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Chemical characterization of acidic oligosaccharides in milk of the red kangaroo (*Macropus rufus*)

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Abstract In the milk of marsupials, oligosaccharides usually predominate over lactose during early to mid lactation. Studies have shown that tammar wallaby milk contains a major series of neutral galactosyllactose oligosaccharides ranging in size from tri- to at least octasaccharides, as well as β(1-6) linked *N*-acetylglucosamine-containing oligosaccharides as a minor series. In this study, acidic oligosaccharides were purified from red kangaroo milk and characterized by ^{1}H-nuclear magnetic resonance spectrometry and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, to be as follows: Neu5Ac(α2-3)Gal(β1-4)Glc (3′-SL), Neu5Ac(α2-3)Gal(β1-3)Gal(β1-4)Glc (sialyl 3′-galactosyllactose), Neu5Ac(α2-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc, Neu5Ac(α2-3)Gal(β1-3)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)[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(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc, Gal(β1-3)(-3-*O*-sulfate)Gal(β1-3)Gal(β1-4)Glc, Gal(β1-3)(-3-*O*-sulfate)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc, Gal(β1-3)(-3-*O*-sulfate)Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc, Gal(β1-3)(-3-*O*-sulfate)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc, Gal(β1-3)(-3-*O*-sulfate)Gal(β1-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc. These acidic oligosaccharides were shown to be sialylated or sulfated in the non-reducing ends to the major linear and the minor branched series of neutral oligosaccharides of tammar wallaby milk.

Keywords Red kangaroo · Milk oligosaccharides · Sialyl oligosaccharide · Oligosaccharide sulfate · Galactosyllactose · Lacto-N-novopentaose I · *Macrupus rufus*

Introduction

Mammalian milk or colostrum contain from a trace to 10 % of carbohydrate in which the disaccharide lactose (Gal(β1-4)Glc) usually dominates along with lower concentrations of many varieties of oligosaccharides that have a lactose unit at their reducing ends [1, 2]. In the milk/colostrum of monotremes, marsupials and some Canoidea species of Carnivora, however, oligosaccharides usually predominate over lactose [2, 3]. Among macropod marsupials, the tammar wallaby has been subjected to detailed studies with respect to its milk oligosaccharides at various stages of lactation [4–8]. It was found that tammar milk contains a major series of oligosaccharides that comprise galactoyllactoses ranging from Gal(β1-3)Gal(β1-4)Glc [4] to Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc [5], and a minor series including Gal(1-3)[GlcNAc(β1-6)]Gal(β1-4)Glc (lacto-N-novotetraose) [6], Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc

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(lacto-N-novopentaose I) as well as Gal(β1-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc [7]. In addition, high molecular branched sialyl oligosaccharides, which contain Gal(β1-4)GlcNAc(β1-6) as well as Gal(β1-3)$_n$Gal(β1-4)Glc, have been partially characterized in tammar milk [8]. It also is noteworthy that the Grey kangaroo appears to be very similar to the tammar wallaby in its milk oligosaccharides, based on comparisons of monosaccharide compositions, gel filtration profiles, and thin layer chromatograms [9, 10]. The chemical structures of Grey kangaroo milk oligosaccharides have not, however, been characterized as yet.

Although there is much information on neutral tammar wallaby milk oligosaccharides, the main acidic oligosaccharides of marsupial milk have never been characterized. In this study, acidic oligosaccharides with *N*-acetylneuraminyl or sulfate groups were characterized in milk of the Red Kangaroo (*Macropus rufus*).

Materials and methods

Milk sample and chemicals

A pooled sample of milk (approx 13 ml) from one Red kangaroo that had been milked at various times between 24 and 35 weeks post partum was kindly provided by the CSIRO Division of Wildlife Research Canberra. Its carbohydrate fraction, extracted using chloroform-methanol as described by Messer and Mossop [9], was stored in a sealed tube at -20°C for about 25 years prior to analysis. 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 (sialyllacto-N-novopentaose a) and Gal(β1-3)[Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (sialyllacto-N-novopentaose b) were isolated from bactrian camel colostrum [11]. Gal(β1-4)Glc-3′-*O*-sulfate (lactose sulfate) was purified from a dog milk [12]. Neu5Ac(α2-3)Gal(β1-4)Glc (3′-SL) was purchased from Sigma Co. (St. Louis, MO, USA).

Preparation of acidic oligosaccharides

Of the carbohydrate fraction of Red kangaroo milk, 100 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 each of galactose (monosaccharide), lactose (disaccharide), and raffinose (trisaccharide). 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 [13] and for sialic acid with periodate-resorcinol [14]. Peak fractions were pooled and freeze-dried. This gel filtration was repeated with another 100 mg of the carbohydrate fraction and the corresponding peak fractions were combined.

The components of peak Mr-1 (see Fig. 1), which reacted positively with both periodate-resorcinol (630 nm) and phenol-H_2SO_4 (490 nm), were pooled, lyophilized, dissolved in 2 mL of 50 mmol/L Tris hydroxyaminomethane-HCl buffer (pH 8.7) and subjected to anion exchange chromatography on a DEAE-Sephadex A-50 column (1.5×20 cm; GE Healthcare, Uppsala, Sweden), which was equilibrated and eluted with the same buffer. Elution was done 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 using the phenol-H_2SO_4 method [13]. Figure 2 shows that the ion exchange chromatography had separated the Mr-1 fraction into two peaks. The components in the second peak, designated as Mr-1-1, 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 Mr-1-1 were then subjected to high performance liquid chromatography (HPLC) on a TSK gel Amide-80 column (4.6×250 mm, pore size 80 Å, particle size 5 μm; Tosoh, Tokyo, Japan) (chromatogram in Fig. 3). The mobile phase was 50 % and 80 % (vol/vol) acetonitrile (CH_3CN) 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. Figure 3 shows that Mr-1-1 resolved into numerous peaks. The contents of the eight peaks designated as Mr-1-1-1 to Mr-1-1-8 were each pooled, concentrated by rotary evaporation, and subjected to ^{1}H-NMR spectroscopy and mass spectroscopy to determine their structures.

