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A Novel Oligosaccharide on Bovine Peripheral Myelin Glycoprotein P0

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Abstract: Sulfated oligosaccharides have found in glycoproteins in myelin in the PNS, such as P0 and PASII/PMP22. These glycoproteins are involved in the interaction of cell membranes and play important roles in the formation and maintenance of myelin sheaths. These functions are due in part to the oligosaccharides in their molecules. Recently, the structures of the oligosaccharides of bovine peripheral myelin P0 were reported, and some of these carry the HNK-1 carbohydrate epitope (SO_4-3GlcAβ1-3Galβ1-4GlcNAc). In this paper, we identified a novel multisulfated oligosaccharide, MS2, using one-dimensional ^{1}H-NMR, mass spectrometry, and chemical and enzymatic analyses. MS2, which contains a HNK-1 epitope, was from a single N-glycosylation site of P0 glycoprotein. The structure of the novel oligosaccharide was: Manα1-3Manα1-6[(3SO_4)GlcAβ1-3(6SO_4)Galβ1-4(6SO_4)GlcNAcβ1-2Manα1-3]Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc. In addition to the 3-*O*-sulfate present in the HNK-1 epitope, the oligosaccharide contained a 6-*O*-sulfated N-acetylglucosamine residue and a 6-*O*-sulfated galactose residue. This implies that both Gal6-*O*-sulfotransferase, which has not been reported in the PNS, and GlcNAc 6-*O*-sulfotransferase are active in the PNS tissues.

Keywords: Myelin, Glycoprotein, P0, Sulfated oligosaccharide, HNK-1, sulfotransferase.
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Introduction

The P0 glycoprotein has an apparent molecular mass of 28-30 kDa and is a glycosylated member of the immunoglobulin superfamily [1-3]. It is the most abundant protein constituent of peripheral myelin [4] and consists of a single, extracellular immunoglobulin-like domain, a transmembrane domain, and a cytoplasmic tail. P0 is believed to contribute to the formation and maintenance of myelin compaction as an adhesion molecule and the essential function of P0 has been demonstrated in studies of P0-deficient mice [5, 6]. Mutations of the P0 gene lead to inherited human neuropathies, such as Dejerine-Sottas disease and Charcot-Marie-Tooth disease [7].

The glycan moiety of P0 plays an important role in cell-to-cell adhesion via homophilic interactions [8, 9]. P0 contains a single N-glycosylation site (Asn[93]) and has a heterogenous glycosylation pattern. The glycosylation pattern of P0 originates from variable fucose, galactose, sialic acid, and sulfate groups and the HNK-1 carbohydrate epitope [10-13]. The glycopeptide consisting of residues 91-95 (GDNGT) markedly inhibits cell aggregation compared with that consisting of residues 90-96 (YGDNGTF) without glycan [14]. In addition, non-glycosylated P0 does not show homophilic adhesion [9]. Age-dependent changes in P0 oligosaccharide in peripheral nerves [10] and the mammalian spinal cord [5, 6, 15] have been reported, suggesting that the glycosylation pattern is regulated by alterations in physiological conditions.

Sulfate residue is an integral part of the structure of PNS myelin. It is present both in glycolipids, in the form of cerebroside sulfate, which constitute about 6 % of the dry weight of myelin lipids [16], and in the glycoproteins such as P0 and PASII/PMP22. Mathieu *et al.* showed that [^{35}S]O_4 co-localized with rat P0 on polyacrylamide gels [17], but the site of sulfation was unknown until we reported that the oligosaccharide chain of bovine

The abbreviations used are: 1D, one-dimensional; MALDI-TOF, matrix assisted laser desorption ionization time-of-flight; PA, 2-aminopyridine; CNS, central nervous system; PNS, peripheral nervous system; ppm, parts per million.

