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Chemical characterization of milk oligosaccharides of the koala (*Phascolarctos cinereus*)

Tadasu Urashima · Epi Taufik · Rino Fukuda · Tadashi Nakamura · Kenji Fukuda · Tadao Saito · Michael Messer

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Abstract Previous structural characterizations of marsupial milk oligosaccharides had been performed in only two macropod species, the tammar wallaby and the red kangaroo. To clarify the homology and heterogeneity of milk oligosaccharides among marsupial species, which could provide information on their evolution, the oligosaccharides of the koala milk carbohydrate fraction were characterized in this study. Neutral and acidic oligosaccharides were separated from the carbohydrate fraction of milk of the koala, a non-macropod marsupial, and characterized by ^{1}H-nuclear magnetic resonance spectroscopy. The structures of the neutral saccharides were found to be Gal(β1-4)Glc (lactose), 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(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (lacto-N-novopentaose I) and Gal(β1-3){Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-6)}Gal(β1-4)Glc (fucosyl lacto-N-novopentaose I), while those of the acidic saccharides were Neu5Ac(α2-3)Gal(β1-4)Glc (3′-SL), Neu5Ac(α2-3)Gal(β1-3)Gal(β1-4)Gal (sialyl 3′-galactosyllactose), 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), Gal(β1-3)[Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (sialyl lacto-N-novopentaose c), and Neu5Ac(α2-3)Gal(β1-3){Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-6)}Gal(β1-4)Glc (fucosyl sialyl lacto-N-novopentaose a). The neutral oligosaccharides, other than fucosyl lacto-N-novopentaose I, a novel hexasaccharide, had been found in milk of the tammar wallaby, a macropod marsupial, while the acidic oligosaccharides, other than fucosyl sialyl lacto-N-novopentaose a had been identified in milk carbohydrate of the red kangaroo. The presence of fucosyl oligosaccharides is a significant feature of koala milk, in which it differs from milk of the tammar wallaby and the red kangaroo.

Keywords Koala · Tammar wallaby · Milk oligosaccharides · Galactosyllactose · Lacto-N-novopentaose I · Fucosyl lacto-N-novopentaose I

T. Urashima (✉) · E. Taufik · R. Fukuda · T. Nakamura · 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

E. Taufik
Faculty of Animal Science, Bogor Agricultural University, Bogor 16680, Indonesia

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

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, which mostly have a lactose unit at their reducing ends [1, 2]. In the milk of monotremes, marsupials and some Arctoidea species of Carnivora, however, oligosaccharides usually predominate over free lactose [2, 3]. Among marsupial species, oligosaccharide structures have been characterized in only two closely related macropod species, the tammar wallaby, *Macropus eugenii* and the red kangaroo, *Macropus rufus*. The neutral oligosaccharides of milk of the tammar wallaby have been separated [4–8], and are characterized by the presence of a major series of galactosyllactoses ranging from Gal(β1-3)Gal(β1-4)Glc [5] to Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc [6], and a minor series of branched

oligosaccharides containing β(1–6) linked GlcNAc [7, 8], as shown in Fig. 1. In addition acidic oligosaccharides have been identified in the milk carbohydrate fraction of the red kangaroo [9] as shown in Fig. 2. These are sialylated or sulfated at the non – reducing ends of the major linear and the minor branched series of the neutral oligosaccharides found in tammar wallaby milk. Fucosyl oligosaccharides have never been detected in the carbohydrate fraction of either milk.

There have not so far been any detailed studies on the structures of the oligosaccharides in milk of marsupials other than the two macropods. To clarify the homology and heterogeneity of milk oligosaccharides among marsupial species, in this study we have characterized the neutral as well as acidic milk oligosaccharides of the koala.

Materials and methods

Milk sample and chemicals

Six koala milk samples were collected between 19/9/1977 and 11/11/1977 in New South Wales, Australia. The carbohydrate fraction of the mixed milk, extracted using chloroform-methanol as described by Messer and Mossop [10], was stored in a sealed tube at −20 °C for about 35 years prior to analysis. Gal(β1-3)Gal(β1-4)Glc (3′-galactosyllactose) and Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc (3′,3″-digalactosyllactose) 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) were separated from brown capuchin colostrum [11]. Neu5Ac(α2-3)Gal(β1-3)Gal(β1-4)Gal (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 separated from Bactrian camel colostrum [12]. Neu5Ac(α2-3)Gal(β1-4)Glc (3′-SL) was purchased from Sigma Co, (St. Louis, MO, USA).

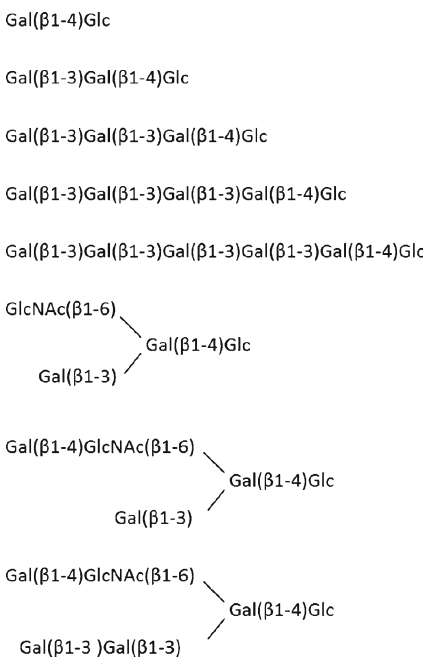

Fig. 1 Tammar wallaby neutral oligosaccharides

Neutral oligosaccharides

Of the carbohydrate fraction of koala milk, 25 mg were dissolved in 2 mL of water and the solution passed though 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 as shown in Fig. 3 and freeze-dried. The saccharides in the peak fractions KM-1 to KM-7 (see Fig. 3) were checked by thin layer chromatography using acetone/2-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 repeated with another 25 mg of the carbohydrate fraction and the corresponding peak fractions were combined.

