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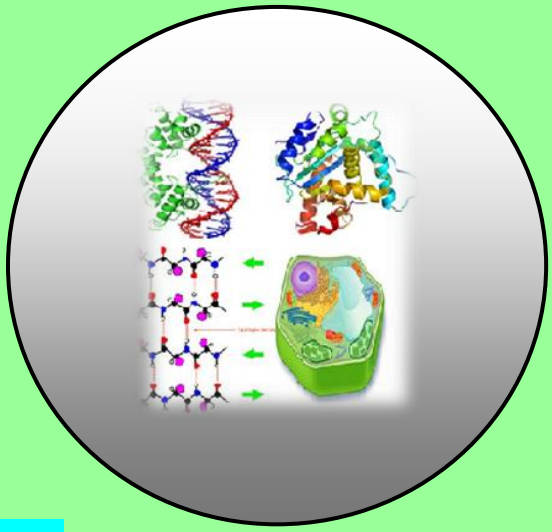

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Structure Elucidation of Novel Milk Oligosaccharide (Osiose) from Sheep Milk

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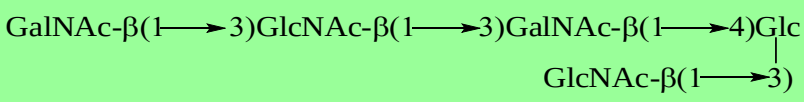
ABSTRACT

Milk is natural sources which provide components that give selective advantage to the growing neonate and execute a number of functions including serving as prebiotics to stimulate the growth of beneficial intestinal bacteria, as receptor analogs to inhibit binding of pathogens, and as substances that promote postnatal brain development. Evidence is also available to support the role of milk oligosaccharides as antitumor, anticancer, antigenic and immuno stimulant activities. Sheep milk which is a rich source of amino acid, calcium, fucose which is beneficial for skin diseases and cosmetic purposes, and aggravates hiccup, dyspnoea and also eliminates pitta, kapha and fat. In the present study, keeping in mind the biological and medicinal importance of milk, sheep milk was taken and was processed by method of Kobata and Ginsburg followed by different chromatographic techniques TLC, CC, HPLC etc. which have been used in the purification of milk oligosaccharides. The structures of purified milk oligosaccharides were elucidated by the using of the data generated from chemical degradation, chemical transformation, spectroscopic techniques like NMR (^{1}H, ^{13}C and 2D-NMR) and mass spectrometry. On the basis of results obtained from above experiments, the structure of the novel oligosaccharide was confirmed as-

Osiose



Keywords- Milk oligosaccharides, Sheep milk, HPLC, NMR and Osiose

INTRODUCTION

Milk contains a variety of components including proteins, endogenous peptides, lipids, carbohydrates and minerals, all of which fulfill the nutritional needs for growth and

development of any mammalian newborns (Gopal et al., 2000, Ashish et al., 2016). Colostrums and milk include oligosaccharides, glycoproteins, glycolipids and antibodies are also protecting infants by reducing the number of pathogen infections and promoting the development of the intestinal epithelium (Coppa et al., 2006 and Zivkavic et al., 2010). Many mammalian milk contains biologically active milk oligosaccharide which play a key role in various physiological, pathological and biological activities such as biological recognition, anticomplementary, anticoagulant, antiinflammatory, antiviral and immunological activities (Schwonzen et al., 1992, Abe et al., 1983 and Srivastava et al., 1989). Goat milk oligosaccharide has played an important role in intestinal protection and repair after a damage caused by DSS (Dextran Sodium Sulphate) induced colitis and their implication in human intestinal inflammation (Villoslada et al., 2006). The oligosaccharide found in Donkey milk have ability to stimulate non-specific and specific immunological resistance (Deepak et al., 1998) and Donkey milk oligosaccharide may be used for prevention of atherosclerosis (Tafaro et al., 2007). Human breast milk playa very important key role in gut colonization and modulation of the infants guts (Coppa et al., 2004). Further fucosylated human milk oligosaccharide and related glycoconjugates can used for several specific disease by inhibition of enteric pathogens such as stable toxin of Escherichia coli(in vitro and its toxin induced secretory diarrhea in vitro and in vivo), noroviruses and Campylobacter (Sudarmo et al.,2003 and Guillermo et al., 2003). Sheep milk aggravates hiccup and dyspnoea, and also eliminates pitta, kapha and fat. It is used against tuberculosis in folk medicine and also helps in the stimulation of platelets count in dengue.

