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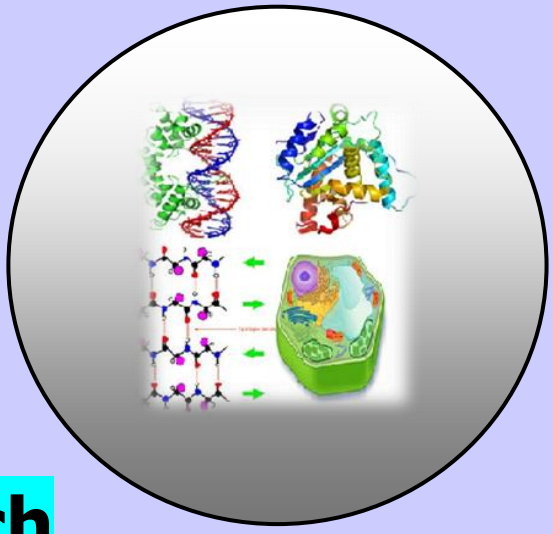

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RESEARCH PAPER

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Isolation and Structure Elucidation of Novel Milk Oligosaccharide from Shyama Dhenu (Black Cow) Milk

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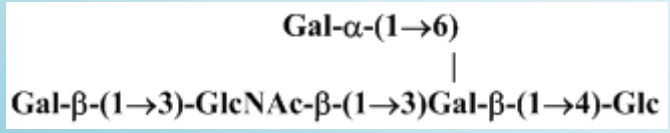
ABSTRACT

Milk is an essential bioactive fluid containing mainly lactose, lipids and oligosaccharides which are responsible for growth and development of mammalian neonates. Oligosaccharides represent a group of bioactive molecules which are present in plant, bacteria and milk. Oligosaccharides having biological importance have been isolated from milk of different origin like human, cow, goat, buffalo, donkey etc that have shown antitumor, anticancer, antigenic and immuno stimulant activities. In all type of mammalian's milk, cow milk is as a drink that provides vitality and inner strength to fight diseases. By considering the physiological, biological & medicinal importance and ancient uses of Shyama Dhenu (black cow) milk which is described in our Ayurvedic system and Charak Sanhita and to search more novel biologically active milk oligosaccharide, Shyama Dhenu milk was collected and processed by Kobata and Ginsburg method followed by gel filtration, HPLC and CC which resulted in the isolation of a novel milk oligosaccharide, namely Dicusose structure of which was established by using ^{1}H NMR, COSY techniques and FAB mass spectrometry as-



Keyword: Shyama Dhenu milk, oligosaccharide, Kobata and Ginsburg.

INTRODUCTION

Milk is the food for neonates, which contains a variety of essential components including proteins, lipids, carbohydrates, and minerals, that fulfill the nutritional needs for growth and development of any mammalian newborns (A. K. Singh. et.al. 2015). Oligosaccharides

