Distinguishing of Linkage Isomers of Lactotetra Oligosaccharides by Using the Relative Ion Intensity Analysis of Post-source Decay Fragment Ions in Curved-field Reflectron Matrix-assisted Laser Desorption/ionization Time-of-flight Mass Spectrometry

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The native oligosaccharides of lacto-*N*-neotetraose (Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc; LNnT) and lacto-*N*-tetraose (Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc; LNT) were analyzed by using curved-field reflectron matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS). Since a curved-field reflectron TOFMS enables a simultaneous focusing of a wide mass range of metastable fragment ions, the relative ion intensities in the post-source decay (PSD) mass spectra can be discussed. The PSD mass spectra of LNnT and LNT were distinguishable in their relative ion intensities. In the case of LNT, β -elimination could occur in the *N*-acetyl glucosamine (GlcNAc) at the C-3 position, which was bonded by galactose (Gal); however, it did not occur in LNnT. The 3-*O* elimination caused a difference in the relative ion intensities in the PSD mass spectra of LNnT and LNT. The β 1-3 glycosyl linkage cleaved more easily than the β 1-4 glycosyl linkage in the MALDI-PSD fragmentation. An analysis of the relative ion intensities in the MALDI-PSD mass spectra of oligosaccharides was very useful for distinguishing the linkage isomers and for characterizing the types of glycosyl linkages.

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Introduction

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) was applied to analyses of biomolecules, such as proteins, peptides and oligosaccharides.¹⁻⁵ The post-source decay (PSD) fragmentation of peptides and oligosaccharides occurred at peptide bonds and at glycosyl linkages in MALDI-MS, respectively. Thus, the PSD mass spectra are useful for the sequencing of peptides and saccharides. The sequencing of peptides could be determined by this method because the peptide bond is just one type, and because almost all amino acids have different molecular weights. On the other hand, it is very difficult to determine the fine structure of the saccharide chains by MS because of the existence of many structural isomers with different linkage types. We could obtain and discuss only the simplest sequence information of oligosaccharides, like Hex-HexNAc-Hex-Hex. In the MALDI-PSD fragment mass spectra of some isomers of oligosaccharides, different tendencies in the spectral patterns were observed;⁶⁻⁸ however, it has been very difficult to quantitatively discuss the ion intensity by using a conventional TOFMS.⁸ Unfortunately, it is impossible to compare the ion intensities in the total PSD spectra because these spectra are usually acquired in segments by stepping the reflectron voltage. Sequence information can be extracted from the reconstructed

time-of-flight mass spectrum by scanning the voltage of the ion reflector and bringing narrow segments of the product spectrum into focus. Consequently, the signal strength might differ in different segments where the ionization conditions might differ. Although it might be possible to compare the absolute intensities of PSD ions in only a narrow segment, huge accumulations of the actual measurements were required to obtain high reproducibility in the absolute intensity; also, the actual spectra of the huge measurements were hardly measured under the same conditions. A logical and actual solution to this problem would be to use an instrument equipped with a reflectron, which records all fragment ions in a single spectrum.

A new type of reflector of curved-field reflectron in a TOF mass detector enables the simultaneous focusing of a wide mass range of metastable fragment ions and the observation of the entire PSD fragment spectrum in a single experiment.^{9,10} Thus, the product-ion mass spectra can be collected without an adjustment under the same conditions, thereby eliminating the need to scan the reflectron voltage. Therefore, the relative intensity of the PSD fragment ions can be discussed at an accurate level. In this work, we studied a new method of a structure analysis for the glycosylation and/or glycoconjugates using the ion intensities of the PSD fragment ions in the curved-field reflectron MALDI-TOF mass spectra.

Previously, we studied the PSD fragment spectra of sugar-branched cyclodextrin derivatives by using curved-field reflectron MALDI-TOFMS.¹¹⁻¹⁵ The PSD ions produced from the branch had higher intensities than those from the part of the cyclodextrin, which is cyclo-malto-oligosaccharide. The former

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ions were produced by the one-site cleavage of the glycosyl linkage; the latter ions were produced by two-site cleavage of the glycosyl linkages. Therefore, the relative intensities of the ions produced by one-site cleavage were higher than those of the ions produced by two-site cleavage.¹¹ It was revealed that the relative ion intensities depended on the structure of the analyte. We also studied the influence of glycosyl donors on the PSD fragmentation of the glycosyl linkages. When the glycosyl donors were glucopyranose and galactopyranose, the glycosyl linkages cleaved much more easily than did the mannosyl linkages, in which the glycosyl donor was mannopyranose.¹²

