

- **In Vitro Isotopic Label**
  ($^{13}$O-H2O, ICAT, BEMAD, etc.)

- **In Vivo Isotopic Label**
  (SILAC)

- **$^{13}$C-CH3¹ ²**
  (in vitro isotopic labeling)

- **IDAWG³**
  (in vivo isotopic labeling)


N- and O-glycan structures determined by “Total-ion monitoring”

- Full MS and TIM spectra obtained
- Spectra analyzed, glycans identified, and quantitated
- Identification based on accurate mass and fragmentation
- Expressed as “% of total” or “Fold change from standard sample”

Relative quantification between 2 samples of released and permethylated N-glycans via isotope labeling with light/heavy iodomethane.
**IDAWG: Isotopic Detection of Aminosugars with Glutamine**

```
Glc
↓
Glc-6-P

Fru-6-P  →  GlcN-6-P  →  GlcN-1-P  →  GlcNAc-1-P  →  UDP-GlcNAc

GLN  GLU  →  Multiple Other AAs

NH4+

GLYCOCONJUGATE

+1 Da
```

**Harvest & Combine**

Homogenization and delipidation

**Mixed Protein Powder**

Tryptic digestion

- C18 reverse phase
- PNGase F digestion

**Peptide Mixture**

- Amide-^{15}N L-glutamine "GLN-15"
- Amide-^{13}N L-glutamine "Gln-14"

**N-linked Glycan Mixture**

O-linked Glycan Mixture

C18 reverse phase

Permethylation

Permethylation

Deionization

**Permethylated Glycans**

**Permethylated Glycans**

**Mass Spectrometry**

Quantification by Full MS

Characterization by MS/MS

Light Medium

Heavy Medium
G. [M+Na]^+; 1256.636 + 1259.627 m/z (mono)

Amide-[^15]N-GLN
Amide-[^14]N-Gln = 0.83
94% incorporation

Glycoanalytics

-Glycan Characterization and Site Mapping
  --robust methods
  --sensitivity & quantification in progress

-Intact glycopeptide analysis
  --very difficult, only pure samples, in infancy