**Tohru Yamagaki[1], Hiroshi Nakanishi[2]**

[1]Department of Chemistry, The University of Tokyo, Tokyo, Japan
[2]National Institute of Bioscience and Human-Technology, Ibaraki, Japan

# Ion intensity analysis of post-source decay fragmentation in curved-field reflectron matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of carbohydrates: For structural characterization of glycosylation in proteome analysis

The various kinds of oligosaccharides were analyzed by using post-source decay (PSD) fragmentation method of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Since the curved field reflectron MALDI-MS can record all fragment ions in a single measurement, we can discuss the fragment ion intensity in PSD mass spectrum more accurately. The intensities of the PSD fragment ions indicate the fine structure of the saccharide chains. The type of glycosyl linkages could be determined by the ion intensity analysis, and the stereo isomers of monosaccharides were distinguished by the MALDI-PSD fragment ion analysis. The linkage isomers and structural isomers were also distinguished by this method. The ion intensity analysis of curved-field reflectron MALDI-MS could be a powerful tool for glycosylation analysis.

**Keywords:** Fragment / Glycosylation / Oligosaccharide / Matrix-assisted laser desorption / Ionization mass spectrometry / Post-source decay PRO 0022

## 1 Introduction

Proteome analysis has identified the target proteins for some diseases and the proteins related to the important biological activities. This approach is thought to be one of the most promising techniques to resolve the complex life systems. The sequencing of the proteins is the first step for their identification after being separated by 2-D electrophoresis. Mass spectrometry, such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and electrospray ionization mass spectrometry (ESI-MS), is one of the most useful methods for this purpose [1, 2]. In the MS/MS analysis of the peptide sequencing using MALDI-TOF-MS, the post-source decay (PSD) fragment ions give much sequence information of the analyte peptides [3, 4]. It is essential in proteome research to perform the sequencing of the digested peptides using the PSD fragment method of MALDI-MS. Recently, we reported the improvement of the sensitivity of the PSD fragment ions of peptides using fluorescent modification [5]. Ideally, these fragment ions should be sufficient to sequence the peptide analyte. For some peptides, however, the fragment ions were not amenable to complete sequence interpretation. The fluorescent modification of the peptide analyte greatly improved the interpretability of the MALDI-PSD mass spectrum and it was useful in sequencing peptides using MALDI-TOF-MS in proteome research.

The analysis of the post-translational modification of proteins is another important step. The glycosylation of proteins relate to their biological activities and they play an important role in expressing their biological functions. The glycosylation can be identified by proteins ladder sequencing using MALDI-MS [6]. The sequencing of the saccharide chains can also be performed easily by the MALDI-PSD fragmentation method [7–13]. The glycosyl linkages in the saccharides were cleaved in the PSD fragmentation, and the product ions indicate a sequence like Hex-HexNAc-Hex-Hex. MALDI-TOF-MS is useful for the sequencing of the saccharide, however, it is difficult to

**Correspondence**: Dr. Tohru Yamagaki, Department of Chemistry, School of Science, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
**E-mail**: yamagaki@chem.s.u-tokyo.ac.jp
**Fax**: + 81-3-5841-8380

**Abbreviations: Hex,** Hexose; **Glc,** D-glucopyranose; **Gal,** D-galactopyranose; **GlcNAc,** *N*-acetyl glucosamine; **Neu5Ac,** *N*-acetylneuraminic acid, **CD,** cyclodextrin, ***m/z***, mass to charge ratio; **s-Le$^x$,** sulfated Lewis$^x$ trisaccaride; **s-Le$^a$,** sulfated Lewis$^a$ trisaccaride; **DHBA,** 2,5-dihydroxybenzoic acid

 1615-9853/01/0202–329 $17.50+ .50/0

determine the fine structure of the saccharide chains by MS due to many structural isomers with different linkage positions. For example, the glycosyl linkages even in a biose between same sugar residues, like Glc-Glc, have eight variations from four linkage types and two configurations (1-2, 1-3, 1-4, and 1-6 linkage types and $\alpha$-, $\beta$-configurations). In addition to that, the structural isomers of monosaccharide units with the same mass numbers also make it difficult to analyze the structure using MS. The following three issues are thought to be important in the structure analysis of saccharide chains by MS; 1) the analysis of the highly branched structures, 2) the linkage analysis and distinguishing of the linkage isomers, 3) the distinguishing between monosaccharide isomers.

In the MALDI-PSD mass spectra of some isomers, the different tendencies in the spectral patterns were observed [7–13], however, it has been very difficult to discuss the ion abundance [13]. Unfortunately, comparison of ion abundance in most PSD spectra is not an absolute measurement because these spectra are usually acquired in segments by stepping the reflectron voltage. Sequence information can be extracted from the reconstructed TOF mass spectrum by scanning the voltage of the ion reflector and bringing narrow segments of the product spectrum into focus. Consequently, the ions in question might appear in different segments where ionization conditions and, consequently, the signal strength, might differ. A logical solution to this problem would be to use an instrument equipped with a reflectron, which records all fragment ions in a single spectrum [13].

A new type reflector of curved-field reflectron [14, 15] in TOF mass detector enables a simultaneous focusing of a wide mass range of metastable fragment ions and observation of the entire PSD fragment spectrum in a single experiment. Thus, the product-ion mass spectra can be collected without the adjustment under the same conditions, thereby, eliminating the need to scan the reflector voltage. Therefore, the relative intensity of the PSD fragment ions can be discussed in more detail. In this paper, we studied the new method of the structure analysis for the glycosylation and/or glycoconjugates by the ion intensity of the PSD fragment ions using curved-field reflectron MALDI-TOF-MS.

## 2 Materials and methods

### 2.1 MALDI-TOF-MS

Kompact MALDI-IV and Discovery instruments with curved-field reflectron (Kratos-Shimadzu Corp., Japan) were used to obtain all MALDI-TOF and MALDI-PSD fragment mass spectra. Since this new type of reflector permits simultaneous focusing of a wide mass range of fragment ions formed by metastable decay, the product-ion mass spectrum can be collected without stepwise adjustment of the reflectron under unchanged conditions [14, 15]. Operating conditions were as follows; nitrogen laser (337 nm); reflectron mode; positive ion mode. The ion detector of this instrument is a electron multiplier type. The acceleration potential was set to 20 kV by a grid type electrode. 2,5-Dihydroxybenzoic acid (DHBA) was used as matrix, dissolved in 10 % ethanol aqueous solution at a concentration of 10 mg/mL [16]. After mixing the sample solution with 0.5 $\mu$L of the matrix solution and about 0.5 % NaCl solution to assist the formation of sodium adducts, the sample plate was dried completely. The sample plate having a textured surface was used to promote reproducibility and the best crystallization of matrix.

Each spectrum was obtained as the average of one hundred shots. To detect the fragment ions generated by the PSD method, the laser power was adjusted to about 40 $\mu$J (well above the threshold) to promote self-decay after acceleration. Comparing the ion intensities, the same value of the laser power was used. MALDI-PSD mass spectra were also measured as the averages of one hundred shots at different spots. The reproducibility of the spectral patterns in the MALDI-PSD mass spectra (*i. e.* relative intensities) were satisfactorily high for the present purposes, checking the measurements after one week and one month. All ions were labelled with the nominal mass of the monoisotope in all MALDI-TOF and MALDI-PSD mass spectra in this paper.