The components in KM-4 to KM-7 were characterized by ^{1}H-NMR spectroscopy. The components in KM-2 and KM-3 were separated by high-performance liquid chromatography (HPLC). 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 components in KM-2-1 to KM-2-4 (from KM-2; see Fig. 4a), and in KM-3-1 to KM-3-10 (from KM-3; see Fig. 4b) were each collected, concentrated by rotary evaporation, and subjected to ^{1}H-NMR spectroscopy.

Acidic oligosaccharides

The components of peak KM-1 of the gel chromatogram (see Fig. 3), which reacted positively with both periodate-resorcinol (630 nm) and phenol-H_2SO_4 (490 nm), were dissolved in 2 mL 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,

Fig. 2 Red kangaroo acidic milk oligosaccharides

Neu5Ac(α2-3)Gal(β1-4)Glc

SO_3-3Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc

Neu5Ac(α2-3)Gal(β1-3)Gal(β1-4)Glc

Neu5Ac(α2-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc

SO_3-3Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc

Gal(β1-4)GlcNAc(β1-6)
Gal(β1-4)Glc
Neu5Ac(α2-3)Gal(β1-3)

Gal(β1-4)GlcNAc(β1-6)
Gal(β1-4)Glc
SO_3-3Gal(β1-3)Gal(β1-3)

Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6)
Gal(β1-4)Glc
Gal(β1-3)

Neu5Ac(α2-3)Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc

SO_3-3Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc

Gal(β1-4)GlcNAc(β1-6)
Gal(β1-4)Glc
Neu5Ac(α2-3)Gal(β1-3)Gal(β1-3)

Gal(β1-4)GlcNAc(β1-6)
Gal(β1-4)Glc
SO_3-3Gal(β1-3)Gal(β1-3)Gal(β1-3)

Uppsala, Sweden), which 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 the phenol-H_2SO_4 method [13]. Figure 6 shows that the ion exchange chromatography had separated the KM-1 fraction into two peaks. The components in the second peak, designated as KM-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 KM-1-1 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. 7). 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 KM-1-1 to KM-1-10 (Fig. 7) were each pooled, concentrated by rotary evaporation, and subjected to ^{1}H-NMR spectroscopy to determine their structures.

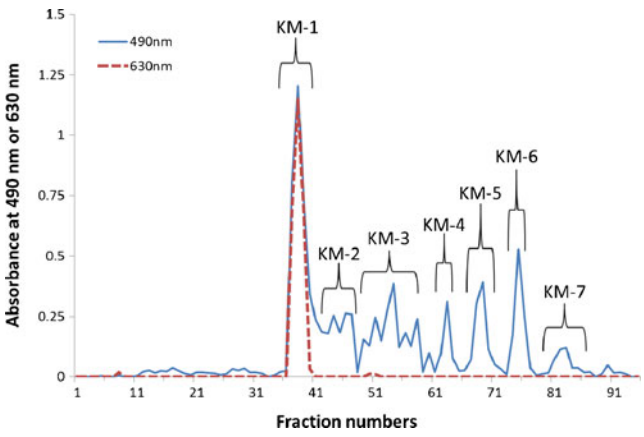

Fig. 3 Gel chromatogram of the carbohydrate fraction from koala milk on a BioGel P-2 column (2.5 X 100 cm). Elution was done with distilled water at a flow rate of 15 mL/h and fractions of 5.0 mL were collected. Each fraction was monitored by the phenol-H_2SO_4 method at 490 nm (solid line) and the periodate-resorcinol method at 630 nm (dotted line)

^{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 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 KM-3-3, KM-1-1-3 and KM-1-1-5, using an Autoflex II TOF/TOF mass spectrometer (Brucker Daltonics, Bremen, Germany). Lyophilized oligosaccharide fractions were dissolved in 10 μl of milli-Q water. The oligosaccharide solution was mixed with equal