^{1}H-NMR spectroscopy

Nuclear magnetic resonance spectra were recorded in D_2O (99.999 atom D%; Aldrich, Milwaukee, WI) at 500 or 600 MHz for ^{1}H-NMR with a JEOL ECP-500 Fourier transform-NMR (Jeol, Tokyo, Japan) or a Varian INOVA 600 spectrometer (Varian Inc., Palo Alto, CA) operated at 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 of Mr-1-1-4, Mr-1-1-5, Mr-1-1-6, Mr-1-1-7, and Mr-1-1-8 (Fig. 3), using an AutoflexII TOF/TOF mass spectrometer (Bruker Daltonics, Bremen,

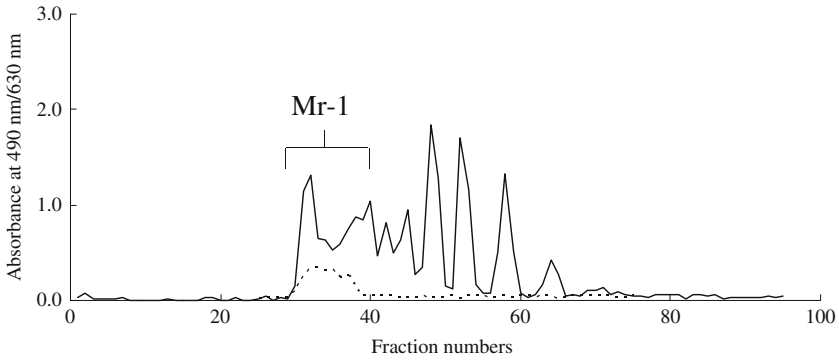

Fig. 1 Gel chromatogram of the carbohydrate fraction from Red kangaroo milk on a BioGel P-2 column (2.5×100 cm). Elution was done with distilled water at a flow rate of 15 mL/h and fractions of 5.0 mL were collected. Each fraction was monitored by the phenol-H_2SO_4 method at 490 nm (solid line) and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

Germany). The sample solution (0.5 μL) was mixed on a target plate (MTP 384 target plate ground steel, T F, Bruker), with an equal volume of 10 mg/mL of 2,5-dihydroxybenzoic acid (DHB) saturated in distilled water. After the solvent had dried, the target plate was loaded into the mass spectrometer. Mass spectra were obtained using a reflector positive ion mode optimized to the mass range of 0 to 3 kDa. Sialyl Lewis X {Neu5Ac(α2-3)Gal(β1-4)[Fuc(α1-3)GlcNAc]} and 3′-SL, which were kind gifts from Dr. Takashi Terabayashi in Kitasato University (Kanagawa, Japan), were used as external mass calibrants.

Results

The crude carbohydrate fraction (total 200 mg) from Red kangaroo milk was separated into several peaks during gel filtration on BioGel P-2 as shown Fig. 1. As this study focused on acidic oligosaccharides, the fraction designated as Mr-1, which reacted positively with the periodate – resorcinol method, was subjected to further purification using ion exchange chromatography. It separated into two peaks as shown in Fig. 2. The first peak was thought to contain high molecular weight neutral oligosaccharides, which were not investigated in this study. The components in the second peak, designated as Mr-1-1, were further separated by HPLC as shown in Fig. 3. The oligosaccharides in Mr-1-1-1 to Mr-1-1-8 were characterized mainly by ^{1}H-NMR.

Mr-1-1-1

As the ^{1}H-NMR spectrum of Mr-1-1-1 (chemical shifts in Table S1) was completely identical with that of authentic 3′-SL, the oligosaccharide in this fraction was characterized to be Neu5Ac(α2-3)Gal(β1-4)Glc.

Mr-1-1-2

The oligosaccharide in fraction Mr-1-1-2 was characterized by its ^{1}H-NMR spectrum, which was compared with those of Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc (digalactosyllactose) [15] and Gal(β1-4)Glc-3′-O-sulfate (lactose-sulphate) [12]. Its spectrum (Fig. 4, chemical shifts in Table S1) had the characteristic doublet doublet and doublet shifts of H-3 and H-4 of β-linked Gal at δ 4.345 and 4.302, respectively, which moved downfield from the usual H-3 and H-4 shifts of β-linked Gal residue. As these downfield shifts were observed in H-3 and H-4 of β(1-4) linked Gal of the ^{1}H-NMR spectrum of lactose-sulfate, it was concluded that the above signals moved downfield by the substitution of sulfate at OH-3 of a β-linked Gal residue in the oligosaccharide in Mr-1-1-2.

