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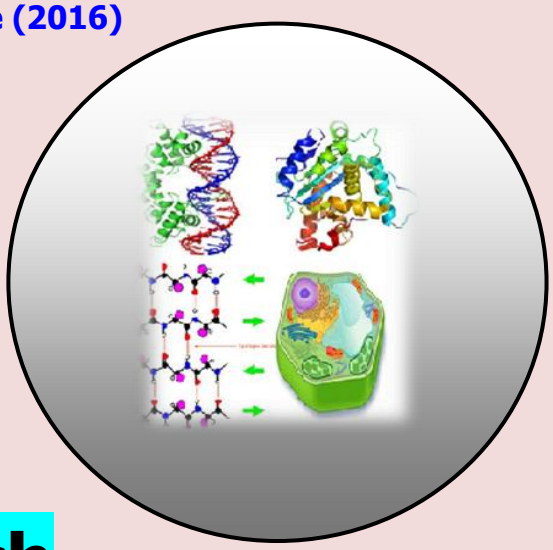

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RESEARCH PAPER

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Isolation and NMR Characterization of Riesose- A Novel Oligosaccharide from Gaddi Sheep's Milk

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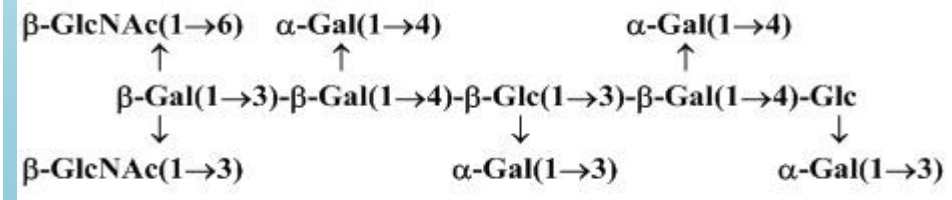
ABSTRACT

Oligosaccharides are natural constituents of all bacteria, fungi, plants, placental mammal and bovine milk that stimulate the growth of beneficial microorganisms in the infant colon, act as receptor analogs that inhibit the attachment of pathogenic microorganisms to colonic mucosa and small amounts are absorbed into the circulation where they modulate immunoreactivity. Oligosaccharides isolated from sheep milk strongly stimulate the immune system, and are used for treatment of immune system related disorders. Sheep milk elevates pitta and kapha, aggravates hiccup and dyspnoea and also reduces the fat. In continuation to our previous studies and keeping above mentioned biological activities of Gaddisheep's (Gaddi is a breed of sheep found at higher altitudeof Himalayan region) milk oligosaccharides in mind we have isolated a novel oligosaccharide namely Riesose from sheep's milk and elucidated its structure by chemical degradation and spectroscopic techniques (like ^{1}H NMR, ^{13}C NMR, COSY, TOCSY, HSQC and Mass). The structure of Riesose was established by comparing the chemical shift (^{1}H NMR and ^{13}C NMR) of anomeric signals and other important signals of isolated milk oligosaccharide with the chemical shifts of known milk oligosaccharides and 2D-NMR and mass of Riesose and structure was deduced as follows-



Keywords: Sheep milk, Oligosaccharides and Riesose.

INTRODUCTION

Milk is secreted by all classes of mammals to provide nutritional and immunological protection to their neonates (Singh et al. 2017). The components present in milk are known to modulate the developing microbiota within the infant gastrointestinal tract (GIT), including immunoglobulins, lactoferin, lysozyme, bioactive lipids and various glycans (free oligosaccharides, glycolipids and glycoproteins) (Newburg et al. 2004). In these functional ingredients, oligosaccharides are arguably the most important because they act as prebiotics and show biological activities likeanti-tumor, immunological, anti-complimentary (Saksenaet al. 1999), anti-cancer, anti-inflammatory, anti-coagulant, hypoglycemic and antipathogenic activities (Srivastava et al. 2016).Milk oligosaccharides are non-digestible due to the presence of β-glycosidic linkage. So this β-glycosidic linkage plays an important role for its prebiotic activity (Kim et al. 2005 and Ben et al.

2004).Prebiotics are selectively fermented ingredient that allows specific changes in both the composition and activity in the gastrointestinal microflora that confers benefits upon host well-being and health. Galactose and sialic acid present in milk oligosaccharide are required for optimal development of the infant's brain (Urashima et al., 2001 and Sharan et al., 2000).Sheep's milk has the natural ability to moisturize and nourish the skin and is safe on even most delicate skin. According to 'Ayurveda and Unani' system of medicine, the sheep's milk has various medicinal importance, aggravates hiccup and dyspnoea, elevates pitta and kapha and decreases fat (Shahu et al. 2017). It is used against tuberculosis in folk medicine and also helps in the enhancement of platelets count during dengue (Shahi et al. 2017). Keeping these activities in mind Gaddi sheep's milk was collected and processed by Modified method of Kobata and Ginsburg and then it was purified by Sephadex G-25 Gel column. Further the acetylation of oligosaccharides mixture followed by the silica gel chromatography led to isolation of a novel oligosaccharide Riesose which gave positive chemical test for normal and amino sugars. Comparison between ^{1}H and ^{13}C signals of natural and acetylated Riesose and data generated from 2D-NMR studies involving COSY, TOCSY andHSQC techniques along with mass spectrometry dataconfirmed the position of linkage in oligosaccharides.

Experimental

General procedures

Same as described in our previous articles (Singh et al. 2017).

