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Structure determination of a sulfated N-glycans, candidate for a precursor of the selectin ligand in bovine lung

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Abstract To clarify the structure of non-sialic acid anionic residue on N-glycans in the mammalian tissues, we have isolated sialidase-resistant anionic residue on N-glycans from bovine lung. Analyses by partial acid hydrolysis and glycosidase digestions combined with a two-dimensional HPLC mapping method revealed that the major sialidase-resistant anionic N-glycan had a fucosylbianntenary core structure. The anionic residue was identified as a sulfate ester by methanolysis, anion-exchange chromatography, and mass spectrometry. The linkage position of the sulfate ester was the 6-position of the GlcNAc residue on the Manα1-6 branch. This conclusion was based on the results of glycosidase digestions followed by two-dimensional HPLC mapping. Furthermore, the disialylated form of this sulfated glycan was dominant, and no asialo form was detected. The structure of the major anionic N-glycan prepared from bovine lung and having a sulfate was proposed to be the pyridylamino derivative of Siaα2-3Gαlβ1-4(HSO_3-6)GlcNAcβ1-2Manα1-6(Siaα2-3Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc.

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Abbreviations

HPLC	high performance liquid chromatography
MALDI	matrix assisted laser desorption ionization
MS	mass spectrometry
PA	pyridylamino
TOF	time of flight

Introduction

The anionic character of N-glycans in mammals is mainly due to sialic acid residues [1, 2]. However, this anionic character can also be due to phosphate esters [3–5], sulfate esters [6–12], or uronic acids [13, 14]. In recent years, a number of anionic glycans that have negative charges, but which have no sialic acid residues have been reported. In particular, sulfated complex-type N-glycans were detected in various glycoproteins of mammals [8–11]. Bovine lung is a rich source of anionic N-glycans and novel anionic sugar chains with negative charges contributed by carboxylic acids other than sialic acids [12]. Owing to their heterogeneity, analyses were performed on a mixture of these glycans. The glycans were treated with exoglycosidases and/or chemical reagents, but their structures were not determined completely. In this study, a major anionic N-glycan with a non-sialic acid-derived negative charge was prepared from bovine lung and then analyzed. It was found to be a disialofucosylbiantennary glycan with a 6-sulfated *N*-acetylglucosamine (SO_4-6GlcNAc) residue attached to the Manα1-6 branch.

Experimental procedures

Materials and reagents

An acetone powder of bovine lung was purchased from Sigma (St. Louis, MO); a mixture of PA-isomaltooligosaccharides was from Takara Biomedicals (Kyoto, Japan); Shodex Asahipak NH2P-50 columns (4.6×50 mm and 4.6×100 mm) were from Showa Denko (Tokyo, Japan); Cosmosil 5C18-P columns (1.5×250 mm and 4.6×150 mm) were from Nacalai Tesque (Kyoto, Japan); a Q-Sepharose 16/10 column (16×100 mm) was from Amersham Pharmacia Biotech; Dowex 50W-X2 (200–400 mesh) was from Dow Chemicals (Richmond, VA); a TSKgel sugar AXI column (4.6×150 mm) and a Toyopearl HW-40F were from Tosoh (Tokyo, Japan); and an IonPac AS4A column (0.4×25 cm) was from Dionex (Sunnyvale, CA). *Arthrobacter ureafaciens* sialidase was purchased from Nacalai Tesque, Newcastle disease virus (NDV) sialidase, *Streptococcus pneumoniae* β1,4-galactosidase and *Streptococcus* β-*N*-acetylhexosaminidase were from Boehringer Mannheim (Germany); bovine epididymis α-fucosidase, jack bean β-galactosidase, human placental *N*-acetylhexosaminidase A, HSO_3-3GlcNAc and HSO_3-6GlcNAc were from Sigma. Insulin chain B (oxidized, from bovine insulin) and 2,5-dihydroxybenzoic acid were also purchased from Sigma, and Neurotensin was from the Peptide Institute Inc (Osaka, Japan).

Preparation of PA-glycans from bovine lung

N-Glycans were released from 100 mg of an acetone powder of bovine lung by hydrazinolysis at 100°C for 10 h [15]. The liberated glycans were *N*-acetylated by acetic anhydride in saturated sodium bicarbonate solution [16]. The sodium cation was removed by Dowex 50W-X2 (H^+ form). The purified glycans were tagged with a fluorophore 2-aminopyridine as described elsewhere [17]. The excess reagents were removed by phenol/chloroform extractions [18]. The aqueous phase was further purified on an HW-40F column (4.7×54 cm) equilibrated with 10 mM ammonium acetate buffer, pH 6.0. The fraction containing PA-oligosaccharide was collected and analyzed as the PA-glycan fraction.

Exoglycosidase digestion of PA-glycans

A PA-glycan (20 pmol) was digested with the following enzymes at 37°C for 12 h; 20 mU α-fucosidase in 100 µl of 40 mM sodium citrate–phosphate buffer, pH 6.0; 100 mU of *Arthrobacter* sialidase in 50 µl of 100 mM ammonium acetate buffer, pH 5.0; 0.5 mU of NDV sialidase in 25 µl of 50 mM ammonium acetate buffer, pH 6.5; 5 mU of *Streptococcus* β-galactosidase in 100 µl of 50 mM ammonium acetate buffer, pH 6.0; 5 mU of *Streptococcus* β-*N*-acetylhexosaminidase in 50 µl of 50 mM sodium citrate–phosphate buffer, pH 4.8; 70 mU of jack bean β-galactosidase in 20 µl of 50 mM sodium citrate buffer, pH 3.5; 40 mU of human placental β-*N*-acetylhexosaminidase A in 100 µl of 50 mM sodium citrate buffer, pH 4.3. The reactions were terminated by heating the solutions at 100°C for 3 min.

Reducing-end analysis of PA-glycans

The reducing ends of PA-glycans were analyzed according to a reported method [19]. PA-glycans (approximately 50 pmol) were hydrolyzed with 100 µl of 4 M HCl at 100°C for 8 h. The reaction mixture was then lyophilized, and free amino groups were acetylated by acetic anhydride in saturated sodium bicarbonate solution for 30 min at room temperature. The PA-monosaccharides were adsorbed to

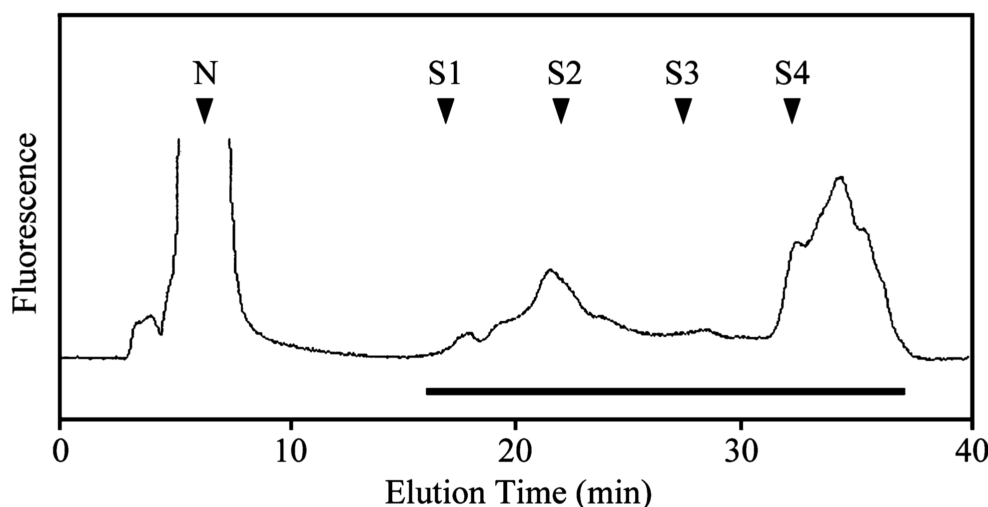


Fig. 1 Separation by Q-Sepharose anion-exchange HPLC of sialidase-digested PA-glycans from bovine lung. An anionic fraction of sialidase-digested glycans was isolated as indicated by the *bar*. *Arrowheads N*, *S1–S4* indicate the elution positions of authentic neutral, monosialo, disialo, trisialo and tetrasialo PA-N-glycans, respectively, obtained from α_1-acid glycoprotein