P0 was heterogeneous, and that at least three of the five components contained sulfate[18]. Sulfation of the oligosaccharide of P0 occurs after nerve crush injury and in myelin assembly during development, and ceases in adults[19]. Recently, Gallego *et al*[20]. reported a detailed structure analysis of about 90 % of the oligosaccharides in bovine P0, identifying the core structure of the carbohydrates and several epitopes, including HNK-1 epitope[21] and 6-*O*-sulfo sialyl Lewis X[22]. Among them, the HNK-1 carbohydrate, is now known to be widely distributed in neural cell adhesion molecules in both the CNS and PNS and, therefore, has become the most possible candidate for a key substance to the cell adhesion event[23-29].

Here, we describe the structure of the most acidic oligosaccharide, which comprises approximately 1 % of the oligosaccharides in P0, and reveal a novel multisulfated oligosaccharide that contains the HNK-1 epitope.

Experimental procedure

Preparation of glycopeptides – Bovine peripheral nerve myelin was obtained according to the method as reported previously[1]. The myelin was delipidated using two chloroform/methanol (2:1) extractions, and the resulting fat-free myelin was digested with *Streptomyces griseus* protease (Sigma, St. Louis, MO) for 24 h at 37℃[18, 30]. After successive digestion of the protein, glycopeptides were recovered in the void volume of Sephadex G-15 gel chromatography eluted with 0.05 % trifluoroacetic acid, and subsequently by Asahipak GS-310 size-exclusion HPLC (7.6×500 mm; Showa Denko, Tokyo) eluted with 0.05 % trifluoroacetic acid at a flow rate of 1 ml/min at room temperature and monitored by a refractive index monitor (RID-6A, Shimadzu Co, Kyoto)[31]. The glycopeptide fraction was applied to a ProteinPak DEAE-8HR ion-exchange column (10.0×100 mm; Waters, MA) and eluted for 60 min with a linear gradient of 0-0.2 M NaCl at a flow rate of 1 ml/min at room temperature. The peptides were monitored at 215 nm by a UV monitor (SPD-6AV, Shimadzu Co, Kyoto). A portion of the most acidic glycopeptide fraction (MS2p) was subjected to amino acid sequence analyses, and the remainder was used for the preparation of oligosaccharide.

Amino acid sequence determination of the glycopeptide – Automatic Edman degradation of glycopeptide MS2p (30 pmol) was carried out in a model PPSQ-10 gas-phase sequence analyzer (Shimadzu).

Preparation of pyridylaminated oligosaccharide – The glycopeptide, MS2p (25 pmol) was digested with 0.5 mU of glycopeptidase-A (EC 3.51.52; Seikagakukogyo, Tokyo) in 100 μl of 0.1 M citrate-phosphate buffer (pH4.5) for 2 days at 37℃[32]. The released oligosaccharide was separated from the peptides and salts with an Asahipak GS-310 column. The oligosaccharide was lyophilized and then pyridylaminated with 2-aminopyridine (PA), as previously reported[31]. After passage through a GS-310 column, the PA-oligosaccharide was applied to a Capcell Pak ODS column (4.6×150 mm Shiseido, Tokyo), and eluted for 60 min with a linear gradient of 0.25 to 5 % (vol/vol) 1-butanol in 0.1 % (vol/vol) acetic acid/triethylamine (pH 4.0) at a flow rate of 1 ml/min at 40℃ . The chromatogram was recorded with a model 821-FP monitor (JASCO Co.,Tokyo). The major oligosaccharide, PA-MS2, was collected.

^{1}H-NMR measurements – ^{1}H-NMR analysis followed a previously reported method[33]. Pyridylaminated MS2 (PA-MS2) was lyophilized with D_2O at least three times and dissolved in 450 μl of 99.96 % D_2O (Sigma). Spectra were recorded at 27 and 60℃ with an EX-400 NMR spectrometer (JEOL Co., Tokyo). Chemical shifts were recorded with reference to acetone {δ =2.2180 parts per million (ppm) at 27℃ and 2.2143 ppm at 60℃ }.

