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Application News

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MALDI-TOF Mass Spectrometry

Characterization of Glycan Binding Site of O-Linked Glycopeptides Using MALDI-7090 High-Resolution MALDI-TOF MS

The majority of proteins synthesized in biological organisms undergo glycosylation. Glycosylation is modification with glycans of high structural heterogeneity composed of multiple monosaccharides, such as glucose and galactose, which are bonded together. These glycans classified into *N*-linked types and *O*-linked types. These types of glycan are known to play an important role in regulating protein function, and obtaining information on protein glycosylation is essential to the development of biopharmaceuticals.
One piece of this information related to glycosylation is where the glycan is bonded to the protein. Almost all *N*-linked glycans have an amino acid consensus sequence of -NXS/T-, which is used to discover potential *N*-linked glycan binding sites. *N*-linked glycan binding sites can be determined by digesting a glycoprotein with a protease, collecting glycopeptides from the digested material using lectin that binds specifically to glycans, cleaving the glycans from the peptides using the *N*-glycosidase (PNGase F) in the presence of $H_2{}^{18}O$, labeling the *N*-linked glycan binding sites with a stable isotope, and then performing analysis.[1] *N*-linked glycan binding sites can also be determined by MS^n analysis using product ions that are specific to *N*-linked glycopeptides.[2] The group of glycans called *O*-linked glycans are known to bond to a serine or threonine amino acid on the protein. Though often little information is available on the amino acid sequence around this *O*-linked glycan binding area, there is no enzyme suitable for cleaving the bond as with *N*-linked glycans, and also no specific product ions have yet been discovered, which makes determination of the binding site of *O*-linked glycans difficult.
In this article, we present a method for determining the *O*-linked glycan binding site that uses partial digestion with multiple enzymes and the MALDI-7090 high-resolution MALDI-TOF MS.

Partial Glycan Digestion with Glycosidases

The *O*-linked FAM-labeled glycopeptides shown in Fig. 1 were used as model glycopeptides. This model glycopeptide (100 pmol) was added to the below cocktail of enzymes in buffer solution and reacted for 16 hours at 37 ˚C.

Enzyme:
*β*1-3 Galactosidase (10000 U/mL, 1 µL)
*α*1-3,6 Galactosidase (4000 U/mL, 1 µL)
*α*1-2,4,6 Fucosidase (8000 U/mL, 1 µL)
β-N-Acetylglucosaminidase (4000 U/mL, 1 µL)

Buffer solution:
50 mM sodium acetate/5 mM calcium chloride aqueous solution

The glycopeptide before addition to the enzyme cocktail and the solution obtained after reaction with the above enzyme cocktail were subject to MS and MS/MS analysis using the MALDI-7090 system following desalination with a ZipTip µC18.

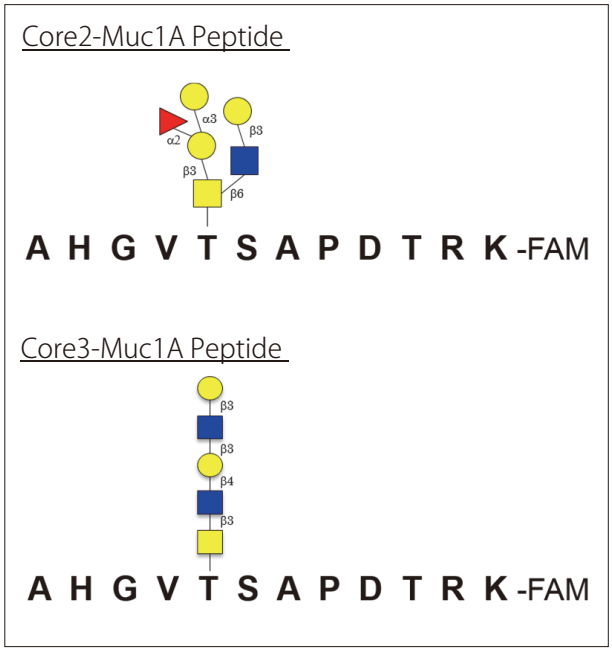

Fig. 1 Model Glycopeptide Structures
Symbol Key: ; N-acetylgalactosamine, ; N-acetylglucosamine, ; Galactose, ; Fucose, FAM; Fluorescent label

