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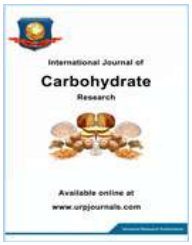

## Original Article

# Isolation of Novel Oligosaccharide from *Bos indicus* (Black Cow) Milk and their DFT Studies

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**Abstract**

The carbohydrate content of milk is comprised of lactose and oligosaccharide. The oligosaccharides present in milk have numerous biological activities such as antitumor, anticancer, antiviral, anti-inflammatory, anticoagulant, antioxidant, antihepatitis, antihypertensive, antifungal, antibacterial, antimicrobial and immunostimulant activities. Cow milk protects the human body from heart diseases and leucoderma. Keeping in mind the biological activity of Shyama dhenu milk and oligosaccharide present therein, it was collected in bulk and was processed by method of Kobata and Ginsburg for obtaining its oligosaccharides content. During the process the milk was deproteinated, filtered by microfilter and lyophilized followed by the gel filtration, HPLC, column chromatography and thin layer chromatography, which resulted into the isolation of a novel milk oligosaccharides namely **Indinose**. The stereoscopic structure of this purified compound was elucidated with the help of chemical degradation, chemical transformation and spectroscopic techniques like NMR ($^{1}H$, $^{13}C$, COSY, TOCSY, and HSQC) and mass Spectrometry.

**Indinose** was confirmed as under;

**Glc–β–(1→3)–GlcNAc–β–(1→6)Gal–β–(1→4)Glc**

**INDINOSE**

The optimized geometry of compound **Indinose** at B3LYP method and 6-311+G basis set on Gaussian 09 program; show that the compound Indinose was stable compound.



**Keywords:** Bovine milk, oligosaccharide, Indinose, NMR, configuration.

## INTRODUCTION

The carbohydrate content of milk is comprised of lactose and oligosaccharide. The oligosaccharides compounds have numerous biological nutrients and show various activities such as anti-tumour, anti-cancer, anti-viral, anti-inflammatory, anticoagulant, antioxidant and immuno--stimulant activity (1,2). The oligosaccharides present in the milk of different animals show fair versatility in their properties. Buffalo milk oligosaccharides have ability to stimulate non-immunological resistance of the host against parasitic infections (3). Cow milk oligosaccharides reduce the adhesion of enterotoxic Eschererchia coli strains of the calf (4). Goat milk oligosaccharides play important roles in intestinal protection and repair after damage caused by DSS (Dextron sodium sulphate) induced colitis and their implication in human intestinal inflammation (5). 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(6). Donkey milk oligosaccharide have ability to stimulate non-specific and specific immunological resistance (7) and proposed to be very helpful in cure of AIDS patients and in prevention of atherosclerosis (8). The oligosaccharides isolated from elephant milk contained a high ratio of sialyl oligosaccharide; this may be significant with respect to the formation of brain components, such as gangliosides of the suckling calves (9). Mare's milk has shown anti-oxidant, lipid lowering and post heparin lipolytic activity (10). N-acetylneuraminlactose sulphate is the dominant oligosaccharide in the Dog milk (11) which plays an important role in the nutrition of the rat pups. Human breast milk play a key role in gut colonization and modulation of the infants guts (12). Fucosylated human milk oligosaccharide and related glyconjugates can used for several specific disease by inhibition of enteric pathogens such as stable toxin of Escherichia coli( in vitro and its toxin induced secretory diarrhea in vitro and in vivo), noroviruses and Campylobactoer (13, 14). They also have inhibitory effect on certain virulence-related abilities of

monocyte, lymphocyte and neutrophill adhesion to endothelial cells and act as anti-inflammatory agents (15, 16).

There are many scriptures like Ayurveda, Rigveda etc. which described the medicinal importants and value of cow milk for human life. According to Rigveda cow milk is Amrita, which is curative and prophylactic from diseases. Dhanvantri was an ancient physician who stated that it protects the human body from vita, pitta, heart diseases and leucoderma.

