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Original Research

Selective deglycosylation of lactoferrin to understand glycans' contribution to antimicrobial activity of lactoferrin

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Abstract: Lactoferrin is a highly glycosylated antimicrobial protein that contains multiple glycan types. In this research, recombinantly produced three forms of novel endo-*β*-*N*-acetylglucosaminidase (free, genetically attached Glutatiohine-S-transferase and polyhistide) were used for selective release of lactoferrin glycans to understand the contribution of specific glycan types to the antimicrobial function of lactoferrin. Three lactoferrin forms with different glycan profile were obtained by treatment with these fusion tagged enzymes; native, fully deglycosylated and sialylated glycan enriched lactoferrin. The released glycan structures were analyzed and confirmed with mass spectrometry. The results showed that native and sialylated glycans enriched lactoferrin have similar minimum inhibitory concentration (MIC) values against *E.coli DH5a* (1 mg/ml), whereas the MIC value for fully deglycosylated lactoferrin was 6mg/ml. These results suggest that sialylated glycans play important role in the antimicrobial function of lactoferrin.

Key words: Glycans; Endo-*β*-*N*-acetylglucosaminidase; Lactoferrin; Antimicrobial activity.

Introduction

Glycans exist as freely or bound to the other structures such as proteins in nature. N-linked glycans (N-glycans) are attached (where a specific amino acid sequence aspragine (AsN)-X-serine (Ser)/threonine (Thr) exists) to an asparagine of glycoproteins with an N-acetylglucosamine monosaccharide (HexNAc) (1). N-glycans share a five monosaccharide long core that contains 2 HexNAcs and extended by 3 mannoses. Further extension of the glycan core determines the type of N-glycans. Elongation of the core with only mannose residues forms high monnaose type glycans, where as the attachment of other monosaccharides such as N-acetylglucoseamine, fucose, galactose and sialic acid produce complex of hybrid type N-glycans (Figure 1). Common N-glycosylated milk proteins are immunoglobulins (IgA, IgG and IgM) and lactoferrin with multiple potential sides in human and other mammalian milks (2, 3).

The biological roles of free or attached glycans can be divided into two main aspects; structural contributions and modulatory characteristics (4), but the exact mechanism of the role has not fully discovered yet. These small chain carbohydrates play active role in many cellular mechanisms, which are closely linked to health and disease. Barboza et al. demonstrated that the attached glycans provide a protection against most bacteria and viruses for the host (5). A recent study also suggest that these compounds inhibit *P. aeruginosa*, which is responsible for epithelial cell invasion and bacterial keratitis (6). Moreover, a study on glycans role in microbe-cell connection showed that microbes interact with lectins through the glycans found on the cell membrane (7). As well as glycans' contribution to the folding and conformation of proteins, they increase the antigenicity, solubility and protein resistance to proteolysis (8). In addition to the mutual roles of glycans, some individual glycans have unique functions. Among these, sialylated glycans are extremely attractive for scientist since they play important role in brain development and learning skills (9). These compounds are also shown to mimic surface glycans that pathogens target for invasion, so the pathogens are no longer able to bind the cells (10).

Lactoferrin is a highly glycosylated protein that contains multiple biological roles including bifidogenic, antimicrobial, immunomodulatory, anti-inflammatory and anticarcinogenic activity (11-13). It is proposed that the antimicrobial activity of lactoferrin is based on two mechanisms; direct interaction with cells and depletion of iron ions (14-16). Although lactoferrins from different mammalians have different biological roles, they have very similar amino acid sequence. Therefore, the

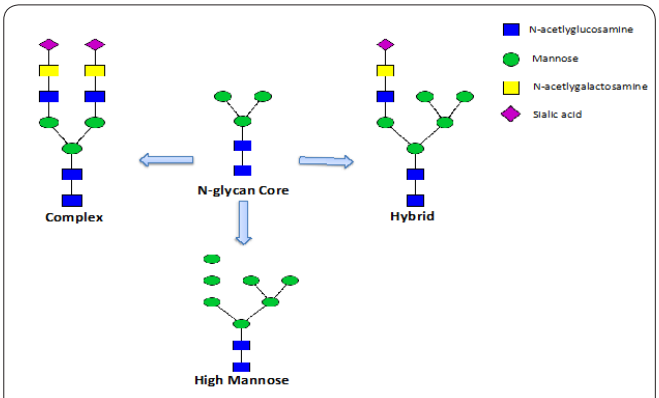

Figure 1. N-glycan core and glycan types.

