Characterization of oligosaccharides in milk of a mink, *Mustela vison*

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Abstract

Carbohydrates were extracted from a sample of milk from a mink, *Mustela vison* (Family Mustelidae). Free neutral and acidic oligosaccharides were isolated from the carbohydrate fraction and their chemical structures were compared with those of the white nosed coati (Procyonidae) and the harbour seal (Phocidae), which we had studied previously. The ratio of free lactose to milk oligosaccharides was similar to that in milk of the white nosed coati; in both species this ratio was much lower than that in the milk of most eutherians. The neutral oligosaccharides of mink milk had α(1-3) linked Gal or α (1-2) linked Fuc residues at their non–reducing ends, as in the neutral oligosaccharides of white-nosed coati milk. Some of the neutral and acidic oligosaccharides, determined here, had been found also in harbour seal milk, but the harbour seal oligosaccharides did not contain α(1-3) linked Gal residues.

Keywords: Mink; *Mustela vison*; Mustelidae; Milk oligosaccharides; Neutral oligosaccharides; Sialyl oligosaccharides

Introduction

Although the dominant sugar in eutherian milk is generally the disaccharide lactose (Jenness et al., 1964), the milks of some of the Carnivora are exceptional in that other saccharides predominate. For example, the milks of three species of bears (Ezo brown bear, Japanese black bear and polar bear) (Urashima et al., 1997, 1999a, 2000; 2003b, 2004b) and of the giant panda (Nakamura et al., 2003) contain isoglobotriose (Gal(α1-3)Gal(β1-4)Glc) as a dominant saccharide as well as other oligosaccharides and free lactose. Lactose is only a minor component in the milks of these species. Bear milks characteristically contain oligosaccharides similar to those of human blood group A (GalNAc(α1-3)[Fuc(α1-2)]Gal-R), blood group B (Gal(α1-3)[Fuc(α1-2)]Gal-R) and H (Fuc(α1-2)Gal-R), and the α-Gal epitope (Gal(α1-3)Gal(β1-4)GlcNAc-R). In humans, blood group A or B antigens are particularly evident in erythrocyte glycolipids (Hanfland et al., 1984; Fukuda and Hakomori, 1982), but are only minor components among the milk oligosaccharides. Bear milk, however, contains relatively high concentrations of oligosaccharides of these types, this being a significant difference between the milks of humans and bears.

The milks of other carnivores, such as the white-nosed coati (Urashima et al., 1999b) and three species of seals (hooded seal, harbour seal and bearded seal) (Urashima et al., 2001, 2003a, 2004a), contain almost equal amounts of 2'-fucosyllactose (Fuc(α1-2)Gal(β1-4)Glc) and lactose, along with other oligosaccharides. Domestic dog milk contains lactose as the dominant saccharide with only small amounts of other saccharides (Bubb et al., 1999). These observations suggest considerable interspecific variation in milk oligosaccharide levels even within the family Carnivora, but whether this is associated with phylogeny or some other aspect of the biology of these species is not known. From these observations, the finding that lactose is not the dominant saccharide in eutherian milk may, for as yet unknown reasons, be restricted to some related species of the Carnivora.

Why should the milks of some carnivores be low in lactose but high in oligosaccharides? Lactose serves as a significant energy source for the neonates of most eutherian mammals, except species of marine mammals in which milks are low in carbohydrate but high in fat (Oftedal et al. 1987, Oftedal 1997). In eutherians, milk

oligosaccharides are thought to more important as anti-infection factors than as sources of energy (Messer and Urashima, 2002), but no research has been done on oligosaccharide digestion or utilization in carnivores. Further research is needed on the phyletic distribution of oligosaccharides to answer this question.

Our objective was to examine the milk carbohydrates in a representative of another Carnivore family which has not previously been investigated, the Mustelidae. The Mustelidae comprise about 65 species, including weasels, skunks, otters, badgers and honey badgers (Wilson and Reeder 1993), and are most closely related to the Procyonidae and the Phocidae (Wozencraft 1989). In this report, we compare the neutral and acidic oligosaccharides of the milk of the mink (*Mustela vison*, Mustelidae [Lariviere, S. 1999]) with the neutral oligosaccharides of the milk of the white-nosed coati milk (*Nasua narica*, Procyonidae), and the neutral and acidic oligosaccharides of the milk of the harbour seal (*Phoca vitulina*, Phocidae). The results show that the oligosaccharides/lactose pattern of mink milk is very similar to that of the white-nosed coati.

2. Materials and methods

2.1. Materials

Milk was collected at 15 days post partum from a lactating mink of the pastel strain maintained at the U.S. Sheep and Fur Animal Experiment Station in Ithaca, New York. Lactation was considered normal, based on growth of the kits, and the milk was not contaminated with blood. The milk sample was stored at –20℃ until analyzed.

Lacto-N-neotetraose (Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc) and lacto-N-neohexaose (Gal(β 1-4)GlcNAc(β 1-3)[Gal(β 1-4)GlcNAc(β 1-6)]Gal(β 1-4)Glc) were purchased from Seikagaku, Tokyo, Japan. 2'-fucosyllactose (Fuc(α 1-2)Gal(β 1-4)Glc) was obtained from Sigma, St. Louis, MO. Isoglobotriose (Gal(α 1-3)Gal(β 1-4)Glc) was separated from milk of the Ezo brown bear (Urashima et al., 1997), Japanese black bear, polar bear (Urashima et al., 1999a) and white-nosed coati (Urashima et al., 1999b), while Galili pentasaccharide (Gal(α 1-3)Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc) was isolated from a sample of white-nosed coati

milk (Urashima et al., 1999b). Lacto-N-fucopentaose Ⅳ (Fuc(α 1-2)Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc) was separated from hooded seal milk (Urashima et al., 2001). The acidic octasaccharide Neu5Ac(α 2-6)Gal(β 1-4)GlcNAc(β 1-3)[Fuc(α 1-2)Gal(β 1-4)GlcNAc(β 1-6)]Gal(β 1-4)Glc was isolated from harbour seal milk. (Urashima et al., 2003a)

2.2. Isolation of neutral oligosaccharides and lactose from a mink milk

The milk was thawed, diluted with 3.5 ml of water and then extracted with four volumes of chloroform/methanol 2:1 (v/v). The emulsion was centrifuged at 4℃ and 4,000 x g for 30 min, the lower chloroform layer and the denatured protein were discarded, the methanol was removed from the upper layer by rotary evaporation, and the residue was dissolved in 2 ml water and freeze–dried. The resulting white powder was called the 'carbohydrate fraction'.

The carbohydrate fraction from 1.7 ml of milk was dissolved in 2 ml of water and the solution passed through a Bio Gel P-2 (＜45 μm) column (2.5 X 100 cm) 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 analysed for hexose with phenol–H_2SO_4 (Hodge and Hofreiter, 1962) and for sialic acid with periodate–resorcinol (Jourdian et al., 1971). Peak fractions were pooled and freeze–dried. The saccharide in the peak fraction, MNK8 was subjected to ^{1}H-NMR to determine its chemical structure. The peak fractions MNK4 and MNK7 were subjected to preparative thin layer chromatography with acetone/2-propanol/0.1 M lactic acid (2/2/1, v/v) as a developing solvent to isolate their saccharides. Saccharides were detected by spraying with 5% H_2SO_4 in ethanol and heating the TLC plate over flame. The saccharides with R_{Lac} = 0.76 and 0.62, designated as MNK4-1 and MNK4-2, respectively, were isolated from the fraction MNK4, while the saccharides with R_{Lac} = 0.96 and 0.81, designated MNK7-1 and MNK7-2, were isolated from fraction MNK-7. Each oligosaccharide was purified by passage through the Bio Gel P-2 column as described above and its chemical structure determined by ^{1}H-NMR spectroscopy.

