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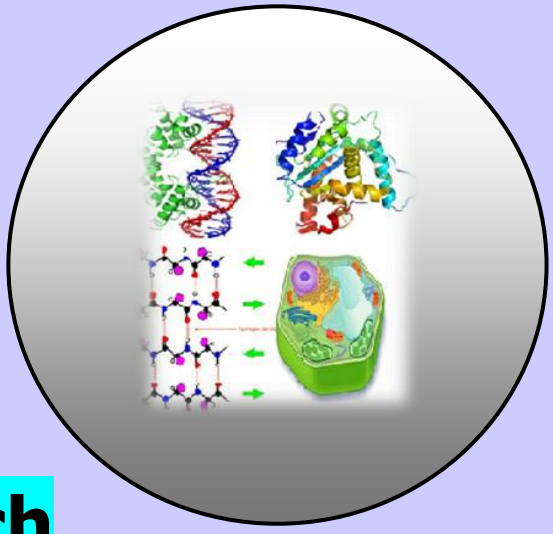

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RESEARCH PAPER

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Isolation and Structure Elucidation of Novel Oligosaccharide Bosnose from Shyama Dhenu (Black cow) Milk

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ABSTRACT

Milk is more than a source of nutrients for any neonate of mammalian species, as well as for growth of children and nourishment of adult human. Recent researches have advanted a large number of bioactive compound in milk which have led to the discovery of specific biochemical, physiological and nutritional functionalities and characteristics that have strong potential for beneficial effects on human health. Likely oligosaccharides from bovine milk could be a first choice for biological functions includes prebiotic effects as well as antipathogenic effects and involvement in immune modulation; they compete with pathogenic bacteria, bacterial toxins and virus for attachment to gastrointestinal receptor sites and inhibit their actions. CLA holds anti- cancer properties which is naturally present in cow's milk and shows a positive impact on cardiovascular health. The medicinal importance of the cow milk in the ancient Indian medicinal system is enormous. So, to increase the activity of the milk oligosaccharide for medicinal importance, shyamadhenu milk was collected and processed by modified method of Kobata and Ginsburg consisting of deproteination, centrifugation and gel chromatography followed by acetylation and silica gel column chromatography of derivatized oligosaccharides while their homogeneity was confirmed by HPLC. Then the identification of the structure of milk oligosaccharide was followed by the various spectroscopic method of 1 D NMR (^{1} H and ^{13} C) and 2 D NMR (HSQC, COSY, TOCSY) and mass spectrometry. So on the basis of this technique the structure of novel oligosaccharide Bosnose was established as:

$$\text{Gal-}\alpha\text{-}(1\rightarrow6)\text{–Gal-}\beta\text{-}(1\rightarrow4)\text{ Glc}$$

Bosnose

Keywords: Novel Oligosaccharide, Black Cow, Bosnose and Prebiotic effects.

INTRODUCTION

Milk is often referred to as the "gold standard" of nutrition for the first few months of life. It contains all essential nutrients for the infant to grow and develop, but it also contains ingredients that may provide health benefits beyond traditional nutrients (Lars Bode 2009). Milk proteins, oligosaccharide, lipids etc, for instance, are known to exert various actions that promote human health. These actions include stimulation of the immune system, digestion and absorption of nutritional elements, development of endogenous defense mechanisms against bacteria, fungi and viruses, prebiotic effects and others (Lönnerdal 2003). It is widely accepted that milk oligosaccharides play several important protective, physiological and biological roles including selective growth stimulation for beneficial gut microbiota, inhibition of pathogen adhesion and immunoregulation (K Mariño et al. 2011, Kleine et al. 2000; Martin-Sosa et al. 2002; Hakkarainen et al. 2005; Coppa et al. 2006; Lane et al. 2010). Many important activities are shown by the different kind of animal milk because they contain a different amount of oligosaccharide as well as different kinds of oligosaccharides like goat milk contain fucosylated as well as sialylated oligosaccharide which shows the supplementary and therapeutic applications (Chaturvedi and Sharma 1990, Kumar et.al 2016). Donkey milk oligosaccharides have ability to stimulate non – specific and specific immunological resistance (Desh Deepak et al., 1998). Mare milk oligosaccharides promote cellular immune response in vitro both in terms of cellular proliferation and reactive oxidative burst. So it show the activation of one of the innate immune defense mechanism (A.Srivastava et al. 2014). Mare milk has a highest antioxidant properties more than sheep and chauri milk, which have positive health promoting factors which not only help against oxidative stress but also have remarkable immune stimulant properties (Tulika et.al 2014). Buffalo milk works as a immunostimulant to migration of macrophages (Saksena R. et. al 1999).