difference in biological activity is thought to be modulated by their unique glycan profile. However the exact contributions of glycans to the functions of glycoproteins are not well elucidated due to the limitations of deglycosylation methods (17, 18). Glycan release is mostly performed with various chemical and enzyme based techniques (19, 20). Combination of one or more of these strategies can provide high efficiency with wide diversity, low cost and easy glycan release from glycoproteins. The biggest challenge of these methods is that they require high heat and detergent application for denaturation of glycoproteins. These harsh conditions may disrupt the glycan and remaining protein part that results in loss of biological roles. Moreover, selective glycan production is not feasible by the application of these approaches. We recently isolated a novel endo-*β*-*N*-acetylglucosaminidase (EndoBI-1) for glycan release from a probiotic from infant gut; *Bifidobacterium longum* subsp. *Infantis* ATCC 15697. This enzyme is able to cleave on the *N*-glycan core of all glycan types (high mannoses, hybrids as well as complex *glycans*). In this study, as a model protein, a highly antimicrobial lactoferrin was selected to investigate the contribution of glycans to the antimicrobial activity of lactoferrin. For this purpose, we used different genetically attached fusion tags (His-tagged, GST-tagged and free form of EndoBI-1) to prepare lactoferrins with different glycan profile. These enzymes' activities were compared in terms of both glycan release efficiency and the substrate specificity. This glycan release manipulation will be an opportunity to study on both individual glycans functions as well as their contributions to the glycoproteins.

Materials and Methods

Molecular cloning and protein purification of endo-β-N-acetylglucosaminidase

Molecular cloning of endo-*β*-*N*-acetylglucosaminidase was performed by pEcoTM-T7-cHis and pEcoTM-T7-nGST, Eco Cloning kits ((GeneTarget Inc, San Diego, CA, USA). The coding sequence of endo-*β*-*N*-acetylglucosaminidase was amplified from the genomic DNA of *B. longum* subsp. *infantis* ATCC 15697 with appropriate primers (Figure 2). The engineered plasmid transformed into *E. coli* Dh5α and into then *E. coli* BL21* for high protein expression as described before (21). Recombinant cells are grown in LB media with a final purified protein yield of 1.6 his-tagged EndoBI-1/L media and 1.9 mg GST-tagged EndoBI-1/ L media. The conditions for bacterial grown were 0.5 mM IPTG, 37 ^{0}C for 4 hours as optimized before (21).

The recombinant proteins were purified by a method described previously (21). Briefly, after grown bacterial cell was lysed, the mixtures were loaded into Ni- columns (for His-tagged EndoBI-1) and 1 mL prepacked glutathione sepharose columns (Bio-Rad, Hercules, CA, USA). The bound proteins were isolated with appropriate reagents from the column (imidazole for His-tagged EndoBI-1 and reduced glutathione for GST-tagged EndoBI-1).

Reagents, Enzymes and Substrates

Ribonuclease A (control glycoprotein), isopropyl β-D-1-thiogalactopyranoside (IPTG), human lactoferrin and Trifluoroacetic acid were obtained from Sigma Aldrich, St. Louis MO, USA. Acetonitrile and Formic acid were LC/MC grade and purchased from Fisher Chemical, Fair Lawn, NJ, USA. Control enzyme PNGase F was obtained from New England BioLabs (Ipswich, MA, USA). Free form of EndoBI-1 was obtained by the cleavage of GST tag from the protein with a GST removal kit from Sigma Aldrich, St. Louis MO, USA.

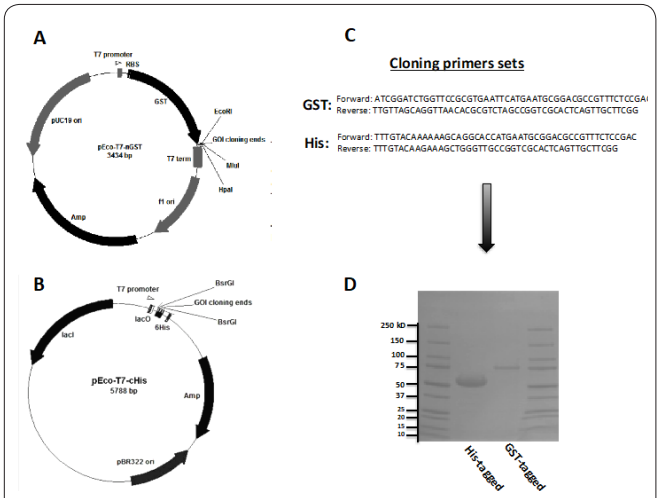

Figure 2. A- GST- tag included cloning vector. **B-**PolyHis included cloning vector. **C-**cloning primers. **D-** Purified GST and His-tagged EndoBI-1.