2.3. Preparation of sialyl oligosacccharides

The components of peak MNK1 obtained during gel chromatography on Bio Gel P-2 (Fig. 1), which gave positive reactions with both the periodate–resorcinol method (630 nm) and the phenol–sulfuric acid method (490 nm), were each dissolved in 2 ml of 50 mM Tris hydroxyaminomethane–HCl buffer (pH 8.7) and were each subjected to anion exchange chromatography on DEAE–Sephadex A-50. The unadsorbed components were eluted with 250 ml of the same buffer and the adsorbed components were then eluted with a linear gradient of 0 – 0.5 M NaCl in the Tris buffer solution. 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-sulfuric acid method. The fractions in MNK11, MNK12 and MNK13 (Fig. 2a) were pooled, lyophilized, dissolved in 2 ml of water and passed through a Bio Gel P-2 column to remove salts, as described above. Peak fractions (Fig. 2b) were pooled and lyophilized.

The components in MNK121 (see Fig. 2b) were further subjected to high performance liquid chromatography (HPLC) which was performed using a Toso CCPM-Ⅱ intelligent pump with a TSK gel Amido–80 column (4.6 X 250 mm, pore size 80 Å, particle size 5 μm, Tosoh Co., Tokyo, Japan). The mobile phase was 50 and 80 % (v/v) acetonitrile (CH_3CN) in 15 mM potassium phosphate buffer (pH 5.2). Elution was done using a linear gradient of CH_3CN from 80 to 50% at 40℃ at a flow rate of 1 ml/min. Eluted materials were detected by measuring the absorbance at 195 nm. The peak fractions of oligosaccharides (Fig. 3) were pooled, concentrated by rotary evaporation and lyophilized. Each component was treated with water to remove salts, using a MDS-8 microdialysis system (National Labnet Inc., NJ. USA), and lyophilized.

2.3. ^{1}H-NMR spectroscopy

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

3. Results

During column chromatography on Bio Gel P-2 the carbohydrate fraction of the mink milk separated into nine peaks as shown in Fig. 1. Since the fractions in MNK1 and MNK2 reacted positively with periodate–resorcinol (absorbance at 630 nm), it was assumed that they contained sialyl oligosaccharides. The peaks designated as MNK3 to MNK8 did not react positively with periodate–resorcinol and were therefore assumed to contain only neutral oligosaccharides. The relative ratio of MNK1/MNK2/MNK4/MNK5/MNK7/MNK8 was estimated to be 19:7:2:1:9:9 from the peak areas in Fig. 1.

3.1. Neutral oligosaccharides

3.1.1. MNK8

The saccharide in MNK-8 had the same Rf value as lactose during TLC. The ^{1}H-NMR of MNK8 (chemical shifts in Table 1) had the anomeric resonance of reducing α-Glc and β-Glc, and β(1-4) linked Gal at δ 5.223, 4.665 and 4.450, respectively. Since this pattern was essentially similar to that of lactose, MNK8 was characterized to be lactose: Gal(β 1-4)Glc.

3.1.2. MNK7

As it was shown that this fraction contained two saccharides by TLC, each component, designated as MNK7-1 and MNK7-2, was subjected to preparative TLC.

3.1.2.1. MNK7-1

The ^{1}H-NMR spectrum of MNK7-1 (chemical shifts in Table 1) had the anomeric signals of reducing α-Glc and β-Glc, α(1-2) linked Fuc and β(1-4) linked Gal at δ 5.226, 4.638, 5.314 and 4.527, respectively. The spectrum had the characteristic shifts of H-5 and H-6 of α(1-2) linked Fuc at δ 4.260 (α), 4.231 (β) and 1.225, respectively. The pattern represents that of 2'-fucosyllactose: Fuc(α 1-2)Gal(β 1-4)Glc.

3.1.2.2. MNK7-2

The ^{1}H-NMR spectrum of MNK7-2 (chemical shifts in Table 1) had the anomeric signals of reducing α-Glc and β-Glc, α(1-3) linked Gal and β(1-4) linked Gal at δ 5.225, 4.669, 5.146 and 4.524, respectively. The spectrum had the characteristic shifts of H-5 of α(1-3) linked Gal at δ 4.195 and H-4 of β(1-4) linked Gal, which was substituted by α(1-3) linked Gal, at δ 4.184. The pattern was essentially that of authentic isoglobotriose: Gal(α1-3)Gal(β1-4)Glc.

3.1.3. MNK5

The ^{1}H-NMR spectrum of MNK5 (chemical shifts in Table 1) had the anomeric resonances of reducing α-Glc and β-Glc at δ 5.219 and 4.663, respectively, β(1-3) linked GlcNAc at δ 4.703 (α) and 4.700 (β), β(1-4) linked Gal at δ 4.479 and another β(1-4) linked Gal, which linked to reducing Glc, at δ 4.436. The spectrum had the characteristic resonances of NAc of β(1-3) linked GlcNAc at δ 2.033 and H-4 of β(1-4) linked Gal, which was substituted by β(1-3) linked GlcNAc, at δ 4.157. This pattern was essentially similar to that of lacto-N-neotetraose: Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc.

3.1.4. MNK4

As it was shown that this fraction contained two saccharides by TLC, each component, designated as MNK4-1 and MNK4-2, was isolated by preparative TLC.

3.1.4.1. MNK4-1

The ^{1}H-NMR spectrum of MNK4-1 (chemical shifts in Table 2) had the anomeric resonances of reducing α-Glc and β-Glc at δ 5.220 and 4.663, respectively, β(1-3) linked GlcNAc at δ 4.702 (α) and 4.698 (β), β(1-4) linked Gal which was substituted, at δ 4.549, and β(1-4) linked Gal, which was linked to a reducing Glc, at δ 4.441. The spectrum had the characteristic resonances of NAc of β(1-3) linked

GlcNAc at δ 2.039 and H-4 of β(1-4) linked Gal, which was substituted by β(1-3) linked GlcNAc, at δ 4.147. This pattern showed that the saccharide had a lacto-N-neotetraose unit. The spectrum also had the H-1, H-5 and H-6 of α(1-2) linked Fuc at δ 5.308, 4.220 and 1.228, respectively. From these assignments and the agreement of the NMR spectrum with that of authentic lacto-N-fucopentaose Ⅳ, we conclude this oligosaccharide was lacto-N-fucopentaose Ⅳ: Fuc (α 1-2)Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc.

3.1.4.2. MNK4-2

The ^{1}H-NMR spectrum of MNK4-2 (chemical shifts in Table 2) had the anomeric resonances of reducing α-Glc and β-Glc at δ 5.219 and 4.663, respectively, β(1-3) linked GlcNAc at δ 4.708 (α) and 4.705 (β), β(1-4) linked Gal, which was substituted, at δ 4.553, and β(1-4) linked Gal, which was linked to a reducing Glc, at δ 4.438. The spectrum had the characteristic resonances of NAc of β(1-3) linked GlcNAc at δ 2.035 and H-4 of β(1-4) linked Gal, which was substituted by β(1-3) linked GlcNAc, at δ 4.156. This pattern showed that the saccharide had a lacto-N-neotetraose unit. The spectrum had also the resonences of H-1 and H-5 of α(1-3) linked Gal at δ 5.146 and 4.187, respectively. The signal at δ 4.184 was assigned to H-4 of a β(1-4) linked Gal which was substituted by an α(1-3) linked Gal. From these assignments and the agreement of the NMR spectrum with that of authentic Galili pentasaccharide, it was characterized to be Galili pentasaccharide: Gal(α 1-3)Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc.

