Title

Discovery of Novel High-Molecular Weight Oligosaccharides Containing N-Acetylhexosamine in Bovine Colostrum Whey Permeate Hydrolyzed with Aspergillus oryzae β-Galactosidase

Journal

Journal of Agricultural and Food Chemistry, 67(12)

ISSN

0021-8561

Authors

Lee, Hyeyoung
de Moura Bell, Juliana Maria Leite Nobrega
Barile, Daniela

Publication Date

2019-03-27

DOI

10.1021/acs.jafc.8b06965

Peer reviewed

Published in final edited form as:

J Agric Food Chem. 2019 March 27; 67(12): 3313–3322. doi:10.1021/acs.jafc.8b06965.

Discovery of Novel High-Molecular Weight Oligosaccharides Containing *N*-Acetylhexosamine in Bovine Colostrum Whey Permeate Hydrolyzed with *Aspergillus oryzae* β-Galactosidase

Hyeyoung Lee[†,‖], **Juliana Maria Leite Nobrega de Moura Bell**[†,‡], and **Daniela Barile**[*,†,§]

[†]Department of Food Science and Technology, University of California—Davis, Davis, California 95616, United States

[‡]Department of Biological and Agricultural Engineering, University of California—Davis, Davis, California 95616, United States

[§]Foods for Health Institute, University of California—Davis, Davis, California 95616, United States

Abstract

Bovine milk oligosaccharides (BMOs) that resemble human milk oligosaccharides are found in whey permeate, indicating that dairy streams can be used as a potential source of bioactive oligosaccharides. Recovery of oligosaccharides from whey permeate is hindered by their low abundance and high concentration of lactose. In the present work, lactose in bovine colostrum whey permeate was hydrolyzed by *Aspergillus oryzae* β-galactosidase to facilitate subsequent monosaccharide removal by membrane separation. Chromatographic separation coupled with high-resolution mass spectrometry revealed β-galactosidase degradation of several β-linkage-containing BMOs and production of novel oligosaccharides that ranged in size from 5 to 11 monosaccharide units containing several galactose repeating units and *N*-acetylhexosamine at their reducing ends. Optimization of BMO hydrolysis and separation methodology could generate high amounts of hetero-oligosaccharides for improved recovery of potentially biotherapeutic oligosaccharides.

Keywords

whey permeate; oligosaccharide; N-acetylhexosamine; Aspergillus oryzae; β-galactosidase; membrane separation

INTRODUCTION

Human milk oligosaccharides (HMOs) constitute a family of nearly two hundred structurally diverse carbohydrates that participate in numerous protective and physiological roles in breast-fed infants.[1–3] In particular, HMOs help establish the intestinal flora of infants by

[*]**Corresponding Author** Phone: +1-530-752-0976. Fax: +1-530-752-4759. dbarile@ucdavis.edu.
[‖]**Present Address** Division of Applied Bioengineering, Dong-Eui University, Busanjin-gu, Busan 47340, Republic of Korea.

Notes

The authors declare no competing financial interest.

selectively stimulating growth of beneficial bacteria.[4,5] Bifidobacteria account for most of the microbiota in healthy breast-fed infants, whereas formula-fed infants tend to have a more diverse microbiota composition with greater numbers of potentially harmful organisms.[6,7] Recent studies show that the initial colonization of beneficial bacteria might have a long-lasting influence on decreasing the risk of gastrointestinal, allergic, autoimmune, and metabolic diseases later in life.[8] Additionally, HMOs serve as pathogen decoys and inhibit the binding of pathogens to intestinal mucosal surfaces, thus blocking their ability to infect hosts.[1] As a consequence, enteric disease morbidity and mortality rates are much lower in breast-fed infants than in those fed infant formula.[9,10]

Many studies have been carried out to find a source of complex HMO-like oligosaccharides for industrial-scale production. A handful of structures have been produced by chemical synthesis; however, their overall low production yields and the use of toxic reagents are not suitable for food applications.[11] Great effort has been invested recently in biotechnological production using microbial and enzymatic methods resulting in single oligosaccharides.[12,13] Alternatively, various mammalian milks are considered as natural sources of oligosaccharides structurally similar to HMOs.[14] Bovine milk and colostrum are potential sources due to their ease of accessibility; however, finding appropriate enrichment and isolation methods for bovine milk oligosaccharides (BMOs) is extremely difficult, necessitating research directed toward the development of methods for large-scale separation and enrichment of the contained biomolecules. The use of membrane technology has gained recognition because it can be implemented at the large scale required for most dairy applications.[15] Indeed, we have observed that whey permeate, a coproduct of whey, generated during the ultrafiltration of whey proteins contains most of milk carbohydrates, including lactose and BMOs.[16] Nanofiltration membranes can then be used to further concentrate the target oligosaccharides that are too diluted in whey permeate. Although filtration membranes discriminate between molecules of different sizes, the selectivity of nanofiltration membranes does not enable adequate fractionation of oligosaccharides, such as sialyllactose, amid a large excess of lactose. Lactose therefore needs to be hydrolyzed into monosaccharides before separation by nanofiltration to increase the purity of the final oligosaccharide fraction.

A previous investigation demonstrated the recovery of HMOs by combining lactose hydrolysis and nanofiltration.[17] Enzymatic hydrolysis of lactose significantly improved the efficiency and selectivity of membrane-based separations. Most of the simple sugars were removed by four nanofiltration cycles, and the oligosaccharide recovery was greater than 50%. Altmann et al. were the first to employ ultrafiltration and nanofiltration along with a β-galactosidase treatment of bovine milk to enrich oligosaccharides.[18] In that work, the membrane process was scaled up from laboratory (0.2 L) to pilot (6 L) and industrial scale (1000 L). The oligosaccharides were enriched by a factor of 100, and the amounts of carbohydrates in nanofiltration retentate were 2.6% oligosaccharides and 28.3% mono- and disaccharides. It was recently demonstrated that under optimum processing conditions, monosaccharides can permeate the nanofiltration membrane, thus allowing the recovery of a monosaccharide-free BMO concentrate.[19] Alternatively, fermentation of the target monosaccharides arising from the lactose hydrolysis was performed prior to final concentration by nanofiltration, enabling the recovery of a BMO fraction with nearly 100%

purity in relation to simple sugars.[20] Regardless of the approach used, enzymatic lactose hydrolysis represents an essential step for the subsequent isolation of oligosaccharides with a high degree of purity. In view of the importance of upstream lactose hydrolysis, we recently optimized processing conditions leading to complete hydrolysis of lactose in bovine colostrum whey permeate using β-galactosidase and demonstrated that sialic acid-containing oligosaccharides are not damaged by the enzyme.[21]

Interestingly, β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) used for the hydrolysis of lactose is known to also possess transgalactosylation activity. A galactose moiety of lactose can be transferred to an acceptor, e.g., any sugar present in the *medium,* thus forming a different oligosaccharide.[22] When lactose is the initial substrate, a transgalactosylation activity usually results in the production of galactooligosac-charides (GOS), which are notably a mixture of di-, tri-, and higher degree of polymerization (DP) oligosaccharides characterized by the presence of a glucose at the reducing end and galactose repeating units.[22,23] Indeed, the formation of GOS in a lactose-rich substrate such as whey permeate using yeast- or fungal-sourced β-galactosidase is well established.[24,25] Disaccharides 6′-galactosyllactose and 3′-galactosyllactose also have been detected, albeit in low concentration, in skimmed bovine milk permeate after β-galactosidase treatment.[18,26,27] However, a detailed investigation of the potential presence of larger and more diverse oligosaccharide structures has not been performed. The objective of this study was to investigate how β-galactosidase affects the profile of naturally occurring oligosaccharides, which are found in bovine colostrum in significant amounts. A commercial *Aspergillus oryzae (A. oryzae)* β-galactosidase was used because the fungal enzyme is widely used in the food and dairy industries, and it is less expensive than β-galactosidases from other sources.[22] An indepth analysis of the oligosaccharide structures was performed using a quadrupole time-of-flight (Q-TOF) mass spectrometer coupled with graphite carbon nano high performance liquid chromatography (nano HPLC) because of its ability to resolve and characterize numerous isomeric oligosaccharides having a high degree of polymerization (DP) and wide mass range.[28]

MATERIALS AND METHODS

Materials and Chemicals.

