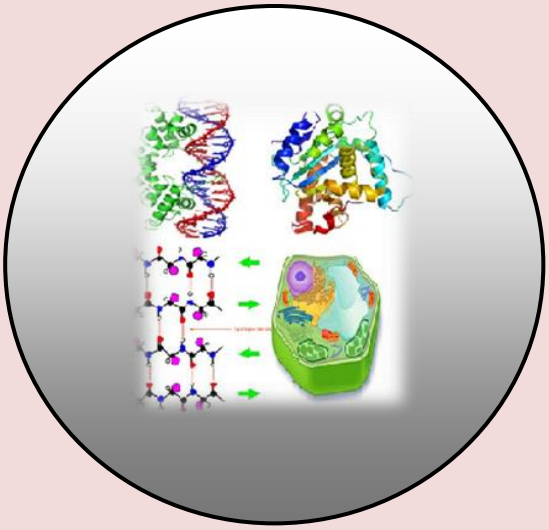
Isolation and Structure Elucidation of Novel Tetrasaccharide from Gaddi Sheep Milk

By

Muzeeb Khan, Sanyogita Shahi and Desh Deepak

ISSN 2319-3077 Online/Electronic
ISSN 0970-4973 Print

UGC Approved Journal No. 62923
MCI Validated Journal
Index Copernicus International Value
IC Value of Journal 82.43 Poland, Europe (2016)
Journal Impact Factor: 4.275
Global Impact factor of Journal: 0.876
Scientific Journals Impact Factor: 3.285
InfoBase Impact Factor: 3.66



J. Biol. Chem. Research
Volume 36 (1) 2019 Part D, Pages No. 149-157

Journal of Biological and Chemical Research

An International Peer Reviewed / Referred Journal of Life Sciences and Chemistry

Indexed, Abstracted and Cited in various International and National Scientific Databases

Published by Society for Advancement of Sciences®

J. Biol. Chem. Research. Vol. 36, No. 1: Part D, 149-157, 2019
(An International Peer Reviewed / Refereed Journal of Life Sciences and Chemistry)
Ms 36/02/111/2019

ISSN 2319-3077 (Online/Electronic)
ISSN 0970-4973 (Print)

Muzeeb Khan
http:// www.sasjournals.com
http:// www.jbcr.co.in
jbiolchemres@gmail.com

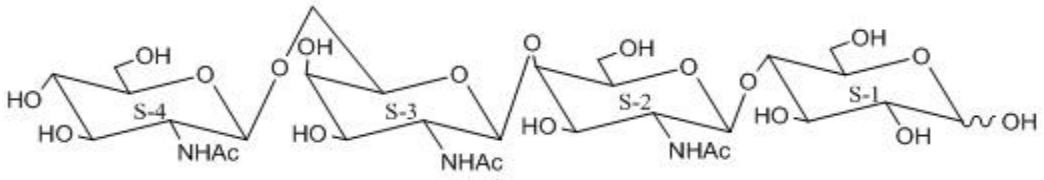
RESEARCH PAPER
Received: 05/05/2019 Revised: 19/06/2019 Accepted: 20/06/2019

Isolation and Structure Elucidation of Novel Tetrasaccharide from Gaddi Sheep Milk

Muzeeb Khan, Sanyogita Shahi and Desh Deepak
Department of Chemistry, Lucknow University, Lucknow 226007, India

ABSTRACT

Milk oligosaccharides are a unit of non digestible carbohydrate consisting of at least three monosaccharides linked by diverse glycosidic bond. Oligosaccharides are an important class of bioactive natural products and are emerging as potent drugs against fatal diseases like cancer and AIDS. Many oligosaccharides have been isolated from various milk which exhibited potent biological activities such as anti-tumor, anti-viral, anti cancer, anti-inflammatory, anti-coagulant, immunostimulant and hypoglycemic activities and they are also used as certain biomarkers. Keeping in mind the biological activities of sheep milk as bioactive inhibitory, hypertensive defense, control of microbial infection, affect in cardiovascular, nervous and immune system, it was collected in bulk and processed by modified method of Kobata and Ginsburg involving deproteination, centrifugation and lyophilzation followed by gel filtration, column chromatography and HPLC, affording crude oligosaccharide mixture which on acetylation and silca gel column chromatography afforded a purified oligosaccharide Isose. The structure of isolated oligosaccharides was elucidated by chemical transformations, chemical degradation, NMR (^{1}H, ^{13}C and 2D COSY, TOCSY HSQC) and mass spectrometry. The structure of novel oligosaccharide Isose was elucidated as under:



βGlcNAc(1→6)βGalNAc(1→4)βGalNAc(1→4)Glc

ISOSE

Keywords: Isolation, Oligosaccharides, Gaddi Sheep Milk, Isose and Tetrasaccharide.

INTRODUCTION

Carbohydrates are one of the most dominant solid fractions of both bovine and human milk, besides the main milk sugar, lactose an energy source for the neonate, number of more complex free oligosaccharides were present in milk at lower abundance (Oddy, 2002). Oligosaccharides of bovine milk were shown to have complex structures closely related to human milk oligosaccharides, therefore bovine milk is currently being used in a variety of health promoting supplements worldwide (Desh Deepak et. al. 1998, Srivatava et. al. 2012, Boehm et. al. 2007).

