Chemical characterization of milk oligosaccharides of the common wombat (*Vombatus ursinus*)

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Abstract

Previous structural characterizations of marsupial milk oligosaccharides have been performed in the tammar wallaby, red kangaroo, koala, common brushtail possum and the eastern quoll. To clarify the homology and heterogeneity of milk oligosaccharides among marsupial species, which could

provide information on their evolution, the oligosaccharides of wombat milk carbohydrate were characterized in this study. Neutral and acidic oligosaccharides were isolated from the carbohydrate fractions of two samples of milk of the common wombat and characterized by ^{1}H-nuclear magnetic resonance spectroscopy. The structures of six 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(β1-3)Gal(β1-3)Gal(β1-4)Glc, Gal(β1-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (galactosyl lacto-N-novopentaose I) and Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4) Glc (lacto-N-novooctaose), while those of six acidic saccharides were 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 (sialyl 3',3"-digalactosyllactose), 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(α2-3)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (sialyl lacto-N-novopentaose c), Neu5Ac(α2-3)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 and Gal(β1-3)Gal(β1-3)[Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc. In addition, small amounts of sulfated oligosaccharides but no oligosaccharides containing Neu5Gc or α(2-6) linked Neu5Ac were detected.

Keywords: common wombat, marsupial, milk oligosaccharide, *Vombatus ursinus*

Introduction

Mammalian milks or colostrum contain from a trace to over 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 (Jenness et al. 1964;

Urashima et al. 2007). However, in the milk of monotremes, marsupials and some Arctoidea species of Carnivora, oligosaccharides usually predominate over free lactose (Urashima et al. 2001, 2007). Among marsupials, oligosaccharide structures have been characterized in only four species: the tammar wallaby (*Macropus eugenii*,), the red kangaroo (*Macropus rufus*), the koala (*Phascolarctos cinereus*), the common brushtail possum (*Trichosurus vulpecula*), and the eastern quoll (*Dasyurus viverrinus*) (Messer & Green 1979; Messer et al. 1980, 1982, Collins et al. 1981, Bradbury et al. 1983; Anraku et al. 2012; Urashima et al. 2013, 2014a, 2015). The predominant neutral milk oligosaccharides of these marsupials, other than the eastern quoll, consist of a series of linear galactosyllactoses, which can be expressed as $[Gal(\beta1\text{-}3)]_nGal(\beta1\text{-}4)Glc$,. In addition these milks contain branched oligosaccharides containing β(1-6) linked GlcNAc, as well as acidic oligosaccharides whose core structures are similar to those of the neutral oligosaccharides found in the milks of these species. However, branched oligosaccharides predominate over the linear ones in the milk carbohydrate fraction of the eastern quoll and neutral and acidic oligosaccharides containing two Gal(β1-4)GlcNAc(β1-6) units were detected (Urashima et al. 2015). Marsupial milk acidic saccharides contain Neu5Ac or sulfate (Anruku et al. 2012; Urashima et al. 2014a), but in koala milk sulfated oligosaccharides were not detected (Urashima et al. 2013). Koala milk exceptionally contains neutral as well as acidic fucosyl oligosaccharides (Urashima et al. 2013).

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 wombat (*Vombatus ursinus*).

Materials and methods

Milk sample and chemicals

The two wombat milk samples were both collected in the ACT, Australia, from one animal, on 20/12/1978 and 12/7/1979, during a study on the

composition of marsupial milk (Green & Merchant 1988). They were frozen and sent to the University of Sydney, where their carbohydrate fractions were extracted using chloroform-methanol (Messer & Mossop 1977). These were freeze-dried and stored in sealed tubes at -20℃ for about 35 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 (Messer & Green 1979; Collins et al. 1981), while Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (lacto-N-novopentaose I) was isolated from koala milk carbohydrate (Urashima et al. 2013) and brown capuchin colostrum (Urashima et al. 1999). Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4) Glc (lacto-N-novooctaose) was isolated from eastern quoll milk carbohydrate (Urashima et al. 2015). 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 (Fukuda et al. 2010), 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 the carbohydrate fraction of koala milk (Urashima et al. 2013). Several sialyl or sulfated galactosyllactoses as well as sialyl or fulfated lacto-N-novopentaose I derivatives were separated from red kangaroo milk carbohydrate (Anraku et al. 2012).

Neutral oligosaccharides

The carbohydrate fractions of wombat milk collected on 12/7/1979 (sample A, 150 mg) and 20/12/1978 (sample B, 150 mg) were each 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 of galactose (monosaccharide), lactose (disaccharide) and

raffinose (trisaccharide). The gel has been 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 (Dubbois et al. 1956) and for sialic acid with periodate – resorcinol (Jourdian et al. 1971). Peak fractions separated from sample A were pooled as shown in Figure 1 and freeze-dried. The saccharides in the peak fractions WM-1 to WM-7 (see Figure 1) were checked using thin layer chromatography with acetone/2-propane/0.1 M 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.

The components in WM-5, WM-6 and WM-7 were characterized by ^{1}H-NMR spectroscopy. Those in WM-2 to WM-4 were subjected to high-performance liquid chromatography (HPLC) (Figure 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℃. 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 and MALDI-TOF mass spectroscopies.

Acidic oligosaccharides

The components in peak WM-1 (Figure 1) from sample A and the first eluted peak fraction from sample B of the gel chromatogram, which reacted positively with both periodate-resorcinol (630 nm) and phenol-H_2SO_4 (490 nm), were mixed and dissolved in 2 mL of 50 mM 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), which had been equilibrated and was 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 (Dubbois et al. 1956). Figure 3 shows that the ion exchange chromatography had separated the WM-1 fraction into three peaks. The components in the peak designated WM-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 WM-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 Figure 4). The mobile phase was 50% and 80% (vol/vol) acetonitrile in 15 mM potassium phosphate buffer (pH 5.2). Elution was done using a linear gradient of acetonitrile from 80% to 50% at 60℃ at a flow rate of 1 mL/min. The eluates were monitored by measuring the absorbance at 195 nm. The peaks designated as WM-1-2-1 to WM-1-2-10 (Figure 4) were each pooled, concentrated by rotary evaporation, and subjected to ^{1}H-NMR and MALDI-TOF mass spectroscopies to determine their structures.