3.1.5. MNK3

The spectrum (chemical shifts in Table 2) of the oligosaccharides in fraction MNK3 had the anomeric signals of α-Glc, β-Glc, β(1-3) linked GlcNAc at δ 5.222, 4.665 and 4.699, respectively, as well as of β(1-6) linked GlcNAc at δ 4.637 and 4.630, indicating that it contained a lacto-N-neohexaose unit. The resonances at δ 5.326, 4.235 and 1.228 arose from H-1, H-5 and H-6 of α(1-2) linked Fuc. The spectrum had the H-1 signals of a β(1-4) linked Gal residue at δ 4.428, 4.472, 4.481,

4.545 and 4.551. The signals at δ 4.545 and 4.551 were assigned to H-1 of a β(1-4) linked Gal which was substituted by α(1-2) linked Fuc, while the resonances at δ 4.481 and 4.472 were assigned to H-1 of β(1-4) linked Gal residues which were unsubstituted. This showed that this fraction contained α (1-2) fucosylated lacto-N-neohexaose as well as lacto-N-neohexaose. The intensities of H-1, H-5 and H-6 of α(1-2) linked Fuc should have shown that the oligosaccharide had one residue of this unit but not two. From these observations, the fraction MNK3 was concluded to be a mixture of monofucosyl lacto-N-neohexaose (MNK3-1) (i.e. both Fuc(α1-2)Gal(β1-4)GlcNAc(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc and Gal(β1-4)GlcNAc(β1-3)[Fuc(α1-2)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc) as well as lacto-N-neohexaose: (Gal(β1-4)GlcNAc(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc) (MNK3-2).

3.2. Sialyl oligosaccharide

The fraction MNK1, which had been obtained from the carbohydrate fraction by gel chromatography on Bio Gel P-2 (Fig. 1), was further separated into fraction MNK11, MNK12 and NMK13 by anion exchange chromatography on DEAE–Sephadex A-50 (Fig. 2a). From these peak areas and those of MNK1~MNK9 in Fig. 1, the relative ratio of acidic oligosaccharides to neutral oligosaccharides was estimated to be around 1:3. MNK12 was then separated into MNK121 and MNK122 by the gel chromatography on Bio Gel P-2 (Fig. 2b). MNK121 was then subjected to HPLC (see Fig. 3) and two sialyl oligosaccharides obtained thereby, designated as MNK121-2 and MNK121-3 were characterized by ^{1}H-NMR. The oligosaccharides in MNK11, MNK13 and MNK122 were not characterized in this study.

3.2.1. MNK121-2

As the ^{1}H-NMR spectrum of MNK121-2 (chemical shifts in Table 3) was essentially identical with that of ZA-1 from harbour seal milk (Urashima et al., 2003a), it was characterized to be monofucosyl monosialyl lacto-N-neohexaose: Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)[Fuc(α1-2)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc.

The anomeric shifts at δ 5.221, 4.667, 4.724, 4.596, 4.454 and 4.436 arose from a reducing α-Glc, a reducing β-Glc, a β(1-3) linked GlcNAc, a β(1-6) linked GlcNAc and two of β(1-4) linked Gal, respectively. The shift at δ 4.541 was assigned to H-1 of a β(1-4) linked Gal; this Gal was substituted by a non–reducing α(1-2) linked Fuc. The shift at δ 4.151 was assigned H-4 of a β(1-4) linked Gal which was substituted at OH-3. The shifts at δ 5.309, 4.223 and 1.229 were assigned to H-1, H-5 and H-6 of α(1-2) linked Fuc, respectively. The H-3 axial and equatorial shifts at δ 1.720 and 2.665 arose from an α(2-6) linked Neu5Ac residue. The NAc shifts at δ 2.029, 2.050 and 2.064 were assigned to α(2-6) linked Neu5Ac, β(1-3) linked GlcNAc and β(1-6) linked GlcNAc, respectively.

3.2.2. MNK121-3

It was shown that MNK121-3 contained small amounts of a minor oligosaccharide together with a major saccharide, because the spectrum had the low intensity of a H-1 shift of an α(1-2) linked Fuc at δ 5.309.

The spectrum (Fig. 4, chemical shifts in Table 3) had the anomeric shifts at δ 5.221, 4.668, 4.725, 4.641, 4.454 and 4.434 of α-Glc, β-Glc, β(1-3) linked GlcNAc. β(1-6) linked GlcNAc and two of β(1-4) linked Gal, respectively, showing that the oligosaccharide contained a lacto-N-neohexaose unit. The shifts at δ 5.145, 4.194 and 4.183 arose from H-1 and H-5 of an α(1-3) linked Gal, and H-4 of a β(1-4) linked Gal, which was substituted by an α(1-3) linked Gal, respectively; this indicated the presence of an α(1-3) linked Gal residue. The signal at δ 4.544 was assigned to H-1 of a β(1-4) linked Gal which was substituted by an α(1-3) linked Gal residue.

The H-3 axial, H-3 equatorial and NAc shifts at δ 1.721, 2.667 and 2.027, respectively, arose from an α(2-6) linked Neu5Ac residue. The NAc shifts at δ 2.050 and 2.062 were assigned to a β(1-3) and β(1-6) linked GlcNAc, respectively. The shift at δ 2.062 indicated the presence of a Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3) unit as in MNK121-2 but not a Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-6) unit, because, had the saccharide contained the latter unit, the spectrum would have had the signal at δ 2.088（Gronberg et al., 1989）. From these considerations, the major saccharide in MNK121-3 was characterized to be galactosyl monosialyl

lacto-N-neohexaose: Neu5Ac(α 2-6)Gal(β 1-4)GlcNAc(β 1-3)[Gal(α 1-3)Gal(β 1-4)GlcNAc(β 1-6)]Gal(β 1-4)Glc.

4. Discussion

The gel chromatogram of the carbohydrate fraction of mink milk as well as the identification of each component showed that, unlike in most eutherian milks, lactose is not a dominant saccharide and that its content in the carbohydrate fraction is similar to that of the trisaccharides. The content of lactose was estimated from Fig. 1 to be about 12% of milk carbohydrate, which is similar to white-nosed coati milk (see Fig. 1 in Urashima et al., 1999b). As the total carbohydrate in mink milk, measured by the phenol-sulfuric acid method, is 4.5% (Oftedal and Iverson 1995), this milk contains only about 0.5% lactose. The finding that lactose is not the predominant saccharide in milk has been previously reported in only a few eutherians, such as bears (Urashima et al., 1997, 1999a, 2000, 2003b, 2004b), the white-nosed coati (Urashima et al., 1999b) and seals (Urashima et al., 2001, 2003a, 2004a). Although these are all in the order Carnivora, this is not a universal feature of this order, since in the domestic dog (*Canus familiaris*) lactose constitutes more than 80% of the milk carbohydrate fraction (Bubb et al., 1999).