isolated from milk have shown structural homology with the carbohydrates carried by glycoproteins and glycolipids on cell surface and also play an essential role in many molecular processes impacting eukaryotic biology and diseases. Oligosaccharides having biological importance have been isolated from milk of different origin like Human, Bovine, Goat, Buffalo, Donkey etc. which of them have shown antitumor, anticancer, antigenic and immuno stimulant activities. N-acetylneuramine lactose sulphate/ plays an important role in the nutrition of the rat pups, which is the dominant oligosaccharide in the Dog milk **(**A. Bubb William et.al 1999). Ability to stimulate non-immunological resistance of the host against parasitic infections has found in Buffalo Milk oligosaccharides (D. Deepak et.al. 1998). Oligosaccharide components of human milk are known to protect breast fed infants from a host of bacterial infection. 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 (P. Johansson et.al. 2005). Cow milk oligosaccharides reduce the adhesion of enterotoxic Escherichia coli strains of the calf (AR Euler et.al. 2005). Human milk oligosaccharides affect the gastrointestinal flora of infants. Previous studies in adults have demonstrated that fructo-oligosaccharides increase potentially beneficial fecal bacteria, including bifid bacteria (AR Euler et.al. 2005, S Fanaro et.al. 2005). Indian Physicians and Ancient Vedas have stated that cow milk is desirable and preferred diet in all types of ailments. There are a number of Mantras in all four Vedas that describe the importance of cow milk not only a complete food but also a curative drink. Apart from curative properties described in ancient medicinal system, cow milk is extensively used in infant nutrition as best alternative to human milk, till date. There are many scriptures which show the importance and value of cow milk for human life. The medicinal importance of cow milk particularly the black cow is very well defined in Ayurveda. Cow milk used for the immuno stimulant, nourishes the body tissues, acts as natural aphrodisiac, does rejuvenation and improves intelligence, in heart diseases and leucoderma, increase breast milk in feeding mother, assists in easy movement of intestine and bleeding disorders. Keeping in mind the ancient uses and recent chemical investigation of cow milk, we have chemically investigated the oligosaccharides contents of Shyama Dhenu milk for the biological activities. For this milk of Shyama Dhenu was collected in bulk and processed by method of Kobata and Ginsburg (Kobata et.al. 1970). In continuation to our previous work on isolation of Shyama Dhenu milk oligosaccharides (Gunjan et.al. 2016) another novel milk oligosaccharide was isolated from the black cow's milk and then its structure was elucidated with the help of chemical degradation, chemical transformation and spectroscopic methods like ^{1}H NMR, and 2DNMR i.e. COSY techniques as well as FAB mass spectrometry.

MATERIALS AND METHOD

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 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 CARLO-ELBA 1108 an elemental analyzer. The sugars were visualized on TLC with 50% 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 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 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 diode array detector and G.P. 100 printer plotter. Authentic samples of glucosamine, galactosamine, galactose and glucose were purchased from Aldrich Chemicals.

Isolation of Black Cow milk oligosaccharide by Kobata and Ginsberg method

12 liters cow milk was collected from a black cow and then its oligosaccharides isolated by Kobata and Ginsberg method. For this method, milk was stored at -20°C and centrifuged for 15 min at 5000 rpm at 4°C. The solidified lipid layer was removed by filtration through glass wool column in cold. Ethanol was added to clear filtrate to a final concentration of 68% and the resulting solution was left overnight at 0°C. The white precipitate formed, mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0°C. The supernatant and washings were combined and filtered through a microfilter and lyophilized affording crude oligosaccharide mixture (120g). The lyophilized material responded positively to Morgan-Elson test (S.M. Partridge et.al. 1948) and thiobarbituric acid assay suggesting the presence of N-acetyl sugars 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 (M. Dubois et.al. 1956) for the presence of neutral sugar.

Acetylation of Black Cow milk oligosaccharide mixture

Dry oligosaccharides of pooled fractions (12 gm) which gave positive phenol-suphuric acid test were acetylated with pyridine (12ml) and acetic anhydride (12ml) at 60° C for 24 hr. The mixture was evaporated under reduced pressure and viscous residue was taken in $CHCl_3$ and washed in sequence with 2 N 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 (15.5g). Non-polar acetyl derivative of oligosaccharides 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

Separation and purification of acetylated derivative were 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 twelve fractions namely I (259mg), II (92mg), III (164mg), IV (2.05gm), V (1.95gm), VI (2.82gm), VII (120mg), VIII (286mg), IX (726mg), X (187mg), XI (342mg) and XII (55mg) respectively. These fractions were containing mixture of two to three compounds. Repeated column chromatography of fraction VI led to the isolation of one chromatographically pure compound "E" (47mg).

Deacetylation of Compound

Deacetylation of acetylated oligosaccharide "E" (47mg) was carried out in 2ml acetone and 13ml NH_3 for 24hrs in a stoppered hydrolysis flask. After 24hrs ammonia was removed under reduced pressure, equal volume of $CHCl_3$ and water were added and the compound was recovered in the aqueous phase and the water layer was finally freeze dried giving the deacetylated oligosaccharide Dicusose (27mg).