MS Analysis of Glycopeptides Before and After Enzymatic Treatment

The mass spectra of each labeled model glycopeptide before and after enzymatic treatment are shown in Fig. 2.
For the two glycopeptides (Core3-Muc1A peptide/ Core2-Muc1A peptide) before enzymatic treatment, signals were detected with *m/z* values corresponding to each complete glycopeptide. For the two glycopeptides after enzymatic treatment, the only glycopeptide signals detected were from glycopeptides with just the root GalNAc remaining on the labeled peptide.
Based on these results, we confirmed the enzyme cocktail treatment cleaved all sugars other than the root GalNAc from glycans of differing structures.

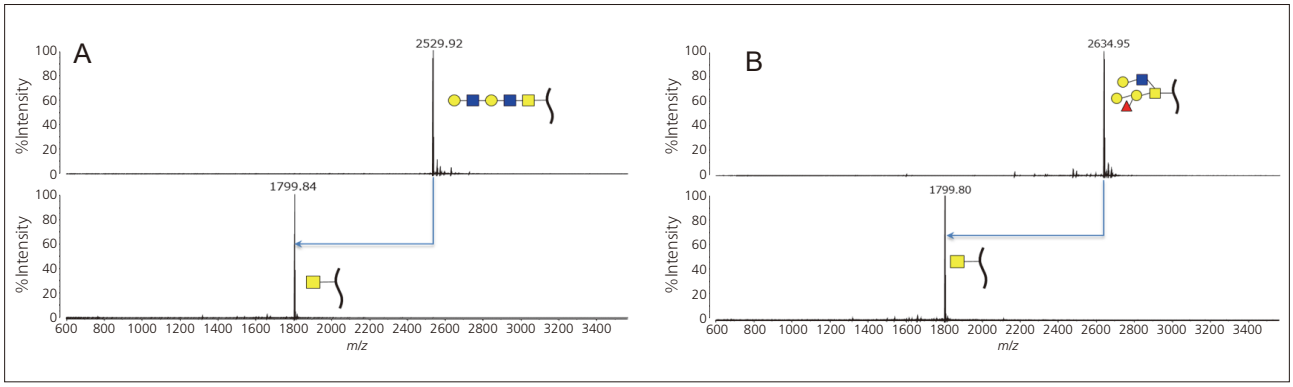

Fig. 2 Mass Spectra of Glycopeptides Before and After Enzymatic Treatment
A: Core3-Muc1A (Top: Before enzymatic treatment, Bottom: After enzymatic treatment),
B: Core2-Muc1A (Top: Before enzymatic treatment, Bottom: After enzymatic treatment)

MS/MS Analysis of Glycopeptide Ions

MS/MS analysis was performed on each ion obtained from MS analysis performed before and after enzymatic treatment. MS/MS analysis of the ions detected in the Core3-Muc1A peptide sample before enzymatic treatment provided information related to glycan composition and amino acid sequence, but information was not obtained that could be used to determine the glycan binding site. Consequently, we could not determine to which of the three potential binding sites on the peptide (threonine at position 5, serine at position 6, or threonine at position 10) the *O*-linked glycan was bonded.

MS/MS analysis of the ions detected after enzymatic treatment resulted in detection of product ions with a mass difference corresponding to threonine with GalNAc bonded to a side chain together with product ions corresponding to peripheral amino acid sequences, as shown in Fig. 4. By analyzing these product ions in order from the precursor ion, we confirmed that of the three potential binding sites, GalNAc was bonded to the threonine at position 5.

