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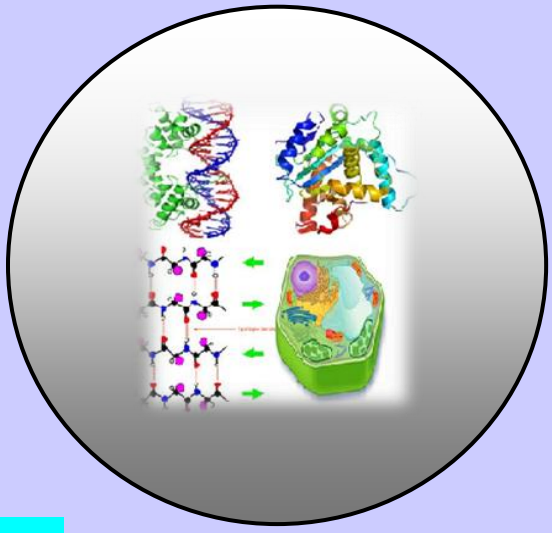

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RESEARCH PAPER

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

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ABSTRACT

Milk is a unique bio-fluid mainly composed of lactose, lipids and oligosaccharides. Milk Oligosaccharides show important role for establishing the gut flora of infants. Numbers of biologically active Oligosaccharides have been isolated from various milk sources which have shown various biological activities such as anti-tumor, anti-inflammatory, anti-cancer, anti-microbial activities. In Indian medicinal system like Ayurveda and Charak Shastra, medicinal importance of black cow (Shyama Dhenu) is described. In order to search for more novel biologically active milk oligosaccharide, Black cow (Shyama Dhenu) milk was collected and processed by Kobata and Ginsburg method followed by gel filtration, HPLC and CC which resulted into the isolation of a novel milk oligosaccharide, namely INDICOSE structure of which was established by using 1D (1H, 13C) and 2D NMR (COSY, TOCSY, HSQC) techniques as well as mass spectrometry as

INDICOSE
Keyword: Bovine milk, oligosaccharide, Kobata and Ginsburg.

INTRODUCTION

Milk is secreted by all species of mammals to supply nutrition and immunological protection to the mammalian neonates. It performs these functions with a large array of distinctive compounds (Wang N P. et.al., 1988). The major components of milk are carbohydrates, lipid, proteins, fats, immunoglobins etc. Each component is present in a specific amount and have specific functions. One of the major component is carbohydrate contain lactose and oligosaccharide. So, the oligosaccharides are a class of bioactive macromolecule found in mammalian milk that are receiving a lot of commercial attention. These complex carbohydrates (oligosaccharides) are known to be responsible for the beneficial effects of

breastfeeded newly borns and perform a number of bioactive functions including prebiotic enrichment of a protective micro biota, limiting the virulence of several pathogens and increasing postnatal neural development (Barile D. et al., 2009) and also inhibit the adhesion of pathogenic micro organism to the intestinal and urinary tract by acting receptor analogues to preventing gastric and urinary infections. So these oligosaccharide exhibits varied biological activity such as a anti-inflammatory, anti-tumor, antithrombotic, immunostimulant, anti-cancer, antiviral, antimicrobial and cardioprotective activities (Ehresmann et al., 1979, Yamada and Haruki 1986, Piere 1982). Moreover, these oligosaccharides have been isolated from various mammalian milk of different origin e.g. buffalo, equine, caprine, elephant, donkey, rat, goat, camel and human etc. The bovine milk is a source of simple as well as complex oligosaccharides. Recent research has demonstrated that bovine milk contains oligosaccharide that are analogues to Human milk oligosaccharide (HMO), suggesting a similar protective and nutritional role in a immune system of infants and human being (Zivkovic A.M. et al., 2011). Bovine milk oligosaccharide (BMO) is an inhibitor of the binding of this toxin to the intestinal mucosa in the suckling young of these species. There are many scriptures which shows 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. It is said that it improves vitality and immunity, nourishes the body tissues, acts as natural aphrodisiac, does rejuvenation and improves intelligence, increase breast milk in feeding mother, assists in easy movement of intestine and bleeding disorders. Like as, according to Rigveda, Cow milk is Amrita, protects human being from diseases, its milk have the curative and prophylactic effects. Charak sutradhan shows that Cow milk as a drink provides vitality or OJA in man, the inner strength to fight diseases. It is a complete diet which gives the right thinking power or wisdom. It gives us tranquility and cheerfulness. Indian ancient Physician Dhanvantri stated that it protects the human body from vata, pitta, heart diseases and leucoderma. Various studies supported the beneficial effects of supplementation of bovine milk in diarrhea in persons with immune- deficiency syndrome, NSAID-induced gastrointestinal disturbances. Keeping this in mind the milk of shyama Dhenu was collected in bulk and processed by method of Kobata and Ginsburg for isolation of novel milk oligosaccharide (Kobata A. et al., 1970). After following the process, a novel milk oligosaccharide was isolated from the cow's milk and then its structure was elucidated with the help of chemical degradation, chemical transformation and spectroscopic method like ^{1}H NMR, ^{13}C NMR and 2DNMR 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 $25^{0}C$ 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 5ul 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, glucose, fucose and silalic acid were purchased from Aldrich Chemicals.