In the present study, the structure of one novel sheep milk oligosaccharide (Osiose) was elucidated with the help of spectroscopic techniques(^{1}H, ^{13}C, HSQC, COSY and TOCSY)and other techniques like deacetylation, hydrolysis, chemical degradation and ESI-MS (mass spectrometry).

MATERIAL AND METHODS

General procedure

The ^{1}H and ^{13}C NMR spectra of oligosaccharides were recorded in D_2O and the spectra of acetylated oligosaccharides were recorded in $CDCl_3$ at 25 ^{0}C on a Bruker AM 300 FT NMR spectrometer. The electrospray mass spectra were recorded on a MICROMASS QUATTRO II triple quadruple mass spectrometer. The sample (dissolved in suitable solvents such as methanol/acetonitrile/water) was introduced into the ESI source through a syringe pump at the rate 5µl per min. The ESI capillary was set at 3.5 KV and the cone voltage was 40 V. The spectra were collected in 6s scans and the print outs are averaged spectra of 6-8 scans. The C, H and N analysis were recorded on elemental analyzer CARLO-ELBA 1108. The sugars were visualized on TLC with 30% aqueous H_2SO4 reagent and on paper chromatography sugars were visualized with acetyl acetone and p-dimethyl amino benzaldehyde reagents. The absorbent for TLC was silica gel G (SRL) and CC silica gel (SRL, 60-120 mesh). PC was performed on Whatman No.1 filter paper using solvent system ethyl acetate-pyridine (2:1) saturated with H_2O.

Sephadex G–25 (PHARMACIA) was used in gel permeation chromatography. Freeze drying of the compound was done with the help of CT 60e (HETO) lyophylizer and centrifuged by a cooling centrifuge Remi instruments C-23 JJRCI 763.

To check the homogeneity of the compounds reverse phase HPLC system was used equipped with Perkin Elmer 250 solvent delivering system, 235 diode array detector and G.P. 100 printer plotter. Authentic samples of glucosamine, galactosamine, galactose, glucose and fucose were purchased from Aldrich Chemicals.

Isolation of sheep milk oligosaccharides by Kobata and Ginsberg method

10 L milk was collected from a sheep and was stored at -20 ^{0}C until use. The milk was processed by the method of Kobata and Ginsberg (Kobata et al., 1969). It was centrifuged for 15 min at 5000 rpm at -4 ^{0}C. The solidified lipid layer was removed by filtration through glass wool column in cold atmospheric condition. Ethanol was added to the clear filtrate (supernatant) to a final concentration of 68% for precipitating out the lactose and proteins and the resulting solution was left overnight at 0 ^{0}C. The white precipitate of lactose and protein was formed and removed by centrifugation for 15 min at 5000 rpm at -4 ^{0}C and washed twice with 68% ethanol. Further for complete removal of remaining lactose the supernatant was passed through a micro filter (0.24 mm) and lyophilized to get the crude oligosaccharide mixture (12.0 gm). The lyophilized material responded positively to Morgan Elson test (Partrige et al., 1948) and thiobarbituric-acid assay (Bryent et al., 1953) suggesting the presence of N-acetyl sugars and in oligosaccharide mixture. This lyophilized material (mixture of oligosaccharide) was further purified by fractionating it on Sephadex G-25 chromatography using glass triple distilled water as eluant at a flow rate of 3 mL/m. Each fraction was analyzed by phenol sulphuric acid reagent (Dubois et al., 1956) for the presence of neutral sugar. After drying 8.0 g of crude oligosaccharide mixture were obtained.

Acetylation of oligosaccharide mixture

6.4 gm of pooled fractions (Sheep Milk) of Sephadex chromatography which gave positive phenol-sulphuric acid test were acetylated with pyridine 7 ml and acetic anhydride 7 ml respectively at 60 °C and the solution was stirred overnight. The mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ (350 ml) and it was washed in sequence with 2N-HCl (1 x 25 ml), ice cold 2N-$NaHCO_3$ (2 x 25 ml) and finally with H_2O (2 x 25 ml). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acetylated mixture 7.1 gm.

