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Journal

Biotechnology Progress, 31(5)

ISSN

8756-7938

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Publication Date

2015-09-01

DOI

10.1002/btpr.2133

Peer reviewed

HHS Public Access

Author manuscript

Biotechnol Prog. Author manuscript; available in PMC 2015 October 28.

Published in final edited form as:

Biotechnol Prog. 2015 September ; 31(5): 1323–1330. doi:10.1002/btpr.2133.

A Novel Endo-β-*N*-Acetylglucosaminidase Releases Specific *N*-Glycans Depending on Different Reaction Conditions

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Abstract

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Milk glycoproteins are involved in different functions and contribute to different cellular processes, including adhesion and signaling, and shape the development of the infant microbiome. Methods have been developed to study the complexities of milk protein glycosylation and understand the role of N-glycans in protein functionality. Endo-β-N-acetylglucosaminidase (EndoBI-1) isolated from Bifidobacterium longum subsp. infantis ATCC 15697 is a recently isolated heat-stable enzyme that cleaves the N-N′-diacetyl chitobiose moiety found in the N-glycan core. The effects of different processing conditions (pH, temperature, reaction time, and enzyme/protein ratio) were evaluated for their ability to change EndoBI-1 activity on bovine colostrum whey glycoproteins using advanced mass spectrometry. This study shows that EndoBI-1 is able to cleave a high diversity of N-glycan structures. Nano-LC-Chip–Q-TOF MS data also revealed that different reaction conditions resulted in different N-glycan compositions released, thus modifying the relative abundance of N-glycan types. In general, more sialylated N-glycans were released at lower temperatures and pH values. These results demonstrated that EndoBI-1 is able to release a wide variety of N-glycans, whose compositions can be selectively manipulated using different processing conditions.

Keywords

N-glycans; deglycosylation; whey

Introduction

Protein glycosylation plays a key role in structural conformation and bioactivity of proteins, including adhesion, targeting, folding, and stability.[1] Glycans can be linked to the protein through *O*-glycosidic or *N*-glycosidic bonds. *O*-linked glycans (O-glycans) are frequently attached to the polypeptide via *N*-acetylgalactosamine to a hydroxyl group of a serine or threonine residue, and can be extended into a variety of different structural core classes. *N*-linked glycans (*N*-gly-cans) are linked, via *N*-acetylglucosamines (HexNAc), to an asparagine residue of proteins in the specific amino acid sequence As*N*-X-Ser/Thr (where X can be any amino acid except proline).[2] The *N*-glycan core is composed of two HexNAc and three mannose residues and is sometimes fucosylated at the HexNAc residue in the *N*-glycan core.[2,3] The *N*-glycan core is assembled in the endoplasmic reticulum and elongated by other monosaccharides via the actions of glycosyltransferases and glycosidases, which determine the degree of branching and the type of linkage.[4] Elongation with fucose and sialic acid increases the complexity and diversity of *N*-glycan structures. *N*-glycans are divided into three main classes: high mannose, complex, or hybrid, based on the number of sialylated and fucosylated sites.[2]

Milk contains a variety of components, including proteins, peptides, lipids, and carbohydrates, that contribute to the development of a newborn.[5] Whey, a by-product of cheese production from bovine milk that represents an attractive source of milk compounds, contains milk glycoproteins, including lactoferrin, immunoglobulins, and α-lactalbumin.[6] These glycoproteins contain covalently attached oligosaccharides (glycans).[7] It has been shown that these proteins have several biological activities. For example, lactoferrin protects intestinal cells against viral and bacterial infections, stimulates immune cells, and is

implicated in the inhibition of the production of pro-inflammatory molecules.[8–10] Other studies reveal that milk glycoproteins contribute to the bifidogenic effect of mother's milk, stimulating the growth of beneficial bacteria by providing a diverse array of complex glycans as a carbon source.[11] However, the function of specific conjugated *N*-glycans is not well elucidated. Some structures may have similar functions to those described for free milk oligosaccharides, including inhibition of pathogen adhesion to intestinal epithelial cells and promoting the growth of a beneficial microflora in the infant intestine.[12,13] We recently demonstrated that a key infant gut microbe, *Bifidobacterium longum subsp. infantis, consumes* these conjugated *N*-gly-cans. However, we found that *B. longum* subsp. *infantis* preferentially consumes some of these milk glycans from a mixed pool of all *N*-glycans found on milk glycoproteins.

We recently showed a novel Endo-β-*N*-acetylglucosaminidase isolated from *B. longum* subsp. *infantis* ATCC 15697 cleaves the *N*-*N*′-diacetyl chitobiose moiety found in the *N*-glycan core of high mannose, hybrid and complex *N*-gly-cans.[14,15] Its activity is not affected by a fucosylated *N*-glycan core and it was also shown to be heat resistant, which enables use during 95°C heat treatments, in contrast to the currently available *N*-acetylglucosaminidases such as PNGase F. We recently demonstrated that temperature, pH, reaction time, and enzyme/protein ratio significantly affected the yield of released *N*-glycans by EndoBI-1.[16] In this work, we demonstrate that varying these conditions (pH, temperature, reaction time, and enzyme/protein ratio) affects the structural diversity of released *N*-glycans from bovine colostrum whey protein concentrate as determined by advanced mass spectrometry. This selective release of *N*-glycans from bovine colostrum whey glycoproteins allows for further study of their biological activity. Moreover, the possibility of producing select cohorts of free milk oligosaccharides, a high-value bioactive compound from colostrum whey, adds value to a commonly discarded milk product.

