J. Biochem. 2009;145(6)739–749
doi:10.1093/jb/mvp031

Evaluation of Conditions for Release of Mucin-Type Oligosaccharides from Glycoproteins by Hydrazine Gas Treatment

Yukinobu Goso*, Daigo Tsubokawa and Kazuhiko Ishihara

Department of Biochemistry, Kitasato University Graduate School of Medical Sciences, Sagamihara 228-8555, Japan

Received February 2, 2009; accepted February 17, 2009; published online February 23, 2009

By using commercially available anhydrous hydrazine in the gas-phase, mucin-type oligosaccharides were released from porcine gastric mucin (PGM) and bovine fetuin. The data indicated that a certain amount of the oligosaccharides from PGM were further degraded. Despite this, the HPLC elution profile of the anthranilic acid (AA)-derivatized oligosaccharides obtained by the treatment with hydrazine at 65°C for 6 h resembled those obtained from the alkaline-borohydride treatment, except for the additional disaccharide fractions derived from the core 1 side of the oligosaccharides by further degradation. The other degraded products derived from the core 2 side could not be derivatized by AA, therefore, not visible by fluorescence detection. Liberation of the oligosaccharides was incomplete by the hydrazine treatment for 6 h. Although almost complete liberation was achieved by extending the treatment to 18 h, the degraded products also increased. In this case, the addition of barium oxide to the reaction vessel decreased the degree of further degradation. Results similar to PGM were obtained from bovine fetuin, but with less degradation. Application of this method for the analysis of rat gastric mucin (RGM) obtained from the corpus and antral region revealed that RGM has a large oligosaccharide portion on the core 1 side.

Key words: anthranilic acid, fetuin, hydrazine, mucin-type oligosaccharide, porcine gastric mucin, rat gastric mucin.

Abbreviations: AA, anthranilic acid; GalNAc-ol, *N*-acetylgalactosaminitol; MALDI-TOF/MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PGM, porcine gastric mucin; RGM, rat gastric mucin.

Recent research has shown that the saccharide portion of glycoproteins has many biological functions (*1–3*), but to clarify the role of these oligosaccharides, their structural information is required. For this purpose, the peptide *N*-glycosidase and hydrazinolysis are used to liberate the *N*-glycosidically linked Asn-type oligosaccharides from glycoproteins (*4*), whereas for the preparation of the *O*-glycosidically linked mucin-type oligosaccharides, alkaline-borohydride treatment is used because there is less degradation of the oligosaccharides (*5*, *6*). However, the alkaline-borohydride treatment loses the reducing property of the reducing-terminal GalNAc, which can be derivatized with either fluorescence (*7–9*) or immobilizing tags (*10*). Therefore, many methods have been developed to obtain mucin-type oligosaccharides bearing the reducing-terminal GalNAc; for instance, methods using hydrazine in both liquid and gas phases (*7*, *11*, *12*), ethylamine (*13*), alkali in-line flow (*14*), ammonia with ammonium carbonate (*15*) and so on. Of these techniques, hydrazine treatment is the most widely used.

During the course of characterizing oligosaccharide epitopes recognized by anti-mucin monoclonal antibodies (*16*, *17*), we tried to use commercially available anhydrous hydrazine in the gas phase to prepare a significant amount of oligosaccharides from porcine gastric mucin (PGM) and rat gastric mucin (RGM). However, we found that many of the obtained oligosaccharides were degraded, contrary to a previous report that hydrazine treatment provided intact oligosaccharides (*18*). Therefore, in the present study, we examined the reaction conditions for the hydrazine gas treatment using two model glycoproteins, PGM and bovine fetuin. Furthermore, these methods were used to examine RGM oligosaccharides in two gastric regions, the corpus and antrum.

MATERIALS AND METHODS

Treatment of Mucins with Anhydrous Hydrazine Gas or Alkaline-Borohydride—PGM (Type I, Sigma, St Louis, MO, USA) was partially purified by pepsin digestion followed by ethanol precipitation, as described previously (*19*). Ten milligrams of PGM or bovine fetuin (Spiro methods, Sigma) in glass tubes (10 mm I.D., 75 mm) were thoroughly dried over P_2O_5 *in vacuo* for more than 72 h. After the tubes were placed in a Waters Pico-Tag vessel (Millipore, Milford, MA, USA), 0.5 ml of anhydrous hydrazine (J-Oil Mills, Tokyo, Japan) was added to the outside of the tubes under dry nitrogen atmosphere in a plastic glove bag to avoid humidity. The vessel was then quickly vacuumized using a Waters Pico-Tag

*To whom correspondence should be addressed: Fax: +81-42-778-8441; E-mail: gosoy@nifmail.jp

Workstation, the cock was closed at ~200 Pa, and the vessel was heated at 65°C for 6 or 18 h. In some cases, another tube containing ~2 g of barium oxide (BaO) was placed in the vessel. After the hydrazine was removed thoroughly *in vacuo* with a cold trap and then over H_2SO_4, the released oligosaccharides were re-acetylated with acetic anhydride in saturated sodium bicarbonate (*20*), applied to a column of Dowex 50 (H^+ form, Bio-Rad, Richmond, CA, USA), and the passed fraction was then applied to a column of QAE-Toyopearl (acetate form, Tosoh, Tokyo, Japan). The passed fraction from the column (neutral oligosaccharides) and the fraction eluted from the column with 2 M pyridinium acetate, pH 5.0, (i.e. acidic oligosaccharides) was collected and lyophilized. A similar hydrazine treatment at 65°C for 6 h was performed using 0.5 mg of highly purified mucin, which was obtained from the corpus or antral region of the rat stomach as preciously described (*21*).

Alkaline-borohydride treatment of the PGM or fetuin (10 mg each) was performed according to the method of Carlson and his group (*6*) using 0.05 M NaOH containing 1 M $NaBH_4$ at 45°C for 15 h. After acidification and borate removal, the neutral and acidic oligosaccharides were obtained similar to the hydrazine-treated oligosaccharides.

Bio-Gel P-6 Chromatography of the Oligosaccharides—The oligosaccharides were fractionated by gel-permeation chromatography with a column (1.5 cm I.D., 45 cm) of Bio-Gel P-6 (Bio-Rad) using 0.1 M pyridinium acetate (pH 5.0) as the eluent. Each fraction collected was analyzed by hexose assay using the phenol/sulfuric acid method (*22*) or by sialic acid assay using the resorcinol method (*23*). The column was calibrated with Dextran T-500 (GE Healthcare Bioscience Japan, Tokyo, Japan), isomaltotridecaose and isomaltohexaose (Seikagaku Kogyo, Tokyo, Japan) and galactose.

TSKgel Amide-80 Normal-Phase HPLC Analysis of Underivatized Oligosaccharides—Oligosaccharides obtained from the Bio-Gel P-6 column were purified by thin-layer chromatography using a cellulose plate (Merk, Darmstadt, Germany) and 1-butanol/ethanol/water (4:1:1, by volume) as the eluting solvent. The oligosaccharides were further purified by solid-phase extraction with a graphitized carbon column (GL-Pak Carbograph, 150 mg/3 ml, GL-Science, Tokyo, Japan) using 25% acetonitrile for the neutral oligosaccharides or 25% acetonitrile containing 0.05% trifluoroacetic acid for the acidic ones as the eluting solvent (*24*). The oligosaccharides (1–10 μg) were injected into a TSKgel Amide-80 column (4.6 mm I.D., 25 cm, Tosoh) equipped with 15 mM potassium dihydrogen phosphate/acetonitrile (20/80, by volume), eluted with a 120 min linear gradient of 80–50% or 80–65% acetonitrile at 80°C at the flow rate of 0.7 ml/min and detected at 213 nm. For preparative purposes, 10–100 μg of the oligosaccharides was injected and eluted under the same conditions. The oligosaccharides were collected under the monitor and then desalted with a graphitized carbon column. The column was calibrated with GalNAc, Galβ1-3GalNAc (Sigma), Fucα1-2Galβ1-3GlcNAcβ1-6(GlcNAcα1-4Galα1-3)GalNAc-ol and GlcNAcα1-4Galβ1-3GlcNAcβ1-6(GlcNAcα1-4Galβ1-3)GalNAc-ol (Kanto Chemical, Tokyo, Japan) and chito-oligosaccharides that had a 1–6 degree of polymerization (Seikagaku Kogyo).