The linkage isomers cause one of the difficulties in the structure analysis of oligosaccharides. The linkage analysis of α 1-4 and α 1-6 linkages was performed by an ion intensity analysis of the PSD mass spectra. The intensity of the ion produced by cleavage of the α 1-4 linkage was higher than that of the ion produced by cleavage of the α 1-6 linkage. The results indicated that the α 1-4 glycosyl linkage cleaved more easily than the α 1-6 linkage in MALDI-PSD fragmentation.¹³ In studies of highly branched oligosaccharides of the cell-wall polysaccharides of xyloglucan,¹⁶⁻¹⁸ we could detect three-site cleavages and distinguished positional isomers in galactose substitution.¹⁷ In the PSD mass spectra of the oligosaccharides, the PSD ions produced by cleavage of the α 1-6 linkages had higher intensities than those of the β 1-4 glycosyl linkage, indicating that the α 1-6 glycosyl linkages cleaved much more readily than the β 1-4 glycosyl linkage.¹⁸

Sialyl linkages were also analyzed by this method.¹⁹ α 2-3 and α 2-6 sialyl linkages play an important role in many biological activities, such as infection of the influenza virus. The α 2-3 sialyl linkage in 3'-sialyllactose cleaved much more easily than the α 2-6 sialyl linkage in 6'-sialyllactose. Since the results make it possible to distinguish sialyl linkages between α 2-3 and α 2-6 in the glycoconjugates, there are many medical applications using this analytical method of MALDI-MS.

Lacto-*N*-neotetraose (LNnT) and lacto-*N*-tetraose (LNT) are human milk oligosaccharides (Fig. 1), which are linkage isomers each other. These oligosaccharides are general motifs in the oligosaccharides of glycoproteins and glycoconjugates. In LNnT and LNT, commonly, the reducing-end glucose (Glc) is linked to galactose by the β 1-4 linkage, which is linked to *N*-acetylglucosamine (GlcNAc) by the β 1-3 linkage. The non-reducing-end galactose (Gal) is linked by a different linkage between LNnT and LNT. LNnT has a branched Gal to GlcNAc by the β 1-4 linkage, and LNT has a branched Gal to GlcNAc at the non-reducing end by the β 1-3 linkage. The glycosyl linkages of -(Gal β 1-4GlcNAc)- and -(Gal β 1-3GlcNAc)- are related to important biological activities and molecular recognitions in a cell.

LNnT and LNT were analyzed by curved-field reflectron MALDI-TOF mass spectrometry. Until now, the native and derived oligosaccharides, such as permethylation, peracetylation, and benzylamination, have been analyzed by conventional reflectron MALDI-TOFMS.^{6,20,21} We have tried to analyze the native oligosaccharides without any chemical derivatization to elucidate the PSD fragmentation mechanisms in MALDI-MS by an analysis of the relative ion intensities.

Experimental

MALDI-TOF mass spectrometry

KOMPACT MALDI discovery instruments employing a curved-field reflectron (Shimadzu Corp., Japan) were used to

Fig. 1 Structure of lacto-*N*-neotetraose and lacto-*N*-tetraose.

obtain all of the MALDI-TOF and MALDI-PSD fragment mass spectra. Because this type of reflector permits the simultaneous focusing of a wide mass range of fragment ions formed by metastable decay, the product ion mass spectrum can be collected without requiring any stepwise adjustment of the reflectron under unchanged conditions.^{9,10} The operating conditions were as follows: nitrogen laser (337 nm); reflectron mode; detection of positive ions. The ion detector of this instrument is an electron multiplier type. The acceleration potential was set to 20 kV by a grid-type electrode. 2,5-Dihydroxybenzoic acid (DHBA) was used as a matrix, and was dissolved in a 10% ethanol aqueous solution at a concentration of 10 mg/mL. After mixing a sample solution with 0.5 μ L of the matrix solution and 0.5% NaCl solution to assist in the formation of the sodium adduct, the sample plate was dried completely.

Each obtained spectrum was the average of 100 shots. To detect the fragment ions generated by the PSD method, the laser power was adjusted to about 40 μ J (well above the threshold) to promote self-decay after acceleration. These MALDI-PSD fragment spectra were also measured as the averages of 100 shots at different spots. By checking the measurements after one week and one month, the reproducibility of the MALDI-PSD fragmentation patterns presented (*i.e.* the relative intensities) was satisfactorily high for the purposes of this study. In this paper, all ions are labeled with the nominal mass of the isotopomer containing only ¹²C, ¹H, and ¹⁶O atoms in all MALDI-TOF and MALDI-PSD fragment spectra.

Sample

Lacto-*N*-neotetraose and lacto-*N*-tetraose were obtained commercially (Funakoshi, Co., Japan).