Deacetylation of compound 'B'

Compound 'B' Osiose (98 mg) was obtained from column chromatography of acetylated oligosaccharide mixture. Compound 'B'(50 mg) was dissolved in acetone (3 ml) and 3.5 ml of NH_3 was added in it and was left overnight in a stoppered hydrolysis flask. After 24 h ammonia was removed under reduced pressure and the compound was washed with (3 x 5 ml) $CHCl_3$ (to remove acetamide) and the water layer was finally freeze dried giving the deacetylated oligosaccharide compound 'B' (39 mg).

Methyl glycosidation/Acid hydrolysis of compound 'B'

Compound 'B' Osiose (8 mg) was ref1uxed with absolute MeOH (2 ml) at 70 ^{0}C for 18 h in the presence of cation exchange IR-l20 (H) resin. The reaction mixture was filtered while hot and filtrate was concentrated. In the solution of methylglycoside of A, 1, 4-dioxane (1 ml), and 0.1N H_2SO4 (1 ml) was added and the solution was warmed for 30 minutes at 50 ^{0}C. The hydrolysis was complete after 24 h. The hydrolysate was neutralized with freshly prepared $BaCO_3$ filtered and concentrated under reduced pressure to afford α-and β-methyl glucosides along with the Glc, GalNAc and GlcNAc. Their identification was confirmed by comparison with authentic samples (TLC, PC).

Kiliani hydrolysis of compound 'B'

Compound 'B' (5 mg) was dissolved in 2 ml Kiliani mixture (AcOH-H_2O-HCl, 7:11:2) and heated at 100 ^{0}C for 1 h followed by evaporation under reduced pressure. It was dissolved in 2 ml of H_2O and extracted twice with 3 ml $CHCl_3$. The aqueous residual solution was made neutral by addition of 1-2 drops of 2N NaOH and was evaporated under reduced pressure to afford glucose, GalNAc and GlcNAc on comparison with authentic samples of glucose, GalNAc and GlcNAc.

Description of Isolated Compound

Compound (Osiose)

^{1}H NMR: δ in D_2O (300 MHz)

5.73 [d, 1H,J=4 Hz, α-Glc(S-1) H-1], 5.22 [d,1H, J=3.9Hz, α-GalNAc (S-5)H-1], 4.66 [d,1H, J=8 Hz, β-Glc (S-1), H-1] , 4.52 [d,1H, J=8 Hz, β-GlcNAc (S-3), H-1], 4.45[d, 2H, , J=8 Hz β-GalNAc (S-2) β-GlcNAc (S-4)H-1], 3.55 [t,1H, J=8 Hz, β-Glc (S-1), H-2], 2.23[s, 3H, $NHCOCH_3$, β-GalNAc(S-2)], 2.01[s, 6H, $NHCOCH_3$, β-GlcNAc(S-3,S-4)], 1.91[s, 3H, $NHCOCH_3$, α-GalNAc(S-5)]

^{1}H NMR (Acetylated): δ in $CDCl_3$

6.26 [d, 1H, J=3.3 Hz, α-Glc (S-1), H-1], 5.68 [d,1H, J=8.1 Hz, β-Glc (S-1), H-1], 4.51[d,J=8Hz, β-GlcNAc (S-4), H-1], 4.50 [d, 1H, J=8 Hz, β-GalNAc (S-2), H-1], 4.48 [d, 2H, J=8.1 Hz, β-GlcNAc (S-3), H-1, β-GalNAc (S-5)],

^{13}C NMR (Acetylated): δ in $CDCl_3$

101.2 [3C, α-GalNAc (S-2, S-5), β-GlcNAc(S-3),C-1], 100.9 [1C,β-GlcNAc (S-4), C-1], 91.5 [β-Glc (S-1), C-1], 88.9 [1C, α-Glc (S-1), C-1]