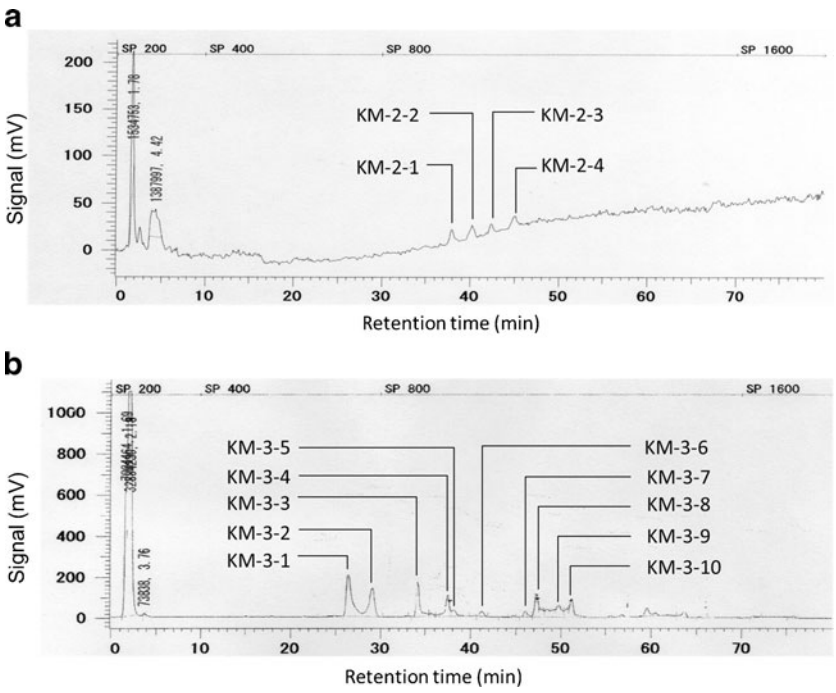

Fig. 4 High performance liquid chromatograms of the neutral oligosaccharide fractions KM-2 (A) and KM-3 (B) separated from the carbohydrate fraction of koala milk by gel chromatography. The Hitachi 7,000 series HPLC system (Tokyo) consisted of autosampler L-7,200, a column oven L-7,300, a pump L-7,100, 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

volume of 10 mg/mL 2,5-dihydroxybenzoic acid (Bruker Daltonics) 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 50 mg) from koala milk separated into into several peaks during gel filtration on BioGel P-2 (Fig. 3). The fractions in each peak were pooled. As the first eluted peak designated KM-1 reacted positively with periodate – resorcinol, it was concluded that the components in this fraction contained sialic acid. The components in KM-2 and KM-3 were subjected to HPLC using a Hypercarb column, as shown in Fig. 4. The resulting peaks were designated as KM-2-1 to KM-2-4 (Fig. 4a), and KM-3-1 to KM-3-10 (Fig. 4b). The separated peak components obtained by gel filtration and HPLC were characterized by ^{1}H-NMR spectroscopy.

KM-7

The ^{1}H-NMR spectrum of the component in KM-7 showed that it was not a free saccharide. Therefore it was not characterized in this study.

KM-6

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

KM-5

As the ^{1}H-NMR spectrum of KM-5 (chemical shifts in Table 1) was identical to the published data [15] for authentic 3′-galactosyllactose, the oligosaccharide in this fraction was characterized to be Gal(β1-3)Gal(β1-4)Glc.

KM-4

As the ^{1}H-NMR spectrum of KM-4 (chemical shifts in Table 1) was identical to the published data [15] for authentic 3′,3″-digalactosyllactose, the oligosaccharide in this fraction was characterized to be Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc.

KM-3-1, KM-3-2

The oligosaccharides in KM-3-1 and KM-3-2 could not be characterized, because the relevant NMR spectra were not available for comparison with the ^{1}H-NMR spectra for these peaks.

KM-3-8, KM-3-10

As the ^{1}H-NMR patterns of KM-3-8 and KM-3-10 were identical, it was concluded that these two peaks contained

Table 1 ^{1}H-NMR chemical shifts of the oligasaccharides KM-3 to KM-6, separated from koala milk carbohydrate by gel filtration and HPLC

Reporter group	Residue	Chemical shift, δ (coupling constants, Hz)				
		KM-3-3, KM-3-4	KM-3-8, KM-3-10	KM-4	KM-5	KM-6
H-1	Glcα	5.223 (3.8)	5.224 (3.9)	5.223 (4.0)	5.224 (4.0)	5.221 (4.0)
	Glcβ	4.670 (8.0)	4.670 (8.0)	4.666 (8.0)	4.660 (8.0)	4.666 (7.4)
	Gal(β1-3)	4.610 (7.7)	4.611 (7.6)	4.615 (7.4) 4.678 (7.9)	4.611 (7.4)	—
	Gal(β1-4)	4.499 (7.9)	4.500 (8.0)	4.511 (6.8)	4.511 (7.4)	4.450 (8.0)
		4.453 (7.8)	4.472 (7.8)		—	—
	GlcNAc(β1-6)	4.643 (8.0)	4.647 (7.8) 4.651 (7.6)	—	—	—
	Fuc(α1-3)	5.106 (4.0)	—	—	—	—
H-4	Gal(β1-4)	4.175 (3.3)[a]	4.181 (3.3)[a]	4.197 (2.3)[a]	4.198 (2.3)[a]	—
	Gal(β1-3)			4.203 (4.6)[a]		—
H-6	Fuc(α1-3)	1.174 (6.6)[a]	—	—	—	—
NAc	GlcNAc(β1-6)	2.054	2.060	—	—	—

[a] $J_{4,3}$, [b] $J_{6,5}$

the same saccharide, which separated into α- and β-anomer isomers during HPLC using the Hypercarb column. As the ^{1}H-NMR spectra of KM-3-8 and KM-3-10 (chemical shifts in Table 1) were identical to the published data [11] for authentic lacto-N-novopentaose I, the oligosaccharide in these peaks was characterized to be Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc.

KM-3-3, KM-3-4

As the ^{1}H-NMR patterns of KM-3-3 and KM-3-4 were identical, it was concluded that these two peaks contained the same saccharide. The MALDI-TOF mass spectrum of KM-3-3 had the MS ions at 1054.463 and 1038.457; these might have arisen from M+K and M+Na of $(Hex)_4(Deoxy\ Hex)_1(HexNAc)_1$. The oligosaccharides in KM-3-3 and KM-3-4 were identified by comparing their ^{1}H-NMR spectra with those of KM-3-8 and KM-3-10. The spectrum (Fig. 5, chemical shifts in Table 1) had the H-1 and H-6 shifts of α(1–3) linked Fuc at δ 5.106 and 1.174, respectively. It 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 5.223, 4.670, 4.643, 4.610, 4.499 and 4.453, respectively, H-4 of β(1–4) linked Gal, which was substituted at OH-3, at δ 4.175, and NAc shift of β(1–6) linked GlcNAc at δ 2.054. The NAc shift of β(1–6) linked GlcNAc shifted up field, compared with that (δ 2.060) of KM-3-8 and KM-3-10, consistent with the view that this residue was substituted by α(1–3) linked Fuc. From these assignments, the oligosaccharide in these fractions was characterized to be Gal(β1-3){Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-6)}Gal(β1-4)Glc (fucosyl lacto-N-novopentaose I).