^{1}H-NMR spectroscopy

Nuclear magnetic resonance spectra were recorded in D_2O (99.96 atom D%; Aldrich, Milwaukee, WI, USA) 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 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 an 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, and 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 oligosaccharides

The crude carbohydrate fractions from the wombat milk samples separated into several peaks during gel filtration on BioGel P-2. The result obtained with sample A is shown in Figure 1; an essentially similar result was obtained with sample B. The fractions in each peak were pooled. As the first eluted peak designated WM-1 reacted positively with periodate – resorcinol, it was concluded that its components contained sialic acid. The components in WM-2 to WM-4 were separated by HPLC using a Hypercarb column as shown in Figure 2. The resulting peaks were designated as WM-2-1 to WM-2-2, WM-3-1 to WM-3-8, and WM-4-1 to WM-4-4. The separated peak components obtained by gel filtration and HPLC were characterized by ^{1}H-NMR and MALDI TOFMS spectroscopies.

WM-7

As the ^{1}H-NMR spectrum (chemical shifts in Table 1) of WM-7 was identical with that of lactose, the saccharide in this fraction was characterized to be Gal(β1-4)Glc.

WM-6

As the ^{1}H-NMR spectrum (chemical shifts in Table 1) of WM-6 was identical with that of authentic 3'-galactosyllactose and its published data (Urashima et al. 1992), the oligosaccharide in WM-6 fraction was characterized to be Gal(β1-3)Gal(β1-4)Glc.

WM-5

As the ^{1}H-NMR spectrum (chemical shifts in Table 1) of WM-5 was identical with that of authentic 3',3"-digalactosyllactose and its published data (Urashima et al. 1992), the oligosaccharide in WM-5 fraction was characterized to be Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc.

WM-4-3, WM-4-4

As the ^{1}H-NMR spectra of WM-4-3 and WM-4-4 were very similar, it was concluded that these fractions contained the α- and β-anomers of the same saccharide. The spectrum (chemical shifts in Table 1) of WM-4-3 had the H-1 shifts of α-Glc, two β(1-3) linked Gal, β-Glc and β(1-4) linked Gal at δ 5.224, 4.681 and 4.617, 4.667 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 and 4.197, respectively. From the signal intensities, it was concluded that the shifts at δ 4.681 and 4.203 corresponded to two protons. From these observations, the oligosaccharides in WM-4-3 and WM-4-4 were characterized to be Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc. In addition, the spectra were similar to that of authentic Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc which had been isolated from tammar wallaby milk (Messer & Green 1979).

WM-3-6, WM-3-8

As the ^{1}H-NMR patterns of WM-3-6 and WM-3-8 were identical, it was concluded that these two peaks contained the same saccharide which separated into α- and β-anomer isomers during HPLC using the Hypercarb column. The ^{1}H-NMR spectrum (chemical shifts in Table 1) had the H-1

shifts of α-Glc, two β(1-3) linked Gal, β-Glc, β(1-6) linked GlcNAc, and two β(1-4) linked Gal at δ 5.224, 4.676, 4.616, 4.670, 4.652 and 4.644, 4.501, and 4.473, respectively, and H-4 of β(1-3) and β(1-4) linked Gal, which were substituted at OH-3, at δ 4.204 and 4.180, respectively. The H-1 shift at δ 4.616 arose from non reducing β(1-3) linked Gal, while that at δ 4.676 corresponded to penultimate β(1-3) linked Gal. From these observations, the oligosaccharides in WM-3-6 and WM-3-8 were characterized to be Gal(β1-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc.

WM-3-1, WM-3-2

The oligosaccharides in these fractions could not be characterized by ^{1}H-NMR in this study.

WM-3-3, WM-3-4, WM-3-5, WM-3-7, WM-4-1, WM-4-2

Because of insufficient amounts of the oligosaccharides in these fractions, they could not be characterized in this study.

WM-2-1, WM-2-2

As the ^{1}H-NMR patterns of WM-2-1 and WM-2-2 were identical, it was concluded that these two peaks contained the same saccharide which separated into α- and β-anomer isomers during HPLC using the Hypercarb column. The MALDI-TOF mass spectrum of WM-2-1 and WM-2-2 had the MS ions at 1419.570 which might have arisen from M+Na of $[Hex]_6[NexNAc]_2$. As the spectrum (chemical shifts in Table 1) was essentially similar to that of the DV-2-1 fraction isolated from the milk carbohydrate of the eastern quoll (Urashima et al. 2015), the oligosaccharides in WM-2-1 and WM-2-2 were both characterized to be Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4) Glc (lacto-N-novooctaose).

Characterization of acidic oligosaccharides

Fraction WM-1 separated into three peaks during ion exchange chromatography, as shown in Figure 3. The first, designated as WM-1-1, was thought to contain a mixture of high molecular weight neutral oligosaccharides which were not investigated, while the third peak, designated as WM-1-3, was thought to contain oligosaccharides with more than two sialic acid residues; these were also not investigated in this study. The components in the second peak, designated as WM-1-2, were further separated by HPLC as shown in Figure 3. The oligosaccharides in WM-1-2-1 to WM-1-2-5 were characterized by ^{1}H-NMR spectroscopy.

WM-1-2-1

As the ^{1}H-NMR spectrum (chemical shifts in Table 2) of this fraction was identical with the published data for sialyl 3'-galactsyllactose (Fukuda et al. 2010; Anraku et al. 2012), the oligosaccharide in WM-1-2-1 was characterized to be the tetrasaccharide Neu5Ac(α2-3)Gal(β1-3)Gal(β1-4)Glc.

