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Chemical characterization of milk oligosaccharides of the common brushtail possum (*Trichosurus vulpecula*)

Tadasu Urashima · Saori Fujita · Kenji Fukuda · Tadashi Nakamura · Tadao Saito · Phil Cowan · Michael Messer

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Abstract Structural characterizations of marsupial milk oligosaccharides have been performed in only three species: the tammar wallaby, the red kangaroo and the koala. To clarify the homology and heterogeneity of milk oligosaccharides among marsupials, 21 oligosaccharides of the milk carbohydrate fraction of the common brushtail possum were characterized in this study. Neutral and acidic oligosaccharides were separated from the carbohydrate fraction of mid-lactation milk and characterized by ^{1}H-nuclear magnetic resonance spectroscopy and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The structures of the 7 neutral oligosaccharides were Gal(β1-3)Gal(β1-4)Glc (3'-galactosyllactose), Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc (3", 3'-digalactosyllactose), Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc, Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc, Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (lacto-N-novopentaose I), Gal(β1-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (galactosyl lacto-N-novopentaose I), Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3)Gal(β1-4)Glc (galactosyl lacto-N-novopentaose II). The structures of the 14 acidic oligosaccharides detected were Neu5Ac(α2-3)Gal(β1-3)Gal(β1-4)Glc (sialyl 3'-galactosyllactose), Gal(β1-3)(O-3-sulfate)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (lacto-N-novopentaose I sulfate a) Gal(β1-3)[Gal(β1-4)(O-3-sulfate)GlcNAc(β1-6)]Gal(β1-4)Glc (lacto-N-novopentaose I sulfate b), Neu5Ac(α2-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc, Neu5Ac(α2-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (sialyl lacto-N-novopentaose a), Gal(β1-3)(−3-O-sulfate)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc, Gal(β1-3)Gal(β1-3)[Gal(β1-4)(−3-O-sulfate)GlcNAc(β1-6)]Gal(β1-4)Glc, Gal(β1-3)[Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (sialyl lacto-N-novopentaose b), Neu5Ac(α2-3)Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc, Gal(β1-3)(−3-O-sulphate)Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc, Neu5Ac(α2-3)Gal(β1-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc, Gal(β1-3)(−3-O-sulphate)Gal(β1-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc, Gal(β1-3)Gal(β1-3)Gal(β1-3)[Gal(β1-4)(−3-O-sulphate)GlcNAc(β1-6)]Gal(β1-4)Glc and Gal(β1-3)Gal(β1-3)[Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (galactosyl sialyl lacto-N-novopentaose b). No fucosyl oligosaccharides were detected. Galactosyl lacto-N-novopentaose II, lacto-N-novopentaose I sulfate a, lacto-N-novopentaose I sulfate b and galactosyl sialyl lacto-N-novopentaose b are novel oligosaccharides. The results are compared with those of previous studies on marsupial milk oligosaccharides.

Keywords Common brushtail possum · Milk oligosaccharides · Marsupials

T. Urashima (✉) · S. Fujita · K. Fukuda
Graduate School of Animal and Food Hygiene, Obihiro University of Agriculture & Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan
e-mail: urashima@obihiro.ac.jp

T. Nakamura
Departmenbt of Food Science, Obihiro University of Agriculture & Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

T. Saito
Graduate School of Agriculture, Tohoku University, Sendai, Miyagi 981-8555, Japan

P. Cowan
Wildlife Ecology & Management, Landcare Research, Lincoln 7640, New Zealand

M. Messer
School of Molecular Biosciences, The University of Sydney, Sydney NSW 2006, Australia

Introduction

Mammalian milk or colostrum contains from a trace to 10 % of carbohydrate in which the disaccharide lactose (Gal(β1-4)Glc) usually predominates over lower concentrations of a variety of oligosaccharides; these mostly have a lactose unit at their reducing ends [1, 2]. In the milk of monotremes, marsupials and some Arctoidea species of Carnivora (ursids, mustelids, pinnipeds), however, oligosaccharides usually predominate over free lactose [2, 3]. Among marsupial species, oligosaccharides have been characterized in the tammar wallaby [4–8], the red kangaroo [9] and koala [10]. The neutral oligosaccharides of milk of the tammar wallaby have been isolated and are characterized by the presence of a major series of galactosyllactoses ranging from Gal(β1-3)Gal(β1-4)Glc to Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc [5, 6], and a minor series of branched oligosaccharides containing β(1–6) linked GlcNAc including Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (lacto-N-novopentaose I) [7, 8]. The acidic oligosaccharides of milk of the red kangaroo were found to contain non reducing *N*-acetylneuraminic acid or sulphate at OH-3 of non reducing Gal residues; their core structures were similar to the core structures of the neutral milk oligosaccharides of the tammar wallaby [9]. Some neutral and acidic milk oligosaccharides of the koala are similar to those of the tammar wallaby and red kangaroo, but koala milk uniquely contains two fucosyl oligosaccharides, *viz.* fucosyl lacto-N-novopentaose I and fucosyl sialyl lacto-N-novopentaose I [10].

The milk oligosaccharides of marsupials other than the above three species have not so far been characterized in detail. To clarify the homology and heterogeneity of milk oligosaccharides among marsupials, in this study we have characterized the neutral and acidic milk oligosaccharides of the common brushtail possum (*Trichosurus vulpecula*).

Materials and methods

Milk carbohydrate sample and chemicals

The sample used in this investigation originated from a study on changes in milk carbohydrates during lactation in the common brushtail possum [11, 12]. This had shown that milk samples obtained during mid-lactation contained a variety of oligosaccharides whereas those obtained early and late in lactation contained mostly free lactose. For more detailed analysis the milk samples obtained from 60 to 147 days post partum [11, 12] were combined and the carbohydrate fraction from this pooled mid-lactation milk was extracted as described by Messer and Mossop [13]. The freeze-dried fraction (440 mg) was stored in a sealed tube at −20 °C for about 26 years, prior to analysis.

Gal(β1-3)Gal(β1-4)Glc (3'-galactosyllactose), Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc (3',3"-digalactosyllactose) and Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc were isolated from tammar wallaby milk [5, 6], while Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (lacto-N-novopentaose I) was isolated from koala milk [10] and brown capuchin colostrum [14]. Neu5Ac(α2-3)Gal(β1-3)Gal(β1-4)Glc (sialyl 3'-galactosyllactose), Neu5Ac(α2-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (sialyl lacto-N-novopentaose a) and Gal(β1-3)[Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (sialyl lacto-N-novopentaose b) were obtained from Bactrian camel colostrum [15], and the mixture of lacto-N-novopentaose a and Gal(β1-3)[Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (sialyl lacto-N-novopentaose c) was isolated from koala milk [10]. Several sialyl or sulfated galactosyllactoses as well as sialyl or sulfated lacto-N-novopentaose I derivatives were separated from red kangaroo milk [9].

Neutral oligosaccharides

Of the carbohydrate fraction of common brushtail possum milk, 150 mg were dissolved in 2 mL of water and the solution passed through a BioGel P-2 column (<45 μm, 2.5×100 cm; Bio-Rad Laboratories, Hercules, CA) that had been calibrated with 2 mg of each galactose (monosaccharide), lactose (disaccharide), and raffinose (trisaccharide). The gel was washed with 0.1 M HCl and 0.1 M NaOH before use. Elution was done with distilled water at a flow rate of 15 mL/h, and fractions of 5 mL were collected. Aliquots (0.5 mL) of each fraction were analyzed for hexose with phenol – H_2SO_4 [16] and for sialic acid with periodate-resorcinol [17]. Peak fractions were pooled as shown in Fig. 1 and freeze-dried. The saccharides in the peak fractions BP-1 to BP-6 (see Fig. 1) were checked by thin layer chromatography using acetone/2-

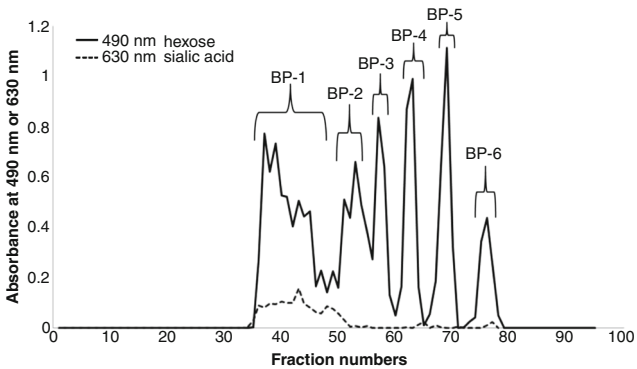

Fig. 1 Gel chromatogram of the carbohydrate fraction from brushtail possum milk on a BioGel P-2 column (2.5×100 cm). Elution was done with distilled water at a flow rate of 15 mL/h and fractions of 5.0 mL were collected. Each fraction was monitored by the phenol-H_2SO_4 method at 490 nm (solid line) and the periodate-resorcinol method at 630 nm (dotted line)

propanol/0.1 mol lactic acid (2:2:1, v/v/v) as a developing solvent. Detection of the spots was done by spraying with 5 % H_2SO_4 in ethanol and heating. Gel filtration was performed three times, each with 150 mg of the carbohydrate fraction, and the corresponding peak fractions were combined.

