ELSEVIER

Carbohydrate Research 268 (1995) 313–318

CARBOHYDRATE RESEARCH

Note

Disialyl lactose from buffalo colostrum: isolation and characterization

Huligerepura S. Aparna, Paramahans V. Salimath *

Department of Biochemistry & Nutrition, Central Food Technological Research Institute, Mysore-570 013, India

Received 26 May 1994; accepted in revised form 20 September 1994

Keywords: Colostrum, buffalo; Disialyl lactose; Isolation and characterization

Colostrum (early milk) contains a complex mixture of oligosaccharides in the free form [1]. These oligosaccharides are of considerable interest because of their ability to enhance growth of Bifidobacterium [2], serve as substrates of glycosyl transferases and hydrolases [3,4] and serve as inhibitors of infectious agents of the neonatal gut [2,5], and because of their role in the development of immunity in newborns. The analysis of these oligosaccharides is confined mainly to human [6], cow [7], and marsupial milk [8]. Herein we report on the composition of oligosaccharides, and isolation and structural elucidation of disialyl lactose, from the colostrum of buffalo, a major milk producing mammal of Asia.

The chloroform–methanol extract of colostrum (yield, 3.3–4%) fractionated on Sephadex G-25 gave three fractions eluting prior to lactose. The first fraction contained glycopeptides (0.2–0.8%), while the second (0.3–1.5%) and third (2.2–2.8%) contained oligosaccharides reported previously from bovine milk [9]. The average yield of these complex saccharides relative to lactose was 5% in the first day sample and decreased to 4.4 and 3.5% in second and third–fifth day samples, respectively. The compositional analyses of fractions (I–III) are summarized in Table 1. The protein content was significantly high in fraction I (38%) compared to the other two (II, 9%; III, 4%), while a wide variation was observed in sugar composition. In the first-day sample, neutral sugars increased from fractions I–III, whereas sialic acid was highest in fraction II (36%) and hexosamines highest in fraction I (29%). A similar pattern was also evident in the second-day sample. The third–fifth day sample contained mainly neutral sugars.

* Corresponding author.

SSDI 0008-6215(94)00329-7

Table 1
Composition (%) of fractions I–III obtained from Sephadex G-25 column chromatography

Fraction	Protein	Saccharide ratios (neutral sugar : sialic acid : hexosamine)		
		1 day	2 day	3–5 day
I	38	53:18:29	74:12:14	85:8:9
II	9	54:36:10	65:26:9	84:9:8
III	4	67:12:21	84:9:8	87:5:3

These data indicate a decrease in the concentration of higher oligosaccharides and sialyl oligosaccharides during early lactation in buffalo colostrum, unlike in human milk where they remain significantly high over a long period [1].

When fraction II was chromatographed on QAE-Sephadex A-25, unbound fractions were eluted as three peaks with water (Fig. 1, a–c) and the bound fractions were subsequently eluted into seven fractions in 0.1 M (d–j), three in 0.25 M (k–m) and three in 0.5 M (n–p) buffer. Overall, fraction II contained 12% unbound saccharides, and 43, 33, and 7% bound fractions in 0.1 M, 0.25 M, and 0.5 M buffer, respectively. These fractions (Fig. 1, a–p; yield, $< 4\%$) were subjected to paper chromatography (PC) and cellulose acetate membrane electrophoresis. Fraction m, eluting with 0.25 M buffer, was homogeneous, while all the other fractions appeared to be heterogeneous (data not shown). The pure fraction (m), obtained in an average yield of 60 mg per litre colostrum, was found to be significantly lower compared to that obtained from bovine colostrum (285 mg) [9]. The relative mobility of this fraction on PC was slower ($R_{lac} = 0.69$), compared to sialyl lactose ($R_{lac} = 0.87$), suggesting that it had a higher molecular mass. But its movement ahead of sialyl lactose on the electrophoregram clearly indicated the presence of additional negative group(s). The purity of fraction m

