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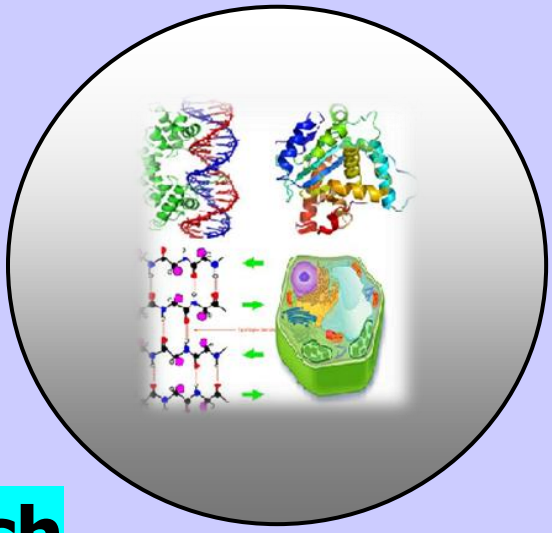

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RESEARCH PAPER

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Isolation and Structure Elucidation of Caprose (Novel Oligosaccharide) from Goat Milk

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ABSTRACT

Milk oligosaccharides have excellent medicinal properties in treatment of allergies, arthritis, cancer, coronary heart disease, dental caries, diabetes, hypertension, lactose intolerance, osteoporosis, bone health and probiotics. It helps in development of immunological & neurological system, defence against viral & bacterial infections and provides strength to the body. Milk oligosaccharides are complex sugars contain fucose, sialic acid (N-acetylneuraminic acid), galactose, mannose, N-acetylglucosamine and N-acetylgalactosamine. Oligosaccharides are third major component in milk of mammals having immunostimulant, anti-cancer, anti-inflammatory and anti-microbial potential. Keeping in the mind, physiological, biological and medicinal importance of milk oligosaccharides, goat milk was taken, which is used for treatment of mal-absorption syndromes, coronary disease, intestinal disorder, premature infant nutrition, infant allergy, cystic fibrosis, inhibiting and dissolving cholesterol deposits. For this purpose goat milk was taken and processed by method of Kobata and Ginsburg followed by different chromatographic techniques like TLC, CC, HPLC etc. which led to the isolation of a novel milk oligosaccharide namely Caprose. The structure elucidation of isolated and purified goat milk oligosaccharide was performed by chemical degradation, chemical transformation, spectroscopic techniques like NMR (^{1}H, ^{13}C and 2D NMR) and ES mass spectrometry. The sequence of monosaccharides and structure of isolated novel oligosaccharide Caprose was deduced as-

GalNAc-β-(1 → 3)-GalNAc-β-(1 → 6) Gal-β-(1 → 4) Glc

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

INTRODUCTION

Mammalians have a common feature that the females have mammary glands to produce milk during the lactation period (Tao et al., 2010). Milk is a complete food for mammalian

neonates and it has many nutritional and medicinal values (Singh et al., 2016). The medicinal value of milk is well reported in recent as well as in ancient medicinal literatures i.e., Ayurveda and Unani medicinal literature (Somvanshi et al., 2006). Milk is a rich source of bioactive oligosaccharides depends on the nature of their origin to which mammals they belongs (Miller et al., 1994 and Ranjan et al., 2015). The enormous biological activity of oligosaccharides such as immunostimulant, anti-tumour, anti-cancer, anti-inflammatory, anti-complementary (Saksena et al., 1999), antiviral, antimicrobial (Yang et al., 2012), antioxidant, hypoglycemic activity, lipid lowering (Halas et al., 2012) and regulation of mineral absorption are reported in medicinal literature (Poeikhampha et al., 2011). Goat milk contain riboflavin, phosphorous, vitamin B_{12}, protein and potassium. Goat milk also containis galacto-oligosaccharides which could be recommended for infant allergy and diseases. Goat milk shows therapeutic values for individuals with certain dietetic problems. 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 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). In the present study, we have studied the oligosaccharides content of goat milk. In continuation to our previous work on isolation of goat milk oligosaccharides (Kumar et al., 2016 and Srivastava et al., 2016) another novel milk oligosaccharide (Caprose) was isolated from the goat milk and then its structure was elucidated with the help of spectroscopic techniques like ^{1}H, ^{13}C, and 2D NMR i.e., HSQC, COSY, TOCSY and other techniques like chemical degradation, chemical transformation as well as ESI-MS 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 $25^{0}C$ 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^{o}C 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 “C”.