We have shown that mink milk contains neutral oligosaccharides with a non-reducing α(1-2) linked Fuc or α(1-3)linked Gal residue attached to lactose or a lacto-N-neotetraose core unit. Nearly all of the neutral oligosaccharides in mink milk are also found in milk of a procyonid, the white-nosed coati (Urashima et al., 1999b) as shown in Fig. 5. The neutral and acidic oligosaccharides of mink milk determined in this study are compared in Fig. 6 with those of milk of the harbor seal (Phocidae; Urashima et al., 2003a). Several of the oligosaccharides are present in both mink and harbor seal milk but only mink milk was found to contain oligosaccharides with an α (1-3) linked Gal residue at the reducing end, such as isoglobotriose, galili pentasaccharide, and galactosyl monosialyl lacto-N-neohexose. It appears that milks of phocid seals lack oligosaccharides with a terminal α(1-3) linked Gal residue (Urashima et al., 2001, 2003a, 2004a). The greater similarity of the neutral oligosaccharides of mink milk to those of a procyonid than to those of phocids is consistent with phylogenetic analyses that indicate mustelids to be more closely related

to procyonids than to phocids (Wozencraft 1989). Unfortunately, we cannot compare acidic oligosaccharides among these taxa as acidic oligosaccharides have not been characterized for any procyonid.

The milk oligosaccharides of three species of bears (Ezo brown, Japanese black and polar bear) are more complex than those of mink, whitenosed coati and phocid seals. For example, the milk oligosaccharides of most bear milks (except one individual polar bear) contained the Lewis x unit (Gal(β 1-4)[Fuc(α 1-3)]GlcNAc), while some contained A antigen (GalNAc(α 1-3)[Fuc(α 1-2)]Gal) and others contained B antigen (Gal(α 1-3)[Fuc(α 1-2)]Gal) (Urashima et al., 1997, 1999a, 2000, 2004b). None of these constituents have been found in mink, coatis or phocid seals.

It has been suggested that milk oligosaccharides serve as anti-infection components for human infants (Messer and Urashima, 2002; Dai et al., 2000; Sharon and Ofek, 2000) because they are likely to act as soluble receptor analogues and compete with pathogenic bacteria, bacterial toxins and viruses for attachment to gastrointestinal receptor sites. Some of the human milk oligosaccharides that have been shown to have antimicrobial effects include fucosylated oligosaccharides, lacto-N-neotetraose and sialyllactose while the pathogenic organisms whose actions are inhibited include *E. coli*, *Vibrio cholera*, *Helicobacter pyroli*, *Streptococcus pneumoniae*, *Campylobacter jejuni* and influenza virus (Dai et al., 2000). Isoglobotriose, which we found in mink milk, has been shown to be a possible inhibitor of the binding of toxin A produced by *Clostridium difficile* (Clark et al., 1987). In human infants, much of the milk oligosaccharide is not absorbed from the small intestine, whereas lactose is both digested and absorbed, providing a valuable source of energy.

What biological functions do lactose and milk oligosaccharides have in eutherian carnivores? As bear milks contain only small amounts of lactose, even during early lactation when carbohydrate is moderately high (Oftedal et al., 1993; Urashima et al., 1997, 1999a, 2000), this saccharide is unable to be a significant energy source for the cubs. Lactating bears fast during early lactation, when they are denned up with their cubs (Oftedal et al., 1993). The need to minimize gluconeogenesis in an animal with limited glycogen and protein reserves, and an ability to mobilize lipids from large fat stores (Iverson and Oftedal, 1992), may underlie the evolution of relatively high-fat and low-carbohydrate milks in bears (Ramsay and Dunbrack, 1986; Oftedal et al., 1993; Oftedal, 1993). There is also a premium on production of milks low in water, since

denned bears do not leave their dens to drink for several months. Lactose synthesis draws water into Golgi vesicles in mammary secretory cells by an osmotic effect; by producing milks high in oligosaccharides, bears can incorporate more carbohydrate into milk relative to milk water. However, it is not known whether bear cubs can digest and utilize oligosaccharides, as has been suggested occurs in marsupials (Messer et al. 1989). On the other hand, the oligosaccharides of bear milk may act as anti-infection factors for bear cubs, as suggested for humans.

In the milks of the white-nosed coati and mink, the ratio of lactose to milk oligosaccharides is somewhat greater than that in bear milks (Urashima et al., 1997, 1999a, 1999b, 2000), but is nevertheless lower than in most eutherians. As these species do not give birth during a fasting period, the selective advantage of a low ratio of lactose to oligosaccharides is less evident. Other taxa in which the free lactose content of milk is also extremely low, both absolutely and in relation to oligosaccharides, include monotremes and marsupials (Messer and Kerry, 1973; Green and Merchant, 1988). What is the common feature of marsupials, monotremes and these species of eutherian carnivores? All produce young that are more or less altricial at birth (or hatching, in the case of monotremes), being blind, hairless or nearly so, and with immature physiological functions (Griffiths, 1978; Tyndale-Biscoe and Janssens, 1988; Oftedal et al., 1993; Gompper, 1995; Lariviere, 1999). Do such immature young have a greater requirement for protection against gastrointestinal pathogens than the more developed young of primates, ungulates and other taxa? Or are their digestive systems more able to absorb macromolecules and thus degrade oligosaccharides by intracellular mechanisms (Messer et al. 1989)? Unfortunately relatively little is known about digestive or immune function in the immediate postnatal period in these species, and further research is needed to resolve this issue.

It is also unclear why the milk carbohydrates of the dog, which also gives birth to quite altricial young, should contain more lactose and less oligosaccharide than mink and other eutherian carnivores that have been studied.

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495-535.

Fig. 1 Gel chromatogram of the carbohydrate fraction from mink milk on Bio Gel 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 ml were collected. Aliquots (0.5 ml) of each fraction were analysed for hexose with phenol – H_2SO_4 at 490 nm and for sialic acid with periodate – resorcinol at 630 nm.

Fig. 2 Preparation of oligosacccharide fractions from the fraction MNK1 separated from mink milk. (a) Anion exchange chromatogram of MNK1 usiing a DEAE – Sephadex A-50 column (1.5 X 35 cm) equilibrated with 50 mM Tris – hydroxyaminomethane – HCl buffer (pH 8.7). The unadsorbed components were eluted with 300 ml of the same buffer and the adsorbed components were then eluted with a linear gradient of 0 – 0.5 M NaCl in the buffer. (b) Gel chromatogram of the MNK12 fraction separated by anion exchange chromatography on Bio Gel P-2 (2.5 X 100 cm).

Fig. 3 HPLC of the acidic oligosaccharide fraction MNK12 separated from mink milk. HPLC was performed using a Toso CCPM-Ⅱ intelligent pump with a TSK gel Amido –80 column (4.6 X 250 mm, pore size 80 Å, particle size 5 μm, Tosoh Co., Tokyo, Japan). The mobile phase was 50 and 80% (v/v) acetonitrile (CH_3CN) in 15 mM potassium phosphate buffer (pH 5.2). Elution was done using a linear gradient of CH_3CN from 80 to 50% at 40℃ at a flow rate of 1 ml/min. Eluted materials were detected by measuring the absorbance at 195 nm.

Fig. 4 Six hundred megahertz ^{1}H-NMR spectrum of the oligosaccharide in MNK121-3 separated by HPLC.

Fig. 5 Comparison of neutral milk oligosaccharides of mink and white-nosed coati.

Fig. 6 Comparison of neutral and acidic milk oligosaccharides of mink and harbor seal.