Derivatization of the Oligosaccharides with Anthranilic Acid (AA) and HPLC Analysis—The oligosaccharides (4–40 μg) obtained from the Bio-Gel P-6 column were derivatized with anthranilic acid (AA; 2-aminobenzoic acid, Wako Chemical, Osaka, Japan) by the method of Anumula and Dhume (*9*). After purification with a DPA-6S column (*25*), the AA-derivatized oligosaccharides (40–400 ng) were separated using a TSKgel Amide-80 column equipped with water containing 0.2% acetic acid and 0.2% triethylamine (Buffer A)/acetonitrile containing 0.1% acetic acid (Buffer B) (15/85, by volume), eluted with a 120 min linear gradient of 85–50% or 85–65% Buffer B at 35°C and the flow rate of 0.7 ml/min, then detected by fluorescence at 360 nm/425 nm (Ex/Em). For preparative purposes, 0.4–4 μg of the AA-derivatized oligosaccharides was applied and eluted under the same conditions, and the peak fractions were collected.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF/MS) Analysis of the Oligosaccharides—MALDI-TOF/MS spectra were acquired on a Voyager DE-Pro mass spectrometer (PE Biosystems, Framingham, MA, USA) using the following conditions: delay time of the grid wire and the guide wire voltages were 65% and 0.1% of the accelerating voltage (20 kV), respectively; 2,5-dihydro benzoic acid was used as the matrix; the positive ion mode was used; and all spectra were measured in the refrectron mode. A portion of the matrix solution (1 μl) was applied to a stainless steel target, to which was added a solution (1 μl) of the oligosaccharides. The target was dried at ambient temperature for several minutes. Lacto-*N*-fucopentaose I was used for the mass calibration.

Sugar Composition and Reducing-End Sugar Analysis—The hexose and hexosamine analysis of the oligosaccharides was performed by HPLC using a TSKgel ODS-80Ts column (4.6 mm I.D., 7.5 cm, Tosoh) after pre-labelling the sugar with AA (*26*). Water containing 0.2% 1-butylamine, 0.5% phosphoric acid and 1% tetrahydrofuran (Buffer C) and the solution of Buffer C/acetonitrile, 50/50, by volume (Buffer D) were used as the solvent system. Detection was performed with fluorescence at 360 nm/425 nm (Ex/Em). The hexosamine and hexosaminitol analysis was performed by the phenylisothiocyanate derivatization method as described previously (*27*) and the sialic acid analysis was performed according to the method of Hara *et al*. (*28*).

For the sugar analyses, the oligosaccharides were hydrolyzed by 20% (v/v) trifluoroacetic acid at 100°C for 6 h for hexose and hexosamine, by 4 M HCl at 100°C for 6 h for hexosamine and hexosaminitol, and by 25 mM H_2SO_4 at 80°C for 1 h for sialic acid.

The reducing-end sugar moiety was determined similar to the hexose and hexosamine analysis after hydrolysis of the AA-derivatized oligosaccharides.

RESULTS

Evaluation of the Hydrazine Treatment Conditions for PGM by Bio-Gel P-6 Chromatography—PGM was treated with anhydrous hydrazine gas, and after re-acetylation

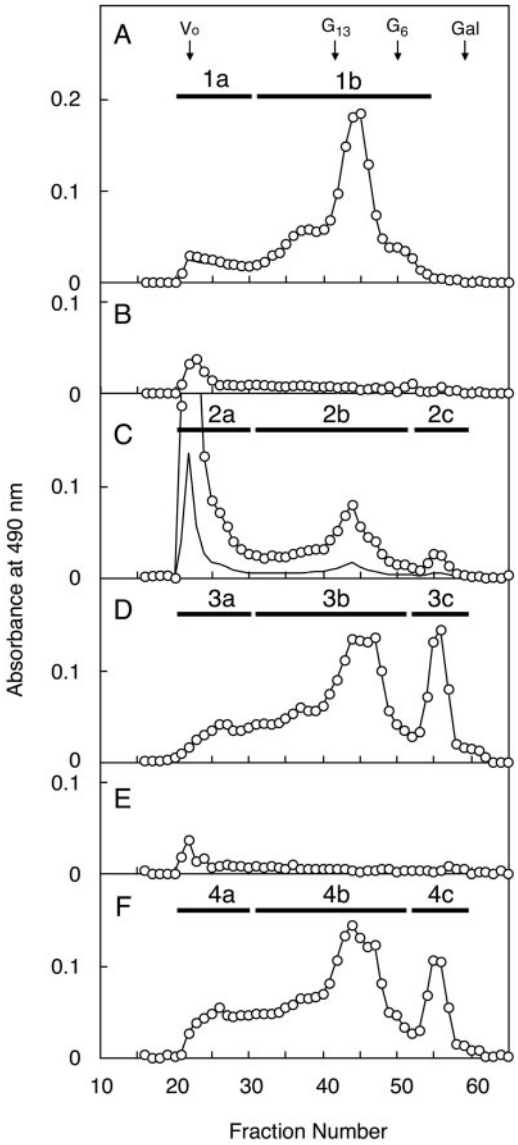


Fig. 1. **Bio-Gel P-6 chromatography of the oligosaccharides obtained from PGM.** (A, B) Neutral and acidic oligosaccharides liberated by alkaline-borohydride treatment, respectively. (C) Neutral oligosaccharides liberated by anhydrous hydrazine gas treatment at 65°C for 6 h. (D, E) neutral and acidic oligosaccharides liberated by anhydrous hydrazine gas treatment at 65°C for 18 h, respectively. (F) neutral oligosaccharides liberated by anhydrous hydrazine gas treatment at 65°C for 18 h in the presence of BaO in the reaction vessel. Oligosaccharides were detected by their hexose content measured by the phenol/sulfuric acid method. The straight line in C shows one-fifth of the absorbance. The fractions indicated by the bars were pooled and subjected to further analysis. The column was calibrated with Dextran T-500 (Vo), isomaltotridecaose (G_{13}), isomaltohexaose (G_6) and galactose (Gal).

the reactant was fractionated into neutral and acidic fractions by ion-exchange chromatography. Both fractions were then analysed using Bio-Gel P-6 chromatography. As the control, PGM was also treated with the alkaline-borohydride solution and analyzed.

A neutral oligosaccharide fraction (designated **1b**) of appropriate size was obtained from the PGM by alkaline-borohydride treatment (Fig. 1A), with a small amount of the larger fractions (**1a**), and only a small amount of the hexose-positive material detected at the position of the column volume. Acidic oligosaccharides were mainly eluted as the excluded fraction, but small amounts of the intermediate-sized oligosaccharides were also present (Fig. 1B).

In contrast to the alkaline-borohydride-released oligosaccharides, different patterns were obtained using the hydrazine-treated PGM. The shorter hydrazine treatment time (6 h) at 65°C resulted in significant amounts of neutral oligosaccharides (**2b**), with a few small oligosaccharides (**2c**), and a large amount of hexose-positive material (**2a**) present in the excluded fraction from the column (Fig. 1C). Figure 1D shows the neutral oligosaccharides obtained from the PGM by hydrazine treatment at 65°C for 18 h. Under these conditions, the large amount of the hexose-positive material in the excluded fraction disappeared and more oligosaccharides (**3b**) were obtained; however, there was a significant amount of small oligosaccharides (**3c**), thought to be degraded products produced by the so-called 'peeling' reaction. In contrast, acidic oligosaccharides obtained by the hydrazine treatment under the same conditions showed an elution pattern similar to that of the alkaline-borohydride-released oligosaccharides (Fig. 1E). Because the appearance of the degradation products (Fig. 1D) seemed to be caused by the presence of water, which is produced during the hydrazine treatment as discussed later, hydrazine treatment was performed in the presence of BaO as a moisture absorption material. Under these conditions, the amount of degraded product (**4c**) decreased with no change in the amount of the medium-sized oligosaccharides (**4b**), although the large amount of the hexose-positive material (**4a**) further increased (Fig. 1F).

These results indicate that significant amounts of the oligosaccharides released by the prolonged anhydrous hydrazine gas treatment might be degraded, whereas fewer degraded oligosaccharides are produced by the shorter treating times, and the presence of BaO may decrease the degradation reaction.