Mass spectrometry – The molecular weight was determined by reflection time-of-flight (TOF) mass spectrometry (Voyager Elite, Perspective Biosystems, Framingham, MA) in the negative mode. 2,5-Dihydroxybenzoic acid was used as the matrix solution at a concentration of 10 mg/ml in a water-MeOH mixture (9:1, v/v). An aliquot (25 pmol/0.5 μl) of glycopeptide or PA-oligosaccharide solution was deposited on a sample plate with an equal volume of the matrix solution and allowed to dry at ambient temperature.

Glycosidase Treatment – PA-oligosaccharide (PA-MS2) was digested with *Escherichia freundii* endo-β-galactosidase (10 mU) in 0.1 M citrate/phosphate buffer (pH 5.0).

Solvolytic desulfation – Sulfates were removed from the PA-oligosaccharide by solvolysis with 0.1 ml of dimethyl sulfoxide containing 10 % methanol for 2-4 h at 100℃ according to the method of Nagasawa *et al*[34].

Results

The glycopeptide pool released from bovine PNS myelin was fractionated into many fractions by ion-

exchange chromatography on DEAE-8HR. We focused on a glycopeptide, MS2p, in the most acidic fraction (Fig. 1). The acidic compound comprised approximately 1 % of the total glycopeptides recovered. The amino acid sequence of MS2p was Gly-Asp-Asn-Gly-Thr, which is consistent with that of the N-glycosylation site of bovine P0. Therefore, MS2p was thought to be from P0 protein. This was confirmed by mass spectroscopy.

The N-linked oligosaccharide was released from MS2p by glycopeptidase-A digestion and subsequently labeled with the fluorescence probe 2-aminopyridine. The pyridylaminated (PA-) oligosaccharide was purified to homogeneity by ODS-HPLC, as shown in Fig. 2-A (PA-MS2). The purified PA-MS2 was analyzed by ^{1}H NMR spectrometry, mass spectrometry, and chemical and enzymatic analyses.

400-MHz ^{1}H NMR Analysis

In the one-dimensional ^{1}H NMR spectrum, the N-acetyl proton signals around 1.95-2.10 ppm clearly demonstrated the presence of three N-acetylhexosamine residues. The methyl proton with double peaks, 1.160/1.144 ppm, showed the presence of one α-fucose residue. In the high field region of the ^{1}H NMR spectrum, the resonances between 4.5 and 5.3 ppm were due to anomeric protons, and those at 5.140, 5.108, 4.891, 4.870/4.860, 4.765/4.744, 4.765, 4.621/4.604, 4.604/4.584, and 4.563/4.584 ppm were identified as H-1 resonances of Man-4, Man-A, Man-4', Fuc α / β, SO_4GlcA-7, Man-3, GlcNAc-2, SO_4Gal-6, and SO_4GlcNAc-5, respectively. The GlcNAc-1 H-1 proton was not expected in this region, because GlcNAc-1 was pyridylaminated. The NMR findings are summarized in Table 1. Comparing the NMR data with those for Q2.10 (SO_4-3GlcAβ1-3Galβ1-4(6-SO_4)GlcNAcβ1-2-Manα1-3(Manα1-3Manα1-6)Man1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-PA) reported by Gallego *et al.*[20] suggested that MS2 is a hybrid type oligosaccharide.

