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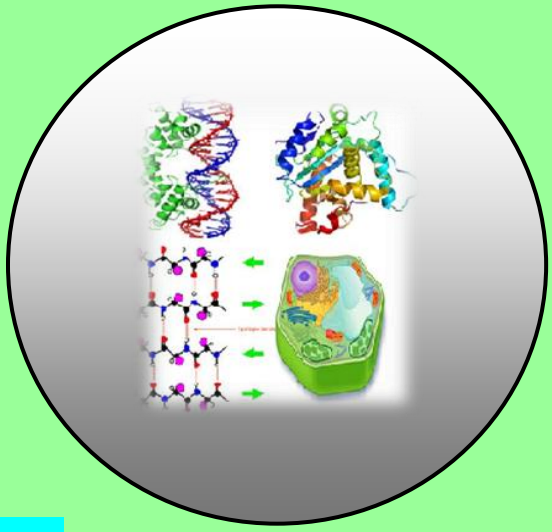

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RESEARCH PAPER

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Isolation of a Novel Oligosaccharide from Goat Milk

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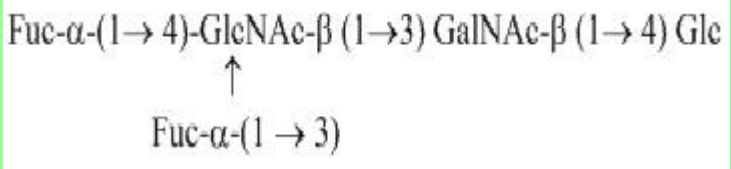
ABSTRACT

Milk is an important fluid in glycobiology, contains a number of carbohydrate chains either free or as glycoconjugates. These compounds are the most abundant component which benefit the infant by developing and maintaining the infant's gut flora. It is one of the important source of biologically active oligosaccharides, which have shown unique biological activities such as anti-tumor, anti-cancer, anti-viral, anti-inflammatory, anticoagulant, antioxidant and immunostimulant. In the present work goat milk was collected and processesed by modified method of Kobata and Ginsburg followed by gel filtration, HPLC and column chromatography. Besides the number of oligosaccharide reported earlier, we have isolated a novel oligosaccharide from Goat milk. The structure of purified oligosaccharide was confirmed by chemical degradation/transformation along with 2-D NMR and Mass spectrometry. Moreover Structure Reporter Group theory was also incorporated for structure elucidation of oligosaccharide along with 1 D NMR experiments. A comparative study of ^{1}HNMR of acetylated and natural oligosaccharide was used to confirm the glycosidic linkages. The structure of novel oligosaccharide was also confirmed by ES MS. On the basis of the result obtain from the above experiment structure of novel oligosaccharide Gaurose was established as:

Compound- Gaurose



Key words- Goat milk, Oligosaccharide, Kobata and Ginsburg and NMR.

INTRODUCTION

The role of milk in the first few days of any newborn is to provide nutrition and protect against infection while the immune system was still developing. It is a natural example of a prebiotics diet of mammals during infancy (Gunther Boehan and Beard et al., 2007). It is a nutritious food containing numerous essential nutrients for the metabolism, growth, development and well-being of the young mammal. Milk supplies energy amino-acid, vitamins, and minerals to the suckling during an important part of life (Jenness R.1986). Milk oligosaccharides are mainly derived from lactose and therefore almost all carry the lactose unit at the reducing end. Oligosaccharides are the third largest solute in human milk after lactose and fat (Coppa et al., 1993). Human milk oligosaccharides play important anti-infective roles in the intestinal, respiratory and urinary tracts (Newburg 1999). In the biological significance, the cow milk oligosaccharides reduce the adhesion of enterotoxic Escherichia coli strains of the calf (Johansson P. et al., 2005). Goat milk oligosaccharides possess 'prebiotic' properties i.e they promote the growth of beneficial Bifido bacteria in the intestine (Raynal–Ljutovac et al., 2008). The benefits of bifidobacteria used to be primarily attributed to their role in promoting neonates immunity and protection against intestinal pathogens as well as brain development (Raynal–Ljutovac et al., 2008). Goat milk is known to contain fucosylated and sialylated oligosaccharide, making them similar in composition to HMO (Chaturvedi and Sharma 1990; Urashima et al., 1994). The wide variety of oligosaccharides present in goat milk are 6'-5L, 3'-5L disialyllactose, N-glycolylneuraminyllactose, 3'-galactosyl lactose, N- acetylglucosaminyllactose, LNH and additional high molecular oligosaccharides (Urashima and Taufik 2010). Goat milk also has stronger immunological and antibacterial effect than bovine milk (Slacanac et al., 2010). Therefore, goat milk may represent an ideal source of oligosaccharide for supplementary and therapeutic application. Keeping in mind the ancient uses and biological activities reported in recent chemical investigation, we have isolated a novel oligosaccharide from the goat milk and then its structure was elucidated with the help of chemical degradation, chemical transformation and spectroscopic method like ^{1}H NMR ^{13}C NMR and 2DNMR i.e COSY TOCSY, HSQC technique and mass spectrometry.