Isolation of sheep milk oligosaccharide

Sheep milk (10 L) was collected from a domestic sheep and was stored at -20^{0}C. The milk was processed by the method of Kobata and Ginsburg. 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. Ethanol was added to the clear filtrate to a final concentration of 68% and the resulting solution was left overnight at 0^{0}C. The white precipitate formed, mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0^{0}C. The supernatant and washings were combined and filtered through a micro filter (0.24 mm) (to remove remaining lactose) and lyophilized affording crude oligosaccharide mixture (162 g). This lyophilized material (mixture of oligosaccharide) was further purified by fractionating it on Sephadex G-25 chromatography using glass triple distilled water as eluent at a flow rate of 5 mi/mm. Each fraction was analysed by phenol sulphuric acid reagent for the presence of neutral sugar.

Sheep milk oligosaccharide mixture chromatographed over Sephadex G-25 (1.6 × 40 cm) column

The repeated gel filtration was performed by Sephadex G-25 chromatography of crude sheep milk oligosaccharide mixture. The oligosaccharide mixture (21.5 g) was packed in a column (1.6 × 40 cm) (void volume = 25 mL) equilibrated with glass triple distilled water (TDW) and left for 10-12 h to settle down (17.2 g). The material was applied onto a Sephadex G-25 column and was eluted for separation of protein and glycoprotein from oligosaccharide mixture (low molecular weight component). Presence of neutral sugars was monitored in all eluted fractions by phenol-sulphuric acid test. The sephadex G-25 chromatography of sheep milk oligosaccharide mixture which was monitored by UV spectrophotometry showed five peaks i.e. I, II, III, IV and V. A substantial amount of proteins, glycoproteins and serum albumin were eluted in the void volume which was confirmed by positive colouration with p-dimethylaminobenzaldehyde reagent and phenol-sulphuric acid reagent. Fractions under peaks II, III and IV gave a positive phenol-sulphuric acid test which showed the presence of oligosaccharide mixture in sheep milk. They were pooled together and lyophilized.

Acetylation of oligosaccharide mixture

The pooled fraction (II, III and IV) (11.3 g) which gave positive phenolsulphuric acid test was acetylated with pyridine (11mL) and acetic anhydride (11 mL) at 60^{0}C and the solution was stirred overnight. The mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ (250 mL) and washed in sequence with 2N HCl (1 × 25 mL), ice cold 2N $NaHCO_3$ (2 × 25 mL) and finally with H_2O (2 × 25 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acetylated mixture (12.1 g). The acetylation converted the oligosaccharides into their nonpolar acetyl derivatives which were resolved very nicely on TLC.

Purification of acetylated milk oligosaccharide on silica gel column

Separation of the acetylated products (12.1 g) was carried over silica gel using varying proportions of C_6H_{12}:$CHCl_3$, $CHCl_3$ and $CHCl_3$:CH_3OH mixture which was resolved into eleven fractions. Repeated column chromatography of fraction II led to the isolation of one chromatographically pure compound Riesose (136 mg).

Deacetylation of compound

The Compound Riesose (28 mg) obtained from column chromatography of acetylated oligosaccharide mixture was dissolved in acetone (2 mL) and NH_3 (2 mL) was added and left overnight in a stoppered hydrolysis flask. Ammonia was removed under reduced pressure and the product was washed with $CHCl_3$ (3 × 3 mL) (to remove acetamide) and was finally freeze dried giving the deacetylated oligosaccharide (21 mg).

Methylglycosidation/Acid hydrolysis of compound Riesose

The compound Riesose (10 mg) was ref1uxed with absolute MeOH (2 ml) at 70°C for 20 h in the presence of cation exchange !R-l40 (H) resin. The reaction mixture was filtered while hot and filtrate was concentrated. To a solution of methylglycoside of Riesose in 1, 4-dioxane (1 ml), 0.1 N H_2S0_4 (1 ml) was added and the solution was warmed for 45 minutes at 50^0C. The hydrolysis was complete after 26 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, Gal and GlcNAc. Their identification was confirmed by comparison with authentic samples (TLC, PC).

Kiliani hydrolysis of compound Riesose

The compound Riesose (5 mg) was dissolved in 2 ml Kiliani mixture (AcOH-H_2O-HCl, 9:13:3) and heated at 100^0C 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 to it and was evaporated under reduced pressure to afford glucose, galactose and GlcNAc on comparison with authentic samples of glucose, galactose and GlcNAc on PC.

Description of isolated compound Riesose

^{1}H NMR of Riesose in D_2O

δ5.271 [d, 1H, J=3.9Hz, αGlc (S_1) & αGal (S_9), H-1], δ5.235 [d, 1H, J=3.6Hz, αGal (S_{11}), H-1], δ5.185 [d, 1H, J=3.8Hz, αGal (S_8), H-1], δ5.155 [d, 1H, J=3.8Hz, αGal (S_{10}), H-1], δ4.725[d, 1H, J=7.8Hz, βGlc (S_1), H-1], δ4.710 [d, 1H, J=7.6Hz, βGlcNAc (S_7), H-1], δ4.695 [d, 1H, J=7.8Hz, βGlc (S_3), H-1], δ4.610 [d, 1H, J=7.6Hz, βGlcNAc (S_6), H-1], and δ4.501 [d, 1H, J=8.4Hz, βGal (S_2) &βGal (S_5), H-1], δ4.100 [t, 1H, J=6.1, βGlcNAc (S_6), H-2], δ3.980 [t, 1H, J=5.8, βGlcNAc (S_7), H-2], δ3.405 [t, 2H, J=5.9, βGlc (S_1) &βGlc (S_3), H-2], δ1.960 [s, 3H, βGlcNAc (S_6), NHCO$\underline{CH_3}$] and δ1.848 [s, 3H, βGlcNAc (S_7), NHCO$\underline{CH_3}$].

