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Understanding of the Contribution of Fetuin O-glycans for the Release of New Bioactive Compounds by a Novel Endo-β-N-acetylglucosaminidase

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ABSTRACT

Bovine fetuin is a model protein to study the activity of various glycosidases since it contains both N- and O- glycans attached to the polypeptide chain. We recently showed a novel glycosidase, endo-*β-N*-acetylglucosaminidase isolated from an infant gut microbe, *Bifidobacterium infantis.* This enzyme is capable of cleaving the *N-N'*-diacetyl chitobiose moiety found in the *N*-glycan core of a wide variety of proteins. It is considered a promising approach to release N-glycans from complex substrates such as whey proteins due to its high activity and wide substrate specificity. Moreover, it also maintains its activity at high temperatures enabling the use of this enzyme in thermal dairy processes such as during the pasteurization. Bovine whey is a potential source of glycans providing million tons of glycoproteins annually. Application of EndoBI-1 on bovine whey is challenging due to the complexity of the whey proteins and their O-glycosylation pattern. O-glycans are considered to be a protective agent for N-deglycosylation that hinders the isolation of these recently found novel compounds. In this study, O-glycans were removed from fetuin (both O- and N- glycosylated model glycoprotein) and the contribution of O-glycans to the accessibility of EndoBI-1 to bovine fetuin N-glycans were tested. Released glycans were characterized by advanced mass spectrometry and 22 different N-glycans (including isomers) were monitored. According to the results, it was shown that removing O-glycans from Fetuin increases the Kcat/Km value 0.52 to 1.54 ml/mg x min^{-1} and the affinity of EndoBI-1 (Km value from 0.32 to 0.22 mg/ml) to target N-glycans enabling more feasible application of this enzyme in dairy streams.

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Fetuin O-glikanlarının, Bioaktif N-Glikanların Yeni Endo-Β-N-asetilglukozaminidaz Tarafından İzole Edilmesindeki Katkısının Belirlenmesi

ÖZET

İnek fetuini N- ve O- glikanları içerdiğinden dolayı farklı glikosidazların aktivitesini test etmek için kullanılan model bir proteindir. Yeni bir glikosidaz olan endo-Β-N-asetilglukozaminidaz enzimi (EndoBI-1) bebek bağırsaklarında bulunan *Bifidobakteri infantis*'ten tarafımızca daha önceden izole edilmiştir. Bu enzim farklı protein yapılarında bulunan N-glikan merkezlerini kesebilmektedir. Enzimin yüksek aktivite ve geniş substrat aralığından dolayı, peynir altı suyu proteinleri gibi karmaşık yapılarda aktivite gösterebilmektedir. Ayrıca, bu enzim aktivitesini yüksek sıcaklıklarda koruyabildiği için, pastörizasyon gibi ısıl işlem gerektiren süreçlerde de kullanılabilmektedir. İnek peynir altı suyu, yıllık milyonlarca ton üretilen bir glikoprotein kaynağıdır. Fakat, EndobI-1 enziminin bu substrata uygulanması, peynir altı proteinlerinin O-glikanları tarafından bloke edilip aktiviteyi düşürdüğü düşünülmemektedir. O-glikanların, proteinleri bir korucuyu görevi ile koruyarak, yeni bir prebiyotik kaynağı olarak kabul edilen N-glikanların izolasyonunu zorlaştırmakta olduğu

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sanılmaktadır. Bu çalışmada, O-glikanlar fetuinden ayrılarak, bunun N-glikanların enzimatik olarak EndoBI-1 tarafından ayrılmasına olan katkısı incelenmiştir. Ayrılan glikanlar kütle spektrometresi ile analiz edilmiş ve izomerler dahil 22 farklı yapı gözlemlenmiştir. Sonuçlara göre, O-glikanların fetuinden ayrılması, N-glikan izolasyon etkinliğini (Kcat/Km) 0.52 'den 1.54 ml/mg x min^{-1}'a çıkarırken, Km değerini 0.32 'den 0.22 mg/ml'ye düşürmüştür. Bu sonuçlara göre, EndobI-1 süt endüstrisinde çok daha etkili bir şekilde kullanabilmesinin yolu açılmıştır.

