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Characterization of a heat-active archaeal β-glucosidase from a hydrothermal spring metagenome

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ABSTRACT

Thermostable enzymes are required for application in a wide range of harsh industrial processes. High stability and activity at elevated temperatures, as well as high tolerances toward various reagents and solvents, are needed. In this work, a glycoside hydrolase family 1 β-glucosidase (Bgl1) of archaeal origin was isolated from a hydrothermal spring metagenome. The enzyme showed a broad substrate spectrum with activity toward cellobiose, cellotriose and lactose. Compared to most enzymes, extremely high specific activity with 3195 U/mg was observed at 90 °C and pH 6.5. Bgl1 was completely stable at pH 4.5–9.5 for 48 h at 4 °C. More than 40% of activity was measured at 105 °C. A thermal activation was observed at 90 °C after 30 min. Enzyme stability was enhanced (5- and 7-fold) after applying pressure of 100 and 200 bar at 90 °C for 2 h, respectively. The affinity of the β-glucosidase to its substrate was significantly increased in the presence of $AlCl_3$. The K_i value for glucose was 150 mM. These distinctive characteristics distinguish Bgl1 from other enzymes described so far and make this enzyme suitable for application in numerous processes that run at high temperatures.

1. Introduction

Microorganisms exhibiting high growth temperature are considered as producer of extreme thermostable enzymes (extremozymes). The majority of these enzymes are more resistant to chemical denaturation compared to their counterparts produced from mesophiles [1]. Of special interest for industrial application are hypertherophilic Archaea since they grow optimally between 80 and 110 °C and produce unique thermostable enzymes [2]. Industrial processes that run at elevated thermal conditions have many advantages. The solubility of complex substrates increases due to lower viscosity and higher diffusion rate of the substrate. Furthermore, the risk of contamination is significantly reduced [3].

In the last decades, many biocatalysts from Archaea exhibiting remarkable characteristics, such as optimal function at 100–110 °C, were discovered [4]. Especially glycoside hydrolases are suitable for a wide range of biotechnological applications. For the efficient bioconversion of lignocellulose to biofuels and chemicals novel glycoside hydrolases that act synergistically are needed [5]. This will lead to the development of biorefinery of the 2nd generation that is competitive to the petroleum-based plants [6]. Endoglucanases (EC 3.2.1.4) cleave within the amorphous regions of long glucose chains. Cellobiohydrolases (EC 3.2.1.91) act on reducing or non-reducing ends of accessible polysaccharides. Cellobiose, cellotriose or longer oligosaccharides that are produced from this reaction represent the substrates for β-glucosidases (EC 3.2.1.21). These enzymes hydrolyze terminal, non-reducing β-D-glucose residues and produce glucose monomers [7]. The limiting factor in the conversion of cellulose to glucose is the inhibition of endoglucanase activity by oligosaccharides [8].

Other applications of β-glucosidases include the cleavage of phenolic and phytoestrogen glucosides from fruits and vegetables for medical purposes or to enhance the quality of beverages. Furthermore, the enzyme is used to hydrolyze naringin resulting in the reduction of fruit bitterness or gellan hydrolysis resulting in the reduction of viscosity [8,9].

Since most microorganisms, particularly extremophiles, are not cultivable under laboratory conditions, the metagenomic approach was introduced. Metagenomic libraries from high temperature habitats offer a great potential for the identification of novel heat-stable enzymes [10]. Using function-based or sequence-based methods putative genes, e.g. β-glucosidase-encoding genes can be identified [11,12]. A number of novel biocatalysts with remarkable properties have been already detected from unknown microorganisms in such metagenomes [13].

In this work, a metagenome was recovered from a hot spring at the island São Miguel (Azores, Portugal). The prevalent elevated temperatures of up to 70 °C favored the presence of thermophilic Bacteria and Archaea. A novel β-glucosidase was purified and characterized.

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2. Materials and methods

2.1. Starting material

Samples were collected at Furnas Valley from different locations of a hot spring named Caldeirão on the island São Miguel (Azores, Portugal, 37°46′ N, −25°18′ E) in September 2010 [14]. Environmental samples included water, mud and sediment and were taken at locations with temperatures ranging from 60 to 70 °C. The pH varied from pH 6 to 7. The collected material was transported and stored at 4 °C under anoxic conditions by adding Na_2S (2.5%, w/v, pH 7, final concentration 0.01%, w/v).

2.2. DNA isolation and amplification

The DNA was isolated through direct lysis [15]. Due to low amounts of recovered DNA, the entire genetic information was amplified by multiple displacement procedure. Therefore, 1 μL of the template DNA was denatured for 3 min at 94 °C with 1 μL random hexamer primer (500 pmol), 2.5 μL phi29 buffer (10×) and 1 μL DTT. Subsequently, isothermal amplification was performed by addition of 2 μL phi29 buffer (10×), 2 μL dNTPs (25 mM each), 10 U phi29 DNA polymerase (Thermo Scientific, Schwerte, Germany) in a volume of 15 μL filled up with deionized water for 12–16 h at 30 °C. The resulting hyperbranched structures were dissolved [16]. Subsequently, single-stranded overhangs were digested, emerging gaps were filled with nucleotides and the DNA was finally dephosphorylated. After each step the amplicon was purified using SureClean solution (Bioline, Luckenwalde, Germany).