Several oligosaccharides have been detected in the milk of many mammalian species such as domestic herbivorous animals, carnivores, non human primates, marsupials, caprines, monotremes, equines, bovine and marine mammals. Milk oligosaccharides function as a receptor for many pathogenic bacteria and lower the pH in the gut which favours the growth of bacteria, avails the mineral absorption and destroys harmful microorganism. Milk oligosaccharides have potential to produce prebiotics (Kunz et. al. 2000), anti adhesion (Hakkarainen et. al. 2005, Zivkovic et. al. 2011), anti-inflammation (Mills et. al. 2011, S. Rudloff et. al. 1996), brain development (Wang et. al. 2003, Bogoch et. al. 1977), mineral absorption (G. Boehm et.al. 2003) and immuno-modulation effects (Schumacher et al. 2006). Milk oligosaccharides can bind to endogenous lectins like siglecs and galectins, which are expressed on mucosal immune cells that participate to the maturation of intestinal immunity and recognition of carbohydrate antigens which are taken orally. Human milk oligosaccharides containing α 1, 2-linked fucose inhibits the stable toxin-producing escherichia coli in vitro and its toxin-induced secretary diarrhea in vitro and in vivo, and the content of 2-linked fucosyl oligosaccharides in human milk is significantly associated with lower risk of diarrhea in breastfed infants, suggesting a major role for these oligosaccharides in immunity (Wu et. al. 2010). Sheep milk is a balanced nutrient and exhibit various course of action such as absorption of nutrients, digestion, growth, plays a specific character to the resist outer infection and development of various organs (Egito et. al. 2002). It contains peptides, proteins, amino acids and low fat content (Ranjan et. al. 2015). The peptides of sheep milk affect the cardiovascular, nervous and immune system. The protein present in Sheep milk is an essential source of bioactive inhibitory, control of microbial infection and hypertensive defense. Sheep milk is a rich source of fucosylated oligosaccharides which constitute a potent innate immune system of human (Sharon et. al. 2000). Keeping in mind the biological activities of Sheep milk and oligosaccharide present therein. The milk of a rare species of sheep i.e. Gaddi sheep, which is found at high altitude of Himalayan resion i.e. mountain of Bhadarwah of Jammu, Kallu, Kangra, Chamba and Mandi, Kinnour, Shimla, it was collected in bulk and was processed by modified method of Kobata and Ginsburg (Kobata et. al. 1970) for obtaining its oligosaccharides constituents. We have isolated a novel oligosaccharide from the Gaddi 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 2D NMR (COSY TOCSY, HMBC, HSQC) technique as well as mass spectrometry. The structure of these basic core oligosaccharides were assigned by chemical degradation and spectroscopic techniques by previous workers (Dorland et. al. 1977) and further, the structures of various milk oligosaccharides were determined by comparing the (^{1}H NMR) chemical shifts of anomeric signals and other important signals of unknown milk oligosaccharide with the chemical shifts of LNT and LNnT (Dua et. al. 1983). In the present study, analogies between chemical shifts of certain 'structural reporter group resonances' were used to make protons resonance assignments as well as structural assignments of the oligosaccharides, ancillary techniques such as Deacetylation, chemical degradations, NMR technique and FAB mass spectroscopy were used for unambiguous determination of the structure.

MATERIAL AND METHODS

General procedure

General procedures were same as described in our previous articles **(**Arjita Mani et. al. 2017).

Isolation of Gaddi milk oligosaccharide by Modified method of Kobata and Ginsburg -

10 litres milk was collected from a Gaddi Sheep then processed by modified method of Kobata and Ginsberg. 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).

Acetylation of Gaddi milk oligosaccharide mixture

60.00 g of pooled fractions which gave positive phenol-sulphuric acid test were acetylated with pyridine (60ml) and acetic anhydride (60ml) at 60°C and the solution was stirred overnight.

The mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ (3 x 300ml) and washed in sequence with 2N-HCl (1x 100ml), ice cold 2N-$NaHCO_3$ (2 x 100ml) and finally with H_2O (2 x 100ml). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acetylated mixture (65.50g).

Purification of Acetylated milk oligosaccharide on Silica Gel Column

The acetylated oligosaccharide mixture (22 g) was purified by column chromatography. The silica was used in the ratio of 1:100 using various proportions of Hexane $CHCl_3$, $CHCl_3$, $CHCl_3$:MeOH mixture which was resolved into twelve fractions namely I(1.045g), II(0.154g), III(0.220g), IV(280), V(1.280m), VI(0.350m), VII(0.520g), VIII(4.510g), IX(4.850g), X(2.250g), XI(0.100g) and XII(2.800g) respectively. These fractions were containing mixture of two to four compounds. Repeated column chromatography of fraction IX led to the isolation of one chromatographically pure compound e (24.0mg).

Deacetylation of Compound e

Compound e (24.0mg) obtained from column chromatography 2 of acetylated oligosaccharide mixture was dissolved in acetone (2ml) and 3 ml of NH_3 was added and left overnight in a stoppered hydrolysis flask. After 24h ammonia was removed under reduced pressure and the compound was washed with (3 x 5ml) $CHCl_3$ (to remove acetamide) and the water layer was finally freeze dried giving the deacetylated oligosaccharide E (21.0mg).