The components in BP-5 and BP-6 were characterized by ^{1}H-NMR spectroscopy. The components in BP-2 – BP-4 were subjected to high-performance liquid chromatography (HPLC) (chromatograms in Fig. 2). The Hitachi 7,000 series HPLC system (Tokyo) consisted of autosampler L-7,200, a column oven L-7,300, a pump L-7,100, and an evaporation light scattering detector SEDEX-75 with a system controller of D-7,100. The HPLC stationary phase was a 7 μm Hypercarb column (100×4.6 mm i.d.; Thermo Fisher Scientific), and the mobile phase was acetonitrile in distilled water run at 40 °C. The LC gradient was delivered at 1.0 mL/min and consisted of an initial linear increase from 5 to 30 % acetonitrile over 80 min. The oligosaccharides in the separated fractions were pooled, lyophilized and characterized by ^{1}H-NMR spectroscopy and MALDI-TOF mass spectrometry.

Acidic oligosaccharides

The components in peak BP-1 of the gel chromatogram (Fig. 1) that reacted positively with both periodate-resorcinol (630 nm) and phenol-H_2SO_4 (490 nm) were dissolved in 2 mL

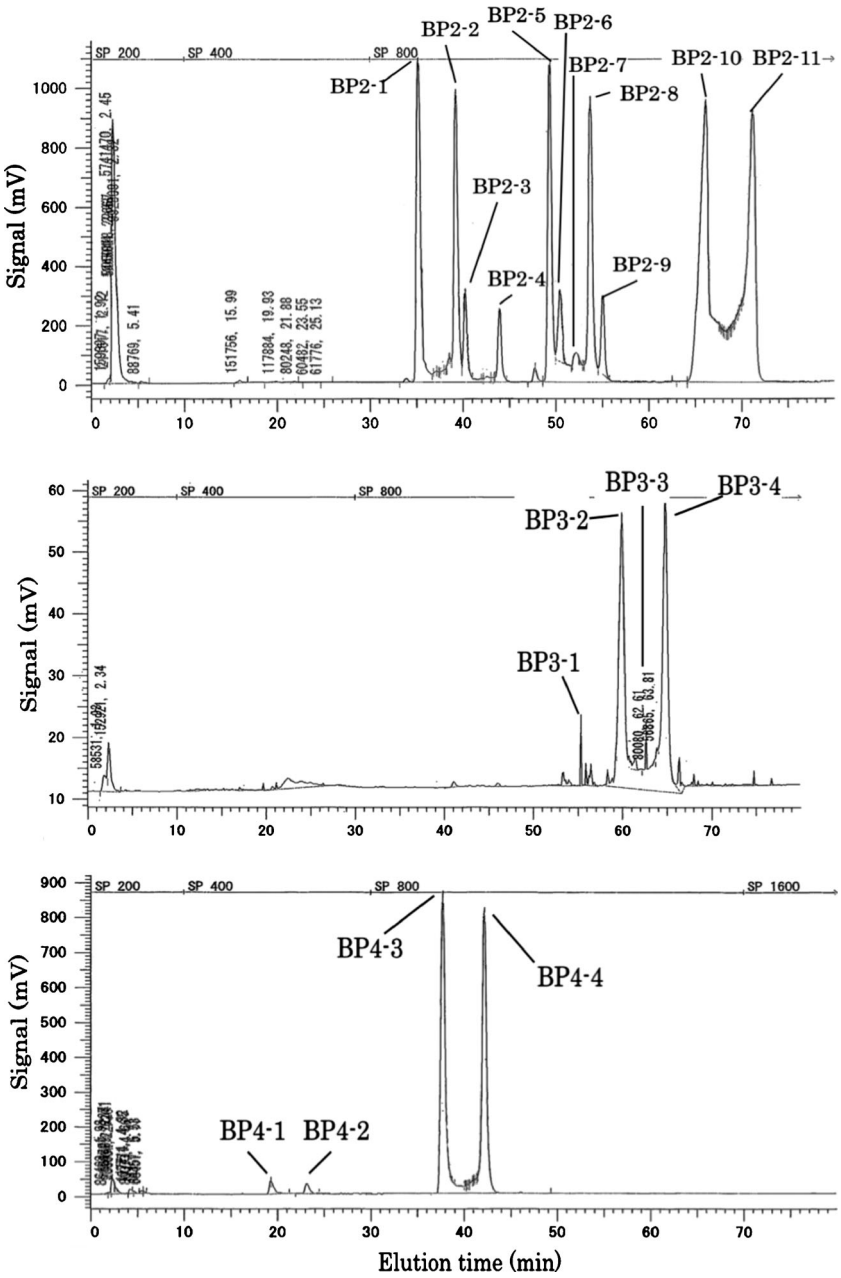

Fig. 2 High performance liquid chromatography of the neutral oligosaccharide fractions BP-2, BP-3 and BP-4 separated from the carbohydrate fraction of brushtail possum milk by gel chromatography (Fig. 1). The Hitachi 7,000 series HPLC system (Tokyo) consisted of autosampler L-7,200, a column oven L-7,300, a pump L-7,100, and an evaporation light scattering detector SEDEX-75 with a system controller D-7,100. The stationary phase was a 7 μm Hypercarb column (100×4.6 mm i.d.; Thermo Fisher Scientific), while the mobile phase was acetonitrile in distilled water run at 40 °C. The LC gradient was delivered at 1.0 mL/min and consisted of an initial linear increase from 5 to 30 % acetonitrile over 80 min

of 50 mmol/L Tris hydroxyaminomethane-HCl buffer solution (pH 8.7) and subjected to anion exchange chromatography on a DEAE-Sephadex A-50 column (2.0×35 cm; GE Healthcare, Uppsala, Sweden) that was equilibrated and eluted with the same solution. Elution was done at a flow rate of 15 mL/h and fractions were analyzed for hexose using phenol-H_2SO_4 method [16]. Fig. 3 shows that the ion exchange chromatography had separated the BP-1 fraction into two peaks. The components in the peak designated BP-1-2 were pooled, lyophilized, dissolved in 2 mL of water, and passed through a column (2.0×35 cm) of BioGel P-2 to remove salts, as described above.

The components in BP-1-2 were then subjected to HPLC on a TSK gel Amide-80 column (4.6×250 mm, pore size 80 Å, particle size 5 μm; Tosoh, Japan (chromatogram in Fig. 4). The mobile phase was 50 % and 80 % (vol/vol) acetonitrile in 15 mmol/L potassium phosphate buffer (pH 5.2). Elution was done using a linear gradient of acetonitrile from 80 to 50 % at 60 °C at a flow rate of 1 mL/min. The eluates were monitored by measuring the absorbance at 195 nm. The peaks designated as BP-1-2-1 to BP-1-2-14 (Fig. 4) were each pooled, concentrated by rotary evaporation, and subjected to ^{1}H-NMR spectroscopy and MALDI-TOF mass spectrometry to determine their structures.

