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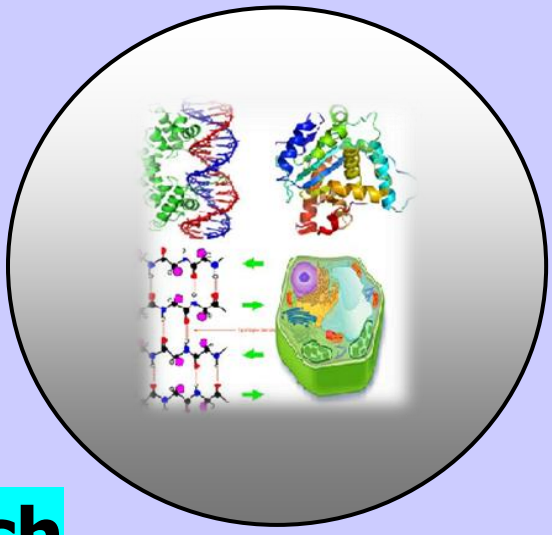

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RESEARCH PAPER

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Structure Elucidation of Novel Oligosaccharide from Yak Milk

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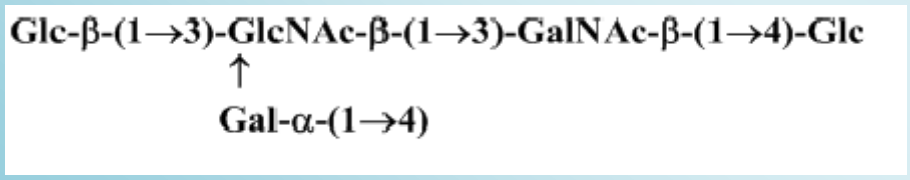
ABSTRACT

Oligosaccharides are amongst the most biologically diverse and important carbohydrates in biological system impacting various molecular processes such as signal transduction, molecular recognition, differentiation and developmental events. In present work, we have focused on isolation and structure elucidation of novel milk oligosaccharide from Yak milk. For this purpose Yak milk was taken, which is a rich source of oligosaccharide, protein, fat, lactose, minerals, amino acid, antioxidant vitamins, oligosaccharide and used as antihypertensive, antioxidative, heart strengthening agent in folk medicinal system and also used in the treatment of enemia therapy along with other drugs. In search for biologically active milk oligosaccharide, yak milk was collected and processed by method of Kobata and Ginsburg, involving deproteination, centrifugation and lyophilization followed by gel filtration and HPLC. Further for isolation, mixture of milk oligosaccharide was acetylated and chromatographed over silica column. The structure of isolated pentasaccharide was elucidated by the result obtained from chemical degradation, chemical transformation, spectroscopic techniques like NMR (^{1}H, ^{13}C and 2D-NMR) and mass spectrometry. In the light of foregoing evidence obtained from above experiments, the structure of novel pentasaccharides Yakose (G) was confirmed as:



Keywords: Oligosaccharides, Yak Milk, Pentasaccharide and Enemia Therapy.

INTRODUCTION

Oligosaccharides are one of the substantial bioactive components, established themselves as an effective class of organic biomolecules impacting various physiological and

pathological processes and shown varied biological activities such as antitumor, anticancer, anticomplementary, anticoagulant, anti-inflammatory, immunostimulant, hypoglycemic, antiviral & immunological activities. Milk oligosaccharides have a unique structure to act as a soluble ligand against infective bacteria to protect the gastrointestinal, respiratory, and urinary tracts. It is recently discovered that a trace amount of milk oligosaccharides are absorbed through the gut and interact directly with the immune system to modulate the immune response (Kunz and Rudloff, 2006). Milk oligosaccharides are reported to play an essential role to stimulate the absorption and transport of some minerals in the gastrointestinal tract through direct and indirect mechanisms (Mills et al., 2011).