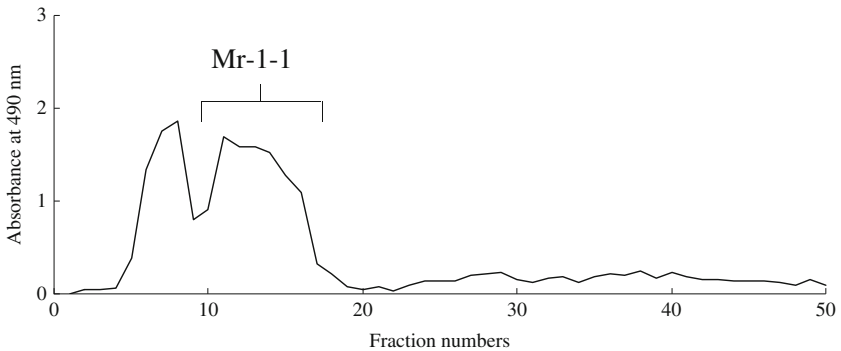

Fig. 2 Anion exchange chromatogram of Mr-1 (Fig. 1) separated from Red kangaroo milk by chromatography on BioGel P-2. A DEAE-Sephadex A-50 column (1.5×20 cm) equilibrated with 50 mmol/L Tris hydroxyaminomethane-HCl buffer (pH 8.7) was used. Elution was done with 250 mL of the buffer. The flow rate was 15 mL/h and fractions of 5 mL were collected. They were monitored by the phenol-H_2SO_4 method

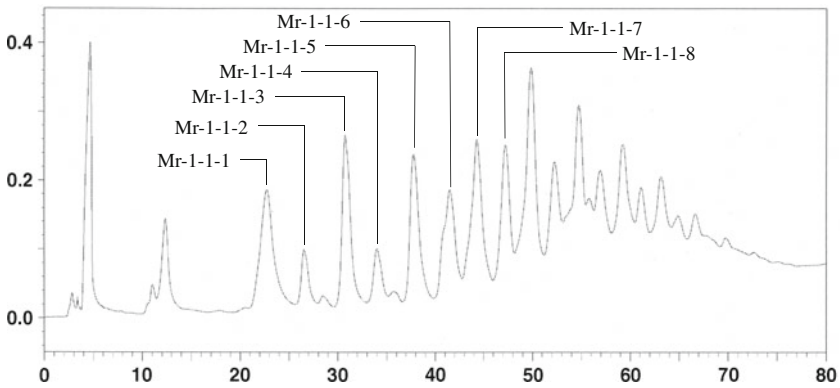

Fig. 3 High-performance liquid chromatography of the fraction of Mr 1-1 (Fig. 2). The HPLC was done using a Shimadzu LC-10 ATVP pump (Shimadzu, Tokyo, Japan) on a TSK-gel Amide-80 column (4.6×250 mm, pore size 80 Å, particle size 5 μm; Tosoh, Tokyo, Japan). The mobile phase was 50 % and 80 % (v/v) acetonitrile (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 of peaks was done by UV absorption at 195 nm

The spectrum had the anomeric shifts of α-Glc, β-Glc and β(1-4) linked Gal at δ 5.230, 4.673 and 4.518, respectively, and two other anomeric shifts at δ 4.738 and 4.687. The spectrum had the characteristic H-4 shift of β-linked Gal, which was substituted at OH-3, at δ 4.207. From its signal intensity, there were two chemical shifts at this position, showing the presence of a Gal(β1-3)Gal(β1-3)Gal(β1-4) unit as in digalactosyllactose. It was concluded that the shift at δ 4.687 arose from H-1 of penultimate β(1-3) linked Gal, because this was fairly close to that (δ 4.679) of digalactosyllactose. The shift at δ 4.738 was concluded to be due to a non-reducing β(1-3) linked Gal residue, whose amoneric shift moved down field from that (δ 4.620) of digalactosyllactose, by the attachment of sulfate to the OH-3 position. From these assignments, the oligosaccharide in Mr-1-1-2 was characterized to be Gal(β1-3)(-*O*-3-sulfate)Gal(β1-3)Gal(β1-4)Glc.

Mr-1-1-3

As the ^{1}H-NMR spectrum (chemical shifts in Table S1) was completely identical with that of sialyl 3′-galactosyllactose of bactrian camel colostrum [11], the oligosaccharide in Mr-1-1-3 was characterized to be Neu5Ac(α2-3)Gal(β1-3)Gal(β1-4)Glc.