WM-1-2-2

The ^{1}H-NMR spectrum (chemical shifts in Table 2) of WM-1-2-2 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, 4.681, 4.669 and 4.512, respectively, H-4 of β(1-3) and β(1-4) linked Gal, which were substituted at OH-3, at δ 4.200 and 4.195. The spectrum had H-3 axial and equatorial of α(2-3) linked Neu5Ac at δ 1.802 and 2.763, respectively, and its NAc shift at δ 2.029, 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 the same as those of Mr-1-1-5-1 derived from red kangaroo milk (Anraku et al. 2012), the oligosaccharide in WM-1-2-2 was characterized to be a pentasaccharide, Neu5Ac(α2-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc.

WM-1-2-3

The ^{1}H-NMR spectrum (chemical shifts in Table 2) of WM-1-2-3 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 5.224, 4.687, 4.671, 4.651 and 4.644, and 4.504, and 4.471, respectively, H-4 of β(1-4) linked Gal, which was substituted at OH-3, at δ 4.174 and NAc of β(1-6) linked GlcNAc at δ 2.061. The spectra had the H-3 axial, H-3 equatorial and NAc of α(2-3) linked Neu5Ac at δ 1.802, 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 4.115. As this pattern was essentially similar to the published data for sialyl lacto-N-novopentaose a (Fukuda et al. 2010; Urashima et al. 2013), one oligosaccharide in this fraction was characterized to be a hexasaccharide, Neu5Ac(α2-3)Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc.

However, the spectrum had a small H-1 shift of non reducing β(1-3) linked Gal, and two small shifts of β(1-4) linked Gal at δ 4.610, 4.552 and 4.500, respectively. It was concluded that these H-1 shifts arose from sialyl lacto-N-novopentaose c (Gal(β1-3)[Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc), which was a minor saccharide in this fraction.

WM-1-2-4

The ^{1}H-NMR spectrum (chemical shifts in Table 2) of WM-1-2-4 had the H-1 shifts of α-Glc,, three β(1-3) linked Gal, β-Glc, and β(1-4) linked Gal at δ 5.224, 4.693, 4.686 and 4.681, 4.669 and 4.513, respectively, H-4 of β(1-3) or β(1-4) linked Gal, which was substituted at OH-3, at δ 4.201. The spectrum had H-3 axial, H-3 equatorial and NAc of α(2-3) linked Neu5Ac, at δ 1.802, 2.763 and 2.028, respectively, and H-3 of β(1-3) linked Gal, which was substituted by α(2-3) linked Neu5Ac, at δ 4.116. From these observations, an oligosaccharide in this fraction was characterized to be Neu5Ac(α2-3)Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc, similar to Mr-1-1-7-2 in red kangaroo milk (Anraku et al., 2012). The MALDI-TOF mass spectrum

had the MS ion at 1158.588 of [M+K].

In addition, the spectrum had the characteristic down field shifts of H-3 and H-4 of ß linked Gal, which was substituted by sulfate at OH-3, at δ 4.340 and 4.296, respectively (see Anraku et al. 2012), at low intensities. This showed that this fraction also contained a small amount of a sulfated oligosaccharide.

WM-1-2-5

The ^{1}H-NMR spectrum (chemical shifts in Table 2) of WM-1-2-5 had the H-1 shifts of α-Glc, ß(1-3) linked Gal, ß-Glc, ß(1-6) linked GlcNAc and two ß(1-4) linked Gal at δ 5.225, 4.692, 4.700, 4.644, and 4.500 and 4.474, respectively, H-4 of ß(1-3) linked Gal, which was substituted at OH-3, at δ 4.200, H-4 of ß(1-4) linked Gal, which was substituted at OH-3, at δ 4.175. The spectrum also had H-3 of ß(1-3) linked Gal, which was substituted by α(2-3) linked Neu5Ac, at δ 4.115, H-3 axial and equatorial of α(2-3) linked Neu5Ac at δ 1.802 and 2.763, respectively, NAc of ß(1-6) linked GlcNAc and α(2-3) linked Neu5Ac at δ 2.063 and 2.029, respectively. The MALDI TOF mass had the MS ions at 1361.425 and 1399.392 of [M+K] and [M+2K-H], respectively. From these observations, one oligosaccharide in WM-1-2-5 was characterized to be Neu5Ac(α2-3)Gal(ß1-3)Gal(ß1-3)[Gal(ß1-4)GlcNAc(ß1-6)]Gal(ß1-4)Glc (WM-1-2-5-1) as in Mr-1-1-8-1 of the red kangaroo (Anraku et al. 2012). In addition, the H-1 shifts of non reducing ß(1-3) linked Gal at δ 4.617 and ß(1-4) linked Gal at δ 4.553 showed the presence of Gal(ß1-3)Gal(ß1-3)[Neu5Ac(α2-3)Gal(ß1-4)GlcNAc(ß1-6)[Gal(ß1-4)Glc (WM-1-2-5-2).

However, the spectrum had another NAc shift of ß(1-6) linked GlcNAc at δ 2.041 and H-1 of ß(1-4) linked Gal at δ 4.514; this might have arisen from another oligosaccharide, which contained Gal(ß1-3)[Gal(ß1-4)GlcNAc(ß(1-6))Gal(ß1-3)Gal(ß1-4)Glc, as in the oligosaccharide fraction from brushtail possum milk (Urashima et al. 2014a). In addition, the spectrum had the H-3 and H-4 of ß(1-3) or ß(1-4) linked Gal at δ 4.341 and 4.297, respectively (see Anraku et al. 2012), at low intensities,

which indicated the presence of a minor sulfated oligosaccharide.

WM-1-2-6 to WM-1-2-10

The oligosaccharides in WM-1-2-6 to WM-1-2-10 could not be characterized with 1H-NMR in this study.

