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Structure elucidation of two novel yak milk oligosaccharides and their DFT studies

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ABSTRACT

Milk is a primary dynamic biological fluid responsible for development of neonates. Besides the other regular constituents it have oligosaccharides in it which are responsible for antitumor, anticancer, antigenic and immunostimulant activities. In our endeavor to find biologically active novel oligosaccharides, yak milk was taken, which is a rich source of oligosaccharide and its milk is used as antihypertensive, antioxidative and heart strengthening agent in folk medicine. For this purpose yak milk was processed by method of **Kobata and Ginsburg** followed by gel filtration HPLC and CC which resulted in the isolation of two novel milk oligosaccharides namely (I) Grunniose and (II) Vakose. The structure of purified milk oligosaccharides were elucidated with the help of chemical degradation, chemical transformation, spectroscopic techniques like NMR (^{1}H, ^{13}C and 2D-NMR), structure reporter group theory and mass spectrometry. The optimized geometry of compound Grunniose and Vakose, at B3LYP method and 6-311 + G basis set on Gaussian 09 program, show that the compound Grunniose is lower in energy as compared to compound Vakose.

Compound Grunniose -

Gal-α(1→3) GlcNAc-β(1→6) Gal-β(1→4) Glc
↑
GalNAc-α(1→3)

Compound Vakose -

Glc-β(1→3) Gal-β(1→3) GlcNAc-β(1→6) Gal-β(1→3) GlcNAc-β(1→3) Gal-β(1→4) Glc

1. Introduction

Milk is nature's designer food and contains all necessary nutrients for growth and development of any mammalian neonate [1]. 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 [2,3]. Many mammalian milk e.g. donkey [4], goat [5], elephant [6], human [7], buffalo [8], mare [9], yak [10] etc contains high concentration of bioactive oligosaccharides [11,12]. Milk oligosaccharide play a key role in various physiological, pathological and biological activities such as biological recognition, anticomplementary, anticoagulant, antiinflammatory, antiviral and immunological activities [13–15]. Donkey milk oligosaccharides have ability to stimulate non-specific and specific immunological resistance [4] and Donkey milk oligosaccharide may be used for prevention of atherosclerosis [16]. Goat milk oligosaccharide have important role in intestinal protection and repair after a damage caused by DSS(Dextran Sodium Sulphate) induced colitis and their implication in human intestinal inflammation [5]. 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 [6].

Human breast milk play a key role in gut colonization and modulation of the infants guts [7,17]. Fucosylated human milk oligosaccharide and related glycoconjugates can used for several

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specific disease by inhibition of enteric pathogens such as stable toxin of *Escherichia coli*(in vitro and its toxin induced secretory diarrhea in vitro and in vivo), noroviruses and Campylobacter [18,19]. They also have inhibitory effect on certain virulence-related abilities of monocyte, lymphocyte and neutrophil adhesion to endothelial cells and act as anti inflammatory agents [20,21]. Buffalo milk oligosaccharides have ability to stimulate non-immunological resistance of the host against parasitic infections [8]. Mare's milk has shown anti oxidant, lipid lowering and post heparin lipolytic activity [9].

The yak (Bos Grunniens) milk play a very important role in traditional Tibetan medicinal system and used in enema therapy as solution along with other drugs [22] and is a rich source of protein, fat, lactose, minerals, amino acid, calcium and vitamin A [23,24]. The bioactive components derived from yak milk possess antihypertensive and immunomodulatory properties [25,10]. We worked on the isolation of novel milk oligosaccharide from yak milk. In the present study, we are describing the structure elucidation of two novel yak milk oligosaccharides, (B) Grunniose and (F) Vakose, and their D.F.T. studies. The names Grunniose and vakose are proposed by us for the first time.

2. Theoretical study

The quantum chemical calculation have been performed on B3LYP functional and 6-311 + G(d,p) basis set. Geometries of compound B and F have been first optimized and the presence of positive wave numbers values for all the optimized geometry indicates stability of the compounds. All computations were performed using the Gaussian 09 program package [26].

