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Carbohydrate Research 318 (1999) 123–128

CARBOHYDRATE RESEARCH

Occurrence of an unusual lactose sulfate in dog milk

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Received 22 January 1999; accepted 19 April 1999

Abstract

The milk of a beagle dog (*Canis familiaris*) was extracted and fractionated to yield, inter alia, β-D-Gal*p*3*S*-(1→4)-D-Glc (lactose 3′-sulfate), which does not appear to have previously been isolated from milk or other natural sources. The structure was established by 2D NMR spectroscopy and mass spectrometry. By contrast with the milk of some closely related Carnivora, the major constituent of the dog milk was lactose, with minor amounts of 2′-fucosyllactose and sialyl oligosaccharides. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Dog milk; Lactose 3′-sulfate; Oligosaccharide structure; NMR data

1. Introduction

Although the dominant carbohydrate in milk is generally lactose, the free disaccharide is only a minor component of the carbohydrate fraction of the milk of several species of mammals [1–11]. The best examples of this are monotremes [6,7] and marsupials [8,9], whose milk contains only small amounts of lactose relative to much higher concentrations of a variety of oligosaccharides.

Among eutherian mammals, the milks of several species of bears contain significantly less lactose than other oligosaccharides [5,12]. For the brown bear (*Ursus arctos yesoensis*) [12], the latter chiefly comprise the trisaccharides α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-D-Glc (2′-fucosyllactose) and α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc, in addition to higher oligosaccharides whose core units are lacto-*N*-neotetraose and lacto-*N*-neohexaose.

Carnivora that are closely related to the bear (family Ursidae), including Procyonidae, Canidae and Mustelidae [13], might be expected to have relatively high concentrations of oligosaccharides in their milks. We have examined the oligosaccharide fraction of milk collected from a beagle dog (*Canis familiaris*) in order to determine if there are any similarities with the milk of the previously studied Carnivora and with a view to establishing some of the constituents of a possible milk formula for pups. One of the isolated oligosaccharides, β-D-Gal*p*3*S*-(1→4)-D-Glc (lactose 3′-sulfate), does not appear to have been previously found in milk or other natural sources.

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PII: S0008-6215(99)00102-0

2. Experimental

Materials.—Milk (2 mL) was collected at 13 days post partum from a beagle dog bred at Obihiro University and the sample was stored at −20 °C for 2 months until use. 2′-Fucosyllactose, *N*-acetylneuraminic acid, and α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-Glc (3′-SL) were obtained from Sigma (St. Louis, MO, USA).

Isolation of oligosaccharides from dog milk.—The milk was thawed and extracted with 4 vol of 2:1 $CHCl_3$–MeOH (v/v). The resultant emulsion was centrifuged at 4 °C and 3500 rpm for 30 min, after which the lower $CHCl_3$ layer and denatured protein were discarded. The MeOH was removed from the upper layer by rotary evaporation and the residue redissolved in 2 mL of water and freeze-dried to give a carbohydrate fraction as a white powder. A solution of the carbohydrate fraction from 2 mL milk in 2 mL of water was passed through a Bio-Gel P-2 (<45 μm) column (2.5×100 cm), which had been calibrated with 2 mg each of galactose, lactose, and raffinose, representing mono-, di-, and trisaccharides, respectively. The column was eluted with distilled water at a flow rate of 15 mL/h and 5 mL fractions were collected. Fractions were pooled and freeze-dried after 0.5 mL aliquots of each had been analysed for hexose by the phenol–H_2SO_4 method [8], and for sialic acids by the resorcinol–HCl method [14].

Thin-layer chromatography.—The components of each column peak from the Bio-Gel P-2 chromatography were examined by TLC using 2:2:1 acetone–isopropanol–0.1 M lactic acid (v/v; solvent A) or 50:10:10:10:3 EtOH–BuOH–pyridine–H_2O–AcOH (v/v; solvent B) as the solvent systems. Saccharides were located by spraying with 5% H_2SO_4 in EtOH and heating on a burner.

NMR samples and spectra.—Each oligosaccharide was treated with ^2H_2O (99.75 atom% ^2H), freeze-dried and finally dissolved in ^2H_2O (99.96 atom% ^2H) and examined by ^1H NMR using a Varian INOVA-600 spectrometer at 599.92 MHz, with the probe temperature set to 20 °C. Chemical shifts are expressed relative to internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), but were actually measured by reference to internal acetone (^1H, δ 2.225; ^{13}C, δ 30.5).

