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Application News

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AXIMA-QITMALDI-QIT-TOF

The Analysis of N-linked Glycans by MALDI QIT TOF Mass Spectrometry

- Structural Characterization of glycan PTMs acheived by step-wise MS fragmentation
- Easier spectral interpretation due to singly-charged ion species and high resolution, high mass accuracy in all modes of operation
- High sensitivity and low chemical noise permits access to fragmentation spectra beyond MS^3 on low abundance glycans

The Analysis of N-linked Glycans by MALDI-QIT-TOF Mass Spectrometry

Introduction

In recent years enormous research efforts have been devoted to a fuller understanding of the expressed proteome of a wide range of organisms. This has been accelerated by the publication of the genome sequences of human, mouse, rice, Arabidopsis and so on. It is already clear that the dynamic nature of the proteome makes its correlation to cell biology, disease and drug development very challenging. This complexity is added to by the presence of post-translational modifications (PTM). Of these, perhaps the best understood are the phosphorylations which are largely transient and under the discrete control of kinases and phosphorylases. Despite the understandable interest in the phosphorylation state of proteins, a more significant class of PTM exists, namely carbohydrates (or glycans). These are present on more than 50% of eukaryotic proteins and can infer considerable changes in the properties of proteins. They can be split into three classes: N-linked glycan structures which are attached to an asparagine in the amino acid sequence motif Asn-X-Ser/Thr (e.g. fetuin, tPA); O-linked glycan structures coupled to Serine or Threonine residues (e.g. mucins); and GPI anchored proteins which incorporate a central carbohydrate segment linking the protein moiety to an interaction with the cell membrane.

Within each of these classes there is enormous potential for heterogeneity since carbohydrates can form branched as well as linear structures and the substitution of one sugar for another e.g. hexoses such as glucose, galactose and mannose. For this reason, here we focus on the N-linked glycans solely.

There are many questions that one would want to address with regard to a protein's glycosylation state, including: Is it glycosylated? What linkages are possible and where according to the amino acid sequence? Which structures are present and where is each one located? Accordingly, a wide number of techniques may need to be applied to deliver the answers and each of these will make a different contribution but might include HPLC, gel electrophoresis, NMR, enzymatic digestion, chemical deglycosylation and mass spectrometry (MS).

As the area of proteomics has developed, the key techniques that have emerged are 2D gel electrophoresis and tryptic-digest peptide mass fingerprinting combined with MALDI mass spectrometry. This gives access to gel-based arrays of proteins which can be stained to reveal presence of glycoproteins and these can then be identified and interrogated using the high sensitivity of MALDI mass spectrometry. However, the two overlapping sets of peptide and glycan masses can occlude each other. To overcome this in the case of N-linked glycans, a general enzymatic cleavage can be performed that removes all of the glycans. The enzyme used to achieve this, Protein N-Glycosidase F (PNGase F, Sigma Aldrich Product Code P 7367), results in the deamidation of the Asparagine to an Aspartate and an increase in mass of 1 Da for the associated peptide. It is then possible to compare the glycosylated and deglycosylated mass patterns. This will allow undisturbed peptide mass fingerprinting to identify the protein and then assist in the localization of likely glycosylation sites. Additionally, the PNGase F -released glycans can be recovered and analyzed independently by various methods including further mass spectrometry and targeted deglycosylations to determine the detailed structure.

Despite its speed, convenience, simplicity and sensitivity, conventional MALDI-TOF has not had the ability to reveal the detailed structure of N-linked glycans. This is in part due to the fact that most sugars share common residue masses and cannot be identified uniquely in a facile manner. This is shown in Table 1.

Monosaccharide	Residue average mass
fucose	146.14
galactose	162.14
mannose	162.14
glucose	162.14
N-acetylglucosamine	203.19
N-acetylgalactosamine	203.19
N-acetylneuraminic acid	291.26

Table 1. Residue masses for common sugars

Additionally, determination of the linkage between sugar residues in a glycan requires cross-ring cleavages of the sugars. To achieve this requires MS instrumentation which retains all the features mentioned above but is able to perform step-wise fragmentation of individual precursor ions and ideally through so called MS^n technology.