Materials and Methods

Bacteria and media

B. longum subsp. *infantis* ATCC 15697 used in this study was obtained from the University of California Davis Viti-culture and Enology Culture Collection (Davis, CA). *Bifido-bacterium* was grown in de Man–Rogose–Sharp (MRS) broth supplemented with 0.05% (w/v) L-cysteine (Sigma-Aldrich). The cells were grown anaerobically (5% H_2, 5% CO_2, 90% N_2, Coy Laboratory Products, Grass Lake, MI) at 37°C for 24 h. *Escherichia coli BL21** was used for protein expression and grown in Luria broth (LB) containing carbenicillin (100 μg/mL) in an Inova 4000 shaker (New Brunswick Scientific, New Jersey) at 200 rpm and 37°C.

Gene cloning, expression, and purification

A pEcoTM-T7-cHis, Eco cloning Kit (GeneTarget Inc, San Diego, CA, USA) was used for gene cloning in *Escherichia coli* DH5α strain (*E. coli*). Genomic DNA coding for EndoBI-1 in *B. infantis* ATCC 15697 was amplified using appropriate primers (Table S1). Signal peptide and transmembrane domains were not amplified to facilitate protein expression and

purification from *E. coli*. Gene amplification and protein expression in *E. coli BL21** strain were performed as described by Garrido et al.[16]

A single colony was used to inoculate a 20 mL LB containing carbenicillin at 100 μg/mL. Cells were incubated overnight at 37 °C with shaking at 200 rpm. Five hundred milliliters of LB with 100 μg/mL carbenicillin was inoculated with 1% of overnight culture and grown for 3 h at 37 °C and 200 rpm to reach a cell density of ~0.6 OD at 600 nm. Protein expression was induced by the addition of IPTG (Roche, San Francisco, CA, USA) to a final concentration of 0.5 mM and cells were incubated at 37 °C for 6 h. Cells were collected by centrifugation at 4,000 rpm for 15 min at 4 °C and the pellet was washed in phosphate buffered saline (pH 7.0). All subsequent steps for bacterial cell lysis were performed at 4 °C. The cell pellet was incubated in 100 mL of Bugbuster (Novagen, Billerica, MA, USA) for 10 min at 24 °C. Two hundred microliters of DNase I (Roche, San Francisco, CA, USA) and 100 μL of lysozyme (100 mg/mL) as well as a protease inhibitor cocktail (Roche, San Francisco, CA, USA) were added and the mixture placed on ice for 30 min. The lysed cells were centrifuged at 13,000 rpm (Ependorf rotor model F45-24-11) for 30 min to remove cell debris.

Expressed protein was purified by affinity chromatography using 5 mL prepacked Ni-charged columns (Bio-Rad, Hercules, CA, USA). All chromatographic steps were performed using EP-1 model Bio-Rad Econo Pump and model 2110 Bio-Rad fraction collector at 5 mL/min flow rate. The column was equilibrated with 25 mL of 300 mM KCl, 50 mM KH_2PO_4, and 5 mM imidazole buffer (pH 8). Fifty milliliters of sample were loaded into the column. The flow-through was collected and the column was washed with 30 mL of 300 mM KCl, 50 mM KH_2PO_4, 5 mM imidazole buffer (pH 8), and 300 mM KCl, 50 mM KH_2PO_4, and 10 mM imidazole buffer (pH 8). The bound protein was eluted with a step-wise gradient using imidazole concentrations ranging from 100 to 300 mM. The purity of EndoBI-1 fractions was evaluated by SDS-PAGE. Purified protein was concentrated using a 15 mL 30-kDa molecular weight cut-off centrifugal filter device (Amicon, Millipore, Billerica, MA, USA) and buffer was exchanged for saline sodium citrate 1× using Bio-Gel P-30 in SSC buffer columns (Bio-Rad). Protein concentration was determined by Qubit Protein Assay Kit (Life Technologies, Grand Island, NY, USA). The purified enzyme was kept at –80 °C.

Pilot-scale production of protein concentrate from bovine colostrum whey

Protein concentration from bovine colostrum whey was carried out in a pilot-scale tangential membrane system (Model L, GEA Filtration, Hudson, WI, USA). The system was composed of a 2.5″ diameter spiral membrane housing (1–2 m^2 area), a 95 L jacketed stainless-steel reactor, a Proline Promass 80 E flow-meter (Endress+Hauser, Reinach, Switzerland), a heat exchanger, and a 7.0 HP feed pump (Hydra-Cell $_{TM}$ Pump, model D10EKSGSNECF, Minneapolis, MN, USA).

