Journal of Molecular Catalysis B: Enzymatic 116 (2015) 39–44

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Characterization of an epilactose-producing cellobiose 2-epimerase from *Thermoanaerobacterium saccharolyticum*

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ARTICLE INFO

Article history:
Received 31 October 2014
Received in revised form 10 March 2015
Accepted 10 March 2015
Available online 19 March 2015

Keywords:
Cellobiose 2-epimerase
Characterization
Epilactose
Epimerization
Thermoanaerobacterium saccharolyticum

ABSTRACT

In this study, a recombinant cellobiose 2-epimerase (GenBank accession number, YP_006392930.1) was characterized from a thermophilic bacterium, *Thermoanaerobacterium saccharolyticum* JW/SL-YS485. The enzyme was metal independent, showed maximal epimerization activity at pH 7.0 and 60 °C, and displayed 29.8, 15.48, and 13.5 U mg^{-1} for mannobiose, cellobiose, and lactose under optimum conditions, respectively. It exhibited promising thermostability under incubation below 60 °C. The K_m, turnover number (k_{cat}), and catalytic efficiency (k_{cat}/K_m) for lactose were 124.7 mM, 30.9 s^{-1}, and 0.248 mM^{-1} s^{-1}, respectively. At pH 7.0 and 60 °C, 50 mM epilactose was produced from 200 mM lactose by a 0.6 μM of enzyme concentration after reaction for 4 h.

1. Introduction

Epilactose (4-*O*-β-D-galactopyranosyl-D-mannose), an epimer of lactose, is an important bioactive lactose derivative and has recently attracted considerable attention due to its promising physiological effects [1]. It is a type of non-digestible disaccharide with good prebiotic properties [2,3], and promotes intestinal mineral absorption [4]. Experimental results obtained *in vivo* indicate that epilactose metabolism increases the level of beneficial short-chain fatty acids and reduces the risk of arteriosclerosis [5]. Epilactose exists in extremely small quantities in nature, and generally is chemically formed in a small amount by heating or sterilization of solutions of lactose [6], especially milk [7].

Recently, a great deal of attention has been focused on its biological production. Cellobiose 2-epimerase (CE, EC 5.1.3.11) is a potential biocatalyst for industrial production of epilactose from the cheap material lactose [1]. CE mainly catalyzes the reversible epimerization between disaccharide cellobiose and 4-O-β-D-glucopyranosyl-D-mannose and generally has broad substrate specificity toward various disaccharides including mannobiose, lactose and epilactose [8]. In 2008, epilactose-producing CE was first characterized from a ruminal anaerobe, *Ruminococcus albus* (Rual-CE) [2]. Since then, epilactose-producing CE was identified from another five anaerobic strains, namely *Eubacterium cellulosolvens* (Euce-CE) [9], *Bacteroides fragilis* (Bafr-CE) [10], *Caldicellulosiruptor saccharolyticus* (Casa-CE) [11], *Dictyoglomus turgidum* (Ditu-CE) [8], and *Spirochaeta thermophila* (Spth-CE) [12]. The CE from a thermohalophilic aerobe, *Rhodothermus marinus* (Rhma-CE), was also proven to be able to produce epilactose effectively [13]. In addition, Ojima et al. recently identified and characterized seven epilactose-producing CEs from various mesophilic aerobes: *Flavobacterium johnsoniae* (Fljo-CE), *Pedobacter heparinus* (Pehe-CE), *Dyadobacter fermentans* (Dyfe-CE), *Herpetosiphon aurantiacus* (Heau-CE), *Saccharophagus degradans* (Sade-CE), *Spirosoma linguale* (Spli-CE), and *Teredinibacter turnerae* (Tetu-CE) [14].

In this study, a recombinant CE was characterized from a thermophilic anaerobe, *Thermoanaerobacterium saccharolyticum* JW/SL-YS485 (Thsa-CE), which showed high epimerization activity toward lactose. The epilactose production from lactose by the enzyme was studied.

2. Materials and methods

2.1. Chemicals and reagents

Lactose and cellobiose were from Sinopharm Chemical Reagent (Shanghai, China). Mannobiose was from Megazyme International

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http://dx.doi.org/10.1016/j.molcatb.2015.03.005
1381-1177/

Ireland Ltd. (Wicklow, Ireland). Epilactose and lactulose were purchased from Sigma (St Louis, MO, USA). Chelating Sepharose Fast Flow resin was from GE Healthcare (Uppsala, Sweden). Electrophoresis reagents were purchased from Bio-Rad. Isopropyl β-D-1-thiogalactopyranoside (IPTG) and all chemicals used for enzyme assays and characterizations in this study were of analytical grade or higher obtained from Sangon Biotech (Shanghai, China).

2.2. Gene cloning

The complete genome of the *T. saccharolyticum* JW/SL-YS485 chromosome was obtained from GenBank (NCBI accession number: NC_017992). The full-length gene (locus_tag: Tsac_2329), encoding a putative protein with ID YP_006392930.1, was synthesized and incorporated with *Nde*I and *Xho*I sites at the 5′- and 3′-termini and then was introduced into the pET-22b(+) plasmid with the same restriction sites to create a reconstructed plasmid, pET-Thsa-CE. An in-frame 6 × His-tag sequence was provided at the C-terminal sequence of the open reading frame for the simple purification of the recombinant protein.