Lactoferrin digestion by three forms of EndoBI-1 and glycan quantification

The concentration of three forms of enzyme and lactoferrin were measured by Qubit Protein Assay Kit (Life Technologies, Grand Island, NY, USA). Lactoferrin samples with a concentration range of 0.1-0.8 mg/mL were mixed with 0.025 mg/ml of three forms of EndoBI-1 in a 0.02 M Na_2HPO_4 for various incubation time starting from 0 to 45 min at 37 ^{0}C with buffer solution at pH 5. 1M of Na_2CO_3 was used to terminate the reaction. 4 volumes to 1 volume cold ethanol were applied into the samples for protein precipitation and supernatant was collected since it contained the released glycans. All experiments were carried out in triplicate. To measure the amount of released glycans, a Carbohydrate Assay Kit, from Biovision, (Milpitas, CA, USA), was used where mannose was used

Calculation of Michaelis-Menten parameters

To evaluate the performance of EndoBI-1 with different tags as well as its free form, kinetic parameters; V_{max}, K_m, K_{cat} and K_{cat}/K_m values on lactoferrin were determined. As a concentration range for lactoferrin 0.1-0.8 mg/mL and incubation time 20 minutes were chosen as we optimized previously for lactoferrin-EndoBI-1 complex (21). The initial rates of reactions were determined by the slopes obtained from the mixtures for the first 15 min of incubation. Then, Graph Prism 6.0.1 software was used to calculate the kinetic parameters by using the initial rates at different substrate concentrations.

Characterization of released N-glycans by mass spectrometry

Released glycans were purified by graphitized carbon plates (Glygen, Columbia, MD, USA) prior to MS analysis as described previously (22). LC-MSMS 8040

(Shimadzu, Kyoto, Japan) was used for the characterization of released glycans. MassHunter Qualitative Analysis software (version B.06.00 SP2, Agilent Technologies) was used to identify the released glycan compositions. The relative abundance of released *N*-glycans was measured by MassHunter Profinder software, where the structures were compared to a *N*-glycan library prepared from all potential bovine N-glycans (consist of hexose (Hex), HexNAc, fucose, *N*-acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc)) by using a mass error tolerance of 20 ppm (23). For the compound detection, minimum 600 counts were selected to remove dirtiness and untargeted structures.

Antimicrobial test of lactoferrin

Antimicrobial activity of lactoferrin samples were determined as described by Conesa et al. with minor modifications (24). Stock solutions of native LF, full deglycosylated LF and sialylated glycans enriched LF were prepared in distilled water and sterilized with a 0.22 um pore size filter (Milipore) before addition the medium. LB agar plates were prepared for *E.coli DH5a* by dissolving various LF concentrations 0.5, 1, 2, 3, 4, 6 and 8 mg/ml in triplicate (three plates for each concentration). In addition, three plates were prepared as positive control with carbenicillin and three plates without any antimicrobial agent as negative control were prepared. After incubation (from logarithmic phase with ~1/10^9 dilution), the MIC value was taken as the lowest concentration that caused complete bacterial growth inhibition.

Statistical analysis

One-way ANOVA was used to determine the difference ($p \leq 0.05$) among N-glycan release efficiency and diversity of free, His-tagged and GST-tagged forms of EndoBI-1 on lactoferrin. Tukey's multiple comparison test was used to compare the means of different groups. NCSS 11.0 was used for optimization study.

Results and discussion

Production of His and GST-tagged EndoBI-1 and their activity control on a model protein

The yield of protein purification steps for His and GST-tagged EndoBI-1 were found to be 1.65 mg/ L and 1.97 mg/ L of LB media. GST-tagged enzyme production resulted in higher yield since the portion of GST-enzyme complex contains 23k Da GST tag that causes the overestimation of the protein concentration. The purified enzymes' activity was confirmed with a 17kDa model glycoprotein ribonuclease B. Rnase B contains high mannose type N-glycans with a single N-glycosylation site. Deglycosylation of RNase B resulted in a 14 kDa molecular weight molecule that could be visualized by gel electrophoreses mobility shift assay (data not shown).