Bovine colostrum whey permeate was kindly provided by La Belle Colostrum (Bellingham, WA). Samples were kept frozen at −20 °C prior to analysis. Food-grade *A. oryzae* β-galactosidase (100 000 ALU/g) was obtained from BIO-CAT, Inc. (Troy, VA). Sodium hydroxide solution (50%, w/w) was obtained from Fisher Scientific (Fair Lawn, NJ). Sodium acetate, sodium borohydride, and trifluoroacetic acid were purchased from Sigma Aldrich (St. Louis, MO). All other reagents were of either analytical or HPLC grade. Oligosaccharide standards were purchased from V-Labs, Inc. (Covington, LA).

Enzymatic Hydrolysis of Lactose in Bovine Colostrum Whey Permeate.

Thirteen milliliters of whey permeate was adjusted to pH 4.5 with 0.1 N HCl, and 0.2% (*w/v*) β-galactosidase was added to the reaction mixture. The whey permeate samples were incubated at 50 °C with gentle agitation, and the reaction was stopped at 19 or 45 min. The

lactose hydrolysis conditions were selected based on our previous work.[21] All experiments were conducted in triplicate.

Quantification of Mono-, Di-, and Oligosaccharides in Bovine Colostrum Whey Permeate.

Simple sugars and the major oligosaccharides, 3′-sialyllactose (3′SL), 6′-sialyllactose (6′SL) and 6′-sialyllactosamine (6′SLN), were quantified by high-performance anion-exchange chromatography with pulsed ampero-metric detection (Thermo Scientific, HPAE-PAD ICS-5000, Sunnyvale, CA). Whey permeate samples were diluted 10-fold for oligosaccharide analysis and 1000-fold for simple sugar analysis, and filtered through a 0.2-μm membrane (Pall, Port Washington, NY). For mono- and disaccharide analysis, a 25-μL aliquot was injected into a CarboPac PA10 analytical column (3 × 250 mm, Dionex, Sunnyvale, CA) and guard column (3 × 50 mm, Dionex) at a 1.2 mL/min flow rate with an isocratic eluent of 10 mM NaOH for 12 min and a gradient that increased to 100 mM sodium hydroxide over the next 13 min. For oligosaccharide analysis, a CarboPac PA200 column (3 × 250 mm, Dionex) and guard column (3 × 50 mm, Dionex) were used. The isocratic eluent was 10 mM sodium acetate in 100 mM sodium hydroxide at 0.5 mL/min. More details were described in a previous publication.[29]

Reduction and Purification of Oligosaccharides from Colostrum Whey Permeate for Mass Spectrometry (MS) Analysis.

Carbohydrates in all samples were reduced to their alditol forms by adding sodium borohydride. First, 0.5 mL of sample was reduced by the addition of 0.5 mL of 2.0 M sodium borohydride and incubation at 65 °C for 1 h. The samples were subsequently purified by solid-phase extraction (SPE) using graphitized carbon cartridges (Carbograph, 4 mL, Alltech, Deerfield, IL). Prior to use, the cartridges were conditioned with three column volumes of 80% acetonitrile in 0.05% aqueous trifluoroacetic acid and three column volumes of deionized water. The oligosaccharide-containing samples were loaded and washed with five column volumes of deionized water. The oligosaccharides retained by SPE were eluted with five column volumes of 40% acetonitrile in 0.1% aqueous trifluoroacetic acid. The samples were dried in a vacuum centrifuge (Genevac miVac, Ipswich, U.K.) prior to MS analysis.

Analysis of Oligosaccharides by nano HPLC-Chip/Q-TOF MS.

Oligosaccharides were analyzed using an Agilent 6520 HPLC-Chip/Q-TOF (Chip/Q-TOF) MS system (Agilent Technologies, Inc., Santa Clara, CA) as described in a previous publication.[30] The chromatography chip consisted of a 9 × 0.075 mm internal diameter enrichment column and a 43 × 0.075 mm internal diameter analytical column, both packed with 5-μm porous graphitized carbon as the stationary phase. Chromatographic separation was performed by a binary gradient consisting of mobile phase A, which was 3% acetonitrile in water (v/v) with 0.1% formic acid, and mobile phase B, which was 90% acetonitrile in water (v/v) with 0.1% formic acid. The elution profile was 0–2.5 min, 0% B; 2.5–20 min, 0–16% B; 20–30 min, 16–44% B; 30–35 min, 44–100% B; and 35–45 min, 100% B. The elution gradient was followed by a column re-equilibration at 0% B for 10 min. The nanopump flow rate was 0.3 μL/min. The dried samples were dissolved in 1 mL of water and diluted 100-fold, and 1-μL aliquots were injected. The mass acquisition range was

from *m/z* 450–2500 in MS mode, and the instrument was operated in 2 GHz extended dynamic range with a dual nebulizer ESI source. The drying gas temperature and flow rate were 350 °C and 3 L/min, respectively. Sample data were collected in positive-ion mode with an acquisition time of 2.0 s per spectrum. Oligosaccharide identification was performed using Agilent Mass Hunter Qualitative Analysis software (version B.06.01). The monosaccharide compositions of individual oligosaccharides were determined using an in-house search algorithm (glycan finder) within a 10 ppm mass error. BMOs were identified by matching experimentally obtained retention times and oligosaccharide masses with those compiled in an annotated BMO library.[30] Tandem MS (MS/MS) analysis was also performed for structural characterization, and the collision energy (CE) that was applied was based on the following equation:

$$\text{collision energy} = \frac{m/z}{100} \times 1.3 - 3.5$$

Relative quantification was performed by measuring the area of the peak of each individual extracted compound in chromatograms, which represented the total ion count associated with a distinct oligosaccharide.

RESULTS AND DISCUSSION

Lactose Hydrolysis and Carbohydrate Composition of Colostrum Whey Permeate.

Aspergillus oryzae β-galactosidase was used to enzymatically digest lactose into monosaccharides to improve the elimination of simple sugars in further processing, for example to increase permeation during nanofiltration of whey permeate or to enable monosaccharides consumption by yeast-based fermentation. In the hydrolysis conditions employed, almost 100% of lactose was digested without detrimental effects on the major acidic oligosaccharides.[21] The concentration of simple sugars and major oligosaccharides found in bovine colostrum whey permeate, before and after lactose hydrolysis, is presented in Table 1. Because of the intrinsic composition of colostrum, acidic oligosaccharides (i.e., containing at least one sialic acid moiety) were the predominant oligosaccharides identified. The unhydrolyzed whey permeate (control) contained 17.40 ± 0.62 g/L lactose, 0.0370 ± 0.0010 g/L 3′SL, 0.0105 ± 0.0002 g/L 6′SL, and 0.0137 ± 0.0015 g/L 6′SLN, measured by HPAE-PAD. The free monosaccharides glucose and galactose were not detected in the starting material. The lactose content decreased with increased reaction time, demonstrating the effectiveness of the β-galactosidase activity used. At the enzymatic reaction conditions applied, the increase of three major sialylated oligosaccharides (3′SL, 6′SL, and 6′SLN) was observed.

Mass Spectrometry Characterization of Oligosaccharides in Bovine Colostrum Whey Permeate with and without β-Galactosidase Treatment.

