Isolation and Structure Elucidation of Novel Pentasaccharide "Primose" from Cow Colostrum

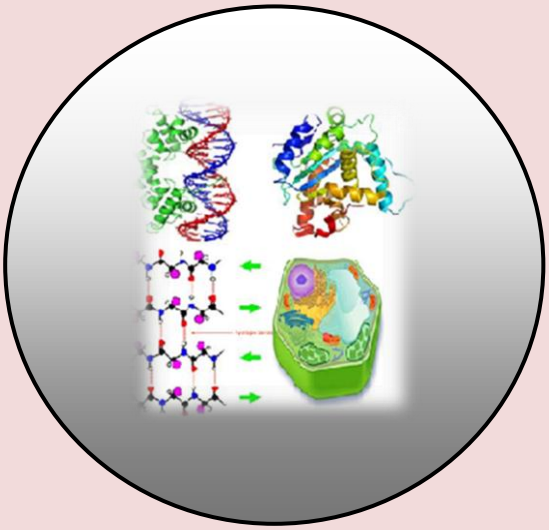
By

Mayank Agnihotri, Shraddha Rathor 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), Part C, 2019 Pages No. 234-241



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 C, 234-241, 2019
(An International Peer Reviewed / Refereed Journal of
Life Sciences and Chemistry)
Ms 36/02/0620/2019

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


Prof. Desh Deepak Shraddha Rathor
http:// www.sasjournals.com
http:// www.jbcr.co.in
jbiolchemres@gmail.com

RESEARCH PAPER
Received: 29/04/2019 Revised: 30/05/2019 Accepted: 01/06/2019

Isolation and Structure Elucidation of Novel Pentasaccharide "Primose" from Cow Colostrum

Mayank Agnihotri, Shraddha Rathor and Desh Deepak
Department of Chemistry, University of Lucknow, Lucknow-226007 India

ABSTRACT

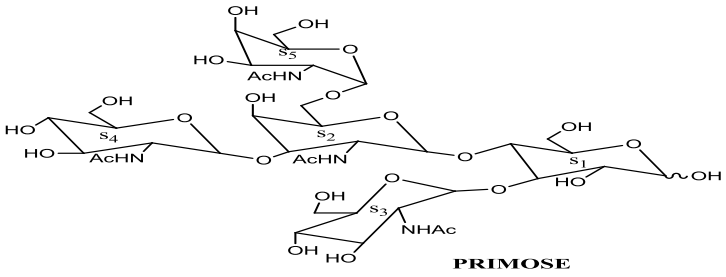
Milk is an important fluid in glycobiology which contains number of carbohydrate chains either in free form or as glyconjugates. The milk is one of the important sources of biologically active oligosaccharides which have shown various biological activities such as anti-tumor, anti-cancer, anti-microbial and anti-inflammatory agents. It protects any newborn against infections and provides nutrition. Milk obtained from any mammal is of two type i.e. normal milk and colostrum. Colostrum means early milk which mammal provides to its new born within 5-7 days. It contains significant quantities that act as natural antimicrobial agent to stimulate the maturation of an infant's immune system. Cow's milk is a good source of calcium, vitamin D and K as well as contains nutrients essential to bone health. It is beneficial against breast cancer, and plays an integral role in blood clotting, muscle contraction, blood pressure regulation and cell membrane function. Keeping in mind the biological activities of cow's milk and importance of oligosaccharides, cow's colostrum was collected and processed by modified method of Kobata and Ginsberg which was further purified by sephadex chromatography and acetylated by acetic anhydride and pyridine to yield acetylated oligosaccharide mixture which was further purified by silica gel column chromatography to obtain novel milk oligosaccharide Primose. The novel milk oligosaccharide was isolated from cow's colostrum by silica gel column chromatography and its homogeneity was confirmed by HPLC. Further the structure of Primose was elucidated by chemical transformation, chemical degradation, ^{1}H NMR, 2-D NMR (COSY, TOCSY and HSQC) and mass spectrometry. The sequence of isolated pentasaccharide molecule was deduced as under.

Keywords: Cow Colostrum, Isolation, Oligosaccharides and Primose.