Methylglycosidation/ Acid Hydrolysis of Compound E

Compound E (5mg) was ref1uxed with absolute MeOH (2ml) at 70°C for 18h in the presence of cation exchange IR-120 (H) resin. The reaction mixture was filtered while hot and filtrate was concentrated. To a solution of methylglycoside of E in 1, 4-dioxane (1ml), 0.1 N H_2SO_4 (1ml) was added and the solution was warmed for 30 minutes at 50^0C and solution was left over night. The hydrolysis was complete after 24h. The hydrolysate was neutralized with freshly prepared $BaCO_3$ filtered and concentrated under reduced pressure to afford α-and β-methylglucosides along with the Glc, GalNAc, GlcNAc. Their identification was confirmed by comparison with authentic samples (TLC, PC).

Killiani Hydrolysis of Compound E

Compound E (3mg) was dissolved in 2ml Killiani mixture (AcOH-H_2O-HCI, 7:11:2) and heated at 100^0C for 1h followed by evaporation under reduced pressure. It was dissolved in 2ml of H_2O and extracted twice with 3ml $CHCl_3$. The aqueous residual solution was made neutral by addition of 1-2 drops of 2N NaOH, to it and was evaporated under reduced pressure to afford glucose, GalNAc and GlcNAc on comparison with authentic samples of glucose, GalNAc and GlcNAc.

Mannich-Siewert Hydrolysis of Compound E

To a solution of compound E in acetone (3ml), conc. HCl (0.05ml, 0.1N) was added. The solution was kept under carbon-dioxide (CO_2) in dark room at room temperature. After three days paper chromatogram showed three spots, mobility of one spot was identical in mobility with authentic sample of GlcNAc, and the other spot with the lowest mobility was identical with unreacted compound E (I), further the compound with the intermediate mobility may be the Trisaccharide(II). Further after seven days two new spots were observed of which one was identical in mobility with authentic sample of GalNAc and other with lower mobility may be the disaccharide (III) which was having faster mobility with trisaccharide (II).The hydrolysis was partially completed in ten days and showing a new spot, which was found identical with authentic sample of Glc showing that after hydrolysis of disaccharide (III) the spot of Glc observed and spot of GalNAc was mersed with already existing spot of GalNAc observed earlier. The hydrolysis was completed in fifteen days and showing three spots, which were found identical with authentic sample of GlcNAc ,GalNAc and Glc on TLC and PC may be confirm in that the sequence of monosaccharide in tetrasaccharide as-

GlcNAc-GalNAc-GalNAc-Glc.

The hydrolyzates were isolated for these compounds as GlcNAc, GalNAc and Glc and it was compared with authentic sample GlcNAc, GalNAc and Glc on paper chromatography. The results obtained from this type of acid hydrolysis confirmed that the sequence of monosaccharide in this tetrasaccharide was-

GlcNAc-GalNAc-GalNAc-Glc.

Description of compounds

COMPOUND E (ISOSE)

Substance E (182.00mg) obtained from fraction 47-55 of column chromatography-9. On deacetylation of 24mg of acetylated compound e with NH_3/ acetone it afforded substance E (21mg) as a viscous mass, $[\alpha]_D$ +42.41^0(c, 2, H_2O). For experimental analysis, this compound was dried over P_2O_5 at 100^0 C and 0.1 mm pressure for 8 hr.

$C_{30}H_{51}N_3O_{21}$	%C	%H	%N
Calcd.	45.63	6.46	5.32
Found	45.61	6.45	5.32

It gave positive Phenol-sulphuric acid test, Feigl test and Morgon-Elson test.

δ in D_2O: ^{1}H NMR

δ5.191 [d, 1H, J=3.8Hz, αGlc (S_1), H-1], δ4.632 [d, 1H, J=7.8Hz, βGlc (S_1'), H-1], δ4.489 [d, 1H, J=7.8Hz, βGlcNAc (S_4), H-1] and δ4.420 [d, 1H, J=7.8Hz, βGalNAc (S_2) & βGalNAc (S_3), H-1], δ3.857 [t, 1H, J=6.1, βGlcNAc (S_4), H-2], δ3.210 [t, 1H, J=5.8, βGlc (S_1'), H-2], δ2.052 [s, 6H, βGalNAc (S_2) & βGalNAc (S_3), NHCO$\underline{CH_3}$] and δ2.044 [s, 3H, βGlcNAc (S_4), NHCO$\underline{CH_3}$].

δ in D_2O: ^{13}C NMR

δ171.47 [βGalNAc (S_2) & βGalNAc (S_3), NH$\underline{CO}CH_3$], δ169.92 [βGlcNAc (S_4), NH$\underline{CO}CH_3$], δ102.00 [βGlcNAc (S_4), C-1], δ101.60 [βGalNAc (S_2) & βGalNAc (S_3), C-1], δ89.90[βGlc(S_1'), C-1] and δ88.20 [αGlc (S_1), C-1], δ20.81 [βGalNAc (S_3), NHCO$\underline{CH_3}$], δ20.61 [βGalNAc (S_2), NHCO$\underline{CH_3}$] and δ20.37 [βGlcNAc (S_4), NHCO$\underline{CH_3}$].

