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GLYCOPEPTIDE DISCOVERY AND CHARACTERISATION USING PARENT ION DISCOVERY ON THE Q-TOF

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OVERVIEW

- Glycopeptide analysis on a Q-Tof mass spectrometer using Parent Ion Discovery
- Detection and characterisation of glycopeptides from a fetuin tryptic digest using LC/MS/MS
- Glycosylation site, composition and partial structure characterised in a single LC/MS/MS experiment
- Glycopeptide MS/MS data analyzed using MaxEnt3 and CarboTools

INTRODUCTION

Importance of characterising Glycosylation

- The study of post-translational modifications (PTMs) is of ever increasing importance in the study of proteins. Physiological changes in an organism can often leave their mark upon proteins in the form of altered PTMs most notably changes in phosphorylation and glycosylation.
- Glycosylation changes have been noted in many diseases including Arthritis[1] and Transmissible Spongiform Encephalopathies[2,3]

Approaches to glycosylation analysis

- Analytical approaches to glycosylation analysis often assume a "global" approach; glycans are released from the glycoprotein either enzymatically or chemically. This gives no information of glycosylation site occupancy, and will not distinguish the source of the glycan in a protein/glycoprotein mixture[4].
- Proteins are identified following mass spectrometric analysis of their enzymatic digest fragments. Identification and characterisation of glycosylation along with routine analysis would save the use of expensive exoglycosidases, glycan release protocols and would provide site specific information.
- Parent ion discovery (PID) previously successfully applied to detection of phosphopeptides[5], is utilized here for glycosylation detection. It is a function of the Micromass Q-Tof mass spectrometer (Waters Corp., Milford, MA) which allows specific species with a common structural motif to be targeted for MS/MS analysis during an on line HPLC experiment.

Glycopeptides

- N-linked glycopeptides are typically large, the carbohydrate portion often being 2-3 kDa. These large and mobile moieties hinder protease hydrolysis, hence trypsin or other proteases may miss a cleavage site close to a glycosylated asparagine, resulting in an unusually large peptide portion[2].
- These large species are only detected as highly charged ions, hence 4,5 or 6+ ions are characteristic of glycopeptides[3].

EXPERIMENTAL

Sample

- 1 mg (approx. 23 nMoles) of bovine fetuin (Sigma, UK), dissolved in 0.1 M NH_4HCO_3 was reduced with 3 µL 1M DTT at room temperature for 90 mins and akylated with 15 µl 1M IAA for 90mins at room temperature.
- The fetuin was then digested over night (16 hours) with 5% (w/w) TCPK treated trypsin (Sigma, UK)

MICROMASS® MS TECHNOLOGIES

Chromatography

- The column was equilibrated with 5% ACN for 10 mins.
- Samples were injected using an autosampler onto a Symmetry 300TM C18 OPTI-PAK (Waters Inc, MA) and on-line separation was carried out on a 75µm column (C18 PepMap, LCPackings, Amsterdam, Netherlands).
- A gradient from 0-40% B (B=95% ACN 0.1% FA) over 40 minutes was provided using a Micromass CapLC.

MASS SPECTROMETRY

- All data was acquired on a Micromass Q-Tof Ultima API mass spectrometer with a nano ESI Z-spray source.

Data Acquisition -Parent Ion Discovery

- A flow chart of the operation of Glyco PID is shown in chart 1.