Isolation of Shyamadhenu (Black Cow) milk oligosaccharide by Kobata And Ginsberg method-

12 liter cow milk was collected from a Shyamadhenu and then isolated by method of Kobata and Ginsberg method (Kobata A et al. 1970). 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.

The lyophilized material responded positively to Morgan-Elson test (Partridge S.M. et al., 1948) and thiobarbituric acid assay suggesting the presence of N-acetyl sugars and sialic acid 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 (Dubois M. et al., 1956) for the presence of neutral sugar.

Acetylation of Shyamadhenu Cow milk oligosaccharide mixture

Dry oligosaccharides of pooled fractions (12 gm) were acetylated by treatment with pyridine (12ml) and acetic anhydride (12ml) at 60° C for 24 hr. Further the, reaction 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

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 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 A (62mg).

Deacetylation of Compound

Deacetylation of acetylated oligosaccharide A (62mg) was carried out in 2ml acetone and 13ml NH_3 for 24hr in a stoppered hydrolysis flask. After 24 h 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 Indicose (36mg).

Description of Isolated Compound Indicose

^{1}H NMR: δ in D_2O (ppm)

δ5.16(d,1H J= 3.9Hz), δ4.60(d,1H J=8.1 Hz), δ4.45(d,1H J= 7.5Hz), δ4.39(d,1H J=7.5Hz), δ4.38(d,1H J=7.5 Hz), δ3.98(t,1H J=5.1Hz), δ3.86(d,1H J=2.7Hz), δ3.22(t,1H J=8.7Hz), δ1.94(s,3H $NHCOCH_3$), δ1.86(s,3H $NHCOCH_3$)

^{13}C NMR: δ in D_2O (ppm)

δ173.4, δ 102.6, δ 95.5, δ 91.6, δ 78.5, δ 78.1, δ77.9, δ76.00, δ75.1, δ 74.8, δ 74.6, δ 74.1, δ 73.6 , δ 72.3, δ 71.5, δ 71.1, δ 70.8, δ 70.7, δ 69.9, δ 69.5, δ 69.1, δ 68.8, δ 68.3, δ 68.2, δ 61.8, δ 60.8, δ 60.3, δ 56.4.

FAB-MS

810 $[M+Na+K]^+$, 787 $[M+K]^+$, 771$[M+Na]^+$ and other fragment ions at 774, 742, 714, 686, 656, 632, 625, 614, 603, 583, 566, 538, 508, 413, 406, 391, 365, 345, 329, 279, 244, 242, 222, 209, 192, 180, 149, 107.