After upstream lactose hydrolysis (0.1% lactase, 30 min, 40–43 °C), 74 L of bovine colostrum whey was ultrafiltered in single batch with a 10 kDa molecular weight cut-off polyethersulfone spiral-wound membrane (effective area of 1.86 m^2) up to a concentration factor of 5.4 (concentration factor = volume of feed/volume of retentate). Whey protein

concentration was performed at 40–43 °C, with a transmembrane pressure of 3.0 bar, and a recirculation flow rate of 10 L/min. After a concentration factor of 5.4 was achieved, the protein-rich retentate was diluted back to its original volume with water. Two discontinuous diafiltrations by volume reduction were performed to increase the removal of residual of simple sugars and free oligosaccharides from the ultrafiltration retentate. In this study, we used three different reaction condition combinations (Table 1), which produced varying yields of released glycans from concentrated bovine colostrum whey by EndoBI-1 in a previous study[16] and the reactions were terminated by 1 M Na_2CO_3.

Glycan purification

Samples were loaded in duplicates on a PGC SPE plate (Glygen, Columbia, MD, USA) that were conditioned using 3 × 100 μL of 80% ACN containing 0.1% TFA in water, followed by 3 × 100 μL of water. After sample loading, wells were washed using 6 × 200 μL of water and *N*-glycans were eluted using 3 × 200 μL of 40% ACN containing 0.1% TFA in water. The enriched *N*-glycans fractions were dried during overnight in vacum. Samples were rehydrated in 50 μL of water, vortexed, and sonicated prior to mass spectrometry analysis. For each condition tested, the same quantity of total carbohydrate was analyzed by mass spectrometry.

Nano-LC-Chip-Q-TOF MS

N-glycans were analyzed using the Agilent 6520 accurate-mass Q-TOF LC/MS with a microfluidic nano-electrospray chip (Agilent Technologies, Santa Clara, CA, USA). *N*-glycans were separated using an HPLC-chip with a 40-nL enrichment column and a 43-mm × 75-μm analytical column, both packed with 5 μm of porous graphitized carbon (PGC). The system was composed of a capillary and nanoflow pump, and both used binary solvents consisting of solvent A (3% ACN, 0.1% formic acid in water (v/v)) and solvent B (90% ACN, 0.1% formic acid in water (v/v)). Two microliters of sample were loaded with solvent A at a capillary pump flow rate of 4 μL/min. *N*-glycan separation was performed on a 65-min gradient delivered by the nanopump at a flow rate of 0.3 μL/min. The 65-min gradient followed this program: 0% B (0.0 – 2.5 min), 0–16% B (2.5 – 20.0 min), 16–44% B (20.0 – 30.0 min), 44–100% B (30.0 – 35.0 min), and 100% B (35.0 – 45.0 min). The gradient was followed by equilibration at 0% B (45.0–65.0 min). Data were acquired within the mass range of 450–3,000 *m/z* for *N*-glycans in the positive ionization mode with an acquisition rate of 2.01 spectra/s for *N*-gly-cans. An internal calibrant ion of 922.010 *m/z* from the tuning mix (ESI-TOF Tuning Mix G1969 – 85000, Agilent Technologies) was used for continual mass calibration. For tandem MS analysis, *N*-glycans were fragmented with nitrogen as the collision gas. Spectra were acquired within the mass range of 100–3,000 *m/z*. The collision energies correspond to voltages (*V*collision) that were based on the equation: $V_{\text{collision}} = m/z$ (1.5/100 Da) Volts—3.6 V; where the slope and offset of the voltages were set at (1.5/100 Da) and (–3.6) respectively. Acquisition was controlled by MassHunter Workstation Data Acquisition software (Agilent Technologies).

N-glycan identification

Compounds were identified with MassHunter Qualitative Analysis software (version B. 04.00 SP2, Agilent Technologies). Compounds were extracted using the Molecular Feature Extractor algorithm. The software generated extracted compound chromatograms (ECCs) in a range of 400–3,000 *m/z* with a ≥1,000 ion count cut-off, allowing charge states of +1–3, a retention time from 5–40 min, and a typical isotopic distribution of small biological molecules. The resulting compounds were matched to a bovine milk *N*-glycan library[17] using a mass error tolerance of 20 ppm. The *N*-glycans from the library were composed of hexose (Hex), HexNAc, Fuc, *N*-acetylneuraminic acid (NeuAc), and *N*-glycolylneuraminic acid (NeuGc). The relative abundance of *N*-glycans was performed by MassHunter Profinder software using Batch Targeted Feature Extraction algorithm. A database was built containing the molecular formula, the mass, and the retention time of identified *N*-glycans in samples. This library was used in combination with Batch Targeted Feature Extraction algorithm. A minimum abundance of 1,000 counts was used to filter out low-abundance compounds. Identified compounds were extracted allowing charge states of +1–3, mass error tolerance of 20 ppm, and retention time tolerance of 1 min. The relative amount of each *N*-glycan was calculated by to the total *N*-glycan area in each sample. The relative amount was expressed in percentage of the total. The assignment of *N*-glycans was confirmed by tandem mass spectrometry

Statistical analyses

Triplicates of each treatment (processing or biological replicates) were analyzed by using Analysis of Variance (ANOVA) with GLM models from the SAS system (version 8.2, SAS Institute, Inc., Cary, NC, USA). One-way ANOVA was used for evaluating the number of identified compositions of *N*-glycans, neutral *N*-glycans, sialylated *N*-glycans, and total possible compositions of *N*-glycans. Relative abundance of neutral and sialylated *N*-glycans were also evaluated by one-way ANOVA. Multiple comparisons of least-square means were made by Tukey's adjustment with the level of significance set at $P < 0.05$. Principal component analysis (PCA) was conducted in *R* via prcomp, to describe the different glycan structure populations released by each treatment and graphed using ggplot2 (H. Wickham. ggplot2: elegant graphics for data analysis. Springer New York, 2009).

