IDENTIFICATION OF N-LINKED GLYCOSYLATION SITES USING MALDI TIME-OF-FLIGHT MASS SPECTROMETRY

Waters

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INTRODUCTION

Glycopeptides are typically generated using proteolytic enzymes such as trypsin, to cleave at specific sites. The resulting glycopeptides tend to give weak ion signals upon mass spectrometry analysis (ESI or MALDI) due to their large molecular weight. The glycosylation site(s) identification using tandem mass spectrometry is hindered by the low ion intensity and complicated by the fragment ions generated from both the peptide back bone and the glycosidic cleavages from the sugar moiety.

We explored a method that uses unspecific enzyme, pronase, to generate smaller glycopeptides; the majority of resulting glycopeptides has an amino acid sequence between two to six. The glycopeptides were enriched and desalted using a micro scale hydrophilic interaction solid phase extraction device (HILIC SPE) prior to MALDI-Q-Tof MS analysis. The protonated glycopeptide ions were enhanced dramatically using ammonium citrate doped MALDI Matrix, 2, 5-Dihydroxybenzoic Acid (Fig. 1). Sodiated ions were also observed as minor ions. Collision induced dissociation was performed on both the protonated and sodiated ions. MS/MS fragmentation spectra revealed that the proton tends to retain on the peptide moiety while the sodium ion tends to retain on the sugar moiety (Fig. 2, 3). Characteristic fragment ion pairs were observed and used to identify glycosylation sites for both the protonated and the sodiated ions. Model proteins, horseradish peroxidase (HRP) and α1- acid glycol proteins (AGP) were used as examples to illustrate how N-linked glycosylation sites can be identified with minimum sample manipulations and data interpretation.

METHODS

Pronase Digestion

Glycoprotein (50 – 100 mg) was solubilized in the surfactant, RapiGest™ SF (0.1%, w/v) solution with 50 mM ammonium bicarbonate. The sample was reduced using 10mM dithiothretol and alkylated using 15 mM iodoacetamide. The pronase to protein concentration is 1 to 50. Enzymatic digestion was performed at 37°C overnight. In the case of AGP, the sialic acid residues were removed by adding 1 unit of α_2(3,6,8,9)-neuraminidase after the pronase digestion and incubated for another 12 hours.

MALDI MS/MS

Micromass MALDI-QTof Ultima™ (Waters) was equipped with a fixed nitrogen laser source. Argon gas was used as the collision gas. The typical collision energy used was in the range of 70 to 120 V depends on the size of the analyte ions. MALDI Matrix used was 2,5-dihydroxy benzoic acid, DHB (MassPREP™ Waters).

FindPept tool in the SwissProt database was used to identify the possible peptide sequences by submitting the peptide masses and searching against a protein with a known primary sequence.

RESULTS

Ammonium tris citrate enhances the MALDI signal of glycopeptides

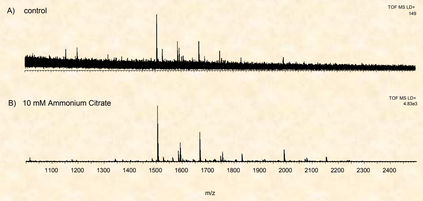
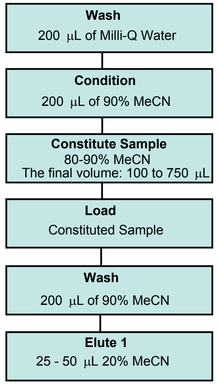


Figure 1. MALDI-QTof MS analysis of glycopeptides generated from HRP. A) The DHB matrix was solubilized in 100% ethanol without any additives. B) DHB matrix contains 10mM ammonium tris citrate.

HILIC SPE Protocol

The glycopeptides were desalted using HILIC SPE (MassPREP™, Waters) before MALDI analysis.



MS/MS fragmentation of the **HRP** glycopeptides (protonated and sodiated) reveals characteristic ion pair

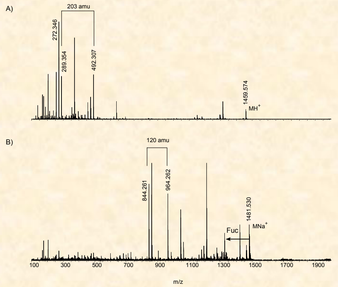
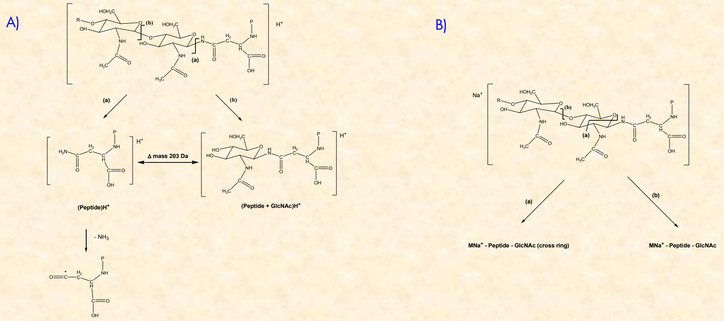


Figure 2. MS/MS fragmentation spectra of the same HRP glycopeptides. A) The precursor ion is protonated. B) The precursor ion is sodiated. Characteristic ion pairs were labeled.

Proposed fragmentation pathway for the protonated glycopeptides (A) and the sodiated glycopeptides (B)

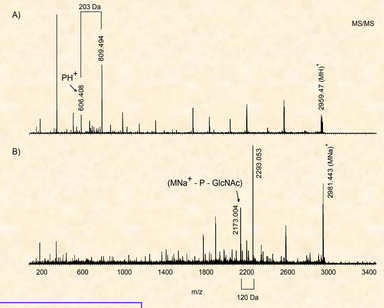


Glycosylation sites identified for HRP (Swiss Prot entry number: P00433)

Precursor ion (MH)+ (m/z)	Peptide mass (m/z Experiment	Actual	Peptide sequence	Sequence number
1459.54	288.35	288.15	NR	188 - 189
1513.55[a)]	320.06	320.13	NST	298 - 300
1525.50[a)]	332.11	332.12 332.17	CPN NLS	41 - 43 244 - 246
1527.50[a)]	334.11	334.15	NTT	87 – 89 228 - 230
1584.50[a)]	391.11	391.17	ANST	297 - 300
1660.59	401.30	401.19 401.24	PNAT LNR	284 - 287 187 - 189
1629.67	458.30	458.26	GLNR	186 - 189
1651.56	488.30	488.22 488.27	SPNAT LNRS	283 - 287 187 - 190
1810.60	617.25	617.26	PNATDT	284 - 289
1920.76	575.29	575.25 575.30	SSPNAT LNRSS	282 - 287 187 - 191

a) The precursor ion is sodiated instead of protonated.

MS/MS fragmentation of the **AGP** glycopeptides (protonated and sodiated)



P is **N**$_{72}$KTED (amino acid residue 72—76) or FTP**N**$_{72}$K (amino acid residue 69—73)

Figure 3. MALDI-Q-Tof MS/MS fragmentation on A) a protonated glycopeptide, B) a sodiated glycopeptide. The characteristic ion-pairs were labeled along with their identity. FindPept tool identifies the glycosylation site at amino acid residue number 72.