^{13}C NMRof Riesose in D_2O

δ166.68 [βGlcNAc (S_6) NH$\underline{CO}CH_3$], δ165.98 [βGlcNAc (S_7) NH$\underline{CO}CH_3$], δ103.40[βGal (S_5), C-1], δ101.50 [βGal (S_2), C-1], δ99.70[βGal (S_4) & βGlcNAc (S_6), C-1], δ97.80 [βGlcNAc (S_7), C-1], δ94.20 [βGlc (S_3), C-1], δ92.00 [αGal (S_9), C-1], δ89.50 [αGal (S_{11}), C-1], δ89.00 [αGal (S_{10}), C-1], δ88.50 [αGal (S_8), C-1], δ88.12 [βGlc(S_1'), C-1] and δ86.00 [αGlc (S_1), C-1], δ20.89 [βGlcNAc (S_6), NHCO$\underline{CH_3}$] and δ20.19 [βGlcNAc (S_7), NHCO$\underline{CH_3}$].

^{1}HNMR of Acetylated Riesose in $CDCl_3$

δ6.185 [d, 1H, J=3.9Hz, αGlc (S_1), H-1], δ5.655 [d, 1H, J=7.8Hz, βGlc (S_1), H-1], δ5.351 [d, 1H, J=3.8, αGal (S_8) & αGal (S_{10}), H-1], δ5.328 [d, 1H, J=3.8Hz, αGal (S_9), H-1], δ5.273[d, 1H, J=3.6Hz, αGal (S_{11}), H-1], δ4.750 [d, 1H, J=7.8Hz, βGlc (S_3), H-1], δ4.725 [d, 1H, J=7.6Hz, βGlcNAc (S_7), H-1], δ4.623 [d, 1H, J=7.6Hz, βGlcNAc (S_6), H-1], δ4.555 [d, 1H, J=8.4Hz, βGal (S_5), H-1] and δ4.504 [d, 1H, J=8.4Hz, βGal (S_2), H-1], δ2.034 [s, 3H, βGlcNAc (S_6), NHCO$\underline{CH_3}$] and δ1.985 [s, 3H, βGlcNAc (S_7), NHCO$\underline{CH_3}$].

^{13}C NMR AcetylatedRiesose in $CDCl_3$

δ166.80 [βGlcNAc (S_6) NH$\underline{CO}CH_3$], δ166.00 [βGlcNAc (S_7) NH$\underline{CO}CH_3$], δ104.50 [βGal (S_5), C-1], δ104.04 [βGal (S_2) & βGal (S_4), C-1], δ103.50 [βGlcNAc (S_6), C-1], δ98.70 [βGlcNAc (S_7), C-1], δ95.00 [βGlc (S_3), C-1], δ92.20 [αGal (S_9), C-1], δ90.10 [αGal (S_{11}), C-1], δ89.90 [αGal (S_{10}), C-1], δ88.70 [αGal (S_8), C-1], δ88.20 [βGlc(S_1'), C-1] and δ86.20 [αGlc (S_1), C-1], δ21.00 [βGlcNAc (S_6), NHCO$\underline{CH_3}$] and δ20.99 [βGlcNAc (S_7), NHCO$\underline{CH_3}$]

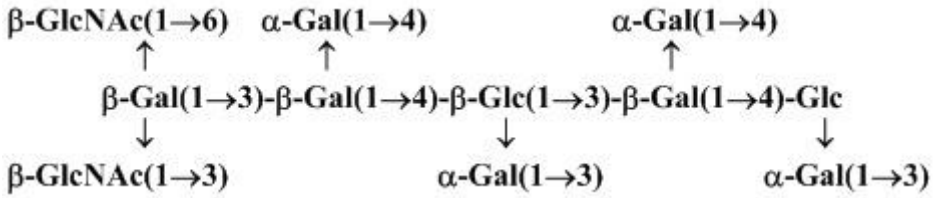
ES-Mass spectral fragments of compound Riesose

m/z 1944 $[M+Na+K]^+$, m/z 1906$[M+Na+H]^+$, m/z 1882$[M]^+$, m/z 1852, m/z 1822, m/z 1821, m/z 1789, m/z 1769, m/z 1720, m/z 1679, m/z 1666, m/z 1662, m/z 1623, m/z 1601, m/z 1591, m/z 1565, m/z 1554, m/z 1542, m/z 1508, m/z 1501, m/z 1476, m/z 1431, m/z 1396, m/z 1378, m/z 1354, m/z 1314, m/z 1298, m/z 1278, m/z 1256, m/z 1234, m/z 1214, m/z 1200, m/z 1172, m/z 1152, m/z 1119, m/z 1103, m/z 1072, m/z 1042, m/z 942, m/z 932, m/z 911, m/z 883, m/z 865, m/z 828, m/z 813, m/z 798, m/z 771, m/z 747, m/z 737, m/z 691, m/z 670, m/z 666, m/z 633, m/z 610, m/z 603, m/z 580, m/z 572, m/z 565, m/z 547, m/z 526, m/z 504, m/z 490, m/z 465, m/z 440, m/z 423, m/z 408, m/z 380, m/z 367, m/z 365, m/z 342, m/z 312, m/z 292, m/z 260, m/z 242, m/z 203, m/z 180, m/z 162 and m/z 144.

