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Isolation of novel heptasaccharide from oligosaccharide fraction of Chauri milk

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Abstract

Oligosaccharide components of cells play essential roles in physiological and pathological processes such as molecular recognition, signal transduction, differentiation, and developmental events.[1]The milk oligosaccharides recognize cancer associated antigens, used as antimicrobial agents; tumor associated antigens and has physiological significance in infants. Recently Buffalo and Donkey milk oligosaccharides have shown promising immunostimulant activity. The medicinal importance of the Chauri milk in the ancient Indian and Tibetans medicinal system is enormous. It has been found that the amino acid, calcium and vitamin A in Chauri milk are comparatively higher than in cow's milk and yak milk casein could become a resource to generate antihypertensive peptides and be used as multifunctional active ingredients for many value-added functional foods as well as a traditional food protein. We have isolated novel heptasaccharide and used recent technique for structure interpretation like 1D NMR (^{1}H, ^{13}C) 2D NMR (HSQC, COSY, TOCSY) & Mass spectrum.

Key wards: NMR Spectroscopy, Mass spectrum, Heptasaccharide, Nienose.

1. Introduction

Carbohydrates are the most abundant natural products. Besides their role in metabolism and as structural building blocks, they are fundamental constituents of every cell surface, where they are involved in vital cellular recognition processes[2]. The tremendous interest in this area stems from the fact that oligosaccharides and glycan-decorated molecules pervade all biological systems. The oligosaccharides which are present in glycoprotein represent the determinant, which is responsible for the specific biological action and determines the antigenic properties of the cells[3]. Oligosaccharides along with lactose may play a role in postnatal brain development. Many newborn mammals undergo a period of rapid postnatal brain development that requires large amount of glycolipid, which are components of cell membranes of neurons and myelin.

2. Materials and Methods

Isolation of Chauri Milk Oligosaccharides

10 liter Chauri milk was collected in about 10 milking as domestic Chauri Gai from Mountains of Dhawalagiri in Nepal at 5000 fts. The milk was fixed by addition of equal amount of ethanol. The preserved milk was taken to laboratory and there it was centrifuged for 30 min at 5000 rpm at 4^0C. The solidified layer was removed by filtration through glass wool column in cold. More ethanol was added to clear filtrate to a final concentration of 68% and the resulting solution was left over night at 0^0C. The white precipitate formed, mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0^0C. The supernatant and washings were combined and filtered through a micro filter and lyophilized affording crude oligosaccharide mixture.

3. Results and Discussion

Compound Nienose

Compound **D** Nienose $C_{46}H_{78}O_{35}N_2$, $[\alpha]_D$ $+70.88^0$ gave positive Phenol-sulphuric acid test[4], Feigl test[5], Morgon-Elson[6] test showing the presence of normal and amino sugar(s) in the compound. The 800 MHz 1HNMR spectra of compound D in D_2O showed the presence of eight doublet in the anomeric proton region at δ4.315(1H), 4.426(1H), 4.441(1H), 4.530(1H), 5.085(1H), 5.133(1H), 5.433(1H) 5.603(1H) which could be interpreted for presence of a heptasaccharide in its reducing form, giving signals for α and β anomers of reducing monosaccharide in the heptasaccharide. The heptasaccharide nature of Nienose was further supported by the presence of eight anomeric carbons in the 13C NMR of compound D at δ 91.73 (1C), 92.19(1C), 95.67 (1C), 96.36(1C), 98.0(1C), 100.74(1C), 102.74, 102.77, confirming the heptasaccharide nature of Nienose its reducing form. Further the HSQC spectrum of acetylated Nienose exhibited the cross peak of α and β anomers at δ [5.35 x 90.20] and δ [5.25 x 92] indicating that the compound D was a heptasaccharide in its reducing form, giving signals for α and β anomers of glucose at its reducing end. The reducing nature of compound Nienose was further confirmed by its methylglycosidation by MeOH/H+ followed by its acid hydrolysis which led to the isolation of α and β-methyl glucoside leading to the presence of glucose at the reducing end in the oligosaccharide. Further the ES mass spectrum of Nienose showed the highest mass ion peak at m/z 1257 assigned to [M+K]+ and m/z 1241 assigned to [M+Na]+,confirming the molecular weight of Nienose 1218 which was in agreement of derived composition C_{46} H_{78} O_{35} N_2 with the molecular ion expected at m/z 1218.The seven monosaccharide present in Nienose have been designated as S1, S2, S3, S4, S5, S6 and S7 for convenience from the reducing end. To confirm the monosaccharide constituents in Nienose, it was hydrolyzed under strong acidic conditions (Kiliani hydrolysis).[7] In Kiliani hydrolysis the reducing heptasaccharide gave five spots on paper chromatography, which were identified as Glc, Gal, GlcNAc, GalNAc and Fuc by co-chromatography with authentic samples (paper chromatography) suggesting that the reducing heptasaccharide was made up of these four monosaccharide units. The