Discussion

The structures of the milk oligosaccharides of the common wombat characterized in this study are shown in Figure 5. The four neutral oligosaccharides have been previously found in milk carbohydrate of the tammar wallaby (Messer et al. 1980, 1982; Collins et al. 1981; Bradbury et al. 1983) and the brushtail possum (Urashima et al. 2014a). Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (lacto-N-novopentaose I), which has been found in the milk carbohydrate fractions of the tammar wallaby (Bradbury et al. 1983), koala (Urashima et al. 2013), brushtail possum (Urashima et al. 2014a) and eastern quoll (Urashima et al. 2015), was not detected in this study, but as it is a precursor of galactosyl lacto-N-novopentaose I, it can be hypothesized that small amounts of this saccharide occur also in wombat milk..

The octasaccharide Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (lacto-N-novooctaose) has been previously detected in eastern quoll milk carbohydrate (Urashima et al. 2015). This type of saccharide was found to be present in the sialyl oligosaccharide fraction of tammar wallaby milk (Urashima et al. 1994), but awaits a complete structural characterization. It is also possible that a similar oligosaccharide was present in the uncharacterized fractions of red kangaroo, koala and common brushtail possum milk carbohydrate.

The acidic oligosaccharides characterized in this study, have been previously detected in the milk carbohydrates of the red kangaroo (Anraku et al. 2012) and/or the brushtail possum (Urashima et al. 2014a). Those of

the red kangaroo and brushtail possum contained sulfated oligosaccharides whose core structures were similar to those of the sialyl oligosaccharides (Anraku et al. 2012; Urashima et al. 2014a). Sulfated oligosaccharides were detected in this study as well, but the ratio of sulfated to sialylated oligosaccharides was smaller in wombat than in red kangaroo and brushtail possum.

The red kangaroo and brushtail possum milk carbohydrates contained Gal(β1-3)[Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (Anraku et al. 2012; Urashima et al. 2014a), but this hexasaccharide was not detected in this study, suggesting that the lactating mammary glands of the wombat lacked α2-6sialyltransferase activity.

We found that in the wombat milk carbohydrate, as in that of tammar wallaby, brushtail possum and koala, the linear oligosaccharides predominate over branched ones, which is unlike the situation in eastern quoll milk carbohydrate, in which branched oligosaccharides were found to be predominant over linear ones (Messer et al. 1980, 1982; Brudbury et al. 1983; Collins et al. 1981; Urashima et al. 2013, 2014a, 2015). In this respect the eastern quoll appears to be unique among those mausrpials whose milk carbohydrates have been studied to date.

It is of interest also to compare the wombat milk oligosaccharides with those of the koala, which is known to be the closest living relative of wombats. Two fucosyl oligosaccharides, Gal(β1-3){Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-6)}Gal(β1-4)Glc and Neu5Ac(α2-3)Gal(β1-3){Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-6)}Gal(β1-4)Glc, which were characterized in koala milk carbohydrate in our previous study (Urashima et al. 2013), were not detected in that of the wombat. Among marsupial species so far studied, fucosyl milk oligosaccharides have notably been found only in the koala. Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc and its sialyl derivative were found in the present study, but had not been detected in koala milk carbohydrate (Urashima et al. 2013); these saccharides may, however, have been present in the unidentified fractions. Sulfated oligosaccharides that we detected in wombat milk carbohydrate were not found in that of the koala. Thus there appear to be some differences

between wombat and koala in their milk oligosaccharides. Nevertheless, it can be concluded that the milk oligosaccharides of the common wombat are similar to those of the tammar wallaby, red kangaroo, brushtail possum and koala, even though some heterogeneity in both neutral and acidic oligosaccharides was observed.

Even though some heterogeneity was found in milk oligosaccharides between the six marsupial species that have so far been studied, this heterogeneity is small when compared with that between eutherian species. The most notable example of such heterogeneity within marsupial oligosaccharides is that between the eastern quoll, a carnivorous marsupial (Order Dasyuromorphia), and the tammar wallaby and the other herbivorous marsupials including the wombat (Order Diprorodontia) (see Urashima et al., 2015). However, support for speculations concerning the possible significance of these findings in terms of marsupial evolution would require further studies on the milk oligosaccharides of other marsupial species such as the Tasmanian devil (Dasyuromorphia) and the bilby and bandicoots (Paramelemorphia).

A significant feature of milk oligosaccharides common to all these marsupials is the presence of a series of linear β(1-3)galactosyllactoses, ranging in size from tri- to at least octa-saccharides. Members of this series greater than the trisaccharide have not been found in the milk/colostrum of any eutherian or monotreme (Urashima et al. 2014b). Therefore, in terms of milk oligosaccharides, eutherians resemble monotremes more than they do marsupials. It is noteworthy, in addition, that lacto-N-neotetraose (Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc) and lacto-N-neohexaose (Gal(β1-4)GlcNAc(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc) have been found as core structures of milk oligosaccharides in both eutherians and monotremes but not in marsupials (Urashima et al. 2014b). It would, of course, be too simplistic to suggest that these data support the notion of eutherians having evolved from monotremes.

The neonates of almost all eutherians are able to digest milk lactose to glucose and galactose due to the activity of the enzyme lactase which is located in the microvilli of the brush border of their small intestinal

epithelial cells. These monosaccharides then enter the circulation, ultimately to be used as energy sources. In contrast, the neonates of the tammar wallaby and other marsupials, as well as of the echidna, a monotreme, lack the eutherian small intestinal brush border lactase (Messer et al. 1989), a finding which is presumably related to the near absence of free lactose in marsupial and monotreme milks. In addition, the small intestinal brush borders of neonatal tammars and echidnas lack the other enzymes that would be required to hydrolyze the oligosaccharides found in their mother's milk (Messer et al. 1989). Therefore these oligosaccharides, to have a nutritional function, must be digested and absorbed by a mechanism that is different from that of eutherians. It has been suggested that this mechanism is most likely based on the absorption of intact marsupial and monotreme milk oligosaccharides via pinocytosis or endocytosis, followed by their digestion to monosaccharides by the actions of a member of lysosomal enzymes including an acid ß-galactosidase, fucosidases, sialidases and N-acetylglucosaminidases (Messer et al. 1989). In eutherian neonates, most of the milk oligosaccharides remain undigested within the small intestine and thus reach the colon. It is assumed, therefore, that they act as prebiotics, stimulating the growth of beneficial colonic bacteria and acting as decoy receptors that inhibit the attachment of pathogenic microorganisms to the colonic mucosa (Urashima et al. 2007, 2009). It is possible that some undigested milk oligosaccharides that had escaped absorption in the small intestine could similarly act as prebiotics in suckling marsupials.