^{1}H-NMR spectroscopy

Nuclear magnetic resonance spectra were recorded in D_2O (99.96 atom D%; Aldrich, Milwaukee, WI) at 500 or 600 MHz for ^{1}H-NMR with a JOEL ECP-500 Fourier transform-NMR (Jeol, Tokyo, Japan) or a Varian INOVA 600 spectrometer (Varian Inc., Palo Alto, CA) operated at

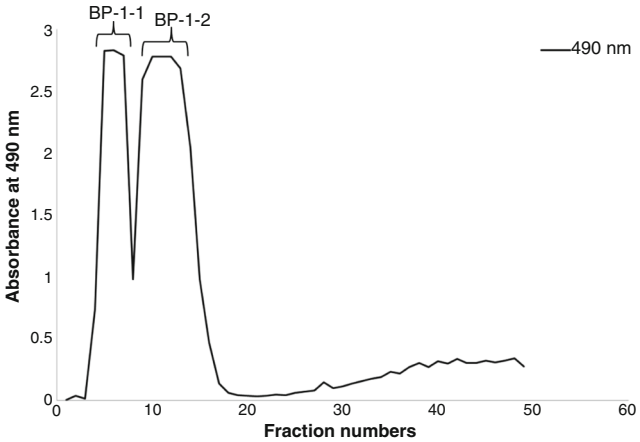

Fig. 3 Anion exchange chromatography of BP-1 (Fig. 1) separated from brushtail possum milk carbohydrate by chromatography on BioGel P-2. A DEAE-Sephadex A-50 column (2.0×35 cm) equilibrated with 50 mmol/L Tris hydroxyaminomethane-HCl buffer (pH 8.7) was used. Elution was done with 250 mL of the buffer. The flow rate was 15 mL/h and fractions of 5 mL were collected. They were monitored by the phenol-H_2SO_4 method

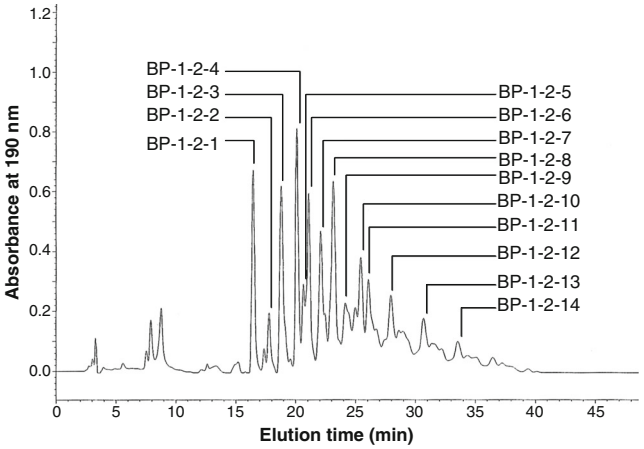

Fig. 4 High performance liquid chromatogram of fraction BP-1-2 (see Fig. 3). The HPLC was done using Shimadzu LC-10 ATVP pump (Shimadzu, Tokyo, Japan) on a TSK-gel Amide-80 column (4.6×250 mm, pore size 80 Å, particle size 5 μm; Tosoh, Tokyo, Japan). The mobile phase was 50 and 80 % (v.v) acetonitrile (CH_3CN) in 15 mmol/L potassium phosphate buffer (pH 5.2). Elution was done using a linear gradient of CH_3CN from 80 to 50 % at 60 °C at a flow rate of 1 mL/min. The detection was done by UV absorption at 195 nm

293.1 K. Chemical shifts are expressed as change relative to internal 3-(trimethylsilyl)-1-propane sulfuric acid, sodium salt, but measured by reference to internal acetone (δ=2.225).

Mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on the oligosaccharide fractions, using an Autoflex II TOF/TOF mass spectrometer (Brucker Daltonics, Bremen, Germany). Lyophilized oligosaccharide fractions were dissolved in 5 μl of milli-Q water. The oligosaccharide solution was mixed with equal volume of 10 mg/mL SDHB (Bruker Daltonics), which is a mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid, saturated in milli-Q water, spotted on a MTP 384 target plate ground steel T F (Bruker Daltonics), and dried. Mass spectra were obtained using a pre-installed method, RP_0–2 kDa (a reflector positive ion mode focusing on the mass range up to 2 kDa). Peptide calibration standard II (Bruker Daltonics) was used for external calibration of the mass spectrometer.

Results

Characterization of neutral saccharides

The crude carbohydrate fraction (total 450 mg) from brushtail possum milk separated into several peaks during gel filtration on BioGel P-2. Since the components in BP-2 to BP-6 (Fig. 1) did not react positively with periodate – resorcinol they were

considered to be neutral oligosaccharides. The components in BP-2 to BP-4 were subjected to HPLC using a Hypercarb column, as shown in Fig. 2. The resulting peaks were designated as BP-2-1 to BP-2-11, BP-3-1 to BP-3-4 and BP-4-1 to BP-4-4. The separated peak components obtained by gel filtration and HPLC were characterized by ^{1}H-NMR and MALDI-TOF MS spectra.

BP-6

As the ^{1}H-NMR spectrum of BP-6 (chemical shifts in Supplemental Table 1) was identical to that of authentic lactose, the saccharide in this fraction was characterized to be lactose.

BP-5, BP-4-1, BP-4-2

As the ^{1}H-NMR spectrum of BP-5 (chemical shifts in Supplemental Table 1) was identical to the published data [18] for authentic 3'-galactosyllactose, the oligosaccharide in this fraction was characterized to be Gal(β1-3)Gal(β1-4)Glc. In addition, since the ^{1}H-NMR spectra of BP-4-1 and BP-4-2 were both identical with that of BP-5, these were also identified to be Gal(β1-3)Gal(β1-4)Glc. The peaks BP-4-1 and BP-4-2 contained the same saccharides, which separated into α- and β-anomer isomers during HPLC using the Hypercarb column.

BP-4-3, BP-4-4

As the ^{1}H-NMR patterns of BP-4-3 and BP-4-4 were identical, it was concluded that these two peaks contained the same saccharides, which separated into α- and β-anomer isomers during HPLC using the Hypercarb column. As the ^{1}H-NMR spectrum of BP-4-3 and BP-4-4 (chemical shifts in Supplemental Table 1) was identical to the published data [18] for authentic 3',3"-digalactosyllactose, the oligosaccharide in these fractions was characterized to be Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc.

BP-3-2. BP-3-4, BP-2-6, BP-2-9

As the ^{1}H-NMR patterns of BP-3-2 and BP-3-4 were identical, it was concluded that these two peaks contained the same saccharides. The oligosaccharides in these fractions were characterized by comparing their ^{1}H-NMR spectra with those of BP-4-3 and BP-4-4. The spectrum of BP-3-2 (Fig. 5, chemical shifts in Supplemental Table 1) had the H-1 of α-Glc, β-Glc, internal β(1–3) linked Gal, non reducing β(1–3) linked Gal, and β(1–4) linked Gal at δ 5.224, 4.667, 4.678, 4.618 and 4.512, respectively, similar to those of BP-4-3 and BP-4-4. However, the spectrum of BP-3-2 had the additional internal β(1–3) linked Gal at δ 4.683, indicating the presence of an additional β(1–3) linked Gal residue. The total signal intensity of H-4 of β(1–3) or β(1–4) linked Gal, which were substituted at OH-3, at δ 4.203 and 4.198 of BP-3-2, was relatively higher than that of BP-4-3; this is consistent with the additional presence of an internal β(1–3) linked Gal. From these observations, the oligosaccharides in BP-3-2 and BP-3-4 were characterized to be Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc. The ^{1}H-NMR spectrum was essentially similar to that of authentic Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc, which had been separated from tammar wallaby milk. As the ^{1}H-NMR spectra of BP-2-6 and BP-2-9 were identical with those of BP-3-2 and BP-3-4, the saccharides in BP-2-6 and BP-2-9 were also identified to be Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc; evidently a small quantity of this pentasaccharide was contained in peak BP-2 as well as BP-3.

BP-2-1

As the ^{1}H-NMR spectrum of BP-2-1 (chemical shifts in Supplemental Table 1) was identical to the published data for authentic lacto-N-novopentaose I [10, 14], the oligosaccharide in this fraction was characterized to be Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc.

BP-2-5, BP-2-8

As the ^{1}H-NMR and MALDI TOF mass spectra of BP-2-5 were essentially similar to those of BP-2-8, it is concluded that these two peaks contained the same saccharides. The oligosaccharide in this fraction was characterized by comparison of its ^{1}H-NMR with that of BP-2-1 and the published data for authentic lacto-N-novopentaose I [10, 14]. The spectrum (Fig. 6, Chemical shifts in Supplemental Table 1) had the H-1 shifts of α-Glc, β-Glc, β(1–6) linked GlcNAc, non reducing β(1–3) linked Gal, and two β(1–4) linked Gal at δ 5.224, 4.670, 4.651 and 4.644, 4.616, and 4.500 and 4.472, respectively, and H-4 of β(1–3) linked Gal, which was substituted at OH-3, at δ 4.180, and NAc of β(1–6) linked GlcNAc at δ 2.063, similar to those in BP-2-1. In addition, the spectrum had H-1 of internal β(1–3) linked Gal at δ 4.677, and H-4 of β(1–3) linked Gal, which was substituted at OH-3, at δ 4.204. From these observations, the saccharide in BP-2-5 was characterized to be Gal(β1-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (galactosyl lacto-N-novopentaose I).