Results

MS analysis of bovine milk N-glycans released by EndoBI-1

Endo-BI-1 cleaves between the two HexNAc residues of the *N*-glycan core, leaving a HexNAc attached to the glycosylated asparagine residue. To identify *N*-glycans, the neutral masses of each compound from the bovine milk *N*-glycan library were subtracted by the mass of one HexNAc residue. 10, 20, and 20 chemically distinct compositions were determined in the samples 1, 2, and 3, respectively (Table 1). These results suggest that the conditions tested for samples 2 and 3 (pH 5.3 or 6.15; incubation time of 245 or 360 min; temperature at 40 or 52.5 °8C; and enzyme/protein ratio of 1/500 or 1/1,000) allowed the release of a higher diversity of *N*-glycans than the conditions tested for sample 1 (pH 7, incubation time of 130 min, temperature at 65 °C, and enzyme/protein ratio of 1/500) (Table 1). Figure 1 shows the extracted compound chromatograms (ECCs) of released *N*-glycans

for each sample. The neutral complex/hybrid glycans were eluted first, followed by sialylated glycans eluted later as the concentration of ACN in the mobile phase increased, demonstrating that their interaction with the PGC was stronger than neutral complex/hybrid glycans, consistent with previous observations of elution patterns from PGC.[18]

Identification of bovine milk N-glycans released by EndoBI-1 by LC-MSMS

Tandem MS was performed to confirm *N*-glycan compositions. MS/MS analysis generated specific fragment ions that are common to all *N*-glycans, including 204.09 *m/z* [HexNAc +H]$^{+1}$ and 366.14 *m/z* [HexNAc-Hex+H]$^{+1}$. To facilitate data analysis, spectra were screened for the presence of these fragment ions (Figure 2). Spectra in Figure 2A represent the fragment pattern of a neutral *N*-glycan with 557.71 *m/z*, *z* = 2 and sialylated *N*-glycan with 784.28 *m/z*, *z* 5 2. The spectra show the presence of the fragment ions described above and other fragments specific to their structure. For example, MS/MS spectra for NeuAc-containing *N*-glycans includes fragments with 292.10 *m/z* [NeuAc+H]$^{+1}$ and 657.23 *m/z* [Hex-HexNAc-NeuAc+H]$^{+1}$ (Figure 2B). 18, 37, and 41 total compounds were identified in the samples 1, 2, and 3, respectively, resulting from the separation of structural and/or linkage isomers or anomers. These results demonstrate that a high diversity of *N*-glycans, including neutral and sialylated complex/hybrid *N*-glycans, were released from bovine colostrum whey protein concentrate using EndoBI-1. The identified compositions are described in Tables S2–S4 for each sample. A higher diversity of released *N*-glycans (statistically different at $P < 0.05$) was observed for Treatments 2 and 3 compared with Treatment 1. Although statistically different, the small variations of the number of released neutral *N*-glycans for Treatments 1, 2, and 3 are likely not of practical relevance. However, a higher number of sialylated structures were released for Treatments 2 and 3 (14 and 13) compared with Treatment 1 (Figure 3). The number of total compositions was also statistically higher for Treatments 2 and 3 (30 and 31) compared with Treatment 1 (18). Lower pH, longer incubation times, and higher enzyme/protein ratio of Treatments 2 and 3 likely favored the enzyme performance resulting in a higher number of identified compositions and sialylated structures for Treatments 2 and 3. Individual *N*-glycan compositions found in each treatment and their relative abundances were shown in Table 2. It was shown that the *N*-glycan diversity and their abundances were significantly different for each treatment. PCA identified these differences between Treatment 1 and Treatments 2 and 3 as the principle driver of differences between samples, explaining 99.21% of total variance in the first principal component, while the differences between Treatment 2 and 3 explained only 0.63% of total variance in the second principal component (Figure 4).

EndoBI-1 cleaves between the two HexNAc residues of the *N*-glycan core, leaving HexNAc residue attached to the glycosylated asparagine residue and an α1-6 fucose residue for the fucosylated core.[14] Thus, some *N*-glycan released by EndoBI-1 have two possible *N*-glycan compositions, one composition may have a core with 2HexNAc-3Hex while another may have a fucosylated core with 2HexNAc–3Hex–1Fuc. The spectra in Figure 2 show one neutral and one sialylated *N*-glycan composition that correspond to the neutral masses 1,113.42 and 1,566.56 Da, respectively. Two putative structures, one with 2HexNAc–3Hex core and another with 2Hex-NAc–3Hex–1Fuc core, are represented in Figure 2 for each *N*-glycan.

Effect of the physicochemical parameters on the released N-glycans

The effects of different combinations of processing conditions (pH, incubation time, temperature, and enzyme/protein ratio) on the release of *N*-glycans were evaluated. Relative abundance of neutral and sialylated *N*-glycans in samples was calculated based on the mass spectrometry analysis (Figure 3). Glycan peak areas were used to compare the amounts of neutral and sialylated *N*-glycans between the conditions tested. The relative abundance of each *N*-glycan type released by each treatment was statistically different at $P < 0.05$. While 87% and 76% of the total *N*-glycan abundances corresponded to sialylated glycans in samples 2 and 3, respectively, only 28% of the total *N*-glycan abundance in bovine milk corresponded to sialylated *N*-glycans in Sample 1. These results reveal that more sialylated *N*-glycans are released when enzymatic release is performed at pH 6.15 or 5.3, temperatures of 40 or 52.5 °C, enzyme ratio of 1/1,000 or 1/500 and incubation time of 245 or 360 min.