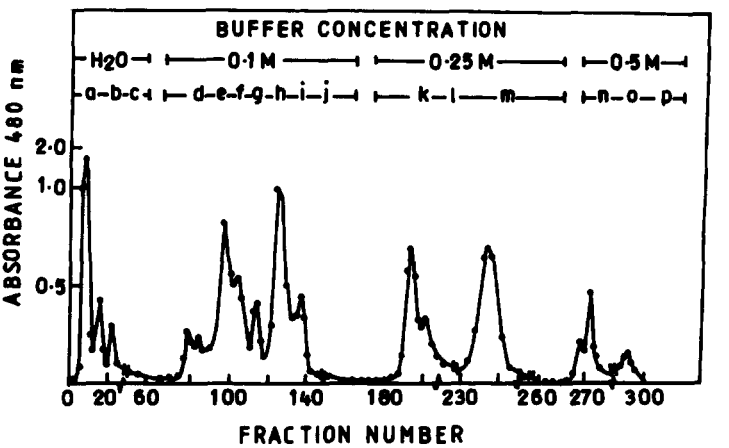

Fig. 1. Fractionation of sialyl oligosaccharides on a QAE Sephadex A-25 column.

Table 2
^{1}H and ^{13}C chemical shifts of structural reporter groups of constituent monosaccharides of disialyl lactose

Residue	Reporter group	Chemical shifts (ppm)					
		^{1}H [a]			^{13}C		
		α	α/β [b]	β	α	α/β [b]	β
D-Glc	1	5.23			93.0		97.0
	2	3.59		3.28	73.8		76.4
	3	3.84		3.63	75.0		76.7
	4	3.69		3.07	80.5		80.5
β-D-Gal	1		4.53				103.9
	2		3.57			72.9	
	3			4.10		75.2	
	4		3.96			70.5	
α-^{3}NeuNAc	3a	1.76			40.9		
	3e	2.78					
	NAc	2.05			23.3		
α-^{8}NeuNAc	3a	1.76			41.7		
	3e	2.68					
	NAc	2.09			23.6		

[a] HOD signals were suppressed by selective saturation.
[b] Data in this column correspond to protons which do not undergo the anomeric effect.

was also evident by HPLC analysis where it eluted as a single peak at 15.07 min compared to sialyl lactose (14.27 min).

Compositional analysis revealed the presence of sialic acid, galactose, and glucose in 2 : 1 : 1 ratio, suggesting it to be a disialyl-type oligosaccharide. Based on the above data, its electrophoretic mobility ahead of sialyl lactose and elution later than sialyl lactose on PC and HPLC could be attributed to the presence of additional sialic acid residues. The partially methylated alditol acetates obtained were identified as 2,4,6-tri-*O*-methyl-galactitol and 1,2,3,5,6-penta-*O*-methyl-glucitol by their characteristic fragments (*m/z*) at 101, 117, 129, 161, 201, and 233, and 101, 117, 129, 145, 189, 205, and 249, respectively. These two derivatives indicate a lactose backbone and substitution of NeuNAc at C-3 of galactose. Fragmentation patterns of sialic acid could not be achieved due to the hydrolytic condition employed [10].

In order to elucidate the primary structure of the oligosaccharide, both ^{1}H and ^{13}C NMR spectra were recorded. The relevant reporter groups are summarised in Table 2. In the spectrum, two sets of NeuNAc structural reporter group resonances were observed, namely, two coincident H-3a triplets at 1.76 ppm and two H-3e doublets of doublets at 2.68 and 2.78 ppm, respectively. These chemical shifts were found to be characteristic of α-(2 → 3) and α-(2 → 8) substituted sialic acids as they were in agreement with signals of sialyl oligosaccharides isolated from gangliosides [11,12]. It should be mentioned that the chemical shift of H-3e for the internal α-(2 → 3) linked NeuNAc (2.68 ppm) is in the range usually observed for NeuNAc residues in α-(2 → 6) linkage. But, the presence of an H-3a signal at 1.76 ppm instead of 1.73 ppm of α-(2 → 6) [8]

confirms an α-(2 → 3) linkage, and substitution of α-(2 → 8) NeuNAc to sialyl lactose causes a considerable downfield shift in H-8 and H-9 from ~ 3.9 to 4.14 ppm and from ~ 3.9 to 4.18 ppm [12]. The presence of α-(2 → 3) substitution was also confirmed by the generation of 2,4,6-tri-*O*-methyl-galactitol (see above). The presence of two distinctly different values for *N*-acetyl signals at 2.09 and 2.05 ppm indicate the presence of two-*N*-acetyl neuraminic acids [11]. This fact was confirmed by the identification of only NeuNAc residues after sialidase treatment.