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INTRODUCTION

Human milk has evolved for million years and scientists have not fully identified all milk bioactive molecules yet (Jenness 1988). It has been demonstrated that breastfed infants have less risk of incidence of diseases such as diarrhea and eczema compared to formula fed babies (Hamosh 2001, Holman, Stoll et al. 2006). Recently, an interesting characteristic of milk being now recognized is the extent of glycosylation of its proteins, which is commonly found in human and bovine milk that is thought to contribute the beneficial effects of milk (O'riordan, Kane et al. 2014). Glycosylation is a post-translational modification where small carbohydrate molecules are attached to the protein during the synthesis (Karav, German et al. 2017). Interestingly, the milk glycans are indigestible by infants/adults, where as they have been shown to promote the growth of beneficial intestinal bacteria (especially Bifidobacteria) thus further protecting the infant from pathogens and stimulating a healthy intestinal environment (Marcobal and Sonnenburg 2012, Karav, Le Parc et al. 2016).

Milk proteins are grouped as caseins and whey proteins. Caseins are mostly O-glycosylated glycoproteins, whereas whey proteins contains both O- and N- glycosylated ones. Whey is a by-product of cheese making process (it is also considered as waste product) is rich in glycoproteins possessing a wide variety of both *N*-linked and *O*-linked glycans. The general content of whey is mostly water (~95%), lactose (~5%), and proteins (0.6-0.7%) (Hejtmánková, Pivec et al.). The production of whey is over 200 million tons globally every year, which serves 1 million tons of free protein (Mollea, Bosco et al. 2013). Common whey glycoproteins are glycomacropeptide, osteopontin, lactoferrin, immunoglobulins, α-lactalbumin and transferrin (Walstra, Walstra et al. 2005, Horowitz 2012).

Glycans have many important roles in cellular mechanisms that are directly linked to health. Barboza et al. showed that glycans are active in cell adhesion and receptor that provides protection against viruses and most bacteria (Barboza, Pinzon et al. 2012). Glycans also interact with proteins helping behavior and structural properties of proteins (Spik, Coddeville et al. 1994). It is also shown that glycans regulate the folding and conformation of proteins as well as the support the protein resistance against proteolysis (Wormald, Petrescu et al. 2002). We also recently showed that glycans released from milk glycoproteins act as selective prebiotic sources for Bifidobacteria species (Karav, Le Parc et al. 2016). The production of these bioactive glycans requires an efficient deglycosylation strategy to study the biological roles of glycans and potentially commercialization. Deglycosylation of glycoproteins is currently performed with two approaches; chemically and enzymatically (Electricwala, Wright et al.). However, there is no available single method that provides efficient, low cost and wide diverse glycan release without disrupting the native form of glycan or the remaining the polypeptide chain (Takahashi 1977, Sojar and Bahl 1987, Nuck, Zimmermann et al. 1990, Altmann, Schweiszer et al. 1995). The harsh conditions that are required for these methods might result in the disruption of the released glycan and remaining polypeptide structures, which is directly associated with the biological roles of these compounds. To solve these problems, we discovered an endo-*β-N*-acetylglucosaminidase (EndoBI-1) isolated from *Bifidobacterium longum* subsp. *Infantis* ATCC 15697 that is active on glycoproteins with any limitations.

There is a great interest in biological roles of glycans (Morgan and Winick 1980, Yolken, Peterson et al. 1992, Bode, Kunz et al. 2004). To further studies on glycans are very limited due to the lack of deglycosylation strategies in large scale. As a scale up approach, bovine where is a promising source, but its O-glycan complexity hinders the accessibility of glycans. Here, we show how removal of O-glycans affects the N-glycan release efficiency of a novel N-glycosidase (EndoBI-1) to achieve the production of these bioactive molecules in large scale.

MATERIALS and METHODS

Reagents

Fetuin from bovine serum, Isopropyl β-D-1-thiogalactopyranoside (IPTG) and Trifluoroacetic acid were purchased from Sigma Aldrich, St. Louis MO,

USA. Acetonitrile and Formic acid were LC/MC grade and obtained from Fisher Chemical, Fair Lawn, NJ, USA. PNGase F was purchased from New England BioLabs (Ipswich, MA, USA).

