Chemical Characterization of Oligosaccharides in the Milk of Water Buffalo (*Bubalus bubalis*)

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ABSTRACT

It has recently been recognized that human milk oligosaccharides (HMOs) have several biological functions. They stimulate the growth of beneficial microorganisms in the infant colon, they act as receptor analogs that inhibit the attachment of pathogenic microorganisms to colonic mucosa, and small amounts are absorbed into the circulation, where they modulate immunoreactivity. Infant formulas areproduced from mature bovine milk. Since HMOs haveseveral important biological functions, other materialswhose functions are similar to those of HMOs, couldprofitably be incorporated into the milk replacer. Therefore studies on milk oligosaccharides of domestic animals such as cow, water buffalo, goat and sheep have been recently progressed, especially in structural characterization. The present study was aimed to chemically characterize milk oligosaccharides from water buffalo (*Bubalus bubalis*) milk samples. Neutral and acidicoligosaccharides were separated from the carbohydrate fraction of water buffalo milk by gel filtration which was followed by high performance liquid chromatography (HPLC) and characterized by^{1}H-nuclear magnetic resonance spectroscopy (^{1}H NMR). In water buffalo milk samples, three oligosaccharides were identified: 3'-sialyllactose, 6'-sialyllactose and α3'-N-acetylgalactosaminyllactose. The first two oligosaccharides were also identified in cow milk, and as it is in cow milk, lactose is also predominant saccharide in water buffalo milk.

Key Words: Milk, Oligosaccharide, Water buffalo, Proton nuclear magnetic resonance spectroscopy (^{1}H NMR)

INTRODUCTION

As milk is the only source of food for the newborn, it contains various nutrition contents and also biofunctional components readily available for the young neonates to maintain their body metabolism and protect them from diseases. One of the major milk components is carbohydrate, the percentage of carbohydrate in the milk or colostrum of the mammalian is range from trace to over 10%, of which disaccharide lactose (Gal(β1-4) Glc; Gal, D-galactose; Glc, D-glucose) is usually but not always constitutes the major part. Apart from lactose, the rest of carbohydrate components are composed of a variety of sugars commonly named as milk oligosaccharides.Almost all of these oligosaccharides have a lactose unit at their reducing end and are built by the attachment of N-acetylglucosamine (GlcNAc), galactose, fucose, and/or sialic acid to lactose (Urashima et al. 2007). Those oligosaccharides which contain sialic acid residue are categorized as acidic oligosaccharides, whereas other oligosaccharides are consider as neutral oligosaccharides.

Various studies reported that human milk oligosaccharides(HMO) are resistant to digestion enzymes of infant gastrointestinal tract, thus they are recognized to have prebiotic effect that stimulate the growth of some beneficial microorganisms. They also act as receptor analogs that inhibit the attachment of pathogenic microorganisms to colonic mucosa, and small amounts are absorbed into the circulation, where they modulate immunoreactivity (Bode

2006). It is generally believed that milk oligosaccharidesfrom other mammals, especially domesticated dairy animals such as cows, possess similar characteristics with HMO (Urashima et al. 2012). Therefore studies on milk oligosaccharides of domestic animals such as cow, water buffalo, goat and sheep have been recently progressed, especially in structural characterization. The present study was aimed to chemically characterize milk oligosaccharides from water buffalo (*Bubalus bubalis*) milk samples.

MATERIALS AND METHODS

Milk samples were collected by hand milking from 3 lactating animals at about 2 -3 months after parturition. The water buffaloes live in colony on the bank of big river in south Sumatera, Indonesia. The collected samples were immediately kept in the cool box. After sampling, the samples were frozen at -20^{o}C before shipped to Obihiro University of Agriculture and Veterinary Medicine, Japan for further analysis.

3'-sialyllactose [3'-NAc-SL; Neu5Ac(α2-3)Gal(β1-4)Glc] and 6'-sialyllactose [6'-NAc-SL; Neu5Ac(α2-6)Gal(β1-4)Glc]; were obtained from Sigma Co. (St. Louis, MO, USA). α3'-N-acetylgalactosaminyllactose [α 3'GalNAcLac; GalNAc(α 1-3)Gal(β1-4)Glc] were isolated from yak mature milk (Taufik 2012).

Milk sample were thawed and extracted with four volumes of chloroform/ methanol (2:1, v/v). About 100 ml emulsions were centrifuged at 4°C and 4000 X *g* for 30 min, and the lower chloroform layer and the denatured proteins were discarded. The methanol was removed from the upper layer by rotary evaporation, and the residues were dissolved in 30 mL water and freeze-dried. The resulting white powders were called the 'carbohydrate fractions'. Based on the experience, the oligosaccharides content of bovine species is low. Therefore in order to isolate enough amount of pooled fractions from peak of gel filtration, 500 mg of carbohydrate fractions was dissolved in 12 mL of water and the solutions passed through a Bio Gel P-2 (<45 mm, Bio-Rad Laboratories, Hercules, CA, USA) column (2.5 X 100 cm) that had been calibrated with 2 mg each of galactose (monosaccharide), lactose (disaccharide) and raffinose (trisaccharide). Elutions were done with distilled water at a flow rate of 15 mL/h and fractions of 5 mL were collected. Aliquots (0.5 mL) of each fraction were analyzed for hexose with phenol-sulfuric acid test (Dubois et al. 1956) and for sialic acid with periodate–resorcinol (Jourdian et al. 1971). Those tests produced six peaks in the chromatogram (chromatogram is not shown), peak fractions were labeled as BM-1, BM-2, BM-3, BM-4, BM-5 and BM-6. Peak fractions were then pooled and freeze-dried.