δ in $CDCl_3$: ^{1}H NMR (Acetylated)

δ6.261 [d, 1H, J=3.8Hz, αGlc (S_1), H-1], δ5.660 [d, 1H, J=7.8Hz, βGlc (S_1'), H-1], δ4.503 [d, 1H, J=7.8Hz, βGlcNAc (S_4), H-1], δ4.499 [d, 1H, J=7.8Hz, βGalNAc (S_2), H-1] and δ4.439 [d, 1H, J=7.8Hz, βGalNAc (S_3), H-1], δ2.052 [s, 6H, βGalNAc (S_2) & βGalNAc (S_3), NHCO$\underline{CH_3}$] and δ2.044 [s, 3H, βGlcNAc (S_4), NHCO$\underline{CH_3}$].

δ in $CDCl_3$: ^{13}C NMR (Acetylated)-

δ171.47 [βGalNAc (S_2) & βGalNAc (S_3), NH$\underline{CO}CH_3$], δ169.92 [βGlcNAc (S_4), NH$\underline{CO}CH_3$], δ104.34[βGalNAc (S_2) & βGalNAc (S_3), C-1], δ104.20 [βGlcNAc (S_4), C-1], δ91.54 [βGlc(S_1'), C-1] and δ89.20 [αGlc (S_1), C-1], δ20.81 [βGalNAc (S_3), NHCO$\underline{CH_3}$], δ20.61 [βGalNAc (S_2), NHCO$\underline{CH_3}$] and δ20.37 [βGlcNAc (S_4), NHCO$\underline{CH_3}$].

ES Mass

m/z 850[M+Na+K]$^+$, m/z 790[M+H]$^+$, m/z 789[M]$^+$, m/z 758, m/z 716, m/z 714, m/z 597, m/z 586, m/z 528 , m/z 510, m/z 468, m/z 461, m/z 449, m/z 430, m/z 390, m/z 387, m/z 372, m/z 352, m/z 330, m/z 310, m/z 294, m/z 276, m/z 271, m/z 211, m/z 180, m/z 162 and m/z 144.

