International Journal of Biological Macromolecules 99 (2017) 594–599

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Characterization of novel thermophilic alpha-glucosidase from *Bifidobacterium longum*

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ARTICLE INFO

Article history:
Received 2 February 2017
Received in revised form 1 March 2017
Accepted 2 March 2017
Available online 7 March 2017

Keywords:
Alpha glucosidase
Bifidobacterium longum
Glycoside hydrolase
Thermophile
Thermostability

ABSTRACT

In this study, the gene encoding α-glucosidase from *Bifidobacterium longum* subsp. *longum* JCM1217 (BLAG) was cloned and expressed in *Escherichia coli*. The amino acid sequence alignment demonstrated that BLAG belongs to glycoside hydrolase (GH) family 13. The optimal temperature for enzyme activity was 75 °C; about 80% of the catalytic activity was lost at 50 °C, which is very unusual for enzymes from the *Bifidobacterium* genus. In the presence of 5 mM of Co^{2+} and Ca^{2+}, enzyme activity was reduced to 47% and 48%, respectively. Furthermore, BLAG lost catalytic activity following the addition of 5 mM of Fe^{2+} ion. The BLAG enzyme was able to hydrolyze α-1,2, α-1,3, α-1,4, and α-1,6 glycosidic *O*-linkages and liberated glucose from the non-reducing end of substrates. The kinetic study revealed that among the maltooligosaccharides, BLAG showed the highest k_{cat}/K_m value to maltotriose (G3), and had relatively low k_{cat}/K_m values on long-chain maltooligosaccharides. This is the first report describing the production of a thermophilic α-glucosidase from the Bifidobacterium genus.

1. Introduction

Bifidobacteria are anaerobic, Gram-positive microorganisms commonly found in the intestinal tracts of mammals [1]. Bifidobacteria are considered to be beneficial commensal microorganisms because they prevent the growth of harmful bacteria [2]. Especially, bifidobacteria, including *Bifidobacterium bifidum*, *Bifidobacterium longum* subsp. *longum*, *B. longum* subsp. *infantis*, *Bifidobacterium breve*, and *Bifidobacterium animalis* subsp. *lactis*, are recognized as probiotics [3]. Practically all bifidobacterial species utilize compounds such as lactose, sucrose, glucose, galactose, and fructose. Furthermore, some species are able to ferment mannitol and sorbitol [4,5].

α-Glucosidase (EC 3.2.1.20, α-D-glucoside glucohydrolase) is a universal enzyme with high abundance in microorganisms, plants, and animals tissues [6]. This enzyme participates in the glycogen metabolism of higher organisms, as well as in the nutrient absorption and processing of bacteria [7–9]. The α-glucosidases may be classified into three types according to substrate specificity [10]. Based on sequence homology, these enzymes are part of glycoside hydrolase (GH) families 13 and 31 within the CAZy classification system [11–14]. Type I α-glucosidases belong to GH family 13, which can hydrolyze heterogeneous substrates such as aryl glucosides and phenyl α-D-glucoside, faster than short-chain maltooligosaccharides [6,10,14,15]. On the other hand, type II and III α-glucosidases are part of GH family 31. Type II α-glucosidases have a maltosyl-structure and prefer homogeneous substrates, such as G2 and IG2, to heterogeneous substrates. Similar to type II α-glucosidases, type III enzymes can be recognized by their maltosyl and oligomeric structure. Therefore, type III enzymes show high catalytic activity toward polysaccharides such as glycogen and soluble starch [6,10,14,15].

In this study, we cloned an α-glucosidases from *B. longum* JCM 1217 and characterized the catalytic properties of this enzyme.

2. Material and methods

2.1. Chemicals and reagents

B. longum subsp. *longum* JCM 1217 was purchased from the Korean Collection for Type Culture (KCTC). *Escherichia coli* MC1061 (F±, *ara*D139, *rec*A13, D [*ara*ABCleu] 7696, *gal*U, *gal*K, Δ*lac*X74, *rps*L, *thi*, *hsd*R2, *mcr*B) was used as a host for gene cloning and expression. Maltose (G2), isomaltose (IG2), kojibiose, nigerose, maltotriose (G3), maltotetraose (G4), maltopentaose (G5), and *p*-nitrophenyl α-D-glucoside (*p*NPG) were obtained from Sigma-Aldrich Co. (St.

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http://dx.doi.org/10.1016/j.ijbiomac.2017.03.009
0141-8130/

Louis, MO, USA). Nickel-nitrilotriacetic acid (Ni-NTA; purified by affinity chromatography) was supplied by Qiagen Inc. (Hilden, Germany). $CoCl_2$, $CaCl_2$, $ZnCl_2$, $MgCl_2$, $MnCl_2$, $FeCl_2$, $CuCl_2$, and ethylenediaminetetraacetic acid (EDTA) were obtained from Junsei Chemical Co. (Tokyo, Japan), Shinyo Pure Chemicals (Tokyo, Japan), and Promega (Madison, WI, USA). *n*-Butanol and ethanol were obtained from Junsei Chemical Co. (Tokyo, Japan).

2.2. Analysis of phylogeny and amino acid sequence

Previously reported α-glucosidase amino acid sequences of 13 GH members were collected using the CAZy database (http://www.cazy.org/). Multiple sequence alignments were executed with the Align X program, part of Vector NTI Suite 5.5 (Informax Inc., North Bethesda, MD, USA). The MEGA6 program (http://www.megasoftware.net/) was used to create a phylogenetic tree [16].

