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RESEARCH ARTICLE

Preparation and Structural Analysis of a Sialo-Oligosaccharide from Glycophorin in Carp Red Blood Cell (RBC) Membranes

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ABSTRACT

Glycophorins (GPs) in the red blood cell (RBC) membranes of carp (*Cyprinus carpio* L.) exhibit bacteriostatic activity against various gram-negative and gram-positive bacteria. This antibacterial property is attributed to a sialo-oligosaccharide. For a structural analysis of this O-linked sialo-oligosaccharide, it is necessary to prepare and purify the oligosaccharide from carp GPs. To prepare sialo-oligosaccharides, RBC membrane proteins were solubilized, and anion exchange column chromatography was performed to obtain the GPs. The GP yield from carp RBC membranes was ca.100% as total sialic acid. Then, released oligosaccharides were obtained from GP preparation by β-elimination. Second, the resulting oligosaccharide fraction was separated into two sialo-oligosaccharide components (P-1 and P-2) using the same anion exchange column. The oligosaccharide preparations that contained high amounts of NaCl were desalted with an activated charcoal column combined with ammonium bicarbonate in acetonitrile as an eluent. Using NMR to analyse the structure of the antimicrobial P-1, we determined the alignment of each proton and C1 carbon. To determine the linkage sites, we performed GC-MS using the permethylated P-1. Lastly, we determined that the structure of P-1 was NeuGcα2→6 (Fucα1→4) (Glcα1→3) Galβ1→4GalNAc-ol.

Keywords: carp, glycophorin, sialo-oligosaccharide, column chromatography, NMR, GC–MS.

Introduction

Glycophorins (GPs) are transmembrane glycoproteins that contain sialic acid. These glycoproteins are found in red blood cell (RBC; erythrocyte) membranes in humans [1-4], other mammals [5-8] and birds [9, 10]. We demonstrated that GPs in the RBC membranes of carp (*Cyprinus carpio* L.) exhibit bacteriostatic activity against various gram-negative and gram-positive bacteria, including fish pathogens[11]. This physiological property is caused by the monosialyl oligosaccharide (P-1) from carp GP[12]. The fraction containing sialic acid (*N*-glycolylneuraminic acid: NeuGc) exhibited antimicrobial activity whereas the GP fraction without NeuGc and human GP containing *N*-acetylneuraminic acid (NeuAc) did not display this activity[11]. This antimicrobial activity is also observed for yellow tail (*Seriola quinqueradiata*) and red sea bream (*Pagrus major*)[13]. Carp are freshwater fish, and yellow tail and red sea bream are marine red-flesh and white-flesh fish, respectively. Thus, it is assumed that the antimicrobial activity of sialo-oligosaccharides from GP is not confined to these teleost species but can be found in all fish[14].

For a structural analysis of the O-linked sialo-oligosaccharide, the preparation of carp GPs following the oligosaccharide fraction with high yield and high purity is important. Compared to human GPs, carp GPs were difficult to isolate from other RBC membrane proteins. This is because nucleic acids contaminate the membrane preparation. Moreover, sialic acid tends to be released from RBC membranes, leading to a low GP yield. It was also difficult to purify sialo-oligosaccharides. This purification is difficult largely because these oligosaccharides contain sialic acids, which are negatively charged (normally, oligosaccharides are neutral). Sialo-oligosaccharide fractions were difficult to desalt, because the molecular weight of oligosaccharides is close to that of various salts.

It was also difficult to analyse P-1 using NMR because the characterized proton signals revealed an overall downfield shift in the resonance of αGlc and αFuc, except for the H-1 signals. These characteristic signals occurred because the P-1 oligosaccharide exhibited a nonchain-like structure, which differed from those of other mammals[8].

Methods

Collection of carp blood

The study carp were anaesthetized with ethyl 3-aminobenzoate methanesulfonate (MS-222). Once anaesthetized, each fish was placed upside down and blood was collected from the dorsal aorta by inserting a heparinized syringe (22G × 70 needle with a 25 mL syringe; Terumo Co. Japan) through the mouth (Figure 1). After the blood was collected, each anaesthetized carp was waved back and forth by the head so that tank water entered the gills to revive the fish. The blood was diluted 1:1 with Fish Ringer (145 mM NaCl, 5 mM $CaCl_2$, 1 mM $MgSO_4$, 4 mM KCl, 10 mM HEPES, and 5 mM glucose, pH 7.9)[15]. Haemagglutination was not observed when each individual carp blood sample was mixed. To remove white blood cells and plasma from the RBC preparation, we used Ficoll-Paque PLUS (GE Healthcare, Sweden). The diluted blood was placed on Ficoll-Paque and then centrifuged at 400 × g for 40 min at room temperature, and the bottom layer of RBCs was collected[11]. All subsequent procedures were performed at 4 °C.

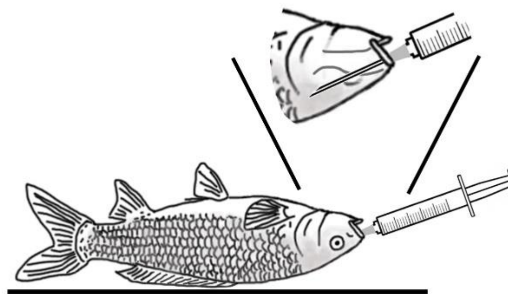

Figure 1. Method of carp blood collection.

Preparation of RBC membranes from carp blood

Haemolysis of the packed RBCs was performed by dilution in a 1:10–15 mixture of ice-cold 5 mM Tris-HCl (pH 7.6) containing 5 mM $CaCl_2$ and 0.15 mM phenylmethylsulfonyl fluoride (PMSF). The suspension was placed on ice for 5 min and then centrifuged at 40,000 × g for 20 min. The upper precipitate layer was collected with a pipette, suspended in ice-cold 5 mM Tris-HCl (pH 7.6) containing 0.15 mM PMSF, and then centrifuged at 40,000 × g for 20 min. The precipitate was collected and suspended in a twofold dilution of Buffer A (75 mM Tris, 12.5 mM $MgCl_2$ and 15 mM EDTA, pH 7.5)[15] containing 5 mM $CaCl_2$ and 0.15 mM PMSF. The suspension was then homogenized with a tight-fitting Dounce homogenizer (10 strokes) and centrifuged at 40,000 × g for 20 min. The resulting membrane pellet was resuspended in Buffer A and homogenized (20 strokes). The membrane suspension was then

placed on a sugar cushion (40% sucrose, 10 mM Tris-HCl and 10 mM $MgCl_2$, pH 7.5) and centrifuged at 700 × g for 15 min in a swing-out rotor. The overlay and interphase fractions were collected and centrifuged at 40,000 × g for 20 min. The membrane pellets were resuspended in Buffer B (20 mM Tris-HCl, 2 mM EDTA, pH 7.5)[15] and homogenized (10 strokes). The final membrane preparation was stored at −20 °C.