Discussion

In this study, we investigate how different reaction conditions affect the diversity of *N*-glycans released by EndoBI-1 from concentrated bovine colostrum whey glycoproteins. These released glycans are structurally similar to human milk oligosaccharides (HMO), which are known to promote the development of a beneficial infant intestinal microbiota, in particular, enriching for *Bifidobacterium*.[19,20] However, the production of these glycans is limited, preventing their use in both research and commercial applications.

PNGase F is the most commonly used enzyme to remove *N*-linked oligosaccharides from glycoproteins.[21] However, *N*-glycans containing a fucose α(1-3)-linked to the glycan core are resistant to this enzyme.[22] EndoBI-1 cleaves the *N*-*N*′-diacetyl chitobiose moiety found in the *N*-glycan core, even if fucosylated.[23] Nwosu et al.[17] identified 50 *N*-glycan compositions released from bovine milk using PNGase F. This previous work was performed on denatured proteins overnight, whereas this study was performed on native proteins in a shorter time (130–365 min). Milk glycoproteins exhibit a complex *N*-glycan structure that is resistant to commercial endoglycosidases under native conditions.[24] Additionally, steric hindrance can inhibit the activity of an enzyme, so denaturation or overnight incubation is needed to make these proteins less resistant to deglycosylation.[25] For these reasons, the majority of glycomics studies denature proteins by heating, by adding detergents or reducing agents, or a combination thereof. One of the objectives of this study was to develop a method that is scalable and compatible with current food processing to release *N*-glycans with high diversity and yet preserve the native protein state, and most importantly, the biological properties of milk. With this objective, denaturation of biological molecules from whey was not performed. Under native conditions common to current processing strategies, this study shows that EndoBI-1 is still able to release a variety of *N*-glycans, with one or two sialic acid and fucose residues decorating the glycan chains. Additionally, given EndoBI-1 is heat stable,[14] this enzyme could be used with high-temperature treatment, including pasteurization, to selectively release these glycans.

The selective release of *N*-glycans with structural similarity to human milk oligosaccharides (HMOs) provides an opportunity to examine the importance of these bioactive compounds to supporting the growth of key members of the gastrointestinal microbiome, such as

Bifidobacterium. Specific HMOs structures are known to be preferentially consumed by bifidobacteria.[26] Similarly, our recent findings on the consumption of released *N*-glycans shows that *B. infantis* preferentially consumes structurally similar classes of *N*-gly-cans. Thus, the selective production of a preferentially consumed bovine milk glycan provides an attractive alternative to HMOs. Our results here suggest that this is possible by altering production conditions using EndoBI-1 and whey protein as a substrate.

In addition to their bioactive role in stimulating the growth of *Bifidobacterium*, previous studies have shown that sialylated glycans protect against rotavirus infection, which is one cause of infant diarrhea.[27] Other studies suggest that sialic acid is an essential monosaccharide for brain development and learning.[28] They reduce the binding of leukocyte to endothelial cells while the neutral glycans do not have any effect.[29] Recombinant human lactoferrins, expressed in *Pichia pistoris*, with or without sialylated glycan structures provided evidence that *N*-acetylneuraminic acid is involved in the immune function of these lactoferrins.[30] Recent *in vitro* studies revealed that the glycan part of human lactoferrin decreases pathogen infections, including *Salmonella*, of cultured human intestinal epithelial cells.[31]

Statistical analysis shows that *N*-glycan production can be manipulated in terms of diversity or relative abundance. Although, Treatments 2 and 3 enabled the release of similar *N*-glycan structures, they differ significantly in terms of the relative abundance of each *N*-glycan type produced. For example, 14 and 13 sialylated *N*-glycans can be produced by Treatment 2 and Treatment 3, respectively. However, because Treatment 2 releases less neutral *N*-glycans compared to Treatment 3 in terms of relative abundance, it is possible to produce a product dominated by sialylated *N*-glycan by using Treatment 2 conditions with fewer neutral *N*-glycans. Moreover, the statistical analysis of the individual *N*-glycans shows that Treatment 1 releases only nonfucosylated glycans. Therefore, it is also possible to produce fucose-free *N*-glycans by using Treatment 1.

Due to the accessibility limitations to human milk in large scale, these released *N*-glycans might be considered as new class of bioactive molecules, both structurally and compositionally analogous to HMOs, and potential alternatives. Because different types of *N*-glycans have different functions, successfully manipulation of *N*-glycan released by EndoBI-1 from concentrated bovine colostrum whey is important to investigate their biological activity in detail.

Moreover, using a waste product, whey, as a substrate for *N*-glycan production will provide entirely new opportunity for its use in dairy industry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work has been supported by the UC Davis RISE program, the Bill and Melinda Gates Foundation, National Institutes of Health awards R01AT008759, R01AT007079, and the Peter J. Shields Endowed Chair in Dairy Food

Science. SK is supported the Education Ministry of the Republic of Turkey. SAF is supported by a National Institutes of Health Ruth Kirschstein National Research Service Award, F32AT008533.

