Automated glycopeptide analysis system combining glycopeptide analysis software and MALDI-DIT MS.

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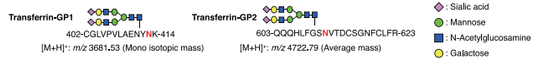
1. Introduction

Glycosylation is one of the most common post-translational modifications of proteins and has important roles in various cell functions. Analysis of glycosylation using tandem mass spectrometry at glycopeptide level can reveal glycosylation sites, glycan components and peptide sequences. However it's difficult to elucidate the structure of glycopeptides by manual analysis due to its complexity. In ASMS2010, we reported an integrated approach, which combines our in-house *de novo* sequencing software (Shimadzu Sequence Explorer; SIMSE) with protein database searching to support manual analysis of the glycopeptide[1].
In this study, we developed automated N-linked glycopeptide analysis software based on SIMSE and established an automated N-linked glycopeptide analysis system with MALDI digital-ion-trap (DIT) MS (DIT is a 3D-quadrupole ion trap (QIT) driven by a rectangular wave high voltage[2]). We demonstrate N-linked glycopeptide analysis with this system.

2. Materials and Methods

Sample:
Human transferrin was digested by trypsin, and glycopeptides GP1 ([M+H]$^+$: *m/z* 3682) and GP2 ([M+H]$^+$: *m/z* 4720) were prepared with reverse-phase HPLC.



Matrix:
purified 2,5-dihydroxybenzoic acid (DHB) (LaserBio labs)
Sample target:
μFocus MALDI plate with 600μm diameter (Hudson Surface Technology)
Instrument:
High vacuum MALDI digital ion trap (DIT) mass spectrometer developed in our laboratory, equipped with 337 nm nitrogen laser
Database engine:
X!Tandem (Global Proteome Machine Organization).
The automated glycopeptide analysis software:
consisted of DIT-Automation, glycopeptide analysis software and MySQL database software.
- DIT-Automation (control software of DIT-MS):
  - > perform MS^n measurements according to requests from glycopeptide analysis software.
  - > provide the DIT-Automation API for glycopeptide analysis software.
  (NOTE: It is not limited to glycopeptide analysis. It can be easily used for other analyses)
- Glycopeptide analysis software:
  - > perform structural analysis of glycopeptides for MS^n spectra based on analysis workflow.
  - > obtain MS^n spectra from DIT-Automation and send requests for next MS^n measurements based on the result of structural analysis.

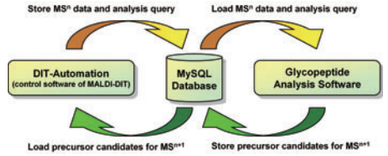
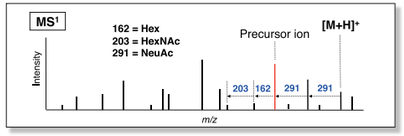


Fig.1 Scheme of automated glycopeptide analysis system

3. Workflow of Automated Glycopeptide Analysis

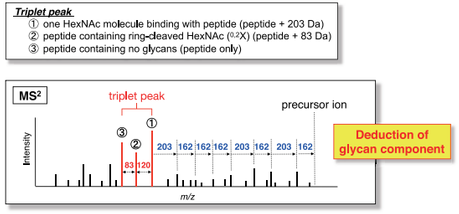
MS^1 analysis

> Assignment of glycopeptide ion peaks by deducing partial glycan sequences from the neutral loss of sugar with *de novo* sequencing.
> The sum total of the intensity of assigned peaks is used as the score in scoring of the partial glycan sequencing.
> Determination of precursor ions with the highest peak intensity among the assigned glycopeptide peaks.



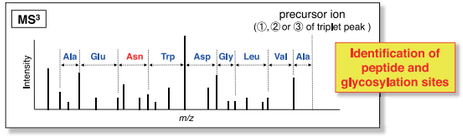
MS^2 analysis

> Assignment of triplet peak, which is characteristic of the N-linked glycopeptide and used as precursor ions for MS^3.
> Deducation of glycan components by *de novo* sequencing from the triplet peak, which is followed by higher masses. The score is a sum total of the intensity of assigned peaks.
> Database searching with X!Tandem, in which the triplet peak was used as precursor ion mass, is also performed.