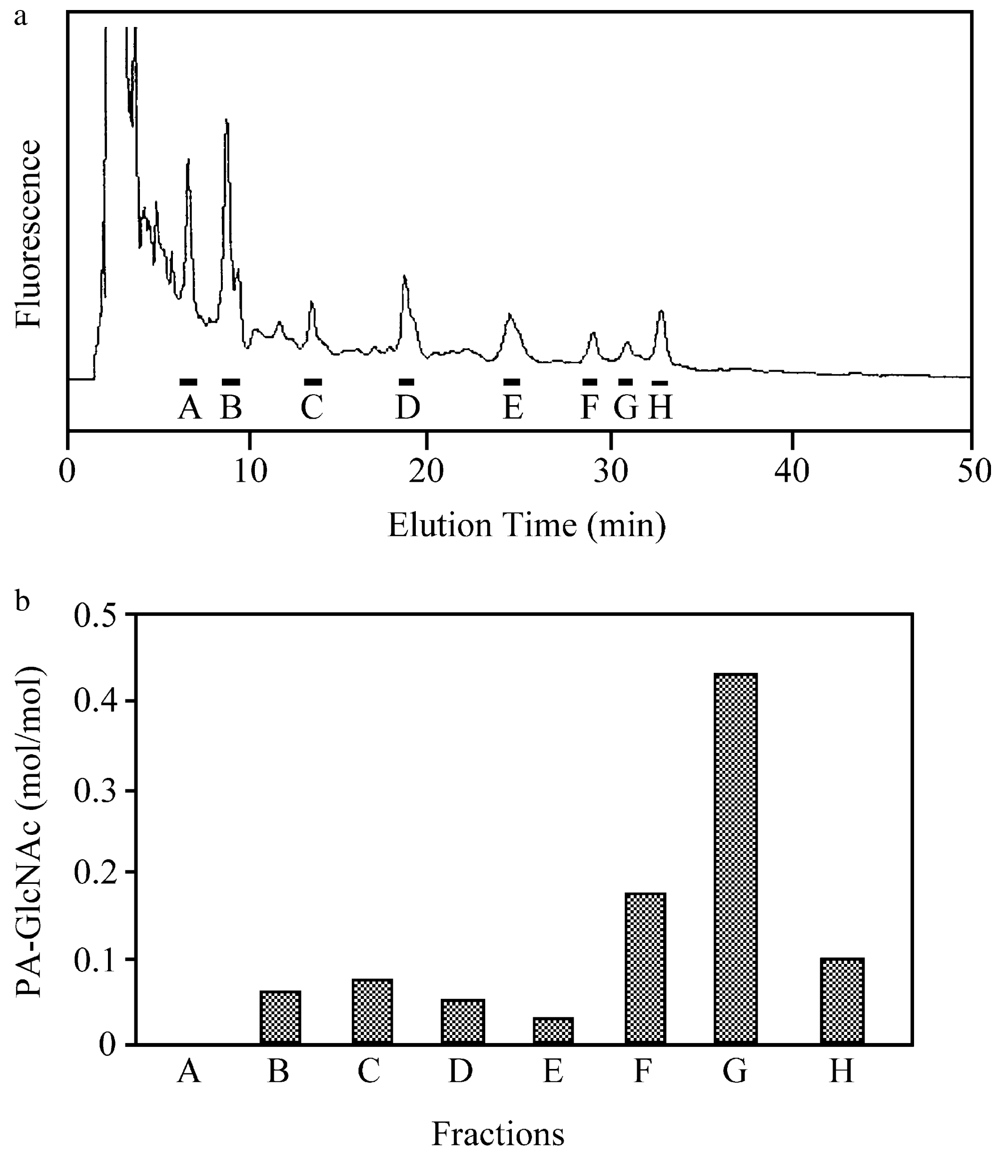


Fig. 2 Separation by reversed phase HPLC of the anionic fraction and reducing-end analysis of the fractions obtained. **a** Fractions were pooled as indicated by *bars A–H*. Peaks that appeared at 2–5 min were due to contaminating materials. **b** Reducing-end PA-GlcNAc in each fraction was quantified after acid hydrolysis. The value of *vertical axis* shows the molar ratio of PA-GlcNAc amount detected from each fraction against total PA-GlcNAc amount in the all fractions

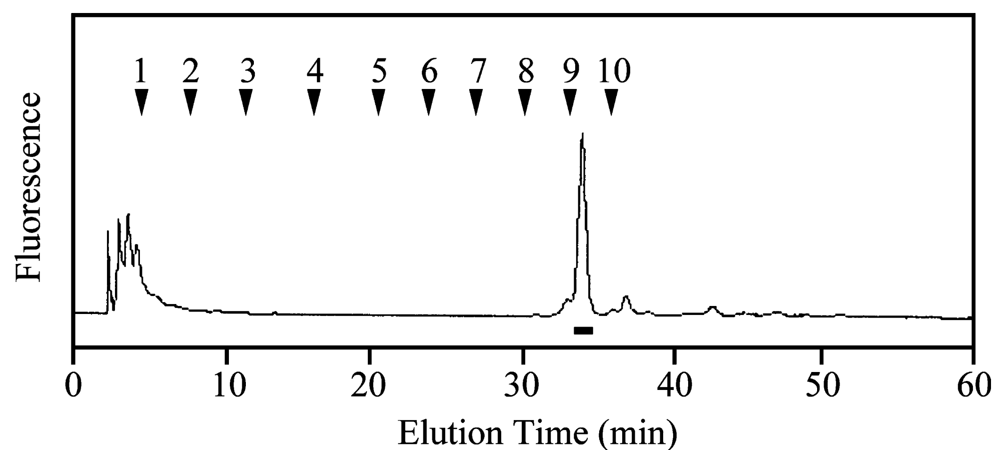


Fig. 3 Separation by size-fractionation HPLC of fraction G. A major fraction indicated by a *bar* was collected as fraction GA. *Arrowheads* 1–10 indicate the elution positions of PA-Glc–PA-isomaltodecaose, respectively. Peaks that appeared at 2–6 min were due to contaminating materials

