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# CARBOHYDRATE STRUCTURES OF BOVINE SUBMAXILLARY MUCIN*†

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ABSTRACT

The structures of carbohydrate chains derived from bovine submaxillary mucin (BSM) were investigated. Oligosaccharide-alditols released from BSM by alkaline borohydride treatment were separated into three acidic (*A*-**1**–*A*-**3**) and five neutral (*N*-**1**–*N*-**5**) oligosaccharide-alditol fractions by liquid chromatography on columns of an ion-exchange resin and Bio-Gel P-4. On the basis of the data obtained on compositional and methylation analyses, and digestion by exoglycosidase, the following structures were assigned to these oligosaccharide-alditols: *A*-**1**, α-NeuAc (or NeuGc)-(2→6)-GalNAc-ol; *A*-**2**, β-Gal-(1→3)-[α-NeuAc-(2→6)]-GalNAc-ol; *A*-**3**, β-GlcNAc-(1→3)-[α-NeuAc-(2→6)]-GalNAc-ol; *N*-**1**, GalNAc-ol; *N*-**2**, β-Gal-(1→3)-GalNAc-ol; *N*-**3**, β-GlcNAc-(1→3)-GalNAc-ol; *N*-**4**, β-Gal-(1→3)-[β-GlcNAc-(1→6)]-GalNAc-ol; and *N*-**5**, α-Fuc-(1→2)-β-Gal-(1→3)-[β-GlcNAc-(1→6)]-GalNAc-ol. These results showed the heterogeneous nature of the carbohydrate chains of BSM.

## INTRODUCTION

A wide variety of sugar chains have been isolated from various mucin glycoproteins. Bovine submaxillary mucin (BSM) and related glycopeptides prepared by proteolytic digestion have been widely used as haptenic inhibitors of lectin–glycoprotein interactions[1,2], as affinity adsorbents for the purification of lectins[2–4], and as substrates for sialidases[5,6], because of the commercial availability of BSM. The predominant carbohydrate chain of BSM was found to be a disaccharide, NeuAc-(2→6)-GalNAc[7–9]. However, Bertolini and Pigman[10] suggested the existence in BSM of more-complex oligosaccharides containing galactose, fucose, and/or 2-acetamido-2-deoxyglucose as well as *N*-acetylneuraminic acid and 2-acetamido-2-deoxygalactose. We also found the carbohydrate chains of BSM to be hetero-

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geneous when we analyzed the sugar chains released on alkaline borohydride treatment of BSM with a newly developed liquid-chromatographic system[11]. The purpose of this study was to extend the findings reported previously by Bertolini and Pigman and by us, and to elucidate the structures of the carbohydrate chains derived from BSM.

## EXPERIMENTAL

*Isolation of carbohydrate moieties from BSM.* — BSM was prepared from bovine submaxillary glands as described by Tettamanti and Pigman[12]. Carbohydrate chains were liberated from BSM by alkaline borohydride treatment. BSM (120 mg) was incubated with 12 mL of 0.2M NaOH–0.4M $NaBH_4$ for 48 h at 20° in the dark. After neutralization by dropwise addition of acetic acid to pH 6.0, the mixture was filtered through a collodion membrane (Sartorius GmbH, Göttingen, FRG) to remove polypeptides. The filtrate was then passed through a Dowex 50WX8 ($H^+$) column, which was then washed with water. The effluent was collected and methanol evaporated repeatedly from it to remove the remaining boric acid. The residue was dissolved in a small amount of 50mM pyridine–25mM acetic acid, pH 5.3, and then applied to a column (1.5 × 100 cm) of Sephadex G-50. Elution was performed with 50mM pyridine–25mM acetic acid, pH 5.3, and fractions were monitored by means of the $NaIO_4$–resorcinol reaction[13] for sialic acid. About 82% of the carbohydrate moieties of BSM were recovered after chromatography on a Sephadex G-50 column, as determined by the $NaIO_4$–resorcinol reaction.

*Analytical methods.* — Neutral sugars and sialic acids were assayed by means of the phenol–$H_2SO_4$ reaction[14] and the $NaIO_4$–resorcinol reaction[13], respectively. The carbohydrate compositions were determined by g.l.c., with a column (0.3 × 100 cm) of 0.1% ECNSS-M, after conversion into the respective alditol acetates as described by Spiro[15]. Hydrolysis for this assay was conducted with 2M HCl for 2.5 h at 100°. The terminal alditol of an oligosaccharide-alditol was determined in the same manner, except for the omission of the $NaBH_4$-reduction step. Sialic acids were identified by g.l.c. on a column (0.3 × 200 cm) of 2% OV-1, after methanolysis followed by trimethylsilylation according to Yu and Ledeen[16].

