Quantitative Derivatization of Sialic Acids for the Detection of Sialoglycans by MALDI MS

ARTICLE *in* ANALYTICAL CHEMISTRY · AUGUST 2008

Impact Factor: 5.64 · DOI: 10.1021/ac800457a · Source: PubMed

5 AUTHORS, INCLUDING:

Akihiko Kameyama

National Institute of Advanced Industrial S…

73 PUBLICATIONS **1,922** CITATIONS

SEE PROFILE

Anal. Chem. **2008,** *80,* 5211–5218

Quantitative Derivatization of Sialic Acids for the Detection of Sialoglycans by MALDI MS

Masaaki Toyoda, Hiromi Ito, Yu-ki Matsuno, Hisashi Narimatsu, and Akihiko Kameyama*

Research Center for Medical Glycoscience, National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Umezono, Tsukuba, Ibaraki 305-8568, Japan

Recently, glycans have been recognized as valuable biomarkers for various disease states. In particular, sialoglycans, which have sialic acids at their terminal end, are likely to have relevance to diseases such as cancer and inflammation. Mass spectrometry (MS) has become an indispensable tool for biomarker discovery. However, matrix-assisted laser desorption ionization (MALDI) MS of sialoglycans normally causes loss of sialic acid. Methylesterification or amidation of carboxyl functionality in sialic acid has been reported to suppress the loss of sialic acids. We found that the modifications of α2,3-linked sialic acids proceed less efficiently than those at α2,6-linkages. Furthermore, the modifications of the α2,3-linked sialic acids are incomplete. This variability in the extent of derivatization presents a major problem in terms of glycan biomarker discovery using MALDI MS. In this study, we developed a novel amidation using acetohydrazide which can completely modify both types of linkages of sialoglycans. With the use of this method, we demonstrate MS profiling of N-linked glycans released from a bovine fetuin which is rich in α2,3-linked sialic acids.

Structural alteration of glycans is recognized to be associated with cell differentiation and certain disease states such as cancer.[1–4] As a result, glycans can be used as a target molecule for biomarker discovery. In particular, sialoglycans, which have sialic acids at their terminal end, are one of the major classes of glycans used for biomarker discovery. Indeed, most tumor-associated glycan antigens reported in the last few decades, such as sialyl Lewis X or sialyl Lewis a, are included in this class.[5,6] Mass spectrometry (MS), which has become an indispensable tool for the discovery of protein biomarkers, has recently been used for glycan biomarker discovery.[7–9] In some papers, comparative analyses of glycans for biomarker discovery were performed with a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS) because of its ease of operation and high-throughput performance.[8,9] However, sialic acid residues of sialoglycans are particularly labile to the ionization process of MALDI.[10,11] Although neutral glycans usually afford stronger signals as $[M + Na]^+$ ions in positive ion mode by MALDI MS, sialoglycans afford stronger signals as $[M - H]^-$ ions in negative ion mode. Because glycans obtained from biological samples usually comprise a mixture of neutral glycans and sialoglycans, spectra in both positive and negative ion mode are required for comparative analysis for glycan biomarker discovery. In addition, sialic acids readily form salts with alkali metals. Therefore, the spectra of sialoglycans often affords multiple peaks as a result of ion formation from both the free acid and the corresponding salts.[11] Thus, accurate comparative analysis of sialoglycans by MS is significantly difficult. Modifications of the carboxyl functionality of sialoglycans, such as methylesterification[11] or amidation,[12] could assist in the analysis of glycans by MS because these modifications stabilize the sialic acid residues and allow the simultaneous detection of sialoglycans and neutral glycans in the positive ion mode.

Permethylation, which is a commonly used method of derivatization for glycan analysis by MS, also gives methylesters of sialic acids.[13–15] Unfortunately, permethylation is unsuitable for the analysis of biologically important sialoglycans containing partially acetylated or methylated sialic acids.[16,17] This is because *O*-acetyl groups are easily decomposed under the harsh conditions used for permethylation, making it difficult to assess which positions were originally methylated. In contrast, methylesterifications and amidations selectively modify carboxyl functionality under rela-

* To whom correspondence should be addressed. Phone: +81-29-861-3123. E-mail: aki-kameyama@aist.go.jp.

(1) Mackiewicz, A.; Mackiewicz, K. *Glycoconjate J.* **1995**, *12*, 241–247.
(2) Dennis, J. W.; Granovsky, M.; Warren, C. E. *Biochim. Biophys. Acta* **1999**, *1473*, 21–34.
(3) Dube, D. H.; Bertozzi, C. R. *Nat. Rev. Drug Discovery* **2005**, *4*, 477–488.
(4) Okuyama, N.; Ide, Y.; Nakano, M.; Nakagawa, T.; Yamanaka, K.; Moriwaki, K.; Murata, K.; Ohigashi, H.; Yokoyama, S.; Eguchi, H.; Ishikawa, O.; Ito, T.; Kato, M.; Kasahara, A.; Kawano, S.; Gu, J.; Taniguchi, N.; Miyoshi, E. *Int. J. Cancer* **2006**, *118*, 2803–2808.
(5) Narayanan, S. *Ann. Clin. Lab. Sci.* **1994**, *24*, 376–384.
(6) Hakomori, S. *Adv. Exp. Med. Biol.* **2001**, *491*, 369–402.
(7) Ressom, H. W.; Varghese, R. S.; Goldman, L.; An, Y.; Loffredo, C. A.; Abdel-Hamid, M.; Kyselova, Z.; Mechref, Y.; Novotny, M.; Drake, S. K.; Goldman, R. *J. Proteome Res.* **2008**, *7*, 603–610.
(8) Kirmiz, C.; Li, B.; An, H. J.; Clowers, B. H.; Chew, H. K.; Lam, K. S.; Ferrige, A.; Alecio, R.; Borowsky, A. D.; Sulaimon, S.; Lebrilla, C. B.; Miyamoto, S. *Mol. Cell. Proteomics* **2007**, *6*, 43–55.
(9) Kam, R. K.; Poon, T. C.; Chan, H. L.; Wong, N.; Hui, A. Y.; Sung, J. J. *Clin. Chem.* **2007**, *53*, 1254–1263.
(10) Talbo, G.; Mann, M. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 100–103.
(11) Powell, A. K.; Harvey, D. J. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 1027–1032.
(12) Sekiya, S.; Wada, Y.; Tanaka, K. *Anal. Chem.* **2005**, *77*, 4962–4968.
(13) Hakomori, S. *J. Biochem.* **1964**, *55*, 205–208.
(14) Ciucanu, I.; Costello, C. E. *J. Am. Chem. Soc.* **2003**, *125*, 16213–16219.
(15) Ciucanu, I. *Anal. Chim. Acta* **2006**, *576*, 147–155.
(16) Varki, A. *Glycobiology* **1992**, *2*, 25–40.
(17) Kohla, G.; Stockfleth, E.; Schauer, R. *Neurochem. Res.* **2002**, *27*, 583–592.