### 2.2 Materials

Malto-cyclo-hexaose ($\alpha$-cyclodextrin; $\alpha$CD), malto-cyclo-heptaose ($\beta$-cyclodextrin; $\beta$CD), glucosyl-$\beta$CD, and maltosyl-$\beta$CD are commercially available (Bio-Research Corp. of Yokohama, Japan). Xyloglucan oligosaccharides were obtained from tamarind seed enzymatically. They consist of four $\beta$-D-glucopyranoses (Glc) bonded by $\beta$1-4 glycosyl linkage, three $\alpha$-D-xylopyranose (Xyl) substituted to each Glc by $\alpha$1-6 linkage, and one $\beta$-D-galactopyranose (Gal) substituted to Xyl by $\beta$1-2 linkage. Maltotriosyl-$\alpha$CD and panosyl-$\alpha$CD are gifts from Nikken Kagaku Co., Japan. Pullulan have repeating units of maltotriose ($\alpha$1-4 linked glucotriose) which are bonded by $\alpha$1-6 linkage each other -(-6Glc$\alpha$1-4Glc$\alpha$1-4Glc$\alpha$1-)-$_n$. The pullulan oligosaccharides are gifts from Dr. Yasushi Mitsuishi (National Institute of Bioscience and Human-technology, Japan). 3'-Sialyllactose (Neu5Ac($\alpha$2-3)Gal($\beta$1-4)Glc) and 6'-sialyllactose (Neu5Ac($\alpha$2-6)Gal($\beta$1-4)Glc) are commercially available (Funakoshi Co. Japan). Glucosyl-$\alpha$CD, galactosyl-$\alpha$CD, and mannosyl-$\alpha$CD are gifts from Bio-Research Corp. of Yokohama, Japan. Sulfated Lewis$^X$

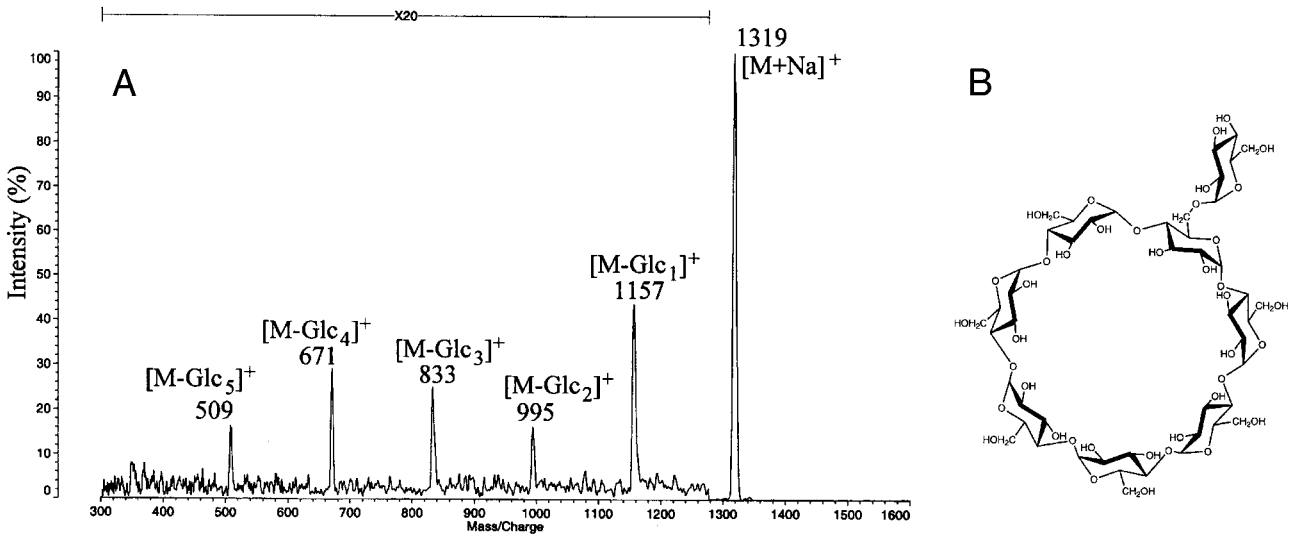


**Figure 1.** The MALDI-PSD Mass Spectrum (A) of Glucosyl-$\beta$CD (B).

and Lewis[a] trisaccharides are commercially available (Funakoshi Co., Japan).

## 3 Results and discussion

### 3.1 The analysis of the highly branched structure

Many *N*-linked oligosaccharides in glycoproteins have complex and highly branched structures. At first, the simple branched neutral oligosaccharides were analyzed by MALDI-PSD fragmentation method. Glucosyl-$\beta$CD ($Glc_1$-$\beta$CD) and maltosyl-$\beta$CD ($Glc_2$-$\beta$CD) are sugar branched cyclodextrins. In the MALDI-PSD mass spectrum of $Glc_1$-$\beta$CD, the precursor ion $[M+Na]^+$ at *m/z* 1 319 and the fragment ions at *m/z* 1 157, 995, 833, 671 and 509, were observed as a sodium adduct (Fig. 1). The intervals of these ions were 162 Da corresponding to the loss of glucose. Each fragment ion corresponded to the chemical species of $[M+Na\text{-}Glc_1]^+$, $[M+Na\text{-}Glc_2]^+$, $[M+Na\text{-}Glc_3]^+$, $[M+Na\text{-}Glc_4]^+$, and $[M+Na\text{-}Glc_5]^+$, which were described for convenience as abbreviations such as $[M\text{-}Glc_1]^+$ to $[M\text{-}Glc_5]^+$.

The intensity of the fragment ion at *m/z* 1 157 ($[M\text{-}Glc_1]^+$) was higher than those of the ions $[M\text{-}Glc_{2\text{-}5}]^+$ as shown in Fig. 1. The ion $[M\text{-}Glc_1]^+$ having higher intensity was produced by the one-site cleavage from the branch, and the ions $[M\text{-}Glc_{2\text{-}5}]^+$ having lower intensity were produced by the two-site cleavages from the cyclodextrin part. In the PSD fragment spectrum of $Glc_2$-$\beta$CD, the fragment ions $[M\text{-}Glc_1,Glc_2]^+$ had higher intensity than the ions $[M\text{-}Glc_{3\text{-}6}]^+$ (Fig. 2). The ions $[M\text{-}Glc_1,Glc_2]^+$ having higher intensity were produced from the branched part, and the ions $[M\text{-}Glc_{3\text{-}6}]^+$ having lower intensity were produced from the cyclodextrin part. These results were reasonable because the fragment ions produced by one-site cleavage of glycosyl linkage are more probable than those produced by two-site cleavage in MALDI-MS [10, 17]. In the FAB-MS spectra of sugar branched cyclodextrins, only the fragment ions produced from the branch were observed [18]. In the MALDI-PSD mass spectra, the fragment ions were produced both from the branch and cyclodextrin parts with intensity difference. It was found that the one-site cleavage occurs easier than two-site cleavage in the MALDI-PSD fragmentation. The relative intensities of the PSD fragment ions give important information about the fine structures of the molecules.

In more highly branched oligosaccharides, the fragment ions produced by multisite cleavages of the glycosyl linkages were clearly observed [19–21]. The one-site cleavage ions have higher intensities than two-site cleavage ions, which were higher than three-site cleavage ions. These results indicate that the one-site fragmentation occurs easier than two- or three-site fragmentation in MALDI-PSD mass measurements [21]. This method was applied to the determination of branched structures, and branched isomers were distinguished by this method. For example, the highly branched oligosaccharides **1**–**3** (Fig. 3) are the analogous isomers with the different positions of the galactose substitution. These isomers were distinguished by the ion intensity analysis. The fragment ion $[Hex_2,Pen_2]^+$-**1** was produced by the one-site cleavage of the glycosyl linkage (vertical lines in Fig. 3). In the