The oligosaccharide content of cow milk is qualitatively and quantitatively different from that of human milk; bovine milk has an oligosaccharide content several times lower than that of human milk, acidic oligosaccharides (sialic acid-containing species) being predominant (Kunz and Rudloff, 1993).Cow milk is particularly high in protein, vitamin B, vitamin D, various minerals, organic compounds and antioxidants that can affect and stimulate the body in many different ways. In Ancient Indian literature (Upnishads, Puranas, and Vedas) gives full of evidence of the beneficial and therapeutic properties of milk. The milk of Surabhi, which is considered the celestial cow and supreme among all bovines, is useful to humans, as mentioned in the Mahabharata. According to Bhava Prakasha, Cow milk is a remedy for the patients of chronic diseases such as epilepsy, jaundice, heart ailments, suppression of stool and urine, spleen enlargement, and piles (R. Nagpal et al.2012). CLA (conjugated linoeic acid) which is naturally present in cow's milk, holds anti-cancer properties, and has a positive impact on cardiovascular health (Nagpal et al., 2007b). According to Ayurveda, Cow milk has been described as nutritive and good for the vital organs such as the Eyes, Brain and the Heart. Cow Milk promotes immunity and acts as rasayana and ojovardhaka as a has better immune booster. Cow milk has the effect of improving medha or intellect as a good memory booster. It is ideally suited as a brain tonic because according to Ayurveda it has the ability to pacify Vata dosha which is responsible for the proper functioning of Nervous system. Cow milk will be a better natural alternative to digest and more nutritive.

According the Charaka Samhita Sutrasthana 27: Cow milk has ten properties viz, Sweetness, cold, soft, unctuous, oily, density, thick, smoothness, slimeness, stickiness heavy, slowness, calming and clarity. Cow milk is very important for women who are nearing menopause because menopause increases the susceptibility to Osteoporosis. It also acts as natural aphrodisiac and assist in peristalsis of intestines. Many diseases like severe debility, relieving stage of fever, diseases related to urinary system, bleeding disorders such as nasal bleeding, heavy menstrual bleeding etc are cure with the help of cow milk. Keeping in mind the above properties, shyama Dhenu cow milk was collected in bulk and processed by method of Kobata and Ginsburg for isolation of novel milk oligosaccharide (Kobata A. et al., 1970). After following the process, Keeping a basic core units in mind the structures of various milk oligosaccharides were established by comparing the chemical shift (1H NMR and 13C NMR) of anomeric signals and other important signals in 2D NMR (COSY TOCSY HSQC and Mass spectrometry) of isolated milk oligosaccharides with the chemical shifts of known milk oligosaccharides.

MATERIAL AND METHODS

General procedure

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

Isolation of Shyamadhenu (Black Cow) milk oligosaccharide by Kobata and Ginsburg method-

Processes of isolation of shyamadhenu milk oligosaccharide were same as described in our previous article (Gunjan et al., 2016).

Acetylation of Shyamadhenu Cow milk oligosaccharide mixture

Dry oligosaccharides of pooled fractions (12 gm) were acetylated by treatment with pyridine (12ml) and acetic anhydride (12ml) at 60° C for 24 hr. Further the, reaction mixture was evaporated under reduced pressure and viscous residue was taken in $CHCl_3$ and washed in sequence with 2N 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 heating.

Purification of Acetylated milk oligosaccharide on Silica Gel Column

Purification and isolation of acetylated oligosaccharide mixture were carried out over silica gel column chromatography: silica ratio of 1:100 was used and 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 Bosnose (128mg).