Expression and purification of novel endo-β-N-acetylglucosaminidase

A cloning kit from Gene Target that has poly his tag was carried out to clone EndoBI-1 coding sequence. Protein expression and purification were performed as decribed before in *E. coli* BL21* (Karav, Bell et al. 2015). The eluted protein was kept at -80 ^{0}C till enzymatic release experiment.

Removal O-glycans

O-glycan removal from bovine fetuin was carried out with a previously described method with minor modifications (Windwarder and Altmann 2014). Briefly, Fetuin (10 mg in 10 ml) was applied into 1ml, 1M NaBH4 in 0.1 M NaOH to reduce the O-glcyans and stored at 55 °C for 18h. The glycans then purified by porous graphitized carbon cartridges and kept for mass analysis. De-O-glycosylated fetuin kept at -80 for kinetics study.

N-glycan isolation by endo-β-N-acetylglucosaminidase

Fetuin N-glycan release was performed enzymatically by using a novel endo-*β-N*-acetylglucosaminidase. As an initial activity test, pretreated fetuin (removed O-glycans) and positive control fetuin were digested under optimal conditions that previously characterized (37 ^{0}C, pH:5 and overnight incubation). The results were consistent with previous studies that we tested the enzyme on pure lactoferrin and concentrated bovine whey colostrum (data not shown).

Released N-glycan quantification

Released *N*-glycans quantification were performed with Life Technologies, USA Qubit. Fetuin substrates (pre-treated and positive control fetuin (0.1-1 mg/mL) were applied under different incubation times (0-240 min) where the incubation conditions were 37 ^{0}C and 0.025 mg/ml EndoBI-1 to determine the appropriate range of fetuin concentration for kinetics study. Adding 1M Na_2CO_3 ended the reactions. Completed reactions were then precipitated by the application of cold ethanol. Biovision, USA Carbohydrate Assay Kit was applied to measure the released *N*-glycans amount where mannose was used as a standard for N-Glycans calibration curves since the majority of the monosaccharides of glycans is mannose.

Determination of kinetic parameters

Prepared fetuin samples with concentrations changing from 0.05 to 1 mg/mL were used to figure out how the reactions take place for a total time of 4 hours (enough incubation time for full release based on a previous study) (Karav, Bell et al. 2015). The slopes were used to calculate the rate of initial reaction took place in 75 minutes. Kinetic values (K_m, V_{max} and K_{cat}) were calculated by using Graph Prism 7.0 software with direct plotting of the data.

Characterization of released glycan by mass spectrometry

MassHunter Qualitative Analysis software (version B.06.00 SP2, Agilent Technologies) was used to identify the compounds with find by formula option after the analysis of the compounds in mass spectrometry. 1000 counts limit were selected to filter the low abundant compounds. The found compounds were match with a library created by Nwosu et al. (Nwosu, Aldredge et al. 2012).

Statistical Analysis

To understand the significant difference ($p \leq 0.05$) between N-glycan release efficiency of EndoBI-1 on fetuin and pretreated fetuin, one way ANOVA (analysis of variance) was applied. Means of different groups were compared by Tukey's multiple comparison test. Graph prism 7.0 software was used for direct plotting of kinetics data.

RESULTS and DISCUSSION

Production of novel endo-β-N-acetylglucosaminidase

Recombinant endo-β-N-acetylglucosaminidase was produced with a method previously described (Karav, Bell et al. 2015). The poly-histidine tag containing protein was purified with a nickel column and the purity of the protein was visually controlled by SDS-PAGE gel electrophoresis. Purified enzyme's activity was monitored by using a model protein RNase B that provides a molecular shift from 17 kDa to 14 kDa after removal of glycans. Both purity and the activity of the enzyme were found as expected to previous studies (Karav, Bell et al. 2015, Karav, Parc et al. 2015). The purity of the recombinant enzyme obtained in these studies was above %90 (controlled with visual observation on SDS-PAGE gel). In present study, the purity was closer to %99 since the volume of the Nickel columns used here had larger volume and the number of washes of the native proteins from the column was higher (3 to 5). The enzyme showed full activity on the control model proteins as it was observed in the previous study (Karav, Parc et al. 2015)