The three major acidic BMOs were quantified as described above, and nano HPLC/Q-TOF MS analyses were performed to determine the overall oligosaccharide profile of control and β-galactosidase-treated whey permeate (Figure 1). Chromatographic separation coupled with high-resolution MS yielded a comprehensive profile of oligosaccharides with relative

quantitation. A list of oligosaccharides identified in the starting material (colostrum whey permeate) and the enzyme treated samples, along with their mass, retention time, abundance and tentative structure, is presented in Table 2. A macroscopic evaluation of the two chromatograms (Figure 1) suggests a similar profile, yet novel low-abundance oligosaccharides were noted. Figure 1A and Table 2 evidence that sialylated species were dominant in the starting material, colostrum bovine whey permeate, which is in agreement with previously published data.[16,29] Disialyllactose, a tetrasaccharide with two sialic acids, eluted at 22.7 min. In addition to these sialic acid-containing oligosaccharides, 19 neutral oligosaccharides were found. Most of the neutral oligosaccharides had a relatively small DP ranging from 3 to 6 (Figure 1, Table 2). However, further detailed evaluation showed that several neutral oligosaccharides containing galactose at their nonreducing end were degraded by the enzyme activity and were not found in the β-galactosidase-treated sample. For example, the trisaccharide Gal(β1–6)Gal(β1–4)Glc with *m/z* 507.19, visible as one of the tallest peaks in Figure 1A eluting at 11.2 min, is missing entirely from Figure 1B, after the lactose hydrolysis reaction. To further investigate this matter, we looked in greater detail at the presence and absence of the various isomers with that same *m/z*. The disappearance of several β-linked isomers in a trisaccharide are shown in the extracted ion chromatograms (EICs) for *m/z* 507.19 (Figure 2). Four trisaccharide isomers were identified in the unhydrolyzed bovine colostrum whey permeate by nano HPLC MS (Figure 2A). The four isomers have a lactose core with an additional galactose attached to the nonreducing end via different linkages. All these trisaccharides except for the early eluting isomer (Gal-Gal α3 linkage) (retention time = 9.8 min) were hydrolyzed by *A. oryzae* β-galactosidase. The three β-linked (β3, β6, and β4) trisaccharides that eluted at retention times 10.7, 11.2, and 14.4 min disappeared after enzymatic reaction in the hydrolyzed samples.

Six trisaccharides with the composition of 2Hex+1HexNAc were found for the peak *m/z* 548.21 in the retention time range from 9–15 min (Figure 3). Four of the trisaccharides had a lactose core, whereas two had a lactosamine core composed of an *N*-acetylglucosamine (GlcNAc) attached to Gal with a β1–4 linkage (retention time of 11.4 and 14.7 min). Their putative structures, elucidated in previous publications, are shown in Figure 3.[14,31] Upon β-galactosidase treatment, the trisaccharides containing a lactosamine core completely disappeared, indicating that the linkages between the two galactoses at the nonreducing end were β form. The trioses with a lactose core, e.g., HexNAc(α1–3)Gal(β1–4)Glc and HexNAc(β1–3)Gal(β1–4)Glc, were not affected by β-galactosidase as the enzyme can only hydrolyze a β-galactose units at the nonreducing end.

A large fraction of oligosaccharides in bovine colostrum is sialylated. Since β-galactosidase specifically cleaves β-galactose at the nonreducing end, most of sialylated BMOs were not affected by the enzyme (Figure 1B, Table 2). For example, the trisaccharides with 1NeuAc + 2Hex (*m/z* 636.23) correspond to sialyllactoses in which one NeuAc is attached to lactose. While it had previously been demonstrated that sialyllactoses were not degraded by β-galactosidase treatment,[21] our MS analysis also revealed for the first time that other sialic acid-containing oligosaccharides such as Neu5Gc-containing sialyllactose and sialyllactosamine were also not affected by the β-galactosidase (Table 2). Sialylated oligosaccharides of greater complexity such as those with *m/z* 798.28 (3Hex + 1Neu5Ac), *m/z* 839.30 (2Hex + 1HexNAc + 1Neu5Ac), and *m/z* 927.32 (2Hex + 2Neu5Ac) were also

preserved. These results indicate that the enzyme used in this study showed specificity toward lactose and modified some neutral oligosaccharides but not sialylated oligosaccharides. The only sialylated oligosaccharide that was hydrolyzed by the enzyme was with an *m/z* of 683.74 (4Hex + 2HexNAc + 1NeuAc) because a galactose (not a sialic acid) was located at its nonreducing end.

One of the well-known neutral oligosaccharides in bovine colostrum is lacto-*N*-neotetraose (LNnT), detected at *m/z* 710.26, whose terminal Gal at the nonreducing end is attached via a β1–4 linkage. Further examination of the EIC of *m/z* 710.26 (Figure 4) revealed that of the three tetrasaccharide isomers, LNnT was digested by β-galactosidase, confirming the presence of a terminal Gal with a β1–4 linkage. The first eluting isomer (retention time = 11.4 min) was not hydrolyzed by the enzyme, indicating that the structure does not have a β-linkage at the nonreducing end.

Oligosaccharides containing HexNAc with higher molecular weights, e.g., peaks at *m/z* 872.32 (4Hex + 1HexNAc), *m/z* 913.34 (3Hex + 2HexNAc; 30200a), *m/z* 538.20 (4Hex + 2HexNAc) and *m/z* 558.71 (3Hex + 3HexNAc) were also digested by β-galactosidase as they contain β1–4 linkages in their nonreducing ends (Table 2).

The high-resolution MS analyses employed in the present study revealed, for the first time, that oligosaccharides with galactose at their nonreducing end were hydrolyzed and that some novel hetero-oligosaccharides were synthesized in small amounts, indicating that the enzyme exhibited transgalactosylation as well as hydrolytic activities.

In previous works, several GOS were found after lactose hydrolysis.[18,26,27,32] However, we did not observe synthesis of GOS, but rather the GOS that originally existed in bovine milk such as Gal(β1–3)Gal(β1–4)Glc, Gal(β1–6)Gal(β1–4)Glc, and Gal(β1–4)Gal(β1–4)Glc were degraded by the enzyme (Table 2). The discrepancy in findings may be due to the low lactose concentration in the colostrum whey permeate in our study, as the balance between hydrolytic and transgalactosylation activities is highly dependent on the concentration of the galactose donor, which was lactose (17.4 g/L in our study vs 40 g/L in the literature) (Table 1).[18] High lactose concentrations are generally required for transgalactosylation, and 500 g/L lactose has been used for maximum oligosaccharide conversion.[33] During lactose conversion, the measurement of the glucose/galactose ratio also provides a good estimate of the extent of competition between transgalactosylation and hydrolysis.[22,34] The low glucose/galactose ratio (6.99/6.86 = 1.02) in this study implied the predominance of hydrolytic activity over transgalactosylation (Table 1, retention time = 45 min). Using other dairy streams with a higher lactose concentration (e.g., mother liquor) would likely yield more oligosaccharides and possibly in higher concentration.

Instead of GOS, which are usually generated during lactose hydrolysis, several novel oligosaccharides (*m/z* 872.32, 517.69, 598.71, 679.74, 760.76, and 841.78—note that several of the oligosaccharides have a double charge, since they have high molecular weight) were generated by β-galactosidase digestion as indicated in solid lined-EICs (Figure 5, Table 2). Their oligosaccharide compositions were $(Hex)_n(HexNAc)_1$, where *n* ranges from 4 to 10. In Figure 5A, one EIC peak of *m/z* 872.32 (4Hex + 1HexNAc, retention time = 15.4

min) was observed in the unhydrolyzed control sample. After enzyme reaction, the peak disappeared, whereas several other isomeric oligosaccharides were synthesized. In the case of $(Hex)_5(HexNAc)_1$~$(Hex)_{10}(HexNAc)_1$ as shown in Figure 5B–F, several isomeric oligosaccharides were newly generated. As the *A. oryzae* enzyme transferred galactose via both β1–4 and β1–6 linkages, but preferred β1–6 linkages, the major peaks at a retention time of 13 min are expected to be the oligosaccharides containing β1–6 linkages.[35] As shown in Table 2, the proportion of synthesized oligosaccharides to total oligosaccharides was 4.73% at 19 min and 4.90% at 45 min of reaction.

