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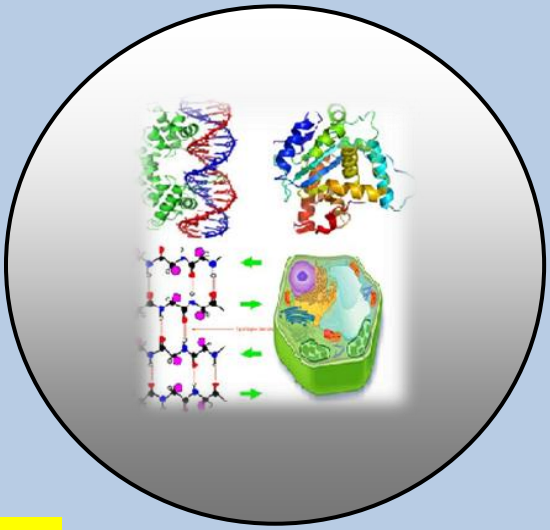

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RESEARCH PAPER
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Novel Heptasaccharide isolated from Sheep Milk

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ABSTRACT

Milk is a natural food for neonates of any mammal which is also responsible for development and immunological support. It contains proteins, fat and carbohydrates including oligosaccharides which have varied biological activities viz Antioxidant, Anti cancer, lipid lowering and post heparin lipolytic activities. Since sheep milk oligosaccharides also contains fucose which is beneficial for skin diseases and cosmetic purposes. With a view to isolate biologically active novel oligosaccharides, Sheep's milk was taken and processed by modified method of Kobata and Ginsburg leading to isolation of oligosaccharide mixture which on gel filtration, HPLC and column chromatography delivered novel oligosaccharide. With help of NMR, ES Mass spectroscopy and given literature the structure of novel oligosaccharide was deduced as-

Oviose

βFuc(1-4)-βGal(1-3)-βGlcNAc(1-3)-βGal(1-4)Glc
| |
αFuc(1-3) βGlcNac(1-3)

Keywords- Milk oligosaccharides, Sheep milk, HPLC and Oviose.

INTRODUCTION

Free oligosaccharides are natural constituents of all bacteria, fungi, plants and placental mammals' milk. The milk is a rich source of bioactive oligosaccharides which contain number of novel oligosaccharides depending on the nature of their origin to which mammals they belongs (Miller et al., 1994). Many oligosaccharides exhibit potent biological activities such as anti-tumor, immunological, anti-complimentary, anti-cancer, anti-inflammatory, hypoglycemic and antiviral activities (Chihara et al., 1969 and Liu et al., 2007). Numerous oligosaccharides have been isolated in milk of many mammalian species including equine bovine and marine mammals (Kunz et al., 2000, Urashima et al., 2001,

Nakamura et al., 2004 and Urashima et al., 2008). A survey of the literature showed that some common basic core units like lacto-N-tetraose (LNT) and lacto-N-neotetraose (LNnT) (Chaturvedi et al., 1988), lacto-N-fucopentaose-1 (LNF-1), lacto-N-fucopentaose-2 (LNF-1I) and lacto-N-fucopentaose-3 (LNF111) (Dua et al., 1983 and Reddy et al., 1991) were found in most of the milk oligosaccharides. The earlier workers have elucidated the structure of milk oligosaccharides by chemical degradation and spectroscopic techniques (NMR and FABMS) (Reddy et al., 1991 and Dorland et al., 1977). Keeping above mentioned basic core units in mind the structure of milk oligosaccharide was established by comparing the chemical shift (^{1}H and ^{13}C-NMR) of anomeric signals and other important signals of isolated milk oligosaccharide with the chemical shifts of known milk oligosaccharides. The oligosaccharides structure was further confirmed by 2D NMR experiments (HSQC, COSY and TOCSY). Other techniques like deacetylation, methylation, hydrolysis, chemical degradation and FAB mass spectrometry were also helpful for elucidation of the structure.

MATERIAL AND METHODS

General procedure

The optical rotations of oligosaccharides were measured with AA-5 series automatic apolarimeter in 1cm tube. 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 ^{o}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_2SO_4 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.

Acetylation of oligosaccharide mixture

6.4 gm of pooled fractions 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 'a'

Compound 'a' Oviose (88 mg) was obtained from column chromatography of acetylated oligosaccharide mixture. 52 mg of compound 'a' 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 A (46 mg).

Methyl glycosidation/Acid hydrolysis of compound A

Compound 'A' Oviose (8 mg) was ref1uxed with absolute MeOH (2 ml) at 70 °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_2SO_4 (1 ml) was added and the solution was warmed for 30 minutes at 50 ^oC. 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-methylglucosides along with the Glc, GalNAc, GlcNAc and Fuc. Their identification was confirmed by comparison with authentic samples (TLC, PC).