Preparation of glycophorins from carp RBC membranes

At the beginning of our research, we prepared GP from carp RBC membranes according to the human GP preparation method[16]. GPs are extracted from RBC membranes in the aqueous phase using phenol with lithium 3,5-diiodosalicylate (LIS). Furthermore, we added the titration step with streptomycin solution to the extract method for removing nucleic acids[11]. Although this extraction method was effective in preparing human GP from RBC membranes, the yield of carp GP from its RBC membranes was extremely low (ca. 1.1% as total sialic acid). This is because sialic acid was released from the GPs during the solubilization process. It is presumed that some sort of sialidase is contained in carp RBC membranes.

Honma et al. reported using anion exchange column chromatography with DEAE-cellulose DE 52 gel (Whatman Inc., NJ, USA) in the presence of a nonionic detergent[17]. However, this procedure was primarily aimed at removing glycolipids from rabbit GP preparations. Gel-filtration chromatography was not adequate to generate a large amount of GP preparation.

Instead of the extraction method using phenol, we developed a method for preparing a large amount of carp GP in which a desalting column was used to remove excess LIS from the fraction, followed by anion exchange column chromatography in the presence of an ionic detergent, sodium cholate[18]. The carp RBC membranes were solubilized in a final concentration of 40 mM Tris-HCl (pH 7.5 containing 0.3 M LIS) and centrifuged at 160,000 × g for 30 min at 4°C. The supernatant was applied to an Econo-Pac 10DG desalting column (Bio-Rad Laboratories) and washed with 50 mM Tris-HCl, pH 7.5. The eluent was fractionated into 1 mL/tube (Figure 2). Then, 2 M NaCl and 10% sodium cholate solutions were added to each fraction containing GP at a final concentration of 130 mM NaCl and 0.4% sodium cholate. The solution of the collected fraction was applied to a column (1.5 × 3.5 cm) of DEAE Toyopearl 650M (Tosoh Corp., Tokyo, Japan) that was equilibrated with 50 mM KPi (pH 7.5, containing 130 mM NaCl and 0.4% sodium cholate). After being washed with five bed volumes of the same buffer, the column was eluted with a continuous linear gradient of 130–500 mM NaCl and 0.4–0.7% sodium cholate in 50 mM KPi, pH 7.5. One fraction containing both neutral sugar and sialic acid by the bar in the figure was pooled in this experiment (Figure 3). The collected solution was dialyzed against deionized water. The Coomassie brilliant blue R-250 (CBB) and periodic acid-Schiff (PAS) staining patterns on the SDS-gels of the fraction (Figure 4) show the same pattern as that of the GP prepared by the extraction method[11]. The resulting GP fraction was stored at −80 °C until use.

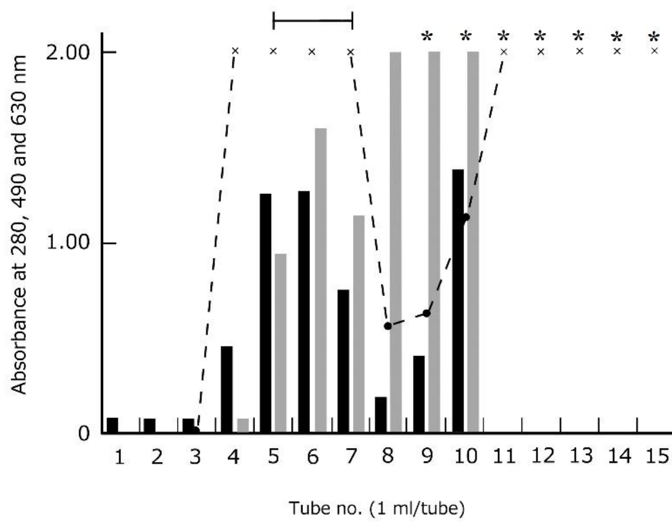

Figure 2. Chromatogram of solubilized RBC membrane preparation on an Econo-Pac 10DG column. The proteins were monitored at 280 nm (-●-); total carbohydrate at 490 nm (■); sialic acid at 490 nm (■). * Precipitation formation. The total carbohydrate and sialic acid contents were measured according to a previous study11.

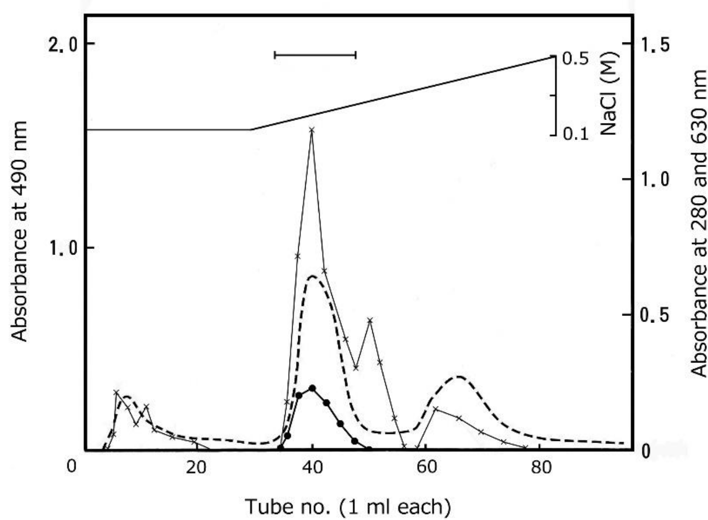

Figure 3. Chromatogram of the Econo-Pac fractions on a DEAE Toyopearl 650M column. The proteins were monitored at 280 nm (- - -); total carbohydrate at 490 nm (- × -); and sialic acid at 490 nm (-●-).

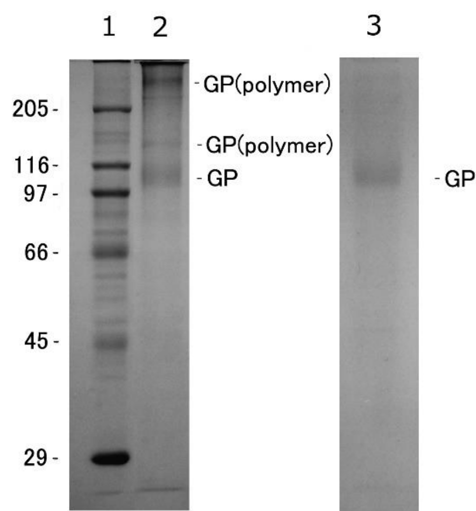

Figure 4. SDS-polyacrylamide gel electrophoresis of carp GP.
Coomassie brilliant blue R-250 (CBB)- and periodic acid-Schiff (PAS)-stained carp GP membranes. Lane 1, CBB-stained molecular mass standards: myosin (205 kDa); β-galactosidase (116 kDa); phosphorylase b (97 kDa); bovine albumin (66 kDa); egg albumin (45 kDa); and carbonic anhydrase (29 kDa). Lane 2, CBB-stained carp GP. Lane 3, PAS-stained carp GP. CBB and PAS staining methods were performed according to a previous study11. Approximately 30 µg of membrane protein was applied per lane.