Keeping in mind the biological activity of Shyama dhenu milk and oligosaccharide present therein, it was collected in bulk and was processed by method of Kobata and Ginsburg (Kobata A. et al 1970) for obtaining its oligosaccharides constituents. In continuation to our previous work on isolation of shyama Dhenu (17,18,19) 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 method like $^{1}H$ NMR, $^{13}C$ NMR and 2DNMR (COSY TOCSY, HSQC) technique as well as mass spectrometry.

**Material and method**

**General procedure**

Optical rotations were measured with an AA-5 series automatic polarimeter in 1dm 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°C on a Bruker AM 300 and 400 FT NMR spectrometer. The ES-MS were recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. 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 N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), galactose (Gal), glucose (Glc), fucose (Fuc) and silalic acid were purchased from Aldrich Chemicals.

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

12 liter Black cow milk was collected from a Shyamadhenu and then processesed by method of Kobata and Ginsberg (20). For this method, milk was collected and stored at -20°C for 12 hours 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 was 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 (267 gm). The lyophilized material responded positively to Morgan-Elson test (21) and thiobarbituric acid assay suggesting the presence of N-acetyl sugars. This lyophilized material (30gm) was further purified by it 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 (22) for the presence of neutral sugar. Finally, 26 gm of the oligosaccharide mixture was obtained.

**Acetylation of Shyamadhenu Cow milk oligosaccharide mixture**

12g of oligosaccharide mixture was acetylated with pyridine (12 ml) and acetic anhydride (12 ml) at $60^0$C and solution was stirred overnight. Further the mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ (250ml) and washed with water (25 ml). The organic layer was dried over anhydrous $Na_2SO_4$, filtered and evaporated to dryness yielding the acetylated mixture (15.5g). The acetylation converted the free sugars into their nonpolar acetyl derivatives which were resolved nicely on TLC, giving six spots on TLC i.e. A, B, C, D, E and F.

**Purification of Acetylated milk oligosaccharide on Silica Gel Column**

Separation of the acetylated products (10 g) was purified by column chromatography. the silica was used in the ratio of 1:100 over Kg silica gel using various proportions 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 VII led to the isolation of one chromatographically pure compound F (55mg).

**Deacetylation of Compound**

Deacetylation of acetylated compound F (45mg) was carried out in 3ml acetone and 4ml $NH_3$ for 24hr in a stoppered hydrolysis flask. After 24 h ammonia was removed under reduced pressure, equal volume of $CHCl_3$ and water was added and the compound was recovered in the aqueous phase and the water layer was finally freeze dried giving the deacetylated natural oligosaccharide Indinose (31mg).

**Description of Isolated Compound Indicose**

**$^{1}H$ NMR: δ in $D_2O$ (ppm)**

δ5.25(d,1H J= 3.6Hz), δ4.65(d,1H J=8.1 Hz), δ4.52(d,1H J= 7.5Hz), δ4.45(d,1H J=7.5Hz), δ4.05(t,1H J=4.9 Hz), δ3.92(d,1H J=2.7Hz ), δ3.28(t,1H J=8.7Hz), δ3.20(t,1H J=8.7Hz), δ2.09(s,3H $NHCOCH_3$)

**$^{13}C$ NMR: δ in $D_2O$ (ppm)**

δ 101.4 δ 101.1, δ 91.7, δ 89.2, δ76.8, δ76.0, δ 75.9, δ 73.7, δ 72.8, δ71.8, δ71.2, δ70.9, δ70.7, δ 69.8, δ 69.6, δ

69.3, δ 69.2, δ 66.8, δ 61.9, δ61.7, δ 61.0

**FAB-MS**

1276, 1243, 1192, 1165, 1119, 1031, 1014, 970, 922, 893, 865, 834, 747, 724, 659, 637, 634, 619, 617, 577, 517, 475, 426, 403, 391, 373, 331, 289, 229, 221, 169, 137.