ES Mass

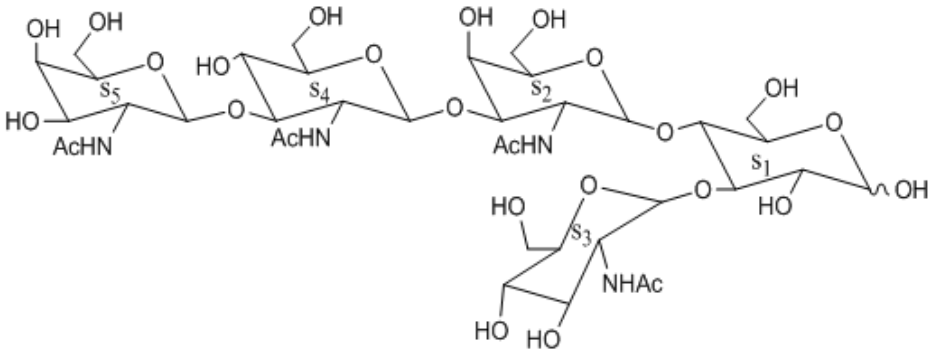
1054[M+Na+K], 1015[M+Na], 992[M]+,963, 946, 934, 929, 874, 826, 816, 789,729, 731, 714, 706, 696, 679, 677, 619, 568, 460, 457, 383, 331, 307, 162.

RESULT AND DISCUSSION

Compound 'B' Osiose $C_{38}H_{64}O_{26}N_4$ gave positive Phenol sulphuric acid test (Dubois et al., 1956), Fiegl test (Fiegl., 1975) and Morgan Elson test (Partridge et al., 1984), showing the presence of normal and amino sugars in the compound. The ^{1}H NMR of Osiose in D_2O at 300 MHz showed five anomeric signals for six anomeric protons at δ5.73(1H), δ5.22(1H), δ4.66(1H), δ4.52(1H) and δ4.45(2H) suggesting it to be a pentasaccharide in its reducing form. The pentasaccharide nature of Osiose was further confirmed by the presence of 4 cross peaks at δ4.02x71.0, δ3.87x71.0, δ3.793x73.80, δ3.827x76.00 in the HSQC spectrum of Osiose acetate at 300MHz. The pentasacchride nature of Osiose was also confirmed by the ^{1}H NMR of Osiose acetate in $CDCl_3$ at 300MHz containing signals for α and β Glucose at δ6.26 (J=3Hz) and δ5.68 (J=8Hz) respectively in the anomeric region . The reducing nature of Osiose was also confirmed by the presence of α and β signals of anomers of glucose in ^{1}H NMR of Osiose and Osiose acetate. The methylglycosydation of Osiose followed by its acid hydrolysis gave α and β methyl glucoside along with GlcNAc and GalNAc, suggesting the glucose was present at the reducing end of the oligosaccharide.