RESULT AND DISCUSSION

Structure elucidations of the isolated Camel milk oligosaccharide

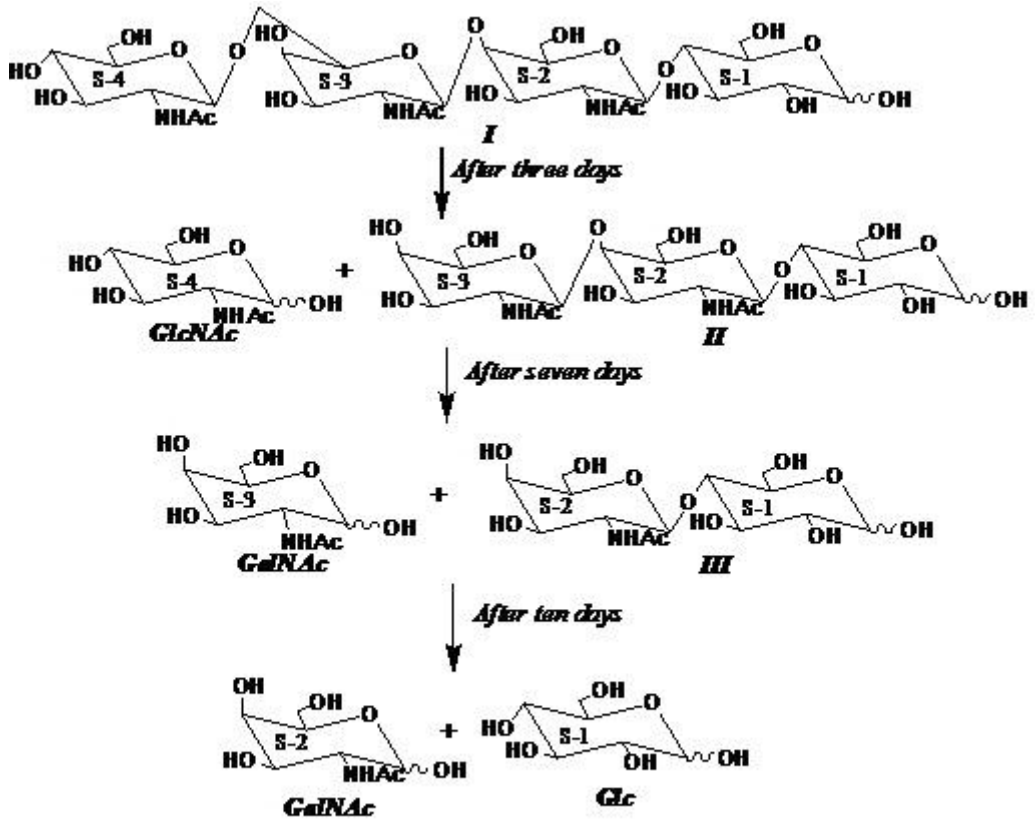
NMR spectroscopy

Compound Isose, $C_{30}H_{51}N_3O_{21,}$ $[\alpha]_D$ +42.41, gave positive Phenol- sulphuric acid test (M. Dubois et. al. 1956), Feigl test (F. Fiegl, 1975), Morgon-Elson test (Partridge et. al. 1948) showing the presence of normal and amino sugar(s) in the compound. The HSQC spectrum of acetylated compound at 300 MHz exhibited five cross peaks for four anomeric proton signals at δ 6.261 x 89.20, δ 5.660 x 91.54, δ 4.503 x 104.34, δ 4.499 x 104.20 and δ 4.439 x 104.34 indicating that the Isose may be a tetrasaccharide, in its reducing form giving signals for α and β anomers of glucose in its reducing end. The tetrasaccharide nature of acetylated compound Isose was further confirmed by the presence of five-anomeric carbon and proton at δ 89.20(1C), δ 91.54(1C),δ 104.20(1C), and δ 104.34(2C), in ^{13}C NMR and δ 6.261(1H), δ 5.660(1H), δ 4.503(1H), δ 4.499(1H) and δ 4.439(1H) in ^{1}H NMR, respectively. Methylglycosidation of Isose by MeOH/H$^+$ followed by its acid hydrolysis led to isolation of α and β-methyl glucoside, which confirmed the presence of glucose at the reducing end of the oligosaccharide. It was further confirmed by the presence of two anomeric proton signals at δ 5.191 and δ 4.632 for α- and β-Glc in ^{1}H NMR of Isose in D_2O.

The four monosaccharides present in compound have been designated as S_1, S_2, S_3 and S_4 for convenience starting from reducing end. To confirm the monosaccharide constituents in compound, it was hydrolysed under strong acidic conditions. In Killiani hydrolysis under strong acid condition, it gave three monosaccharides i.e. glucose, N-acetylgalactosamine and N-acetylglucosamine, confirming that the tetrasaccharide is consist of three types of monosaccharide units i.e. glucose, N-acetylgalactosamine and N-acetyl-glucosamine. To confirm the monosaccharide constituents and their sequence in Isose, it was hydrolysed under mild acidic conditions (Mannich-Siewert method) followed by paper chromatography and TLC. In this hydrolysis after three days paper chromatogram showed three spots, mobility of one spot was identical in mobility with authentic sample of GlcNAc, and the other spot with the lowest mobility was identical with unreacted compound E (I), further the compound with the intermediate mobility may be the Trisaccharide(II). Further after seven days two new spots were observed of which one was identical in mobility with authentic sample of GalNAc and other with lower mobility may be the disaccharide (III) which was having faster mobility with trisaccharide (II).The hydrolysis was partially completed in ten days and showing a new spot, which was found identical with authentic sample of Glc showing that after hydrolysis of disaccharide (III) the spot of Glc observed and spot of GalNAc was merged with already existing spot of GalNAc observed earlier. The hydrolysis was completed in fifteen days and showing three spots, which were found identical with authentic sample of GlcNAc ,GalNAc and Glc on TLC and PC may be confirm in that the sequence of monosaccharide in tetrasaccharide as-

GlcNAc-GalNAc-GalNAc-Glc.

The hydrolyzates were isolated for these compounds as GlcNAc, GalNAc and Glc and it was compared with authentic sample GlcNAc, GalNAc and Glc on paper chromatography. The results obtained from this type of acid hydrolysis confirmed that the sequence of monosaccharide in this tetrasaccharide was-



Scheme -1 Mannich-Siewert Hydrolysis of Isose (Compound E)

Since the glucose was present in its reducing form which was supported by ^{1}H NMR of Isose which contains two anomeric proton signals for α- and β-Glc at δ 5.191 (J=3.9) and at δ 4.632 (J=7.8) (T. Urashima et. al. 2002, T. Urashima et. al. 2004b, T. Urashima et. al. 2007).

Further the presence of another anomeric proton doublet signal at δ 4.420 (J=7.8) along with signal of amide methyl group at δ 1.964, was due to presence of β-GalNAc moiety (Saito T et. al. 1984) in the Isose. β-Glc (S_1) H-2 signal as a triplet at δ3.210 in the ^{1}H NMR of Isose showed that the equatorial orientation of hydroxyl group at C-4 of the reducing β-Glc (S_1) was substituted and involved in glycosidation, suggested the presence of β-GalNAc(1→4) Glc moiety at reducing end (T. Urashima et. al. 1997a, Yusuke Uemura et. al. 2006). The splitting pattern of anomeric signal with J value of δ7.8 shows the β-configuration of anomeric linkage at S_2→S_1. It was further supported by the presence of β-Glc H-4 proton resonance at δ3.967 in acetylated derivative of Isose. Another anomeric proton signal, which appeared at δ4.420(J= 7.8) along with signal of amide methyl group at δ 1.964 was due to presence of β-GalNAc (S_3) moiety. The coupling constant of anomeric signal with J value of δ7.8 shows the β-configuration of anomeric linkage at S_3→S_2. The presence of H-4 and C-4 resonances of β-GalNAc (S_2) of Isose acetate, at δ 3.954 and δ 76.58 confirm that β-GalNAc (S_3) was (1→4) linked to β-GalNAc (S_2), respectively. Further the presence of another anomeric signal at δ 4.489(J=7.8) along with signal of amide methyl group at δ 1.877 was due to presence of β-GlcNAc (S_4) moiety. The position of anomeric proton at δ 4.489(J=7.8) confirmed that β-GlcNAc (S_4) (1→6) linked to β-GalNAc (S_3) (SRG) [24]. The splitting pattern of anomeric signal with J value of δ7.8 shows the β-configuration of anomeric linkage at S_4→S_3. It was also confirmed by the presence of H-6 and C-6 resonances of β-GalNAc (S_3) of Isose acetate, at δ 3.961 and δ 77.00 respectively. This was confirmed on the basis of assignments made by 2D NMR spectra of acetylated compound.