The MALDI-TOF mass spectrum of this saccharide had the MS ions at 1054.318 and 1070.318 of [M + Na] and [M + K], respectively.

BP-2-2

The oligosaccharide in this fraction was characterized by comparison of its ^{1}H-NMR with that of the published data [18] for authentic lacto-N-novopentaose II (Gal(β1-3)[GlcNAc(β1-6)]Gal(β1-3)Gal(β1-4)Glc). The spectrum of BP-2-2 (Fig. 7, chemical shifts in Supplemental Table 1) had

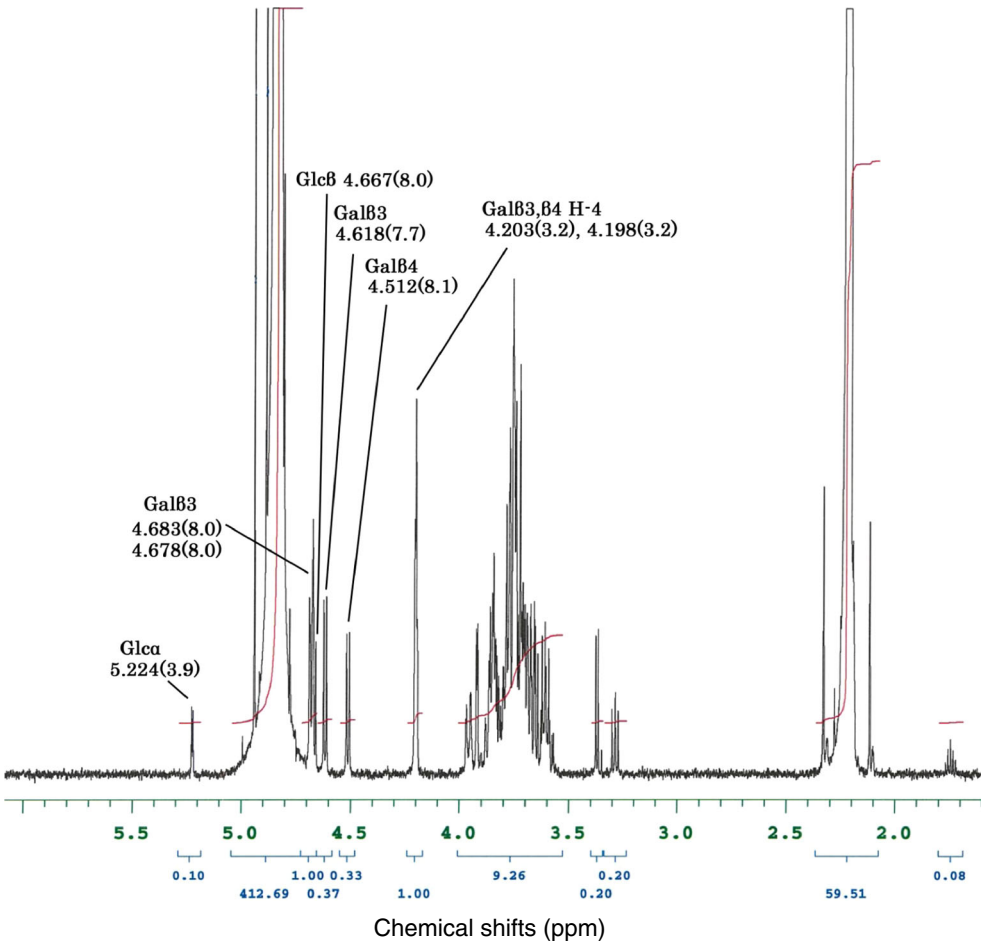

Fig. 5 ^{1}H-NMR spectrum of the oligosaccharide in BP-3-2 isolated from brushtail possum milk carbohydrate by HPLC (Fig. 2). The spectrum was obtained in D_2O at 600 MHz with a Varian INOVA spectrometer operated at 293.1 K. Chemical shifts are expressed relative to internal 3-(trimethylsilyl)-1-propane sulfuric acid, sodium salt

the H-1 shifts of α-Glc, β-Glc, internal β(1–3) linked Gal, non reducing β(1–3) linked Gal, and two of β(1–4) linked Gal at δ 5.225, 4.668, 4.681, 4.616, and 4.513 and 4.471, respectively, and H-4 of β(1–4) and β(1–3) linked Gal, which were substituted at OH-3, at δ 4.181 and 4.171, respectively. In addition, the spectrum had the H-1 shift at δ 4.591 and NAc shift at δ 2.044, which arose from β(1–6) linked GlcNAc, as those of lacto-N-novopentaose II were observed at δ 4.568 and 2.047, respectively [18]. The H-1 chemical shift of β(1–6) linked GlcNAc at δ 4.591 shifted down field from that of lacto-N-novopentaose II at δ 4.568 due to substitution of this residue by β(1–4) linked Gal. From these observations, the saccharide in BP-2-2 was characterized to be Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3)Gal(β1-4)Glc (galactosyl lacto-N-novopentaose II).

The MALDI-TOF of this saccharide mass spectrum had the MS ions at 1054.337 and 1070.312 of [M + Na] and [M + K], respectively.

BP-2-10, BP-2-11

As the ^{1}H-NMR patterns of BP-2-10 and BP-2-11 were identical, it was concluded that these two peaks contained the same saccharides. The oligosaccharides in these fractions were characterized by comparison of their ^{1}H-NMR with that of BP-2-9. The spectrum of BP-2-10 (Supplemental Fig. 1, chemical shifts in Supplemental Table 1) had the H-1 shifts of α-Glc, β-Glc, two internal β(1–3) linked Gal, non reducing β(1–3) linked Gal and β(1–4) linked Gal at δ 5.224, 4.667, 4.683 and 4.679, 4.617, and 4.512, respectively, and H-4 of β(1–3) and β(1–4) linked Gal, which were substituted at OH-3, at δ 4.203. However, the relative intensity of the shift at δ 4.683 was higher than that of BP-2-9, corresponding to two internal β(1–3) linked Gal residues. In addition, the relative intensity of the shift at δ 4.203 was higher than that of BP-2-9. From these observations, the oligosaccharides in BP-2-10 and BP-2-11 were characterized to be Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc.

The MALDI-TOF mass spectrum of this saccharide had the MS ions at 1013.287 and 1029.280 of [M + Na] and [M + K], respectively.

BP-3-1, BP-3-3, BP-2-3, BP-2-4, BP-2-7

The oligosaccharides in these fractions were not characterized in this study, as the sample amounts were too small.

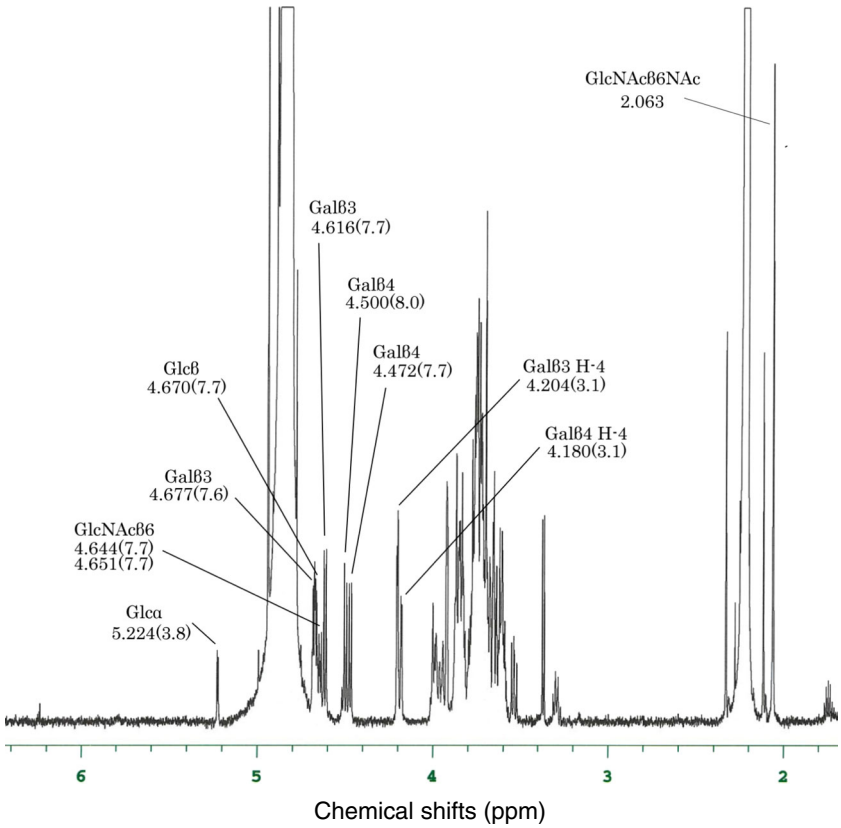

Fig. 6 ^{1}H-NMR spectrum of the oligosaccharide in BP-2-5 isolated from brushtail possum milk carbohydrate from by HPLC (Fig. 2)

Characterization of acidic oligosaccharides

Fraction BP-1 separated into two peaks during ion exchange chromatography, as shown in Fig. 3. The first peak designated as BP-1-1, was thought to contain a mixture of high molecular weight neutral oligosaccharides, which were not investigated in this study. The components in the second peak, designated as BP-1-2, were further separated by HPLC, as shown in Fig. 4. The separated oligosaccharides were characterized by ^{1}H-NMR and MALDI TOF mass spectrometry.

