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ORIGINAL ARTICLE

Chemical characterization of milk oligosaccharides of the island flying fox (*Pteropus hypomelanus*) (Chiroptera: Pteropodidae)

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ABSTRACT

Although a considerable amount of information has accumulated about oligosaccharides in the milk and colostrum of representatives of various mammalian orders, nothing is so far known concerning these sugars in the milk of any bat species (order Chiroptera). In this study, we determined that the following oligosaccharides occur in milk of the island flying fox, *Pteropus hypomelanus* (Chiroptera: Pteropidae): Gal(α1–3)Gal(β1–4)Glc (isoglobotriose), Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)Glc (lacto-N-neotetraose), Gal(β1–4)GlcNAc(β1–3)[Gal(β1–4)GlcNAc(β1–6)]Gal(β1–4)Glc (lacto-N-neohexaose) and Neu5Gc(α2–3)Gal(β1–4)Glc (3′-NGc-SL). However, lactose was found to be the dominant saccharide in this milk, as in most eutherian mammals. The biologic importance of oligosaccharides in Chiropteran milks warrants further study.

Key words: *bat, Chiroptera, island flying fox, milk oligosaccharides, Pteropidae.*

INTRODUCTION

The milk of eutherian mammals usually contains lactose as a dominant saccharide (Jenness *et al.* 1964) in addition to low concentrations of a variety of oligosaccharides, most of which contain a lactose unit at their reducing ends. For example, human milk carbohydrate is composed of about 80% lactose and 20% of more than 130 different oligosaccharides. The structures of the milk or colostrum oligosaccharides of many mammalian species have been elucidated; these include representatives of several mammalian orders, including Monotremata, Diprotodontia, Proboscidea, Pilosa, Primates, Rodentia, Cetartiodactyla, Carnivora and Perissodactyla (Urashima *et al.* 2007).

However, att present there is no information on oligosaccharides that may be found in the milk of any species of the order Chiroptera (bats), even though this is the second-most predominant species mammalian order, containing about 1120 species (Wilson & Reeder 2005). Systematic studies of the proximate composition of bat milks have included temperate and neotropical ‘microchiropteran’ genera, such as *Artibeus* and *Phyllostomus* (Phyllostomatidae) (Oftedal & Iverson 1995; Stern *et al.* 1997), *Eptesicus* and *Myotis* (Vespertilionidae) (Kunz *et al.* 1983, 1995; Hood *et al.* 2001) and *Tadarida* (Molossidae) (Kunz *et al.* 1995) as well as ‘megachiropteran’ genera of Africa, Asia and Australia, such as *Pteropus* and *Rousettus* (Pteropidae) (Messer & Parry-Jones 1997; Korine & Arad 1999; Hood *et al.* 2001). These studies indicate that the concentration of carbohydrate in milk of the Pteropid bats may be somewhat higher (about 5.5–6.5%) than in the three families of microchiropterans (about 3% to 5%, depending on the species). In all these studies the carbohydrate content was determined in relation to lactose standards, even though some of this carbohydrate may be comprised of

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oligosaccharides, that is, saccharides other than free lactose.

In this study of oligosaccharides in bat milk, we chose a species of Pteropid bat (*Pteropus hypomelanus*, the island flying fox) that is relatively large (0.57 kg) and thus enabled substantial milk samples to be obtained (Hood *et al.* 2001). This species is native to Southeast Asia, including New Guinea, Indonesia, Thailand, Vietnam and the Phillipines. Milk was obtained from bats in a breeding colony in Florida, as described by Hood *et al.* (2001). At peak lactation (45–90 days post partum), the milk of this species contains a total of 6.2% ± 1.2 SD carbohydrate.

MATERIALS AND METHODS

Materials

The milk samples (total 2.9 mL) were collected at 87, 101 and 132 days post partum from seven lactating *Pteropus hypomelanus* maintained at the Lubee Foundation, Gainesville, Florida, USA. Lactating bats were separated from their pups for 3 h prior to milking and injected with oxytocin prior to manual expression (Hood *et al.* 2001). The samples were immediately frozen at −20°C in sealed tubes. They were shipped on dry ice to the Nutrition Laboratory of the Smithsonian National Zoological Park (Washington, DC, USA) where they were stored at −20°C until use.

Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)Glc (lacto-N-neotetraose) and Gal(β1–4)GlcNAc(β1–3)[Gal(β1–4)GlcNAc(β1–6)]Gal(β1–4)Glc (lacto-N-neohexaose) were purchased from Seikagaku Co. (Tokyo, Japan). Neu5Gc(α2–3)Gal(β1–4)Glc (3′-NGc-Lac) and Gal(α1–3)Gal(β1–4)Glc (isoglobotriose) were purified from ovine colostrum (Nakamura *et al.* 1998) and caprine colostrum (Urashima *et al.* 1994), respectively.

Isolation of milk oligosaccharides and lactose

The milk samples were thawed, combined and extracted with four volumes of chloroform/methanol (2:1, v/v). The emulsion was centrifuged at 4°C and 4000 × *g* for 30 min, and the lower chloroform layer and the denatured proteins were discarded. The methanol was removed from the upper layer by rotary evaporation, and the residue was dissolved in 10 mL water and freeze-dried. The resulting white powder was called the 'carbohydrate fraction'.

The carbohydrate fraction was dissolved in 2 mL of water and the solution passed through a Bio Gel P-2 (<45 μm; GE Healthcare, Hercules, CA, USA) column (2.5 × 100 cm) that had been calibrated with 2 mg each of galactose (monosaccharide), lactose (disaccharide) and raffinose (trisaccharide). Elutions were 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 (Dubois *et al.* 1956) and for sialic acid with periodate – resorcinol (Jourdian *et al.* 1971). Peak fractions were pooled and freeze-dried. The saccharides in the peak fractions Fly-10, Fly-9, Fly-7 and Fly-4 were subjected to ^{1}H–nuclear magnetic resonance (^{1}H-NMR) spectroscopy to determine their chemical structures.

The components of peak Fly-1 (see Fig. 1), which gave positive reactions with both periodate-resorcinol (630 nm) and phenol-H_2SO_4 (490 nm), were further separated by high performance liquid chromatography (HPLC) on a TSKgel® Amido-80 column (4.6 × 250 mm, pore size 80 Å, particle size 5 μm; Tosoh, Tokyo, Japan) using a LC-10ATVP pump (Shimadzu, Kyoto, Japan) (chromatogram on Fig. 2). 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 acetonitrile from 80% to 50% at 60°C at a flow rate of 1 mL/min. The peak fractions of oligosaccharides were pooled, concentrated by rotary evaporation, and subjected to ^{1}H-NMR spectroscopy to determine the structures.

^{1}H-NMR spectroscopy

NMR spectra were recorded in D_2O (100.00 atom D%; Aldrich, Milwaukee, WI, USA) at 500 or 600 MHz for ^{1}H-NMR with a JEOL ECD-500 FT-NMR or a Varian INOVA 600 spectrometer operated at 293.1 K. Chemical shifts are expressed in δ relative to internal 3-(trimethylsilyl)-1-propane sulfonic acid, sodium salt (TPS), but actually measured by reference to internal acetone (δ = 2.225).