Table 1.- ^{1}H and ^{13}C NMR values of Isose in D_2O -

Moieties	^{1}H NMR	^{13}C NMR	Coupling Constt.(J)
α-Glc (S_1)	5.191	88.20	3.8
β-Glc (S'_1)	4.632	89.90	7.8
β-GalNAc (S_2)	4.420	101.60	7.8
β-GalNAc (S_3)	4.420	101.60	7.8
β-GlcNAc (S_4)	4.489	102.00	7.8

Table 2.- ^{1}H NMR values of acetylated Isose in $CDCl_3$-

Moieties	H-1	H-2	H-3	H-4	H-5	H-6	-CH_3
α - Glc (S_1)	6.261	5.0532	5.416	3.967			
β - Glc (S'_1)	5.660	5.130	5.365	3.872			
β - GalNAc (S_2)	4.499	3.854	5.080	3.954			2.052
β - GalNAc (S_3)	4.439	3.806	5.053	4.400	5.212	3.961	2.052
β - GlcNAc (S_4)	4.503	3.857	5.285	4.518			2.044

The ^{13}C NMR values of anomeric carbons and ring carbons of Isose are given in table. The various values of ring carbons are in accordance with ^{13}C value of their respective monosaccharides, which also supports the derived structure.

Table 3.- ^{13}C NMR values of acetylated Isose in $CDCl_3$-.

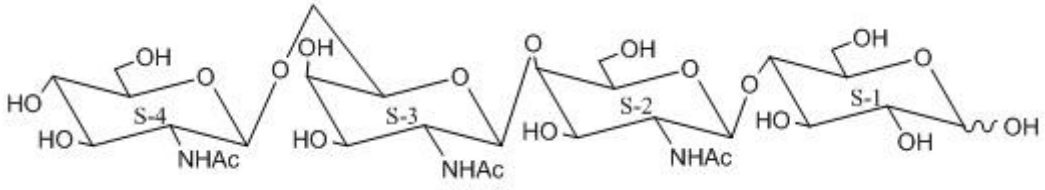
Moieties	C-1	C-2	C-3	C-4	C-5	C-6	-$CONH_2$	-CH_3
α - Glc (S_1)	89.20	62.79	63.00	77.43				
β - Glc (S'_1)	91.54	61.85	63.29	77.21				
β - GalNAc (S_2)	104.34	68.83	68.15	76.58			171.47	20.61
β - GalNAc (S_3)	104.34	62.79	68.98	62.00	61.85	77.00	171.47	20.81
β - GlcNAc (S_4)	104.20	68.94	65.00	66.98			169.92	20.37

The glycosidic linkages were assigned by the cross peaks for glycosidically linked carbons with their protons in HSQC spectrum of Isose acetate. The values of these cross peaks were as α-Glc (S_1) H-4 and C-4 at (δ3.967 x 77.43) showed (1→4) linkage of S_2 and S_1, β-Glc (S_1) H-4 and C-4 at (δ3.872 x 77.21) showed (1→4) linkage of S_2 and S_1 ,β-GalNAc (S_2) H-4 and C-4 at (δ3.954 x 76.58) shows (1→4) linkage of $S_3 \rightarrow S_2$. β-GalNAc(S_3) H-6 and C-6 at (δ3.961 x 77.00) shows (1→6) linkage of S_4 and S_3. The tetrasaccharide nature of compound was further confirmed by the spectral studies of acetylated derivative of compound. Ring hydrogens involved in linkage at δ3.872 (4-position) for $S_1 \rightarrow S_2$, δ3.954(4-position) for $S_2 \rightarrow S_3$, δ3.961(6-position) for $S_3 \rightarrow S_4$, showing same chemical shift in acetylated and deacetylated spectra. These studies were made on the basis of HOMOCOSY, TOCSY and HSQC connectivities. It was further confirmed by the presence of same peaks in COSY and TOCSY spectrum. Based on the pattern of chemical shifts of ^{1}H , ^{13}C, HOMOCOSY, TOCSY and HSQC NMR experiments it was interpreted that the compound was tetrasaccharide having structure as-