BP-1-2-1

As the ^{1}H-NMR spectrum (chemical shifts in Supplemental Table 2) of BP-1-2-1 was identical with the published data [9, 10, 15] for sialyl 3'-galactosyllactose, the oligosaccharide in this fraction was characterized to be Neu5Ac(α2-3)Gal(β1-3)Gal(β1-4)Glc.

BP-1-2-2

As the ^{1}H-NMR (Fig. 8, chemical shifts in Supplemental Table 2) had the characteristic down field resonances of H-3 doublet and H-4 doublet doublet of β-Gal at δ 4.340 and 4.297, respectively, as found in that of lactose 3'-O-sulfate [19], it was concluded that the saccharide in BP-1-2-2 contained sulfate linked to OH-3 of a β-Gal residue. The ^{1}H-NMR spectrum had the H-1 shifts of α-Glc, β-Glc, β(1–6) linked GlcNAc, and two β(1–4) linked Gal at δ 5.223, 4.671, 4.654 and 4.646, and 4.504 and 4.473, respectively, NAc of β(1–6) linked GlcNAc at δ 2.063, and H-4 of β(1–4) linked Gal, which was substituted at OH-3, at δ 4.182. The down field chemical shift at δ 4.729 arose from H-1 of β(1–3) linked Gal, resulting from the attachment of sulfate to this residue. From these observations, one oligosaccharide in BP-1-2-2 was characterized to be Gal(β1-3)(O-3-sulfate)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (BP-1-2-2-1, lacto-N-novopentaose I sulfate a).

On the other hand, the spectrum had two minor H-1 shifts at δ 4.610 and 4.590, which arose from the minor oligosaccharide in this fraction. The shift at δ 4.610 was assigned to H-1 of non reducing β(1–3) linked Gal, which was not substituted by sulfate. The shift at δ 4.590 arose from H-1 of β(1–4) linked Gal, which was substituted by sulfate. From these observations, another saccharide in this fraction was characterized to be Gal(β1-3)[Gal(β1-4)(O-3-sulfate)GlcNAc(β1-6)]Gal(β1-4)Glc (BP-1-2-2-2, lacto-N-novopentaose I sulfate b).

The MALDI-TOF mass spectrum of these saccharides had the MS ions at 1010.252, 1026.232, 1087.120 and 1103.053 of [M + K + Na]. [M+2 K], [M+3 K+Na-H] and [M+4 K-H], respectively.

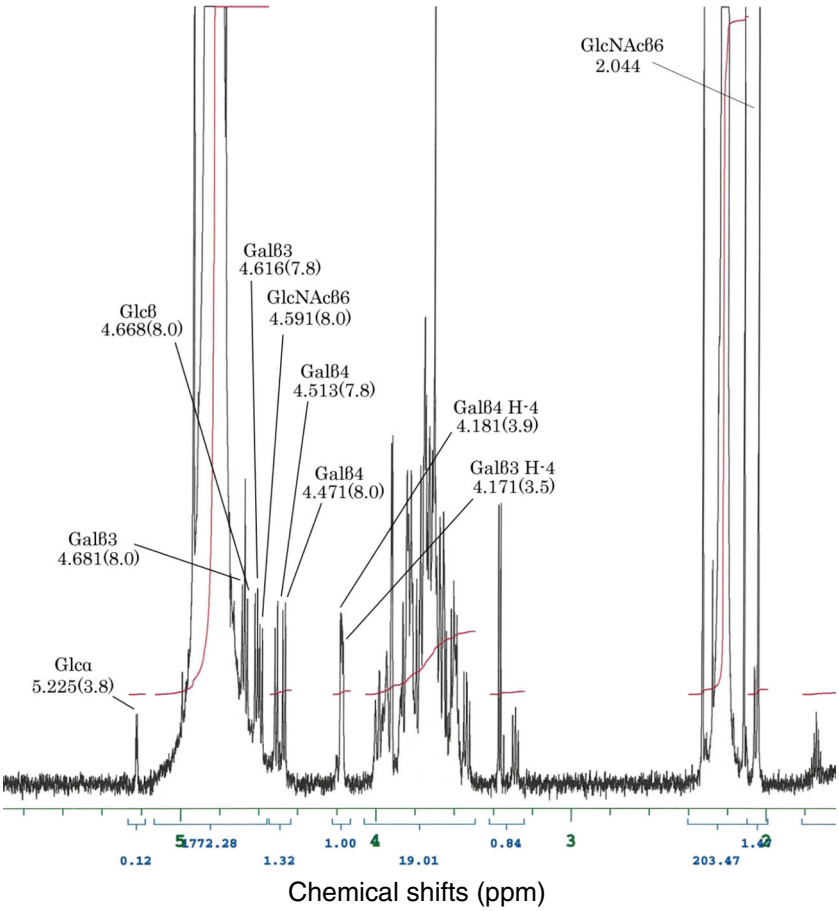

Fig. 7 ^{1}H-NMR spectrum of the oligosaccharide in BP-2-2 isolated from brushtail possum milk carbohydrate by HPLC (Fig. 2)

From comparison of the signal heights of δ 4.729 and δ 4.590, the ratio of BP-1-2-2-1 to BP-1-2-2-2 was estimated to be 3.6 : 1.

BP-1-2-3

The ^{1}H-NMR spectrum (chemical shifts in Supplemental Table 2) had the H-1 shifts of α-Glc, two β(1–3) linked Gal, β-Glc and β(1–4) linked Gal at δ 5.225, 4.693 and 4.681, 4.665 and 4.513, respectively, H-4 of β(1–3) and (β1-4) linked Gal, which were substituted at OH-3, at δ 4.201 and 4.195. The spectrum had H-3 axial and equatorial of α(2–3) linked Neu5Ac at δ 1.804 and 2.763, respectively, and its NAc shift at δ 2.028, and H-3 of β(1–3) linked Gal, which was substituted by α(2–3) linked Neu5Ac, at δ 4.116. As these chemical shifts were essentially similar to those of Mr-1-1-5-1, separated from red kangaroo milk [9], the oligosaccharide in BP-1-2-3 was characterized to be Neu5Ac(α2-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc (BP-1-2-3-1). The MALDI-TOF mass spectrum had the MS ions at 996.034, 1034.267 and 1075.301 of [M + K,]M+2 K-H] and [M+3 K-H], respectively.

However, the spectrum had other shifts at δ 4.444 (doublet), 4.166 (doublet doublet), and 4.076 (doublet), suggesting that BP-1-2-3 contained another saccharide. In addition, the H-1 shift of β(1–3) linked Gal at δ 4.733, doublet doublet shift of H-3 of β(1–3) linked Gal at δ 4.341, and doublet shift of H-4 of β(1–3) linked Gal at δ 4.297 at low intensities confirmed the presence of another oligosaccharide, in which non reducing Gal was sulfated at OH-3. This might be Gal(β1-3)(*O*-3-sulfate)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc, the same as in Mr-1-1-5-2 from red kangaroo milk [9].