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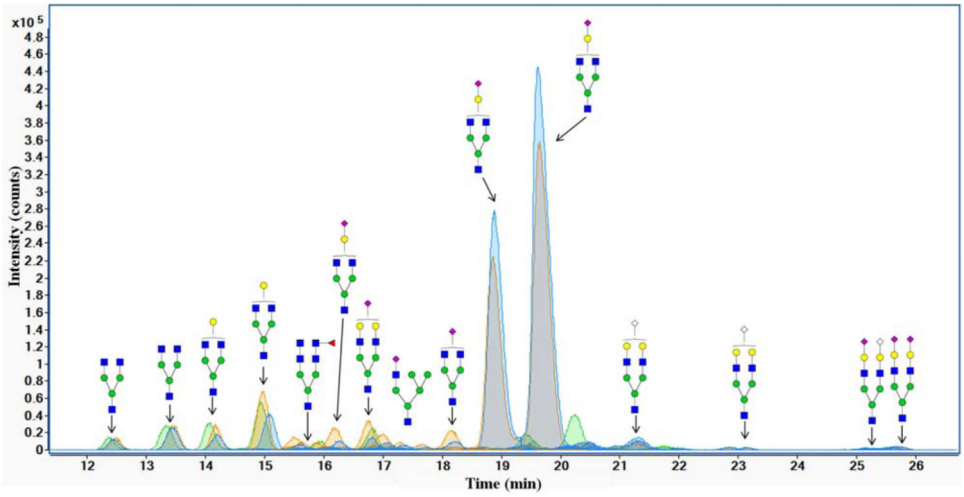

Figure 1.

Extracted compound chromatograms (ECCs) of *N*-glycans from bovine colostrum whey concentrate. The samples are differentiated by color: 1 is green, 2 is blue, and 3 is orange. Endo-β-*N*-acetylglucosaminidase was incubated with bovine colostrum whey protein concentrate under different conditions. Sample 1: pH 7, 130 min, 65 °C, enzyme/protein ratio 1/500; Sample 2: pH 6.15, 245 min, 52.5 °C, enzyme/protein ratio 1/1,000; Sample 3: pH 5.30, 360 min, 40 °C, enzyme/protein ratio 1/500. °-glycans were purified by solid-phase extraction and analyzed by Nano-LC-Chip-Q-TOF MS.

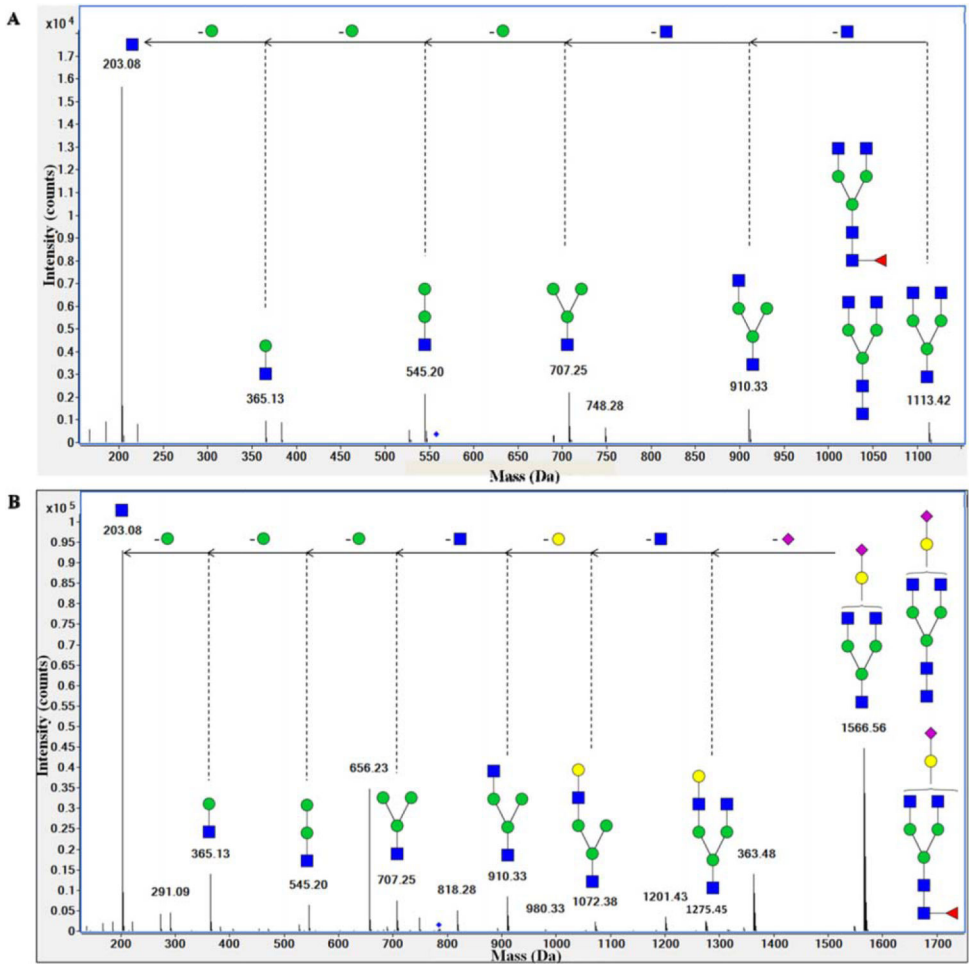

Figure 2.