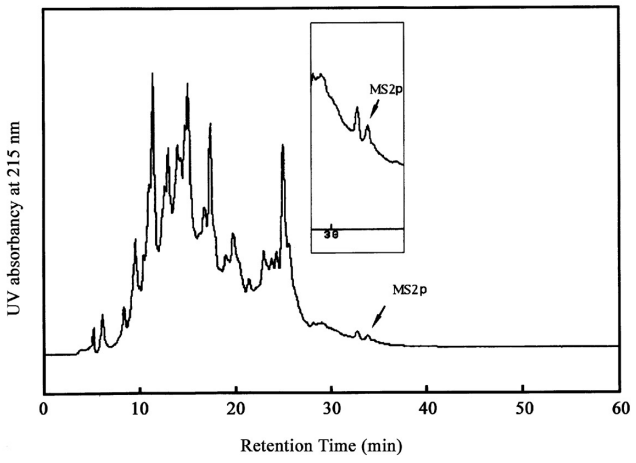

Fig. 1. Fractionation pattern of the glycopeptides derived from bovine myelin protein on a DEAE-ProteinPak 8HR column. Glycopeptides were eluted for 60 min with a linear concentration gradient of 0-0.2 M NaCl at a flow rate of 1 ml/min at room temperature and monitored with an UV monitor at 215 nm. The most acidic area was magnified and is shown in the insert.

Mass spectrometry of the PA-oligosaccharides

The negative-ion mode TOF mass spectrum of PA-MS2 (Fig. 3) showed about 18 signals in the mass area between 1700 and 2200 (Fig. 3-A). These were divided into four groups, indicated by bars. Since the mass difference between the prominent signals in each group matched the molecular weight of sulfate (MW =80), the signals in these four groups were suggested to be non-(S0 form), mono-(S1 form), di-(S2 form), and tri-(S3 form) sulfated oligosaccharide derivatives of PA-MS1. In each group, six signals were expected at most: signals of Na, K, 2Na, both Na and K, 2K, and no cation. For example, in the mono-sulfated (S1 form) oligosaccharide group, the major signals at m/z 1917.76, 1939.66, 1955.90, and 1977.28, corresponded to HexUA-Hex_5-dHex-$HexNAc_3$-PA ($[M-(SO_3)_2-H]^-$) containing no-cation, Na, K, and both Na and K, respectively. The molecular weight difference between consecutive groups was the molecular weight of sulfate; *e.g.*, m/z 1837.91 $[M-(SO_3)_3-H]^-$ in the non-sulfated group and m/z 1917.76 $[M-(SO_3)_2-H]^-$ in the mono-sulfated group. The MALDI-TOF mass spectrum of PA-MS2 indicating that the structure contained three sulfates: HexUA-Hex_5-dHex-$HexNAc_4(SO_3)_3$.

We also compared the mass spectra of the pyridylaminated oligosaccharide (PA-MS2) and glycopeptide (MS2p) forms of MS2, shown in Fig. 3-A and Fig. 3-B, respectively. Since the overall patterns of the two forms were similar, it was easy to assign most of the signals observed in one form to their counterparts in the other form. The mass difference between the two forms was calculated to be 366, which exactly matches the molecular weight of Gly-Asp-Asn-Gly-Thr minus aminopyridine.

HPLC analyses

PA-MS2 and PA-GP5/Q2.10 oligosaccharides were desulfated by solvolysis (Fig. 2-C and D, respectively). As the solvolytic desulfation seemed to be insufficient under the conditions used, intact PA-GP5 and PA-MS2 were still evident in the reaction mixture. Note that the retention times of the desulfated products of

Table 1. Comparison of the reporter group signals of MS2 and GP5/Q2.10

		Chemical shift (ppm)	
Reporter group	residue	MS2	Q2.10[c]
H-1	GlcNAc-1	/	5.179/4.692
	GlcNAc-2	4.621/4.604[a]	4.658
	Man-3	4.765[a]	4.783
	Man-4	5.140[a]	5.125
	Man-4'	4.891[a]	4.895
	Man-A	5.108[a]	5.093
	GlcNAc-5(6SO4)	4.563/4.584[a]	4.576
	Gal-6	/	4.597
	Gal-6(6SO4)	4.604/4.584[a]	/
	GlcUA	4.765/4.744[a]	4.758
	Fuc α / β	4.870/4.860[a]	4.895/HOD
H-2	Man-3	4.233[b]	4.253
	Man-4	4.186[b]	4.210
	Man-4'	4.130[b]	4.141
	Man-A	4.060[b]	4.060
	GlcUA-7	ND	3.580
NAc	GlcNAc-1 α	1.952[b]	2.036
	GlcNAc-2	2.084[b]	2.077
	GlcNAc-5(6SO4)	2.042[b]	2.051
CH_3	Fuc α / β	1.160/1.144[b]	1.207/1.218