Putative Structure of Newly Generated Oligosaccharides in β-Galactosidase-Treated Whey Permeate.

The structures of the major transgalactosylated oligosaccharides were elucidated with the aid of MS/MS (Figure 6A–C). As an example, the MS/MS annotated spectrum of $(Hex)_7(HexNAc)_1$ with the precursor ion at *m/z* 679.73 (retention time = 13.0 min) is shown in Figure 6A. Reading it from right to left, the first 2Hex unit was lost which was followed by the loss of consecutive Hex units, leaving the reducing end HexNAc at *m/z* 224.12 which is indicative of a lactosamine core.

$(Hex)_8(HexNAc)_1$ has several isomeric oligosaccharides (EIC of *m/z* 760.76) as shown in Figure 5E. The MS/MS spectra of the two most abundant isomers (retention times of 10.9 and 13.0 min) are shown in Figure 6B,C. In both spectra, consecutive Hex losses were observed, and the reducing end HexNAc fragment was also found, confirming the presence of a lactosamine core. The peaks at *m/z* 325.11 and 487.16 correspond to $[(Hex)_2 + H]^+$ and $[(Hex)_3 + H]^+$, respectively, suggesting that several hexoses are directly linked. A diagnostic ion for a lactosamine core (*m/z* 386.16) was found in Figure 6C.

Several researchers reported that microbial β-galactosidases transfer galactose from lactose to *N*-acetyllactosamine (LacNAc) or GlcNAc. The transgalactosylation reaction was well studied using *Bacillus circulans* β-galactosidase.[36,37] This enzyme preferentially synthesized β-1,4-linkages during transgalactosylation, and the main reaction product was thus LacNAc, with smaller amounts of GlcNAc containing higher oligosaccharides (tri- and tetrasaccharides) with β1–4 linkages. Multiple β-galactosidases were discovered in bifidobacteria and lactobacilli, and the enzymes showed some transgalactosylation activity, yielding di- and trisaccharides[38,39] The fungus *A. oryzae* was also shown to form oligosaccharides containing GlcNAc, especially with a higher DP; however, the composition and the structure of the individual oligosaccharides formed were not well characterized.[35] In our study, high-resolution mass spectrometry analyses revealed the DP and the position of GlcNAc in the oligosaccharides generated from *A. oryzae* β-galactosidase-treated whey permeate.

The addition of HexNAc to oligosaccharides is of interest because of the potential of extending the functionality of commercial GOS. The generated oligosaccharides contained the HMO core structure, LacNAc, and the possible physiological functions of the produced oligosaccharides may therefore be related to their structural similarities to HMOs. HMOs that contain GlcNAc, fucose, and sialic acid are a preferred substrate for infant-associated bifidobacteria and selectively stimulate their growth.[4] The novel oligosaccharides may also

serve as pathogen decoys as pathogens recognize and attach to GlcNAc-containing oligosaccharides on the cell surface.[40,41] GOS possess the greatest inhibitory effect among various commercial oligosaccharides due to their multiple galactose residues.[42] Further modification with GlcNAc could provide additional adherence inhibition. While functional studies would be required to determine the functional and biological properties on the GlcNAc-containing oligosaccharides, their complex structure could lead to potential improvement for the gut microbiome.

From an industrial perspective, the results of this study can be applied to the food-grade conversion of colostrum whey permeate to produce novel bioactive oligosaccharides. HMOs cannot currently be industrially produced, and infant formula is supplemented with simpler GOS structures. These new oligosaccharides are of interest because of the potential extension of GOS functionality. However, an adequately high acceptor/donor ratio, reaction time control, continuous product removal, and enzyme recycling may be employed to improve the biocatalytic productivity of the target oligosaccharides. Kinetic studies could help elucidate enzyme substrate preference and further explain why HexNAc-containing oligosaccharides rather than simpler GOS are the main transgalactosylation products under the tested reaction conditions. Therefore, selection of the appropriate reaction condition could generate high amounts of potentially biotherapeutic oligosaccharides.

ACKNOWLEDGMENTS

This research was supported in part by funding from the National Institutes of Health awards R01AT007079, R01AT008759; USDA:NIFA 2014-05266; the National Research Foundation of Korea grant No. 201700620001 and the Peter J. Shields Endowed Chair in Dairy Food Science. The authors thank Cora Dillard for editing this manuscript.

REFERENCES

(1). Newburg DS; Ruiz-Palacios GM; Morrow AL Human milk glycans protect infants against enteric pathogens. Annu. Rev. Nutr 2005, 25, 37–58. [PubMed: 16011458]

(2). Bode L Human milk oligosaccharides: every baby needs a sugar mama. Glycobiology 2012, 22, 1147–1162. [PubMed: 22513036]

(3). Barile D; Rastall RA Human milk and related oligosaccharides as prebiotics. Curr. Opin. Biotechnol 2013, 24, 214–219. [PubMed: 23434179]

(4). Zivkovic AM; German JB; Lebrilla CB; Mills DA Human milk glycobiome and its impact on the infant gastrointestinal microbiota. Proc. Natl. Acad. Sci. U. S. A 2011, 108, 4653–4658. [PubMed: 20679197]

(5). Marcobal A; Barboza M; Froehlich JW; Block DE; German JB; Lebrilla CB; Mills DA Consumption of human milk oligosaccharides by gut-related microbes. J. Agric. Food Chem 2010, 58, 5334–5340. [PubMed: 20394371]

(6). Stark PL; Lee A The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life. J. Med. Microbiol 1982, 15, 189–203. [PubMed: 7143428]

(7). Penders J; Thijs C; Vink C; Stelma FF; Snijders B; Kummeling I; van den Brandt PA; Stobberingh EE Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics 2006, 118, 511–521. [PubMed: 16882802]

(8). Rautava S; Luoto R; Salminen S; Isolauri E Microbial contact during pregnancy, intestinal colonization and human disease. Nat. Rev. Gastroenterol. Hepatol 2012, 9, 565–576. [PubMed: 22890113]

(9). Howie PW; Forsyth JS; Ogston SA; Clark A; Florey CD Protective effect of breast feeding against infection. Br. Med. J. 1990, 300, 11–16. [PubMed: 2105113]

(10). Duijts L; Ramadhani MK; Moll HA Breastfeeding protects against infectious diseases during infancy in industrialized countries. A systematic review. Matern. Child Nutr 2009, 5, 199–210. [PubMed: 19531047]

(11). Routenberg Love K; Seeberger PH Automated solid-phase synthesis of protected tumor-associated antigen and blood group determinant oligosaccharides. Angew. Chem., Int. Ed 2004, 43, 602–605.

(12). Han NS; Kim TJ; Park YC; Kim J; Seo JH Biotechnological production of human milk oligosaccharides. Biotechnol. Adv 2012, 30, 1268–1278. [PubMed: 22119239]

(13). Petschacher B; Nidetzky B Biotechnological production of fucosylated human milk oligosaccharides: Prokaryotic fucosyltransferases and their use in biocatalytic cascades or whole cell conversion systems. J. Biotechnol 2016, 235, 61–83. [PubMed: 27046065]

(14). Urashima T; Saito T; Nakamura T; Messer M Oligosaccharides of milk and colostrum in non-human mammals. Glycoconjugate J. 2001, 18, 357–371.