KM-3-5, KM-3-6, KM-3-7, KM-3-9, KM-2-2, KM-2-2, KM-2-3 and KM-2-4

The oligosaccharides in these fractions could not be characterized by ^{1}H-NMR in this study, as clear chemical shifts were not observed because of a sensitivity problem.

Characterization of acidic oligosaccharides

The fraction KM-1 separated into two peaks during ion exchange chromatography, as shown in Fig. 6. The first peak was thought to contain a mixture of high molecular weight neutral oligosaccharides, which were not investigated in this

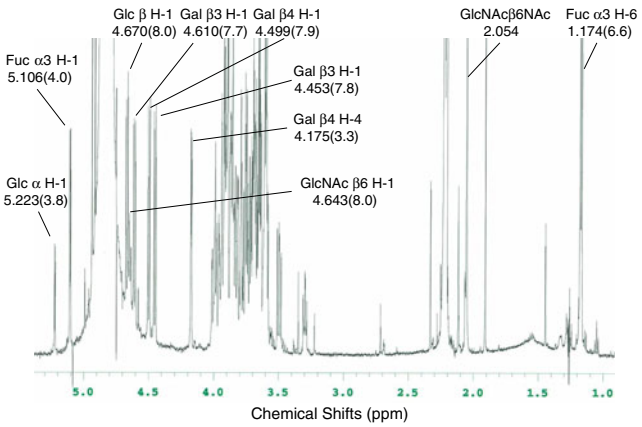

Fig. 5 ^{1}H-NMR spectrum of the oligosaccharide in KM-3-3 isolated from koala milk carbohydrate by HPLC (Fig. 4). The spectrum was obtained in D_2O at 600 MHz with a Varian INOVA spectrometer operated at 293.1 K. Chemical shifts are expressed relative to internal 3-(trimethylsilyl)-1-propane sulfuric acid, sodium salt

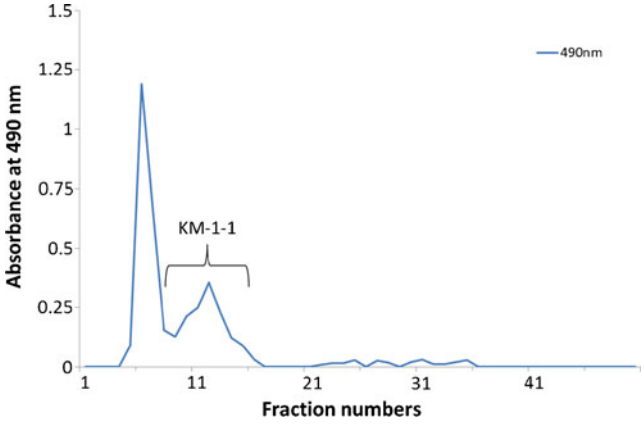

Fig. 6 Anion exchange chromatogram of KM-1 (Fig. 1) separated from koala milk carbohydrate by chromatography on BioGel P-2. A DEAE-Sephadex A-50 column (2.0×35 cm) equilibrated with 50 mmol/L Tris hydroxyaminomethane-HCl buffer (pH 8.7) was used. Elution was done with 250 mL of the buffer. The flow rate was 15 mL/h and fractions of 5 mL were collected. They were monitored by the phenol-H_2SO_4 method

study. The components in the second peak, designated as KM-1-1, were further separated by HPLC as shown in Fig. 7. The oligosaccharides in KM-1-1-1 to KM-1-1-10 were characterized by ^{1}H-NMR.

KM-1-1-1

As the ^{1}H-NMR spectrum (chemical shifts in Table 2) of KM-1-1-1 was identical with that of authentic 3′-SL, the oligosaccharide in this fraction was identified as Neu5Ac(α2-3)Gal(β1-4)Glc.

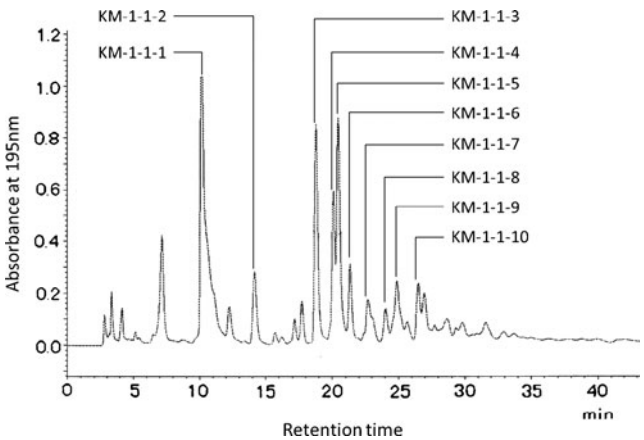

Fig. 7 High performance liquid chromatogram of the fraction KM-1-1 (Fig. 6). The HPLC was done using a Shimadzu LC-10 ATVP pump (Shimadzu, Tokyo, Japan) on a TSK-gel Amide-80 column (4.6×250 mm, pore size 80 Å, particle size 5 μm; Tosoh, Tokyo, Japan). The mobile phase was 50 % and 80 % (*v/v*) acetonitrile (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 was done by UV absorption at 195 nm

KM-1-1-2

As the ^{1}H-NMR spectrum (chemical shifts in Table 2) of KM-1-1-2 was identical with the published data [12] for sialyl 3′-galactosyllactose, the oligosaccharide in this fraction was characterized to be Neu5Ac(α2-3)Gal(β1-3)Gal(β1-4)Glc.