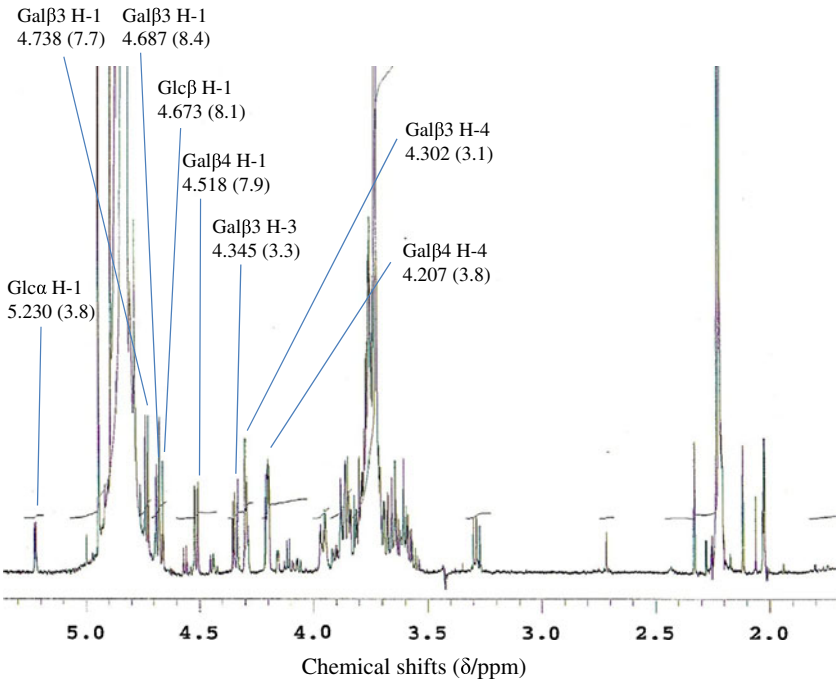

Fig. 4 ^{1}H-NMR spectrum of the oligosaccharide in Mr 1-1-2 isolated from Red kangaroo milk by HPLC (Fig. 3). The spectrum was obtained in D_2O at 600 MHz with a Varian INOVA spectrometer operated at 293.1 K. Chemical shifts are expressed relative to internal 3-(trimethylsilyl)-1-propane sulfuric acid, sodium salt

Mr-1-1-4

Clear M+2K or M-H+2K ions were not obtained in its MALDI-TOF mass spectrum. The ^{1}H-NMR spectrum of the components in this fraction showed H-1 shifts of α-Glc, β-Glc, and several β(1-3) linked Gal, β(1-6) linked GlcNAc and β(1-4) linked Gal, H-3 axial and equatorial shifts of α(2-3) linked Neu5Ac and characteristic down field shifts of H-3 and H-4 of β linked Gal, which was substituted by sulfate at OH-3. It was assumed that this fraction was composed of a complicated mixture of several oligosaccharides. Consequently, the oligosaccharides in this fraction were not characterized.

Mr-1-1-5

The MALDI-TOF mass spectrum of Mr-1-1-5 had the MS ions at 985.260 and 1034.318; these might have arisen from M+2K of $(Hex)_5$-SO_3 and M-H+2K of $(Hex)_4(Neu5Ac)_1$, suggesting a mixture of two oligosaccharides with these monosaccharide compositions.

The ^{1}H-NMR spectrum (Fig. 5, chemical shifts in Table S1) had the characteristic H-3 doublet doublet and H-4 doublet of β-linked Gal at δ 4.341 and 4.297, respectively, showing the presence of sulfate in one component of this fraction, as in Mr-1-1-2. The spectrum had the anomeric shifts of α-Glc, β-Glc and β(1-4) linked Gal at δ 5.225, 4.665, and 4.512, respectively. It had the characteristic downfield H-1 shift of a non reducing β(1-3) linked Gal residue whose OH-3 was substituted by sulfate, at δ 4.733, as in the similar shift of Mr-1-1-2. The spectrum had another anomeric shift of β(1-3) linked Gal at δ 4.694. From these assignments and monosaccharide composition estimated from the MS ion, one of the oligosaccharides in Mr-1-1-5, designated as Mr-1-1-5-2, was characterized to be Gal(β1-3)(-*O*-3-sulfate)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc.

The spectrum also had the H-3 axial and equatorial shifts of α(2-3) linked Neu5Ac at δ 1.803 and 2.763, the NAc shift of this residue at δ 2.029, and H-3 shift of a β-linked Gal residue, whose OH-3 was substituted by α(2-3) linked Neu5Ac, at δ 4.116; these showed the presence of a non reducing Neu5Ac(α2-3)Gal unit in another saccharide in this fraction. In addition, the spectrum had the anomeric shift of a penultimate β(1-3) linked Gal residue, which was substituted by α(2-3) linked Neu5Ac, at δ 4.688, as in the similar shift of Mr-1-1-3, showing the presence of a non reducing Neu5Ac(α2-3)Gal(β1-3) unit. The other H-1 shift of β(1-3) linked Gal at δ 4.681 was assumed to have overlapped with that of the above other saccharide in this fraction. From these assignments and the monosaccharide composition estimated from the MS ion, another saccharide in Mr-1-1-5, designated Mr-1-1-5-1, was characterized to be Neu5Ac(α2-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc.

The overlapped shifts at δ 4.207 and 4.202 were assigned to H-4 of β(1-4) and/or β(1-3) linked Gal, substituted by β-linked Gal at OH-3 in both oligosaccharides.

Mr-1-1-6

The MALDI-TOF mass spectrum of Mr-1-1-6 had the MS ions at 1188.345 and 1237.471; these might have arisen from M+2K of $(Hex)_5(HexNAc)_1$-SO_3 and M-H+2K of $(Hex)_4(HexNAc)_1(Neu5Ac)_1$, suggesting a mixture of two oligosaccharides with these monosaccharide compositions.

The corresponding signals in the ^{1}H-NMR spectrum (Fig. 6, chemical shifts in Table S2) of one oligosaccharide in Mr-1-1-6 were essentially similar to those of siallyllacto-N-novopentaose a from Bactrian camel colostrum [11]; it