10.1021/ac800457a CCC: $40.75
Published on Web 05/17/2008

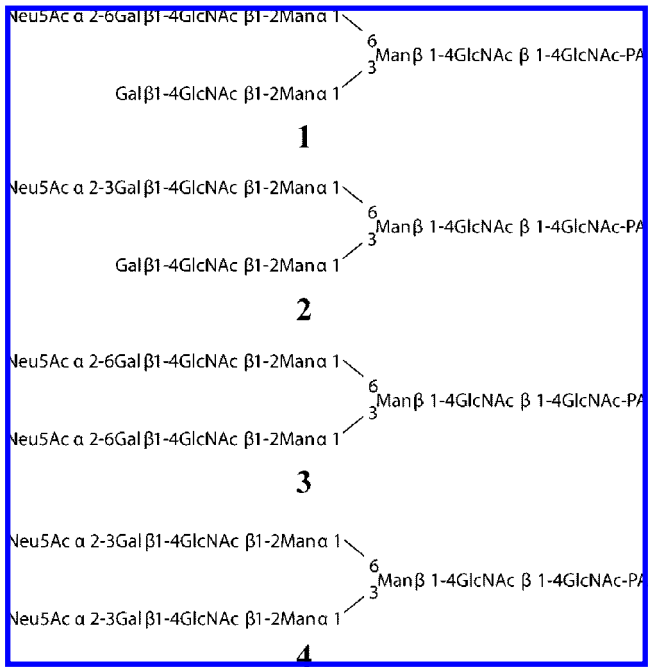


Figure 1. Structures of sialoglycans used for the modification study on sialic acids. PA refers to pyridyl amino group.

tively mild conditions so that these modifications are free from the flaws described for permethylation. Sialic acids are usually found at the nonreducing end of sialoglycans in the form of α2,3- or α2,6-linked to a β-D-galactopyranose residue, or in a lateral position α2,6-linked to a GalNAc or a GlcNAc residue. Chemical modification of sialic acids for glycan biomarker discovery is required to be a quantitative reaction regardless of whether the linkage type is α2,3 or α2,6. A difference in reactivity between linkage types of sialic acids has been reported for perbenzoylation.[18] However, no such difference in reactivity for methylesterification and amidation as a function of sialic acid linkage type has been reported.

In this study, we show that modification at α2,3-linkages of sialic acids by the reported methylesterification and amidation reaction is more difficult than those at α2,6-linkages. Consequently, both methods result in incomplete modifications at α2,3-linkages. We have, however, developed a novel amidation method using acetohydrazide that can completely modify both types of linkages of sialoglycans. And the stabilizing effect for sialic acids was evaluated by MALDI MS of the modified sialoglycans. With the use of this method, we demonstrate MALDI MS profiling of N-linked glycans released from bovine fetuin which is rich in α2,3-linked sialic acids.

EXPERIMENTAL SECTION

Materials and Reagents. Glycopeptidase F was purchased from Takara Bio Inc. (Otsu, Japan). Sialoglycans labeled with 2-pyridylamine (PA) were purchased from Masuda Chemical Industries Co., Ltd. (Takamatsu, Japan) and Takara Bio Inc. Bovine fetuin was purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals and solvents were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

(18) Chen, P.; Werner-Zwanziger, U.; Wiesler, D.; Pagel, M.; Novotny, M. V. *Anal. Chem.* **1999**, *71*, 4969–4973.

Methylesterification of Sialoglycans. Sialoglycans were methylesterified according to the procedure by Powell and Harvey.[11] Briefly, the sialoglycans (4 pmol) were applied to a short column (bed volume 100 μL) containing a cation-exchange resin AG-50 (Bio-Rad, Hercules, CA) conditioned to the Na^+ form, eluted with double-distilled water, and then dried. The residues were dissolved in 1 μL of dimethyl sulfoxide (DMSO), mixed with 1 μL of methyl iodide (MeI), and allowed to react for 2 h at room temperature. Unreacted MeI was removed under a gentle stream of nitrogen, and the sample was finally dried in vacuo. The dried samples were analyzed as described below.

Amidation of Sialoglycans. Sialoglycans were amidated according to the procedure described by Sekiya et al.[12] A volume of 4 μL of the solutions of sialoglycan (1 pmol/μL in 10 mM ammonium bicarbonate) was dissolved in 50 μL of 1 M ammonium chloride (pH 6.2) and then mixed with 30 μL of 1 M 4-(4,6-dimethoxy-1,3,5-triazin-2yl)-4-methylmorphonium chloride (DMT-MM). After incubation at 50 °C for 24 h, the reaction mixtures were desalted using a Sep-Pak C18 cartridge (50 mg, 1 mL; Waters Corp., Milford, MA) with distilled water. The glycans were eluted with 1 mL of 0.1% trifluoroacetic acid (TFA) in 80% acetonitrile (ACN), dried in vacuo, and then analyzed as described below.

Modification of Sialoglycans with Acetohydrazide. Sialoglycans (4 pmol) were dissolved in 50 μL of 1 M acetohydrazide, and the solutions were adjusted to pH 2.5 with 1 N HCl. Ten microliters of 2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was added to the solutions, and the mixtures were incubated at room temperature for 2 h. After incubation, the mixtures were loaded onto a Sep-Pak C18 cartridge equilibrated with 0.1% TFA and washed with 0.1% TFA followed by elution with 0.1% TFA in 80% ACN. Each eluate was dried in vacuo and analyzed by reversed-phase high-performance liquid chromatography (HPLC) and MS.

Reversed-Phase HPLC. The modified sialoglycans were separated on a TSKgel ODS-80Ts QA column (4.6 mm i.d. × 150 mm; Tosoh Corp., Tokyo, Japan). The mobile phases were the following: solvent A, 10 mM triethylammonium acetate, pH 4.0; solvent B, 0.5% 1-butanol in solvent A. The samples were injected onto the column equilibrated with an 80:20 mixture of solvent A and solvent B. The reaction mixtures were separated with a linear gradient of 20−98% solvent B in solvent A at a flow rate of 0.7 mL/min for 52 min at 32 °C. The eluate was monitored by fluorescence intensity at 400 nm (excitation, 320 nm) with a fluorescence detector RF-10AXL (Shimadzu Corp.).