Results and Discussion

In the MALDI-PSD mass spectrum of LNnT, the precursor ion at m/z 730 and the fragment ions at m/z 568 (H), 550 (I), 406 (J), 388 (K), and 365 (L) were observed as a sodium adduct (Fig. 2-A). The fragment ion H at m/z 568 was produced by the loss of anhydrogalactose (anGal) at the non-reducing end, corresponding to the species [(M-anGal)+Na]⁺, as shown in Fig. 3 (Y-type fragmentation; Y₄) (the nomenclature for carbohydrate fragmentation B, C, Y and Z, initially proposed by Domon and Costello, is used).²² The fragment ion L at m/z 365 was produced by the loss of the anhydrogalactose and anhydro-*N*-acetylglucosamine (anGal and anGlcNAc), corresponding to the species [(M-anGal-anGlcNAc)+Na]⁺ (Y₃; ion L). The fragment ions at m/z 550 (I) and 388 (K) were produced by the loss of glucose (Glc), and by the loss of glucose and galactose

Fig. 2 MALDI-PSD mass spectra of LNnT (A), and LNT (B). *The ions at m/z 670 labeled by an asterisk was produced by the cross-ring cleavage of ^{0,2}A fragmentation in the reducing-end glucose.

Fig. 3 PSD fragmentation of LNnT.

(Glc and Gal), corresponding to the species [(M-Glc)+Na]⁺ (B₃; ion I) and [(M-anGlc-Gal)+Na]⁺ (B₂; ion K), respectively. There was a possibility that the ion K could be produced by the loss of Glc at the reducing end and the loss of Gal at the non-reducing end, which was a two-site cleavage of the glycosyl linkages. Since the ion intensity of the two-site cleavage ions are lower than that of the single-cleavage ions,¹¹ the ion K at m/z 388 was mainly from the chemical species [(M-anGlc-Gal)+Na]⁺ (B₂) produced by a one-site cleavage. The ion J at m/z 406 was mainly produced by a one-site cleavage and by the loss of Glc and anGal, corresponding to the species [(M-anGlc-anGal)+Na]⁺ (C₂; ion J). These assignments are summarized in Fig. 3.

In the MALDI-PSD mass spectrum of LNT, the precursor ion

at m/z 730 and the fragment ions at m/z 568 (H), 550 (I), 406 (J), 388 (K), and 365 (L) were observed (Fig. 2-B). The number and m/z values of the fragment ions were the same as in the spectra of LNnT and LNT (Fig. 2), because the sequences of LNnT and LNT were the same (Hex-HexNAc-Hex-Hex). It was difficult to distinguish LNnT and LNT at this stage. However, the spectrum pattern of LNnT and LNT differed from each other in the MALDI-PSD mass spectra (Fig. 2).

From the structural differences of LNnT and LNT, it was expected that the intensity of Y₄ (ion H) would show a difference between the 1-3 and 1-4 glycosyl linkages. The intensity of the ion H of LNT was higher than that of LNnT, indicating that the 1-3 linkage cleaved more easily than the 1-4 linkage. These results confirmed those in the PSD mass spectra of Lewis-type of oligosaccharides.^{15,23} This difference at the ion H was small in the PSD mass spectra between LNnT and LNT.

We focused our attention on the relative intensities between ion I and ion K. These ions showed the largest difference in the PSD mass spectra between LNnT and LNT (Fig. 2). In the spectrum of LNnT, the intensity of ion K was higher than that of ion I. The ion K at m/z 388 (B₂ ion) was produced by a cleavage of the glycosyl linkage between GlcNAc and Gal, in which GlcNAc is a glycosyl donor (B₂ ion in Fig. 3). In FAB-MS studies, the aminoglycoside linkage cleaved specifically and predominantly in lactosaminoglycans, which consist of the repeating units (-Galβ1-4GlcNAcβ1-3Gal-)_n.^{24,25} Since the repeating unit is a part of the structure of LNnT and the ion K was produced by cleavage of the aminoglycoside linkage, it was reasonable that the ion K had the highest intensity in the MALDI-PSD mass spectrum of LNnT.

In the PSD mass spectrum of LNT, the intensity of the ion K was lower than that of ion I (Fig. 2-B). The relative ion intensity of the ion K of LNT was much lower than that of LNnT, although the ion K of LNT was also produced by cleavage of the aminoglycoside linkage. In the structure of LNT, the non-reducing end Gal is linked to GlcNAc at the C-3 position (Fig. 1). In negative-ion FAB-MS studies, Z-type fragmentation at 1-3 linkage specifically occurred in branched oligosaccharides containing a 3-O glycosidic linkage.²⁶ It was caused by β-elimination at the C-3 position in the glycosyl acceptor. In the MALDI-PSD fragmentation, 3-O elimination also occurred in the N-acetyl glucosamine branched at the C-3 position in native oligosaccharides.^{15,23} Thus, the 3-O elimination in GlcNAc was expected to occur much more easily in the PSD fragmentation of LNT, because the Gal at the non-reducing end of LNT was bonded to GlcNAc by the 1-3 linkage.