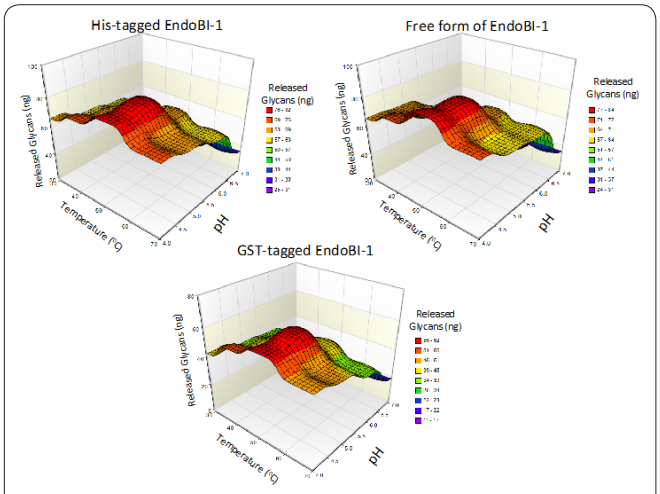

Figure 3. Working condition optimization of His-tagged, GST-tagged and free form of EndoBI-1.

Optimization of working conditions of three forms of EndoBI-1 on lactoferrin

We previously optimized the working conditions of his-tagged EndoBI-1 that were found to be pH: 5 and 52.5 ^{0}C (22). Since the activity of enzymes might alter when a fusion tag attached, other two forms of the enzyme (free and GST-tagged) were also characterized. The results showed that all three forms of EndoBI-1 had the same trend in terms of pH and temperature (Figure 3). It was found that the optimum pH and incubation temperature were 5 and 50 ^{0}C, respectively. Although the optimal conditions were found to be same for all enzyme forms, GST-tagged EndoBI-1 showed lower enzymatic performance compared to free and His-tagged EndoBI-1.

Calculation of kinetik parameters; V_{max}, K_m, K_{cat} and K_{cat}/K_m

The kinetics parameters of three forms of EndoBI-1 on lactoferrin were calculated by using various initial concentrations of each form of enzyme for 60 min for each reaction. The initial rates of each reaction were observed from the slopes and the rates were constant at the first 20 min of reactions. Working on initial rates is critical since the assumption of (S)>>(E) is valid only for initial rates of reaction. Otherwise, the enzyme amount becomes limited that causes low reaction rate estimation.

Both the direct and linearized methods (Lineweaver-Burke) were used to evaluate the kinetic parameters. The direct plotting was performed by plotting of the reaction rate (v) versus the initial substrate concentration (S), whereas 1/(S) vs 1/(v) was used for linearized Lineweaver-Burke method (Table 1). Linearized results

Table 1. Kinetic parameters of free, GST and His tagged EndoBI-1 based on Lineweaver-Burke linearization plot (V in mg/ml x min, K_m in mg/ml, K_{cat} in min^{-1} and K_{cat}/K_m in ml/mg x min^{-1}).

	Free	His-tagged	GST-tagged
V_{max}	$4.5\ 10^{-2} \pm 1.1\ 10^{-3}$ [a]	$4.2\ 10^{-2} \pm 1.1\ 10^{-3}$ [a]	$1.8\ 10^{-2} \pm 1.1\ 10^{-3}$ [b]
K_m	3.35 ± 0.26 [a]	3.50 ± 0.17 [a]	10.12 ± 0.29 [b]
K_{cat}	1.8 ± 0.05 [a]	1.68 ± 0.09 [a]	0.72 ± 0.08 [b]
K_{cat}/K_m	0.53 ± 0.03 [a]	0.48 ± 0.04 [a]	0.07 ± 0.001 [b]

Table 2. N-glycan composition (including isomers) from lactoferrin released by free, His and GST-tagged EndoBI-1.

	Total released N-glycans	Neutral released N-glycans	Sialylated released N-glycans
Free	38 ± 1 [a]	23 ± 1 [a]	15 ± 0 [a]
His-tagged	37 ± 1 [a]	22 ± 1 [a]	15 ± 0 [a]
GST-tagged	25 ± 2 [a]	23 ± 1 [a]	2 ± 1 [b]

were used to evaluate the enzyme efficiencies since the error was smaller then non-linear method. Based on the results, V_{max} values for free, His- and GST tagged enzymes on lactoferrin found to be $4.5x10^{-2}$, $4.2x10^{-2}$ and $1.8x10^{-2}$ mg/ml, respectively. K_m value (indicates the enzyme affinity to the substrate) was 3.35 mg/ml for free enzyme, whereas it was found to be 3.50 and 10.12 mg/ml for His and GST tagged enzyme, respectively. Vmax and Km values were similar between Free and His-tagged enzymes whereas GST tagged enzyme values were significantly different from the others.