Deacetylation of Compound Bosnose

Deacetylation of acetylated oligosaccharide D (128mg) was carried out in 3ml acetone and 3ml 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 natural oligosaccharide Bosnose (89mg).

Description of Isolated Compound Bosnose

^{1}H NMR of compound Bosnose

δ 3.27 (t, 1H, J=8.2 Hz), δ 3.91 (d, 1H, J=2.7 Hz), δ 4.44 (d, 1H, J=7.5 Hz), δ 4.65 (d, 1H, J=8.1Hz), δ 5.20 (d, 1H, J=3.3Hz), δ 5.25 (d, 1H, J=3.6 Hz).

^{13}C NMR of compound Bosnose Acetate

δ 61.6, δ 62.4, δ 66.7, δ 68.6, δ 68.7, δ 69.1, δ 69.5, δ 70.5, δ 70.7, δ 71.2, δ 71.3, δ 72.3, δ 74.4, δ 82.4, δ 82.5, δ 90.0, δ 91.4, δ 95.1, δ 101.7.

FAB MS of compound Bosnose Acetate

967 $[M+H]^{+}$,779, 755,732, 710, 682, 659, 637, 619, 595, 577, 559, 535, 517, 488, 457, 426, 391, 360, 331, 307, 289, 254, 229, 209, 187, 169, 109, 89.

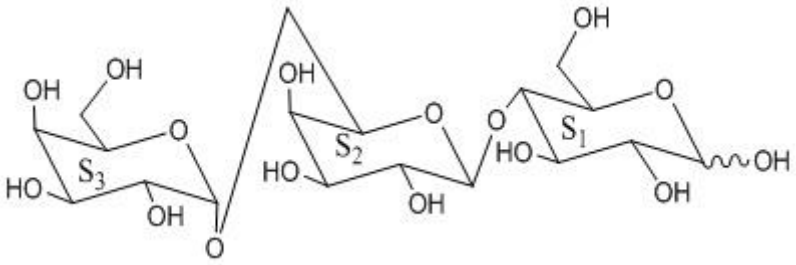
RESULT AND DISCUSSION

Compound Bosnose $C_{18}H_{32}O_{16}$ gave positive Phenol-sulphuric acid test (Dubois M et al., 1956) and Fiegl test (Fiegl F. et al., 1975) indicating the presence of normal sugar moieties in the compound Bosnose. The structure of the oligosaccharide was assigned by chemical degradation, spectroscopic techniques (1D and 2D NMR) and structure reporter group theory. The HSQC spectrum of acetylated Bosnose showed the presence of three cross peaks of four anomeric proton and carbons in the respective region at δ93.1 x 5.58, δ101.7 x 4.65, δ90 x 5.32 suggesting the presence of four anomeric proton and carbon in it. Further the presence of four anomeric protons were separately confirmed by the presence of four anomeric doublets at δ 4.44(1H), δ4.65(1H), δ5.20(1H) and δ5.25(1H) in the ^{1}H NMR spectrum of compound Bosnose in D_2O at 300 MHz. It was further supported by the appearance of four signals for four anomeric carbons at δ 101.7(1C), 95.1(1C), 91.4(1C) and 90.0(1C) in the ^{13}C NMR spectrum of its acetylated Bosnose. ^{1}H and ^{13}C NMR spectrum justify the four anomeric signal for trisaccharide with total integral intensity of three anomeric protons/carbons. These data suggested that compound Bosnose may be a trisaccharide in its reducing form. ^{1}H NMR and ^{13}C NMR spectrum contained downfield shifted α and β anomeric proton and carbon confirming that compound Bosnose as trisaccharide in its reducing. Further the presence of two cross peak at δ3.78x77 and δ3.53 x 82.2 in the HSQC spectrum of Bosnose acetate shows that their were two glycosidic linkages in Bosnose confirming it to be a trisaccharide. The reducing nature of Bosnose was further confirmed by its methylglycosidation by $MeOH/H^{+}$ followed by its acid hydrolysis which led to the isolation of α and β–methylglucoside, and Gal which confirmed the presence of glucose at the reducing end and Gal at the nonreducing end in the oligosaccharide. The three monosaccharide units present in compound Bosnose have been designated as S_1, S_2, and S_3 for convenience starting from the reducing end. Further the acid hydrolysis of compound Bosnose gave two spots on the paper chromatography, which were identified as Glc and Gal by co-chromatography with authentic samples confirming the presence of Glc and Gal in the Bosnose. 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 δ 5.25(1H) (J= 3.6Hz) and δ 4.65 (1H)(J= 8.1 Hz) respectively in the ^{1}H NMR of Bosnose. Presence of two doublets of anomeric protons at δ 4.44 (1H) J=7.5 Hz and δ 4.65 (1H) J=8.1 Hz in the compound Bosnose showed the presence of Glc and Gal residue as a Lactosyl moiety i.e Gal β (S_2) (1→4) Glc (S_1).

