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Cloning with kinetic and thermodynamic insight of a novel hyperthermostable β-glucosidase from *Thermotoga naphthophila* RKU-10^T with excellent glucose tolerance

Fatima Akram, Ikram ul Haq*, Mahmood Ali Khan, Zahid Hussain, Hamid Mukhtar, Kaleem Iqbal

Institute of Industrial Biotechnology, GC University, Lahore 54000, Pakistan

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ABSTRACT

With a paradigm shift in industry, moving from natural fuels to alternative renewable resource utilization, the need of efficient thermostable cellulases are expected to increase in future. β-glucosidase, an essential member of cellulases that plays a critical role in cellulosic biomass degradation and in many biological processes. Therefore, a novel β-glucosidase gene encodes a protein (*Bgl*A) of 446 amino acid, belonging to glycoside hydrolase family 1 (GH1), was cloned from a hyperthermophilic bacterium *Thermotoga naphthophila* RKU-10^T and over-expressed in *Escherichia coli* BL21CodonPlus. An extracellular *Bgl*A with a molecular weight of 51.50 kDa, was purified to homogeneity by ion-exchange and hydrophobic interaction chromatography after heat treatment. Purified enzyme displayed optimal activity at pH 7.0 and 95 °C. It was quite stable over a broad range of pH (6.0 –9.0) and temperature (60–90 °C), fairly stable up to 8 h at 80 °C. Enzyme activity was stimulated by glucose concentration up to 600 mM and exhibited high glucose tolerance with a K_i value of 1200 mM. *Bgl*A showed great affinity toward *p*-nitrophenyl substrates and cellobiose. The K_m, V_{max} and K_{cat} values, against pNPG as substrate, were 1.5 mM, 297 mmol mg^{-1} min^{-1} and 1527778 s^{-1}, respectively. Thermodynamic parameters for pNPG hydrolysis by *Bgl*A like ΔH^*, ΔG^* and ΔS^* were calculated at 95 °C as 25.7 kJ mol^{-1}, 47.24 kJ mol^{-1} and −58.6 J mol^{-1} K^{-1}, respectively. It displayed a half-life ($t_{1/2}$) of 5.21 min at 97 °C with denaturation parameters of enzyme including ΔH^*_D, ΔG^*_D and ΔS^*_D were 662.04 kJ mol^{-1}, 110.10 kJ mol^{-1} and 1.491 kJ mol^{-1} K^{-1}, respectively. This is the first ever report on a highly glucose and thermotolerant β-glucosidase from *T. naphthophila* with high catalytic efficiency and low product inhibition, also exhibited independence of detergents and metal cations. All these significant features make *Bgl*A an appropriate candidate for biotechnological and industrial applications.

1. Introduction

The widening gap between energy demand and supply has led to power outages, and has caused disruption in the economy and development of countries. Despite having abundant renewable energy resources, the sharp growing demand has led to country-wide energy crisis which threatens the economic growth. These challenges require a multipronged approach that would address both the immediate energy shortage, help to build long-term energy sufficiency and reduce environmental pollution by adopting an alternative energy source like biofuel. Therefore, lignocellulosic plant biomass is an essential renewable and economical energy source, which can be used as a substrate for biofuel production, composite with three biopolymers i.e., cellulose (approximately 40–55%), hemicellulose (25–50%) and lignin (10–40%) [1,2]. Cellulose, the most abundant natural carbohydrate and the main component of plant cell walls, is composed of D-glucose units linked together to form linear chains via β-1,4-glycosidic linkages [3]. Non-edible cellulosic plant biomass is a significant alternative energy source to cope with the problem of food and energy with explosive increase in human population. Cellulosic wastes are the chief source of biomass which constitutes switch grasses, agricultural residues (rice hulls, corn fiber, sugarcane bagasse and wheat bran etc.), municipal and industrial superfluous material. These raw materials are inexpensive, easily accessible, environmentally sound, unexploited, and inexhaustible great potential resource for biofuel production [4]. The limiting factor of bioethanol production is the conversion of cellulose to fermentable sugars; however,

* Corresponding author.
E-mail addresses: ikmhaq@yahoo.com, ravianiib@yahoo.com (I.u. Haq).

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the rigid and complex structure of cellulose is a major bottleneck preventing the development of cost-effective production methods. Consequently, cellulosic biodegradation using thermostable cellulolytic enzymes is the most suitable way. Cellulases require less energy and mild environmental conditions to convert biomass into fermentable sugars that are used to make many important bio-based industrial products, which can replace fossil fuels [1,2].

β-glucosidases (EC 3.2.1.21), widely distributed in nature, are considered to be a significant component of cellulase system. It acts on cellobiose, alkyl- and aryl-β-D-glucosides in β-1,4-glucosidic linkage, exhibits transglycosylase and alkyl transferase activities, and catalyzes the synthesis of glycoconjugates and alkyl glucosides [5]. During saccharification of cellulosic biomass, cellobiose and cello-oligosaccharides are produced by synergistic action of cellobiohydrolases (CBHs) and endoglucanases (EGs), which are subsequently hydrolyzed by β-glucosidases to produce glucose. EGs and CBHs activities are inhibited by cellobiose (product inhibitor), therefore, β-glucosidase stimulates and regulates the cellulose hydrolysis more efficiently by relieving cellobiose inhibition [6,7]. It plays a significantly essential role in detoxification of cyanogenic glycosides, seed development, hormone production, in regulating chemical defense against pathogen attack in plants; also exhibits a vital role in hydrolysis of glucosylated flavonoids, synthesis of biodegradable surfactants and biomass conversion [8,9]. Furthermore, β-glucosidase has gained more attention due to liberation of flavor from tasteless glycosylated precursors. Hence, widely used to improve flavor and aroma of fruit juices, wines and tea [6].