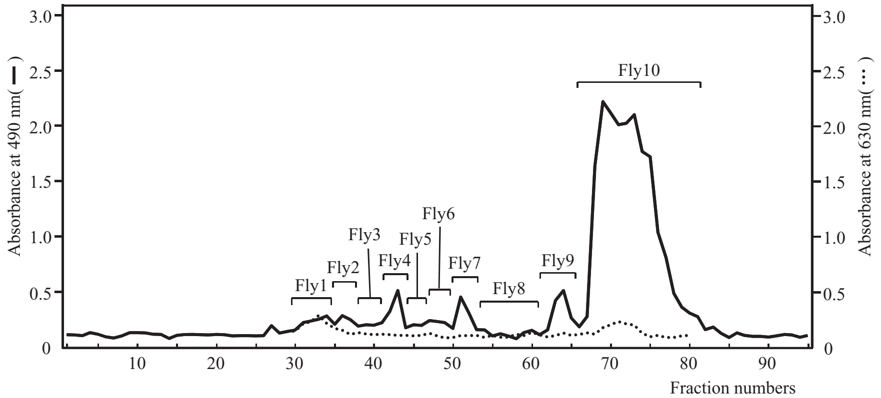

Figure 1 Gel chromatogram of the crude carbohydrate fraction extracted from the island flying fox milk using a Bio Gel P-2 extra-fine column (2.5 × 100 cm). Fractions were monitored by the phenol-H_2SO_4 method (OD_{490}) and the periodate-resorcinol method (OD_{630}).

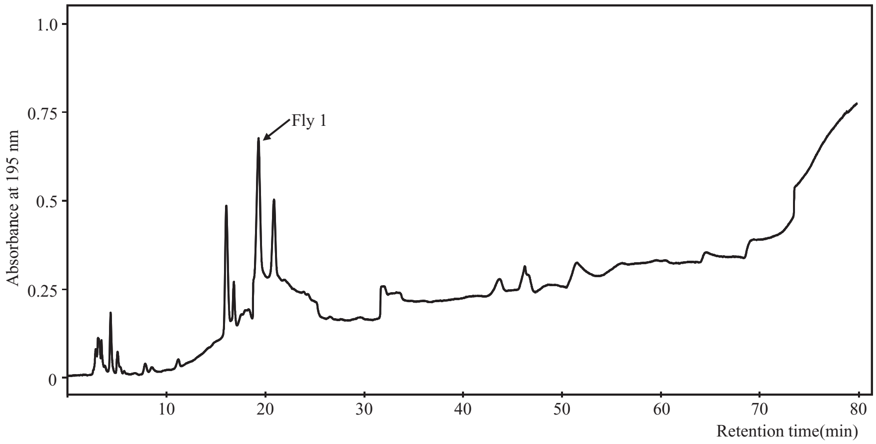

Figure 2 High performance liquid chromatography (HPLC) profile of the acidic oligosaccharide fraction, Fly-1, separated from island flying fox milk. HPLC was done using a Shimadzu LC-10ATVP pump on a TSK gel Amido 80 column (4.6 × 250 mm, pore size = 80 Å, particle size = 5 μm). The mobile phase was 50% and 80% acetonitrile in 15 mmol potassium phosphate buffer. Elution was done using a linear gradient of acetonitrile from 80% to 50% at 60°C at a flow rate on 1 mL/min. The detection of peaks was done by UV absorption at 195 nm.

RESULTS

The carbohydrate fraction of the small flying fox milk was resolved into several peaks by chromatography on Bio Gel P-2 as shown in Figure 1. The components in each fraction were pooled and designated as in Figure 1. The saccharides in Fly-10, Fly-9, Fly-7 and Fly-4 were characterized by ^{1}H-NMR spectroscopy.

Neutral oligosaccharides

Fly-10

As the ^{1}H-NMR spectrum of the saccharide in Fly-10 (chemical shifts in Table 1) was identical with that of lactose, it was characterized as Gal(β1–4)Glc.

Fly-9

As the ^{1}H-NMR spectrum of the saccharide in Fly-9 (chemical shifts in Table 1) was identical with that of authentic isoglobotriose, it was characterized as Gal(α1–3)Gal(β1–4)Glc.

The spectrum had the anomeric signals of α-Glc, α(1–3) linked Gal, β-Glc and β(1–4) linked Gal at δ 5.225, 5.146, 4.669 and 4.524, respectively; H-5 of α(1–3) linked Gal at δ 4.198, and H-4 of β(1–4) linked Gal, which was substituted by α(1–3) linked Gal, at δ 4.184, similar to the spectrum of isoglobotriose.

Fly-7

As ^{1}H-NMR spectrum of the saccharide in Fly-7 (chemical shifts in Table 1) was identical with that of authentic lacto-N-neotetraose, the saccharide in this fraction was identified as Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)Glc.