**RESULT AND DISCUSSION**

Compound F (Indinose) $C_{28}H_{48}O_{21}N$ gave positive Phenol sulphuric acid test (22), Fiegl test (23) and Morgan-Elson test (21), showing the presence of normal and amino sugars in the compound. The HSQC spectrum of acetylated Indinose showed the presence of four cross peaks of anomeric protons and carbons in the respective region at δ 6.26 x 89.2(1C), δ 5.67 x 91.7(1C), δ 4.48 x 101.4(2C), δ 4.48 x 101.1(1C) suggesting presence of five anomeric protons and carbons in it. $^1$H NMR spectrum of F in $D_2O$ at 300 MHz showed four anomeric proton signal at δ 5.25(1H), δ 4.60(1H), 4.65(1H), 4.52(1H), and 4.45(2H) indicating that the compound F may be tetrasaccharide in its reducing form. It was further supported by appearance of four signals for the five anomeric carbons at δ 101.4(2C), 101(1C), 91.7.4(1C), and 89.2(1C), in the $^{13}$C NMR spectrum of acetylated compound F. The four monsaccharide units present in the compound F 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 F gave three spots on the paper chromatography which were identified as Glc, Gal and GlcNAc by paper chromatographic with authentic samples showing that the compound F (Indinose) was comprised of Glc, Gal and GlcNAc. Methylglycosidation of F by MeOH/$H^+$ and 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 5.25(1H)(J= 3.6Hz) and δ 4.65 (1H)(J= 8.1 Hz) respectively. Further the presence of another anomeric proton doublet at δ 4.65 (J=8.1Hz) showed the presence of β-Gal(S-2) residue as the next monosaccharide unit. The appearance of β-Glc(S-1) H-2 signal as triplet at δ 3.28 (SRG) suggested the presence of 1→4 glycosidic linkage between Gal (S-2) and β-Glc (S-1) i.e., Gal(1→4)β-Glc in compound Indinose and hence the presence of lactosyl moiety in it at the reducing end. Further the $^1$H NMR spectrum of Indinose showed another anomeric proton signal appeared as a doublet at δ4.45 (1H) (J=7.5 Hz) along with a signal of three protons at δ 2.09 for NHAc group which confirmed the presence of only GlcNHAc ($S_3$) unit in the compound F. The large coupling constant of $S_3$ (J= 7.5 Hz) confirmed the β glycosidic linkage between $S_3$ and $S_2$. TOCSY spectrum of Indinose acetate showed a doublet at δ 5.67 corresponding to Gal (S-2) showed four cross peaks for ring protons of S-2, further the connectivity of ring protons in COSY spectrum showed that H-6 of Gal S-2 was linked with GlcNAc S-3. Further presence of another anomeric proton doublet at δ 4.52 (1H) (J=7.5 Hz) in the $^1$H NMR of Indinose was due to presence of Glc S-4 the large coupling constant of 7.5 Hz showed Glc S-4 was glycosydically linked by β glycosydic linkage to GlcNAc S-3 and the linkage between $S_3$ and $S_4$ was established on the basis of presence of GlcNAc H-3 proton appearing as a triplet in the downfield region at δ 4.05 (J=5.1Hz) which indicates that GlcNAc ($S_3$) was substituted at 3-position by a β-Gal ($S_4$). The 1→3 linkage between β-Gal ($S_4$) and β-GlcNAc ($S_3$), It was further confirmed by the COSY and TOCSY spectrum of Indinose acetate. The fourth monosaccharide unit i.e. glucose was present at the non-reducing end of the tetrasaccharide which was confirmed by the TOCSY spectrum of Indinose acetate which showed all the methine signals between δ 4.2-δ 5 hence no linkage was available in $S_4$. All the $^1$H NMR assignments F were confirmed by structural reporter group and COSY, TOCSY and HSQC experiments of the acetylated compound F. Based on the pattern of chemical shift of $^1$H, $^{13}$C, HOMOCOSY, TOCSY and HSQC experiments, it was interpreted that the compound F was a tetrasaccharide having the following structure.

