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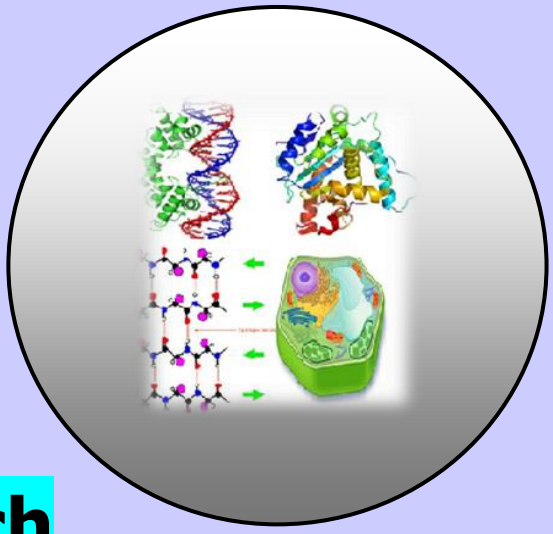

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RESEARCH PAPER

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Isolation and Structure Elucidation of Novel Oligosaccharide Aminose from Mare Milk

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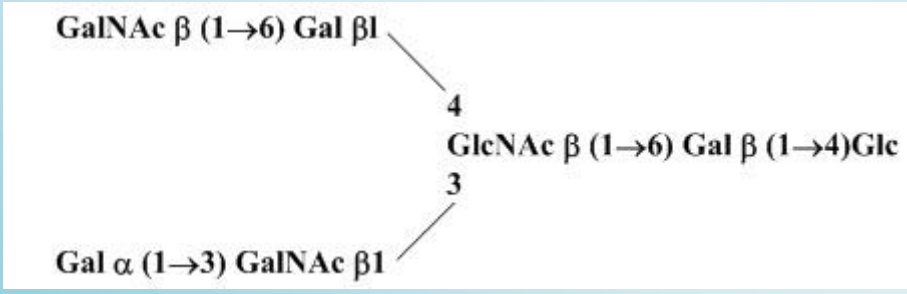
ABSTRACT

Milk is an essential bioactive fluid which is responsible for growth and development of mammalian neonates. Milk is the only naturally occurring product which contains nearly all components necessary for human health like carbohydrates, proteins, fatty acids, lactose and vitamins responsible for growth and immunological support. Oligosaccharides which are present in milk in high ratio having immuno-stimulant, anti-cancer, anti-oxidant,lipid lowering and anti-inflammatory activities. In the present study, to find more biologically active novel milk oligosaccharides which have medicinal importance, mare milk was collected and processed by the method of Kobata and Ginsburg followed by gel filtration HPLC and column chromatography which resulted in the isolation of a novel milk oligosaccharide named Aminose. The structure of isolated and purified milk oligosaccharide was elucidated with the help of chemical degradation, chemical transformation, spectroscopic techniques like NMR (^{1}H, ^{13}C and 2D NMR) and mass spectrometry. The isolated oligosaccharide (Aminose) was interpreted as heptasaccharide having branched structure as-



AMINOSE

Key words: Mare milk, Oligosaccharides, Kobata and Ginsburg method.

INTRODUCTION

Milk furnishes a broad range of biologically active compounds that guard neonates, adult pathogens and illness such as immunoglobulin peptides, antimicrobial proteins, lipids and oligosaccharides besides many other components at low concentration. Milk contains a wide range of prebiotic oligosaccharides impacting various physiological and pathological process like molecular reorganisation, signal transaction and exhibit varied biological activities such as anti-tumour (Schwonzen et al 1992), anticancer (Fang et al 1985), immuno-stimulant (Abe et al 1983) and immunological activities (Srivastava et al 2012). They also have inhibitory effect on certain virulence-related abilities of monocytes, lymphocytes and neutrophils adhesion to endothelial cells (Singh et al 2016). The cow's milk oligosaccharide reduces the adhesion of enterotoxic E.coli strain of the calf (Johnsson et al 2005). Goat milk oligosaccharides have been important role in intestinal protection and repair after a change caused by DSS (dextron sodium sulphate) induced colitis and their implication in human intestinal inflammation (Villoslada et al. 2006). The development of carbohydrates and glycoconjugates as therapeutics continues to gain interest when biological relevance of these biopolymers were further revealed and understood (Singh et al 2016). The oligosaccharide isolated from elephant milk contained a high ratio of sialyl oligosaccharides which may be significant with respect to the formation of brain components such as gangliosides of suckling calves (Ostho et al 2007). Donkey milk oligosaccharides have ability to stimulate nonspecific and specific immunological resistance (Deepak et al 1998). Mare milk oligosaccharide fractions are having multi-fold properties such as anti-oxidant and lipid lowering activities (Srivastava et al 2012). Numerous oligosaccharides have been isolated from milk of many mammalian species including equine, bovine and marine mammals (Kunz et al 2000, Urashima et al 2008 and Nakamura et al 2004). Mare milk includes numerous biologically active oligosaccharides, it have an excellent medicinal importance usually in treatment of metabolic gastrointestinal and liver problems and for recovering after surgery and severe illness. Because of its importance in treatment of inflammatory disorder (especially inflammation of intestine), in liver disorder in cholesterol and skin disorders, we worked on isolation oligosaccharide from mare milk. In the present study, we have elucidated the structure of mare milk oligosaccharide namely Aminose with the help of chemical degradation, chemical transformation, spectroscopic techniques like (^{1}H NMR, ^{13}C NMR and 2D NMR).