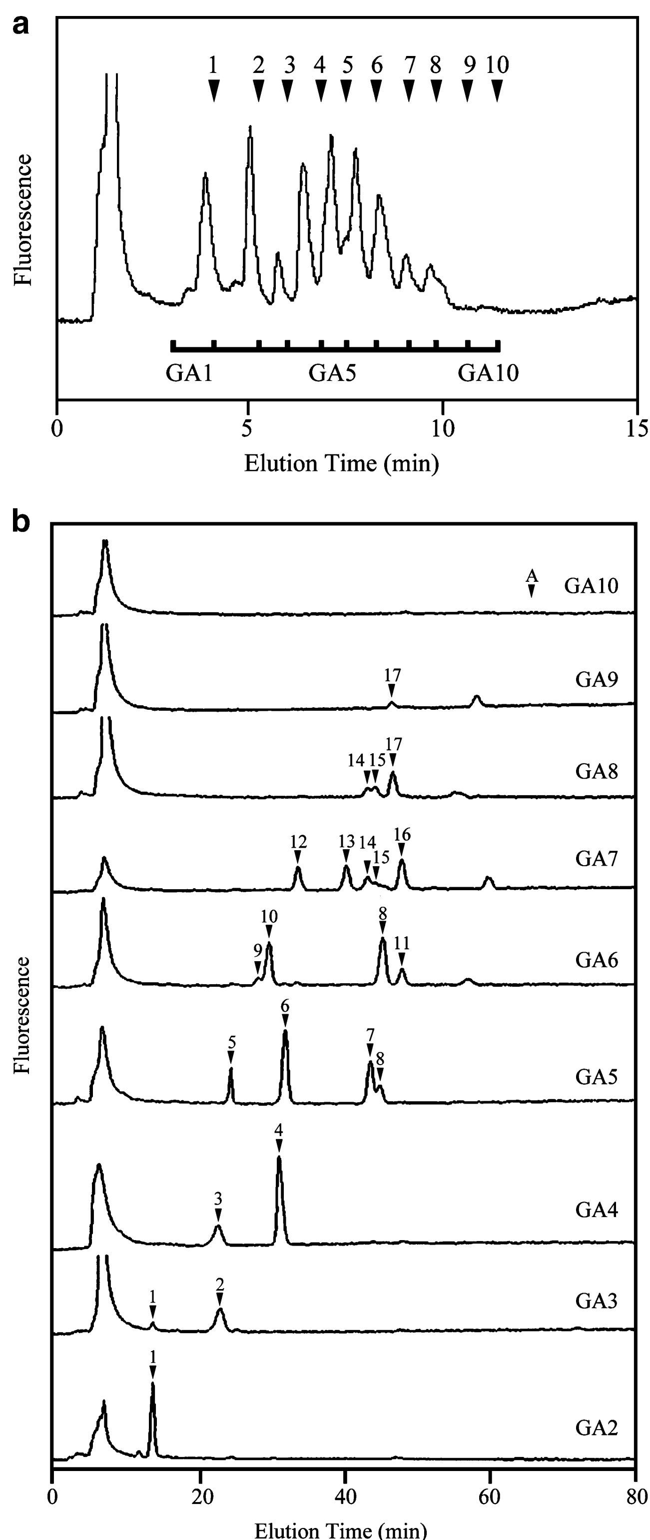


Fig. 4 Analysis of the partial acid hydrolysates of GA by HPLCs. **a** The partial acid hydrolysates of GA (200 pmol) were fractionated on a Shodex NH2P-50 column (4.6×50 mm). *Arrowheads* 1–10 indicate the elution positions of PA-Glc–PA-isomaltodecaose, respectively. Fractions were pooled as indicated by *bars GA1–GA10*. Peaks that appeared at around 2 min were due to contaminating materials. **b** Fractions GA2–GA10 were analyzed by reversed phase HPLC. *Numbered arrowheads* indicate the elution positions of authentic PA-glycans, of which structures are listed in Table I. *Arrowhead A* indicates the elution position of GA

Table 1 Structures of standard PA-glycans used for partial acid hydrolysis analysis

No.	Structure
1	GlcNAcβ1-4GlcNAc-PA
2	Manβ1-4 GlcNAcβ1-4GlcNAc-PA
3	Manβ1-4 GlcNAcβ1-4GlcNAc-PA Manα1-3
4	Manα1-6 Manβ1-4 GlcNAcβ1-4GlcNAc-PA
5	Manβ1-4 GlcNAcβ1-4GlcNAc-PA GlcNAcβ1-2Manα1-3
6	Manα1-6 Manβ1-4 GlcNAcβ1-4GlcNAc-PA Manα1-3
7	GlcNAcβ1-2Manα1-6 Manβ1-4 GlcNAcβ1-4GlcNAc-PA
8	GlcNAcβ1-2Manα1-6 Manβ1-4 GlcNAcβ1-4GlcNAc-PA Manα1-3
9	Manβ1-4 GlcNAcβ1-4GlcNAc-PA Galβ1-4GlcNAcβ1-2Manα1-3
10	Manα1-6 Manβ1-4 GlcNAcβ1-4GlcNAc-PA GlcNAcβ1-2Manα1-3
11	Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4 GlcNAcβ1-4GlcNAc-PA
12	Manα1-6 Manβ1-4 GlcNAcβ1-4GlcNAc-PA Galβ1-4GlcNAcβ1-2Manα1-3
13	GlcNAcβ1-2Manα1-6 Manβ1-4 GlcNAcβ1-4GlcNAc-PA GlcNAcβ1-2Manα1-3
14	GlcNAcβ1-2Manα1-6 Manβ1-4 GlcNAcβ1-4GlcNAc-PA Galβ1-4GlcNAcβ1-2Manα1-3
15	Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4 GlcNAcβ1-4GlcNAc-PA GlcNAcβ1-2Manα1-3
16	Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4 GlcNAcβ1-4GlcNAc-PA Manα1-3
17	Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4 GlcNAcβ1-4GlcNAc-PA Galβ1-4GlcNAcβ1-2Manα1-3

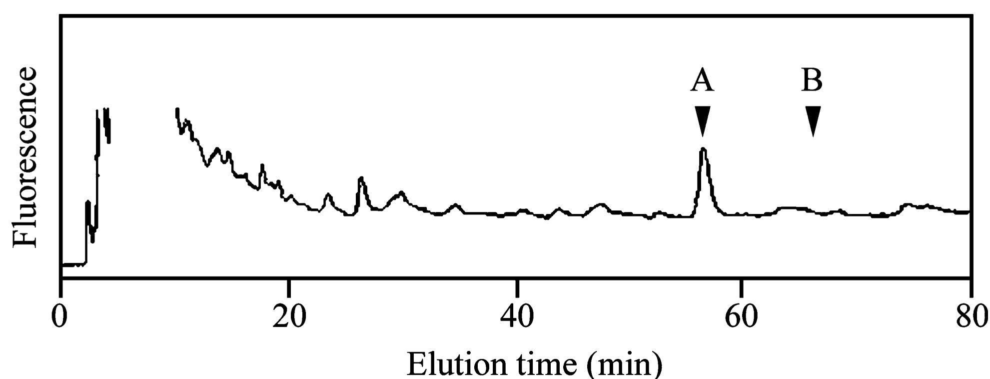


Fig. 5 Reversed phase HPLC of the methanolysates of GA. *Arrowheads A* and *B* indicate the elution positions of PA-fucosylbiantennary glycan and GA, respectively

Dowex 50W-X2 (H^+ form) and eluted with 2.5% aqueous ammonia. The purified PA-monosaccharides were separated and quantified by anion-exchange HPLC using a TSKgel Sugar AXI column.