βGlcNAc(1→6)βGalNAc(1→4)βGalNAc(1→4)Glc

The result obtained from the ES mass spectrum further substantiated the structure of Isose which was derived by its ^{1}H and ^{13}C NMR Spectra. The highest mass ion peak were recorded m/z 850 which was due to $[M+Na+K]^+$. Other mass ion peak recorded at m/z 790 was due to $[M+H]^+$, confirmed that the molecular weight of compound was 789. Further the mass fragments were formed by repeated H transfer in the oligosaccharide and was accompanied by the elimination of terminal sugar less water. The fragmentation pathway confirmed the sequence of monosaccharide units in the tetrasaccharide (scheme: 2 & 3). The result obtained from ES mass spectrum further sustained the structure of Isose which was derived by its ^{1}H and ^{13}C NMR spectra. The highest mass ion peak was recorded at m/z 850 for [M+Na+K] and other was at m/z 790 [M+H], further confirmed the molecular weight of compound was 789. Further the mass fragments were formed by repeated H transfer in the oligosaccharide and was accompanied by the elimination of terminal sugar less water. The fragmentation pathway confirmed the sequence of monosaccharide units in tetrasaccharide (scheme 3). The tetrasaccharide on fragmentation gave a mass ion peak at m/z 586(I), which was due to loss S-4 sugar unit i.e. GlcNAc (S-4) sugar unit linked to the S-3 of tetrasaccharide unit. The trisaccharide (I), on fragmentation gave a mass ion peak at m/z 383(II), which was due to loss S-3 sugar unit i.e. GalNAc (S-3) sugar unit linked to the S-2 of trisaccharide unit. This disaccharide on further fragmentation gave a mass ion peak at m/z 180(III), which was due to loss of S-2 sugar unit i.e. GalNAc (S-2) sugar unit linked to the S-1 of disaccharide. The other supporting mass fragments obtained at m/z 714 (789- $NHCOCH_3$, OH), m/z 758 (789-CH_2OH), m/z 716 (758-CH_2CO), m/z 597 (758- CH_2OHCHO, $NHCOCH_3$, H^+), confirm tetrasaccharide nature of compound. The tetrasaccharide m/z 789 on fragmentation gave trisaccharide m/z 586 (M-S_4), which was further confirmed by its other fragments ions at m/z 528 (586-$NHCOCH_3$), m/z 461 (528-CH_2OH,$2H_2O$), m/z 430 (461-CH_2OH), m/z 372 (430-$NHCOCH_3$), m/z 510 (586- H_2O,$NHCOCH_3$), m/z 468 (510-CH_2CO), m/z 449 (468-H_3O^+), m/z 390 (449-CH_2OH,$2H_2O$) and m/z 372 (390- H_2O). The trisaccharide m/z 586 on fragmentation gave disaccharide m/z 383, which was further confirmed by its other fragments ions at m/z 352 (383-CH_2OH), m/z 310 (352- $2H_3O^+$), m/z 271 (310-CH_2OH), m/z 330 (387-$2H_2O$,OH), m/z 294 (330-$2H_2O$), m/z 276 (294- H_2O),and m/z 211 (276- CH_2OH,H_2O,OH and 271-CH_2OHCHO). The disaccharide m/z 347 on fragmentation gave monosaccharide m/z 180, it was supported by m/z 162 (180-H_2O) and m/z 144 (162-H_2O).

Based on the results obtained from chemical degradation chemical transformation, mass spectrometry and ^{1}H , ^{13}C, HOMOCOSY, TOCSY , HSQC NMR, the structure of the isolated novel tetrasaccharide, Isose was deduced as-



CONCLUSION

In summary, the novel milk oligosaccharides namely **E** Isose has been isolated from Gaddi Sheep milk and the structure of Isose was elucidated with the help of ^{1}H, ^{13}C, 2D NMR spectroscopy and mass spectrometry as-

βGlcNAc(1→6)βGalNAc(1→4)βGalNAc(1→4)Glc

ACKNOWLEDGMENTS

Authors are thankful to UGC New Delhi for financial assistance and gratefully acknowledge to Prof. Raja Roy CBMR SGPGI for providing spectral data. We are also gratefully to University of Lucknow, Lucknow for providing lab facility and computational help.

REFERENCES

Oddy, W.H. (2002). The impact of breastmilk on infant and child health. *Breastfeed Rev*. 10(3): 5-18.

Desh Deepak, Rina Saksena and Anakshi Khare (1998). Indian Patent no.3044/oct/98 Serial no.189748,

Srivatava, A., R. Tripathi, G. Bhatia, A.K. Khanna and D. Deepak (2012). Antioxidant, lipid lowering and post heparin lipolytic activity of mare milk oligosaccharides in tritan treated hyperlipidemic rats. *Asian Pac. J. Trop. Biomed*. 1-6.

Boehm, G. and B. Stahl (2007). Oligosaccharides from milk. *J. Nutr.* 137 (3): 847-849.

Kunz, C., S. Rudloff, W. Baier, N. Klein and S. Strobel (2000). Annu. Oligosaccharides in human milk. Structural, functional, and metabolic aspects. *Rev. Nutr.* 20: 699-722.

Hakkarainen, J., M. Toaiven, A. Leinonen, L. Frangsmyr, N. Stromberg, S. Lapinjoki, X. Nasssif, and C. Tikkanen-Kaukanen (2005). Human and bovine milk oligosaccharides inhibit *Neisseria meningitidis* pili attachment in vitro. *J. Nutr.* 135: 2445-2448.

Zivkovic, A. M. and D. Barile (2011). Bovine milk as a source of functional oligosaccharides for improving human health. *Adv. Nutr.* 2: 284-288.

Mills, S., R. P. Ross, C. Hill, G. F. Fitzgerald and C. Stanton (2011). Milk intelligence: Mining milk of bioactive substances associated with human health. *Int. Dairy J.* 21: 382-386.