Mass Spectrometry. Spectra were acquired in positive ion mode using a MALDI-TOF mass spectrometer (Reflex IV; Bruker Daltonik, Bremen, Germany). Ions were generated by a pulsed 337 nm nitrogen laser and were accelerated to 20 kV. All the spectra were obtained using reflectron mode with delayed extraction of 200 ns. For sample preparation, 2 μL of the glycan in matrix solution (1 mg/mL 2,5-DHB in 30% ethanol) was mixed with 2 μL of 6.5 mM NaCl. Then, 0.5 μL of the mixed solution was spotted onto an Anchorchip plate (Bruker Daltonik) and dried. Tandem mass spectrometry (MS/MS) spectra were acquired in reflectron positive ion mode with a MALDI quadrupole ion trap TOF instruments (AXIMA-QIT; Shimadzu Corp., Kyoto, Japan).

Preparation of N-Linked Glycans from Fetuin. An amount of 40 μL of glycopeptidase F (0.5 U/mL of 20 mM $NaHCO_3$) was

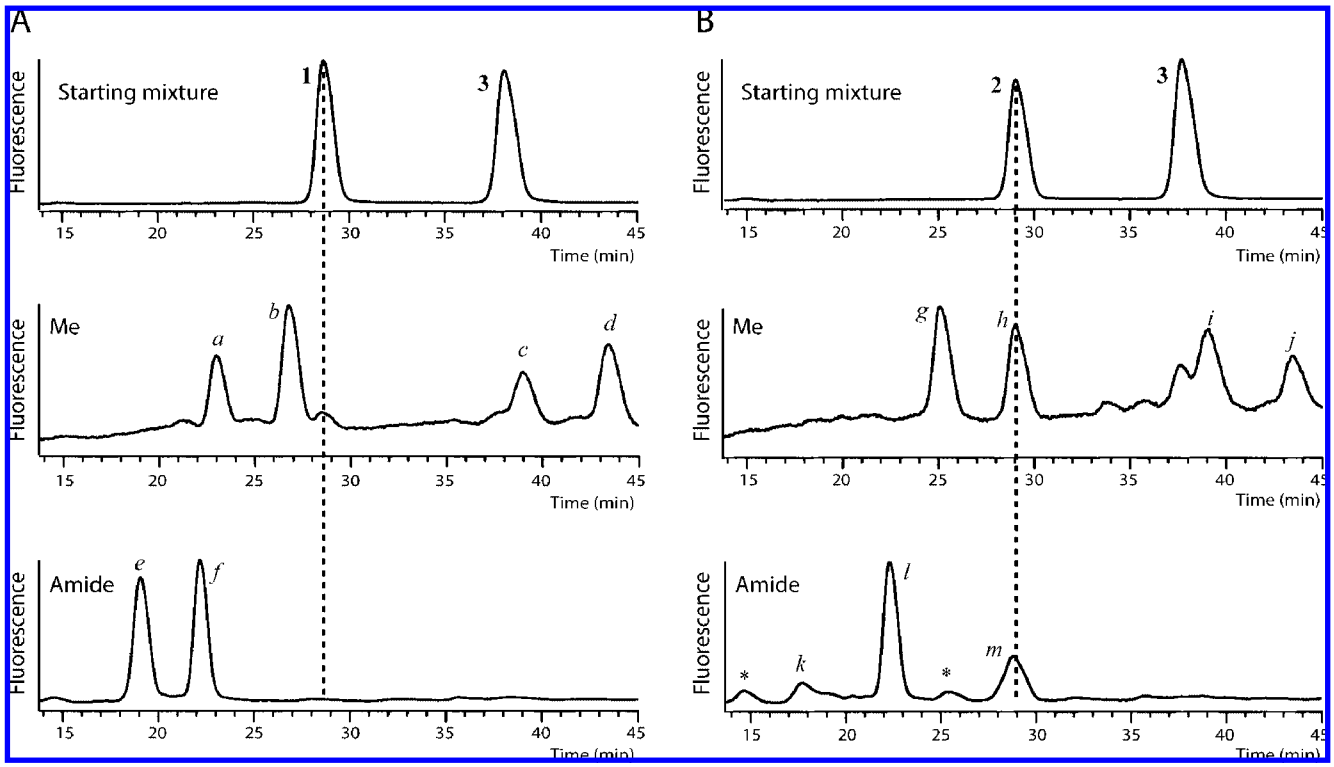


Figure 2. HPLC chromatograms of mixtures of modified sialoglycans. (A) Modifications of a mixture of sialoglycans **1** and **3**: upper, the starting mixture; middle, methylesterified; bottom, amidated. (B) Modifications of a mixture of sialoglycans **2** and **3**: upper, the starting mixture; middle, methylesterified; bottom, amidated. MS spectral data of the peaks in the chromatograms (a−m) are summarized in Table 1. Asterisks indicate unidentified byproducts.

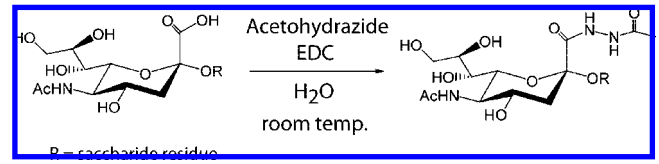
Table 1. MS Spectral Data for Each of the Peaks Shown in the Chromatograms of Figure 2

peak	obsd *m/z*	calcd *m/z*	rel intensity (%)[a]	glycan	ion species
a	2038.85	2038.78	47.1	**1**	$[M^{diMe} + H]^+$
	2060.83	2060.77	52.9	**1**	$[M^{diMe} + Na]^+$
b	2046.83	2046.75	100	**1**	$[M^{monoMe} + Na]^+$
c	2343.98	2343.90	32.3	**3**	$[M^{triMe} + H]^+$
	2365.92	2365.88	67.7	**3**	$[M^{triMe} + Na]^+$
d	2352.21	2351.86	100	**3**	$[M^{diMe} + Na]^+$
e	2031.95	2031.75	100	**1**	$[M^{monoAmide} + Na]^+$
f	2322.21	2321.86	100	**3**	$[M^{diAmide} + Na]^+$
g	1733.75	1733.67	12.2	**2**	$[M^{monoMe} + H - Neu5Ac]^+$
	1755.79	1755.66	13.6	**2**	$[M^{monoMe} + Na - Neu5Ac]^+$
	2024.77	2024.77	25.5	**2**	$[M^{monoMe} + H]^+$
	2046.79	2046.75	27.1	**2**	$[M^{monoMe} + Na]^+$
	2068.76	2068.73	21.5	**2**	$[M^{monoMe} - H + 2Na]^+$
h	1741.68	1741.64	34.5	**2**	$[M + Na - Neu5Ac]^+$
	2032.94	2032.74	25.6	**2**	$[M + Na]^+$
	2054.77	2054.72	39.9	**2**	$[M - H + 2Na]^+$
i	2343.94	2343.90	25.4	**3**	$[M^{triMe} + H]^+$
	2365.91	2365.88	74.6	**3**	$[M^{triMe} + Na]^+$
j	2352.16	2351.86	100	**3**	$[M^{diMe} + Na]^+$
k	2031.97	2031.75	100	**2**	$[M^{monoAmide} + Na]^+$
l	2321.94	2321.86	100	**3**	$[M^{diAmide} + Na]^+$
m	1741.86	1741.64	19.8	**2**	$[M + Na - Neu5Ac]^+$
	2014.96	2014.72	26.7	**2**	$[M + Na - H_2O]^+$
	2032.96	2032.74	24.9	**2**	$[M + Na]^+$
	2054.96	2054.72	28.6	**2**	$[M - H + 2Na]^+$

