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By

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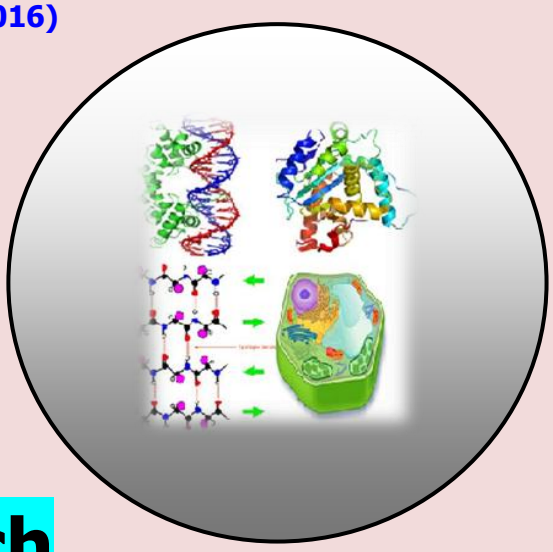
ISSN 2319-3077 Online/Electronic
ISSN 0970-4973 Print

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 35 (1) 2018 Pages No. 241-247



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. 35, No. 1: 241-247, 2018
(An International Peer Reviewed / Refereed Journal of Life Sciences and Chemistry)
Ms 35/01/0801/2018

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

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RESEARCH PAPER

Received: 03/02/2018 **Revised: 27/03/2018** **Accepted: 28/03/2018**

Isolation, Purification and Structure Elucidation of Novel Donkey Milk Oligosaccharide

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ABSTRACT

A large number of oligosaccharides have been isolated from milk of different ruminants and they have shown unique biological activities such as anti-tumor, anti-cancer, anti-inflammatory, antioxidant. Donkey's milk oligosaccharides have ability to stimulate non-specific and specific immunological resistance and proposed to be very helpful in cure of AIDS patients and in prevention of atherosclerosis. Simultaneously it also contains sialytated and fucosylated oligosaccharide, which are useful for cosmetic purpose. So, donkey's milk processed by Kobata and Ginsburg method followed by Gel filteration and column chromatography techniques which resulted in to the isolation of a novel milk oligosaccharide, VULGOSE. 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 sturucture of isolated novel oligosaccharide (VULGOSE) was interpreted as tetrasaccharide having disaccharide repeating unit structure as -

GalNAc-β-(1→4)-Glc-β-(1→3)-GalNAc-β-(1→4)Glc

VULGOSE

Key words- Milk Oligosaccharides, 2D NMR and Vulgose, Donkey's milk.

INTRODUCTION

Oligosaccharides of natural origin are an important class of bioactive natural products which are used for medicinal purpose and emerging as potent drugs against fatal diseases like cancer and AIDS. Oligosaccharides, glycoproteins and antibodies are present in milk also protect infants by reducing the number of pathogen infections and promoting the development of the intestinal epithelium (Coppa et al., 2006 and Zivkavic et al., 2010). Any mammalian milk e.g. donkey (Deepak et al., 1998), goat (Pooja et al.2017), elephant (Osthoff et al., 2007), human (Coppa et al., 2004), buffalo (R. Saksena, et. al.1999), mare (Srivastava et al., 2012) and yak (G. Boehm, et. al. 2007) etc contains high concentration of bioactive oligosaccharides (Mujeeb et al.2017). Milk oligosaccharide play a key role in various physiological, pathological and biological activities such as biological recognition, anti complementary, anticoagulant, anti inflammatory, antiviral and immunological activities (M. Schwonzen, et. al.1992, K. Abe et al. 1983, R. Srivastava, 1989). Donkey milk oligosaccharide has ability to stimulate non-specific and specific immunological resistance and (Deepak et al., 1998), proposed to be very helpful in cure of AIDS patients and in prevention of atherosclerosis (A. Tafaro, et al., 2007). Goat milk

oligosaccharide have important role in intestinal protection and repair after a damage caused by DSS (Dextron Sodium Sulphate) induced colitis and their implication in human intestinal inflammation (F. Lara-Villoslada et al. 2006). The oligosaccharides isolated from elephant milk contained a high ratio of sialyl oligosaccharide; this may be significant with respect to the formation of brain components, such as gangliosides of the suckling calves (Osthoff et al., 2007). Keeping in mind the biological activity of donkey milk oligosaccharide present there in, it was collected in bulk and was processed by method of Kobata and Ginsburg (A. Kobata et.al. 1970). followed by different chromatographic techniques like gel filtration, TLC, CC, HPLC etc. which resulted into the isolation of new milk oligosaccharides namely Vulgose.