To evaluate the performance of all three forms of enzyme, K_{cat}/K_m was used. This value indicated amount of released glycans from certain concentrated substrate in one minute. Based on the calculations, Free and his-tagged enzyme showed similar performance (0.53 and 0.48 ml/mg x min^{-1}, respectively), while GST-tagged enzyme resulted in K_{cat}/K_m value of 0.07 ml/mg x min^{-1}. The findings show that free and His-tagged EndoBI-1 exhibit similar enzymatic activity, whereas GST tagged enzyme shows lower affinity to the substrate and low performance of glycan release. The low activity of GST-tagged EndobI-1 can be explained by the size of the tag that hinders the accessibility of the enzyme to glycan found in various potential N-glycosylation sites. GST is a large tag with 211 amino acid length protein compared to polyhis tag that contains only 6 amino acids (25).

Glycoprofiling of released N-glycans by three forms of EndoBI-1

In addition to enzymatic release efficiency, substrate specificity is another important criterion to evaluate an enzyme's performance. Many research have focused on the manipulation of enzyme activity to release specific substrates from a complex mixtures. One way to alter the enzymes target is performed with the genetically addition of various fusion tags. Lee et. al. (26) characterized biochemical properties of polyhis-tagged and free form of thioesterase I expressed in Coli. The findings on the comparison of two forms of the enzyme on seven substrates suggest that polyhis-tagged enzyme target more shortest chain-lenght substrate; p-nitrophenyl, where as free form of the enzyme's activity has shifted to more palmitoyl-CoA and p-nitro-phenyl dodecanoate. In conclusion, Studies showed that though C-terminal His-tag did not influence seriously all structure of enzyme, substrate specificity and catalytic activity of enzyme are changed by His-tag. The study concludes that the eznyme's activity can be redirected without affecting its efficiency. However, enzyme activity manipulation by fusion tags is enzyme-substrate specific. Araujo et. al. (27) expressed poly-histagged tagged chlorocatechol 1,2-dioxygenase (CCD) and compared the activity with free form. The findings indicate that both forms of the enzyme have similar substrate specificity, but polyhis-tagged enzyme has 5-fold lower activity than the free form. The study suggest that the low activity of the tagged enzyme might be the result of the interference of the tag with the active site of the enzyme that limits the enzyme's activity.

Since glycoproteins contain multiple glycan structures, substrate specificity of glycosidases is critical to study the individual compositions' biological functions. In addition to mutual benefits of glycans, different glycans types (high mannose complex or hybrid) possess unique biological roles. For example, glycans with sialic acid (neuraminic acid with a nine-carbon backbone) play important role in the prevention of pathogen binding as well as the contribution to brain development and increase in learning skills (28, 29). Therefore, the isolation of unique glycans by enzymatically enables further investigations on the discovery of new functions of these structures. Another aspect of this technology is that glycoproteins containing multiple N-glycan structures such as immunoglobulins and lactoferrin can be partially deglycosylated that enables to determine which glycan types are responsible for the function of the glycoproteins. Previously, it was shown that various combinations of time, temperature, pH, enzyme protein ratio results in different glycan profile from concentrated bovine colostrum (30). However, the conditions used for the isolation of different glycan types are not under the optimal conditions of the enzyme that hinders the high enzymatic release efficiency. The compositional analysis of released glycan by advanced mass spectrometry was performed to understand the substrate specificity of the three forms of EndoBI-1. As a mass filter, a library previously produced from bovine milk was used for accurate and reproducible N-glycan identification. Based on the mass analysis, free, His- and GST tagged enzymes resulted in 38, 37 and 25 different N-glycan structures respectively (Table 2). The released glycans were also grouped into neutral or sialylated glycans. Among the structures, all forms of the enzyme released same number of neutral glycans (22 ±1), whereas the sialylated glycans release of free, His- and GST-tagged EndoBI-1 were 15, 15 and 2 structures respectively. The results showed that all forms of EndoBI-1 were able to release all neutral glycan types. However, sialylated N-gycan release was very limited by GST-tagged EndoBI-1. Figure 4 illustrates extracted compound chromatograms (ECCs) of isolated glycans from lactoferrin by three forms of the enzyme.

The present study suggests that specific N-glycan isolation can be manipulated by changing the geneticall-ly attached fusion tags under optimal EndobI-1 working conditions that was previously optimized (31). Interestingly, when GST tag is attached to EndoBI-1, enzyme loses its ability to target sialylated glycans, where as it still maintains full activity on neutral glycans compared to free and His-tagged enzyme. In addition to the comparison of number of released glycans, relative abundance of these compositions confirms that the activity of GST-tagged EndoBI-1 is extremely limited on sia-