Sequential analysis of PA-glycan structures from the reducing-end terminal [20]

To 200 pmol of a PA-glycan, 100 µl of 1 M trifluoroacetic acid aqueous solution was added; half of this amount was hydrolyzed at 100°C for 10 min, and the other half for 1 h. The combined hydrolysates were lyophilized, and re-*N*-acetylated. The sample obtained was then size-fractionated with separations at every one glucose unit using PA-isomaltooligosaccharides as an elution scale. After lyophilization of each fraction, the monosaccharide fraction was analyzed by anion-exchange HPLC with a TSKgel Sugar AXI column, and the other fractions were analyzed by reversed phase HPLC.

Analysis of PA-glycan structures by two-dimensional HPLC mapping

The structures of the PA-glycans were assessed by two-dimensional HPLC mapping. The elution positions of 93 standard PA-N-glycans have already been reported, and the introduction of a reversed phase scale made it possible to predict the elution positions, even if the standard PA-N-glycans had not been available [21]. A PA-glycan was separated by reversed phase HPLC and size-fractionation HPLC, and its elution position was compared with those of standard PA-glycans on the two-dimensional HPLC map.

Analysis of sulfated GlcNAc obtained from Fraction GA

The sulfated GlcNAc obtained after sequential exoglycosidase digestions of Fraction GA was pyridylaminated as described previously [22]. Excess reagents were evaporated once with 40 µl of triethylamine:methanol (1:1, v/v) at 60°C for 20 min, five times with 60 µl of toluene:methanol (2:1, v/v) at 60°C for 10 min and once with 40 µl of toluene at 60°C for 10 min. The residue was dissolved in 20 µl of water, and the solution was analyzed by reversed phase HPLC.

High-performance liquid chromatography of PA-glycans

Size-fractionation HPLC for sequential analysis starting from the reducing-end terminal was carried out using a Shodex Asahipak NH2P-50 column (4.6×50 mm) run at 25°C with a flow rate of 0.8 ml/min. Two eluents, A and B, were used. Eluent A was acetonitrile:water:acetic acid (200:800:3, v/v/v) titrated to pH 7.0 with aqueous ammonia; Eluent B was acetonitrile:water:acetic acid (930:70:3, v/v/v) titrated to pH 7.0 with aqueous ammonia. The column was equilibrated with Eluent B:Eluent A (32:1, v/v). After injecting a sample, a linear gradient elution was performed as follows: to Eluent B:Eluent A (2:1, v/v) in 1 min and then to Eluent B:Eluent A (3:7, v/v) in the next 34 min. PA-glycans were detected by fluorescence (excitation wavelength, 310 nm; emission wavelength, 380 nm).

Size-fractionation HPLC for other analyses was carried out using a Shodex Asahipak NH2P-50 column (4.6×100 mm) run at 25°C with a flow rate of 1.0 ml/min. Two eluents, C and D, were used. Eluent C was acetonitrile:water:acetic acid (20:80:3, v/v/v) titrated to pH 7.3 with triethylamine; Eluent D was acetonitrile:water:acetic acid (80:20:3, v/v/v) titrated to pH 7.3 with triethylamine. The column was equilibrated with Eluent D. After injecting a sample, a linear gradient elution was performed to Eluent D:Eluent C (1:1, v/v) in 60 min.

Reversed phase HPLC for preparation of the PA-glycans was performed on a Cosmosil 5C18-P column (4.6×150 mm) run at a flow rate of 1.5 ml/min. The column was equilibrated with 100 mM acetic acid containing 0.025% 1-butanol. After injecting a sample, the concentration of 1-butanol was raised linearly to 0.5% in 55 min. Elution was monitored by measuring the fluorescence (excitation wavelength, 315 nm; emission wavelength, 400 nm).

Reversed phase HPLC for two-dimensional HPLC mapping was performed on a Cosmosil 5C18-P column

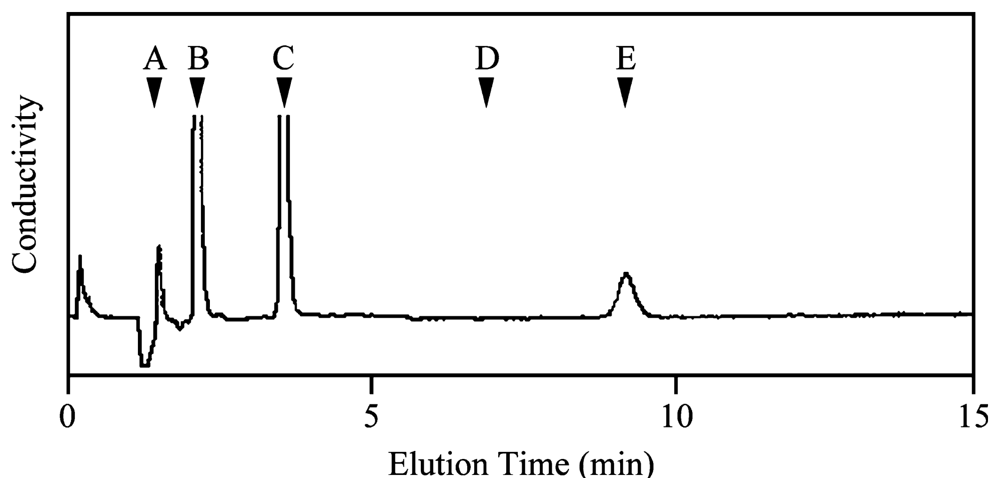


Fig. 6 Quantitative analysis of sulfate ion of GA. The acid hydrolysate of GA was analyzed by ion chromatography. *Arrowheads A–E* indicate the elution positions of fluoride, chloride, bromide, phosphate and sulfate ions, respectively. The peaks that appeared at 1, 2 and 4 min were due to contaminating fluoride, chloride and bromide ions, respectively

(1.5×250 mm) at a flow rate of 150 µl/min. The column was equilibrated with 20 mM ammonium acetate buffer, pH 4.0, containing 0.075% 1-butanol. After injecting a sample, the concentration of 1-butanol was raised linearly to 0.4% over 90 min.

Reversed phase HPLC to analyze sulfated GlcNAc-PA was carried out on a Cosmosil 5C18-P column (4.6× 150 mm) run at a flow rate of 1.5 ml/min, with isocratic elution carried out using 100 mM acetic acid.

Anion-exchange HPLC was carried out on a Q-Sepharose 16/10 column run at 25°C at flow rates of 1.0 and 3.0 ml/min, respectively. Two eluents, E and F, were used. Eluent E was aqueous ammonia, pH 9.0; Eluent F was 500 mM ammonium acetate solution, pH 9.0. The column was equilibrated with Eluent E. After injecting a sample, a linear gradient elution was performed to Eluent E:Eluent F in the following proportions (v/v) and for the following times: 3:22 in 3 min, 2:3 in 14 min and to 100% Eluent F in 5 min. PA-glycans were detected as described for size-fractionation HPLC.

Anion-exchange HPLC for PA-monosaccharides was performed on a TSKgel Sugar AXI column at 73°C run at a flow rate of 0.3 ml/min, with isocratic elution using a mixture of 9 parts of 0.8 M potassium borate, pH 9.0 and 1 part of acetonitrile [19].