Isolation and preparation of sialo-oligosaccharides (P-1, P-2) from carp GPs

We routinely obtained O-linked oligosaccharides from the GP preparation by β-elimination. However, the GP preparation obtained by column chromatography easily leads to the aggregation of GPs with each other through hydrophobic areas within the protein moiety. Due to this tendency, the aggregated GP molecules are larger than those prepared by using the extraction method with phenol. It was more difficult for the larger aggregated GP forms to release oligosaccharides from their protein moiety. In a previous study, it seemed that the smallest GP molecules (40 nm diameter) selectively possessed bacteriostatic activity[11]. It is necessary to denature GP protein with phenol prior to β-elimination. Prior to β-elimination, the GP fraction was mixed with an equal volume of freshly prepared 50% phenol in water. This suspension was stirred vigorously for 15 min and centrifuged at 4,000 × g for 1 h in a swinging-bucket rotor. The upper phase was collected and dialyzed against water. Following dialysis, the inner solution was freeze-dried as the GP preparation.

The oligosaccharides were released from the GPs by β-elimination according to the method by Carlson[19]. The carp GP preparation was incubated in 1.0 M $NaBH_4$ and 0.1 M NaOH in the dark at 37 °C for 48 h under N_2 gas. The reaction was neutralized by carefully adding 1 M acetic acid at 0 °C. The mixture was centrifuged at 2,500 × g for 30 min, and the supernatant was evaporated. The concentrate was then dissolved in 5 mL of water and evaporated. This procedure was repeated three times. The preparation was then washed with water and evaporated to form a syrup that was washed with 5 mL of methanol followed by 5 mL of ethanol. The concentrated oligosaccharide alditol preparation was dissolved in 5 mL of water[11].

To separate the sialo-oligosaccharides, we performed anion exchange column chromatography according to the negative charge of sialic acid. The resulting oligosaccharide alditol preparation was separated into two components (P-1 and P-2) using a DEAE Toyopearl 650M column. The oligosaccharide fraction was applied to a column (1.5 × 1.5 cm) equilibrated with 5 mM Tris-HCl buffer (pH 7.5) containing 40 mM NaCl. After being washed with 15 bed volumes of the same buffer at a flow rate of 0.6 mL/min, the column was eluted with the same buffer containing 120 mM NaCl. The UV detector was set at 205 nm. Peak fractions (P-1 and P-2) were pooled and freeze-dried (Figure 5).

Our previous study reported that the P-1 oligosaccharide contained one NeuGc residue, whereas P-2 contained two residues (Figure 6)[12].

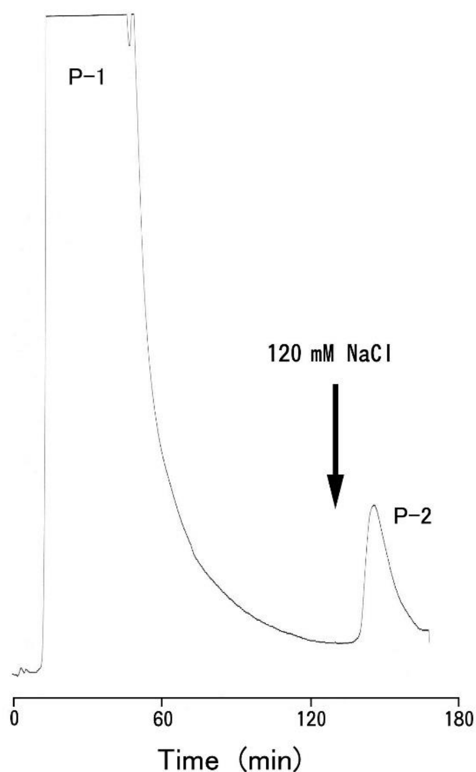

Figure 5. Chromatogram of carbohydrate preparation on a DEAE Toyopearl 650M column. The oligosaccharides were monitored at 205 nm (—).

Figure 6. Thin-layer chromatograms of the P-1 and P-2 fractions. Approximately 10 μg of oligosaccharide was applied to each TLC12.

Desalting the sialo-oligosaccharide (P-1, P-2) fraction

High amounts of NaCl and salts were present in the oligosaccharide fractions obtained by ion exchange chromatography and other preparation steps. The method of desalting the oligosaccharide fraction was primarily gel-filtration chromatography with a Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, CA, USA) column[20-24].

However, Bio-Gel P-2 gel adsorbs specifically to a terminal site on the GalNAc of the P-1 oligosaccharide and it is difficult to release it from the gel. Lectin columns are very effective for selecting specific oligosaccharides. However, with this type of column, it is difficult to elute an oligosaccharide once it is adsorbed. We developed the desalting method by using a graphitized carbon column normally used to prepare neutral oligosaccharides[25]. The adsorption efficiency was strictly related to the concentration of NaCl and oligosaccharides.

Prior to use, a GL-Pak Carbograph cartridge column (300 mg/6 mL, GL Sciences Inc., Tokyo, Japan) was washed with 40% (v/v) acetonitrile and then rinsed with water. The carbon gel was swollen by storage in water for 12 h. The charcoal column was washed with three bed volumes of 80% acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA). After being washed with water, the activated column was washed with 45% (v/v) acetonitrile in 2.5% (w/v) ammonium bicarbonate (one bed volume), rinsed with water and then washed with 80% acetonitrile in 0.1% (v/v) TFA followed by water and 1% NaCl. The freeze-dried oligosaccharide fraction was dissolved in 4 mL of 1% NaCl (ca. 10–20 μg carbohydrate/mL) and applied to the column. The column was washed with 5 bed volumes of water and then eluted with 45% acetonitrile in 2.5% ammonium bicarbonate. Ammonia solution, as well as ammonium bicarbonate, is also effective at eluting oligosaccharides. The eluate (ca. 30 mL) was pooled and evaporated. The resulting sialo-oligosaccharide fraction (P-1) was stored at −80 °C until use.