[a] The relative intensity of a signal is indicated as a percentage of the total intensities of the observed glycan signals in the MS spectrum of each peak in the chromatogram.

added to a solution of bovine fetuin (50 μg in 10 μL of 1 M Tris−HCl pH 8.6), and the mixture was incubated for 17 h at 37 °C. The reaction mixture was then loaded onto an Oasis MCX cartridge (50 mg, 1 mL; Waters), and the fraction passed through the cartridge was dried in vacuo. The dried glycans were labeled by reductive amination with 2-aminopyridine using a pyridylamination apparatus, GlycoTAG (Takara).

Scheme 1. General Scheme for the Derivatization of Sialic Acid Using Acetohydrazide[a]



[a] EDC refers to 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride.

RESULTS AND DISCUSSION

Methylesterification and Amidation of Sialoglycans. To evaluate the methylesterification[11] and amidation[12] of sialoglycans for glycan biomarker discovery we first examined the modifications of sialoglycans (**1**, **2**, and **3**) with different linkage types of sialic acids (Figure 1). These glycans were labeled with a pyridyl amino group (PA) at their reducing ends so that the reaction could be monitored by HPLC with a fluorescence detector. Glycans **1** and **2** are monosialo biantennary N-linked glycans with α2,6- and α2,3-linkages, respectively. Glycan **3**, having two sialic acids with α2,6-linkage, was used to ensure the reactions were equivalent to those reported in the original papers in which sialoglycans only having α2,6-linkage were used as substrate.[11,12]

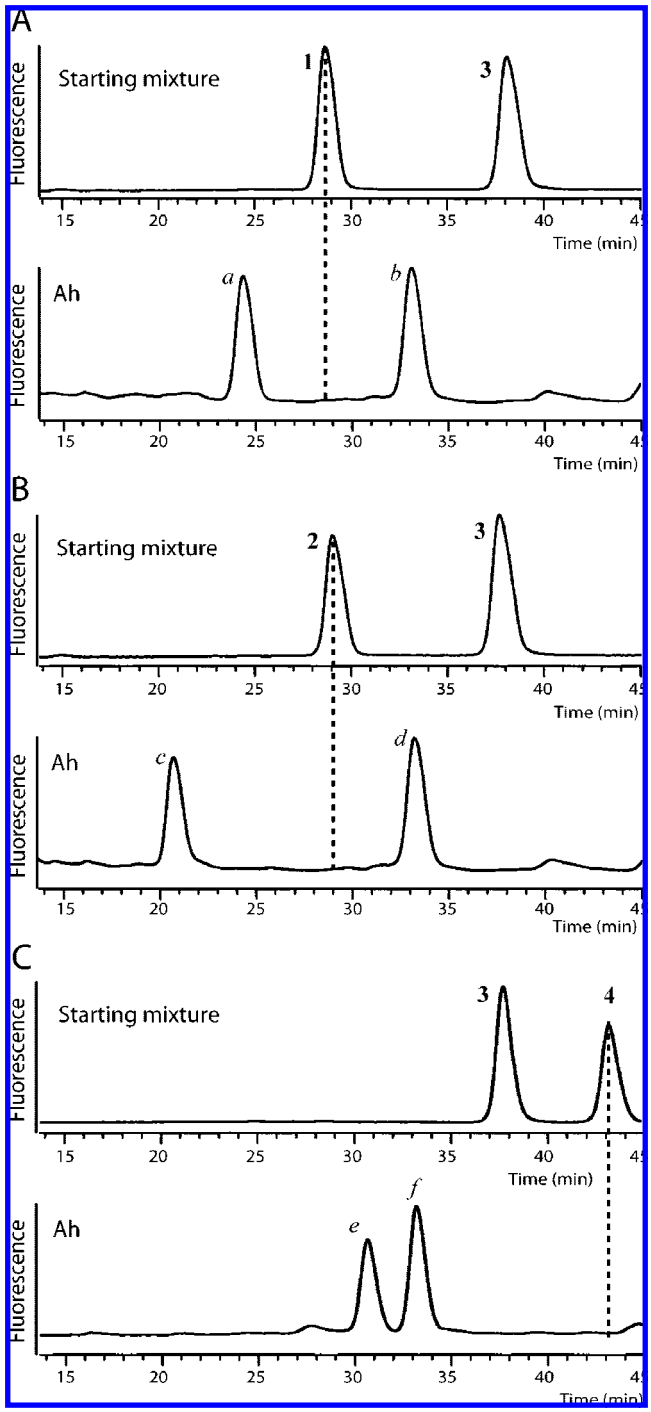


Figure 3. HPLC chromatograms of the sialoglycan mixtures modified with acetohydrazide. (A) Modifications of a mixture of sialoglycans **1** and **3**: upper, the starting mixture; bottom, modified with acetohydrazide. (B) Modifications of a mixture of sialoglycans **2** and **3**: upper, the starting mixture; bottom, modified with acetohydrazide. (C) Modifications of a mixture of sialoglycans **3** and **4**: upper, the starting mixture; bottom, modified with acetohydrazide. MS spectral data of the peaks in the chromatograms (a–f) are summarized in Table 2.

