

**Selective Prebiotic Activity of *N*-glycans Released From Milk
Glycoproteins by Novel Endo- β -*N*-acetylglucosaminidase**

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Abstract

Human milk is the primary source of nutrition for newborns before they are able to digest any other food. Interestingly, some glycosylated milk components are completely indigestible to the infant, and only select gut bacteria possess the enzymes to release and utilize such compounds. Glycans are important biological components involved in cell-to-cell interaction, signaling, structural integrity of cells, and proper protein formation. Glycans are found linked to other compounds (lipids and proteins) as well as in a free form. Studies on glycosylated proteins indicate that glycans can affect many biological and nutritional properties. However, the challenges in isolating glycans from glycoproteins have so far limited detailed investigations of these compounds. Glycosidases are widely used for *N*-glycan release from glycoproteins. The efficient use of glycosidases is essential to scale up the release of these bioactive compounds from protein-rich dairy streams. We recently isolated a novel *N*-glycosidase that cleaves the *N*-*N'*-diacetyl chitobiose moiety found in the *N*-glycan core of a wide variety of proteins. Sophisticated analytical platforms are enabling food scientists to develop detailed molecular-level understanding of functional compounds in milk and other foods. Mass spectrometry is the preferred technique for glycans analysis as the high mass accuracy improves the ease of identification of compounds based on mass to charge ratio. By determining the microstructures

of these compounds, one can determine specific activities associated with the free oligosaccharides as well as the glycoconjugates. Membrane filtration can then be used to scaled-up production and purification of the released glycans. With all these technologies in place, is possible to assess and identify which foods and food streams yield the highest concentrations of functional glycans to obtain the necessary amounts for functional assays and clinical trials. Successful application of the novel enzymes in the dairy industry will allow the conversion of whey, a waste product, into a substrate for selective modulation of the gut microbiota, mimicking what happens in the gut of a healthy breast-fed infant.

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TABLE OF CONTENTS

CHAPTER I. Food digestion: what milk can teach us from the infant's perspective 1

1. Introduction.....	2
2. Bovine whey as a glycan source	10
2.1 Purification.....	10
2.2 Substrate denaturation.....	11
3. Deglycosylation strategies	12
3.1 Chemically deglycosylation.....	12
3.2 Enzymatically deglycosylation	13
4. Glycan purification, quantification and characterization.....	15
4.1 Glycan purification	15
4.2 Glycan quantification.....	15
4.3 Characterization of glycans by advanced mass spectrophotometry	16
5. Determination of kinetic parameters.....	18
5.1 Basis of Michaelis-Menten Kinetics.....	18
5.2 Determination of the initial rates	18
5.3 Determination of the kinetic constants: K_m and v_{max}	19
6. Conclusion	20
References.....	22

Chapter II. Characterizing the release of bioactive N-glycans from dairy products by a novel endo- β -N-acetylglucosaminidase 28

Summary.....	29
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1. Introduction.....	30
2. Materials and methods	33
2.1 Bacteria, media and substrates	33
2.2 <i>N</i> -glycan release of RNase B:	33
2.3 Experimental Design and Statistical Analysis	34
2.4 <i>N</i> -glycan release comparison of EndoBI-1 and PNGase F on denatured/native substrates and pasteurization conditions	36
2.5 Glycan quantification.....	36
2.6 Glycan purification	37
2.7 <i>N</i> -glycan analysis by Nano-LC-Chip-Q-TOF MS	37
3. Results.....	39
3.1 Production of EndoBI-1 and activity of the enzyme on RNase B.	39
3.3 Optimization of the release of <i>N</i> -glycans from bovine colostrum whey by EndoBI-1	41
3.3 Statistical Analysis.....	41
3.4 <i>N</i> -glycan release comparison of EndoBI-1 and PNGase F on denatured/native substrates and pasteurization conditions	47
3.5 <i>N</i> -glycan analysis by Nano-LC-Chip-Q-TOF MS	47
4. Discussion	53
Acknowledgements.....	56
References	57
Chapter III. A novel endo-β-N-acetylglucosaminidase releases specific N-glycans depending on different reaction conditions.....	61
Summary	62

1. Introduction.....	63
2. Materials and methods	65
2.1 Bacteria and media.....	65
2.2 Gene cloning, expression and purification.....	65
2.3 Pilot-scale production of protein concentrate from bovine colostrum whey	67
2.4 Glycan purification	67
2.5 Nano-LC-Chip-Q-TOF MS	68
2.6 <i>N</i> -glycan identification.....	69
2.7 Statistical Analyses	70
3. Results.....	70
3.1 MS analysis of bovine milk <i>N</i> -glycans released by EndoBI-1	70
3.2 Identification of bovine milk <i>N</i> -glycans released by EndoBI-1 by LC-MSMS	75
3.3 Effect of the physico-chemical parameters on the released <i>N</i> -glycans.....	79
4. Discussion.....	82
Supplemental information.....	85
Acknowledgements.....	91
References.....	92
Chapter IV. Kinetic characterization of a novel endo-β-N-acetylglucosaminidase on concentrated bovine colostrum whey to release bioactive glycans.....	94
Summary	Error! Bookmark not defined.
1. Introduction.....	96
2. Materials Methods	98
2.1 Gene Cloning, expression and purification.....	98

2.2 Substrates	99
2.3 Glycoprotein digestion by EndoBI-1 and glycan quantification	100
2.4 Determination of kinetic constants	100
2.5 Glycan purification for Nano-LC-Chip-Q-TOF MS.....	101
2.6 Nano-LC-Chip-Q-TOF Mass spectrometry analysis	101
2.7 <i>N</i> -glycan identification.....	102
3. Results.....	103
3.1 EndoBI-1 production and confirmation of its activity on RNase B.....	103
3.2 Determination of K_m and V_{max} by non-linear and linearized plotting.....	105
3.3 <i>N</i> -Glycan structures	111
4. Discussion	114
Acknowledgements.....	117
References.....	118
Chapter V. Selective prebiotic activity of N-glycans released from milk glycoproteins by novel endo-β-N-acetylglucosaminidase	121
Summary	Error! Bookmark not defined.
1. Introduction.....	123
2. Materials and Methods.....	126
2.1 Bacteria and media.....	126
2.2 Gene cloning, protein expression and purification	127
2.3 Gene Expression	128
2.4 Pilot-scale concentration of bovine milk glycoproteins.....	129
2.5 <i>N</i> -glycan release.....	130

2.6 Glycan quantification	131
2.7 Bacterial fermentation of <i>N</i> -glycans.....	131
2.8 Glycan purification for Nano-LC-Chip-Q-TOF MS	132
2.9 Nano-LC-Chip-Q-TOF MS	132
2.10 <i>N</i> -glycan identification	133
3. Results.....	135
3.1 Release and purification of <i>N</i> -glycans from concentrated whey proteins	135
3.2 Growth of <i>B. infantis</i> and <i>B. lactis</i> on whey, deglycosylated whey proteins, and <i>N</i> -glycans.....	137
3.3 Glycoprofiling of <i>N</i> -glycans consumption by <i>B. infantis</i> and <i>B. lactis</i>	140
4. Discussion	145
Acknowledgements.....	147
Supplemental data.....	148
References.....	149
Chapter VI. Conclusions and Future Directions	153
1. Introduction.....	Error! Bookmark not defined.
2. EndoBI-1 is an effective tool for deglycosylation	155
3. Bovine whey is a good source of glycans.....	156
4. Specific <i>N</i> -glycan production by EndoBI-1	156
5. <i>B. infantis</i> utilizes released <i>N</i> -glycans	157
6. Future Directions	158
References	159

Chapter I

Food digestion: what milk can teach us from the infant's perspective

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Abbreviations: N-glycans: N-linked glycans; O-glycans: O-linked glycans; GlcNAc: N-acetylgalactosamine; AsN: Asparagine; Ser: Serine; Thr: Threonine; PNGases: Peptidyl-N-glycosidases; TFA: trifluoromethanesulfonic acid; EndoBI-1: endo- β -N-acetylglucosaminidase; MALDI: Matrix-assisted laser desorption ionization.

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1.Introduction

Mammalian milks have evolved for more than 200 million years under the constant Darwinian selective pressure to be nourishing and protective. To date, scientists have yet to fully identify all of the biomolecules of human milk. Breastfed infants have reduced incidence of diseases such as diarrhea and eczema compared to infant formula fed infants [1, 2]. One striking property of milk being recognized now is the extent of glycosylation of its proteins, which is common to human and bovine milk and may be responsible for the beneficial effects observed. Glycosylation is a post-translational modification where sugar residues are added after the protein is synthesized. Importantly, while the milk glycans are indigestible to the host, they have been shown to promote the growth of beneficial intestinal bacteria thus further protecting the infant from deleterious bacteria and stimulating a favorable intestinal environment [3].

Glycans are found as free or attached to the other structures such as proteins, lipids or peptides in nature. Free glycans (oligosaccharides) in milk are composed of a lactose core (galactose and glucose units) and extended by N-acetylglucosamine, sialic acids and fucoses. Glycans are also linked to proteins via O- or N- glycosidic bonds. The appearance of each type of glycan in glycoproteins can be exclusive but both types can co-exist on the same protein. O-linked glycans (O-glycans) are mostly attached to the proteins through N-acetylgalactosamine to a hydroxyl group of a serine or threonine residue, and can be modified into various core classes [4]. N-linked glycans (N-glycans) are linked via N-acetylglucosamines (HexNAc) to an asparagine residue of proteins in the particular amino acid sequence AsN-X-Ser/Thr (Table 1) [4]. Glycans have various physico-chemical and biological functions such as signaling, structural integrity, cell-to-cell interaction and proper protein formation [5, 6].

N-glycans have a single common core composed of 2 N-acetylglucosamines (GlcNAc) followed by three mannoses. Further glycosylation of the mannoses determines the types of N-glycans. The presence of only mannose residues yields high mannose-type oligosaccharides. The core mannoses may be extended by other monosaccharides such as N-acetylglucosamine, fucose, galactose and sialic acid to produce hybrid or complex types. O-glycans have a higher number of core structures with at least eight known. O-glycans are linked to either serine or threonine through an N-acetylgalactosamine without a consensus sequence. Free oligosaccharides in milk are made of a lactose core decorated by N-acetylglucosamine, fucose, glucose, galactose and sialic acid and are more structurally similar to *O*-glycans than to *N*-glycans.

While the backbone of milk proteins has been extensively elucidated via proteomics studies, the roles of glycosylation in contributing to protein bioactivities have largely been ignored. For example, it is well known that the milk protein lactoferrin is highly glycosylated; while intact lactoferrin has been shown to induce bacterial growth; the role played by lactoferrin's *N*-glycans on the biological activity has been typically neglected [7].

Glycosylation protects the peptide backbone by covering enzymatic cleavage sites and making it more resistant to proteases during digestion. Glycosidases are enzymes widely used for *N*-glycan release from glycoproteins. The efficient use of glycosidases with broad activity is essential to scale up the release of these bioactive compounds from protein-rich dairy streams. Common highly *N*- and/or *O*- glycosylated proteins found in nature are lactoferrins, immunoglobulins, caseins, mucin and fetuin [8-10]. Glycan diversity of these proteins and glycosylation sites have been widely investigated [11-13]. Some proteins such as lactoferrins show dissimilar *N*-glycan patterns among mammals, but their functions are not well elucidated due to the limitations of large-scale production of these glycans [14, 15].

Enzymes are biological catalysts that speed up biochemical reactions. An important focus in enzyme research is the determination of kinetic parameters, which are described by the analysis of the rate of enzymatic reactions. Kinetic characterization of an enzyme is an essential step of understanding biological processes in a living organism, and in development of novel bioactive compounds [16, 17]. The first leap in the history of enzyme kinetics was taken by Victor Henri [18], who derived an equation to relate the rate of sucrose inversion to the sucrose (substrate) concentration: a model involving the formation of an enzyme-substrate complex. Later, Henri's model was significantly improved by Leonor Michaelis and Maud Leonora Menten [19], which constructed the very foundation of the famous Michaelis-Menten equation. These scientists measured the initial rate of sucrose inversion at variable sucrose concentrations and fit their data to theory by assuming enzyme-substrate binding is in equilibrium and the rate of the overall reaction was proportional to the concentration of the enzyme-substrate complex. The later alteration of the Michaelis-Menten model came from Briggs and Haldane [20], who instead of equilibrium assumption, stated that the rate of change of the enzyme-substrate complex concentration is negligible as compared to rate of change in the substrate or product concentration (also known as the “quasi steady-state assumption”). With the advent of this new derivation the Michaelis-Menten equation which relates the initial rate of an enzymatic reaction to the substrate concentration is given by $v = \frac{v_{max}[S]}{K_m + [S]}$, where v is the initial rate, $[S]$ is the substrate concentration and K_m and v_{max} are named the Michaelis-Menten constant and the maximum reaction rate, respectively. Determination of these parameters and the adaptation of Michaelis-Menten equation in various experimental set ups has been a robust way to understand enzymatic reactions and characterize novel enzymes [21-24]. There are various methods to determine the parameters of the Michaelis-Menten equation based on linearization of the

equation and non-linear methods [25, 26]. In the present review, we describe the methods based on linearization of the Michaelis-Menten equation. The methods described above have both advantages and disadvantages due to the errors coming from the measurement of v and $[S]$, which will be described later.



Whey, a by-product of cheese making process, is a promising glycan source in terms of its availability in large-scale and the wide variety of glycoprotein diversity contained therein. Whey is the liquid part of milk that separates from the curd following acid or enzymatic coagulation, and has traditionally been considered a by-product of the cheese industry. During the last three decades, whey production has grown enormously and will continue to grow along with cheese production. Ultrafiltration of whey has become an economic and efficient means to recover a valuable product—whey protein concentrate—from whey. Bovine whey is composed primarily of the proteins Beta-lactoglobulin and alpha-lactalbumin. In addition to these highly abundant proteins, whey also contains several glycoproteins, including lactoferrin, lactoperoxidase, and lactophorin, all of which present interesting biological activities. For example, there is increasing evidence that both human and bovine lactoferrin have antimicrobial, antiviral, anti-inflammatory, antioxidative, anticancer, and immunomodulating activities while lactoperoxidase has a bacteriostatic effect. We recently reported glycan purification from whey at the pilot scale [23, 27, 28].

The release of glycan structures from glycoproteins can also be achieved using various chemical methods. Hydrazinolysis, beta-elimination, and trifluoromethanesulfonic acid are commonly used chemical methods for deglycosylation that result in total destruction of the proteins [29-31]. Although these methods are faster and cheaper compared to enzymatic methods, the chemicals and required high temperature used for the deglycosylation limit the high quality glycan production. Enzymes such as Peptidyl-*N*-glycosidases (PNGases) are commonly used for the release of *N*-glycans from glycoproteins for analytical studies [32, 33]. They cleave the glycans from the asparagine residue by hydrolyzing the amide side chain. However, glycans that have α -linked fucose to *N*-acetylglucosamine are resistant to PNGases [33].

Importantly, we recently isolated a novel N-glycosidase that cleaves the *N*-*N'*-diacetyl chitobiose moiety found in the *N*-glycan core including fucosylated glycans. Release of O-glycans enzymatically is limited compared to N-glycans due to the complexity of O-glycan cores, lack of commercially available O-glycosidases and their incomplete activity [33, 34]. Therefore more research is needed to identify O-glycanases with broader cleaving activity.

There is a great interest in the biological functions of glycans [35-37]. However, the investigations are limited due to their low abundance in glycoprotein substrates and the lack of the enzymatic deglycosylation methods with broad activity. Therefore, the determination of kinetic parameters of deglycosylation process is critical. Deglycoylsation of proteins involves various challenging steps that hinder accurate kinetic parameter determination of deglycosidases. The overall kinetic characterization of a glycosidase enzyme is shown in Figure 2.

```
graph TD; A[Step 1: Substrate preparation] --> B[Step 2: Deglycosylation]; B --> C[Step 3: Purification, quantification and characterization]; C --> D[Step 4: Determination of kinetic parameters: K_m and V_max]; D --> E[Linear Plots:]; D --> F[Nonlinear Plotting:];
```

Step 1: Substrate preparation

- Removal of free oligosaccharides
- Denaturation

Step 2: Deglycosylation

Enzymatic Deglycosylation Chemical Deglycosylation

Step 3: Purification, quantification and characterization

Step 4: Determination of kinetic parameters: K_m and V_{max}

- Plot $[P]$ vs time for various $[S]$
- Determine initial reaction rate
- Plot v vs $[S]$

Linear Plots:

- *Lineweaver-Burk Plot:*
 $1/v$ vs $[S]$, y intercept: V_{max} , x intercept: $-1/K_m$
- *Eadie-Hofstee Plot:*
 v vs $v/[S]$, y intercept: V_{max} , slope: K_m
- *Hanes and Woolf Plot:*
 $[S]/v$ vs $[S]$, y intercept: K_m/V_{max} , slope: $1/V_{max}$

Nonlinear Plotting:
 v vs $[S]$ is directly plotted.

Figure

2. Bovine whey as a glycan source

Milk and milk products are rich in glycoproteins. Bovine whey, a by-product of cheese-making process, is an especially promising glycan source due to its high glycoprotein content (~4.5 g glycoproteins/ L whey) [38]. However, the determination of kinetic parameters of a glycosidase on complex substrates like whey is very challenging. Bovine whey contains free oligosaccharides that are structurally similar to conjugated glycans. These oligosaccharides might result in the overestimation of kinetic parameters if they are not removed prior to the enzymatic reaction. Moreover, the presence of free glycans hinders the characterization of released glycan due to the similarity in monosaccharidic composition. Denaturation of substrates for deglycosylation is also an essential step for enzymatic release of glycans from glycoproteins; however, when studying functional activities denaturation is highly undesirable as it may mask/compromise some tests. Interestingly, our recently isolated endo- β -N-acetylglucosaminidase shows higher activity compared to other glycosidases even in absence of denaturation [39].

2.1 Purification

The preparation of glycoprotein substrates for enzymatic release of glycans plays an important role in the accurate determination of kinetic parameters, especially for complex substrates such as whole milk or whey that contains several glyco-forms: conjugated glycans and free oligosaccharides [40, 41]. These oligosaccharides have similar monosaccharidic composition and molecular weight (1-3kDa) as conjugated glycans; this hinders the determination of kinetic parameters and the characterization of only the released glycans [42].

Therefore, all the free oligosaccharides should be removed from the substrates to obtain accurate kinetic parameters.

Purification of released glycans (and separation from free glycans) can be achieved by membrane filtration or ethanol precipitation techniques [39, 43]. The selection of appropriate membranes is based on the molecular size differences between free glycans and intact glycoproteins. The molecular weight of glycoproteins varies between 12-13 kDa to 200 kDa, while the molecular weight of free oligosaccharides is smaller than 3 kDa [44]. Therefore, membranes with molecular weight cut off of 10 kDa are widely used to separate free glycans from complex substrates [45].

2.2 Substrate denaturation

The denaturation of substrates is an essential step prior to the release of conjugated glycans from glycoproteins by glycosidases [32, 46]. The secondary and tertiary structure of the polypeptide chain prevents the glycan accessibility of the glycosidases [47]. Therefore, heat denaturation ($\sim 95^{\circ}\text{C}$) for 5 min is widely used prior to the enzymatic digestion of glycoproteins [48, 49]. The reagent dithiothreitol is also frequently combined with the heat treatment to reduce the sulfide bonds of proteins [50]. Despite the necessity of the denaturation of glycoproteins for commercial enzymes' activity, the high heat treatment and the detergents used for the denaturation result in the degradation of released N-glycans that hinders further investigation of the biological functions of these bioactive compounds. To this effect, we recently isolated a novel endo- β -N-acetylglucosaminidase (EndoBI-1) from an infant gut microbe, *Bifidobacterium longum* subsp. *Infantis*, that is active on native milk proteins [27]. We compared the activity of

the commercial enzyme PNGase F and our EndoBI-1 (cloned at the lab) and we demonstrated a comparable activity on ribonuclease B (a simple glycoprotein). Interestingly, when using a real substrate such as bovine whey and the milk protein lactoferrin, our EndoBI-1 enzyme yielded significantly more *N*-glycans than PNGase F [27].

3. Deglycosylation strategies

3.1 Chemically deglycosylation

Chemical methods are commonly used for deglycosylation of glycoproteins due to their easy application, low cost, and wide substrate specificity [51]. In addition, lack of specific enzymes for O-glycan cleavage leads to the further requirement for chemical deglycosylation [52, 53]. Commonly used chemical deglycosylation methods, especially for O-glycan cleavage, are β -elimination and hydrazination [54]. β -elimination is based on the liberation of glycans via exposing the glycoprotein to alkaline conditions. The resulting free glycans might be degraded by subsequent β -elimination reactions (peeling action), which can be avoided by addition of reducing agents such as sodium borohydride [52, 53, 55]. The disadvantage of this method is that oligosaccharides have a single group for labeling that is converted to alditols by the reductive agent, which limits the ability to attach a fluorophore or a chromophore. Consequently, it becomes challenging to monitor glycan release [52, 53]. β -elimination also leads to sample losses during the cleaning of high salt content [53]. In hydrazine treatment, the hydrolysis reaction is initiated by the addition of anhydrous hydrazine to a salt free and lyophilized glycoprotein. This method is advantageous in that it allows controlled release of both N- and O-glycans, by adjusting the reaction conditions such as temperature [56]. The higher potential of hydrazine treatment has been indicated by Turyan et al.[53], who showed that hydrazinolysis is

more efficient than ammonium based β -elimination to release O-glycans in terms of activity and selectivity. Interestingly, combinations of both of these methods have also been demonstrated as efficient techniques for release of O-glycans from pig gastric mucin [57]. In addition to β -elimination and hydrazine treatment, anhydrous forms of HF and TMFS have also been employed for chemical deglycosylation [46, 51, 58].

3.2 Enzymatic deglycosylation

Enzymatic deglycosylation of glycoproteins is mostly achieved by Peptidyl-*N*-glycosidases (PNGases) [24]. A PNGase (peptide- N^4 - (N-acetyl- β -glucosaminyl), glycopeptidase, N-glycanase, glycoaminidase or N-glycosidase) removes asparagine-linked glycans by hydrolyzing the amide side chain [59, 60]. Although commercially available PNGases release *N*-linked glycans irrespective of their size or charge [61-63], if fucose is α 1-6-linked to *N*-acetylglucosamine, the *N*-glycans are resistant to hydrolysis by PNGase F [64]. We recently showed that an endo- β -*N*-acetylglucosaminidase (EndoBI-1) isolated from *Bifidobacterium longum* subsp. *Infantis* ATCC 15697 has the ability to cleave the *N*-*N'*-diacetyl chitobiose moiety found in the *N*-glycan core of high mannose, hybrid and complex *N*-glycans and is not affected by fucosylation of the *N*-glycan core [65]. Endoglycosidases F1, F2 and F3 show more activity on native protein proteins compared to PNGase F, but they have a limited activity on the tri- and tetra-antennary glycans. The various glycosidases and their activity on *N*-glycans are shown in Figure 3.

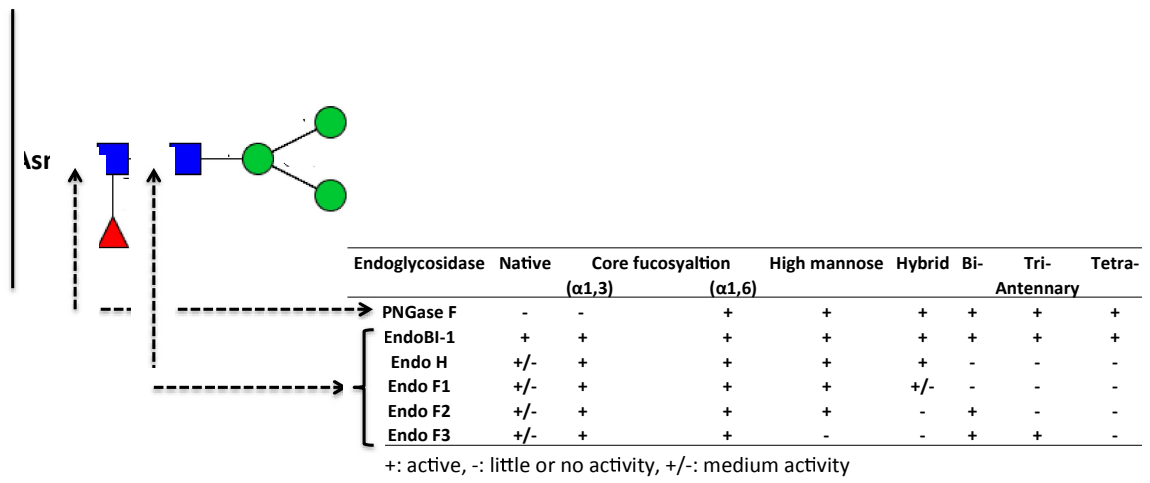


Figure 3. Endoglycosidases and their specificity on *N*-glycans.