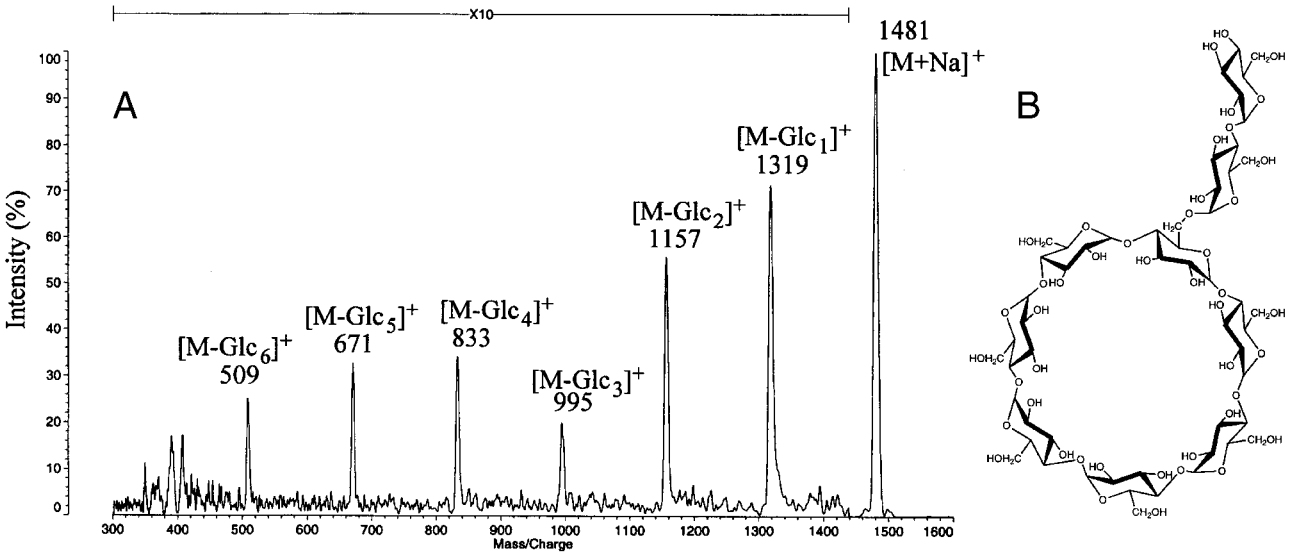


**Figure 2.** The MALDI-PSD Mass Spectrum (A) of Glucosyl-$\beta$CD (B).

case of **2** and **3**, the fragment ions $[Hex_2,Pen_2]^+$-**2** and -**3** were produced by the two-site cleavages of the glycosyl linkages as shown by the vertical lines in Fig. 3. Thus, the intensity of the $[Hex_2,Pen_2]^+$-**1** ion was much higher than those of the $[Hex_2,Pen_2]^+$-**2** and -**3** ions in the MALDI-PSD mass spectra [21] and the oligosaccharide **1** could be distinguished from **2** and **3**. The oligosaccharides **2** and **3** could be differentiated by the ion intensities of the $[Hex_2,Pen_1]^+$ ions as shown by the horizontal line in Fig. 3. The $[Hex_2,Pen_1]^+$-**3** was produced by one-site cleavage, which resulted in higher ion intensity than that of the $[Hex_2,Pen_1]^+$-**2** ion which was produced by two-site cleavage. Thus, these three isomers could be distinguished using the ion intensity analysis of the MALDI-PSD mass spectra, which give us much sequence and branching information. It was thought that *N*-Linked oligosaccharides can be also analyzed effectively by this method. The analysis of ion intensities of MALDI-PSD mass spectra could be a very useful technique in analyzing even highly branched tri- and/or tetra-antennary oligosaccharides in glycoproteins.

## 3.2 Distinction of linkage isomers and the linkage analysis

### 3.2.1 $\alpha$1-4, $\alpha$1-6, and $\beta$1-4 linkages, and $\alpha$-, $\beta$-configurations

One of the difficulties for the structure analysis of oligosaccharides are caused by the existence of various kinds of linkage isomers. We tried to perform the linkage analysis and to distinguish those linkage isomers by the ion intensity analysis in the MALDI-PSD fragmentation method. Maltotriosyl-$\alpha$CD and panosyl-$\alpha$CD differ only at the glycosyl linkage of the nonreducing end of the branch ($\alpha$1-4 and $\alpha$1-6 linkage, Fig. 4). Comparing their PSD fragment spectra, the relative intensity of the $[M+Na\text{-}Glc_1]^+$ of maltotriosyl-$\alpha$CD was much higher than that of panosyl-$\alpha$CD as shown in Fig. 5. The $[M+Na\text{-}Glc_1]^+$ ion of maltotriosyl-$\alpha$CD was produced by the cleavage of $\alpha$1-4 glycosyl linkage, and that of panosyl-$\alpha$CD was produced by the cleavage of $\alpha$1-6 linkage. The results indicate that the glycosidic linkage of $\alpha$1-4 cleaves much easier than that of $\alpha$1-6 in MALDI-PSD fragmentation. In the case of pullulan that has $\alpha$1-4 and $\alpha$1-6 linkages in the molecule, $\alpha$1-4 glycosidic linkage also cleaves easier than $\alpha$1-6 linkage. The result was consistent with those of maltotriosyl and panosyl oligosaccharides [22].

In the studies of a xyloglucan heptasaccharide, we investigated the fragmentation of $\alpha$1-6 and $\beta$1-4 glycosyl linkages [23]. A xyloglucan heptaose consists of $\beta$1-4 linked four glucose residues (cellotetraose back-born) and three xylose residues branching at the glucoses by $\alpha$1-6 linkage (the structure of the octaoses are shown in Fig. 3). In the MALDI-PSD mass spectrum of the xyloglucan heptaose, almost of all fragment ions, which were produced by one- to three-sites cleavage of the glycosyl linkage, were observed. The intensities of the ions produced by the cleavage of $\alpha$1-6 linkage were much higher than those of the ions produced by the cleavage of $\beta$1-4 linkage. These results indicate that the glycosidic linkage of