Methanolysis of sulfated PA-glycan

A PA-glycan (20 pmol) was methanolyzed with 150 µl of 50 mM HCl in methanol at 37°C for 3 h [23]. After concentrating to dryness three times with 100 µl methanol, the products were *N*-acetylated as described for reducing-end analysis of PA-glycans.

MALDI-TOF MS of PA-glycan

A PA-glycan was co-crystallized in a matrix of 2,5-dihydroxybenzoic acid and analyzed with a Voyager-DE-RP BioSpectrometry Workstation (Perseptive Biosystems, Framingham, MA) equipped with delayed extraction operated in the reflector mode. Peptide standards (Neurotensin and Insulin chain B) were used to achieve a two-point external calibration for mass assignment of ions.

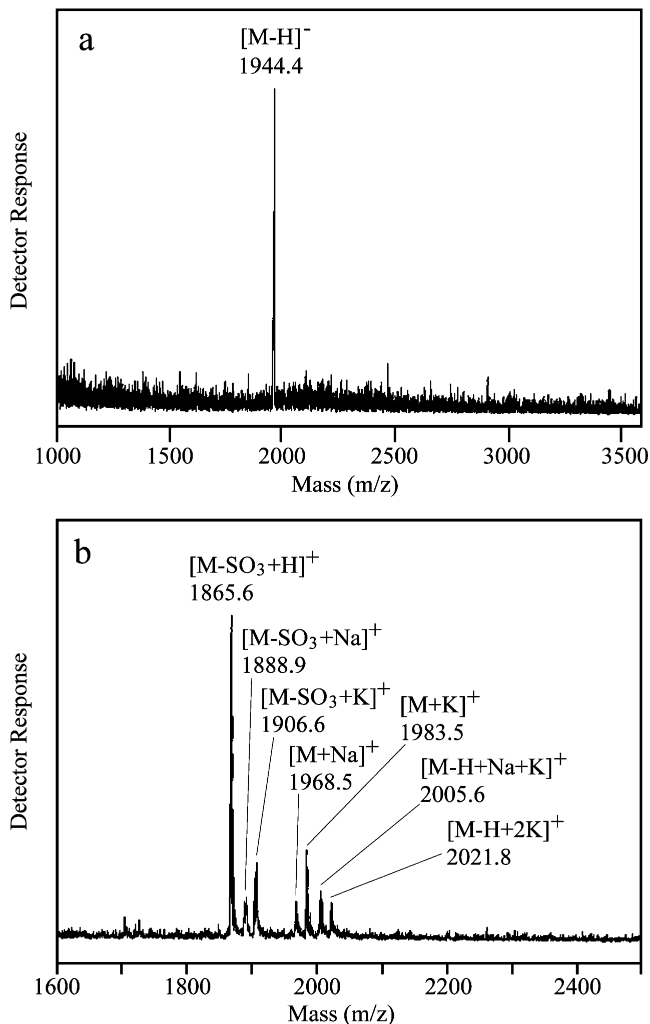
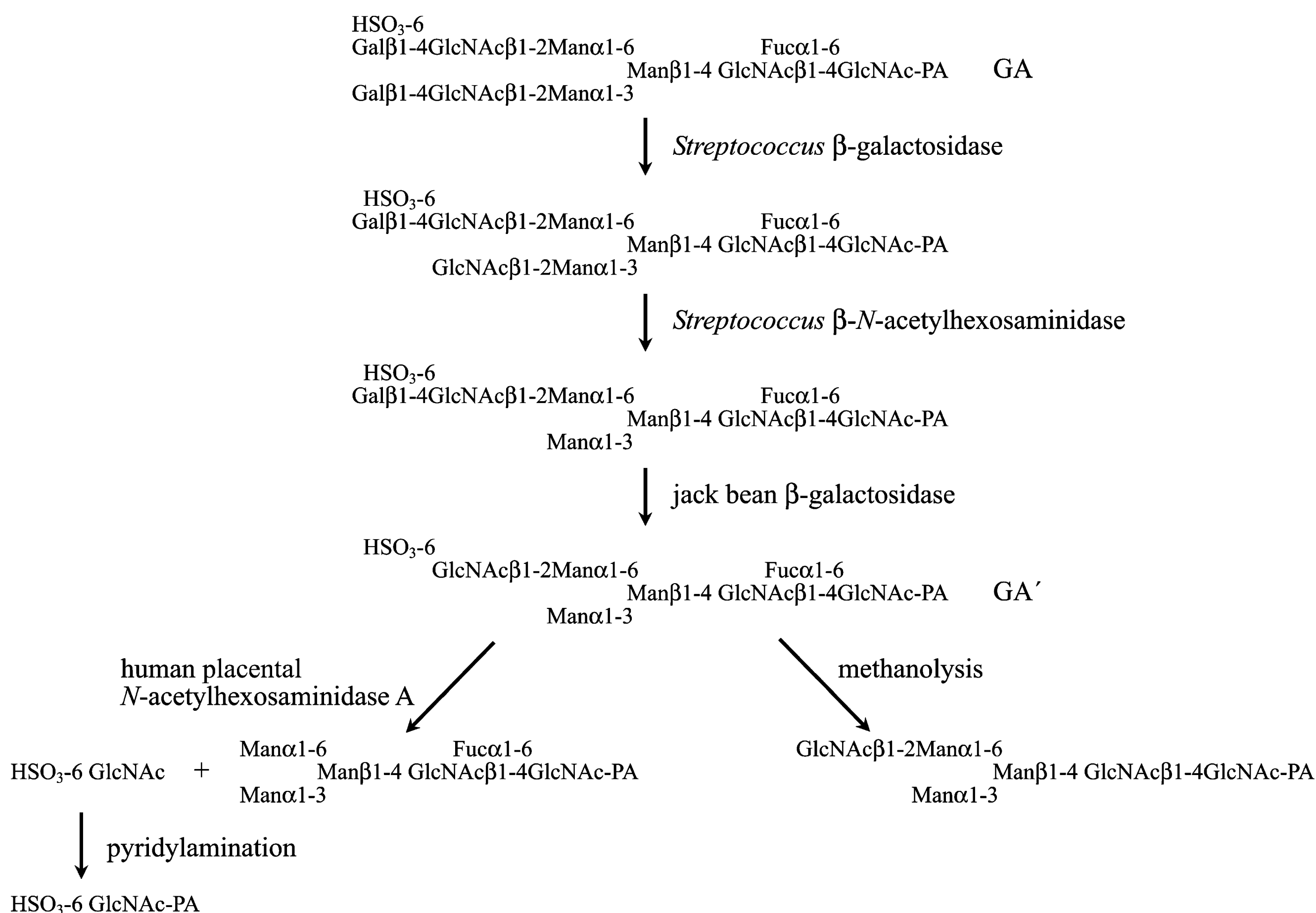


Fig. 7 MALDI-TOF MS spectra of Fraction GA. **a** negative ion mode; **b** positive ion mode



Scheme 1 Strategy to determine the binding position of the sulfate group in GA

Quantitative analysis of sulfate ion

Sulfate ion was measured by ion chromatography. A PA-glycan (200 pmol) was heated in 20 μl of 1 M HCl at 100°C for 1 h, and the resulting sulfate ion was measured with a Dionex 20210i ion chromatography system using an IonPac AS4A column and 2.8 mM Na_2CO_3, 2.25 mM $NaHCO_3$ as a solvent, with a flow rate of 1.5 ml/min at 25°C [24].