MALDI Analysis of N-linked Glycans

The AXIMA-QIT is an instrument which has the capabilities to satisfy these criteria. The configuration MALDI - Quadrupole Ion Trap - Time Of Flight (MALDI-QIT-TOF) takes advantage of: i) the time independent nature and sensitivity of the MALDI ionization process; ii) the ability to see in real-time the full mass range of the ions formed using a TOF mass analyzer; and iii) the possibility to perform the controlled and step-wise fragmentation of precursor ions in an ion trap device while retaining resolution in all MS modes. This ion trap also acts as a powerful precursor ion selection device with a resolution of 1000 FWHM at 1000 Da permitting the isolation of individual isotopes.

An example of the fragmentation process is shown in Figure 1. The analyte is a typical high mannose triantennary N-linked glycan derivatized with a 2AB coupling group. The MALDI matrix used in all these examples is 2,5-dihydroxybenzoic acid (DHB). The predominating $[M+Na]^+$ ion (2026 m/z; Resolution = 9000 FWHM) is chosen as a precursor for the subsequent MS/MS and MS^n experiments since such neutral sugars do not readily produce the typical $[M+H]^+$ MALDI ions seen in peptide-based proteomics. The figure clearly shows the ability to select specific precursor ions for further investigation and the typical fragmentation pattern (for example, the loss of 162 Da for each single mannose residue from 1481 Da (Figure 1a) and losses of 203 Da for a N-Acetyl Glucosamine from 1684 Da, and the loss of the GlcNAc-2AB to the original precursor mass in the MS/MS spectrum (not shown)). A further precursor (671 m/z) was selected from the MS^3 experiment thereby generating an MS^4 fragmentation spectrum showing two mannose residue losses down to a mannose dimer $[M+Na]^+$ at 347 m/z (Figure 1b).

In addition to precursor ion selection and step-wise fragmentation it is also possible to attenuate the energy of fragmentation applied in the ion trap. An example of this is shown in Figure 2 where spectrum A shows a lower energy of fragmentation and spectrum B a higher energy for the analyte maltopentaose. As expected, greater structural information is generated at the low mass end with increasing fragmentation energy.

One specific example of an aberrant glycan structure is shown in Figure 3. The precursor ion at 1972 m/z has an additional galactose residue to the normal form shown at 1810 m/z. However, the MS result does not indicate whether this is a terminal addition or a further branching structure. The MS^3 results for the two precursor ions were compared and only the higher m/z precursor generates the possible di-hexose product at 347 m/z. This confirms that the extra galactose residue is located at a terminal position. It should also be noted that this MS^3 result was achieved on just 20 fmol of sample showing the excellent sensitivity of the AXIMA-QIT.

Ultimately, however, the challenge is to be able to differentiate between different sugar residues of the same nominal mass and the form that any branching takes. The difficulty with the hexoses is described in the following example: the three structures [galactose-β(1-4)glucose], [glucose-β(1-4)glucose] and [mannose-α(1-2)mannose] are unique di-hexoses with an indistinguishable nominal mass of 445 Da that can be generated by fragmentation. This is replicated with N-Acetyl Glucosamine and N-Acetyl Galactosamine, both with a residue mass of 203 Da.

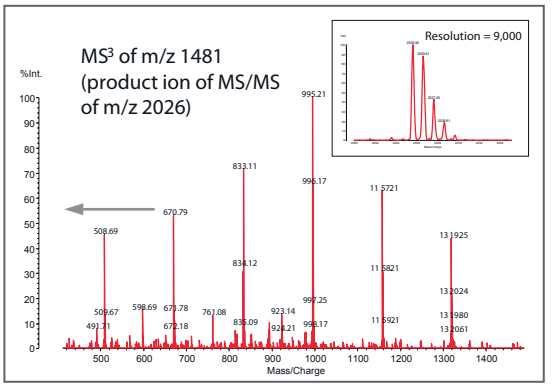

Figure 1a. Analysis of $(Man)_9(GlcNAc)_2$2AB from MS to MS^3 showing the sequential loss of hexose (mannose) and the high resolution of the precursor ion