4. Glycan purification, quantification and characterization

4.1 Glycan purification

After enzymatic release of glycans from glycoproteins, the released glycans need to be purified from the complex mixture to ensure accurate results for the quantification. This step is also essential for the analysis of these released glycans by mass spectrophotometer in order to reveal the number of isomeric forms and monosaccharide composition. Various techniques have been developed for the isolation of glycans from mixtures including precipitation, paper chromatography, gel filtration and solid-phase extraction (SPE) [66-69]. Among these methods PGC (Porous Graphitic Carbon) SPE is a widely used approach for glycan purification. We also showed that application of ethanol precipitation prior to PGC SPE improved the glycan recovery from concentrated bovine whey colostrum [39].

4.2 Glycan quantification

Quantification of *N*-glycans after their release is an important step in the kinetic and structural characterization of a glycosidase enzyme. Mass spectrometry has been employed in relative or absolute quantification of glycans despite various challenges [70]. One problem in this type of analysis is that sialic acid can be lost due to the decay of the ion-source. However, adaption of ion sources such as intermediate pressure MALDI are now being used to minimize this decay [71]. Moreover, permethylation has also been used to stabilize sialic acids [72, 73]. For absolute quantification, internal standards are commonly used since they promote the accuracy and linearity of quantitation [70, 74]. Isotope labeled 2-aminopyridine has been reported as an internal standard yielding high sensitivity [74-76]. Alternatively, Orlando et al. [77] used ^{15}N as a means of stable isotope labeling, although this method is only suitable for cell

cultures [70]. In addition to MS, simpler approaches such as quantifying the total carbohydrate are possible. We demonstrated the use of UV spectrophotometry with a colorimetric reaction to aid the kinetic and structural characterization of released high-mannanose *N*-glycans. Despite the advantages of this rapid and economical method, the complex structure of glycans hinders the selection of the appropriate monosaccharides for the preparation of standard curves. Although mannose can be considered an appropriate standard monosaccharide for high mannose glycans, it might also be representative enough for use with other types of glycans.

4.3 Characterization of glycans by advanced mass spectrophotometry

Glycans are structurally diverse and very challenging to analyze. Mass spectrometry is the *de riguer* technique for glycans analysis as the high mass accuracy improves the ease of identification of compounds based on mass-charge ratio. Mass spectrometric platforms are capable of detecting more compounds in a single run than other traditional analytical tools and enable detection of low-abundance molecules at sub-femtomole concentrations. The development of desorption/ionization techniques like matrix-assisted laser desorption ionization (MALDI) and electrospray (ESI), has improved carbohydrates research using mass spectrometry. When the mass of the intact molecule alone is not enough to achieve unambiguous identification, another technique, called collision-induced dissociation (CID), is used to obtain information about glycan composition. In CID, all unknown ions are sequentially isolated and fragmented so that just by observing characteristic patterns of glycan fragmentation one can identify the composition of unknown/novel glycans. Mass spectrometry also provides a sensitive technique to assist in the determination of the structure of carbohydrates, but it is essential that the samples are pure to achieve maximum ionization. Indeed, without separation, all types of molecules

would compete for ionization. Molecules with low ionization efficiency (e.g. carbohydrate) would not be detected as their signals would be suppressed by other molecules that are easily ionized (e.g. peptides or proteins present in the same sample).

Novel instruments such as the nano liquid chromatography Chip Quadrupole Time of Flight MS, have a built-in ability to pre-separate molecules over time based on their affinity to a stationary phase inserted in a Chip. By separating molecules over time with chromatography, fewer molecules are introduced to the mass spectrometer at any one time, decreasing ion suppression and increasing the number of possible compounds detected. This time of liquid chromatography is paired in-line with mass spectrometry via nano-electrospray ionization. This nanoflow chromatography system employs less sample and solvent than normal liquid chromatography and can be packed with a variety of solid phases allowing separation of peptides, glycans, lipids and other classes of molecules just by changing the type of chip, like in a “plug and play” device. Graphitized carbon columns can separate glycans down to the structural isomers level and thus enable structure-specific glycomics for N-glycans, O-glycans and free oligosaccharides. Because of the more reproducible retention times achieved with the nanoLC system, novel bioinformatics libraries based on accurate mass, tandem spectra and retention time can be assembled to aid in the automated identification of known molecules in any chromatogram. These libraries, when integrated with software for data analysis, can also provide each compound's abundance significantly speeding up data analysis time.

5. Determination of kinetic parameters

5.1 Basis of Michaelis-Menten Kinetics

Here, we present a list of steps on how to elucidate the kinetic characteristics of an enzyme via aforementioned methods based on our studies with a novel endo- β -N-acetylglucosaminidase (EndoBI-1) [27]. According to the derivation of the Michaelis-Menten equation introduced by Briggs and Haldane ($v = \frac{v_{max}[S]}{[S]+K_m}$, where $K_m = \frac{k_{-1}+k_2}{k_1}$), the enzyme and substrate form a complex, which is followed by the release of product and the free enzyme molecule [20, 79, 80]. K_m and v_{max} are the parameters used to characterize the kinetic behavior of an enzyme. v_{max} is the theoretical maximum reaction rate when the enzyme is completely saturated by the substrate. K_m represents the substrate concentration at half of the theoretical maximum velocity. The key assumption of this approach is that the change in the concentration of enzyme substrate complex is negligible at the beginning of the reaction, also known as the quasi steady-state assumption as the substrate concentration is much greater than the enzyme concentration ($[S] \gg [E]$) [19, 20, 27, 79]. Thus, the initial rate of the reaction used in Michaelis-Menten equation is valid as long as this assumption holds.

5.2 Determination of the initial rates

In kinetic characterization of an enzyme, initial product formation rate is plotted against the substrate concentrations that is later used to determine K_m and v_{max} [19, 20, 81, 82]. To achieve this, the first step is to plot the change of glycan (product of deglycosylation) concentration versus time for a range of initial substrate concentrations. The range of substrate concentrations should be selected such that the time at which enzyme saturation begins can be identified, where $[S] \gg [E]$ and the quasi steady-state assumption holds [20]. The initial reaction

rate is calculated using the slope until this point [27]. Considering this, selection of substrate concentration range greatly depends on the characteristics of the enzyme under study. For example, N-glycosidase A and F have been shown to have very diverse activities at the same substrate concentrations [24]. As a result, it is possible to see different substrate concentration ranges used for enzymes with similar functions in the literature. Karav et al. [27] demonstrated that a substrate range of 0.1 to 0.8 mg/mL was required to be able to identify the point where enzyme saturation starts for EndoBI-1. On the other hand, Mussar [83] has used 2-6 mg substrate for 3 μ L enzyme preparation of peptide N-glycosidase F to deglycosylate octoglycopeptide derived from ovomucoid.

5.3 Determination of the kinetic constants: K_m and v_{max}

After the initial reaction rates are determined, they can be plotted against the initial substrate concentrations either directly or by linearizing to determine K_m and v_{max} . Here, we focus on linearization techniques. One of the most common linearization techniques is the double reciprocal method proposed by Lineweaver and Burk [81]. The reciprocal of both sides of Michaelis-Menten equation is taken resulting in $\frac{1}{v} = \frac{1}{v_{max}} + \frac{K_m}{v_{max}} \frac{1}{[S]}$. Then, $1/v$ versus $1/[S]$ is plotted, which can be applied to a linear fit. The y-intercept of the resulting line plot gives $1/v_{max}$ while the x-intercept of the line on the second quadrant is $-1/K_m$.

Another technique to linearize the Michaelis-Menten equation was introduced by Eadie-Hoftsee [82, 84]. The linearized version of the equation becomes $v = v_{max} - K_m \frac{v}{[S]}$. When v is plotted against $v/[S]$ and the data is subjected to a linear fit y- intercept represents v_{max} whereas the slope of the linear fit gives $-K_m$. Finally, Hanes and Woolf [85, 86] proposed the linear form

of the Michaelis-Menten equation as $\frac{[S]}{v} = \frac{K_m}{v_{max}} + \frac{1}{v_{max}}[S]$. According to this method, the linear fit of the plot of $[S]/v$ versus $[S]$ has the y-intercept of K_m/v_{max} and slope of $1/v_{max}$.

The methods described above have both advantages and disadvantages due to the errors coming from the measurement of v and $[S]$. In the Eadie-Hofstee plot, v is seen in both coordinates. This introduces the same amount of error in x and y coordinates, inevitably leading to correlation that might be misinterpreted [87]. However, Eadie-Hofstee also allows detection of deviations from the Michaelis-Menten model due to having error in both coordinates [87]. On the other hand, Lineweaver-Burk and Hanes plots may lead to high deviations in $1/v$ when there are small errors in v [87] (or small errors in S). Jericevic & Kuster [26] and Dowd [87] also pointed out that the data transformation via these methods does not always support the assumption of linear regression. Nonetheless, as compared to non-linear determination of Michaelis-Menten constants, linearization methods are more practical as they are easier to handle as non-linear optimization depends on good initial guesses for parameter values and accurate balancing of parameters [26]. Furthermore, it is more challenging to realize the differences of experimental data from the model in a non-linear fit [87]. According to Tummler et al. [79] it is also problematic to accurately detect the asymptotes when v vs $[S]$ is not linearized. Considering these differences, we have tested all the linearization methods explained above to ascertain the kinetic parameters of EndoBI-1 [27].

6. Conclusion

Glycans have various biological functions such as the protection against pathogens and supporting immune system [88-90]. Because they are structurally similar to human milk oligosaccharides (HMO), that are known to promote the development of beneficial infant

intestinal microbiota by enriching for *Bifidobacterium* [91, 92], there is great interest in obtaining large amounts of pure glycans to develop novel prebiotics and symbiotic products. Currently, despite their important beneficial effects, glycans are not yet commercially available in large-scale. The limitation in deglycosylation techniques and the lack of substrates hinder the production these bioactive compounds in larger scale. In this work, we demonstrate that the combination of the novel enzyme EndoBI-1 and bovine whey as a substrate can yield large-scale glycan production.

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Chapter II

Characterizing the release of bioactive N-glycans from dairy products by a novel endo- β -N-acetylglucosaminidase

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Summary

Endo- β -*N*-acetylglucosaminidase isolated from *B. infantis* ATCC 15697 (EndoBI-1) is a novel enzyme that cleaves *N*-*N*'-diacetyl chitobiose moieties found in the *N*-glycan core of high mannose, hybrid and complex *N*-glycans. These conjugated *N*-glycans are recently shown as a new prebiotic source that stimulates the growth of a key infant gut microbe, *Bifidobacterium longum* subsp. *Infantis*. The effects of pH (4.45-8.45), temperature (27.5-77.5 °C), reaction time (15-475 min), and enzyme/protein ratio (1:3000-1:333) were evaluated on the release of *N*-glycans from bovine colostrum whey by EndoBI-1. A central composite design was used, including a two-level factorial design (2⁴) with four center points and eight axial points. In general, low pH values, longer reaction times, higher enzyme/protein ratio and temperatures around 52 °C resulted in the highest yield. The results demonstrated that bovine colostrum whey, considered to be a by/waste product, can be used as a glycan source with a yield of 20 mg *N*-glycan/ g total protein under optimal conditions for the ranges investigated. Importantly, these processing conditions are suitable to be incorporated into routine dairy processing activities, opening the door for an entire new class of products (released bio-active glycans and glycan-free milk). The new enzyme's activity was also compared with a commercially available enzyme: showing that EndoBI-1 is more active on native proteins than PNGase F and can be efficiently used during pasteurization, streamlining its integration into existing processing strategies.

1. Introduction

Milk contains a variety of components, including proteins, peptides, lipids, carbohydrates and minerals that contribute to the growth and development of newborns. Whey proteins represent 18-20% of total milk protein and have high nutritive and functional properties for the young [93]. During biosynthesis in the mammary epithelial cell, some milk proteins are decorated with long carbohydrate chains (glycoproteins). Glycoproteins contain a variety of oligosaccharides (glycans) covalently attached to the polypeptide side chains [94]. The most abundant bovine milk glycoproteins are lactoferrin, immunoglobulins and α -lactalbumin [95]. These proteins have interesting biological activities including antioxidant, antibacterial and antiviral action, as well as immunomodulatory activity [96-99]. These functions are supposedly modulated by both the polypeptide chain and the glycans. However, the function of glycoproteins' *N*-glycans is not well elucidated. They may have similar functions to those described for free milk oligosaccharides from human milk, including inhibition of pathogen adhesion to intestinal epithelial cells and promoting the growth of beneficial commensal microbes in the infant intestine [100-103]. In fact, we have recently shown that a key infant gut microbe, *Bifidobacterium longum* subsp. *infantis*, is able to consume these conjugated *N*-glycans as a sole carbon source while other less important microbes do not.

Glycans are 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. *N*-linked glycans (*N*-glycans) are linked, via *N*-acetylglucosamines (HexNAc), to an asparagine residue of proteins in the specific amino acid sequence AsN-X-Ser/Thr (where X can be any amino acid except proline). The *N*-glycan core is composed of two HexNAc and three mannose residues. This core is elongated by other

monosaccharides, including fucose and sialic acid, via the actions of glycosyltransferases and glycosidases, which determine the degree of branching and the type of linkage [104]. *N*-glycans are divided into three main classes: high mannose, complex and hybrid.

Whey, a by-product of cheese production from bovine milk, is an attractive source of milk compounds with interesting biological properties. It contains 93-94% water, 4.5-5% lactose, 0.8-1% total protein, 0.5-0.7% minerals and others [105]. Whey accounts for 90% of the initial milk volume processed, which means that approximately 9 liters of whey are produced from 10 liters of bovine milk during cheese-making. Whey worldwide production is estimated to be 190,000,000 ton/year [106, 107]. The underutilization of the organic components in whey means that only 50% of the whey produced is used in food applications or for animal feed. The unused waste stream causes serious economic and environmental problems, as cheese processors have to dispose of this highly organic wastewater stream. In addition to containing proteins with high functionality, whey is also a potential source of *N*-glycans, with concentrations of ~4.5 g glycoproteins/L [38]. Therefore, the recovery of whey bioactive compounds is an opportunity for the development of new high-value products for the dairy industry while reducing the industry's environmental pollution.

Hydrazinolysis and alkali/reducing conditions (β -elimination) are commonly used techniques for deglycosylation of glycoproteins [108, 109]. However, the chemicals used for glycan release may result in the degradation of glycoproteins and are potentially hazardous [110-112]. There are also commercially available enzymes (endoglycosidases, glycosamidases) that can be used for *N*-glycan release [110]. However, commercial enzymes are affected by fucosylation of the *N*-glycan core resulting in limited glycan release. For example, peptidyl-*N*-glycosidase F (PNGase F), one the most commonly used enzymes [113], catalyzes the cleavage

between HexNAc and asparagine residues, but it is not able to cleave *N*-glycans from glycoproteins when an α 1-3 fucose residue is linked to the core HexNAc [114]. Additionally, the release of *N*-glycans at large-scale has been impaired by the price of commercial enzymes. We previously showed that a novel Endo- β -*N*-acetylglucosaminidase isolated from *B. infantis* ATCC 15697 cleaves the *N*-*N*'-diacetyl chitobiose moiety found in the *N*-glycan core of high mannose, hybrid and complex *N*-glycans. Its activity is not affected by a fucosylated *N*-glycan core, and it was also shown to be heat resistant which enables its use during heat treatments at 95°C [115, 116], a common practice is milk processing.

Improving the release of *N*-glycans is an essential step to enable the large-scale production of *N*-glycans. This will also enable a better understanding of their biological properties and source of new bioactive ingredients. In the present work, the effects of pH, temperature, reaction time, and enzyme/protein ratio on the total release of *N*-glycans by EndoBI-1 were evaluated. In order to identify the optimal combination of pH, reaction time, temperature, and enzyme/protein ratio within the most likely processing range, a central composite rotatable design was employed including a four-factor, two-level full factorial design (2^4) along with four center points and eight axial points. Because it was previously shown that EndoBI-1 is a heat stable enzyme [65], we investigated how efficiently it can be applied within pasteurization (62 °C, 30 min) so that no additional heating step would be required for deglycosylation. We also demonstrated how denaturation of substrates affected *N*-glycan release by EndoBI-1 and commercial enzyme PNGase F.

2. Materials and methods

2.1 Bacteria, media and substrates

The *B. longum subsp. Infantis* ATCC 15697 strain used in this study was obtained from the University of California Davis Viticulture and Enology Culture Collection (Davis, CA). Bifidobacterium was grown in Man-Rogose-Sharp (MRS) broth supplemented with 0.05% (w/v) L- cysteine (Sigma-Aldrich). The cells were anaerobically grown in a vinyl chamber (Coy Laboratory Products, Grass Lake, MI) at 37 °C for 24 h, in an atmosphere consisting of 5% carbon dioxide, 5% hydrogen, and 90% nitrogen. The *E.coli* Bl21* strain used for protein expression was grown in Luria-Broth (LB) media containing Carbenicillin (100µg /ml). All incubations were carried out in an Innova 4000 shaker (New Brunswick Scientific, New Jersey) at 200 rpm and 37 °C. Bovine lactoferrin and RNase B were obtained from Sigma-Aldrich (St. Louis, MO, USA). Gene cloning, expression and purification and pilot-scale production of protein concentrate from bovine colostrum whey were performed as described before [49].

2.2 N-glycan release of RNase B:

20 µg of Ribonuclease B (RNase B) (Sigma-Aldrich, San Diego, CA, USA) was reconstituted in 20 µL of 0.2 M Na₂HPO₄ (pH 5) and 200 µg of EndoBI-1 were added to the sample. PNGase F (New England BioLabs, Ipswich, MA, USA) was used as a positive control. Two microliters of 500,000 units/mL PNGase F were added to 20 µg of RNase B reconstituted in 20 µL of commercial denaturing buffer (New England BioLabs). The two mixtures were incubated 2h at 37°C. The enzyme activity was analyzed by SDS-PAGE on 4–15% precast polyacrylamide gels.

2.3 Experimental Design and Statistical Analysis

In order to find the optimal combination of pH, reaction time, temperature and enzyme/protein ratio, a complete 2^4 factorial design of the central rotational type was established, with four central points and eight axial points, based on Response Surface Methodology [27]. The effects of pH (4.45-8.45), temperature (27.5-77.5°C), reaction time (15-475 min), and enzyme/protein ratio (1:3000-1:333) on the release of *N*-glycans from bovine colostrum whey by EndoBI-1 were tested. The independent variables (pH, reaction time, temperature and enzyme/protein ratio) were evaluated according to coded levels ($-\alpha$, -1 , 0 , $+1$, $+\alpha$). Center points are located at the average of levels -1 and $+1$ for each factor, while axial points were determined by interpolation ($\alpha = \pm 2.0$) for each factor. Coded and uncoded levels and their corresponding independent variables are shown in Table 1. The dependent variable (i.e., evaluated response) was the release of *N*-glycans from bovine colostrum whey by EndoBI-1. Data were analyzed by Statistica version 8.0 software. The significance of the model was tested by Analysis of Variance (ANOVA).

Table 1. Experimental design for optimizing the release of *N*-glycans from bovine colostrum whey by EndoBI-1

Treatments	Coded Levels				Uncoded Levels				
	pH (X ₁)	Time (min) (X ₂)	Temperature (°C) (X ₃)	Enzyme/protein ratio (X ₄)	pH (X ₁)	Time (min) (X ₂)	Temperature (°C) (X ₃)	Enzyme/protein ratio (X ₄)	Glycans release (µg)
1	+1	+1	+1	+1	7.00	360	65.0	1:500	9.35
2	+1	+1	-1	-1	7.00	360	40.0	1:1500	3.85
3	+1	+1	-1	+1	7.00	360	40.0	1:500	9.85
4	+1	-1	-1	-1	7.00	130	40.0	1:1500	3.97
5	-1	-1	-1	-1	5.30	130	40.0	1:1500	12.87
6	-1	-1	-1	+1	5.30	130	40.0	1:500	23.33
7	-1	-1	+1	+1	5.30	130	65.0	1:500	17.62
8	-1	+1	+1	+1	5.30	360	65.0	1:500	25.84
9	+1	-1	+1	-1	7.00	130	65.0	1:1500	3.15
10	-1	+1	-1	+1	5.30	360	40.0	1:500	54.75
11	+1	-1	+1	+1	7.00	130	65.0	1:500	3.98
12	+1	+1	+1	-1	7.00	360	65.0	1:1500	5.50
13	+1	-1	-1	+1	7.00	130	40.0	1:500	5.60
14	-1	+1	-1	-1	5.30	360	40.0	1:1500	24.60
15	-1	-1	+1	-1	5.30	130	65.0	1:1500	8.47
16	-1	+1	+1	-1	5.30	360	65.0	1:1500	13.32
17	0	0	0	0	6.15	245	52.5	1:1000	27.47
18	0	0	0	0	6.15	245	52.5	1:1000	27.29
19	0	0	0	0	6.15	245	52.5	1:1000	27.36
20	0	0	0	0	6.15	245	52.5	1:1000	27.48
21	+2	0	0	0	7.85	245	52.5	1:1000	4.78
22	-2	0	0	0	4.45	245	52.5	1:1000	36.13
23	0	+2	0	0	6.15	475	52.5	1:1000	38.32
24	0	-2	0	0	6.15	15	52.5	1:1000	4.03
25	0	0	+2	0	6.15	245	77.5	1:1000	5.64
26	0	0	-2	0	6.15	245	27.5	1:1000	8.43
27	0	0	0	+2	6.15	245	52.5	1:333	43.49
28	0	0	0	-2	6.15	245	52.5	1:3000	6.43

Complete 2^4 factorial design parameters, with four independent variables in two levels, four repetitions in the central point and eight in the axial points

For each experiment, 450 μ l of concentrated whey protein were diluted in 50 μ l of 0.2 mM Na_2HPO_4 . Each experiment was performed in duplicate. Five mL of cold pure ethanol were added into the samples to stop the reactions, precipitate proteins and collect the released glycans. Samples were dried overnight by vacuum centrifugation. Samples were rehydrated in 100 μ L of water.

2.4 *N*-glycan release comparison of EndoBI-1 and PNGase F on denatured/native substrates and pasteurization conditions

500 μ l concentrated bovine colostrum whey, bovine lactoferrin (5 mg/ml) or RNase B (1 mg/ml) were used to examine how the denaturation state of substrates affects the deglycosylation efficiency of glycoproteins by EndoBI-1 and PNGase F. Samples were denatured at 95 $^{\circ}\text{C}$ for 5 min in a 10 mM final concentration of DTT. 250 μ g EndoBI-1 and PNGase F were added into samples and enzymatic digestions were performed under the conditions described before by Garrido et al. [65] and incubated for 8 hours.

The *N*-glycan release comparison of EndoBI-1 and PNGase F on concentrated bovine colostrum whey under pasteurization conditions (62 $^{\circ}\text{C}$, 30 min) were also investigated. 250 μ g of enzyme was added into 500 μ L of whey when the temperature reached the 62 $^{\circ}\text{C}$ and incubated for 30 min. All the reactions were stopped with 1 M Na_2CO_3 . Total released *N*-glycans by each enzyme were calculated after glycan purification.

2.5 Glycan quantification

A total carbohydrate Assay Kit, (Biovision Milpitas, CA, USA), was used to quantify released *N*-glycans. To create a standard curve, 0, 2, 4, 6, 8 and 10 μ l of mannose standard (2 mg/mL) were added into a series of wells in a 96-well microplate (supplemental Figure S1). This

generated 0, 4, 8, 12, 16 and 20 μg /well of mannose standard. The volume of each sample was adjusted to 30 μl per well with water. 30 μl of sample was used. 150 μl of concentrated sulfuric acid (98%) were added to each well. Samples were mixed on a shaker for ~ 1 min and then incubated at 85°C for 15 min. After incubation, 30 μl of Developer (provided by the manufacturer) was added to each well. Samples were again mixed on the shaker for 5 min. OD of each sample was measured at 490 nm. Sample OD was applied to the mannose standard curve linear function to calculate the quantity of carbohydrate in the sample.

2.6 Glycan purification

Samples 9,10 and 17 were loaded on PGC SPE plates (Glygen, Columbia, MD, USA) that were conditioned using 3 x 100 μL of 80% ACN (acetonitrile) containing 0.1% TFA in water, followed by 3 x 100 μL of water. After sample loading, wells were washed using 6 x 200 μL of water and *N*-glycans were eluted using 3 x 200 μL of 40% ACN containing 0.1% TFA (trifluoroacetic acid) in water. The enriched *N*-glycan fractions were dried overnight under vacuum. 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.