2.3. Metagenomic library construction and screening

Fragments with sizes in the range 4–10 kb were purified from an agarose gel (1%) using the GeneJET Gel Extraction kit (Thermo Scientific, Schwerte, Germany). The addition of a single 3′-deoxyadenosine overhang enabled the ligation into the pCR-XL-TOPO vector. *E. coli* Top10 cells were transformed with ligation mixture according to the manufacturer's instructions (Invitrogen, Karlsruhe, Germany). The cells were plated as recommended. After sweeping off the cells, the plasmids were isolated employing the Qiagen Plasmid Plus Midi Kit (Hilden, Germany). Screening for activity-conferring genes took place by using clones derived from transformation of *E. coli* DH5α cells with 1 μL of metagenomic plasmid library. The cells were grown on LB agarplates with 50 μg/mL kanamycin. The colonies were replicated by stamping on velvet with a subsequent transfer on a new plate. After growth of colonies, screening top-agarose (50 mM sodium acetate, 2.5 mM calcium chloride x 2 H_2O, 170 mM NaCl, 2.5 mM esculin hydrate, 0.4 mM ammonium ferric citrat, 1.2% agarose) was used to overlay cells. Clones were incubated at 70 °C over night.

2.4. Analysis of activity-conferring clones

Plasmids were isolated employing the GeneJET Plasmid Miniprep Kit (Thermo Scientific, Schwerte, Germany). Inserts of pCR-XL-TOPO were sequenced by Eurofins MWG Operon (Ebersberg, Germany). Potential activity-conferring open reading frames (ORFs) were identified with frameplot 4.0 beta (http://nocardia.nih.go.jp/fp4/). BLAST (Basic Local Alignment Search Tool) analyses were done to determine protein sequence identities employing GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/). A hypothetical model was constructed on the basis of the deduced amino acid sequence using the SWISS-MODEL workspace (http://swissmodel.expasy.org/) [17].

2.5. Gene expression and purification

The gene *bgl1* was amplified (for: GGATCCGTAAAGTTCCCTAAAGG, *Bam*HI recognition site is underlined, rev: GTCGACCTAAGTAAGAACGTTTGG, *Sal*I recognition site is underlined) and ligated into subcloning vector pJET (Thermo Scientific, Schwerte, Germany) prior to transformation of *E. coli* NovaBlue. The plasmids were isolated and *bgl1* was recovered by restriction excision. The linear gene *bgl1* was ligated into *Bam*HI/*Sal*I linearized pQE-80L resulting in plasmid pQE-80L::*bgl1*. Subsequently, *E. coli* C43 cells were transformed. 1 L of *E. coli* C43 pQE-80L::*bgl1* culture was grown to an optical density (λ = 600 nm) of 0.6. Gene expression was induced with 0.1 mM IPTG. After 12 h of growth, cells were harvested by centrifugation (7000 × *g*, 4 °C, 10 min). Resuspension of sedimented cells was achieved with 15 mL lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8). For cell disruption, pressure of 1000 psi was applied five times using a French Pressure Cell Press (SLM-Aminco, Maryland, USA). After centrifugation (13,000 × *g*, 4 °C, 15 min), Ni-NTA-affinity chromatography was conducted with 4 mL of supernatant using Ni-NTA Agarose and Ni-NTA Spin Columns according to the manufacturer's instructions (Qiagen, Hilden, Germany). Pure elution fractions were dialyzed (Visking® dialysis tubing, Serva, Heidelberg, Germany) in 20 mM maleate buffer, pH 6.5 (20 mM maleic acid, 20 mM NaOH). Protein was visualized and the molecular mass was surveyed by SDS-PAGE (12%, sodium dodecyl sulphate-polyacrylamide gel electrophoresis) [18]. The quaternary structure was investigated by native PAGE using the Pro Gel Tris Glycin gel (4–20%) as recommended by the manufacturer (Anamed Elektrophorese, Groß-Bieberau/Rodau, Germany).

The identified potential catalytic glutamate residues were mutated by PCR using degenerated primers exhibiting one exchanged base (E209 to G209 – for: GCACAATGAATGGACCAAATGTAAC, rev: GTTACATTTGGTCCATTCATTGTGC; E395 to G395 – for: GATAACTGGAAATGGAGTAGC, rev: GCTACTCCATTTCCAGTTATC; exchanged base is underlined). The constructs were fused using the flanking primers of the gene and the purified fragments as template.