RESULT AND DISCUSSION

Compound A $C_{28}H_{48}O_{21}N_2$ gave positive Phenol sulphuric acid test(Dubois M et al., 1956), Fiegl test (Fiegl F. et al., 1975) and Morgan-Elson test(Partridge S.M. et al., 1948), showing the presence of normal and amino sugars in the compound.^{1}H NMR spectrum in D_2O at 300 MHz showed five anomeric proton signal at δ 5.16(1H), δ 4.60(1H), 4.45(1H), 4.39(1H), 4.38(1H) for five protons leading to the presence of five anomeric proton in it. It was further supported by the appearance of three signals for five anomeric carbons at δ 102.6(3C), 95.5(1C) and 91.6(1C) in the ^{13}C NMR spectrum. These data led to the suggestion that it may be a tetrasaccharide in its reducing form. The FAB mass spectrum of compound Indicose showed the highest mass ion peak at m/z 771 $[M+ Na]^+$, m/z 787$[M+K]^+$, and m/z 810 $[M+Na+K]^+$ quasi molecular ion peak, which was indicative of the composition $C_{28}H_{48}O_{21}N_2$ with the molecular ion expected at m/z 748 for a tetrasaccharide. The four monosaccharide units present in compound Indicose have been designated as S_1, S_2, S_3 and S_4 for convenience starting from the reducing end. The acid hydrolysis of compound Indicose gave three spots on the paper chromatography, which were identified as Glc, Gal and GlcNAc by co-chromatography with authentic samples. Methylglycosidation of compound by $MeOH/H^+$ followed by its acid hydrolysis led to the isolation of α and β–methyl glucoside, GlcNAc and Gal which suggested the presence of glucose at the reducing end in the oligosaccharide. 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 δ 5.16(1H)(J= 3.9Hz) and δ 4.60 (1H)(J= 8.1 Hz) respectively. Further another anomeric proton doublet at δ 4.39 (J=7.5Hz) was due to presence of β Gal unit suggesting the presence of Lactosyl moiety into compound Indicose.

The presence of Lactose moiety was further confirmed by β Glc(S_1) H-2 signal (a structural reporter group) which appeared as a triplet at δ 3.22, (J=8.7Hz) (Dua, and Bush 1983, Gronberg G et al., 1992 and Dorland L et al., 1977). Further, the ^{1}H NMR spectrum showed two other anomeric proton signals appearing as a doublet at δ4.45 (1H) (J=7.5 Hz), 4.38 (1H)(J=7.5 Hz) along with two singlets of three protons at δ 1.94 and δ1.86 which were assigned to two NHAc groups, thereby confirming the presence of two GlcNAc moieties in the compound Indicose. Thus the first anomeric proton of GlcNHAc which appeared at δ 4.45 was due to third monosaccharide (S-3) present in the tetrasaccharide Indicose was linked to Gal (S-2). The absence of a downfield shifted H-4 proton resonance of β-Gal (S_2) which appeared as a doublet at δ3.86 (J=2.7Hz), instead of the downfield shifted chemical shift shown by this proton in the range δ 4.13-4.15 ppm (Gronberg G et al., 1992) confirmed that β-Gal (S_2) was not substituted at C-3 by a GlcNAc (S_3) moiety, this implies that the GlcNAc was 1-6 linked to Gal (S-2)(Chaturvedi P and Sharma C.B. et al., 1988). The large coupling constant (7.5Hz) of the anomeric proton of GlcNAc indicated that GlcNAc (S-3) unit was β linked to S_2 of lactosyl moiety by β glycosidic linkage. The fourth anomeric proton which appeared at δ4.38 (7.5 Hz) as a doublet confirmed the presence of another GlcNHAc moiety in the Indicose. The linkage between S_3 and S_4 was established on the basis of presence of GlcNAc H-3 proton of S_3, appearing as a triplet in the region δ 3.98 which indicates that GlcNAc (S_3) was substituted at 3- position by another β-GlcNAc (S_4), which was the fourth monosaccharide present at the non-reducing end of the tetrasaccharide. The large coupling constant of 7.5 Hz of anomeric proton implies that it was also linked by a β glycosidic linkage to S-3 of tetrasaccharide. All the ^{1}H NMR assignments for structural reporter protons of monosaccharide units of compound Indicose were confirmed by HOMOCOSY spectrum. The chemical shifts of the anomeric carbons of compound Indicose at δ 91.6 (1C, α-Glc), 95.5 (1C, β-Glc) and 102.6 (3C, 2β-GlcNAc, 1β-Gal) present in the ^{13}C NMR spectrum are in accordance with the anomeric carbon values of Glc, Gal and GlcNAc (Bush C.A. et al.,1985). The tetrasaccharide nature of compound Indicose was further confirmed by the spectral studies of acetylation product of compound Indicose, which contained twelve singlets of methyl protons of acetyl groups in its ^{1}H NMR spectrum besides the signals of ring protons and anomeric protons present in acetate. HSQC spectrum of acetylated compound Indicose confirmed anomeric assignments in ^{1}H and ^{13}C NMR spectra of compound by showing the ^{1}H and ^{13}C cross peaks of α-Glc (δ6.25× δ88.8) and β-Glc (δ5.68×91.3). It also contains a cross peak of one β-Gal and two β-GlcNAc moieties at δ4.5× δ100.7 (1C), 4.5× δ101.0 (2C) respectively. It also showed presence of three cross peaks for four glycosidically linked carbons at δ3.90 ×δ70.3, δ3.79× δ73.3, δ3.85× δ75.6, δ3.82× δ75.5. Based on the pattern of chemical shift of ^{1}H, ^{13}C, HOMOCOSY, TOCSY and HSQC experiments, it was interpreted that the compound Indicose was a tetrasaccharide having structure.