Kiliani hydrolysis of compound A

Compound A (5 mg) was dissolved in 2 ml Kiliani mixture (AcOH-H_2O-HCl, 7:11:2) and heated at 100 ^oC 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, GlcNAc and Fuc on comparison with authentic samples of glucose, GalNAc, GlcNAc and Fuc.

Description of isolated substance

Compound (Oviose)

^{1}H NMR: δ in D_2O

5.71 [d, 1H, J=3.0 Hz, α -Glc (S-1) H-1] , 4.81 [d, 1H, J=8Hz, β-Glc(S-1) H-1], 4.79[s,2H J=3.0Hz, α-Fuc (S-5,S-6)H-1], 4.68[d,2H(J=8Hz)β-GlcNAc (S-3,S-7) H-1] , 4.41 [d, 2H, J=8.1Hz, β-Gal (S-2,S-4) H-1] , 3.54[t, 1H ,J=8.0Hz, β-Glc(S-1) H-2], 2.003[s,3H, $NHCOCH_3$,β-GlcNAc(S-3)], 1.91[s,3H, $NHCOCH_3$, β-GlcNAc(S-7)]

^{13}C NMR: δ in $CDCl_3$

100.9 [4C,α-Fuc(S-5, S-6), β-Gal(S-2, S-4)C-1], 91.9 [2C, β-GlcNAc (S-3, S-7), C-1], 91.5 [1-C, β-Glc (S-1), C-1], 89.5 [1C, α-Glc (S-1), C-1]

^{1}H NMR (Acetylated): δ in $CDCl_3$

6.30 [d, 1H, J=3.0Hz, α-Glc (S-1), H-1], 5.71 [d, 1H, J=8.0Hz, β-Glc (S-1), H-1], 5.357 [d, 2H, J=8Hz, β-GlcNAc (S-3, S-7) H-1], 4.97 [d, 2H, J=3.0Hz, α-Fuc (S-5,S-6), H-1], 4.45 [d, 2H, J=8.4 Hz, β-Gal (S-2, S-4), H-1]

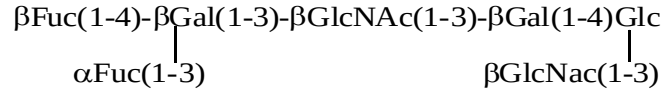
ES Mass

1264[M+Na+K],1241[M+K], 1202 [M]+, 1184,1173,1142,1111, 1105,1075, 1057, 1053, 1038, 1001,996, 979, 960, 938, 923, 910, 871, 813, 789, 748, 720, 670, 545, 506, 460, 429, 391, 342, 307, 289, 209, 180, 162 .

