Carbohydrate Research, 165 (1987) 43-51
Elsevier Science Publishers B.V., Amsterdam - Printed in The Netherlands

CHEMICAL STRUCTURE OF THREE NEUTRAL TRISACCHARIDES ISOLATED IN FREE FORM FROM BOVINE COLOSTRUM*

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(Received July 7th, 1986; accepted for publication, August 25th, 1986)

ABSTRACT

Three neutral trisaccharides, which comprise 25.1% of the neutral oligosaccharide other than lactose, were isolated from bovine colostrum, obtained 6 h after parturition, by l.c. on amino silica gel. The chemical structures were identified, by methylation analysis with direct m.s. and g.l.c.-m.s., and by structural analysis with ^{13}C-n.m.r., as β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)-]-D-GlcNAc (3-fucosyl-*N*-acetyllactosamine), β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc (3′-galactosyllactose), and β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-D-Glc (6′-galactosyllactose). The first-named compound was a novel oligosaccharide from mammalian milk.

INTRODUCTION

Lactose [β-D-Gal*p*-(1→4)-D-Glc] is the main carbohydrate in nearly all milks of placental mammals. A wide variety of oligosaccharides generally occur in milk, especially human[1,2] and marsupials[3-6]. Bovine colostrum also contains eight acidic oligosaccharides (a di-, six tri-, and a tetra-saccharide) having one or two *N*-acylneuraminic acid residues[7-10]. However, the presence of neutral oligosaccharides other than lactose in bovine milk had not been extensively investigated. Recently, we have isolated two neutral disaccharides containing an *N*-acetylhexosamine residue from bovine colostrum[11]. However, there were indications that additional oligosaccharides were still present in the unfractionated colostrum.

We describe herein the isolation of additional neutral oligosaccharides from bovine colostrum by use of liquid chromatography (l.c.) on amino silica gel, which has been employed recently for the separation of several oligosaccharides[12-14], as well as their chemical structures.

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* This study was supported, in part, by research grant (No. 5 8760 098) from the Ministry of Education of Japan.

0008-6215/87/$ 03.50

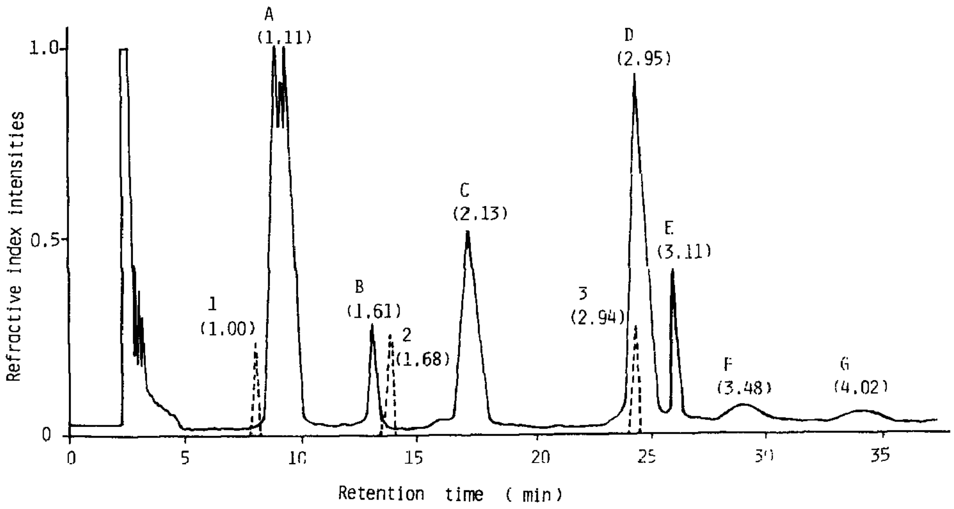

Fig. 1. Elution diagram of liquid chromatogram of neutral oligosaccharide fraction (other than lactose) isolated from bovine colostrum taken 6 h after parturition. Chromatography was run on a Hibar column EC (0.4 × 25 cm, Merck) containing Lichrosorb-NH$_2$ (5 μm, Merck). Isocratic elution for 40 min at 1 mL/min was carried out at 25° with 4:1 acetonitrile-water. The working pressure was 9.8 MPa. The retention times relative to D-Glc are shown in parentheses. The position of elution of reference sugars (1, D-glucose; 2, lactose; and 3, rafinose) is represented by dotted line.