2.7 *N*-glycan analysis by Nano-LC-Chip-Q-TOF MS

N-glycans were analyzed using an 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 x 75- μm analytical column, both packed with 5 μm 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 μ L/min. *N*-glycan separation was performed on a 65-min gradient delivered by the nanopump at a flow rate of 0.3 μ L/min. The 65-min gradient followed this program: 0% B (0.0–2.5 min), 0 to 16% B (2.5–20.0 min), 16 to 44% B (20.0–30.0 min), 44 to 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 450–3000 *m/z* for *N*-glycans in the positive ionization mode with an acquisition rate of 2.01 spectra/s for *N*-glycans. 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 100–3000 *m/z*. The collision energies correspond to voltages (*V*_{collision}) that were based on the equation: *V*_{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, Santa Clara, CA, USA).

Released glycans 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 ≥ 1000 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 [117] 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).

3. Results

3.1 Production of EndoBI-1 and activity of the enzyme on RNase B.

The production of poly-histidine-tagged EndoBI-1 was achieved with a yield of 1.7 mg/L using LB media under optimum induction conditions. The activity of recombinant EndoBI-1 was tested on RNase B, an *N*-glycosylated protein of 17 kDa that contains high mannose *N*-linked glycans [118], and compared to commercial enzyme, PNGase F (Figure 1). Cleavage of RNase B by an endoglycosidase results in a 14 kDa deglycosylated protein. This experiment was performed under non-denaturing conditions. After two hours of incubation with the enzymes, the band corresponding to glycosylated RNase B disappeared. The protein was detected at lower molecular weight in presence of EndoBI-1 and PNGase F. The bands observed in lane 2 and 3 corresponded to the deglycosylated RNase B. These data indicate that the recombinant EndoBI-1 was as efficient as PNGase F in releasing *N*-glycans.

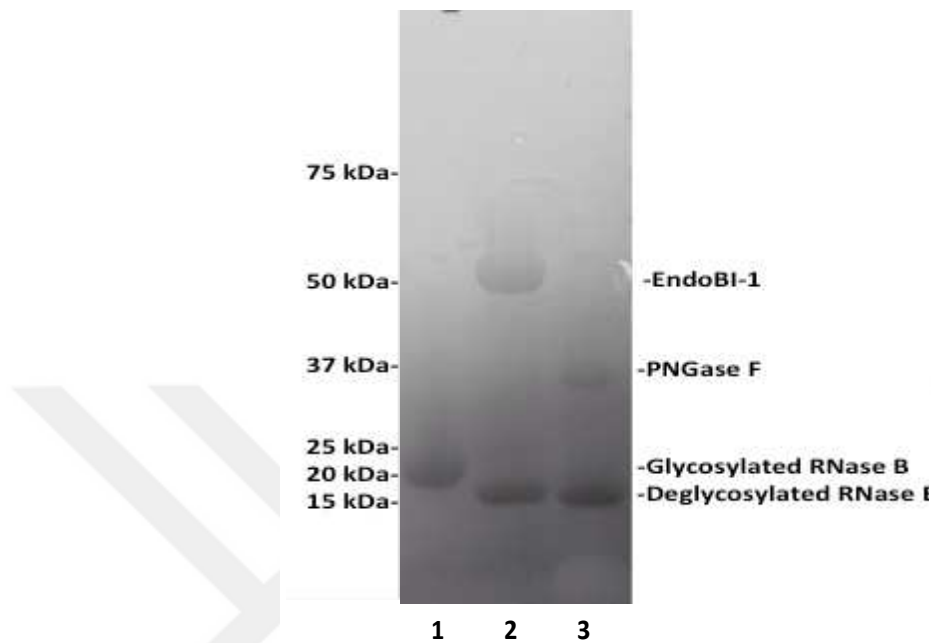


Figure 1. Enzymatic deglycosylation of RNase B by Endo- β -*N*-acetylglucosaminidase and PNGase F on 12% SDS-PAGE gel. Lane 1: glycosylated RNase B (17kDa). Lane 2: deglycosylated RNase B by Endo- β -*N*-acetylglucosaminidase (47kDa). Lane 3: deglycosylated RNase B by PNGase F (36kDa).

3.3 Optimization of the release of *N*-glycans from bovine colostrum whey by EndoBI-1

In Table 1, the effects of pH, incubation time, temperature, and enzyme/protein ratio on the release of *N*-glycans from bovine colostrum whey protein concentrate are presented. In general, deglycosylation activity was reduced by higher pH values regardless of the other operation parameters evaluated. The lowest values of *N*-glycan release (3.97 – 9.35 µg of *N*-glycans) were observed at pH 7.0 and 7.85, in agreement with the qualitative results presented by Garrido et al. (2012). The highest *N*-glycan release (54.7 µg) was observed at pH 5.3, the longer incubation time (360 min), a temperature of 40 °C, and an enzyme/protein ratio of 1:500. Enzymatic activity reduction was observed when temperature was increased from 40°C to 65°C. *N*-glycan release was reduced from 24.6 to 13.2 µg when temperature increased from 40°C to 65°C, respectively (Treatments 14 and 16).

3.3 Statistical Analysis

The estimated regression model and coefficient of determination (R^2) for total release of *N*-glycans from bovine colostrum whey by EndoBI-1 was determined as described in Table 2. According to the model, higher glycan release will happen at lower pH values ($-2 = 4.45$); longer reaction time ($+2 = 475$ min); temperature in the center point ($0 = 52.5$ °C) and higher enzyme/protein ratio ($+2 = 1:333$). Only parameters significant at $p < 0.05$ were used in the regression model. The coefficient of determination (R^2) of the predictive model for release of *N*-glycans from bovine colostrum whey by EndoBI-1 was 0.74, indicating that the regression model was able to explain 74% of the total variation between the observed values for release of *N*-glycans from bovine colostrum whey by EndoBI-1.

Table 2. Estimated regression model and coefficient of determination for the release of *N*-glycans from bovine colostrum whey by EndoBI-1

Estimated Regression Model*	R ²
Estimated release of <i>N</i>-glycans from whey by <i>EndoBI-1</i> (μg) $= 21.4 - 8.3 X_1 + 5.7 X_2 - 4.8 X_3^2 + 6.2 X_4$	0.74
X₁ = coded level corresponding to pH; X₂ = coded level corresponding to time; X₃= coded level corresponding to temperature; X₄= coded level corresponding to enzyme/protein ratio. * Only parameters with a confidence level above 95% (<i>P</i> < 0.05) were considered as significant.	

Table 3. Analysis of variance of the estimated model for the release of *N*-glycans from bovine colostrum whey by EndoBI-1

Source of variation	Sum of squares	Degrees of freedom	Mean Squares	F-test
Regression	3968.56	4	992.14	16.33^a
Residual	1397.249	23	60.75	
Lack of fit	1397.223	20	69.86	8123.2^b
Pure error	0.026	3	0.0086	
Total	5365.809			

Values in bold are statistically meaningful ($P < 0.05$). Coefficient of determination: $R^2 = 0.74$; $F_{0.95-4, 23} = 2.80$; $F_{0.95-20,3} = 8.66$

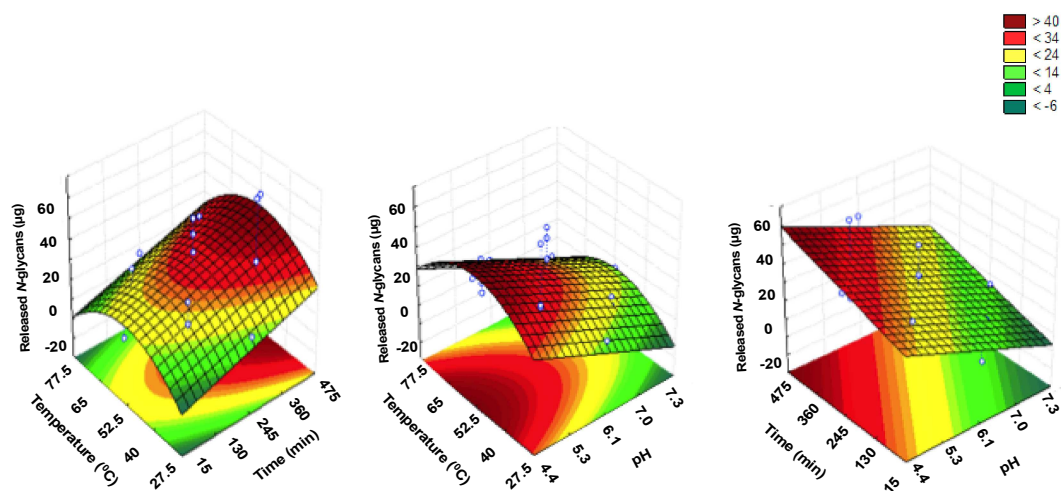


Figure 2. Effects of temperature vs time, temperature vs pH and time vs pH on the release of *N*-glycans from bovine colostrum whey by EndoBI-1.

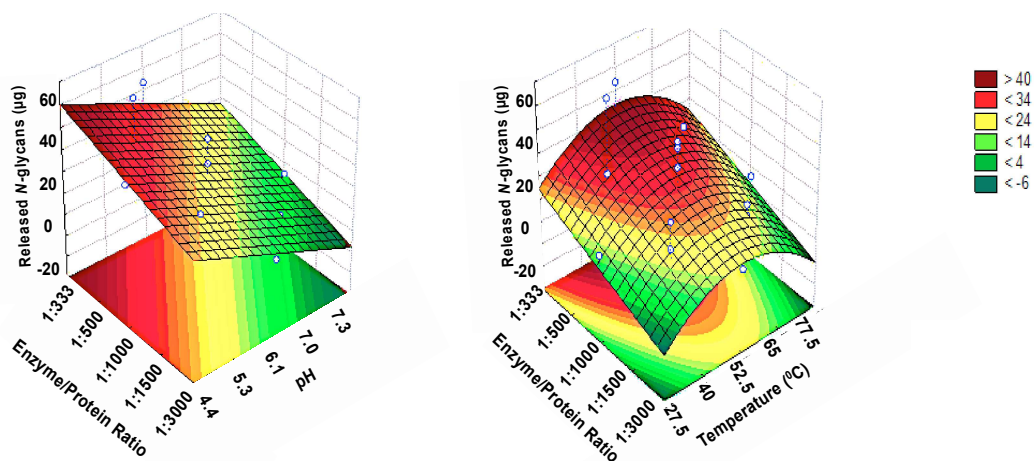


Figure 3. Effects of enzyme/protein ratio vs pH and enzyme/protein ratio vs temperature on the release of *N*-glycans from bovine colostrum whey by EndoBI-1.

The analysis of variance of the estimated regression model is presented in Table 3. The regression was significant ($F_{\text{calculated}} > F_{\text{table}}$) and thus could be used for predictive goals in the range of parameters evaluated since the ratio $F_{\text{calculated}} / F_{\text{table}}$ is greater than 3 [27]. Although the F-test for the lack of fit was statistically significant ($F_{\text{calculated}} > F_{\text{table}}$), the high lack of fit of the model was associated with the extremely low value of the pure error (0.026) likely due to the low degrees of freedom (3).

Based on the estimated regression model generated, response surfaces were built to express the release of *N*-glycans from bovine colostrum whey by EndoBI-1. According to the estimated regression model and Figure 2, the release of *N*-glycans is favored by increased reaction time and low pH values. The highest release of *N*-glycans was observed in the axial points or both variables: 4.45 pH and 475 min. Based on the estimated regression model and Figure 2, EndoBI-1 activity was favored by temperature values ranging from 40 to 65 °C, with the highest activity observed at the temperature of the central point (52.5 °C) and lowest pH values. Reduction of EndoBI-1 activity when moving further away from the center point is likely due to some protein denaturation effects at higher temperature (+1, 65 °C) and to temperatures that are below its optimum value (-1, 40 °C).

According to the estimated regression model and Figure 3, EndoBI-1 activity increases when enzyme/protein ratio increases from -2.0 to +2.0 (1/3000 to 1/333), with highest values at lowest pH (-2.0, 4.45 pH). Based on the estimated regression model and Figure 2, temperature in the central point (52.5 °C) and longer extraction times (+1 to 2.0, 360 to 475 min) did favor EndoBI-1 activity, with highest glycan release observed at 52.5 °C and 475 min of reaction time. According to the estimated regression model and Figure 3, EndoBI-1 activity was favored by

temperature in the central point (52.5 °C) and higher enzyme/protein ratio (+1 to 2.0, 1/500 to 1/333 min), with highest glycan release observed at 52.5 °C and 1/333 enzyme/protein ratio. Overall, for the conditions tested, optimal conditions for glycan release were observed for low pH, a temperature of 52.5°C, a high enzyme/protein ratio, and longer reaction times.

3.4 *N*-glycan release comparison of EndoBI-1 and PNGase F on denatured/native substrates and pasteurization conditions

The yield of *N*-glycans released by EndoBI-1 and PNGase F from native or denatured bovine colostrum whey, bovine lactoferrin or RNase B is shown in Figure 4. The treatment of native whey, lactoferrin and RNase B by EndoBI-1 resulted in 80, 103 and 49 µg *N*-glycans, respectively, while the yield after denaturation was 157, 203 and 56 µg, respectively. In comparison, the yield of *N*-glycans from native whey, lactoferrin and RNase B after treatment with PNGase F was found to be only 34, 31 and 44 µg, respectively. After denaturation, treatment with PNGase F produced 155, 203 and 58 µg *N*-glycans, respectively.

Similarly, the *N*-glycan yield of EndoBI-1 and PNGase F on 500 µL concentrated bovine whey under pasteurization conditions (62 °C, 30 min) showed that EndoBI-1 yield was 87 µg, while the PNGase F yield was only 18 µg (Figure 5).

3.5 *N*-glycan analysis by Nano-LC-Chip-Q-TOF MS

After digestion by EndoBI-1, released *N*-glycans from the bovine milk glycoproteins were purified by solid-phase extraction and analyzed by nano-LC-Chip-Q-TOF MS for a sample from experimental treatment 10 (see Table 1). The PGC chip was able to separate *N*-glycans based on their structures and their monosaccharide compositions.

Figure 5 shows the extracted ion chromatogram (EIC) for one neutral and one sialylated oligosaccharide. For each of them, the composition was determined based on the mass and data on bovine milk *N*-glycans from the literature [26]. EIC of these *N*-glycans shows 2 peaks at different retention times for the same mass. These results suggest two structural isomers or α or β anomers for the same composition.

The analytical platform used enables measurement of the presence of *N*-glycans in a complex mixture, bovine colostrum whey protein concentrate, with high resolution and separation of isomeric forms. The *N*-glycan composition was identified by an automated data analysis using a library for bovine milk glycoproteins. A complete *N*-glycan profiling was performed using LC-MS and LC-MS/MS on samples 9, 10 and 17 in another study to evaluate the effect of pH, reaction time, temperature and enzyme/protein ratio on the *N*-glycan release diversity [49].

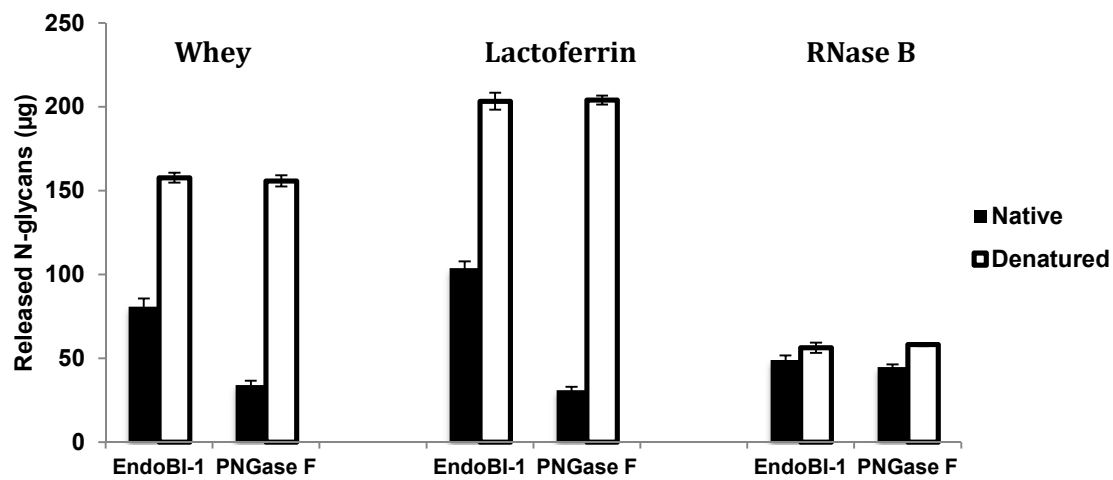


Figure 4. Denaturation affect of concentrated bovine colostrum whey, bovine lactoferrin and RNase B on *N*-glycan release by EndoBI-1 and PNGase F.

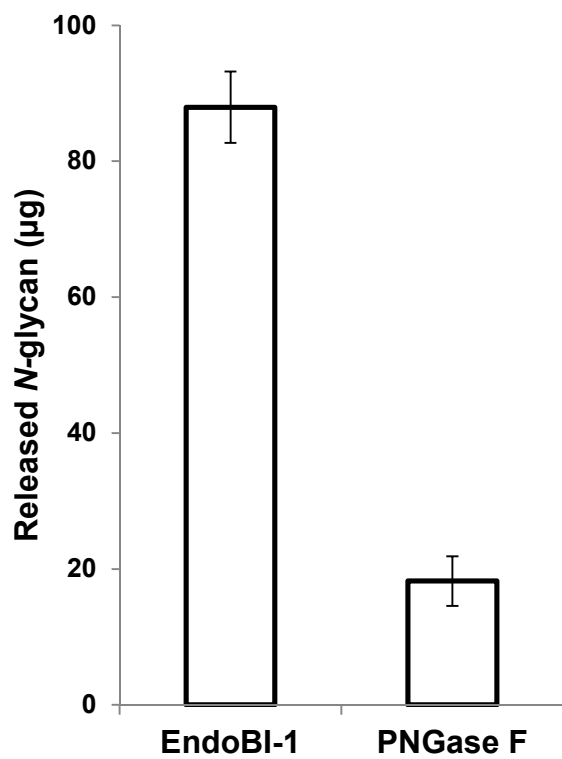


Figure 5. *N*-glycan release efficiency comparison of EndoBI-1 and PNGase F on concentrated bovine whey colostrum under pasteurization conditions (62 °C, 30 min).

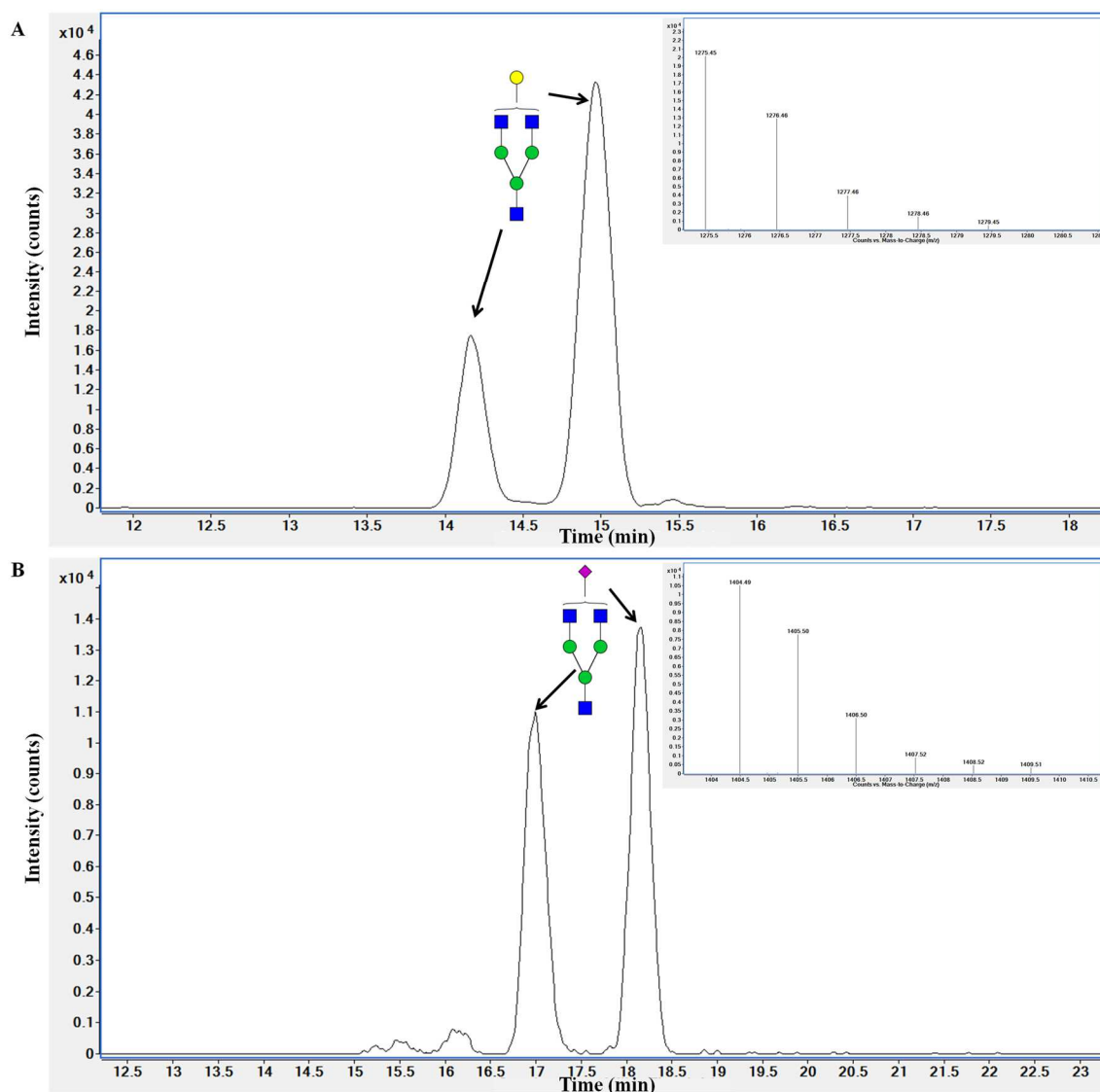


Figure 5. Extracted ion chromatogram (EIC) of two isomers or anomers for neutral *N*-glycans with 638.73 *m/z* (*z*=1) and sialylated *N*-glycan with 703.23 *m/z* (*z*=1). Deconvoluted CID Endo-B-*N*-acetylglucosaminidase was incubated with bovine colostrum whey protein concentrate at pH 5.30, 360 min, 40°C, enzyme/protein ratio 1/500. *N*-glycans were purified by solid phase extraction and analyzed by Nano-LC-Chip-Q-TOF MS. Green circles, yellow circles,

blue squares, red triangles and purple diamonds represent mannose, galactose, HexNAc, fuc, and NeuAc residues, respectively.



4. Discussion

In this study, we investigate how different reaction conditions affect the efficiency of *N*-glycan release by EndoBI-1 from concentrated bovine colostrum whey glycoproteins. These released glycans are structurally similar to human milk oligosaccharides (HMO) that are shown to stimulate the development of a beneficial infant intestinal microbiota, in particular enriching for *Bifidobacterium* [91, 92]. However, the large-scale isolation of these glycans is not feasible. Therefore, their use in both research and commercial applications are limited.

Currently, *N*-glycans are not commercially available, despite their biological activity, in large part because of the difficulty in producing them. Available technologies for releasing *N*-glycans from glycoproteins relies on the use of PNGase F, an enzyme derived from *Flavobacterium* [110], whose activity is limited by core-fucosylation, is heat-sensitive, active at a pH higher than that of most physiological systems, denaturation is required for activity, and the enzyme is expensive. These limitations restrict its use in the production of *N*-glycans from common sources, including whey protein.

Protein denaturation, especially, before enzymatic treatment plays an important role for efficient enzymatic release [119]. However, the native environment of *B. infantis* from which EndoBI-1 was isolated [65] likely contains few denatured glycoproteins and as a result, evolutionary pressures have shaped this enzyme's activity to act efficiently on native-state glycoproteins. To investigate this possibility, we compared the ability to release *N*-glycans from native proteins, in comparison to PNGase F. The results show that both enzymes were more active on denatured proteins compared to native proteins. PNGase F showed 4.55, 7.41 and 1.31-fold reduced activity on native whey, lactoferrin and RNase B, respectively, compared to denatured conditions, while the yield of *N*-glycans released from EndoBI-1 treatment of native

whey, bovine lactoferrin and RNase B was only 1.9, 1.95 and 1.14-fold reduced under native conditions, respectively. These results suggest that denaturation of substrates for *N*-glycan release by EndoBI-1 is not critical for *N*-glycan release, compared to PNGase F. Importantly, these results demonstrates that EndoBI-1 can be used in dairy industry to efficiently release *N*-glycans from milk proteins.