The neutral saccharide residue chemical shifts were similar to those observed in sialyl lactose [12,13]. The resonances of β-D-galactopyranose at 4.53, 4.10 and 3.96 ppm for H-1, H-3, and H-4, respectively, were only moderately affected by the elongation to sialyl lactose. Signals at 5.23, 3.28, 3.84, and 3.69 ppm for H-1, H-2, H-3, and H-4 of β-D-glucopyranose appeared in close agreement with corresponding signals in lactose or sialyl lactose [13].

In accordance with ^{1}H-NMR results, ^{13}C NMR analysis also indicated the presence of a lactose backbone in the structure from characteristic chemical shifts as indicated in Table 2. The reporter signals for C-1, C-2, C-3, and C-4 at 93.0, 73.8, 75.0, and 80.5 ppm, respectively, were similar but not identical to those observed in oligosaccharides derived from gangliosides [14,15]. Similarly, C-1 to C-4 of the β-D-galactopyranosyl ring were also in good agreement with acidic oligosaccharides of gangliosides [14]. The neuraminic acid residues ^{3}NeuNAc and ^{8}NeuNAc were identified by C-3 signals at 40.9 and 41.7 ppm, and their *N*-acetyl signals at 23.3 and 23.6 ppm, respectively [15,16]. Like GD1b, the linkage of the second NeuNAc to sialyl lactose results in an upfield shift of C-8 from 77.0 to 72.4 ppm and downfield shifts in C-9 from 62.5 to 63.8 ppm [15]. The remaining NeuNAc carbons were found to resonate at field positions not significantly different from those of oligosaccharides described previously.

From methylation analysis and NMR data, the following structure of the oligosaccharide is proposed:

$$\alpha\text{-NeuNAc-}(2 \rightarrow 8)\text{-}\alpha\text{-NeuNAc-}(2 \rightarrow 3)\text{-}\beta\text{-Gal-}(1 \rightarrow 4)\text{-Glc}$$

The disialyl lactose isolated in the present investigation has a primary sequence identical to that of GD3, which may reflect its role during growth and development of newborns. Hitherto disialyl lactose has been isolated and identified only from bovine milk and not from human milk, further suggesting their specific role in milking mammals and in ruminants.

1. Experimental

Materials.—Fresh buffalo colostrum was collected locally and stored at −20°C. The saccharides were extracted with 2 : 1 $CHCl_3$–MeOH and were fractionated on a Sephadex G-25 column (150 × 2.5 cm) using water as the eluent [17]. Peak II obtained from this column was fractionated on a QAE-Sephadex A-25 column (40 × 2.5 cm) using water and ammonium acetate buffer (pH 5.4, 0.1–0.5 M) as eluents. The resulting fractions were desalted on a Biogel P-2 column (40 × 2.5 cm) and lyophilized.

Methods.—Paper chromatography was carried out in 5:5:1:3 EtAc–PrOH–AcOH–H_2O and spots were detected with $AgNO_3$–NaOH reagent. Electrophoresis was carried out on cellulose acetate membranes using acetate buffer (pH 5.0, 50 mM), and the electrophoregrams were stained with $AgNO_3$ reagent. HPLC was performed on a Maxil NH_2 column using 18:7 CH_3CN–potassium phosphate buffer (pH 5.2, 15 mM) as eluents isocratically at a flow rate of 1.5 mL/min using a UV detector at 195 nm [18].

Sialic acid was identified and quantified by chromatographic characterization of the products after sialidase (*Clostridium perfingens*,, type V, 25 mU, 37°C, 60 min) treatment, as well as after hydrolysis using H_2SO_4 (0.05 M) [19]. Identification and quantitation of neutral sugars were performed by GLC as alditol acetates on 3% OV-225 [20]. Samples were methylated [21] and analysed by GLC–MS on a CP-SIL-5 column [22].