HPLC chromatograms of the reaction products of the methylesterification of mixtures of **1** and **3**, or **2** and **3**, clearly show that after methylesterification, the starting glycans, having an α2,6-linkage, had almost disappeared, whereas a substantial amount of the starting glycan **2**, having an α2,3-linkage, was resistant to the reaction (Figure 2, parts A and B). The results indicated that the methylesterification of sialic acid in the form of an α2,3-linkage was more difficult than the corresponding reaction involving an α2,6-linkage. Furthermore, even in the case of the modification of glycans **1** and **3**, which were completely derivatized in the methylesterification reaction, byproducts of the reaction can be readily detected in the chromatograms. MS spectra of the peaks a, c, and i in Figure 2 revealed that the byproducts were overmethylated glycans, which were probably derivatized at the amino functionality of PA in the glycans (Table 1). MS/MS spectra of them indicated that the overmethylation occurred at the reducing end of the modified glycans. Because a nitrogen atom is a better nucleophile than an oxygen atom, overmethylation probably occurred at the amino functionality of PA in the glycans (data not shown). The MS spectrum of g gave five peaks all of which could be assigned to monomethylated glycan **2** and its decay products. Two peaks among them were assigned to ions arising from a loss of free sialic acid but not methylesterified sialic acid. The ion of m/z 2068.8 has two Na, suggesting that peak g contains free sialic acid. These results indicate that the amino functionality of PA was more susceptible to methylation than the carboxylic group of the α2,3-sialic acid. To increase the yield of methylesterification, we repeated the procedure under the same conditions. However, repeated methylesterification of sialoglycans containing an α2,3-linkage afforded an even greater amount of byproducts (data not shown). It is possible that alternative methods for the methylesterification of sialoglycans may have overcome this problem.[19] However, we did not examine all the different methylesterification protocols in this study.

The sialoglycans containing α2,3-linkages were also difficult to derivatize in the amidation reaction. HPLC chromatograms of the glycans after amidation showed that the starting glycan **2** remained a major peak (m) in the reaction mixture after amidation, whereas glycans **1** and **3** were completely derivatized into peaks e, f, and l (Figure 2, parts A and B). Unidentified byproducts, indicated by asterisks in Figure 2B, were also detected in the reaction mixture. Moreover, increasing the reaction temperature to obtain a better yield of amidation generated dehydration products (data not shown). In the overreaction, both the α2,3- and α2,6-linked sialoglycans gave undesirable overmethylated

Table 2. MS Spectral Data for Each of the Peaks Shown in the Chromatograms of Figure 3

peak	obsd m/z	calcd m/z	rel intensity (%)[a]	glycan	ion species
a	2088.77	2088.77	83.6	**1**	$[M^{monoAh} + Na]^+$
	2110.77	2110.75	16.4	**1**	$[M^{monoAh} - H + 2Na]^+$
b	2436.14	2435.91	81.1	**3**	$[M^{diAh} + Na]^+$
	2458.09	2457.89	18.9	**3**	$[M^{diAh} - H + 2Na]^+$
c	2089.02	2088.77	100	**2**	$[M^{monoAh} + Na]^+$
d	2435.84	2435.91	86.7	**3**	$[M^{diAh} + Na]^+$
	2457.81	2457.89	13.3	**3**	$[M^{diAh} - H + 2Na]^+$
e	2436.23	2435.91	85.0	**4**	$[M^{diAh} + Na]^+$
	2458.30	2457.89	15.0	**4**	$[M^{diAh} - H + 2Na]^+$
f	2436.30	2435.91	83.7	**3**	$[M^{diAh} + Na]^+$
	2458.29	2457.89	16.3	**3**	$[M^{diAh} - H + 2Na]^+$

[a] The relative intensity of a signal is indicated as a percentage of the total intensities of the observed glycan signals in the MS spectrum of each peak in the chromatogram.

(19) Miura, Y.; Shinohara, Y.; Furukawa, J.; Nagahori, N.; Nishimura, S.-I. *Chem. Eur. J.* **2007**, *13*, 4797–4804.

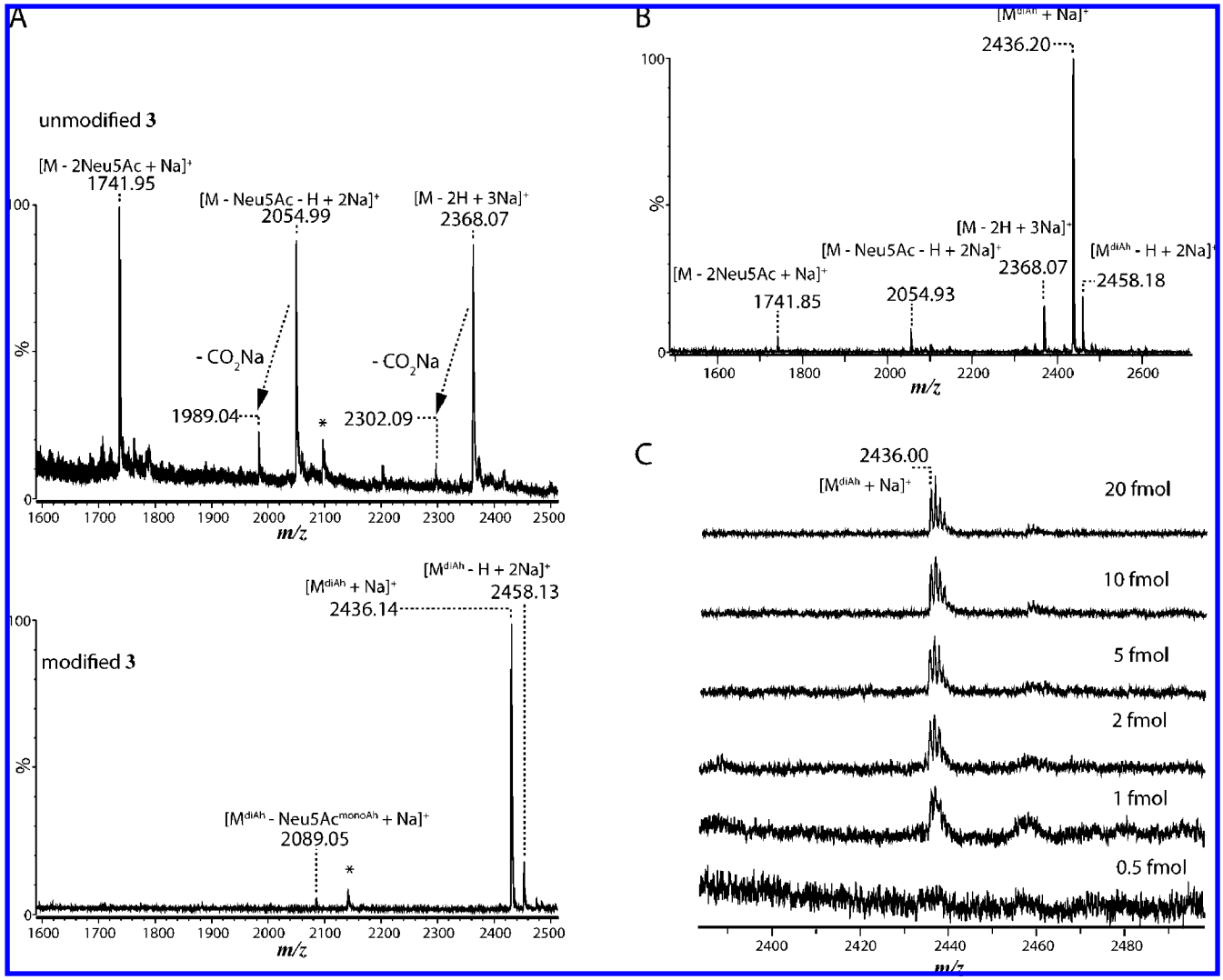


Figure 4. Mass spectra of unmodified and acetohydrazide-modified sialoglycan **3**. All spectra were acquired in positive reflectron mode by MALDI-TOF MS. (A) Comparison of the mass spectra of unmodified and modified sialoglycan **3**: upper, unmodified sialoglycan **3**; bottom, acetohydrazide-modified **3**. (B) Mass spectrum of an equimolar mixture of unmodified and modified **3**. (C) Mass spectra of acetohydrazide modified **3** at various different concentrations. M^{Ah} refers to an acetohydrazide-derivatized molecule. Asterisks indicate metastable ions.