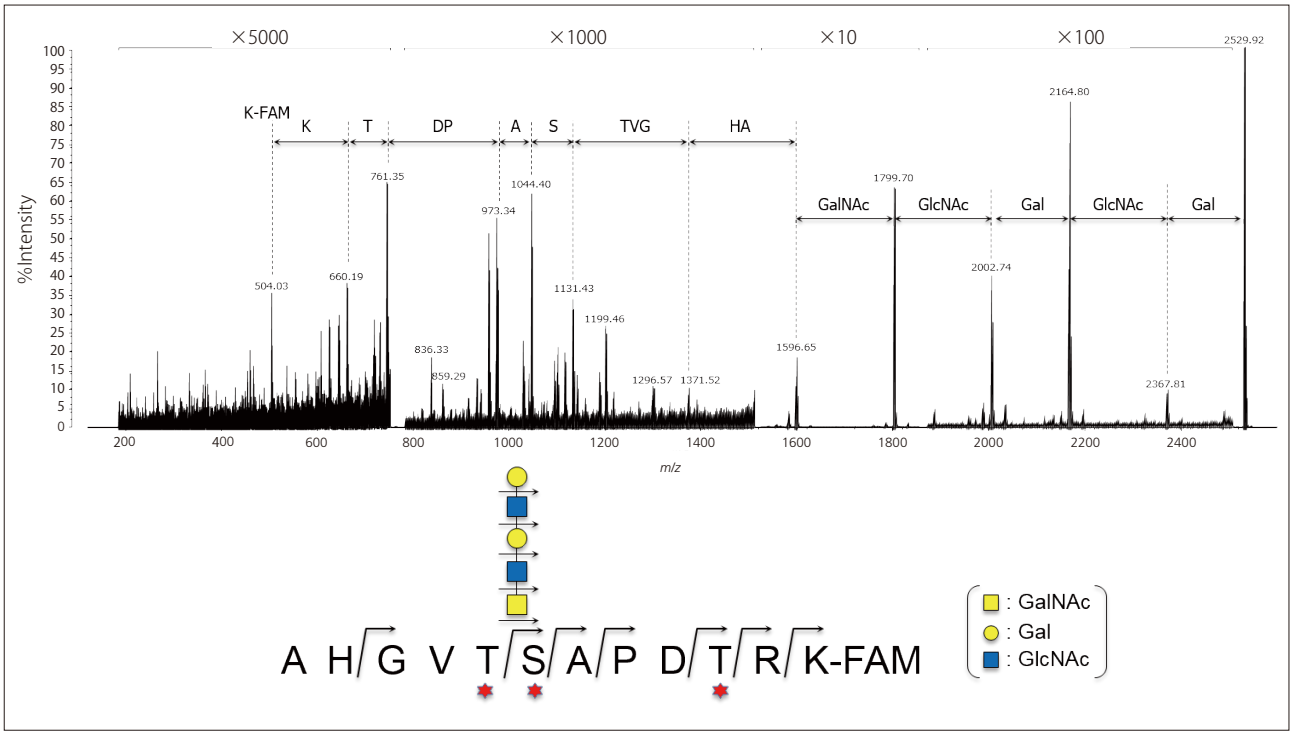

Fig. 3 Glycopeptide (Core3-Muc1A peptide) MS/MS Spectra After Enzymatic Treatment
✸: Potential Glycan Binding Site Obtained from MS/MS Analysis

Next, MS/MS analysis of the ions detected in the Core2-Muc1A peptide sample before enzymatic treatment (Fig. 5) provided information related to glycan composition and amino acid sequence, but information was not obtained that could be used to determine the glycan binding site. However, by performing MS/MS analysis of the ions detected after enzymatic treatment (Fig. 6) with analysis of product ions in order from the precursor ion, we were able to easily determine that GalNAc was bonded to the threonine at position 5 from the N-terminal of the peptide.

In this way, for glycans with complex structures where the glycan binding site cannot be determined without treatment, glycosidases can be used to determine the binding sites of these glycans with relative ease.

