Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract

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ABSTRACT

Background: Human milk oligosaccharides (HMOs) show a complexity and variety not found in milk of any other species. Although progress has been made in the past 3 decades with regard to identification and structural characterization of HMOs, not much is known about the physiologic functions of HMOs.

Objective: As a prerequisite for biological activity in infant metabolism, HMOs have to resist enzymatic hydrolysis in the gastrointestinal tract. To assess the extent to which selected HMOs are hydrolyzed, we carried out in vitro digestion studies using enzyme preparations of human and porcine pancreas and intestinal brush border membranes (BBMs).

Design: Fractions of HMOs, including structurally defined isolated oligosaccharides, were digested for up to 20 h with human pancreatic juice and BBMs prepared from human or porcine intestinal tissue samples. HMOs were incubated by using a porcine pancreatic homogenate and BBMs as enzyme sources. HMOs and digestion products were identified by mass spectrometry and anion-exchange chromatography. Additionally, free N-glucose, l-fucose, and N-acetylnearuminic acid were determined enzymatically.

Results: Whereas maltodextrin (control) was rapidly and completely hydrolyzed, neutral and acidic HMOs showed a profound resistance against pancreatic juice and BBM hydrolases. However, cleavage of most of the HMOs was achieved by using a pancreatic homogenate containing intracellular, including lysosomal, enzymes in addition to secreted enzymes.

Conclusions: The results of this study strongly suggest that HMOs are not hydrolyzed by enzymes in the upper small intestine. Although intact HMOs may be absorbed, we postulate that a majority of HMOs reach the large intestine, where they serve as substrates for bacterial metabolism. Therefore, HMOs might be considered the soluble fiber fraction of human milk.

INTRODUCTION

Knowledge about a carbohydrate fraction distinct from lactose in human milk goes back > 100 y (1), whereas the structures of human milk oligosaccharides (HMOs) were unknown for a long time. In the past couple of years, > 90 HMOs have been characterized and, as a result of methodologic improvements, many more are expected to be identified, particularly those with high molecular weights (2). The highest concentrations of HMOs can be found in colostrum (20 g/L), but even mature milk contains oligosaccharides in concentrations up to 13 g/L (3). All HMOs have a core structure consisting of a lactose unit at the reducing end linked to N-acetyllactosamine units (type 1 and 2), with branching occurring frequently. Residues of l-fucose, N-acetylnearuminic acid (NANA), or both can be found linked to the core without further elongation. Highest concentrations of the acidic HMOs, characterized by ≥ 1 NANA residues, are found in colostrum (4). As a result of multiple linkage sites, even small HMOs can exist in various isomeric forms. This is shown as an example of the monofucosylated pentasaccharide lacto-N-fucopentaose (LNFP) in Figure 1.

As far as the physiologic role of HMOs in infants is concerned, it has been suggested that HMOs serve as soluble ligands that prevent pathogenic microorganisms from adhering to and invading into the epithelia of the gastrointestinal, urogenital, and respiratory tracts (5–8). Antisecretory effects in the intestine have also been described (9). Only small amounts of intact HMOs were found in the feces of term and preterm breast-fed infants (10–12). This suggests that most of the HMOs are hydrolyzed in the upper gastrointestinal tract to the constituent monomers, absorbed as intact molecules, or degraded by the colonic microflora. Fermentation of HMOs in the large intestine of infants was shown recently on the basis of a hydrogen breath test (13). Although there have been no studies of the absorption of HMOs, small quantities of HMOs have been identified in the...
were immediately frozen at –20°C were collected over a 2-h period with a nasoduodenal tube and many) and cholecystokinin (Fering, Malmoe, Sweden), HDAs after intravenous infusion of Frankfurt, Germany) were collected routinely after stimulation of exocrine pancreatic secretion. After intravenous infusion for preparation of BBMs and a sample was taken for assessment of morphologic unaltered (BBMs) were prepared according to the method of Hopfer et al (18) as modified by Luecke et al (19). Morphologically unaltered, (1). This might explain why the colon of most breast-fed children is colonized predominantly by bifidobacteria, a species with proposed health-promoting effects in children and adults (15, 16). Although there have been numerous attempts to promote selectively the growth of bifidobacteria in formula-fed infants, no major advances have been made until now (17).
samples and blanks (controls) be treated in the same way and that they contain enzymes as well as oligosaccharides. For this reason, controls always consisted of oligosaccharides added to the incubation solution containing the enzyme preparations that had been heat-inactivated before incubation.