Determination of kinetic constants K_m, V_{max}, K_{cat} and K_{cat} / K_m

To evaluate the contribution of removal of O-glycans on the release of N-glycans by EndBI-1, kinetic parameters of EndoBI-1 on pre-treated fetuin with O-glycans removal and non-treated fetuin were calculated. Direct plotting (Lineweaver Burke) reaction rate vs treated and non-treated fetuin resulted in a considerable different trend (Figure 1). Parham et al (2000) used different linearization

techniques to decrease the calculation error including Lineweaver Burke, Hanes Woolf and Eadie Hofstee. It was shown that Lineweaver-Burke method was the most promising approach for the enzymes that are similar to EndoBI-1. The results showed that EndoBI-1 show higher affinity on treated form of fetuin with a K_m value of 0.22 mg/ml compared to 0.32 mg/ml on native form of fetuin (Table 1). Low K_m value suggests that removing O-glycans increases the accessibility of EndoBI-1 on fetuin. We previously characterized the kinetic parameters of EndobI-1 on both complex substrates (concentrated bovine whey) and simple glycoproteins (RNase B) (Karav, Parc et al. 2015). The results showed that the enzyme had extremely low affinity on bovine whey that contained both N- and O-glycoproteins compared to the simple proteins. Based on these two studies, it suggests that O-glycans act as protective agents for the deglycosylation of the protein. V_{max} values of EndoBI-1 on non-treated and treated fetuin were found to be 4.2x10^{-3} and 8.69x10^{-3} mg/ml x min, and K_{cat} values were 0.168 and 0.34 min^{-1}, respectively. The results on native form of fetuin were consistent with our previous study that was focused on the heat denaturation of glycoproteins and its effect on glycan release efficiency (Karav, Parc et al. 2015). Karav et al. (2015) showed that the EndoBI-1 yielded considerably high glycan release on denatured bovine whey compared to native form the glycoproteins. In the study, the activity of EndoBI-1 was tested on native and denatured Rnase B, lactoferrin and concentrated bovine whey. The results showed that the denaturation step is crucial for complex substrates such as lactoferrin and bovine. The most efficient way to compare an enzyme's activity on two different substrates is using K_{cat} / K_m value since it represents both enzyme affinity and the glycan release rate in certain time. It was shown that removal of O-glycans increased K_{cat} / K_m from 0.52 to 1.54 mg/ml. The increase suggests that enzyme EndoBI-1 shows three fold more activity on pretreated fetuin compared to the native from. Application of various O and N-glycosidases was to glycoproteins widely investigated with other groups.

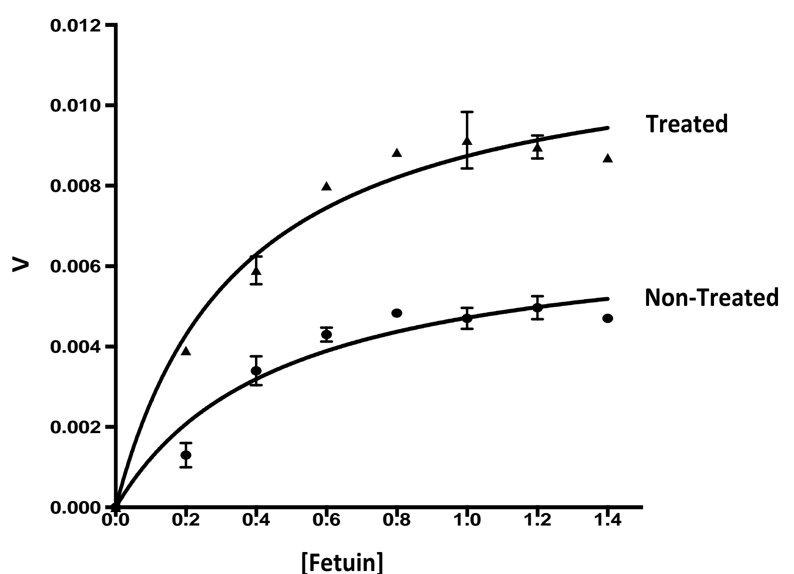

Figure 1. Plotting of rate of reaction (mg/ml x min) vs various non-treated and treated fetuin concentrations (mg/ml).