RESULTS AND DISCUSSION

The neutral oligosaccharide fraction, other than lactose, was isolated from bovine colostrum by dialysis, removal of peptides, Bio-Gel filtration, and by ion-exchange chromatographies. The oligosaccharides were further fractionated by l.c. on amino silica gel and were eluted to give seven peaks (A-G) with well base-line separation (Fig. 1). Preparative l.c. was performed for the collection of components A–G, followed by lyophilization after removal of the solvent by evaporation. The elution values, abundance in the milk, sugar composition, and reducing-end group analysis are shown in Table I.

The data for components A and B agreed in relative retention times on l.c., p.c., and ^{13}C-n.m.r. spectra with those of β-D-Gal*p*-(1→4)-D-GlcNAc and β-D-GalNAc-(1→4)-D-Glc, respectively, the isolation of which from bovine colostrum was reported in our previous paper[11]. Component C was a neutral trisaccharide containing an L-fucose residue. Components D,E,F, and G were composed of only D-galactose and D-glucose, and were estimated to be a tri-, tri-, tetra-, and pentasaccharide, respectively. Oligosaccharide C gave one mole of 2-acetamido-2-deoxyglucitol and each D–G saccharide gave one mole of glucitol by reduction, as determined by g.l.c.. The amount of components F and G was too small for further structural analysis. Fractions C,D, and E, which comprised 25.1% of the neutral oligosaccharides other than lactose, were subjected to methylation and n.m.r.

TABLE I

R_{lactose} VALUES, CONTENT, SUGAR COMPOSITION, AND REDUCING-END ANALYSES OF SEVEN *OLIGOSACCHARIDES* ISOLATED FROM BOVINE COLOSTRUM BY L.C.

Oligosaccharide fractions on p.c.	$R_{lactose}$ *p.c.*	*Content (mg/L)*	*Weight ratio (% w/w)*	*Molar values (mol/mol)*					*Sugar in the reducing position*
				L-Fuc	*D-Glc*	*D-Gal*	*D-GlcNAc*	*D-GalNAc*	
A	1.36	103.3	70.3	0	0	1.00	1.13	0	D-GlcNAc
B	1.10	5.4	3.7	0	1.00	0	0	0.93	D-Glc
C	0.88	10.6	7.2	1.05	0	1.00	1.96	0	D-GlcNAc
D	0.75	17.8	12.1	0	1.01	2.17	0	0	D-Glc
E	0.64	8.5	5.8	0	1.00	2.10	0	0	D-Glc
F	0.51	0.9	0.6	0	2.00	2.18	0	0	D-Glc
G	0.38	0.4	0.3	0	3.10	2.33	0	0	D-Glc

TABLE II

PARTIAL MASS SPECTRA OF PERMETHYLATED OLIGOSACCHARIDE ALDITOLS C,D, AND E ANALYZED WITH DIRECT-INLET SYSTEM

Oligosaccharide alditols	m/z *(Intensities)*
Fraction C	686(M^+, 8), 655(M^+ −31,9), 641(M^+ −45, 32), 627(16) 597(M^+ −89,7), 542(30), 481(M^+ −205,30), 465(24) 451(M^+ −235,31), 441(20), 406(20), 349(94), 309(58) 260(95), 219(38), 189(100), 187(85), 157(73), 131(73) 111(58), 101(92), 89(70), 45(55)
Fractions D and E	586(M^+ −89, 4), 552(5), 541(M^+ −134, 6), 500(9) 440(M^+ −235, 9), 423(M^+ −252, 11), 391(9), 359(9) 349(9), 335(9), 260(39), 250(46), 236(100), 219(99) 187(99), 149(61), 134(43), 111(77), 101(96), 90(71) 89(68), 45(61)

analysis.