Bovine milk contains a large amount of protein (32g/L), and a small proportion of free milk glycans, whose structural similarity to human milk oligosaccharides has been previously discussed [120]. The biological activity of these milk oligosaccharides are chiefly responsible for the ability of milk to promote the growth of *Bifidobacterium* in the neonatal gut, and contribute to infant health [91, 92]. We have recently shown that *N*-glycans purified from milk glycoproteins bear structural similarity to the free glycans found in milk. These *N*-glycans also possess similar bifidogenic potential and could be used to improve human health, but these glycans are currently not widely available [48].

The effect of temperature, pH, reaction time, and enzyme/protein ratio was examined to identify the optimum conditions under which a novel enzyme, EndoBI-1 is active on whey protein. In general, a higher glycan yield was obtained at lower pH values (4.45), longer reaction times (475 min), and moderate temperatures in the range studied (52.5 °C), and a higher enzyme/protein ratio (1:333). A yield of 0.12 g L⁻¹ is possible under optimum conditions, which is significantly higher than that obtained with PNGase F under its optimum conditions. Also, unlike PNGase F, these data also demonstrate that EndoBI-1 is a versatile enzyme, active under a wider variety of incubation time, temperature, and pH combinations that can be used in large-scale *N*-glycan production strategies. For example, a protein ratio of 1:1000 enzyme/protein and

18 hours incubation time was predicted to result in approximately the same yield as the optimum conditions (1/ 500 ratio and 360 min).

As a conclusion, the large scale *N*-glycan production can be achieved with a novel enzyme EndoBI-1 and processing technique. These *N*-glycans will enable us to study their biological functions. Moreover, the use of whey as a *N*-glycan source provides added value for a former waste product, contributing to improved economic and environmental sustainability with the added benefit of the production of a beneficial bioactive food additive.

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Supplemental information

Table S1: Primers used in this study.

Primer name	Sequence (5'-3')
a) Cloning Primers	
2468-fwd	TTTGTACAAAAAAGCAGGCACCATGAATGCGGACGCCGTTTCTCCGAC
2468-rev	TTTGTACAAGAAAGCTGGGTGCGCGTCGCACTCAGTTGCTTCGG
b) Sequencing Primers	
IC-1006-fwd	TAATACGACTCACTATAGGG
IC-1006-rev	TGCTAGTTATTGCTCAGCGG

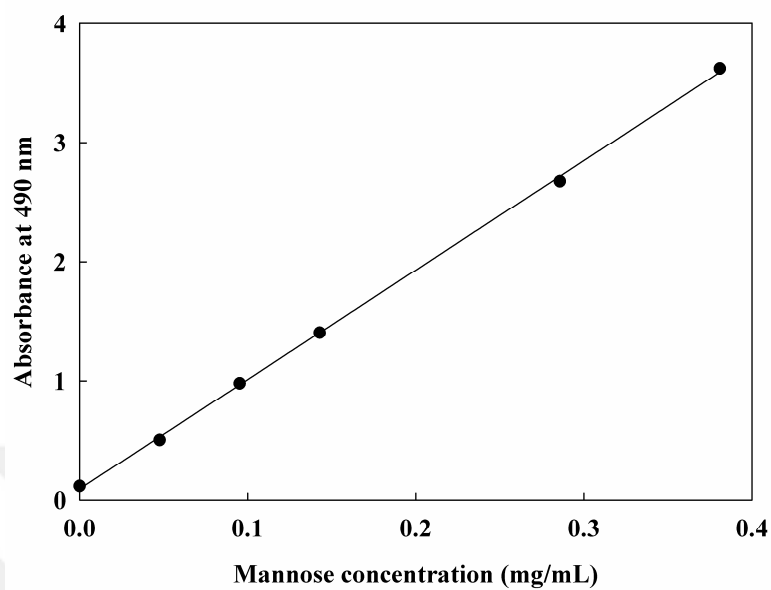


Figure S1. Calibration curve of D-mannose. The concentration range studied was from 0 to 0.38 mg/mL. The linear equation was: $y = 9.1848x + 0.0919$, $R^2 = 0.9995$.

Chapter III

A novel endo- β -N-acetylglucosaminidase releases specific N-glycans depending on different reaction conditions

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Contributions: SK %35 %, ALP 35 %, JMLNMB 5 %, SAF 5 %, YL 5 %, DAM 5 %, DEB 5 %, DB 5 %.

*These authors contributed equally to this work

Summary

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

1. Introduction

Protein glycosylation play a key role in structural conformation and bioactivity of proteins, including adhesion, targeting, folding and stability [5]. 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*-glycans) 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) [121]. 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 [121, 122]. 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 [104]. 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 [121].

Milk contains a variety of components, including proteins, peptides, lipids, carbohydrates that contribute to the development of a newborn [123]. 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 [124]. These glycoproteins contain covalently attached oligosaccharides (glycans) [125]. 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 [126-128]. Other studies reveal that milk glycoproteins contribute to the bifidogenic effect of mother's milk by stimulating the growth of beneficial bacteria by providing a diverse array of complex glycans as a carbon source [129]. 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 [130, 131]. We recently demonstrated that a key infant gut microbe, *Bifidobacterium longum* subsp. *infantis*, consumes these conjugated *N*-glycans. 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 [48].

We recently showed a novel endo-*B-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*-glycans [116, 132]. 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 temperature, pH, reaction time, and enzyme/protein ratio significantly affected the yield of released *N*-glycans by EndoBI-1 [27]. In the present 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.

2. Materials and methods

2.1 Bacteria and media

B. longum subsp. *infantis* ATCC 15697 used in this study was obtained from the University of California Davis Viticulture and Enology Culture Collection (Davis, CA). *Bifidobacterium* was grown in Man-Rogose-Sharp (MRS) broth supplemented with 0.05% (w/v) L-cysteine (Sigma-Aldrich). The cells were grown anaerobically (5% H₂, 5% CO₂, 90% N₂, Coy Laboratory Products, Grass Lake, MI) at 37 °C for 24 hr. *E.coli* BL21* strain for protein expression was grown in Luria broth (LB) containing carbenicillin (100µg/ml) in an Inova 4000 shaker (New Brunswick Scientific, New Jersey) at 200 rpm and 37 °C.

2.2 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. Genomic DNA coding for EndoBI-1 in *B. infantis* ATCC 15697 was amplified using appropriate primers (Table S1). Signal peptide and trans-membrane 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 ¹⁷.

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. 500 mL of LB with 100 µg/ml carbenicillin was inoculated with 1% of overnight culture and grown for 3h 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 6h. 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. 200 µl 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 5ml/ min flow rate. The column was equilibrated with 25 mL of 300 mM KCl, 50 mM KH₂PO₄, 5 mM imidazole buffer (pH 8). 50 mL 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 KH₂PO₄, 5 mM imidazole buffer (pH 8) and 300 mM KCl, 50 mM KH₂PO₄ 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 1X 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.

2.3 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 m² 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), seventy four liters 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 m²) 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 [27] and the reactions were terminated by 1 M Na₂CO₃.

2.4 Glycan purification

Samples were loaded in duplicate on a PGC SPE plate (Glygen, Columbia, MD, USA) that were conditioned using 3 x 100 µL of 80% ACN containing 0.1% TFA in water, followed

by 3 x 100 μ L of water. After sample loading, wells were washed using 6 $\times$ 200 μ L of water and *N*-glycans were eluted using 3 $\times$ 200 μ L of 40% ACN containing 0.1% TFA in water. The enriched *N*-glycans fractions were dried during overnight in vacuum. Samples were rehydrated in 50 μ L 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.

2.5 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 $\times$ 75- μ m analytical column, both packed with 5 μ m 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 μ L/min. *N*-glycan separation was performed on a 65-min gradient delivered by the nanopump at a flow rate of 0.3 μ L/min. The 65-min gradient followed this program: 0% B (0.0–2.5 min), 0 to 16% B (2.5–20.0 min), 16 to 44% B (20.0–30.0 min), 44 to 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 450–3000 m/z for *N*-glycans in the positive ionization mode with an acquisition rate of 2.01 spectra/s for *N*-glycans. 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

100–3000 m/z . The collision energies correspond to voltages ($V_{\text{collision}}$) that were based on the equation: $V_{\text{collision}} = m/z (1.5/100 \text{ Da}) \text{ Volts} - 3.6 \text{ V}$; 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).

2.6 *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 ≥ 1000 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 [117] 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 1000 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

2.7 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 precomp, 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.)

3. Results

3.1 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 *N*-glycan chemically distinct compositions were determined in the samples 1, 2 and 3 respectively (Table 1). These results suggest that the conditions tested for the samples 2 and 3 (pH 5.3 or 6.15; incubation time of 245 or 360 min; temperature at 40 or 52.5°C; and enzyme/protein ratio of 1/500 or 1/1000) 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 [133].



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 <i>N</i> -glycans	Sialylated <i>N</i> -glycans	Total possible compositions
7	130	65	1/1500	10 ± 0 ^A	7 ± 0 ^A	3 ± 0 ^A	18 ± 0 ^A
6.15	245	52.5	1/1000	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,B,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. Shown are the neutral mass, charge (z), m/z, monosaccharide composition, glycan type, Hex, Hexose; HexNAc, *N*-acetylglucosamine; Fuc, Fucose; NeuAc, *N*-acetylneuraminic acid, NeuGc; *N*-glycolylneuraminic acid.

Neutral Mass	Composition					% Relative Abundance		
	Hex	HexNAc	Fuc	NeuAc	NeuGc	Treatment 1	Treatment 2	Treatment 3
1113.42	3	3	0	0	0	46.84% ± 0.50% ^A	13.75% ± 0.17% ^B	15.86% ± 0.25% ^C
1275.47	4	3	0	0	0	7.77% ± 0.44% ^A	2.71% ± 0.07% ^B	3.64% ± 0.16% ^C
1437.51	5	3	0	0	0	6.22% ± 0.41% ^A	2.33% ± 0.07% ^B	1.66% ± 0.13% ^C
1478.54	4	4	0	0	0	6.29% ± 0.32% ^A	0.00% ^B	2.90% ± 0.19% ^C
1519.54	3	5	0	0	0	9.27% ± 0.53% ^A	3.11% ± 0.11% ^B	3.86% ± 0.22% ^C
1599.57	6	3	0	0	0	4.72% ± 0.25% ^A	1.53% ± 0.09% ^B	1.97% ± 0.09% ^C
1665.62	3	5	1	0	0	0.00% ^A	3.88% ± 0.37% ^B	4.83% ± 0.28% ^C
1404.51	3	3	0	1	0	0.00% ^A	3.10% ± 0.33% ^B	2.98% ± 0.18% ^B
1525.53	5	2	0	1	0	0.00% ^A	1.46% ± 0.10% ^B	1.15% ± 0.07% ^C
1566.56	4	3	0	1	0	6.97% ± 0.12% ^A	41.16% ± 0.46% ^B	37.66% ± 0.58% ^C
1687.59	6	2	0	1	0	0.00% ^A	1.39% ± 0.15% ^B	1.52% ± 0.09% ^C
1728.61	5	3	0	1	0	5.11% ± 0.50% ^A	3.68% ± 0.14% ^B	4.55% ± 0.18% ^C
1744.64	5	3	0	0	1	2.10% ± 0.20% ^A	4.21% ± 0.28% ^B	3.06% ± 0.24% ^C
1769.64	4	4	0	1	0	0.00% ^A	1.46% ± 0.03% ^B	1.84% ± 0.04% ^C
1810.66	3	5	0	1	0	0.00% ^A	1.97% ± 0.18% ^B	2.37% ± 0.10% ^C
1874.67	5	3	1	1	0	0.00% ^A	2.35% ± 0.25% ^B	1.94% ± 0.10% ^C
1890.67	5	3	1	0	1	0.00% ^A	2.69% ± 0.11% ^B	1.80% ± 0.18% ^C
1931.7	5	4	0	1	0	0.00% ^A	1.71% ± 0.08% ^B	1.83% ± 0.08% ^B
2019.74	5	3	0	2	0	0.00% ^A	2.09% ± 0.16% ^B	2.27% ± 0.03% ^B
2035.73	5	3	0	1	1	0.00% ^A	2.71% ± 0.05% ^B	2.31% ± 0.09% ^C
1640.6	5	4	0	0	0	4.73% ± 0.33%	0.00% ^B	0.00% ^B
2051.73	5	3	0	0	2	0.00% ^A	2.71% ± 0.20% ^B	0.00% ^A

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

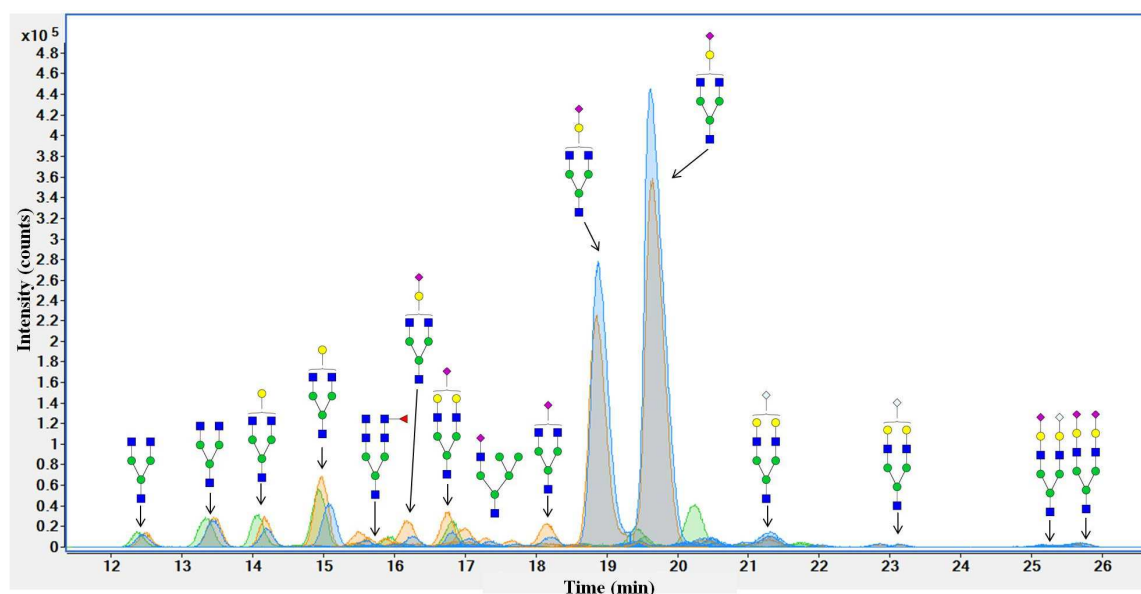


Figure 1. Extracted compound chromatograms (ECCs) of *N*-glycans from bovine colostrum whey concentrate. The samples are differentiated by color: 1 is green, 2 is blue and 3 is orange. Endo- β -*N*-acetylglucosaminidase was incubated with bovine colostrum whey protein concentrate under different conditions. 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 1/1000, 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.

3.2 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*=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 Table S2, S3 and 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 4). 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 α 1-6 fucose residue for the fucosylated core [132]. 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 1113.42 and 1566.56 Da, respectively. Two putative structures, one with 2HexNAc-3Hex core and another with 2HexNAc-3Hex-1Fuc core, are represented in Figure 2 for each *N*-glycan.

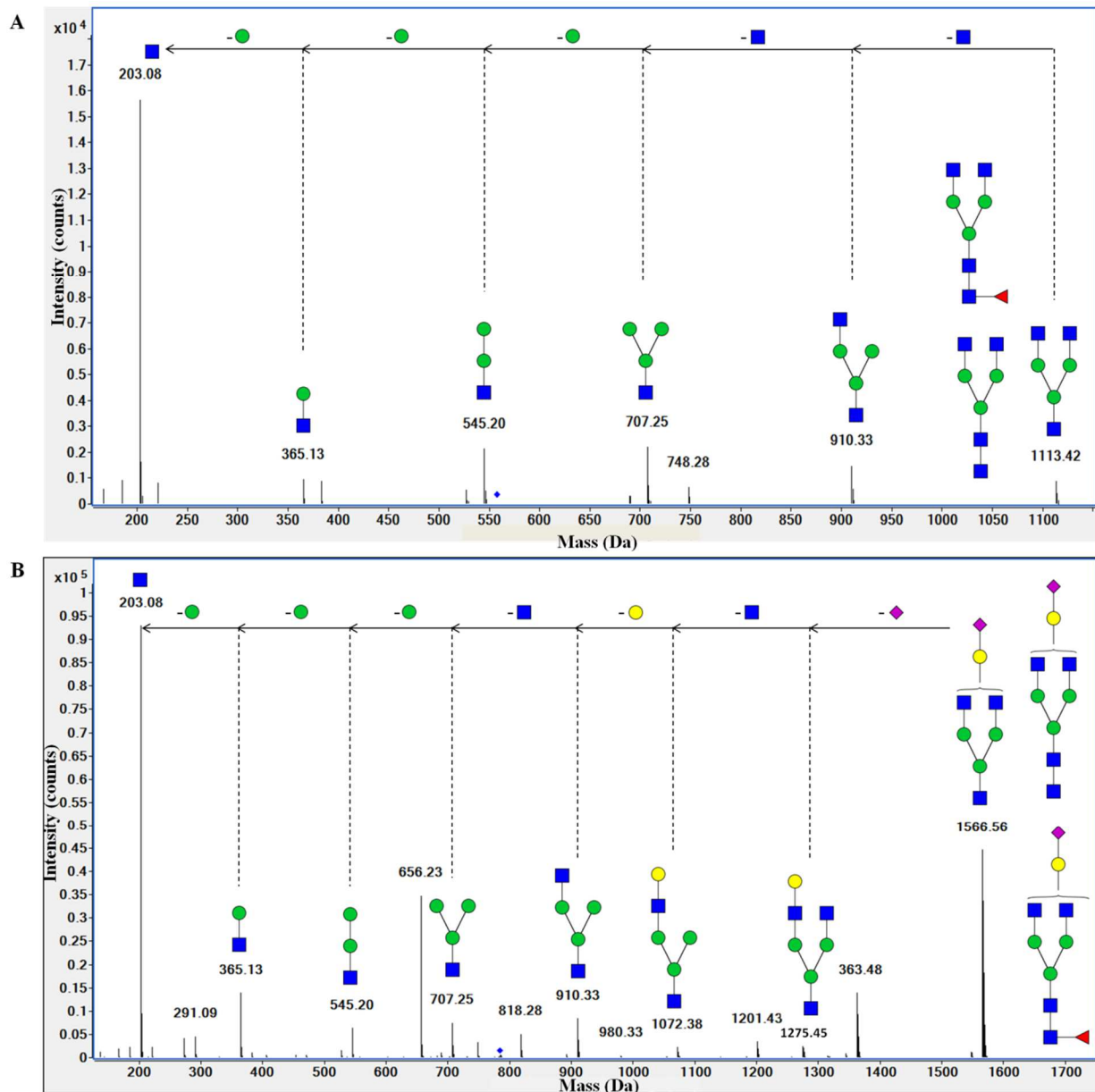


Figure 2. MS/MS spectra of neutral and sialylated *N*-glycans. (A) Deconvoluted tandem spectrum of the neutral *N*-glycan 3Hex-3HexNAc from bovine milk glycoproteins. This glycan corresponded to 557.71 m/z with $z = +2$. (B) Deconvoluted tandem spectrum of the sialylated *N*-glycan 4Hex-3HexNAc-1NeuAc 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.



3.3 Effect of the physico-chemical 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 1/1000 or 1/500 and incubation time of 245 or 360 min.

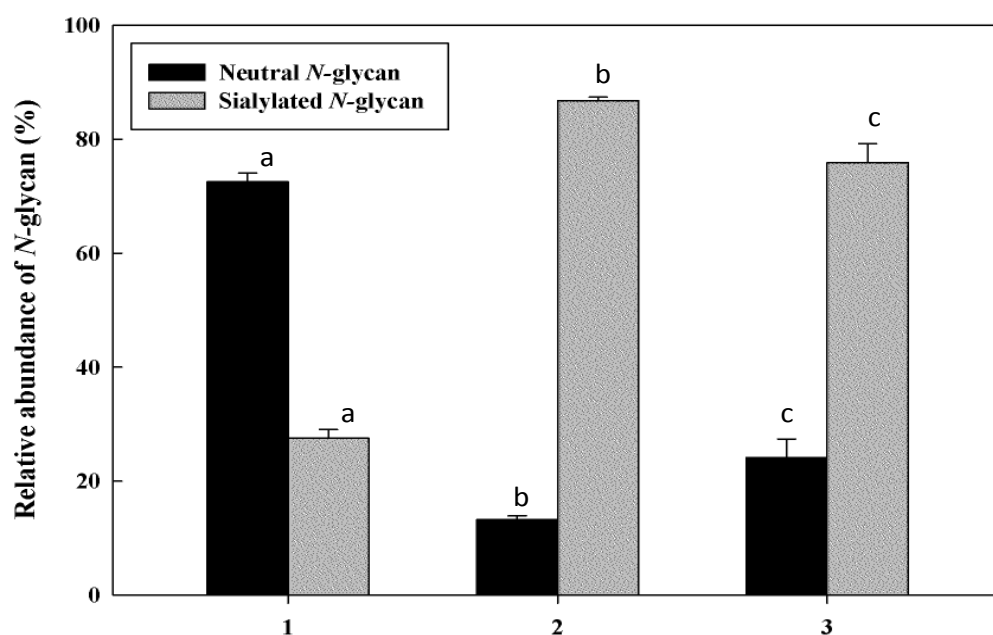


Figure 3. Relative abundance of neutral and sialylated *N*-glycans in sample 1, sample 2 and sample 3. Endo- β -*N*-acetylglucosaminidase was incubated with bovine colostrum whey protein concentrate under different conditions. Sample 1: pH 7, 130 min, 65°C, enzyme/protein ratio 1/500, Sample 2: pH 6.15, 245 min, 52.5°C, enzyme/protein ratio 1/1000, 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 $P < 0.05$.

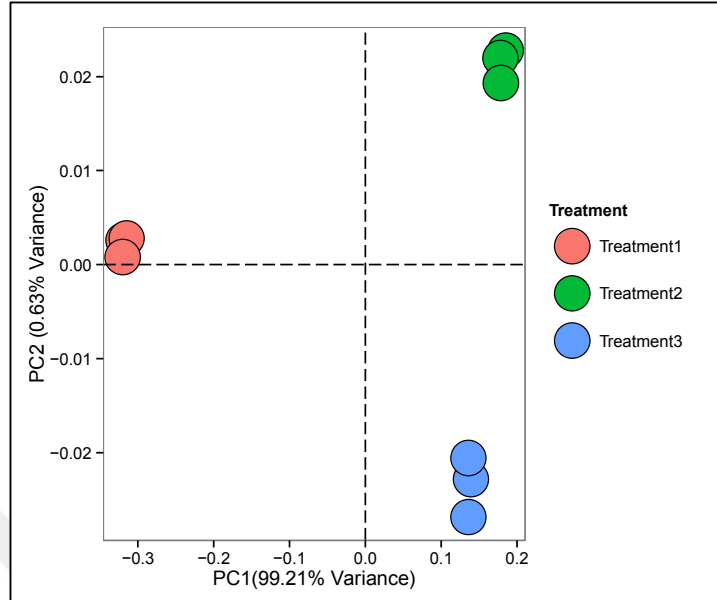


Figure 4. Treatment conditions shape populations of released *N*-glycans. PCA plot of composition of released *N*-glycans shows differences between Treatment 1 and Treatments 2 and 3. Nearly all variance between samples is explained in the x-axis differentiating Treatment 1 from Treatments 2 and 3 (PC1; 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%).

4. 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* [91, 92]. 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 [24]. However, *N*-glycans containing a fucose $\alpha(1-3)$ -linked to the glycan core are resistant to this enzyme [134]. EndoBI-1 cleaves the *N*-*N'*-diacetyl chitobiose moiety found in the *N*-glycan core, even if fucosylated [135]. Nwosu et al., [117] identified 50 *N*-glycan compositions released from bovine milk using PNGase F. This previous work was performed on denatured proteins overnight whereas the current 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 [136]. 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 [137]. 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 [132] 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 [138]. Similarly, our recent findings on the consumption of released *N*-glycans shows that *B. infantis* preferentially consumes structurally similar classes of *N*-glycans [48]. 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 [88]. Other studies suggest that sialic acid is an essential monosaccharide for brain development and learning [89]. They reduce the binding of leukocyte to endothelial cells while the neutral glycans do not have any effect [139]. Recombinant human lactoferrins, expressed in *Pichia pastoris*, with or without sialylated glycan structures provided evidence that *N*-acetylneuraminic acid is involved in the immune function of these lactoferrins[90]. Recent *in vitro* studies revealed that the glycan part of human lactoferrin decreases pathogen infections, including Salmonella, of cultured human intestinal epithelial cells[140].