BP-1-2-4

The ^{1}H-NMR spectrum (Supplemental Fig. 2, chemical shifts in Supplemental Table 2) had the H-1 shifts of α-Glc, β(1–3) linked Gal, β-Glc, β(1–6) linked GlcNAc, and two β(1–4) linked Gal at δ 5.224, 4.686, 4.671, 4.644, and 4.504 and 4.472, respectively, H-4 of β(1–4) linked Gal, which was substituted at OH-3, at δ 4.176, H-3 of β(1–3) linked Gal, which was substituted by α(2–3) linked Gal at δ 4.116, H-3 axial and equatorial of α(2–3) linked Neu5Ac at δ 1.802 and 2.763, respectively, and NAc of β(1–6) linked GlcNAc and α(2–3) linked Neu5Ac at δ 2.061 and 2.029. As these chemical shifts were essentially similar to those of KM-1-1-3-2 separated from koala milk [10], and of CC-1-2-5 separated from Bactrian camel colostrum [15], one oligosaccharide in

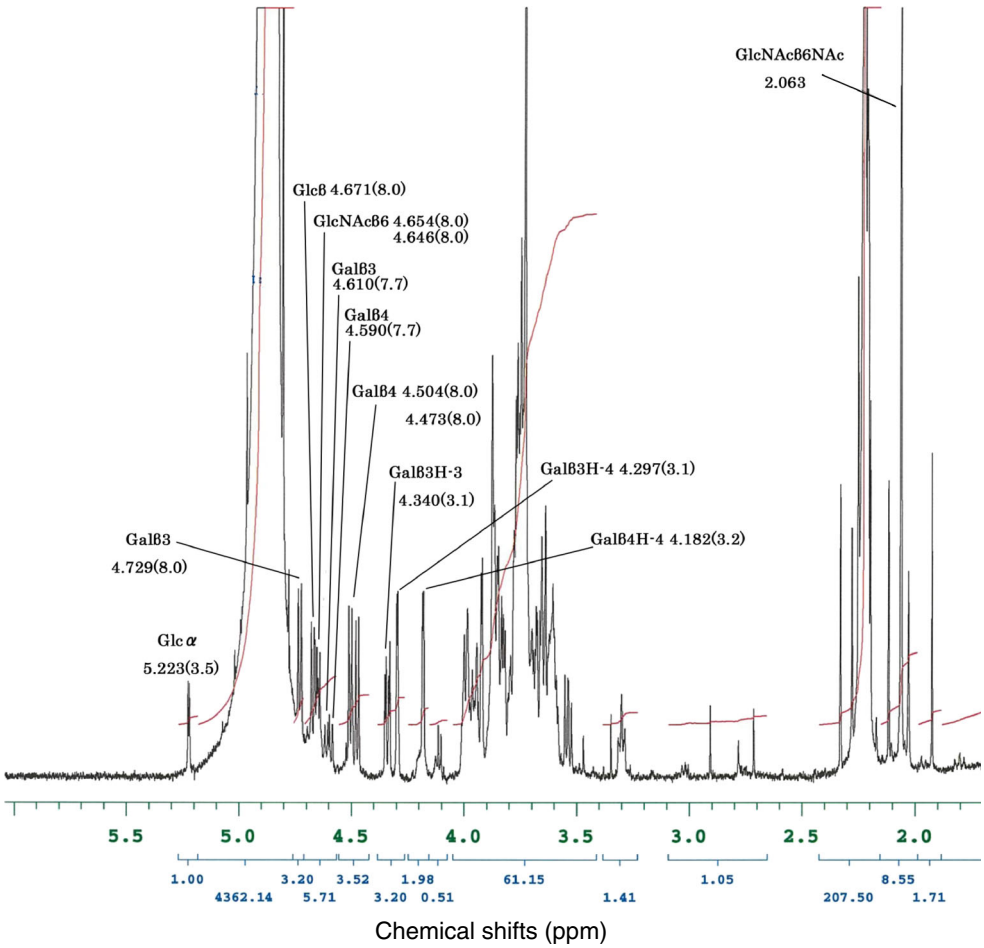

Fig. 8 ^{1}H-NMR spectrum of the oligosaccharides in BP-1-2-2 isolated from brushtail possum milk carbohydrate by HPLC (Fig. 4)

BP-1-2-4 was characterized to be Neu5Ac (α2-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (BP-1-2-4-1, sialyl lacto-N-novopentaose a). The MALDI-TOF mass spectrum of this saccharide had the MS ion at 1237.343 of [M+3 K-H].

On the other hand, the spectrum had the characteristic down field shifts of H-3 and H-4 of β-Gal, which was substituted by sulfate at OH-3, at 3.440 and 4.298, respectively, showing the existence of another saccharide which contained sulfate. The spectrum had the H-1 of β(1–3) linked Gal and β(1–4) linked Gal, which were substituted by sulfate, at δ 4.733 and 4.592, respectively. From these observations, the fraction BP-1-2-4 contained Gal (β1-3)(−3-O-sulfate)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (BP-1-2-4-2) and Gal(β1-3)Gal(β1-3)[Gal(β1-4)(−3-O-sulfate)GlcNAc(β1-6)]Gal(β1-4)Glc (BP-1-2-4-3) as in Mr-1-1-6 of the red kangaroo [9]. The MALDI-TOF mass spectrum of these saccharides had the MS ion at 1188.269 of [M+2 K].

Furthermore, the small NAc shift of β(1–6) linked GlcNAc at δ 2.046 suggested the presence of low concentrations of one or more other oligosaccharides, which contained Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3)Gal(β1-4)Glc unit.

From comparison of the signal heights of δ 4.116 and δ 4.440, the ratio of sialyl oligosaccharide (BP-1-2-4-1) to sulfated oligosaccharides (BP-1-2-4-2 and BP-1-2-4-3) was estimated to be 2.0 : 1.

BP-1-2-5

This fraction did not yield a clearly resolved ^{1}H-NMR spectrum because of insufficient material.

BP-1-2-6

The ^{1}H-NMR (chemical shifts in Supplemental Table 3) had the H-1 shifts of α-Glc, β-Glc, β(1–6) linked GlcNAc, β(1–3) linked Gal and two β(1–4) linked Gal at δ 5.225, 4.667, 4.659, 4.611, and 4.505 and 4.445, H-4 of β(1–4) linked Gal, which was substituted at OH-3, at δ 4.187, H-3 axial and equatorial of α(2–6) linked Neu5Ac at δ 1.719 and 2.665, respectively, and NAc of β(1–6) linked GlcNAc and α(2–6) linked Neu5Ac at δ 2.089 and 2.028, respectively,. As these were essentially similar to those of Mr-1-1-7-1 and CC-1-2-6 from red kangaroo milk [9] and Bactrian camel colostrum

[15], respectively, an oligosaccharide in BP-1-2-6 was characterized to be Gal(β1-3)[Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (BP-1-2-6-1, sialyl lacto-N-novopentaose b). The MALDI-TOF mass spectrum had the MS ion at 1237.355 of [M+2 K-H].

The spectrum had other H-3 axial and equatorial shifts of α(2–3) linked Neu5Ac at δ 1.803 and 2.763, respectively, and H-3 of β(1–3) linked Gal, which was substituted by α(2–3) linked Neu5Ac, at 4.118. The spectrum also had H-1 of β(1–3) linked Gal at δ 4.696 and 4.678, of β(1–4) linked Gal at δ 4.513, and H-4 of β(1–3) Gal, which was substituted at OH-3, at δ 4.200. From these observations, another oligosaccharide in this fraction was characterized to be Neu5Ac(α2-3)Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc (BP-1-2-6-2), similar to Mr-1-1-7-2 in red kangaroo milk [9]. The MALDI-TOF mass spectrum had the MS ion at 1158.317 of [M + K].

In addition, the spectrum had the characteristic down field shifts of H-3 and H-4 of β linked Gal, which was substituted by sulfate at *O*H-3, at δ 4.341 and 4.297, respectively, and H-1 of β(1–3) linked Gal, which was substituted by sulfate, at δ 4.734. As these were similar to the chemical shifts of Mr-1-1-7-3 of red kangaroo milk [9], it is concluded that the fraction contained Gal(β1-3)(−3-O-sulfate)Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc (BP-1-2-6-3).

From comparison of the signal heights of δ 1.719 and δ 1.803, the ratio of BP-1-2-6-1 to BP-1-2-6-2 was estimated to be 1.4 : 1. From comparison of the signal heights of δ 4.118 and δ 4.341, the ratio of BP-1-2-6-2 to BP-1-2-6-3 was estimated to be 1.8 : 1. Thus, the ratio of BP-1-2-6-1 : BP-1-2-6-2 : BP-1-2-6-3 was calculated to be 2.5 : 1.8 : 1.