TSKgel Amide-80 Normal-Phase HPLC Analysis of the PGM Neutral Oligosaccharides—The neutral oligosaccharides obtained from the Bio-Gel P-6 chromatography were further analysed by normal-phase HPLC using a TSKgel Amide-80 column. The mass and sugar composition of each oligosaccharide separated by the preparative HPLC were determined by MALDI-TOF/MS and sugar analyses, respectively. The acidic oligosaccharides were not further analysed due to insufficient amounts.

Five neutral oligosaccharyl alditols, designated from **1b-1** to **5**, were obtained as major components of the PGM by the alkaline-borohydride treatment (Fig 2A). The mass and sugar composition analyses showed that these were tri- to hepta-saccharides (Table 1); for example, **1b-3** was considered to be ($Fuc_1Gal_2GlcNAc_2$)-GalNAc-ol based on the following data: (*1*) its mass of 1122.3 corresponded to that of the Na-adducted

$dHex_1Hex_2HexNAc_2HexNAc\text{-}ol_1$; (*2*) the hexose and hexosamine analysis showed that Fuc:Gal:GlcN:GalN was 1.0:2.0:2.0:0.0; (*3*) the hexosamine and hexosaminitol analysis showed that GlcN:GalN:GalN-ol was 2.0:0.0:1.0; (*4*) Fucα1-2Galβ1-3GlcNAcβ1-6(GlcNAcα1-4Galβ1-3)GalNAc-ol was obtained from the PGM as a major component (*16*); and (*5*) this oligosaccharyl alditol (m3) eluted at the same position as **1b-3**. Therefore, **1b-3** seemed to have this oligosaccharide, although it is possible that other oligosaccharides with the same sugar composition were present.

In contrast, a different elution profile was obtained from the hydrazine-released neutral oligosaccharides **3b** (Fig. 2D). Although **3b-12** to **15** seemed to be oligosaccharides corresponding to **1b-2** to **5**, because of their mass and sugar compositions (Table 1), **3b-12** to **15** were the minor components, therefore **3b-1** to **11** were the

Table 1. **Structures of oligosaccharides prepared from PGM by alkaline-borohydride or hydrazine treatment.**

Fractions	*m/z* $[M+Na]^+$	Proposed structures[a]
1b-1	611.5	$(Gal_1GlcNAc_1)$-GalNAc-ol[b]
1b-2	814.5	$(Gal_1GlcNAc_2)$-GalNAc-ol
1b-3	1122.3	$(Fuc_1Gal_2GlcNAc_2)$-GalNAc-ol
1b-4	1177.5	$(Gal_2GlcNAc_3)$-GalNAc-ol
1b-5	1325.6	$(Fuc_1Gal_2GlcNAc_2GalNAc_1)$-GalNAc-ol
3b-1	485.5	GlcNAc-**X**[c]
3b-2	485.5	GlcNAc-**X**
3b-3	688.3	$(GlcNAc_1GalNAc_1)$-**X**
3b-4	647.5	$(Gal_1GlcNAc_1)$-**X**
3b-5	688.6	$(GlcNAc_1GalNAc_1)$-**X**
3b-6	647.6	$(Gal_1GlcNAc_1)$-**X**
3b-7	793.4	$(Fuc_1Gal_1GlcNAc_1)$-**X**
3b-8	793.4	$(Fuc_1Gal_1GlcNAc_1)$-**X**
	850.5	$(Gal_1GlcNAc_2)$-**X**
3b-9	850.6	$(Gal_1GlcNAc_2)$-**X**
3b-10	996.5	$(Fuc_1Gal_1GlcNAc_1GalNAc_1)$-**X**
3b-11	996.4	$(Fuc_1Gal_1GlcNAc_1GalNAc_1)$-**X**
3b-12	812.4	$(Gal_1GlcNAc_2)$-GalNAc[d]
3b-13	1120.4	$(Fuc_1Gal_2GlcNAc_2)$-GalNAc
3b-14	1175.5	$(Gal_2GlcNAc_3)$-GalNAc
3b-15	1323.6	$(Fuc_1Gal_2GlcNAc_2GalNAc_1)$-GalNAc
3b-16	1282.5	$(Fuc_1Gal_2GlcNAc_2GalNAc_1)$-Gal
3b-17	1339.4	$(Gal_2GlcNAc_3GalNAc_1)$-Gal
3c-1	485.5	GlcNAc-**X**
3c-2	485.5	GlcNAc-**X**
3c-3	406.4	GlcNAc-Gal

[a]Structures were estimated due to the mass value and sugar composition analysis as described in the text. [b]GalNAc-ol represents *N*-acetylgalactosaminitol. [c]**X** is an unknown compound having a mass value of 259.3. [d]Reducing ends are estimated to be GalNAc or Gal by the reducing-end sugar analyses as described in the text.

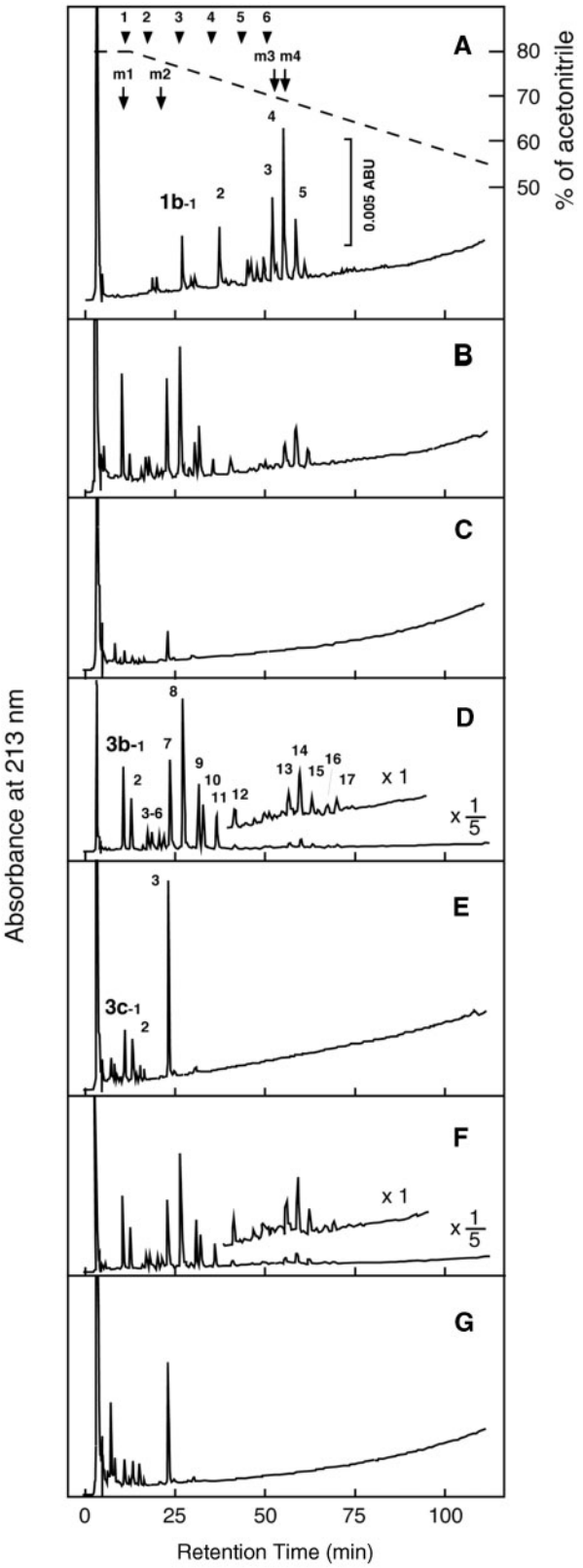
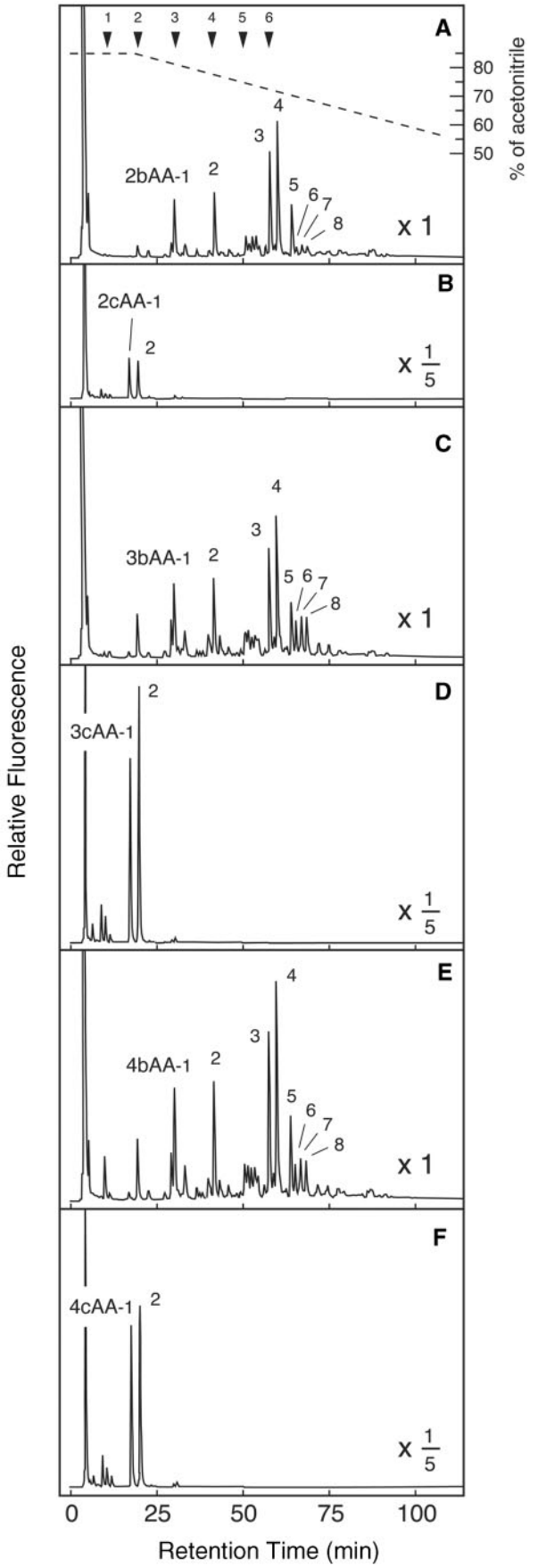