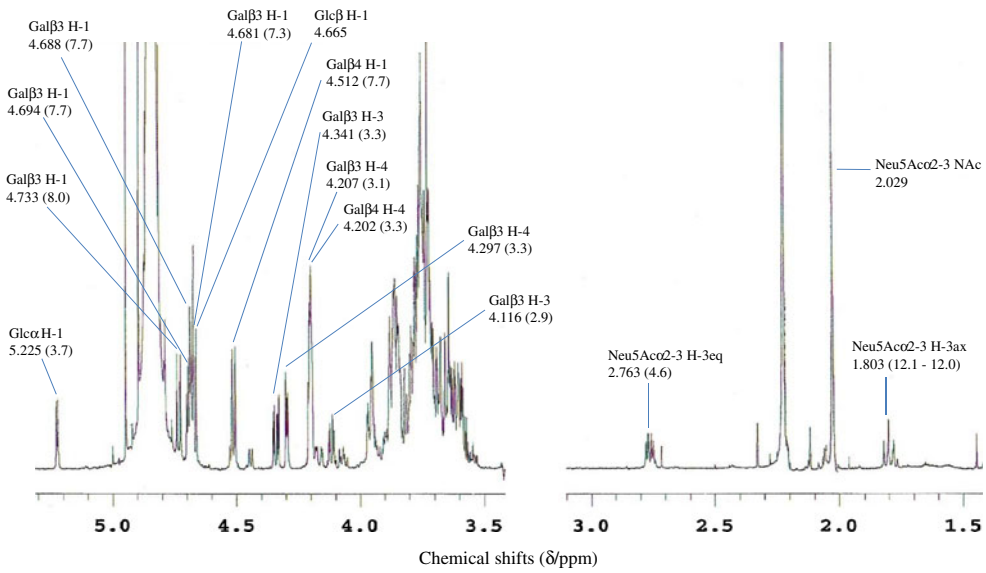

Fig. 5 ^{1}H-NMR spectrum of the oligosaccharides in Mr-1-1-5 isolated from Red kangaroo milk (Fig. 3)

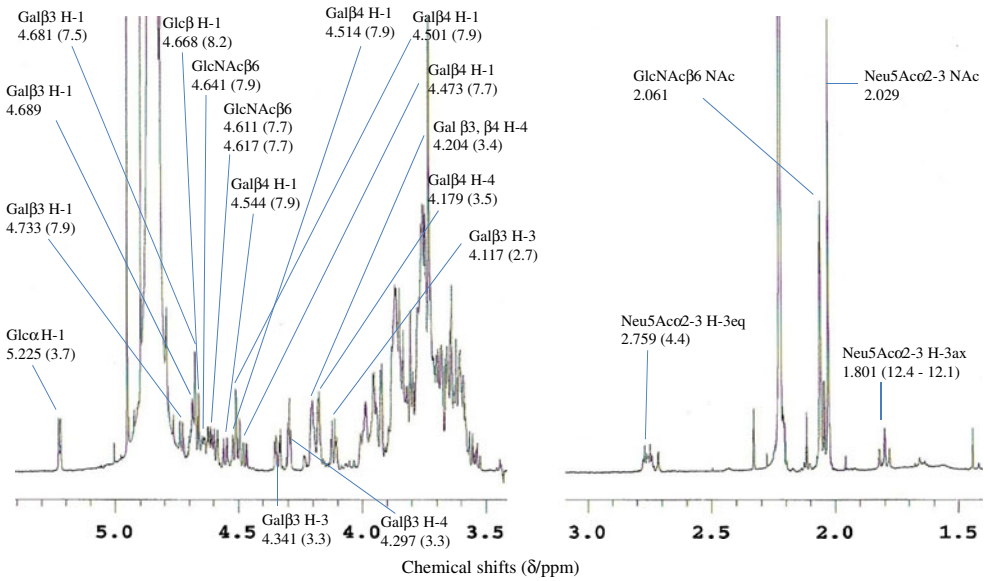

Fig. 6 ^{1}H-NMR spectrum of the oligosaccharides in Mr-1-1-6 isolated from Red kangaroo milk (Fig. 3)

was therefore characterized to be Neu5Ac(α2-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc, designated Mr-1-1-6-1.

The ^{1}H-NMR spectrum also had the characteristic H-3 doublet doublet and H-4 doublet of β-linked Gal at δ 4.341 and 4.297, respectively, showing the presence of sulfate in the other compound in this fraction. The characteristic downfield shift at δ 4.733 was assigned to H-1 of non-reducing β(1-3) linked Gal, which was substituted by sulfate at OH-3, as in Mr-1-1-2 and Mr-1-1-5-2. The spectrum had other anomeric shifts of two β(1-4) linked Gal at δ 4.544 and 4.514, β(1-3) linked Gal at δ 4.681, and β(1-6) linked GlcNAc at δ 4.617 and 4.611. From these assignments and the monosaccharide composition data from MS, the other oligosaccharide in this fraction was characterized to be Gal(β1-3)(-3-O-sulfate)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc, designated Mr-1-1-6-2. The shifts at δ 4.204 and 4.297 arose from H-4 of β(1-4) and/or β(1-3) linked Gal, which were substituted by β-linked Gal at OH-3.

Mr-1-1-7

The MALDI-TOF mass spectrum of Mr-1-1-7 had MS ions at 1147.352, 1196.425 and 1237,441, which might have arisen from M+2K of $(Hex)_6$-SO_3, M-H+2K of $(Hex)_5$ $(Neu5Ac)_1$, and M-H+2K of $(Hex)_4(HexNAc)_1(Neu5Ac)_1$, suggesting a mixture of three oligosaccharides with the above monosaccharide compositions.

As the corresponding signals in the ^{1}H-NMR spectrum (Fig. 7, chemical shifts in Table S2) of one oligosaccharide in Mr-1-1-7 were essentially similar to those of sialyllacto-N-novopentaose b from Bactrian camel colostrum [11], it was characterized to be Gal(β1-3)[Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (Mr-1-1-7-1).