The milk/colostrum of a few eutherian species, including the dog (Bubb et al. 1999), Hamadryas baboon (Goto et al. 2010), sifaka (Taufik et al. 2012) and rat (Sturman et al. 1985), contain lactose sulfate or sialyl lactose sulfate, while the milk of marsupials other than koala contain sulfated oligosaccharides in which non reducing galactose is substituted by sulfate. The presence of this type of sulfated oligosaccharide is a notable feature of marsupial milks. The sulfate in these oligosaccharides can be utilized for the synthesis of proteoglycans such as chondroitin sulfate and dermatan sulfate that are essential components in the body tissues of marsupial neonates. It is well known that marsupial neonates are very immature compared with

eutherian neonates. Thus it is likely that marsupial neonates utilize the sulfate of sulfated oligosaccharides, that are found in their mother's milk, as a nutritional source for their growth.

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Figure 1　Gel chromatogram of the carbohydrate fraction from wombat 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).

Figure 2　High performance liquid chromatography of the neutral oligosaccharide fractions WM-2 to WM-4 separated from the carbohydrate fraction of wombat 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℃. 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

Figure 3　Anion exchange chromatography of WM-1 (Fig. 1) separated from wombat 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.

Figure 4　High performance liquid chromatogram of fraction WM-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℃ at a flow rate of 1 mL/min. The detection was done by UV absorption at 195 nm.

Figure 5　The chemical structures of wombat milk oligosaccharides characterized in this study.

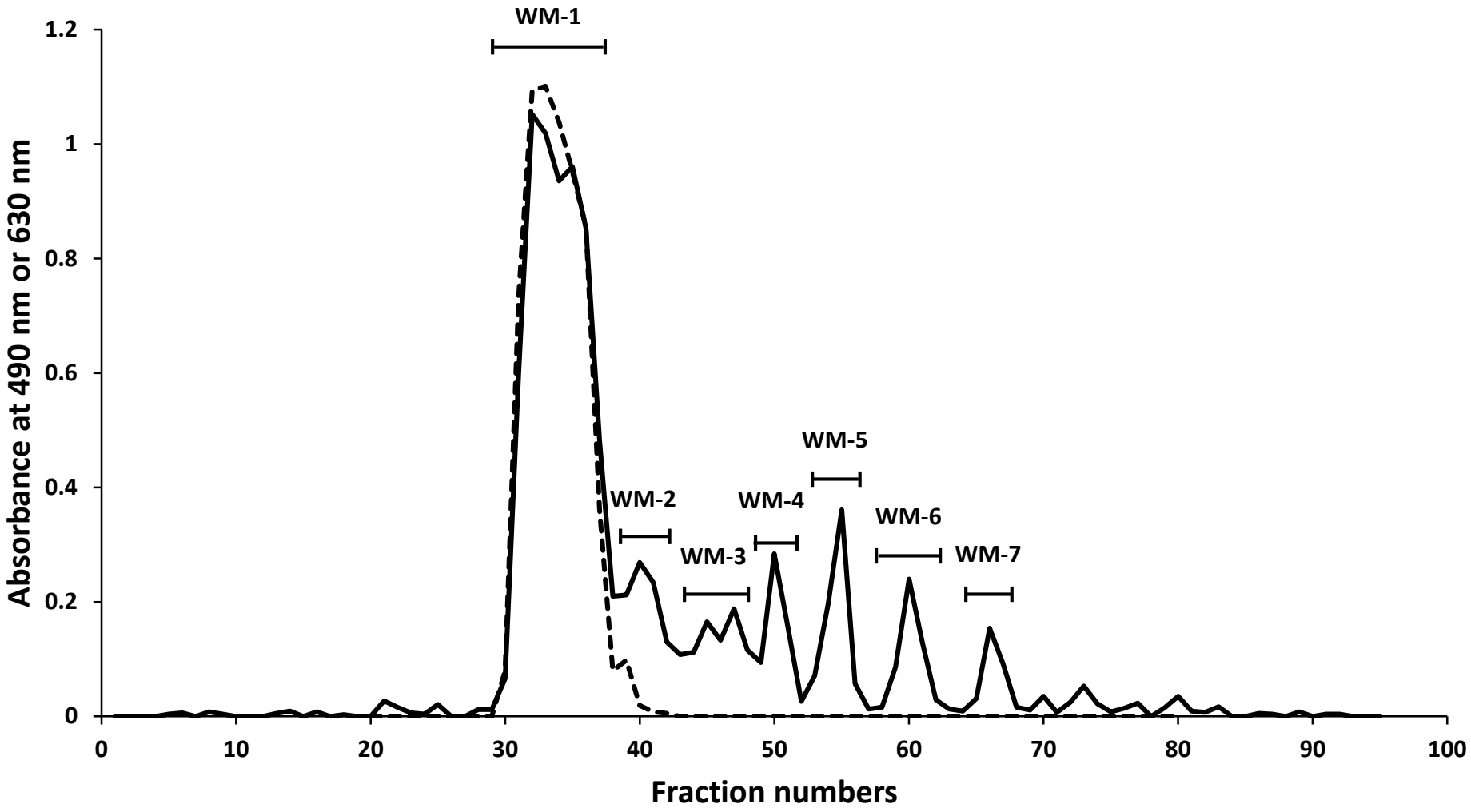
WM-1
WM-2
WM-3
WM-4
WM-5
WM-6
WM-7
Absorbance at 490 nm or 630 nm
Fraction numbers
0
0.2
0.4
0.6
0.8
1
1.2
0
10
20
30
40
50
60
70
80
90
100