The large coupling constant J=8.1 Hz of anomeric signal of S2 at δ 4.65 (J=8.1Hz) confirmed the β glycosidic linkage between Glc (S_1) and Gal (S_2). This was further supported by the presence of β Glc (S_1) H-2 signal (a structural reporter group) which appeared as a triplet at δ 3.27, (J=8.2Hz) (Gronberg G et al., 1992). The ^{1}H NMR spectrum of Bosnose besides containing two anomeric proton signals for α and β Glc, also showed another anomeric proton appearing as a doublet in the downfield region δ 5.20(1H) (J=3.3 Hz) which was assigned for α Gal (S_3) residue (Urashima T et. al 1997, Urashima T et. al 1999) and its small coupling constant (3.3 Hz) of anomeric signal present at δ5.21 confirms α-configuration of glycosidic linkage. The linkage between S_2 and S_3 was established on the basis of chemical shift of H-4 proton resonance of the β Gal (S_2). The absence of a downfield shifting of the H-4 proton resonance of β- Gal (S_2) which appeared as a doublet at δ3.91, J=2.7 Hz, instead of being shifted down field to δ4.13-4.15 as a result of substitution at the 3- position (Gronberg G et al., 1992), confirmed that β-Gal (S_2) was not substituted at C-3 this implies that α Gal was 1→6 linked to β-Gal. In the light of foregoing evidences the structure Bosnose was derived as:

Gal-α-(1→6)–Gal-β-(1→4) Glc

The results obtained from the FAB mass spectrum of the acetylated trisaccharide further substantiates the structure of compound Bosnose as derived by its ^{1}H and ^{13}C NMR spectra and also helped in substantiating the sequence of monosaccharide units in it. The highest mass ion peaks were recorded at m/z 967 which were due to $[M+H]^+$ confirming the molecular weight of Bosnose acetate as 966. The fragment ion at m/z 967 further fragmented to give fragment ion at m/z 637 with the subsequent loss of terminal α Gal residue from the non reducing terminal. The presence of fragment ion m/z 619 also confirms the presence of lactosyl moiety in the trisaccharide. The lactosyl disaccharide unit at m/z 619 further fragmented to give fragment ions at m/z 289 (S_2) and m/z 331 (S_1) which also confirmed the presence of Glc at the reducing terminal of the trisaccharide. The FAB mass spectrum of Bosnose Acetate also contained other mass ion peaks at m/z 779[966-CH_3COOH, -CH_3CO, -2CH_2=C=O], 595[637-2CH_2=C=O], 577[619-CH_2=C=O], 559[619-CH_3COOH], 535[577- CH_2=C=O], 517[577- CH_3COOH], 457[577-2 CH_3COOH], 391[577-CH_3COOH,-CH_3CO, 2 CH_2=C=O], 229[391- 2CH_3COOH-CH_2=C=O], 187[229-CH_2=C=O], 169[229-CH_3COOH], 109[169-CH_3COOH]. This fragmentation pattern further confirmed the presence of Gal (1 → 4) Glc at the reducing end and terminal galactosyl residue at the non-reducing end of the trisaccharide. On the basis of results obtained from physico-chemical techniques and chemical transformation, the structure of compound Bosnose was determined as:



Bosnose

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