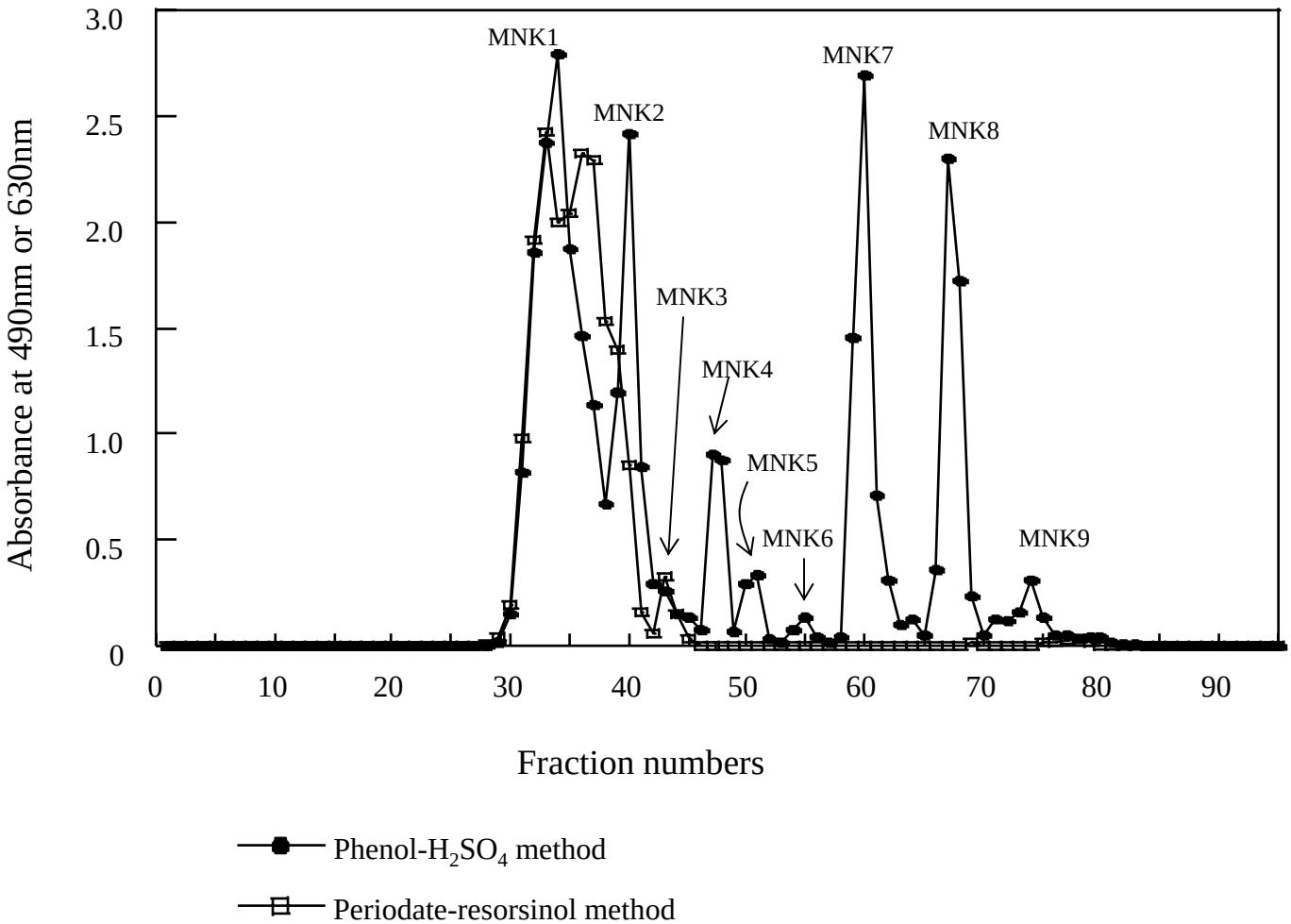
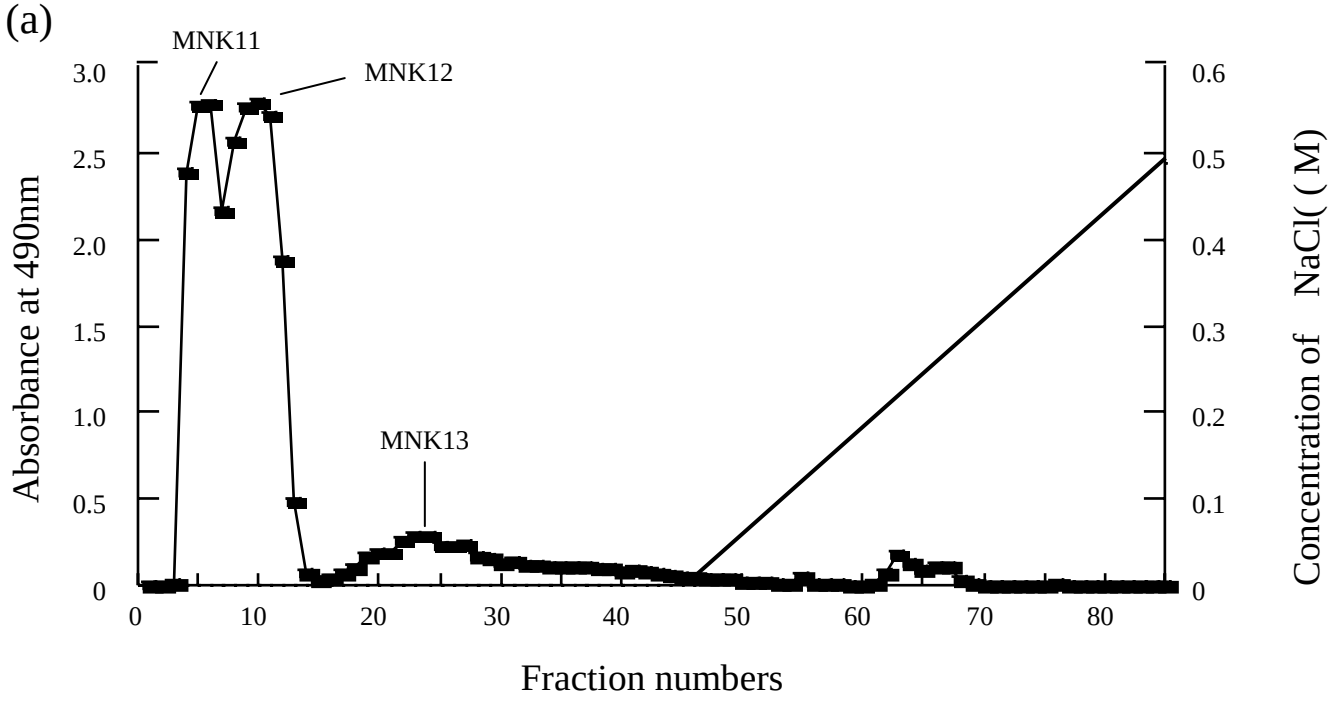

3.0
2.5
2.0
1.5
1.0
0.5
0
Absorbance at 490nm or 630nm
MNK1
MNK2
MNK3
MNK4
MNK5
MNK6
MNK7
MNK8
MNK9
0
10
20
30
40
50
60
70
80
90
Fraction numbers
Phenol-H2SO4 method
Periodate-resorsinol method

Fig. 1

(a)


