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Anupam Kumar Srivastava, Pushpraj Singh and Desh Deepak

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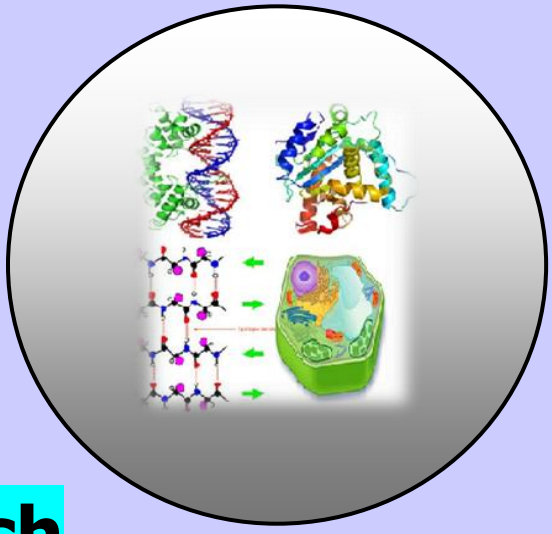

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Prof. Desh deepak
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RESEARCH PAPER

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Isolation and NMR Studies of Novel Oligosaccharide from Goat Milk

Anupam Kumar Srivastava, Pushpraj Singh and Desh Deepak
Department of Chemistry, University of Lucknow, Lucknow 226007, U.P., India

ABSTRACT

Milk is a primary source of nutrition for mammalian infants before they are able to digest other types of foods. Milk contains different carbohydrate units including lactose and oligosaccharides. Milk oligosaccharides are most important carbohydrates in biological system which exhibit potent biological activities such as anti-cancer, anti-inflammatory, anti-tumour, anti-coagulant and anti-viral activities etc. To find biologically active novel oligosaccharides, goat milk was taken, which is used for treatment of mal-absorption syndromes, intestinal disorder, coronary disease, premature infant nutrition, cystic fibrosis, infant allergy, inhibiting and dissolving cholesterol deposits. For this purpose goat milk was processed by method of Kobata and Ginsburg followed by gel filtration HPLC and column chromatography which resulted in the isolation of one novel milk oligosaccharide named Apriose. The structure of isolated and purified goat milk oligosaccharide was elucidated with the help of chemical degradation, chemical transformation, spectroscopic techniques like NMR (^{1}H, ^{13}C and 2D NMR), structure reporter group theory and mass spectrometry. The sequence of monosaccharides and structure of isolated novel oligosaccharide molecule Apriose was deduced as-

Keywords: Goat milk, Oligosaccharide, Kobata and Ginsburg method.

INTRODUCTION

Milk is nature's designer food and contains all necessary nutrients for growth and development of any mammalian neonate (Singh et al., 2015). Milk is made up of milk fat, protein, milk carbohydrates (lactose and oligosaccharides), water, vitamins and minerals