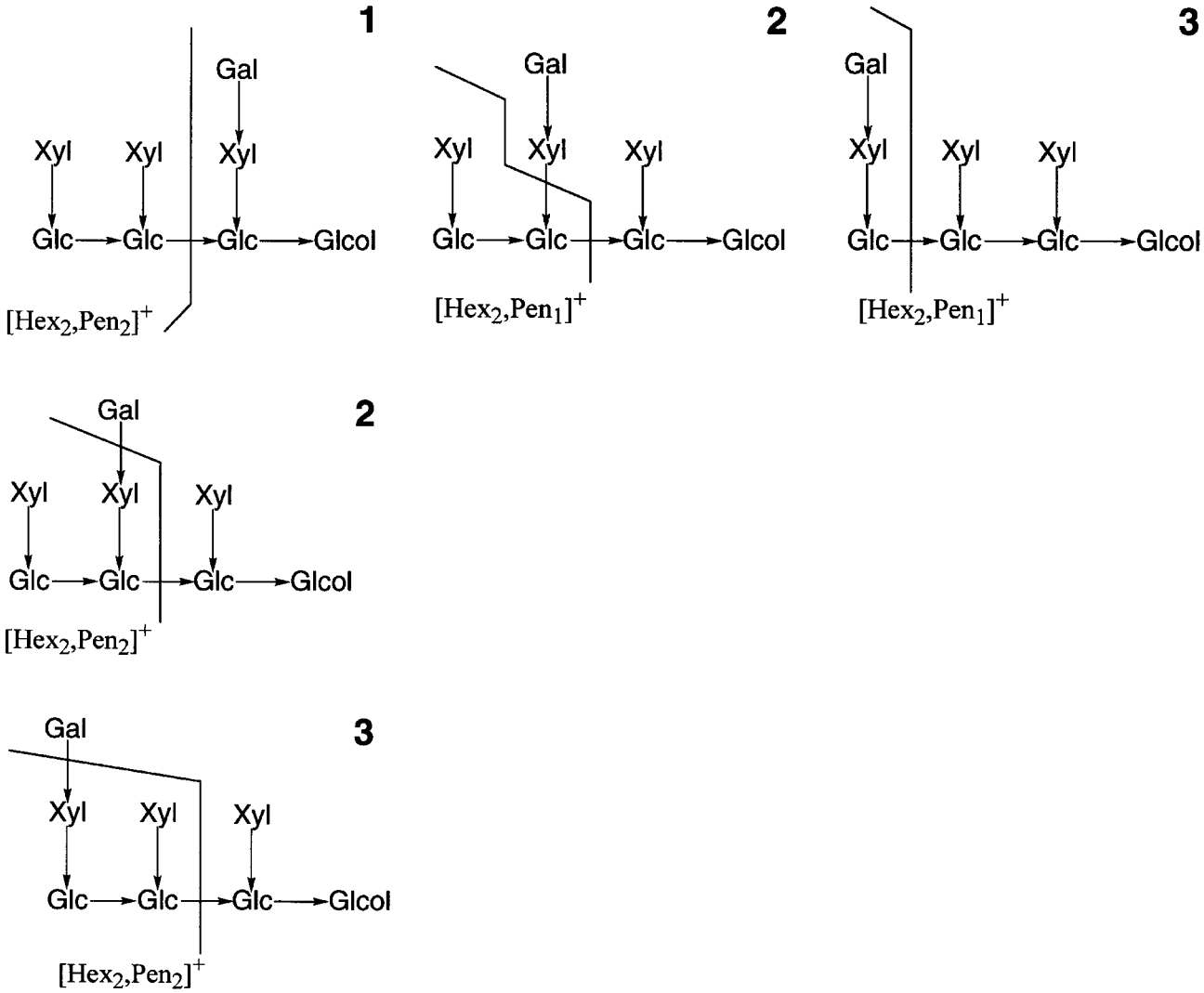


**Figure 3.** Structures of highly branched xyloglucan oligosaccharides.

$\alpha$1–6 cleaves much more easily than that of $\beta$1-4 linkage. Therefore, in the MALDI-PSD fragmentation, the $\alpha$1-4 glycosyl linkages cleave easier than that of $\alpha$1-6, and the glycosyl linkages of $\alpha$1-6 cleave easier than that of $\beta$1-4. These results lead to the following assumption; the fragmentation of $\alpha$1-4 glycosyl linkage occurs easier than that of $\beta$1-4 glycosyl linkage ($\alpha$1-4 linkage $>$ $\beta$1-4 linkage). The assumption will be proved by the data of the actual measurements. These results and discussions indicated that the fragmentation of MALDI-MS also depended on $\alpha$- and $\beta$-configuration. The $\alpha$-linkage cleaved easier than $\beta$-linkage in the PSD-fragmentation of 1-4 glycosyl linkage.

Moreover, we will also discuss in detail the fragmentation of the 1-3 and 1-4 glycosyl linkages using ion intensity analysis in Section 3.4.2 of sulfated Lewis oligosaccharides. The fine analyses of their PSD mass spectra showed that the fragmentation of both $\alpha$ and $\beta$ 1-3 linkages occurs easier than that of the corresponding 1-4 linkages (1-3 linkages $>$1-4 linkages). It was shown that the $\alpha$1-4 glycosyl linkages cleaved easier than the $\beta$1-4 glycosyl linkages in the above discussions. The results indicated that the $\alpha$1-3 glycosyl linkages cleaved easier than the $\beta$1-4 glycosyl linkages.

From these analyses, it was concluded that the MALDI-PSD fragment ions reveal different intensities based on the kinds of glycosyl linkages. Therefore, the detailed analysis of the intensities of the PSD fragment ions using MALDI-TOF-MS is a very powerful tool in estimating the type of glycosyl linkage in saccharide compounds.

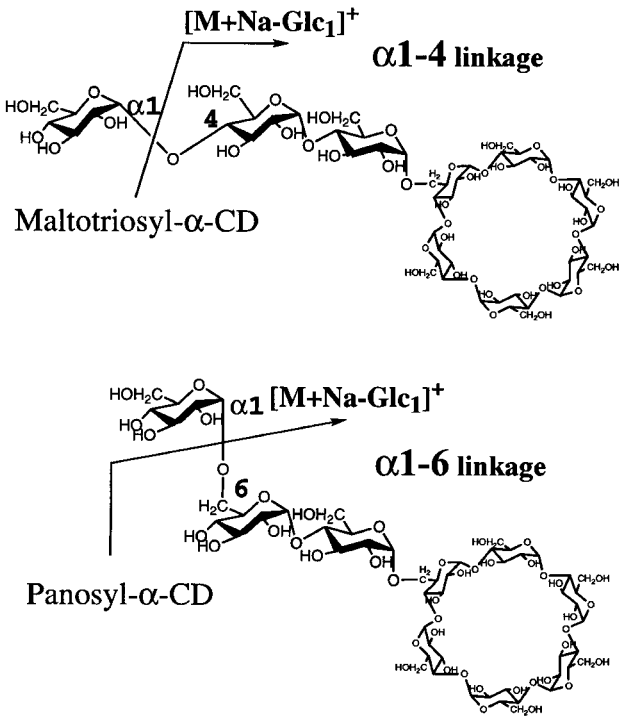


**Figure 4.** Structures of Maltotriosyl-αCD and Panosyl-αCD.

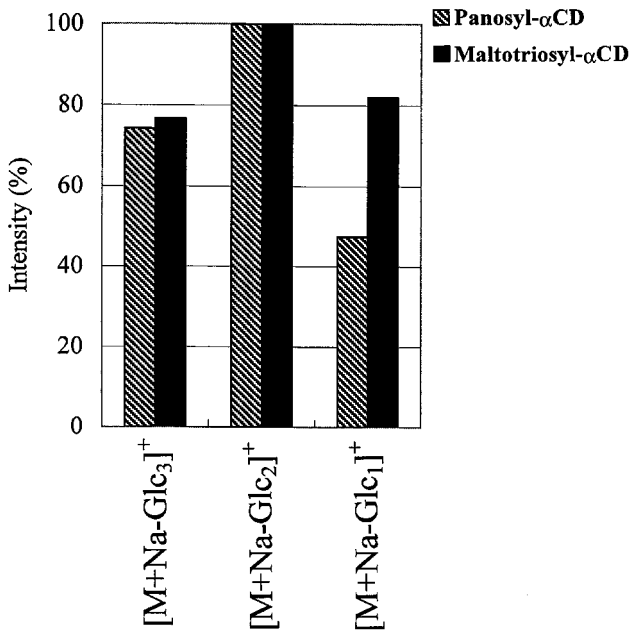


**Figure 5.** The Intensity of the Fragment Ions Produced from the Branch of Maltotriosyl-αCD and Panosyl-αCD.

### 3.2.2 *α*2-3/*α*2-6 Sialyl linkage analysis

Sialyl linkages are one of the most important glycosyl linkages for the biological activities. *N*-acetyl neuraminic acid (Neu5Ac) substitutes at the non-reducing end by α2-3 or α2-6 sialyl linkages in gangliosides and glycoproteins. The Neu5Ac residues play an important role in the molecular recognition because they are located at the nonreducing end. These linkages are thought to have the special functions in the biological activities due to the carboxyl group at the C-1 position. In many cases, the expression of the biological activities depends on the types of sialyl linkages. For example, the types of influenza virus were classified by the recognition of the α2-3/α2-6 sialyl linkages [24].