NMR and GC-MS

^{1}H- and ^{13}C-NMR and 2D NMR experiments (^{1}H-^{13}C HSQC, COSY, H2BC, HMBC, TOCSY and ROESY) were performed using JNM-α500 or JNM-ECA920 spectrometers (JEOL Ltd., Tokyo, Japan). The oligosaccharide preparation was permethylated according to Ciucanu and Kerek[26] prior to GC-MS analysis. The hydrolysis of the permethylated oligosaccharide was conducted with 2 M TFA at 121 °C for 1 h. Reduction was performed by adding DMSO-$NaBD_4$ at 40 °C for 1.5 h, and peracetylation was performed by adding acetic acid, 1-methyl imidazole and acetic anhydride successively. The GC-MS system consisted of an HP5890 gas chromatograph (Hewlett-Packard Co., Palo Alto, PA, USA), a JMS DX-303 mass spectrograph and a JMA DA5000 data module (JEOL).

Results and Discussion

Preparation of O-linked sialo-oligosaccharides

In the case of a carp with 1 kg of body weight, ca. 25 mLof blood per carp was obtained by blood collection. We obtained 3-4 mg of total membrane protein from 1 mL of packed RBCs[11]. Using chromatography methods, we obtained ca. 15 mg of total GP protein from 1 g of total membrane protein. The GP yield from carp RBC membranes was 103% as total sialic acid. We desalted the oligosaccharide fractions by graphitized carbon column chromatography. From 450 µg of GP protein, we obtained 230 µg of P-1 and 15 µg of P-2 as total neutral sugar.

Analysis of an asialo-oligosaccharide from P-1 by NMR

Before the P-1 sialo-oligosaccharide could be analysed, the sialic acid had to be removed because there were too many proton signals on the NMR spectra, preventing detection. Sialic acid was released from the P-1 fraction by adding 5 mM HCl at 80 °C for 50 min under N_2 gas. The reaction was terminated by adjusting the pH to 7.5 at 0 °C followed by desalting.

The ^{1}H-NMR, COSY, TOCSY, HSQC, ROESY and H2BC spectra of the asialo P-1 fraction were obtained. Vliegenthart et al. proposed the concept of a "structural reporter group" as a method to analyse structures in terms of primary structural assignments[27]. Through this concept, the structure of O-linked oligosaccharides can be analysed first from anomeric protons (H-1 signals) and then by assigning other protons for each constituent monosaccharide. We detected the H-1 signals of Glc, Fuc, and Gal, in addition to H-2 of the acetyl group of GalNAc-ol [H-N(-)-C(-CH3)=O]. This identification of the H-2 of GalNAc-ol was also suggested by observing the H-1a and H-1b in the COSY and TOCSY spectra (Figures 7, 8) The presence of an acetyl group suggested that the site was exposed to the outside of the globular-like oligosaccharide structure unlike the chain-like structure (Chart 1). However, we could not assign all the protons of each constituent monosaccharide. Therefore, a structural model of the oligosaccharide had to be studied, and each proton site had to be analysed by NMR spectra[28].

The TOCSY (Figure 8) and HSQC (Figures 9,10) spectra revealed that the asialo P-1 fraction contained Glc, Fuc, Gal and GalNAc-ol in a molar ratio of 1:1:1:1. Based on the 1H-NMR spectrum, the H-1 signal of the Gal residue exhibited a large coupling constant ($J1, 2$ = 8.0 Hz), which indicates β-coupling to Gal. The proton signal proportions on the TOCSY spectrum revealed an overall downfield shift in the resonance of αGlc and αFuc, except for the H-1 signals. All the protons of the asialo P-1 fraction were characterized by TOCSY, COSY, HSQC, ROESY and H2BC (datum was not shown) spectra[12] (Figure 7) (Figure 8) (Figure 9) (Figure 10).

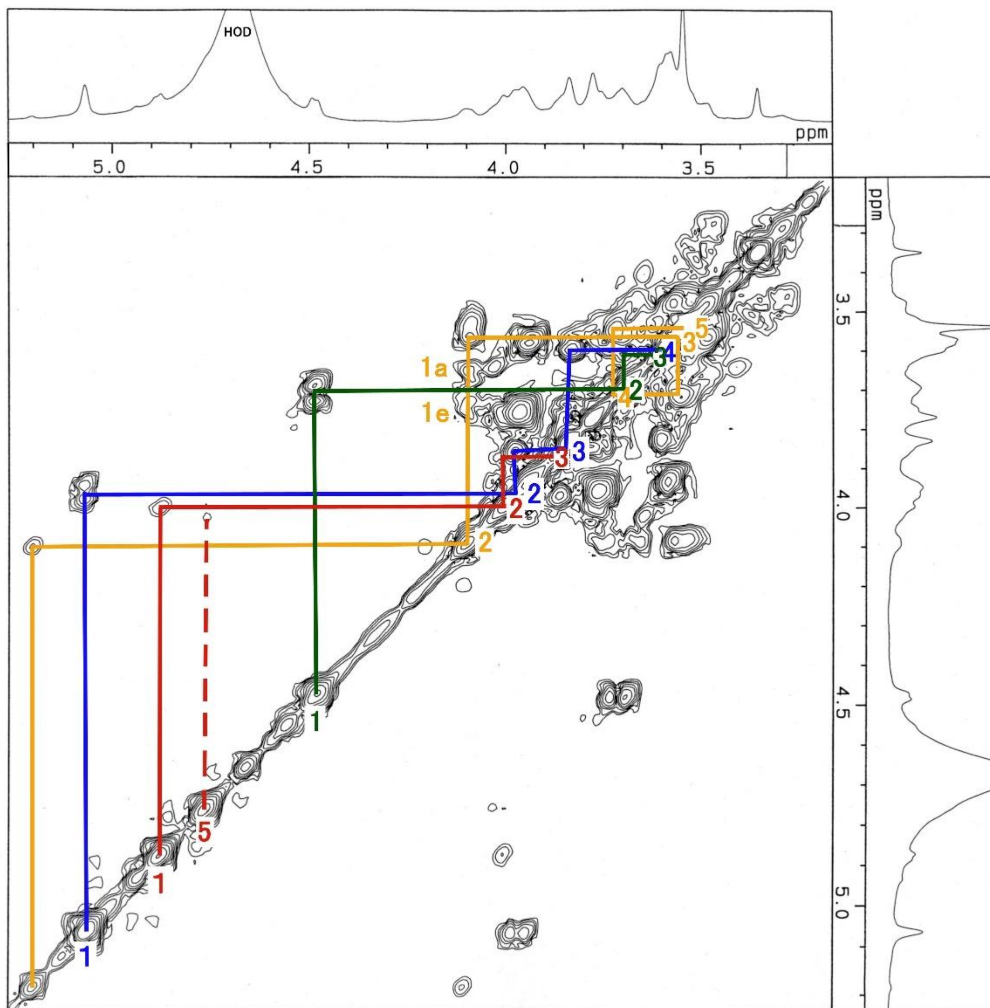

Figure 7. COSY spectrum of the asialo P-1 fraction. The blue line denotes the Glc residue; red line, Fuc; green line, Gal; and brown line, GalNAc-ol. The number denotes the position of the proton. The spectrum was recorded over 256 scans at 500 MHz at 22.5 °C.