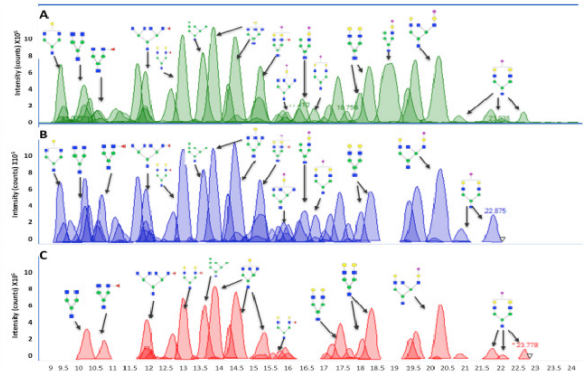

Figure 4. Extracted compound chromatograms (ECCs) of glycans from lactoferrin released by Free (A), His-tagged (B) and GST-tagged EndoBI-1 (C). Green circles, yellow circles, blue squares, red triangles, purple diamonds and gray diamonds represent mannose, galactose, HexNAc, fuc, NeuAc and NeuGc residues, respectively.

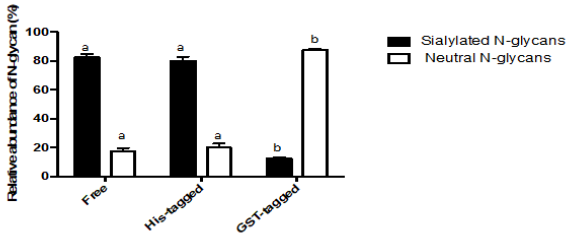

Figure 5. Determination of abundance of both neutral and sialylated glycan compositions released by Free, His-tagged and GST-tagged EndoBI-1. Results are given as the mean of biological triplicates, where different letters refers statistically different within the same class (sialylated or neutral) at $P < 0.05$.

lylated glycans. However this limitation of the enzyme can be utilized to remove only neutral glycans (sialylated glycans will be still attached to the protein) that will enable to study the contributions of attached sialylated to the protein.

Relative abundance of released glycans

Relative abundances of released N-glycans by the three forms of EndoBI-1 from lactoferrin determined as well as detailed glyco-profiling. This information provided not only the differences of *N*-glycans types found in each sample, but also their abundance. Figure 5 shows abundances of released neutral and sialylated from each sample. Based on the results, the releative abundance of sialylated an neutral glycans released bu GST-tagged EndoBI-1 was significantly different from other treatments. The sialylated N-g;ycans respresented only 10% in glycan pool in GST-tagged enzyme release, whereas free and His-tagged EndoBI-1 resulted in 81 and 90%, respectively. Similarly, relative abundance of neutral glycans found in GST tagged enzyme was significantly higher than other samples with a 89 % abundance compared to free (18%) and His-tagged (19%) EndoBI-1 release.

Antimicrobial activity of Lactoferrin

Antimicrobial activity of three different forms of lactoferrin was shown in Table 3. The MIC value for native LF and sialylated glycans enriched LF were 1mg/mL, whereas fully deglycosylated LF had 6 mg/mL MIC value against *Escherichia coli DH5a.* Our results suggest that sialylated glycans are responsible for the binding of glycans to the pathogen cell surface that results in cell lysis. However, removal of neutral glycans didn't affect the antmicrobial activity of lactoferrin (Table 3). Similar to our results, It was previously reported that glycans were responsible for cell to cell or cell to microbe interactions in other systems (4). Conesa et al. demonstrated that recombinant lactoferrin produced in rice showed 1mg/mL MIC value against *E.coli O157:H7* (24). Theolier et al. investigated the antimicrobial activity of lactoferricin (a digested form of lactoferrin) on fourteen different *E.coli* strains and the results showed that MIC value of lactoferricin against these strains were between 0.002-0.03mg/mL. It was shown that lactoferricin form of lactoferrin had extremely high antimicrobial activity compared to native lactoferrin (32). Shin et al. showed that MIC value of bovine lactoferrin against four clinical isolates of *E.coli O157:H7* were 3mg/mL. Transmission electron microscopy findings in this study showed that lactoferrin acts on the bacterial surface that resulted in cell lysis (33).

Table 3. Minimum Inhibitory Concentration of three glycan forms of Lactoferrin against *Escherichia coli DH5a*.

	Native LF	Fully Deglycosylated LF	Sialylated Glycans Enriched LF
MIC (mg/ml)	1± 0 [a]	6± 0 [b]	1± 0 [a]

Conesa et al. investigated the antimicrobial activity of lactoferrin isolated from different animals including sheep, goat, camel, alpaca, elephant and grey seal as well as human. It was shown that lactoferrin isolated from camel was the most effective lactoferrin against *E. coli* 0157:H7 with a 0.5mg/mL MIC value, whereas alpaca and human lactoferrins were the least active (5 and 6mg/mL, respectively) (34). Despite the high amino acid sequence homology found in different lactoferrins (up to 99%) (35), they have a different antimicrobial activities due to their different glycosylation patterns.