In order to determine the sugar sequence, permethylated, deuterium-reduced oligosaccharides C,D, and E were analyzed by direct m.s.; *m/z* values of conspicuous fragments and relative intensities on the mass fragmentograms are shown in Table II. The mass spectrum of component C gave a molecular ion (M^+) at *m/z* 686, which agreed well with the calculated value. The lack of a peak at *m/z* 277 indicated a double substitution of the reducing 2-acetamido-2-deoxyglucose residue. A branched structure was confirmed by the presence of two peaks at *m/z* 451 and 481. Fragments at *m/z* 219 and 189 corresponded to terminal nonreducing galactosyl and fucosyl groups, respectively. The reducing 2-acetamido-2-deoxyglucose residue was not substituted at O-6 because of the presence of fragments *m/z* 89 and 597 (M^+ − 89). Furthermore, the presence of a fragment at *m/z* 349 showed that a fucosyl group was attached to O-3 of a 2-acetamido-2-deoxyglucose residue and, by consequence, a galactosyl residue was linked to O-4.

Components D and E gave nearly the same mass spectra for fragmentation patterns and peak intensities. The characteristic fragment at *m/z* 423 corresponded to a terminal hexosyl→hexose (Gal→Gal) disaccharide. The absence of a branched structure was demonstrated by the presence of an ion, at *m/z* 236, from the reducing glucose residue. The ions at *m/z* 541 and 586 proved the linkage of the disaccharide unit to the glucose residue to be at O-4.

3,4-Di-*O*-acetyl-2-deoxy-1,5,6-tri-*O*-methyl-2-(N-methylacetamido)glucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol, and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylgalactitol were also identified by g.l.c.-m.s. as alditol acetate derivatives of partially methylated sugars on the chromatograms of the oligosaccharide alditols C, D, and E, respectively. These data led to the conclusion that components C, D, and E are trisaccharides having the sequences Gal-(1→4)-[Fuc-(1→3)]-GlcNAc, Gal-(1→3)-Gal-(1→4)-Glc and Gal-(1→6)-Gal-(1→4)-Glc, respectively.

TABLE III

^{13}C-CHEMICAL SHIFTS (δ)[a] AND ASSIGNMENTS FOR NEUTRAL OLIGOSACCHARIDES C,D, AND E, AND RELEVANT COMPOUNDS

Oligosaccharides	*Residues*		*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	-CH_3 (*$NHCOCH_3$*)	-*C=O* (*$NHCOCH_3$*)
Fraction C	Gal-(1→4)		105.7	73.8	75.4	71.3	78.2	63.0		
	[Fuc-(1→3)]		96.9	70.5	72.5	73.8	67.7	23.5		
	-GlcNAc	α	94.7	52.5	76.7	81.3	72.5	63.9	24.9	177.5
		β	98.6	53.7	77.3	81.5	77.7	63.9	24.9	177.5
Fraction D	Gal-(1→3)		105.7	73.7	75.4	72.0	77.9	63.4		
	-Gal-(1→4)		105.5	73.7	80.2	71.1	77.9	63.4		
	-Glc	α	94.3	74.3	72.2	81.3	72.5	63.8		
		β	98.6	76.7	77.3	81.4	77.9	63.8		
Fraction E	*Gal-(1→6)-*		*107.2*	*73.7*	*75.4*	*71.5*	78.2	62.9		
	-Gal-(1→4)		105.4	73.4	75.4	71.5	77.9	69.4		
	-Glc	α	94.3	74.3	73.9	81.1	73.1	63.9		
		β	98.6	76.7	77.3	81.4	77.7	63.9		
Lactose										
Nonreducing-end unit			105.8	73.8	75.4	71.4	78.2	63.0		
Reducing-end unit		α	94.7	74.3	74.0	81.3	73.0	63.9		
		β	98.6	76.7	77.2	81.4	77.7	63.9		
N-Acetyllactosamine										
Nonreducing-end unit			105.8	73.9	75.5	71.5	78.3	62.9		
Reducing-end unit		α	93.4	56.6	72.2	81.4	73.2	63.9	24.9	177.3
		β	97.8	59.1	75.5	81.8	77.8	63.9	25.2	177.3

[a] Downfield from signal for internal ($^2H_4$)TPS.