Statistical analysis shows that *N*-glycan production can be manipulated in terms of diversity or relative abundance. Although, treatments 2 and treatment 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 or 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 non-fucosylated 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 release 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.

Supplemental information

Table S1: Primers used in this study

Primer name	Sequence (5'-3')
a) Cloning Primers	
2468-fwd	TTTGTACAAAAAAGCAGGCACCATGAATGCGGACGCCGTTTCTCCGAC
2468-rev	TTTGTACAAGAAAGCTGGGTGCGCGTTCGCACTCAGTTGCTTCGG
b) Sequencing Primers	
IC-1006-fwd	TAATACGACTCACTATAGGG
IC-1006-rev	TGCTAGTTATTGCTCAGCGG

Table S2. Identified bovine milk glycoproteins *N*-glycans released by endo- β -*N*-acetylglucosaminidase. The enzyme digestion was performed at pH 7 during 130 min at 65°C with a ration enzyme:protein of 1:1500. Shown are the neutral mass, charge (z), m/z, monosaccharide composition, glycan type, Hex, Hexose; HexNAc, *N*-acetylglucosamine; Fuc, Fucose; NeuAc, *N*-acetylneuraminic acid, NeuGc; Nglycolylneuraminic acid.

Neutral mass	Composition					<i>N</i> -glycan type
	Hexose	HexNAc	Fucose	NeuAc	NeuGc	
1113.41	3	3	0	0	0	Neutral
1275.49	4	3	0	0	0	Neutral
1437.52	5	3	0	0	0	Neutral
1478.54	4	4	0	0	0	Neutral
1519.57	3	5	0	0	0	Neutral
1599.58	6	3	0	0	0	Neutral
1640.60	5	4	0	0	0	Neutral
1566.57	4	3	0	1	0	Sialylated
1728.63	5	3	0	1	0	Sialylated
1744.63	5	3	0	0	1	Sialylated

Table S3. Identified bovine milk glycoproteins *N*-glycans released by endo- β -*N*-acetylglucosaminidase. The enzyme digestion was performed at pH 6.15 during 145 min at 52.5°C with a ration enzyme:protein of 1:1000. Shown are the neutral mass, charge (z), m/z, monosaccharide composition, glycan type, Hex, Hexose; HexNAc, *N*-acetylglucosamine; Fuc, Fucose; NeuAc, *N*-acetylneuraminic acid, NeuGc; *N*-glycolylneuraminic acid.

Neutral mass	Composition					<i>N</i> -glycan type
	Hex	HexNAc	Fuc	NeuAc	NeuGc	
1113.41	3	3	0	0	0	Neutral
1275.47	4	3	0	0	0	Neutral
1437.51	5	3	0	0	0	Neutral
1519.55	3	5	0	0	0	Neutral
1599.57	6	3	0	0	0	Neutral
1665.62	3	5	1	0	0	Neutral
1404.51	3	3	0	1	0	Sialylated
1525.53	5	2	0	1	0	Sialylated
1566.56	4	3	0	1	0	Sialylated
1687.59	6	2	0	1	0	Sialylated
1728.62	5	3	0	1	0	Sialylated
1744.64	5	3	0	0	1	Sialylated
1769.66	4	4	0	1	0	Sialylated
1810.67	3	5	0	1	0	Sialylated
1874.67	5	3	1	1	0	Sialylated

1890.68	5	3	1	0	1	Sialylated
1931.70	5	4	0	1	0	Sialylated
2019.74	5	3	0	2	0	Sialylated
2035.74	5	3	0	1	1	Sialylated
2051.73	5	3	0	0	2	Sialylated



Table S4. Identified bovine milk glycoproteins *N*-glycans released by endo- β -*N*-acetylglucosaminidase. The enzyme digestion was performed at pH 5.30 during 360 min at 40°C with a ration enzyme:protein of 1:500. Shown are the neutral mass, charge (z), m/z, monosaccharide composition, glycan type, Hex, Hexose; HexNAc, *N*-acetylglucosamine; Fuc, Fucose; NeuAc, *N*-acetylneuraminic acid, NeuGc; *N*-glycolylneuraminic acid.

Neutral mass	Composition					<i>N</i> -glycan type
	Hex	HexNAc	Fuc	NeuAc	NeuGc	
1113.42	3	3	0	0	0	Neutral
1275.47	4	3	0	0	0	Neutral
1437.51	5	3	0	0	0	Neutral
1478.54	4	4	0	0	0	Neutral
1519.54	3	5	0	0	0	Neutral
1599.57	6	3	0	0	0	Neutral
1665.62	3	5	1	0	0	Neutral
1404.51	3	3	0	1	0	Sialylated
1525.53	5	2	0	1	0	Sialylated
1566.56	4	3	0	1	0	Sialylated
1687.59	6	2	0	1	0	Sialylated
1728.61	5	3	0	1	0	Sialylated
1744.64	5	3	0	0	1	Sialylated
1769.64	4	4	0	1	0	Sialylated
1810.66	3	5	0	1	0	Sialylated

1874.67	5	3	1	1	0	Sialylated
1890.67	5	3	1	0	1	Sialylated
1931.70	5	4	0	1	0	Sialylated
2019.74	5	3	0	2	0	Sialylated
2035.73	5	3	0	1	1	Sialylated



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Chapter IV

Kinetic characterization of a novel endo- β -N-acetylglucosaminidase on concentrated bovine colostrum whey to release bioactive glycans

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Contributions: SK % 40, ALP 10 %, JMLNMB 10 %, CQ 10 %, DAM 10 %, DB 10 %, DEB 10 %.

Summary

EndoBI-1 is a recently isolated endo- β -*N*-acetylglucosaminidase, which cleaves the *N*-*N*'-diacetyl chitobiose moiety found in the *N*-glycan core of high mannose, hybrid and complex *N*-glycans. These *N*-glycans have selective prebiotic activity for a key infant gut microbe, *Bifidobacterium longum* subsp. *infantis*. The broad specificity of EndoBI-1 suggests the enzyme may be useful for many applications, particularly for deglycosylating milk glycoproteins in dairy processing. To facilitate its commercial use, we determined kinetic parameters for EndoBI-1 on the model substrates ribonuclease B and bovine lactoferrin, as well as on concentrated bovine colostrum whey. K_m values ranging from 0.25-0.49, 0.43-1.00 and 0.90-3.18 mg/mL and V_{max} values ranging from 3.5×10^{-3} - 5.09×10^{-3} , 4.5×10^{-3} - 7.75×10^{-3} and 1.9×10^{-2} - 5.2×10^{-2} mg/mL*min were determined for ribonuclease B, lactoferrin and whey, respectively. In general, EndoBI-1 showed the highest apparent affinity for ribonuclease B, while the maximum reaction rate was the highest for concentrated whey. EndoBI-1-released *N*-glycans were quantified by a phenol-sulphuric total carbohydrate assay and the resultant *N*-glycan structures monitored by Nano-LC-Chip-Q-TOF MS. The kinetic parameters and structural characterization of glycans released suggest EndoBI-1 can facilitate large-scale release of complex, bioactive glycans from a variety of glycoprotein substrates. Moreover, these results suggest that whey, often considered as a waste product, can be used effectively as a source of prebiotic *N*-glycans.

Keywords: Deglycosylation, *N*-glycans, Endo- β -*N*-acetylglucosaminidase

1. Introduction

Glycosylation of human and bovine milk proteins is a post-translational modification that results from enzymatic processing of polypeptide chains in mammary epithelial cells. Protein glycosylation influences the biological and physicochemical properties of the protein including targeting, folding and stability [65]. Glycans are linked to proteins 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*-glycans) are linked via *N*-acetylglucosamines (HexNAc) to an asparagine residue of proteins in the specific amino acid sequence AsN-X-Ser/Thr (where X can be any amino acid except proline) [141].

Human milk oligosaccharides (HMO) are known to guide the development of the infant intestinal microbiota, in particular enriching a beneficial bifidobacterial population [130, 131], which has recently been implicated in a range of health benefits including anti-adhesive antimicrobial activity, positive modulation of intestinal epithelial cell responses, immune modulation, protection against necrotizing enterocolitis and stimulation of brain development [142-146]. Unfortunately, many infants do not have access to human milk and, consequently, there have been numerous efforts to identify functionally equivalent molecules for infant formula and other prebiotic, therapeutic uses. Bovine milk represents one possible source of structurally similar oligosaccharides [147], however the concentration of these oligosaccharides in fluid milk is 20-fold lower than HMOs in human milk—a fact that limits their usage commercially [101, 148]. Alternatively, bovine milk glyco-conjugates such as glycoproteins might be considered as an additional source of bovine glycans with desirable bioactivities. In fact, we have recently shown that a key infant gut microbe, *Bifidobacterium longum* subsp. *infantis*, consumes these

conjugated *N*-glycans as a carbon source while other less desirable microbes do not (unpublished results). Whey, a by-product of cheese-making, contains ~4.5 g of glycoproteins per L [38]. Because only half of the total whey (190,000,000 ton/year) is currently utilized by the food industry, it is still considered a waste product [105]. Therefore, the use of whey as a source of bioactive glycans may also improve the sustainability of the dairy industry. However, robust enzymes with the appropriate activity to release the conjugated glycans are necessary for this process to become a commercial reality.

Peptidyl-*N*-glycosidases (PNGases) are widely used for deglycosylation of glycoproteins for analytical research purposes [24]. A PNGase (peptide- N^4 - (N-acetyl- β -glucosaminyl), glycopeptidase, N-glycanase, glycoaminidase or N-glycosidase) cleaves asparagine-linked oligosaccharides from glycoproteins by hydrolyzing the amide side chain [59, 60]. Commercially available PNGases (PNGase A from sweet almond and PNGase F from *Falvobacterium meningosepticum*) release several kinds of *N*-linked glycans irrespective of their size or charge [61-63]. However, if fucose is α -linked to *N*-acetylglucosamine, the *N*-glycans are resistant to hydrolysis by PNGase F [64]. We recently showed that an endo- β -*N*-acetylglucosaminidase (EndoBI-1) isolated from *Bifidobacterium longum* subsp. *infantis* ATCC 15697 has the ability to cleave the *N*-*N'*-diacetyl chitobiose moiety found in the *N*-glycan core of high mannose, hybrid and complex *N*-glycans [65]. EndoBI-1 activity is not affected by fucosylation of the *N*-glycan core, and it is heat resistant making it attractive for use in various industrial processes. Despite the wide potential use of this enzyme, data are not yet available on its kinetics and substrate specificity in terms of initial maximum rates and K_m -values.

In this study, we examine the kinetic parameters of EndoBI-1 for three purified, glycosylated substrates: model proteins ribonuclease B (RNase B) and bovine milk lactoferrin

(bLF), as well as bovine colostrum whey protein concentrate. A goal was to investigate the potential use of EndoBI-1 for large-scale *N*-glycan production of released glycans from whey. Different direct and linearized plotting methods [149-151] were used to facilitate the prediction of kinetics parameters of the EndoBI-1. The values obtained enable comparison to other enzymes and estimations of optimal combinations of enzyme concentration and incubation time to yield complete *N*-glycan release from whey at a commercial-scale. These well determined kinetic parameters will enable us to produce a new prebiotic source efficiently. Finally, the structures of the released *N*-glycans were elucidated for each substrate by advanced mass spectrometry methods.

2. Materials Methods

2.1 Gene Cloning, expression and purification

A pEcoTM-T7-cHis, Eco Cloning Kit (GeneTarget Inc, San Diego, CA, USA) was used for gene cloning. The EndoBI-1 coding sequence was amplified from *B. longum* subsp. *infantis* ATCC 15697 genomic DNA with primers; 5'-TTTGTACAAAAAAGCAGGCACCATGAATGCGGACGCCGTTTCTCCGAC-3' and 5'-TTTGTACAAGAAAGCTGGGTTGCCGGTCGCACTCAGTTGCTTCGG-3' and then cloned into the pEco-T7-cHis vector. The vector was transformed into *E. coli* Dh5a and *E. coli* BL21* for protein expression. The sequence was confirmed (IC-1006-forward; TAATACGACTCACTATAGG, IC-1006-reverse; TGCTAGTTATTGCTCAGCGG). Poly-histidine-tagged EndoBI-1 was produced in the *E. coli* host with a yield of ~1.7 mg/L on LB media under optimal induction conditions (0.5 mM IPTG, 37 °C for 4 hours). The protein was purified following bacterial lysis using affinity chromatography with 5 mL prepacked Ni-

charged columns (Bio-Rad, Hercules, CA, USA). The bound protein was eluted with 80 mM imidazole with high purity (data not shown).

2.2 Substrates

The concentration of bovine colostrum whey proteins was carried out using a pilot-scale cross-flow membrane system (Model L, GEA Filtration, Hudson, WI, USA). The system was composed of a 6.4 cm diameter spiral membrane housing (1-2 m² area), a 95 L jacketed stainless steel hold tank, a Proline Promass 80 E flow-meter (Endress+Hauser, Reinach, Switzerland), a heat exchanger, and a 7.0 HP feed pump (Hydra-CellTM Pump, model D10EKSGSNECF, Minneapolis, MN, USA). After upstream lactose hydrolysis (0.1% lactase, 30 min, 40-43°C), seventy-four liters of bovine colostrum whey were ultrafiltered using this system in single batch with a 10 kDa molecular weight cut-off polyethersulfone spiral-wound membrane (effective area of 1.86 m²) up to a 5.4 concentration factor (concentration factor = volume of feed/volume of retentate). Whey protein concentration was performed at 40-43°C with a transmembrane pressure of 3.0 bars 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 diafiltrations were performed to increase the removal of monosaccharides and oligosaccharides from the ultrafiltration retentate. RNase B from bovine pancreas and bLF were obtained from Sigma-Aldrich (St. Louis, MO, USA). To directly compare the pure glycoproteins (bLF and RNase B) to concentrated whey protein, five times the whey protein mass was used in reactions, to account for the mixed population of glycosylated (20%) and un-glycosylated (80%) proteins in whey [38, 152].

2.3 Glycoprotein digestion by EndoBI-1 and glycan quantification

Enzyme and substrate concentration were determined by a Qubit Protein Assay Kit (Life Technologies, Grand Island, NY, USA). RNase B, bLF and concentrated bovine whey (0.1-0.8 mg/mL) were incubated for various times from 0 to 45 min at 37 °C with 0.025 mg/ml EndoBI-1 in a 0.02 M Na₂HPO₄ buffer solution at pH 5. The reactions were terminated by the addition of 1M Na₂CO₃. Protein precipitation was carried out using a ratio of 4:1 cold pure ethanol added into the samples to precipitate proteins and collect the released *N*-glycans. Samples were dried overnight by vacuum centrifugation and were rehydrated in 100 µL of water, vortexed and sonicated. All experiments were performed in at least triplicate. A Carbohydrate Assay Kit, from Biovision, (Milpitas, CA, USA), was used to quantify released *N*-glycans with mannose as a standard. Aliquots of 30 µl of sample and 150 µl of concentrated sulfuric acid (98%) were added to each well. Samples were mixed on a shaker for ~1 min and incubated at 85°C for 15 min. After incubation, 30 µl of developer (provided by Biovision) were added to each well. Samples were mixed on the shaker for 5 min and the OD of each sample was measured at 490 nm. Sample OD was converted to carbohydrate concentration using a mannose standard curve linear function.

2.4 Determination of kinetic constants

Because of the complex nature of glycosylated and non-glycosylated proteins in whey, we first tested the activity of the EndoBI-1 on the purified model glycoproteins RNaseB and Lactoferrin. This allowed us to calculate kinetic parameters that could be compared to those for the more complex substrate found in whey, as well as identify a concentration range for the whey protein that would facilitate measurement of kinetic parameters and the heterogeneous structures of glycans released.

In this manner, RNase B, bLF and bovine whey concentrate with initial concentrations ranging from 0.1 to 0.8 mg/mL were chosen to determine the reaction curves for a total time of 45 min. The initial rate of reaction was obtained from the slopes calculated from samples taken in the first 20 min of reaction for each of the three substrates. Kinetic values (K_m and V_{max}) were determined from the initial rates at various initial substrate concentrations by using Graph Prism 6.0 software with non-linear and linearized plotting techniques including Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstie [149, 150, 153].

2.5 Glycan purification for Nano-LC-Chip-Q-TOF MS

After enzymatic digestion and protein precipitation by ethanol, 100 μ L of samples in water were loaded in duplicate on PGC SPE plates (Glygen, Columbia, MD, USA) that were conditioned using 3 x 100 μ L of 80% ACN containing 0.1% TFA in water, followed by 3 x 100 μ L of water. After sample loading, wells were washed using 6 x 200 μ L of water and *N*-glycans were eluted using 3 x 200 μ L of 40% ACN containing 0.1% TFA in water. The enriched *N*-glycans fractions were dried overnight in vacuum. Samples were rehydrated in 50 μ L of water, vortexed, sonicated and diluted 50 times prior to mass spectrometry analysis. For each sample, duplicates were analyzed by mass spectrometry.

2.6 Nano-LC-Chip-Q-TOF Mass spectrometry analysis

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 x 75- μ m analytical column, both packed with 5 μ m 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 μ L/min. *N*-glycan separation was performed on a 65-min gradient delivered by the nanopump at a flow rate of 0.3 μ L/min. The 65-min gradient followed this program: 0% B (0.0–2.5 min), 0 to 16% B (2.5–20.0 min), 16 to 44% B (20.0–30.0 min), 44 to 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 450–3000 *m/z* for *N*-glycans in the positive ionization mode with an acquisition rate of 1 spectra/s. 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 100–3000 *m/z*. The collision energies correspond to voltages ($V_{\text{collision}}$) that were based on the equation: $V_{\text{collision}} = m/z (1.5/100 \text{ Da}) \text{ Volts} - 3.6 \text{ Volts}$; where the slope and offset of the voltages were set at (1.5/100 Da) and (–3.6) respectively. Data acquisition was controlled by MassHunter Workstation Data Acquisition software (Agilent Technologies).

2.7 *N*-glycan identification

Compounds were identified using MassHunter Qualitative Analysis software (version B.06.00 SP2, Agilent Technologies) and the Find by Formula algorithm. The compounds were matched to a bovine milk *N*-glycan library using a mass error tolerance of 20 ppm [154]. The *N*-glycans from the library were composed of hexose (Hex), HexNAc, fucose, *N*-acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc). For compound identification, a minimum

abundance of 1000 counts was used to filter out low-abundance compounds. Identified compounds were extracted allowing charge states of +1 to +3 and the isotope model was “glycans.” Extracted ion chromatogram peaks were smoothed with the Gaussian function, and the resulting peaks were integrated via the “agile” algorithm. The assignment of *N*-glycans was confirmed by tandem mass spectrometry.

3. Results

3.1 EndoBI-1 production and confirmation of its activity on RNase B

The activity of recombinant EndoB-1 was tested on model glycoprotein RNase B that contains only high mannose *N*-linked glycans. RNase B is a 17 kDa *N*-glycosylated protein and its deglycosylation results in a 14-15 kDa deglycosylated protein. Confirmation of the deglycosylation activity of RNase B by purified EndoB-1 and a control commercial enzyme PNGase F are shown in Figure 1. Because EndoBI-1 cleaves one HexNAc residue less than PNGase F, the molecular weight of deglycosylated RNase B is slightly higher than 14 kDa for the EndoBI-1 enzyme. Substrates were denatured at 95 °C for 5 min in a final concentration of 10 mM DTT prior to the hydrolysis.

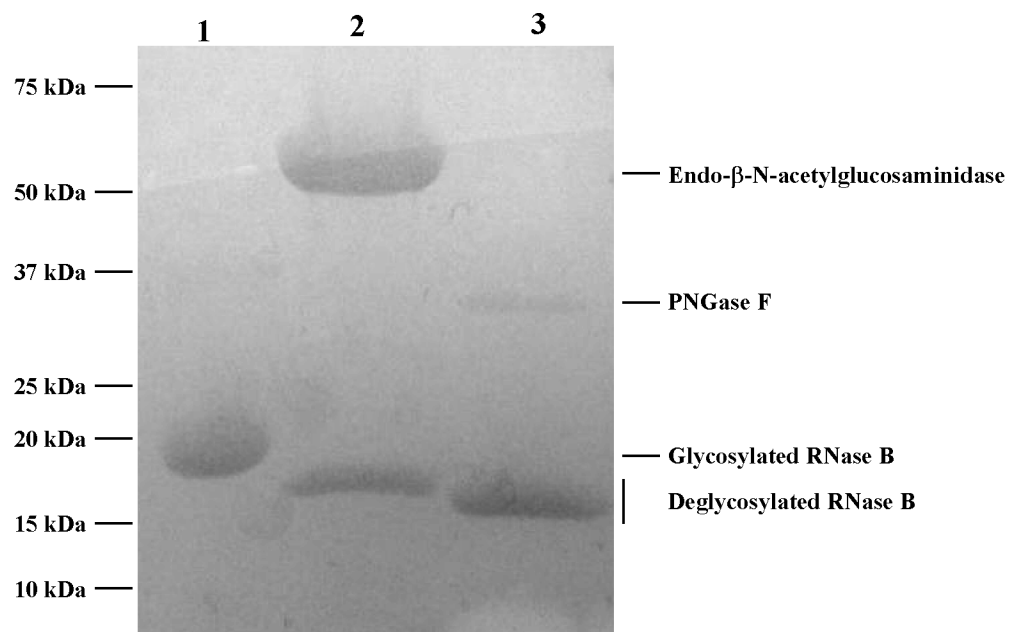


Figure 1. Enzymatic deglycosylation of RNase B by Endo-β-N-acetylglucosaminidase and PNGase F. on 12% SDS-PAGE gel. Lanes 1 :glycosylated RNase B (17kDa) . Lane 2:deglycosylated Rnase B by Endo-β-N-acetylglucosaminidase (47kDa). Lane 3: de glycosylated Rnase B by PNGase F (36kDa).

3.2 Determination of K_m and V_{max} by non-linear and linearized plotting

Using the Michaelis-Menten model, the velocity of the reaction is obtained from the initial linear decrease of the substrate concentration or the increase in product concentration. K_m , the substrate concentration when the reaction reaches half of V_{max} , can also be calculated from the obtained curve [19]. The direct plot may be obtained by plotting the initial reaction rate (v) against the corresponding initial substrate concentration (S) that yields a hyperbolic curve of saturation. However, it may be difficult to locate the asymptotes properly or potential deviations from the expected curve may be difficult to monitor. Thus, there are several linearized plotting methods that have been developed including Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstman, each with their own advantages and disadvantages [149-151, 155].

The kinetics of EndoBI-1 deglycosylating RNase B, bLF and whey glycoproteins were determined using various initial concentrations of each substrate over 45 min for each reaction (Figure 2). The reaction rate for each substrate was constant (i.e. slope of concentration versus time was constant) for the first 20 min, and initial rates of reaction were therefore calculated from the slopes of these data. The assumption of $[S] \gg [E]$ is not valid after 20 minutes. Therefore, the enzyme concentration becomes limited and reaction rate gets slower.

The direct plot was created by plotting the reaction rate (v) against the initial substrate concentration (S). These plots all resulted in a hyperbolic or saturation relationship (Figure 3). K_m values of 0.25, 0.43 and 0.90 mg/mL and V_{max} values of 5.09×10^{-3} , 7.75×10^{-3} and 5.2×10^{-2} mg/mL*min were calculated for RNase B, bLF and bovine whey, respectively. The linearization methods, Lineweaver-Burk ($1/S$ vs $1/v$), Hanes-Woolf (S vs S/v) and Eadie-Hofstee (v/S vs. v), plotting techniques were also used (Figure 4) to estimate the kinetic parameters for each substrate. K_m and V_{max} values estimated from these plotting techniques and a comparison with the

non-linear curve fitting is shown in Table 1. In general, there is a significant difference in the parameters derived from the non-linear and linearized fitting techniques for each substrate. It was shown that K_m values estimated from non-linear fitting are 1.77, 2 and 2.92 fold lower than the average of the three linearized fitting methods for RNase B, bLF and bovine whey, respectively, though the general trends are the same. K_m refers to the minimum substrate concentration that achieves half of V_{max} . Thus, the results demonstrate that the enzyme has a higher apparent affinity for RNase B than for the other substrates. V_{max} values ranging from 3.5×10^{-3} - 5.09×10^{-3} , 4.5×10^{-3} - 7.75×10^{-3} and 1.9×10^{-2} - 5.2×10^{-2} mg/mL*min were found for RNase B, bLF and bovine whey, respectively. These results indicate that the maximum rate of reaction is higher with the concentrated bovine colostrum whey as substrate compared with the model glycoprotein substrates and that the concentrated whey prepared at the UC Davis Milk Processing Lab can be used as *N*-glycan source at a rate of 2.08 mg *N*-glycan/ml*min per 1 mg/ml EndoBI-1 under optimal conditions.