EXPERIMENTAL

General procedures

General procedures were same as described in our previous article (A.K.singh et al.2016)

Isolation of Donkey milk oligosaccharides by Kobata and Ginsburg method

25 litres milk was collected from a Donkey and was stored at -20°C until use. The milk was processed by the method of Kobata and Ginsburg**.** It was centrifuged for 15 min. at 5000 rpm at -4°C. The solidified lipid layer was removed by filtration through glass wool column in cold atmospheric condition. Ethanol was added to the clear filtrate (supernatant) to a final

Concentration of 68% for precipitating out the lactose and proteins and the resulting solution was left overnight at 0°C. The white precipitate of lactose and protein was formed and removed by centrifugation for 15 min. at 5000 rpm at -4°C and washed twice with 68% ethanol. Further for complete removal of remaining lactose the supernatant was passed through a microfilter (0.24 µm) and lyophilized to get the crude oligosaccharide mixture (205g). The lyophilized material responded positively to Morgan-Elson (Partridge S.M. et al. 1948) and thiobarbituric-acid assay suggesting the presence of N-acetyl sugars 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 (Dubois M et al. 1956) the presence of neutral sugar.

Acetylation of oligosaccharide mixture

11g of crude oligosaccharide mixture was acetylated with pyridine (11 ml) and acetic anhydride (11 ml) at 60° C and solution was stirred overnight. Further the mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ (250 ml) and washed with ice cold water (25 ml). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness yielding the acetylated mixture (2.3g). 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 of which one compound was finally separated by column chromatography over silica gel (60-120 mesh) using $CHCl_3$ and MeOH: $CHCl_3$ as eluents.

Purification of acetylated milk oligosaccharide

Separation of the acetylated oligosaccharide mixture (10 g) was purified by column chromatography. The silica was used in the ratio of 1:100 using various proportions of Hexane $CHCl_3$, $CHCl_3$ and $CHCl_3$: MeOH mixture which resolved into eight fractions namely I(24mg), II(27 mg), III(33 mg), IV(37 mg), V(49mg), VI(280), VII(54mg) and VIII(54 mg) respectively. These fractions were containing a mixture of three to four compounds. Repeated column chromatography of fraction III, led to the isolation of one chromatographically pure compound C (83 mg).

Deacetylation of compounds

Compound Vulgose (21 mg) was dissolved in acetone (6 ml) and NH_3 (7 ml) and left overnight, in hydrolysis flask and ammonia was removed under reduced pressure, washed with $CHCl_3$ and was finally freeze dried giving the natural oligosaccharide C (14 mg).

Killiani Hydrolysis

Vulgose (5 mg) was dissolved in 2 ml Kiliani mixture (AcOH-H_2O-HCl, 7: 11:2) and heated at l00^oC 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 to it and was evaporated under reduced pressure to afford Glc and GalNAc.Their identification was confirmed by comparison with authentic samples of Glc and GaNAc on TLC, PC.

Methylglycosidation/ acid hydrolysis of compound Vulgose.

Vulgose (8 mg) was refluxed with absolute MeOH (2 ml) at 70°C for 18 h 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 C in 1, 4-dioxane (1 ml), 0.1 N H_2SO_4 (1 ml) was added and the solution was warmed for 30 minutes at 50°C. The hydrolysis was complete after 22 h. The hydrolysate were neutralized with freshly prepared $BaCO_3$, filtered and concentrated under reduced pressure to afford α-and β-methylglucosides along with the Glc. and GalNAc. Their identification was confirmed by comparison with authentic samples on TLC, PC.

Description of compounds

COMPOUND C (Vulgose):

Compound Vulgose (85mg) obtained from fractions 22-48 of column chromatography-5, on deacetylation with NH_3/ Acetone, it afforded substance C (49 mg) as a viscous mass. $[\alpha]_D$ +64 ^{0}(c, 4, H_2O). For elemental analysis, this compound was dried over P_2O_5 at 100^0 C and 0.1 mm pressure for 8 hr.