Results

Preparation and separation of anionic PA-N-glycans from bovine lung

PA-Glycans were prepared from an acetone powder of bovine lung. After digestion of the PA-glycans by *Arthrobactor* sialidase, the digests were separated on a Q-Sepharose column (Fig. 1) and the anionic fraction was collected. Since negative charges of this anionic fraction were not removed by mild acid hydrolysis (1 M HCl, 80°C for 45 min) (data not shown), the negative charges of the anionic fraction were evidently not due to sialic acid residues that were resistant to *Arthrobactor* sialidase. The anionic fraction thus obtained was separated by reversed phase HPLC and fractions A to H were collected (Fig. 2a). Reducing-end PA-monosaccharides of each fraction were analyzed as described under “Experimental Procedures,” and PA-glycans with PA-GlcNAc at their reducing end were detected mainly in fraction G (Fig. 2b). Fraction G was further separated by size-fractionation HPLC, and a major fraction named GA was collected (Fig. 3).

Analysis of the core structure of Fraction GA

To determine the structure of GA, reversed phase HPLC and size-fractionation HPLC for two-dimensional HPLC mapping were performed. Since GA was not identified as one of the authentic 93 PA-N-glycans on the map, the core structure of GA was analyzed by combining partial acid hydrolysis and two-dimensional HPLC mapping [20]. The result of size-fractionation HPLC is shown in Fig. 4a. Fractions GA1–GA10 were collected. PA-GlcNAc was detected in fraction GA1, as indicated by anion-exchange

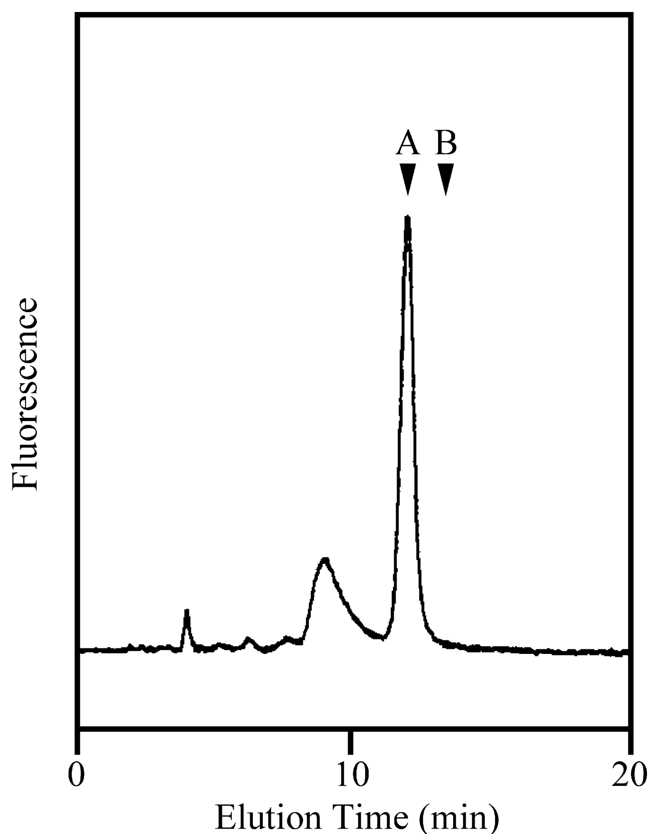


Fig. 8 Structural analysis of sulfated GlcNAc-PA. Sulfated GlcNAc released from GA was pyridylaminated, and the product was analyzed by reversed phase HPLC. *Arrowhead A* and *B* indicate the elution positions of HSO_3-6GlcNAc-PA and HSO_3-3GlcNAc-PA, respectively. The peaks that appeared at 9 min were due to contaminating materials

HPLC on a TSKgel Sugar AXI column (data not shown). Fractions GA2–GA10 were analyzed by reversed phase HPLC (Fig. 4b). Sugar chains 1 and 2 (Table 1) were detected in fractions GA2 and GA3, respectively. Sugar chains 3 and 4 were detected in fraction GA4. These findings indicated that fraction GA5 might contain sugar chain 6, and this was in fact confirmed by detecting a peak at the elution position of sugar chain 6 (Fig. 4b, GA5). The above results suggested that Fraction GA had the trimannosyl core structure (sugar chain 6). Detection of a peak at the elution position of sugar chain 17 in fraction GA9 indicated that GA contained a biantennary structure. The structure of sugar chain 17 as a component of GA was further confirmed by detection of peaks at the elution positions of sugar chains 5–16 in fractions GA5–GA8, and by identifying PA-glycan fragments (sugar chains 1–17) derived from authentic sugar chain 17 by applying the same procedure (data not shown). The peaks that appeared at around 55 min (Fig. 4b, GA6–GA9) were not identified, but they were likely due to anionic sugar chain fragments. Absence of a peak at the elution position of GA indicated that GA had acid-labile substituent(s).

The core structure of GA was further analyzed by exoglycosidase digestion combined with two-dimensional HPLC mapping. The GA was susceptible to digestion with α-fucosidase, and the difference in the elution position between GA and the product was characteristic of the Fucα1-6 residue (data not shown) [21]. The GA was methanolyzed and the product appeared at the position of PA-fucosylbiantennary glycan [Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-PA] on the map. The result of reversed phase HPLC is shown in Fig. 5. These results demonstrated that GA had the fucosylbiantennary core structure.

Analysis of the anionic character of GA

The result of methanolysis suggested that GA was a sulfated form of a PA-fucosylbiantennary glycan. The presence of a sulfate group was confirmed by ion chromatography after acid hydrolysis (Fig. 6). The amount of sulfate ion detected was 0.94 mole per mole of GA. The molecular weight of GA was analyzed by MALDI-TOF MS (Fig. 7). The negative-ion mass spectrum (Fig. 7a) showed a quasimolecular ion, $[M\text{-}H]^-$ (*m/z* 1944.4, calculated 1943.8 for a PA-monosulfated fucosylbiantennary glycan). In the positive-ion mass spectrum (Fig. 7b), expected peaks of molecule-related ions, $[M\text{-}SO_3\text{+}H]^+$ (m/z 1865.6, calculated 1865.8), $[M\text{-}SO_3\text{+}Na]^+$ (m/z 1888.9, calculated 1887.8), $[M\text{-}SO_3\text{+}K]^+$ (m/z 1906.6, calculated 1903.9), $[M\text{+}Na]^+$ (m/z 1968.5, calculated 1967.8), $[M\text{+}K]^+$ (m/z 1983.5,

Table 2 Proposed structures for sulfated N-glycans

Name	Structure
GA	HO_3S-6 Galβ1-4GlcNAcβ1-2Manα1-6 Fucα1-6 Manβ1-4 GlcNAcβ1-4GlcNAc-PA Galβ1-4GlcNAcβ1-2Manα1-3
SiaGA	HO_3S-6 Siaα2-3Galβ1-4GlcNAcβ1-2Manα1-6 Fucα1-6 Manβ1-4 GlcNAcβ1-4GlcNAc-PA Siaα2-3Galβ1-4GlcNAcβ1-2Manα1-3