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 Caprose.

Description of Isolated Compound Caprose

^{1}H NMR: δ in $CDCl_3$ (ppm)

δ6.25(d,1H), δ5.66(d,1H), δ5.43(d,1H), δ5.28(m,2H), δ5.21(d,1H), δ5.08(m,1H), δ5.03(d,1H), δ4.98(d,1H), δ4.95(d,1H), δ4.50(d,1H), δ4.49(d,1H), δ4.43(d,1H), δ4.10(d,1H), δ4.08(d,1H), δ3.99(m,1H), δ3.82(m,2H), δ3.78(d,1H), δ3.53(d,1H), δ2.093(s,3H), δ2.058(s,3H), δ2.001(s,3H), δ1.947(s,3H), δ1.946(s,3H), δ1.944(s,3H)

^{13}C NMR: δ in $CDCl_3$ (ppm)

δ173.5, δ170.5, δ169.9, δ169.7, δ101.6, δ100.9, δ91.46, δ88.9, δ83.45, δ75.71, δ73.41, δ72.56, δ71.76, δ70.26, δ69.04, δ68.91, δ60.89, δ60.56, δ20.91, δ20.82, δ20.8, δ20.5, δ20.4

^{1}H NMR: δ in D_2O (ppm)

δ5.25(d,1H), δ4.70(d,1H), δ4.55(d,1H), δ4.48(d,1H), δ4.44(d,1H), δ4.07(d,1H), δ4.03(d,1H), δ4.01(d,1H), δ3.89(m,1H), δ3.82(d,1H), δ3.76(d,1H), δ3.72(d,1H), δ3.66(d,1H), δ3.62(m,2H), δ3.59(d,1H), δ3.57(m,1H), δ3.51(d,1H), δ3.49(m,2H), δ3.24(d,1H), δ2.01(s,3H), δ1.99(s,3H)

^{13}C NMR: in D_2O (ppm)

δ 173.0, δ102.8, δ101.57, δ95.7, δ91.8, δ81.6, δ 74.96, δ74.7, δ73.79, δ72.56, δ72.51, δ71.3, δ70.94, δ 70.59, δ 69.81, δ 69.26, δ68.53, δ 68.37, δ 62.06, 61.02, 60.48, 21.7, 21.3

ES-MS

771$[M+Na]^+$,748 $[M]^+$.

RESULT AND DISCUSSION

Compound "C" $C_{28}H_{48}O_{21}N_2$, gave positive Phenol-sulphuric acid test, Feigl test and Morgon-Elson test, indicating the presence of normal and amino sugar(s) in the moiety. The ^{1}H NMR spectrum of Caprose at 300 MHz exhibited five signals in the anomeric proton region as doublets at δ5.25 (1H), 4.70(1H), 4.53 (1H) and δ4.48 (1H) and δ4.44 (1H) for five anomeric protons indicating the presence of five anomeric protons in it. It was further supported by the appearance of five anomeric carbons at δ91.8 (1C), 95.7 (1C), 102.8 (2C), 101.5(1C) in the ^{13}C NMR spectrum of compound Caprose. These data led to the suggestion that "C" may be a tetrasaccharide in its reducing form .The presence of anomeric signals at δ5.25 and δ 4.70 for α and β glucose respectively showed that glucose unit was present at the reducing end. The four-monosaccharide units present in compound Caprose have been designated as S_1, S_2, S_3 and S_4 for convenience starting from the reducing end. The reducing nature of glucose was confirmed by the Methylglycosidation of Caprose by $MeOH/H^+$ followed by its acid hydrolysis led to the isolation of α and β methyl glucosides. Further, for confirmation of the monosaccharide constituents in it Caprose was hydrolyzed under strong acidic condition (Kiliani hydrolysis) followed by paper chromatography showed the presence of GalNAc, Gal, Glc moieties, which confirmed that these moieties has participated in the building of the compound "C".

The chemical shifts of anomeric carbons observed in ^{13}C NMR spectrum and anomeric protons observed in ^{1}H NMR spectrum of Caprose were also in agreement with the reported values of ^{1}H and ^{13}C anomeric chemical shifts of Glc, Gal and GalNAc. The presence of four monosaccharides and its molecular formula suggested that there may be two GalNAc, one Gal and one Glc unit was present in Caprose. To confirm the monosaccharide constituents and their sequence in Caprose, it was hydrolysed under mild acidic conditions (Mannich-Siewert method) followed by Paper Chromatography and TLC.