(including calcium). It is a highly nutritious and quality product, naturally containing over 10 essential nutrients for good health and wellbeing. Each component is present in specific amount and has specific function. One of the major components is carbohydrate which contains lactose and oligosaccharides. Milk oligosaccharides are the important class of complex carbohydrates as supplements for the food and the pharmaceutical industries. Milk oligosaccharide plays a key role in various physiological and biological activities such as biological recognition, anti-complementary, anti-coagulant, anti-inflammatory and immunological activities (Singh et al., 2016). More than 250 milk oligosaccharides have been isolated from mammalian (Urashima et al., 2001) milk of different origin eg., human, elephant, bovine, rat, cow, mare, buffalo, donkey, goat, yak, camel, sheep, etc. Elephant milk oligosaccharides fraction contained a high ratio of sialyl oligosaccharide; this may be significant with respect to the formation brain components, such as gangliosides of the suckling calves (Ostho et al., 2007). N-acetylneuraminlactose sulphate may play an important role in the nutrition of the rat pups, which is the dominant oligosaccharide in the Dog milk (Bubb et al., 1999). Buffalo milk oligosaccharides have ability to stimulate non-immunological resistance of the host against parasitic infections (Saxena et al., 1999). Donkey milk oligosaccharides have ability to stimulate non-specific and specific immunological resistance (Deepak et al., 1998). Bovine milk oligosaccharides have several potentially important biological activities including the prevention of the pathogen binding to the intestinal epithelial and as nutrients for beneficial bacteria. Mare milk has shown anti oxidant, lipid lowering and post heparin lipolytic activity (Srivastava et al., 2012). Camel milk oligosaccharides show potent activity against gonorrhoea septic and have hysteric properties as well as anti-tuberculosis activity. Sheep milk aggravates hiccup and dyspnoea. It also eliminates pitta, kapha and fat. It also contains fucose in its oligosaccharides which causes various biological activities. Cow milk oligosaccharides reduce the adhesion of enterotoxic Escherichia coli strains of the calf (Johansson et al., 2005). In Naturopathic medicine, goats' are referred to as bioorganic sodium animals. They are also associated with vigour, flexibility and vitality. Bioorganic sodium is an important element in keeping joints mobile and limber. Goat milk has traditionally been used in medicinal cultures to nourish and regenerate an over-taxed nervous system. Goat's milk is also extremely nutrient dense. It has almost 35% of daily needs for calcium in one cup. It is extremely high in riboflavin, phosphorous, vitamin B_{12}, protein and potassium. Fresh goats' milk is sometimes substituted for breast milk. This reduces the risk of the child developing electrolyte imbalances, metabolic acidosis, megaloblastic anaemia, and a host of allergic reactions. Goat milk has many advantages in terms of percentage of an over sized fat droplets smaller than cow's milk (Silanikove et al*.,* 2010). Because the goat milk fat globule has a greater surface area, and lipases in the gut are sup-posedly able to attack the lipids more rapidly. Almost 20% of the fatty acids of goat milk fall into the short-chain fatty acids category (C4:O to C12:O) compared with l0- 20% for cow milk. Lipases attack the ester linkages of the shorter-chain fatty acids more readily, so these differences may contribute to more rapid digestion and absorption of goat milk fat (Arora et al*.,* 2013). Some physicochemical properties of goat milk such as smaller fat globules, higher percentage of short and medium chain fatty acids, and softer curd formation of its proteins are advantageous for higher digestibility and healthier lipid metabolism relative to cow milk (Park, 1994).

Thus goat milk has been recommended for either infants, old, convalescent people (Kompan and Komprej, 2012), and goat milk-based diet (animal fat) has a benefecial effect and thus its consumption may be recommended especially in cases of malabsorption syndromes (Alferez et al., 2001). Goat milk oligosaccharides play an important role in intestinal protection and repair after damage caused by DSS (Dextron sodium sulphate) induced colitis and their implication in human intestinal inflammation (Villosladaa et al., 2006). Goat milk oligosaccharides have anti-inflammatory effects in rats with trinitrobenzenesulfonic (T) acid induced colitis and may be useful in the management of inflammatory bowel disease (Hakkarainen et al., 2005). Goat milk containing galacto-oligosaccharides could be recommended to decrease most of infant allergy and diseases. Goat milk shows therapeutic virtues for individuals with certain dietetic problems. Keeping this in mind goat milk was collected in the bulk and processed by method of Kobata and Ginsburg for isolation of novel milk oligosaccharide (Kobata A. et al., 1970). In continuation to our previous work on isolation of goat milk oligosaccharides (Kumar et al., 2016) another novel milk oligosaccharide was isolated from the goat milk and then its structure was elucidated with the help of chemical degradation, chemical transformation and spectroscopic methods like ^{1}H NMR, ^{13}C NMR and 2D NMR i.e., COSY, TOCSY, HSQC technique as well as mass spectrometry.

MATERIAL AND METHODS

General procedure

Optical rotations were measured with a PERKIN-ELMER 241 automatic polarimeter in 1cm tube. ^{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 25^0C on a Bruker AM 300 FT NMR spectrometer. The electrospray mass spectra were recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. The samples (dissolved in suitable solvents such as methanol/acetonitrile/water) were 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 CARLO-ELBA 1108 an elemental analyzer. The sugars were visualized on TLC with 30% aqueous H_2SO_4 reagent and on Paper Chromatography 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 ethylacetate-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) lyophilized and centrifuged by a cooling centrifuged 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 diod array detector and G.P. 100 printer plotter. Authentic samples of glucosamine, galactosamine, galactose and glucose were purchased from Aldrich Chemicals.

Isolation of Goat milk oligosaccharide by Kobata and Ginsburg method

15 litres goat milk was collected from a domestic goat and equal amount of ethanol was added and stored at -20^oC until used.