MS^3 analysis

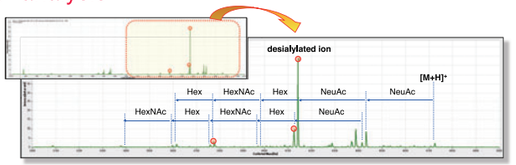
> Identification of peptide and glycosylation sites by database searching with X!Tandem.



4. Results

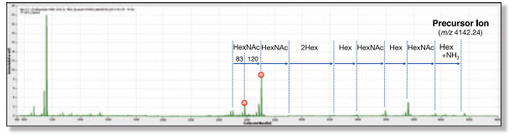
4-1. Transferrin-GP2 analysis

MS^1 analysis



> Neutral loss of sugar from the intact glycopeptide was detected from fragment ions.
  √ Glycan Component: 2Hex, 2HexNAc, 2NeuAc (sequenced with mass tolerance 1,5 Da).
> Desialylated ions are properly selected as MS^2 precursor ions : *m/z* 3776.70, 4125.64, 4142.24 (red circles;○).
  √ Sialylated ions with higher intensity are automatically removed so that they are not to be selected as precursor ions because CID of such ions results in poor fragmentation.

MS^2 analysis



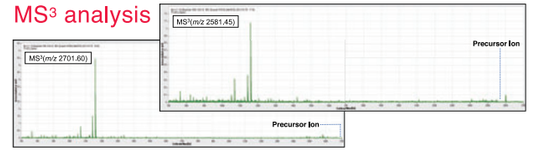
> MS^2 spectrum was acquired from desialylated ion (*m/z* 4142.24) automatically.
> Peaks from the characteristic triplet peak were properly selected as precursor ions for MS^3 : *m/z* 2701.60, 2581.45 (red circles;○).
> Neutral loss of sugar was sequenced between estimated peptide ion and precursor ion with mass tolerance 0.5 Da.
  √ Combined with result from MS^1 analysis, correct glycan composition of transferrin-GP2 was obtained: 5Hex, 4HexNAc, 2NeuAc.
  √ Correctly treated NH_3 loss of product ions generated by the cyclization of N-terminal glutamine.

Precursor ion mass (Da)	Score	Expect	Sequence
2701.60	19.2	3.6e-1	qQQHLFGSnVTDcSGNFcLFR+HexNAc
2581.45	19.2	9.5e-1	qQQHLFGSnVTDcSGNFcLFR+^{0,2}X (HexNAc)

Identification: 0.01 ≥ expectation value (Expect), Homology: 0.1 ≥ expectation value > 0.01

Database searching with peak list generated from MS^2 spectrum resulted in the 1st candidate hitting the correct peptide for both peak lists, though neither identification nor homology were achieved. This result suggests the plausibility of precursor selection for MS^3.

MS^3 analysis



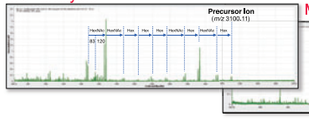
Precursor ion mass (Da)	Score	Expect	Sequence
2701.60	31.3	3.5e-3	qQQHLFGSnVTDcSGNFcLFR+HexNAc
2581.45	34.2	2.1e-4	qQQHLFGSnVTDcSGNFcLFR+^{0,2}X (HexNAc)

MS^3 spectra were acquired automatically from precursor ions selected in the previous MS^2 analysis. From both spectra, glycopeptides were identified with higher confidence. The identified peptides and glycosylation sites were correct.

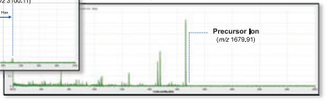
4-2. Transferrin-GP1 analysis

Transferrin-GP1 was also analyzed with the automated glycopeptide analysis system. Combining MS^1 and MS^2 analysis correct glycan component was deduced: 5Hex, 4HexNAc, 2NeuAc. From MS^3 spectra, correct peptide and glycosylation site were identified by the database searching.

MS^2 analysis



MS^3 analysis



Precursor ion mass (Da)	Score	Expect	Sequence
1679.91	28.7	1.6e-3	cGLVPVLAENYnK+HexNAc

4. Conclusion

The automated glycopeptide analysis system could correctly achieve the deduction of glycan components, determination of glycosylation sites and protein identification of transferrin-GP1 and -GP2.

5. Future works

- Developing this strategy by applying it to a wider variety of N-linked glycopeptides.
- Developing an analytical strategy for O-linked glycopeptides.

Reference
1) M. Murase et al., 58th ASMS Conference, TP148 (2010)
2) L. Ding. et al., J. Mass Spectrom., 39: 471-484 (2004).