The spectrum had the anomer signals of α-Glc, β(1–3) linked GlcNAc, β-Glc and two of β(1–4) linked Gal at δ 5.219, 4.701, 4.663, 4.479 and 4.438, respectively; H-4 of β(1–4) linked Gal, which was substituted at OH-3 by β(1–3) linked GlcNAc, at δ 4.156, and NAc of β(1–3) linked GlcNAc at δ 2.034, similar to the spectrum of lacto-N-neotetraose.

Fly-4

As the ^{1}H-NMR spectrum of the saccharide in Fly-4 (chemical shifts in Table 1) was identical with that of authentic lacto-N-neohexaose, the saccharide in this fraction was identified as Gal(β1–4)GlcNAc(β1–3)[Gal(β1–4)GlcNAc(β1–6)]Gal(β1–4)Glc.

The spectrum had the anomer signals of α-Glc, β(1–3) linked GlcNAc, β-Glc, β(1–6) linked GlcNAc, and three of β(1–4) linked Gal at δ 5.219, 4.700 and 4.665, 4.644 and 4.637, 4.481, 4.471, and 4.427, respectively; H-4 of β(1–4) linked Gal, which was substituted at OH-3 by β(1–3) linked GlcNAc, at δ 4.147, and NAc of β(1–6) and β(1–3) linked GlcNAc at δ 2.061 and 2.031, respectively, similar to the spectrum of lacto-N-neohexaose.

Acidic oligosaccharide

Each oligosaccharide in the fraction Fly-1 was separated by HPLC (chromatogram in Fig. 2) prior to characterization by ^{1}H-NMR. Only the component in Fly-1 (Fig. 2) was characterized in this study, as the amounts of other components were too small to be characterized.

Fly-1

As the ^{1}H-NMR spectrum of the saccharide in Fly-1 (chemical shifts in Table 1) was identical with that of authentic 3′-NGc-SL, the saccharide in this fraction was identified as Neu5Gc(α2–3)Gal(β1–4)Glc.

Table 1 1H-NMR chemical shifts of the oligosaccharides separated from flying fox milk

Reporter group	Residue	Chemical shifts, δ (coupling constants, Hz)				
		Fly-1	Fly-4	Fly-7	Fly-9	Fly-10
H-1	Glc α	5.221 (3.8)	5.219 (4.0)	5.219 (4.0)	5.225 (4.0)	5.223 (4.0)
	Glc β	4.664 (7.9)	4.665 (7.4)	4.663 (8.0)	4.669 (8.0)	4.664 (8.0)
	Gal α3	–	–	–	5.146 (4.0)	–
	Gal β′4	4.536 (7.6)	4.427 (8.0)	4.438 (8.0)	4.524 (7.4)	4.447 (8.0)
	Gal β‴4	–	4.471 (7.4), 4.481 (8.0)	4.479 (7.4)	–	–
	GlcNAc β3	–	4.699 (8.0), 4.700 (8.0)	4.701 (8.6)	–	–
	GlcNAc β6	–	4.637 (8.0), 4.644 (7.4)	–	–	–
H-3$_{ax}$	Neu5Gc(α2-3)	1.817 (12.6, −12.0)	–	–	–	–
H-3eq	Neu5Gc(α2-3)	2.775 (4.3)	–	–	–	–
H-4	Gal β4	–	4.147 (3.4)	4.156 (3.4)	4.184 (2.9)	–
H-5	Gal α3	–	–	–	4.198	–
NAc	GlcNAc β3	–	2.031	2.034	–	–
	GlcNAc β6	–	2.061	–	–	–
NGc	Neu5Gc(α2-3)	4.119	–	–	–	–

Fly1, Neu5Gc(α2-3)Gal(β1-4)Glc; Fly4, Gal(β1-4)GlcNAc(β1-3)[Gal(β1-4)GlcNAc(β 1-6)]Gal(β1-4)Glc; Fly7, Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc; Fly9, Gal(α1-3)Gal(β1-4)Glc; Fly10, Gal(β1-4)Glc.

The spectrum had the anomeric signals of α-Glc, β-Glc and β(1–4) linked Gal at δ 5.221, 4.664 and 4.536, H-3 axial, H-3 equatorial and NGc shifts of α(2–3) linked Neu5Gc at δ 1.817, 2.775 and 4.119, respectively, similar to the spectrum of 3′-NGc-SL.

