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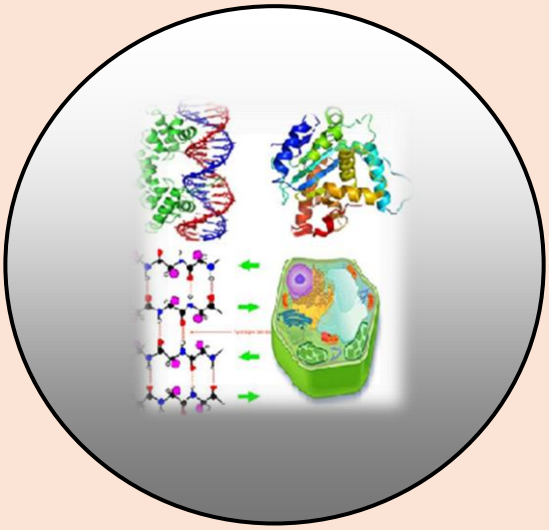

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RESEARCH PAPER

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Structure Elucidation of Novel pentasaccharide Ubaliose from *Bubabus bubalis* Colostrum

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ABSTRACT

Oligosaccharides present in the milk of different origin have shown varied biological activities. Oligosaccharide isolated from Buffalo milk has shown encouraging immuno stimulant activities. With a view to isolate more biologically active oligosaccharides from colostrum of buffalo (Babalus bubalis). Buffalo Colostrum was collected and processed by modified method of Kobata and Ginsberg, involving deproteination, centrifugation and lyophilization followed by acetylation and silica gel column chromatography of derivatized oligosaccharides. The structure of isolated pentasaccharide was elucidated by chemical transformation, chemical degradation, ^{1}H NMR, ^{13}C NMR, 2D NMR (HSQC, TOCSY, COSY and HMBC) and mass spectrometry. In the light of results obtained from above experiments, the structure of novel pentasaccharides Ubaliose was confirmed as α-Gal(1→3)-α-GalNAc(1→3)-β-Glc(1→3)-βGal(1→4)-Glc

UBALIOSE

Keywords: Carbohydrate, Babalus bubalis, Colostrum, Spectroscopy, Pentasaccharide and Ubaliose.

INTRODUCTION

Milk is a natural source for the physiological and biological development of any neonate provided to him by his mother in any mammal. It is a biological fluid of unique complexity and richness (Casado et al., 2009). It contains all necessary nutrients for the growth and development of the newborn. Besides the normal milk, the milk of early days i.e. 1-5 days which is called colostrum have important role in the development of infant. Both milk and colostrum are rich resource of oligosaccharides. Oligosaccharides have established themselves as an effective class of organic bio molecules impacting various physiological and pathological processes such as molecular recognition, signal transaction, differentiation and developmental events and exhibit varied biological activities such as antitumor

(Schwonzen et al. 1992), immuno stimulant (Abe et al., 1983) anticancer (Fang et al., 1985) and immunological activities (Srivastava and Kulshretha, 1989). Oligosaccharides isolated from various milk sources are categorized under classes i.e. sialylated and non sialylated. Both these classes of oligosaccharide have been tested for their varied biological activities (Ferdouse and Monirujjaman, 2014). Milk oligosaccharides are important parts of these functional ingredients (Peterson et al., 2013, Barboza et al., 2012). Numbers of biologically active oligosaccharides have been isolated from human, buffalo, donkey, cow, mare, sheep and goat milk (Claeys et al., 2014). The milk oligosaccharides recognize cancer associated antigens, used as antimicrobial agents; tumor associated antigens and has physiological significance in infants (Fujimura et al., 2010). Donkey and Buffalo milk oligosaccharides have shown promising immuno stimulant activity (Deepak et al., 1998, Dubois et al., 1956). Immunostimulants can enhance body's resistance against various infections through increasing basal levels of immune response. Keeping in mind the immunostimulant activity of buffalo milk and importance of colostrum it was thought to isolate some novel, biologically active oligosaccharide from colostrum of buffalo (*Bubalus bubalis*). Present research work includes isolation of novel oligosaccharide from colostrum of buffalo and elucidation of its three dimensional structure by chemical degradation, chemical transformation and spectroscopic techniques like ^{1}H NMR, ^{13}C NMR, 2D NMR (HSQC, TOCSY, COSY and HMBC) and mass spectrometry, Further, quantum chemical calculation has been performed to determine the lower geometry structure and stability of the isolated and characterized compounds.