**Glc**–β–(1→3)–**GlcNAc**–β–(1→6)**Gal**–β–(1→4)**Glc**

The tetrasaccharide nature of F was also further confirmed by the FAB-MS spectrum analysis of acetylated compound F which showed the highest mass ion peaks at m/z 1276 $[M+Na]^+$ as Pseudomolecular ion peak, which was in agreement of derived composition with the molecular ion expected at m/z 1253 acetylated terasaccharide and molecular formula $C_{52}H_{71}NO_{34}$. It also helped in substantiating the sequence of monosaccharide units in the tetrasaccharide F. The fragment ion at m/z 331showed the presence of terminal hexosyl moiety in the tetrasaccharide which was produced by the cleavage of terminal non reducing Glc($S_4$) with the subsequent formaation of fragment ion at m/z 922[$S_1$-$S_2$-$S_3$]. The presence of fragment ion at m/z 922 showed the presence of trisaccharide containing GlcNAc($S_3$)-Gal($S_2$)-Glc($S_1$) sugar moieties in the tetrasaccharide. Consequent loss of a $NHCOCH_3$ from fragment ion at m/z 922[$S_1$-$S_2$-$S_3$] leading to the fragment ion at m/z 864 showed the presence of another amino sugar in the tetrasaccharide. The presence of lactosyl moiety ($S_2$-$S_1$) is further confirmed by the presence mass ion fragment ion at m/z 619 which was produced by the fragmentation of molecular ion at m/z 1253 at N-acetylglucosamine residue starting from non reducing terminal of the tetrasaccharide, along with the formation complementary fragment ion at m/z 634 that corresponds to the disaccharide Glc($S_4$)-GlcNAc($S_3$). This confirms that the disaccharide Glc-GlcNAc was linked to the lactosyl moiety. The lactosyl disaccharide at m/z 619 further fragmented to give fragment ions at m/z 289($S_2$) and m/z 331($S_1$) which also confirmed the presence of Glc at the reducing terminal of the tetrasaccharide. The FAB mass spectrum of compound F also contained other mass ion peaks at m/z 1192[1276-2$CH_2$=C=O], 1119[1192 -$CH_2$=C=O, -$OCH_3$], 1031[1192- $CH_3COOH$, -$CH_3CO$, $NHCOCH_3$], 1014[1031-OH], 970[1120-$CH_3COO$-$CH_3COOH$-$OCH_3$], 865[1014-2$CH_3COO$-$OCH_3$] 834[865-$OCH_3$], 747[865-2$CH_3COO$], 705[747-$CH_2$=C=O] 659[865-2$CH_3COOH$-2$CH_3CO$], 637[679-$CH_2$=C=O], 577[619-$CH_2$=C=O], 517[577-$CH_3COOH$], 475[517-$CH_2$=C=O], 426[517-$CH_3COOH$-$OCH_3$], 391[475-2$CH_2$=C=O], 373[475-$CH_3COOH$-$CH_2$=C=O], 211[331-

$2CH_3COOH]$, 229[331-$CH_3COOH$-$CH_2$=C=O] 169[229-$CH_3COOH$] and 109[169-$CH_3COOH$]. This fragmentation also supported the formation of various fragment mass ion that served as anchoring units of the tetrasaccharide. On the basis of results obtained from physico-chemical techniques like $^1$H NMR, $^{13}$C NMR and 2DNMR (COSY, TOCSY, HSQC), chemical transformation and chemical degradation the structure of compound F was determined as-

**Glc–β–(1→3)–GlcNAc–β–(1→6)Gal–β–(1→4)Glc**

**2. Theoretical study**

The quantum chemical calculations have been analyzed on basis set of B3LYP functional and 6-311+G (d, p). Geometry of compound F has been first optimized and the presence of positive wave numbers values for all the optimized geometry indicates stability of the compounds. All the isolated compounds were described by computational data using the Gaussian 09 program package (24).

**Stability of Molecular geometries of the isolated compounds**

The geometry optimization of indinose has been done using B3LYP method at 6-311G basis set employing density functional theory (DFT). The theoretical calculations have been performed using Guassian 09W package. The optimized geometry is visualized using Gauss View 5.0.9 utility software. The indinose molecule possesses C1 symmetry. All the rings of indinose are present in the most stable chair form. The molecule is highly polar in nature with the total dipole moment of 10.0954 Debye. The molecule has total energy of -2651.3601 a. u. The optimized geometry shows that the compound Indinose was stable compound.

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