In order to isolate milk oligosaccharide it was centrifuged for 15 min at 5000 rpm at 4^{o}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^{o}C. The white precipitate formed, mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0^{o}C. The supernatant and washing were combined and filtered through a microfilter (0.24 µ, to remove remaining lactose) and lyophilized affording crude oligosaccharide mixture (13 g). The lyophilized material which responded positively to phenol-sulphuric acid test and Morgon-Elson test were taken for further studies. The lyophilized material (mixture of oligosaccharides) of goat milk was further purified on Sephadex G-25 column chromatography for separation of thousands of enzymes, nucleic acids, peptide, glycoproteins, free proteins and other biological macromolecules from oligosaccharide (low molecular weight component) by using glass distilled water as eluent at a flow rate of 5 ml/min. Goat milk oligosaccharide mixture was packed in a column (1.6x40 cm) (void volume = 25 ml) equilibrated with glass triple distilled water (TDW) and it was left for 10-12 hrs to settle down. Presences of neutral sugars were monitored in all eluted fractions by phenol-sulphuric acid test.

Acetylation of Goat milk oligosaccharide mixture

13 gm Goat milk oligosaccharides of pooled fractions, which gave positive phenol-sulphuric acid test, were acetylated with pyridine (12ml) and acetic acid (11.5ml) at 60 ^{0}C and the reaction mixture was stirred overnight. The mixture was evaporated under reduced pressure and viscous residue was taken in $CHCl_3$ and washed in sequence with 2N 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 (15.5gm). The acetylation converted the free oligosaccharides into their non-polar derivatives which were resolved nicely on TLC using $CHCl_3$: MeOH as eluent. Detection of the spots was done by spraying with 50% H_2SO_4 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(56mg), II(650mg), III(1.414g), IV(216mg), V(2.959g), VI(832mg), VII(701mg), VIII(932mg), IX(1.209g), X(811mg) and XI(514mg) respectively. These fractions were containing mixture of two to three compounds. Repeated column chromatography of fraction III led to the isolation of one chromatographically pure compound B.

Deacetylation of Compound

The compound obtained from the column chromatography of acetylated oligosaccharide mixture was dissolved in acetone and NH_3 and left overnight in a stoppered hydrolysis flask. Ammonia was removed under reduced pressure and the compound was washed thrice with $CHCl_3$ (to remove acetamide) and was finally freeze dried giving the deacetylated oligosaccharide Apriose.

Description of Isolated Compound Apriose

^{1}H NMR: δ in $CDCl_3$ (ppm)

δ6.170(d,1H), δ5.686(d,1H), δ5.437(t,1H), δ5.351(d,1H), δ5.144(d,1H), δ5.017(t,1H), δ4.950(d,1H), δ4.844(d,1H), δ4.827(d,1H), δ4.620(d,1H), δ4.508(d,2H), δ4.487(d,2H), δ4.117(d,1H), δ4.086(d,1H), δ3.78(m,2H), δ3.67(d,1H), δ3.66(d,1H), δ3.57(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), δ1.944(s,1H), δ1.990(s,1H).

^{13}C NMR: δ in $CDCl_3$ (ppm)

δ173.2, δ170.0, δ169.9, δ169.8, δ101.05, δ100.97, δ 95.25, δ91.41, δ90.09, δ73.45, δ72.93, δ72.03, δ70.95, δ 70.76, δ 68.26, δ 65.53, δ61.16, δ 60.89, δ 60.56, δ 20.91, δ20.82, δ20.8, δ 20.5, δ20.4.

^{1}H NMR: δ in D_2O (ppm)

δ5.250(d,1H), δ5.021(d,1H), δ4.681(d,1H), δ4.575(d,1H), δ4.563(d,1H), δ4.537(d,1H), δ4.501(d,1H), δ4.475(d,1H), δ4.061(d,1H), δ3.956(d,1H), δ3.898(t,1H), δ3.884(m,1H), δ3.832(d,1H), δ3.767(d,1H), δ3.714(d,1H), δ3.683(d,1H), δ3.628(m,1H), δ3.593(m,2H), δ3.573(m,1H), δ3.503(d,1H), δ3.21(t,1H), δ2.26(s,3H), δ2.03(s,3H), δ2.01(s,3H)

^{13}C NMR: δ in D_2O (ppm)

δ172.3, δ101.0, δ100.9, δ95.2, δ91.8, δ91.4, δ 90.0, δ81.6, δ76.0, δ75.2, δ74.9, δ73.2, δ71.2, δ70.0, δ69.08, δ69.7, δ68.9, δ59.7, δ23.7

ES-MS

1338$[M+Na+K]^+$, 1275 $[M]^+$.