The analysis using GC/MS and FAB-MS for permethylated or peracetylated sialo-oligosaccharides have been reported [25–27]. Recently, the derivatization of the sialo-oligosaccharides for MALDI-MS measurements were also reported [28]. The MALDI-PSD fragmentation method [29] was used to distinguish between α2-3 and α2-6 sialyl linkages based upon ion intensity analysis. Sialyllactoses have the linkage isomers with α2-3 and α2-6 sialyl linkages at the non-reducing end (Figs. 6 and 7). The sialyllactoses were not derivatized for the measurements. In the positive MALDI-PSD mass spectra of 3'- and 6'-sialyllactoses, the precursor ions at m/z 678 ($[M+2Na-H]^+$) and the fragment ions at *m/z* 336 ($B_1$) were observed as a sodium adduct ($[Neu5Ac+2Na-H]^+$) as shown in Figs. 6 and 7. The fragment ions at *m/z* 336 were produced by the cleavage of the sialyl linkages (B-type fragmentation, $[B_1-H+2Na]^+$; the nomenclature for carbohydrate fragmentation [30]).

The $B_1$ fragment ion at *m/z* 336 of 3'-sialyllactose had the higher intensity than the precursor ion (Fig. 6). The intensity of the precursor ion was about 10 % to the ion $B_1$. One of the reasons is that the matrix of DHBA can promote the cleavage of the glycosyl linkage, and that the sialyl linkages cleave much easier in MALDI-MS [31]. The $B_1$ fragment ion at *m/z* 336 of 6'-sialyllactose was lower than that of the precursor ion under the same measurement conditions of 3'-sialyllactose (Fig. 7), and the intensity of the ion $B_1$ was about 60 % to the precursor ion. These results indicate that the relative intensity of the $B_1$ fragment ion of 3'-sialyllactose was considerably higher than that of 6'-sialyllactose. The fragmentation of α2-3 sialyl linkage occurs much easier than that of α2-6 sialyl linkage in MALDI-TOF-MS. Therefore, it was possible to distinguish between α2-3 and α2-6 sialyl linkages using MALDI-PSD method without any derivatization. Sialo-oligosaccharides are one of the most interesting biomolecules due to their many kinds of important functions such as molecular recognition. This distinguishing method of sialyl linkages

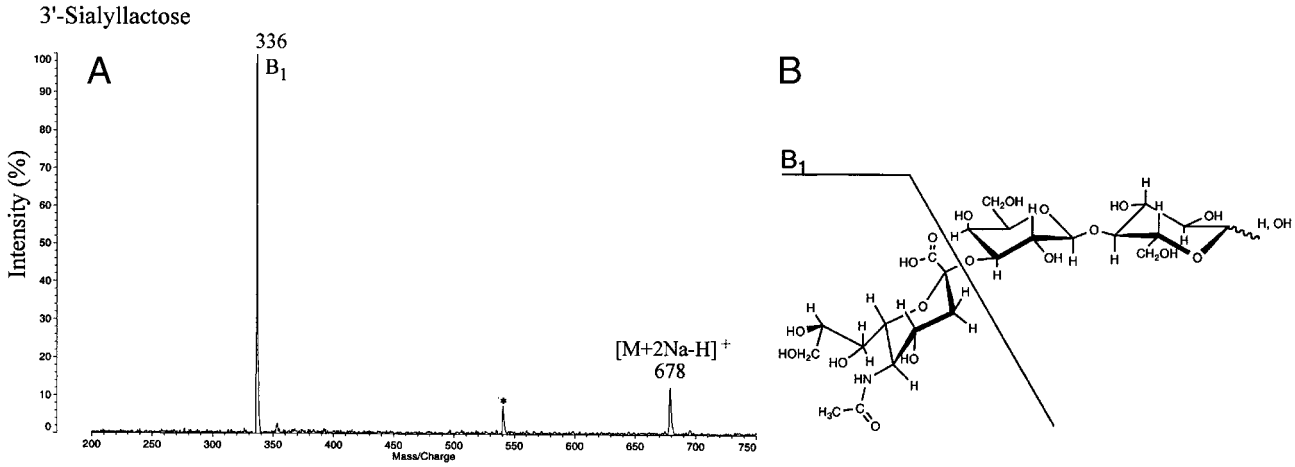


**Figure 6.** The MALDI-PSD Mass Spectrum (A) of 3'-Sialyllactose (B). * The ion produced by cross-ring fragmentation.

is also a potentially powerful tool for many medical applications.

### 3.3 Distinction of the stereo isomeric monosaccharides

We studied the influence of the stereo isomeric monosaccharides on the MALDI-PSD fragmentation [32]. The monosaccharides which consist of saccharide chains are structural isomers. For example, galactose (Gal) and mannose (Man) are epimers of glucose (Glc) (Fig. 8). These stereo isomers cause the difficulty of the structure analysis of oligosaccharides. To clarify the influence of the glycosyl donor variations of Glc, Gal, and Man, it would be required to compare the fragment ions produced from Glc(α1-6)Glc, Gal(α1-6)Glc, and Man(α1-6)Glc units, which have the same glycosyl acceptor Glc. The aglycon as a glycosyl acceptor and the glycosyl linkage should be the same in the analytes. Glycosyl-αCD, galactosyl-αCD, and mannosyl-αCD (Fig. 8) were analyzed by the MALDI-PSD mass spectra. The glycosyl donors of α-D-glucopyranose, α-D-galactopyranose, and α-D-mannopyranose differ in the MALDI-PSD fragmentation although they are isomers (Fig. 8). The fragment ions $[M+Na\text{-}Glc]^+$ and $[M+Na\text{-}Gal]^+$ were more intense than the $[M+Na\text{-}Man]^+$ ion in the MALDI-PSD mass spectra (Fig. 9). All hydroxyl groups of C-2 to C-4 in glucose are at the equatorial orientations. Galactose is the C-4 epimer of glucose and mannose is the C-2 epimer of glucose. The C-2 hydroxyl groups in glucose and galactose are in the same orientation (equatorial), however, that of mannose is in the axial orientation. It was reported that the hydrogen atoms at C-2 in the glycosyl donors relate to Y-type fragmentation mechanism as the glycosyl linkage cleaves at the non-reducing end side [33]. Thus, it was reasonable that the fragmentation of the glycosidic linkages depended on the orientation of the hydroxyl groups at C-2 in glycosyl donors. We should consider the influences of different glycosyl donors on the ion intensities to interpret the MALDI-PSD mass spectra. These fragmentation roles in MALDI-MS potentially enable us to distinguish even between monosaccharide epimers.

### 3.4 The analysis of the structural isomers of sulfated Lewis$^X$ and Lewis$^a$ trisaccharides

#### 3.4.1 The fragmentation analysis

Lewis-type glycoconjugates relate to very important biological activities such as molecular recognition in the cell and they relate to some disease, such as sialyl Lewis$^X$ in tumor [34–36]. Lewis$^X$ trisaccharide is a part of sialyl Lewis$^X$, and Lewis$^a$ trisaccharide is a Lewis-type blood antigen. The Lewis-type oligosaccharides are isomers and corresponding glycosphingolipids have been studied by FAB-MS and ESI-MS [37–42]. In this paper, we studied the PSD fragmentation of sulfated Lewis-type oligosaccharides using MALDI-TOF-MS.