INTRODUCTION

Milk is a complex mixture of lipids, proteins, carbohydrates and smaller metabolites, and thus represents a key role in infant nourishment and development (Sundekilde et al, 2012). Oligosaccharides, glycoproteins and anti-bodies present in milk protect infant by reducing the number of pathogen infections and promotes the development of intestinal epithelium.

Oligosaccharides play an essential role in many molecular processes impacting eukaryotic biology and diseases and exhibit varied biological activity such as anti-tumor (Schwonzen et al, 1992), immunostimulant (Abe et al, 1983), anti-cancer (Fang et al, 1985), anti-complementary, anti-inflammatory (Maslowski et al, 2009), anticoagulant, hypoglycaemic, antiviral and immunological activity (Ehresmann et al, 1979). 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 (Euler AR et al, 2005). Indian Physicians and Ancient Vedas have stated that the cow milk is desirable and preferred diet in all types of ailments. Apart from its curative properties described in ancient medicinal system, cow milk is extensively used in infant nutrition as best alternative to human milk. 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 (Gunjan et al, 2016). Cow milk used as the immunostimulant, nourishes the body tissues, acts as natural aphrodisiac, does rejuvenation and improves intelligence, in heart diseases and leukoderma, increase breast milk in feeding mother, assists in easy movement of intestine and bleeding disorders (Gangwar et al, 2017).Rigveda says that the cow milk is Amrita i.e. protects human beings from diseases. Several studies supported the constructive effects of supplementation of cow milk in diarrhoeain human with immune deficiency syndrome, NSAID-induced gastrointestinal disturbances. Cow milk oligosaccharides reduce the adhesion of enterotoxic E.coli strains of the calf (Euler AR et al, 2005). We have already isolated Aurose (Gunjan et al, 2018), Indinose (Khan M. et al, 2017), Dicusose (Gangwar et al, 2017), Indose (Khan M. et al, 2017), Indicose (Gunjan et al, 2016) from cow milk. In continuation to our previous work we have collected cow's milk (Jursi cow) and processed it by modified method of Kobata and Ginsburg (Shahi S. et al, 2017) yielding oligosaccharide mixture. This oligosaccharide mixture on acetylation and purification by column chromatography and deacetylation yielded a novel oligosaccharide namely Primose. Structure elucidation of this novel milk oligosaccharide was done by the chemical degradation, chemical transformation and by the modern spectroscopic techniques (^{1}H, ^{13}C, 2-D NMR, HSQC, COSY, TOCSY) and mass spectrometry.



PRIMOSE

Experimental

General Procedure

The ^{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 and 400 FT NMR spectrometer. The electrospray mass spectra were recorded on a MICROMASS QUATTRO II triple quadruple 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 elemental analyzer CARLO-ELBA 1108. 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 ethyl acetate-pyridine (2:1) saturated with H_2O.

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 sialic acid were purchased from Aldrich Chemicals.

Acetylation of oligosaccharide mixture

12 gm of pooled fractions obtained from sephadex column which gave positive phenol-sulphuric acid test were acetylated with pyridine (12ml) and acetic anhydride (12ml) at 60°C and the solution was stirred overnight. The mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ (500 ml) and it was washed with 2N-HCl (1 x 25 ml), ice cold 2N-$NaHCO_3$ (2 x 25 ml) and finally with H_2O (2 x 25 ml). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acetylated mixture (12.1gm). The acetylation converted the free sugars into their nonpolar acetyl derivatives which were resolved nicely on TLC, giving eight spots on TLC i.e. a,b,c,d,e,f,g and h.

Purification of acetylated oligosaccharide mixture by column chromatography

Acetylated Cow's milk oligosaccharides mixture (12.10gm) gave eight spots a,b,c,d,e,f,g and h, on TLC which on column chromatography over slica gel by various proportion of $CHCL_3$and CHCL3:MeOH resulted into isolation of compound primose (65mg) in pure form.

Deacetylation of compound d

25mg of compound d was dissolved in acetone (3 ml) and 3.5 ml of NH_3 was added in it and was left overnight in a stoppered hydrolysis flask. After 24 hrs ammonia was removed under reduced pressure and the compound was washed thrice with $CHCl_3$ (5 ml) (to remove acetamide) and water layer was finally freeze dried giving the deacetylated oligosaccharide primose (19 mg).