(15). Pouliot Y Membrane processes in dairy technology—From a simple idea to worldwide panacea. Int. Dairy J. 2008, 18, 735–740.

(16). Barile D; Tao N; Lebrilla CB; Coisson JD; Arlorio M; German JB Permeate from cheese whey ultrafiltration is a source of milk oligosaccharides. Int. Dairy J. 2009, 19, 524–530. [PubMed: 20161544]

(17). Sarney DB; Hale C; Frankel G; Vulfson EN A novel approach to the recovery of biologically active oligosaccharides from milk using a combination of enzymatic treatment and nanofiltration. Biotechnol. Bioeng 2000, 69, 461–467. [PubMed: 10862685]

(18). Altmann K; Wutkowski A; Kämpfer S; Klempt M; Lorenzen PC; Clawin-Rídecker I Comparison of the efficiency of different NF membranes for the enrichment of milk oligosaccharides from bovine milk. Eur. Food Res. Technol 2015, 241, 803–815.

(19). Cohen JL; Barile D; Liu Y; de Moura Bell J Role of pH in the recovery of bovine milk oligosaccharides from colostrum whey permeate by nanofiltration. Int. Dairy J. 2017, 66, 68–75. [PubMed: 28652648]

(20). de Moura Bell J; Cohen JL; de Aquino L; Lee H; de Melo Silva VL; Liu Y; Domizio P; Barile D An integrated bioprocess to recover bovine milk oligosaccharides from colostrum whey permeate. J. Food Eng 2018, 216, 27–35. [PubMed: 29217872]

(21). de Moura Bell JMLN; Aquino LFMC; Liu Y; Cohen JL; Lee H; de Melo Silva VL; Rodrigues MI; Barile D Modeling lactose hydrolysis for efficiency and selectivity: Toward the preservation of sialyloligosaccharides in bovine colostrum whey permeate. J. Dairy Sci 2016, 99, 6157–6163. [PubMed: 27236766]

(22). Torres DPM; Gonçlalves M. d. P. F.; Teixeira JA; Rodrigues LR Galacto-oligosaccharides: production, properties, applications, and significance as prebiotics. Compr. Rev. Food Sci. Food Saf 2010, 9, 438–454.

(23). Gänzle MG Enzymatic synthesis of galacto-oligosaccharides and other lactose derivatives (hetero-oligosaccharides) from lactose. Int. Dairy J. 2012, 22, 116–122.

(24). López Leiva MH; Guzman M Formation of oligosaccharides during enzymic hydrolysis of milk whey permeates. Process Biochem 1995, 30, 757–762.

(25). Rustom IYS; Foda MI; López-Leiva MH Formation of oligosaccharides from whey UF-permeate by enzymatic hydrolysis — analysis of factors. Food Chem 1998, 62, 141–147.

(26). Rodriguez-Colinas B; Fernandez-Arrojo L; Ballesteros AO; Plou FJ Galactooligosaccharides formation during enzymatic hydrolysis of lactose: towards a prebiotic-enriched milk. Food Chem 2014, 145, 388–394. [PubMed: 24128493]

(27). Rodriguez-Colinas B; Poveda A; Jimenez-Barbero J; Ballesteros AO; Plou FJ Galacto-oligosaccharide synthesis from lactose solution or skim milk using the beta-galactosidase from *Bacillus circulans*. J. Agric. Food Chem 2012, 60, 6391–6398. [PubMed: 22676418]

(28). Ruhaak LR; Lebrilla CB Advances in analysis of human milk oligosaccharides. Adv. Nutr 2012, 3, 406S–414S. [PubMed: 22585919]

(29). Lee H; MeloSilva V. L. d.; Liu Y; Barile D Quantification of carbohydrates in whey permeate products using high-performance anion-exchange chromatography with pulsed amperometric detection. J. Dairy Sci 2015, 98, 7644–7649. [PubMed: 26364096]

(30). Lee H; Cuthbertson DJ; Otter DE; Barile D Rapid screening of bovine milk oligosaccharides in a whey permeate product and domestic animal milks by accurate mass database and tandem mass spectral library. J. Agric. Food Chem 2016, 64, 6364–6374. [PubMed: 27428379]

(31). Albrecht S; Lane JA; Marino K; Al Busadah KA; Carrington SD; Hickey RM; Rudd PM A comparative study of free oligosaccharides in the milk of domestic animals. Br. J. Nutr 2014, 111, 1313–1328. [PubMed: 24635885]

(32). Urrutia P; Rodriguez-Colinas B; Fernandez-Arrojo L; Ballesteros AO; Wilson L; Illanes A; Plou FJ Detailed analysis of galactooligosaccharides synthesis with β-galactosidase from *Aspergillus oryzae*. J. Agric. Food Chem 2013, 61, 1081–1087. [PubMed: 23330921]

(33). Neri DFM; Balcão VM; Costa RS; Rocha ICAP; Ferreira EMFC; Torres DPM; Rodrigues LRM; Carvalho LB; Teixeira JA Galacto-oligosaccharides production during lactose hydrolysis by free *Aspergillus oryzae* β-galactosidase and immobilized on magnetic polysiloxane-polyvinyl alcohol. Food Chem 2009, 115, 92–99.

(34). Arreola SL; Intanon M; Wongputtisin P; Kosma P; Haltrich D; Nguyen T-H Transferase activity of Lactobacillal and Bifidobacterial β-galactosidases with various sugars as galactosyl acceptors. J. Agric. Food Chem 2016, 64, 2604–2611. [PubMed: 26975338]

(35). Reuter S; Rusborg Nygaard A; Zimmermann W β-Galactooligosaccharide synthesis with β-galactosidases from Sulfolobus *solfataricus, Aspergillus oryzae*, and *Escherichia coli*. Enzyme Microb. Technol 1999, 25, 509–516.

(36). Li W; Sun Y; Ye H; Zeng X Synthesis of oligosaccharides with lactose and N-acetylglucosamine as substrates by using β-d-galactosidase from *Bacillus circulans*. Eur. Food Res. Technol 2010, 231, 55–63.

(37). Hernaiz MJ; Crout DHG A highly selective synthesis of N-acetyllactosamine catalyzed by immobilised β-galactosidase from *Bacillus circulans*. J. Mol. Catal B: Enzym 2000, 10, 403–408.

(38). Hung M-N; Lee BH Purification and characterization of a recombinant beta-galactosidase with transgalactosylation activity from *Bifidobacterium infantis* HL96. Appl Microbiol. Biotechnol 2002, 58, 439–445. [PubMed: 11954789]

(39). Splechtna B; Nguyen T.-h.; Steinböck M; Kulbe KD; Lorenz W; Haltrich D Production of prebiotic galacto-oligosac-charides from lactose using β-galactosidases from *Lactobacillus reuteri*. J. Agric. Food Chem 2006, 54, 4999–5006. [PubMed: 16819908]

(40). Kulkarni AA; Weiss AA; Iyer SS Glycan-based high-affinity ligands for toxins and pathogen receptors. Med. Res. Rev 2010, 30, 327–393. [PubMed: 20135686]

(41). Hyland RM; Sun J; Griener TP; Mulvey GL; Klassen JS; Donnenberg MS; Armstrong GD The bundlin pilin protein of enteropathogenic *Escherichia coli* is an N-acetyllactosamine-specific lectin. Cell. Microbiol 2007, 10, 177–187. [PubMed: 17697132]

(42). Shoaf K; Mulvey GL; Armstrong GD; Hutkins RW Prebiotic galactooligosaccharides reduce adherence of enteropatho-genic *Escherichia coli* to tissue culture cells. Infect. Immun 2006, 74, 6920–6928. [PubMed: 16982832]