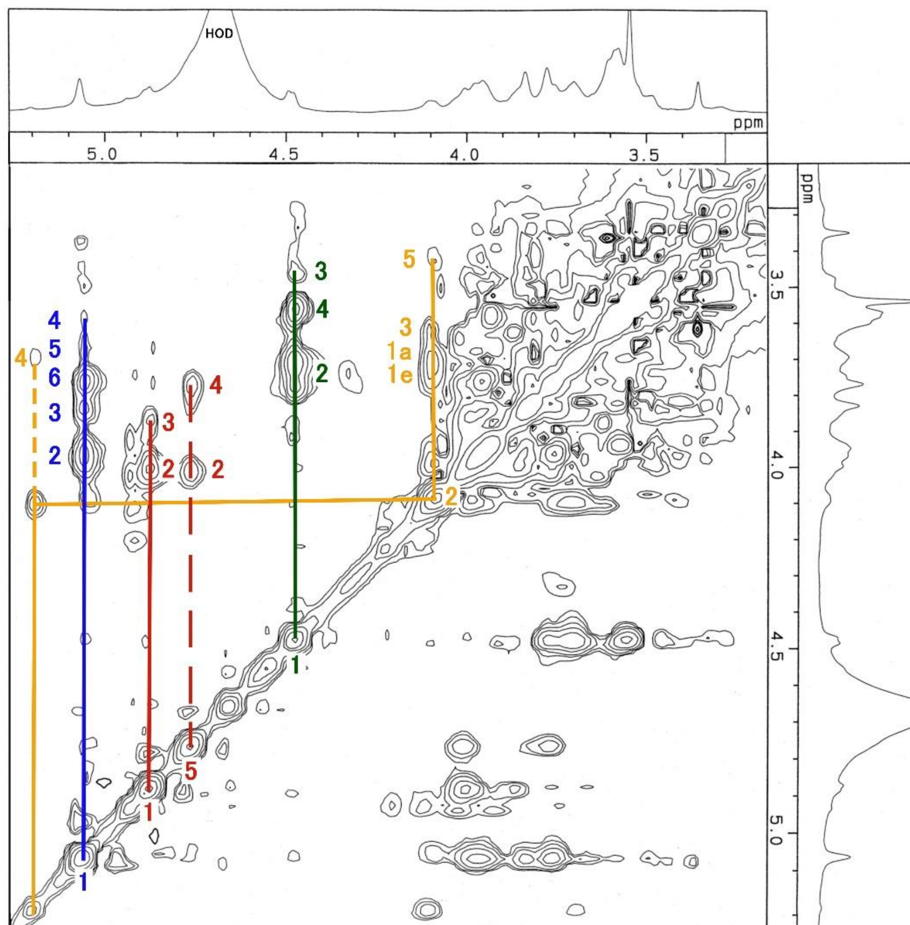

Figure 8. TOCSY spectrum of the asialo P-1 fraction. The blue line denotes the Glc residue; red line, Fuc; green line, Gal; and brown line, GalNAc-ol. The number denotes the position of the proton. The spectrum was recorded over 64 scans at 500 MHz at 22.9 °C.

Analysis of P-1 by NMR

We obtained the TOCSY and HSQC spectra using the intact P-1 fraction (the HSQC spectrum is not shown). In the TOCSY spectrum from the intact P-1 fraction, the H-3e signal of NeuGc (δ = 2.651 ppm) was detected (Figure 11). However, the H-3a signal was not observed. These results suggest that the structure of P-1 is not chain-like, and two hexoses and one hexosamine are attached to a Gal residue.

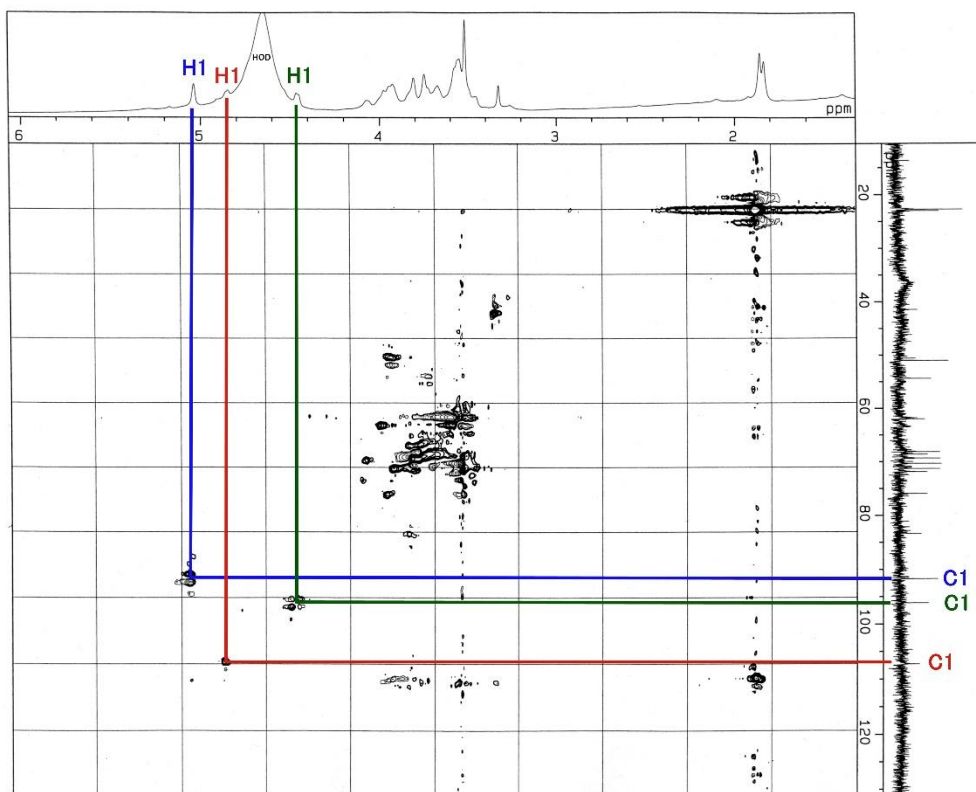

Figure 9. HSQC spectrum of the asialo P-1 fraction. The blue line denotes the Glc residue; red line, Fuc; and green line, Gal. The number denotes the position of the proton and the carbon. The spectrum was recorded over 128 scans at 500 MHz at 22.3 °C.