*Liquid chromatography.* — A Jasco Tri-rotar high pressure liquid chromatograph (Japan Spectroscopic Co., Ltd., Tokyo, Japan) with a variable-wavelength ultraviolet spectrophotometer (Uvidec 100-II, Jasco) was used. Two liquid-chromatographic methods were employed in the present study. One was anion-exchange chromatography on a column (4.6 × 250 mm) of Hitachi Custom Resin no. 2630 (Hitachi Ltd., Tokyo, Japan). Elution was performed with a concave gradient of aqueous sodium chloride at a flow rate of 1.0 mL/min. The column temperature was maintained at 55°. This system was effective for the separation of acidic oligosaccharides[11].

The other method was gel-permeation chromatography on two serial columns

(7.2 × 500 mm) of Bio-Gel P-4 (~400 mesh) as described previously[17]. The columns were eluted with distilled water at a flow rate of 0.3 mL/min and maintained at 55°. This method was used for estimation of the molecular size of an oligosaccharide. Mixtures of oligomers of glucose and 2-acetamido-2-deoxyglucose prepared by partial hydrolysis of dextran and chitin, respectively, were used as standards.

*Methylation analysis.* — Methylation of oligosaccharide-alditols was performed according to Hakomori[18], and the permethylated sugars were purified on a small column of silica gel[19]. Alditol acetates of the partially methylated sugars were prepared by hydrolysis of the methylated oligosaccharide-alditol with 3M HCl for 3 h at 80°, followed by reduction with $NaB^2H_4$ and acetylation with acetic anhydride as described by Stellner *et al.*[20], and then analyzed by gas chromatography–mass spectrometry (Shimadzu QP-1000; Shimadzu Corp., Kyoto, Japan) on a column (0.26 × 100 cm) of 2% OV-1 or 2% OV-17. For the methylation study on neutral oligosaccharide-alditols, the unhydrolyzed samples were also analyzed with the same gas chromatograph–mass spectrometer on a column (0.3 × 40 cm) of 2% OV-1. The column temperature was programmed from 140–285° at the rate of 5°/min. The conditions for mass spectrometry were as follows: separator temperature, 290°; ion-source temperature, 290°; ionization potential, 70 eV.

*Glycosidase treatment.* — Sialidase from *Arthrobacter ureafaciens* was obtained from Nakarai Chemicals, Ltd. (Kyoto, Japan), and α-L-fucosidase from *Charonia lampas* from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). 2-Acetamido-2-deoxy-β-D-hexosidase was purified from jack bean meal[21]. β-D-Galactosidase from *Aspergillus niger* was purchased from Sigma Chemical Co. (St. Louis, MO), and further purified by chromatography on Sephacryl S-200, DEAE-Sephadex A-50, and hydroxyapatite[22].

Each oligosaccharide-alditol (10–20 μg) was digested at 37° with a glycosidase (0.5–5 units) in 0.1 mL of 0.05M sodium acetate buffer, pH 4.0, under a toluene layer for 16–40 h, followed by heating for 3 min at 100° to terminate the reaction. The monosaccharides liberated through the action of the glycosidases were determined by g.l.c. after conversion into alditol acetates, with 2-deoxy-D-*arabino*-hexose as the internal standard.

## RESULTS

*Fractionation of oligosaccharide-alditols liberated from BSM.* — The oligosaccharide-alditol fraction obtained from BSM on treatment with alkaline borohydride was subjected to anion-exchange liquid chromatography, with monitoring on the basis of the absorbance at 210 nm and the $NaIO_4$–resorcinol reaction. The oligosaccharide-alditols from BSM were separated into one neutral fraction (designated as fraction *N*), which was eluted at the void volume, and three acidic fractions (designated as *A*-**1**–*A*-**3**), which emerged on NaCl-gradient elution (Fig. 1). Analysis of the terminal alditol showed that 2-acetamido-2-deoxygalactitol was the terminal alditol residue of all of the oligosaccharide-alditols. By determina-