KM-1-1-3

The MALDI-TOF mass spectrum of KM-1-1-3 had the MS ions at 1199.420 and 1183.456; these might have arisen from M+K and M+Na of $(Hex)_4(HexNAc)_1(Neu5Ac)_1$. The ^{1}H-NMR spectrum (Fig. 8, chemical shifts in Table 2) of KM-1-1-3 showed that this fraction contained two oligosaccharides one major and the other minor, as concluded from the intensity of each chemical shift. As the chemical shifts that arose from the minor saccharide were essentially similar to the published data for the saccharide designated CC-1-2-5 from Bactrian camel colostrum [12], this was identified to be Neu5Ac(α2-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (sialyl lacto-N-novopentaose a).

In addition, the spectrum had other chemical shifts at δ 4.667, 4.634, 4.610, 4.552 and 4.499. The following chemical shifts overlapped those of the minor saccharide; δ 5.223 of H-1 of Glc α, δ 4.179 of H-4 of β(1–4)linked Gal, which was substituted at OH-3, δ 4.117 of H-3 of β linked Gal, which was substituted by α(2–3) linked Neu5Ac at OH-3, δ 2.759 of H-3 equatorial of α(2–3) linked Neu5Ac, δ 1.800 of H-3 axial of α(2–3) linked Neu5Ac, δ 2.057 of β(1–6) linked GlcNAc and δ 2.030 of α(2–3) linked Neu5Ac. The shift at δ 4.610, which was assigned to H-1 of β(1–3) linked Gal, showed that this residue was unsubstituted by Neu5Ac, while the shift of β(1–4) linked Gal at δ 4.552 showed that this residue was substituted by α(2–3) linked Neu5Ac. The shifts at δ 4.667, 4.634 and 4.499 could be assigned to H-1 of Glc-α, β(1–6) linked GlcNAc and β(1–4) linked Gal, respectively. From these assignments of the chemical shifts, the major saccharide in this fraction was identified to be Gal(β1-3)[Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (sialyl lacto-N-novopentaose c).

KM-1-1-4

As the ^{1}H-NMR (chemical shifts in Table 2) of KM-1-1-4 was identical with the published data [12] for sialyl lacto-N-novopentaose b, the oligosaccharide in this fraction was characterized to be Gal(β1-3)[Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc.

KM-1-1-5

The MALDI-TOF mass spectrum of KM-1-1-5 had the MS ions at 1345.514 and 1329.545; these might have arisen from M+K

Table 2 [1]H-NMR chemical shifts of the oligasaccharides KM-1-1-1 to KM-1-1-5, separated from koala milk carbohydrate by gel filtration, ion exchange chromatography and HPLC

Reporter group	Residue	Chemical shift, δ (coupling constants, Hz)					
		KM-1-1-1	KM-1-1-2	KM-1-1-3-1	KM-1-1-3-2	KM-1-1-4	KM-1-1-5
H-1	Glcα	5.220 (4.0)	5.224 (3.6)	5.223 (3.7)	5.223 (3.7)	5.225 (3.7)	5.223 (3.7)
	Glcβ	4.663 (8.0)	4.667 (7.7)	4.667 (8.1)	4.671 (8.1)	4.666 (7.0)	4.670 (7.9)
	Gal(β1-4)	4.531 (7.4)	4.515 (8.0)	4.499 (7.7)	4.472 (7.7)	4.445 (8.1)	4.453 (7.9)
				4.552 (7.7)	4.504 (7.7)	4.505 (7.7)	4.504 (7.9)
	Gal(β1-3)	—	4.689 (8.1)	4.610 (7.7)	4.688 (8.0)	4.611 (7.7)	4.687 (7.8)
	GlcNAc(β1-6)	—	—	4.634 (8.1)	4.641 (7.7)	4.661 (8.1)	4.644 (7.7)
	Fuc(α1-3)	—	—	—	—	—	5.104 (4.1)
H-3	Gal(β1-4)	4.130 (1.7)[a]		4.117 (3.0)			
	Gal(β1-3)	—	4.119 (3.3)	—	4.117 (3.0)		4.113
H-4	Gal(β1-4)	—	4.195 (3.3)[e]	4.179 (3.3)[e]	4.179 (3.3)[e]	4.187 (2.9)[e]	4.168 (3.3)[e]
$H\text{-}3_{ax}$	Neu5Ac(α2-3)	1.813 (12.6[b],-12.0[c])	1.803 (12.2[b],-12.1[c])	1.800 (12.2[b],-12.4[c])	1.800 (12.2[b],-12.4[c])	—	1.801 (12.3[b],-12.0[c])
	Neu5Ac(α2-6)	—	—	—	—	1.718 (12.5[b],-12.1[c])	—
$H\text{-}3_{eq}$	Neu5Ac(α2-3)	2.771 (5.2[d])	2.763 (4.7)[d]	2.759 (4.4)[d]	2.759 (4.4)[d]	—	2.762 (4.6)[d]
	Neu5Ac(α2-6)	—	—	—	—	2.666 (4.7)[d]	—
H-6	Fuc(α1-3)	—	—	—	—	—	1.172 (6.6)[f]
NAc	Neu5Ac(α2-3)	2.029	2.029	2.030	2.030	—	2.029
	Neu5Ac(α2-6)	—	—	—	—	2.028	—
	GlcNAc(β1-6)	—	—	2.057	2.057	2.080	2.053