MATERIAL AND METHODS

Collection and storage of buffalo colostrum

Twelve (12) liter of buffalo colostrum (1 to 5 day) was collected from a domestic buffalo (*Babalus bubalis*) from the Kharika village near Telibagh of district Lucknow, Uttar Pradesh. The colostrum was fixed by addition of equal amount of ethanol (12 liter).

Isolation of colostrum oligosaccharides by modified method of Kobata and Ginsburg

Isolation of colostrum oligosaccharides was done by modified Kobata and Ginsburg (1970) method. The preserved colostrum was taken to laboratory and there it was cooled at 4°C and centrifuged for 30 min at 5000 rpm at 4°C. The solidified lipid layer was removed by filtrating 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°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 washing were combined and filtered through a micro filter and lyophilized affording crude oligosaccharides mixture.

Acetylation of oligosaccharide mixture

The oligosaccharides are very polar in nature hence their isolation is not an easy task. Therefore, oligosaccharide mixture (8 gm) was acetylated by acetic anhydride and pyridine for getting the oligosaccharides as its acetyl derivative (9.60 gm). The acetylated oligosaccharide mixtures were purified by column chromatography.

The structure elucidation of isolated and purified novel oligosaccharide was performed by chemical transformation, chemical degradation and spectroscopic technique ^{1}H NMR, ^{13}C NMR, 2D NMR (COSY, TOCSY and HSQC) and mass spectrometry.

Further, quantum chemical calculation was performed to determine the lower geometry structure and stability of the isolated and characterized compound.

Deacetylation of compound

Compound (44 mg) was obtained from column chromatography of acetylated oligosaccharide mixture. 30 mg of compound was dissolved in acetone and NH_3 was added in it and was left overnight in a stopper hydrolysis flask. After 24h ammonia was removed under reduced pressure and the compound was washed with $CHCl_3$ (to remove acetamide) and the water layer was finally freeze dried giving the deacetylated oligosaccharide (25 mg).

General Procedure for structure elucidation

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°C on a Bruker AM 300 and 400 FT NMR spectrometer. The electro spray 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 printouts are recorded 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 (PC) 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 H_2O. To check the homogeneity of the compound 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.

Methyl glycosidation/Acid hydrolysis of compound

Compound (5 mg) was refluxed with absolute MeOH 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 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, GalNAc and GlcNAc. Their identification was confirmed by comparison with authentic samples (TLC, PC).

Kiliani hydrolysis of compound

Compound (5 mg) was dissolved in 2 ml Kiliani mixture (AcOH-H_2O-HCl, 7:11:2) and heated at 100°C for 1 h 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, Galactose, GalNAc and GlcNAc on comparison with authentic samples of Glucose, Galactose, GalNAc and GlcNAc (Killiani, 1930).

RESULT AND DISCUSSION

Structure elucidation of compound Ubaliose

Compound, Ubaliose $C_{32}H_{55}O_{27}N$, gave positive Phenol-sulphuric acid test[13], Fiegl test[14] and Morgon-Elson test[15] showing the presence of normal and amino sugar moieties in the compound. The HSQC spectrum of acetylated Ubaliose showed the presence of six cross peaks of anomeric protons and carbons in the respective region at δ 6.19 x 88.82, δ 5.62 x 91, δ 5.05 x 100.5, δ 5.01 x 101, δ 4.45 x 101, δ 4.45 x 101 suggesting the presence of six anomeric protons and carbons in it. The presence of six anomeric protons were further confirmed by the presence of six anomeric proton doublets at δ 6.19 (1H), δ 5.62 (1H), δ 5.05 (1H), δ 5.01 (1H), δ 4.45 (2H) in the ^{1}H NMR spectrum of acetylated Ubaliose in $CDCl_3$ at 300 MHz. The presence of six anomeric carbons were also confirmed by the presence of six anomeric carbon signals at δ 101 (3C), δ 100.5(1C), δ 91.0 (1C) and δ 88.82 (1C) in the ^{13}C NMR spectrum of acetylated Ubaliose in $CDCl_3$ at 300 MHz. The presence of downfield chemical shift of α and β anomeric protons and carbons in ^{13}C NMR of Ubaliose acetate, respectively, suggested that compound may be a pentasaccharide in its reducing form. The reducing nature of compound was further confirmed by its methylglycosylation of Ubaliose by $MeOH/H^+$ followed by its acid hydrolysis which led to the isolation of α and β-methyl glucosides, suggested the presence of glucose at the reducing end of Ubaliose. For convenience the five monosaccharides present in compound have been designated as S-1, S-2, S-3, S-4 and S-5, respectively, starting from glucose (S-1) at the reducing end. The monosaccharide constituents of compound were confirmed by its Killiani hydrolysis under strong acidic conditions, followed by paper chromatography (PC) and thin layer chromatography (TLC). In its hydrolysis three spots were found on PC and TLC which were found identical with glucose (Glc), galactose (Gal), and GalNAc by co-chromatography with the authentic samples, thus confirming that the pentasaccharide contained four types of monosaccharide units i.e., Glc, Gal and GalNAc in it. The chemical shifts values of anomeric protons and carbon observed in ^{1}H and ^{13}C NMR spectra of compound were also in agreement with the reported values of ^{1}H and ^{13}C anomeric chemical shifts of Glc, Gal and N-acetylgalactosamine (GalNAc) confirming the presence of these monosaccharide unit in the compound Ubaliose.

