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A novel cold-adapted and glucose-tolerant GH1 β-glucosidase from *Exiguobacterium antarcticum* B7

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ABSTRACT

A novel GH1 β-glucosidase (*Ea*Bgl1A) from a bacterium isolated from Antarctica soil samples was recombinantly overexpressed in *Escherichia coli* cells and characterized. The enzyme showed unusual pH dependence with maximum activity at neutral pH and retention of high catalytic activity in the pH range 6 to 9, indicating a catalytic machinery compatible with alkaline conditions. *Ea*Bgl1A is also a cold-adapted enzyme, exhibiting activity in the temperature range from 10 to 40 °C with optimal activity at 30 °C, which allows its application in industrial processes using low temperatures. Kinetic characterization revealed an enzymatic turnover (K_{cat}) of 6.92 s^{-1} (cellobiose) and 32.98 s^{-1} (*p*NPG) and a high tolerance for product inhibition, which is an extremely desirable feature for biotechnological purposes. Interestingly, the enzyme was stimulated by up to 200 mM glucose, whereas the commercial cocktails tested were found fully inhibited at this concentration. These properties indicate *Ea*Bgl1A as a promising biocatalyst for biotechnological applications where low temperatures are required.

1. Introduction

The enzymes with β-glucosidase activity (EC 3.2.1.21) catalyze the hydrolysis of β-1,4 glucosidic bonds in cellobiose and other short oligosaccharides derived from cellulose [1,2]. Regarding their evolutionary and structural properties, they can be classified into one of five glycoside hydrolase families (GHs): 1, 3, 5, 9 and 30 [3,4]. The vast majority of β-glucosidases (BGLs) characterized so far belong to family GH1 [4,5]. Proteins belonging to this group are able to hydrolyze different kinds of substrates and exhibit a $(\beta/\alpha)_8$-barrel structure, with two conserved carboxylic acid residues in their active sites on β-strands 4 and 7 [4,6,7].

β-Glucosidases are ubiquitous in most living kingdoms, from bacteria to higher eukaryotes, and are involved in a wide variety of natural processes, which are extensively explored in biotechnological procedures in pharmaceutical and food industries (production of bioactive agents, antimicrobial properties for use in cosmetics, aroma enhancement, winemaking), as well as in biofuels production [6,8–10]. In the context of second generation bioethanol production, these enzymes play an important role in breaking down glucose oligomers and cellobiose, producing glucose and alleviating the inhibition of cellobiohydrolases and endoglucanases by these oligosaccharides during enzymatic hydrolysis [11,12]. On the other hand, β-glucosidases are themselves inhibited by an increasing glucose concentration, which makes the search for highly glucose-tolerant enzymes crucial for an effective hydrolysis of plant biomasses [11–13].

Nowadays, most of the β-glucosidases, as well as enzymatic preparations studied and commercialized, have been isolated from fungal strains [2]. Among them, the enzymatic cocktails most commonly employed for biomass hydrolysis in second generation ethanol production are usually active at 50 °C [2,14,15], which requires the cooling down of the hydrolysate for further yeast fermentation. This comprises a significant bottleneck in lignocellulosic ethanol production, as industrial yeasts grow at lower temperatures (around 32 °C) [16]. Consequently, the steps of saccharification and fermentation have to be performed separately, generating expenses to the industries that could be reduced in case

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these two steps were combined into a single stage (simultaneous saccharification and fermentation).

In this context, the present work was elaborated aiming at the identification and characterization of a cold-active β-glucosidase, obtained from *Exiguobacterium antarcticum* B7, a bacterium belonging to Phylum Firmicutes isolated from Antarctica soil samples.

2. Material and methods

2.1. Microbial strains, vectors and reagents

E. antarcticum B7 was isolated from a microbial biofilm at Ginger Lake (King George Island, Antarctic Peninsula) in 2005 [17,18]. Plasmid vectors employed in this work were the cloning vector pJET (CloneJET Cloning Kit, Thermo Scientific) and the expression vector pET28a (Novagen). *Escherichia coli* strains DH5α and pRARE2 were used as cloning and expression hosts, respectively. All substrates used for the biochemical characterization assays were purchased from Sigma–Aldrich.

2.2. Molecular Cloning and sequence analysis

The gene *bglA*, encoding a β-glucosidase from family GH1, was amplified from the *E. antarcticum* B7 genome (GenBank accession number: CP003063) using the following primers: (ExiguoGH1-F, 5′-ATAGCTAGCATGAAATTTGCA-3′ and ExiguoGH1-R, 5′-TATGGATCCTTACGCTTCTTG-3′). These primers have restriction sites for the enzymes *Nhe*I and *Bam*HI (underlined), respectively, for further subcloning of the ORF into a pET28a expression vector with an N-terminal His_6-tag.

The nucleotide sequence obtained for the cloned *bglA* sequence was employed for similarity search in NCBI database (http://www.ncbi.nlm.nih.gov/) using the online tools BlastN and BlastP. The BglA (in this work referred to as *Ea*Bgl1A, accession number AFS69459) sequence was compared to other glucosidases from *Humicola insolens* [19], *Humicola grisea* var. *thermoidea* [20], *Trichoderma reesei* [21] and *T. harzianum* [22] and other bacterial species belonging to the Genera *Exiguobacterium* and *Clostridium*, the last belonging to the same phylum (Firmicutes) as *Exiguobacterium* spp. and widely used in the biofuels industry [23]. Amino acid sequence comparison was performed both by multiple alignment using Clustal Omega [24] and phylogenetic analysis using the program MEGA 6.06 [25].