Rudloff, S., G. Phlentz, L. Diekmann, H. Egge, and C. Kunz (1996). Urinary excretion of lactose and oligosaccharides n preterm infants fed human milk or infant formula. *Acta Paedeatr.* 85: 598-603.

Wang, B. and J. Brand-Miller (2003). The role and potential of sialic acid in human nutrition. *Eur. J. Clin. Nutr.* 57: 1351-1369.

Bogoch, S., F. V. DeFeudis and J. M. Delgado (1977). Recognins and their chemoreciprocals. In: Behavioural neurochemistry. (eds) Spectrum Publishers, New York, NY, USA, p. 270.

Boehm, G., B. Stahl and T. Mattila-Sandholm (2003). Woodhead publishers, Cambridge, UK, 203-243.

Schumacher, G., G. Bendas, B. Stahl and C. Beermann (2006). Human milk oligosaccharides affect P-selectin binding capacities: in vitro investigation. *Nutr.* 22: 620-627.

Wu, S., N. Tao, J.B. German, R. Grimm and C. B. Lebrilla (2010). The development of an annotated library of neutral human milk oligosaccharides**,** *J Proteome Res*. (9): 4138–4151.

Egito, A.S., L. Miclo, C. Lopez, A. Adam, J.M. Girardet and J.L. Gaillard (2002). Separation and characterization of mare's milk alpha (s1)-beta-,kappa- caseins, gamma-casein-like, and proteose peptone component 5-like peptides. *J. Dairy Sci.*, 85: 697-706.

Ranjan, A. K. and Desh Deepak (2015). Isolation and Purification of Sheep Milk Oligosaccharide as Therapeutic Agents *J. Biol. Chem. Research*. 32(2): 455-465.

Sharon, N. and I. Ofek (2000). Safe as mother's milk: carbohydrates as future anti-adhesion drugs for bacterial diseases. *J. Glucoconj*. 17: 659-64.

Kobata, A. and V. Ginsburg (1970). An enzymatic basis for Lewis blood types in man. *J.Biol.Chem*. 245: 1484.

Dorland, L., J. Haverkamp, B. Schut, J.F.G. Vliegenthart, G. Spik, G. Strecker and B. Fournet (1977). The primary structure of the asialo-carbohydrate units of the first glycosylation site of human plasma α1-acid glycoprotein *J. Montreuil FEBS Lett.* 77: 15-20.

Dua, V.K. and C.A. Bush (1983). Identi¢cation and fractionation of human milk oligosaccharides by proton-nuclear magnetic resonance spectroscopy and reverse-phase high performance liquid chromatography, *Anal. Biochem.* 133 1-8.

Arjita Mani, Muzeeb Khan, Anil Mishra and Desh Deepak (2017). Isolation, Purification and Structure elucidation of novel camel milk oligosaccharides and their DFT studies. *Chemistry & Biology Interface*, 7(6): 328-337.

Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith (1956). Colorimetric method for determination of sugars and related substances, *Anal. Chem.* 28: 350.

Fiegl, F. (1975). Spot test in organic analysis. Elsevier Publication, Amsterdam, pp.337.

Partridge, S. M. and R.G. Westall (1948). Filter paper partition chromatography of sugars (I) General description and application to the qualitative analysis of sugars in apple juice, egg white and fetal blood of sheep, *J. Biochem.* 42: 238-250.

Urashima, T., H. Sato, J. Munakata, T. Nakamura, I. Arai, T. Saito, M. Tetsuka, Y. Fukui, H. Ishikawa, C. Lydersen and K.M. Kovacs (2002). Chemical characterization of the oligosaccharides in beluga (*Delphinapterus leucas*) and Minke whale (*Balaenoptera acutorostrata*) milk. Comp. *Biochem. Physiol. B* 132: 611–624.

Urashima, T., T. Nakamura, K. Teramoto, I. Arai, T. Saito, T. Komatsu and T. Tsubota (2004b). Chemical characterization of sialyl oligosaccharides in milk of the Japanese black bear, Ursus thibetanus japonicus. *Comp. Biochem. Physiol*. B 139: 587–595.

Urashima, T., Mami Kobayashi , Sadaki Asakuma , Yusuke Uemura , Ikichi Arai , Kenji Fukuda , Tadao Saito ,Toshihiro Mogoe , Hajime Ishikawa and Yutaka Fukui (**2007**). Chemical characterization of the oligosaccharides in Bryde's whale (*Balaenoptera edeni*) and Sei whale (Balaenoptera borealis lesson) milk Comparative, *Biochemistry and Physiology*, Part B 146: 153–159.

Saito, T., Ito T. and Adachi S. (1984). Bovine Colostrum or Milk, BBA; 801: 147-50.

Urashima, T., Y. Kusaka, T. Nakamura, T. Saito, N. Maeda, M. Messer (1997a). Chemical characterization of milk oligosaccharides of the brown bear, Ursus arctos yesoensis, *Biochim. Biophys. Acta*. 1334: 247–255.

Yusuke Uemura, Sadaki Asakuma, Lisa Yon, T adao Saito, Kenji Fukuda, Ikichi Arai and Tadasu Urashima (2006). Structural determination of the oligosaccharides in the milk of an Asian elephant (Elephas maximus) Comparative *Biochemistry and Physiology*, Part A 145: 468–478.

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