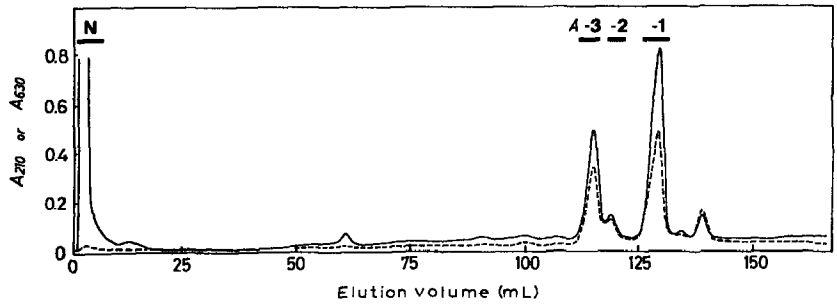


Fig. 1. Ion-exchange liquid chromatography of BSM oligosaccharide-alditols. The experimental details are given in the text. The oligosaccharide-alditol fraction obtained from BSM on treatment with alkaline borohydride was subjected to liquid chromatography on a column (4.6 × 250 mm) of Hitachi Custom Resin no. 2630. Elution was performed with a concave gradient of aqueous sodium chloride at a flow rate of 1.0 mL/min. The first and second of the three compartments of the gradient device were filled with distilled water (120 mL), and the third with 0.15M NaCl (120 mL). Sugars were monitored on the basis of the absorbance at 210 nm (——), and the $NaIO_4$–resorcinol reaction (-----).

tion of the 2-acetamido-2-deoxygalactitol in each fraction, the proportions of these four fractions were estimated to be as follows: *N*, 16.0%; *A*-**1**, 53.3%; *A*-**2**, 4.1%; and *A*-**3**, 22.3%. Neither *A*-**1** nor *A*-**3** was found to contain any neutral sugars, although *N* and *A*-**2** did contain some, as determined by the phenol–$H_2SO_4$ method. A small peak that was eluted at ~132 mL was identified by g.l.c. as *N*-acetylneuraminic acid. Both *N*-acetyl- and *N*-glycolylneuraminic acids were detected, in the molar ratio of ~2:1, in the *A*-**1** fraction, whereas the sialic acid in the *A*-**2** and *A*-**3** fractions was determined to be mainly *N*-acetylneuraminic acid.

The neutral oligosaccharide-alditols were further fractionated according to molecular size by gel-permeation chromatography on a Bio-Gel P-4 column (Fig. 2B), and five fractions (*N*-**1**–*N*-**5**) were separately pooled as indicated by the bars shown at the top of Fig. 2b. When aliquots of the fractions (0.45 mL) were assayed for neutral sugars by the phenol–$H_2SO_4$ method, *N*-**2**, *N*-**4**, and *N*-**5** were found to contain neutral sugars, however, neither *N*-**1** nor *N*-**3** did. Each fraction was rechromatographed on the same Bio-Gel P-4 column, as some peaks slightly overlapped each other. The eight oligosaccharide-alditol fractions (*A*-**1**–*A*-**3** and *N*-**1**–*N*-**5**) thus prepared were used for further analyses.

*Structures of acidic oligosaccharide-alditols.* — The elution profiles of desialylated *A*-**1**–*A*-**3** on a column of Bio-Gel P-4, after sialidase treatment, are shown in Fig. 2a. The elution position of asialo-*A*-**1** was found to be identical with that of 2-acetamido-2-deoxygalactitol. However, asialo-*A*-**2** was found to be larger in size, and its elution position was the same as that of Gal-GalNAc-ol. Asialo-*A*-**3** was eluted faster from a Bio-Gel P-4 column, than the other two asialo-oligosaccharide-alditols, at a position corresponding to HexNAc-HexNAc-ol.

Asialo-*A*-**1** was identified as 2-acetamido-2-deoxygalactitol on compositional analysis (Table I). As methylation of *A*-**1** gave 2-deoxy-1,3,4,5-tetra-*O*-methyl-2-(methylacetamido)galactitol (Table II), *A*-**1** was assigned as NeuAc(or NeuGc)-(2→6)-GalNAc-ol.

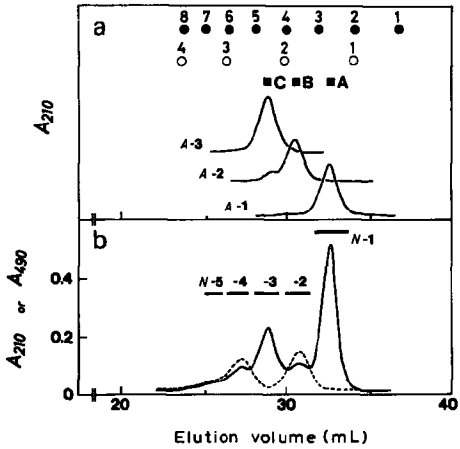


Fig. 2. Gel-permeation liquid chromatography of BSM oligosaccharide-alditols on a column of Bio-Gel P-4. The experimental details are given in the text. (*a*) Desialylated oligosaccharide-alditols *A*-**1**–*A*-**3**. (*b*) Neutral oligosaccharide-alditol fraction. Sugars were monitored on the basis of the absorbance at 210 nm (——), and the phenol–$H_2SO_4$ reaction (-----). A–C indicate the eluted positions of authentic standard mono- and di-saccharide-alditols: A, GalNAc-ol; B, Gal-GalNAc-ol; C, GlcNAc-GlcNAc-ol. Closed circles (1–8) and open circles (1–4) indicate the elution positions of oligomers of glucose and 2-acetamido-2-deoxyglucose, respectively.

