Food Chemistry 240 (2018) 422–429

Contents lists available at ScienceDirect

Food Chemistry

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

β-Glucosidase from *Thermotoga naphthophila* RKU-10 for exclusive synthesis of galactotrisaccharides: Kinetics and thermodynamics insight into reaction mechanism

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ARTICLE INFO

Keywords:
β-Glucosidase
Prebiotics
Thermotoga naphthophila
Galactooligosaccharides (GOS)
Catalytic selectivity

ABSTRACT

This work reports a novel thermophilic β-glucosidase (TN0602) from *Thermotoga naphthophila* RKU-10, demonstrating exceptionally high catalytic selectivity (100%) for the exclusive synthesis of prebiotic galactotrisaccharides (GOS3) in a high volumetric production yield of 23.28 g L^{-1} h^{-1} (higher than the highest value ever reported) at pH 6.5 and 75 °C, with milk processing waste lactose as both the galactosyl donor and acceptor. A comparative study with commercial β-galactosidase from *Aspergillus oryzae* (AO) with respect to reaction kinetics, enzyme-substrate thermodynamic binding (substrate induced fluorescence quenching) and molecular docking simulation studies showed that β-glucosidase TN0602 has a deep catalytic "pocket" with a narrow entrance that prevents simultaneous access of lactose and GOS3 to the catalytic site, explaining its distinct catalytic specificity and reaction kinetics. The findings revealed in this work offer an improved understanding of how enzyme protein structure determines catalytic specificity, which serves as new knowledge to engineer β-glucosidase for the biosynthesis of designer GOS.

1. Introduction

Galactooligosaccharides (GOS) belong to a group of prebiotic compounds that are non-digestible but that stimulate the growth of certain types of colon microbiota associated with bifidogenic effects (Muir et al., 2009; Slavin, 2013). GOS can be fermented by specific colon microbes to produce short chain fatty acids (butyrate, propionate and acetate), inhibiting the growth and reproduction of some harmful bacteria (Puertollano, Kolida, & Yaqoob, 2014). GOS are generally found in legumes, seaweed and many other foods, but the content of GOS is too low to have a significant beneficial effect (Muir et al., 2009). Therefore, straightforward ways to supply GOS are to either use nutritional supplements or add GOS to foods directly. Many countries allow GOS as a dietary fibre additive to food products (Slavin, 2013). Chemically speaking, GOS are a kind of oligomer in which one to four molecules of galactose are bound mainly to the galactose residue of lactose. Galactose molecules are linked through β-(1–3), β-(1–4) and β-(1–6) glycosidic bonds, while galactose and glucose molecules are linked by β-(1–4) glycosidic bonds (Scheme S1, Supporting Information) (Oku, 1996). GOS are also quite stable during high temperature storage, even under acidic conditions. Thus, enzymatic conversion other than chemical synthesis is the main production method of GOS, and lactose is the main substrate feedstock (Mlichova & Rosenberg, 2006; Sako, Matsumoto, & Tanaka, 1999).

Lactose is a naturally occurring sugar found mainly in milk that can be hydrolysed by intestinal lactase. This enzyme is expressed in young mammals, but its activity declines following weaning. Thus, adult mammals are normally incapable to digest lactose, which leads to potential intestinal disorders following milk ingestion (Vilotte, 2002). Lactose intolerance is widespread throughout the world; a large proportion of the world's population would not benefit from putative milk nutrition (Visioli & Strata, 2014). Delactosed or low-lactose milk is therefore commercially produced, leading to large quantities of lactose as biowaste. Lactose can be comprehensively utilized to synthesize GOS and reclaim lactose, reducing food waste (Harnpicharnchai, Champreda, Sornlake, & Eurwilaichitr, 2009; Mahoney, 1998; Yu & O'Sullivan, 2014).

β-Glucosidase, displaying β-galactosidase activity, is ubiquitous in

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http://dx.doi.org/10.1016/j.foodchem.2017.07.155
Received 21 March 2017; Received in revised form 26 July 2017; Accepted 28 July 2017
Available online 29 July 2017

nature and is found in plants, mammals and bacteria (Harnpicharnchai et al., 2009). β-Glucosidase has two typical catalytic activities: hydrolysis and transglycosylation, corresponding to two main biotechnological applications in the dairy industry: the removal of lactose from milk for lactose-intolerant people and the production of GOS from lactose as prebiotic food stuffs, respectively (Mahoney, 1998; Torres, Goncalves, Teixeira, & Rodrigues, 2010; Yu & O'Sullivan, 2014). There are numerous reports on the synthesis of GOS by β-galactosidase with high lactose conversion (15–95%). Palai, Mitra, and Bhattacharya (2012) synthesized GOS using commercial grade β-galactosidase from *Bacillus circulans*; the product contains trisaccharides, tetrasaccharides and pentasaccharides, accounting for approximately 24%, 12% and 3%, respectively. Moreover, Tokošová, Hronská, and Rosenberg (2015) produced GOS by transgalactosylation with three different commercial sources of fungal β-galactosidases. The maximum GOS concentration (70.9 g L^{-1}, from trisaccharides to heptasaccharides) was obtained in the second hour, which is the highest productivity yet reported. Yu and O'Sullivan (2014) produced GOS using a hyperthermophilic β-galactosidase in permeabilized whole cells of *Lactococcus lactis* at 85 °C. A maximum production of 197 g L^{-1} of GOS tri- and tetrasaccharides was obtained after 55 h of incubation. The critical drawbacks of the current reaction systems are low volumetric productivity and poor enzyme selectivity; the enzymatic GOS products usually consist of different degrees of polymerization and add additional cost for purification. Therefore, screening and discovery of a highly active/selective β-galactosidase is of paramount importance to establish a highly efficient/selective system for GOS production (Choi et al., 2003; Hung & Lee, 2002; Mahoney, 1998; Torres et al., 2010; Yu & O'Sullivan, 2014). More importantly, current knowledge for the design a highly specific β-galactosidase is very limited; hence, a fully kinetic characterization of a highly selective β-galactosidase will provide precise knowledge and improved understanding for designing and engineering novel enzymes towards the designer synthesis of GOS with the desired structure and functionality.