BP-1-2-7

The ^{1}H-NMR spectrum (Supplemental Fig. 3, chemical shifts in Supplemental Table 3) had the H-1 shifts of α-Glc, β(1–3) linked Gal, β-Glc, β(1–6) linked GlcNAc and two β(1–4) linked Gal at δ 5.225, 4.680, 4.669, 4.644, and 4.501 and 4.473, respectively, H-4 of β(1–3) linked Gal, which was substituted at OH-3, at δ 4.200, H-4 of β(1–4) linked Gal, which was substituted at OH-3, at δ 4.180. The spectrum also had H-3 of β(1–3) linked Gal, which was substituted by α(2–3) linked Neu5Ac, at δ 4.116, H-3 axial and equatorial of α(2–3) linked Neu5Ac at δ 1.802 and 2.763, respectively, NAc of β(1–6) linked GlcNAc and α(2–3) linked Neu5Ac at δ 2.062 and 2.029, respectively. The MALDI TOF mass had the MS ions at 1361.444 and 1399.407 of [M + K] and [M+2 K-H], respectively. From these observations, one oligosaccharide in BP-1-2-7 was characterized to be Neu5Ac(α2-3)Gal(β1-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (BP-1-2-7-1) as in Mr-1-1-8-1 of the red kangaroo [9].

The spectrum had the H-3 and H-4 shifts of β-linked Gal, which was substituted at OH-3 by sulfate, at δ 4.340 and 4.297, respectively, showing the presence of another sulfated saccharide in this fraction. The spectrum had other shifts of H-1 of β(1–3) linked Gal, which was substituted by sulfate, at δ 4.733, H-1 of β(1–3) linked Gal at δ 4.617, H-1 of β(1–4) linked Gal, which was substituted by sulfate, at δ 4.590. The MALDI TOF mass had the MS ion at 1350.336 of [M+2 K]. From these observations, this fraction also contained Gal(β1-3)(−3-*O*-sulfate)Gal(β1-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (BP-1-2-7-2) and Gal(β1-3)Gal(β1-3)Gal(β1-3)[Gal(β1-4)(−3-*O*-sulfate)GlcNAc(β1-6)]Gal(β1-4)Glc (BP-1-2-7-3).

The spectrum also had another NAc shift of β(1–6) linked GlcNAc at δ 2.041 and H-1 of β(1–4) linked Gal at δ 4.514, showing the presence of other saccharides, which contained a Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3)Gal(β1-4)Glc unit.

From comparison of the signal heights of δ 4.116 and δ 4.340, the ratio of sialyl oligosaccharide (BP-1-2-7-1) to sulfated oligosaccharides (BP-1-2-7-2 and BP-1-2-7-3) was estimated to be 1.2 : 1.

BP-1-2-8

The ^{1}H-NMR spectrum (Fig. 9, chemical shifts in Supplemental Table 3) of BP-1-2-8 had the H-1 shifts of α-Glc, β-Glc, β(1–6) linked GlcNAc, non-reducing β(1–3) linked Glc, two β(1–4) linked Gal at δ 5.226, 4.670, 4.660, 4.617, and 4.501 and 4.445, respectively, H-4 of β(1–4) linked Gal, which was substituted at OH-3, at δ 4.178, H-3 axial and equatorial of α(2–6) linked Neu5Ac at δ 1.718 and 2.666, respectively, and NAc of β(1–6) linked GlcNAc and α(2–6) linked Neu5Ac at δ 2.089 and 2.028, respectively. In addition, this spectrum also had H-1 shift of another β(1–3) linked Gal, which was not in non reducing end, at δ 4.684. The MALDI-TOF mass had the MS ions at 1361.446 and 1399.419 of [M + K] and [M+2 K-H], respectively. From these observations, one oligosaccharide in this fraction was characterized to be Gal(β1-3)Gal(β1-3)[Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (BP-1-2-8-1, galactosyl sialyl lacto-N-novopentaose b).

However, the spectrum had other chemical shifts of β(1–3) linked Gal and three β(1–4) linked Gal at δ 4.734, and 4.593, 5.513, and 4.476, respectively, H-3 and H-4 of β-linked Gal, which was substituted by sulfate at OH-3, at δ 4.341 and 4.298, respectively. The spectrum also had H-4 of β(1–3) linked Gal, which was substituted at OH-3, at δ 4.202, H-3 of β(1–3) linked Gal, which was substituted by α(2–3) linked Neu5Ac, at δ 4.108, H-3 axial and equatorial of α(2–3) linked Neu5Ac at δ 1.803 and 2.763, respectively, and NAc of β(1–6) linked GlcNAc at δ 2.068 and 2.044. This fraction contained other oligosaccharides which were not, however, characterized at this stage.

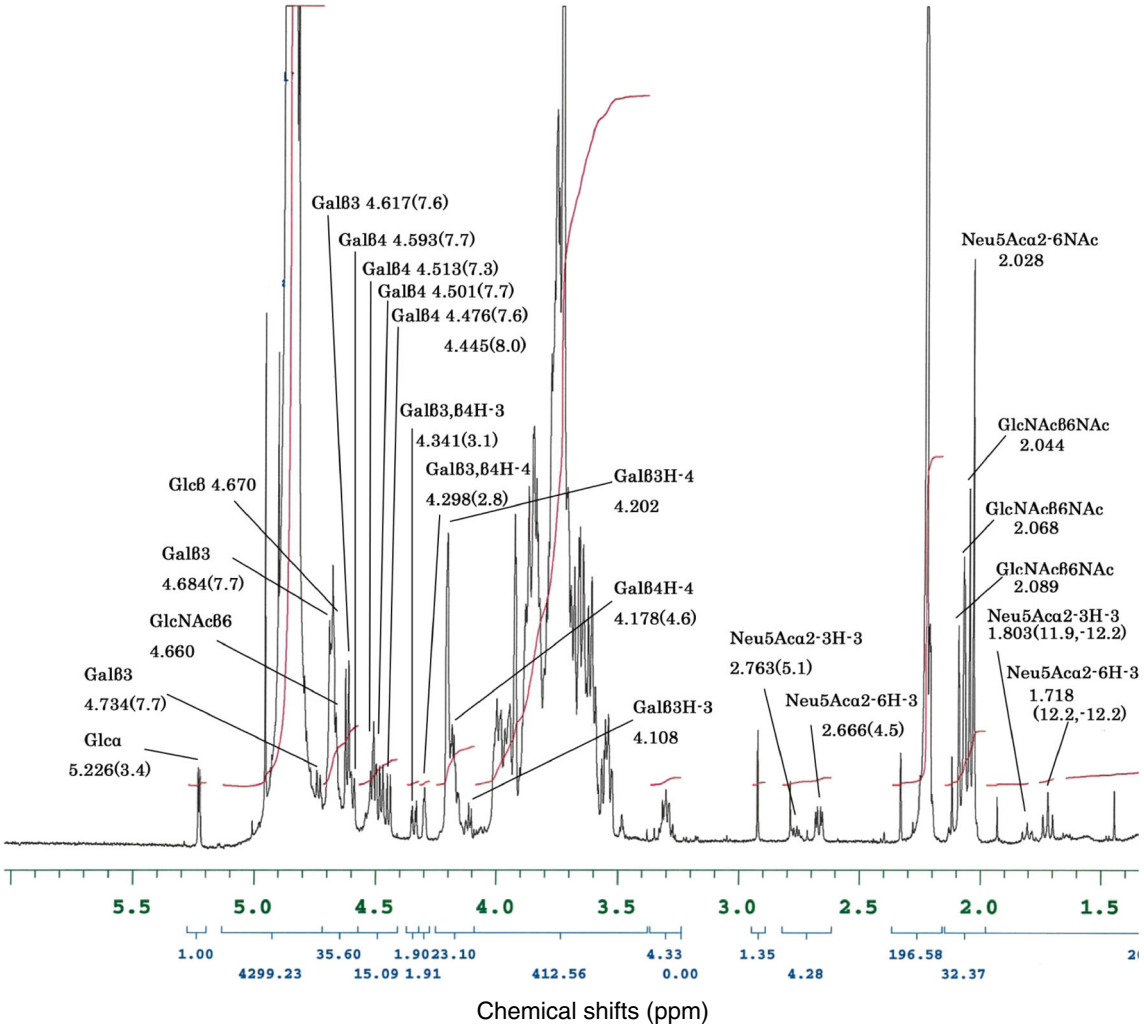

Fig. 9 ^{1}H-NMR spectrum of the oligosaccharides in BP-1-2-8 isolated from brushtail possum milk carbohydrate by HPLC (Fig. 4)

BP-1-2-9 to BP-1-2-14

The oligosaccharides in BP-1-2-9 to BP-1-2-14 were not characterized in this study, as the chemical shifts in their ^{1}H-NMR spectra were not well resolved.