DISCUSSION

The milk oligosaccharides described in this paper are the first to have been found in any species of the order Chiroptera. However, these oligosaccharides comprised only a relatively small proportion of the total milk carbohydrates. By comparing the peak areas of the Bio Gel P-2 column chromatograph in Figure 1, the ratio of milk oligosaccharides to lactose was roughly estimated to be 1:3 (w/w). This ratio is somewhat similar to the ratios reported for the milk or colostrum of other species, including giant anteater (Urashima *et al.* 2008), chimpanzee (Urashima *et al.* 2009) and bonobo (Urashima *et al.* 2009).

The milk oligosaccharides of *Pteropus hypomelanus* were characterized to be isoglobotriose, lacto-N-neotetraose, lacto-N-neohexose and 3′-NGc-SL; this pattern is rather simple compared with those of other species such as humans, bears, elephants and seals (Urashima *et al.* 2001, 2007). It is noteworthy that no fucosyl oligosaccharides were detected in this milk: the only other species whose milk unequivocally lacks fucose-containing oligosaccharides are marsupials (Urashima *et al.* 2007). However, it is possible that the milk sample contained traces of fucosyl oligosaccharides that could not be detected using our methods, since the neutral fraction contained other very small peaks that we did not study (Fig. 1). Isoglobotriose, which is the only neutral trisaccharide in this milk, has been previously detected in milk or colostrum of cows, goats, sheep, bears, mink, white-nosed coati (Urashima *et al.* 2001, 2007), elephants (Uemura *et al.* 2006; Osthoff *et al.* 2008), giant anteaters (Urashima *et al.* 2008) and spotted hyenas (Uemura *et al.* 2009), but has not been found in humans, horses, dogs, seals and bottlenose dolphins (Urashima *et al.* 2001, 2007), among others. It is of interest that the milk of this species was found to contain only type II oligosaccharides (that have Gal(β1–4)GlcNAc) and no type I oligosaccharides such as lacto-N-tetraose (Gal(β1–3)GlcNAc(β1–3)Gal(β1–4)Glc) which has Gal(β1–3)GlcNAc. In all these respects the flying fox oligosaccharides differ from those of humans, chimpanzees, bonobo and orangutans (Urashima *et al.* 2009). This may be of interest insofar as phylogenetically the Chiroptera have been suggested to be distantly related to Primates within the superorder Archonta (Springer & de Jong, 2001; Sargis 2002). It is also worth noting that the acidic oligosaccharide found in this flying fox milk contained the N-glycolyl unit instead of the more common N-acetyl unit. N-Glycolyl-neuraminic acid-containing saccharides, although found in the milk of several other species, including some primates (Urashima *et al.* 2009), are absent from human milk and colostrum (Urashima *et al.* 2007). Since the acidic oligosaccharide fraction contained other components that we could not characterize, it is possible that our milk sample contained small amounts of other sialyl oligosaccharides such as Neu5Ac(α2–3)Gal(β1–4)Glc.

In other mammals, including humans, oligosaccharides in consort with other constituents are believed to be important in restricting pathogenic invasion of the mammary gland and/or the digestive tract of the neonates, and oligosaccharide levels are often elevated in the initial secretion or colostrum (Newburg & Neubauer 1995; Urashima *et al*. 2007). It is not known if pteropid colostrum has elevated oligosaccharide levels. Total carbohydrate did not change over the course of lactation in four species of pteropids (including Pteropus hypomelanus) studied by Hood *et al*. (2001) but milk carbohydrate was found to decrease over lactation in *P. poliocephalus* (Messer & Parry-Jones 1997). In the absence of any information about oligosaccharides in any other bat species, the degree to which *P. hypomelanus* is typical of other pteropids or chiropterans is not known. Further discussion concerning the possible biological or phylogenetic significance of our results must await studies on the milk oligosaccharides of additional species of Chiroptera. It will be particularly interesting to determine whether microchiropteran species also produce oligosaccharide-containing milks.

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