2.3. Cloning of the BLAG gene and nucleotide sequence analysis

For expression of recombinant protein in *E. coli*, the coding region of the α-glucosidase (*bllj_0112*) was amplified by polymerase chain reaction (PCR), with genomic DNA from *B. longum* (KCTC 3127) used as a template. The gene-specific primers 5′-GGA ATC CAT ATG ACC GCA AAT AAC CTC-3 and 5′-CCA AGG CTC GAG CTT GAT AAC CCA CG-3′ were used in this reaction, which introduced *Nde*I and *Xho*I restriction sites into the sequences (underlined). The PCR was performed as follows: denaturation at 98 °C for 3 min, followed by 30 cycles of denaturation at 98 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min 48 s. The *Nde*I- and *Xho*I-digested PCR products (1.8 kb) were ligated into the pTKNd119 vector bearing the *Bacillus licheniformis* maltogenic amylase (BLMA) promoter and a C-terminal hexahistidine tag [17–19]. The pTKNd *bllj_0112* was transformed into *E. coli* MC1061 and then spread on LB agar plates, including kanamycin (50 μg/mL) and 1% (w/v) agarose.

2.4. Expression and purification of the enzyme

The transformants were incubated in LB medium (10 g/L bactotryptone, 5 g/L yeast extract, and 5 g/L NaCl) containing 100 mM sorbitol and kanamycin (50 μg/mL) for 12 h at 37 °C with shaking at 200 rpm. The purification steps were performed using Ni-NTA chromatography as described previously [18]. The protein pattern of cell fractions was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The purified recombinant enzyme was named BLAG.

2.5. Enzyme assay

The BLAG enzyme activity assays were performed using G2, G3, G4, *p*NPG, nigerose, kojibiose, and IG2 as substrates. BLAG activity was determined by measuring the amount of glucose produced, or the reduction in substrate during the enzyme reaction. The enzymatic reaction was performed using 50 mM maleic acid-Tris-NaOH buffer (pH 6.5) at 65 °C. The amount of glucose produced was measured using high-performance anion exchange chromatography (HPAEC) and the glucose oxidase-peroxidase (GOD-POD) method [20,21]. HPAEC (DX-500 system; Dionex, Sunnyvale, CA, USA) used a CarboPac™ PA1 column (4 × 250 mm; Dionex) and a pulsed amperometric detector (ED40; Dionex). A flow rate of 1 mL/min was used with a gradient of 600 mM sodium acetate and 150 mM NaOH in water (0–10 min, 10–30%; 10–20 min, 30–100%). GOD-POD solution was reacted with D-glucose at 570 nm. One unit of enzyme activity was defined as the amount of enzyme needed to liberate 1 μmol of glucose per minute. BLAG concentrations were measured using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Two microliters of protein solution were analyzed with a suitable extinction coefficient of BLAG (123,850 cm^{-1} M^{-1}).

2.6. Differential scanning calorimetry (DSC) analysis

The melting temperature (T_m) of BLAG was determined using DSC 1 (Mettler Toledo, Greifensee, Switzerland). The purified enzyme (50 mg/mL) was heated from 30 to 130 °C at a rate of 1 °C/min and 50 mM Tris-HCl buffer (pH 7.5) was used as a reference [14].

2.7. BLAG enzyme activity: temperature, pH, and the thermostability effect

The optimal temperature of BLAG was determined by performing the enzymatic reactions at various temperatures (50–82 °C). The relative enzyme activity at various pHs was measured using 50 mM sodium acetate buffer (pH 5.0–6.0), 50 mM maleic acid-Tris-NaOH buffer (pH 6.0–8.0), and 50 mM Tris-HCl buffer (pH 7.0–9.0) at 65 °C. The highest enzymatic activity was defined as 100%. To examine the thermal stability of BLAG, the purified enzyme (0.0447 mg/mL) was pre-incubated at 30–80 °C for 3–60 min in 50 mM maleic acid-Tris-NaOH buffer (pH 7.5). Then, the BLAG enzyme activity was measured under standard condition with pNPG as a substrate. The activity of the enzyme without pre-incubation was designated as 0 min.

2.8. Effects of metal ions on enzymatic activity

The effects of various metals and chemical agents on the purified enzyme were determined. $MgCl_2$, $FeCl_2$, $CuCl_2$, $MnCl_2$, $CoCl_2$, $ZnCl_2$, $CaCl_2$, and EDTA were assayed at concentrations of 5 mM in the reaction mixture. BLAG enzyme activity without metal ions was considered to be 100%. Activity was measured under pH 7.5 at 65 °C with *p*NPG used as a substrate.

2.9. Reaction product analysis using thin-layer chromatography (TLC)

TLC was performed to assay the reaction products using Whatman silica gel 60 TLC plates (Whatman, Maidstone, UK). The reaction mixture, containing enzyme and a substrate at a concentration of 0.5% (w/v) in 50 mM maleic acid-Tris-NaOH buffer (pH 7.5), was incubated at 65 °C for 12 h. The mixture was spotted on the plate, which was developed with a solvent system of ethanol, *n*-butanol, and water (5:5:3, v/v/v). Reaction products on the plate were visualized by a dipping solution consisting of 0.3% (w/v) N-1-naphthylethylene diamine dihydrochloride and 5% (v/v) H_2SO_4 and 15% (v/v) methanol in water, followed by heating at 110 °C for 10 min [22].

2.10. Kinetic analysis of BLAG

A kinetic study of BLAG on G2, G3, G4, *p*NPG, nigerose, kojibiose, and IG2 was performed using GOD-POD and HPAEC under optimal conditions, as described previously. For the enzyme kinetic analysis, a Michaelis–Menten equation was fitted with GraFit software (ver. 7.0; Erithacus Software Ltd., Staines, UK). When saturation was not observed, the k_{cat}/K_m value was extrapolated from the slope of the plot of V_o versus "S" ($V_o = k_{cat}E_o[S]/K_m$) measured over a range of substrate concentrations (0.9–15 mM) [22,23].