Description of Isolated Compound Dicusose

^{1}H NMR: δ in D_2O (ppm)

δ 1.99 (s,3H, $NHCOCH_3$), δ3.29 (t,1H, J=8.4 Hz), δ4.12 (d,1H J= 2.7Hz), δ4.45 (d,1H J=7.8Hz), δ4.50 (d,1H, J=7.5 Hz), δ 4.67 (d,2H, J=8.1Hz), δ5.20 (d,1H J=3.3Hz), δ5.25 (d,1H J=3.9Hz).

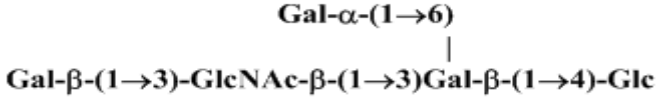
FAB-MS

1565, 1507, 1434, 1347, 1320, 1275, 1234, 1211, 1153, 1129, 1069, 1043, 981, 924, 880, 867, 803, 731, 718, 691, 617, 595, 577, 556, 535, 493, 460, 391, 289, 247, 229, 169, 107.

RESULT AND DISCUSSION

Compound E $C_{32}H_{55}NO_{26}$ gave positive Phenol sulphuric acid test (M. Dubois et.al. 1956), Fiegl test (F. Fiegl 1975) and Morgan-Elson test (S.M. Partridge et.al. 1948), showing the presence of normal and amino sugars in the compound. The ^{1}H NMR spectrum of compound E at in 300 MHz exhibited five doublet for six anomeric protons signal at δ 4.45(1H), 4.50(1H), 4.67(1H), 4.67(2H), 5.20(1H) and 5.25(1H) indicating that the compound E may be a pentasaccharide in its reducing form. The five monosaccharides present in compound E have been designated as S_1, S_2, S_3, S_4, and S_5 for convenience, starting from the reducing end. Methylglycosidation of E by MeOH/H^+ followed by its acid hydrolysis led to the isolation of α and β-methyl glucoside, Gal and GlcNAc which suggested the presence of glucose at the reducing end of the oligosaccharide. The reducing and free nature of glucose was further supported by the two anomeric proton signals as doublet and their coupling constant, for α and β-Glc (S_1) at δ 5.25 (J= 3.9 Hz) and δ 4.67 (J= 8.1 Hz) respectively. The presence of a lactosyl moiety i.e. Gal β (1→4) Glc in the compound E was also indicated by the two doublets of anomeric protons resonating at δ 4.45 (J=7.5 Hz) and δ 4.67 (J= 8.1) Hz for β-Gal (S_2) and β-Glc (S_1) residues respectively present in lactosyl moiety. The presence of a lactosyl unit was further substantiated by presence of β-Glc (S_1) H-2 signal (a structural reporter group) which appeared as a triplet at δ 3.29, J= 8.4 Hz (Gronberg et. al. 1992). The typical downfield shifted signal of H-4 proton of β-Gal (S_2) which appeared as a doublet at δ 4.12, J = 2.7 Hz, confirmed that β-Gal (S_2) is 3,6-di substituted and is substituted at C-3 by a GlcNAc (S_3) moiety (G. Gronberg et.al. 1992, V.K. Dua et.al. 1983). This was supported by the presence of anomeric proton signal of S_3 (Glc-NAc) as doublet at δ 4.67 (J= 8.1 Hz) which was overlapped with the anomeric proton signal of βGlc (S_1).