The carbohydrate compositions of asialo-*A*-**2** and asialo-*A*-**3** indicated that the former was composed of one residue each of galactose and 2-acetamido-2-deoxygalactitol and the latter of one residue each of 2-acetamido-2-deoxyglucose and 2-acetamido-2-deoxygalactitol (Table I). On methylation analysis of the two oligosaccharide-alditols, 2-deoxy-1,4,5-tri-*O*-methyl-2-(methylacetamido)galactitol was obtained from both *A*-**2** and *A*-**3** (Table II), indicating that 2-acetamido-2-deoxygalactitol with substituents at O-3 and O-6 was located at the reducing terminus of each oligosaccharide-alditol. In addition, *A*-**2** and *A*-**3** gave 2,3,4,6-tetra-*O*-methylgalactitol and 2-deoxy-3,4,6-tri-*O*-methyl-2-(methylacetamido)-glucitol, respectively (Table II), indicating that galactose and 2-acetamido-2-deoxy-glucitol occupied the non-reducing terminals of the respective oligosaccharide-alditols. Methylation of asialo-*A*-**2** and asialo-*A*-**3** revealed that each 2-acetamido-2-deoxygalactitol residue that originally had substituents at O-3 and O-6 was converted into 2-acetamido-2-deoxygalactitol with only one substituent at O-3, namely, 2-deoxy-1,4,5,6-tetra-*O*-methyl-2-(methylacetamido)galactitol, after treatment with sialidase. Accordingly, an *N*-acetylneuraminic acid residue is linked to O-6 of the 2-acetamido-2-deoxygalactitol residue in each oligosaccharide-alditol.

The anomeric configurations of the galactose residue in *A*-**2** and 2-acetamido-2-deoxyglucosyl residue in *A*-**3** were examined with the use of glycosidases. Treatment with 2-acetamido-2-deoxy-β-D-hexosidase (jack bean) quantitatively released 2-acetamido-2-deoxyglucose from asialo-*A*-**3**. Similarly, treament with a high concentration (50 units/mL) of β-D-galactosidase (*A. niger*) liberated galactose from asialo-*A*-**2**. Based on these results, the structures of *A*-**2** and *A*-**3** were deduced to

TABLE I

CARBOHYDRATE COMPOSITIONS OF OLIGOSACCHARIDE-ALDITOLS

| *Fraction* | *Molar ratio*[a] | | | |
|---|---|---|---|---|
| | *Fuc* | *Gal* | *GlcNAc* | *GalNAc-ol* |
| Asialo-*A*-**1** | | | | + |
| Asialo-*A*-**2** | | 0.87 | 0.14 | 1.00 |
| Asialo-*A*-**3** | | | 1.04 | 1.00 |
| *N*-**1** | | | | + |
| *N*-**2** | 0.03 | 0.82 | 0.11 | 1.00 |
| *N*-**3** | 0.08 | 0.12 | 0.94 | 1.00 |
| *N*-**4** | 0.21 | 0.93 | 1.07 | 1.00 |
| *N*-**5** | 0.88 | 1.12 | 1.18 | 1.00 |

[a]The molar ratios are expressed in relation to GalNAc-ol taken as 1.00.

TABLE II

METHYLATION ANALYSIS OF OLIGOSACCHARIDE-ALDITOLS FROM BSM

| *Methylated sugar* | *Molar ratio*[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *A*-**1** | *A*-**2** | *Asialo-A*-**2** | *A*-**3** | *Asialo-A*-**3** | *N*-**2** | *N*-**3** | *N*-**4** | *N*-**5** |
| 2,3,4-Tri-*O*-methylfucitol | – | | | | | | 0.1 | 0.2 | 0.7 |
| 2,3,4,6-Tetra-*O*-methylgalactitol | – | 1.0 | 1.0 | | | 0.8 | 0.1 | 0.8 | 0.2 |
| 3,4,6-Tri-*O*-methylgalactitol | – | | | | | | 0.1 | 0.2 | 1.1 |
| 2-Deoxy-3,4,6-tri-*O*-methyl-2-(methylacetamido)glucitol | – | 0.2 | 0.1 | 1.0 | 1.0 | 0.2 | 1.1 | 1.2 | 1.0 |
| 2-Deoxy-1,4,5,6-tetra-*O*-methyl-2-(methylacetamido)galactitol | – | | 1.2 | | 1.2 | 1.0 | 1.0 | | |
| 2-Deoxy-1,3,4,5-tetra-*O*-methyl-2-(methylacetamido)galactitol | + | | | | | | | | |
| 2-Deoxy-1.4.5-tri-*O*-methyl-2-(methylacetamido)galactitol | – | 1.3 | | 1.0 | | | | 1.0 | 1.0 |

[a]The molar ratios are expressed relative to the methylated sugar derivatives underlined.

be β-Gal-(1→3)-[α-NeuAc-(2→6)]-GalNAc-ol and β-GlcNAc-(1→3)-[α-NeuAc-(2→6)]-GalNAc-ol, respectively.