Methyl glycosidation/Acid hydrolysis of compound Primose

Primose (8 mg) was ref1uxed with absolute MeOH (2 ml) at 70°C for 18 h in the presence of cation exchange IR-l20 (H) resin. The reaction mixture was filtered while hot and filtrate was concentrated. In the solution of methylglycoside of D, 1,4-dioxane (1 ml), and 0.1N H_2SO_4 (1 ml) was added and the solution was warmed for 30 minutes at 50˚C. The hydrolysis was complete after 24 h. 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, Gal and GlcNAc. Their identification was confirmed by comparison with authentic samples (TLC, PC).Compound (8 mg) was ref1uxed with absolute MeOH (2 ml) at 70°C for 18 h in the presence of cation exchange IR-l20 (H) resin. The reaction mixture was filtered while hot and filtrate was concentrated. In the solution of methylglycoside of D, 1,4-dioxane (1 ml), and 0.1N H_2S0_4 (1 ml) was added and the solution was warmed for 30 minutes at 50˚C. The hydrolysis was complete after 24 h. 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, Gal and GlcNAc. Their identification was confirmed by comparison with authentic samples (TLC, PC).

Kiliani hydrolysis of compound Primose

Primose (5 mg) was dissolved in 2 ml Kiliani mixture (AcOH-H_2O-HCI, 7:11:2) and heated at 100^0C for 1 hours followed by evaporation under reduced pressure. It was dissolved in 2 ml of H_2O and extracted twice with 3 ml $CHCl_3$. The aqueous residual solution was made neutral by addition of 1-2 drops of 2N NaOH and was evaporated under reduced pressure to afford glucose, Gal and GlcNAc on comparison with authentic samples of glucose, Gal and GlcNAc.

Description of compound Primose

Compound (31.5 mg) was obtained from fraction 20-35 of column chromatography 5. On deacetylation of 25 mg of substance with NH_3 / acetone it afforded compound (19.0 mg) as a viscous mass. For experimental analysis, this compound was dried over P_2O_5 at 100˚C and 0.1 mm pressure for 8 hour.

$C_{38}H_{64}O_{26}N_4$	%C	%H	%N
Calculated	45.96	6.45	5.64
Found	45.95	6.45	5.63

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

^{1}H NMR: in D_2O

δ5.09 [d, 1H, J=3Hz, α -Glc (S-1) H-1], δ4.53 [d, 1H, J=8Hz, β -Glc (S-1) H-1], δ4.44 [d, 1H, J=8Hz, β-GalNAc (S-2)H-1], δ4.43 [d, 1H, J=8Hz, β-GlcNAc(S-3) H-1], 4.39[d,1H(J=8Hz)β-GlcNAc (S-4) H-1], δ4.32 [d,1H,J=8Hz, β-GalNAc (S-5) H-1],δ3.16 [t, 1H, J=8Hz, β-Glc(S-1) H-2], 2.10[s, 3H, NHCOCH3, β-GalNAc (S-2)], 1.96[s,3H, NHCOCH3 β-GlcNAc (S-3)], 1.88[s, 3H, NHCOCH3 β-GlcNAc(S-4)], 1.86[s, 3H, NHCOCH3 β-GalNAc(S-5)],

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

102.3 [2C,β-GlcNAc (S-3,S-4), C-1], 102.0 [2C,β-GalNAc (S-2,S-5), C-1], 91.9 [1C,β-Glc (S-1),C-1], 89.0 [1C α-Glc (S-1), C-1]