Fig. 2. **TSKgel Amide-80 HPLC of the oligosaccharides of PGM.** The following oligosaccharide fractions were analyzed: A, **1b**; B, **2b**; C, **2c**; D, **3b**; E, **3c**; F, **4b** and G, **4c**. After injection, the oligosaccharides were eluted with a linear gradient of acetonitrile (dashed line), as described in the text, and detected at 213 nm. The column was calibrated with chitooligosaccharides having a degree of polymerization of 1–6 (1–6), GalNAc (m1), Galβ1-3GalNAc (m2), Fucβ1-2Galα1-3GlcNAcβ1-6(GlcNAcα1-4Galβ1-3)GalNAc-ol (m3) and GlcNAcα1-4Galα1-3GlcNAcβ1-6(GlcNAcα1-4Galβ1-3)GalNAc-ol (m4). The absorbance unit is indicated by the bar in A. Absorbances in D and F are expressed as one-fifth of the magnitude, except for the inserts.



major components. An almost similar elution profile was obtained for **2b**, except for the lower amounts of the oligosaccharides corresponding to **3b-1** to **11** (Fig. 2B). The mass and sugar composition analyses of the oligosaccharides obtained by preparative HPLC showed that **3b-1** to **11** probably contained the unknown compound **X**, the mass of which was 259.3 (Table 1). The small fractions, **2c** and **3c**, were mainly composed of three oligosaccharides (Fig. 2C and E), which were estimated to be GlcNAc-**X** and GlcNAc-Gal (Table 1). Figure 2F and G show the respective elution profiles of **4b** and **4c**. The oligosaccharides corresponding to **3b-12** to **15** increased, but those corresponding to **3b-1** to **11** and **3c-1**, **2** and **3** decreased.

These results indicate that **3b-12** to **15**, and the corresponding ones in **2b** and **4b**, were undegraded oligosaccharides corresponding to **1b-2** to **5**, whereas **3b-1** to **11** and **3c-1** to **3** seemed to be degraded products. As compared to the absorbance of **1b-2** to **5** in Fig. 2A, it was estimated that ~30%, 30% and 40% of the oligosaccharides in PGM were obtained in an undegraded form by hydrazine treatment at 65°C for 6 h, 18 h and 18 h in the presence of BaO, respectively. Although the absorbance of the degraded products containing unknown product **X** was quite high, this seemed to be related to their large molecular extinction coefficient, as described later.

HPLC Analysis of AA-Derivatized Oligosaccharides— For the microscale analysis of the oligosaccharides, various fluorescent labels are used and we chose the AA-derivatization for this purpose. Figure 3A shows the elution profile of the AA-derivatized **2b**, which was quite similar to the elution profile of the oligosaccharides obtained by the alkaline-borohydride treatment (Fig. 2A); namely, five major oligosaccharides, **2bAA-1** to **5** corresponding to **1b-1** to **5** were seen. Compared with this, the elution profile of the AA-derivatized **3b** was somewhat different (Fig. 3C); namely, although almost the same amounts of **3bAA-1** to **5** were observed, additional oligosaccharides, **3bAA-6** to **8**, were seen. Mass measurement and reducing-end sugar analyses of the AA-derivatized oligosaccharides obtained by the preparative HPLC showed that **3bAA-1** to **5** were undegraded products corresponding to **1b-1** to **5**, while **3bAA-6** to **8** were degraded products, because their reducing end was not GalNAc, but Gal (Table 2). **3cAA-1** and **2** (Fig. 3D) were estimated to be Fuc-**Gal-AA** and GlcNAc-**Gal-AA**, respectively, because of their mass and reducing-end sugar moieties. Therefore, they were thought to be degraded products. These two oligosaccharides were significantly increased in the AA-derivatized **3c**

Fig. 3. **TSKgel Amide-80 HPLC of the AA-derivatized oligosaccharides of PGM.** The following oligosaccharide fractions, after being derivatized with AA, were analyzed: A, **2b**; B, **2c**; C, **3b**; D, **3c**; E, **4b** and F, **4c**. After injection, the AA-derivatized oligosaccharides were eluted with a linear gradient of acetonitrile (dashed line), as described in the text, and detected by fluorescence at 360 nm/425 nm (Ex/Em). The column was calibrated with AA-derivatized chito-oligosaccharides having a degree of polymerization of 1–6 (1–6). The relative fluorescence intensity in B, D and F is expressed as one-fifth of the fluorescence intensity of A, C and E.

Table 2. **Structures of AA-derivatized oligosaccharides prepared from PGM**

Fractions	*m/z* [M + Na]$^+$	*m/z* [M − H + 2Na]$^+$	Proposed structures[a]
3bAA-1	730.3		($Gal_1GlcNAc_1$)-**GalNAc-AA**[b]
3bAA-2	933.5		($Gal_1GlcNAc_2$)-**GalNAc-AA**
3bAA-3	1241.6	1263.6	($Fuc_1Gal_2GlcNAc_2$)-**GalNAc-AA**
3bAA-4	1298.5	1320.5	($Gal_2GlcNAc_3$)-**GalNAc-AA**
3bAA-5	1444.2	1466.2	($Fuc_1Gal_2GlcNAc_2GalNAc_1$)-**GalNAc-AA**
3bAA-6	1346.4	1368.3	($dHex_2Hex_2HexNAc_2$)-**Gal-AA**
3bAA-7	1403.5	1425.5	($Fuc_1Gal_2GlcNAc_2GalNAc_1$)-**Gal-AA**
3bAA-8	1460.7		($Gal_2GlcNAc_3GalNAc_1$)-**Gal-AA**
3cAA-1	470.4		Fuc-**Gal-AA**
3cAA-2	527.5	549.2	GlcNAc-**Gal-AA**

[a]Structures were estimated due to the mass value and reducing-end sugar moiety and comparison with the sugar composition of **3b-12** to **17** and **3c-3**. [b]**Gal-AA** and **GalNAc-AA** are AA-derivatized Gal and GalNAc, respectively.