Thermotolerant and highly active cellulases that have received considerable attention, in these days, are extremely desirable to attain a higher degree of efficiency on the industrial scale and for biotechnological applications. Thermostable cellulolytic enzymes, especially β-glucosidase, with higher specific activity, have the ability to function under extreme conditions, a longer hydrolysis time due to stability, lesser amount used simultaneously and directly along with other thermostable cellulases for saccharification purpose, as this decreases processing time (by eliminating pre-cooling step where steam is applied to make biomass more accessible to degradation), improves fermentation yields, reduces contamination risk, less energy consumption, enhances enzyme activity and solubility of reactants and products [7]. Thus, the potential commercial advantages of efficient and cost-effective thermostable cellulases with low degree of inhibition would reduce the cost of bioethanol production from cellulosic materials significantly in a simultaneous saccharification and fermentation (SSF) process [10]. Biomass conversion into simpler components for bio-sourced energy products using cellulases has become a major research area and thermophilic bacteria are excellent source of these enzymes [8]. Demand of thermostable β-glucosidases is growing rapidly because of its usage in food processing and biofuel production. Therefore, the search for β-glucosidases, insensitive to product inhibition with high specific activity and thermostability, has increased worldwide. Some thermostable and glucose tolerant β-glucosidases from bacteria have been studied [6,11,12] but there are limited reports on kinetic and thermodynamics study of β-glucosidases.

The quest for thermotolerant and acidic-alkali resistant glycoside hydrolases (GHs) especially cellulases led the way toward hyperthermophiles; genus *Thermotoga* have distinct characteristics from other bacteria, they are strictly anaerobes, optimally grow at 80–90 °C, and are believed to be an excellent resource of highly active, resistant to denaturing agents and heat-stable enzymes (GHs) [8,9,13,14]. The present study describes cloning, over-expression, purification and biochemical characterization of a highly thermostable β-1,4-glucosidase (*Bgl*A) from a hyperthermophilic bacterium *Thermotoga naphthophila* RKU-10^T (ATCC BAA-489/DSM 13996/JCM 10882) in a mesophilic expression host (*Escherichia coli* BL21CodonPlus), together with its kinetic and thermodynamic analysis which give insights about the thermostability of the enzyme. This is the first-ever report of kinetic, thermodynamics and characterization-based study of cloned β-glucosidase from *T. naphthophila.*

2. Materials and methods

2.1. Chemicals and enzymes

Enzymes used in molecular cloning, such as T4 DNA ligase, restriction endonucleases, *Taq* polymerase and InsTAclone PCR Cloning Kit (Catalog # K1214) were purchased from Thermo Fisher Scientific Inc. (Waltham, USA). For PCR amplicons and restricted DNA fragments purification, QIAquick Gel Extraction kit (Catalog # 28704), was purchased from Qiagen (Hilden, Germany). Expression vector pET-21a(+), host *E. coli* BL21CodonPlus (RIPL) and Protein molecular Marker (Catalog # 69079-3) were obtained from Novogen (Madison, WI, USA). All other chemical reagents, salts and buffers were purchased from Sigma chemicals Co., (St. Louis, MO, USA) and Merck (Darmstadt, Germany) unless otherwise stated.

2.2. Bacterial strain, plasmids and growth conditions

T. naphthophila RKU-10^T (ATCC BAA-489/DSM 13996/JCM 10882), employed as a source of the β-1,4-glucosidase (*Bgl*A) gene, was cultivated anaerobically in MMI medium by the method of Haq et al. [15]. Initially, pTZ57R/T cloning vector and *E. coli* DH5α were used to clone PCR amplicons and for transformation purpose, respectively. Then *E. coli* BL21CodonPlus (RIPL) and expression vector pET-21a(+) were employed for over-expression and purification of recombinant enzyme. Luria-Bertani (LB) medium supplemented with ampicillin (100 μg/mL) was used to grow recombinant *E. coli* strain, at 37 °C.

2.3. DNA manipulation/β-1,4-glucosidase gene amplification

Genomic DNA of *T. naphthophila* was isolated by phenol-chloroform method and used as a template to amplify coding region of the β-1,4-glucosidase *Bgl*A gene (ADA66698.1; GenBank Accession No. CP001839.1; Tnap_0602) by a pair of oligonucleotide primers designed by using software OligoCalc [16]. Primers F-5'-CATATGAACGTGAAAAAGTTCCCTGAAGG-3' and R-5'-TCAATCTTCCAGACTGTTGCTTTTGACC-3', with *Nde* I restriction site upstream (underlined) in forward primer but no restriction site was added in reverse primer because *Hind* III restriction site already present in multiple cloning site (MCS) of pTZ57R/T (cloning vector). Primers were used to amplify *Bgl*A gene using polymerase chain reaction (PCR) with the following conditions: initial denaturation at 95 °C for 05 min, then 25 cycles for 01 min at 95 °C, 01 min at 52 °C and 01 min at 72 °C with a final extension phase at 72 °C for 10 min. Amplified PCR product was purified using QIAquick Gel Extraction kit and ligated into pTZ57R/T vector. Ligation product was used to transform *E. coli* DH5α competent cells according to the standard protocol [17]. Positive clones were identified by colony PCR, restriction analysis and sequencing. The insert (*Bgl*A) in vector was sequenced by Dye Terminator Cycle sequencing kit (PerkinElmer). Nucleotide and amino acid sequences were analyzed (compared and aligned) to the sequences available in GenBank database by utilizing Blast server at NCBI and CLUSTALW2. Theoretical molecular mass, isoelectric point (pI), total number of negatively and positively charged residues, instability index and aliphatic index was calculated for amino acid sequence using ProtParam tool, and some tools of ExPASy were also used to study the peptide infor-