MS/MS spectra of neutral and sialylated *N*-glycans. (A) Deconvoluted tandem spectrum of the neutral *N*-glycan 3Hex-3HexNAc from bovine milk glycoproteins. This glycan corresponded to 557.71 *m/z* with *z* = +2. (B) Deconvoluted tandem spectrum of the sialylated *N*-glycan 4Hex-3HexNAc-1NeuAc from bovine milk glycoproteins. This glycan corresponded to 784.28 *m/z* with *z* = +2. Green circles, yellow circles, blue squares, red triangles, purple diamonds, and gray diamonds represent mannose, galactose, HexNAc, fuc, NeuAc, and NeuGc residues, respectively.

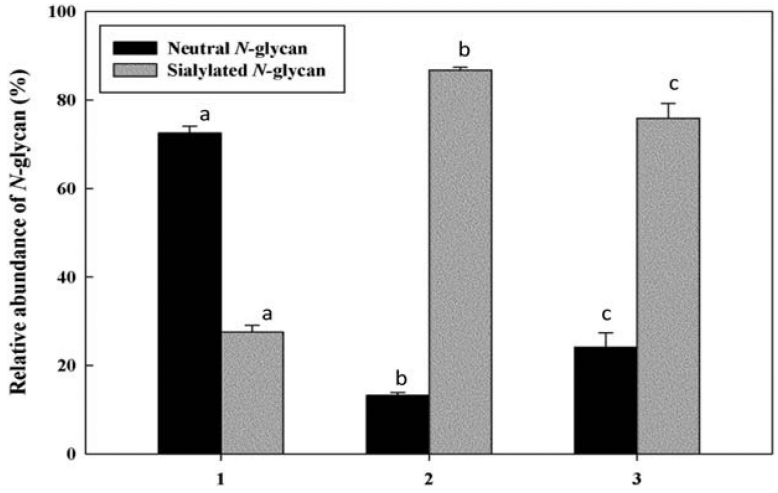

Figure 3.

Relative abundance of neutral and sialylated *N*-glycans in sample 1, sample 2 and sample 3. Endo-β-*N*-acetylglucosaminidase was incubated with bovine colostrum whey protein concentrate under different conditions. Sample 1: pH 7, 130 min, 65 8C, enzyme/protein ratio 1/500; Sample 2: pH 6.15, 245 min, 52.5 °C, enzyme/protein ratio 1/1,000; Sample 3: pH 5.30, 360 min, 40 °C, enzyme/protein ratio 1/500. *N*-glycans were purified by solid-phase extraction and analyzed by Nano-LC-Chip-Q-TOF MS. ^{A,B,C} Means (three biological replicates) within the same class (neutral or sialylated) followed by different superscripts are statistically different at $P < 0.05$.

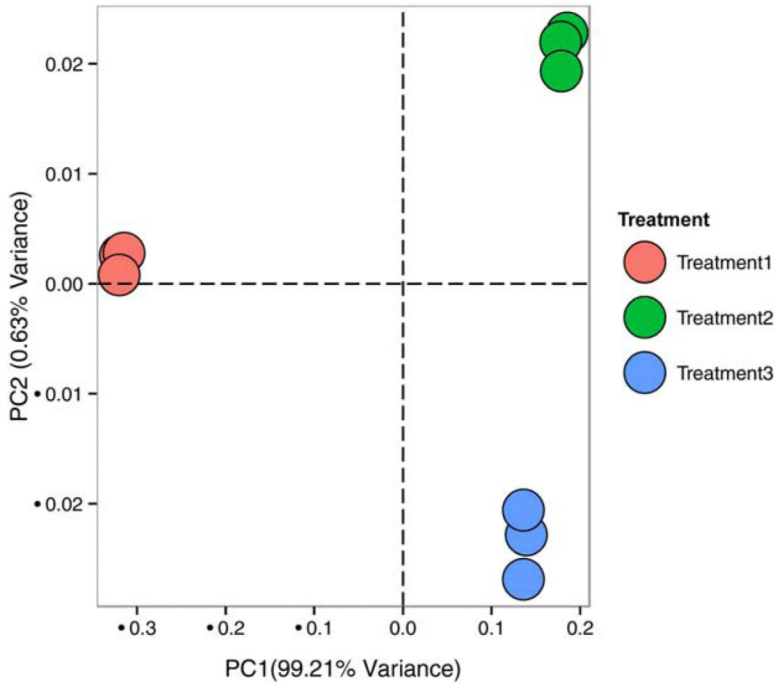

Figure 4.

Treatment conditions shape populations of released *N*-glycans. PCA plot of composition of released *N*-glycans shows differences between Treatment 1 and Treatments 2 and 3. Nearly all variance between samples is explained in the *x*-axis differentiating Treatment 1 from Treatments 2 and 3 (PC1; 99.21%), which is primarily the release of sialylated *N*-glycans in Treatments 2 and 3. Differences between Treatment 2 and 3 are observed on the *y*-axis and are comparatively small (PC2, 0.63%).