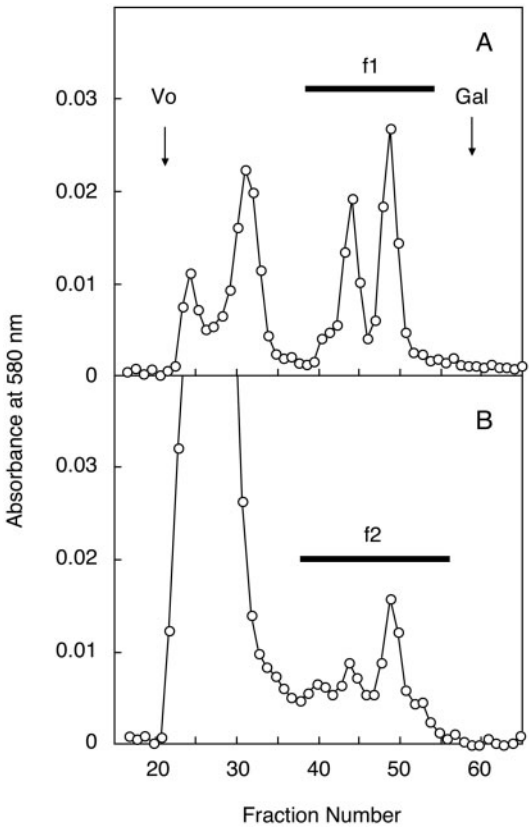


Fig. 4. **Bio-Gel P-6 chromatography of the fetuin acidic oligosaccharides obtained by alkaline-borohydride treatment (A) and by anhydrous hydrazine gas treatment at 65°C for 6 h (B).** Oligosaccharides were detected by their sialic acid content determined by the resorcinol method. The fractions indicated by the bars were pooled and subjected to further analysis. The column was calibrated with Dextran T-500 (Vo) and galactose (Gal).

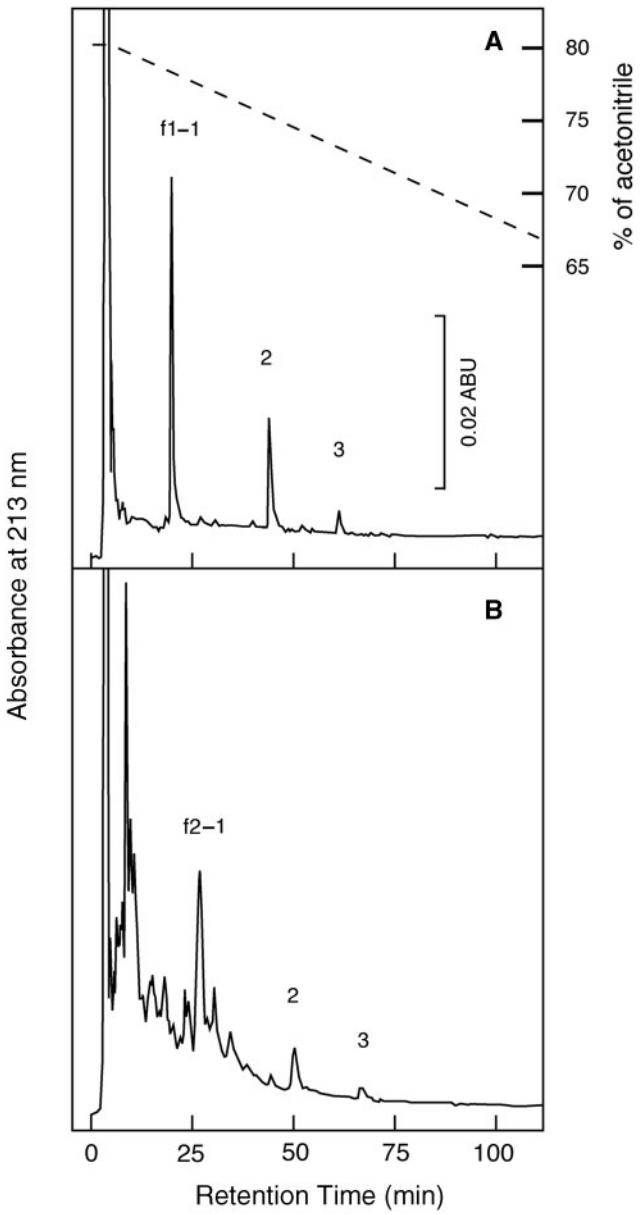


Fig. 5. **TSKgel Amide-80 HPLC of the fetuin oligosaccharide fractions f1 (A) and f2 (B).** After injection, the oligosaccharides were eluted with a linear gradient of acetonitrile (dashed line), as described in the text, and detected at 213 nm. The absorbance unit is indicated by the bar in A.

compared to the AA-derivatized **2c**. Because Fuc-Gal has no absorbance at 213 nm, it was apparently not detected in Fig. 2.

These results indicate that the oligosaccharides having unknown compound **X** were not derivatized with AA, whereas the degraded oligosaccharides bearing the Gal residue at the reducing end were derivatized, and the shorter hydrazine treatment produced less of the degraded oligosaccharides than did the longer treatment, agreeing with the results for the underivatized oligosaccharides.

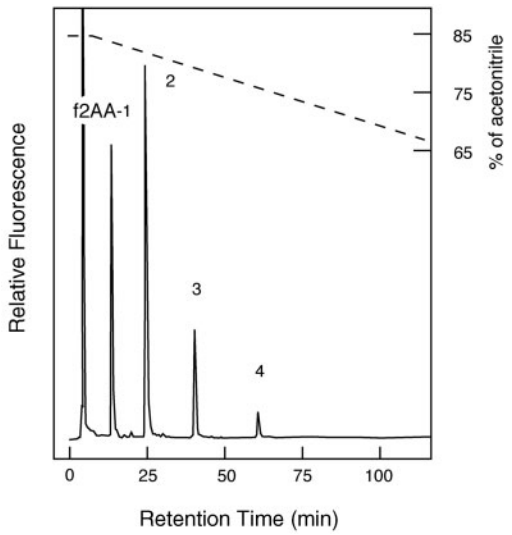


Fig. 6. **TSKgel Amide-80 HPLC of the AA-derivatized fetuin oligosaccharide fractions prepared from f2.** After injection, the AA-derivatized oligosaccharides were eluted with a linear gradient of acetonitrile (dashed line), as described in the text, and detected by fluorescence at 360 nm/425 nm (Ex/Em).

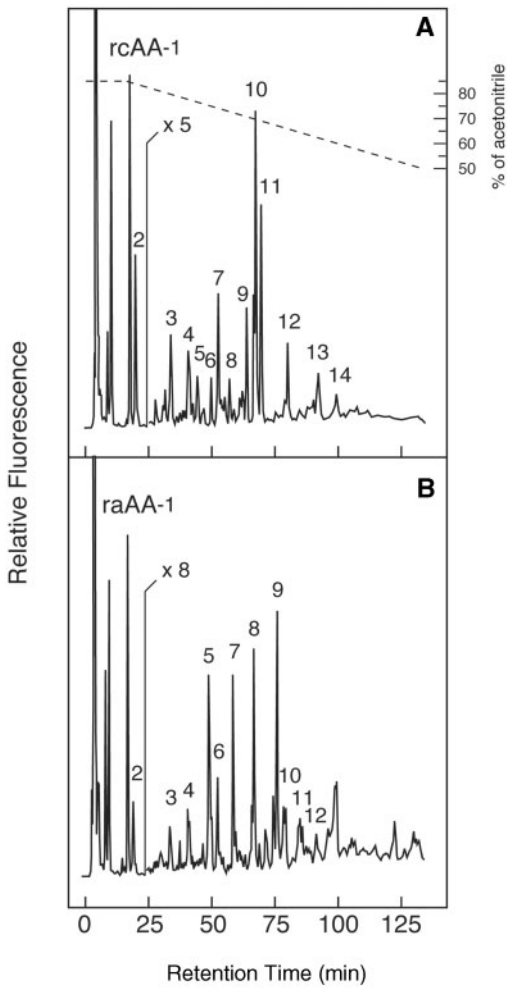


Fig. 7. **TSKgel Amide-80 HPLC of the AA-derivatized oligosaccharide fractions prepared from the rat gastric corpus (A) or antral (B) mucin.** After injection, the AA-derivatized oligosaccharides were eluted with a linear gradient of acetonitrile (dashed line), as described in the text, and detected by fluorescence at 360 nm/425 nm (Ex/Em).