*Structures of neutral oligosaccharide-alditols.* — The major oligosaccharide-alditol in the neutral fraction, *N*-**1**, was eluted from a Bio-Gel P-4 column at a position corresponding to 2-acetamido-2-deoxygalactitol (Fig. 2b). *N*-**1** was identified as GalNAc-ol from the result of g.l.c. analysis (Table I).

*N*-**2**, the elution position of which from a Bio-Gel P-4 column was identical with that of authentic β-Gal-(1→3)-GalNAc-ol (Fig. 2b), contained galactose in addition to 2-acetamido-2-deoxygalactitol (Table I). Methylation of *N*-**2** yielded 2,3,4,6-tetra-*O*-methylgalactitol and 2-deoxy-1,4,5,6-tetra-*O*-methyl-2-(methylacetamido)galactitol as the principal methylated sugars (Table II), indicating that

*N*-2 has a disaccharide-alditol structure, Gal-(1→3)-GalNAc-ol. This sequence was confirmed by mass-spectrometric analysis of the permethylated and unhydrolyzed derivative of *N*-2. The mass spectrum of permethylated *N*-2 was almost identical with that reported for permethylated Gal-(1→3)-GalNAc-ol[23–26] (Fig. 3 and 4A). Since the galactosyl linkage was cleaved by β-D-galactosidase (*A. niger*), *N*-2 was assigned as β-Gal-(1→3)-GalNAc-ol.

*N*-3, the elution position of which from a Bio-Gel P-4 column corresponded to Hex-HexNAc-ol, consisted of one residue each of 2-acetamido-2-deoxyglucose and 2-acetamido-2-deoxygalactitol (Table I). Methylation of *N*-3 yielded 2-deoxy-3,4,6-tri-*O*-methyl-2-(methylacetamido)glucose and 2-deoxy-1,4,5,6-tetra-*O*-methyl-2-(methylacetamido)galactitol in equimolar proportions (Table II), indicating that *N*-3 has a disaccharide-alditol structure, GlcNAc-(1→3)-GalNAc-ol. The mass spectrum of permethylated and unhydrolyzed *N*-2 was essentially identical with the spectrum reported for permethylated GlcNAc-(1→3)-GalNAc-ol or GalNAc-(1→3)-GalNAc-ol[27] (Fig. 3 and 4B), confirming the sequence already described. As the 2-acetamido-2-deoxyglucosyl linkage was susceptible to 2-acetamido-2-deoxy-β-D-glucosidase (jack bean), *N*-3 was assigned as β-GlcNAc-(1→3)-GalNAc-ol.

*N*-4, which was shown to be larger in size by one hexose unit than *N*-3, as determined from its elution position on a Bio-Gel P-4 column (Fig. 2b), was composed of one residue each of galactose, 2-acetamido-2-deoxyglucose, and 2-acetamido-2-deoxygalactitol (Table I). On methylation of *N*-4, 2,3,4,6-tetra-*O*-

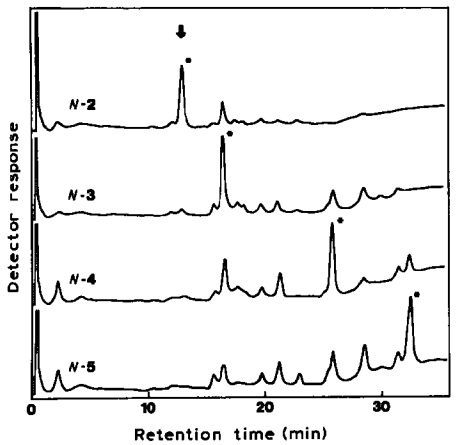


Fig. 3. Gas chromatography–mass spectrometry of permethylated derivatives of BSM oligosaccharide-alditols. The experimental details are given in the text. Permethylated neutral oligosaccharide-alditols (*N*-2–*N*-5) were subjected to gas chromatography–mass spectrometry on a column (0.3 × 40 cm) of 2% OV-1 and the total ions were monitored. The column temperature was programmed between 140–285° at the rate of 5°/min. The arrow at the top of the panel indicates the retention time for the permethylated derivative of authentic Gal-GalNAc-ol. The mass spectrum of the peak marked with an asterisk in each chromatogram is shown in Fig. 4.