MATERIAL AND METHODS

General procedures

Centrifugation of crude milk was done with the help of cooling centrifuge remi instruments C-23 JJRCI 763. The evaporation of alcohol from crude extract of milk oligosaccharides was done on Buchi Rotatry evaporator. The freeze drying of the compounds was done with the help of CT 60e (HETO) lyophilizer. NMR experiments were recorded in solvent $CDCl_3$ and D_2O at 25^{o} on a Bruker AM 300 MHz FT NMR spectrometer. The Electrospray mass spectra were recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. The milk oligosaccharide sample (dissolved in water as solvent) was introduced into the ESI source through a syringe pump at the rate of 5ul/min. The ESI capillary was set at 3.5 kV and the cone voltage was 40 V. Sephadex G-25 (PHARMACIA) was used in gel permeation chromatography. The HPLC system was equipped with Shimadzu CLASS-VP V6.13 solvent delivering system, 235-diode array detector and G.P 100 plotter. Authentic samples of glucosamine, galactosamine, glucose and fucose were purchased from Aldrich Chemicals.

Isolation of goat milk oligosaccharides by modified method of Kobata and Ginsburg

15 litre goat milk was collected from a domestic goat and equal amount of C_2H_5OH was added and filtered then treated in accordance with the modified method of Kobata and Ginsberg (Narendra M. Tripathi et al., 2014). Milk was centrifuged for 15 min at 5000 rpm at 4^oC and was filtered with glass wool column under cold atmoshpheric condition. After removing the lipid, and protein, lactose were precipited with 68% ethanol and the resulting solution was left overnight at 0^oC. The white precipitate formed, mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0^oC. Further for complete removal of remaining lactose the supernatant was passed through a microfilter (0.24 µ) and lyophilized to get the crud oligosaccharide mixture (15gm). The lyophilized material responded positively to phenol-sulphuric acid test (Dubios et.al., 1956), and Morgon-Elson test (Patridge et al., 1948).

Sephadex G-25 gel filtration of crude Goat milk oligosaccharide

Lyophilized material (15gm) was further purified through sephadex G-25 chromatography using glass triple distilled water as eluant at a flow rate of 5ml/m. Presence of natural sugars was analyzed by Phenol-sulphuric acid test. Fractions were polled & lyophilized and used for further investigation.

FRACTION NO.	**SOLVENT**	**COMPOUND (in grams)**	**PHENOL-H_2SO_4 TEST FOR SUGAR**	**FURTHER INVESTIGATION**
1-60	Glass triple Distilled H_2O	0.83	-ve [I]	
61-171	”	3.54	**+ve** [II]	
172-275	”	4.70	**+++ve** [III]	(13.0 gm)
276-343	”	3.48	**+++ve** [IV]	Purified by
344-374	”	1.28	**++ve** [V]	Chromatograpy
375-389	”	0.52	-ve [VI]	
390-398	”	0.75	-ve [VII]	

Confirmation of homogeneity of goat's milk oligosaccharide by reverse phase HPLC-

Pooled fractions obtained from Sephadex G-25 column, containing oligosaccharide mixture (13.1 g) were qualitatively analyzed by reverse phase HPLC. The column used for this purpose was C18 Purosphere 25 cm × 0.4 cm × 5-µ m (from E. Merck). A binary gradient system of acetonitrile: 0.5% trifluro-acetic acid (5:95) in triple distilled water (TDW) to CH3CN: 0.5 % TFA (60:40) within 25 min at a flow rate of 1 ml/min was used. The eluants were detected at 220 nm. Twelve peaks were noticed in the sample (pooled fraction II and III) at the varied retention times from 1.092 min to 20.675 in, for convenience the peaks were numbered in their increasing order of retention time *i.e.* [.299(R_1), 1.323(R_2), 2.272(R_3), 3.093(R_4), 3.605(R_5), 3.968(R_6), 4.320(R_7), 5.067(R_8), 5.483(R_9), 5.728(R_{10}), 5.877(R_{11}) and 6.165(R_{12}).