The FAB mass spectrum of Indicose helped in substantiating the sequence of monosaccharide units in it. The highest mass ion peaks were recorded at m/z 787, 771 and 810 which were due to $[M+K]^+$, $[M+Na]^+$ and $[M+ Na +K]^+$ respectively, confirming the molecular weight of Indicose as 748. The mass ion at m/z 810 further fragmented to give fragment ion at m/z 566[S_1-S_2-S_3], which was produced by cleavage of the glycosidic bond at N-acetylglucosamine residues at the non reducing terminal of the tetrasaccharide i.e. M-S_4 indicating the presence of GlcNAc (S_4) at the non reducing end.

Subsequent loss of a $NHCOCH_3$ from fragment ion at m/z 566 [S_1-S_2-S_3] leading to ion at m/z 508 confirms the presence of another amino sugar in the tetrasaccharide. It was further supported by the formation of mass ion peaks at m/z 774[810-2H_2O], 714[774-CH_2OHCHO], 656[714-$NHCOCH_3$], 625[656-CH_2OH], 583[625-CH_2=C=O], 523[583-CH_2OHCHO], 465[523-$NHCOCH_3$], 430[465-H_2O-OH], 413[430-OH], 329[413-2CH_2=C=O], 192[329-CH_2OHCHO-CH_2=C=O-H_2O-OH]. The pseudomolecular mass ion fragment at m/z $[M+K]^+$ further fragmented to produce mass ion m/z 583 and m/z 345 by the successive loss of two GlcNAc residues , confirming the presence of two GlcNAc units in the tetrasaccharide. Similarly pseudomolecular mass ion fragment at m/z 771 $[M+Na]^+$ fragmented to give mass ion fragment at m/z 406(S_3-S_4) and m/z 365 (S_1-S_2) which were assigned to the two disaccharide units produced predominantly by cleavage of the glycosidic bond between S_2 and S_3 at N-acetylglucosamine residues which further confirming that the two GlcNAc (S_3& S_4) residues were glycosidically linked and lactosyl moiety was present at the reducing terminal of the tetrasaccharide. This fragmentation pathway confirmed the sequence of monosaccharides in the oligosaccharide. It was further supported by the formation of mass ion peaks at m/z 345 [406- CH_2OH-CHOH], 329[365-2H_2O], 329[406- CH_2OHCHO-OH], 279[345-2CH_3CO]. The FAB mass spectrum of compound Indicose also contained other mass ion peaks at m/z 742[771-CHO], 686[M-2CH_2OH], 632 [M-2$NHCOCH_3$], 614[632-H_2O], 583[614-CH_2OH], 566[810-GlcNAc-H_2O], 465[686-GlcNAc-H_2O], 430[465-H_2O-OH], 244 [465-GlcNAc-H_2O], 209[244-H_2O-OH], 167[209-CH_2=C=O], 149[180-CH_2OH], 107[149-CH_2=C=O] and many other fragment ion peak are obtained from the further fragmentation of various fragment ions. On the basis of results obtained from physico-chemical techniques and chemical transformation, the structure of compound Indicose was determined as-

Indicose

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