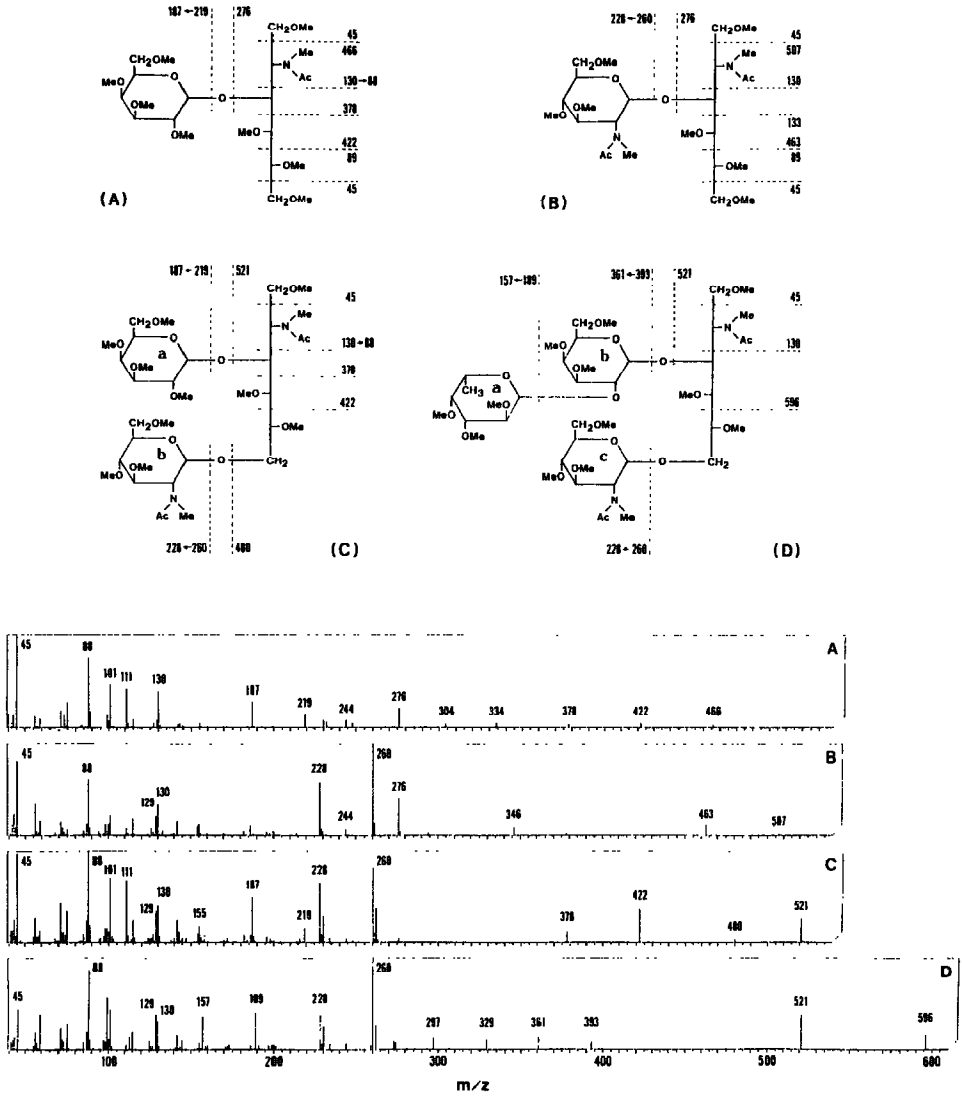


Fig. 4. Mass spectra of permethylated derivatives of neutral BSM oligosaccharide-alditols. Some important fragmentations are also shown. (A), *N*-**2**; (B), *N*-**3**; (C), *N*-**4**; and (D), *N*-**5**.

methylgalactitol, 2-deoxy-3,4,6-tri-*O*-methyl-2-(methylacetamido)glucitol and 2-deoxy-1,4,5-tri-*O*-methyl-2-(methylacetamido)galactitol were detected as principal methylated sugars in approximately equimolar proportion (Table II). These results indicated that *N*-**4** has a trisaccharide-alditol structure, in which galactose and 2-acetamido-2-deoxyglucose are linked to O-3 and O-6 of a common 2-acetamido-2-deoxygalactitol residue. This was confirmed by the mass spectrum of permethylated *N*-**4** (Fig. 3 and 4C). The observation of two A-series fragments of $aA_1$ (*m/z* 219), $aA_2$ (187) and $aA_3$ (155), and $bA_1$ (260), and $bA_2$ (228) indicated the presence of