Acetylation of Goat's milk oligosaccharide mixture

Dry oligosaccharides of pooled fractions were acetylated by treatment with pyridine (12ml) and acetic anhydride (11.5ml) at 60° C for 24 hr. In order to remove reagent,

reaction mixture was evaporated under reduced pressure and viscous residue was taken in $CHCl_3$ and washed in sequence with 2 N HCl, ice cold 2N $NaHCO_3$ and finally with H_2O. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acetylated mixture (15.5g). Non-polar acetyl derivative of oligosaccharides were resolved nicely on TLC using $CHCl_3$: MeOH as developing solvent. Detection of the spots were done by spraying with 30%H_2SO_4 in distil water and heating.

Purification of acetylated milk oligosaccharide on silica gel column

Purification of acetylated derivative was carried over silica gel column chromatography into compounds : silica ratio of 1:100 using various proportion of Hexane: $CHCl_3$, $CHCl_3$, $CHCl_3$:MeOH mixture which was resolved into eleven fractions namely I(56gm), II(650mg), III(1.414g), IV(216mg), V(2.959gm), VI(823mg), VII(701mg), VIII(932mg), IX(1.209g), X(811mg), XI(514mg) respectively. These fractions were containing mixture of two to three compounds. Repeated column chromatography of fraction II led to the isolation of one chromatographically pure compound A (112mg).

Deacetylation of compound

Deacetylation of acetylated oligosaccharide A (112mg) was carried out in 2ml acetone and 13ml NH_3 for 24hr in a stoppered hydrolysis flask. After 24 h ammonia was removed under reduced pressure, equal volume of $CHCl_3$ and water were added and the compound was recovered in the aqueous phase and the water layer was finally freeze dried giving the deacetylated oligosaccharide Gaurose (78 mg).

Description of isolated compound Gaurose

^{1}H NMR : δ in $CDCl_3$ (ppm)

δ6.350(d,1H), δ5.730(d,1H), δ5.387(m,2H), δ5.339(t,1H), δ5.335(d,1H), δ5.307(d,1H), δ5.204(d,1H), δ5.199(d,1H), δ5.013(d,1H), δ5.002(d,1H), δ4.507(d,2H), δ4.481(d,1H), δ4.204(m,1H), δ4.189(m,1H), δ4.090(t,1H), δ3.962(m,1H), δ3.950(d,1H), δ3.921(d,1H), δ3.901(t,1H), δ3.882(d,1H), δ3.650(d,1H), δ3.628(d,1H), δ3.581(t,1H), δ2.115(s,1H), δ2.093(s,1H), δ2.058(s,1H), δ2.001(s,1H), δ1.967(s,1H), δ1.947(s,1H), δ1.946(s,1H), δ1.244(d,1H), δ1.190 (d,1H).

^{13}C NMR : δ in $CDCl_3$ (ppm)

δ173.2, δ170.0, δ169.9, δ169.8, δ100.88, δ99.85, δ 91.95, δ89.51, δ71.49, δ70.82, δ70.64, δ70.39, δ68.73, δ68.65, δ66.26, δ65.53, δ61.16, δ60.89, δ60.56, δ20.91, δ20.82, δ20.8, δ 20.5, δ20.4.