Fig. 1

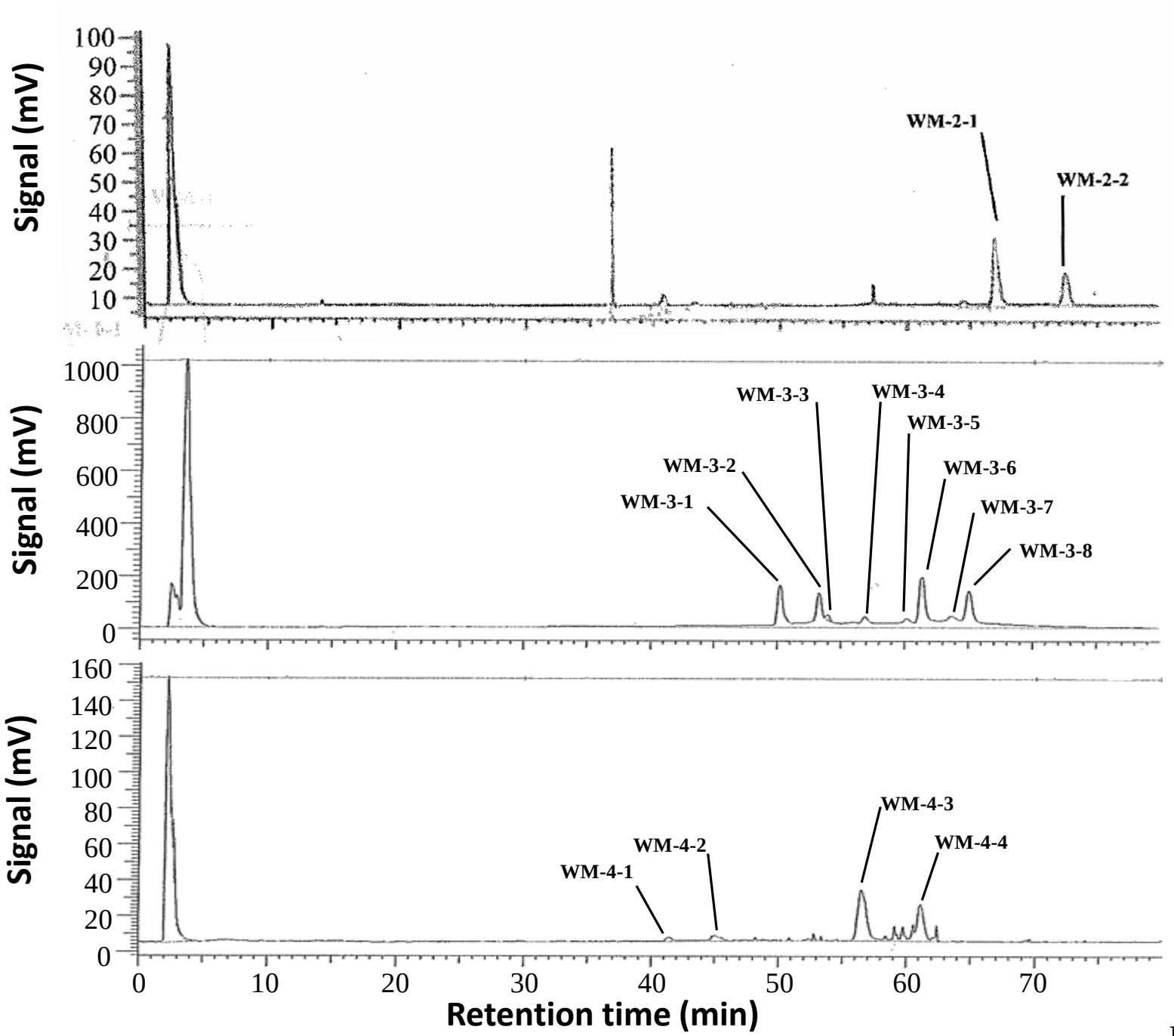
Signal (mV)
100
90
80
70
60
50
40
30
20
10
WM-2-1
WM-2-2
Signal (mV)
1000
800
600
400
200
0
WM-3-1
WM-3-2
WM-3-3
WM-3-4
WM-3-5
WM-3-6
WM-3-7
WM-3-8
Signal (mV)
160
140
120
100
80
60
40
20
0
WM-4-1
WM-4-2
WM-4-3
WM-4-4
0
10
20
30
40
50
60
70
Retention time (min)