Table 1

N-Glycans Identification from Bovine Colostrum Whey Concentrate Released by Endo-B-*N*-Acetylglucosaminidase

pH	Time (min)	Temperature (°C)	Enzyme/ Protein Ratio	Identified Compositions	Neutral *N*-Glycans	Sialylated *N*-Glycans	Total Possible Compositions
7	130	65	1/1,500	10 ± 0^{A}	7 ± 0^{A}	3 ± 0^{A}	18 ± 0^{A}
6.15	245	52.5	1/1,000	20 ± 0^{B}	6 ± 0^{B}	14 ± 0^{B}	30 ± 0^{B}
5.3	360	40	1/500	20 ± 0^{B}	7 ± 0^{A}	13 ± 0^{C}	31 ± 0^{C}

^{A}Means (three biological replicates), within the same column, followed by different superscripts are statistically different at $P < 0.05$.

^{B}Means (three biological replicates), within the same column, followed by different superscripts are statistically different at $P < 0.05$.

^{C}Means (three biological replicates), within the same column, followed by different superscripts are statistically different at $P < 0.05$.

Table 2

Individual *N*-Glycan Structures Found in Each Treatment

	Composition					% Relative Abundance		
Neutral Mass	**Hex**	**HexNAc**	**Fuc**	**NeuAc**	**NeuGc**	**Treatment 1**	**Treatment 2**	**Treatment 3**
1,113.42	3	3	0	0	0	46.84% ± 0.50%^{A}	13.75% ± 0.17%^{B}	15.86% ± 0.25%^{C}
1,275.47	4	3	0	0	0	7.77% ± 0.44%^{A}	2.71% ± 0.07%^{B}	3.64% ± 0.16%^{C}
1,437.51	5	3	0	0	0	6.22% ± 041%^{A}	2.33% ± 0.07%^{B}	1.66% ± 0.13%^{C}
1,478.54	4	4	0	0	0	6.29% ± 0.32%^{A}	0.00%^{B}	2.90% ± 0.19%^{C}
1,519.54	3	5	0	0	0	9.27% ± 0.53%^{A}	3.11% ± 0.11%^{B}	3.86% ± 0.22%^{C}
1,599.57	6	3	0	0	0	4.72% ± 0.25%^{A}	1.53% ± 0.09%^{B}	1.97% ± 0.09%^{C}
1,665.62	3	5	1	0	0	0.00%^{A}	3.88% ± 0.37%^{B}	4.83% ± 0.28%^{C}
1,404.51	3	3	0	1	0	0.00%^{A}	3.10% ± 0.33%^{B}	2.98% ± 0.18%^{B}
1,525.53	5	2	0	1	0	0.00%^{A}	1.46% ± 0.10%^{B}	1.15% ± 0.07%^{C}
1,566.56	4	3	0	1	0	6.97% ± 0.12%^{A}	41.16% ± 0.46%^{B}	37.66% ± 0.58%^{C}
1,687.59	6	2	0	1	0	0.00%^{A}	1.39% ± 0.15%^{B}	1.52% ± 0.09%^{C}
1,728.61	5	3	0	1	0	5.11% ± 0.50%^{A}	3.68% ± 0.14%^{B}	4.55% ± 0.18%^{C}
1,744.64	5	3	0	0	1	2.10% ± 0.20%^{A}	4.21% ± 0.28%^{B}	3.06% ± 0.24%^{C}
1,769.64	4	4	0	1	0	0.00%^{A}	1.46% ± 0.03%^{B}	1.84% ± 0.04%^{C}
1,810.66	3	5	0	1	0	0.00%^{A}	1.97% ± 0.18%^{B}	2.37% ± 0.10%^{C}
1,874.67	5	3	1	1	0	0.00%^{A}	2.35% ± 0.25%^{B}	1.94% ± 0.10%^{C}
1,890.67	5	3	1	0	1	0.00%^{A}	2.69% ± 0.11%^{B}	1.80% ± 0.18%^{C}
1,931.7	5	4	0	1	0	0.00%^{A}	1.71% ± 0.08%^{B}	1.83% ± 0.08%^{B}
2,019.74	5	3	0	2	0	0.00%^{A}	2.09% ± 0.16%^{B}	2.27% ± 0.03%^{B}
2,035.73	5	3	0	1	1	0.00%^{A}	2.71% ± 0.05%^{B}	2.31% ± 0.09%^{C}
1,640.6	5	4	0	0	0	4.73% ± 0.33%	0.00%^{B}	0.00%^{B}
2,051.73	5	3	0	0	2	0.00%^{A}	2.71% ± 0.20%^{B}	0.00%^{A}

Shown are the neutral mass, charge (*z*), *m/z*, monosaccharide composition, and glycan type.

Hex, Hexose; HexNAc, *N*-acetylglucosamine; Fuc, Fucose; NeuAc, *N*-acetylneuraminic acid, NeuGc; *N*-glycolylneuraminic acid.

^{A}Means (three biological replicates), within the same row, followed by different superscripts are statistically different at $P < 0.05$.

^{B}Means (three biological replicates), within the same row, followed by different superscripts are statistically different at $P < 0.05$.

^{C}Means (three biological replicates), within the same row, followed by different superscripts are statistically different at $P < 0.05$.