For reference, the structure and the numbering of the residues are given in Fig. 5. ND, not determined, [a] Measured at 60℃, [b] Measured at 27℃, [c] Data from reference 20.

GP5 corresponded to those of four of the eight peaks observed in the case of desulfated PA-MS2 (indicated by the arrowheads in Fig. 2-C). The retention time of the completely desulfated form of PA-MS2 (peak a in Fig. 2-C) corresponded to that of PA-GP5 (peak c in Fig. 2-D). This was confirmed by the co-elution experiment shown in Fig. 2-E. From these results, we speculated that the structure of MS2 was the same as that of GP5, except for an additional mole of sulfate.

In order to determine the location of the additional sulfate, we focused on one of the monosulfated isoforms produced from PA-MS2 by solvolysis, peak b in Fig. 2-C, which was not observed in the solvolytic products of GP5. The purified PA-sugar corresponding to peak d (Fig. 2-F) was not susceptible to endo-β-galactosidase digestion (Fig. 2-G). PA-GP5 (Fig. 4-A), whose oligosaccharide part corresponds to Q2.10 reported by Gallego et al.[20], was susceptible to endo-β-galactosidase digestion (Fig. 4-B), whereas PA-MS2 was not (Fig. 4-C and D). However, endo-β-galactosidase acted on the completely desulfated form of PA-MS2 (data not shown). These results indicated that the galactose residue of MS2 might be sulfated, because endo-β-galactosidase can not act on sulfated galactose[36]. The proposed structure of MS2 oligosaccharide is shown in Fig. 5.

DISCUSSION

The isolation of membrane glycoproteins, such as myelin P0 and PASII/PMP22, has proved to be extremely difficult and laborious. Therefore, only a limited amount of pure oligosaccharides have been obtained from the isolated glycoproteins. To overcome these problems and to prevent the loss of precious material, we developed a method to isolate N-linked oligosaccharides from a certain glycoprotein. First, the glycopeptide fraction was prepared from whole tissue, fat-free peripheral nervous tissue in this case, by non-specific protease digestion. The resultant glycopeptides were fractionated by chromatography; then, N-glycans were obtained from the isolated glycopeptides by glycopeptidase-A digestion, which specifically liberates N-linked oligosaccharides from glycopeptides. By comparing the amino acid sequence of an isolated glycopeptide and those listed in protein sequence databases, we should be able to determine what kind

of glycoprotein the glycopeptide originates from. To analyze the structures of the librated oligosaccharides, we used pyridylamination to label oligosaccharides and ODS-HPLC to isolate them in highly purified form. Based on the hypothesized sugar compositions from mass spectrometric analyses of the purified PA-oligosaccharides, we converted the PA-sugars into a structurally known one by chemical and enzymatic derivatization and compared the derivatives with the known oligosaccharide on ODS-HPLC.

In this paper, we focused on an extremely acidic glycopeptide, MS2p (the suffix p stands for peptide), which was observed in the most retained fraction on anion-exchange chromatography, as shown in Fig. 1. Based on its molecular weight and amino acid sequence, we assigned the origin of the glycopeptide to P0 glycoprotein.