Fig. 2

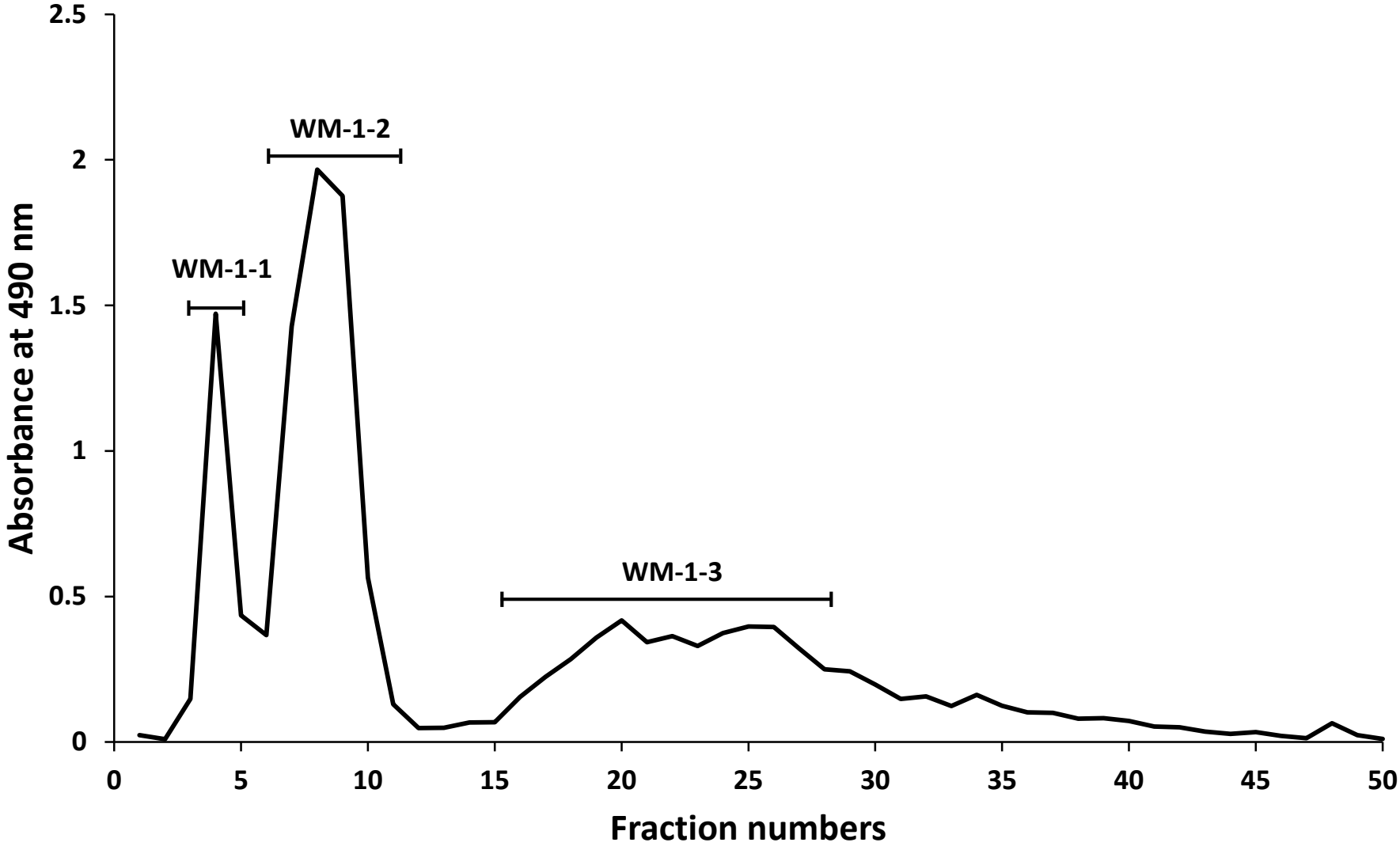
2.5
2
1.5
1
0.5
0
Absorbance at 490 nm
WM-1-1
WM-1-2
WM-1-3
0
5
10
15
20
25
30
35
40
45
50
Fraction numbers

Fig. 3

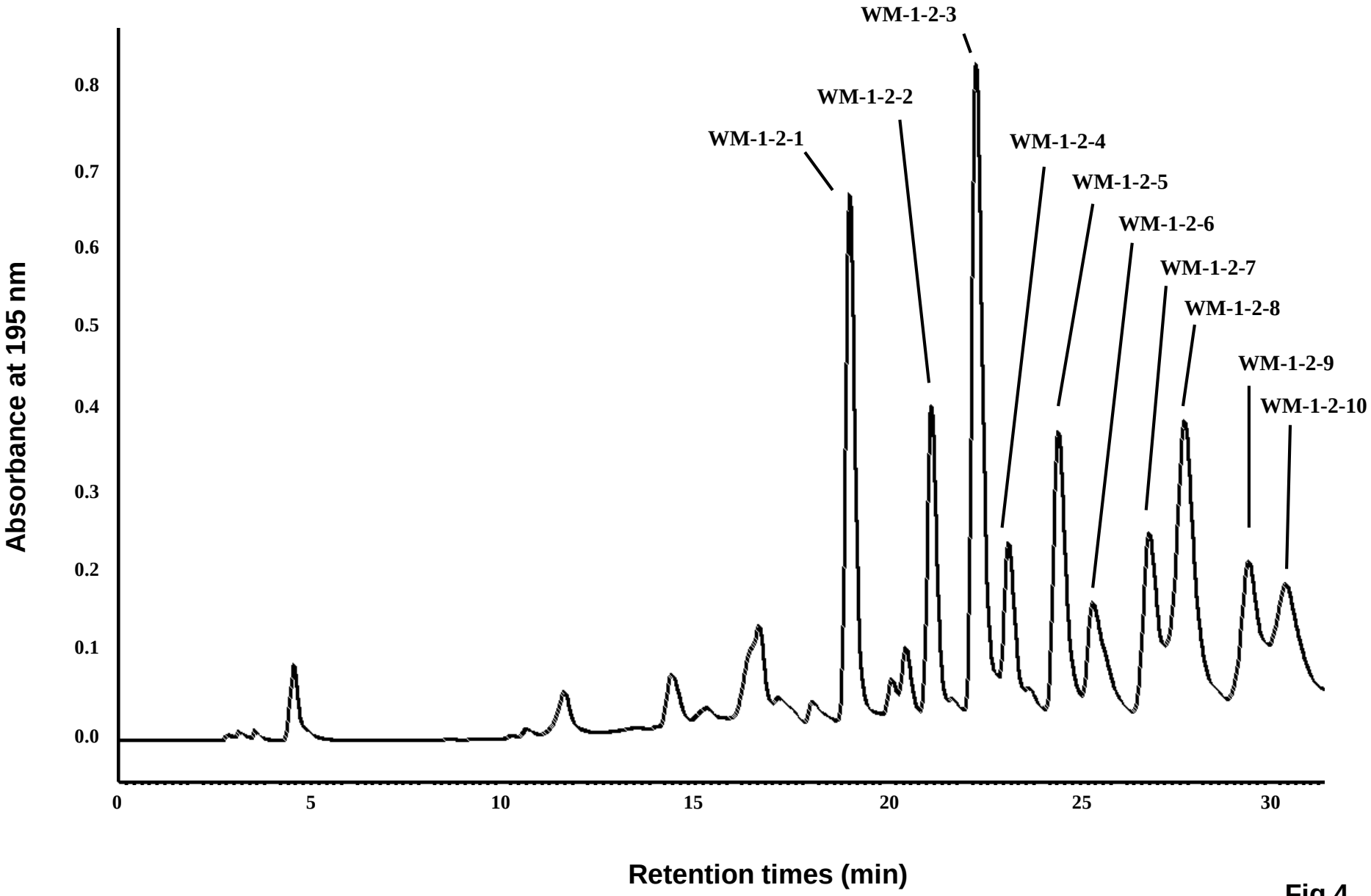
WM-1-2-3
WM-1-2-2
WM-1-2-1
WM-1-2-4
WM-1-2-5
WM-1-2-6
WM-1-2-7
WM-1-2-8
WM-1-2-9
WM-1-2-10
Absorbance at 195 nm
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
5
10
15
20
25
30
Retention times (min)