MATERIAL AND METHODS

General procedure

General procedure was same as in our previous articles (Ranjan et al 2015).

Isolation of Mare milk oligosaccharide by Kobata and Ginsberg method

10 litres milk was collected from a female horse and then isolated by method of Kobata and Ginsburg (Kobata 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 (S.M. Partridge 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 (M. Dubois et.al. 1956) for the presence of neutral sugar.

Acetylation of Mare milk oligosaccharide mixture

Dry oligosaccharides of pooled fractions (12 gm) which gave positive phenol-suphuric acid test were acetylated with pyridine (12ml) and acetic anhydride (12ml) at 60° C for 24 hr. The mixture was evaporated under reduced pressure and viscous residue was taken in $CHCl_3$ and washed in sequence with 2 N HCl, ice cold 2N $NaHCO_3$ and finally with H_2O. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acetylated mixture (15.5g). Non-polar acetyl derivative of oligosaccharides were resolved nicely on TLC using $CHCl_3$: MeOH as eluent. Detection of the spots was done by spraying with 50% H_2SO_4 and heat.

Purification of Acetylated milk oligosaccharide on Silica Gel Column

Separation and purification of acetylated derivative were carried over silica gel column chromatography into compounds : silica ratio of 1:100 using various proportion of Hexane: $CHCl_3$, $CHCl_3$, $CHCl_3$:MeOH mixture which was resolved into twelve fractions namely I(259mg), II(92mg), III(164mg), IV(2.05gm), V(1.95gm), VI(2.82gm), VII(120mg), VIII(286mg), IX(726mg), X(187mg), XI(342mg) and XII(55mg) respectively. These fractions were containing mixture of two to three compounds. Repeated column chromatography of fraction VI led to the isolation of one chromatographically pure compound "D" (47mg).

Deacetylation of Compound

Deacetylation of acetylated oligosaccharide "D" (47mg) was carried out in 2ml acetone and 1.3ml NH_3 for 24hrs in a stoppered hydrolysis flask. After 24hrs ammonia was removed under reduced pressure, equal volume of $CHCl_3$ and water were added and the compound was recovered in the aqueous phase and the water layer was finally freeze dried giving the deacetylated oligosaccharide Aminose (27mg).

Description of Isolated Compound Aminose

Compound D (37 mg) obtained from fraction 40-48 of column chromatography-5, on deacetylation with NH_3/ acetone it afforded compound D (16 mg), as a viscous mass. For elemental analysis, this compound was dried over P_2O_5 at 100°C and 0.1 mm pressure for 8 hr.

$C_{48}H_{81}N_3O_{36}$		% C	% H	% N
	Calcd.	45.17	6.35	3.29
	Found	45.19	6.36	3.28

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

^{1}H NMR: δ in $CDCl_3$ (ppm)

δ 4.51 (d, 1H, J=8.4 Hz β-GlcNAc (S_7), H-1), 4.54 (d, 1H, J = 7.5 Hz, β-Gal(S_2), H-1), 4.57 (d, 2H, J = 7.5 Hz, β-Gal(S_4) & β-GalNAc(S_5), H-1), 4.63 (d, 1H, J = 8.1 Hz, β-GlcNAc (S_3), H-1), 5.4 (d, 1H, J = 3.0 Hz, α Gal (S_6), H-1), 5.76 (d, 1H, J = 8.4 Hz, β-Glc (S_1), 6.37 (d, 1H, J = 3.3 Hz, α-Glc (S_1), H-1).