The ^{1}H-NMR spectrum also had the H-3 doublet doublet and H-4 doublet of β-linked Gal at δ 4.341 and 4.295, respectively, showing the presence of sulfate in another compound in this fraction. The downfield shift at δ 4.731 was assigned to H-1 of non-reducing β(1-3) linked Gal, which was substituted by sulfate at OH-3, as in Mr-1-1-2, Mr-1-1-5-2 and Mr-1-1-6-2. The spectrum had other anomeric shift of β(1-4) linked Gal at 4.511, and three β(1-3) linked Gal at δ 4.694, 4.686 and 4.681. From these assignments and the monosaccharide composition data from MS, another oligosaccharide in this fraction was characterized to be Gal(β1-3)(-3-*O*-sulfate)Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc (Mr-1-1-7-3).

The spectrum had other H-3 axial and equatorial shifts of α(2-3) linked Neu5Ac at δ 1.800 and 2.760, respectively, and H-3 of β-linked Gal, which was substituted by α(2-3) linked Neu5Ac, at δ 4.114, showing another saccharide in this fraction contained a non reducing Neu5Ac(α2-3)Gal unit. It was assumed that the H-1 shift of β(1-4) linked Gal and β(1-3) linked Gal at δ 4.511, and δ 4.686 and 4.681 in this compound overlapped with that of Mr-1-1-7-3. From these assignments and the monosaccharide composition derived from MS, this oligosaccharide was characterized to be Neu5Ac(α2-3)Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc (Mr-1-1-7-2).

The overlapped shifts at δ 4.200 and 4.195 were assigned to H-4 of β(1-4) and/or β(1-3) linked Gal, which were substituted by β-linked Gal at OH-3, in these oligosaccharides in this fraction.

Mr-1-1-8

The MALDI-TOF mass spectrum of Mr-1-1-8 had MS ions at 1350.364 and 1399.485; these might have arisen from M +2K of $(Hex)_6(HexNAc)_1$-SO_3 and M-H+2K of

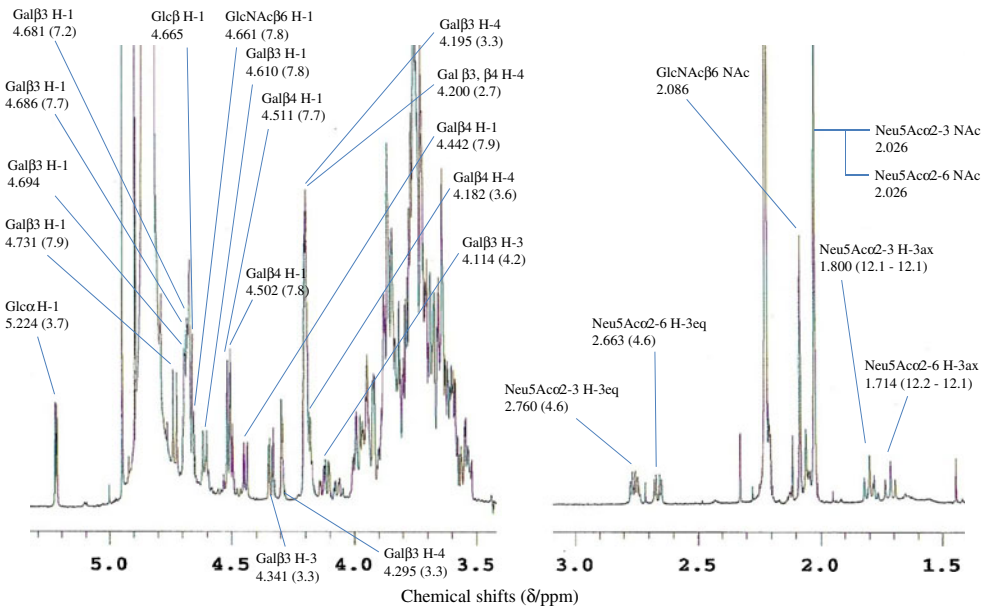

Fig. 7 ^{1}H-NMR spectrum of the oligosaccharides in Mr-1-1-7 isolated from Red kangaroo milk (Fig. 3)

$(Hex)_5(HexNAc)_1(Neu5Ac)_1$, suggesting a mixture of two oligosaccharides with these monosaccharide compositions.

As the spectrum (Fig. 8, chemical shifts in Table S3) had the H-3 doublet doublet and H-4 doublet of H-3 of β-linked Gal, which was substituted by sulfate at OH-3, at δ 4.341 and 4.297, respectively, and the downfield anomeric shift of β(1-3) linked Gal at δ 4.734, it was concluded that one of the oligosaccharides in this fraction contained non-reducing β(1-3) linked Gal-3-*O*-sulfate. The spectrum had the anomeric shifts of α-Glc, β-Glc, β(1-6) linked GlcNAc, two β(1-4) linked Gal, and another β(1-3)linked Gal at δ 5.225, 4.669, 4.617, 4.553 and 4.590, and 4.681, respectively. The NAc shift at δ 2.061 arose from a β(1-6)· linked GlcNAc residue and suggested that this residue is attached to the β(1-4) linked Gal of the lactose unit, because if it were attached to other β(1-3) linked Gal residue, the chemical shift would be at δ 2.04 but not 2.06 [15]. From these assignments and the monosaccharide composition derived from MS, one oligosaccharide in this fraction was characterized to be Gal(β1-3)(-*O*-3-sulfate)Gal(β1-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc, designated Mr-1-1-8-2.