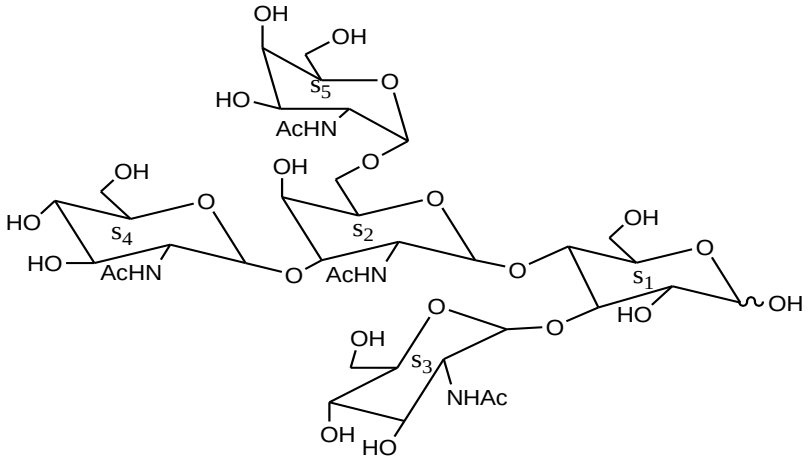
ES Mass

1054[M+Na+K+], 1031[M+K], 1015[M+Na], 991 [M]+,992, 977, 960, 956, 926, 789, 754, 739, 713, 667, 666, 585, 492, 440, 384, 366, 326, 162.

RESULTS AND DISCUSSION

Compound Primose $C_{38}H_{64}O_{26}N_4$ gave positive Phenol Sulphuric acid test(Dobois et al, 1956), Feigl test(Feigl et al, 1975) and Morgon-Elson test (Gey et al, 1996) showing the presence of normal and amino sugar(s) in the moiety. The ^{1}H NMR of Primose in D_2O at 400 MHz showed six anomeric proton signal for six anomeric protons in Primose at δ5.09. δ4.53, δ4.44, δ4.43, δ4.39, δ4.32 suggesting it to be pentasaccharide in its reducing form. The pentasaccharide nature of Primose was also confirmed by the presence of six crosspeaks in the anomeric region of HSQC spectrum of Primose at 400 MHz. The values of crosspeaks were present at δ 5.09x93, δ4.53x97, δ4.44x99, δ4.43x104, δ4.39x105 and δ4.32x105. The glycosidic region of HSQC spectrum of Primose Acetate contains four cross peaks for glycosidically linked carbons i.e. the region δ70-80x δ3.5-δ4.1 confirming the nature of Primose as pentasaccharide. The presence of α and β anomeric protons in the ^{1}HNMR of Primose and Primose Acetate confirmed the reducing nature of Primose. Further presence of four singlets of three protons each in the region 1.80-1.98 confirm the presence of four N-Acetyl group in Primose. The reducing nature of Primose was also confirmed by its methyl glycosidation followed by acid hydrolysis which led to the isolation of α and β methyl glucosides along with GalNHAc and GlcNHAc suggesting the presence of Glucose at the reducing end and presence of GalNAc and GlcNAc as its constituents.The five-monosaccharide present in Primose have been designated as S-1, S-2, S-3, S-4,and S-5 for the convenience starting from the reducing end. To confirm the monosaccharide constituents in Primose it was hydrolysed under strong acidic condition by Killiani Hydrolysis (Killiani et al**,** 1930**)** which gave three type of monosaccharide units i.e. Glc, GlcNHAc and GalNHAc supporting the fact that Primose consists of these monosaccharide units. ^{1}H NMR of Primose in D_2O at 400 MHz contains two anomeric proton signals as doublets at δ5.09 (J=4Hz) and δ4.53(J=8Hz) confirming the presence of Glucose at the reducing end, which was also confirmed by the ^{1}H NMR data of acetylated Primose in $CDCl_3$ at 400 MHz which contained two anomeric proton signal at δ6.22(J=4Hz) and δ5.63(J=8Hz). The anomeric proton of β-Glc at δ5.63 of Primose Acetate showed three cross peaks for ring protons with respect to anomeric proton at δ5.63, which were at δ3.58, δ3.81, δ4.90 in the TOCSY spectrum of Primose Acetate. In the TOCSY spectrum two cross peaks present at δ3.58 and δ3.81 showed that two of the OH- groups of reducing Glc S-1 were involved in glycosidation by the next monosaccharide unit. These positions of δ3.58 and δ3.81 were later identified as H-3 and H-4 of Glc(S-1) by COSY spectrum of Primose Acetate.

