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Improvement in thermostability of xylanase from *Geobacillus thermodenitrificans* C5 by site directed mutagenesis

Muhammad Irfan[a,b,c], Claudio F. Gonzalez[b], Saad Raza[a], Muhamamd Rafiq[a,d], Fariha Hasan[a], Samiullah Khan[a], Aamer Ali Shah[a,*]

[a] Department of Microbiology, Faculty of Biological Sciences, Quaid-I-Azam University, Islamabad, Pakistan
[b] Department of Microbiology and Cell Science, Genetics Institute and Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL, USA
[c] Institute of Biological Sciences, Sarhad University of Science and Information Technology, Peshawar, Khyber-Pakhtunkhwa, Pakistan
[d] Department of Microbiology, Abdul Wali Khan University, Mardan, Pakistan

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ABSTRACT

Enzymes activity and stability at extreme temperature can be intensified by regularly applying protein engineering. In the present study, two amino acids were perceived to mark the temperature dependability of xylanase from *Geobacillus thermodenitrificans* C5. Six mutants of *G. thermodenitrificans* C5 were built through site-directed mutagenesis by interchanging the residue with proline and glutamic acid (R81P, H82E, W185P, D186E, double mutant W185P/D186E and triple mutant H82E/W185P/D186E). Both mutant and wild type enzymes were quantified in host *E. coli* BL21. In comparison to wild type, the temperature was enhanced by 4 °C, 5 °C and 11 °C in H82E, W185P/D186E and H82E/W185P/D186E mutant models, respectively. The mutant H82E and the combined substitutions (H82E/W185P/D186E) showed the most pronounced shifts in their half-lives for thermal inactivation. Half-life was increased 13 times at 60 °C, 15 times at 65 °C, 9 times at 70 °C and 5 times at 75 °C by H82E/W185P/D186E mutant. Mutations in xylanase enzyme causes rigidification of essential chain and filling of groove that leads to stabilization of mutants and finally resulted into enhancement in their thermostability.

1. Introduction

Xylan is a heterogeneous polymer and one of the key constituents of hemicellulose [1]. Owing to its complex structure, complete degradation of xylan requires a synergistic action of several hydrolytic enzymes which include endo-1,4-β-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), α-glucuronidase (EC 3.2.1.139), α-arabinofuranosidase (EC 3.2.1.55), and acetyl xylan esterase (EC 3.1.1.72) with endo-xylanase and β-xylosidase [2]. Last decade has seen a successful use of xylanase such as its application in food, feed, textile, paper and pulp industries. Xylanases also show guaranteeing potential for bioconversion and biodegradation of waste released from agro-industries, into value added compounds [3]. Enzymes stable at high temperature are favored over their mesophilic counterparts as high temperature yields enhanced mass transfer rates and reduction in substrate viscosity and contamination risk [4]. For example, pulp bleaching and bioconversion process applies xylanases prior to or during processes taking place at high temperatures. Similarly in animal feed, the production feed is mixed with xylanases before pelleting process which is typically carried out at 70–95 °C. Xylanases have already been reported from thermophilic bacteria; *Thermotoga maritima* MSB8 (90 °C) [5], *Thermobifida alba* UL JB1 (80 °C) [6], *Thermotoga petrophila* RKU-1 (90 °C) [7], and for acidomesophilic *Bispora* sp. strain MEY-1 (85 °C) [8]. The structure and existing sequences of these xylanases (i.e., 1VBU and 3NIY) [5,7], provide important supportive details of function and structure relationship of thermostable enzymes.

Another strategy to obtain thermophilic xylanases is to engineer mesophilic protein in such a way that its stability at high temperature is increased. For this purpose, the general approaches used include replacing the N terminus, introducing disulfide bridges, and increasing the number of salt bridges or hydrogen bonds [9]. DNA shuffling technique was employed by Wintrode et al. [10] for development of a mutant protease having increased melting temperature (*Tm*) of 25 °C and increased half-life at 60 °C (1200-fold). Combining this technique with computer aided prediction, rational and random design enhances its efficiency. Fenel et al. [11] and Wang et al. [12] shifted the temperature optima of xylanases produced by *Trichoderma reesei* and *Thermomyces lanuginosus* to at least 10 °C higher via introducing disulfide bridges into the protein. *Bacillus circulans* xylanase's melting temperature was improved to 4.2 °C via thermal fluctuation analysis by

* Corresponding author.
E-mail address: alishah@qau.edu.pk (A.A. Shah).

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Joo et al. [13].

A GH10 xylanase, GthC5Xyl produced by *Geobacillus thermodenitrificans* C5 depicted exceptional characteristics like stability over a broad range of pH. It has also been expressed in *Escherichia coli* [14]. However, at temperature above 70 °C, which is commonly required by most of the industrial processes, GthC5Xyl showed poor stability. This necessitates the improvement of GthC5Xyl thermal properties. The present study aims to employ bio-informatics driven rational engineering to make GthC5Xyl, thermostable. A significant improvement in thermostability of GthC5Xyl is expected by replacement of certain key residues.