chemical shifts values of anomeric carbons observed in 13C NMR spectrum of Nienose and of anomeric protons observed in 1H NMR spectrum of Nienose were also in agreement with the reported values of 1H and 13C anomeric chemical shifts of Glc, Gal, Fuc and GlcNAc confirming the presence of these monosaccharide in the oligosaccharide. Further the Presence of two anomeric proton doublet signals at 800 MHz in D_2O in the ^{1}HNMR spectrum of Nienose at δ 5.604 (J=4.0 Hz and δ 5.435 (J=6.4 Hz) were assigned to the presence of α and β anomers of glucose at the reducing end in the heptasaccharide that also confirmed the presence of glucose at the reducing end[8] in compound D.The anomeric proton of β-Glc (S-1) at 5.433 showed two consequent complementary signals in the linkage region at 3.55 and 3.82 in the TOCSY spectrum of Nienose acetate. These signals were identified as H-3 and H-4 of β-Glc (S-1) by the COSY spectrum of Nienose acetate suggesting that H-3 and H-4 of S-1 were available for glycosidic linkage by the next monosaccharide unit.

1H and 13C NMR values of compound D in D_2O

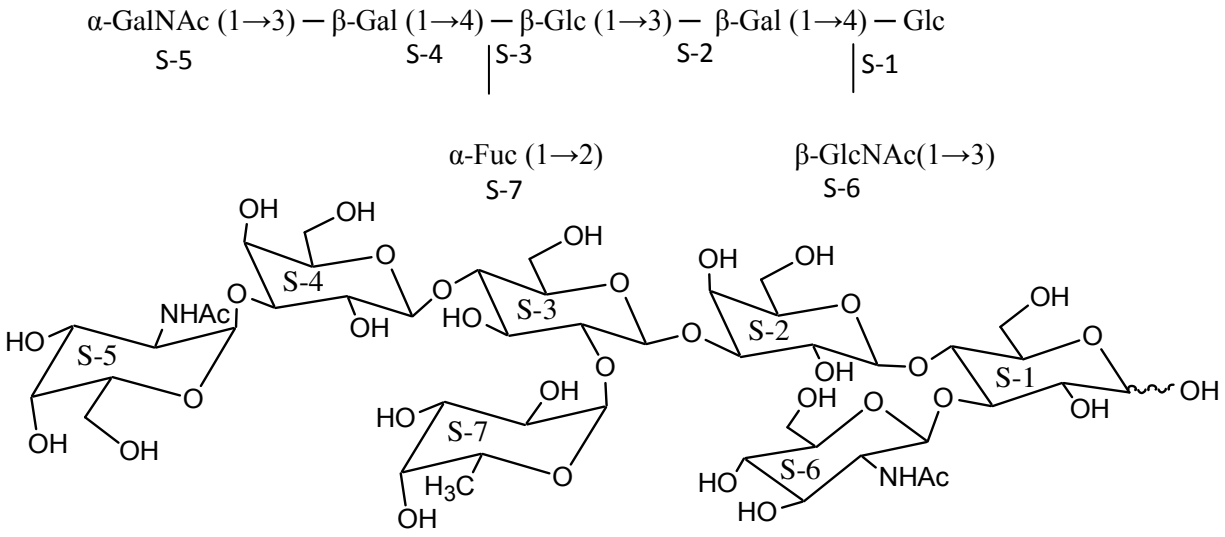
Moieties	1H NMR	Coupling Cons.(J)	13C NMR
α-Glc (S-1)	5.603	4.0	91.73
β-Glc (S-1)	5.433	6.4	96.36
β-Gal (S-2)	4.441	8.0	102.74
β-Glc (S-3)	4.530	8.0	98.00
β-Gal (S-4)	4.315	8.0	102.77
α-GalNAc (S-5)	5.133	4.0	92.19
β-GlcNAc (S-6)	4.426	7.2	100.74
α-Fuc (S-7)	5.085	4.0	95.67