2.1. Experimental

2.1.1. General procedures

Optical rotations were measured using an AA-5 automatic polarimeter in a 1 dm tube for water solutions whose concentrations are expressed in g/100 mL. NMR spectra were recorded in $CDCl_3$ and D_2O on Bruker DRX-spectrometer at 300 and 400 MHz. The ESMS spectra were recorded on a MICROMASS QUATTRO II triple quadruple mass spectrometer. The C, H and N analysis were recorded on CARDO-ELBA 1108 elemental analyzer. The detection of new spots for carbohydrates was monitored by thin layer chromatography (TLC) using Silica Gel 60 F254 plates. TLC plates were visualized by exposure to them in with 50% aq. H_2SO_4 reagent.

Spots for carbohydrates were monitored by paper chromatography (PC) with acetyl acetone and p-dimethyl amino benzaldehyde reagents. PC was performed on Whatman No.1 filter paper using ethylacetate-pyridine (2:1) saturated with H_2O as solvent system. Sephadex G-25 (Pharmacia) was used in gel permeation chromatography. Freeze drying of the compounds was performed with the help of a CT 60e (Heto) lyophilizer and centrifuged by a cooling centrifuge Remi Instrument C-23 JJRCI 763. To check the homogeneity of the compounds, reverse phase HPLC(Supplementary file given) system was used equipped with Perkin–Elmer 250 solvent delivering system, 235 diode array detector and G.P. 100 printer plotter. Authentic sample of glucosamine, galactosamine, galactose, and glucose were purchased from Aldrich Chemicals.

2.2. Isolation of yak milk oligosaccharides by Kobata and Ginsberg method

10 L milk was collected from a yak and was stored at −20 °C until use. The milk was processed by the method of Kobata and Ginsberg [27]. 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 (12.0 g).

The lyophilized material responded positively to Morgan–Elson test [28] and thiobarbituric-acid assay [29] suggesting the presence of N-acetyl sugars and sialic acid 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 [30] for the presence of neutral sugar.

2.3. Acetylation of oligosaccharide mixture

The pooled fraction (6.5 g) of sephadex chromatography which gave positive phenol-sulphuric acid test [30] was acetylated with pyridine and acetic anhydride at 60 °C and the solution was stirred overnight. The mixture was evaporated under reduced pressure and the viscous residue was taken in $CHCl_3$ and washed in sequence with 2 N HCl, ice cold 2 N $NaHCO_3$ and finally with H_2O. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness yielding the acetylated mixture (6.7 g). By acetylation the oligosaccharides were converted into their non-polar acetyl derivatives which resolved nine spots on TLC i.e. a, b, c, d, e, f, g, h and i given in Fig. 4(Supplementary file).

2.4. Purification of acetylated milk oligosaccharide on silica gel column

Separation of the acetylated products (6.7 g) 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 gm), II(455 mg), III(143 mg), IV(517 mg), V(1.252 gm), VI(650 mg), VII(515 mg) and VIII(513) respectively. These fractions contained mixture of two to three compounds. Repeated column chromatography of fractions II and VI, led to the isolation of two chromatographically pure compounds B (90 mg) and F (55 mg).

2.5. Deacetylation of compounds

Compound B(45 mg) was dissolved in acetone (3 mL) and NH_3 (3.5 mL) and left overnight in a stoppered hydrolysis flask and same method were used for compound F(35 mg). Ammonia was removed under reduced pressure and the compounds were washed with $CHCl_3$ and were finally freeze dried giving the deacetylated oligosaccharide B (38 mg) and F (28 mg).

2.6. Killiani hydrolysis

Dry compounds B and F each were placed separately in hydrolysis flasks with 1 mL of Kiliani mixture (AcOH–H_2O–HCl, 7:11:2) and heated on a boiling water bath for half an hour. After evaporation they were checked by paper chromatography with the authentic samples of sugars. It was observed that compound **B** on Kiliani hydrolysis [32] gave Glc, Gal, GalNAc, and GlcNAc, and compound **F** gave Glc, Gal, and GlcNAc confirming the presence of these sugar units in it, given in Schemes 1 and 2 (Supplementary file).