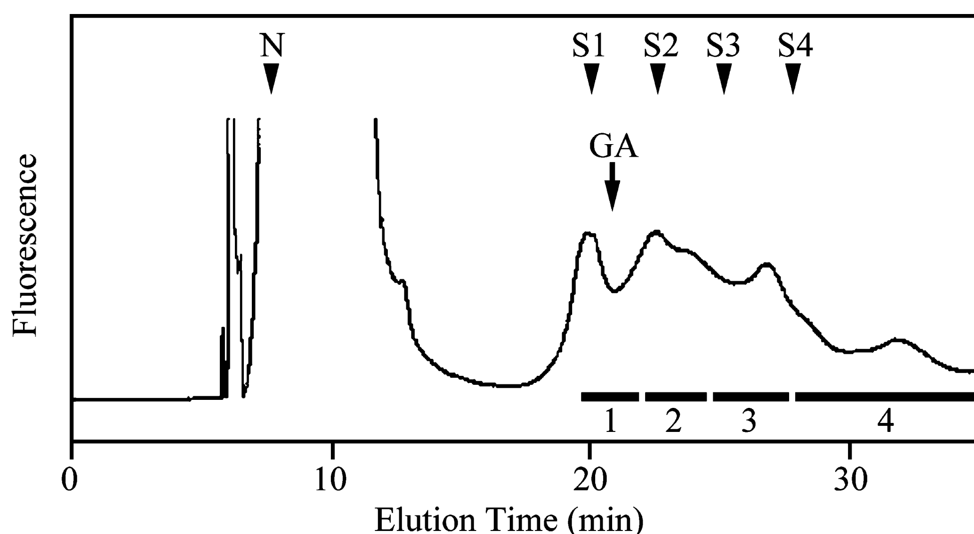


Fig. 9 Separation by Q-Sepharose anion-exchange HPLC of PA-glycans from bovine lung. Fractions 1–4 were pooled as indicated by *bars*. *Arrowheads N*, *S1–S4* indicate the elution positions of authentic neutral, monosialo, disialo, trisialo and tetrasialo PA-N-glycans, respectively, obtained from α_1-acid glycoprotein. *Arrow GA* indicates the elution position of GA. Peaks that appeared at 5–15 min were due to neutral PA-glycans and contaminating materials

calculated 1983.9), $[M-H+Na+K]^+$ (m/z 2005.6, calculated 2005.9), and $[M-H+2K]^+$ (*m/z* 2021.8, calculated 2022.0) were detected. We concluded from these results that the anionic character of GA was due to a sulfate ester.

Determination of the linkage position of the sulfate group in GA

To determine the linkage position of the sulfate residue, exoglycosidase digestions and methanolysis were combined with two-dimensional HPLC mapping. It has been reported that jack bean β-galactosidase can hydrolyze Galβ1-4 (HSO_3-6)GlcNAc, whereas the β1,4-galactosidase from *S. pneumoniae* can not hydrolyze the linkage [25]. Further structural analysis of GA took advantage of the differential activities of these two β-galactosidases. After *Streptococcus* β1,4-galactosidase digestion of GA, the difference in glucose units corresponded to the removal of only one galactoside. On the other hand, there was a decrease in glucose units corresponding to the removal of two galactosides after jack bean β-galactosidase digestion of GA (data not shown). Those results suggested that GA had the HSO_3-GlcNAc structure. To determine whether the HSO_3-GlcNAc residue is bound to the Manα1-3 or the Manα1-6 branch of the biantennary chain, sequential exoglycosidase digestions and methanolysis were done (Scheme 1). The

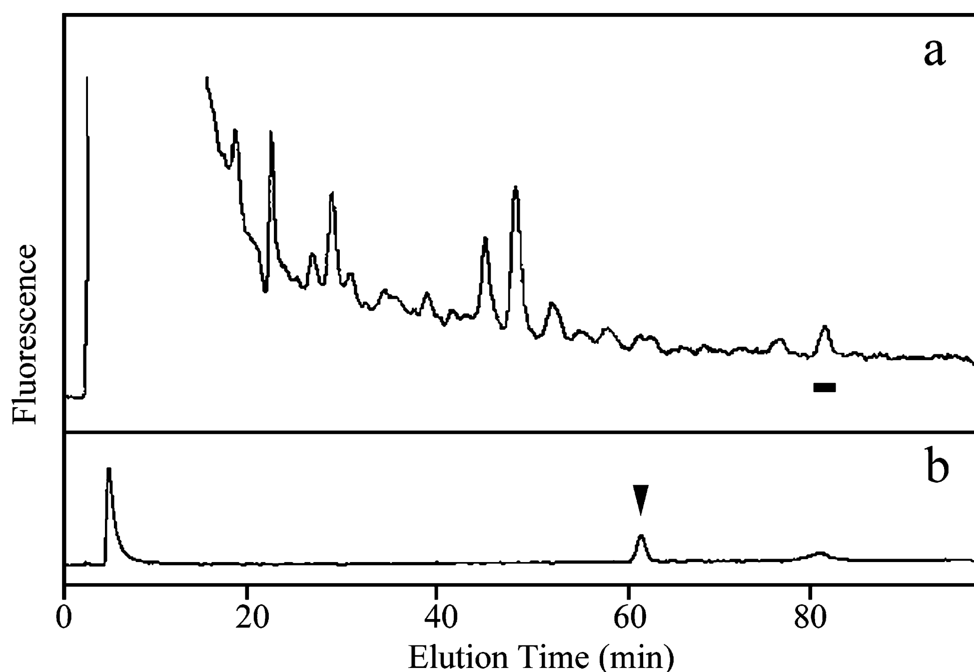


Fig. 10 Separation by reversed phase HPLC of sialylated glycans. **a** Fraction 4 in Fig. 9 was separated by reversed phase HPLC. The fraction indicated by the *bar* was collected and named as SiaGA. **b** Sialidase digest of SiaGA was separated by reversed phase HPLC. The *arrowhead* indicates the elution position of GA. Peaks that appeared at 2–15 min were due to contaminating materials

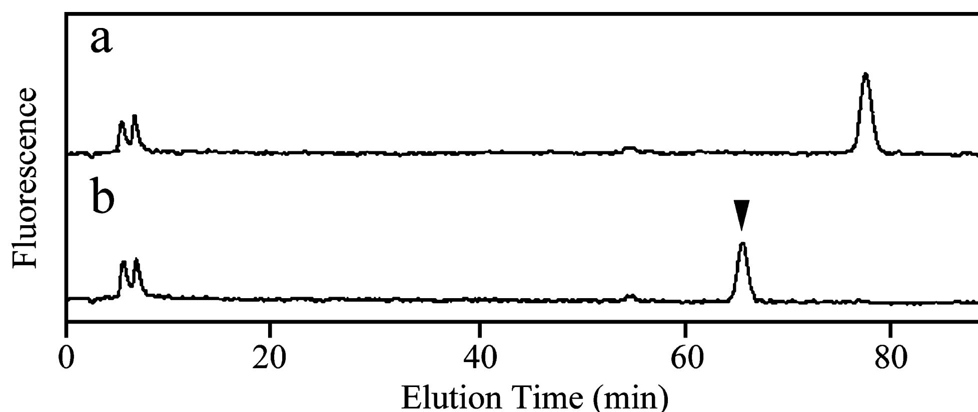


Fig. 11 Reversed phase HPLC analysis of SiaGA digested by α(2,3)specific sialidase. **a**, SiaGA; **b**, NDV sialidase-digested SiaGA. The *arrowhead* shows the elution position of GA. Peaks that appeared at around 5 min were due to contaminating materials