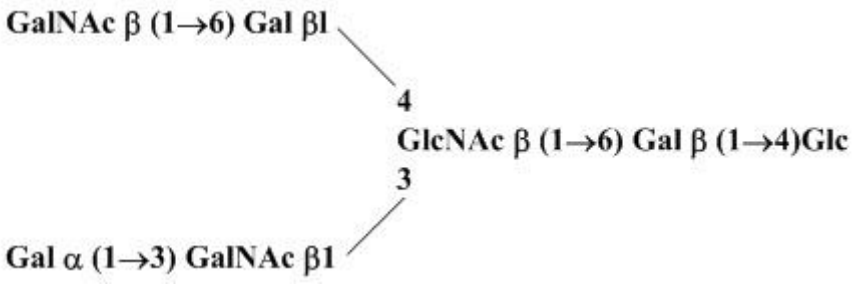
^{13}C NMR: δ in $CDCl_3$ (ppm)

δ20.5[NHCO<u>CH</u>$_3$,β-GalNAc (S_5& S_7)], 20.6[NHCO<u>CH</u>$_3$,β-GlcNAc(S_3)], δ 89.2, α Glc (S_1) C1, δ 90.3, α Gal (S_6) C1, 91.5 β Glc (S_1) C-1, 100.8 β Gal (S_2) C-1, 101.1 β Gal (S_4) C-1, 103.5 β-GlcNAc (S_3) C-1, 104.2 β-GalNAc (S_7) C-1, 104.4 β-GalNAc (S_6) C-1.

RESULT AND DISCUSSION

Compound 'D' Aminose, $C_{48}H_{81}O_{36}N_3$ gave positive Phenol-sulphuric acid test, Fiegl test (Fiegl et al 1975) and Morgon-Elson test indicating the presence of normal and amino sugar(s) in the moiety. The ^{1}H NMR spectrum of acetylated compound 'D' at 300 MHz exhibited Eight signals in the anomeric proton region as doublets at δ6.37 (1H), δ5.76 (1H), δ5.4 (1H), δ 4.63 (1H), δ4.57 (2H), δ4.54 (1H) and δ4.51 (1H) for Eight protons leading to the presence of eight anomeric protons in compound. It was further supported by the appearance of eight signals for eight anomeric carbons at δ 89.2 (1C), 91.5 (1C), 90.3 (1C), 103.5 (1C), 100.8 (1C), 101.1 (1C), 104.4 (1C) and 104.2 (1C) in the ^{13}C NMR spectrum of Aminose. These data led to the suggestion that 'D' may be a heptasaccharide in its reducing form. The seven-monosaccharide units present in compound 'D' have been designated as S_1, S_2, S_3, S_4, S_5, S_6 and S_7 for convenience starting from the reducing end. The Kiliani acid hydrolysis (Killiani et al 1930) of compound 'D' gave four spots on the paper chromatography, which were identified as Glc, Gal, GlcNAc and GalNAc 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, which suggested the presence of glucose at the reducing end in the oligosaccharide molecule. 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 6.37 (1H, J=3.0 Hz) and δ 5.76 (1H,J=8.4 Hz) respectively. The presence of a lactosyl moiety i.e. β Gal β (1→4) Glc in compound D was confirmed by two doublets of anomeric protons present at δ 4.54 (1H, J=8.1 Hz) and δ 5.76 (1H, J=7.8 Hz) for β Gal (S_2) and β Glc (S_1) residues respectively which is present in lactosyl moiety in compound Aminose. This linkage was further confirmed by H-2 signal β Glc (S_1), which appeared as a triplet at δ 5.276, J=8.7 Hz in acetylated spectrum led to suggestion for lactose in compound of 'D'. Further, the ^{1}H NMR spectrum showed another anomeric proton signal appeared as a doublet at δ 4.63 (1H, J=8.1) along with singlet signal of NHAc group of three protons at δ 2.17 was due to the presence of β GlcNAc (S_3) which is present next in sequence with lactose moiety. The linkage of GlcNAc to the lactose moiety, β GlcNAc (S_3) [1→6] β-Gal (S_2) was confirmed by acetylated ^{1}H NMR spectrum of Aminose, In which H-6 of Gal (S_2) of lactose appeared at δ 3.66 ppm. The acetylated ^{1}H-^{1}H COSY spectrum of Aminose also showed the H-6 proton signal of β Gal (S_2) at up field position and this confirms that the oxygen atom of C-6 of β Gal (S_2) was involved in inter residual glycosidation with β GlcNAc (S_3) [1→6] β-Gal (S_2). This linkage was also confirmed with the HSQC spectrum of acetylated compound D, In this spectrum the H-6 proton of β Gal (S_2) was present at δ 3.66 in ^{1}H axis and its cross peak with C-6 on ^{13}C axis present at δ 72.5 (δ 3.66 x δ 72.5). In ^{1}H-^{1}H COSY and TOCSY spectrum of acetylated compound 'D', H-2 and H-3 proton of GlcNAc (S_3) appeared on upfield region at δ3.82 ppm and δ3.75 ppm respectively, this implies that β-GlcNAc (S_3) was equatorially linked at C-3 and C-4 positions.