With respect to the other saccharide, the spectrum had H-3 axial and equatorial shifts of α(2-3) linked Neu5Ac at δ 1.801 and 2.756, respectively, and H-3 of β-linked Gal, which was substituted by α(2-3) linked Neu5Ac, at δ 4.119, showing the presence of a non reducing Neu5Ac (α2-3)Gal unit in this fraction. The spectrum showed the presence of another β(1-6) linked GlcNAc, and of two β (1-4) linked Gal residues at δ 4.641, and 4.501 and 4.514, respectively. From the signal intensity, the shift at δ 4.681,

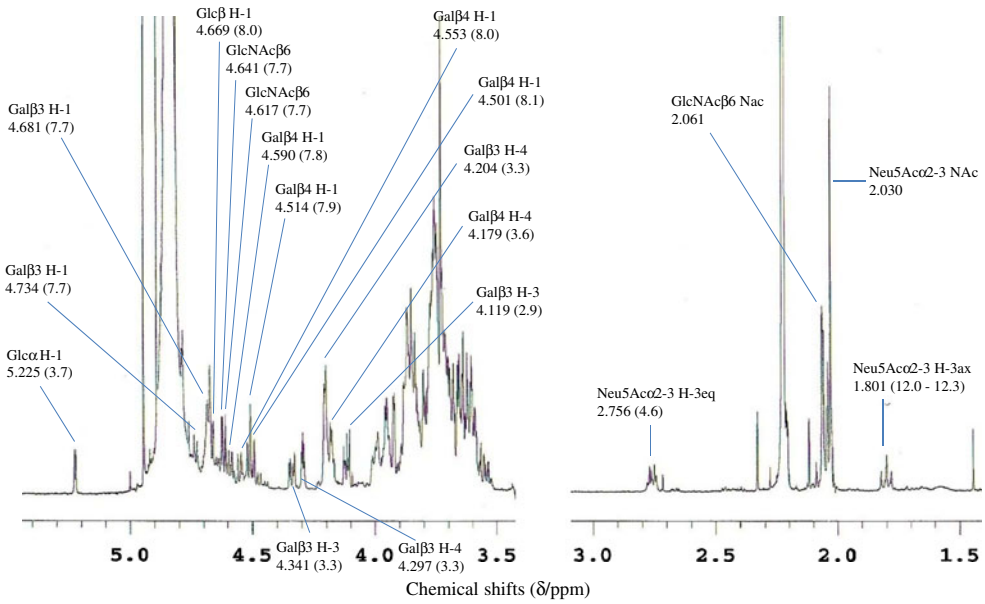

Fig. 8 ^{1}H-NMR spectrum of the oligosaccharides in Mr-1-1-8 isolated from Red kangaroo milk (Fig. 3)

which was assigned to H-1 of a β(1-3) linked Gal residue, was assumed to overlap the corresponding chemical shifts that arose from Mr-1-1-8-2. From these assignments and the monosaccharide composition derived from MS, the other oligosaccharide was characterized to be Neu5Ac(α2-3)Gal(β1-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc, designated Mr-1-1-8-1.

The shifts at δ 4.179 and 4.204 were assigned to H-4 of β(1-4) linked Gal and of β(1-3) linked Gal, both of which were substituted at OH-3, respectively, of Mr-1-1-8-1 and Mr-1-1-8-2.

Oligosaccharides in the peaks eluted after Mr-1-1-8 in the HPLC

The components in the peak fractions which eluted after Mr-1-1-8 in the HPLC were subjected to ^{1}H-NMR. It was considered that among some of these peak components β-GlcNAc residues were linked to β(1-3) linked Gal but not to β(1-4) linked Gal residues. A few components contained more than one GlcNAc residue. As we do not at this stage have a suitable ^{1}H-NMR chemical shifts database to characterize such complex structures, the components in these peak fractions were not characterized in this study.

Discussion

In this study, 12 of the acidic oligosaccharides of Red kangaroo milk were characterized as shown in Fig. 9. We found that they were either sialylated or sulfated to lactose or to the tri- to hexasaccharides of the β(1-3)galactosyllactose series or to lacto-N-novopentaose I, Gal(β1-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc and Gal(β1-3)Gal(β1-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc. As their core neutral oligosaccharides units, other than that of the heptasaccharide in Mr-1-1-8-2, have been previously identified in tammar wallaby milk [4–7], our characterization of the structures of these acidic oligosaccharides is consistent with the previous data on the milk oligosaccharides of the tammar wallaby, a species that is closely related to the Red kangaroo. A significant feature of these sialyl oligosaccharides was the presence of α(2-3) Neu5Ac linked to a penultimate Gal(β1-3) residue and of α(2-6) Neu5Ac linked to a Gal(β1-4) residue of *N*-acetyllactosamine of the branched unit.