RESULT AND DISCUSSION

Compound "B" $C_{48}H_{81}O_{36}N_3$ gave positive Phenol-sulphuric acid test, Fiegl test and Morgon-Elson test indicating the presence of normal and amino sugar(s) in the moiety. The ^{1}H NMR spectrum of compound Apriose acetate at 300 MHz exhibited eight signals in the anomeric proton region as doublets at δ6.17(1H), δ5.68 (1H), 5.35 (1H),4.62 (1H), 4.50 (2H) and 4.48 (2H) for eight protons leading to the presence of eight anomeric protons in compound Apriose acetate. This was further supported by the appearance of seven signals for eight anomeric carbons at δ90.0 (1C), δ91.4 (1C), δ91.8 (1C), δ95.2 (1C), δ100.9 (2C), δ101.05 (1C) and δ101.06 (1C) in the ^{13}C NMR spectrum of Apriose acetate. These data led to the suggestion that compound 'B' may be Heptasaccharide in its reducing form with a glucose unit at the reducing end. The reducing nature of glucose was further confirmed by the Methylglycosidation of 'B' by $MeOH/H^+$ followed by its acid hydrolysis which led to the isolation of α and β-methyl glucoside, which suggested the presence of glucose at the reducing end in the oligosaccharide The seven-monosaccharide units present in compound 'B' 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. Further, for confirmation of the monosaccharide constituents in it, Apriose was hydrolyzed under strong acidic condition (Kiliani hydrolysis) followed by paper chromatography which showed the presence of Glc, Gal, GlcNAc, GalNAc, which confirmed that these moieties has participated in the building of the compound 'B'.

The reducing and free nature of glucose was further supported by the presence of two anomeric proton signals as doublets and their coupling constants, for α and β Glc at 5.25 ppm (1H, J=3.0 Hz) and δ 4.68 ppm (1H, (J=7.8 Hz) respectively. Beside, α and β Glucose (S_1), another anomeric proton doublet present at δ4.47 ppm (1H) J=7.5 Hz for Gal (S_2) residue suggested the presence of a lactosyl moiety i.e. Gal β (1→4) Glc in compound Apriose. This was further confirmed by β Glc (S_1) H-2 signal appeared as a triplet at δ3.296, J=8.4 Hz, these ^{1}H NMR of data of lactosyl moiety was compared with structural reporter group. Further in the ^{1}H NMR spectrum of Apriose, another anomeric proton appeared as a doublet at δ 4.57 (J=8.4Hz) along with signal of NHAc group at 2.26 was due to the presence of GlcNAc which was present next in sequence with lactosyl moiety. The presence of downfield shifted H-4 proton of β-Gal(S_2) at δ4.11 confirmed that this β-Gal(S_2) was substituted at C-3 position by GlcNAc (S_3) moiety which is structure reporter group in the ^{1}H NMR of acetylated Apriose. Further in ^{1}H-^{1}H COSY and TOCSY NMR spectrum of acetylated compound 'B' showed that H-3 and H-4 proton of GlcNAc (S_3) appeared in upfield region at δ3.80 ppm and δ3.67 ppm respectively, this suggested that β-GlcNAc (S_3) was glycosidically linked at C-3 and C-4 positions. Further, the fourth anomeric proton which was present as a doublet at δ4.50 (1H, J=7.5 Hz) is due to β-Gal (S_4),The downfield shift of β-Gal (S_4), H-1 suggested that β-Gal (S_4) was glycosidically linked to β GlcNAc (S_3) It was further confirmed that the β-Gal (S_4) was glycosidically linked with the C-4 of β GlcNAc (S_3) because H-4 proton of S_3 was present at δ3.65 ppm in acetylated spectrum of Apriose acetate. Further, the fifth anomeric proton of Apriose which appeared as a doublet at δ4.47 ppm (J=7.8 Hz) along with a singlet of three protons at δ 2.02 showed the presence of β GalNAc (S_5) in Apriose, structure reporter group value of anomeric proton at δ 4.47 suggested that it was glycosidically linked to the C-3 of GlcNAc (S_3). This linkage was confirmed by the acetylated ^{1}H NMR spectrum data, which showed that H-3 proton of GlcNAc (S_4) was present at upfield region at δ 3.66 ppm, this value was further confirmed by ^{1}H-^{1}H COSY and TOCSY spectrum of Apriose actate. In ^{1}H NMR spectrum of compound 'B'**,** showed the sixth anomeric proton which also appeared as a doublet at δ 4.49 (1H, J=7.8 Hz) along with singlet of three protons at δ 2.03 showed the presence of β GalNAc (S_6) which was glycosidically linked to the Gal (S_4) by [1→6]linkage. This linkage was confirmed by acetylated ^{1}H-^{1}H COSY and TOCSY spectrum region of β-Gal (S_4), In which H-6 protons signal appear at δ 3.78 ppm, further the value of H-2, H-3 and H-4 protons were assigned at 5.14, 4.18, 5.37 respectively which confirmed that H-2 and H-4 were not involved in linkage and H-3 was involved in linkage by the next sugar that is (S_4) .The seventh anomeric proton which appeared as a doublet at δ 5.35 ppm (J=3.3Hz) showed the presence of α -Gal (S_7) that was glycosidically linked to the C-3 of Gal (S_4) because H-3 proton of (S_4) was present at δ 4.18 ppm in ^{1}H NMR of Apriose acetate, which was confirmed by acetylated ^{1}H-^{1}H COSY and TOCSY spectra of acetylated compound of Apriose, In which H-3 proton of (S_4) appear at upfield position δ 4.18 ppm indicates that C-3 of Gal (S_4) was involved in inter residual glycosidation with S_7 sugar, this implies that β-Gal (S_4) glycosidically linked to α -Gal (S_7) and the linkage α Gal (S_7) [1→3] β-Gal (S_4) was confirmed by ^{1}H-^{1}H COSY, TOCSY as well as HSQC spectrum of acetylated compound of Apriose. The Heptasaccharide nature of isolated oligosaccharide Apriose was further confirmed by spectral studies of acetylated Apriose**.**