After five days paper chromatogram showed three spots, the faster moving spots was identical in mobility with authentic sample of GalNAc and the spot with lowest mobility was identical with unreacted: (1) further the compound with intermediate mobility may be the Trisaccharide (2), Further after seven days another spot was developed which was having faster in mobility then Trisaccharide (3) and its mobilitly was found identical to the authentic sample of lactose , which suggested that second sugar residue in sequence was also GalNAc (S_3) which was linked to lactose. The hydrolysis was partially completed in ten days and shows two more spot on TLC which were found identical with Galactose (S_2) and Glucose (S_1) on TLC and PC confirming the sequence of monosaccharide in Tetrasaccharide as GalNAc-GalNAc-Gal-Glc. The hydrolysis was completed in 20 days which led to the isolation of GalNAc, Gal and Glc and it was compared with authentic sample of GalNAc, Gal and Glc on paper chromatography. The ^{1}H NMR of Caprose not only supported the tetrasaccharide nature of 1 but also confirmed the free anomeric nature of glucose was further supported by presence of two anomeric proton signals as doublets and their coupling constants for αGlc δ 5.25 (1H J=3.6 Hz) and βGlc δ4.70 (1H J=8.4 Hz) respectively. Further the presence of another anomeric signal at δ 4.44 (1H) J=8.1 Hz suggested the presence of β Gal moiety in Caprose led to the suggestion for the presence of lactose molecule in it. This was further confirmed by β Glc (S_1) H-2 signal (a structural reporter group) which appeared as a triplet at δ3.28 (J=8.4Hz). Further, another anomeric protons appeared at δ4.36 (J=7.4Hz) along with signal of NHAc group at δ1.996 was due to the presence of GalNAc which is present next in sequence with lactose moiety. The linkage of GalNAc to the lactose moiety GalNAc (S_3) [1→6] β-Gal (S_2) was confirmed by acetylated ^{1}H-^{1}H COSY and TOCSY spectrum of Caprose, in which H-6 of Gal (S_2) appeared upfield in the region δ 3.82 ppm and H-2, H-3 and H-4 of Gal appear at δ4.47, δ 5.11 and δ 5.24 respectively, these data confirms that C-2, C-3 and C-4 positions of Gal (S_2) were not involved in glycosidic linkages with β-GalNAc (S_3) which was also supported by structural reporter group. This was also confirmed by the HSQC spectrum of acetylated compound "C" in HSQC spectrum the H-6 proton of Gal (S_2) present at δ 3.82 in ^{1}H axis and its cross peak with the ^{13}C axis was present at (δ3.82 x δ 73.4). The fourth anomeric proton of Caprose which also appeared as a doublet at δ 4.50 (1H, J=8.4 Hz) along with a singlet of three protons at δ 2.01 showed the presence of another β-GalNAc (S_4) in Caprose, which was glycosidically linked to the C-3 of GalNAc (S_3). This linkage GalNAc (S_4) [1→3] β-GalNAc (S_3) was confirmed by the acetylated ^{1}H-^{1}H COSY and TOCSY spectrum of Caprose, which showed H-4, and H-6 proton signals at δ5.28, and δ5.03 respectively but H-3 proton was present at δ3.88 .The HSQC spectrum also confirms that the H-3 proton of S_3 was gave cross peak at (δ3.88 x δ72.56) which indicated that GalNAc (S_4) glycosidically linked to S_3 at C-3 position by a glycosidic linkage.

The chemical shifts of the anomeric carbons of compound "C**"** at δ91.8 (1C, α-Glc), 95.7 (1C, β-Glc), 101.5 (1C, β-Gal) and δ102.98 (2C, β-GalNAc) are in accordance with the anomeric carbon values of Glc, Gal and GalNAc present in "C". The tetrasaccharide nature of Caprose was further confirmed by spectral studies of acetylated derivative of Caprose. The heteronuclear single quantum-coherence (HSQC) spectrum of acetylated product of compound "C" confirms the anomeric assignments in ^{1}H and ^{13}C NMR spectra of "C" which showed the ^{1}H and ^{13}C cross peaks of α-Glc (S_1) at δ6.25 x δ88.9 and β-Glc (S_1) at δ5.6 x δ91.4.