[a] $J_{3,4}$, [b] $J_{3ax,4}$, [c] $J_{3ax,3eq}$, [d] $J_{3eq,4}$, [e] $J_{4,3}$, [f] $J_{6,5}$

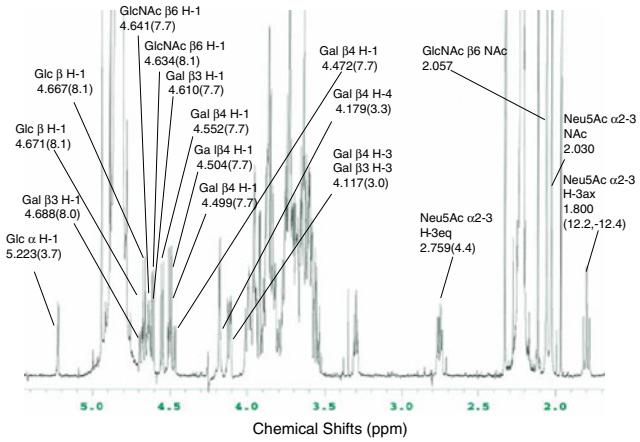

Fig. 8 ^{1}H-NMR spectrum of the oligosaccharides in KM-1-1-3 isolated from koala milk carbohydrate (Fig. 7)

and M+Na of $(Hex)_4(deoxyHex)_1(HexNAc)_1(Neu5Ac)_1$. The oligosaccharide in fraction KM-1-1-5 was characterized by its ^{1}H-NMR spectrum (Fig. 9, chemical shifts in Table 2), when compared with those of CC-1-2-5 from Bactrian camel colostrum, KM-1-1-3 and the published data [16] for oligosaccharides separated from human milk. The spectrum had the H-1 and H-6 of α(1–3) linked Fuc at δ 5.104 and 1.172, respectively as in KM-3-3. The H-1 shift of β(1–3) linked Gal at δ 4.687 shifted down field compared with that (δ 4.610) of KM-3-3, showing that this residue was substituted by α(2–3) linked Neu5Ac. The spectrum had H-1 of Glc α, Glc β, β(1–6) linked GlcNAc, two β(1–4) linked Gal at δ 5.223, 4.670, 4.644, 4.504 and 4.453, respectively and H-4 of β(1–4) linked Gal, which was substituted at OH-3, at δ 4.168. The NAc shift of β(1–6) linked GlcNAc at δ 2.053 shifted more up field compared with that (δ 2.057) of KM-1-1-3, showing that this residue was substituted by α(1–3) linked Fuc. The spectrum had the H-3 axial, H-3 equatorial and NAc shift of α(2–3) linked Neu5Ac at δ 1.801, 2.762 and 2.029, respectively, and H-3 of β(1–3) linked Gal, which was substituted by α(2–3) linked Neu5Ac, at δ 4.113. From these observation and the agreement of the published data [16], the oligosaccharide in this fraction was characterized to be Neu5Ac(α2-3)Gal(β1-3){Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-6)}Gal(β1-4)Glc (fucosyl sialyl lacto-N-novopentaose a).

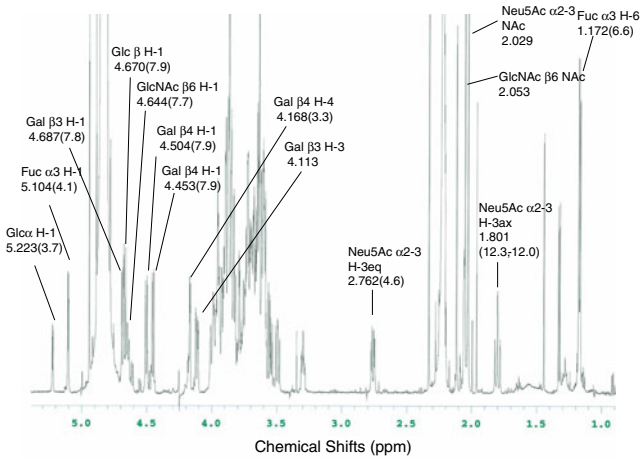

Fig. 9 ^{1}H-NMR spectrum of the oligosaccharides in KM-1-1-5 isolated from koala milk carbohydrate (Fig. 7)

KM-1-1-6 to KM-1-1-10

The oligosaccharides in these fractions were not characterized as clear chemical shifts were not observed because of a sensitivity problem.