Further the presence of another anomeric proton doublet at δ 4.441 (J=8.0 Hz) showed the presence of β-Gal S-2 residue as the next monosaccharide. The linkage between S-1 and S-2 was confirmed by the appearance of β-Glc (S-1) H-2 signal which appeared as a triplet at δ 3.18 (SRG) confirmed (1→4) linkage between S-2 and S-1, hence confirming the presence of lactosyl moiety i.e. β Gal (1→4) Glc in compound D at the reducing end. The linkage was further confirmed by presence of β-Glc H-4 signal at δ 3.81 in the ^{1}H NMR of acetylated D which was assigned by the COSY and TOCSY spectrum of acetylated D. The next anomeric proton signal which appeared at δ 4.426 (J= 7.2 Hz) along with a singlet of amide methyl at δ 1.89 was due to the presence of β-GlcNAc moiety[9] S-6. As already suggested by TOCSY spectrum of Nienose acetate that positions 3 and 4 of glucose (S-1) were vacant for glycosidic linkages and position 4 was already occupied by Gal S-2, GlcNAc must belinked to H-3 of S-1. This linkage was further supported by the presence of ^{1}H NMR of acetylated Nienose in which the signal for H-3 of S-1 appeared at δ 3.55 confirming the 1→3 linkage between S-

6 andS-1. The coupling constant of anomeric signal (S_3) with J value 7.2 Hz confirmed the β-configuration of the linkage between $S_6 \rightarrow S_1$. The next anomeric proton signals, which appeared at δ 4.53 was due to presence of Glc (S-3) moiety. The coupling constant of anomeric signal of S-3 with J values of 8.0 Hz shows β-configuration of anomeric linkage between S3→S2. In the TOCSY spectrum of Nienose acetate the anomeric proton signal at 4.41 showed that only one position of Gal (S-2) was involved in glycodic linkage by the chemical shift at 3.90 which was assigned at H-3 of S-2 by COSY spectrum of Nienose acetate. Further the anomeric proton of β-Glc (S-3) at 4.530 showed two consequent complementary signals in the linkage region at δ 3.41 and δ 3.75 in the TOCSY spectrum of Nienose acetate. These signals were identified as H-2 and H-4 of β-Glc (S-3) by the COSY spectrum of Nienose acetate suggesting that H-2 and H-4 of S-3 were available for glycosidic linkage by the next monosaccharide units. The next anomeric proton signal which appeared at δ 5.085 (J=4.0 Hz) in the heptasaccharide was identified as α-Fuc (S_7) the J value of anomeric signal J= 4.0 Hz. suggested the α glysodic linkage for fucose, the 1HNMR of Nienose at 800 MHz also contain the secondary methyl doublet of Fucose at δ 1.12 (J=6.0 Hz.). The position of linkage H-2 of S-3 was confirmed by the H-2 signal at δ 3.41 by the COSY spectra of Nienose acetate. The position of H-2 of S-3 at δ 3.41 confirmed the 1→2 linkage between S-7 and S-3. Since the anomeric proton of α-Fuc (S_7) at δ5.085 in TOCSY spectrum of Nienose acetate does not show any signal in linkage region confirming that α -Fuc (S_7) was present at non –reducing end and none of its OH group was available for glycosidic linkage. The next anomeric proton signal which appeared as a doublet at 4.315 (J=8.0 Hz) was due to presence of of β-Gal (S-4) in 1HNMR of Nienose in D_2O. As already suggested by TOCSY spectrum of Nienose that position 2 and 4 of glucose (S-3) were vacant for glycosidic linkages and position 2 was already occupied by Fuc S-7, β-Gal (S-4) may be linked to H-4 of S- .