NMR spectra of lactose and lactose 3′-sulfate.—All spectra were recorded at 25 °C on a Bruker DRX-600 spectrometer with a 5 mm triple-resonance inverse-detection *xyz*-gradient probe, employing standard Bruker pulse sequences (XWIN-NMR version 2.1), modified for off-resonance water suppression (2 s) in the homonuclear experiments as described previously [15]. Apart from the heteronuclear multiple bond correlation (HMBC) [16] experiment, all spectra were recorded in phase-sensitive mode and routinely processed with one level of zero-filling in F1 and a $\pi/2$ shifted sine-bell or −2 Hz Gaussian enhancement in each dimension.

Chemical shifts for anomeric protons were determined from a 1D spectrum but all other ^1H and all ^{13}C chemical shifts were obtained from a gradient-selected sensitivity-enhanced heteronuclear single quantum coherence (HSQC) [17] spectrum, optimised for $^1J_{CH}$ of 145 Hz, with GARP decoupling [18] during acquisition, and recorded over spectral widths of 2694 Hz (^1H) and 13582 Hz (^{13}C), with 512 t_1 increments of 1616 data points and 68 scans per increment. A similar experiment without ^{13}C-decoupling was recorded for measurement of ^{13}C–^1H coupling constants.

The COSY spectrum was recorded using the pulse sequence of Derome [19], the TOCSY spectrum [20] for a mixing time of 170 ms with the spin-lock field strength adjusted for a 90° pulse-length of 25 μs, and the HMBC spectrum with a delay of 80 ms for evolution of long-range ^{13}C–^1H couplings.

Mass spectrum of lactose 3′-sulfate.—An electrospray spectrum (negative-ion mode) was recorded from a solution of the oligosaccharide in CH_3OH on a Finnigan MAT LCQ instrument with a capillary temperature of 200 °C and spray voltage of 5 kV.

3. Results and discussion

Isolation and characterisation of oligosaccharides.—Analysis of the dog milk revealed 4.7 g/100 mL of hexose and 310 mg/100 mL of

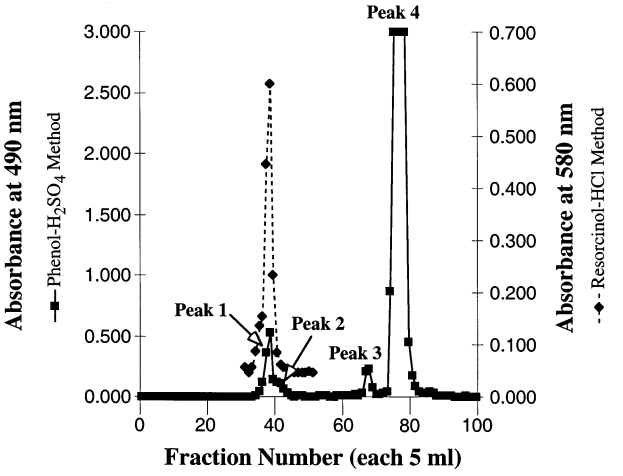

Fig. 1. Bio-Gel P-2 elution profile of the carbohydrate fraction from the milk of a beagle dog.

sialic acids as measured by the phenol–H_2SO_4 method [8] and resorcinol–HCl method [14], with lactose and *N*-acetylneuraminic acid as standards, respectively. The oligosaccharides were separated into four fractions by Bio-Gel P-2 chromatography of a $CHCl_3$–MeOH extract (Fig. 1). Only Peak 1, whose constituents contained 91 and 320 μg/mg of hexose and sialic acid, respectively, gave a positive result with resorcinol–HCl. TLC with solvent B showed that it contained at least four oligosaccharides whose $R_{3'\text{-SL}}$ values were 1.0, 0.82, 0.55 and 0.43. The substance with $R_{3'\text{-SL}}$ 1.0 was dominant relative to the other saccharides, but the total yield of Peak 1 was too small to permit the isolation and identification of any pure compounds. Peak 4, which constituted more than 90% of the total saccharide, was assigned to lactose since it co-eluted with lactose on Bio-Gel P-2, had the same R_f value as lactose in TLC with solvent A, and an identical ^{1}H NMR spectrum to that of an authentic sample of lactose [21], with resonances for anomeric protons at δ 5.223 (α-Glc, $J = 3.8$ Hz), δ 4.664 (β-Glc, $J = 8.0$ Hz), δ 4.453 (β-Gal (with α-Glc), $J = 7.8$ Hz) and δ 4.451 (β-Gal (with β-Glc), $J = 7.8$ Hz), the separate β-Gal resonances only being evident with Gaussian enhancement prior to Fourier transformation of the spectrum. Thus lactose is a dominant oligosaccharide in dog milk but only a minor component of the milks of closely related Carnivora, including several species of bears [5,12].