With hydrazine treatment performed in the presence of BaO, much higher amounts of the intact oligosaccharides **4bAA-1** to **5** were obtained when compared to **3bAA**, without an increase in the amounts of the degraded products **4bAA-6** to **8** (Fig. 3E). In addition, **4cAA-1** and **2** decreased when compared to **3cAA-1** and **2**. These results seem to indicate that the removal of water from the reaction vessel during the hydrazine treatment prevents the 'peeling' reaction. Based on the relative fluorescence intensities, the ratio of the intact oligosaccharides (**nbAA-1** to **5** plus minor components among these major ones, $n = 2$, 3 and 4) to the degraded ones (**nbAA-6** to **8** plus **ncAA-1** and **2**, $n = 2$, 3 and 4) were calculated to be 62:38, 34:66 and 42:58, respectively.

Evaluation of the Hydrazine Treatment Conditions with Fetuin—Bovine fetuin is used as a model glycoprotein for evaluating the release of the mucin-type oligosaccharides, because of its availability as a commercial source and the reported structures of its mucin-type oligosaccharides (*29*). Therefore, we also performed hydrazine treatment of the fetuin. Bio-Gel P-6 chromatography showed that the **f1** fraction, which seemed to be composed of mucin-type oligosaccharides, was obtained after the alkaline-borohydride treatment (Fig. 4A). Similarly, the mucin-type oligosaccharide fraction, **f2**, was obtained from the hydrazine-treated samples, but there were large oligosaccharide fractions that seemed to contain *N*-glycans (Fig. 4B).

HPLC analysis showed that **f1** was mainly composed of three oligosaccharyl alditols (Fig. 5A) and from the sugar composition analysis (data not shown) and the reported structures, **f1-1**, **2** and **3** were identified as Neu5Acα2-3Galβ1-3GalNAc-ol, Neu5Acα2-6(Neu5Acα2-3Galβ1-3)GalNAc-ol and Neu5Acα2-3Galβ1-3GlcNAcβ2-6(Neu5Acα2-3Galβ1-3)GalNAc-ol, respectively. Similarly, the three oligosaccharides, **f2-1**, **2** and **3**, were observed in the hydrazine-treated sample, although some additional peaks were present (Fig. 5B). It was calculated from the absorbance of the oligosaccharide peaks at 213 nm in Fig. 5A and B that ~70% of the mucin-type oligosaccharides was recovered intact from fetuin by the hydrazine treatment under these conditions.

In contrast, the AA-derivatized oligosaccharides comprised of four oligosaccharides (Fig. 6) and the MS and reducing-end sugar analyses showed that **f2AA-2**, **3** and **4** were AA-derivatized oligosaccharides corresponding to **f2-1**, **2** and **3**; namely, the tri-, tetra- and hexasaccharides. **f2AA-1** seemed to be Neu5Ac α 2-3Gal, which was a degraded product from the hydrazine treatment. **f2AA-1** was comprised ~30% (mol/mol) of the AA-derivatized oligosaccharides based on its fluorescence intensity, which again indicates that ~70% of the mucin-type oligosaccharides was recovered intact under these conditions.

Table 3. **Structures of AA-derivatized oligosaccharides prepared from RGM**

Fractions	*m/z* $[M+Na]^+$	Fractions	*m/z* $[M+Na]^+$	Proposed structures[a]
rcAA-1	470.1	**raAA-1**	469.9	Fuc-**Gal-AA**[b]
rcAA-2	527	**raAA-2**	526.9	HexNAc-**Gal-AA**
rcAA-3	730.2	**raAA-3**	730.6	($Gal_1HexNAc_1$)-**GalNAc-AA**
rcAA-4	835.2	**raAA-4**	835.3	($Fuc_1Gal_1HexNAc_1$)-**Gal-AA**
		raAA-4	876.7	($Fuc_1Gal_1HexNAc_1$)-**GalNAc-AA**
rcAA-5	892.3			($Gal_1HexNAc_2$)-**Gal-AA**
rcAA-6	1038.4	**raAA-5**	1038.4	($Fuc_1Gal_2HexNAc_1$)-**GalNAc-AA**
rcAA-7	1038.9	**raAA-6**	1038.4	($Fuc_1Gal_1HexNAc_2$)-**Gal-AA**
rcAA-8	1054.3			($Gal_2HexNAc_2$)-**Gal-AA**
		raAA-7	1241.5	($Fuc_1Gal_1HexNAc_3$)-**Gal-AA**
rcAA-9	1257.8			($Gal_2HexNAc_3$)-**Gal-AA**
rcAA-10	1346.7	**raAA-8**	1346.6	($Fuc_2Hex_2HexNAc_2$)-**Gal-AA**
rcAA-11	1460.6			($Gal_3HexNAc_3$)-**GalNAc-AA**
		raAA-9	1752.7	($Fuc_2Gal_2HexNAc_4$)-**Gal-AA**
rcAA-12	1712	**raAA-10**	1711.6	($Fuc_2Gal_3HexNAc_3$)-**Gal-AA**
		raAA-11	2117.9	($Fuc_2Gal_3HexNAc_5$)-**Gal-AA**
rcAA-13	2223.1	**raAA-12**	2222.8	($Fuc_3Gal_4HexNAc_4$)-**Gal-AA**
rcAA-14	2587.9			($Fuc_3Gal_5HexNAc_5$)-**Gal-AA**

[a]Structures were estimated due to the mass value and reducing-end sugar moiety. [b]**Gal-AA** and **GalNAc-AA** are AA-derivatized Gal and GalNAc, respectively.

These results indicate that degradation also occurs during the hydrazine gas treatment of fetuin, but a much higher amount of intact oligosaccharides is recovered compared to the PGM.

HPLC Analysis of AA-Derivatized Oligosaccharides of RGM—The methods described here were used for the analyses of the RGM's oligosaccharides. Figure 7A shows the elution profile of the AA-derivatized neutral oligosaccharides obtained from the corpus mucin. Mass measurement showed that the major components were the AA-derivatized di- (**rcAA-1** and **2**) to tetradecasaccharide (**rcAA-14**) (Table 3). In spite of their large size, reducing-end analysis showed that many of them were degraded products, because their reducing-end was not GalNAc but Gal, although the intact oligosaccharides, for instance, **rcAA-6** and **11**, were also present as major components. Some minor components between **rcAA-4** and **14** also seem to be the intact AA-derivatized oligosaccharides, because they possessed GalNAc at their reducing-end (data not shown). As the oligosaccharides having the Gal residue at the reducing end were thought to be derived from the core 1 side of the oligosaccharides, the results seem to indicate that many oligosaccharides of the corpus mucins consist of oligosaccharides having a large saccharide portion on their core 1 side.

The AA-derivatized oligosaccharides from the antral mucins, **raAA-1** to **12**, have sizes almost similar to **rcAA-1** to **13**, and many of them were derived from the degraded products having Gal at their reducing-end (Fig. 7B and Table 3). However, some **raAAs** were different from the **rcAAs** (Table 3).

DISCUSSION

Hydrazine treatment has often been used for releasing mucin-type oligosaccharides from glycoproteins. Many reports showed that the condition, 6 h at 60–65°C, was sufficient for complete liberation of the oligosaccharides without further degradation. Based on this condition, we aimed to obtain the mucin-type oligosaccharides from the PGM, but we found that the release of the oligosaccharides was incomplete with a reaction time of 6 h at 65°C (Fig. 1B). The discrepancy between our results and those of others may be related to differences in the reaction phase (i.e. the gas-phase in ours versus the liquid-phase in others). However, this is unlikely because the liquid-phase reactions using the same hydrazine reagent gave results similar to those for the gas-phase reactions; that is, the incomplete release of the oligosaccharides from the PGM after treatment for 6 h (data not shown).

We proposed that this discrepancy may be explained by the degradation of the released oligosaccharides. Our data showed that the amount of the intact oligosaccharides did not increase by extending the reaction time from 6 to 18 h (Figs 2B and D, and 3A and C), therefore, the reaction appeared to be complete, although the degraded ones increased. This indicates that the liberated intact oligosaccharides might be further degraded during the prolonged reaction time. Because many degraded oligosaccharides, except for the small ones, could not be derivatized with a fluorescent-tag as will be described later, degradation of the oligosaccharides might not be detected in the experiment using the fluorescent-tag.