**Enzymatic detection of monosaccharides**

D-Glucose was measured by the hexokinase reaction and free L-fucose was quantified according to the colorimetric method of Horiuchi et al (22) by using L-fucose dehydrogenase (Sigma, Deisenhofen, Germany). Free sialic acid (NANA) was determined according to the method of Horiuchi and Kurokawa (23) with NANA aldolase (Sigma) and acylmannosamin dehydrogenase (Funakoshi, Tokyo). Maltodextrin served as a readily hydrolyzable control oligosaccharide and was treated as described above. Glucose liberation from maltodextrin was determined at various times (10 min to 20 h).

**Matrix-assisted laser desorption ionization mass spectrometry**

Ethanol was added to 20 μL of incubation samples to yield a final concentration of 66% (by volume). After centrifugation, the supernate was freeze-dried and resuspended in aqueous bidest. To remove salts, 10 mg of a Dowex ion exchanger (50WX8–200; Sigma, Deisenhofen, Germany) was added and samples were mixed for 1 h. The supernate then was removed, diluted with aqua bidest (to yield 0.5–1.0 g HMOs/L), and submitted to MALDI-MS. One-microliter samples were applied together with aqua bidest. To remove salts, 10 mg of a Dowex ion exchanger (50WX8–200; Sigma, Deisenhofen, Germany) was added and samples were mixed for 1 h. The supernate then was removed, diluted with aqua bidest (to yield 0.5–1.0 g HMOs/L), and submitted to MALDI-MS. One-microliter samples were applied together with 1 μL of 2.5-dihydroxybenzoic acid (10 g/L; Aldrich, Deisenhofen, Germany), serving as matrix (24). Oligosaccharides were analyzed by using time-of-flight mass spectrometers (Voyager RP and Voyager DE STR; Perseptive Biosystems, Framingham, MA) with flight tube lengths of 1.3 and 2 m, respectively. Analysis was performed with a 2-stage ion source and in a linear positive ion mode. A nitrogen laser with an emission wavelength of 337 nm and 3-ns pulse duration was used. Accelerating voltage was adjusted to 20 kV and grid voltage to 60% and 94%, respectively. The recorded mass spectra were smoothed by using a second-order 19-point Savitsky-Golay algorithm. After internal calibration over ≥3 known oligosaccharide masses per spectrum, mass accuracy was determined as follows. The highest and lowest oligosaccharide peak and the 2 oligosaccharide peaks of the highest intensities were selected. The mass differences between theoretical and observed average molecular weights were calculated for each of the selected peaks. The absolute values of the calculated mass differences divided by the theoretical average mass weights were multiplied by 100%. Using this calculation, we found a maximum mass deviation from the theoretical average mass weight of 0.02% in the spectra of neutral oligosaccharides and 0.06% for the spectra of acidic oligosaccharides. The higher maximum mass deviation for acidic oligosaccharides was due to the poorer ratios of signal to noise and poorer intensities than those of the mass spectra of the neutral oligosaccharides.

**Anion exchange chromatography**

After removal of salts from the samples of the digestibility assay by anion exchanger (AG501X8; Bio-Rad), stachyose was added as an internal standard. HPAEC with pulsed electrochemical detection was carried out on a DX-300 Bio-LC-system (Dionex, Idstein, Germany) with sodium hydroxide and sodium acetate as eluents according to the protocol of Thurl et al (21).

**RESULTS**

The applicability of the digestibility assay was shown by a rapid and complete hydrolysis of the control oligosaccharide maltodextrin. Enzymatic determination of free glucose showed that after the preincubation with the HDAs, 13.3 ± 1.2% of the dextrin polymers were hydrolyzed to yield free d-glucose within 30 min. After addition of the BBMs, complete cleavage was obtained within 2 h, as indicated by a rapid increase in glucose concentration corresponding to a complete dextrin cleavage rate of 95.1 ± 2.9%. Even after incubation periods of up to 20 h, the glucose liberated from maltodextrin was recovered completely (97.2 ± 2.3%). This finding is of particular importance with respect to hydrolysis of HMOs because it shows that our preparations were essentially free of enzymatic activity of bacterial origin, which could have utilized the glucose. The hydrolysis of maltodextrin could also be followed by MALDI-MS. Whereas individual oligomers of maltodextrin were easily identified by their distinct masses [degrees of polymerization (DP) 3 to DP 21] in the controls (Figure 2, upper panel), no such mass peaks could be resolved after digestion of maltodextrin with BBMs (Figure 2, lower panel). Although the time for complete hydrolysis of maltodextrin could be shortened drastically by increasing the concentrations of the enzyme sources, sample preparation for MALDI-MS became more difficult because of the high protein concentrations used. Therefore, lower activities (protein content) of enzyme preparations combined with prolonged incubation times proved to be more suitable.