A structural model of the enzyme was obtained by homology molecular modeling using the HHpred server [26] and the homologous structure (41% sequence identity) of the GH1 β-glucosidase from *Micrococcus antarcticus* (PDB code: 3W53, www.rcsb.org/pdb/home). The structure was analyzed using the program PyMoL (http://www.pymol.org).

2.3. Expression and purification

E. coli pRARE2 cells containing the recombinant plasmid were grown in selective medium and induction of protein expression was performed with 0.5 mM IPTG for 4 h at 37 °C. Centrifuged cells were resuspended in 15 mL of a lysis solution (20 mM sodium phosphate buffer, 300 mM NaCl, 5 mM imidazole, pH 7.4) with 30 μg mL^{-1} lysozyme and 1 mM PMSF, following disruption by sonication. For enzyme purification, the suspension was centrifuged (11,000 × *g* for 20 min) and the clarified supernatant was loaded onto a His-Trap Ni^{2+}-chelating affinity column. The bound proteins were eluted with a non-linear imidazole gradient (5-500 mM) and submitted to size-exclusion chromatography (Hiload Superdex 200 16/60) previously equilibrated with 20 mM sodium phosphate buffer and 150 mM NaCl, at pH 7.4.

All steps of heterologous expression and enzyme purification were analyzed by sodium dodecyl sulfate-polyacrylamide gel (12% polyacrylamide) electrophoresis (SDS-PAGE) [27]. Protein concentration was determined by absorbance at 280 nm, considering the molar extinction coefficient as 110,030 M^{-1} cm^{-1} [28].

2.4. Circular dichroism

Circular dichroism (CD) spectra were measured in a JASCO J-815 CD Spectrometer in 1 mm optic path quartz cuvettes using 4.3 μM of the enzyme in 20 mM sodium phosphate buffer, 150 mM NaCl, pH 7.4 at 10 °C. All results were generated from the mean of at least 16 spectra of 200–260 nm measured at 100 nm min^{-1}. An internal control system (Peltier Type Control System PFD 425S-Jasco) was employed for temperature maintenance inside the cell. Data were treated using the software Origin 8.1 (OriginLAB Corporation) and the values (mDeg) obtained in CD measurements were converted to residual molar ellipticity.

2.5. pH and temperature dependence

Unless otherwise stated, all assays were carried out in triplicate, in 100 μL reactions containing a final concentration of 1.1 ηg $μL^{-1}$ (20.75 nM) of purified enzyme, 40 mM sodium phosphate buffer pH 7.0 and 0.5 mM 4-nitrophenyl β-D-glucopyranoside (*p*NPG). The reactions were incubated for 10 min at 30 °C and stopped by the addition of 100 μL of 1 M Na_2CO_3. The relative absorbance to *p*-nitrophenol concentration was measured at 405 nm using an Infinite® 200 PRO microplate reader (TECAN). One unit (U) of enzyme was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per minute and the specific activity was defined as the number of units per mg of protein [29].

pH dependence for enzymatic activity was determined in the pH range from 3.0 to 10.0 using one of the following buffers: citrate-phosphate (pH 3, 4, 5 and 5.5), phosphate (pH 6, 6.5, 7, 7.5 and 8) and glycine (pH 8.5, 9, 9.5 and 10) at a final concentration of 40 mM. The optimal temperature was evaluated under the pH of highest enzymatic activity in assays ranging from 5 to 55 °C at 5 °C intervals. Thermotolerance was determined by incubation of the enzyme (0.5 mg mL^{-1}) at 30 °C for a total of 75 h, with aliquots taken regularly.

2.6. Influence of ions

The effects of metal ions, reducing agents and salts on enzymatic activity was evaluated in individual reactions containing 10 mM (final concentration) of KCl, $CuCl_2$, $MgCl_2$, $MnCl_2$, $CoCl_2$, NaCl, EDTA, $FeCl_3$, LiCl, $ZnSO_4$, DTT (dithiothreitol) and $CaCl_2$, as well as SDS (final concentration of 0.1%) and Tween 80 (final concentration of 0.2%). The activity assayed without any additional reagent was recorded as 100%.

2.7. Determination of kinetic parameters and substrate specificity

Kinetic parameters were determined using a final concentration of 1 ηg $μL^{-1}$ of *Ea*Bgl1A in 40 mM sodium phosphate buffer (pH 7.0) at 30 °C by the rate of *p*NPG and cellobiose hydrolysis at various concentrations, ranging from 0.01 to 60 mM. Data analysis was performed in *Graph*Pad Prism 5 software adjusting the non-linear fit of Michaelis-Menten equation for determination of maximum velocity (V_{max}) and half-saturation coefficient (K_m) based on the value of substrate concentration plotted *versus* the initial velocity of each reaction. All kinetic assays were performed in quadruplicate. Substrate specificity was assessed using the standard assay described above with CMC (0.5%, [30]) and different 2- and

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