Oligosaccharides isolated from various milk sources are categorized in two classes i.e. acidic and neutral types. Neutral oligosaccharides do not contain any charged monohydrate residues while acidic oligosaccharides contain one or more residues of N-acetylneuraminic acid (sialic acid) which are negatively charged. About 200 different human milk oligosaccharides (HMO) have been identified (Urashima and Taufik, 2010). Neutral human milk oligosaccharide structures such as LNFPIII and LNneoT showed an effect on murine IL-10 production. On the other hand, the acidic oligosaccharides might play an important role in the prevention of adhesion of pathogenic bacteria on the intestinal epithelial surface. Acidic oligosaccharides are also involved in reactions of the immune system such as the interaction with selectins, e.g., in inflammation processes. The elephant milk oligosaccharide fraction contained a high ratio of acidic oligosaccharide (sialyl), this may be significant with respect to the formation of brain components such as gangliosides of suckling claves (Osthoff et al., 2007). A study by Srivastava et al.(2012) established that Mare's milk oligosaccharide fractions have multifold properties such as antioxidant and lipid lowering activities. The first ever identified functional role of milk oligosaccharides is denoted as prebiotic effect. In addition to the prebiotic activity, recently, it is well known that oligosaccharides act as a soluble receptor for many pathogenic microorganisms and thus play an important role to protect infections (Barile et al., 2009; Boehm and Stahl, 2007; Gopal and Gill, 2000). Buffalo and Donkey milk oligosaccharides have shown promising immunostimulant activity (Deepak et al.1998 and Saxena et al.,1999). Fucosylated HMOs reduce human immunodeficiency virus (HIV)-1 mother-to-child transmission by competing with the viral envelope glycoprotein (gp120) for binding to dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) (Hong et al., 2009). Human milk oligosaccharides (HMO) both directly and indirectly influence intestinal development by regulating cell proliferation, acting as prebiotics for beneficial bacteria and modulating immune development. Recently, 2'FL and LNnT were shown to promote gut epithelial cell maturation and barrier function *in vitro*, suggesting specific HMO directly promote epithelial cell maturation in the small intestine. *α*-1,2-fucosyllated HMO bind to *Campylobacter jejuni* and inhibit pathogen binding to intestinal epithelial cells, it also block binding of the stable toxin from enterotoxigenic *Escherichia coli* by binding to its target receptor—guanylyl cyclase C. Human milk oligosaccharides enhance innate immunity against respiratory viruses by reducing respiratory viral infection and/or inflammation in human airway epithelialm and peripheral blood mononuclear cells *in vitro*.

In the present study, we have described the isolation and structure elucidation of novel oligosaccharide from yak milk.

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

MATERIAL AND METHODS

General procedures

NMR experiments were recorded in solvent $CDCl_3$ and D_2O at 25º on a Bruker AM 300 MHz FT NMR spectrometer. The Electrospray mass spectra were recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. The milk oligosaccharide sample (dissolved in water as solvent) was introduced into the ESI source through a syringe pump at the rate of 5ul/min. The ESI capillary was set at 3.5 kV and the cone voltage was 40 V. Sephadex G-25 (PHARMACIA) was used in gel permeation chromatography. Centrifugation of crude milk was done with the help of cooling centrifuge remi instruments C-23 JJRCI 763. The evaporation of alcohol from crude extract of milk oligosaccharides was done on Buchi Rotatry evaporator. The freeze drying of the compounds was done with the help of CT 60e (HETO) lyophilizer. The HPLC system was equipped with Shimadzu CLASS-VP V6.13 solvent delivering system, 235-diode array detector and G.P 100 plotter. Authentic samples of glucosamine, galactosamine, glucose and fucose were purchased from Aldrich Chemicals.

Isolation of Yak milk oligosaccharides by method of Kobata and Ginsburg

10 litre yak milk was collected and equal amount of ethanol was added and filtered then treated in accordance with the modified method of Kobata and Ginsberg (Ashish et al., 2016; Minakshi et.al 2016). Milk was centrifuged for 15 min at 5000 rpm at -4ºC and was filtered with glass wool column under cold atmospheric condition. After removing the Lipid, and protein, lactose were precipited with 68% ethanol and the resulting solution was left overnight at 0ºC. The white precipitate formed, mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0ºC. Further for complete removal of remaining lactose the supernatant was passed through a microfilter (0.24 µ) and lyophilized to get the crude oligosaccharide mixture (12gm). The lyophilized material responded positively to phenol-sulphuric acid test (Dubios et.al., 1956), and Morgon-Elson test (Patridge et al., 1948) suggesting the presence of neutral and 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.