Relative incidence of the fractions

The relative incidence of fractions KM-1 to KM-6 was estimated from the peak areas in Fig. 3 to be as follows; KM-1 : KM-2 : KM-3 : KM-4 (3′,3″-galactosyllactose) : KM-5 (3′-galactosyllactose) : KM-6 (lactose)=6.8:3.2:4.9:1.0:2.0:2.2. The relative incidence of fractions KM-3-1, KM-3-2, KM-3-3, KM-3-4, KM-3-8 and KM-3-10 in fraction KM-3 was estimated from the peak areas in Fig. 4 to be: KM-3-1 : KM-3-2 : KM-3-3 : KM-3-4 : KM-3-8 : KM-3-10=6.6:4.6:3.5:2.3:2.0:1.0. The relative ratio of fucossyl lacto-N-novopentaose I (KM-3-4 plus KM-3-5) to lacto-N-novopentaose I (KM-3-8 plus KM-3-10) was estimated to be 1.9:1.0. The relative incidence of the acidic oligosaccharide fraction KM-1-1-1 to KM-1-1-10 was estimated by the peak heights in Fig. 7 to be; KM-1-1-1 : KM-1-1-2 : KM-1-1-3 : KM-1-1-4 : KM-1-1-5 : KM-1-1-6 : KM-1-1-7 : KM-1-1-8 : KM-1-1-9 : KM-1-1-10=9.2:2.5:7.5:5.0:7.7:2.4:1.4:1.0:1.7:1.8. The relative ratio of 3′SL (KM-1-1-1), sialyl 3′-galactosyllactose (KM-1-1-2), sialyl lacto-N-novopentaose a and c (KM-1-1-3), sialyl lacto-N-novopentaose b (KM-1-1-4) and fucosyl sialyl lacto-N-novopentaose a (KM-1-1-5) was estimated to be 3.7:1.0:3.0:2.0:3.1.

Discussion

As in tammar wallaby [4] and grey [10] and red [9] kangaroo milk, oligosaccharides predominate over lactose in koala milk as shown in the gel chromatogram of the milk carbohydrate fraction (Fig. 3). The presence of oligosaccharides in koala milk had previously been demonstrated by thin layer chromatography [17], but their detailed chemical structures had not been explored. The oligosaccharides that were characterized in this study are listed in Fig. 10. Of these, 3′-galactosyllactose, 3′,3″-galactosyllactose and lacto-N-novopentaose I had previously been found in tammar wallaby milk [5, 6, 8]. Lacto-N-novotetraose (Gal(β1-3)[GlcNAc(β1-6)]Gal(β1-4)Glc), 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 and Gal(β1-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-

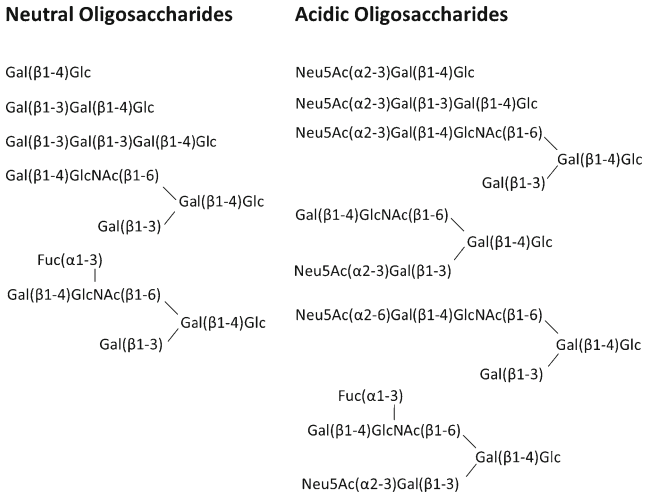

Fig. 10 Structures of the neutral and acidic oligosaccharides of koala milk characterized in this study

6)]Gal(β1-4)Glc, which are present in tammar wallaby milk [6–8], were not detected in koala milk in this study, but it is possible that they are present at low concentrations; the components in the small peaks in KM-2 and KM-3 could not be characterized.

3′-SL, sialyl 3′-galactosyllactose, sialyl lacto-N-novopentaose a and sialyl lacto-N-novopentaose b were identified in red kangaroo milk [9]. Although 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 and Neu5Ac(α2-3)Gal(β1-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc, which were found in red kangaroo milk [9], were not identified in this study, it is again possible that these are present in koala milk at low concentrations.

In our previous study of the red kangaroo milk oligosaccharides, we had not identified sialyl lacto-N-novopentaose c, but we have re-examined the ^{1}H-NMR spectrum of the Mr-1-1-6 fraction separated from red kangaroo milk (Fig. 6 of reference [9]). The oligosaccharides in this fraction had been characterized by assignments of the shifts in the ^{1}H-NMR and also by pseudo molecular ions (1188.345 and 1237.471) in the MALDI-TOF mass spectrum [9]. As the NMR spectrum had the H-1 chemical shifts at δ 4.631 of β(1–6) linked GlcNAc, 4.611 of β(1–3) linked Gal, which was not substituted, and 4.554 of β(1–4) linked Gal, which was substituted by α(2–3) linked Neu5Ac, we have concluded that this fraction contained sialyl lacto-N-novopentaose c as well as sialyl lacto-N-novopentaose a. In addition, the presence of the unusual down field shift of H-1 of β(1–4) linked Gal at δ 4.589 suggested that this fraction contained an oligosaccharide, which contains Gal(β1-4)(−3-*O*-sulfate), because a similar down field shift (δ 4.569) was observed in the ^{1}H-NMR spectrum of Gal(β1-4)Glc-3′-O-sulfate [18]. This suggested that the fraction contained Gal(β1-3)Gal(β1-3)[Gal(β1-4)(−3-*O*-sulfate)GlcNAc(β1-6)]Gal(β1-4)Glc as well as Gal(β1-3)(−3-O-sulfate)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc, which was described in this fraction in the previous paper [9].

Furthermore, we also looked at the ^{1}H-NMR spectrum of the Mr-1-1-8 fraction separated from the red kangaroo milk (Fig. 8 of reference [9]). As the spectrum had H-1 chemical shifts at δ 4.617 of β(1–3) linked Gal and 4.553 of β(1–4) linked Gal, we concluded that this fraction contained Gal(β1-3)Gal(β1-3)[Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc in addition to Neu5Ac(α2-3)Gal(β1-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc, which was described in reference [9]. In addition, the presence of an unusual downfield shift of H-1 of β(1–4) linked Gal at δ 4.590 suggested the presence in Mr-1-1-8 of Gal(β1-3)Gal(β1-3)Gal(β1-3)[Gal(β1-4)(−3-*O*-sulfate)GlcNAc(β1-6)]Gal(β1-4)Glc in addition to Gal(β1-3)(3-*O*-sulfate)Gal(β1-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc, which had been identified in this fraction [9].