$C_{28}H_{48}O_{21}N_2$	%C	%H	%N
Calculated	44.92	6.46	3.74
Found	44.89	6.45	3.74

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

^{1}H NMR of Vulgose

(D_2O, 300MHz): 1.94(s, 3H, $NHCOCH_3$), 2.03(s, 3H, $NHCOCH_3$), 3.32(t, 1H, J=8.1Hz, β-Glc(S_1) H-2), 4.10(d, 1H, J=5.1Hz, β-GalNAc(S_2), H-4), 4.48(d, 1H, J=7.5Hz, β-GalNAc(S_4), H-1), 4.49(d, 1H, J=7.5Hz, β-GalNAc(S_2), H-1), 4.55(d, 1H, J=7.5Hz, βGlc(S_3), H-1) 4.70(d, 1H, J=7.8Hz, β-Glc(S_1), H-1), 5.25(d, 1H, J=3.6Hz, α-Glc(S_1), H-1).

^{13}CNMR of Vulgose

(D_2O, 300 MHz):

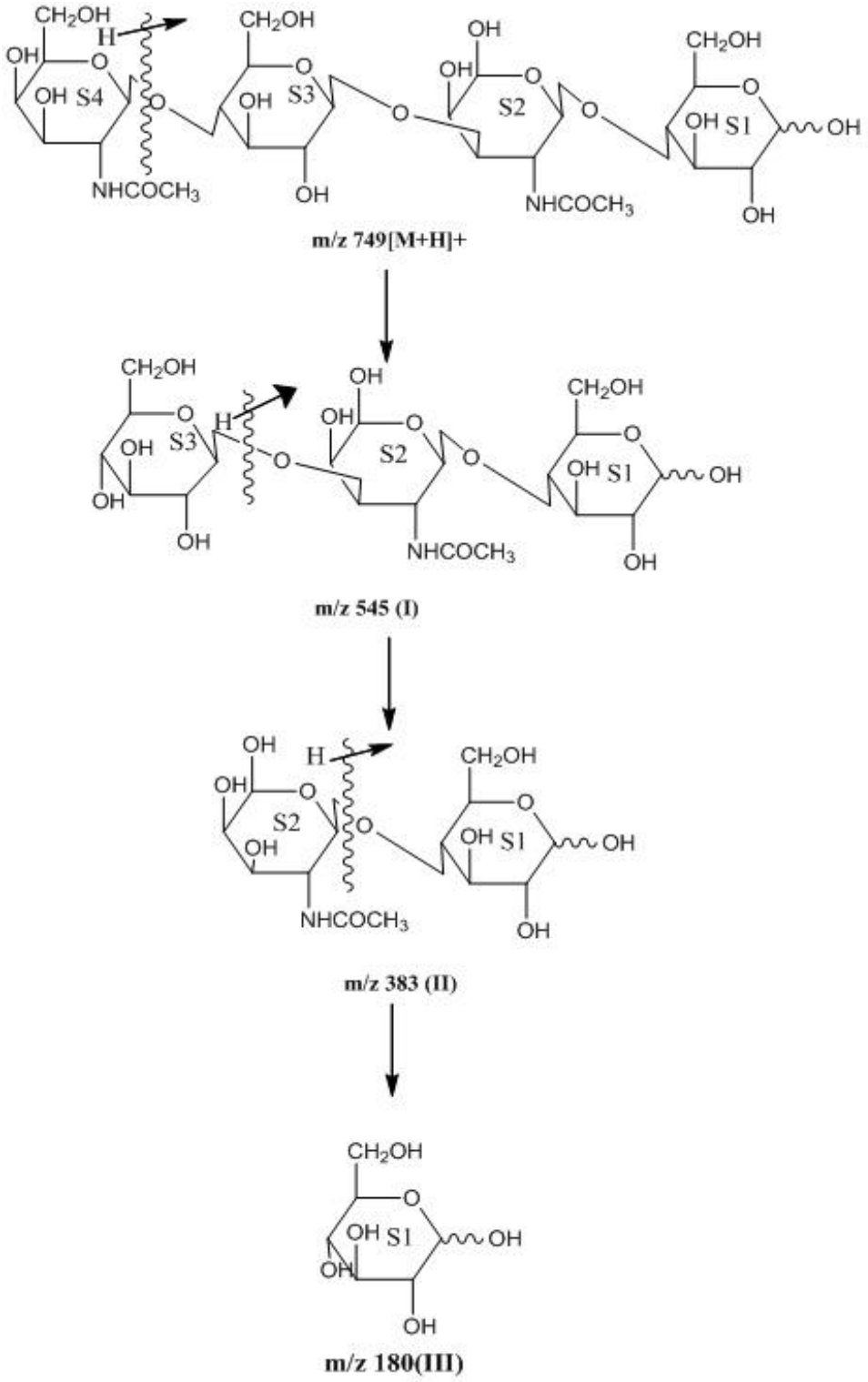
23.0, 23.3, 56.63, 59.87, 59.97, 60.10, 61.04, 69.04, 69.28, 71.17, 71.71, 72.5, 74.38, 74.79, 74.90, 74.98, 75.35, 76.24, 78.12, 78.34, 78.47, 78.72, 91.8, 95.8, 102.9, 176.1, 176.5.