GA was digested with *Streptococcus* β1,4-galactosidase, decreasing the molecular size of the product by 0.9 glucose unit. This indicated that only the galactose residue bound to the unsulfated GlcNAc residue was hydrolyzed. The product was next digested with *Streptococcus* β-*N*-acetylhexosaminidase, which decreased the molecular size of the product by 0.7 glucose unit. This corresponded to the hydrolysis of a terminal GlcNAc residue. The product was then digested with jack bean β-galactosidase, and the resulting decrease in the molecular size (0.8 glucose unit) indicated that this was due to removal of the galactoside bound to the sulfated GlcNAc residue. The sample obtained after these exoglycosidase digestions (referred to as GA′) was methanolyzed, and the desulfated product eluted at 55.3 of reversed phase scale on the reversed phase HPLC, which is corresponding to the position of the standard sugar chain 8 (53.8), not to the position of the standard sugar chain 10 (40.0). This indicated that the sulfated GlcNAc residue was on the Manα1-6 branch. This suggested that the GA had a HSO_3-GlcNAc attached to the Manα1-6 branch. The next step was to determine at which position the GlcNAc residue was sulfated. GA′ was digested with human placental β-*N*-acetylhexosaminidase A, which was able to cleave the terminal HSO_3-6GlcNAc [26]. The product derived from reducing end coincided with Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6) GlcNAc-PA on the 2D-map (data not shown). The product derived from non-reducing end, sulfated GlcNAc, was pyridylaminated, and this product was analyzed by reversed phase HPLC (Fig. 8). The sulfated GlcNAc-PA was eluted at the position of HSO_3-6GlcNAc-PA, but not at the position of HSO_3-3GlcNAc-PA. From the results described above, the structure of GA was proposed to be Galβ1-4(SO_4-6) GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-PA (Table 2).

Detection of sialylated forms of GA in bovine lung

So far we have analyzed the asialo derivative of anionic N-glycans. We next analyzed the native form of GA. PA-N-Glycans from bovine lung were prepared as described above, and were separated on a Q-Sepharose column without the sialidase treatment. Acidic fractions were collected as 1–4 according to the elution positions of sialylated N-glycans, as shown in Fig. 9. Each fraction was separated by reversed phase HPLC. Fraction 1 was presumed to contain the asialo form of GA, judging from the elution position of GA as analyzed by Q-Sepharose anion-exchange HPLC (Fig. 9), but no peak corresponding to GA was detected from Fraction 1 on the reversed phase HPLC (data not shown). This result indicated that GA, which was a non-sialylated form, was not present at detectable levels in bovine lung. Fractions 2–4 were separated by reversed phase HPLC, and major peaks collected were digested with *Arthrobacter* sialidase. Among the peaks, only one peak (SiaGA, indicated by the bar in Fig. 10a) was shifted its elution position to that of GA by the sialidase digestion (Fig. 10b). These results suggested that SiaGA was the predominant structure.

Analysis of the structure of Fraction SiaGA

To determine the structure of SiaGA, exoglycosidase digestions and methanolysis were performed. The SiaGA was digested with jack bean β-galactosidase and no galactose residues were hydrolyzed (data not shown). Fraction SiaGA was next desulfated by methanolysis to determine a number of sialic acid residues. The peak of the product was eluted at the position of disialo N-glycans on Mono-Q anion-exchange HPLC, and eluted at the same position as Siaα2-3Galβ1-4GlcNAcβ1-2Manα1-6(Siaα2-3Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4 (Fucα1-6)GlcNAc-PA on reversed phase HPLC. This suggested that both of the galactose residues of SiaGA were capped by sialic acid residues. To determine the linkages of the sialic acids, SiaGA was digested with NDV sialidase, which cleaves Siaα2-3 residues but not Siaα2-6 residues under the conditions used in this study. The product was eluted at the position of GA on the two-dimensional HPLC map, and the result of reversed phase

HPLC is shown in Fig. 11. These results indicated that the linkages of sialic acid residues bound to SiaGA were both α2-3. From these results, the structure of SiaGA was proposed to be Siaα2-3Galβ1-4(HSO_3-6)GlcNAcβ1-2Manα1-6(Siaα2-3Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-PA (Table 2).

Discussion

Structures of unusual anionic N-glycans in bovine lung were previously reported [12], and it was shown that the glycans participated in acute inflammatory responses [27]. However, analyses were performed on the glycan mixture, and the structures of individual glycans were not determined completely. To clarify the structures of such anionic glycans, anionic N-glycans with non-sialic acid-negative charges from bovine lung were separated and analyzed. In contrast to the results of a previous study showing that the anionic character of N-glycans in bovine lung was mainly due to carboxylic acid residues other than sialic acid [12], a sulfated N-glycan was detected as a major single sugar chain (approximately 90% of Fraction G). This sulfated glycan had a Siaα2-3Galβ1-4(HSO_3-6)GlcNAc structure attached to the Manα1-6 branch of the fucosylbiantennary glycan.

A major fraction of the lung (about 40% of the mass) consists of endothelial cells [28]. The N-glycans from calf pulmonary artery endothelial cells were analyzed by exoglycosidase digestion and were reported to contain the terminal structure: Siaα2-(3)6Galβ1-4(HSO_3-6)GlcNAcβ1- [10]. It is generally supposed that complex-type glycans with sulfosialyllactosamine units exist in endothelial cells. It has been reported that anionic glycans mediate interactions among lymphocytes and endothelial cells, and that this is an important function of the glycans. L-Selectin ligands on the surface of endothelial cells mediate the attachment of circulating leukocytes to endothelium in inflammatory leukocyte trafficking and in lymphocyte homing to the lymph node. O-glycans in GlyCAM-1 (glycosylation-dependent cell adhesion molecule) were identified as L-selectin ligands, and candidates for capping their structures were 6′-sulfosialyl Lewis x [Siaα2-3(HSO_3-6)Galβ1-4(Fucα1-3) GlcNAc], 6-sulfosialyl Lewis x [Siaα2-3Galβ1-4(Fucα1-3) (HSO_3-6)GlcNAc] and 6,6′-disulfosialyl Lewis x [25, 29, 30]. It has also been reported that a major carbohydrate capping group of the L-selectin ligand on high endothelial venules in human lymph nodes was 6-sulfosialyl Lewis x [31]. It has been assumed that the L-selectin ligand was biosynthesized by transferring the Fucα1-3 residue to HSO_3-6GlcNAc of the sulfosialyllactosamine by fucosyltransferase VII (Fuc-TVII) in these particular endotherial cells, and that these were the target of lymphocyte homing [32–34]. The sulfosialylbiantennary sugar chain described in this study may be a precursor of the L-selectin ligand of N-glycans. This sulfated glycan had Fucα1-6 and Siaα2-3 residues, and was a major glycan in non-sialic acid anionic N-glycans in bovine lung. It is also interesting to ask whether sulfation of the glycan is regulated by fucosylation and/or sialylation.

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