Table I. Summary of the regressions shown in fig. 3&4 (linearized equations, quantities plotted on the x and y axis and results for v in mg/mL*min and K_m in mg/ml.).

	Non-linearized plotting	Lineweaver-Burke	Eadie-Hofstee	Hanes-Woolf
Linearized equation	-	$\frac{1}{v} = \frac{K_m}{V_{max}} \left(\frac{1}{[S]} \right) + \frac{1}{V_{max}}$	$v = -K_m \frac{v}{[S]} + V_{max}$	$\frac{[S]}{v} = \frac{1}{V_{max}} [S] + \frac{K_m}{V_{max}}$
x	S	1/S	v/S	S
y	v	1/v	v	S/v
RNase B				
V_{max}	3.5×10^{-3}	5.09×10^{-3}	4.6×10^{-3}	4.6×10^{-3}
K_m	0.2528	0.497	0.420	0.426
Lactoferrin				
V_{max}	4.5×10^{-3}	7.75×10^{-3}	6.7×10^{-3}	6.5×10^{-3}
K_m	0.4316	1.007	0.828	0.785
Whey				
V_{max}	1.9×10^{-2}	5.2×10^{-2}	3.7×10^{-2}	4.3×10^{-2}
K_m	0.9026	3.18	2.16	2.575

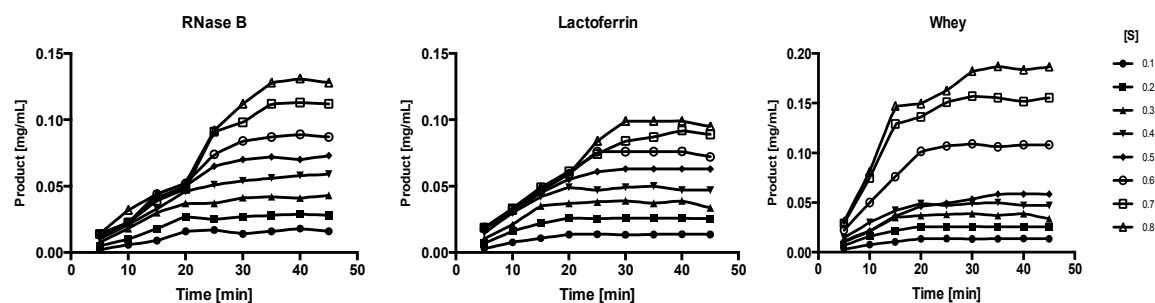


Figure 2. Reaction curves of EndoBI-1 acting on RNase B, bLF and concentrated whey. The initial substrate concentration ranged from 0.1-0.8 mg/ml for and the enzyme concentration for each experiment was 0.025 mg/ml.

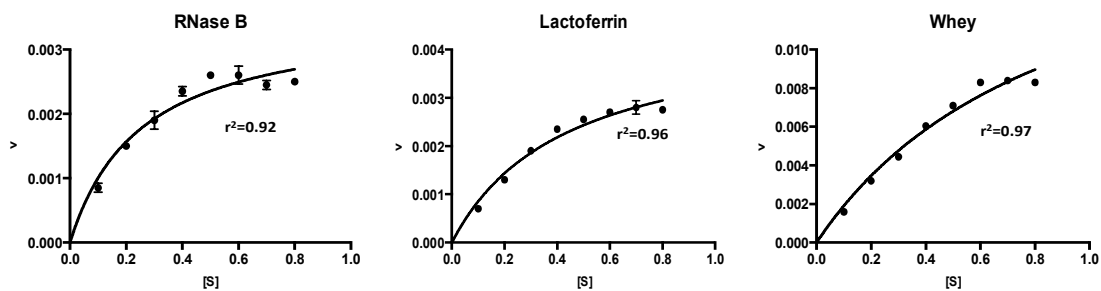


Figure 3. Direct plotting of rate of reaction (v) (mg/mL*min) and substrate concentration $[S]$ (mg/ml).

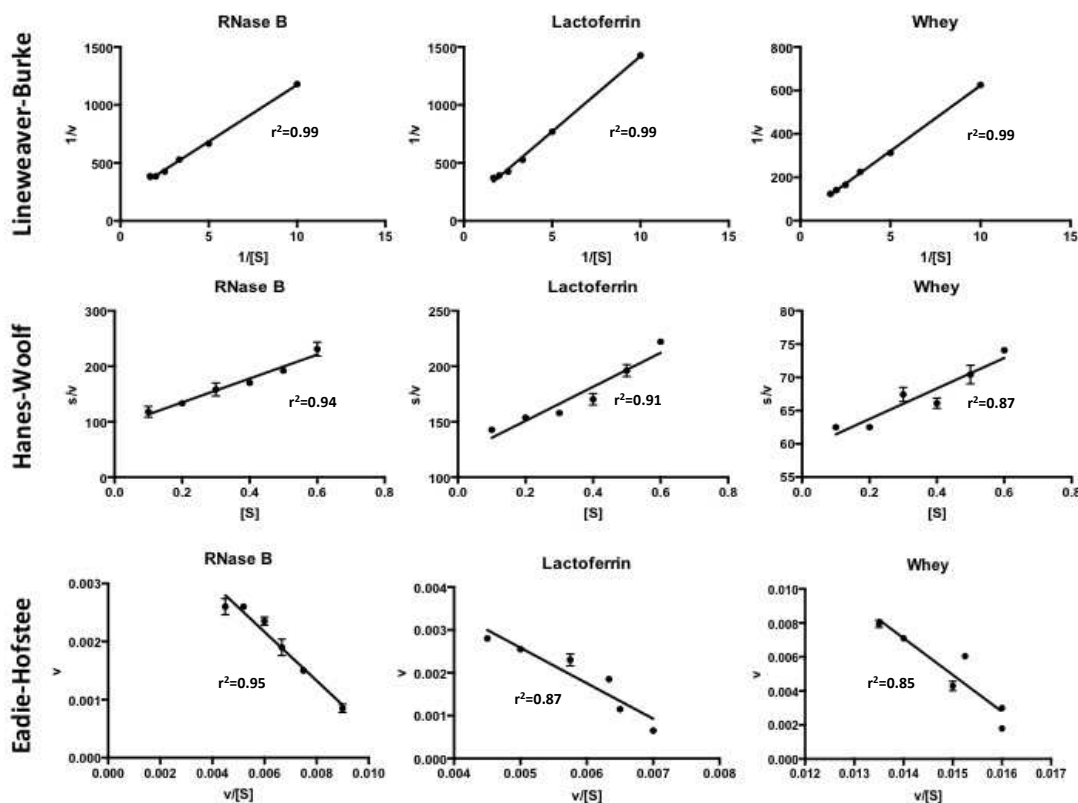


Figure 4. Lineweaver-Burke ($1/[S]$ (mL/mg) vs $1/v$ (mL*min/mg)), Hanes-Woolf ($[S]$ (mg/mL) vs s/v (min)) and Eadie-Hofstee ($v/[S]$ (min^{-1}) vs v (mg/mL*min)) linearized plotting techniques for the estimation of kinetic parameters of EndoBI-1 on RNase B, bLF and concentrated whey.

3.3 *N*-Glycan structures

Mass Spectrometry analysis of released *N*-glycan from RNase B, bLF and bovine colostrum whey protein concentrate after digestion by EndoBI-1 provides information on the glycosylation patterns for the three substrates as well as their potential bioactivity. Application of the bovine milk *N*-glycan library as a mass filter in Find by Formula allowed the identification of *N*-glycan compositions with high mass accuracy and retention time reproducibility [154]. Tandem MS analysis generated specific fragment ions that were common to all *N*-glycans allowing the confirmation of 5, 33 and 18 different oligosaccharide structures for RNase B, bLF and bovine colostrum whey protein concentrate, respectively. The details of all structures, including neutral and sialylated *N*-glycans, are described in the supporting information, (Table S1, S2 and S3).

RNase B is a well-characterized bovine glycoprotein that contains high mannose glycans with five to nine mannoses. Nano-LC-Chip–Q-TOF MS confirmed the presence of only high mannose *N*-glycans that are eluted between 11 and 17 min and are linked to the unique *N*-glycosylation site of the protein, ⁶⁰Asn [156]. The spectrum shown in Figure 5 illustrates the fragment pattern of a high mannose *N*-glycan with 1032.36 m/z, z=1. The spectrum shows a 162 Da difference between each peak that corresponds to the molecular weight of a mannose residue. Different high mannose isomers were resolved by nano-LC-Chip–Q-TOF MS for RNase B.

Figure 6 presents extracted compound chromatograms (ECCs) of bLF and bovine colostrum whey protein concentrate. Glycomics profiling demonstrates different patterns for these two substrates. Whey glycoproteins and pure bLF exhibit high-mannose *N*-glycans and complex/hybrid *N*-glycans, including fucosylated and sialylated *N*-glycans.

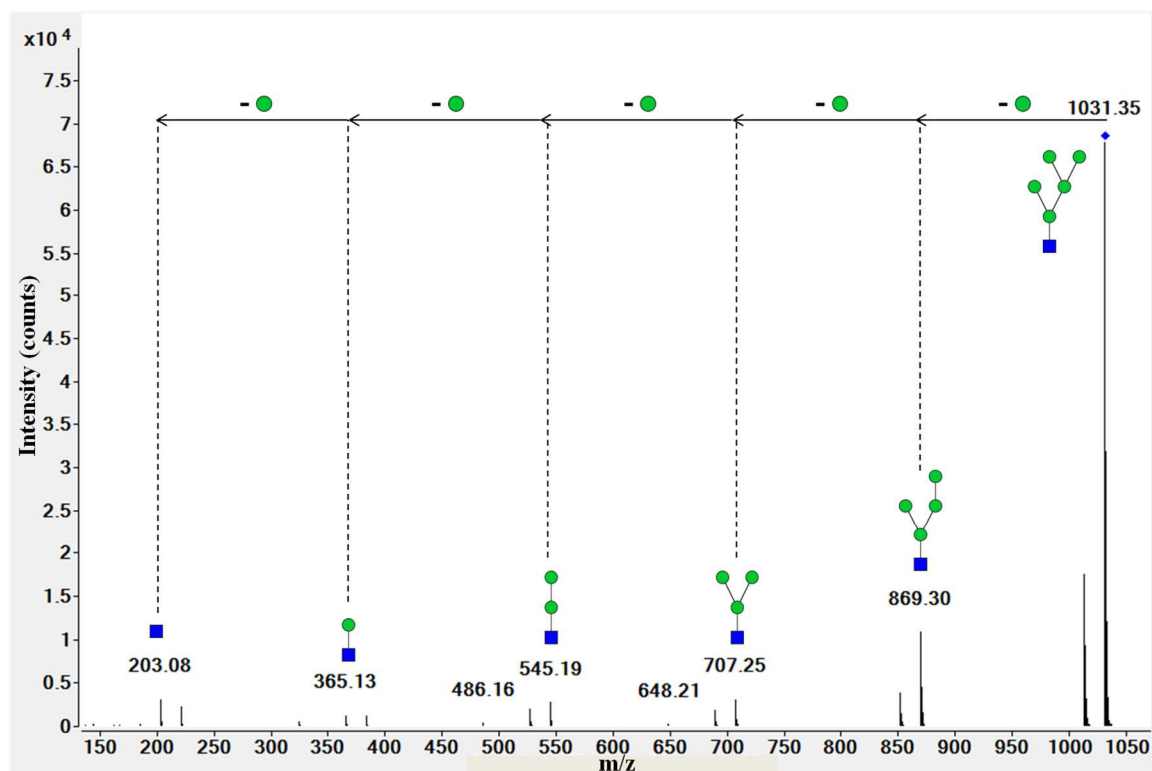


Figure 5. Deconvoluted tandem spectrum of high mannose *N*-glycans 5Hex-1HexNAc from RNase B. This glycan corresponded to m/z 1032.36 with $z = +1$. Green circles and blue squares represent mannose and HexNAc residues, respectively.

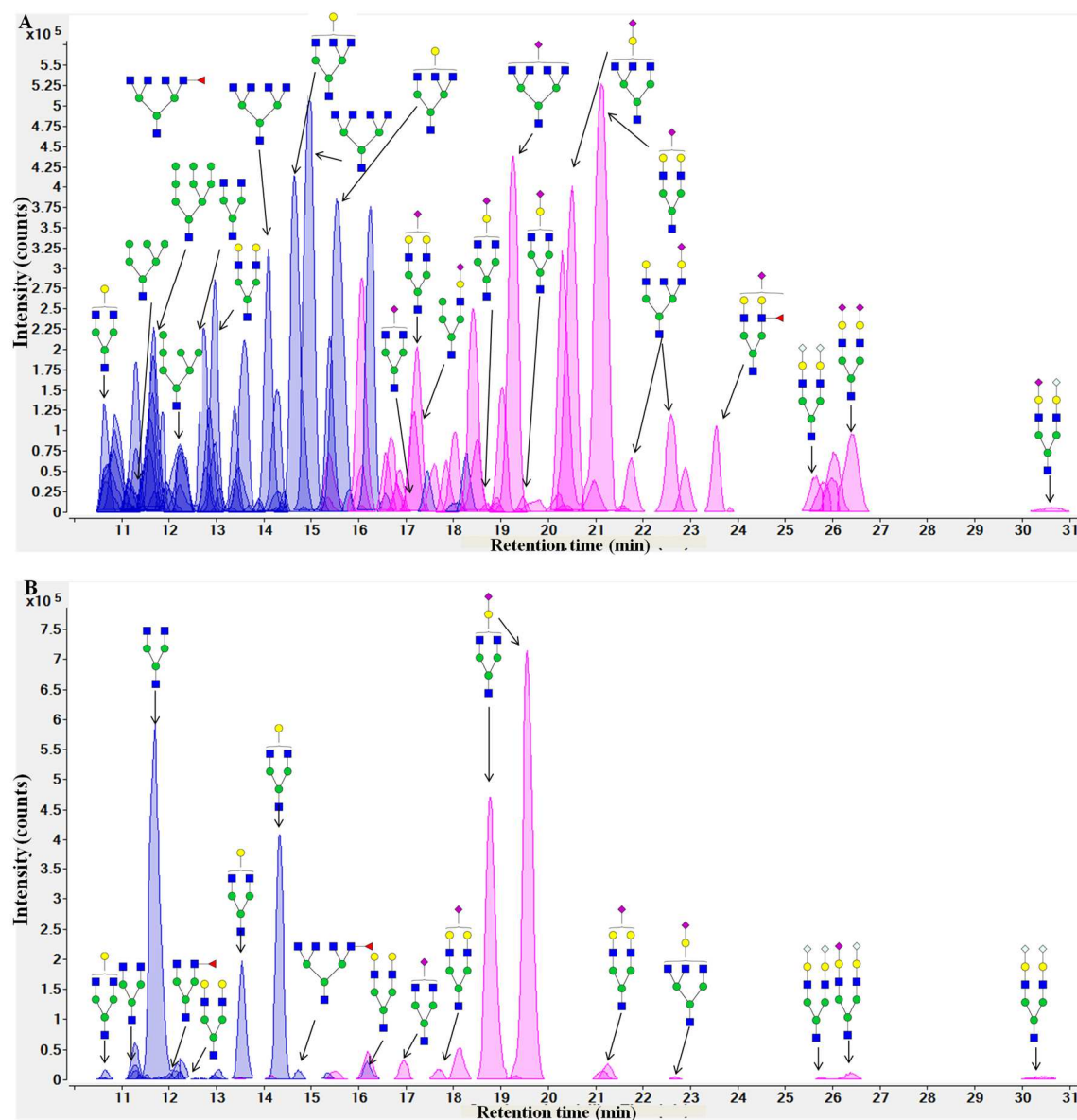


Figure 6. Extracted compound chromatograms (ECCs) of *N*-glycans from bovine lactoferrin (A) and bovine colostrum whey concentrate (B). The glycan types are differentiated by color: neutral glycans are purple and sialylated glycans are pink. Green circles, yellow circles, blue squares, red triangles, purple diamonds and gray diamonds represent mannose, galactose, HexNAc, fucose, NeuAc and NeuGc residues, respectively.

4. Discussion

In the present study, we characterize the kinetics of glycan release from model glycoproteins using a novel endo- β -*N*-acetylglucosaminidase, EndoBI-1, obtained from *B. longum subsp. infantis* ATCC 15697. Importantly, we also evaluated the ability of this enzyme to catalyze the conversion of a dairy waste stream, concentrated bovine colostrum whey prepared at the pilot-scale (UC Davis Milk Processing Lab), into a potential bioactive *N*-glycan source. Using this enzyme, we demonstrated *N*-glycan release from glycoproteins in whey with a maximum rate of 5.2×10^{-2} mg/mL min using 0.025 mg enzyme/mL.

We first showed that *N*-glycans are, in fact, released from both the purified model glycoproteins and the bovine colostrum whey protein concentrate using this novel enzyme. This demonstrates that the EndoBI-1 enzyme has the potential to release bioactive glycans from bovine-milk-related glycoproteins. A wider variety of *N*-glycans, including anomers and isomers, was released from pure bLF than from glycoproteins in bovine colostrum whey protein concentrate. This difference can be explained by the fact that whey is a complex fluid that contains *N*-linked as well as *O*-linked and nonglycosylated proteins, including α -lactoglobulin, α -lactalbumin, serum albumin bovine immunoglobulin and lactoferrin. Among these proteins, lactoferrin and immunoglobulins are some of the most abundant glycoproteins in bovine milk. [152, 157]. Non-glycosylated whey proteins, including α -lactalbumin (1.2 g/L) and α -lactoglobulin 3.2 g/L), are present at higher concentration than whey *N*-linked proteins like lactoferrin and immunoglobulins, [124]. These differences could explain why the *N*-glycan release by EndoBI-1 is more efficient on a pure glycoprotein than a complex mixture that contains lower relative amount of glycoproteins. It should be noted, however, that structure determinations were made using samples taken early on in the reaction corresponding to when

the reactions were still at their initial rate. It is possible that samples taken at a later stage of the reactions could yield a different distribution of released glycans.

To further characterize the reaction kinetics of EndoBI-1, we calculated the Michaelis-Menten kinetic parameters, V_{max} and K_m for two purified glycoprotein substrates and bovine colostrum whey protein concentrate, an example of a potential commercial substrate for this novel enzyme. Interestingly, there are significant differences in the kinetic parameters derived from different fitting techniques for each substrate. These results are consistent with other studies that compared the non-linear and linearized plotting techniques [158-160]. In general, non-linear plotting resulted in the underestimation of the kinetic parameters compared to the linearized methods. It is possible that more sophisticated methods for non-linear fitting [161] would improve the agreement between methods. However, the general trends in kinetic parameters for the three substrates are, in themselves, instructive and consistent between methods.

The lowest K_m was observed for RNase B while the highest K_m value was determined for whey. The lower apparent affinity of the enzyme for whey glycoproteins is explained by the heterologous nature of the substrate as described above. Because whey includes not only glycoproteins but also non-glycosylated proteins, this may result in the overestimation of the K_m value, or may also be a good indicator of the competitive inhibition that might be expected by non-substrate glycoproteins and non-glycosylated proteins [162, 163].

Although, RNase B showed the highest enzyme-protein affinity, it has the lowest V_{max} value compared to bLF and whey-derived glycoproteins. RNase B has a single high mannose *N*-glycosylation site that might result in low *N*-glycan release efficiency per amount of a given protein. While EndoBI-1 showed lower affinity for bLF than RNase B, it has a slightly higher V_{max} for bLF than for RNase B. This might be the result of the multiple and more complex *N*-

glycan content of bLF. The highest V_{max} value was observed for whey. The enzyme exhibited a 5-10 fold higher V_{max} on bovine colostrum whey protein concentrate than on RNase B and bLF depending on the fitting method used to calculate this parameter. Although, EndoBI-1 has *lower apparent affinity* value for whey glycoproteins, its high maximum reaction rate could be a result of a high biantennary *N*-glycan content of whey immunoglobulins. Immunoglobulins mainly contain biantennary *N*-glycans, while the model proteins we used contain mostly tri- and tetra-antennary-type *N*-glycans (Fig. 6) Because most of endo- β -*N*-acetylglucosaminidases prefer the high mannose and biantennary *N*-glycans to tri- and tetra-antennary-type *N*-glycans, EndoBI-1 digests the *N*-glycans of immunoglobulin efficiently, which results in a higher observed activity on whey.

The kinetic parameters of PNGase F for various glycoproteins have been investigated by several groups [24, 164, 165]. In general, it was reported that the denaturation of glycoproteins is essential for PNGase F activity. The K_m values of PNGase F for denatured glycoproteins are higher than EndoBI-1 values that we determined in this study. Moreover, lower V_{max} values observed in these studies compared to our findings indicate that EndoBI-1 is an efficient alternative enzyme that can be used for large-scale glycan production. The low V_{max} of PNGase F can be explained by its limited activity on core fucosylated *N*-glycans.

Successful release of these *N*-glycans from bovine milk glycoproteins, which were recently shown to be a new prebiotic source (submitted), by EndoBI-1, an enzyme active on all *N*-glycan cores, will enable further investigation of the biological and potential nutritional or therapeutic functions of bioactive glycans in bovine milk streams and lead to their larger-scale commercialization. However, a necessary step in this process is to fully characterize the enzymatic behavior and kinetics of the enzyme on a number of food substrates, including various

dairy streams. Moreover, the use of these enzymes to release bioactive glycans from dairy processing waste streams like whey would also make dairy processing more economically and environmentally sustainable.

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Chapter V

Selective prebiotic activity of N-glycans released from milk glycoproteins by novel endo- β -N-acetylglucosaminidase

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Summary

Milk, in addition to nourishing the neonate, provides a range of complex glycans whose construction ensures a specific enrichment of key members of the gut microbiota in the nursing infant, a consortium known as the Milk-Oriented Microbiome (MOM). Milk glycoproteins are thought to function similarly, as specific growth substrates for bifidobacteria common to the breast fed infant gut. However, the exact mechanism of this glycoprotein enrichment is unclear. Recently, a cell wall-associated endo- β -*N*-acetylglucosaminidase (EndoBI-1) found in various infant-borne bifidobacteria was shown to remove a range of intact *N*-linked glycans. It remains unclear if the released oligosaccharide structures can serve as a sole source for the selective growth of bifidobacteria. Here, EndoBI-1 was used to release *N*-glycans from concentrated bovine colostrum at the pilot scale. EndoBI-1-released *N*-glycans that supported the rapid growth of *Bifidobacterium longum* subsp. *infantis* while *Bifidobacterium animalis* subsp. *lactis*, a species which does not grow well on human milk oligosaccharides, did not exhibit significant growth on released milk *N*-glycans. Furthermore, mass spectrometry-based profiling revealed that *B. longum* subsp. *infantis* consumed 73% of neutral and 92% of sialylated *N*-glycans, while *B. animalis* subsp. *lactis* only degraded 11% of neutral and virtually no (<1%) sialylated glycans. These results provide mechanistic support that *N*-linked glycoproteins from milk serve as selective substrates for the enrichment of infant-borne bifidobacteria. Moreover, the variety of bioactive *N*-glycans released from bovine milk glycoproteins suggests that these released *N*-glycans may serve as novel prebiotic substrates with selective properties similar to those of human milk oligosaccharides.

Keywords: Bifidobacterium, endo- β -*N*-acetylglucosaminidase, EndoBI-1, Mass spectrometry, *N*-linked glycan

1. Introduction

Protein glycosylation is a common modification that adds a major dimension of complexity to protein structure [166] and has been linked to significant roles in protein functions, such as protein folding, biological recognition, and enzymatic protection [166, 167]. In eukaryotes, *N*-glycosylation or *O*-glycosylation is the two major types of glycosylation in which glycans can be attached to polypeptides. *N*-linked glycans are linked to a polypeptide at a specific asparagine while *O*-linked glycans occur at a serine/threonine residue. *N*-linked glycans have a standard consensus sequence of Asn-X-Ser/Thr while *O*-linked glycans do not have a common consensus sequence for attachment [166]. Monosaccharides that are components of glycoproteins include mannose, fucose, N-acetylglucosamine, sialic acid, galactose, and N-acetylgalactosamine [166]. High-mannose glycans, hybrid glycans, and complex glycans are different classifications of *N*-linked glycans [166]. The nearly infinite combinations of linkages, compositions, and structures provided by complex glycan synthesis can create a similarly diverse array of possible glycoprotein structures [166].