RESULT AND DISCUSSION

Compound Riesose, $C_{70}H_{118}N_2O_{56}$,$[\alpha]_D$ -2.68, gave positive Phenol-sulphuric acid test, Feigl test (Feigl 1975) and Morgon-Elson test showing the presence of normal and amino sugar(s) in the compound. The HSQC spectrum of acetylated compound at 300 MHz exhibited twelve cross peaks for twelve anomeric proton signals at δ6.185 x 86.20, δ5.655 x 88.20, δ4.450 x 104.04, δ4.504 x 104.04, δ4.750 x 95.00, δ4.555 x 104.50, δ4.623 x 103.50, δ4.725 x 98.70, δ5.273 x 89.90, δ5.351 x 88.70, δ5.351 x 90.10, and δ5.328 x 92.20 indicating that the Riesose may be a undecasaccharide in its reducing form with signals for α and βanomers of glucose showing the presence of Glc at the reducing end. Methylglycosidation of Riesose by $MeOH/H^+$ followed by its acid hydrolysis led to isolation of α and β-methyl glucoside, which confirmed the presence of glucose at the reducing end of the oligosaccharide. It was also confirmed by the presence of two anomeric proton signals at δ5.271 and δ4.725 for α- and β-Glc in ^{1}H NMR of Riesose in D_2O. The undecasaccharide nature of Riesose was further confirmed by the presence of twelve anomeric carbon and proton at δ86.20(1C), δ88.20(1C), δ88.70(1C), δ89.90(1C), δ90.10(1C), δ92.20(1C),δ95.00(1C), δ98.70(1C), δ103.50(1C), δ104.04(2C) and δ104.50(1C) in ^{13}C NMR and δ6.185(1H), δ5.655 (1H), δ4.450(1H), δ4.504(1H), δ4.750(1H), δ4.555(1H), δ4.623(1H), δ4.725(1H), δ5.273(1H), δ5.351(2H), and δ5.328(1H) in ^{1}H NMR, in Riesose acetate, respectively. The eleven monosaccharides in compound were represented as S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_8, S_9, S_{10} and S_{11} for convenience starting from reducing end. To confirm the monosaccharide constituents in compound, it was hydrolysed under strong acidic conditions. In Kiliani hydrolysis under strong acid condition, it gave four monosaccharides i.e. glucose, galactose and N-acetyl-glucosamine, confirming that the undecasaccharide is consist of four types of monosaccharide units i.e. glucose, galactose and N-acetylglucosamine. Since the glucose was present in its reducing form which was supported by ^{1}H NMR of Riesose in D_2O which contains two anomeric proton signals for α- and β-Glc at δ5.271 (J=3.9Hz) and at δ4.725 (J=7.8Hz). It also contains signals for two methyl group at δ1.960 and δ1.848 suggesting the presence of two amino sugars in it. Further the presence of another anomeric proton doublet signals at δ4.501 (J=8.4Hz) was due to presence of β-Gal moiety in the Riesose. The ^{1}H NMR also contains a triplet at δ3.405 for β-Glc (S_1') H-2 signal in the downfield region, which indicated that both the equatorially oriented hydroxyl groups at C-3 and C-4 of the reducing β-Glc (S_1) were substituted and are involved in glycosidation (StructurReporter Group, SRG), confirming the presence of a lactosyl moiety i.eβ-Gal(1→4)Glc with a substitution on 3-position of Glc(S_1) in Riesose. The coupling constant of S_2anomeric signal with J value of 8.4Hz shows the β-configuration of anomeric linkage between S_2→S_1 it in lactosyl moiety. It was also supported by the presence of β-Glc H-4 proton resonance at δ3.800 in acetylated derivative of Riesose. Another anomeric signal, which appeared at δ5.185, was due to presence of α-Gal (S_8) moiety. The anomeric proton chemical shift value at δ5.185 along with SRG value of triplet of S_1 at δ3.405 which suggested that the H-3 and H-4 are involved glycosidic linkages. Since the H-4 of S_1 was already involved in glycosidic linkage with β-Gal (S_2), the α-Gal (S_8) must be linked with H-3 of S_1at non-reducing end. The splitting pattern of anomeric signal with J value of δ3.8Hz shows the α-configuration of anomeric linkage at S_8→S_1.It was further confirmed by the upfield shifted values of H-3 of S_1 at δ4.087 and H-5 of S_8 at δ4.137 (SRG) in Riesose acetate. Further the presence of next anomeric proton doublet at δ4.695(J=7.8) was due to the presence of another β-Glc moiety which is represented as S_3. The position of anomeric proton resonance at δ4.695 suggested that Glc may be (1→3) linked (SRG) to β-Gal(S_2). The coupling constant of anomeric signal with J value of δ7.8Hz shows the β-configuration of anomeric linkage, amongst S_3→S_2. This linkage was further confirmed by the presence of H-3 & C-3 resonance of β-Gal (S_2), in ^{1}H NMR of Riesose acetate at δ3.923 &δ73.30, respectively. The presence of another Gal moiety was suggested by the presence of another anomeric proton signal S_9 at δ5.271was due to presence of α-Gal (S_9) moiety. The position of anomeric proton resonance at δ5.271 suggested that α-Gal may be (1→4) linked (SRG) to β-Gal(S_2). The coupling constant of anomeric signal with J value of 3.6 Hz shows the α-configuration of anomeric linkage, between S_9→S_2. It was further confirmed by the presence of H-4 & C-4 resonance of β-Gal (S_2), of Riesose acetate at δ3.887 &δ73.21, respectively. The next two anomeric proton signal, which appeared at δ4.430 (J= 8.1) and δ5.155 (J=3.8) were due to presence of β-Gal (S_4) and α-Gal (S_{10}) moieties, respectively. The presence of anomeric proton chemical shift at δ4.430 with H-2 triplet of β-Glc (S_3) at δ3.405 in the downfield region, confirmed the presence of repeated lactosyl moiety i.e. βGal (1→4) Glc with substitution on 3-position in Glc moiety. The splitting pattern of anomeric signal with J value of 8.1Hz shows the β-configuration of anomeric likage between S_4→S_3 confirming that the β-Gal (S_4) was linked to Glc (S_3) at H-4.