The singlet at δ 1.99 confirmed the amide methyl of N-acetyl glucoamine. All the above assignments have resemblance with the chemical shift and splitting pattern of the ^{1}H NMR of LNT (V.K. Dua et.al. 1983). The fourth anomeric proton doublet resonating at δ 4.50(J=7.5) was assigned to another β-Gal (S_4). On the basis of its chemical shift comparison with LNT, which helped in its assignment loading to the conclusion that Gal (S_4) was present as the fourth monosaccharide unit after GlcNAc (S_3) and is linked to GlcNAc (S_3) at C-3 position, confirming the 1→3 linkage between S_4 and S_3(G. Strecker et al 1992, V.K. Dua et.al. 1983). All these assignment were also confirmed by the 1H-1H COSY spectrum of acetylated compound E. The ^{1}H NMR spectrum of E also contained a fifth anomeric proton signal appearing as a doublet in the downfield region at δ 5.20(J=3.3 Hz) which indicated the presence of a terminal α-Gal (S_5) (T. Urashima et.al. 1997, T. Urashima et.al. 1999) residue. On the basis of above assignment and the indication of the presence of a 3, 6-disubstituted β-Gal (S_2) residue, it is interfered that the α-Gal (S_5) unit is attached at the C-6 position of the β-Gal (S_2). The sequence of the monosaccharides in the pentasaccharides was further established on the basis of FABMS analysed of the acetylated compound E. The FAB mass spectrum of acetylated compound E helped in substantiating the sequence of monosaccharide units in it. It showed the highest mass ion peak at m/z 1565 as $[M+Na+H]^+$, which was in agreement with the expected molecular ion at m/z 1540 and derived molecular formula ($C_{64}H_{88}N_2O_{41}$). The mass ion fragment at m/z 331 showed the presence of terminal hexosyl moieties present in the pentasaccharide (H. Egg et.al. 1983). The molecular ion at m/z 1541 further fragmented to give mass ion fragment m/z 1211 (S_1-S_2-S_3-S_4) with the loss of terminal αGal residue from the non reducing terminal. Consequent loss of a $NHCOCH_3$ from fragment ion at m/z 1211(S_1-S_2-S_3-S_4) leading to the fragment ion at m/z 1153 showed the presence of an amino sugar in the tetrasaccharide unit. The fragment ion at m/z 1211 further fragmented to the give fragment ion at m/z 880, consisting of trisaccharide S_1-S_2-S_3, formed by the loss of terminal β-Gal unit. It is supported by the formation of fragment ion at m/z 954 (S_1-S_2-S_5) product by the cleavage of the glycosidic bond at N-acetylglucosamine residue (S_3) starting from the non reducing terminal of pentasaccharide i.e. [M-(S_3-S_4)] with its complimentary ion of the loss of terminal S_5, S_4 and S_3 confirmed that the Gal residue of the lactosyl moiety (S_1-S_2) of the pentasaccharide, is 3,6-disubstituted. The FAB mass spectrum of compound E also contained other mass ion peaks at m/z 1507 [1565-$NHCOCH_3$], 1434 [1507 –CH_2=C=O, -OCH_3] 1347 [1507-2CH_3COO, -CH_2=C=O], 1320 [1507-2CH_2=C=O, -CH_3COOH, -OCH_3], 1275 [1434, -$NHCOCH_3$, -CH_2=C=O, -OCH_3], 1234 [1565-Gal], 1172 [1275$CH_3CO(OH)COCH_3$], 1129 [1172-CH_3CO], 1069 [1129-CH_3COOH], 1043 [1129-2CH_3CO], 981 [1234-3CH_3COOH, -OCH_3, -CH_2=C=O], 903 [1234-Gal], 803 [981- CH_3COOH, -2OCH_3], 731 [981- 3CH_3COO, -OCH_3, -CH_2=C=O], 718 [803-CH_3CO, -CH_2=C=O], 595 [M-S_5,S_4,S_3], 577 [M-S_5,S_4,S_3], 535 [577- CH_2=C=O], 493 [535-CH_2=C=O], 391 [493-CH_3COOH, -CH_2=C=O], 247 [M-S_5,S_4,S_3,S_1], 229 [331-CH_3COOH, -CH_2=C=O], 187 [229-CH_2=C=O], 169 [229-CH_3COOH], 109-CH_3COOH]. This fragmentation also supported the formation of various fragment ions of the acetylated pentasaccharide. On the basis of results obtained from physico-chemical techniques and chemical transformation, the structure of compound pentasaccharide was determined as-



Dicusose

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