We hypothesized that water, which is produced by the reaction of the oligosaccharides with hydrazine, enhances the 'peeling' reaction (Fig. 8). If this were the case, removal of the water produced during the reaction may result in less degradation of the oligosaccharides. As expected, less degradation was achieved by the addition of BaO as a desiccant, although the effect was limited (Figs 1F, 2F, 2G, 3E and 3F).

Relatively less degradation was observed with fetuin when compared to PGM, which may be related to the

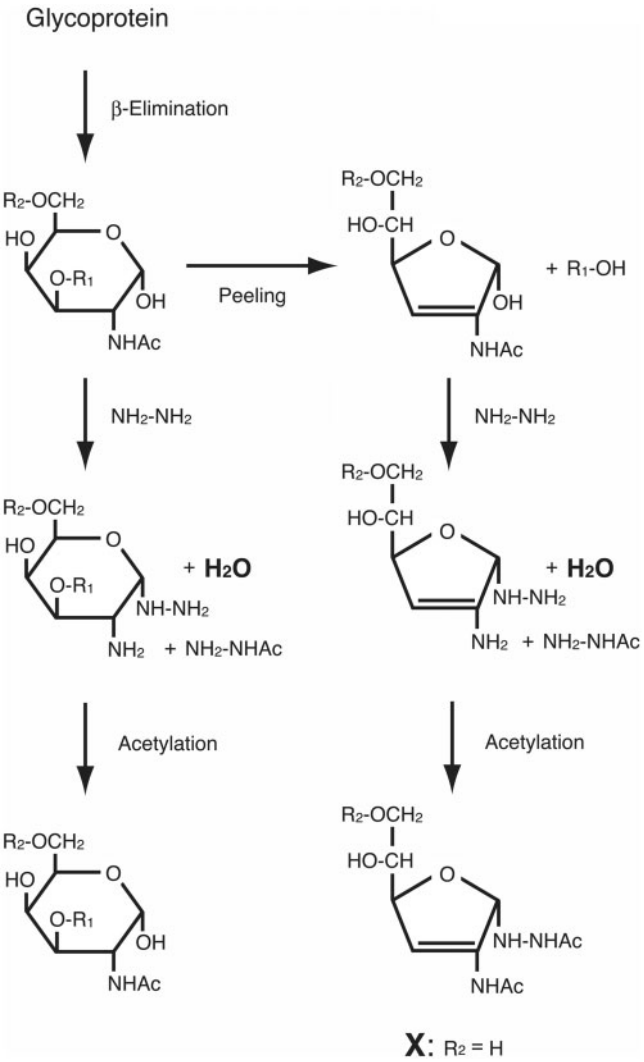


Fig. 8. **Hypothesized mechanism of hydrazine treatment.** Although the sugar residues are represented as the α -anomer, the β-anomer and the acyclic form are present. R_1 and R_2: H, monosaccharide or oligosaccharide. Ac: acetyl group.

lower number of mucin-type oligosaccharides attached to the fetuin. Less water produced from fetuin by hydrazine treatment might produce less of a 'peeling' reaction, as already discussed. The other explanation is that acidic oligosaccharides may be more resistant to the 'peeling' reaction. Analysis of the acidic oligosaccharides obtained from the PGM may clarify this point.

It was reported that complete removal of the water from the reaction system resulted in no degradation at all (*18*, *30*). In that case, our reaction system may have still contained sufficient amounts of water for the 'peeling' reaction to occur, despite using dried glycoprotein samples and anhydrous hydrazine. This may be partly true, because our hydrazine reagent caused degradation even for a short reaction time. However, the abovementioned studies did not state the amount of water produced during the reaction, and a trace amount of water may be needed for β -elimination of the oligosaccharides from the glycoproteins, because the addition of BaO to the reaction vessel partially prevents the release of the oligosaccharides (Fig. 1F), so complete removal of water from the reaction system may prevent the β-elimination reactions.

In spite of the degradation caused by the 'peeling' reaction, which was evident from the elution profiles of the oligosaccharides during the Bio-Gel P-6 chromatography (Fig. 1) and TSKgel Amide-80 HPLC (Fig. 2) and from the presence of oligosaccharides with a Gal residue at the reducing end (Table 1), the elution profile of the AA-derivatized oligosaccharides shown in Fig. 3A was similar to that for the oligosaccharyl alditols obtained by alkaline-borohydride treatment (Fig. 2A). The observed similarity may occur for the following reasons.

First, fewer degradations occurred with the hydrazine treatment shown in Fig. 3A, as already discussed. In addition, degradation of the oligosaccharides seemed to non-selectively occur.

Second, the degraded products produced from the core 2 side of the oligosaccharides, which contain the unknown compound **X** at the reducing end (Table 1), were not derivatized with AA. Based on the reported studies (*6*, *14*, *31*), we hypothesize that **X** may be the compounds shown in Fig. 8, in which we propose the reaction mechanism producing the **X**-containing oligosaccharides by hydrazine treatment. The presence of **X** was supported by the following results. (*1*) The mass value measured by MALDI-TOF/MS coincided with that of this structure. (*2*) A pair of oligosaccharides bearing **X** and the same sugar composition were observed during the HPLC (i.e. **3b-1** and **2**, **3b-3** and **5**, etc.), which seems to indicate the presence of α - and β -configurated oligosaccharides in their reducing end. Usually, α - and β-configurated oligosaccharides having an aldose residue at their reducing end are separated from each other in an Amide-80 column at room temperature, but not at a high column temperature. These pair of oligosaccharides separated from each other even at a high column temperature (80°C), thus the reducing-end sugar moiety may not be aldose. (*3*) Oligosaccharides having **X** at the reducing end were not derivatized with AA, so **X** might not have reducing termini. Although the reason why the unacetylated **X** is not converted to aldose by re-acetylation is unknown, the presence of a double bond between C-2 and C-3 may prevent the conversion. Recently, Anumula reported that the reducing-terminal moiety might be the Morgan-Elson chromogen, and therefore, would not be efficiently derivatized by AA, nor would the fluorescence be quenched (*30*). This idea is in contrast to our hypothesis, so further analysis of **X** may clarify this point.

Third, degraded products originating from the core 1 side of the oligosaccharides are largely disaccharides, which had already been separated by Bio-Gel P-6 chromatography. Other degraded products having an appropriate size for production from the core 1 side (e.g. **2bAA-6** to **8**, which may be produced from relatively large oligosaccharides corresponding to **1a**) were unremarkable. In contrast to PGM, many large AA-derivatized oligosaccharides derived from the degraded components were obtained from the RGM, which seemed to be derived from the core 1 side of the

oligosaccharides. In such a case, the HPLC elution profile should not reflect the appropriate glycoform.

This study reveals that degradation of the oligosaccharides was not avoided in the used methods. Nevertheless, lower amounts of the AA-derivatized oligosaccharides were sufficient for detecting the oligosaccharides in the HPLC analysis compared to the detection of the oligosaccharyl alditols. Furthermore, the AA-derivatized oligosaccharides were more easily detectable using the MS analysis. Therefore, this method is useful for determining the profile of mucin-type oligosaccharides in glycoprotein, except for those bearing a large core 1 branch. In the latter case, however, additional information is obtained, that is, whether or not the oligosaccharides contain a large core 1 branch. Furthermore, this study showed that the different oligosaccharides were present between the corpus and antral regions of the rat stomach even if many products were degraded. This may represent the different staining patterns between corpus and antrum of rat stomach by anti-mucin monoclonal antibodies recognizing saccharide portion (*32*).

This study also showed that much higher amounts of intact oligosaccharides were obtained by hydrazine treatment with a relatively long reaction time (18 h at 65°C) when BaO was added to the reaction vessel, although further degradation of the oligosaccharides could not be avoided. These oligosaccharides are able to be immobilized, therefore, they seem to be useful for examining the oligosaccharide-protein interaction, for example, to determine the oligosaccharide epitope recognized by monoclonal antibodies.

FUNDING

This study was supported in part by Grants-in Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan and by the Integrative Research Program of the Kitasato University Graduate School of Medical Sciences.

CONFLICT OF INTEREST

None declared.

ACKNOWLEDGEMENTS

The authors would like to thank Setsuko Sugawara for her technical assistance.