A series of experiments revealed that MS2 contained sulfated glucuronic acid, which forms the HNK-1 carbohydrate epitope[21]. MS2 contained one mole of HNK-1 carbohydrate epitope, in hybrid-type N-glycan as shown in Fig. 5. Although the HNK-1 epitope is prominent in the nervous system and is part of a variety of cell surface glycoproteins[37] and glycolipids[16], the complete carbohydrate structures carrying the HNK-1 epitope have been reported only for the glycoproteins bovine P0[20, 21] and PASII/PMP22[32]. Schachner's group[20, 21] reported most (up to 90%), but not all of the oligosaccharide structures of bovine P0, and the structure presented in this paper has not been reported before.

The HNK-1 epitope is one of the important mediators of precise cell adhesion and the molecular recognition processes that underlie the interaction of neural cells[38]. It has also been implicated in interactions with laminin and peripheral nerve regeneration[39]. This carbohydrate antigen is also found in demyelinating neuropathies[40, 41]. Recently, Senn C *et al.* reported that they have generated mice deficient for the HNK-1 sulfotransferase (ST)[42]. ST-/-mice showed an impaired long-term memory and a poorer spatial learning when a short inter-trial interval was used, although the

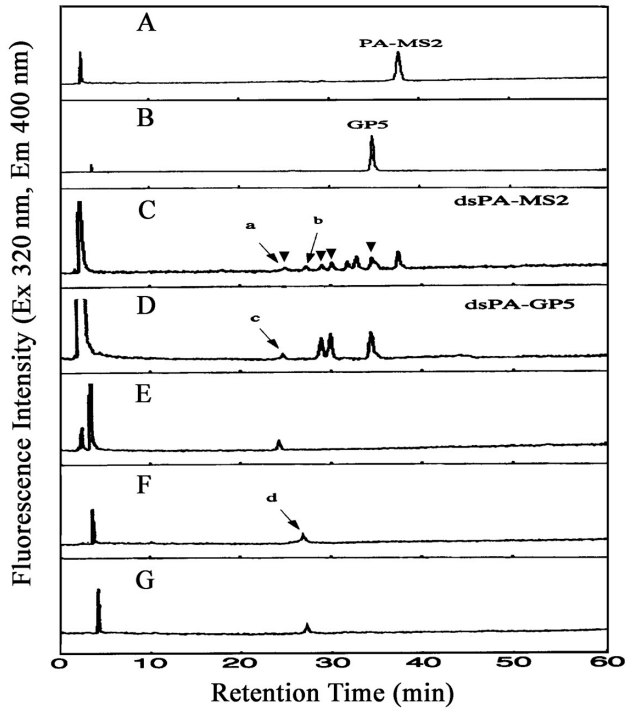

Fig. 2. Comparison of desulfated PA-MS2 with PA-oligosaccharide PA-GP5. A: Intact PA-MS2, B: Intact PA-GP5, C: Desulfated PA-MS2 (dsPA-MS2) after solvolysis, D: Desulfated PA-GP5 (dsPA-GP5) after solvolysis. Peak c corresponds to completely desulfated PA-GP5. Note that the four peaks observed in this panel correspond to the peaks indicated by arrowheads in Panel C, E: A mixture of purified peaks a and c, F: Purified peak b, G: After endo-β-galactosidase digestion of peak d. Note that the enzyme did not act on peak d.

