Enzyme and Microbial Technology 77 (2015) 46–53

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

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

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ARTICLE INFO

Article history:
Received 15 January 2015
Received in revised form 27 May 2015
Accepted 30 May 2015
Available online 3 June 2015

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

ABSTRACT

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 to 0.49, 0.43 to 1.00 and 0.90 to 3.18 mg/mL and V_{max} values ranging from 3.5×10^{-3} to 5.09×10^{-3}, 4.5×10^{-3} to 7.75×10^{-3} and $1.9 \times 10^{-2}$to 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.

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 [1]. 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 As*N*-X-Ser/Thr (where X can be any amino acid except proline) [2].

Human milk oligosaccharides (HMO) are known to guide the development of the infant intestinal microbiota, in particular enriching a beneficial bifidobacterial population [3,4], 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 [5–9]. 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 [10], 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 [11,12]. Alternatively, bovine milk glyco-conjugates such as glycoproteins might be considered as an additional source of

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http://dx.doi.org/10.1016/j.enzmictec.2015.05.007
0141-0229/

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 [13]. 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 [14]. 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 [15]. 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 [16,17]. 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 [18–20]. However, if fucose is α1,3-linked to *N*-acetylglucosamine, the *N*-glycans are resistant to hydrolysis by PNGase F [21]. We recently showed that an endo-β-*N*-acetylglucosaminidase (EndoBI-1) isolated from *B. 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 [1]. 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 [22–24] 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′-TTTGTACAAGAAAGCTGGGTTGCCGGTCGCACTCAGTTGC-TTCGG-3′ and then cloned into the pEco-T7-cHis vector. The vector was transformed into *Escherichia coli* Dh5α 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 h). 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 m^2 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 7.0HP feed pump (Hydra-Cell™ 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 m^2) 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 [13,25].

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 Na_2HPO_4 buffer solution at pH 5. The reactions were terminated by the addition of 1 M Na_2CO_3. 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 pro-