Discussion

Gross and Bolliger, using paper chromatography, were the first to show that milk of the common brushtail possum contains carbohydrates other than lactose [20, 21]. These carbohydrates were found to consist mainly of a compound or compounds which, upon acid hydrolysis, yielded only galactose. The exact nature of this "galactan" was not determined. Subsequent studies on two macropod marsupials showed that their milk carbohydrates consisted of oligosaccharides that were composed mainly of galactose and smaller amounts of glucose, *N*-acetylglucosamine and sialic acid [4, 13].

There were no further studies on the milk of the common brushtail possum until those of Cowan [11] and Crisp *et al.* [12]. The latter authors used thin layer chromatography to show that mid-lactation possum milk contains a variety of oligosaccharides rich in galactose, some of which appeared to be similar, if not identical, to those that had been isolated from milk of the tammar wallaby (*Macropus eugenii*) and whose detailed structures had been determined [5–8].

The present study on the mid-lactation carbohydrate fraction of brushtail possum milk confirms that it consists of a variety of galactosyl oligosaccharides and provides detailed information on the chemical structures of these oligosaccharides. The structures of the 7 neutral and 14 acidic milk

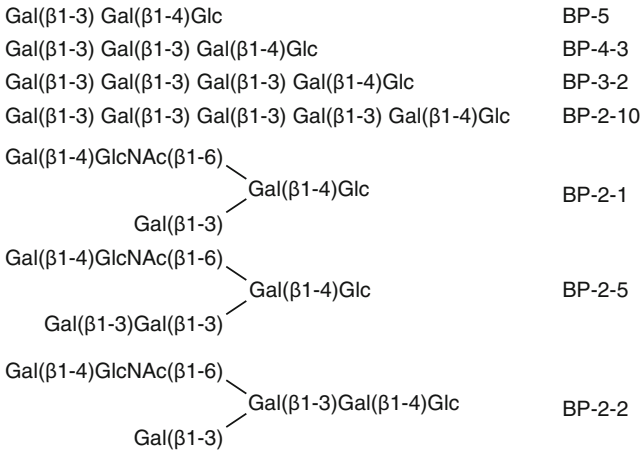

Fig. 10 Structures of the neutral oligosaccharides of brushtail possum milk characterized in this study

oligosaccharides characterized in this study are shown in Figs. 10 and 11, respectively.

The structures of neutral milk oligosaccharides of marsupials have been previously characterized in the tammar wallaby [5–8] and the koala [10]. In tammar wallaby milk, tri to hexasaccharides of [Gal(β1-3)]$_n$Gal(β1-4)Glc as well as Gal(β1-3)[GlcNAc(β1-6)]Gal(β1-4)Glc (lacto-N-novotetraose), Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (lacto-N-novopentaose I) and Gal(β1-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (galactosyl lacto-N-novopentaose I) have been characterized [5–8]. In this study these oligosaccharides, other than lacto-N-novotetraose, were also found in brushtail possum milk. As lacto-N-novotetraose is a presumed precursor of lacto-N-novopentaose I, small amounts of lacto-N-novotetraose may have been present in the brushtail possum milk. A novel hexasaccharide, Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3)Gal(β1-4)Glc (galactosyl lacto-N-novopentaose II) was found in the brushtail possum milk; this hexasaccharide had not been detected in tammar wallaby milk. It is worth noting that a β6*N*-acetylglucosaminyltransferase present within lactating tammar wallaby mammary glands acts on Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc as well as on Gal(β1-3)Gal(β1-4)Glc to synthesize Gal(β1-3)[GlcNAc(β1-6)]Gal(β1-3)Gal(β1-4)Glc (lacto-N-novopentaose II) and lacto-N-novotetraose, respectively [16]. Lacto-N-novopentaose II would be the likely precursor of galactosyl lacto-N-novopentaose II. Overall it appears that the neutral milk oligosaccharides of brushtail possum milk are very similar to those of tammar wallaby milk.

Although fucosyl lacto-N-novopentaose I, Gal(β1-3){Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-6)}Gal(β1-4)Glc has been found in koala milk [10], no fucosyl oligosaccharides were detected in this study, nor in milk of the tammar wallaby and red kangaroo. So far, the koala is the only species of marsupial in which fucosyl milk oligosaccharides have been found.

Most of the brushtail possum acidic milk oligosaccharides characterized in study have been previously found in milk of the red kangaroo [9], with the exception of Gal(β1-3)(*O*-3-sulfate)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (lacto-N-novopentaose I sulfate a) Gal(β1-3)[Gal(β1-4)(*O*-3-sulfate)GlcNAc(β1-6)]Gal(β1-4)Glc (lacto-N-novopentaose I sulfate b), and Gal(β1-3)Gal(β1-3)[Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc, galactosyl sialyl lacto-N-novopentaose b]. However, these oligosaccharides may have been present in the unidentified fractions of red kangaroo milk carbohydrate. Red kangaroo and koala

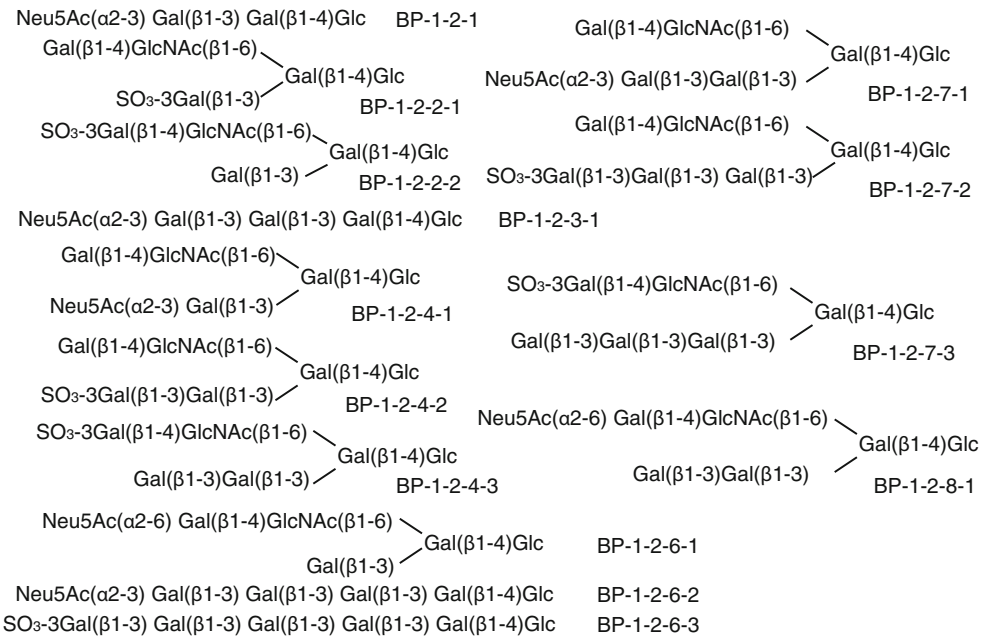

Fig. 11 Structures of the acidic oligosaccharides of brushtail possum milk characterized in this study

milk both contained Gal(β1-3)[Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (sialyl lacto-N-novopentaose c) [9, 10]. The ^{1}H-NMR spectrum of BP-1-2-4 (Supplemental Fig. 2) had the small shift at δ 4.553 of H-1 of β(1–4) linked Gal, suggesting the presence of this saccharide also in brushtail possum milk. However, the ratio of putative sialyl lacto-N-novopentaose c to Neu5Ac(α2-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (sialyl lacto-N-novopentaose a) is smaller in brushtail possum milk than in that of the red kangaroo and koala. This study showed that brushtail possum milk contains acidic oligosaccharides whose core units are Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3)Gal(β1-4)Glc. As the ^{1}H-NMR spectra of Mr-1-1-6 and Mr-1-1-8 of red kangaroo milk had the NAc shifts of β(1–6) linked GlcNAc at δ 2.046 and 2.041, respectively [9], these two fractions of red kangaroo milk probably contained oligosaccharides of this type in addition to those characterized in our previous red kangaroo study [9]. It can be concluded from the above comparisons that the acidic oligosaccharides of brushtail possum milk are very similar to those of red kangaroo milk.

Koala milk was found to contain a fucosyl sialyl oligosaccharide, Neu5Ac(α2-3)Gal(β1-3){Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-6)}Gal(β1-4)Glc [10], but our results indicate that brushtail possum milk contains neither neutral nor acidic fucosyl oligosaccharides.

All of those brushtail possum oligosaccharides that contained acetylglucosamine had the N-acetyllactosamine type II structure, Gal(β1-4)GlcNAc and none had the lacto-N-biose type I structure, Gal(β1-3)GlcNAc that is predominant in human breast milk [22].

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