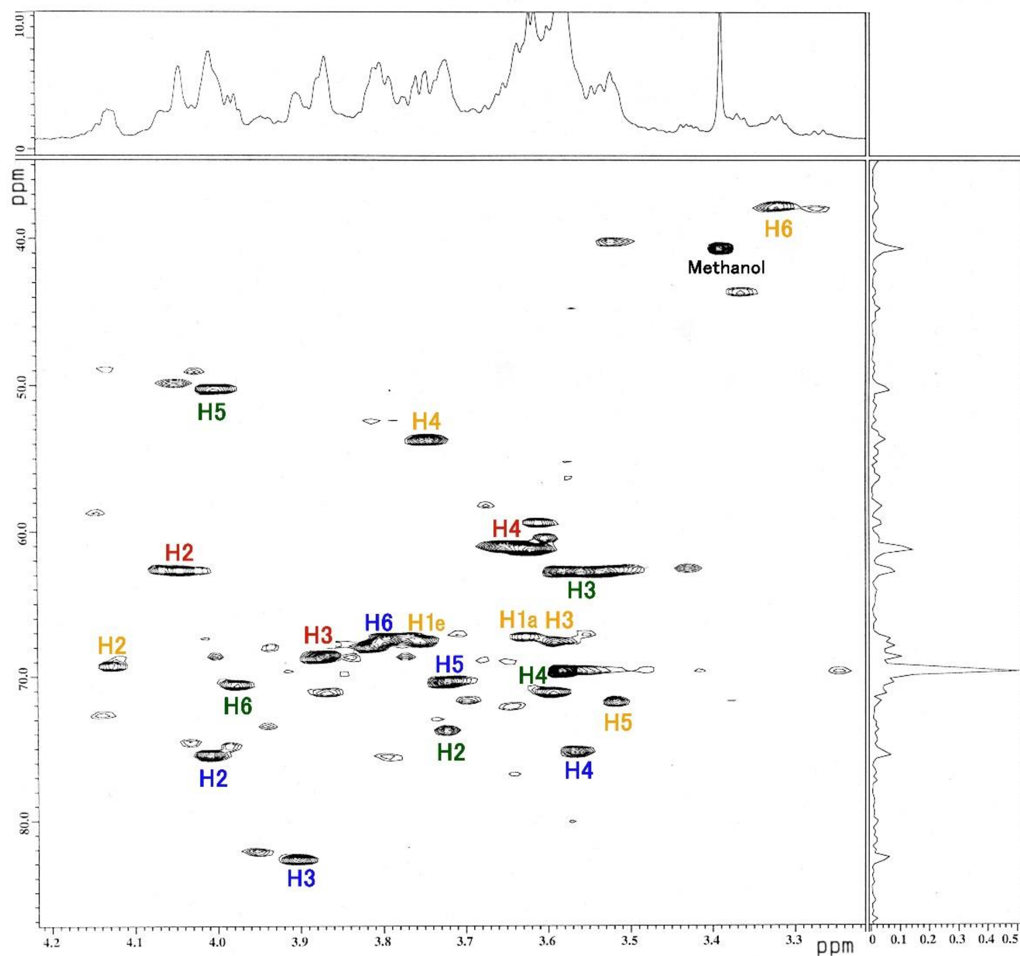

Figure 10. HSQC spectrum of the asialo P-1 fraction. The number denotes the position of the proton. The blue number denotes the Glc residue; red number, Fuc; green number, Gal.; and brown number, GalNAc-ol. The spectrum was recorded over 64 scans at 920 MHz at 25.0 °C.

Analysis of the P-1 linkage site by GC-MS

Although each proton was assigned, the linkage of each constituent monosaccharide was not clear because the P-1 oligosaccharide is unique compared to other chain-like sialo-oligosaccharides. It was necessary to obtain the data by GC–MS.

Based on the GC chromatogram of the permethylated P-1 fraction (Figure 12-a) and the structural elucidation of each methylhexose fraction by MS spectra (Table 1), we determined that the structure of P-1 was NeuGcα2→6(Fucα1→4)(Glcα1→3)Galβ1→4Gal NAc-ol (Chart 1). These determined glycosidic linkages were also supported by the observation of ROESY correlation peaks[12].