Table 1. Kinetic parameters of EndoBI-1 on fetuin (O-glycans removed form and native form) (V_{max} 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}).

	Non-treated	Treated
K_m	0.32 ± 0.02^{a}	0.22 ± 0.02^{b}
V_{max}	4.2x10^{-3} ± 2.3x10^{-4a}	8.69x10^{-3} ±2.6x10^{-4b}
k_{cat}	0.168 ± 0.03^{a}	0.34 ±0.09^{b}
k_{cat}/K_m	0.52 ±0.01^{a}	1.54 ±0.03^{b}

Results are given as the mean of biological triplicates, where different letters refers statistically different within the same **row** at P<0.05.

Characterization of released glycans

In addition to total *N*-glycan release efficiency, the glycan diversity was also characterized. Based on the glyco-profiling, a total of 22 different glycans (including isomers) have been detected (Table 2). The major compounds were 5Hex4HexNac1NeuAc, 5Hex4HexNac1NeuAc, 5Hex4HexNac1NeuAc, 5Hex4HexNac1NeuAc, 5Hex4HexNac1NeuAc and their isomers.

Table 2. N-glycan composition isolated from bovine fetuin. Green circles, yellow circles, blue squares and purple diamonds represent mannose, galactose, HexNAc and NeuAc and residues, respectively.

Code	Structure	Composition	Isomers (Native)	Isomers (Treated)	Abundance (%)
1		5Hex4HexNac1NeuAc	3	3	3
2		5Hex4HexNac1NeuAc	5	5	20
3		5Hex4HexNac1NeuAc	3	3	2.5
4		5Hex4HexNac1NeuAc	2	6	21
5		5Hex4HexNac1NeuAc	3	5	40.5

The monitored glycan structures were similar to other studies (Stavenhagen, Plomp et al. 2015). As expected, majority of the glycans were sialylated. The most abundant structure is 5Hex4HexNac1NeuAc with a 40% percent of total relative abundance, which was consistent with other studies (Stavenhagen, Plomp et al. 2015, Sun, Shah et al. 2016). Although the pretreatment of fetuin did not change the released N-glycan compositions, some isomers were not detected on native form. For example, 5Hex4HexNac1NeuAc was released with 6 isomers from treated fetuin, whereas EndobI-1 was able to released only 2 isomers from native form. Similarly, 5Hex4HexNac1NeuAc was released with 5 isomers from treated and 2 from native form. These results suggest that removing glycans enables the accessibility of EndoBI-1 to certain isomers. O-glycans released from fetuin were similar to the structures found by Altman et al. (2014), which were subjected to LC-ESI-MS analysis. Similar to this study, reporter ions (m/z = 292, 366, 454, 657) in MS/MS spectra were investigated to confirm the glycan composition. The sialylated compositions found in this study were consistent with the study that focused on the sialylated structures by Green et al. (1988).

CONCLUSION

Novel Endo-*β*-*N*-acetylglucosaminidase is a new strategy to release bioactive glycans from milk glycoproteins. One of the biggest challenges for the

isolation of these compounds from whey proteins is that whey contains not only N-glycans but also O-glycans that hinders the N-glycosylation process. Previous studies show that EndoBI-1 show low activity on bovine whey compared to pure proteins. In this study it is demonstrated that low enzyme activity is the result of O-glycosylation pattern. Understanding of this mechanism will allow to produce N-glycans more efficiently in large scale. Increasing the accessibility of endoglycosidases to the target N-glycans by removing O-glycans will also increase the diversity of the released glycans that enable to produce a wider glycan pool. This wide range of glycans might possess different biological roles that can be utilized in biotechnology, pharmaceuticals and as food additives.

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