Further the ^{1}H NMR of Primose in D_2O at 400 MHz contained a triplet at δ3.16 shifted downfield(Urashima T. et al, 2007)indicating both the equatorially oriented hydroxyl groups present at C-3 and C-4 of reducing β-Glc were substituted and were involved in glycosidation and also suggesting that there was a presence of Lactose type of structure in it. Another anomeric proton present at δ4.44 as a doublet along with a singlet for N-Acetyl group at δ2.10 in the ^{1}H NMR of Primose was due to the presence of GalNHAc in the pentasaccharide. The large coupling constant of anomeric proton at δ 4.44(J=8Hz) confirmed β-glycosidiclinkage between GalNHAc(S-2) and Glc(S-1). Since the SRG of Lactose type of structure was supported by bythe triplet present at δ3.16 suggested 1→4 β-glycosidic linkage between GalNHAc(S-2) and Glc(S-1). Further the next anomeric proton which was present at δ4.43 along with a signal for N-Acetyl group at δ1.96 was due to the presence of GlcNHAc(S-3) moiety in the pentasaccharide Primose. As it was mentioned earlier that two hydroxyl groups (H-3 and H-4) of Glc (S-1) were available for glycosidic linkage and since H-4 of Glc(S-1) was already linked with GalNHAc(S-2) at H-4 of Glc(S-1), the GlcNHAc (S-3) must be attached to H-3 of reducing Glc(S-1). The large coupling constant of anomeric proton of GlcNHAc(S-3) at δ4.43(J=8Hz) confirmed the β-glycosidic linkage between GlcNHAc (S-3) and Glc(S-1) hence confirming β 1→3 glycosidic linkage between GlcNHAc (S-3) and Glc(S-1). All these assignments were confirmed by COSY and TOCSY spectrum of Primose Acetate. Further another anomeric proton of GlcNHAc(S-3) at δ4.43 in the ^{1}H NMR of Primose Acetate showed none of its methine proton in the glycosidic region in the TOCSY spectrum of Primose Acetate. Hence confirming the presence of GlcNHAc(S-3) at the non-reducing end. Further the anomeric proton of GalNHAc(S-2) present at δ4.44 as a doublet along with a singlet for N-Acetyl group at δ2.10 in the ^{1}H NMR of Primose suggested the presence of 3,6-disubstituted β-linked GalNHAc(SRG)supported by the signal of H-1 at δ4.4ppm and H-4 at a typical downfield shift around δ4.13-δ4.15ppm due to substitution in the 3-position by a β-linked GlcNHAc residue(S-4). Thus, confirming that two of the hydroxyl group(H-3 and H-6) of GalNAc (S-2) were available for glycosidic linkage. The next anomeric proton which was present at δ4.39 along with a signal at δ1.88 was due to presence of GlcNHAc (S-4) which was linked to H-3 of GalNHAc(S-2)SRG. The large coupling constant J=8Hz of anomeric signal at δ4.39(S-4) confirmed the β Glycosidic linkage between (S-4) and (S-2).The fifth anomeric proton which was present at δ4.32 along with a signal for N-Acetyl group at δ1.86 was due to presence of GalNHAc as a fifth monosaccharide in the pentasaccharide Primose. Since Structure Reporter Group value of GalNHAc(S-2) at δ4.44 suggests a 3,6- disubstituted GalNHAc and position H-3 of GalNHAc(S-2) was already substituted with GlcNHAc(S-4). The H-6 position of GalNHAc(S-2) must be substituted by GalNHAc(S-5). The large coupling constant of anomeric proton for GalNHAc(S-5) present at δ4.32(J=8Hz) confirmed the β-glycosidic linkage between GalNHAc(S-5) and GalNHAc(S-2). In the light of fore given evidences, the structure of Primose was confirmed as under-



PRIMOSE

Mass Fragmentation of Compound d

The Electronspray Mass Spectrometry data of primose not only confirmed the derived structure but also supported the sequence of monosaccharide in primose. The highest mass ion peaks were recorded at m/z 1054, 1031 and 1015 which were due to [M+Na+K], [M+K] and [M+Na] respectively.It alsocontainthe molecular ion peak at m/z 991 as M-H confirming the molecular weight of primose as 992 was in agreement with its molecular formula. Further the mass fragments were formed in the oligosaccharide and was accompanied by the elimination of terminal sugar less water.