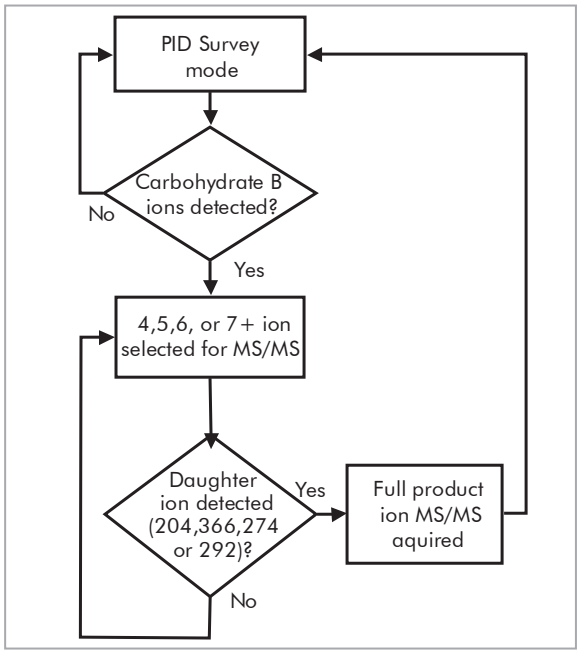

Chart 1. Flow Chart of Glycopeptide PID experiment

- The Micromass Q-Tof was run in Parent Ion Discovery (PID) mode, where the voltage on the gas collision cell is switched alternately between high (30 v) and low (8 v) every second (5). This provides both a standard low energy MS spectrum and a spectrum of all the product ions seen in the standard scan.
- The software was modified to specifically allow the selection for MS/MS of ions with 4^+ to 7^+ charges, to increase the likelihood of selecting the large glycopeptides over the smaller peptides.
- The MS functions are run in centroid mode, for increased accuracy for real time product ion detection.
- Upon detection of carbohydrate B ions ($[Hex]^{1+}$ m/z 204, $[HexHexNac]^{1+}$ m/z 366, $[NeuAc - H_20]^{1+}$ m/z 274; $[NeuAc]^{1+}$ m/z 292) the instrument switches to MS/MS mode and selects the most intense multiply charged ion for fragmentation.
- Once this precursor ion has been selected, MS/MS will only continue if it yields the expected carbohydrate b type product ions (Chart 1).
- During MS/MS a collision energy ramp of 20-40 v was applied to obtain a diverse range of fragment ions, in order to provide as much structural information as possible.
- For comparative purposes, the experiment was repeated but the instrument was prevented from switching into MS/MS. The low energy survey allowed chromatography to be monitored, while a reconstructed mass chromatogram for glycopeptide B ions gives an "pseudo-SIM" chromatogram for glycopeptide detection.

RESULTS

Chromatography

Chromatograms for this experiment are shown in Figure 1

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- All the glycopeptides eluted during the last 10 minutes of the gradient, as seen on the pseudo-SIM scan (Figure 1, a + b).
- The reconstructed mass chromatograms for glycopeptide B ions from all of the 4 MS/MS functions (Figure 1, c-f) show several glycopeptides selected for MS/MS.
- The distinctive shape of the reconstructed MS/MS chromatograms is attributed to the varying collision energy ramp applied.

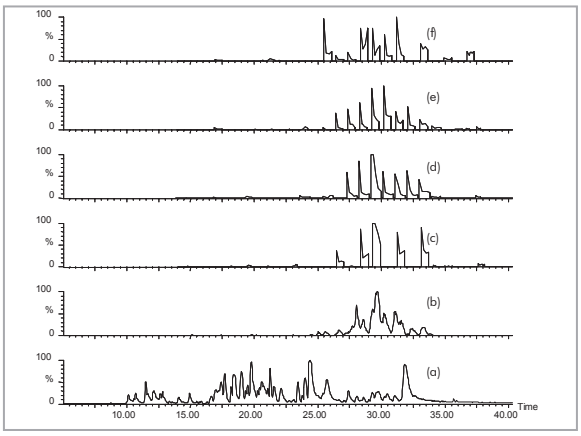

Figure 1. Glycopeptide PID chromatograms obtained from a fetuin tryptic digest run by LC/MS/MS. (a) Low energy survey scan (b) reconstructed high energy mass chromatogram for carbohydrate b ions (m/z 204, 366, 274, 292)(instrument prevented from switching). (c-f) MS/MS reconstructed mass chromatograms for carbohydrate B ions

Data processing

Steps in data processing are shown in Figure 2

- MaxEnt3 was used to de-isotope and deconvolute glycopeptide MS/MS data (see Figure 3 for example).

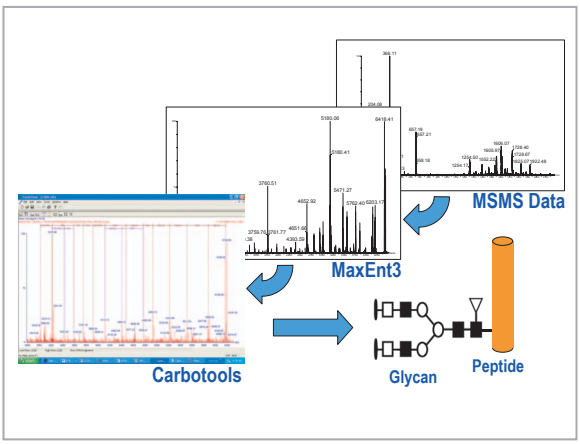

Figure 2. Data processing. Raw data is de-isotoped and deconvoluted using MaxEnt3, and is then analysed using CarboTools. Carbohydrate differences between the fragment ions are measured and assigned through with use of the software