2.6. Biochemical characterization of Bgl1

Unless otherwise noted, the standard activity assay was carried out with 2 mM 4-NP-β-D-glucopyranoside, 20 mM maleate buffer (pH 6.5) in a volume of 1 mL (with deionized water) at 90 °C in a heating block. After 10 min, the assay was stopped by addition of 100 μL 0.1 M Na_2CO_3. The optical density was determined at λ = 410 nm. One unit of β-glucosidase activity is defined as the amount of protein required to release 1 μmol of 4-nitrophenol per min at 90 °C and pH 6.5. Protein concentration was determined using the Bradford assay [19]. When influence of pH values was tested, Britton–Robinson buffer (20 mM) was used for a pH range between 3.0 and 10.0 [20]. Temperatures above 95 °C were adjusted using heated oil.

Influence of pressure was directly tested by performing the activity assay at 90 °C in batch reactors [21]. Diluted enzyme was incubated at 90 °C in a reactor to investigate the influence of 100 and 200 bar on enzyme stability. Subsequently, after slow release of pressure, the activity assay was carried out at 90 °C. Influence of metal ions at a final concentration of 1 or 5 mM ($AgNO_3$, $AlCl_3$ (0.001–3 mM), $CaCl_2$, $CoCl_2$, $CrCl_3$, $CuCl_2$, $FeCl_2$, $FeCl_3$, KCl, $MgCl_2$, $MnCl_2$, NaCl, $NiCl_2$, RbCl, $SrCl_2$ and $ZnCl_2$) was measured in 50 mM maleate buffer. Detergents (CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), CTAB (cetyltrimethyl-ammonium bromide), SDS, Triton X-100, Tween 20 and Tween 80), additives (β-mercaptoethanol, DTT (dithiothreitol), EDTA (ethylenediaminetetraacetic acid), guanidine hydrochloride, urea, iodoacetic acid, sodium azide and Pefabloc SC®) were added to the assay to a final concentration of 5 mM and organic solvents (acetone, acetonitrile, DMF (dimethylformamide), DMSO (dimethyl sulfoxide), ethanol, methanol, 1,2-propanediol, 1-propanol and 2-propanol) were supplemented to 10%. Substrates, such as 4-NP-β-D-glucopyranoside, 4-NP-α-D-glucopyranoside, 4-NP-β-D-galactopyranoside, 4-NP-α-D-galactopyranoside, 2-NP-β-D-galacto-pyranoside, 4-NP-β-D-fucopyranoside, 4-NP-β-D-xylopyranoside, 4-NP-β-D-manno-pyranoside, 4-NP-β-D-cellobioside, 4-NP-α-L-arabinopyranoside and 4-NP-β-D-glucuronide were tested in a final concentration of 2 mM. Cellobiose (1%, w/v), cellotriose (0.1%, w/v) and lactose (1%, w/v) were analyzed by HPLC (high-performance liquid chromatography) after incubation at 70 °C for 3 h using a HPX42A column (Bio-Rad, München, Germany). High purity water was filtrated and flushed (helium, 10 min) before being employed as running solvent with a flow rate of 0.6 mL/min. Enzyme stability was investigated by pre-incubation of pure enzyme supplemented with 0.1 mg/mL BSA (bovine serum albumin) followed by an activity assay at 90 °C.

Kinetic parameters were determined by plotting reaction rates against substrate concentrations followed by nonlinear regression analysis to determine K_m and V_{max} [22]. The inhibition constant K_i was investigated by plotting the inverse reaction rate using different substrate concentrations against inhibitor concentration [23].

2.7. Nucleotide sequence accession number

DNA sequence of *bgl1* including flanking regions was deposited in GenBank (HG326254).

3. Results

3.1. Identification and analysis of the ORF bgl1

The putative activity-conferring open reading frame (ORF) *bgl1* (1488 bp) was identified in a metagenome derived from a hot spring in the Azores (Portugal) by activity-based screening of the respective *E. coli* DH5α library using esculin as substrate. The deduced protein sequence exhibited an identity of 53% (98% sequence overlap) to a putative β-galactosidase from *Thermoproteus uzoniensis* (YP_004338090). The insert of the plasmid pCR-XL-TOPO comprised 3755 bp (Fig. 1). Besides *bgl1*, two further ORFs (*glnS1* complete and *xylA* incomplete) were identified on different reading frames but the insert of the gene library clone *E. coli* DH5α pCR-XL-TOPO::*bgl1* did not show an operon-like organization of ORFs. The deduced amino acid sequence of *glnS1* (1428 bp) exhibited 63% identity in a 98% amino acid overlap to a putative glutamine synthetase from *Caldiarchaeum subterraneum* (BAJ49060). The deduced protein sequence of the incomplete ORF *xylA* (276 bp) exhibited 51% identity to a section of a xylose-isomerase from *Sphingobacterium* sp. (YP_004318167). ORF *bgl1* contained a GC content