Isolation of Milk Oligosaccharides using Solid-Phase Extraction

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Abstract: A method was developed to isolate mono- and disaccharide-free oligosaccharides from human and bovine milk using a combination of enzymatic digestion of lactose and solid-phase extraction. In the initial trial, 2.5 g of oligosaccharides were isolated from one liter of human milk. In subsequent trials this was increased to over 5 g of oligosaccharides per liter. Compared to filtration-based extraction methods, this procedure allows for further isolation of oligosaccharide fractions *via* modulation of the column rinsing solvent. Neutral monosaccharide composition of the oligosaccharide polymers was investigated using gas chromatographic analysis of the monosaccharides as alditol acetate derivatives. Results indicate oligosaccharides are approximately made up of 24% fucose, 41% galactose, 22% glucose and 13% glucosamine. Isolated bovine and human milk oligosaccharides were compared to lactose as fermentation substrates for *Bifidobacterium longum biovar infantis*. Lactose fermentation yielded the greatest production of biomass followed by bovine and human milk oligosaccharides.

INTRODUCTION

Human milk is rich in complex soluble carbohydrate polymers, and little is understood of the function of these molecules. Some analytical studies have indicated that there may be as much as 20 g/L of oligosaccharides in breast milk which would make them the third most concentrated component after lactose and fat [1, 2]. However, unlike lactose and fat, these molecules do not appear to provide energy to infants as they resist the action of human digestive enzymes [3, 4]. Most human milk oligosaccharides (HMO) are elongation products of lactose, and are synthesized from glucose, galactose, glucosamine, fucose and sialic acid [2]. In addition to being concentrated, HMO are also diverse and at least 200 individual structures have been detected using mass spectrometry [5]. The compositions of HMO are also different among women, and any proposed function they may serve must address this heterogeneity [6].

As the composition of milk is the product of millennia of selective pressure, it stands to reason that individual constituents, especially those present in abundant quantities, would be expected to provide some sort of benefit to the infant or the mother or they would tend to be selected against over subsequent generations [7]. Several interesting bioactivities have been attributed to HMO. For example, they antagonize the binding of some strains of bacteria to epithelial cells [8], and a large epidemiologic study indicated that sugar epitopes resulting from an individual genotype appear to protect against diarrhea in breast fed infants [9]. Furthermore, those containing sialic acid may serve as immune modulators [10, 11]. It has also been suggested that HMO may serve as growth factors for colonic microbiota [2], as it has long been known that breast feeding results in increased levels of fecal *bifidobacteria* with respect to infant formula. Based on their structure and composition, HMO may affect the gut microbiota consortium either by selectively providing growth factors and energy substrates to some members, or alternatively *via* binding and eliminating others, or both. However, to begin to address whether HMO might be modulating this activity, it is first necessary to isolate them in large enough quantities to serve as fermentation and or growth factor substrates in microbiological media, and to insure that they are entirely free of milk mono and disaccharides.

Prior to the initiation of this work, it was noted in the literature that several groups have undertaken the task of isolating HMO from other constituents in milk, although mostly for quantification as opposed to the production of substrates for biochemical assays. The general scheme has been to first remove the fat from milk by centrifugation, and then the protein by precipitation with organic solvents [12]. To separate the HMO from lactose, gel filtration has often been used. This is a slow method that requires long separation times and has a low capacity. In contrast, two methods have been published for obtaining HMO in large quantities, one using charcoal column chromatography [13], and the other nanofiltration [14]. In each case the milk fat was first removed using centrifugation, and protein precipitated with organic solvents. Both groups then enzymatically converted lactose to glucose and galactose to facilitate separation. Brand-Miller *et al*. [13] used charcoal column adsorption to retain oligosaccharides on the column, while monosaccharides were eluted. However, little methodological information was provided, and the resulting product was not well characterized. Using nanofiltration, Sarney *et al*. [14] isolated HMO from other milk constituents, and compared the resulting HMO produced with gel filtration. Their yield with nanofiltration was 6.7 grams of HMO from 1L of milk, yet there did appear to be residual lactose in the oligosaccharide fraction produced with nanofiltration, but not in that prepared using gel filtration. Nanofiltration is an attractive method for HMO isolation due to the speed with which separations can be performed and it does not require the use of organic solvents. Nonetheless, nanofiltration does not allow

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tion time to individual monosaccharide standards deritivatized using the same process.

Oligosaccharide Fermentation Assay

The fermentation of human and bovine milk oligosaccharides was measured using *Bifidobacterium longum b.v. infantis* according to the method of Ward *et al.* [17]. In short, MRS media was made up with isolated oligosaccharides instead of glucose at 1% (m/v). Growth was measure with a Klett-Summerson colorimeter (Klett Manufacturing Co., Inc., New York, NY) at specific time points using the no. 45 (green filter).