REFERENCES

1. Gagneux, P. and Varki, A. (1999) Evolutionary considerations in relating oligosaccharide diversity to biological function. *Glycobiology* **9**, 747–755
2. Haltiwanger, R.S. and Lowe, J.B. (2004) Role of glycosylation in development. *Annu. Rev. Biochem.* **73**, 491–537
3. Ohtsubo, K.J. and Marth, D. (2006) Glycosylation in cellular mechanisms of health and disease. *Cell* **126**, 855–867
4. Geyer, H. and Geyer, R. (2006) Strategies for analysis of glycoprotein glycosylation. *Biochim. Biophys. Acta* **1764**, 1853–1869
5. Spiro, R.G. and Bhoyroo, V.D. (1974) Structure of the O-glycosidically linked carbohydrate units of fetuin. *J. Biol. Chem.* **249**, 5704–5717
6. Zinn, A.B., Plantner, J.J., and Carlson, D.M. (1977) Nature of linkages between protein core and oligosaccharides in *The Glycoconjugates* (Horowitz, M.I. and Pigman, W., eds.) Vol. 1), pp. 69–85, Academic Press, New York
7. Kuraya, N. and Hase, S. (1992) Release of O-linked sugar chains from glycoproteins with anhydrous hydrazine and pyridylamination of the sugar chains with improved reaction conditions. *J. Biochem.* **112**, 122–126
8. Royle, L., Mattu, T.S., Hart, E., Langridge, J.I., Merry, A.H., Murphy, N., Harvey, D.J., Dwek, R.A., and amd Rudd, P.M. (2002) An analytical and structural database provides a strategy for sequencing O-glycans from microgram quantities of glycoproteins. *Anal. Biochem.* **304**, 70–90
9. Anumula, K.R. and Dhume, S.T. (1998) High resolution and high sensitivity methods for oligosaccharide mapping and characterization by normal phase high performance liquid chromatography following derivatization with highly fluorescent anthranilic acid. *Glycobiology* **8**, 685–694
10. Chai, W., Stoll, M.S., Galustian, C., Lawson, A.M., and Feizi, T. (2003) Neoglycolipid technology: deciphering information content of glycome in *Methods in Enzymol.* (Lee, Y.C. and Lee, R., eds.) Vol. 362), pp. 160–195, Academic Press, New York
11. Iwase, H., Ishii-Karakasa, I., Fujii, E., Hotta, K., Hiki, Y., and Kobayashi, Y. (1992) Analysis of glycoform of O-glycan from human myeloma immunoglobulin A1 by gas-phase hydrazinolysis following pyridylamination of oligosaccharides. *Anal. Biochem.* **206**, 202–205
12. Patel, T., Bruce, J., Merry, A., Bigge, C., Wormald, M., Jaques, A., and Parekh, R. (1993) Use of hydrazine to release in intact and unreduced form both N- and O-linked oligosaccharides from glycoproteins. *Biochemistry* **32**, 679–693
13. Chai, W., Feizi, T., Yuen, C.-T., and Lawson, A.M. (1997) Nonreductive release of O-linked oligosaccharides from mucin glycoproteins for structure/function assignments as neoglycolipids: application in the detection of novel ligands for E-selectin. *Glycobiology* **7**, 861–872
14. Karlsson, N.G. and Packer, N.H. (2002) Analysis of O-linked reducing oligosaccharides released by an in-line flow system. *Anal. Biochem.* **305**, 173–185
15. Huang, Y., Mechref, Y., and Novotny, M.V. (2001) Microscale nonreductive release of O-linked glycans for subsequent analysis through MALDI mass spectrometry and capillary electrophoresis. *Anal. Chem.* **73**, 6063–6069
16. Ishihara, K., Kurihara, M., Goso, Y., Urata, T., Ota, H., Katsuyama, T., and Hotta, K. (1996) Peripheral α-linked N-acetylglucosamine on the carbohydrate moiety of mucin derived from mammalian gastric gland mucous cells: epitope recognized by a newly characterized monoclonal antibody. *Biochem. J* **318**, 409–416
17. Tsubokawa, D., Goso, Y., Sawaguchi, A., Kurihara, M., Ichikawa, T., Sato, N., Suganuma, T., Hotta, K., and Ishihara, K. (2007) An anti-mucin monoclonal antibody, PGM34, against 6-sulfated blood-group H type 2 antigen on the carbohydrate moiety of mucin: Analysis of the epitope sequence and immunohistochemical study. *FEBS J* **274**, 1833–1848
18. Merry, A.H., Neville, D.C., Royle, L., Matthews, B., Harvey, D.J., Dwek, R.A., and Rudd, P.M. (2002) Recovery of intact 2-aminobenzamide-labeled O-glycans released from glycoproteins by hydrazinolysis. *Anal. Biochem.* **304**, 91–99
19. Iwase, H., Ishii, I., Ishihara, K., Tanaka, Y., Omura, S., and Hotta, K. (1988) Release of oligosaccharides possessing reducing-end N-acetylgalactosamine from mucus

glycoprotein in Streptomyces sp. OH-11242 culture medium through action of endo-type glycosidase. *Biochem. Biophys. Res. Commun.* **151**, 422–428
20. Patel, T. and Parekh, R. (1994) Release of oligosaccharides from glycoproteins by hydrazinolysis in *Methods in Enzymol.* (Lennarz, W.J. and Hart, G.W., eds.) Vol. 230), pp. 57–66, Academic Press, New York
21. Goso, Y., Ishihara, K., Kurihara, M., Sugaya, T., and Hotta, K. (1999) Rat gastric mucins recognized by monoclonal antibodies RGM21 and HIK1083: Isolation of mucin species characteristic of the surface and glandular mucosa. *J. Biochem.* **126**, 375–381
22. Dubois, M., Gilles, K.A., Hamilton, J.K., Roebers, P.A., and Smith, F. (1956) Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **28**, 350–356
23. Tuppy, H. and Gottschalk, A. (1972) The Structure of Sialic Acids and their Quantitation in *Glycoproteins* (Gottschalk, A., ed.) Part A, pp. 403–449, Elsevier, Amsterdam
24. Packer, N.H., Lawson, M.A., Jardine, D.R., and Redmond, J.W. (1998) A general approach to desalting oligosaccharides released from glycoproteins. *Glycoconj. J.* **15**, 737–747
25. Neville, D.C., Coquard, V., Priestman, D.A., te Vruchte, D.J., Sillence, D.J., Dwek, R.A., Platt, F.M., and Butters, T.D. (2004) Analysis of fluorescently labeled glycosphingolipid-derived oligosaccharides following ceramide glycanase digestion and anthranilic acid labeling. *Anal. Biochem.* **331**, 275–282
26. Anumula, K.R. (1994) Quantitative determination of monosaccharides in glycoproteins by high-performance liquid chromatography with highly sensitive fluorescence detection. *Anal. Biochem.* **220**, 275–283
27. Cheng, P.-W. (1987) High-performance liquid chromatographic analysis of galactosamine, glucosamine, glucosaminitol, and galactosaminitol. *Anal. Biochem.* **167**, 265–269
28. Hara, S., Takemori, Y., Yamaguchi, M., Nakamura, M., and Ohkura, Y. (1987) Fluorometric high-performance liquid chromatography of *N*-acetyl- and *N*-glycolylneuraminic acids and its application to their microdetermination in human and animal sera, glycoproteins, and glycolipids. *Anal. Biochem.* **164**, 138–145
29. Edge, A.S. and Spiro, R.G. (1987) Presence of an O-glycosidically linked hexasaccharide in fetuin. *J. Biol. Chem.* **262**, 16135–16141
30. Anumula, K.R. (2008) Unique anthranilic acid chemistry facilitates profiling and characterization of Ser/Thr-linked sugar chains following hydrazinolysis. *Anal. Biochem.* **373**, 104–111
31. Cooper, C.A., Packer, N.H., and Redmond, J.W. (1994) The elimination of O-linked glycans from glycoproteins under non-reducing conditions. *Glycoconj. J* **11**, 163–167
32. Ishihara, K., Kurihara, M., Goso, Y., Ota, H., Katsuyama, T., and Hotta, K. (1996) Establishment of monoclonal antibodies against carbohydrate moiety of gastric mucins distributed in the different sites and layers of rat gastric mucosa. *Glycoconj. J.* **13**, 857–864