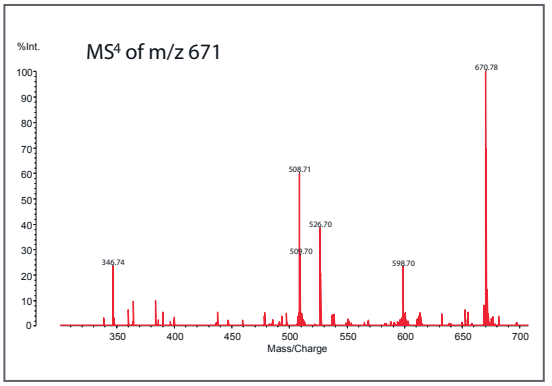

Figure 1b. MS^4 of the MS^3 product ion at 671 m/z revealing the mannose dimer $[M+Na]^+$

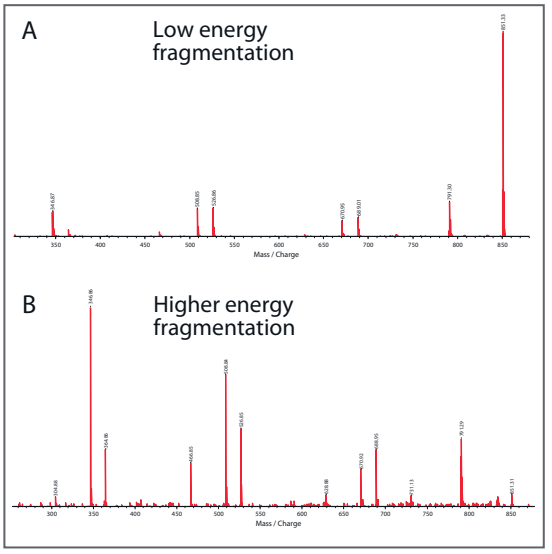

Figure 2. Comparison of fragmentation using different ion trap excitation amplitudes

To overcome this, one needs to perform further fragmentation which results in cross-ring cleavages that give diagnostic ions for the specific sugars and the linkage between them. Figure 4 shows a combination of the information gained from a sugar cross-ring cleavage (A_n) and the fragmentation of the peptide bonds of tripeptide Asn-Ser-Val and a triantennary pentamannose N-linked glycan structure (compound 5-MGP). The cross-ring cleavage information can be used to gain complete structural analysis of complex unknown carbohydrate structures as have been described by Reinhold,et al. (2002) [5] and Harvey et al. (2004) [2] who deal with the processes and analyses in greater detail.

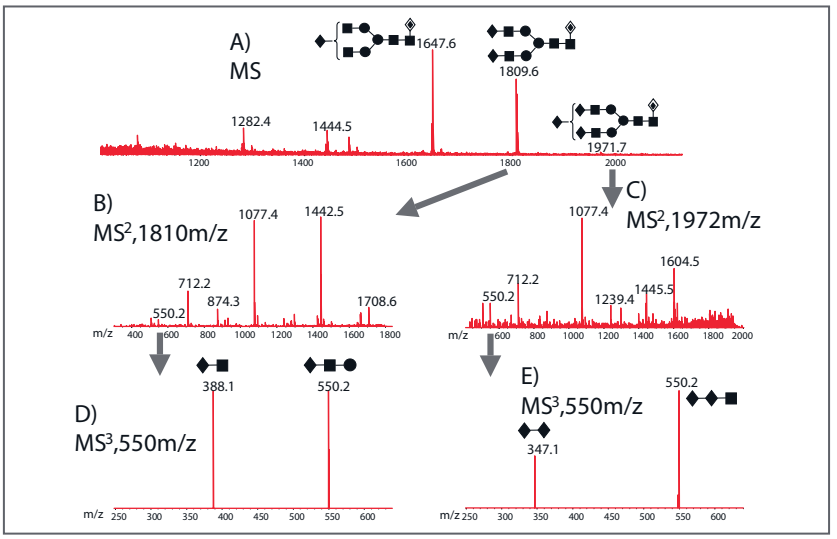

Figure 3. Determination of an aberrant glycan structure on 20 fmol IgA

Spectrum A: MS of the IgA glycans. An unusual feature is seen at 1971.7 m/z. The unresolved question was whether the extra galactose residue was terminal to the antennary structure forming a unique hexose-hexose linkage or if it was an internal branching.