400-MHz ^1H NMR and 100-MHz ^{13}C NMR spectra of saccharides were recorded on a Bruker AMX NMR spectrometer in D_2O at room temperature. Chemical shifts are reported as ppm downfield from an external standard (Me_4Si).

Acknowledgements

The authors thank Dr. K.V. Ramanathan, IISc, Bangalore, for assistance in the NMR analyses, and Dr. N. Chandrasekhara, CFTRI for his valuable suggestions and support. HSA thanks the University Grants Commission for the award of a Fellowship.

References

[1] B. Blanc, *World Rev. Nutr. Diet.*, 36 (1981) 1–89.
[2] P. Gyorgy, R.W. Jeanloz, H.V. Nicolai, and F. Zilliken, *Eur. J. Biochem.*, 43 (1974) 29–33.
[3] G.C. Haunson and D. Zopf, *J. Biol. Chem.*, 260 (1985) 9388–9398.
[4] M.L. Schneir and M.E. Rafelson, *Biochim. Biophys. Acta*, 130 (1966) 1–11.
[5] J. Parkkinen and J. Finne, *J. Biol. Chem.*, 260 (1985) 10971–10975.
[6] G. Gronberg, P. Lipniunas, T. Lundgren, F. Lindh and B. Nilsson, *Arch. Biochem. Biophys.*, 296 (1992) 597–610.
[7] R. Roy, C.A. Laferriere and H. Dettman, *Carbohydr. Res.*, 186 (1989) C1–C5.
[8] J.P. Kamerling, L. Dorland, H. van Halbeek, J.F.G. Vliegenthart, M. Messer and R. Schauer, *Carbohydr. Res.*, 100 (1982) 331–340.
[9] R.W. Veh, J.C. Michalski, A.P. Corfield, S.W. Michael, D. Gies and R. Schauer, *J. Chromatogr.*, 212 (1981) 313–322.
[10] R. Kannagi, S.B. Levery, F. Ishigami, S.I. Hakomori, K.L.H. Shivensky, B.B. Knowles and D. Solter, *J. Biol. Chem.*, 258 (1983) 8934–8942.
[11] H. Friebolin, W. Baumann, M. Hauck, D. Kurz, R. Wajda, G. Weisshaar, G. Keilich, D. Ziegler, R. Brossmer and H.V. Nicolai, *Hoppe-Seyler's Z. Physiol. Chem.*, 365 (1984) 1309–1321.
[12] L. Dorland, H. van Halbeek, J.F.G. Vliegenthart, R. Schauer and H. Wiegandt, *Carbohydr. Res.*, 151 (1986) 233–245.
[13] N. Platzer, D. Davoust, M. Lhermitte, C. Bauvy, D.M. Meyer and C. Derappe, *Carbohydr. Res.*, 191 (1989) 191–207.
[14] J.F.G. Vliegenthart, L. Dorland and H. van Halbeek, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 209–374.

[15] L.O. Sillerud, R.K. Yu and D.E. Schafer, *Biochemistry,* 21 (1982) 1260–1271.
[16] R. Schauer, *Sialic acids – Chemistry, metabolism and function,* Cell Biol. Monogr., Vol. 10, Springer, Vienna, 1982, pp. 263–291.
[17] R. Ohman and O. Hygstedt, *Anal. Biochem.,* 23 (1968) 391–402.
[18] M.L.E. Bergh, P. Koppen and D.H.V. Eijnden, *Carbohydr. Res.,* 94 (1981) 225–229.
[19] D. Aminoff, *Biochem. J.,* 81 (1961) 384–392.
[20] J.S. Sawardeker, L.S. Sloneker and A. Jeanes, *Anal. Chem.,* 37 (1967) 1602–1604.
[21] S.I. Hakomori, *J. Biochem.,* 55 (1964) 205–208.
[22] P.E. Jansson, L. Kenne, H. Liedgren, B. Lindberg and J. Lonngren, *Univ. Stockholm Chem. Commun., 8 (1976) 1–77.*