The five monosaccharides present in osiose have been designated S-1, S-2, S-3, S-4 and S-5 for convenience, starting from reducing end. To confirm the monosaccharide constituent in it, it was hydrolysed under strong acidic condition byKilliani Hydrolysis (Killiani.,1930), which gave three monosaccharide units i.e. Glucose, GlcNHAc and GalNHAc respectively, confirming the pentasaccharide was consisting of three type of monosaccharide units. The 1H NMR of Osiose also contains two singlet of three protons each and one singlet of six protons at δ1.91(3H), δ2.01(6H) and δ2.23(3H) confirming the presence of 4 N-Acetyl group in the moiety, suggesting the presence of 4-N-Acetylated monosaccharide in it. Further the 1H NMR of Osiose in D_2O at 300 MHz contain two anomeric protons signal at δ5.73(J=4Hz) and δ4.66(J=8Hz) confirming the presence of glucose (Fournet et al., 1978, Kitagawa et al., 1991) at reducing end. The presence of glucose at the reducing end was also confirmed by the 1H NMR of acetylated Osiose in $CDCl_3$ at 300MHz which contain two anomeric proton signal at δ6.26(J=3Hz) and δ5.68(J=8Hz) respectively confirming the presence of glucose at the reducing end in the pentasaccharide Osiose. It was also confirmed by the ^{13}C NMR of Osiose, which showed the signals of α and β anomeric carbon at δ88.9 and δ91.5 respectively. Further presence of another anomeric proton doublet at δ4.45(2H)(J=7.5Hz) along with triplet at δ3.55 for βGlc (S-1) suggested the presence of Lactose type of structure, the down field shifted region of triplet at δ3.55 indicated that the both equatorially oriented hydroxyl groups at C-3 and C-4 of reducing β-Glc were substituted and were involved in the glycosidation, suggested the presence of Lactose type of structure (Uemura., 2006) with substitution at position 3 and 4 of reducing glucose into the Osiose. It was further confirmed by the TOCSY spectrum of Osiose Acetate which contain two consecutive complimentary signals at δ3.8 and δ4.1 for anomeric signal of Glc (S-1) showing that two OH groups of reducing glucose were available for glycosidic linkage which were later ascertained as H-3 and H-4 of the β-Glc by the COSY spectrum of Osiose Acetate. Since the 1HNMR of Osiose in D_2O contains signals for four methyl groups for N-Acetyl sugars, it was suggested that in the pentasaccharide Osiose all the monosaccharide besides the reducing glucose S-1 were N-Acetylated sugars i.e. GlcNac and GalNac. This suggested that reducing end of pentasaccharide contains a Lactose type of structure in which instead of Gal, GalNac (S-2) was present. It was supported by a multiplet in the region δ3.8 to δ3.9 for H-2 of β-GalNac, confirming the presence of a lactose type of structure in which GalNac was 1→4 glycosidically linked to Glc with a substitution at C-3 of reducing Glc. The splitting pattern of anomeric proton signal at δ4.45(J=7.5Hz) confirm the β-glycosidic linkage between GalNac (S-2) and β-Glc (S-1). Since the anomeric proton signal at δ4.45(2H) represents two protons which is a SRG for LNT type of structure. It was proposed that this pentasaccharide contained a LNT type of structure with substitution at C-3 of β-Glc (S-1) and monosaccharide besides the reducing Glc all monosaccharides were N-Acetylated monosaccharides. Further another anomeric proton doublet at δ 4.52 (J=7.8Hz) along with signal of N-Acetyl group at δ2.01 suggested the presence of N-acetylglucose. As it was confirmed that the reducing Glc has two OH groups having Glycosidic linkage and H-4 was already occupied by the GalNHAc the left over H-3 of reducing Glc must be linked at H-3 of S-1by GlcNAc. The splitting pattern of anomeric proton signal at δ4.522 (J=7.8Hz) clearly indicates that glycosidic linkage between S-3 and S-1 was β(1→3) linkage in which GlcNAc (S-3) was linked to Glc (S-1) by β(1→3) linkage.

The next anomeric proton at δ4.45 (2H) along with the signal of N-Acetyl at δ2.01 was interpreted for the presence of another GlcNAc in the pentasaccharide Osiose. As the SRG value of anomeric proton at δ4.45 (2H) suggested the presence of LNT structure in the pentasaccharide confirmed the presence of GlcNAc as the next sugar in the series with a (1→3) Glycosidic linkage between S-4 and S-2. Again the large coupling constant of anomeric proton at δ4.45 (J=7.5Hz) could be interpreted for β-configuration of glycosidic linkage between GlcNAc (S-4) and GalNAc (S-2). Further the next anomeric proton present at δ5.22 along with a signal of N-Acetyl group at δ1.91 may be assigned for the presence of another GalNAc (S-5) unit in the pentasaccharide Osiose. The SRG value of α-GalNAc at δ5.22 along with the H-4 doublet of GalNAc (S-5) supports the presence of GalNAc as the last unit of the pentasaccharide Osiose. The small coupling constant of J=3.9Hz confirms the α-Glycosidic linkage between S-5 and S-4. In the light of fore given evidencies the structure of Osiose was confirmed as under-



OSIOSE

The Electronspray Mass Spectrometry data of Osiose not only confirmed the derived structure but also supported the sequence of monosaccharide in Osiose. The highest mass ion peaks were recorded at m/z 1054 and 1015 which were due to [M+Na+K] and [M+Na] respectively. It also contains the molecular ion peak at m/z 992 confirming the molecular weight of Osiose as 992 and was in agreement with its molecular formula.

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