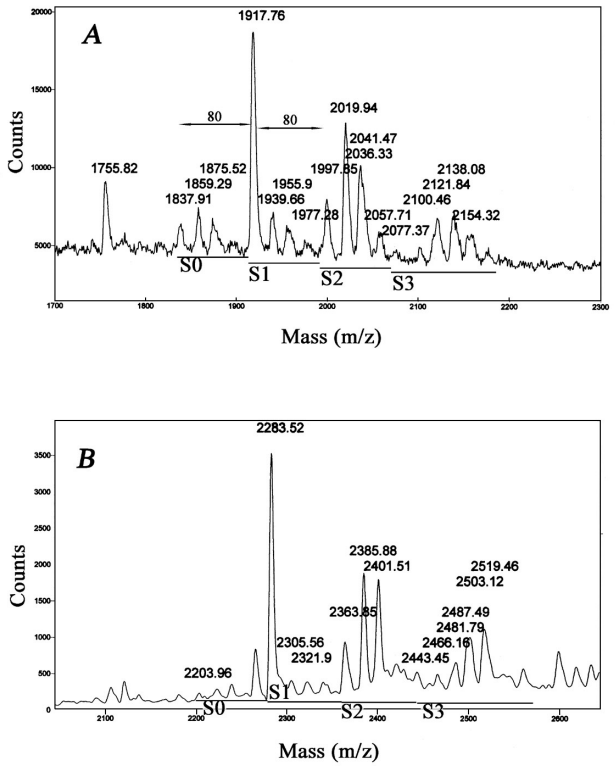

Fig. 3. MALDI TOF mass spectra of PA-MS2 and MS2p. The peaks were divided into four groups; non-(S0 form), mono-(S1 form), di-(S2 form), and tri-(S3 form) sulfated oligosaccharide derivatives of PA-MS1. A: Pyridylaminated oligosaccharide form of MS2 (PA-MS2) B: Glycopeptide form of MS2 (MS2p).

anatomy of all major brain areas appeared histologically normal. These indicate an essential role for the sulfate group of the HNK-1 carbohydrate in synaptic plasticity of the hippocampus. However, the significance of the expression of the HNK-1 epitope should to be determined in further studies.

The other novel feature of the structure reported here is the presence of a sulfate attached to the Gal residue in MS2. Except for this sulfate, the structure is the same as that of a major oligosaccharide of P0 glycoprotein, GP5/Q2.10[20,21]. The sulfate was assigned to the Gal residue, because endo-β-galactosidase acts on desulfated-MS2, but not on intact MS2. The possible sulfation sites are on the 6-, 4-, 3-, and 2-O Gal residues. Of these, site 3-O was substituted by glucuronic acid. 6-O was considered the most likely site of sulfate attachment, because Gal-6-*O*-sulfotransferase activity has been documented in brain[36], while neither Gal-4-nor Gal-2-*O*-sulfotransferases have been reported.

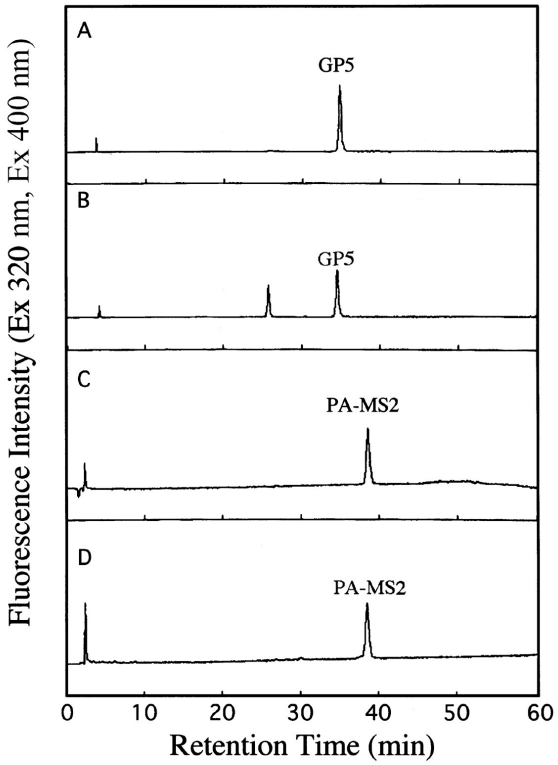

Fig. 4. Endo-β-galactosidase susceptibility of PA-MS2 with GP5. A: Intact PA-GP5, B: PA-GP5 after endo-β-galactosidase digestion, C: Intact PA-MS2, D: PA-MS2 after endo-β-galactosidase digestion, Note that PA-GP5 was susceptible to the enzyme, but not PA-MS2.