MNK11
MNK12
MNK13
Absorbance at 490nm
Concentration of NaCl(M)
Fraction numbers
0
0.5
1.0
1.5
2.0
2.5
3.0
0.1
0.2
0.3
0.4
0.5
0.6
10
20
30
40
50
60
70
80

(b)

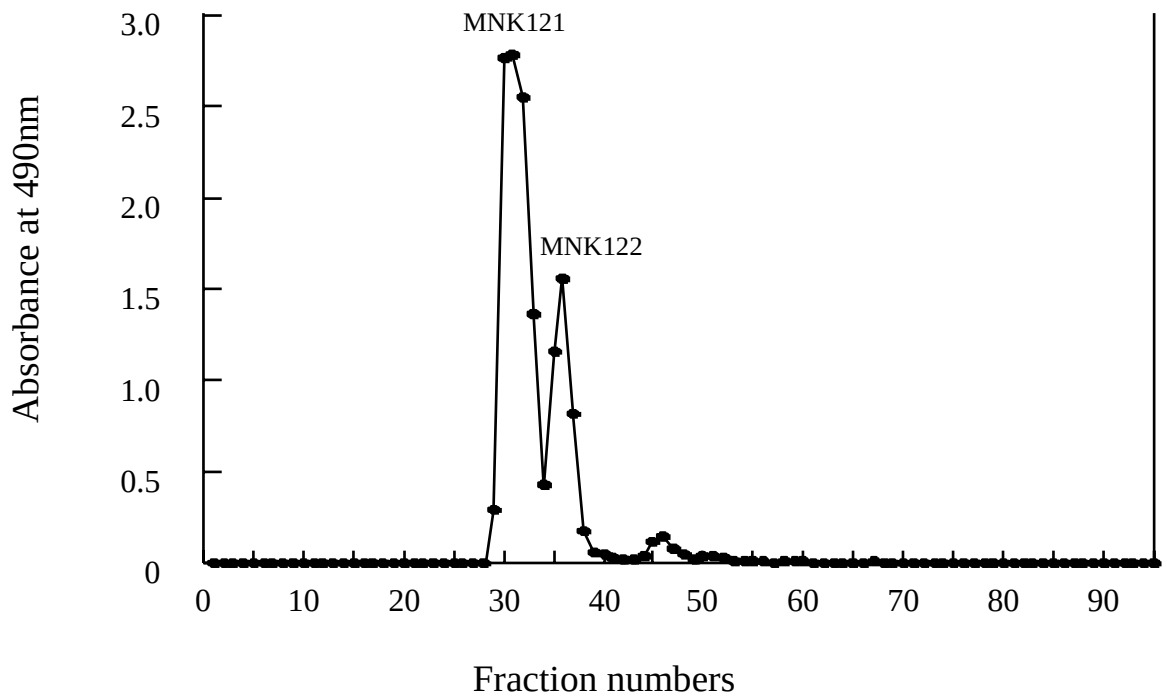

MNK121
MNK122
Absorbance at 490nm
Fraction numbers
0
0.5
1.0
1.5
2.0
2.5
3.0
10
20
30
40
50
60
70
80
90

Fig.2

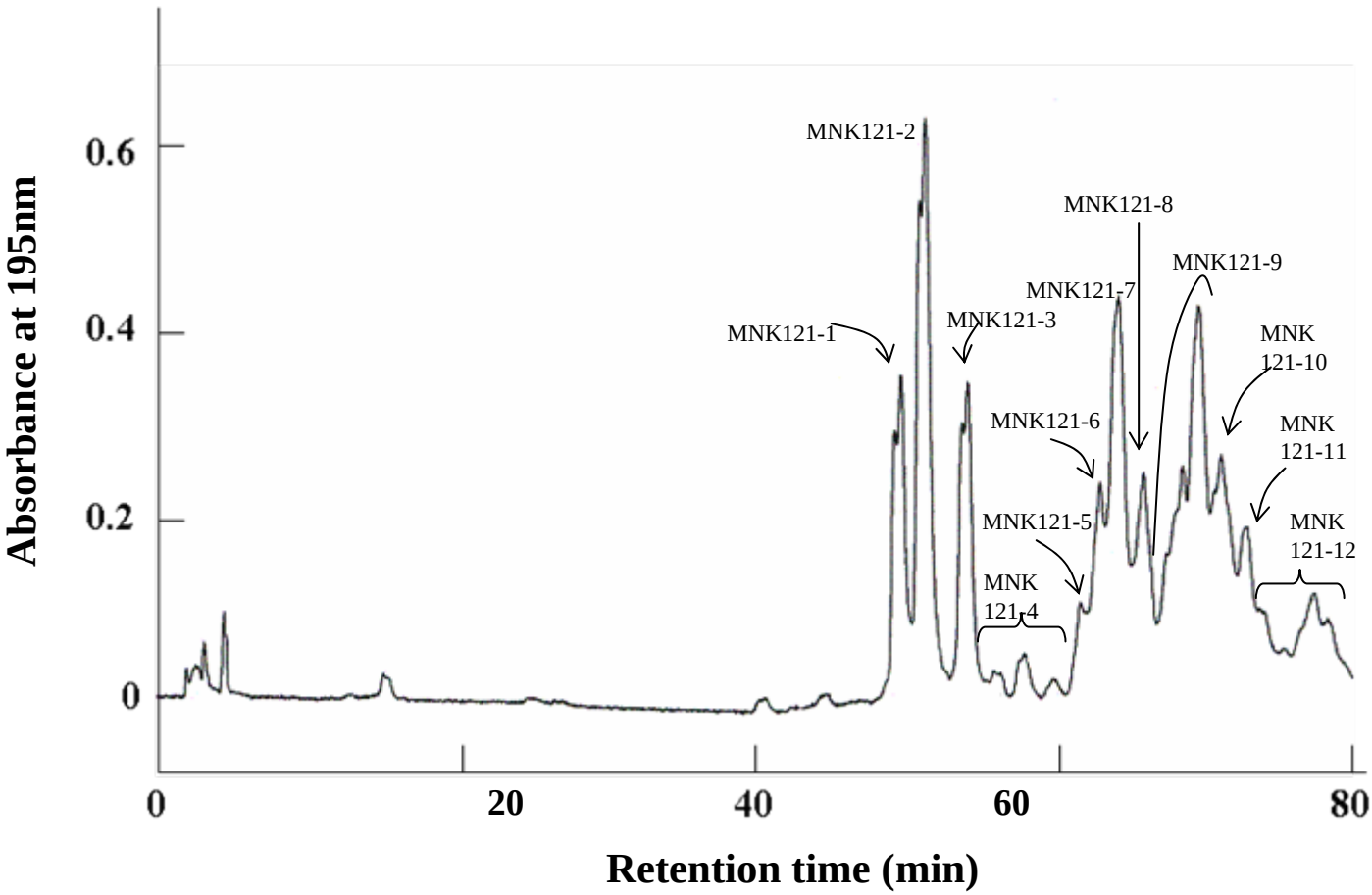
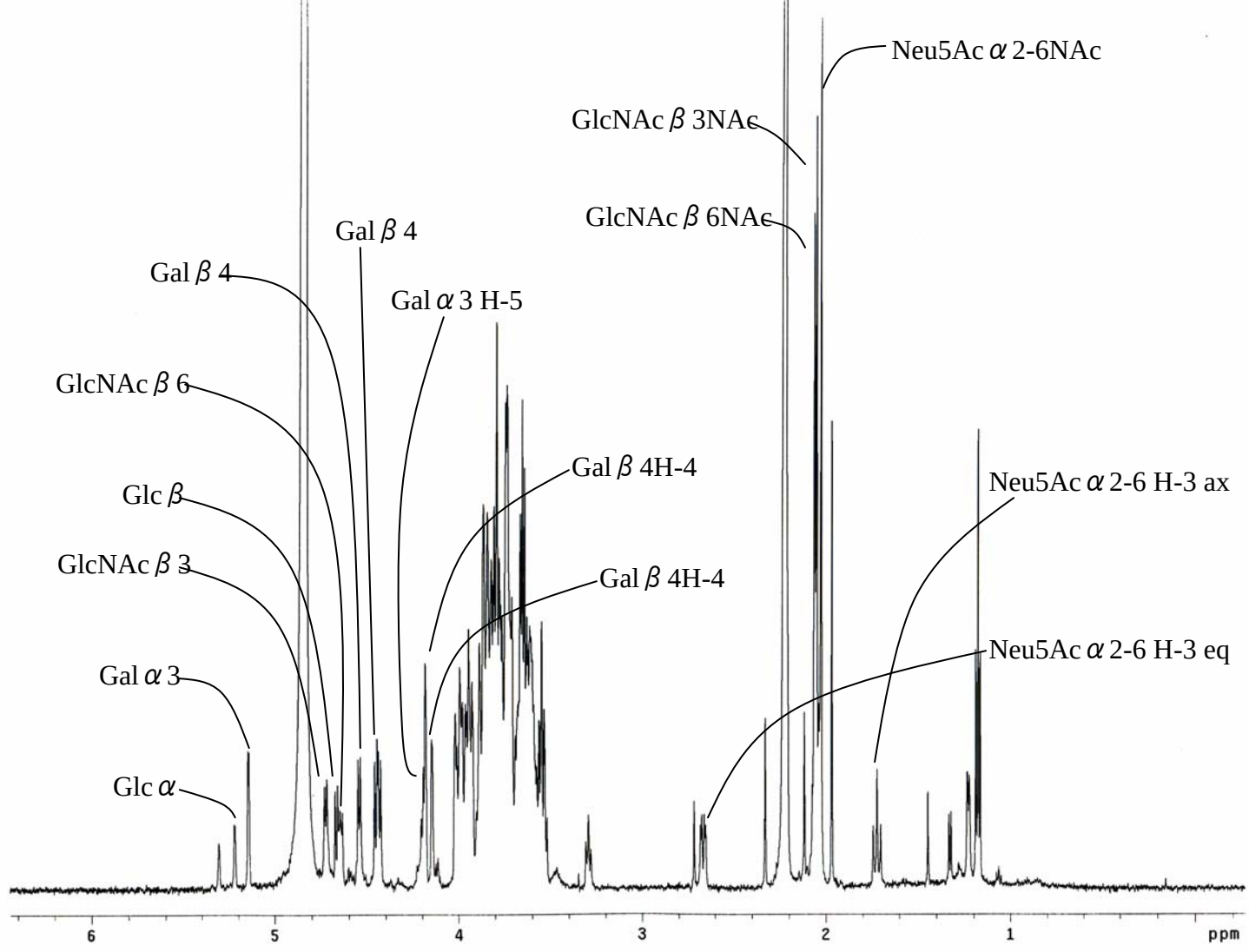