The substance represented by Peak 3 was indistinguishable from lactose on TLC with solvent A, but was identified as 2′-fucosyllactose from its ^{1}H NMR spectrum since the chemical shifts of the anomeric resonances at δ 5.312, 5.226, 4.634 and 4.528, and CH_3 at δ

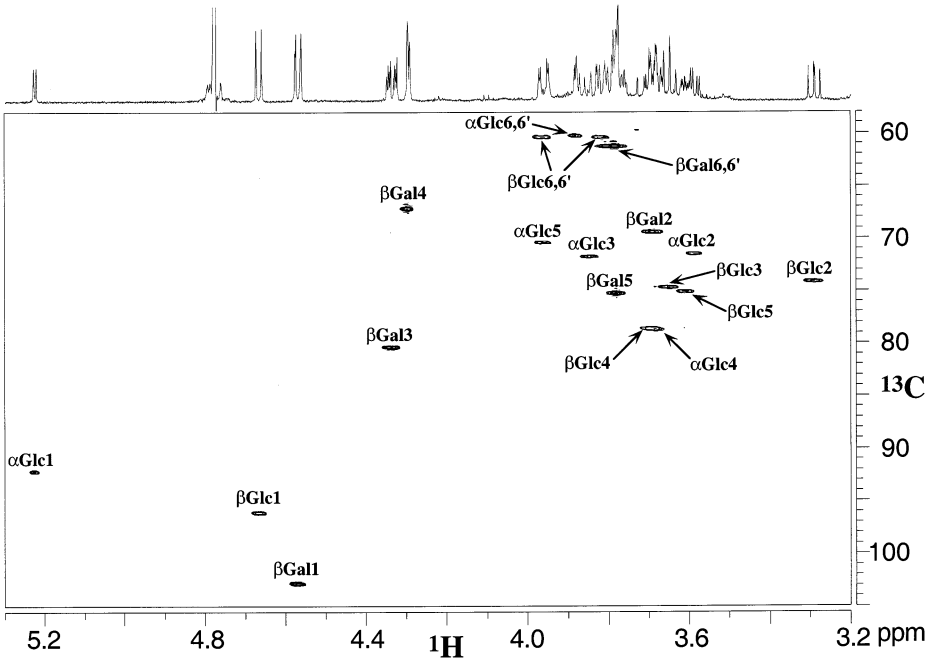

Fig. 2. 600 MHz sensitivity-enhanced HSQC spectrum of lactose 3′-sulfate recorded in ^2H_2O at 25 °C. The spectrum is shown in phase-sensitive mode after processing with 2 Hz Gaussian enhancement in each dimension. Assignments are according to the data presented in Table 1. A projection of the 1D ^{1}H spectrum is shown above the contour plot.

Table 1
NMR data for lactose 3′-sulfate

Quantity	Residue	Designated proton/carbon and value [a]						
		1	**2**	**3**	**4**	**5**	**6**	**6′**
δ (^{1}H)	α-Glc	5.225	3.59	3.84	3.68	3.96	3.88	3.88
	β-Glc	4.665	3.29	3.65	3.70	3.61	3.96	3.82
	β-Gal [b]	4.568 (β), 4.569 (α)	3.69	4.34	4.30	3.78	3.79	3.79
δ (^{13}C)	α-Glc	92.1	71.4	71.7	78.6	70.3	60.2	
	β-Glc	96.0	74.1	74.6	78.5	75.0	60.3	
	β-Gal	102.8	69.4	80.3	67.1	75.2	61.2	
$^1J_{CH}$	α-Glc	169.7	144.7	144.7		142.1		
	β-Glc	161.8	144.7	144.7		143.4		
	β-Gal	163.1	147.3	144.7	148.6			
^nJ (^{13}C)	α-Glc **A**	A3, A5	*A3*	A2, *A4*	*A3*, A5, A6			
	β-Glc **B**		B1, B3	B2, B4	B3, B5, B6			
	β-Gal **C**	A4, B4	C1, C3	C2, *C4*	C2, C3			

[a] Chemical shifts in ppm from internal acetone (^{1}H, δ 2.225; ^{13}C, δ 30.5); $^1J_{CH}$ in Hz; ^nJ (^{13}C), HMBC correlations between ^{1}H designated by column and row headings and ^{13}C identified by appropriate letter and number (relatively weak correlations are italicised).