The saccharides in the peak fractions BM-2, BM-3, BM-4, BM-5 and BM-6 were subjected to proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy to determine their chemical structures.Based on previous experience, to avoid loss of the sample and isomerization of acidic oligosaccharides due to anion exchange process, the components of peak BM-1 which gave positive reactions with both periodate–resorcinol (630 nm) and phenol–sulfuric acid (490 nm) was directly subjected to high performance liquid chromatography (HPLC) on a TSK gel Amide-80 column (4.6 X 250 mm, pore size 80 Å, particle size 5 μm; Tosoh, Tokyo, Japan) (chromatogram not shown). The mobile phase was 50% and 80% (vol/vol) acetonitrile (CH_3CN) in 15 mmol/L potassium phosphate buffer (pH 5.2). Elution was done using a linear gradient of acetonitrile from 80 to 50% at 60^{o}C at a flow rate of 1 mL/min. The eluates were monitored by measuring the absorbance at 195 nm. The peak fractions of oligosaccharides were pooled, concentrated by rotary evaporation, and subjected to ^{1}H-NMR spectroscopy.

Nuclear magnetic resonance spectra were recorded in D_2O (100.00 atom D%; Aldrich, Milwaukee, WI, USA) at 500 or 600 MHz for ^{1}H-NMR with a JEOL ECP-500 FT-NMR

(Akishima, Tokyo, Japan) or a Varian INOVA 600 spectrometer (Palo Alto, CA, USA) operated at 293.1 K. Chemical shifts are expressed in δ relative to internal 3-(trimethylsilyl)-1-propane sulfonic acid, sodium salt (TPS), but actually measured by reference to internal acetone (δ = 2.225).

RESULTS AND DISCUSSION

Acidic oligosaccharides

The results of HPLC analysis showed that peak BM-1 produced two peaks labeled as BM-1-1 and BM-1-2.As the ^{1}H-NMR spectra of BM-1-1 and BM-1-2 were completely identical to those of authentic 3'-NAc-SL and 6'-NAc-SL, respectively, the oligosaccharides in these peaks were characterized to be Neu5Ac(α2-3)Gal(β1-4)Glc and Neu5Ac(α2-6)Gal(β1-4)Glc.

Neutral oligosaccharides

As the ^{1}H-NMR spectra of BM-6 were completely identical to those of authentic lactose the oligosaccharides in this peak was characterized to be Gal (β1-4) Glc. The ^{1}H-NMR spectra of BM-4 and BM-5were completely identical to the chemical shift of α3'-N-acetylgalactosaminyllactose of YM-3 of yak mature milk, therefore the oligosaccharides in these peaks were characterized to be GalNAc(α 1-3)Gal(β1-4)Glc.Based on their ^{1}H-NMR spectra, the other small peak components of BM-2 and BM-3 can not be characterized in this study.

As it is found in other bovine milk samples, the water buffalo mature milk sample contains low content of oligosaccharides, even though the amount of carbohydrate fraction used in gel filtration was 500 mg. All oligosaccharides detected from water buffalo mature milk sample were also found in the cow and yak mature milk (Taufik 2012). By comparing the pattern of mature milk gel chromatogram from other bovine species such as yak (Taufik 2012) and cow (Fukuda et al. 2010), it can be said that water buffalo milk has the same pattern of gel chromatogram with them. The pattern of milk oligosaccharides content depends on the lactation stage, in which colostrum contained more oligosaccharides than that of mature milk is not solely characteristic of bovine species. Data of bactrian camel colostrum and mature milk oligosaccharides reported by Fukuda et al. (2010) showed similar pattern.

Moreover, from the gel chromatograms of those species, it is obvious that in colostrum, acidic oligosaccharides are produced more as compared to mature milk. As Fukuda et al. (2010) noticed in bactrian camel, it can be said also for bovine species that most of sialyl oligosaccharides are synthesized in mammary gland at early lactation. As it is generally believed that milk oligosaccharides protect neonates agains infection (Urashima et al., 2009) andit has also been reported that sialic acid may exhibit a number of health benefits to human infants including the promotion of infant brain development(Nakamura and Urashima 2004); therefore, it can be understood why the pattern of oligosaccharides is higher in colostrum compared with mature milk, especially in bovine species.

It might can be also said, that the synthesis of oligosaccharides by mammary gland is reflecting the steps of fullfilment of the required substances by the young for their growth and protection again diseases.

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