When the total fraction of neutral oligosaccharides isolated from human milk was submitted to hydrolysis, no indication of enzymatic degradation was obtained. The MALDI-MS spectrum obtained after incubation with heat-inactivated enzymes showed the distinct major neutral oligosaccharides in the mass range of 500–2000 Da (Figure 3, upper panel). After 20 h of incubation with active enzyme preparations, all known masses in the entire mass range were still detectable (Figure 3, lower panel). When human instead of porcine BBM preparations were used as the enzyme source, generally the same results were obtained. Although MALDI-MS does not permit one to quantify the compound on the basis of the height of its mass peak, the relation of peak heights from different oligosaccharides (masses) are basically constant within a sample (25). Because relative peak ratios remained essentially unchanged before and at the end of incubation, no indication for significant hydrolysis was obtained. As shown in the mass scan (Figure 3), a few additional mass peaks were observed after incubation of the HMOs. However, neither of these masses correspond to an oligosaccharide of known structure or to a predictable breakdown product. Therefore, they appear to be released from the protein sources during prolonged incubations.

When the purified HMO fractions FL, LNT, LNFP, and LNDFH were submitted to digestion, these oligosaccharides were also recovered intact and no hydrolysis products were detectable by MALDI-MS. Given that monosaccharides that may eventually be released cannot be resolved by MALDI-MS because of matrix effects in the low-molecular-mass ranges (<400 Da), we used enzymatic assays to assess whether there was a release of terminal fucose or core glucose units from the neutral HMO fractions. Enzymatic determination of these 2 monosaccharides in the in vitro digests did not yield a significant increase during incubation above that obtained in the absence of any added HMOs. Depending on the HMO fraction
used, 0.31 ± 0.11 to 2.9 ± 0.93 mg monosaccharide/L was observed, representing <1.2% of the amount that could be released in case of complete breakdown. These results are consistent with the MALDI-MS data in showing no evidence of a breakdown of the oligosaccharides. We therefore conclude that the neutral HMOs are very resistant to hydrolysis by secreted human pancreatic glycosidases and the enzymes bound to porcine or human BBMs.

To verify that no HMO had been lost after incubation, we performed HPAEC as an additional means of characterizing HMO stability. This method generally requires much higher sample loads than does MALDI-MS but it allows a quantitative analysis of the isomeric HMOs and possible breakdown products. Elution profiles of the pentaose LNFP under control conditions and after 20 h of incubation are shown in Figure 4 (upper and lower panel, respectively). Stachyose served as the internal standard. No changes in the peak ratios of the detectable LNFP isomers (LNFP I, II, and III) were observed and no possible breakdown products (eg, lactose and LNT) could be identified. The results of HPAEC therefore corroborated the high stability of the neutral HMOs as found by MALDI-MS and enzymatic analysis.

Hydrolysis studies using the acidic HMOs also did not show evidence of hydrolysis according to the MALDI-MS spectra comparing control and digested samples. At each incubation time, all masses could be detected without any significant change in their peak height ratios. The mass spectra of the control preparation and the total acidic fraction obtained after 20 h of incubation, both measured in the positive ion mode, are shown in Figure 5. A comparison of these 2 spectra shows that, within 1 Da deviation, all acidic structures remained unchanged. Moreover, enzymatic analysis of D-glucose and NANA did not show evidence of hydrolysis: even at incubation times >10 h, both D-glucose and NANA remained between 0.3–1.2 and 0.5–11.6 mg/L, respectively, representing <3.0% of the amount that may have been released in case of complete breakdown.
As a positive control for determining hydrolysis of the apparently nondigestible HMOs, we used a porcine pancreatic tissue homogenate containing the zymogens and intracellular, including lysosomal, enzymes. Additionally, BBM preparations served as the intestinal enzyme source. On the basis of the fact that identical amylase activities were used as in the duodenal aspirates, HMOs were found to be digested rapidly with this pancreas preparation. After only 4 h of incubation, neutral HMO samples did not show major mass peaks when analyzed by MALDI-MS. Only the low-molecular-mass oligosaccharides could still be identified among the background peaks at 4 h of incubation and these vanished after prolonged incubation times. The acidic HMOs showed a similarly pronounced hydrolysis rate when incubated with the pancreas homogenate for > 4 h, as evident by the lack of all characteristic mass peaks of the NANA-containing oligosaccharides.