We detected all three isomers of sialyl lacto-N-novopentaose (a, b and c) in koala milk, in contrast to Bactrian camel colostrum which was found to contain only the a and b isomers. Presumably, the presence (koala and red kangaroo) or absence (Bactrian camel) of sialyl lacto-N-novopentaose c in milk/colostrum is determined by the substrate specificity of α(2–3) sialyltransferase in the lactating mammary glands. It would seem that koala or red kangaroo α(2–3) sialyltransferases catalyse the transfer of Neu5Ac to both Gal(β1-3) and Gal(β1-4) residues, whereas that of Bactrian camel transfers it only to Gal(β1-3) residues. Although koala milk contained two sialyl oligosaccharides containing α(2–3) linked Neu5Ac, *viz*, sialyl lacto-N-novopentaoses a and c, only one fucosyl sialyl oligosaccharide (fucosyl sialyl lacto-N-novopentaose a) was detected in this milk. The absence of Gal(β1-3){Neu5Ac(α2-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-6)}Gal(β1-4)Glc suggests that the koala α(2–3) sialyltransferase does not transfer Neu5Ac to Gal(β1-4)[Fuc(α1-3)]GlcNAc.

Free fucosyl oligosaccharides have never been found in the milk of tammar wallaby or red kangaroo. In addition, fucose was found to be absent from acid hydrolysates of the neutral milk oligosaccharides of the Eastern gray kangaroo, *Macropus giganteus* [10] and from the carbohydrate fraction of the milk of a South American didelphid marsupial, the gray short-tailed opossum, *Monodelphis domestica* [19]. Our finding that koala milk contains fucosyl lacto-N-novopentaose I and fucosyl sialyllacto-N-novopentaose a was therefore a surprise. It can be hypothesized that milk of the common ancestor of marsupials had contained fucosyl oligosaccharides, but during the course of evolution the macropods and possibly other species such as *Monodelphis* had lost these due to loss of the activity of one or more mammary gland fucosyltransferases, which were retained by the koala. It was

recently shown that glycoproteins of tammar wallaby milk contain core fucosylated and O-fucosylated structures [20]. Therefore, lactating mammary glands of the tammar wallaby do contain fucosyltransferases but the ones that catalyse the fucosylation of lacto-N-novopentaose I and sialyl lacto-N-novopentaose a appear to be either absent or poorly expressed. It would clearly be of interest to study the milk of other marsupials in this regard.

Sulfated oligosaccharides, which were identified in red kangaroo milk [9] (see Fig. 2), were not detected in koala milk in this study. In our previous study of red kangaroo milk oligosaccharides, two acidic oligosaccharide fractions, designated as Mr-1-1-6 and Mr-1-1-7, contained oligosaccharides similar to those in KM-1-1-3 and KM-1-1-4. However, it was found that the red kangaroo fractions also contained sulfated oligosaccharides, namely Gal(β1-3)(−3-O-sulfate) Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (Mr-1-1-6-2) and Gal(β1-3)(−3-O-sulfate)Gal(β1-3)Gal(β1-3)Ga(β1-3) Gal(β1-4)Glc (Mr-1-1-7-3). The presence of these sulfated oligosaccharides was detected on the basis of the characteristic doublet doublet shifts at δ 4.341 and doublet shifts at δ 4.297 in their ^{1}H-NMR spectra, because these signals were not observed in the ^{1}H-NMR spectra of KM-1-1-3 and KM-1-1-4. As the HPLC-based purification method used in the koala study was similar to that used in the red kangaroo studies, we conclude that at least these sulfated oligosaccharides are absent from koala milk carbohydrate. Nevertheless, it is possible that some of the small non-identified peak fractions from koala milk may have contained sulfated oligosaccharides.

In our previous papers, we had suggested that eutherians are closer to monotremes than to marsupials with respect to milk oligosaccharides [21, 22], mainly because the milk of both monotremes (echidna and platypus) and of some eutherians is rich in fucosyl oligosaccharides, while the galactosyl oligosaccharides that are characteristic of marsupial milks are absent from the milks of both monotremes and eutherians. In addition the milks of some eutherians and of the platypus 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), whereas marsupial milks do not contain this type of oligosaccharide.

However, our finding of two fucosyl oligosaccharides in koala milk tends to be inconsistent with the above suggestion. Fucosyl sialyl lacto-N-novopentaose a, which had previously been found in human milk [16], was detected in koala milk in this study. It is also noteworthy that platypus milk does not contain oligosaccharides whose core units are lacto-N-novopentaose I, an oligosaccharide which has been found in the milk of several eutherian species such as Bactrian camel [12], cow [23, 24], horse [25], capuchin [11, 26], etc. as well as of marsupials including the tammar wallaby [8] and koala. Furthermore, sialyl lacto-N-novopentaose a and b have been detected in milk of the Bactrian camel [12] and of marsupials including the red kangaroo [9] and now in the koala. These findings suggest that this type of oligosaccharide had occurred in the milk of the common ancestor of marsupials and eutherians and had been lost in some eutherians. It should be noted, however, that this and other suggestions or hypotheses remain to be tested in further studies on a greater number of samples from a variety of species.

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