^{1}H NMR : δ in D_2O (ppm)

δ5.338(d,1H), δ5.316(d,2H), δ4.575(d,1H), δ4.391(d,1H), δ4.364(d,1H), δ4.16(d,1H), δ4.14(d,1H), δ4.06(d,1H), δ4.03(d,1H), δ3.95(t,1H), δ3.92(d,1H), δ3.88(m,1H), δ3.75(m,1H), δ3.73(d,1H), δ3.70(d,1H), δ3.63(d,2H), δ3.60(m,1H), δ3.59(m,1H), δ3.51(d,1H), δ3.50(d,1H), δ3.24(t,1H), δ3.21(t,1H), δ1.22(d,3H), δ1.20(d,3H)

^{13}C NMR : δ in D_2O (ppm)

δ170.2, δ170.1, δ102.4, δ100.8, δ100.4, δ95.84, δ 88.54, δ81.4, δ79.9, δ79.7, δ76.4, δ75.76, δ75.06, δ 74.29, δ 72.34, δ 72.12, δ70.69, δ 69.88, δ 69.18, δ 68.60, δ60.89, δ56.84, δ 20.5, δ14.2

ES-MS

940 $[M+Na+K]^+$, 878 $[M]^+$ and other fragment ions at 860, 842, 775, 732, 722, 687, 677, 673, 622, 618, 617, 608, 607, 586, 583, 575, 566, 551, 547, 527, 518, 496, 462, 383, 361, 334, 304, 244, 231, 185, 180.

RESULTS AND DISCUSSION

Compound "Gaurose", $C_{34}H_{58}O_{24}N_2$ gave positive Phenol-sulphuric acid test, Fiegl test (Fiegl etal., 1975) and Morgon-Elson test indicating the presence of normal and amino sugars in the moiety. The ^1H NMR spectrum of Gaurose acetate at 300 MHz exhibited six doublets for six anomeric proton at δ6.35(1H), 5.73(1H), 5.20(1H), 5.19(1H), 4.50(1H) and 4.48(1H) indicating that the compound Gaurose may be pentasaccharides in its reducing form. It was further supported by the appearance of four signals for six anomeric carbons at δ 89.5(1C), 91.9(1C), 99.8 (1C) and 100.8(3C) in the ^{13}C NMR spectrum of Gaurose acetate. These data suggested that compound may be a pentasaccharide in its reducing form. The five-monosaccharide units present in compound Gaurose have been designated as S_1, S_2, S_3, S_4 and S_5 for convenience starting from the reducing end. The presence of anomeric signal at δ5.33 (J=3.6Hz) and 4.57 (J=8.1Hz) (Urashima et. al., 2002, 2003) $_{in}$ the 1H NMR of Gaurose for α and β glucose respectively showed that glucose unit was present at the reducing end. The reducing nature of glucose was further confirmed by the Methylglycosidation of Gaurose by MeOH /H^+ followed by its acid hydrolysis which led to the isolation of α and β methyl glucoside. Further monosaccharide constituents in compound Gaurose was confirmed by its acidic Kiliani hydrolysis (Killiani, 1930). In its hydrolysis four spots were detected on PC and TLC which were found identical with Glc, Fuc, GlcNac and GalNac by co-chromatography with authentic samples. The reducing and free nature of glucose was further supported by the presence of two anomeric proton signals as doublets for α and β Glc at 5.33 (1H, J=3.6Hz) and δ 4.57 (1H, J=8.1Hz) (Urashima etal.,2002,2004) respectively. Further the presence of another anomeric proton signal as doublet at δ 4.36 (J=7.8Hz), along with a singlet of three proton at δ 1.98 which was assigned as NHAc group. These signals were assigned to the presence of GalNAc (Urashima et.al., 1989) which may be present as second monosaccharide (S_2). The absence of downfield shifting of H-4 methine proton observed as triplet at δ3.59 (J= 7.2 Hz) in ^1H NMR spectrum of Gaurose acetate as compared to Gaurose is in conformity with the chemical shift of H-4 methine proton of Glc (S_1) indicating linkage between S_1 and S_2 as (1→4) that is GalNAc β(1→4) Glc. The linkage of GalNAc with Glc was confirmed by ^1H-^1H COSY and TOCSY spectrum of Gaurose in which H-4 of Glc appear at up field region δ3.59 and H-2, H-3, and H-5 of Glc appear at δ5.30, δ5.33 and δ4.18 respectively, these data confirms that C-2, C-3 and C-5 positions of Glc is not involved in glycosidic linkages with βGalNAc. Another anomeric proton signals which appeared as a doublet at δ4.391 (J=7.8 Hz) along with singlet of three protons at δ 2.03 was due to the presence of β-GlcNAc (S_3) moiety in compound. The H-3 proton of GalNAc (S_2) which appeared at δ3.88 implied that the GlcNAc (S_3) sugar will be [1→3] linked with β-GalNAc (S_2). This was further confirmed by ^1H-^1H COSY and TOCSY spectrum of Gaurose which showed that position of H-4 and H-5 protons of GalNAc (S_2) at δ5.002 and 5.013 respectively, the H-3 proton was present at δ3.88. The HSQC spectrum also confirms that the H-3 proton of S_2 gave cross peak at (δ3.88 x δ66.26) which indicated that GlcNAc (S_3) was linked to S_2 at C-3 position by glycosidic linkage. Another anomeric proton appeared as doublet at δ5.19 (J=3.6) which was due to presence of α fucose (S_4) which was further supported by a doublet of three proton of methyl of α fuc (S_4) δ 1.19 (J= Hz). The H-1 resonance α fuc (S_4) at δ5.19 (J=3.6 Hz) and C-6 at 14.90 was absolutely identical to that observed for α-Fucosyl group (1→3) linked (Nunez et.al 1980) GlcNAc in oligosaccharides bearing the X determinant which confirmed the (1→3) linkage between (S_3) and (S_4).