FAB-MS:

m/z 787, 751, 749, 745, 730, 717, 712, 707, 706, 699, 693, 688, 672, 670, 670, 653, 639, 634, 632, 596, 545, 514, 478, 472, 456, 443, 414, 412, 383, 365, 325, 323, 307, 305,2 89, 287, 221, 180.

RESULT AND DISCUSSION

Compound Vulgose, [α]D +12.5^0 (C,0.4,H_2O),C_{28} $H_{48}O_{21}$ N_2 gave positive phenol sulphuric acid test, Fiegl test and Morgan-Elson test indicating the presence of normal and amino sugar (S) in the moiety. The ^{1}H NMR spectrum of Vulgose at 300MHz exhibited five signals in the anomeric proton region as doublets at δ 5.25 (1H), δ 4.70(1H), δ 4.55(1H), δ 4.49(1H) and δ 4.48(1H) for five protons leading to the presence of five anomeric protons in it. It was further supported by the appearance of three signals for five anomeric carbons at δ 102.9 (3C), δ 95.8(1C), δ 91.8 (1C) in the ^{13} C NMR spectrum of Vulgose. These data led to the suggestion that Vulgose may be a pentasaccharide or a tetresaccharide in its reducing form. The FAB mass spectrum of compound Vulgose showed the highest mass ion peak at m/z 787 $[M+K]^+$, and m/z 749 $[M+H]^+$ **,**which was in agreement of derived composition C_{28} H_{48} O_{21} N_2 with the molecular ion expected at m/z 748 for a tetrasaccharide. The four monosaccharide units present in compound Vulgose have been designated as S-1, S-2, S-3 and S-4 for convinience starting from the reducing end. To confirm the monosaccharide constituent and their sequence in Vulgose, it was hydrolysed strong acidic condition (Kiliani hydrolysis) and mild acidic condition (Mannich Siewert method) respectively. The results obtained from this acid hydrolysis confirmed that the sequence of monosaccharides in this tetrasaccharide is GalNAc-Glc-GalNAc-Glc. The chemical shifts of anomeric carbons obserbed in ^{13}C NMR spectrum and of anomeric observed in ^{1}H NMR of Vulgose is also in agreement with the reported values of ^{1}H and ^{13}C anomeric chemical shift of Glc and GalNAc. The presence of only two monosaccharide units i.e. Glc and GalNAc Vulgose and its molecular formula $C_{28}H_{48}O_{21}N_2$ suggested that there may be two Glc and two GalNAc in Vulgose Methylglycosidation of Vulgose by MeOH/H^+ followed by its acid hydrolysis led to the isolation of α and β-methyl glucoside, GalNAc and Glc confirming the presence of glucose at the reducing end in the oligosaccharide. The free anomeric nature of glucose was further supported by presence of two anomeric proton signals as doublets and their coupling constant, for α and β Glc at δ 5.25 (1H) (J=3.6Hz) and δ 4.70 (1H) (J=7.8Hz). Further the ^{1}H NMR spectrum of Vulgose which showed another anomeric proton doublet appearing as a doublet at δ 4.49 (1H) (J=7.5), along with a singlet of three protons at δ 2.03 which was assigned to NHAc group. These signals were attributed to the presence of a β-GalNAc which may be present as second monosaccharide (S_2) in Vulgose.