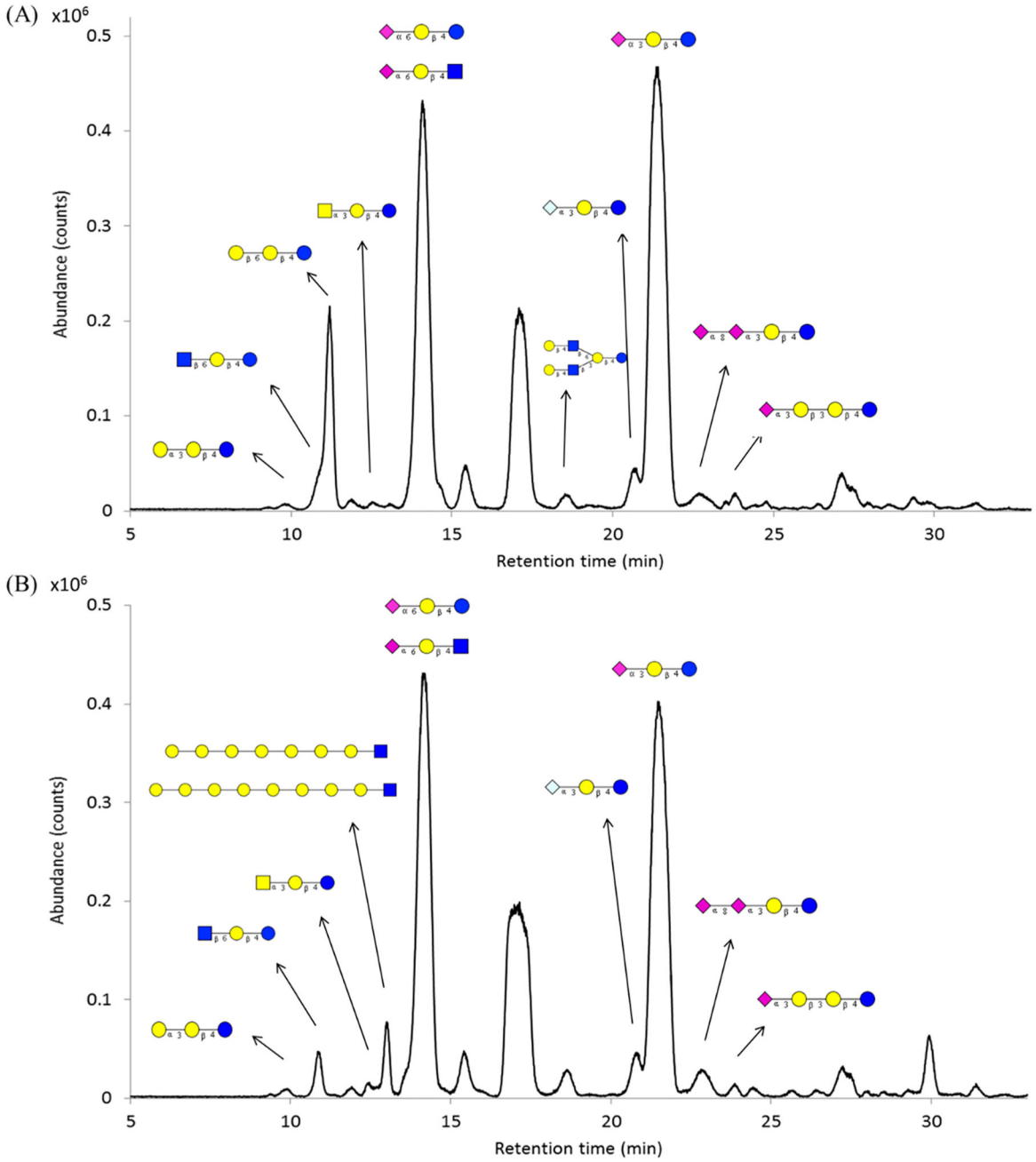


Figure 1.

Nano HPLC-Chip/TOF MS profiles of BMOs in (A) unhydrolyzed bovine colostrum whey permeate (control) and (B) *β*-galactosidase-treated bovine colostrum whey permeate. The enzyme reaction time was 45 min. Major peaks are labeled with structures, where blue circles, blue squares, yellow circles, yellow squares, purple diamonds, and white diamonds represent Glc, GlcNAc, Gal, GalNAc, NeuAc, and NeuGc, respectively.

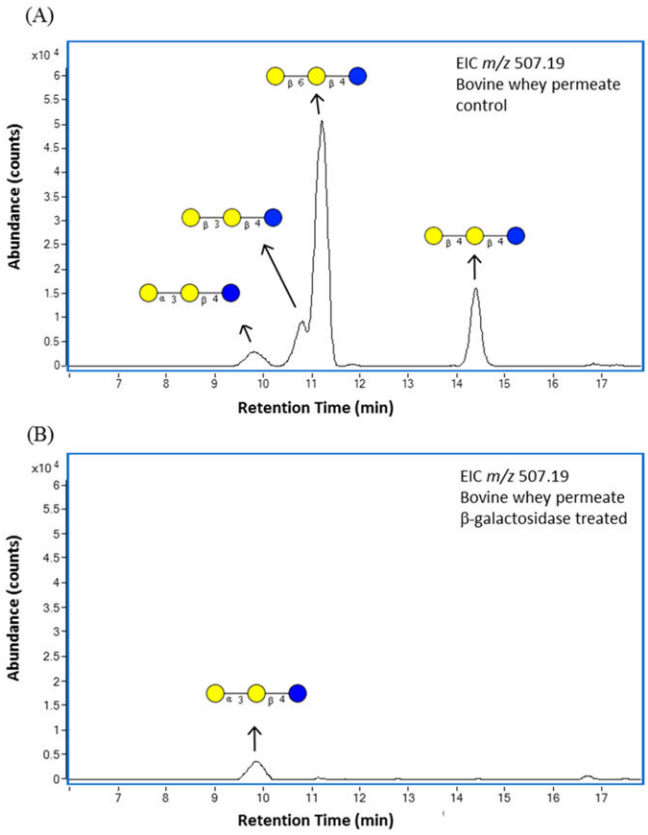


Figure 2.

Nano HPLC-Chip/TOF MS extracted ion chromatograms (EICs) of *m/z* 507.18 from (A) bovine colostrum whey permeate and (B) *β*-galactosidase-treated colostrum whey permeate. The enzyme reaction time was 45 min.

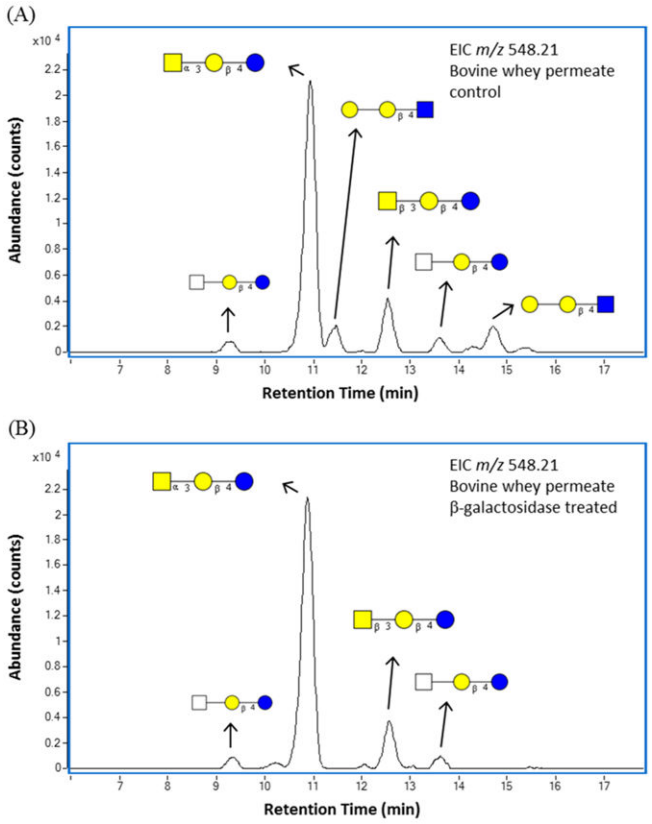


Figure 3.