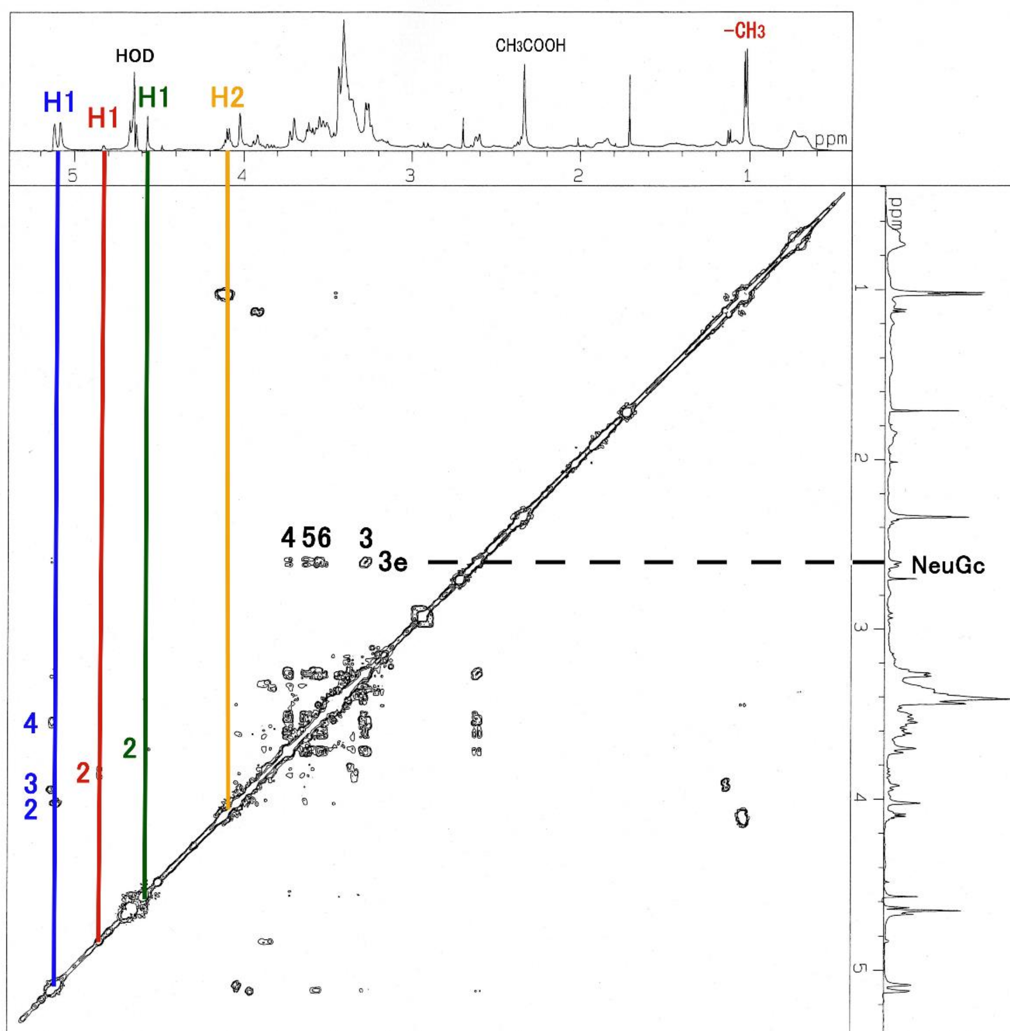

Figure 11. TOCSY spectrum of the intact P-1 fraction. The blue line denotes the Glc residue; red line, Fuc; green line, Gal; and brown line, GalNAc-ol. The number denotes the position of the proton. The spectrum was recorded over 16 scans at 500 MHz at 20.3 °C.

The low reactivity of *N*-acetylhexosamine is caused by the difficulty in detecting hexosamine delivery after permethylation. On the TOCSY spectrum from the intact P-1 fraction, the contaminated acetic acid peak was detected at δ = 2.383 ppm (Figure 11). It was necessary to remove acetic acid from the desalted P-1 preparation for GC–MS analysis. After the successive acetonitrile and methanol washing process[12], hexosamine delivery appeared on the GC chromatogram (Figure 12-a). Compared to the GC chromatogram obtained without the washing process (Figure 12-b), three permethylated pentose structures (peaks 2, 3, and 5) also appeared in the chromatogram. We assumed that these peaks originated from contaminated xylose fibres from the laboratory environment during the washing process.

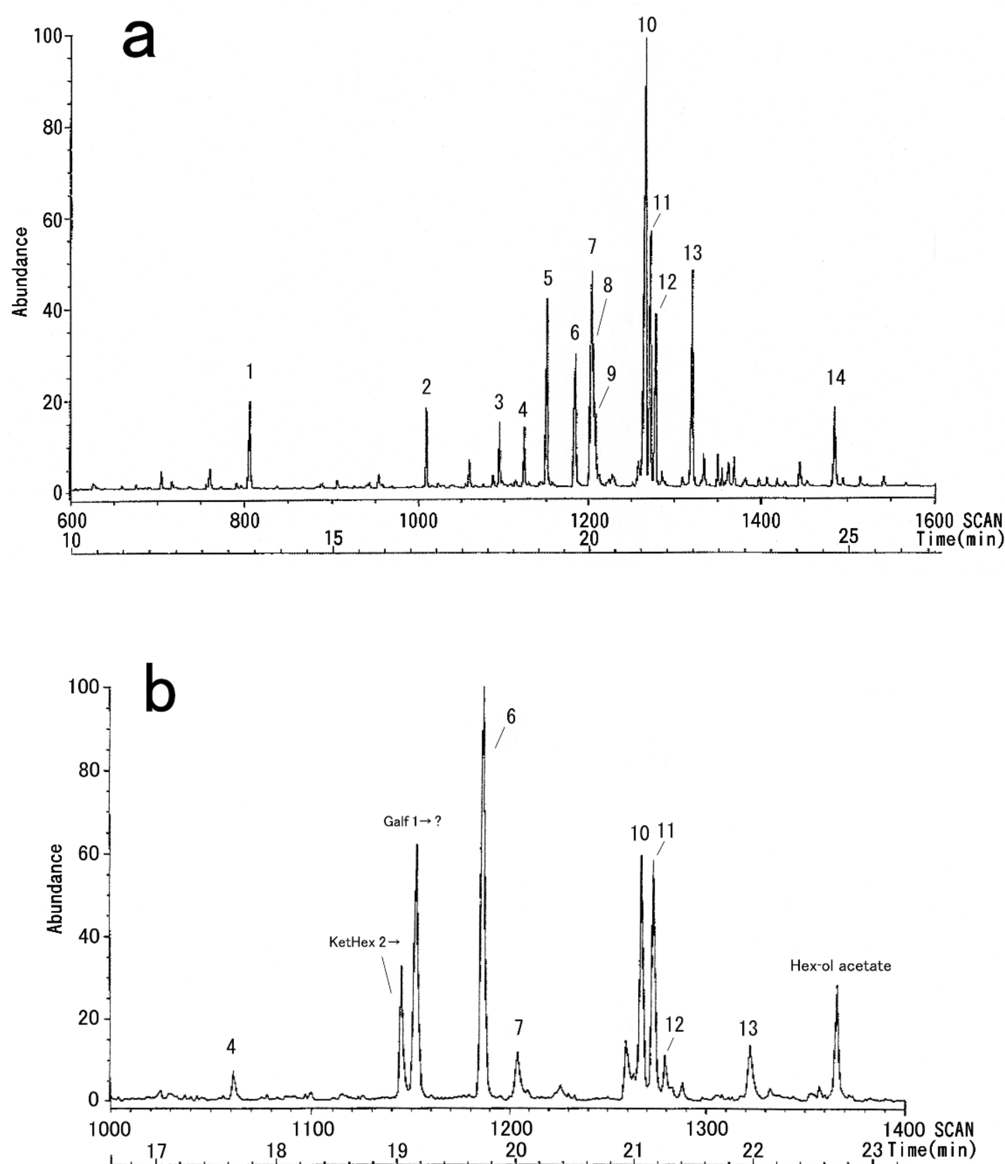

Figure 12. Total-ion chromatogram of permethylated oligosaccharides obtained from the P-1 fraction. (a) GC chromatogram of the P-1 fraction with the washing process. (b) GC chromatogram of the P-1 fraction without the washing process The following peaks were identified: 2, 2,3,5-tri-O-methylpentose; 3, 3,5-di-O-methylpentose; 4, 6-deoxy-2,3,4-tri-O-methylhexose; 5, 2,3-di-O-methylpentose; 6, 2,3,4,6-tetra-O-methylhexose; 7, 2,3,4,6-tetra-O-methylhexose; 8, 2,3,4-tri-O-methylhexose; 9, 2,3,4-tri-O-methylhexose; 10, 2,3,6-tri-O-methylhexose; 11, 2,3,6-tri-O-methylhexose; 12, 2,4,6-tri-O-methylhexose; 13, 2,6-di-O-methylhexose; and 14, 3,6-di-O-methyl-2-N-methylacetoamidehexosamine.