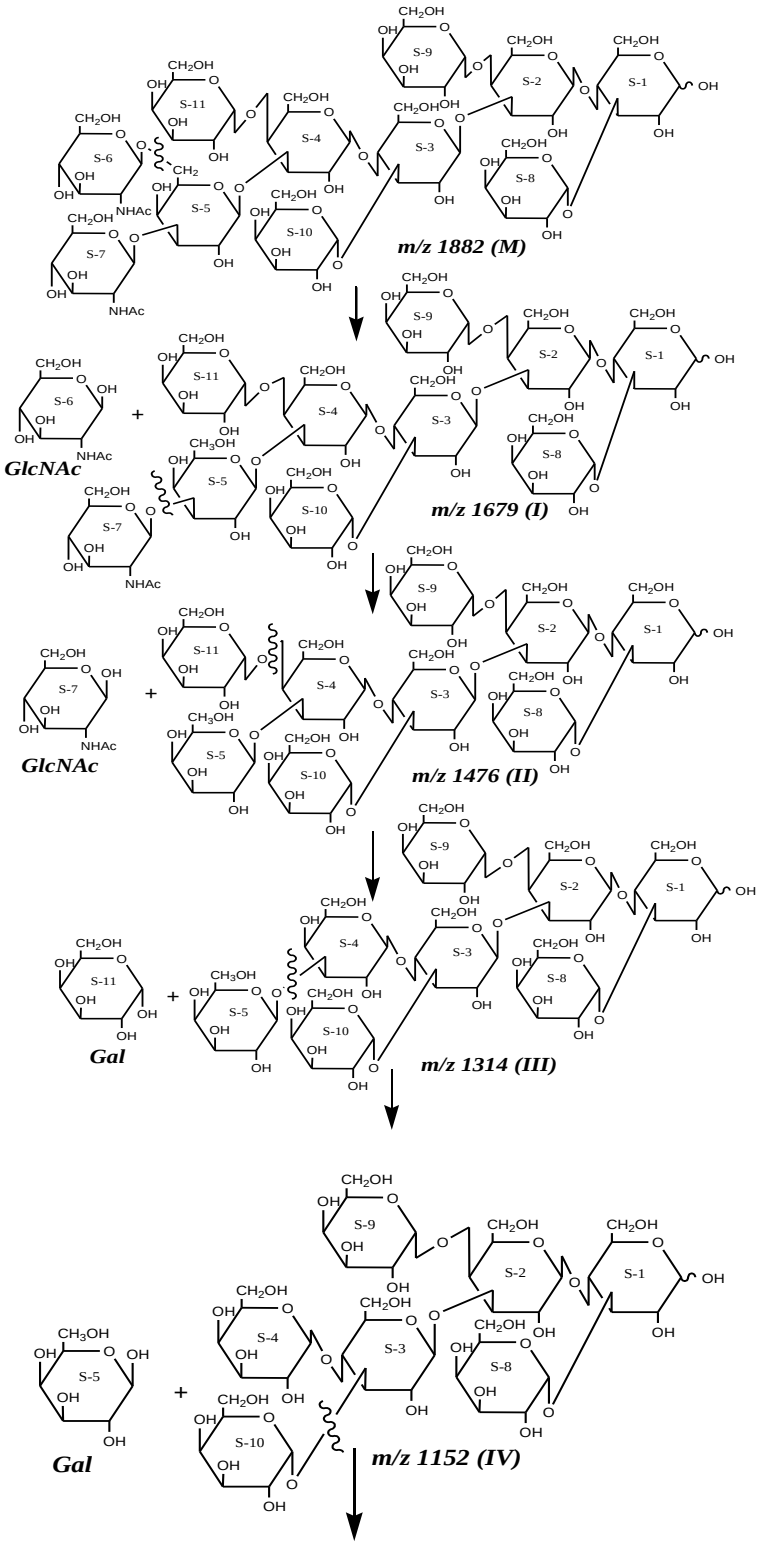
It was further confirmed by the presence of H-4 & C-4 resonances of β-Glc (S_3), of Riesose acetate at δ3.828 &δ76.68, respectively. The second anomeric proton described earlier at δ5.155 (J=3.8Hz) suggested that α-Gal (S_{10}) may be (1→3) linked (SRG) to β-Glc (S_3). The coupling constant of anomeric signal with J value of 3.8Hz shows the α-configuration of anomeric linkage, among S_{10}→S_3 confirming the α-Gal (S_{10}) was glycosidicaly linked to Glc (S_3). It was further confirmed by the presence of H-3 & C-3 resonance of β-Glc(S_3), of Riesose acetate at δ3.762 &δ71.94, respectively. Further the next anomeric proton signal, which appeared at δ4.501(J=8.4) was due to presence of β-Gal (S_5). The splitting pattern of anomeric signal with J value of 8.4Hz suggested the β-configuration of anomeric linkage at S_5→S_4. It was further confirmed by the β-Gal H-3 proton resonance at δ3.817 and C-3 carbon resonance at δ76.77 in Riesose acetate. Another anomeric proton signal, which appeared at δ5.235(J=3.6) was due to presence of α-Gal (S_{11}). The coupling constant of anomeric signal with J value of δ3.6 Hz shows the α-configuration of anomeric linkage at S_{11}→S_4. It was further substantiated by the β-Gal H-4 & C-4 at δ3.986 and δ74.20, along with upfield shifted H-5 value of α-Gal (S_{11}) at δ4.119 in acetylated derivative of Riesose. The next two anomeric proton signal, which appeared at δ4.610(J=7.6) along with signal of amide methyl group at δ1.960 and δ4.710(J=7.6) along with signal of amide methyl group at δ1.848 proposed the presence of β-GlcNAc (S_6) and β-GlcNAc (S_7) moieties, respectively.The position of anomeric proton values of β-GlcNAc(S_6) at δ4.610 & β-GlcNAc (S_7) at δ4.710 with downfield shifted value of H-4 of β-Gal (S_5) (SRG) suggested it may be (1→6) and (1→3) linked, respectively. The coupling constant of anomeric signals with J value of δ7.6Hz andδ7.6Hz shows the β-configurationof anomeric linkages between S_6→S_5 and S_7→S_5, respectively. It was further confirmed by the chemical shift of β-Gal (S_5) of H-3 and C-3 resonances at δ3.910 and δ75.00, respectively and H-6 & C-6 at δ3.775 & δ73.21 respectively, with upfield shifted H-4 value of β-Gal (S_7) at δ4.188 (SRG) in acetylated spectrum of Riesose. The various values of ring carbons are in accordance with ^{13}C value of their respective monosaccharides, which also supports the derived structure. The undecasaccharide nature of compound was further confirmed by the spectral studies of acetylated derivative of compound. The heteronuclear single quantum coherence (HSQC) spectrum of acetylated compound confirmed linkages in ^{1}H and ^{13}C NMR spectra by showing cross peaks of an α-Glc (S_1) H-3 & C-3 at (δ4.044 x 75.00) showed (1→3) linkage of S_8 and S_1 and H-4& C-4 at (δ3.800 x 74.00) showed (1→4) linkage of S_2 and S_1, β-Glc (S_1) H-3 & C-3 at (δ4.087 x 75.50) showed (1→3) linkage of S_8 and S_1 and H-4 & C-4 at (δ3.775 x 77.31) showed (1→4) linkage of S_2 and S_1. It also contains cross peak of H-3 & C-3 of β-Gal (S_2) at (δ3.923 x 73.30) showed (1→3) linkage of S_3 and S_2 and H-4 & C-4 of β-Gal (S_2) at (δ3.887 x 73.21) showed (1→4) linkage of S_9 and S_2, H-3 & C-3 of β-Glc (S_3) at (δ3.762 x 71.94) showed (1→3) linkage of S_{10} and S_3 and H-4 & C-4 of β-Glc (S_3) at (δ3.828 x 76.68) showed (1→4) linkage of S_4 and S_3, H-3 & C-3 of β-Gal (S_4)at (δ3.817 x 76.99) showed(1→3) linkage of S_5 and S_4, and H-4& C-4 of β-Gal (S_4) at (δ3.986 x 74.20) showed (1→4) linkage of S_{11} and S_4 and H-6 & C-6 of β-Gal (S_5) at (δ3.910 x 75.00) showed (1→6) linkage of S_6 and S_5, and H-3& C-3 of β-Gal (S_5) at (δ3.775 x 73.21) showed (1→3) linkage of S_7 and S_5, respectively showing same chemical shift in acetylated and deacetylated spectra. It was further confirmed by the presence of same peaks in COSY and TOCSY spectrum. Thus, based on the pattern of chemical shifts of ^{1}H NMR , ^{13}C NMR, HOMOCOSY, TOCSY and HSQC NMR experiments it was interpreted that the compound was aundecasaccharide having structure as-