It also contains three crosspeaks of one β- Gal (S_2) at δ4.43 x δ101.6, and two β-GalNAc moieties (S_3) & (S_4) present at δ4.50 x δ100.9 and δ4.49 x δ100.9 respectively. The glycosidic linkage were assigned by the cross peaks for glycosidically linked carbon with their proton in HSQC spectrum of Caprose acetate. The values of these cross peaks were as β-Glc(S_1) H-4 and C-4 at δ3.822 x δ73.41 confirms (1→4) linkage between S_1 and S_2 , β- Gal (S_2) H-6 and C-6 at δ 3.789 x δ 73.41 confirms (1→6) linkage between S_2 and S_3 , β-GalNAc (S_3) H-3 and C-3 at δ 3.776 x δ 75.41 confirms (1→3)linkage between S_3 and S_4 respectively. ^{1}H-^{1}H COSY spectrum confirms the assignment of ring proton involved in linkage at δ3.822 (4-position) for β-Glc(S_1) δ 3.789 (6- position) for β- Gal (S_2), δ 3.776 (3- position) for β-GalNAc (S_3) and it was further confirmed by the presence of same chemical shifts in TOCSY spectrum. The respective values of ring proton involved in glycosidic linkage in H-4 of (S_1) , H-6 of (S_2) and H-3 of (S_3) arises at δ3.822, δ 3.789 and δ 3.776 respectively in the ^{1}H NMR of Caprose. Based on the pattern of chemical shift of ^{1}H, ^{13}C, HOMOCOSY, TOCSY and HSQC NMR experiments, it was interpreted that the compound "C" was a tetrasaccharide having one Glc, one Gal and two GalNAc moieties and compound was interpreted as linear tetrasaccharide having GalNAc-β- (1-3)-GalNAc-β-(1-6) Gal-β-(1-4) Glc structure. The Electrospray Mass Spectrometric data of compound not only confirmed the derived structure but also supported the derived sequence of monosaccharides in Caprose. The highest mass ion peaks was recorded at m/z 771 which was due $[M+Na]^+$, the other mass ion peak recorded at m/z 748 which was due to $[M]^+$ confirming the molecular weight of Caprose as 748 and is in agreement with its molecular formulae. Further the mass fragments were formed by repeated H transfer in the oligosaccharide and was accompanied by the elimination of terminal sugar less water. This fragmentation path way also confirmed the sequence of monosaccharides in the tetrasaccharide. The tetrasaccharide m/z 748 (I) fragmented to give mass ion at m/z 545 (II), this fragment corresponded to the loss of terminal GalNAc (S_4) moiety from tetrasaccharide [748-S_4], indicating the presence of GalNAc (S_4) at the non-reducing end. It further fragmented to give mass ion peak at m/z 324 (III) with loss of another GalNAc moiety less water from Trisaccharide [324-S_3 -H_2O] this further fragmented to give mass ion peak at 161 (IV) These three mass ion peaks II, III, IV are appeared due to the consequent loss of GalNAc, GalNAc and Gal from original molecule. The other fragmentation pathway in ES Mass spectrum of compound C at m/z 748 shows the mass ion peak at m/z at 627[M-$NHCOCH_3$], 575[748-3H_2O-2OH], 545[748-S_4], 545[603-$NHCOCH_3$]. The mass ion fragment at m/z 545 (II) was also supported by its respective fragment at m/z 495[545-CH_3OH], in another pathway 440[545-CH_2OH-CH_3OH-CH_2C=O], 365[440-$NHCOCH_3$-OH], 422[440-H_2O], 391[422-CH_2C=O]. In third way of fragmentation pattern of m/z 545 gave mass ion fragments at m/z 441[545-2CH_2OH-CH_2C=O], 346[441-CH_2OHCHO-H_2O-OH] and in fourth fragmentation the fragments m/z at 487[545-$NHCOCH_3$], 455[487-CH_3OH], 407[455-CH_2OH-OH], 406[455-CH_3OH-H_2O], 371[406-H_2O-OH], 303[371-4OH], 225[303-CH_2OHCHO-H_2O], 222[303-CH_2OH-CH_3OH-H_2O], 260[324-2CH_3OH], 225[260-H_2O], 169[262-CH_2OHCHO-CH_3OH] respectively. 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 Caprose and acetylated Caprose, the structure and sequence of isolated Novel oligosaccharide molecule Caprose was deduced as-

GalNAc-β-(1 ➝ 3)-GalNAc-β-(1 ➝ 6) Gal-β-(1 ➝ 4) Glc

Compound Capriose

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