The present study examines the *N*-glycan release efficiency and diversity of free His-tagged and GST-tagged EndoBI-1 from a model glycoprotein; lactoferrin. Application of these fusion tags enables full and/or selective glycan release from lactoferrin. Removal of unique glycans from lactoferrin is crucial to understand the contribution of glycans to the function of glycoproteins. In this study, three glycan forms of lactoferrin have been obtained by this novel technique and investigated how the antimicrobial activity of lactoferrin has been changed by the selective deglycosylation. The findings suggest that sialylated glycans are responsible for the antimicrobial activity of lactoferrin, whereas neutral glycans do not have any effect.

Conflict of interest

There is no conflict of interest to declare.

References

1. Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Marth JD, et al. Symbol nomenclature for glycan representation. Proteomics. 2009;9(24):5398-9. doi: Doi 10.1002/Pmic.200900708. Pub-

Med PMID: ISI:000273515000001.
2. Fuquay JW, Fox PF, McSweeney PL. Encyclopedia of Dairy Sciences 2nd Edition, Four-Volume set: Academic Press; 2011.
3. Recio I, Moreno F, López-Fandiño R. Glycosylated dairy components: their roles in nature and ways to make use of their biofunctionality in dairy products. In Dairy-derived ingredients: food and nutraceutical uses, Editor M Corredig. 2009:170-211.
4. Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, et al. Structures Common to Different Glycans. 2009.
5. Barboza M, Pinzon J, Wickramasinghe S, Froehlich JW, Moeller I, Smilowitz JT, et al. Glycosylation of human milk lactoferrin exhibits dynamic changes during early lactation enhancing its role in pathogenic bacteria-host interactions. Mol Cell Proteomics. 2012;11(6):M111. 015248.
6. Kautto L, Nguyen-Khuong T, Everest-Dass A, Leong A, Zhao Z, Willcox MD, et al. Glycan involvement in the adhesion of Pseudomonas aeruginosa to tears. Experimental eye research. 2016;145:278-88.
7. Nwosu CC, Aldredge DL, Lee H, Lerno LA, Zivkovic AM, German JB, et al. Comparison of the human and bovine milk N-glycome via high-performance microfluidic chip liquid chromatography and tandem mass spectrometry. Journal of proteome research. 2012;11(5):2912-24.
8. Wormald MR, Petrescu AJ, Pao Y-L, Glithero A, Elliott T, Dwek RA. Conformational studies of oligosaccharides and glycopeptides: complementarity of NMR, X-ray crystallography, and molecular modelling. Chemical Reviews. 2002;102(2):371-86.
9. Wang B. Sialic acid is an essential nutrient for brain development and cognition. Annual review of nutrition. 2009;29:177-222.
10. Wang B, Yu B, Karim M, Hu H, Sun Y, McGreevy P, et al. Dietary sialic acid supplementation improves learning and memory in piglets. Am J Clin Nutr. 2007;85(2):561-9.
11. Bezault J, Bhimani R, Wiprovnick J, Furmanski P. Human lactoferrin inhibits growth of solid tumors and development of experimental metastases in mice. Cancer Research. 1994;54(9):2310-2.
12. Qiu J, Hendrixson DR, Baker EN, Murphy TF, Geme JWS, Plaut AG. Human milk lactoferrin inactivates two putative colonization factors expressed by Haemophilus influenzae. Proceedings of the National Academy of Sciences. 1998;95(21):12641-6.
13. Legrand D, Elass E, Carpentier M, Mazurier J. Interactions of lactoferrin with cells involved in immune function This paper is one of a selection of papers published in this Special Issue, entitled 7th International Conference on Lactoferrin: Structure, Function, and Applications, and has undergone the Journal's usual peer review process. Biochemistry and cell biology. 2006;84(3):282-90.
14. García-Montoya IA, Cendón TS, Arévalo-Gallegos S, Rascón-Cruz Q. Lactoferrin a multiple bioactive protein: an overview. Biochimica et Biophysica Acta (BBA)-General Subjects. 2012;1820(3):226-36.
15. MICROBIOL E. Actividades antibacterianas de lactoferrina. Enfermedades infecciosas y Microbiologia. 2006;26(2):58.