The result obtained from the ES mass spectrum further substantiated the structure of Riesose which was derived by its ^{1}H and ^{13}C NMR spectra. The highest mass ion peak were recorded m/z 1944 which was due to $[M+Na+K]^+$. Other mass ion peak recorded at m/z 1906 and m/z 1882, were due to $[M+Na+H]^+$ and [M] respectively, confirmed that the molecular weight of compound was 1882. Further the mass fragments were formed by repeated H transfer in the oligosaccharide and was accompanied by the elimination of terminal sugar less water. The undecasaccharide fragment mass ion peak at m/z 1882 (M) on further fragmentation gave the decasaccharide mass ion peak at m/z 1679 (I)

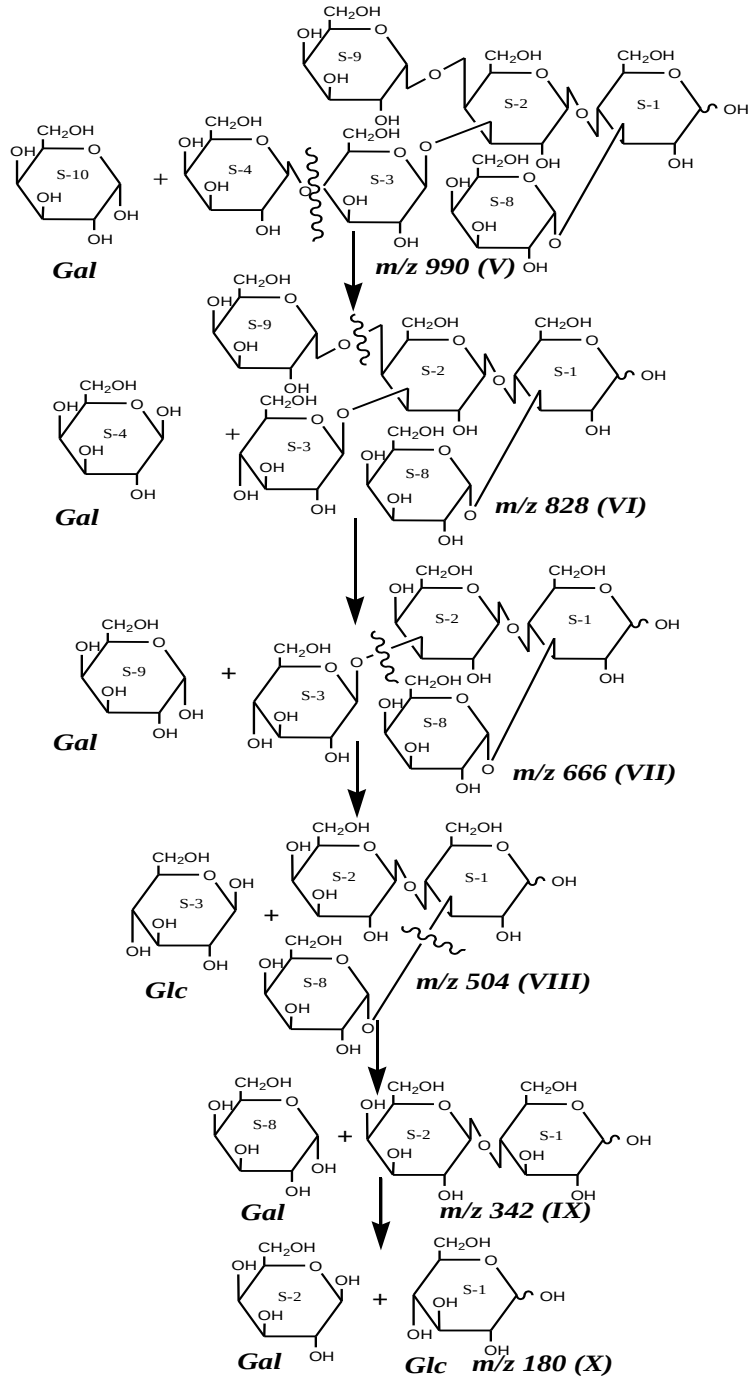
which was obtained by the loss of S_6 sugar unit linked to S_5 of the oligosaccharide, which was supported by its respective fragment at m/z 203, this confirmed the presence of GlcNAc (S_6) at the non-reducing end. Further the decasaccharide mass ion fragmented by the loss of another GlcNAc (S_7), gave the corresponding nonasaccharide at m/z 1476 (II) which was also supported by its complimentary fragment at m/z 203, this confirmed the presence of GlcNAc (S_7) at the non-reducing end. The mass ion peak at 1476 further fragmented to give mass ion fragment for octasaccharide moiety which was arose by loss of sugar Gal (S_{11}). It was accounted for the mass ion fragment at m/z 1314 (III). Further the octasaccharide mass ion fragmented to gave heptasaccharide (IV) fragment at m/z 1152, by loss of Gal (S_5). This heptasaccharide mass ion on further fragmentation gave hexasaccharide segment (V) at m/z 990, by the loss of Gal (S_{10}). This hexasaccharide mass ion on further fragmentation gave pentasaccharide segment (VI) at m/z 828, by the loss of Gal (S_4). This pentasaccharide mass ion on further fragmentation gave tetraccharide segment (VII) at m/z 666, by the loss of Gal (S_9). This tetrasaccharide mass ion on further fragmentation gave trisaccharide segment (VIII) at m/z 504, by the loss of Glc (S_3). This trisaccharide mass ion on further fragmentation gave disaccharide segment (IX) at m/z 342, by the loss of Gal (S_8), which by further fragmentation gave monosaccharide (X) at m/z 180, by loss of Gal (S_2). The undecasaccharide mass ion peak at m/z 1882 in the spectrum of compound M also showed other supporting mass ion peaks which are shown in scheme 1 & 4. Other mass ion fragments was obtained at m/z 1720 [M-S_8], m/z 1679 [M-S_6 or M-S_7], m/z 1558 [M-S_1,S_8], m/z 1396 [M-S_1,S_8,S_9], m/z 1234 [M-S_1,S_2,S_8,S_9], m/z 910 [M-S_1,S_2,S_3,S_8,S_9,S_{10}], m/z 748 [M-S_1,S_2,S_3,S_8,S_9,S_{10},S_{11}], m/z 666 [M-S_3,S_4,S_5,S_6,S_7,S_{10},S_{11} or M-S_1,S_4,S_5,S_6,S_7,S_8,S_{11}], m/z 586 [M-S_1,S_2,S_3,S_4,S_8,S_9,S_{10},S_{11}], m/z 504 [M-S_3,S_4,S_5,S_6,S_7,S_9,S_{10} or M-S_1,S_4,S_5,S_6,S_7,S_8,S_9,S_{11}] and m/z 383 [M-S_1,S_2,S_3,S_4,S_7,S_8,S_9,S_{10},S_{11} or M-S_1,S_2,S_3,S_4,S_6,S_8,S_9,S_{10},S_{11}]which support sequence in oligosaccharide moiety. The other supporting mass fragments obtained at m/z 1852 (1882-HCHO), m/z 1789 (1852-CH_2CHO,CH_2OH), m/z 1720 (1789-H_2O,CH_2OH,HCHO), m/z 1666 (1720-3H_2O), m/z 1601 (1666-HCHO,H_2O,OH), m/z 1565 (1601-H_2O,OH), m/z 1821 (1852-CH_3O), m/z 1679 (1821-CH_2CHO), m/z 1822 (1882-2HCHO), m/z 1769 (1822-2H_2O), m/z 1720 (1769-H_3O^+), m/z 1662 (1720-$NHCOCH_3$) and m/z 1679 (1769-2HCHO). The undecasaccharide m/z 1882 on fragmentation gave decasaccharide m/z 1679 (M-S_6), which was further confirmed by its other fragments ions at m/z 1666 (1679-H_3O^+), m/z 1623 (1666-CH_3CHO), m/z 1565 (1623-$NHCOCH_3$), m/z 1591 (1623-CH_3OH), m/z 1554 (1591-2H_2O,H_3O^+), m/z 1501 (1554-2H_2O,OH), m/z 1542 (1591-OH,CH_2CHO), m/z 1508 (1542-2H_2O) and m/z 1476 (1508-CH_3OH). The decasaccharide m/z 1679 on fragmentation gave nonasaccharide m/z 1476 (1679-S_7), which was further confirmed by its other fragments ions at m/z 1431 (1476-H_2O,OH,HCHO), m/z 1396 (1431-H_2O,OH), m/z 1354 (1396-CH_2CHO), m/z 1298 (1354-$CHCOCH_3$), m/z 1234 (1298-CH_3OH), m/z 1256 (1298-CH_2CHO), m/z 1200 (1256-$CHCOCH_3$), m/z 1214 (1256-CH_2CHO), m/z 1378 (1431-H_2O,OH) and m/z 1314 (1378-2CH_2OH). The nonasaccharide m/z 1476 on fragmentation gave octasaccharide m/z 1314 (1476-S_{11}), which was further confirmed by its other fragments ions at m/z 1278 (1314-2H_2O), m/z 1214 (1278-2CH_3OH), m/z 1172 (1214-CH_2CHO), m/z 1119 (1172-2H_2O, OH) and m/z 1152 (1214-2CH_2OH). The octasaccharide m/z 1314 on fragmentation gave heptasaccharide m/z 1152 (1314-S_5), which was further confirmed by its other fragments ions at m/z 1103 (1152-HCHO, H_3O^+), m/z 1072 (1103-CH_2OH) and m/z 1042 (1072-HCHO). The heptasaccharide m/z 1152 on fragmentation gave hexasaccharide m/z 990 (1152-S_{10}), which was further confirmed by its other fragments ions at m/z 932 (990-CH_3CHO, CH_3), m/z 911 (932-H_3O^+), m/z 942 (990-HCHO, H_2O), m/z 883 (942-2HCHO), m/z 865 (883-H_2O) and m/z 828 (865-H_2O, H_3O^+). The hexasaccharide m/z 990 on fragmentation gave pentasaccharide m/z 828 (990-S_4), which was further confirmed by its other fragments ions at m/z 813 (828-CH3), m/z 747 (813-2CH_2OH), m/z 691 (747-2H_2O), m/z 670 (691-H_3O^+), m/z 771 (828-$CHCOCH_3$), m/z 798 (828-HCHO), m/z 737 (798-CH_2OH), m/z 670 (737-OH,CH_3OH,H_2O), m/z 633 (670-HCHO,OH), m/z 603 (633-HCHO), m/z 572 (603-CH_2OH), m/z 547 (603-$CHCOCH_3$) and m/z 666 (737-2H_2O,2OH). The pentasaccharide m/z 828 on fragmentation gave tetrasaccharide m/z 666 (828-S_9), which was further confirmed by its other fragments ions at m/z 580 (666-$CHCOCH_3$,HCHO), m/z 526 (580-3H_2O), m/z 465 (526-HCHO,CH_2OH), m/z 490 (526-2H_2O), m/z 465 (490-H_2O,OH), m/z 440 (465-CH_3), m/z 408 (440-CH_3OH), m/z 365 (408-CH_3CHO), m/z 610 (666-$CHCOCH_3$), m/z 565 (610-HCHO,CH_3), m/z 504 (565-HCHO), m/z 423 (465-CH_2CHO), m/z 367 (423-$CHCOCH_3$) and m/z 342 (367-CH_3). The tetrasaccharide m/z 666 on fragmentation gave trisaccharide m/z 504 (666-S_3), which was further confirmed by its other fragments ions at m/z 440 (504-2CH_3OH), m/z 423 (440-OH), m/z 380 (423-CH_3CHO), m/z 408 (440-CH_3OH) and m/z 342 (408-HCHO, 2H_2O). The trisaccharide m/z 504 on fragmentation gave disaccharide m/z 342 (504-S_8), which was further confirmed by its other fragments ions at m/z 292 (342-CH_2CHO), m/z 260 (292-CH_2OH), m/z 312 (342-HCHO), m/z 260 (312-CH_2CHO), m/z 203 (260-CH_2CHO,CH_3), m/z 144 (203-CH_2CHO,OH), m/z 242 (260-H_2O) and m/z 180 (242-2CH_2OH).