Further the presence of two anomeric proton signals at δ 6.19 (J = 3.6 Hz) and δ 5.62 (J = 6.0 Hz) in the ^{1}H NMR spectrum of Ubaliose acetate in $CDCl_3$ at 300 MHz for α and β anomers of reducing monosaccharides (S-1) which was later identified as glucose by comparing the chemical shift data of ^{1}H and ^{13}C NMR spectrum and also from the results obtained by methylglycosylation by $MeOH/H^+$ followed by its acid hydrolysis. The reducing end chemical shift of anomeric proton and carbon for S-1 was obtained at δ5.62 × δ91.0 ppm from HSQC Spectra of Ubaliose acetate. This proton signal present at δ5.62 in the TOCSY spectra of Ubaliose acetate contains three cross peaks at δ5.62 x δ3.78, δ5.62 x δ5.0, δ5.62 x δ5.19. The sequence of ring protons in monosaccharide S-1 was then confirmed by COSY spectrum of Ubaliose acetate as (H_2) δ5.0, (H_3) δ5.19, (H_4) δ3.78. Out of which one cross peak arised at δ 5.62 x δ3.78 suggested that the position of linkage into reducing S-1 may be at H-4 of glucose S-1, showing that the H-4 of glucose S-1 was available for glycosidation by the next monosaccharide S-2 suggesting the 1→4 linkage between S-2→S-1. Further the position of linkage at of H-4 was confirmed by HMBC spectrum of Ubaliose Acetate with a cross peak of ^{1}H and ^{13}C at δ 3.78 × δ 100.5 ppm.

Therefore, the anomeric carbon value of S-2 will be δ100.5 ppm. Further the ^{1}H NMR value of S-2 in the accordance to ^{13}C NMR value of δ100.5 was obtained by HSQC spectra of Ubaliose acetate came at δ5.05 ppm.

Further the anomeric proton signal at δ 5.05 (J = 5.1 Hz) contains three cross peaks at δ 5.05 x δ 3.75, δ5.05 x δ4.42, δ5.05 x δ5.05 in its TOCSY spectrum of acetylated Ubaliose. The ring proton sequence of S-2 was confirmed by COSY spectrum of Ubaliose acetate as (H_2) δ5.28, (H_3) δ3.75, (H_4) δ4.42 of Ubaliose acetate. The coupling constant of anomeric proton at δ5.05 (S-2) had a J value 5.1 Hz which confirm the β-configuration of linkage between S-2 ↔ S-1. Here, one cross peak appeared in the upfield region of δ3.75 ppm which corresponds the linkage at H-3 of S-2, which was later confirmed as H-3 of β-Gal (S-2) by COSY spectrum of Ubaliose acetate. Further the position of linkages at H-3 was confirmed by HMBC spectra of Ubaliose acetate with crosspeak of ^{1}H and ^{13}C at δ 3.75 × δ 101 ppm.