2. Materials and methods

2.1. Strains, plasmids, media, and chemicals

Geobacillus thermodenitrificans C5 was the donor of GH10 xylanase gene (GenBank accession number KP203956) [14]. *Escherichia coli* JM101 was used for plasmid amplification, *Escherichia coli* BL21 as the expression host, while Plasmids p15TV-L (Invitrogen) was used for expression. Oat spelt xylan, media supplemented with ampicillin (100 μg/ml) for *E. coli* and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Identification of key residues

Amino acid that might relate to thermophilic properties was identified from hyperthermophile sequence of *Thermotoga maritima* (TmxB) and *Thermotoga petrophila* RKU-1 xylanase (TpXyl10B). The three-dimensional structure of GthC5Xyl and its six mutants (81P, 82E, 185P, 186E, 185P/186E, and 82E/185P/186E mutants) were modeled using Discovery Studio 2.5.5 software (Accelrys, San Diego, CA).

2.3. Generation, gene cloning and expression of wild-type GthC5Xyl and its mutants

The significant residues associated to thermostability were determined from the previous study carried out by Wang et al. [15]. Recombinant articulation was executed as delineated by Pagliai et al. [16]. Standard strategies were utilized for chromosomal DNA isolation, agarose gel electrophoresis, ligation and restriction enzyme digestion [17]. The plasmid was extracted using QIAprep Spin Miniprep Kit and PCR products were purified through QIAquick purification Kit (Qiagen). The specified amino acid residues as identified in previous analysis were replaced to either glutamic acid or proline by site-directed mutagenesis executed by overlap extension PCR. Mutations were introduced in oligonucleotide primers and the overlying ends of fragments were annealed to each other, plasmid p15TV-LdtR was used as template. DNA sequencing was performed for the verification of mutations using M13 and T7 primers.

2.4. Protein purification

The expression and purification of protein was carried out by the method as previously described by Pagliai et al. [16]. A plasmid with His-tagged fusion proteins was cloned and overexpressed in *E. coli* BL21-Star (DE3) cells (Life Technologies, Grand Island, NY, USA). BL21 cells were grown in LB broth medium at 37 °C in shaking water bath for few hours until the OD_{600} became 0.5. 0.5 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the medium and re-incubated overnight at 17 °C. Cells were harvested through centrifugation and re-suspended in binding buffer (500 mM NaCl, 5% glycerol, 50 mM sodium phosphate pH 8.0, 5 mM imidazole, and 0.5 mM TCEP). Cells were lysed by French press and lysate was centrifuged at 14,100 × *g* at 4 °C for 30 min. The cell free extract was applied to a metal chelate affinity-column charged with $Ni^{2}+$ (Qiagen) and equilibrated with binding buffer supplemented with 25 mM imidazole. The proteins were eluted through the column using elution buffer (binding buffer with 250 mM imidazole), and finally dialyzed against same buffer. Protein assay kit (Bio-Rad, Hercules, CA, USA) was used for the determination of concentration of protein, while 12% gel of SDS-PAGE stained with Coomassie Blue R250 (Bio-Rad, Hercules, CA, USA) for determining molecular size as well as purity of protein.

2.5. Activity assay and determination of protein concentration of xylanase

The activity of purified GthC5Xyl was determined by dinitrosalicylic acid (DNSA) technique as previously described [18]. Enzyme activity was calculated after incubation of diluted enzyme at 60 °C in 100 mM sodium phosphate buffer containing 1% xylan. The amount of reducing sugar liberated from oat spelt xylan was estimated by using 3,5-dinitrosalicylic acid (DNSA). One unit of xylanase is characterized as the measure of enzymes that releases 1 μmol of reducing sugar under the test conditions utilizing oat spelt xylan as the substrate. Bradford technique (1976) was utilized for defining protein concentration, using bovine serum albumin as standard protein [19].

2.6. Determination of kinetic parameters of GthC5Xyl and its mutants

The kinetics of GthC5Xyl and its mutants were determined by Lineweaver-Burk plot method. Sodium phosphate buffer with pH 6.0 containing 0.5–30.0 mg/ml of oat spelt xylan was used in order to determine the maximum rate of metabolism (V_{max}) and the kinetic parameters (K_m),

2.7. Measurement of optimal temperature and pH for activity and stability

The activity of GthC5Xyl and its six mutants was measured at wide range of temperature (40–90 °C) in order to determine the optimum range. Thermal stability of both wild as well as mutant xylanases was determined by incubating at temperature 65–80 °C for different durations after dilution to 100 μg/ml in sodium phosphate buffer (pH 6.0). The residual activity was calculated under standard assay conditions in order to measure the half-life of enzyme inactivation ($t1/2$). Similarly, the enzymes activity was calculated at wide range of pH (3.0–12.0) in order to optimize the pH value. The enzymes were incubated in various buffer systems at pH ranges from 5.0–10.0 and 60 °C for 200 mins and finally the residual activity was determined under standard assay conditions.

2.8. Substrate docking analysis

AutoDock 4.0 (http://autodock.scripps.edu/) was utilized as suitable docking instrument to do the docking reproductions of substrate xylohexaose and GthC5Xyl. The GLU 190 side chain atom OE2 was taken as center for docking count and docking grid was set around this point with a boundary of 20 Å in every xyz axis, using Lamarckian Genetic Algorithm. A total of ten docked positions were made by using this software. All modeling as well as docking studies their images were supported with Chimera (http://pymol.en.uptodown.com). The LigPlot program was used in order to relate the hydrophobic and hydrogen-bonding interactions of docked molecules [20].

3. Results

3.1. Identification of key residues

Hyperthermophilic xylanases with optimum temperature over 85 °C have glutamic acid at sites 82 and 186 and proline at 81 and 185 [15] with higher recurrence linking to arginine and tryptophan and histidine and aspartic acid in GthC5Xyl, independently. The homology modeling was applied to generate the 3-D structures of the strain C5 sequence of