This compound has another anomeric proton doublet at δ5.20 (J=3.6 Hz) which was due to presence of α fucose (S_5) which was further supported by a doublet of three proton of methyl of α fucose (S_5) at δ 1.24 and C-6 at 14.90 and since in (S_4) and (S_5) there is not shifting of proton signal in upfield region this show that there is no carbon involved in glycosidation and both the fucose at terminal end attached to the GlcNAc (S_3) in Gaurose.

The pentasaccharide nature of Gaurose was further confirmed by spectral studies of acetylated derivative of Gaurose. The heteronuclear single quantum-coherence (HSQC) spectrum of acetylated product of compound Gaurose confirms the anomeric assignments in ^{1}H and ^{13}C NMR spectra of Gaurose by showing the ^{1}H and ^{13}C cross peaks of α-Glc (S_1) at δ6.35 x δ89.5 and β-Glc (S_1) at δ 5.7 x 91.9. It also contains four crosspeaks of one β-GalNAc (S_2) at δ 4.48 x δ 100.8, and one β-GlcNAc moieties (S_3) present at δ4.50 x δ99.8 and two α-Fuc moieties S_4 and S_5 present at δ 5.19x100.8 and 5.02x 100.8 respectively. The glycosidic linkage were assigned by the cross peaks for glycosidically linked carbon with their proton in HSQC spectrum of Gaurose acetate. The values of these cross peaks were as β-Glc(S_1) H-4 and C-4 at δ3.929 x δ71.49 shows (1→4) linkage between S_1 and S_2 , β- GalNAc (S_2) H-3 and C-3 at δ 3.883 x δ 66.26shows (1→3) linkage between S_2 and S_3 , β-GlcNAc (S_3) H-3 and C-3 at δ 3.906 x δ 66.65 and H-4 and C-4 at 3.596x65.53 shows (1→3)linkage and (1→4)linkage between S_3 and S_4, S_3 and S_5 respectively. ^{1}H-^{1}H COSY spectrum gave assignment of ring hydrogen involved in linkage at δ3.929 (4-position) for β-Glc(S_1) ,δ 3.883 (3- position) for β-GalNAc (S_2) , δ 3.596 (3- position) and 3.596(4- position) for β-GlcNAc (S_3) and it was further confirmed by the presence of same peaks in TOCSY spectrum. Based on the pattern of chemical shift of ^{1}H, ^{13}C, HOMOCOSY, TOCSY and HSQC NMR experiments, it was interpreted that the compound Gaurose was as pentasaccharide having one Glc, one GalNAc, one GlcNAc and two Fuc moieties and compound was interpreted as pentasaccharide having Fuc-α-(1-4)[Fuc-α-(1-3)]-GlcNAc-β-(1-3) GalNAc-β-(1-4) Glc structure. The ES-MS data of compound confirmed the derived structure as well as derived sequence of monosaccharide. The highest mass ion peak at m/z 940 which was due to $[M+Na+K]^{+}$ and m/z 878 which has due to $[M]^{+}$ confirming the molecular weight of Gaurose as m/z 878.

'GAUROSE'

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