The chemical species of the ion K of LNnT was Gal-GlcNAc at the non-reducing end (Fig. 3), and the intensity of the ion K was the highest in the spectrum of LNnT. In the case of LNT, since the linkage of Gal-GlcNAc cleaved by the 3-O elimination (Z₄) as well as Y₄ fragmentation, as shown in Fig. 4, the amount of the chemical species of ion K decreased considerably. Thus, the relative intensity of the ion K of LNT could be much lower than that of LNnT.

The intensity of the ion I was highest in the PSD spectrum of LNT (Fig. 2-B). In the PSD fragmentation of oligosaccharides containing the 3-O branch, the Z-type fragmentation caused by 3-O elimination in the glycosyl acceptor GlcNAc occurred much more easily than Y-type fragmentation.²³ Therefore, in the cleavage of Galβ1-3GlcNAc in LNT, the Z-type fragmentation (Z₄ at m/z 550) could occur much more easily than Y-type fragmentation (Y₄ at m/z 568) (Fig. 4). Since the ion I at m/z 550 of LNT was the sum of two chemical species from large amounts of Z₄ ions [(M-Gal)+Na]⁺ and from B₃ ion [(M-Glc)+Na]⁺, ion I had a higher intensity than ion K, and ion I

Fig. 4 PSD fragmentation of LNT.

was the highest peak in the PSD spectrum of LNT.

The ion intensity of ion H of LNT was slightly higher than that of LNnT, as described above; 1-3 glycosyl linkage in LNT cleaved easier than 1-4 glycosyl linkage in LNnT. The difference between 1-4 and 1-3 glycosyl linkages in the PSD fragmentation was expected to be much larger than that in the observed spectra. We should consider the influence of 3-O elimination on the relative ion intensities. Since the intensity of the ion H in LNT decreased due to 3-O elimination, the amount of the ion intensity produced by the cleavage of the 1-3 linkage should be estimated to be much higher than that of the observed ion H in the PSD spectrum of LNT.

The chemical species of ions J and K in LNT are shown in Fig. 5, which had the glycosyl linkage of Gal-GlcNAc commonly. If the intensity of ion K in the PSD spectrum of LNT decreased by Z₄ and Y₄ fragmentation (Figs. 2-B and 4), the intensity of ion J in the PSD spectrum of LNT should decrease. However, the intensity of ion J did not decrease (Fig. 2-B). One possibility is that the 3-O substitution of Gal at GlcNAc influenced the cleavage of the aminoglycoside linkage of GlcNAc-Gal. As another possibility, we also focused our attention on the difference in the chemical species of ions J and K in LNT (Fig. 5). The GlcNAc had a C-1 hydroxyl group in the chemical species of ion J, because ion J was produced by C-type fragmentation. In the chemical species of ion K produced by B-type fragmentation, a double bond is formed between the C-1 and C-2 atoms in GlcNAc. The ring oxygen atom having nonbonding electron pair and the N-acetyl group at C-2 were neighbors of the double bond between the C-1 and C-2 atoms in the chemical species of ion K. The difference in the chemical properties of these chemical species could influence the secondary fragmentation. Z₄ and Y₄ fragmentation could occur more easily from the chemical species of ion K than from those of ion J. In addition, the stability of the sodium adduct of the chemical species would influence the intensity of ions J and K.

The analyzing of MALDI-PSD mass spectra of oligosaccharides systematically would bring us a clue to clarify the general roles of the fragmentation mechanisms in MALDI-PSD mass spectrometry.

Conclusion

The linkage isomers of LNnT and LNT were distinguished by using relative-intensity analyses of the ion K and ion I in their PSD spectra. Ion K had a higher intensity than ion I in the PSD spectrum of LNnT; in reverse, ion I had a higher intensity than

Fig. 5 Chemical species of ion J and K in LNT. The chemical species of ions J and K are sodium adducts, which correspond to [(M-anGlc-anGal)+Na]⁺ and [(M-anGlc-Gal)+Na]⁺, respectively.

ion K in the PSD spectrum of LNT. The relative ion intensity indicated the fine structure of the analytes. Correlation studies between the ion intensities and the structures of analyte systematically could make it possible to develop a new powerful method for the structure analysis of biologically important oligosaccharides and glycosylation at the glycoproteins.

Recently, a proteome analysis has been used to identify the target proteins for some disease or related to important biological activities. This approach is thought to be one of the most promising techniques to resolve complex life systems. The sequencing of proteins using MS is the first step for their identification after being separated through 2D-electrophoresis. An analysis of the post-translational modification of proteins is another important step. The glycosylation of proteins is related to their biological activities, and they play important roles in expressing their biological functions. In the future, we can identify glycosylation rapidly by using our data base of the relative ion intensities in the PSD mass spectra of the glycosylations. Therefore, the relative ion intensity analysis is a very useful method and a powerful tool for proteome research in post-translational modification analysis.

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