While the construction of glycoproteins may be critical for their function, for a variety of purposes, certain bacteria have been shown to have the ability to deglycosylate and degrade glycoproteins [168-172]. While this has been primarily described in pathogens acting against host defense glycoproteins in deglycosylating human IgG [173], RNaseB [170], or lactoferrin [174], it has also been described in cases when the release of these *N*-linked glycoconjugates serve as an energy source for the pathogen. *Streptococcus pneumoniae* has an endo-beta-*N*-acetylglucosaminidase, EndoD, that can act on asparagine-linked oligosaccharides [169], and *S. pneumoniae* has the capacity to grow on these substrates, but this growth is dependent on sequential degradation by exoglycosidases [175-178]. Commensal *Bacteroides* can degrade the

O-linked glycans in mucin and have the capacity to utilize the released host glycans, which gives them a fitness advantage *in vivo* [179, 180] and also enables these species to consume the free glycans found in milk [181], which are structurally and compositionally analogous to those found conjugated to proteins.

Milk, from many species, contains an abundant amount of complex glycans [182-184]. These glycans can be found free, as human milk oligosaccharides (HMOs), or as conjugated glycans, such as those found attached to proteins (glycoproteins) or lipids (glycolipids) [184]. Human milk oligosaccharides are composed of a pool of carbohydrates composed of diverse structures [185]. HMOs are constructed in a dizzying array of configurations from monosaccharides; glucose, galactose, N-acetylglucosamine, fucose, and N-acetylneuraminic acid. HMOs are clearly a factor that influences populations of beneficial microorganisms such as *Bifidobacterium* in the infant gut [184, 186]. Several *Bifidobacterium* are known to grow to high cell densities during *in vitro* growth on HMO as a sole carbon source [187-190], which mirrors observations of breastfed infants consuming human milk. Of these species of *Bifidobacterium*, only *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) possesses a large cassette of genes which render the subspecies uniquely able to grow to high cell densities on a broad array of HMOs as sole carbon sources, while many other bifidobacteria do not have this ability [191], or are able to consume only a limited diversity of structures [192]. In addition, several different strategies are employed by groups of *Bifidobacterium* to utilize HMO. *B. infantis* ATCC15697 is believed to import HMO intact and subsequently employ intracellular glycosyl hydrolases such as β -galactosidases, N-acetyl- β -D-hexosaminidases, α -fucosidases, and α -sialidases to deconstruct HMO [193-197]. In contrast, several other species of *Bifidobacterium* that do utilize a limited diversity of HMOs, utilize extracellular glycosidases to sequentially degrade these

structures and transport mono-, or di-, or oligosaccharides rather than the complex, compositionally diverse and often-branched structures which compose HMOs [192].

Similar to the diversity of structures of HMOs, many different types of glycoproteins are found in human milk. Given that the same cellular machinery used to synthesis HMOs also produces the structures, which comprise glycoprotein, this is not surprising. Mucins, secretory immunoglobulin A (sIgA), bile salt stimulated lipase (BSSL), lactoferrin, lactoperoxidase, butyrophilin, lactadherin, adiponectin, α -lactalbumin, lysozyme, β -casein, and κ -casein are some of the major glycoproteins found in human milk [198] and appear to vary across genotypes and protein isoforms. While HMOs have long been known to serve as prebiotic substrates for *Bifidobacterium*, it is unclear whether glycoproteins, owing to their structural similarity to HMOs can be similarly utilized.

Glycoconjugates, such as lactoferrin and the κ -casein derived glycomacropeptide, have been previously associated with the enrichment of bifidobacteria [171, 199-202] and strains of bifidobacteria have even been shown to grow on a structurally distant yeast mannoprotein as a sole carbon source [171]. In the case of glycoprotein enrichment of bifidobacteria, it remained unclear to what extent the glycan component and protein component are responsible for the enrichment [186]. The ability to deglycosylate or degrade glycoproteins has been demonstrated in select species of *Bifidobacterium* [171, 203] [204]. We recently showed that *B. infantis* ATCC15697 contains an endoglycosidase, EndoBI-1 (glycosyl hydrolase family 18), that has activity on major types of *N*-linked glycans found in glycoproteins and could release glycans from human lactoferrin and immunoglobulins [171].

Bovine milk glycans have similarities to human milk glycans [205-208]. We recently determined the optimal conditions and kinetic parameters of *N*-glycan release from concentrated

bovine whey for large-scale *N*-glycan production using EndoBI-1 [39, 176]. In this work, EndoBI-1 was used to release *N*-glycans from concentrated bovine colostrum whey. We recovered 80 mg of released *N*-glycans that was sufficient to carry out an investigation of *in vitro* growth by two species of *Bifidobacterium* on *N*-glycans, using one strain that grows well on HMO, *B. infantis*, and one that is unable to consume HMO, *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*). We measured expression of the EndoBI-1 in the presence of glycoproteins and compared this to deglycosylated controls, and the consumption of individual *N*-glycans using advanced mass spectrometry techniques (Nano-LC-Chip-Q-TOF MS), both of which indicated that EndoBI-1 plays a key role in the release of *N*-glycans from milk glycoconjugates which, in turn, facilitates the selective growth of competent *Bifidobacterium* strains.

2. Materials and Methods

2.1 Bacteria and media

Bifidobacterium longum subsp. *infantis* (*B. infantis*) ATCC 15697 and *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) UCD316 cultures were routinely propagated in Mann-Rogosa-Sharpe (MRS) broth (Becton Dickinson, Franklin Lakes, NJ) supplemented with 0.05% (w/v) L-cysteine at 37 °C under anaerobic conditions. *E. coli* strains Top10 (GeneTarget Inc, San Diego, CA, USA) and BL21* (Invitrogen, Carlsbad, CA) used for gene cloning and expression were propagated in Luria Broth (LB) under selective conditions (100 µg/mL carbenicillin).

2.2 Gene cloning, protein expression and purification

Gene amplification and protein expression in *E. coli* BL21* were performed as described by Sela et al. [192]. A pEcoTM-T7-cHis, Eco cloning Kit (GeneTarget Inc, San Diego, CA, USA) was used for gene cloning. Blon_2468 was PCR amplified from *B. infantis* ATCC15697 genomic DNA using a high-fidelity polymerase with the following primers: 5'-TTTGTACAAAAAAGCAGGCACCATGAATGCGGACGCCGTTTCTCC

GAC-3'&5'-TTTGTACAAGAAAGCTGGGTTGCCGGTCGCACTCAGTTGCTTCGG

-3'. Transmembrane domains and signal peptide were not amplified to facilitate protein expression and purification. *E. coli* BL21* containing the vector was grown for 3h at 37°C to reach a cell density of ~0.6 optical density (OD) at 600 nm. Protein expression was induced by the addition of IPTG to a final concentration of 0.5 mM and cells were grown at 37°C for an additional 6h. Bacteria were collected by centrifugation at 4,000 rpm (Eppendorf rotor A-4-81) for 20 min at 4°C. All subsequent steps for bacteria lysis were achieved at 4°C. All solutions were supplemented with a protease inhibitor cocktail (Roche, San Francisco, CA, USA). Cell pellets were incubated in 100 mL of BugBuster (Novagen, Billerica, MA, USA) for 10 min at 24°C. 200 µL of DNase I (Roche, San Francisco, CA, USA) and 100 µL of lysozyme (100 mg/mL) were added into the sample and the mixture was placed on ice for 30 min. Bacteria were centrifuged at 13,000 rpm (Eppendorf rotor model F45-24-11) for 30 min to remove cell debris.

Protein was purified by affinity chromatography using 5 mL prepacked Ni-charged columns (Bio-Rad, Hercules, CA, USA). All chromatographic steps were performed using an EP-1 model Econo Pump (Bio-Rad) and model 2110 fraction collector (Bio-Rad) at 5 mL/min flow rate. The column was equilibrated with 25 mL of a solution containing 300 mM KCl, 50 mM KH₂PO₄, 5 mM imidazole buffer (pH 8). 50 mL 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 KH₂PO₄, 5 mM imidazole buffer (pH 8) and 300 mM KCl, 50 mM KH₂PO₄ 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 1X using Bio-Gel P-30 in SSC buffer columns (Bio-Rad). Protein concentration was determined by using a Qubit Protein Assay Kit (Life Technologies, Grand Island, NY, USA). The purified enzyme was stored at -80°C.

2.3 Gene Expression

Biological triplicate cultures of *B. longum* subsp. *infantis* ATCC15697 were grown to OD=0.6 (early mid-log phase) on de Mann Rogosa Sharpe (MRS) medium containing lactose (2%) as a sole carbon source, harvested by centrifugation and washed in pre-warmed (37 °C) MRS containing no carbohydrates. Cells were pelleted and resuspended in the original volume of pre-warmed media containing lactose (2% w/v), treated whey protein, or untreated whey protein as sole carbon sources. After 2 hours anaerobic incubation at 37 °C, 2 mL aliquots of culture was harvested by centrifugation and immediately resuspended in an equal volume of RNALater (Ambion/Life Technologies Grand Island NY, USA) and frozen at -80 °C for later use. RNA was extracted using the RNAqueous kit according to the manufacturer's instructions, except cell lysis was preceded by a 15 minute incubation with lysozyme (50 mg/mL) and mutanolysin (120 U) at 37 °C, and a 5 minute incubation at 42 °C with 10uL of proteinase K

(Qiagen Valencia, CA USA). RNA was subjected to a DNase treatment using TURBOfree DNase (Ambion/Life Technologies Grand Island NY, USA) before RT-PCR.

2 μ L of DNase-treated RNA was used in a 20 μ L SYBR Mastermix (Applied Biosystems/Life Technologies Grand Island NY, USA) reaction containing 0.1 μ L of RNase inhibitor (Applied Biosystems/Life Technologies Grand Island NY, USA), 10 pmol of each primer, and 0.1 μ L of MultiScribe reverse transcriptase (Applied Biosystems/Life Technologies Grand Island NY, USA). Blon_2468 expression was measured via primers Blon_2468F (5'-ACCGGCAAGATCTACACAGC-3') and Blon_2468R (5'-GCACTCAGTTGCTTCGGTTG-3') and compared to expression of a tRNA-synthase using primers Bl_0301F (5'-CAACCGCCGCGATCTTC-3') and Bl_0301R (5'-CCAGCTGTGAAAGCAACGTGTT-3'), previously described for *B. longum* and used in studies of *B. infantis* expression [209], but were modified to account for a single base mismatch to *B. infantis* ATCC 15697. The RT-PCR reaction was preceded by a synthesis step (48 $^{\circ}$ C for 30 minutes, 95 $^{\circ}$ C for 10 minutes), followed by 40 cycles of 95 $^{\circ}$ C for 15 seconds and 60 $^{\circ}$ C for 60 seconds. A subsequent melting curve step was conducted to ensure amplification product specificity. Normalized expression was calculated as previously described [192], outliers removed by Grubb's test, and compared by a ratio-paired t-test relative to cells incubated on lactose using GraphPad Prism 6.

2.4 Pilot-scale concentration of bovine milk glycoproteins

Protein concentration from bovine colostrum whey was carried out using a pilot-scale cross-flow membrane system (Model L, GEA Filtration, Hudson, WI, USA). The system was composed of a 2.5" diameter spiral membrane housing (1-2 m² area), a 95 L jacketed stainless

steel feed tank, 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) (*Aspergillus oryzae* beta-galactosidase EC 3.2.1.23), seventy four liters of bovine colostrum whey were ultrafiltered in single batch with a 10 kDa molecular weight cut-off polyethersulfone spiral-wound membrane (effective area of 1.86 m²) ^{up} to a 5.4 concentration factor (concentration factor = volume of feed/ volume of retentate). Whey protein concentration was performed at a constant temperature of 40-43°C, transmembrane pressure of 3.0 bars, and 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. Simple sugars (e.g. residual lactose and the free monosaccharides derived from its hydrolysis) lack selective prebiotic activity and can be a confounding factor in all functional studies, so they were removed by diafiltration. Additionally, in order to test only the prebiotic activity of the new released *N*-glycans newly released from glycoproteins, we performed two discontinuous diafiltrations (by volume reduction) to increase the removal of free oligosaccharides from the ultrafiltration retentate.

2.5 *N*-glycan release

100 mL of concentrated bovine colostrum whey were incubated for 18 h at 37°C with 20 mg of EndoBI-1 in 20 mM Na₂HPO₄, pH 5. 700 mL of pure cold ethanol were added into the mixture to precipitate proteins. The mixture was incubated for 2h at -20 °C. The mixture was centrifuged at 4,000 rpm (Eppendorf rotor A-4-81) for 10 min at 4°C. The resulted supernatant containing the soluble released *N*-glycans was dried in a rotary evaporator (Heidolph 36000130

Hei-Vap Value Collegiate Rotary, Fisher Scientific, Waltham, MA, USA). Samples were rehydrated in 100 μ L of water, vortexed, sonicated for glycan quantification and purification.

2.6 Glycan quantification

A microplate colorimetric carbohydrate Assay (Biovision, Milpitas, CA, USA), was used to quantify the purified-glycans. Commercial mannose standard (0, 2, 4, 6, 8 and 10 μ L of a 2 mg/mL solution) were used to create a standard curve. The volume of each sample was adjusted to 30 μ L per well with water. 30 μ L of sample was used. 150 μ L of concentrated sulfuric acid (98%) were added to each well. Samples were mixed on a shaker for ~1 min and then incubated at 85°C for 15 min. After incubation, 30 μ L of Developer (provided by the manufacturer) was added to each well. Samples were again mixed on the shaker for 5 min. OD of each sample was measured at 490 nm. The OD was applied to the mannose standard curve linear function to calculate the quantity of carbohydrate in the sample.

2.7 Bacterial fermentation of *N*-glycans

B. infantis ATCC 15697 and *B. lactis* UCD316 colonies were cultured in MRS broth and incubated overnight at 37°C in an anaerobic growth chamber (Coy Laboratory Products). The resultant cultures were inoculated at 1% (v/v) into 100 μ L of reconstituted MRS, and supplemented with 2% *N*-glycan (w/v), 10% deglycosylated bovine whey (w/v) and 10% bovine whey as the sole carbohydrate (MRS_{*N*-glycan}) and overlaid with 25 μ L of sterile mineral oil in a 96-well microtiter plate to prevent evaporation. Cell growth was monitored in real time by assessing OD at 600 nm using a BioTek PowerWave 340 plate reader (BioTek, Winoosky, VT, USA) every 30 min, preceded by 15s shaking at variable speed. Three biological replicates were

performed for each sample. Once harvested, culture supernatants were centrifuged at 3000 x g for 15 min and filtered through a 0.22- μ m pore membrane (Millipore, Billerica, MA, USA) prior to storage at -80°C . An inoculated sample (control) with no carbon source was performed in parallel as a plate control.

2.8 Glycan purification for Nano-LC-Chip-Q-TOF MS

Recovered *N*-glycans from the bacteria supernatants and control samples were loaded on a C18 plate (Glygen, Columbia, MD, USA). The plate was conditioned using 3 x 100 μ L of 80% ACN containing 0.1% TFA in water, followed by 3 x 100 μ L of water. Samples were loaded and *N*-glycans were eluted with three volumes of water. The *N*-glycan solution was loaded on a PGC SPE (Porous Graphitic Carbon, Solid Phase Extraction) plate (Glygen, Columbia, MD, USA) previously optimized for the purification of free glycans. The plate was conditioned as described previously. After sample loading, wells were washed using 6 $\times$ 200 μ L of water and *N*-glycans were eluted using 3 $\times$ 200 μ L of 40% ACN containing 0.1% TFA in water. The enriched *N*-glycans fractions were dried overnight *in vacuum*. Samples were rehydrated in 50 μ L of water, vortexed, sonicated and diluted 50 times prior to mass spectrometry analysis.

2.9 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 x 75- μ m analytical column, both packed with 5 μ m 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 $\mu\text{L}/\text{min}$. *N*-glycan separation was performed on a 65-min gradient delivered by the nanopump at a flow rate of 0.3 $\mu\text{L}/\text{min}$. The 65-min gradient followed this program: 0% B (0.0–2.5 min), 0 to 16% B (2.5–20.0 min), 16 to 44% B (20.0–30.0 min), 44 to 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 450–3000 m/z for *N*-glycans in the positive ionization mode with an acquisition rate of 1 spectra/s for *N*-glycans. 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 100–3000 m/z . The collision energies correspond to voltages ($V_{\text{collision}}$) that were based on the equation: $V_{\text{collision}} = m/z (1.5/100 \text{ Da}) \text{ Volts} - 3.6 \text{ Volts}$; 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).

2.10 *N*-glycan identification

All compounds in the chromatograms were identified with MassHunter Qualitative Analysis software (version B.06.00 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 ≥ 1000 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

[117] using a mass error tolerance of 20 ppm. The *N*-glycans from the library were composed of hexose (Hex), HexNAc, fucose, NeuAc and *N*-glycolylneuraminic acid (NeuGc). The assignment of *N*-glycans was confirmed by tandem mass spectrometry.

Calculation of the relative abundance of *N*-glycans was performed by MassHunter Profinder software using the Batch Targeted Feature Extraction algorithm. A database was built containing the molecular formula, the mass and the retention time of identified *N*-glycans in the control sample. This library was used in combination with the Batch Targeted Feature Extraction algorithm. A minimum abundance of 1000 counts was used to filter out low-abundance compounds. Compounds were extracted allowing charge states of +1–3, mass error tolerance of 20 ppm and retention time tolerance of 1 min.

N-glycan consumption was calculated with respect to the uninoculated control by normalizing the summed abundance of neutral and sialylated *N*-glycans in ion count in the bacterial supernatant to that of the control using the following equation:

$$= \left[1 - \left(\frac{\sum_{i=1}^n \text{API bacteria sample}}{\sum_{i=1}^n \text{API control sample}} \right) \right] \times 100$$

where API is absolute peak intensity and *n* is the number of identified *N*-glycans. The relative amount was expressed in percentage of the total % of consumption.

3. Results

3.1 Release and purification of *N*-glycans from concentrated whey proteins

Release and purification of *N*-glycans was accomplished using EndoBI-1 as described in Figure 1. Briefly, EndoBI-1 was incubated with concentrated bovine colostrum whey in conditions previously determined to be optimum for enzyme activity (pH 5, overnight, 37 °C) [115]. This experiment was performed under non-denaturing conditions because the goal of this study was to produce the *N*-glycans in a native state and preserve their biological properties. Using this method, a total of 80 mg of *N*-glycans were released from 40 mL of concentrated bovine colostrum whey. A description of the released *N*-glycan compositions is shown in Table 1. Notably, four of the compositions contained fucosylated oligosaccharides, and their detection extends the previous identification of fucosylated oligosaccharides within the bovine milk glycome to *N*-linked glycoproteins [42].

Table 1. Details of released *N*-glycans: neutral mass, monosaccharide composition, glycan type, and whether or not the composition remained in the supernatant of the control sample, *B. lactis* UCD316 and *B. infantis* ATCC 15697 samples. HexNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid, NeuGc; *N*-glycolylneuraminic acid. +: presence, -: absence.

Mass	Composition					Glycan type	Consumption phenotype		
	Hexose	HexNAc	Fucose	NeuAc	NeuGc		Control	<i>B. lactis</i>	<i>B. infantis</i>
								UCD316	<i>ATCC15697</i>
1113.408	3	3	0	0	0	Neutral <i>N</i> -glycan	+	+	+
1275.460	4	3	0	0	0		+	+	+
1421.547	4	3	1	0	0		+	+	-
1437.525	5	3	0	0	0		+	+	+
1519.573	3	5	0	0	0		+	-	-
1665.632	3	5	1	0	0		+	+	-
1404.509	3	3	0	1	0	Sialylated <i>N</i> -glycan	+	+	+
1525.543	5	2	0	1	0		+	+	-
1566.563	4	3	0	1	0		+	+	+
1728.627	5	3	0	1	0		+	+	+
1744.620	5	3	0	0	1		+	+	-
1769.668	4	4	0	1	0		+	+	-
1810.660	3	5	0	1	0		+	+	-
1874.687	5	3	1	1	0		+	+	-
1890.686	5	3	1	0	1		+	+	-
1931.713	5	4	0	1	0		+	+	-
2035.737	5	3	0	1	1		+	+	-
2051.725	5	3	0	0	2		+	-	-

3.2 Growth of *B. infantis* and *B. lactis* on whey, deglycosylated whey proteins, and N-glycans released from whey

Previous work has shown a bifidogenic effect upon ingestion of milk glycoproteins [200, 201, 210]. While milk glycoprotein-derived peptides have been implicated in bifidogenic responses, [211] it is unclear if this effect is a result of the catabolism of the protein itself, or the associated glycan (or both), of these glycoconjugates. To test this, we examined growth of *B. infantis* ATCC 15697 and *B. lactis* UCD316 on 10% whey glycoprotein concentrate, where total glycan concentrations match those used in a previous study [176]. *B. infantis* ATCC15697 grows well on HMOs [187, 188, 212] while *B. lactis* UCD316 does not grow when HMO is provided as a sole carbon source [213]. As shown in Figure 2A, *B. infantis* readily grew to a high cell density on released N-glycans while no growth occurred on 10 % deglycosylated whey protein concentrate. Intact whey protein concentrate supported moderate growth of *B. infantis*. In contrast, none of the substrates tested supported vigorous growth of *B. lactis* UCD316.

To determine how EndoBI-1 expression is related to substrate availability, given the growth of *B. infantis* on whey protein concentrate, we measured relative expression of Blon_2468, the gene encoding the EndoBI-1 endoglycosidase on these substrates. We found that its expression was significantly upregulated during incubation on untreated whey, but not when the whey had been deglycosylated by incubation with EndoBI-1 prior to incubation, or in the presence of lactose (Figure 5).

Glycan Production Process

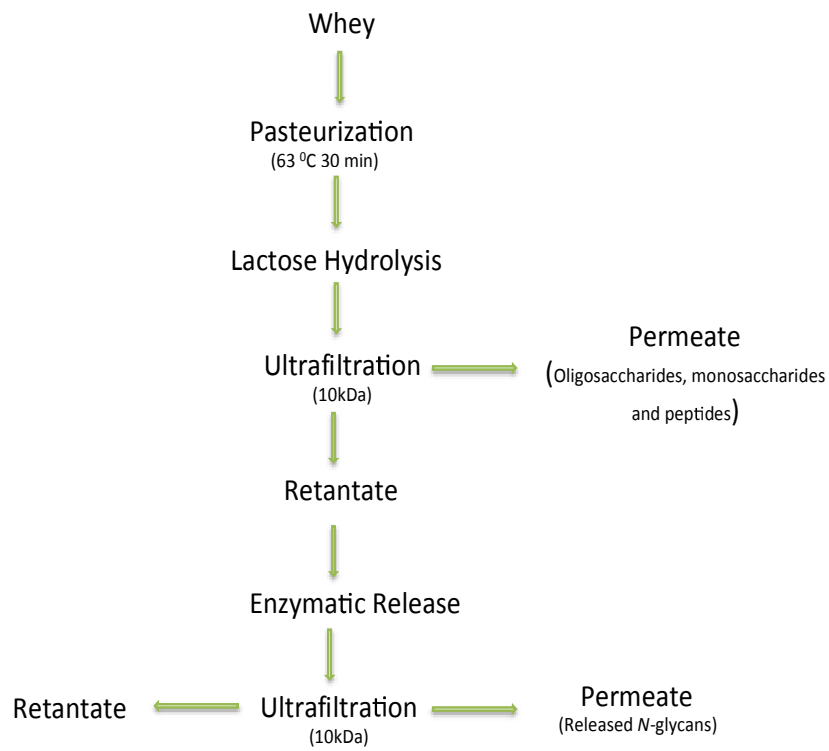


Figure 1: Diagram of methods involved release and purification of N-glycans using purified EndoBI-1. EndoBI-1 was cloned and expressed in *E. coli*. Purified EndoBI-1 was incubated with bovine colostrum whey to release N-glycans.