Extracted ion chromatograms (EICs) of *m/z* 548.21 from (A) bovine whey permeate and (B) *β*-galactosidase-treated whey permeate. The enzyme reaction time was 45 min.

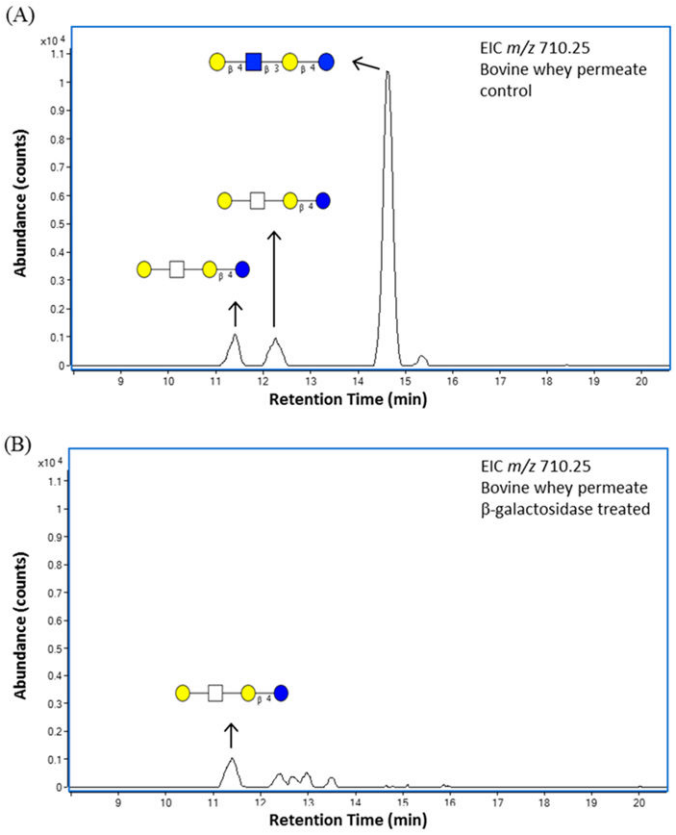


Figure 4.

Extracted ion chromatograms (EICs) of *m/z* 710.26 from (A) bovine whey permeate and (B) *β*-galactosidase-treated whey permeate. The enzyme reaction time was 45 min.

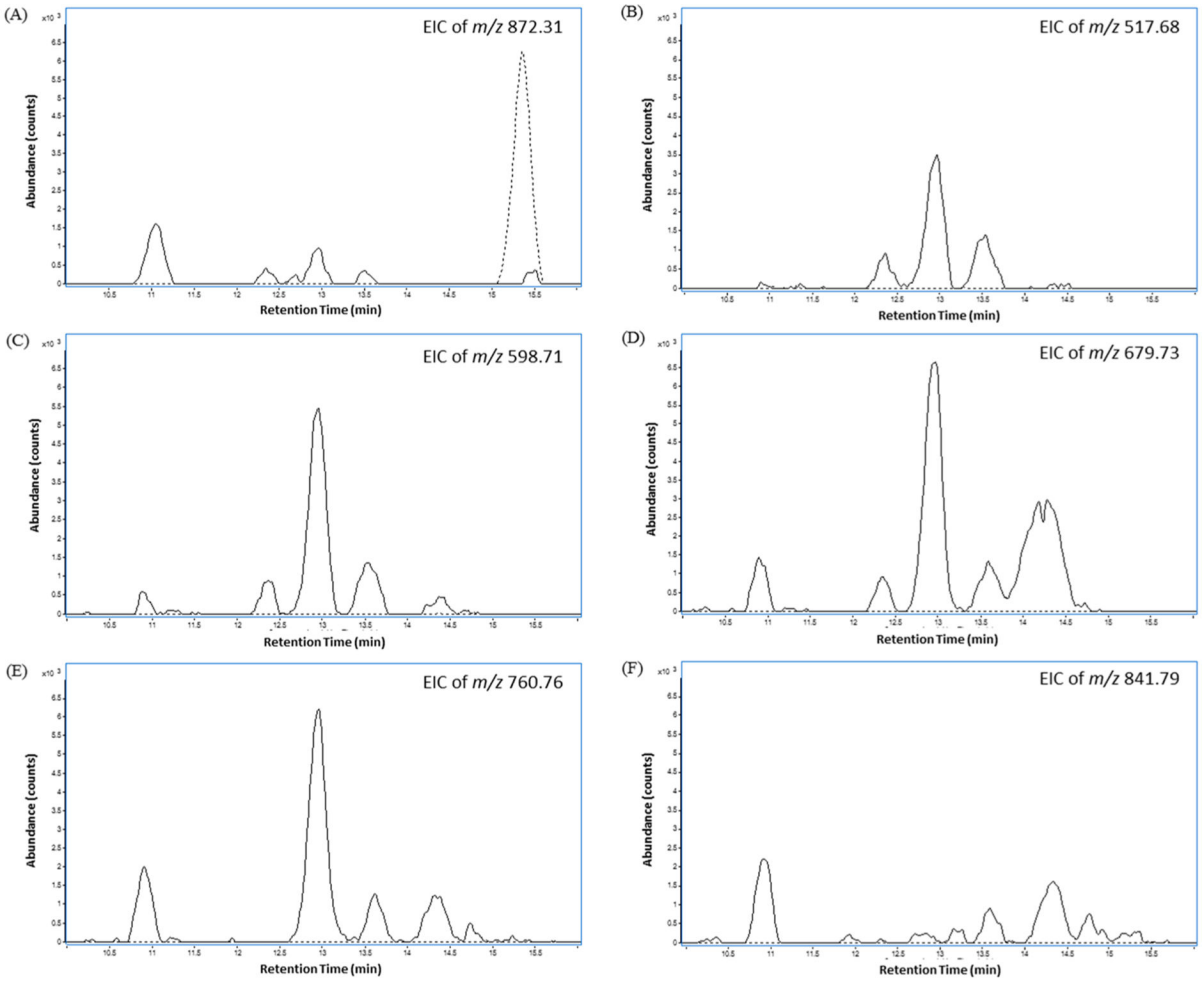


Figure 5.

Extracted ion chromatograms (EICs) of the newly generated oligosaccharides. (A) EIC of *m/z* 872.32, $[(Hex)_4(HexNAc)_1 + H]^+$, (B) *m/z* 517.68, $[(Hex)_5(HexNAc)_1 + 2H]^{2+}$, (C) *m/z* 598.71, $[(Hex)_6(HexNAc)_1 + 2H]^{2+}$, (D) *m/z* 679.73, $[(Hex)_7(HexNAc)_1 + 2H]^{2+}$, (E) *m/z* 760.76, $[(Hex)_8(HexNAc)_1 + 2H]^{2+}$, and (F) *m/z* 841.79, $[(Hex)_9(HexNAc)_1 + 2H]^{2+}$. EICs of control (dotted line) and β-galactosidase-treated sample (solid line) are overlaid.