Therefore, the anomeric carbon value of S-3 will be δ101 ppm. Further the ^{1}H NMR value of S-3 in accordance to ^{13}C NMR value of δ101ppm was obtained by HSQC spectra of Ubaliose acetate came at δ 5.01 ppm. Further the anomeric proton signal which appeared at δ 5.01 (S-3) (J = 11.1 Hz) contains three cross peaks at δ 5.01 x δ 3.73, δ 5.01 x δ4.41, δ 5.01 x δ5.20 in its TOCSY spectrum of Ubaliose acetate. The sequence of ring protons of S-3 was confirmed by COSY spectrum of acetylated Ubaliose as (H_2) δ 5.20, (H_3) δ3.73, (H_4) δ4.41. The coupling constant of anomeric proton at δ5.01 (S-3) had a J value 5.1 Hz confirm the β-configuration of linkage between S-3 ↔ S-2. Out of which the one crosspeaks appeared in the upfield region of δ 3.73 ppm corresponds the linkage at H-3 of β-Glc (S-3) by COSY spectrum of acetylated Ubaliose. Later this signal of δ 3.73 ppm was confirmed as H-3 of β-Glc (S-3) by COSY and TOCSY spectrum of acetylated Ubaliose. Further the position of linkage at H-3 of β-Glc (S-3) was confirmed by HMBC spectra of Ubaliose acetate with a crosspeak of ^{1}H and ^{13}C at δ 3.73 × δ 101 ppm.

Therefore, the anomeric carbon value of S-4 will be δ101 ppm. Further the ^{1}H NMR value of S-4 in the accordance to ^{13}C NMR value of δ 101 ppm was obtained by HSQC spectrum of Ubaliose acetate came at δ 4.45 ppm. The anomeric proton signal present at δ 4.45 (J = 3.3 Hz) along with the singlet amide methyl (- $NHCOCH_3$) at 1.96 was defined for the presence of α-GalNHAc (S-4). This anomeric proton contains three cross peaks at δ4.45 x δ3.80, δ 4.45 x δ4.08, δ4.45 x δ4.48 in its TOCSY spectrum of Ubaliose acetate. This sequence of ring protons was confirmed by COSY spectrum of Ubaliose acetate as (H_2) δ4.08, (H_3) δ3.80, (H_4) δ4.48. Out of which the one cross peaks appeared in the upfiled region of δ 3.80 corresponds the linkage at H-3 of α - GalNHAc (S-4), which was confirmed by COSY spectrumof Ubaliose acetate. Further the position of linkage at H-3 was confirmed by HMBC spectra of Ubaliose acetate by obtaining a cross peak of ^{1}H and ^{13}C at δ 3.80 × δ 101 ppm.

Therefore, the anomeric carbon value of S-5 will be δ101 ppm. Further the ^{1}H NMR value of S-5 in the accordance to ^{13}C NMR value of δ 101 ppm was obtained by HSQC spectrum of Ubaliose acetate came at δ 4.45 ppm. The anomeric proton signal δ 4.45 (J = 3.3 Hz) was assigned as α- Gal (S-5), contains three cross peaks at δ 4.45 x δ 4.46, δ 4.45 x δ 4.91, δ 4.45 x δ5.07 in its TOCSY spectrum of Ubaliose acetate. The ring proton sequence was further confirmed by COSY spectrum of acetylated Ubaliose at (H_2) δ 5.07, (H_3) δ 4.91, (H_4) δ4.46 which also suggested that it does not contain any methyne protons in glycosidic linkage region i.e., δ 3-4 ppm suggesting that none of -OH group of α-Gal (S-5) were involved in glycosidic linkages.

Hence, confirmed that α- Gal (S-5) were present at non-reducing end and none of their -OH group were available for glycosidic linkages, which was confirmed by the TOCSY spectrum of acetylated Ubaliose.