Acetylation of Yak milk oligosaccharide mixture

The pooled fraction (6.5 g) of sephadex chromatography which gave positive phenol-sulphuric acid test was acetylated with pyridine and acetic anhydride at 60ºC and the solution was stirred overnight. The mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ and after usual workup as described earlier (Kumar et.al, 2016) yielding the acetylated mixture (6.7 g). By acetylation the oligosaccharides were converted into their non-polar acetyl derivatives which resolved nicely on TLC using CHCl3: MeOH as developing solvent.

Purification of acetylated milk oligosaccharide on silica gel column

Separation of the acetylated products (6.7 gm) was carried over silica gel column chromatography into compounds: silica ratio of 1:100 using various proportions of Hex:

$CHCl_3$, $CHCl_3$ and $CHCl_3$:MeOH mixture which was resolved into eight fractions namely I(556 mg), II(455 mg), III(143 mg), IV(517 mg), V(1.252 gm), VI(650 mg), VII(515 mg) and VIII(513mg) respectively. These fractions contained mixture of two to three compounds. Repeated column chromatography of fractions VII, led to the isolation of chromatographically pure compounds g (54 mg).

Deacetylation of compound

Deacetylation of acetylated oligosaccharide g (54mg) was carried out in 2ml acetone and 13ml NH_3 for 24hr in a stoppered hydrolysis flask. After 24 h 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 Yakose (G) (31 mg).

DESCRIPTION OF ISOLATED ACETYLATED COMPOUND (g)

^{1}H NMR : δ in $CDCl_3$(ppm)

δ6.22(d,1H), δ5.66(d,1H), δ5.64(d,1H), δ5.37(d,1H), δ5.30(d,1H), δ4.98(d,1H), δ4.89(d,1H), δ4.85(d,1H), δ4.73(d,1H), δ4.52(d,1H), δ4.42(d,1H), δ4.40(m,2H), δ4.08(d,1H), δ4.06(d,1H), δ3.83(m,2H), δ3.80(d,1H), δ3.72(m,2H), δ3.59(d,1H), δ3.57(d,1H), δ2.01(s,3H), δ1.99(s,3H), 1.98(s,3H), δ1.97(s,3H), δ1.95(s,3H).

^{13}C NMR : δ in $CDCl_3$ (ppm)

δ 173.25, δ173.23, δ100.96, δ95.21, δ92.08, δ91.8, δ 81.43, δ73.42, δ72.91, δ72.07, δ71.28, δ71.01, δ70.61, δ 69.53, δ 69.10, δ 68.19, δ66.91, δ 61.84, δ 22.32, 22.26, 22.08, 21.65, 21.31.

DESCRIPTION OF ISOLATED COMPOUND YAKOSE (G)

^{1}H NMR : δ in D_2O (ppm)

δ5.22(d,2H), δ4.67(d,1H), δ4.56(d,1H), δ4.52(d,1H), δ4.45(d,1H), δ4.08(d,1H), δ4.04(d,1H), δ3.92(m,2H), δ3.85(m,2H), δ3.78(d,1H), δ3.72(d,1H), δ3.65(d,1H), 3.63(d,1H), δ3.57(t,1H), δ1.92(s,3H), δ1.90(s,3H).

^{13}C NMR : δ in D_2O (ppm)

δ 170.0, δ101.5, δ94.4, δ91.6, δ88.5, δ77.4, δ 77.3, δ77.0, δ76.5, δ76.2, δ73.4, δ72.9, δ72.0, δ 70.6, δ 69.5, δ 61.89, δ60.8, 22.7, 20.6.

ES-MS:

972 $[M+Na+K]^+$, 910 $[M]^+$ and other fragment ions at, 850, 807, 779, 762, 748, 744, 712, 690, 655, 593, 586, 575, 573, 559, 558, 481, 465, 421, 386, 383, 365, 288, 273, 271, 270, 236, 235, 180, 138.

RESULTS AND DISCUSSION

Compound "Yakose" **G**, $C_{34}H_{58}O_{26}N_2$, gave positive Phenol-sulphuric acid test, Feigl 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 Yakose acetate **"g"** at 300 MHz exhibited six signals in the anomeric proton region as doublets at δ6.26 (1H), 5.66 (1H), 5.30 (1H), 4.73 (1H) and δ 4.52 (1H) and δ 4.42 (1H) for six anomeric protons indicating the presence of six anomeric protons in it.