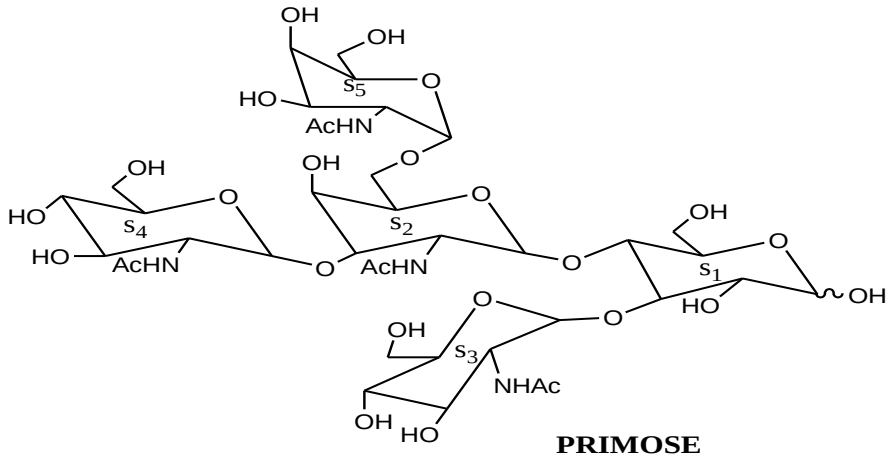
The pentasaccharide m/z 992 (I) fragmented to give mass ion at m/z 771(II) by loss of 221, [992-S5], this fragment was arised due to the loss of terminal α-GalNHAc (S5) moiety from pentasaccharide indicating the presence of GalNHAc (S5) at the non -reducing end. It further fragmented to give mass ion peak at m/z 550 (III) [771-S4] which was due to loss of GlcNAc S-4 moiety from Tetrasaccharide. This fragment of 550 further fragmented to give mass ion peak at m/z 347 (1V) [550-203] which was due to loss of GalNAc (S2) moiety from the trisaccharide, which further fragmented to give mass ion peak at m/z 144 [347-203] which was due to loss of GalNAc moiety from disaccharide.These four-mass ion peak II, III, IV, V, were appeared due to the consequent loss of S5, S4, S3, and S2 from original molecule. Based on result obtained from chemical degradation/acid hydrolysis, Chemical transformation, Electro spray mass spectrometry and ^{1}H, ^{13}C NMR and HOMO, COSY, TOCSY and HSQC2D NMR technique of acetylated Primose and Primose the structure and sequence of isolated Novel oligosaccharide molecule Primose was deduced as-

GalNAc(β 1→5)
(S-5)

GlcNAc- (β 1→3)-GalNAc- (β 1→4)
(S-4) (S-2)

Glc(S-1)

GlcNAc- (β 1→3)
(S-3)



PRIMOSE

CONCLUSION

From the above information, we concluded the structure of isolated Cow milk (Colostrum) oligosaccharide, Primose. This oligosaccharide was reported for the first time from any natural source or any milk and its structure was elucidated with the help of spectroscopic techniques like 1H, 13C, 2D-NMR (COSY, TOCSY and HSQC) spectroscopy and mass spectrometry.

ACKNOWLEDGEMENTS

The authors are thankful to UGC, New Delhi for financial assistance [Ref.No.: 254/(CSIR-UGC NET DEC.2017] and Prof. Raja Roy, CBMR-SGPGI Lucknow, India for providing NMR Spectroscopy facilities.

REFERENCES

Sundekilde, U.K., Larsen, L.B. and Poulsen, N.A. (2012). Natural variability in bovine milk oligosaccharide from Danish Jersey and Holstein-Friesian breeds. *Journal of Agricultural and Food Chemistry*, 60(24):6188-6196.

Schwonzen, M., Schmits, R., Baldus, S.E., Vierbuchen, M. and Hanish, F. G. (1992). Monoclonal antibody FW6 generated against a mucin-carbohydrate of human amniotic fluid recognizes a colonic tumour associated epitope*. Br. J. Cancer.* 65, 559-565.