Sugar	**H_1**	**C_1**
S_1	5.62	91
S_2	5.05	100.5
S_3	5.01	101
S_4	4.45	101
S_5	4.45	101

S_1 **5.62 × 91**			
H_1	5.62	**C_1**	91
H_2	5.0	**C_2**	70
H_3	5.19	**C_3**	72
H_4	3.78	**C_4**	75

For solving the spectra of oligosaccharide molecules I followed the spectra in the order of → HSQC, TOCSY, COSY, HMBC. The glycosidic linkages in Ubaliose were confirmed by the cross peaks for glycosidically linked carbons and protons in the HMBC spectrum of acetylated Ubaliose. The values of these cross peaks were appeared as Glc (S- 1) H-4 x C-4 at δ 3.78 x 100.5 showed (1→4) linkage between S-2 and S-1, β-Gal (S-2) H-3 x C-3 at δ 3.75 x 101 showed (1→3) linkage between S-3 and S-2, β- Glc (S-3) H-3 x C-3 at δ 3.73 x 101 showed (1→3) linkage between S-4 and S-3, α-GalNAc (S-4) H-3 x C-3 at δ3.80 x 101 showed (1→3) linkage between S-5 and S-4. All these values were summarized in the table as-

Chemical shift for Anomeric Proton/ Carbon by HSQC Spectra

S_2 **5.05 × 100.5**			
H_1	5.05	**C_1**	100.5
H_2	5.28	**C_2**	70
H_3	3.75	**C_3**	75.5
H_4	4.42	**C_4**	69

S_3 **5.01 × 101**			
H_1	5.01	**C_1**	101
H_2	5.20	**C_2**	69.5
H_3	3.73	**C_3**	75.5
H_4	4.41	**C_4**	70

S_4 **4.45 × 101**			
H_1	4.45	C_1	101
H_2	4.08	C_2	71
H_3	3.80	C_3	75.5
H_4	4.48	C_4	70.5

S_5 **4.45 × 101**			
H_1	4.45	C_1	101
H_2	5.07	C_2	68
H_3	4.91	C_3	71
H_4	4.46	C_4	69.2

Table for TOCSY correlation of Ubaliose:-

Order of S_1	Order of S_2	Order of S_3	Order of S_4	Order of S_5
3.78	3.75	3.73	3.8	4.45
5.0	4.42	4.41	4.08	4.46
5.19	5.28	5.20	4.45	4.91
5.62	5.05	5.01	4.98	5.07

Correlation obtained by COSY spectra of Ubaliose:-

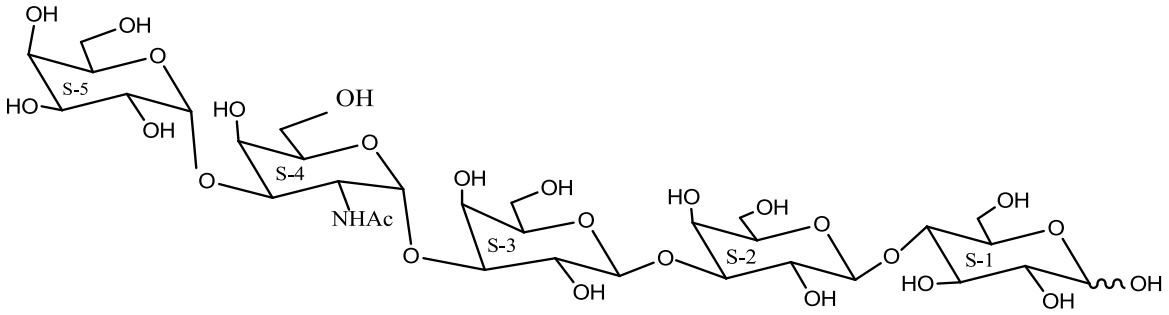
	Order of S_1	Order of S_2	Order of S_3	Order of S_4	Order of S_5
H_1	5.62	5.05	5.01	4.45	4.45
H_2	5.0	5.28	5.20	4.08	5.07
H_3	5.19	3.75	3.73	3.80	4.91
H_4	3.78	4.42	4.41	4.48	4.46

Linkages confirmed by HMBC Spectra:-

Sugar	Position	Type of linkage	value
S_1	H_4	β	3.78 x 100.5
S_2	H_3	β	3.75 x 101
S_3	H_3	α	3.73 x 101
S_4	H_3	α	3.80 x 101

All signals obtained in ^{1}H and ^{13}C NMR of compound Ubaliose was in conformity by 2D ^{1}H-^{1}H COSY, TOCSY HSQC and HMBC spectras.Thus based on the pattern of chemical shifts of ^{1}H, ^{13}C, COSY, TOCSY HSQC and HMBC NMR spectra it was interpreted that the compound 'A' Ubaliose was a pentasaccharide having the following structure as-

α-Gal(1→3)-α-GalNAc(1→3)-β-Glc(1→3)-βGal(1→4)-Glc



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