It was further supported by the appearance of six anomeric carbons at δ 88.5 (1C), 91.8 (1C), 92.0 (1C), 95.21 (1C) and 100.9 (2C) in the ^{13}C NMR spectrum of compound **g**. These data led to the suggestion that **G** may be a pentasaccharide in its reducing form. The presence of anomeric signals at δ 6.26 and δ 5.66 for α and β glucose respectively showed that glucose unit was present at the reducing end. The five monosaccharide units present in **G** have been designated as S_1, S_2, S_3 S_4 and S_5 for convenience starting from the reducing end. The reducing nature of glucose was confirmed by the Methylglycosidation of **G** by MeOH /H^+ followed by its acid hydrolysis led to the isolation of α and β methyl glucosides. Further, for confirmation of the monosaccharide constituents in it, **G** was hydrolyzed under strong acidic condition (Kiliani hydrolysis) followed by paper chromatography showed the presence of GalNAc, GlcNAc, Gal, Glc moieties, which confirmed that these moieties has participated in the building of the **G**. The reducing and free nature of glucose was further supported by the presence of two anomeric proton signals as doublets for α and β Glc at 5.22 (1H, J=3.9Hz) and δ 4.56 (1H, J=9.8Hz) respectively. Further the presence of another anomeric signal at δ 4.45 (1H) J=7.8 Hz along with a signal of three proton at δ 1.90 which was assigned as NHAc group. These signals were assigned to the presence of β-GalNAc which may be present as second monosaccharide (S_2) in **G.** Since there was no downfield shifting of H-4 methine proton of Glc (S_1) observed as triplet at δ 3.57 in ^{1}H NMR spectrum of **g**, it suggested that H-4 of Glc (S_1) was involved in (1→4) linkage with β-GalNAc (S_2). The large coupling constant J=7.8 Hz of anomeric signal of S2 at δ 4.45 (J=7.8Hz) confirmed the β glycosidic linkage between Glc(S_1) and GalNAc(S_2). Thus second monosaccharide in the pentasaccharide sequence was β-GalNAc, which resembles with the pattern of lactose (Galβ1→4Glc) with the extra signal of NHAc of β-GalNAc. This pattern was confirmed by the characteristic signal of H-2 of β-Glc which appeared as triplet at δ3.31 in ^{1}H NMR spectrum of G. Further, another anomeric protons appeared at δ4.45 linkage along with signal of NHAc group at δ1.92 was due to the presence of β-GlcNAc. The linkage of β-GalNAc (S_2) to the β-GlcNAc (S_3) was confirmed by COSY and TOCSY spectrum of **g**, in which H-3 of β-GalNAc (S_2) appeared upfield in the region δ3.57 ppm and H-2, H-4 and H-6 of β-GalNAc appear at δ4.06, δ5.50 and δ4.85 respectively, these data confirms that C-2, C-4 and C-6 positions of β-GalNAc (S_2) were not involved in glycosidic linkages with β-GlcNAc (S_3). This was also confirmed by the HSQC spectrum of **g,** in which the H-3 proton of β-GalNAc (S_2) present at δ 3.57 in ^{1}H axis and its cross peak with the ^{13}C axis was present at δ71.4 (δ3.57 x δ 71.4) confirmed the β-GlcNAc (S_3)[1→3] β-GalNAc (S_2). The large coupling constant J=7.8 Hz of anomeric signal of S_3 at δ 4.45 (J=7.8Hz) confirmed the β glycosidic linkage between GalNAc(S_2) and β-GlcNAc (S_3). Further, the presence of the downfield shifted H-4 proton resonance of β-GalNAc (S_2) confirmed that this β-GalNAc (S_2) was glycosidically linked at C-3 position by β-GlcNAc (S_3). The fourth anomeric proton of **G** appeared as a doublet at δ4.67 (1H) was due to the presence of another β-Glc (S_4) and its large coupling constant (7.8 Hz) confirms β-configuration of glycosidic linkage. In COSY and TOCSY spectrum of **g**, β-GlcNAc (S_3) showed H-3, H-4, H-5, and H-6 proton signals at δ3.70, δ3.83, δ4.22, and δ4.45 suggesting that C-3 and C-4 were glycosidically linked. The HSQC spectrum of g also confirms that the H-3 proton of S_3 was gave cross peak at (δ3.70 x δ72.07) and H-4 proton of S_3 gave cross peak at (δ3.83 x δ73.42), this upfield signal indicated that C-3 and C-4 position of β-GlcNAc (S_3) was involved in glycosidic linkage and thus β-Glc (S_4) was glycosidically linked to β-GlcNAc (S_3) by (1→3) glycosidic linkage.