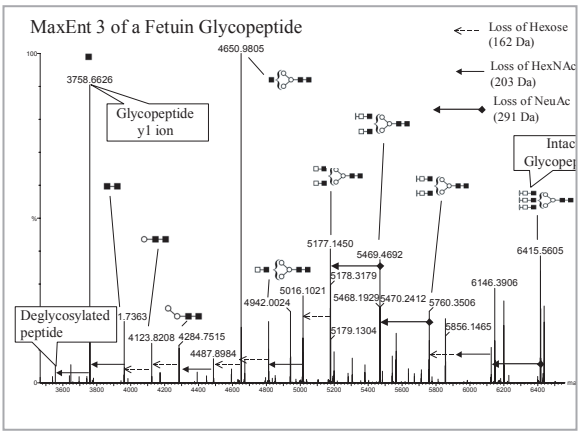

Figure 3. MaxEnt3 spectrum of a fetuin tryptic peptide bearing a tri-sialylated tri-antennary glycan. Fragmentation is preferential to the glycan, and no fragmentation of the peptide is observed. The Y1 ion (peptide + HexNAc) at m/z 3758 is intense and can be used to determine the mass of peptide minus the glycan portion

- CarboTools, from Masslynx 4.0 software (Waters, MA), was used to characterise the carbohydrate moiety from the MaxEnt3 spectra.

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Interpretation

- The smallest y-type fragment ion (nomenclature as Domon and Costello[6]) detected is the usually the glycopeptide y1 ion, (the peptide with the reducing terminus N-acetyl hexosamine attached), Figure 3.
- Using Carbotools and working up from this intense ion a structure for the carbohydrate moiety on the glycopeptide can be established. In the example shown here, a tetra-sialylated triantennary is characterised using CarboTools.
- The charge state and the m/z of the ion can be used to assign a mass to the glycopeptide, which can be checked against Glycomod from the Expasy website (http://ca.expasy.org).

Glycopeptides characterised

- Glycopeptides were observed on all 3 of fetuin's N-linked glycosylation sites.
- The glycopeptides characterised were largely tri-sialylated tri-antennaries, as expected[7].
- A number of sialylated glycopeptide species were also detected in their sodiated form.

Glycopeptide Ave MW (Da)	Precursor Ion (m/z)	Peptide	Carbohydrate
6474.91	1296.66, 5+ 1619.73, 4+	$_{72}$RPTGEVYDIEIDTLETTCHVLDPT PLANCSVR$_{103}$ 1CMC	
6419.00	1605.75, 4+	$_{72}$RPTGEVYDIEIDTLETTCHVLDPT PLANCSVR$_{103}$ No CMC	
5816.42	1459.23, 4+	$_{72}$RPTGEVYDIEIDTLETTCHVLDPT PLANCSVR$_{103}$ 1CMC	
5760.91	1441.23, 4+	$_{72}$RPTGEVYDIEIDTLETTCHVLDPT PLANCSVR$_{103}$ No CMC	
6709.73	1342.95, 5+	$_{72}$RPTGEVYDIEIDTLETTCHVLDPT PLANCSVR$_{103}$ No CMC	
6969.2	1743.30, 4+	$_{68}$VWPRRPTGEVYDIEIDTLET TCHVLDPTPLANCSVR$_{103}$ No CMC	
4696.88	1175.22,4+ (Na adduct)	$_{144}$KLCPDCPLLAPLNDSR$_{159}$ No CMC	
4033.032	1343.34, 3+	$_{145}$LCPDCPLLAPLNDSR$_{159}$ No CMC	
4486.93	1496.53, 3+	$_{145}$LCPDCPLLAPLNDSR$_{159}$ No CMC	
6167.90	1542.97, 4+	$_{160}$VVHAVEVALATFNAES NGSY LQLVEISR$_{187}$ No CMC	
5877.71	1470.43, 4+	$_{160}$VVHAVEVALATFN AESNGSY LQLVEISR$_{187}$ No CMC	

Table 1. Tryptic glycopeptides of fetuin detected and characterised with the glycopeptide PID experiment

CONCLUSION

- From the fetuin tryptic digest 11 glycopeptides were identified in a single Parent Ion Discovery experiment.
- Glycopeptide analysis gives site specific glycoform information, as shown in the results on Table 1, where all three N-linked glycosylation sites were identified.
- Peptide heterogeneity from both incomplete alkylation and missed tryptic cleavage sites was observed, giving rise to a greater number of glycopeptides than expected.
- The method described is a sensitive, highly specific approach to glycopeptide detection and sequencing.
- Result interpretation is greatly simplified by the use of MaxEnt3 and Carbotools.

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