Absorbance at 195nm
0.6
0.4
0.2
0
0
20
40
60
80
Retention time (min)
MNK121-1
MNK121-2
MNK121-3
MNK121-4
MNK121-5
MNK121-6
MNK121-7
MNK121-8
MNK121-9
MNK 121-10
MNK 121-11
MNK 121-12

Fig.3



Chemical Shifts (ppm)

Fig.4

Mink	White nosed coati
(MNK8) lactose	
Gal(β 1-4)Glc	Gal(β 1-4)Glc
(MNK7-1) 2'-fucosyllactose	
Fuc(α 1-2)Gal(β 1-4)Glc	Fuc(1-2)Gal(β 1-4)Glc
(MNK-7-2) isoglobotriose	
Gal(α 1-3)Gal(β 1-4)Glc	Gal(α 1-3)Gal(β 1-4)Glc
(MNK5) lacto-*N*-neotetraose	
Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc	Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc
(MNK4-1) lacto-*N*-fucopentaose IV	
Fuc(α 1-2)Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc	Fuc(α 1-2)Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc
(MNK4-2) Galilipentasaccharide	
Gal(α 1-3)Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc	Gal(α 1-3)Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc
(MNK3-2) lacto-*N*-neohexose	
Gal(β 1-4)GlcNAc(β 1-6) | Gal(β 1-4)Glc | Gal(β 1-4)GlcNAc(β 1-3)	Fuc(α 1-2) Gal(α 1-3) Gal(β 1-4)GlcNAc(β 1-6) | Gal(β 1-4)Glc | Gal(β 1-4)GlcNAc(β 1-3)
(MNK3-1) monofucosyl lacto-*N*-neohexaose	
Fuc(α 1-2) Gal(β 1-4)GlcNAc(β 1-6) | Gal(β 1-4)Glc | Gal(β 1-4)GlcNAc(β 1-3)	