Scheme 8: FAB MASS Fragmentation of compound C (Vulgose)

Since there was no downfield shifting in the chemical shift of H-4 methine proton of Glc (S_1) was observed as a triplet at δ 3.88 (J=9.0Hz) in the ^{1}HNMR spectrum of Vulgose acetate as compared to Vulgose, it lent support to the fact that the H_4 of Glc (S_1) was involved in 1→4 linkage with GalNac (S_2).

Thus the second monosaccharide in the tetrasaccharide sequence was GalNAc which resembles with the pattern of lactose (Galβ 1→4Glc) with an extra signal for NHAc of GalNAc. This pattern was also confirmed by the characteristic signal of H-2 (A structure reporter group) **(**G. Groongerg et al, 1992,C.A. Bush, et al, 1985) of β-Glc which appeard as a triplet at δ 3.32 (J=8.1Hz). The third monosaccharide present in tetrasaccharide (Vulgose) was identified as β Glc (S_3) by a doublet for anomeric proton at δ 4.55 (J=7.5Hz). The downfield shifted signal of H-4 of β-GalNAc (S_2) at δ 4.10(J=5.1Hz) suggested that the equatorially oriented hydroxyl group of C-3 of β-GalNAc (S_2) was substituted and it was involved in glycosidation with β-Glc (S_3). The fourth sugar in the tetrasaccharide was identified as β-GalNAc which gave an anomeric proton doublet at δ 4.48 (J=7.5) Hz and its linkage to S_3 i.e. β-Glc by a (1→4) linkage was confirmed by the presence of another H-4 methine ptoton triplet of β-Glc (S_3) at δ 3.82 (J=9.6Hz) in ^{1}HNMR spectrum of Vulgose acetate .The NHAc group of second GalNAc (S_4) appeared at δ 1.94 as a singlet. These observations confirmed that the tetrasaccharide was made up of of β-GalNAc (1→4) Glc repeating units. All the ^{1}HNMR assignments for ring protons of monosaccharide units of Vulgose were confirmed by HOMOCOSY and TOCSY experiment. The tetrasaccharide nature of compound Vulgose was further confirmed by the spectral studies of acetylation product of Vulgose (Compound c), which contained fifteen singlets of methyl protons of acetyl groups in its ^{1}HNMR spectrum besides the signals of ring protons and anomeric protons present in Vulgose aceate. The chemical shifts of the anomeric carbons of Vulgose at δ 91.8 (1C, α-Glc), 95.8 (1C, β-Glc) and δ 102.9 (3C, 2β-GalNAc, 1β-Glc) present in the ^{13}CNMR spectrum are in accordance with the anomeric carbon values of Glc and GalNAc. The values of chemical shifts of ring carbons of tetrasccharide also supports the derived structure (Table 16).The hetronuclear multiple quantum-coherence (HMQC) spectrum of Vulgose confirmed anomeric assignments ^{1}H and ^{13} C NMR spectra of Vulgose by showing the ^{1}H and ^{13}C cross peaks of α-Glc (δ 5.25× δ 91.8) and β-Glc (δ 4.70× δ 95.8) . It also contains three cross peaks of one β-Glc and two β–GalNAc moieties at δ 4.55× δ 102.9, δ 4.49× δ 102.9 respectively. Based on the pattern of chemical shifts of ^{1}H, ^{13}C, HOMOCOSY, TOCSY and HMQC NMR experiments, it was interpreted that the compound Vulgose was a tetrasaccharide having two Glc and two GalNAc moieties.