hexose and 2-acetamido-2-deoxyhexose at the nonreducing terminals. Furthermore, the fragmentation on $\alpha$-cleavage of the alditol chain (*m/z* 130, 378, 422) showed that the 2-acetamido-2-deoxygalactitol was substituted at O-3 and O-6 by a galactosyl and a 2-acetamido-2-deoxyglucosyl group, respectively. The mass spectrum shown in Fig. 4C is essentially identical with that reported previously for permethylated Gal-(1→3)-[GlcNAc-(1→6)]-GalNAc-ol[25,31]. As the galactosyl and the 2-acetamido-2-deoxyglucosyl linkages were cleaved by a mixture of $\beta$-D-galactosidase and 2-acetamido-2-deoxy-$\beta$-D-glucosidase, the structure of ***N*-4** was deduced to be $\beta$-Gal-(1→3)-[$\beta$-GlcNAc-(1→6)]-GalNAc-ol.

***N*-5**, which was a rather minor fraction of the oligosaccharide-alditols from BSM, was found to be larger in size by about one hexose unit than ***N*-4**, as determined from its elution position on a Bio-Gel P-4 column (Fig. 2b). The major oligosaccharide-alditol in ***N*-5** seemed to be composed of one residue each of fucose, galactose, 2-acetamido-2-deoxyglucose, and 2-acetamido-2-deoxygalactitol (Table I), although the data obtained on methylation analysis (Table II and Fig. 3) suggested that this fraction contained some impurities derived, presumably, from adjacent fractions. Methylation of ***N*-5** yielded 2,3,4-tri-*O*-methylfucitol and 3,4,6-tri-*O*-methylgalactitol, in addition to 2-deoxy-3,4,6-tri-*O*-methyl-2-(methylacetamido)glucitol and 2-deoxy-1,4,5-tri-*O*-methyl-2-(methylacetamido)galactitol, which were observed in the case of ***N*-4** (Table II). These results suggested that the major oligosaccharide-alditol in ***N*-5** was a tetrasaccharide-alditol, in which a galactose residue in a trisaccharide-alditol in the manner that ***N*-4** was substituted at O-2 by a fucose residue. This was confirmed by gas chromatography–mass spectrometric analysis of the permethylated derivative of ***N*-5**. The mass spectrum of the major peak (Fig. 3) detected at 32.4 min after injection is shown in Fig. 4D. As to A-series fragments, $aA_1$ (*m/z* 189), $aA_2$ (157), $baA_1$ (393), $baA_2$ (361), $baA_3$ (329), $cA_1$ (260), and $cA_2$ (228) were observed. These fragments indicated the presence of a 6-deoxyhexosyl-hexose [namely, Fuc-(1→2)-Gal] sequence and a nonreducing terminal 2-acetamido-2-deoxyhexose (namely, GlcNAc) residue. Fragments at *m/z* 521 (aldc) and 596 derived on $\alpha$-cleavage of the alditol chain, together with the detection of 3,6-di-*O*-substituted 2-acetamido-2-deoxygalactitol on methylation analysis, strongly suggested that the Fuc-(1→2)-Gal sequence and the GlcNAc residue are linked to O-3 and O-6, respectively, of the 2-acetamido-2-deoxygalactitol residue. The fucose residue was determined to be of the $\alpha$ configuration with the use of $\alpha$-L-fucosidase (*C. lampas*). Glycosidase digestion of $\alpha$-L-fucosidase-treated ***N*-5** gave the same result as that of ***N*-4**. Based on these results, the structure of the major oligosaccharide-alditol in the ***N*-5** fraction was deduced to be $\alpha$-Fuc-(1→2)-$\beta$-Gal-(1→3)-[$\beta$-GlcNAc-(1→6)]-GalNAc-ol.

## DISCUSSION

The structures of the oligosaccharide-alditols released from BSM on alkaline borohydride treament, together with the proportion of the individual fractions are

summarized in Table III. Three acidic (*A*-**1**–*A*-**3**) and five neutral (*N*-**1**–*N*-**5**) carbohydrate chains, ranging in length from mono- to tetra-saccharides, were isolated. The major carbohydrate chain (*A*-**1**) of BSM was found to be NeuAc(or NeuGc)-(2→6)-GalNAc-ol, which comprised 53.3% of the oligosaccharide-alditols liberated on alkaline borohydride treatment. This was in good agreement with earlier reports[7–9]. Bertolini and Pigman[10] suggested that BSM contained a significant proportion of oligosaccharides containing galactose, fucose, and 2-acetamido-2-deoxyglucose, on the basis of the results of compositional analysis of the alkaline borohydride-released carbohydrates. This suggestion was confirmed and extended by the structural elucidation in the present study. It has been indicated that a common characteristic of porcine and canine submaxillary mucins is the heterogeneous nature of their carbohydrate chains[28–30]. The present study also shows some heterogeneity in the carbohydrate chains of BSM.