Table 1. Structural elucidation of the total ion chromatogram for permethylated oligosaccharides obtained from the P-1 fraction.

Peak no.	Linkage site	GC retention time (min)	Peak area	MS retention time (min)
1	–	15.297	13928	13.23
2	Pen 1 →	18.747	11924	16.47
3	→ 2 Pen 1 →	20.200	7818	18.13
4	Fuc 1 →	20.747	13925	18.42
5	→ 5 Pen 1 →	21.170	30312	19.09
6	Hex 1 →	21.755	26944	19.43
7	Hex 1 →	22.071	41924	20.02
8	→ 6 Hex 1 →	22.153	28993	20.04
9	→ 6 Hex 1 →	22.205	8515	20.07
10	→ 4 Hex 1 →	23.162	91934	21.05
11	→ 4 Hex 1 →	23.257	39668	21.10
12	→ 3 Hex 1 →	23.361	25210	21.17
13	→ 3,4 Hex 1 →	24.083	39414	21.59
14	→ 4 HexNAc 1 →	27.002	11999	24.43

Pen, pentose; Hex, hexose; HexNAc, *N*-acetylhexose

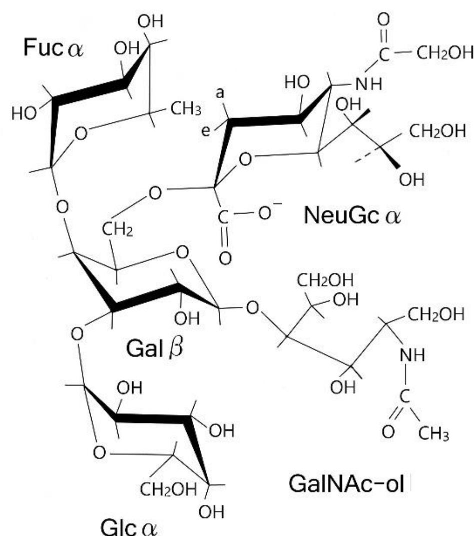

Chart 1. Assumed structure of P-1 oligosaccharide.

Determination of a monosialyl-oligosaccharide (P-1) structure

Human GPs contain O-linked sialo-oligosaccharides, and the structures of these oligosaccharides from RBC membranes have been analysed[29]. The most commonly elucidated GP oligosaccharides include the tetrasaccharide core NeuAcα2→3Galβ1→3(NeuAcα2→6)GalNAc-ol and the trisaccharides Galβ1→3(NeuAcα2→6)GalNAc-ol or NeuAcα2→3Galβ1→3GalNAc-ol[8]. A NeuGc-containing O-linked oligosaccharide has also been reported from horse, pig, goat and rabbit GPs, and the most commonly reported structure is the trisaccharide Galβ1→3(NeuGcα2→6)GalNAc-ol. Other derivatives are synthesized by attaching NeuGc and Gal residues to the trisaccharide core to form a chain-like structure[8].

The carbohydrate fraction of carp GP contained at least two types of O-linked

oligosaccharides (P-1, P-2). The results of several sugar analyses suggested that the P-1 and P-2 fractions were composed of Glc, Fuc, Gal, GalNAc-ol and NeuGc[12].

Glc residue was not detected in the reported O-linked oligosaccharides from mammalian[8] and chicken GPs[30]. Guérardel *et al*. reported that O-glycans synthesized by nematodes contained the Glc residue[31], whereas the Fuc residue was detected in the O-linked oligosaccharides of human GP A[32].

From the NMR spectra, the characterized proton signals of the asialo P-1 fraction revealed an overall downfield shift in the resonance of αGlc and αFuc, except for the H-1 signals. This O-linked oligosaccharide indicates a globular-like structure, not a chain-like one[8].

Furthermore, the linkage between Gal and GalNAc-ol is 1→4, unlike the 1→3 standard linkage for O-linked oligosaccharides. The 1→4 linkage of GalNAc is unique compared with other O-linked oligosaccharides of mammalian origin[8]. Interestingly, the β1→3 glycosidic linkage of xylan, which is a component of the seaweed cell wall, is different from the standard β1→4 linkage of land plants[33]. It is possible to detect the β1→4 linkage of GalNAc in marine organisms.

Conclusion

We found that the GPs in the RBC membranes of carp, yellow tail and red sea bream exhibit bacteriostatic activity[14]. This antibacterial property of carp GPs is caused by the attached sialo-oligosaccharide (P-1)[11]. There is no other report on the physiological activity of sialo-oligosaccharides except some blood group antigens[34].

For the structural analysis of O-linked sialo-oligosaccharides, preparing at high yield and high purity is important. Because the sialo-oligosaccharide is not directly encoded on the carp gene, we cannot adopt the approach by gene analysis. Thus, it is essential to isolate sialo-oligosaccharides from carp GPs and then to desalt them from various salts in the oligosaccharide fraction. We had to develop the method of sialo-oligosaccharide preparation and then the desalting method. To prepare sialo-oligosaccharides, we performed anion exchange column chromatography. The resulting oligosaccharide preparations were desalted using an activated charcoal column combined with ammonium bicarbonate in acetonitrile as an eluent.

The NMR spectra using the desalted oligosaccharide preparation revealed the unique structure of the P-1 oligosaccharide. This O-linked oligosaccharide indicates a globular-like structure unlike other mammalian-origin oligosaccharides[8], and the presence of NeuGc is essential for antimicrobial activity. Compared to the human GP sialo-oligosaccharide, its O-linked tetra-oligosaccharide is a simpler form than that of the carp pentose. Human NeuAc is also simpler than carp NeuGc. It is believed that the antibacterial property of human GPs has been lost over the process of evolution[35].

Abbreviations

GP: glycophorin;
RBC: red blood cell;
NeuGc: *N*-glycolylneuraminic acid;
Ac: acetyl;
Fuc: fucose;
Glc: glucose;
Gal: galactose;
GalNAc-ol: *N*-acetylgalactosaminitol;
NeuAc: *N*-acetylneuraminic acid;
PMSF: phenylmethylsulfonyl fluoride;
LIS: lithium 3,5-diiodosalicylate;
CBB: Coomassie brilliant blue R-250;
PAS: periodic acid-Schiff;
TFA: trifluoroacetic acid.

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