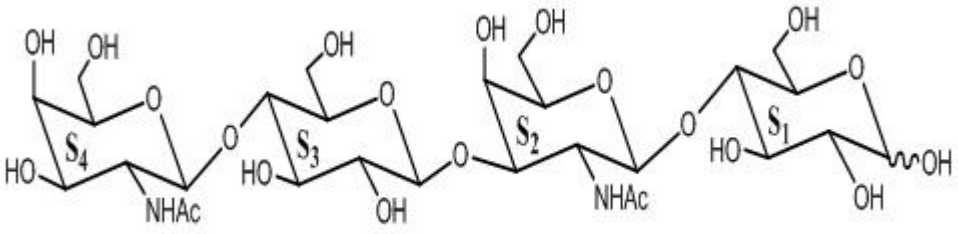
Table 1. ^{13} C NMR Values of Compound Vulgose.

	C-1	C-2	C-3	C-4	C-5	C-6	-CO	-CH_3
αGlc(S_1)	91.8	71.71	72.5	78.12	71.17	61.04		
βGlc($S_{1)}$	95.8	74.38	75.35	78.34	75.35	59.87		
βGalNac(S_2)	102.9	56.63	78.72	69.04	74.79	60.10	176.5	23.3
βGlc(S_3)	102.9	74.98	75.35	78.47	76.24	59.97		
βGalNAc(S_4)	102.9	56.63	78.72	69.28	74.90	61.04	176.1	23.0

The FAB mass spectrum of Vulgose helped in substantiating the sequence of monosaccharide units in it. The highest mass ion peaks were recorded at m/z 787 and 749 which were due to $[M+K]^+$and $[M+H]^+$ confirming the molecular weight of Vulgose as 748.The fragment ion at m/z 749 further fragmented to give mass ion fragment at m/z 545 (scheme8),which was assigned to the trisaccharide unit (1). This fragmentation corresponded to the loss of terminal GalNAc moiety from tetrasaccharide [M-S_4], indicating the presence of GalNAc (S_4) at the non reducing end. The FAB mass spectrum of compound Vulgose also contained other mass ion peaks at m/z 751[787-2H_2O), 745[787-CH_2=C=O], 730[M-H_2O], 717[M-CH_2OH], 712[M-2H_2O], 707[749-CH_2=C=O], 706[M-CH_2=C=O], 699[730-CH2OH], 693[751-$NHCOCH_3$], 688[M-CH_2OHCHO], 672[730-$NHCOCH_3$], 670[712-CH_2=C=O], 653[688-H_2O-OH], 639[688-CH_2OH-H_2O], 634[670-2H_2O], 632[M-2$NHCOCH_3$], 596[632-2H_2O] which were obtained from the molecular ion. The mass ion fragment at m/z 545 (1) further fragmentated to give mass ion peak at m/z 383 (2) assigned to the loss of Glc from fragment 1 confirming that the Glc was the second sugar in sequence from the nonreducing end. The mass ion fragment at m/z 545 was also supported by its respective fragments at m/z 514[545-CH_2OH], 478[514-H_2O], 472[514-CH_2=C=O], 456[514-NHCOCH3], 443[478-H_2O OH], 414[443-CH_2=C=O], 412[443-CH_2OH] or [472-CH_2OHCHO]. On the further fragmentation, the disaccharide fragment (2) at m/z 383 gave a monosaccharide fragment (3) at m/z 180 attributed to the loss of GalNAc (S_2) which confirmed that this disaccharide was comprised of one Glc and one GalNAc moieties and the third sugar in sequence from the nonreducing end of tetrasaccharide (Vulgose) ie. S_2 was GalNAc. This fragmentation also supported the presence of Glc at the reducing end of the tetrasaccharide having alternatively linked Glc and GalNAc i.e. GalNAc – Glc-GalNAc-Glc.

The mass ion fragment m/z 383 was also supported by other mass ion fragment at m/z 365[383-H_2O], 325[383-$NHCOCH_3$], 323[383-CH_2OHCHO], 307[365-$NHCOCH_3$], 305[323-H_2O], 289[307-H_2O], 287[323-$2H_2O$]. On the basis of results obtained from physico-chemical techniques, chemical transformation and chemical degradation, the structure of Vulgose was determined as

GalNAc-β-(1→4)-Glc-β-(1→3)-GalNAc-β-(1→4)-Glc



VULGOSE

ACKNOWLEDGEMENTS

Authors are thankful to Prof. Raja Roy, CBMR-SGPGI Lucknow for providing NMR facilities.

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