In the MALDI-PSD mass spectrum of sulfated Lewis$^X$ trisaccharide (s-Le$^X$), the precursor ion $[M-H+2Na]^+$ at *m/z* 654 and the charge-remote fragment ions at *m/z* 508 ($[Y_{1\beta}+2Na\text{-}H]^+$), 490 ($[Z_{1\beta}+2Na\text{-}H]^+$), and 287 ($[B_{1\alpha}+2Na\text{-}H]^+$) were observed (Fig. 10). The fragment ion at *m/z* 508 was produced by the loss of anhydrofucose because of the interval of 146 Da from the precursor ion (Y-type fragment ion; $[Y_{1\beta}+2Na\text{-}H]^+$). The ion at *m/z* 490 having the interval of 164 Da was produced by the loss of fucose

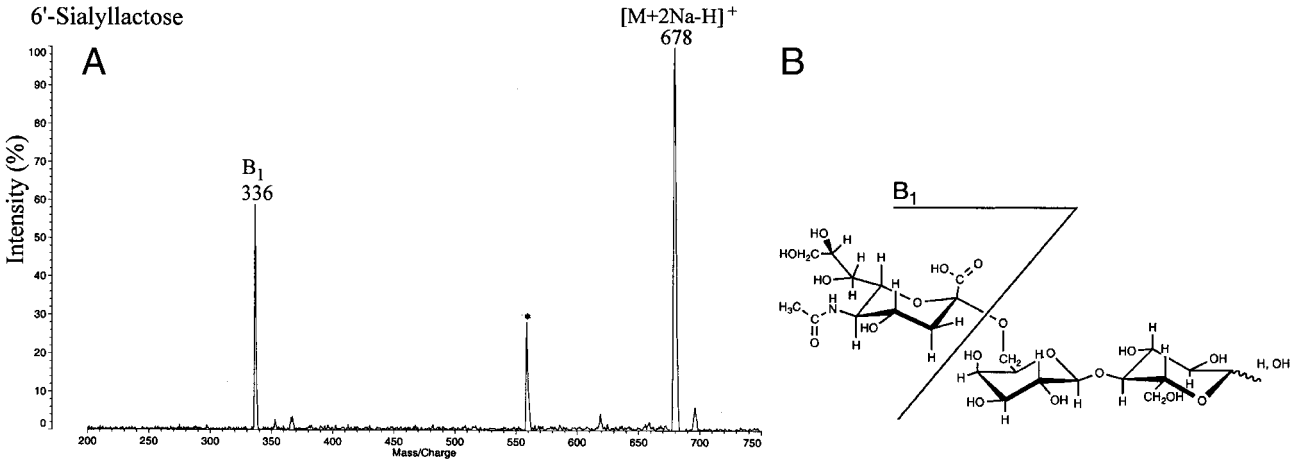


**Figure 7.** The MALDI-PSD Mass Spectrum (A) of 6'-Sialyllactose (B). * The ion produced by cross-ring fragmentation.

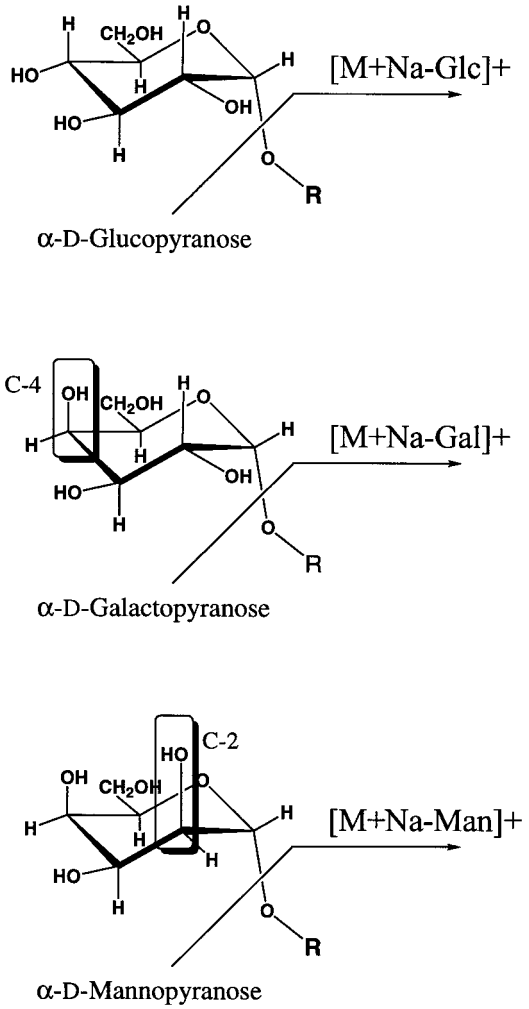


**Figure 8.** Structures of Glucosyl-$\beta$CD, Galactosyl-$\beta$CD, and Mannosyl-$\beta$CD. **R** is $\beta$-cyclodextrin ($\beta$CD).

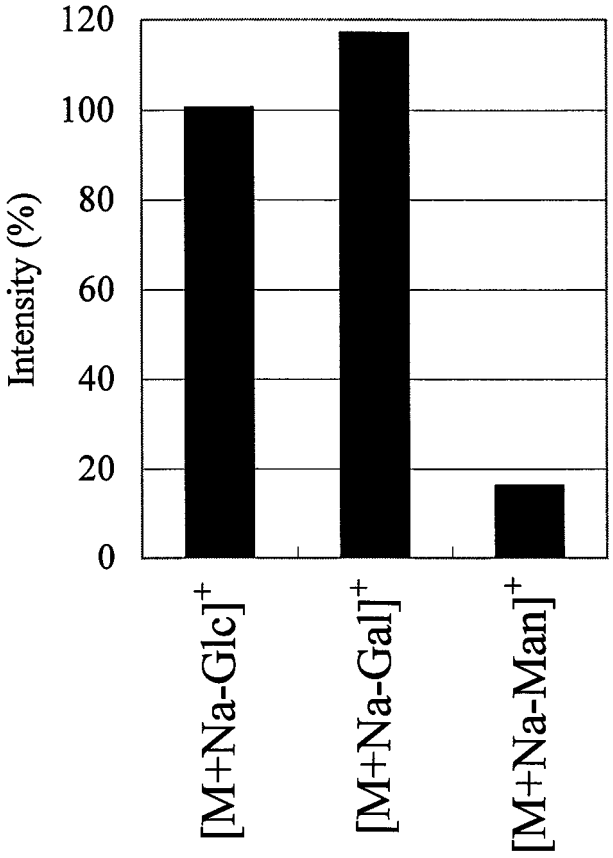


**Figure 9.** The Intensities of the Fragment ions [M+Na-Glc]$^+$ from Glucosyl-$\beta$CD, [M+Na-Gal]$^+$ Ion from Galactosyl-$\beta$CD, [M+Na-Man]$^+$ ion from Mannosyl-$\beta$CD. Each ion was normalized to the intensity of the [M-Hex4]$^+$ ions.