RESULT AND DISCUSSION

Compound Oviose $C_{46}H_{78}O_{34}N_2$ 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. ^{1}H NMR of Oviose in D_2O at 300 MHz showed eight anomeric proton signal at δ 5.7(1H), 4.81(1H), 4.79(2H), 4.68(2H) 4.41(2H) showing it to be a heptasaccharide in its reducing form. The heptasaccharide nature of Oviose was further confirmed by presence of eight anomeric proton signals in the ^{1}H NMR of Oviose acetate at 300 MHz in $CDCl_3$ at 6.30(1H), 5.71(1H), 5.35(2H), 4.97(2H) 4.45(2H) Further ^{13}C NMR of acetylated oviose also supported the heptasaccharide nature of oviose which contains anomeric carbon signals at 89.5(1C), 91.5(1C), 91.9(2C), 100.9(4C). Presence of α and β anomeric protons and carbons in ^{1}H NMR of Oviose and Oviose acetate respectively suggested the presence of Glc at the reducing end. The methylglycosidation of Oviose by $MeOH/H^+$ followed by acid hydrolysis led to the isolation of α and β methylglycosides confirming the presence of Glc at the reducing end. Seven monosaccharide present in Oviose have been designated as S_1, S_2, S_3, S_4, S_5, S_6 and S_7. For convenience starting from the reducing end, confirm the monosaccharide constituents in it. It was hydrolysed under strong acidic condition in Killiani Hydrolysis (Killiani.1930) which gave five monosaccharides units i.e. Glc, Gal, Nacetylglucosamine, Nacetylgalactosamine and Fuc respectively confirming that the heptasaccharide consist of five types of monosaccharide units i.e. Glc, Gal, GlcNHAc, GalNHAc, and Fucose. Further the ^{1}H NMR of oviose in D_2O at 300 MHz contains two anomeric proton signal at δ5.7(J=3Hz), 4.81(J=8Hz), confirm the presence of glucose at the reducing end. Further presence of another anomeric proton doublet of δ4.41 (2H) J=8.1Hz suggested the presence of a βGal Moiety in the heptasaccharide. The β Glc. (S_1) H-2 signal as a triplet at δ3.54 at down field region indicated that both are equatorially oriented hydroxyl group at C-3 and C-4 of the reducing β Glc (S_1) were substituted and were involved in the glycosydation suggested the presence of lactosyl moiety i.e. β Gal (1-4) linkage with the substitution at 3 position of Glc (S_1) (Fournet et al. 1978, Kitagawa et al. 1991). The coupling constant of anomeric signal at δ4.41 (J=8.1Hz) showed the β configuration of anomeric linkage between S-2 and S-1. It was also supported by the presence of β Glc H-4 proton resonace at δ3.6 in ^{1}H NMR of acetylated oviose at 300MHz in $CDCl_3$. Further the signal of H-3 of Glc(S-1) at δ3.9, suggested that the H-3 position of Glc (S-1) was also substituted by another monosaccharide unit. The position of H-3 and H-4 proton of β Glc S-1 at 3.6 and 3.9 were confirmed by COSY spectra of acetylated oviose with reference to anomeric proton derived of β Glc (S1) at δ 5.7. Further the presence of anomeric protons doublet of Gal (S-2), 2H, δ4.41, J=8.1Hz in the ^{1}H NMR of oviose in D_2O suggested that the heptasaccharide oviose may contain a LNT (SRG) value [Gal(β1-3) GlcNAc(β1-3) Gal(β1-4) Glc]. Further another anomeric proton doublet at δ4.68, J=8Hz along with a signal of N-Acetyl group δ2.003 suggested the presence of N-Acetyl Glc as another monomer, Which may be linked at H-3 of Glc(S-1) Which was supported by the structure reporter group value of anomeric proton signal of β-GlcNAc at δ4.68 for 1→3 linkage which was in conformity with the H-3 signal of β-Glc S-1 at δ3.9. Further another anomeric proton signal of βGlc(S-1) at δ5.71 along with a signal of N-Acetyl group at δ1.91 suggested the presence of another GlcNAc moiety in the heptasaccharide leading to the confirmation of LNT structure in the molecule. The J value of anomeric signal of 8 Hz confirmed the β-glycosidic linkage between GlcNAc(S-3) and β-Gal(S-2).

It was supported by the evidences i.e. presence of a doublet at δ4.41(2H) for 2-Gal moiety i.e. S-2 and S-4 in the ^{1}H NMR of Oviose in D_2O along with signal of H-3 of S-2 at δ4.1 in the ^{1}H NMR of Oviose acetate. The Presence of fourth anomeric proton signal which was due to presence of Gal (S-4) was substantiated by the anomeric proton doublet present at δ4.41(2H) containing signal for Gal(S-2) and Gal(S-4), which is also a SRG for the presence of LNT structure in the heptasaccharide. Further the anomeric proton value of Gal (S-2) and (S-4) arise at δ4.45 in the ^{1}H NMR of Oviose acetate. This anomeric proton showed two consecutive complementary signals at δ4.1 and δ3.9 for H-3 and H-4 protons of Gal(S-4) showing that H-3 and H-4 of Gal S-4 was substituted by next monosaccharide units in the heptasaccharide. Further the next anomeric ^{1}H signal which arise at δ4.79 (2H) for two protons along with two doublets at δ1.22 and δ1.252 for two secondary methyl group in the ^{1}H NMR of Oviose in D_2O confirm the presence of two fucose moiety (S-5 and S-6) in the heptasaccharide. The presence of anomeric protons of fucose units was further confirmed by the anomeric proton doublet at δ4.97 in the ^{1}H NMR of Oviose acetate. Since the proton H-3 and H-4 of Gal(S-4) were present at δ4.1 and δ3.9 in the ^{1}H NMR of Oviose acetate compound. These protons (H-3, H-4) were available for glycosidic linkages and were replaced by Fucose units. The small coupling constant of anomeric protons of Fucose δ4.972 J=3Hz showed the α-glycosidic linkage at H-3 and H-4 of Gal(S-4). In the light of foreseeing evidences the structure of Oviose was confirmed as under-



The Electronspray Mass Spectrometry data of Oviose not only confirmed the derived structure but also supported the sequence of monosaccharide in Oviose. The highest mass ion peaks were recorded at m/z 1266 and 1243 which were due to [M+Na+K] and [M+K] respectively. It also contains the molecular ion peak at m/z 1202 confirming the molecular weight of Oviose as 1202 and was in agreement with its molecular formula.

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