**DISCUSSION**

The composition of infant formulas that are based on bovine milk has been adjusted to that of human milk with respect to most of the constituents. However, the complex oligosaccharides are not contained in milk of bovine origin (<0.1 g/L). Therefore, this unique fraction of human milk has attracted considerable scientific interest in the past decade in terms of characterizing it chemically and determining its biological functions (26). Because epidemiologic findings show that breast-fed infants have a lower incidence of bacterial infections within the gastrointestinal, respiratory, and urogenital tracts than do non-breast-fed infants (27), oligosaccharides have been considered as offering protection by serving as soluble ligands for pathogenic bacteria, preventing adhesion to or invasion into the epithelium. For this role to be fulfilled, one of the main requirements

**FIGURE 3.** Matrix-assisted laser desorption ionization mass spectrometry (Voyager DE STR; PerSeptive Biosystems, Framingham, MA) of the neutral fraction of human milk oligosaccharides under control conditions (upper panel) and after 20 h incubation in the presence of human duodenal aspirates and an intestinal brush border membrane preparation (lower panel). Each spectrum corresponds to a sum of 50 single spectra. The abbreviations indicate the individual oligosaccharides at their distinct mass positions. FL, fucosyllactose; LNT, lacto-N-tetraose; LNFP, lacto-N-fucopentaose; LNDFH, lacto-N-difucohexaose; LNHF, lacto-N-hexaose; LNO, lacto-N-octaose; LND, lacto-N-decaose; F, fucose. Peaks in the lower mass range represent matrix signals. Peaks labeled with an asterisk emerged during incubations and represent noncarbohydrate compounds.
The resistance of the milk oligosaccharides to digestion in the upper gastrointestinal tract.

The results of our in vitro studies clearly show a remarkable stability of all neutral and acidic fractions of HMOs against hydrolysis by secreted pancreatic and mucosa-bound glycosidases. Using enzymatic, chromatographic, and mass spectroscopic techniques, we did not find any evidence of hydrolysis of HMOs that would lead to a release of smaller oligosaccharides from the more complex structures, nor did we detect the liberation of monomers such as L-fucose and NANA. Because high-molecular-weight HMOs were not available, we focused on neutral and acidic HMO fractions, with the largest structures represented by fucosylated or siaylated octasaccharides, or both. The purified individual oligosaccharides studied included tri- to hexasaccharides. Although the high-molecular-mass compounds could not be studied, we predict them to be equally resistant to hydrolysis because of the repetitive nature of the structural pattern of the HMOs consisting of N-acetyllactosamine, L-fucose, and NANA units.

Because we applied the enzyme preparations in concentrated form and used very long incubation times, our in vitro conditions represented the worst case compared with the situation in an infant’s gut. There are obviously no enzymes secreted by the adult human pancreas or bound to human intestinal brush borders that are capable of hydrolyzing the complex carbohydrates that are present in human milk. Besides using material of human origin, we also performed digestibility studies using pancreas and duodenal preparations from piglets and fully grown pigs. On the basis of standardized α-amylase and maltase activities, no differences in the capability to hydrolyze of HMOs were observed between piglet samples and samples from adult pigs or humans. Therefore, we propose that the immature upper small intestine of piglets and most likely also of infants—despite the significant lactase activity—are not capable of cleaving HMOs. The low α-amylase activities of saliva and pancreas in infants (28) are compensated mainly by an intrinsic human milk amylase (29). Because the results of our studies show that even very high concentrations of α-amylase are unable to cleave the complex HMOs, hydrolysis by milk amylase appears to be of no relevance. Human milk is also a source of α-fucosidase and sialidase (30, 31). However, activities reported for those enzymes are far too low to substantially affect the stability of ingested HMOs in the gastrointestinal tract. Moreover, salivary and intestinal sialidase activities reported in infants and suckling rats (32–34) suggest only very low catalytic capacity for a release of sialic acid from HMOs in the small intestine. No α-fucosidases or N-acetyllactosaminidases have yet been detected in the saliva or small intestine of infants. Taken together, enzymatic capacities to degrade HMOs in human milk or in the immature orogastrointestinal tract appear to be extremely low. However, we could show that enzymatic digestion of HMOs is generally possible by using homogenized porcine pancreas. The inherent intracellular, including lysosomal, enzyme activities we observed when using pancreatic tissue were capable of splitting most of the glycosidic bonds found in HMOs even during short incubation periods. Nevertheless, these enzymes were not released in significant amounts during stimulation of pancreatic secretion because their activity was essentially absent from the duodenal aspirates.