This compound has another anomeric proton doublet at δ5.22(1H, J=3.9 Hz) which was due to presence of α-Gal (S_5) in **G** which is glycosidically linked to C-4 position of β-GlcNAc (S_3). The small coupling constant J=3.9 Hz of anomeric signal of S5 at δ 5.22 (J=3.9Hz) confirmed the α-glycosidic linkage between β-GlcNAc(S_3) and α-Gal (S_5). This downfield shift of the α-Gal (S_5) H-1 was derived from the comparision with the ^{1}H NMR data given by Prasoon *et al.*, only difference of α-Gal instead of β-Gal which suggested that α-Gal (S_5) was linked to β-GlcNAc (S_3) by (1→4) linkage and its small coupling constant (3.9 Hz) confirms α-configuration, linkage was further confirmed by COSY and TOCSY spectrum of **g.** This was also confirmed by the HSQC spectrum of **g,** in HSQC spectrum the H-4 proton of β-GlcNAc (S_3) present at δ3.83 in ^{1}H axis and its cross peak with the ^{13}C axis was present at δ73.4 (δ3.83 x δ 73.4) confirmed the α-Gal (S_5) [1→4] β-GlcNAc (S_3) linkage.

The chemical shifts of the anomeric carbons of compound **G** at δ 88.5(1C, α-Glc), 94.4(2C, β-Glc), 91.6 (1C, α-Gal), 101.5 (1C, β-GlcNAc) and 101.5 (1C, β-GalNAc) are in accordance[@] with the anomeric carbon values of Glc, Gal GlcNAc and GalNAc present in **G.** The comparison of chemical shifts of ring carbons of this pentasaccharide with the reported values are given in the literature which are in accordance with the **^{13}C** reported value of respective monosaccharide. This also supports the derived structure of derived structure of compound G.

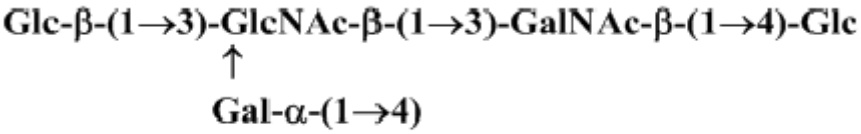
The pentasaccharide nature of **G** was further confirmed by spectral studies of acetylated derivative of G. HSQC spectrum of acetylated product of compound **G** confirms the anomeric assignments in ^{1}H and ^{13}C NMR spectra of **G** which showed the ^{1}H and ^{13}C cross peaks of α-Glc (S_1) at δ6.25 x δ88.9 and β-Glc (S_1) at δ5.6 x δ95.21. It also contains four cross peaks of one β-GalNAc (S_2) at δ4.52 x δ100.9, and one β-GlcNAc moieties (S_3) at δ4.42 x δ100.9, β-Glc (S_4)at δ4.73 x δ92.08 and α-Glc (S_5) at δ5.30 x δ91.8 respectively. The glycosidic linkage were assigned by the cross peaks for glycosidically linked carbon with their proton in HSQC spectrum of **G**. The values of these cross peaks were as β-Glc(S_1) H-4 and C-4 at δ3.72 x δ71.0 confirms (1→4) linkage between S_1 and S_2, β-GalNAc (S_2) H-3 and C-3 at δ3.57 x δ71.4 confirms (1→3) linkage between S_2 and S_3, β-GlcNAc (S_3) H-3 and C-3 at δ3.70 x δ72.9 confirms (1→3)linkage between S_3 and S_4, β-GlcNAc (S_3) H-4 and C-4 at δ3.83 x δ 73.4confirms (1→4)linkage between S_3 and S_5 respectively. COSY spectrum confirms the assignment of ring proton involved in linkage at δ3.72 (4-position) for β-Glc(S_1) δ3.57 (3-position) for β-GalNAc (S_2), δ3.70 (3-position) for β-GlcNAc (S_3), δ3.83 (4-position) for β-GlcNAc (S_3)and it was further confirmed by the presence of same chemical shifts in TOCSY spectrum. The respective values of ring proton involved in glycosidic linkage in H-4 of (S_1) , H-3 of (S_2) and H-3, H-4 of (S_3) arises at δ3.72, δ3.57 and δ3.70, 3.83 respectively in the ^{1}H NMR of G.

