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PRECURSOR ION DISCOVERY - GLYCOPEPTIDE DETECTION AND CHARACTERISATION FROM A SINGLE CAPLC Q-TOF EXPERIMENT

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OVERVIEW

- Parent ion discovery on a Micromass Q-Tof Ultima applied to glycopeptide analysis
- Detection and characterisation of glycopeptides from CapLCMS-MS/MS run of a tryptic digestion of fetuin
- Glycopeptide MS/MS data analysis using MaxEnt3 and CarboTools

INTRODUCTION

The study of post-translational modifications (PTMs) is of ever increasing importance in the study of proteomics. 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.

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 [1].

In a proteomic experiment proteins are often identified by performing tryptic digests of either protein solutions or excised gel bands and analysing by electrospray (ESI) LC-MS/MS. Identification and characterisation of glycosylation in this same experiment would save the use of expensive exoglycosidases, glycan release protocols and would provide site specific information in conjunction with routine peptide analysis.

N-linked glycopeptides are often large, the carbohydrate portion being typically 2-3KDa or more. Often 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]. Large species are only detected in the mass range as highly charged ions, hence 4,5 or 6+ ions are characteristic of glycopeptides [3].

Parent ion discovery (PID) is a function of the Micromass Q-Tof (Waters Corp., Milford, MA) which allows specific targeting for MS/MS of species with a common structural motif during an LC-MS experiment. It has previous been successfully applied to detection of phosphopeptides [4].

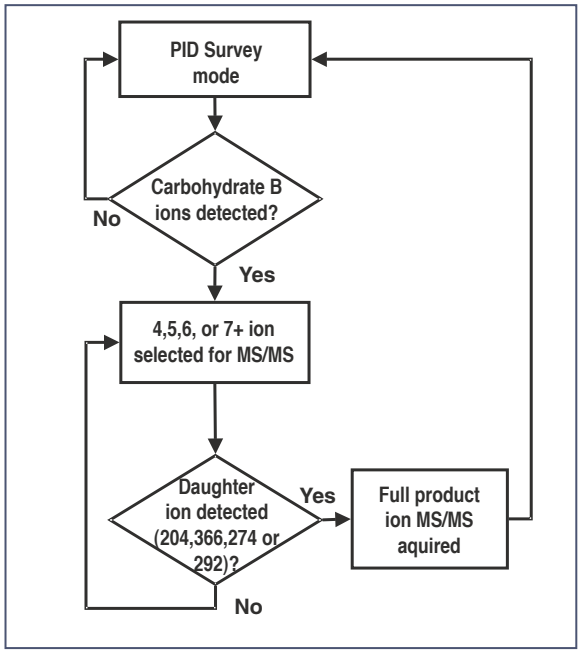
EXPERIMENTAL

Bovine fetuin (Sigma) was reduced and akylated with DTT and IAA respectively, and digested with trypsin before being analysed by ESI LC MS and MS/MS. Samples were injected using an autosampler onto a C18 cartridge pre column 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.

The Micromass Q-Tof Ultima API mass spectrometer with a nano Z-spray source was run in Parent Ion Discovery (PID) mode, where the voltage on the gas collision cell is switched alternately between high (30v) and low (8v) every second [4]. This provides both a standard precursor MS spectrum and a spectrum of all the product ions seen in the standard scan.

MICROMASS MS TECHNOLOGIES

Upon detection of carbohydrate b ions (m/z 204, 366, 274, 292) the instrument switches to MS/MS mode and selects the most intense multiply charged ion for fragmentation. Only if the selected precursor ion yields the expected carbohydrate b type product ions will the MS/MS continue (**scheme 1**). The software was modified to specifically allow the selection of ions with 4 + to 7+ charges for MS/MS. A collision energy ramp of 20-40v was applied to obtain a diverse fragmentation spectra.



Scheme 1. Flow Chart of Glycopeptide PID experiment

For comparative purposes, the experiment was repeated but the instrument was prevented from switching into MS/MS. The low energy survey served as a view of the chromatography, while a reconstructed mass chromatogram for glycopeptide B ions gives an "pseudo-SIM" chromatogram for glycopeptide detection.

RESULTS

Data acquisition

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

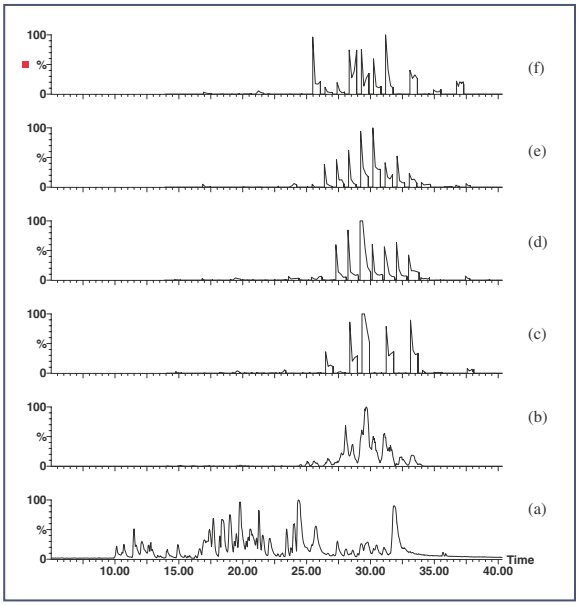


Figure 1. Glycopeptide PID experiment chromatograms. Fetuin tryptic digest run by CapLCMS-MS/MS, in (a) and (b) the instrument was prevented from switching into MS/MS. (a) Low energy survey scan (b) Psuedo-SIM scan, reconstructed high energy mass chromatogram for carbohydrate b ions (m/z 204, 366, 274, 292). (c-f) MS/MS chromatograms reconstructed as mass chromatograms for carbohydrate b ions.