The heteronuclear single quantum-coherence (HSQC) spectrum of acetylated product of compound 'B' confirmed anomeric assignments in ^{1}H and ^{13}C NMR spectra of 'B' by showing the ^{1}H and ^{13}C cross peaks of α-Glc at δ6.17 x δ 90.0 and β-Glc at δ 5.68 x 91.4, and it also contains six other anomeric crosspeaks signals at 5.35x δ91.8, δ4.48 x δ101.0, 4.48 x δ101.0, δ4.62 x δ95.2, δ4.50x δ100.9 and4.50x δ100.9 for α-Gal(S_7) , β-GalNAc(S_5) , β-GalNAc (S_6), β-GlcNAc(S_3), β-Gal(S_2) and β-Gal(S_4) respectively. The glycosidic linkage were assigned by the cross peaks for glycosidically linked carbon with their proton in HSQC spectrum of Apriose acetate. The values of these cross peaks were as β-Glc(S_1) H-4 and C-4 at δ4.077xδ70.95 confirms (1→4) linkage between S_1 and S_2, β-Gal (S_2) H-3 and C-3 at δ3.678 x δ72.03 confirms (1→3) linkage between S_2 and S_3 , β-GlcNAc (S_3) H-3 and C-3 at δ3.661 x δ72.03 confirms (1→3)linkage between S_3 and S_4 and β-GlcNAc (S_3) H-4 and C-4 at δ3.670 x δ72.93 confirms (1→4)linkage between S_3 and S_5, β-Gal (S_4) H-3 and C-3 at δ4.180x δ71.02 confirms (1→3) linkage between S_4 and S_7 , β-Gal (S_4) H-6 and C-6at δ3.78 x δ73.45 confirms (1→6) linkage between S_4 and S_6. The Electrospray Mass Spectrometric data of compound not only confirmed the derived structure but also supported the derived sequence of monosaccharides in Apriose. The highest mass ion peak was recorded at m/z 1338 which was due $[M+Na+K+H]^+$, the other mass ion peak recorded at m/z 1275 which was due to $[M]^+$ confirming the molecular weight of Apriose as 1275 and is in agreement with its molecular formulae. Based on the results obtained from chemical degradation/acid Hydrolysis, Chemical transformation, Electro Spray Mass spectrometry and ^{1}H, ^{13}C NMR and HOMOCOSY, TOCSY and HSQC 2D NMR techniques of Apriose and acetylated Apriose, the structure and sequence of isolated Novel oligosaccharide molecule Apriose was deduced as-

Compound Apriose

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Corresponding author: Dr. Desh Deepak, Department of Chemistry, University of Lucknow, Lucknow 226007, U.P., India
Email: deshdeepakraju@rediffmail.com