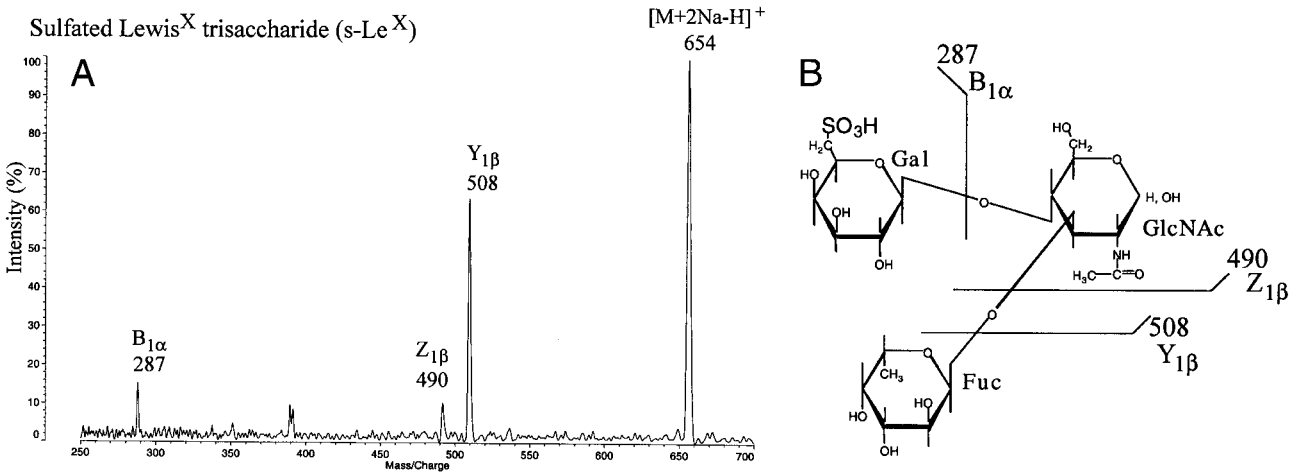


**Figure 10.** The MALDI-PSD Mass Spectrum (A) of Sulfated Lewis$^X$ Trisaccharide (s-Le$^X$) (B).

(Z-type fragmentation; $[Z_{1\beta}+2Na-H]^+$). The ion at *m/z* 287 corresponded to the chemical species $[Gal-SO_3+2Na]^+$ which was produced by the cleavage of the glycosyl linkage between Gal-GlcNAc. In the spectrum of sulfated Lewis$^a$ trisaccharide (s-Le$^a$), the precursor ion $[M+2Na-H]^+$ at *m/z* 654 and the charge-remote fragment ions at *m/z* 508 ($[Y_{1\beta}+2Na-H]^+$), 305 ($[C_{1\alpha}+2Na-H]^+$), and 287 ($[B_{1\alpha}+2Na-H]^+$) were observed (Fig. 11). The fragment ions at *m/z* 508 and 287 of s-Le$^a$ was the same chemical species of s-Le$^X$. The fragment ion at *m/z* 305 was produced by the cleaving of the linkage between Gal-GlcNAc as well as the ion at *m/z* 287. The fragmentation of the Lewis$^X$ and Lewis$^a$ sulfated oligosaccharides could be summarized as follows: the fragment ions were due to charge-remote fragmentation, distant from the charged sulfate group at the galactose. Most abundant were the fragment ions $Y_{1\beta}$ due to the ease of cleavage of the-fucosyl linkage [43]. The presence of $Z_{1\beta}$ in s-Le$^X$ and $C_{1\alpha}$ in s-Le$^a$ could be rationalized by the specific $\beta$-elimination at C-3 of the GlcNAc which probably stabilizes the formation of these ions. The $\beta$-elimination could form the double bond between C-2 and C-3 of the GlcNAc, which were conjugated to amide group at C-2.

### 3.4.2 Distinguishing between 1-3 and 1-4 linkages

We analyzed the intensity of the fragment ions finely to get the information about glycosyl linkages. The fragment ions $Y_{1\beta}$ at *m/z* 508 of s-Le$^X$ and s-Le$^a$ were produced by the cleavage of the fucosyl linkage between Fuc-GlcNAc. The intensity of $Y_1$ of s-Le$^X$ was about 60 % to that of the precursor ion (Fig. 10), and the intensity of $Y_{1\beta}$ of s-Le$^a$ was about 40 % (Fig. 11). Thus, $Y_{1\beta}$ of s-Le$^X$ had higher intensity than that of s-Le$^a$. In addition to that, the sum of the ion intensities of the ions produced by the cleavage of the fucosyl linkage of s-Le$^X$ ($Y_{1\beta}$ and $Z_{1\beta}$) was much higher than that of the ion of s-Le$^a$ ($Y_{1\beta}$). Thus, the fucosyl linkage at C-3 position of GlcNAc cleaved much easier than that at C-4 position of the glycosidic acceptor GlcNAc.

In the case of galactose substitution, the same tendency of the fragmentation at C-3/C-4 positions in glycosyl acceptor GlcNAc was observed. The ions $B_{1\alpha}$ of s-Le$^X$ and s-Le$^a$ were produced by the cleavage of the glycosidic linkage between Gal-GlcNAc. The intensity of the ion $B_{1\alpha}$ of s-Le$^X$ was about 17 % to that of the precursor ion (Fig. 10), and that of the ion $B_{1\alpha}$ of s-Le$^a$ was about 8 % (Fig. 11). Therefore, $B_{1\alpha}$ of s-Le$^X$ had higher intensity than that of s-Le$^a$. The sum of the ion intensities produced by the cleavage of the glycosyl linkage at C-3 position of GlcNAc in s-Le$^a$ ($B_{1\alpha}$ and $C_{1\alpha}$) was much higher than that of the ion which was produced by the cleavage of the glycosyl linkage at C-4 position of GlcNAc in s-Le$^X$ ($B_{1\alpha}$). These results also indicate that the glycosyl linkage at C-3 position cleaved much easier than that at C-4 position of the glycosyl acceptor GlcNAc.

The results in the fragmentation of Gal-GlcNAc were consistent with those of the fragmentation of the fucosyl linkage (Fuc-GlcNAc). Therefore, $\alpha$1-3 linkage cleaved easier than $\alpha$1-4 linkage, and $\beta$1-3 linkage also cleaved easier than $\beta$1-4 linkage in MALDI-PSD fragmentation.

These ion intensity analyses enabled us to distinguish between 1-3 and 1-4 linkages. We could perform the linkage analysis of the structural isomers of these oligosaccharides like s-Le$^X$ and s-Le$^a$ based on the infor-

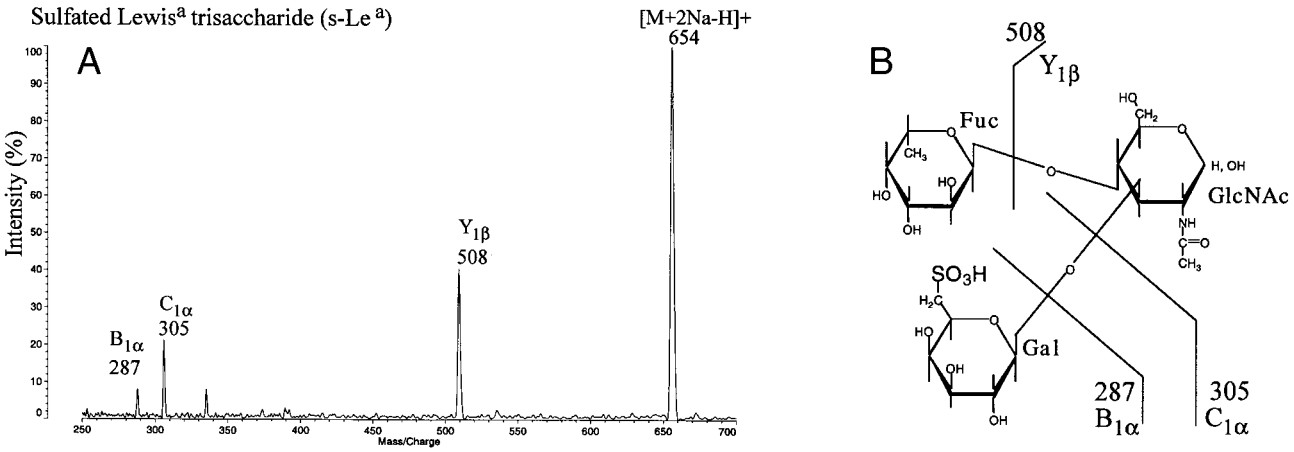


**Figure 11.** The MALDI-PSD Mass Spectrum (A) of Sulfated Lewis[a] Trisaccharide (s-Le[a]) (B).

mation about ion intensity. Therefore, this ion intensity analysis of PSD fragmentation in MALDI-MS will be very useful for the structure analysis of glycosylation as a new method.