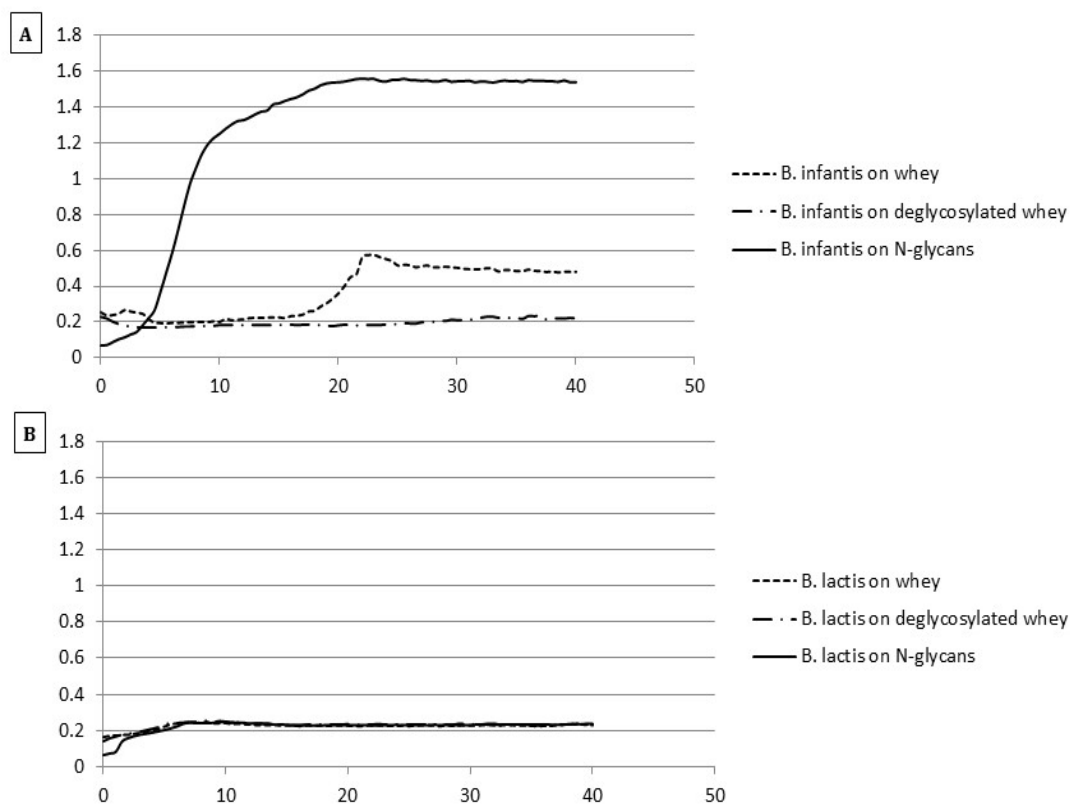


Figure 2. Representative growth curves of *B. infantis* and *B. lactis* on whey, deglycosylated whey, and *N*-glycans. *Bifidobacterium* species were grown in MRS medium supplemented with 2% of *N*-glycans (w/v) as the sole carbon source. The growth responses of each sample were measured by determining the optical density at 600 nm (OD₆₀₀). Growth curves were performed in biological triplicates.

3.3 Glycoprofiling of *N*-glycans consumption by *B. infantis* and *B. lactis*

To determine if there was any preferential consumption of specific *N*-glycan compositions mass spectrometric-based glycoprofiling was performed on culture supernatants. Supernatants were collected after 96 hours of growth on *N*-glycans and residual *N*-glycans from purified by solid phase extraction and profiled by nano-HPLC-chip/TOF MS. The separation of *N*-glycans was performed on a microchip packed with porous graphitized carbon, which enabled isomer-level separation with reproducible retention times. Tandem MS analysis generated specific fragment ions common to all *N*-glycans and allowed confirmation of *N*-glycans compositions, including both neutral and sialylated *N*-glycans. For this study, we specifically profiled bifidobacterial consumption of 6 neutral and 12 sialylated *N*-glycans compositions. Figure 3 shows the extracted compound chromatograms of *N*-glycans obtained from the *B. infantis* and *B. lactis* growth supernatants as well as an uninoculated control. This analysis revealed the presence of 18, 16 and 6 *N*-glycans in the uninoculated, *B. lactis* and *B. infantis* samples, respectively. The majority of the *N*-glycans in the uninoculated control are also present in the *B. lactis* sample with similar peak area suggesting minimal consumption of *N*-glycans by *B. lactis*. Conversely *B. infantis* consumed the majority of the *N*-glycans present. The presence and absence of *N*-glycans in the three samples are presented in Table 1.

The data reveal that *B. infantis* consumed 73% of neutral and 92% of sialylated *N*-glycans, whereas *B. lactis* degraded 11% of neutral and only 0.9% of sialylated *N*-glycans (Supplementary Figure 1). The relative abundance of each *N*-glycan composition is presented in Figure 4 for inoculated sample and for *B. infantis* and *B. lactis* samples. The graphs reveal that the majority of *N*-glycans were nearly entirely consumed by *B. infantis*, except for the neutral *N*-

glycan 3Hexose-3HexNAc. Notably, three *N*-glycans (3Hexose-5HexNAc, 4Hexoses-3HexNAc-1Fucose and 5Hexoses-3HexNAc-2NeuGc) could not be detected after incubation with *B. lactis*.



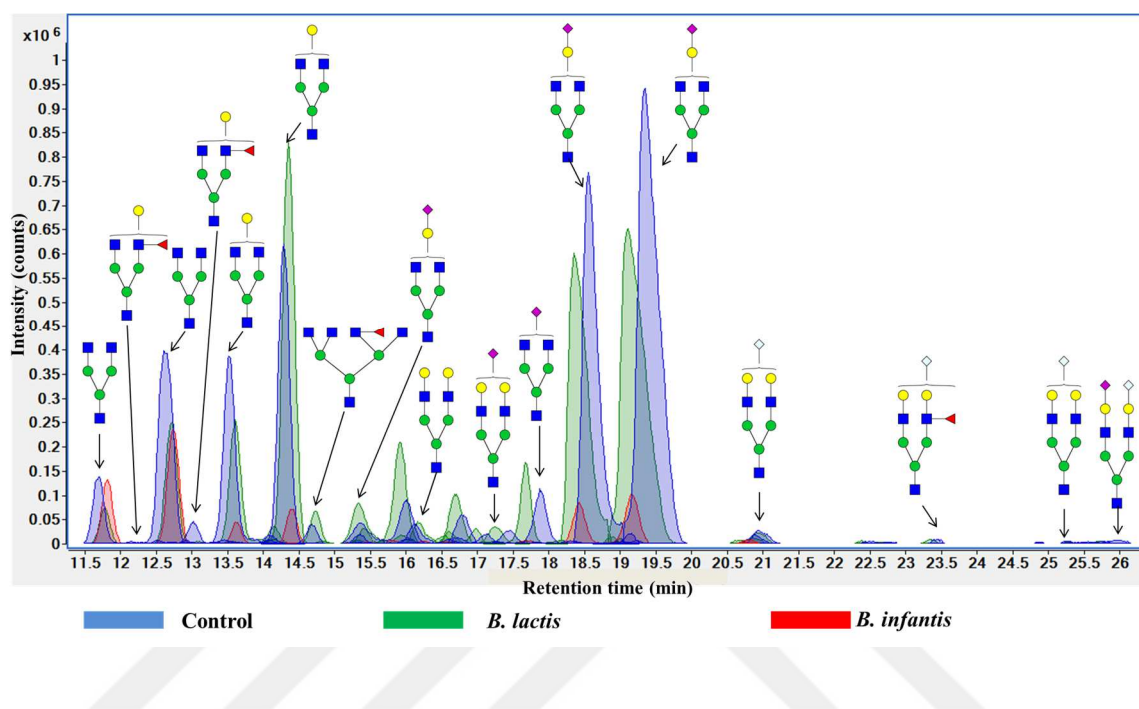


Figure 3. Nano LC Chip Q-ToF extracted compound chromatograms (ECCs) of *N*-glycans from bovine colostrum whey concentrate remaining in media after bacterial fermentation (blue: uninoculated control, green: *B. lactis*, red: *B. infantis*). Green circles, yellow circles, blue squares, red triangles, purple diamonds and gray diamonds represent mannose, galactose, HexNAc, fucose, NeuAc and NeuGc residues, respectively.

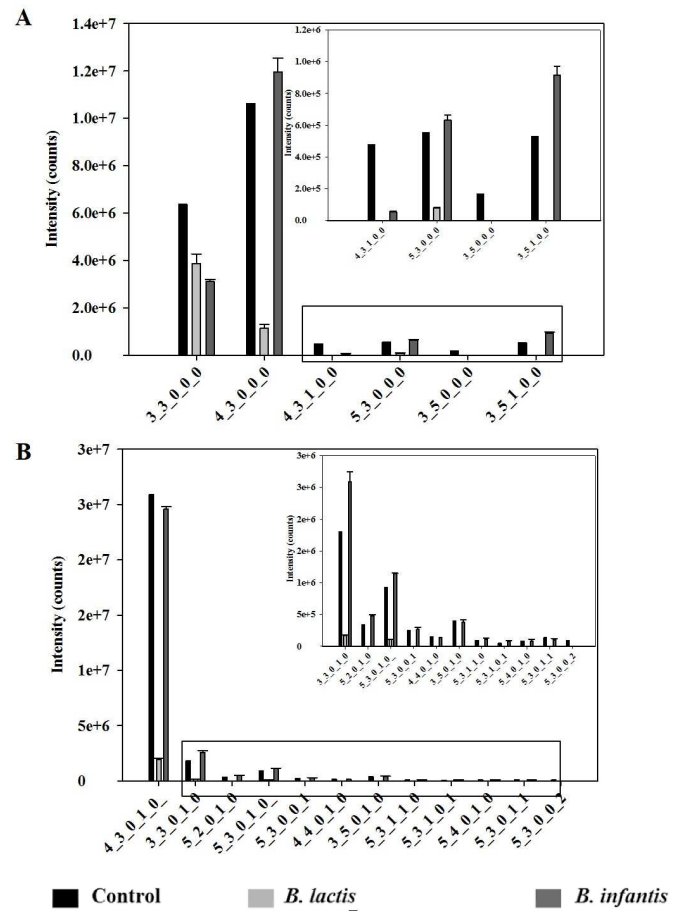


Figure 4. Glycoprofiling of *N*-glycans extracted from media after incubation with or without *Bifidobacterium* species. (A) Neutral *N*-glycans. (B) Sialylated *N*-glycans. Monosaccharide compositions are denoted as Hexose_HexNAc_Fucose_NeuAc_NeuGc.

Blon_2468 Expression is Significantly Increased On Incubation With Untreated Whey

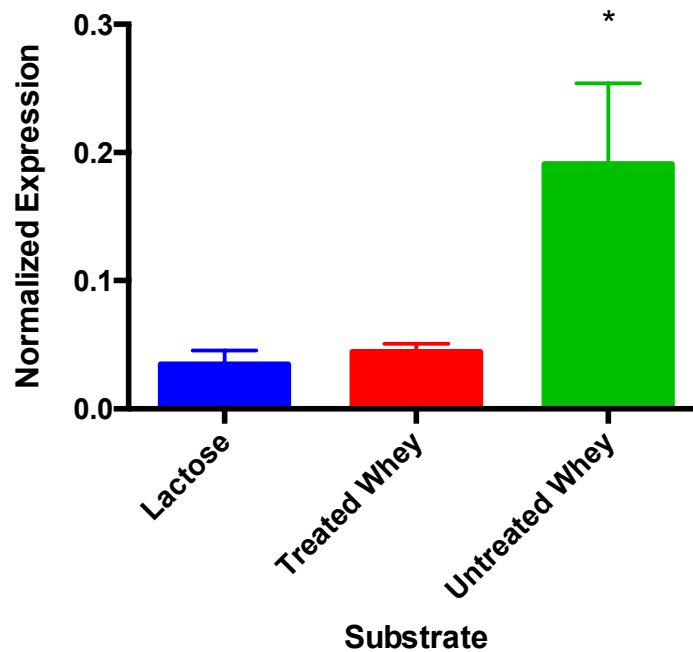


Figure 5. Normalized gene expression levels show a significant increase in Blon_2468 (EndoBI-1) expression when incubated on whey protein concentrate, but not when the same compound was pre-treated with EndoBI-1, to remove complex glycans from the underlying protein skeleton.

4. Discussion

Previous work identified EndoBI-1 as a possible mechanism by which *B. infantis* utilizes milk glycoproteins for growth, however it remained unclear if the oligosaccharide substrates released by EndoBI-1 could serve as a growth substrate. To examine this, purified EndoBI-1 was used to release a diversity of bioactive *N*-glycans from whey glycoproteins at lab scale. *B. infantis* clearly showed an ability to consume these released *N*-glycans as a sole carbon source, growing to a high cell density thus mimicking the strong growth of *B. infantis* on HMO. In contrast, *B. lactis* did not readily consume released *N*-glycans as a sole carbon source, which is not surprising considering that the strain is unable to consume structurally analogous HMOs and lacks a homolog to EndoBI-1 [91].

Only a select assortment of infant-associated strains of *Bifidobacterium* such as *B. infantis*, *B. breve*, and *B. bifidum* have the ability to grow to high cell densities on HMO as a sole carbon source [187-189]. *B. infantis* is believed to import HMO intact and subsequently use intracellular glycaosyl hydrolases to deconstruct HMO. *B. infantis* contains an array of family 1 solute binding proteins that bind to HMO and are associated with the import of HMO [193]. Furthermore, *B. infantis* has a full suite of transporters and intracellular α -fucosidases, and α -sialidases, β -galactosidases, N-acetyl- β -D-hexosaminidases whose activity on HMO has been described [194-197]. Recent work has also suggested that *Bifidobacterium breve* consumes HMO in a similar manner to that of *B. infantis* involving the import of intact HMO and subsequent intracellular deconstruction [190], in contrast the to extracellular degradation of HMO by *B. bifidum*.

In this work, we demonstrate that not only are milk protein glycoconjugates decorated with glycan structures analogous to HMOs, but that these structurally analogous glycans have a

similar bioactive potential; that is, they selectively enrich for infant-associated taxa such as *B. infantis* but not *B. animalis*. While glycoprotein degradation strategies in *Bifidobacterium* have been examined among several species of *Bifidobacterium*, and EndoBI-1 homologs have been identified and characterized from strains of *B. longum* subsp. *longum*, *B. breve* and others [171], here we show that this enzyme is a critical component of accessing a large pool of complex carbon structures inaccessible to other bacteria when conjugated to milk proteins.

Compared to the role of HMOs in shaping the distal gut of infants, which has been described extensively [214], the role of glycoconjugates has been relatively unexplored. Here, we show that glycoproteins in milk provide a possible additional energy source for competent gut bacteria, that may not have been considered in terms of the amount of complex bioactive glycans found in milk [42]. Additionally, the nature of the specific enrichment of these glycans, once freed, suggests that glycoconjugates in milk may also play a key role in shaping the pools of available carbon sources in the distal gut, and that these released glycans emerged as a way to favor the growth of coevolved species of gut microbes, such as *B. infantis*, over less specific colonizers, who are unable to break the complex and varied linkages found in these complex molecules [65], similar to the role of diversity and complexity in the structure of free HMOs.

While synthetic commercial production of HMO-like structures can have challenges due to the complexity of HMO [184], and despite differences between the glycan composition of bovine milk and the glycan composition of human milk [184, 207], we demonstrate here that these selective, bioactive milk glycans can be produced in at least a small scale from a low cost substrate. While previously bovine milk was considered to be a poor source of complex milk oligosaccharides [207], here we show that bovine colostrum whey can be treated to release a significant amount of complex milk glycans, whose biological activity is intact. Future

strategies that utilize existing products with demonstrable safety and low substrate cost may be an attractive alternative to chemical synthesis of complex HMO mimics.

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Supplemental data

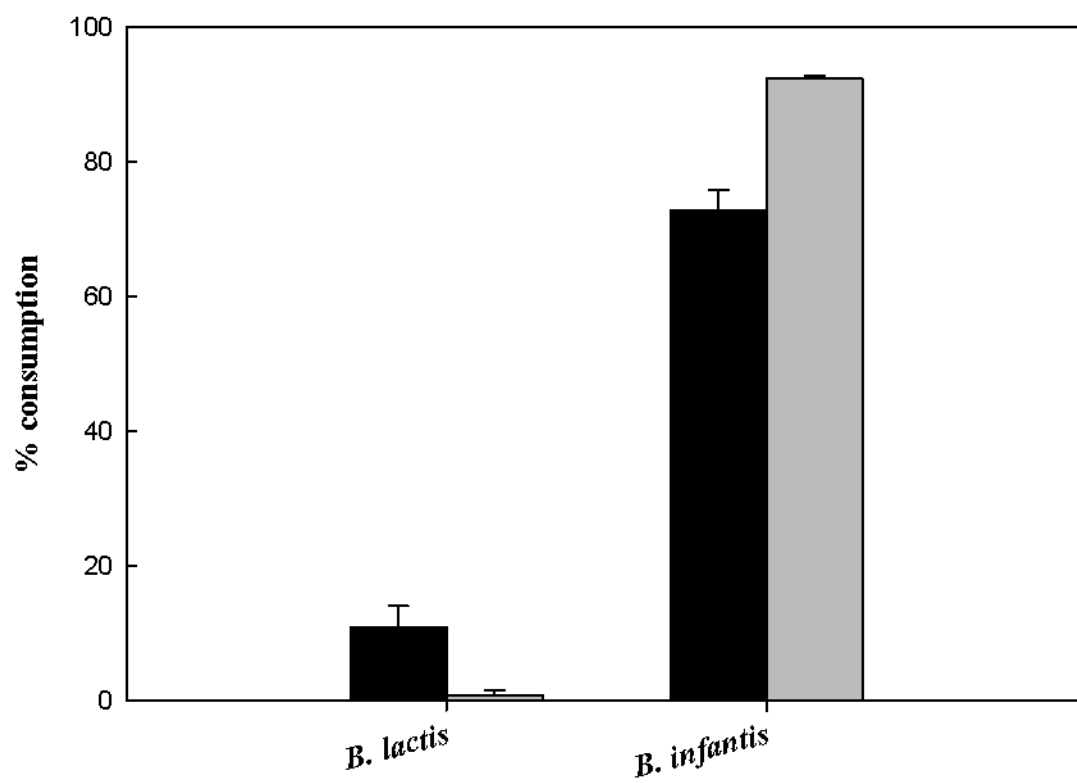


Figure S1. Glycoprofiling of *N*-glycan consumption by *B. lactis* and *B. infantis*. (A) Consumption is calculated based on Equation 1. Black: neutral *N*-glycans, grey: sialylated *N*-glycans.

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Chapter VI

Conclusions and Future Directions

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Human milk oligosaccharides (HMO) are known to promote the development of the infant intestinal microbiota, in particular enriching a beneficial bifidobacterial population [130, 131], which provides health benefits including anti-adhesive antimicrobial activity, positive modulation of intestinal epithelial cell responses, immune modulation, protection against necrotizing enterocolitis and stimulation of brain development [142-146]. Unfortunately, these critical human milk oligosaccharides are not available to formula fed infants and there have been intensive efforts to identify HMO alternative molecules to fill this gap.

Bovine milk contains oligosaccharides similar to HMOs [147], however the concentration of these oligosaccharides (bovine milk oligosaccharides, BMOs) in fluid milk is 20-fold lower than HMOs in human milk—a fact that limits their usage commercially [101, 148]. Alternatively, bovine milk glycoconjugates such as glycoproteins might be considered as an additional source of bovine glycans with desirable bioactivities. While the structure of glycoproteins may be critical for their function for a variety of purposes, certain bacteria have the ability to deglycosylate and degrade glycoproteins utilizing the released glycans as a carbon source [168-172]. However, it is unclear to what extent the glycan component and protein component are responsible for the bacterial growth. Bovine whey, an abundant by-product of the cheese-making process, is rich in these glycoproteins. It contains ~4.5 g of glycoproteins per L [38], and because half of the total whey (190,000,000 ton/year) is not currently utilized by the food industry, it can be considered as a promising source of milk glycans. The use of whey as a source of bioactive glycans may also improve the sustainability of the dairy industry, which is currently faced with the problem of disposing of excess of high-carbon whey.

Previous studies in the Mills lab showed that an endo- β -*N*-acetylglucosaminidase (EndoBI-1) isolated from *Bifidobacterium longum* subsp. *infantis* ATCC 15697 has the ability to cleave the *N*-*N'*-diacetyl chitobiose moiety found in the *N*-glycan core of high mannose, hybrid and complex *N*-glycans [115]. In this dissertation, I showed that this novel enzyme can be used to recover glycans from milk glycoproteins at pilot scale and I tested their consumption by bifidobacterial strains. Then, I showed that the utilization of these recovered glycans suggest that they can be considered as HMO alternatives and can be produced in large quantities using bovine whey as a substrate.

2. EndoBI-1 is an effective tool for deglycosylation

A key finding of this dissertation is that EndoBI-1 is an efficient tool that can be utilized to release *N*-glycans from bovine whey glycoproteins, whereas commercially available enzymes currently cannot to the same extent. Peptidyl-*N*-glycosidases (PNGases) are commonly used for deglycosylation of glycoproteins enzymatically [24]. A PNGase (peptide- N^4 - (N-acetyl- β -glucosaminyl), glycopeptidase, N-glycanase, glycoaminidase or N-glycosidase) removes asparagine-linked glycans by hydrolyzing the amide side chain [59, 60]. Although, commercially available PNGases release *N*-linked glycans irrespective of their size or charge [61-63], if fucose is α -linked to *N*-acetylglucosamine, the *N*-glycans are resistant to hydrolysis by PNGase F [64]. Importantly, our EndoBI-1 isolated from *Bifidobacterium longum* subsp. *Infantis* ATCC 15697 has the ability to cleave the *N*-*N'*-diacetyl chitobiose moiety found in the *N*-glycan core of high mannose, hybrid and complex *N*-glycans and is not affected by fucosylation of the *N*-glycan core [65]. Moreover, in chapter 2, we showed that it is active both on native glycoproteins and during pasteurization conditions. These characteristics enable us to produce *N*-glycans in large scale.

3. Bovine whey is a good source of glycans

Another key development of this dissertation was demonstrating that whey, a by-product of the cheese making process, can be used as an abundant glycan source in terms of its availability and wide variety of glycoprotein diversity. In chapter 2, I showed that EndoBI-1 is an efficient tool to release milk glycans from whey glycoproteins. Given that whey is the liquid part of milk that separates from the curd following acid or enzymatic coagulation, and has traditionally been considered a by-product of the cheese industry, these results show a promising future for its use as a source of novel bovine milk glycans. In chapters 3 and 4, I showed that bovine whey proteins can be used as a rich source of milk glycans. Using advanced mass spectrometry, I showed that a wide variety of *N*-glycans are released from bovine milk glycans and that they can be released efficiently by EndoBI-1.

4. Specific N-glycan production by EndoBI-1

Not only can these bioactive glycans be released by EndoBI-1, as I demonstrated in Chapters 2, 3, and 4, but optimized parameters can be applied to the reaction conditions to specifically release different structures of glycans from milk glycoproteins. Specifically, we can manipulate the reaction conditions to favor sialylated glycans from milk glycoproteins.

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 [88]. Other studies suggest that sialic acid is an essential monosaccharide for brain development and learning [89]. These glycans reduce the binding of leukocytes to endothelial cells while the neutral glycans do not have any effect [139]. Recombinant human lactoferrins, expressed in *Pichia pastoris*, with or without sialylated glycan structures, provided

evidence that *N*-acetylneuraminic acid is involved in the immune function of these lactoferrins[90].

In Chapter 3, I demonstrated that advanced enzyme kinetic analyses could be used to identify conditions under which different glycans could be released from identical starting glycoprotein substrate.

5. *B. infantis* utilizes released N-glycans

Glycoconjugates, such as lactoferrin and the κ -casein derived glycomacropeptide, have been previously associated with the enrichment of bifidobacteria [171, 199-202] and strains of bifidobacteria have even been shown to grow on a structurally distant yeast mannoprotein as a sole carbon source [171]. In the case of glycoprotein enrichment of bifidobacteria, it remained unclear to what extent the glycan component and protein component are responsible for the enrichment [186]. In chapter 5, I used EndoBI-1 to collect glycans from concentrated bovine whey glycoproteins and test the utilization of these glycans by *B.infantis*. I found that *B.infantis* can grow on released *N*-glycans and concentrated bovine whey. However, *B. infantis* did not grow on bovine whey pre-treated with EndoBI-1 (*N*-glycans were removed). Moreover, I also showed that EndoBI-1, encoded by Blon_2468, was expressed on bovine whey, but not on bovine whey from which glycoconjugates had been removed. These findings suggest that *B.infantis* uses EndoBI-1 to cleave to *N*-glycans from glycoproteins to consume the released glycans as a sole carbon sole. We also demonstrated that the utilization of released individual *N*-glycans is highly specific, which suggests that the conditions under which EndoBI-1 is used is critically important to consider when producing these glycans, as the products of different reaction conditions shape the bioactivity of the released glycans.

6. Future Directions

This work suggests a novel enzyme EndoBI-1 for deglycosylation of glycoproteins that has many advantages over existing commercial enzymes and chemical deglycosylation methods that will enable the production of these bioactive glycans at a large scale. Perhaps most importantly, this enzyme can be integrated into existing dairy processing protocols, due to its heat stability and activity on a wide variety of glycan structures, and native protein structures. The simplicity of this scheme to produce bovine milk glycans is an attractive addition enabling the recovery of bioactive compounds from existing waste streams.

The production of these released glycans will enable further investigation into their biological functions. The work presented here allows for the production of structurally specific, and differentially bioactive, glycans from whey, facilitating the use of these compounds in biomedical research. Future work using other enzymes active on similar glycoconjugates such as O-linked glycans, to obtain full glycoconjugate cleavage from bovine milk proteins further extends this work to other glycoconjugate substrates.

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