Spectrum B: MS/MS of the normal glycan structure observed at 1809.6 m/z.

Spectrum C: MS/MS of the aberrant glycan mass observed at 1971.7 m/z.

Spectra D and E are the MS/MS/MS products of the respective ions at 550 m/z (2 hexoses with 1 N-Acetyl Glucosamine) in B and C. The generation of the unique $[M+Na]^+$ at 347 m/z confirms that the additional galactose in the structure is indeed terminal to the normal antennary structure.

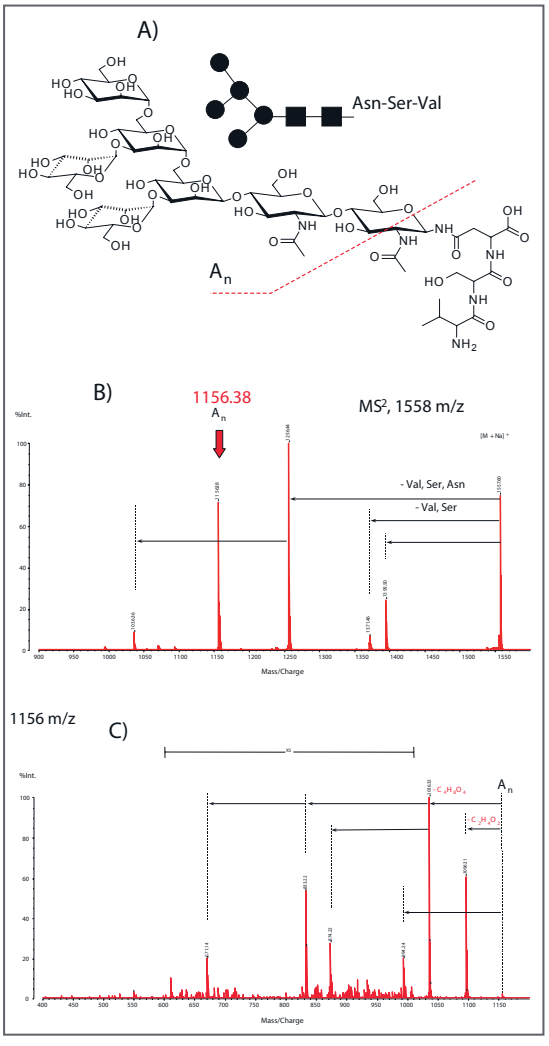

Figure 4. Combination analysis of glycan cross-ring cleavages and associated peptide structure of 5-MGP

A: the structure of a small synthetic peptidoglycan 5-MGP.

B: MS/MS of a precursor ion (1558 m/z) reveals an unexpected fragment ion (postulated as being A_n) which may be the cross-ring cleavage of the first N-Acetyl Glucosamine. Further ions are seen describing the tri-peptide composition and the typical loss of one mannose and also of one N-Acetyl Glucosamine.

C: the suspect ion at 1156 m/z is subjected to MS/MS/MS and the fragmentation confirms this is indeed the cross-ring cleavage at the N-linked N-acetyl glucosamine. The ions at 1096 and 1036 m/z are the loss of the cross-ring fragments from the N-acetyl glucosamine and the loss of the second N-acetyl glucosamine at 833 m/z (equivalent to the residual pentamannose $[M+Na]^+$ ion).

Conclusions

The understanding of glycan structures is a key requirement in protein biochemistry, cell biology and drug discovery. Multiple strategies are evidently required to achieve this. The AXIMA-QIT MALDI-QIT-TOF mass spectrometer is a powerful new tool to aid the structural dissection of these important post-translational modifications. This can easily be combined with upstream enzymatic and proteomic sample preparation technologies.

REFERENCES AND FURTHER READING

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