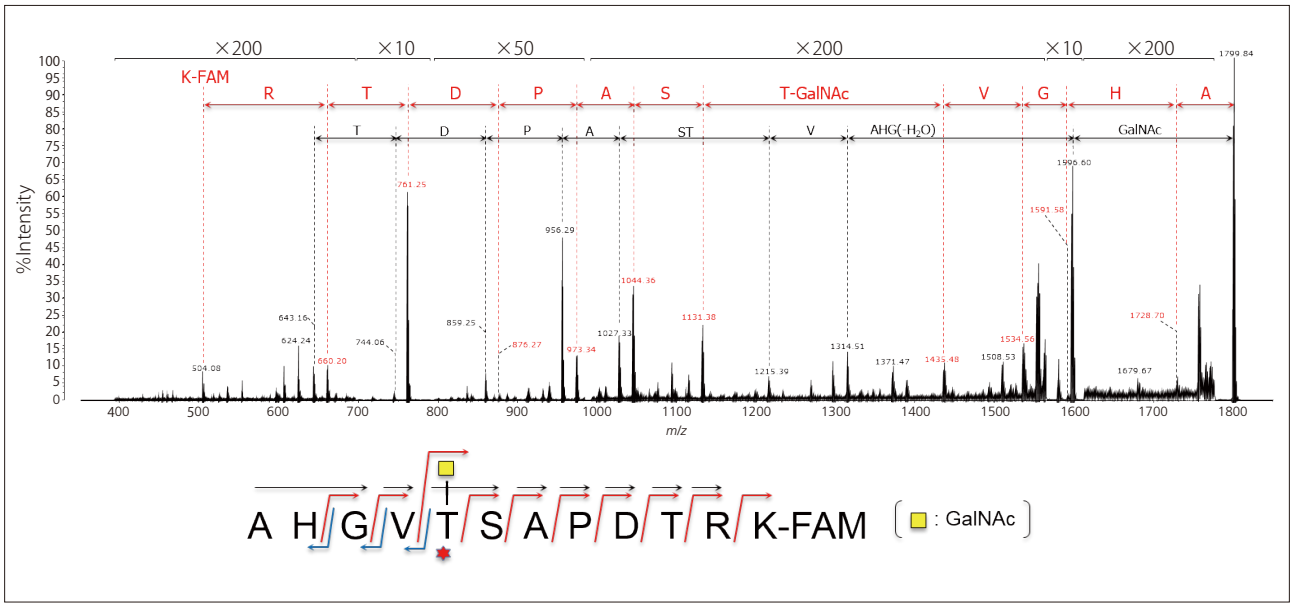

Fig. 4 Glycopeptide (Core3-Muc1A peptide) MS/MS Spectra After Enzymatic Treatment
: Glycan Binding Site Obtained from MS/MS Analysis