## 4 Concluding remarks

Discussion of the intensities of the PSD fragment ions in detail enabled us to elucidate the structure of the analytes in the curved-field reflectron MALDI-MS. Highly branched oligosaccharides were analyzed by using MALDI-PSD fragmentation method. As expected the intensities of the fragment ions produced by one-site cleavage of the glycosyl linkages were much higher than those of the ions produced by two-site and three-site cleavages of glycosyl linkages. The linkage types were determined by using the ion intensity analysis. The glycosyl linkage of $\alpha$1-4 cleaves easier than $\alpha$1-6 linkage, and that of $\alpha$1-6 cleaves easier than $\beta$1-4 linkage in MALDI-PSD fragmentation. Thus, the $\alpha$1-4 glycosyl linkage cleaved easier than that of $\beta$1-4 linkage. The result indicated that the ion intensity also depended on $\alpha$- and $\beta$-configurations. The $\alpha$1-3 linkage cleaved easier than $\alpha$1-4 linkage, and the $\beta$1-3 linkage cleaved easier than $\beta$1-4 linkage in the MALDI-PSD fragmentation. Moreover, the sialyl linkages of $\alpha$2-3/$\alpha$2-6 types were also distinguished by the ion intensity analysis. Therefore, the ion intensity analysis in the MALDI-PSD fragmentation method is very useful for the linkage analysis, and it would be possible to perform the linkage analysis of saccharide chains in glycosylation using MALDI-TOF-MS. The stereoisomers of monosaccharides were also distinguished by MALDI-PSD fragmentation method. The analogous linkage isomers of sulfated Lewis-type oligosaccharides were able to be analyzed by using the ion intensity analysis. These results strongly indicate that the relative intensity of the MALDI-PSD fragment ions strongly depend on the fine structure of the analyte. Consequently, the ion intensity analysis in the MALDI-PSD fragment spectra is a powerful tool for glycosylation analysis and the structure analysis of glycoconjugates and oligosaccharides.

Received February 24, 2000

## 5 References

[1] Ji, H., Whitehead, R., H., Reid, G. E., Moritz, R. L., Ward, L. D., Simpson, R. J., *Electrophoresis* 1994, *15*, 391–405.
[2] Rasmussen, H. H., Mortz, E., Mann, M., Roepstorff, P., Celis, J. E., *Electrophoresis* 1994, *15*, 406–416.
[3] Kaufmann, R., Spengler, B., and Lutzenkirchen, F., *Rapid Commun. Mass Spectrom*. 1993, *7*, 902–910.
[4] Kaufmann, R., Kirsch, D., Spengler, B., *Int. J. Mass Spectrom. Ion Proc.* 1994, *131*, 355–385.
[5] Nakagawa, M., Yamagaki, T., Nakanishi, H., *Electrophoresis* 2000, *21*, 1651–1652.
[6] Chait, B. T., Wang, R., Beavis, R. C., Kent, S. B. H., *Science* 1993, *262*, 89–92.
[7] Harvey, D. J., Naven, T. J. P., Küster, B., Bateman, R. H, Green, M. R., Critchley, G., *Rapid Commun. Mass Spectrom.* 1995, *9*, 1556–1561.
[8] Talbo, G., Mann, M., *Rapid Commun. Mass Spectrom.* 1996, *10*, 100–103.
[9] Spengler, B., Kirsch, D., Kaufmann, R., Lemoine, J., *J. Mass Spectrom.* 1995, *30*, 782–787.
[10] Yamagaki, T., Ishizuka, Y., Kawabata, S., Nakanishi, H., *Rapid Commun. Mass Spectrom.* 1996, *10*, 1887–1890.
[11] Kaufmann, R., *J. Biotechnol.* 1995, *41*, 155–175.
[12] Rouse, J. C., Strang, A-M., Yu, W., Vath, J. E., *Anal. Biochem.* 1998, *256*, 33–46.
[13] Harvey, D. J., *Mass Spectrom. Rev.* 1999, *18*, 349–451.

[14] Cornish, T. J., and Cotter, R. J., *Rapid Commun. Mass Spectrom.* 1993, *7*, 1037–1040.
[15] Cornish, T. J., and Cotter, R. J., *Rapid Commun. Mass Spectrom.* 1994, *8*, 781–785.
[16] Strupat, K., Karas, M., Hillenkamp, F., *Int. J. Mass Spectrom. Ion Proc.* 1991, *111*, 89–102.
[17] Yamagaki, T., Ishizuka, Y., Kawabata, S., Nakanishi, H., *Proc. 9th Int. Symposium on Cyclodextrins*, Santiago de Compostela, Kluwer Academic Publishers, The Netherlands 1999, pp. 145–148.
[18] Koizumi, K., Tanimoto, T., Okada, Y., Nakanishi, N., Kato, N., *Carbohydr. Res.* 1991, *215*, 127–136.
[19] Yamagaki, T., Mitsuishi, Y., Nakanishi, H., *Biosci. Biotechnol. Biochem.* 1997, *61*, 1411–1414.
[20] Yamagaki, T., Mitsuishi, Y., Nakanishi, H., *Chem. Lett.* 1998, 57–58.
[21] Yamagaki, T., Mitsuishi, Y., Nakanishi, H., *Tetrahedron Lett.* 1998, *39*, 4051–4054.
[22] Yamagaki, T., Ishizuka, Y., Kawabata, S., Nakanishi, H., *Rapid Commun. Mass Spectrom.*, 1997, *11*, 527–531
[23] Yamagaki, T., Mitsuishi, Y., Nakanishi, H., *Rapid Commun. Mass Spectrom.* 1998, *12*, 307–311.
[24] Cahan, L. D., Paulson, J. C., *Virology* 1980, *103*, 505–509.
[25] Cointe, D., Leroy, Y., Chirat, F., *Carbohydr. Res.* 1998, *311*, 51–59.
[26] Lemoine, J., Strecker, G., Leroy, Y., Forurnet, B., *Carbohydr. Res.* 1991, *221*, 209–217.
[27] Perreault H., Costello, C. E., *J. Mass Spectrom.* 1999, *34*, 184–197.
[28] Chen, P., Werner-Zwanziger, U., Wiesler, D., Pagel, M., Novotny, M. V., *Anal. Chem.* 1999, *71*, 4969–4973.
[29] Yamagaki, T., Nakanishi, H., *Glycoconjugate J.* 1999, *16*, 385–389.
[30] Domon, B., Costello, C. E., *Glycoconjugate J.* 1988, *5*, 397–409.
[31] Papac, D. I., Wong, A., Jones, A. J. S., *Anal. Chem.* 1996, *68*, 3215–3223.
[32] Yamagaki, T., Nakanishi, H., *Rapid Commun. Mass Spectrom.* 1998, *12*, 1069–1074.
[33] Poulter, L., Burlingame, A. L., *Methods Enzymol.* 1990, *193,* 661–689.
[34] Takada, A., Ohmori, K., Yoneda, T., Tsuyuoka, K., Hasegawa, A., Kiso, M., Kannagi, R., *Cancer Res.* 1993, *53*, 354–361.
[35] Erbe D. V., Watson, S. R., Presta, L. G., Wolitzky, B. A., Foxall, C., Brandley, B. K., Lasky L. A., *J. Cell Biol.* 1993, *120*, 1227–1235.
[36] Lasky, L. A., *Science* 1992, *258*, 964–969.
[37] Li, T., Ohashi, Y., Ogawa T., Nagai, Y., *Glycoconjugate J.* 1996, *13*, 273–283.
[38] Li, T., Ohashi, Y., Ogawa T., Nagai, Y., *J. Mass Spectrom. Soc. Jpn.* 1996, *44*, 183–195.
[39] Li, T., Ohashi, Y., Nunomura, S. Ogawa, T., Nagai, Y., *J. Biochem.* 1995, *118*, 526–533.
[40] Li, T., Ohashi, Y., Nagai, Y., *Carbohydr. Res.* 1995, *273*, 27–40.
[41] Ohashi, Y., in: Cole, R. B., (Ed.) *Electrospray ionization mass spectrometry*, Wiley, New York 1997, pp. 459–498.
[42] Ohashi, Y., Ii, T., Kubota, M., Nunomura, S., Niwa, H., Ohashi, M., Ogawa, T., Nagai, Y., *J. Mass Spectrom. Soc. Jpn.* 1998, *46*, 45–52.
[43] Yamagaki, T., Mitsuishi, Y., Nakanishi, H., *Biosci. Biotechnol. Biochem.* 1998, *62*, 2470–2475.