[b] Small chemical shift differences, associated with each glucose anomer, were evident for H-1 and H-3 in 1D ^{1}H spectra but were not resolved in the HSQC spectra (see Fig. 1 and projection).

1.226 were essentially identical to those for the α-Fuc, α-Glc, β-Glc, β-Gal anomeric protons and fucose methyl group, respectively, of an authentic sample of 2′-fucosyllactose [12,15].

In gel chromatography, Peak 2 eluted near the sialyl oligosaccharides and much sooner than the di- and trisaccharides (Fig. 1). Such rapid elution was assumed to be associated with a negatively charged compound, which might be repelled by the slight negative charge of the Bio-Gel, comparable to the effect observed with Sephadex G-25 [22]. In the ^{1}H NMR spectrum of this substance, the resonances at δ 5.225 and 4.665, which together integrated for one proton, were associated through COSY and TOCSY spectra with a reducing glucose residue. Similarly the remaining anomeric resonance (δ 4.57) was associated with a galactose residue, there being only very weak correlations between H-4 and H-5. A sensitivity-enhanced HSQC experiment (Fig. 2), supported by data from an HMBC experiment, enabled the assignment of all carbon and proton resonances (Table 1) to a 3′-substituted lactose. The significant high-frequency shift of the β-Gal C-3 and smaller shift to low frequency for C-2 and C-4 compared with values for lactose [23] are similar to the effects on ^{13}C chemical shifts caused by sulfation at C-3 of D-glucose [24] and methyl α-D-galactopyranoside [25]. Similarly, the relatively high chemical shifts of the H-3 and H-4 resonances in methyl α-D-galactopyranoside 3-sulfate are comparable with those observed here. The electrospray mass spectrum of the oligosaccharide had $[M-H]^-$ at m/z 421.3 as predicted for a lactose sulfate.

Although one-bond carbon–proton coupling constants are sensitive to substituent effects [26], few data are available on the influence of substituent electronegativity on the magnitude of $^1J_{CH}$ in carbohydrates [27]. We recorded the HSQC spectrum without ^{13}C-decoupling and report in Table 1 all $^1J_{CH}$ values that could be measured from clearly resolved cross-peaks without undue complications from the effects of proton–proton couplings. The data show that determination of $^1J_{CH}$ is clearly not of practical significance for the detection of sulfation.

Occurrence of lactose sulfates.—Lactose 6′-sulfate was isolated from lactating rat mam-

mary gland [28] and the structure confirmed by chemical methods [29]. α-Neu5Ac-(2→3)-β-D-Gal*p*6*S*-(1→4)-D-Glc (designated neuraminlactose sulfate) was also found in lactating rat mammary glands [29] and was the only sulfate ester (named *N*-acetylneuramin lactose sulfate) subsequently found in rat milk [30]. In the latter study, it was suggested that *N*-acetylneuramin lactose sulfate was also present in human milk, but the observation was based on a mass spectrum in which no molecular ion for the sulfate ester could be detected under the conditions used (fast atom bombardment) and this finding must, therefore, be considered tentative.

Galactose sulfated at C-3 has been reported in a variety of oligosaccharides [31–33], glycolipids including β-D-Gal*p*3*S*-(1→4)-β-D-Glc*p*-(1→1)-Cer (sulfolactosyl ceramide) [34], and synthetic 3′-sulfo-β-lactosides [35]. In the last-mentioned paper, treatment of 4-methylumbelliferyl 3′-sulfo-β-lactoside with ceramide glycanase was shown by TLC to produce lactose 3′-sulfate, but neither the source nor properties of the authentic sample were recorded. To our knowledge, this is the only reference in the literature to free lactose 3′-sulfate.

It was considered [30] that *N*-acetylneuramin lactose sulfate may play an important role in the nutrition of rat pups. While lactose is clearly the dominant oligosaccharide in the dog milk examined in this work, the sulfate ester may have a similarly important function for dogs and other mammals. The data we have presented should facilitate the isolation and characterisation of lactose sulfates from other milks and subsequently lead to a better understanding of their significance in infant nutrition.

Acknowledgements

We thank Dr Shigeyuki Tanabe, Dr Yuhji Uzuka and Professor Takao Sarashina of Obihiro University of Agriculture and Veterinary Medicine for supplying the dog milk and Professor Takatoshi Itoh of Tohoku University for the 1D ^{1}H NMR spectra. This study was partially supported by a Grant-in-Aid for Scientific Research (C) in Japan.

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