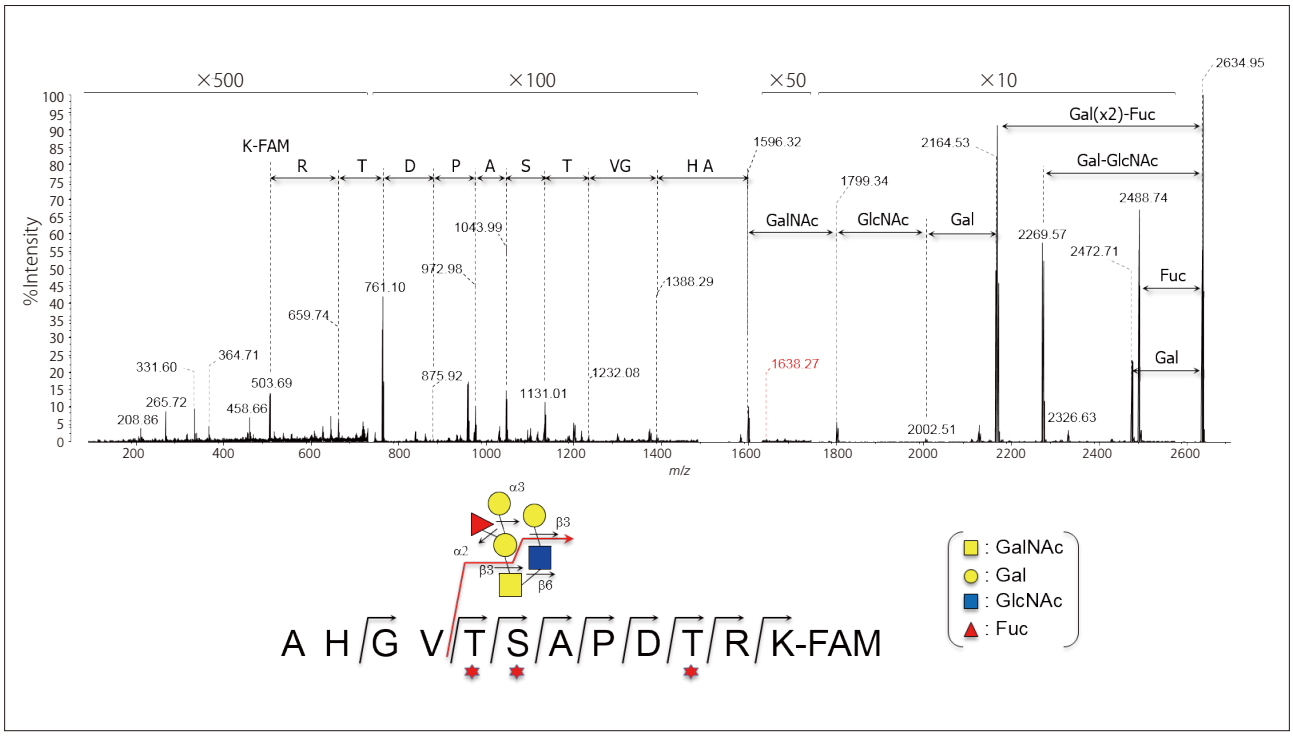

Fig. 5 Glycopeptide (Core2-Muc1A peptide) MS/MS Spectra Before Enzymatic Treatment
: Potential Glycan Binding Site Obtained from MS/MS Analysis

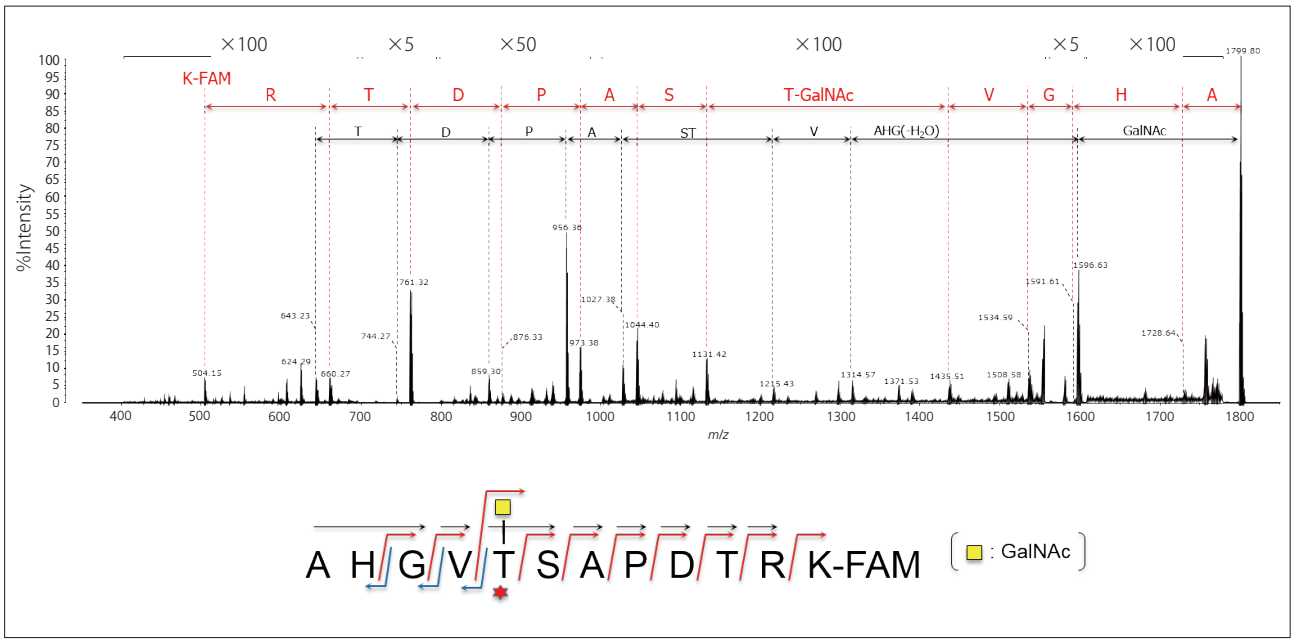

Fig. 6 Glycopeptide (Core2-Muc1A peptide) MS/MS Spectra After Enzymatic Treatment
: Glycan Binding Site Obtained from MS/MS Analysis

[Reference]
1) Kaji, H. et al. Lectin affinity capture, isotope-coded tagging and mass spectrometry to identify *N*-linked glycoproteins. *Nat. Biotechnol*. 21(2003). 667–672.
2) Analysis of Glycopeptides of Monoclonal Antibody Using MALDI-7090 High-Resolution MALDI-TOF MS. B55. Shimadzu Application News.

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