^{13}C-N.m.r. spectroscopy was used for anomeric and structural elucidation of the three components, C, D, and E. The chemical shifts of each sugar units in the three compounds are given in Table III. Resonances of component C were assigned by comparing the spectrum with the data for the corresponding reference sugar of *N*-acetyllactosamine[11] possessing an identical fragment. Resonances of components D and E were also interpreted by comparison with the spectra of lactose[15] and 3′-galactosyllactose[3].

The signals at δ 105.7 and 96.9 in the spectrum of component C were clearly assigned to the anomeric carbon atoms of the galactosyl and fucosyl groups, respectively. The two anomeric configurations of the galactose and fucose units were determined to be in the β-D and α-L forms, respectively. The signals at δ 76.7 and 77.3 for C-3 arising from the α and β anomers, respectively, of the 3-*O*-substituted 2-acetamido-2-deoxyglucose unit, were also assigned by down-field shifts of 4.5 (α) and 1.8 (β) p.p.m. from the signal of the free C-3 of the reducing unit in *N*-acetyllactosamine. The two signals at δ 105.7 and 105.5 in the spectrum of fraction D were assigned to the anomeric carbon atoms of the nonreducing galactosyl group and the internal galactosyl unit, respectively, and could be due to the β-D configurations. Almost all chemical shifts were in agreement with those of 3′-galactosyllactose. The two peaks at δ 107.2 and 105.4 in the spectrum of fraction E were also assigned to the anomeric carbon atoms of the nonreducing galactosyl group and the internal galactosyl unit, respectively, which were interpreted to be in the β-D forms. The peak at δ 69.4 arising from C-6 in a 6-*O*-substituted internal galactose unit was assigned by down-field shifts of 6.4 p.p.m. from the free C-6 of the nonreducing group in lactose. Thus, the above-described structural assignments agree with the chemical structure indicated by the methylation analysis.

In conclusion, the chemical structures of oligosaccharides C, D and E were identified as β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-D-GlcNAc (3-fucosyl-*N*-acetyllactosamine), β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc (3′-galactosyllactose), and β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-D-Glc (6′-galactosyllactose), respectively. The first-named oligosaccharide is a novel one which has not been previously described for mammalian milks.

The l.c. conditions used in this work were very effective for the rapid and complete separation of neutral oligosaccharides, prepared from bovine colostrum, which could not be well isolated by preparative p.c. Kobata *et al.*[2] reported that Bio-Gel P-4 (−400 mesh) is superior for the separation of milk oligosaccharides because a 2-acetamido-2-deoxyglucose residue behaves like two units of hexose. The presence of an *N*-acetylhexosamine residue in components A, B, and C was also reflected in their earlier elution on alkylamine-modified silica. Disaccharide A and trisaccharide C were eluted from the column in the regions of elution of mono- and di-saccharides, respectively. Such an effect was more pronounced in an oligosaccharide containing a reducing *N*-acetylhexosamine residue, as compared to an *N*-acetylhexosamine in a nonreducing position.

N-Acetyllactosamine (component A) has been demonstrated to be the most abundant neutral oligosaccharide (70.3%) after lactose in bovine colostrum[11]. 3-Fucosyl-*N*-acetyllactosamine (component C) may be biosynthesized from *N*-acetyllactosamine by addition of a fucosyl residue. Herlant-Peers *et al.*[16] reported the presence of the same trisaccharide, in new-born meconium, as a sugar-chain fragment formed as a result of catabolism of mucin-type glycoproteins. No report has so far been presented on the presence of such sugar in mammalian milk. Bovine colostrum has now been shown to contain three oligosaccharides having an *N*-acetyllactosaminyl core structure, *N*-acetyllactosamine[11], 3-fucosyl-*N*-acetyllactosamine (this paper), and 6′-sialyl-*N*-acetyllactosamine, which was reported by Kuhn and Gauhe[17].