Data processing and interpretation

The charge state and the m/z of the ion can be used to assign a mass to the glycopeptide, from which a putative carbohydrate composition may be assigned using Glycomod from the Expasy website (http://ca.expasy.org)

Glycopeptides were then characterised from the associated MS/MS data using MaxEnt3 and CarboTools in Masslynx 4.0 software (Waters Corp., Milford, MA). The MaxEnt3 de-isotoped and deconvoluted MS/MS spectra were imported into CarboTools. The smallest y-type fragment ion (nomenclature as Domon and Costello [5]) detected is the usually the glycopeptide y1 ion, (the peptide with the reducing terminus N-acetyl hexosamine attached), **Figure 2**. 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 (**Figure 3**). In the case of insufficient MS/MS data for carbohydrate characterisation, glycan structure and peptides were assigned from Glycomod.

The glycopeptides characterised were largely tri-sialylated triantennaries, as expected [6]. Some tetra-sialylated triantennaries were also found.

A number of sodiated glycopeptide species were selected for MS/MS, but have been omitted from the summary in **table 1**.

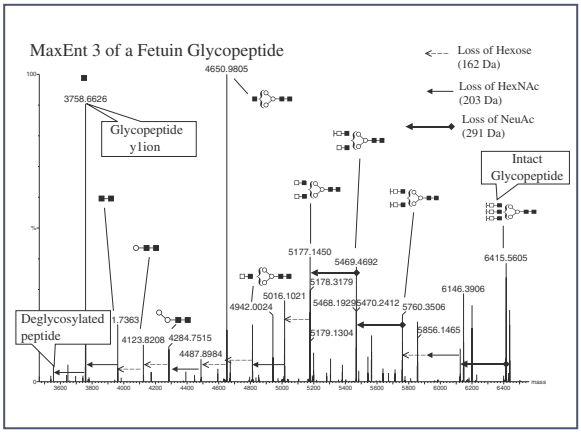


Figure 2. MaxEnt3 deconvoluted deisotoped spectrum of a fetuin tryptic peptide bearing a tri-sialylated triantennary glycan. Fragmentation is preferential to the glycan, and no fragmentation of the peptide is observed. The y1 ion (peptide + HexNAc) at m/z 3758 is intense and can be used to easily determine the peptide mass.

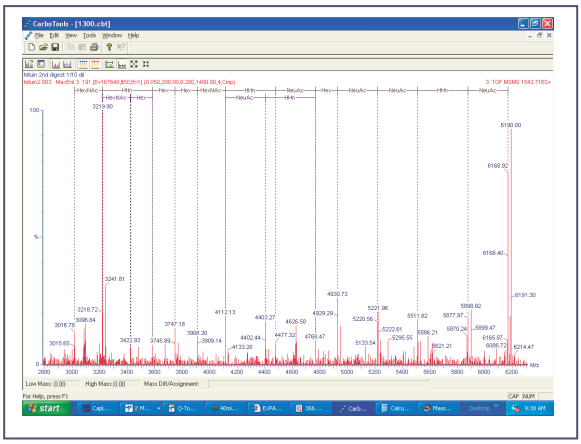


Figure 3. MaxEnt3 data analysed using CarboTools. Carbohydrate differences between the fragment ions are measured and assigned manually with use of the software. Here a tetra-sialylated triantennary is sequenced entirely.

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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	
6533.67	1307.89, 5+ 1634.23, 4+	$_{68}$VWPRRPTGEVYDIEIDTLET TCHVLDPTPLANCSVR$_{103}$ 2 CMC	*
3904.61	1302.53, 3+	$_{145}$LCPDCPLLAPLNDSR$_{159}$ 2CMC	*
4033.032	1343.34, 3+	$_{145}$LCPDCPLLAPLNDSR$_{159}$ No CMC	
4486.93	1496.53, 4+	$_{145}$LCPDCPLLAPLNDSR$_{159}$ No CMC	
7270.52	1455.10, 5+	$_{143}$LCPDCPLLAPLNDSRVVHAV EVALATFNAESNGSYLQLVE ISR$_{187}$ No CMC	*
6167.90	1542.97, 4+	$_{160}$VVHAVEVALATFNAESNGSY LQLVEISR$_{187}$ No CMC	
5877.71	1470.43, 4+	$_{160}$VVHAVEVALATFNAESNGSY LQLVEISR$_{187}$ No CMC	

Table 1. Tryptic glycopeptides of fetuin detected and characterised with the glycopeptide PID experiment. Where MS/MS data was insufficient for characterisation, most likely Glycomod assignments have been chosen.

* *identification done on Glycomod alone, insufficient MS/MS data CMC - cysteines derivatised with IAA.*

CONCLUSION

From the fetuin digest a total of 12 glycopeptides were identified from all three N-linked glycosylation sites. Peptide heterogeneity from both derivatisation and cleavage location were observed, giving rise to a greater number of glycopeptides than expected. The use of an exclude list could possibly increase this number of glycopeptides detected after a subsequent run.

The method described is a sensitive, highly specific approach to glycopeptide detection and sequencing. In combination with other methods, this approach will allow full elucidation of N-linked glycosylaytion structure, including linkage information.

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