products after methylesterification. Furthermore, α2,3-linked sialoglycans gave the dehydrated products after amidation. The α2,3-linked sialoglycans were difficult to derivatize in both reactions. The difference in reactivity between α2,3- and α2,6-linked sialic acid might be attributed to an interaction of the carboxyl group in α2,3-linked sialic acid with adjacent hydroxyl groups: 2-OH or 4-OH of the galactose linked to sialic acid.[18] The carboxyl proton probably forms a hydrogen bond to an oxygen atom of the hydroxyl moiety. This might affect activation of the carboxyl group by the reagents. In conclusion, our results suggest that the methylesterification and amidation methods are unsuitable for the derivatization of sialoglycans containing sialic acids with α2,3-linkage for analyzing glycan biomarker.

Modification Method for Sialoglycans. Given the difficulties of derivatizing sialoglycans using conventional methodologies, we focused on developing a novel modification using an acylhydrazide, which is easily coupled with carboxylic acids. Among the acylhydrazides that have been used to modify carboxylic acids, we chose acetohydrazide to act as the nucleophile because with the use of this reagent there are no problems associated with steric hindrance. It should be noted that glycans having an intact reducing end are not appropriate substrates for the modification of sialic acids using a hydrazide because they also react with aldehydes. EDC was chosen as the coupling reagent, which can be readily removed from the reaction mixture using solid-phase extraction. Scheme 1 shows a representative scheme of the reaction.

Initially, we attempted the modification of the sialoglycans (**1**, **2**, and **3**) by this method without regard to pH. Although the yield of the modified glycan of **2** was moderately higher than that obtained by conventional amidation, HPLC analysis revealed significant amounts of starting material in the resulting mixture (data not shown). Nakajima and Ikada studied the mechanism of amide formation by EDC and reported that reaction with carboxyl groups occurs at a relatively narrow pH range of 3.5—4.5.[20] We examined the pH dependency of the reaction to determine optimal conditions for the modification of sialoglycans. The maximum yield of the modified glycan of **2** was obtained at pH 2.5 at which

(20) Nakajima, N.; Ikada, Y. *Bioconjugate Chem.* **1995**, *6*, 123–130.

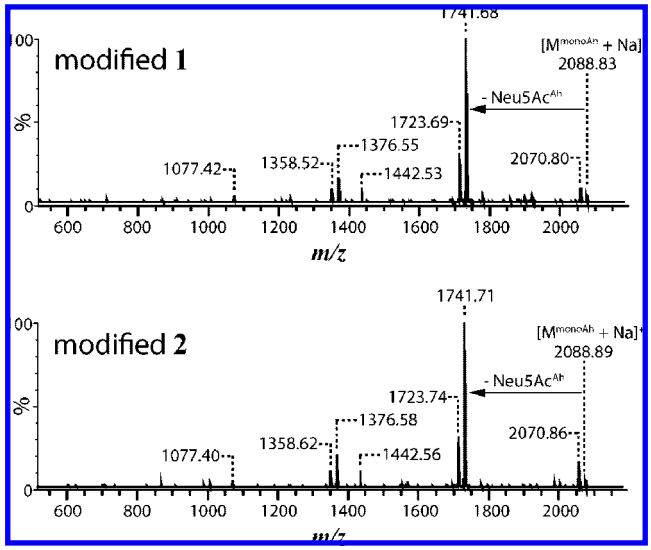


Figure 5. CID spectra of the acetohydrazide-modified sialoglycans **1** and **2**. Both spectra were obtained from $[M^{monoAh} + Na]^+$ ion as a precursor ion. Product ions were assigned as shown in Table 3.

Table 3. Signals Assignment for the MS/MS Spectra of the Modified Sialoglycans with Acetohydrazide in Figure 5

obsd *m/z*			
modified **1**	modified **2**	calcd *m/z*	assigned composition
1077.42	1077.40	1077.36	$[Hex_4HexNAc_2 - H_2O + Na]^+$
1358.52	1358.62	1358.50	$[Hex_4HexNAc_3PA - H_2O + Na]^+$
1376.55	1376.58	1376.51	$[Hex_4HexNAc_3PA + Na]^+$
1442.53	1442.56	1442.49	$[Hex_5HexNAc_3 - H_2O + Na]^+$
1723.69	1723.74	1723.63	$[Hex_5HexNAc_4PA - H_2O + Na]^+$
1741.68	1741.71	1741.64	$[Hex_5HexNAc_4PA + Na]^+$
2070.80	2070.86	2070.76	$[Neu5Ac^{monoAh}Hex_5HexNAc_4PA - H_2O + Na]^+$
2088.83[a]	2088.89[a]	2088.77	$[Neu5Ac^{monoAh}Hex_5HexNAc_4PA + Na]^+$

[a] 2088.83 and 2088.89 are precursor ions for modified **1** and modified **2**, respectively.

EDC would be decomposed within 60 min according to the paper mentioned above.[20] Presumably, low-pH conditions are effective, even for α2,3-linked sialic acids, because of a weakening in the hydrogen bond of the carboxyl proton with the adjacent hydroxyl groups.[21] Moreover, acetohydrazide, unlike ammonium chloride, is still an effective nucleophile at low pH. To confirm the optimal conditions for the reaction, modification of sialoglycans (**1**, **2**, and **3**) was performed at pH 2.5 and the reaction mixture was analyzed by reversed-phase HPLC (Figure 3). Furthermore, peaks in the HPLC chromatograms were examined by MALDI-TOF MS. The data is summarized in Table 2. The chromatograms clearly demonstrate that all three glycans were completely modified by this procedure. Next, we attempted the modification of biantennary N-linked glycan **4** having two sialic acids with an α2,3-linkage using this method. The HPLC chromatograms and MS data (Figure 3C and Table 2) clearly show that sialoglycan **4** was almost completely modified by this procedure. This result suggested that the novel amidation method works well even for sialoglycans having multiple α2,3-linked sialic acid residues. Thus, our novel protocol allows quantitative modification of sialoglycans regardless of linkage type.