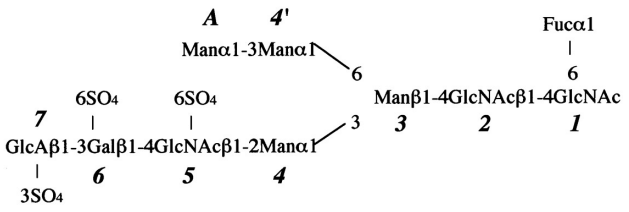

Fig. 5. Proposed structure of MS2 oligosaccharide. The bold italic numbers in the structure refer to the corresponding residues in the Table 1.

The presence of both the GlcNAc-6-*O*-sulfo and Gal-6-*O*-sulfo, in addition to GlcA-3-*O*-sulfo, implies that there are at least three types of sulfotransferase in peripheral nervous tissues. The sulfation of glucuronic acid, which occurs at the onset of myelination and regeneration and ceases after development, is mediated by a highly specific HNK-1 sulfotransferase (HNK-1ST)[43, 44]. Of the various GlcNAc-6-*O*-sulfotransferases identified thus far[45-48], the existence of a nervous system sulfotransferase is described in brain[49].

The finding of a sulfated galactose in MS2 was surprising. A number of specific sulfotransferases have now been characterized, and these include enzymes that attach sulfate to C-3[50] and C-6[36] of Gal. The sulfotransferase responsible for the 6-*O*-sulfation of galactose has a strict requirement for oligosaccharide acceptors that are capped by a α2-3-linked sialic acid residue. This enzyme might be responsible for the 6-*O*-sulfation of 3-*O*-substitued galactose by a glucuronic acid, or another enzyme may be responsible for this. In this context, sulfate blocks the activity of endo-β-galactosidase.

Multiple sulfates and glucuronic acid in MS2 add negative charges to the sugar chains and may alter their functions. Although the relative amount of this type of oligosaccharide in the glycopeptides from whole tissue was small (at most 1.0 %), the proportion in the glycoform of P0 glycoprotein must be much higher. It would be interesting to determine whether its expression is regulated during development and aging, *e.g.*, during Schwann cell development, pre-myelination, myelination, and post-myelination stages. We plan to conduct functional analysis of the oligosaccharide in the future.

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ウシの P0 蛋白の強酸性糖鎖の構造解析

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ミエリン糖タンパク質は細胞膜の相互作用に関係し，ミエリンの形成およびその維持に重要な役割を果たす．P0 蛋白や PASII/PMP22 蛋白のような末梢神経ミエリン糖タンパク質は硫酸化された糖鎖を含有し，これらの糖タンパク質の機能の一部は糖鎖によることが明らかとなっている．最近，ウシの P0 蛋白の糖鎖の構造が報告され，その内のいくつかは HNK-1 エピトープとして知られている硫酸化グルクロン酸を含有する糖鎖構造（SO_4-3GlcAβ1-3Galβ1-4GlcNAc）をもつことが明らかとなった．本論文では，ウシの P0 蛋白の糖鎖のうち最も酸性度の強い糖鎖の構造を一次元の ^{1}H-NMR や質量分析等により解析した．その結果，この糖鎖（MS2）の全構造は，Manα1-3Manα1-6[(3SO_4)GlcAβ1-3(6SO_4)Galβ1-4(6SO_4)GlcNAcβ1-2Manα1-3]Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc. と決定され，今まで報告されたことのない P0 糖タンパク質由来の新奇な硫酸化糖であることが判明した．MS2 には HNK-1 糖鎖中のグルクロン酸 3-O 硫酸に加え，6-O 硫酸化された N-acetylglucosamine および 6-O 硫酸化されたガラクトースを含有していた．このことから，既に報告されているグルクロン酸 3-O 硫酸化転移酵素や GlcNAc 6-O 硫酸化転移酵素に加え，新たに Gal 6-O 硫酸化転移酵素の末梢神経組織における存在が示唆された．

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