The linkage between S-4 and S-3 was further supported by the presence of ^{1}H NMR signal of acetylated Nienose in which the signal for H-4 of S-3 appeared at δ 3.75 confirming the 1→4 linkage between S-4 and S-3. The results arisen from 1H NMR Nienose in D_2O at 800 MHz concluded that Nienose contains a repeating lactosyll moiety in it with the sequence S-4→S-3→S-2→S-1 (Gal→Glc→Gal→Glc) with branching at S-1 and S-3. The linkage between S-3 and S-4 was confirmed by the appearance of β-Glc (S-3) H-2 signal which appeared as a triplet at δ 3.118 (2H) (SRG) confirmed the presence of another lactosyl moiety i.e. Gal β (1→4) Glc in compound D. The next anomeric proton signal appeared at δ 5.138 (J=4.0 Hz) along with a singlet of amide methyl at δ 1.772 was due to the presence of α-GalNAc moiety. Since in the TOCSY spectrum of Nienose acetate the anomeric signal of Gal S-4 at δ 4.315 showed a signal at δ 3.90 which was later assigned for H-3 by COSY spectrum of Nienose acetate of S-4 suggested that S-5 may be linked to S-4 by1→3 linkage. The position of GalNAc (S-5) was confirmed at non reducing end by TOCSY spectrum of Nienose acetate in which the anomeric proton signal of GalNAc S-5 at δ 5.138 does not contain any

of its ring proton in glycosidic region. The heptasaccharide nature of compound D was further confirmed by the spectral studies of acetylated derivative of this compound. The heteronuclear single quantum coherence (HSQC)[10] spectrum of acetylated compound confirmed the position of glycosidic linkages by cross peaks of β-Glc (S1) H-4 and C-4 at (δ 3.82 x 73.0) showed (1→4) linkage of S2 and S1 and β-Glc (S1) H-3and C-3 at (δ 3.55 x 72.0) showed (1→3) linkage i.e. its 4 and 3-positions of Glc (S1) was involved in linkage, β-Gal (S2) H-3 and C-3, at (δ3.966 x 74.82) showed (1→3) linkages of S3 → S2. β-Glc (S3) H-4 and C-4 at (δ 3.75 x 73.0) showed (1→4) linkage of S-4 and S-3, β-Glc (S3) H-2 and C-2 (δ 3.41 X 82.0) showed (1 →2) linkage between S3→S7. β-GalNAc (S5) H-3 and C-3, at (δ 3.9 x 75.0) showed (1→3) linkages of S5 and S4 . These chemical shifts obtained from cross peaks of HSQC were consistent with the COSY and TOCSY spectrum. the pattern of chemical shifts of 1H , 13C, HOMOCOSY, TOCSY and HSQC NMR experiments it was interpreted that the compound Nienose was heptasaccharide comprised of two Glc, two Gal, one GlcNAc, one GalNAc and one Fuc moieties having the structure. The Electronspray Mass Spectrometry data of compound not only confirmed the derived structure but also supported the sequence of monosaccharide in Nienose. The highest mass ion peak was recorded at m/z 1257 which was due to [M+K], the other mass ion peak recorded at m/z 1241 which was due to $[M+Na]^+$ confirming the molecular weight of Nienose as 1218 and was in agreement with its molecular formula. Based on result obtained from chemical degradation/acid hydrolysis, Chemical transformation, Electro spray mass spectrometry and ^1H, ^{13}C NMR and HOMOCOSY, TOCSY and HSQC 2D NMR technique of Nienose and acetylated Nienose the structure and sequence of isolated Novel oligosaccharide molecule Nienose was deduced as-



Experimental

General Procedure

The optical rotations of oligosaccharides were measured with AA-5 series automatic apolarimeter in 1cm tube. The 1H and 13C NMR spectra of oligosaccharides were recorded in D2O and the spectra of acetylated oligosaccharides were recorded in CDCl3 at 250C on a Brukerl AM 400 and 800 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 H2SO4 reagent and on paper chromatography sugars were visualized 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 H2O. 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 centrifuge 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.

Chromatography

Following chromatographic techniques have been used for the isolation and identification of the oligosaccharides

Paper Chromatography (PC)

Paper chromatography was performed on Whatman paper No.1 by the use of a three solvent system of toluene, butanaol and water and spots were detected by appropriate reagent for specific moieties.

Thin Layer Chromatography (TLC)

The glass plates coated with slurry of silica gel G (SRL) in water were used which were dried at room temperature for about 24 h and activated at 100-1100C.

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