Mass Spectrometry of the Modified Sialoglycans. The main aim of this study was to develop a suitable modification protocol of sialic acids that can be used for glycan profiling by MALDI MS. We analyzed the modified sialoglycans by MS analysis in order to assess the performance such as the stabilizing effect for sialic acid. MS spectra of **3** and the modified **3** were acquired with MALDI-TOF MS in positive ion and reflectron mode. As shown in Figure 4A, an acetohydrazide (Ah) derivative of sialoglycan **3** afforded a prominent signal of $[M^{diAh} + Na]^+$ ion in the mass spectrum, whereas the underivatized sialoglycan **3** gave a more complex spectrum which consisted of a $[M - 2H + 3Na]^+$ ion accompanied by signals from loss of sialic acids. The result suggested that this modification could stabilize the glycosidic bond of sialic acid. We also compared the signal intensities in an MS spectrum of a mixture of equal amounts of **3** and modified **3** (Figure 4B). Amidation with acetohydrazide catalyzed by EDC resulted in an enhanced signal intensity of the derivatized sialoglycan that was 6-fold higher than that of the unmodified starting glycans. The results presented here indicate that amidation of sialoglycans with acetohydrazide using EDC contributed to increase the sensitivity of analysis. The detection limit of modified **3** was 1 fmol at a signal-to-noise ratio of 3:1 (Figure 4C).

Multistage tandem MS analysis of glycans allows the discrimination of isomeric structures arising from different patterns of branching, linkage, or anomericity.[22] Collision-induced dissociation (CID) of unmodified sialoglycans in positive ion mode, however, preferentially detaches sialic acid residues from the glycan. As a consequence, structural information concerning the original position of sialic acid units within the glycan is lost. We examined the structural analysis of modified sialoglycans with acetohydrazide by acquiring CID spectra with a MALDI-QIT-TOF mass spectrometer. Figure 5 shows the CID spectra of $[M^{monoAh} + Na]^+$ ions of the acetohydrazide derivatives of sialoglycans **1** and **2**. In both spectra, the fragment ion *m/z* 1741, which corresponds to the value of an asialoglycan, is the most abundant signal. The other signals could also be assigned as fragment ions without sialic acid residue (Table 3). Furthermore, the fragment patterns of the two spectra are quite similar, making it impracticable to distinguish linkage types by CID. These results indicated that the acetohydrazide derivative of silaoglycans can stabilize the glycosidic bond of sialic acid enough to detect by MALDI MS, but not enough to determine the position of attachment of the sialic acids.

Dissociation of the glycoside of sialic acid might be promoted by the acidic proton of the carboxyl group adjacent to the glycosidic bond. The loss of a sialic residue from an acetohydrazide derivative of sialoglycans during CID might also be due to the adjacent proton. Indeed the $[M^{monoAh} - H + 2Na]^+$ ion, generated by exchange of an acidic proton for a sodium ion, was observed in the MS spectrum of the modified sialoglycans (Figure 4A and Table 2). In Figure 4C, the signal corresponding to the $[M^{monoAh} - H + 2Na]^+$ ion gradually increases with decreasing concentration of sample. These findings indicate the proton of

(21) Furutani, Y.; Ikeda, D.; Shibata, M.; Kandori, H. *Chem. Phys.* **2006**, *324*, 705–708.

(22) Kameyama, A.; Kikuchi, N.; Nakaya, S.; Ito, H.; Sato, T.; Shikanai, T.; Takahashi, Y.; Takahashi, K.; Narimatsu, H. *Anal. Chem.* **2005**, *77*, 4719–4725.