2.3. Expression and purification of recombinant Thsa-CE

The pET-Thsa-CE plasmid was transformed into host cell *Escherichia coli* BL21(DE3) (Sangon Biotech, Shanghai, China). The recombinant *E. coli* was cultivated in LB medium containing 100 μg ml^{-1} of ampicillin with shaking (200 rpm) at 37 °C until A_{600} reached 0.6. IPTG was then added to obtain a final concentration of 0.5 mM, and the fermentation was continued at 28 °C for 6 h. The LB medium used was composed of 5 g/l yeast extract, 10 g/l tryptone, and 10 g/l NaCl.

The recombinant enzyme, expressed as a 6 × His-tagged fusion protein, was purified by one-step nickel-affinity chromatography (Novagen) according to the manufacturer's protocol (pET His Taq System; Novagen). The active fractions eluted were pooled and dialyzed overnight against 50 mM sodium phosphate buffer (pH 7.0). The purity and integrity of protein were checked by SDS-PAGE on a 12% gels.

2.4. Enzyme assay

Enzyme activity was measured by determination of the amount of produced epilactose from lactose. The reaction mixture of 1 ml volume contained 100 mM lactose, sodium phosphate buffer (50 mM, pH 7.0), and 1 μM enzyme. The reactions were carried out at 60 °C for 15 min; and were stopped by addition of HCl to the reaction mixture at a final concentration of 500 mM. One unit (U) of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of epilactose per min at pH 7.0 and 60 °C.

2.5. Biochemical characterization

The characterization of the purified enzyme was detected using lactose as a substrate. The effect of pH was determined at 60 °C using four buffer systems (50 mM), including sodium citrate buffer (pH 5.0–6.0), sodium phosphate buffer (pH 6.0–7.5), Tris–HCl buffer (pH 7.5–9.0), and glycine–NaOH buffer (pH 9.0–10.5). The optimum temperature was determined by measuring the activity at different temperatures, ranging from 30 to 80 °C. The thermostability was determined by detecting the residual activity of the enzyme that had been pre-incubated at different temperatures.

Kinetic parameters were determined using 10–100 mM concentrations of substrate. The parameters, including the Michaelis–Menten constant (K_m) and turnover number (k_{cat}) values, were determined by Lineweaver–Burk plots from the Michaelis–Menten equation.

2.6. Analytical methods

To detect the enzyme activity, lactose and epilactose were determined by an HPLC (Agilent 1200 system, Agilent technologies, CA, USA) equipped with a refractive index detector and an Asahipak NH2P-50-4E column (4.6 mm id × 250 mm, Shodex, Tokyo, Japan). The column was eluted with acetonitrile/water (65:35, v/v) at room temperature and 1 ml min^{-1}.

In addition, ion chromatography was used to analyze whether the enzymatic reaction produced lactulose from lactose. The reaction products were analyzed by Dionex ion chromatography ICS-5000 (Sunnyvale, CA, USA) with a Dionex pulsed amperometric detector (HPAEC-PAD) equipped with an Au electrode and a Dionex Carbopac PA20 column (3 mm id × 150 mm, Sunnyvale, CA, USA). The column was eluted with 1.5 mM NaOH as the mobile phase at 30 °C and 0.5 ml min^{-1}.

3. Results and discussion

3.1. Amino acid sequence alignment

The putative protein with accession No. YP_006392930, shown as *N*-acyl-D-glucosamine 2-epimerase (AGE) from *T. saccharolyticum* JW/SL-YS485 in GenBank, was identified as CE and renamed Thsa-CE due to the high specificity toward cellobiose. Similarly, many putative AGEs in GenBank have been identified as CEs without activity toward *N*-acetyl-D-glucosamine in previous reports [8,13,14]. By sequence analysis, Thsa-CE was determined to be a 392 amino acid protein with a calculated molecular mass of 46,516 Da.

Based on the phylogenetic tree analysis, Thsa-CE showed a much closer relationship with Casa-CE (Fig. 1). Homologous comparison of the amino acid sequences of various CEs was analyzed. Thsa-CE (GenBank accession No.: YP_006392930.1) shared 52.8% amino acid identity with Casa-CE (YP_001179132), 40–50% identity with Ditu-CE (YP_002352551), Rual-CE (BAF81108), Euce-CE (BAG68451.1), Bafr-CE (BAH23773), and Rhma-CE (BAK61777), and less than 40% identity with other CEs (Table 1). Although more than 10 microbial CEs were characterized, the amino acid sequence identity between them was interestingly very low, all less than 50% except the identity between Thsa-CE and Casa-CE (52.8%) and the identity between Bafr-CE and Pehe-CE (50.5%) (Table S1). The

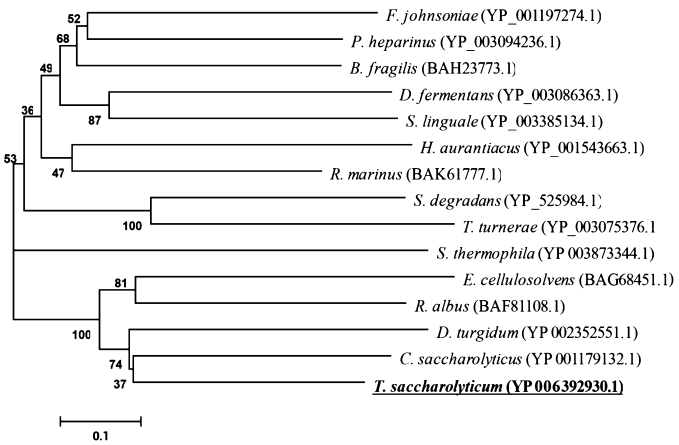

Fig. 1. Phylogenetic tree of the characterized epilactose-producing CEs from various microorganisms. The scale bar indicates the amino acid substitutions per position. GenBank accession numbers of the CEs are given after each species name.