16. Yamauchi K, Wakabayashi H, Shin K, Takase M. Bovine lactoferrin: benefits and mechanism of action against infections This paper is one of a selection of papers published in this Special Issue, entitled 7th International Conference on Lactoferrin: Structure, Functions, and Applications, and has undergone the Journal's usual peer review process. Biochemistry and Cell Biology. 2006;84(3):291-6.
17. Le Parc A, Dallas DC, Duaut S, Leonil J, Martin P, Barile D. Characterization of goat milk lactoferrin N,Äêglycans and comparison with the N,Äêglycomes of human and bovine milk. Electrophoresis. 2014;35(11):1560-70.
18. Royle L, Matthews E, Corfield A, Berry M, Rudd PM, Dwek RA, et al. Glycan structures of ocular surface mucins in man, rabbit and dog display species differences. Glycoconjugate journal. 2008;25(8):763-73.
19. Sojar HT, Bahl OP. A chemical method for the deglycosylation of proteins. Archives of biochemistry and biophysics. 1987;259(1):52-7.
20. Altmann F, Schweiszer S, Weber C. Kinetic comparison of peptide: N-glycosidases F and A reveals several differences in substrate specificity. Glycoconjugate journal. 1995;12(1):84-93. Epub 1995/02/01. PubMed PMID: 7540902.
21. Karav S, Bell JM, Parc AL, Liu Y, Mills DA, Block DE, et al. Characterizing the release of bioactive N-glycans from dairy products by a novel endo-beta-N-acetylglucosaminidase. Biotechnology progress. 2015. Epub 2015/06/23. doi: 10.1002/btpr.2135. PubMed PMID: 26097235.
22. Karav S, Parc LA, Bell MLNMJ, Liu Y, Mills DA, Block DE, et al. Characterizing the release of bioactive N-glycans from dairy products by a novel endo-β-N-acetylglucosaminidase. Biotechnology progress. 2015.
23. Nwosu CC, Aldredge DL, Lee H, Lerno LA, Zivkovic AM, German JB, et al. Comparison of the human and bovine milk N-glycome via high-performance microfluidic chip liquid chromatography and tandem mass spectrometry. J Proteome Res. 2012;11(5):2912-24. Epub 2012/03/24. doi: 10.1021/pr300008u. PubMed PMID: 22439776; PubMed Central PMCID: PMC3345083.
24. Conesa C, Rota C, Castillo E, Perez M-D, Calvo M, SAnchez L. Antibacterial activity of recombinant human lactoferrin from rice: effect of heat treatment. Bioscience, biotechnology, and biochemistry. 2009;73(6):1301-7.
25. Terpe K. Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. Applied microbiology and biotechnology. 2003;60(5):523-33.
26. Lee Y-L, Su M-S, Huang T-H, Shaw J-F. C-terminal His-tagging results in substrate specificity changes of the thioesterase I from Escherichia coli. Journal of the American Oil Chemists' Society. 1999;76(10):1113-8.
27. Araujo A, Oliva G, Henrique-Silva F, Garratt R, Cáceres O, Beltramini L. Influence of the histidine tail on the structure and activity of recombinant chlorocatechol 1, 2-dioxygenase. Biochemical and biophysical research communications. 2000;272(2):480-4.
28. Varki A, Lowe JB. Biological roles of glycans. 2009.
29. Georgi G, Bartke N, Wiens F, Stahl B. Functional glycans and glycoconjugates in human milk. Am J Clin Nutr. 2013;98(2):578S-85S.
30. Parc AL, Karav S, Bell JMLND, Frese SA, Liu Y, Mills DA, et al. A novel endo-β-N-acetylglucosaminidase releases specific N-glycans depending on different reaction conditions. Biotechnology progress. 2015;31(5):1323-30.
31. Karav S, Bell JMLND, Parc AL, Liu Y, Mills DA, Block DE, et al. Characterizing the release of bioactive N-glycans from dairy products by a novel endo-β-N-acetylglucosaminidase. Biotechnology progress. 2015;31(5):1331-9.
32. Théolier J, Fliss I, Jean J, Hammami R. MilkAMP: a comprehensive database of antimicrobial peptides of dairy origin. Dairy Science & Technology. 2014;94(2):181-93.
33. Shin K, Yamauchi K, Teraguchi S, Hayasawa H, Tomita M, Otsuka Y, et al. Antibacterial activity of bovine lactoferrin and its peptides against enterohaemorrhagic Escherichia coli O157: H7. Letters in Applied Microbiology. 1998;26(6):407-11.
34. Conesa C, Sánchez L, Rota C, Pérez M-D, Calvo M, Farnaud S, et al. Isolation of lactoferrin from milk of different species: calorimetric and antimicrobial studies. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 2008;150(1):131-9.
35. Chung M. Structure and function of transferrin. Biochemical Education. 1984;12(4):146-54.