Fig 4

WM-7

Gal(β1-4)Glc

WM-6

Gal(β1-3)Gal(β1-4)Glc

WM-5

Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc

WM-4-3

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

WM-3-1-6

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

WM-2-1

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

WM-1-2-1

Neu5Ac(α2-3)Gal(β1-3)Gal(β1-4)Glc

WM-1-2-2

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

WM-1-2-3-1

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

WM-1-2-3-2

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

WM-1-2-4

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

WM-1-2-5-1

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

WM-1-2-5-2

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

Fig. 5

Table 1 ^{1}H-NMR chemical shifts of the oligosaccharides WM-2 to WM-7, separated from wombat milk carbohydrate by gel filtration and HPLC

Reporter group	Residue	Chemical shift, δ (coupling constants, Hz)					
		WM-2-1	WM-3-6	WM-4-3	WM-5	WM-6	WM-7
H-1	Glcα	5.225 (3.5)	5.224 (3.8)	5.224 (3.8)	5.224 (4.0)	5.225 (4.0)	5.223 (4.0)
	Glcβ	4.671	4.670 (8.4)	4.667 (8.1)	4.668 (8.0)	4.668 (8.0)	4.665 (8.0)
	Galβ4	4.475 (8.0)	4.473 (7.7)	4.512 (8.0)	4.513 (7.4)	4.512 (8.0)	4.451 (8.0)
		4.500 (8.0)	4.501 (7.7)				
	Galβ3	4.616 (7.7)	4.616 (8.0)	4.617 (7.7)	4.617 (7.4)	4.613 (7.4)	—
		4.686	4.676	4.681	4.677 (7.4)		
	GlcNAcβ6	4.655	4.644 (7.7)	—	—	—	—
			4.652 (7.7)				
		4.594 (8.1)					
H-4	Galβ4	4.157 (2.8)[a]	4.180 (3.1)[a]	4.197 (3.2)[a]	4.198 (3.4)[a]	4.200 (3.4)[a]	
	Galβ3	4.172 (2.8)[a]	4.204 (3.1)[a]	4.203 (3.2)[a]	4.204 (2.9)[a]		
NAc	GlcNAcβ6	2.067	2.063	—	—	—	—
		2.043					

[a] $J_{4,3}$

Table 2 ^{1}H-NMR chemical shifts of the oligosaccharides WM-1-2-1 to WM-1-2-5, separated from wombat milk carbohydrate by gel filtration, ion exchange chromatography and HPLC

Reporter group	Residue	Chemical shift, δ (coupling constants, Hz)						
		WM-1-2-5-1	WM-1-2-5-2	WM-1-2-4	WM-1-2-3-1	WM-1-2-3-2	WM-1-2-2	WM-1-2-1
H-1	Glcα	5.225 (3.5)	5.225 (3.5)	5.224 (3.6)	5.224 (3.9)	5.224 (3.9)	5.225	5.225 (4.0)
	Glcβ	4.700	4.700	4.669 (8.0)	4.671 (8.1)	4.671 (8.1)	4.669 (7.9)	4.668 (8.0)
	Galβ4	4.474 (8.0)	4.553 (7.6)	4.513 (7.7)	4.471 (8.0)	4.552 (7.9)	4.512 (7.9)	4.515 (7.4)
		4.500 (8.0)	4.500 (8.0)		4.504 (8.0)	4.500 (8.0)		
	Galβ3	4.692	4.692	4.693 (8.0)	4.687 (8.1)	4.610 (7.6)	4.681 (7.1)	4.688 (8.0)
			4.617 (7.6)	4.686 (7.7)			4.693 (7.6)	
				4.681 (6.6)				
	GlcNAcβ6	4.644 (8.0)	4.644 (8.0)	—	4.651 (7.7)	4.651 (7.7)	—	—
					4.644 (7.7)	4.644 (7.7)		
H-3	Galβ4		4.115			4.115 (3.1)[a]		
	Galβ3	4.115		4.116 (3.1)[a]	4.115 (3.1)[a]		4.116 (3.2)[a]	4.116 (3.4)[a]
H-4	Galβ4	4.175	4.175	4.201	4.174 (3.5)[b]	4.174 (3.5)[b]	4.195 (3.3)[b]	4.195 (3.4)[b]
	Galβ3	4.200	4.200	4.201			4.200 (2.9)[b]	
H-3 ax	Neu5Ac(α2-3)	1.802 (12.2[c], -12.2[d])	1.802 (12.2[c], -12.2[d])	1.802 (12.2[c], -11.9[d])	1.802 (12.1[c], -11.9[d])	1.802 (12.1[c], -11.9[d])	1.802 (12.3[c], -12.1[d])	1.802 (12.6[c], -12.0[d])
H-3 eq	Neu5Ac(α2-3)	2.763 (4.9)[e]	2.763 (4.9)[e]	2.763 (4.7)[e]	2.762 (4.5)[e]	2.762 (4.5)[e]	2.763 (4.7)[e]	2.762 (4.6)[e]
NAc	GlcNAcβ6	2.063	2.063	—	2.061	2.061	—	—
	Neu5Ac(α2-3)	2.029	2.029	2.028	2.027	2.027	2.029	2.027

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