RESULTS

In three subsequent extractions the yield of oligosaccharides per liter of breast milk was approximately 2.5 g, 5.5 g, and 5.2 grams. The ratio of lactose (measured enzymatically) to total sugars (measured colometrically) at various stages in the procedure is shown in Fig. (**3**), and inset photos show HPTLC carbohydrate profiles resulting from these steps. It is worth noting when interpreting this figure, and the other data presented in this study, that of the five monosaccharide constituents of HMO, only glucose and galactose are detected by the colorimetric Dubois method, and only glucose, galactose and fucose stain with the orcinol reagent on TLC plates. From the figure it is clear that the majority of carbohydrate in breast milk is lactose, and approximately 5% of glucose and galactose is bound up in oligosaccharides which is consistent with previous estimates [10].

After the initial extraction with the 2.5 g/L HMO yield, it was reasoned that the process might be improved, and changes to the process outlined in Fig. (**1**) included rextraction of the removed fat (Step 1) and protein (Step 2) to prevent any loss of HMO. Inset A of Fig. (**3**) shows the carbohydrate profiles after various stages of the first extraction, and from the image it appears there is some undigested lactose and some residual monosaccharides in the final HMO (Lane 5). For the second trial this was addressed by optimizing the enzymatic digestion of lactose, and modifying the SPE column loading protocol. According the manufacturer, β-galactosidase from *Kluyveromyces fragilis* has relatively sharp pH and temperature optima (pH 6.5 and 37º C). Care was taken to insure that the reaction was conducted under these conditions, and the amount of enzyme added to the milk extract was doubled. In the second round of purification the progress of hydrolysis was monitored and determined to be complete after 3 hours (data not shown).

According to Redmond and Packer [18], the capacity of graphitized carbon SPE columns to adsorb the trisaccharide raffinose is approximately 27% of its mass. In the first trial, six columns, each with 10 g adsorbent beds and 60 ml volumes, were used to extract 1L of milk. The theoretical capacity of this system for oligosaccharides is over 16 g, which is in excess of the level of HMO in milk, and yet only 2.5 g were isolated. To better understand the interaction of HMO with the adsorbent, a binding study was conducted with a small SPE column (500 mg bed, 6 ml volume) and 5, 10, 20, 25 and 30 ml of milk. The findings (data not shown) indicated that graphitized carbon SPE columns could bind up to approximately 20% of their weight in HMO, and all the HMO in the 5 and 10 ml of milk. As the loading of the column was further increased up 25 ml of milk, no more than 20% of the weight of the column was bound, and the percentage bound per ml of milk went down. Thus, from 10 ml of milk approximately 97 mg of HMO were bound, and 101 mg were extracted from 25 ml of milk. However, when 30 ml was applied to the column, only 39 mg of HMO were adsorbed to the column, which indicates that overloading the column washes off HMO that had been previously bound. In the second and third trial this was addressed by increasing the number of columns on the manifold to 12, by diluting the HMO extract prior to loading columns by doubling the volume, and by alternating rinse steps with deionized water in between column loadings. These changes led to a 50% increase in yield, however, it would appear that the overall yield could still be improved *via* a better understanding of the factors that limit HMO binding as column loading is increased.

To investigate the potential of this method to isolate specific fractions of HMO a second binding study was conducted with a small column (500 mg bed, 6 ml volume). After loading the column, it was rinsed with solutions containing an increasing percentage of butanol to probe the interaction of the carbohydrates with the graphitized carbon adsorbent, and to get a better understand the size distribution of the HMO. This experiment was conducted with butanol, rather than acetonitrile, which was used in the bulk purification, as prior work had shown this solvent system to be effective in isolating specific oligosaccharides [18]. In Fig. (**4**), the carbohydrates in Lane 1 consist of the material that eluted from the column during the application of the sample. Comparing this lane to the standards at the other end of the plate indicates that the eluate consists primarily of glucose and galactose. Lane 2 contains material that was rinsed off the loaded column with water, and consisted primarily of monosaccharides, as well. Lanes 3-9 show the effects of increasing the concentration of butanol in the column rinse, and it is clear that as the concentration increases, different fractions of HMO are released. From this data it appears HMO elute in three broad groups, one near the top (Lanes 3-6) with a similar migration distance to the disaccharides lactose (Lane 11) and sucrose (Lane 12) and the trisaccharide raffinose, a second group (Lanes 3-8) with a similar mobility to the tetrasaccharide stachyose (Lane 12), and a group that remains at the origin (Lanes 6-10). On the bottom of the figure a bar graph of the relative concentration of sugars in each fraction is superimposed, and summing the contribution of Lanes 3-6 indicates that greater than 2/3 of the HMO appear to be tri- and tetrasaccharides. This appears to be in agreement with the observation by LoCascio *et al.* [19] that *B. longum bv. infantis* preferentiall uses small HMO for fermentation substrates, which they find to represent 63.9% of the total. Starting in Lane 6, a band at the origin appears and becomes darker in the subsequent four fractions (Lanes 6-10). , and Lanes 9 and 10 consisted solely of this band. In Lane 10, 0.1% Trifluoroacetic acid (TFA) was added to the 4% butanol in the rinse. According to Redmond *et al.* [18], acidic sugars (including phosphorylated and sulfated monosaccharides) are strongly adsorbed to graphitized carbon, and will not be eluted by the addition of an organic modifier unless a volatile acid, such as TFA, is added. Thus, it would appear that the band at the bottom of Lane 10 represents acidic

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