The Electrospray Mass Spectrometric data (Domen et.al; 1988) of compound not only confirmed the derived structure but also supported the derived sequence of monosaccharides in **G** The highest mass ion peaks was recorded at m/z 972 which was due $[M+Na+K]^+$, the other mass ion peak recorded at m/z 910 which was due to $[M]^+$ confirming the molecular weight of **G** as 910 and is in agreement with its molecular formulae. Further the mass fragments were formed by repeated H transfer in the oligosaccharide and was accompanied by the elimination of terminal sugar less water. This fragmentation path way also confirmed the sequence of monosaccharides in the pentasaccharide.

The pentasaccharide m/z 910 (I) fragmented to give mass ion at m/z 748 (II), this fragment corresponded to the loss of terminal Gal (S_5) moiety from pentasaccharide [910-S_5], indicating the presence of Gal (S_5) at the non-reducing end. It further fragmented to give mass ion peak at m/z 586 (III) with loss of another Glc(S_4) moiety from tetrasaccharide [748-S_4], it was further fragmented to give mass ion peak at 383 (IV) with the loss of GlcNAc (IV). Further it fragmented to give mass ion peak m/z at 180 (V) which was again due to loss of GalNAc (S_2) and it further fragmented to give mass ion peak at 180 which correspond to Glc. These four mass ion peaks II, III, IV and V are appeared due to the consequent loss of Gal, Glc, GlcNAc and GalNAc from original molecule. The mass ion fragment A, B, C, D corresponds to m/z 545, 586, 748 and 586 which confirmed the anchoring nature of S_3 sugar.

The other fragmentation pathway in ES Mass spectrum of compound D at m/z 910 shows the mass ion peak at m/z at 850 [910-CH_2OH-CHO], 807 [850-CH_3CO], 779 [748-3H_2O-OH], 780 [850-2H_2O-OH], 762 [780-H_2O], 744 [762-H_2O]. The mass ion fragment at m/z748 (II) was also supported by its respective fragment at m/z 712 [748-2H_2O], 690[748-$NHCOCH_3$], 655[690-H_2O-OH], 593[712-CH_2OHCHO-CH_2CO-OH], 575[593-H_2O], 573[655-2CH_3OH-H_2O], 559[593-2OH], 558[593-H_2O-OH], 500[558-$NHCOCH_3$], 465[500-H_2O-OH]. The mass ion fragment at m/z 586(III) [748-S_4], was also supported by its respective fragment at m/z 526 [586-CH_2OH-CHO], 481 [526-H_2O-OH], 421 [481-CH_2OH-CHO], 386 [421-H_2O-OH], 273 [386-CH_2OHCHO-2H_2O-OH], 231 [273-CH_3CO], 223 [273-CH_3OH-H_2O]. The mass ion fragment at m/z 748 (II) was also supported by its respective fragment at m/z 383 (IV) [586-S_3], was also supported by its respective fragment at m/z 365 [383-H_2O], 288 [383-CH_3CO-2H_2O-OH], 271 [288-OH], 270 [288-H_2O], 236 [271-H_2O-OH], 235 [270-H_2O-OH], .The mass ion fragment at m/z180 [383-S_2], was also supported by its respective fragment at m/z 138 [180-CH_2CO].

Based on the results obtained from chemical degradation/acid Hydrolysis, Chemical transformation, Electro Spray Mass spectrometry and ^{1}H, ^{13}C NMR, COSY, TOCSY and HSQC NMR techniques of **Yakose** and Yakose acetate, the structure and sequence of isolated Novel oligosaccharide molecule Yakose was deduced as-



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