The disaccharide m/z 342 on fragmentation gave monosaccharide m/z 180 (342-S_2), which was further confirmed by its other fragments ions at m/z 162 (180-H_2O) and m/z 144 (162-H_2O).



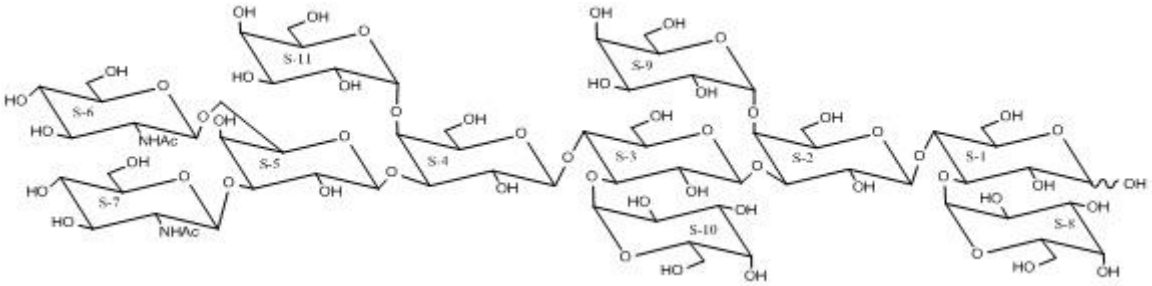
Cont.....

ES Mass fragmentation of Riesose



ES Mass fragmentation of Riesose

Based on the results obtained from chemical degradation/acid hydrolysis, chemical transformation, Electro spray mass spectrometry and 1D-NMR viz.^{1}H NMR, ^{13}C NMR and2D-NMR viz. COSY, TOCSY and HSQC NMR spectra of Riesose acetate and Riesose, the structure and sequence of isolated novel undecasaccharide Riesose was deduced as-



Compound Riesose

CONCLUSION

From the above information, we conclude the structure of isolated sheep milk oligosaccharide, Riesose. This novel oligosaccharide was reported for the first time from any natural source or any milk and its structure was elucidated with the help of spectroscopic techniques like ^{1}H, ^{13}C, 2D-NMR (COSY, TOCSY and HSQC) spectroscopy and mass spectrometry.

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