In view of the metabolic fate of HMOs ingested by infants, only a few studies provided preliminary information. Two studies showed the recovery of small amounts of HMOs in the urine of breast-fed infants (7, 14). The oligosaccharides identified in urine were found to be identical to those in breast milk, suggesting that a minor fraction of the HMOs might be absorbed by the infant and excreted unchanged via the kidneys. However, neither the extent nor the mechanism of intestinal absorption of HMOs by infants is known currently. Although high resistance to hydrolysis in the upper gastrointestinal tract has been shown, large-scale intestinal absorption of intact HMOs appears very unlikely. Even the smaller lactose units, which are an integral part of any HMOs, are not absorbed to a large extent in infants, and not absorbed at all in infants who lack lactase (35). This in turn suggests that most HMOs, the neutral as well as the acidic fraction, pass down the intestine of infants to reach the colon in intact form. Whether intestinal absorption of intact HMOs is higher during the first days or weeks after birth, when an infant’s gut is considered to be more permeable and when the oligosaccharide load from milk is especially high, seems likely but is not known.

Colonic bacteria express a wide range of enzymes, including fucosidases and sialidases (36, 37). Therefore, a substantial degradation of HMOs in the colon is to be expected, with only small amounts of the oligosaccharides escaping bacterial hydrolysis. Up to 8% of ingested HMOs were recovered from infant feces by Coppa et al (12). Sabharwal et al (38) detected different HMOs in fecal samples depending on the blood group secretor type, but in most cases only trace amounts of oligosaccharides were found. Bacterial degradation of HMOs therefore appears to be very efficient considering the large amount of complex oligosaccharides ingested with human milk. A recently published study provided the first evidence of bacterial fermentation of HMOs: Brand Miller et al (13) showed by hydrogen exhalation with 3–8-mo-old infants that HMOs are fermented at a rate similar to that of lactulose. Lactulose is recognized as being nondigestible in the human...
small intestine but is fermented by bacteria with production of hydrogen that is easily detected in exhaled air. In the study by Brand Miller et al., a load of 0.7–1.0 g HMOs/kg body wt produced a hydrogen-exhalation profile in infants that was not significantly different from that after administration of the equivalent amount of lactulose over a 4-h period. This was indirect proof of HMOs reaching the colon, where bacteria generate hydrogen by fermentation. Our studies extend this observation by showing that essentially no hydrolysis occurs in the small intestine, which suggests that the oligosaccharides may reach the colon in intact form. HMOs may, in analogy to the fermentation of other nondigestible complex oligosaccharides, play an important role in the growth of microorganisms such as bifidobacteria or lactobacilli, 2) the production of short-chain fatty acids known to serve as a fuel for colonocytes and to stimulate sodium and water absorption, and 3) the stimulation of cell replication in the colon. Although nondigested lactose serves—at least in part—the same purposes (39), HMOs clearly have the advantage of a lower osmotic load because of their higher molecular weights.

In summary, we showed that oligosaccharides contained in human milk have an extraordinary resistance to hydrolysis by digestive enzymes of the small intestine. Complementary to recent findings in infants, we therefore propose that HMOs may serve predominantly as fermentable substrates in the large intestine.

We thank Anja Pfenninger, Marko Mank, Beate Mueller-Werner, and Elisabeth Fischer for their excellent technical assistance. Human duodenal aspirates were provided by J Stein, Gastroenterology Unit, University of Frankfurt, Germany.

REFERENCES