Abe, K., Mckibbin, J.M.J. and Hakomori, S. (1983). The monoclonal antibody directed to difucosylated type 2 chain (Fuc alpha 1 leads to 2 Gal beta 1 leads to 4 [Fuc alpha 1 leads to 3] GlcNAc; Y Determinant). Eur. *J. Biochem.* 258: 11793-11797.

Fang, J.N., Proksch, H., Wagner, H. (1985). Immunologically active polysaccharides of Acanthopanax senticosus. *Phytochemistry*. 24(11), 2619-2622.

Maslowski, K.M., Vieira, A.T., Ng, A., Kranich, J., Sierro, F., Yu D., Schilter, H.C., Rolph, M.S., Mackay, F., Artis, D., Xavier, R.J., Teixeira, M.M., and Mackay, C.R. (2009). Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature*. 461, 1282-1286.

Ehresmann, D.W., Deig, E.F. and Hatch, M.T. (1979). Antiviral properties of algal polysaccharides and related compounds, *Mar. Algee Pharm. Sci*. 293-302.

Euler, A.R., D.K. Mitchell, R. Kline and L.K. Pickering (2005). Prebiotic effects of fructo-oligosaccharides supplemented term infant formula at two concentrations compared with unsupplemented formula and human milk. *J. Pediatr. Gastroenterol*. *Nature*., 40(2):157-64.

Fanaro, S., G. Boehm, J. Garssen, J. Knol, F. Mosca, B. Stahl and V. Vigi (2005). Galacto oligosaccharides and long-chain fructo-oligosaccharides as prebiotics in infant formulas, *Acta P aediatr Supp. l*, 94(449):22-6.

Gunjan, Narain D., Khare A. and Deepak D. (2016). Isolation of novel oligosaccharide from Shyama Dhenu (Black cow) milk, *J. Biol. Chem. Research*. Vol.33, No. 2: 648-654.

Gangwar, L., Narain D., Khare A. and Deepak D. (2017). Isolation and structure elucidation of novel milk oligosacharide from Shyama Dhenu (Black cow) milk, *J. Biol. Chem. Research*. Vol. 34 (1): 249-255.

Gunjan, Kumar K. and Deepak, D. Structural characterization of Noval Milk Oligosaccharide Aurose from cow colostrums, *Journal of molecular structure*, Volume 1176, 394-401.

Khan, M., Narain D., Mishra A., Khare A. and Deepak D. (2017). Isolation of Noval Oligosaccharide from Bos indicus (Black cow) Milk and their DFT Studies, *International Journal of Carbohydrate Research*, Vol. 7(1): 4-8.

Gangwar, L., Narain D., Khare A. and Deepak D. (2017). Isolation and Structure Elucidation of Novel Milk Oligosaccharide from Shyama Dhenu (Black Cow) Milk, *J. Biol. Chem. Research*. 34, 1, 249-255.

Khan, M., Sharma S., Narain D., Mishra A., Khare A. and Deepak D. (2017). Structure Elucidation of Novel Oligosaccharide from Shyama Dhenu Milk and their DFT studies, *J. Biol.Chem. Research*. Vol. 34 No. 1: 188-195.

Shahi, S., Khan M. and Deepak, D. (2017). Isolation, Purification and NMR Study of a Novel Nonasaccharide (Rieose) from 'Gaddi Sheep' Milk, *J. Biol. Chem. Research.* 34, 2, 557-568.

Dubois, M., Giellet, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956). Colorimetric method for determination of sugars and related substances, *Analytical Chemistry*. 28: 350-356.

Feigl, F. (1975). Spots tests in organic analysis. *Elsvier Publication, Amsterdam*. pp. 337.

Gey, M.H., Unger, K. and Batterman, G. (1996). A Strategy for Chromatoghraphic and Structural analysis of monosaccharide species from glycoproteins, *Journal of Analytical Chemistry*. 356, 488-494.

Killiani, H. (1930). Uber Digitalinum Verum. bet. Deutsch *Chem. Ges*. 63: 2866-2869.

Urashima, T. and Mami Kobayashi (2007). Sadaki Asakuma, Yusuke Uemura, Ikichi Arai, Kenji Fukuda, Tadao Saito, Toshihiro Mogoe, Hajime Ishikawa, Yutaka Fukui, 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.

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