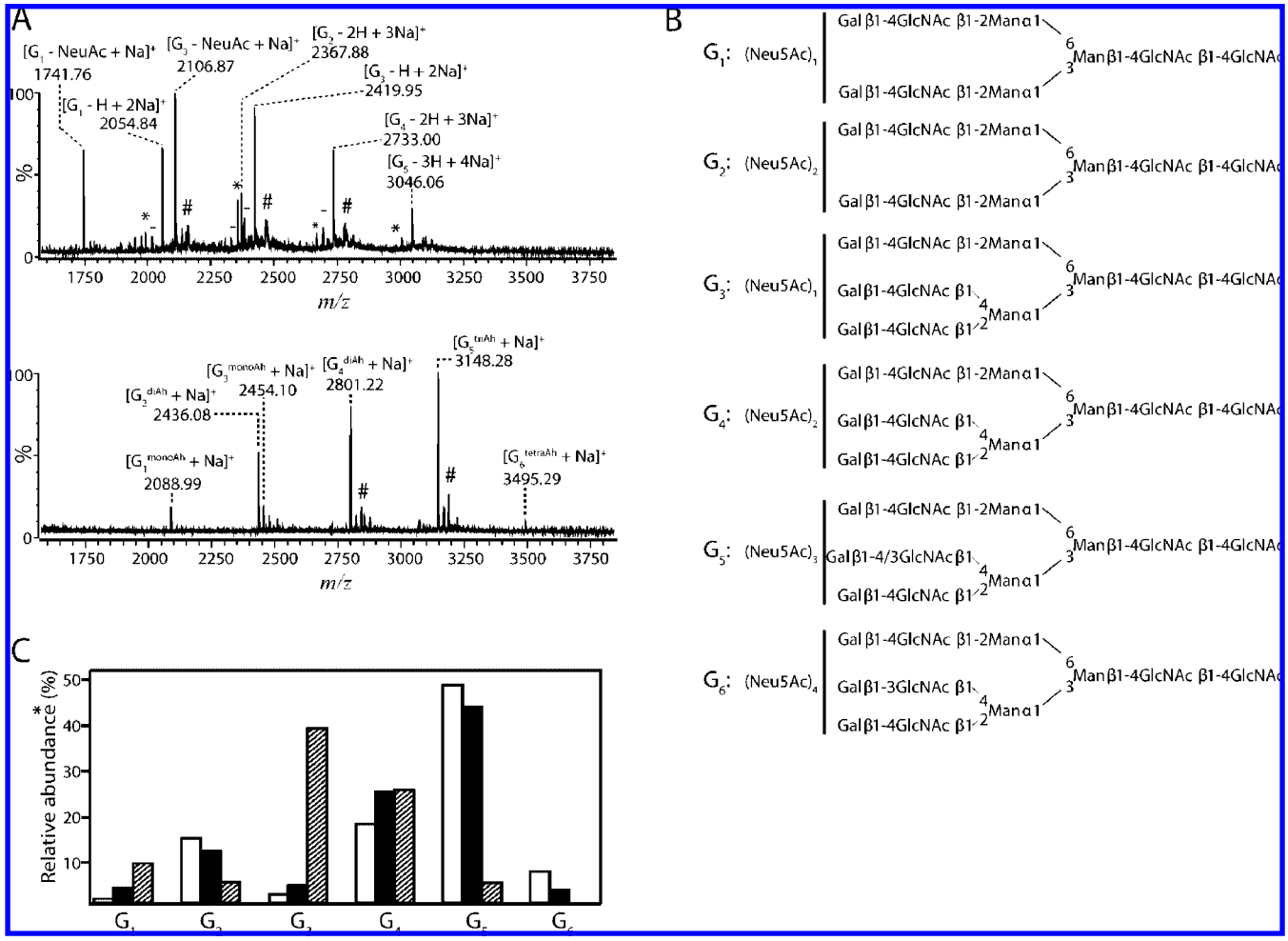


Figure 6. N-Linked glycan profiling of bovine fetuin by MALDI-TOF MS. (A) MS spectra of N-linked glycans released from bovine fetuin: upper, unmodified N-linked glycans; bottom, acetohydrazide-modified N-linked glycans. (B) The reported structures for N-linked glycans of bovine fetuin (ref 24). G_1−G_6 represent isomers of sialic acid linkages. (C) Comparison of the relative abundances of G_1−G_6 between the MS spectral data and HPLC data (ref 24). The relative abundance of each signal in the MS spectrum is indicated as a percentage of the total intensities of G_1−G_6. Blank bar, HPLC; solid bar, MS of modified N-linked glycans; shaded bar, HPLC including only α2,6-linkage in the count. Symbols #, −, and * indicate dehydrated ions, decarboxylated ions, and acetylated ions, respectively.

NH in the sialyl hydrazide is slightly acidic. CID of doubly cationized sialoglycans can avoid the loss of sialic acid and afford the different fragment pattern to distinguish the linkage types.[23,24] We are currently investigating procedures for generating a sufficient amount of doubly cationized ions of the acetohydrazide derivatives to test the discrimination of the linkage types by CID.

Profiling of N-Linked Glycans of Bovine Fetuin by MS. To demonstrate the feasibility of our novel modification methodology, we analyzed N-linked glycans released from bovine fetuin as a model glycoprotein in which α2,3-linked sialic acids dominate over α2,6-linked sialic acids. N-Linked glycans were released by glycopeptidase F and then labeled with PA by a reductive amination. PA is a fluorescence tag which is useful for HPLC separation and also used in the reference glycans for our MS^n spectral library.[22] Indeed, this tag is extremely useful for identifying structures. The PA-labeled N-linked glycans were modified with acetohydrazide by the method described above and then analyzed by MALDI-TOF MS. The MS spectrum of the modified N-linked glycans was compared with that of the unmodified compound (Figure 6A). The reported structures of the N-linked glycans in fetuin are summarized in Figure 6B.[25] The MS spectrum of the unmodified glycan mixture revealed abundant signals with fewer sialic acids. However, numerous signals corresponding to decomposed species, such as dehydrated ions and decarboxylated ions $[M - 44 + Na]^+$, were also detected. By contrast, the modified glycan mixture gave the trisialyl triantennary glycan G_5 (m/z 3148) as the most abundant signal, but no signals from decomposed species were observed. These results indicate that a large proportion of the unmodified sialoglycans were decomposed by in-source and/or postsource decay which could be suppressed by our modification procedure. Small signals of $[M + 42 + Na]^+$ were also observed in both spectra. These signals might be attributed to acetylated byproducts formed during the PA labeling reaction in which acetic acid was used to adjust the pH. The relative intensities of the signals of glycans in

(23) Leavell, M. D.; Leary, J. A. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 528–536.
(24) Von Seggern, C. E.; Zarek, P. E.; Cotter, R. J. *Anal. Chem.* **2003**, *75*, 6523–6530.
(25) Green, E. D.; Adelt, G.; Baenziger, J. U.; Wilson, S.; Van Halbeek, H. *J. Biol. Chem.* **1988**, *263*, 18253–18268.

the MALDI MS spectra are thought to be related to the amount of corresponding glycan. We summarized the relative intensities of the signals from the modified N-linked glycans and compared them with the data previously reported by HPLC analysis in Figure 6C.[25] The relative abundances of each signal in the MS spectrum were calculated as a percentage of the total intensities of G_1-G_6, and are indicated as solid bars. HPLC abundances of G_1-G_6 were calculated from the reported data and are indicated as blank bars. The shaded bars in Figure 6C indicate the calculated abundance in terms of α2,6-linked sialic acid only. These abundances were calculated on the assumption that all the α2,3-linked sialic acids were missing. For example, a disialoglycan G_2 having an α2,3-linked sialic acid and an α2,6-linked sialic acid are considered to be monosialoglycan G_1 in this calculation. The MS spectrum of the modified glycans (Figure 6A, bottom) showed no signal corresponding to glycans bearing unmodified sialic acid moieties. If modification exclusively occurred at the α2,6-linked sialic acid, the glycan profile of the MS spectrum should resemble that of the shaded bar (i.e., loss of α2,3-linked sialic acid by in-source and/or postsource decay). As shown in Figure 6C, the profile of the solid bar is similar to that of the blank bar but completely different from that of the shaded bar. These results suggest that the modification described in this report can be used for glycan profiling by MALDI MS even if the glycans contain α2,3-linked sialic acids.

CONCLUSION

The modification of sialoglycans at pH 2.5 using acetohydrazide and EDC quantitatively converts the sialoglycans to the corresponding acetohydrazide derivatives which stabilize the glycosidic bonds of sialic acids. The reaction proceeds to completion regardless of linkage type (i.e., α2,3- or α2,6-linked sialic acids). These features are ideal for comparative analysis of glycans using MALDI MS. Although the linkage type of sialic acid cannot be distinguished by CID of the acetohydrazide derivatives, a combination of conventional amidation and acetohydrazide derivatization by a stepwise modification might provide a differential profiling capable of discriminating α2,3- and α2,6-linked sialic acid. The modification method described in this study will be utilized for glycan biomarker discovery by MALDI MS.

ACKNOWLEDGMENT

We thank Ms. Azusa Tomioka for technical assistance. We also thank Dr. Sadanori Sekiya (Shimadzu Corporation, Kyoto, Japan) for valuable suggestions regarding practical aspects of the amidation of sialic acids. This work was performed as a part of the R&D Project of the Industrial Science and Technology Frontier Program supported by the New Energy and Industrial Technology Development Organization (NEDO).

Received for review March 5, 2008. Accepted April 11, 2008.

AC800457A