Further, In acetylated spectrum of compound Aminose, the fourth anomeric proton present as a doublet at δ 4.570 (2H, J=7.5 Hz) of β-Gal (S_4), it was glycosidically linked to the C-4 of β GlcNAc (S_3) because H-4 proton of S_3 was present at δ 3.65 ppm. The linkage Gal (S_4) [1→4] β-GlcNAc (S_3) was confirmed by ^{1}H-^{1}H COSY spectra of acetylated compound of Aminose. This linkage was further supported by acetylated HSQC spectrum of Aminose, In which H-4 proton of GlcNAc (S_3) was present at δ 3.65 in ^{1}H axis and its cross peak with C-4 on ^{13}C axis present at δ 73.9 (δ 3.65 x δ 73.9).The fifth anomeric proton appeared as a doublet at δ 4.570 (2H, J=7.5 Hz) along with a singlet of three protons at δ 2.18 of β-GalNAc (S_5), it glycosidically linked to the C-3 of GlcNAc (S_3) because H-3 proton present at δ 3.82 ppm. This linkage further was further supported by COSY spectra of GlcNAc (S_3) which is also discussed in earlier. The acetylated HSQC spectrum of acetylated Aminose also confirms that the H-3 proton of GlcNAc (S_3) give cross peak with C-3 at (δ 3.82 x δ 76.6) which indicated that GalNAc (S_5) moiety β glycosidically linked to S_3 at C-3 position. The sixth anomeric proton of Aminose appeared as a doublet at δ 5.4 (1H, J=3.0 Hz) showed the presence of α Gal (S_6) in compound, which was glycosidically linked to the C-3 of GalNAc (S_5), this linkage α Gal (S_6) [1→3] β-GalNAc (S_5) was confirmed by the acetylated ^{1}H-^{1}H COSY spectrum of Aminose shows that H-3 proton signals of S_5 appeared upfield region at δ 3.85 ppm. The acetylated HSQC spectrum also confirms that the H-3 proton of S_5 given cross peak with C-3 at (δ 3.85 x δ 72.5) which indicated that (S_6) moiety α glycosidically linked to S_5 at C-3 position. In Acetylated ^{1}H NMR spectrum, Seventh anomeric proton appeared as a doublet at δ 4.512 (1H, J=7.5 Hz) along with a singlet of three protons at δ 2.19 of another β-GalNAc (S_7) sugar, it glycosidically linked to the C-6 of Gal (S_4) because H-6 proton present at δ 3.89 ppm because this position are involved in glycosidation with S_7 sugar. It was further supported by ^{1}H-^{1}H COSY and TOCSY spectrum region of Gal (S_4). The HSQC spectrum of acetylated Aminose also confirms that the proton of C-6 of Gal (S_4) give cross peak at (δ 3.89 x δ 61.0) which indicated that GalNAc (S_7) moiety is β glycosidically linked to S_4 at C-6 position. The Heptasaccharide nature of Aminose was further confirmed by spectral studies of acetylated derivative of compound. The heteronuclear single quantum-coherence (HSQC) spectrum of acetylated product of compound 'D' confirms the anomeric assignments in ^{1}H and ^{13}C NMR spectra of 'D' by showing the ^{1}H and ^{13}C cross peaks of α-Glc (S_1) at δ6.37 x δ 89.2 and β-Glc(S_1) at δ 5.76 x 91.5. It also contains other six crosspeaks of anomers of one β- Gal (S_2) at δ 4.54 x 100.8, β-GlcNAc (S_3) at δ 4.63 x 103.5, β-Gal (S_4) at δ 4.57 x 101.1, β-GalNAc (S_5) at δ 4.57 x 104.4, α-Gal (S_6) at δ 5.48 x δ 90.3 and one β-GalNAc (S_7) moiety at δ 4.51 x δ104.2 respectively. The results obtained from pattern of chemical shift of ^{1}H, ^{13}C, ^{1}H-^{1}H HOMOCOSY and HSQC NMR experiments, it was interpreted that the compound Aminose was a Heptasaccharide having one reducing end Glucose (S_1), two β Gal (S_2 & S_4), one β GlcNAc (S_3), one α Gal (S_6) and two β GalNAc (S_5 & S_7) moieties and the isolated oligosaccharide (AMINOSE) was interpreted as Heptasaccharide having branched structure. The results obtained from chemical degradation / acid hydrolysis, chemical transformation and ^{1}H, ^{13}C, HOMOCOSY and HSQC NMR techniques, the structure of acetylated novel oligosaccharide (AMINOSE) was deduced as-



AMINOSE

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