Human milk contains a small quantity of 6′-galactosyllactose[18] and marsupial milk is the only milk containing 3′-galactosyllactose as a preponderant neutral oligosaccharide[3]. Bovine colostrum is, therefore, remarkable for the dual presence of both 3′- and 6′-isomers of galactosyllactose. However, by 7 days after parturition, these unique oligosaccharides have completely disappeared from bovine milk and lactose remains the preponderant sugar[11]. The disappearance of these compounds with the progress of lactation has interesting implications for the time-dependent expression of specific glycosyltransferases in the bovine mammary gland.

EXPERIMENTAL

Materials. — Colostrum was obtained from a Horstein–Friesian cow at 6 h after parturition. β-D-Gal*p*-(1→4)-D-GlcNAc (*N*-acetyllactosamine) and β-D-GalNAc-(1→4)-D-Glc, isolated from bovine colostrum[11], were used as reference sugars for l.c. and n.m.r. analysis.

Preparation of neutral milk oligosaccharides. — Milk carbohydrates from defatted colostrum were separated by dialysis according to the method of Schneir and Rafelson[9]. The complete removal of peptides from the dialyzate by ion-exchange chromatography with SP-Sephadex C-50 and isolation of neutral oligosaccharides other than lactose by gel filtration with Bio-Gel P-2 (200–400 mesh, 2.2 × 90 cm) were performed as described previously[11]. The oligosaccharides were finally purified by ion-exchange chromatography with Dowex 50W-X4 (H^+) and AG-3 (OH^-) ion-exchange resins in order to remove contaminating, acid-soluble nucleotides.

Chromatography. — P.c. was performed on Whatman 3MM paper by the ascending system in 6:4:3 (v/v) butanol–pyridine–water (five times) and spots were detected with alkaline silver nitrate. Analytical and preparative l.c. were performed with a Hitachi Model 638-30 liquid chromatograph on a Hibar column EC (0.4 × 25 cm, Merck) containing Lichrosorb-NH_2 (5 μm, Merck). Isocratic elution was carried out with 4:1 acetonitrile–water for 40 min at room temperature, and a flow rate of 1 mL/min. Elution was monitored with a Shodex differential refractometer Model SE-11.

Analytical methods. — Reduction of oligosaccharides was carried out in M

NaBD$_4$ solution at 4° for 6 h in the dark. The carbohydrate compositions of intact and reduced oligosaccharides were determined by g.l.c. after methanolysis, followed by per(trimethylsilyl)ation according to the method of Clamp *et al.*[19]. The other analytical conditions have been described previously[15] in detail.

Methylation analysis. — Oligosaccharide alditols were permethylated by the method of Hakomori[20]. Both the triphenylmethane test[21] and the purification with a small silica gel column[22] were adopted in the methylation process to assure completion of the reaction. Alditol acetate derivatives of partially methylated sugars were prepared from permethylated oligosaccharide alditols by use of the method of Stellner *et al.*[23]. Permethylated oligosaccharide alditols and alditol acetate derivatives were analyzed by m.s. with a direct inlet system (direct m.s.) and g.l.c.-m.s., respectively, under conditions similar to those described previously[24].

N.m.r. spectroscopy. — ^{13}C-N.m.r. spectra were recorded at 30° for solutions in D$_2$O (99.75%, Merck) with a JEOL-JNM-FX 100 spectrometer, in the pulsed F.t. mode, with a repetition time of 1.18 s, a pulse width of 0.68 s, and an accumulation of 2000–70 000. For structural analyses, spectra were recorded at 25.00 MHz with sodium 4,4-dimethyl-4-sila(2,3-^{2}H$_4$)pentanoate [(^{2}H$_4$)TPS] (Merck) as internal standard.

ACKNOWLEDGMENTS

The authors thank Drs. Taichi Usui (Shizuoka University) and Tateo Suzuki (Tohoku University) for their advice on l.c. and ^{13}C-n.m.r. analyses, and also thank Dr. Minoru Ohta (Kawatabi Farm of Tohoku University) for supplying bovine colostrum.

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