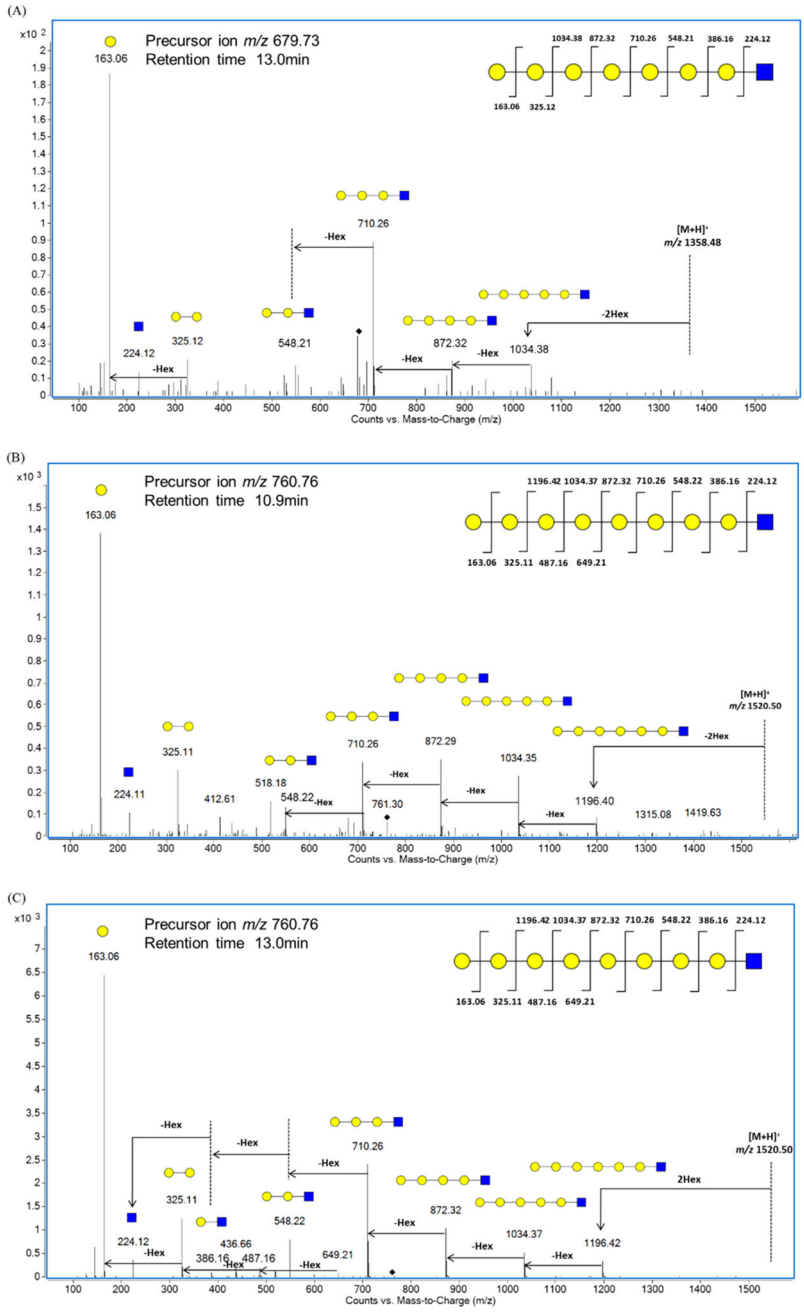


Figure 6.

Nano HPLC-Chip/TOF MS tandem mass spectra of (A) *m/z* 679.73 at 13.0 min, (B) *m/z* 760.76 at 10.9 min, and (C) *m/z* 760.76 at 13.0 min with the resulting putative structures inset. An *m/z* 224.12 peak corresponds to a reducing end HexNAc.

Table 1.

Concentration of Major Carbohydrates in Colostrum Whey Permeate with and without β-Galactosidase Treatment Measured by HPAE-PAD[a]

carbohydrate	unhydrolyzed whey permeate (control)	β-galactosidase treatment (reaction time = 19 min)	β-galactosidase treatment (reaction time = 45 min)
Gal (g/L)	ND	6.76 ± 0.5[b]	6.86 ± 0.43
Glc (g/L)	ND	6.89 ± 0.46	6.99 ± 0.46
Lac (g/L)	17.40 ± 0.62	ND[c]	ND
3′SL (g/L)	0.0370 ± 0.0010	0.0431 ± 0.0008	0.0471 ± 0.0019
6′SL (g/L)	0.0105 ± 0.0002	0.0115 ± 0.0001	0.0123 ± 0.0002
6′SLN (g/L)	0.0137 ± 0.0015	0.0241 ± 0.0024	0.0253 ± 0.0018

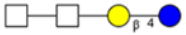
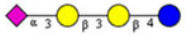
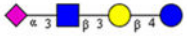
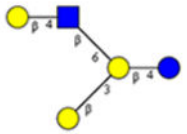
[a]Lactose hydrolysis was performed by the addition of 0.2% (w/v) of β-galactosidase at pH 4.5 and 50 °C under gentle agitation.

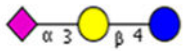
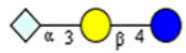
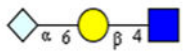
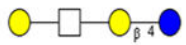
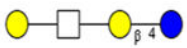
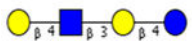
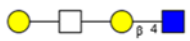
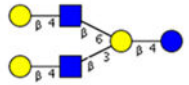
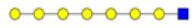
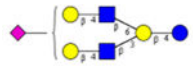
[b]Data are the mean ± standard deviation of three replicates.

[c]ND, not detected.

Table 2.

Bovine Milk Oligosaccharides and Novel Oligosaccharides Generated by β-Galactosidase Reactions in Colostrum Whey Permeate[a]

m/z	neutral mass	retention time (min)	tentative structure	abundance (counts) 0 min	19 min	45 min	*m/z*	neutral mass	retention time (min)	tentative structure	abundance (counts) 0 min	19 min	45 min
507.19	506.18	9.8		156386	171911	114769	751.29	750.28	13.9		24021	15064	14382
507.19	506.18	10.7		424846	ND	ND	798.28	797.27	23.8		138414	92397	101476
507.19	506.18	11.2		3215704	ND	ND	839.30	838.29	14.7		65290	51306	53483
507.19	506.18	14.4		320175	ND	ND	872.32	871.30	11.0		ND	22237	20658
548.21	547.20	9.4		24854	13423	14893	872.32	871.30	15.4		107809	13885	ND
548.21	547.20	10.9		579875	446011	452914	913.34	912.33	14.1		68585	ND	ND
548.21	547.20	11.4		40114	ND	ND	927.32	926.31	22.7		286793	469562	409325
548.21	547.20	12.5		83876	70410	69421	517.69	1033.35	13.0		ND	87933	84094

m/z	neutral mass	retention time (min)	tentative structure	abundance (counts)		
				0 min	19 min	45 min
548.21	547.20	13.6		18909	17234	16348
548.21	547.20	14.7		47226	ND	ND
636.23	635.22	14.2		2931769	3329632	3461666
636.23	635.22	21.4		9188479	8285357	9036998
652.22	651.21	20.7		385330	346929	335528
677.25	676.24	14.1		6421748	5856132	5979629
693.25	692.24	13.7		248711	204862	239808
710.26	709.25	11.4		19752	18356	19950
710.26	709.25	12.3		25562	24550	ND
710.26	709.25	14.7		203517	ND	ND
751.29	750.28	13.0		28303	ND	ND
538.20	1074.38	18.4		38450	ND	ND
558.71	1115.41	16.7		29295	ND	ND
598.71	1195.40	13.0		ND	104632	118302
679.74	1357.45	10.8		ND	125208	134101
683.74	1365.47	24.8		26418	ND	ND
760.76	1519.50	10.9		ND	31586	34260
760.76	1519.50	13.0		ND	354129	358637
760.76	1519.50	14.4		ND	62437	49580
841.78	1681.55	10.9		ND	56084	72628
841.78	1681.55	14.1		ND	85506	136542
922.81	1843.60	14.2		ND	34509	38599

[a] Symbols: Glc (blue circle), Gal (yellow circle), Fuc (red triangle), GlcNAc (blue square), GalNAc (yellow square), Neu5Ac (purple diamond), and Neu5Gc (diamond).