It is of interest that most but not all marsupial milk oligosaccharides differ from those of eutherians. Tammar wallaby milk does not contain oligosaccharides whose core units are lacto-N-neotetraose (Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc) or lacto-N-neohexaose (Gal(β1-4)GlcNAc(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc), unlike the milk of several eutherian species [2, 3, 16, 17]. In addition, galactosyllactoses such as those found in tammar wallaby milk other than Gal(β1-3)Gal(β1-4)Glc, have never been found in any eutherian milk/colostrum [2, 3, 16, 17]. However, lacto-N-novopentaose I has been found in the milk/colostrum of both the tammar wallaby and a few eutherian species including cow [18–20], camel [11], pig [21], horse [22] and capuchin monkey [23]. Moreover, sialyl 3′-galactosyllactose, sialyl lacto-N-novopentaose a, and sialyl lacto-N-novopantaose b, which are found in the milk of Red kangaroo in this study, have been previously found in

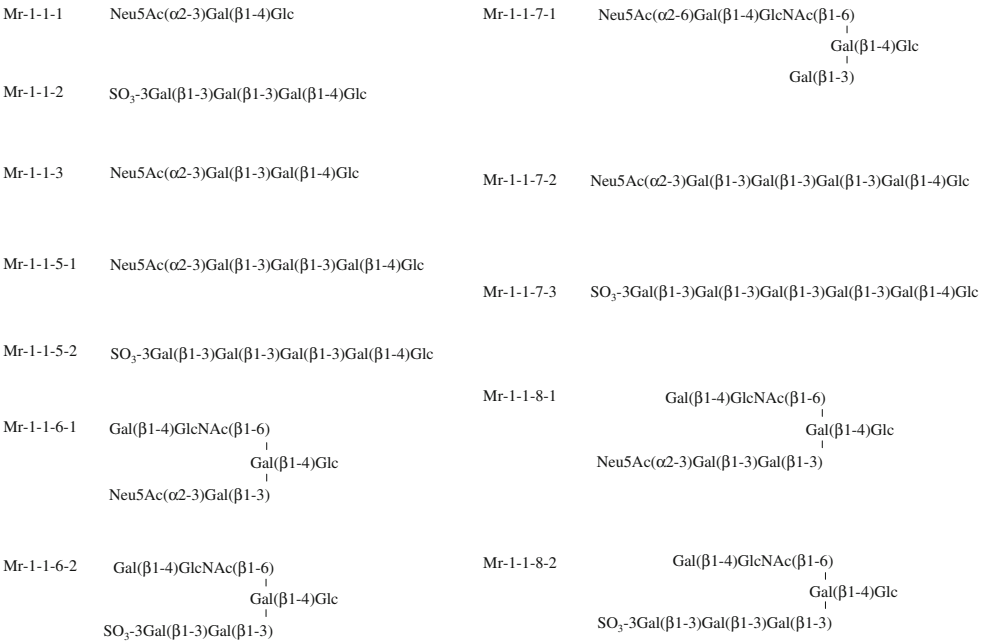

Fig. 9 Structures of the 12 acidic oligosaccharides of Red kangaroo milk characterized in this study

Bactrian camel colostrum [11]. Thus some of these oligosaccharides of kangaroos and wallabies resemble those of some eutherian mammals.

In a previous study, we described the high molecular weight monosialyl and disialyl oligosaccharides of tammar wallaby milk, which were concluded to contain Neu5Ac (α2-3)Gal(β1-4)GlcNAc or Neu5Ac(α2-6)Gal(β1-4) GlcNAc units [8]. These oligosaccharides remained in the retentate following dialysis against distilled water; their molecular weight was estimated by gel filtration HPLC, to be around 3,000, which is higher than those of the sialyl oligosaccharides characterized in this study. At that time (1994), sialyl oligosaccharides in which Neu5Ac is linked to members of the linear galactosyl series, as in Mr-1-1-8-1, had not been found in the milk of wallabies and kangaroos; therefore we were not in a position to conclude the presence of such oligosaccharides in this high molecular weight fraction. At this stage, we assume that, in this fraction, α(2-3) Neu5Ac is linked to a linear galactosyl unit, while α(2-6) Neu5Ac is linked to a branched Gal(β1-4)GlcNAc unit. In this study, it was shown that Red kangaroo milk contains another type of acidic oligosaccharides in which non reducing Gal(β1-3) residues are sulfated through OH-3. The sulfation was confirmed by the characteristic H-3 doublet doublet and H-4 doublet resonances at δ 4.34 and 4.30, respectively, in their ^{1}H-NMR spectra as in 3′-*O*-lactose sulfate of dog milk [12]. The ^{1}H-NMR spectra of the high molecular weight sialyl oligosaccharides from tammar wallaby milk also exhibited these characteristic resonances [8], suggesting that these oligosaccharides had contained sulfate. However, we were unable to conclude this at that time (1994), because the characteristic H-3 doublet doublet and H-4 doublet by sulfation found in the ^{1}H-NMR spectrum in our dog milk oligosaccharide study was not published until 1999 [12].

Lactose 3′-*O*-sulfate has been found in the milk of dog [12] and Hamadryas baboon [24], while lactose 6′-*O*-sulfate and sialyllactose 6′-*O*-sulfate have been described in rat milk [25–27]. Sulfation has also been observed in the milk oligosaccharides of bearded seal [28, 29] and in two human milk oligosaccharides [30] but the sulfated oligosaccharides of kangaroo milk (Fig. 9) have not so far been found in any other milk/colostrum. It is noteworthy that of the twelve structures shown in Fig. 9, seven are sialylated while the other five are sulfated. This high incidence of sulfation has not been found in the milk/colostrum of other mammals, suggesting that it is a specific feature of kangaroo milk, and perhaps of the milk of other marsupials. It may also be worth noting that none of these structures contain both sulfur and sialic acid and that α(2-6)Neu5Ac was found to be linked to Gal(β1-4)GlcNAc(β1-6) while α(2-3)Neu5Ac was linked to lactose or to Gal(β1-3)Gal. In milk of the echidna, a monotreme, the major oligosaccharide is 4-*O*-acetyl-neuraminyl-lactose [31, 32], but we did not observe *O*-acetylated sialic acid in any of the Red kangaroo oligosaccharides.

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