The three acidic carbohydrates (*A*-**1**–*A*-**3**) are considered to be sialylated forms of three neutral carbohydrates (*N*-**1**–*N*-**3**), which may be derived from the three neutral carbohydrates. A sialic acid residue of each acidic oligosaccharide-alditol is exclusively linked to O-6 of the 2-acetamido-2-deoxygalactitol residue. When this residue is substituted by a 2-acetamido-2-deoxyglucose residue at O-6, the sialic acid appears to be no longer attached to the 2-acetamido-2-deoxygalactitol residue, as shown in *N*-**4** and *N*-**5**. One of the structural features of BSM oligosaccharides seems to be the existence, in a rather large proportion, of a disaccharide-alditol, β-GlcNAc-(1→3)-GalNAc-ol, with or without a sialic acid residue (*A*-**3** or *N*-**3**). On the other hand, another disaccharide-alditol, β-Gal-(1→3)-GalNAc-ol, with or without a sialic acid residue (*A*-**2** or *N*-**2**), is a somewhat minor component of BSM. This situation is distinct from in the case of porcine submaxillary mucin, in which this structure with a sialic acid residue is one of the major carbohydrate chains[28,29]. Two of the neutral oligosaccharide-alditols, *N*-**4** and *N*-**5**, have a common β-Gal-(1→3)-[β-GlcNAc-(1→6)]-GalNAc-ol branched-core structure. It has recently been shown that several glycoproteins contain this core structure, such as human secretory immunoglobulin A[31], κ-casein of ewe colostrum[32], plasma membrane glycoproteins from an ascites hepatoma[25] (AH66), and human platelet glycocalicin[26]. However, the 2-acetamido-2-deoxyglucose residue in these sugar chains is often substituted by a galactose residue via a β-(1→4) linkage. The activity of 2-acetamido-2-deoxy-β-D-galactosyltransferase, which catalyzes the synthesis of the β-Gal-(1→4)-GlcNAc sequence, appeared to be relatively low in the bovine submaxillary gland, as no carbohydrate chain with this sequence was isolated in the present study. Alternatively, the substitution of a galactose residue by an L-fucosyl residue was found in the *N*-**5** oligosaccharide-alditol. A carbohydrate chain with this tetrasaccharide structure had also been isolated from swine trachea mucin[33]. As such a structure as that of *N*-**5** is similar to that of an H-determinant, we examined the inhibitory effect of BSM on the hemagglutination induced by an anti-H(O) lectin, *Ulex europeus* lectin[34] I. BSM was found to inhibit the agglutination of blood-group O erythrocytes induced by 4

TABLE III

STRUCTURES OF OLIGOSACCHARIDE-ALDITOLS ISOLATED FROM BSM

| *Acidic oligosaccharide-alditol* | | | *Neutral oligosaccharide-alditol* | | |
|---|---|---|---|---|---|
| *Structure* | | *Proportion*[a] *(%)* | *Structure* | | *Proportion*[a] *(%)* |
| *A*-1 | (NeuGc)<br>NeuAc<br>2<br>↓<br>6<br>GalNAc-ol | 53.3 | *N*-1 | GalNAc-ol | 9.5 |
| | | | *N*-2 | β-Gal-(1→3)-GalNAc-ol | 1.6 |
| | | | *N*-3 | β-GlcNAc-(1→3)-GalNAc-ol | 3.0 |
| *A*-2 | NeuAc<br>2<br>↓<br>6<br>β-Gal-(1→3)-GalNAc-ol | 4.1 | *N*-4 | β-GlcNAc<br>1<br>↓<br>6<br>β-Gal-(1→3)-GalNAc-ol | 1.1 |
| *A*-3 | NeuAc<br>2<br>↓<br>6<br>β-GlcNAc-(1→3)-GalNAc-ol | 22.3 | *N*-5 | β-GlcNAc<br>1<br>↓<br>6<br>α-Fuc-(1→2)-β-Gal-(1→3)-GalNAc-ol | 0.3 |

[a]Values denote the amounts of the respective oligosaccharide-alditols expressed as percentages of the total oligosaccharide-alditols liberated from BSM on alkaline borohydride treatment.

hemagglutinating doses of the lectin at a final concentration of 0.21 mg/mL.

Recently, we purified lectins specific for β-Gal-(1→3)-GalNAc from a mushroom, *Agaricus bisporus*[3]. BSM and related glycopeptides were found to be good inhibitors of the lectin binding. The carbohydrate chains containing this sequence, such as ***N*-2**, ***N*-4**, and ***A*-2**, may be important for the inhibitory activity. The sugar chains obtained from BSM, which can be rather readily prepared on a large scale, are good standards for structural and functional studies on various glycoproteins.

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