On the basis of our recently expressed recombinant thermophilic β-glucosidase from *Thermotoga naphthophila* RKU-10 (TN0602) (Kong et al., 2015), this study shows that this enzyme has extremely high selectivity, which results in an exclusive biosynthesis of galactotrisaccharide (GOS3) via transgalactosylation of lactose. To our knowledge, this is the first example of a β-glucosidase that yields solely GOS3 rather than a GOS mixture. Comparing to β-galactosidase from *Aspergillus oryzae* (β-galactosidase AO) (a widely used commercial enzyme), the effects of key parameters such as reaction time, temperature, pH and substrate concentration on reaction selectivity and productivity are evaluated. To elucidate the catalytic mechanism and understand the molecular structure and chemical interaction leading to the high specificity of β-glucosidase TN0602, kinetic studies, fluorescence quenching spectra of enzyme by substrates and molecular docking of enzyme-substrate interaction were performed. A plausible explanation is thus proposed.

2. Materials and methods

2.1. Materials

All reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). β-Galactosidase from *Aspergillus oryzae* was purchased from Sigma-Aldrich (St. Louis, MO, USA). Silica gel plates were purchased from Merck Ltd. (Darmstadt, Germany).

2.2. Preparation of β-glucosidase

The protocol applied to produce β-glucosidase from *Thermotoga naphthophila* RUK-10 was described previously (Kong et al., 2015). The transformed *E. coli* BL21 (DE3) cells were pre-incubated in LB medium (with 10 mg mL^{-1} kanamycin) with 180 rpm agitation at 37 °C. After the OD_{600} of the culture liquid reached 1.0, IPTG was added to induce enzyme expression, and the cells were grown at 25 °C for 12 h with 150 rpm agitation. The induced cells were harvested by centrifugation and washed with 50 mmol L^{-1} sodium phosphate buffer (pH 7.0) and disrupted by sonication. Cellular debris was removed by centrifugation (15,000 rpm for 15 min at 4 °C) to obtain a crude extract. The crude extract was incubated in a water bath for 10 min at 80 °C to denature the *E. coli* proteins. The extract was then centrifuged to separate the crude enzyme from the heat-denatured cellular components and proteins. The supernatant was loaded onto a Ni^{2+}-NTA agarose resin column. The enzyme was eluted with a linear gradient of 0–200 mmol L^{-1} imidazole in sodium phosphate buffer. The purity was determined by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Finally, the pure protein was lyophilised for subsequent experiments.

2.3. Enzyme assay

The hydrolytic activity of β-glucosidase (TN0602) was determined using 5% (w/w) lactose in 50 mmol L^{-1} sodium phosphate buffer (pH 7.0) as the substrate. The hydrolytic reaction was started by adding the enzyme (1 mg mL^{-1}) and performed at 75 °C with agitation (250 rpm) for 30 min.

The β-Galactosidase activity of β-galactosidase from *Aspergillus oryzae* was measured using lactose as a substrate. β-Galactosidase (1 mg mL^{-1}) was added to a reaction mixture containing 5% lactose and 50 mmol L^{-1} acetate buffer (pH 4.5) followed by incubation at 50 °C and agitation (250 rpm) for 30 min. Analysis of the samples was performed with high-performance liquid chromatography (HPLC), and hydrolytic activity was calculated on the basis of galactose concentration. One unit of enzymatic activity was defined as the amount of enzyme required to liberate 1 μmol of galactose per minute under the experimental conditions (Kong et al., 2015).

2.4. Synthesis and analysis of GOS

The reaction was performed in 20 mL reaction bottles with 5 mL of lactose at 75 °C (β-glucosidase from *Thermotoga naphthophila* (RKU-10)) or 50 °C (β-galactosidase from *Aspergillus oryzae*) with agitation at 250 rpm. Transgalactosylation was initiated by adding 20 Units of enzymes. Samples were taken at 30 min intervals and analysed by HPLC. The production of GOS was quantified by the measurement of GOS concentration in the reaction mixture as the reaction evolved.

The reaction mixture was analysed using thin-layer chromatography (TLC) with butanol-propanol-ethanol-water (2:3:3:2, v/v) as the developing solvent and developed twice. The developed TLC plates were stained with α-naphthol (2.56 g L^{-1}) in an ethanol-sulfuric acid mixture (90:10, v/v). All carbohydrates were visualized by heating the plate at 100 °C for 5 min. Qualitative and quantitative analyses of tetrasaccharide, trisaccharide, lactose, glucose and galactose were determined by HPLC using a SUPELCOGEL™ Ca column (Sigma-Aldrich, St. Louis, MO, USA) conjugated with an evaporative light scattering detector (ELSD). The mobile phase was deionized water and the flow rate was kept constant at 0.5 mL min^{-1}. The column temperature was kept at 80 °C. Fig. S8 shows a typical chromatogram of the reaction mixture with xylitol as the internal standard.

2.5. Structural identification of enzymatically synthesized trisaccharide and tetrasaccharide

Galactooligosaccharides from enzymatic reaction mixtures were separated and purified through column chromatography, and the resulting products were subject to structural identification. The products were characterized through ^{1}H NMR, ^{13}C NMR, and mass spectrometry analysis. ^{1}H NMR and ^{13}C NMR were recorded on a Bruker AVANCE III 400 MHz spectrometer using D_2O as the solvent. MS spectra were