Fig.5

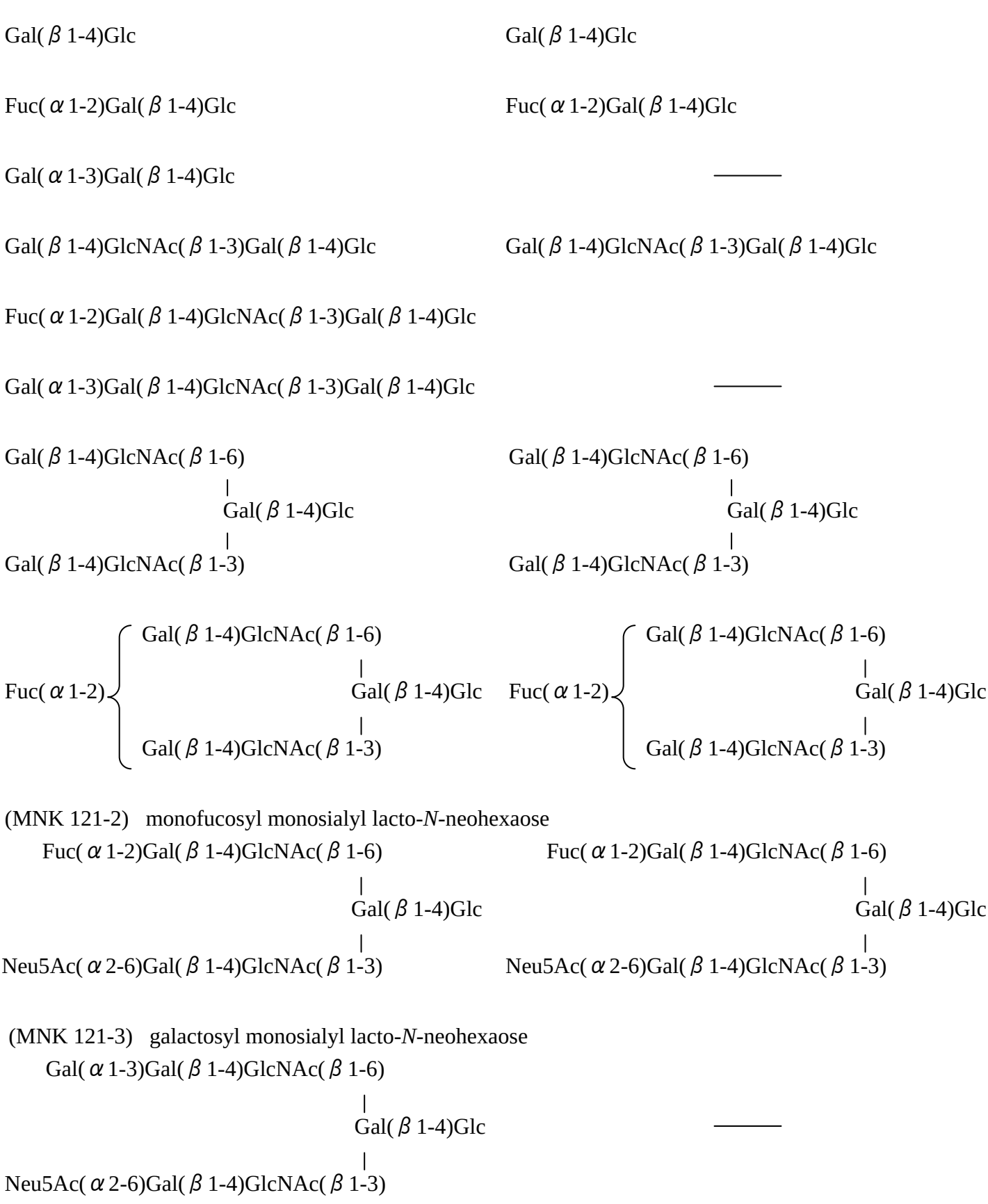

Mink
Harbour seal
Gal(β 1-4)Glc
Fuc(α 1-2)Gal(β 1-4)Glc
Gal(α 1-3)Gal(β 1-4)Glc
Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc
Fuc(α 1-2)Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc
Gal(α 1-3)Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)Glc
Gal(β 1-4)GlcNAc(β 1-6)
Gal(β 1-4)Glc
Gal(β 1-4)GlcNAc(β 1-3)
Fuc(α 1-2)
(MNK 121-2) monofucosyl monosialyl lacto-N-neohexaose
Neu5Ac(α 2-6)Gal(β 1-4)GlcNAc(β 1-3)
(MNK 121-3) galactosyl monosialyl lacto-N-neohexaose

Fig.6

Table 1 ^{1}H-NMR chemical shifts of oligosaccharides in MNK 5 to MNK 8 separated from mink milk.

Reporter gruop	Residue	Chemical shifts, δ (coupling constants,Hz)			
		MNK5	MNK7-1	MNK7-2	MNK8
H-1	Glc α	5.219(4.0)	5.226(4.0)	5.225(3.4)	5.223(4.0)
	Glc β	4.663(8.0)	4.638(8.0)	4.669(8.0)	4.665(8.0)
	Gal'(β 1-4)	4.436(8.0)	4.527(8.0)	4.524(7.4)	4.450(8.0)
	Gal'''(β 1-4)	4.479(8.0)	—	—	—
	Gal(α 1-3)	—	—	5.146(4.0)	—
	Fuc(α 1-2)	—	5.314	—	—
	GlcNAc(β 1-3)	4.700(8.0) 4.703(8.0)	—	—	—
H-4	Gal'(β 1-4)	4.157(2.9[a])		4.184(2.9[a])	
H-5	Gal(α 1-3)	—	—	4.195	—
	Fuc(α 1-2)	—	4.260 4.231	—	—
H-6	Fuc(α 1-2)	—	1.225(6.9[b])	—	—
NAc	GlcNAc(β 1-3)	2.033	—	—	—

[a] $J_{H\text{-}4,H\text{-}3}$

[b] $J_{H\text{-}6,H\text{-}5}$

Table 2 ^{1}H-NMR chemical shifts of oligosaccharides in MNK 3 and MNK 4 separated from mink milk.

reporter group	residue	chemical shifts、δ (coupling constants,$H_{z)}$			
		MNK3-1	MNK3-2	MNK4-1	MNK4-2
H-1	Glc α	5.222(3.4)	5.222(3.4)	5.220(3.4)	5.219(4.0)
	Glc β	4.665(8.0)	4.665(8.0)	4.663(8.0)	4.663(8.0)
	Gal'(β 1-4)	4.428(7.4)	4.428(7.4)	4.441(8.0)	4.438(8.0)
	Gal'''(β 1-4)	4.472(8.0)	4.472(8.0)	4.549(7.4)	4.553(7.4)
		4.481(8.0)	4.481(8.0)		
		4.545(8.0)			
		4.551(8.0)			
	Gal(α 1-3)	—	—	—	5.146(4.0)
	Fuc(α 1-2)	5.326	—	5.308(2.3)	—
	GlcNAc(β 1-3)	4.699(8.0)	4.699(8.0)	4.702(8.0)	4.705(8.0)
				4.698(8.6)	4.708(8.6)
	GlcNAc(β 1-6)	4. 637(8.0)	4. 637(8.0)	—	—
		4. 630(7.4)	4. 630(7.4)		
H-4	Gal'(β 1-4)	4.147(3.4[a])	4.147(3.4[a])	4.147(3.4[a])	4.156(2.3[a])
	Gal'''(β 1-4)				4.184(2.9[a])
H-5	Gal(α 1-3)	—	—	—	4.187
	Fuc(α 1-2)	4.235	—	4. 220	—
H-6	Fuc(α 1-2)	1.228(6.3[b])	—	1.228(6.9[b])	—
		1.225(6.3[b])			
NAc	GlcNAc(β 1-3)	2.031	2.031	2.039	2.035
	GlcNAc(β 1-6)	2.061	2.061	—	—

[a] $J_{H-4,H-3}$

[b] $J_{H-6,H-5}$

Table 3 ^{1}H-NMR chemical shifts of oligosaccharides in MNK 121-2 and MNK 121-3 separated from mink milk.

Reporter group	Residue	Chemical shifts、δ (coupling constants,H_z)	
		MNK121－2	MNK121－3
H-1	Glc α	5.221(3.7)	5.221(3.5)
	Glc β	4.667(8.0)	4.668(7.9)
	Gal'(β 1-4)	4.436	4.434(7.9)
	Gal'''(β 1-4)	4.454(8.0)	4.454(7.9)
		4.541(7.4)	4.544(7.6)
	Fuc(α 1-2)	5.309	—
	Gal(α 1-3)	—	5.145(3.8)
	GlcNAc(β 1-3)	4.724(6.9)	4.725(7.6)
	GlcNAc(β 1-6)	4.596(8.0)	4.641(7.6)
H-3_{ax}	Neu5Ac(α 2-6)	1.720(12.6[a],-11.5[b])	1.721(12.3[a],-12.3[b])
H-3_{eq}	Neu5Ac(α 2-6)	2.665(5.2[c])	2.667(4.7[c])
H-4	Gal'(β 1-4)	4.151	4.148
	Gal'''(β 1-4)		4.183
H-5	Fuc(α 1-2)	4.223	—
	Gal(α 1-3)	—	4.194
H-6	Fuc(α 1-2)	1.229(6.3[d])	—
NAc	GlcNAc(β 1-3)	2.050	2.050
	GlcNAc(β 1-6)	2.064	2.062
	Neu5Ac(α 2-6)	2.027	2.027

[a] $J_{3ax,4}$

[b] $J_{3ax,3eq}$

[c] $J_{3eq,4}$

[d] $J_{6,5}$