Enzyme and Microbial Technology 118 (2018) 50–56

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

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

Rational design of a thermophilic β-mannanase from*Bacillus subtilis* TJ-102 to improve its thermostability

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ARTICLE INFO

Keywords:
β-Mannanase
Thermostability
Rational design
Dynamics transition temperature

ABSTRACT

A rational design method to improve β-mannanase (ManTJ102) thermostability was developed successfully in this study. The flexible area of residues 330–340 in ManTJ102 was firstly selected from analysis of molecular dynamics simulation and then the critical amino acid residue (Ala336Pro) with the lowest mutation energy was determined by virtual mutation, whose mutant was named as Mutant336. Afterward, the dynamics transition temperature (T_{dtt}) of ManTJ102 and Mutant336 was evaluated by simulated annealing and heating, and Mutant336 with higher T_{dtt} was implemented for experimental verification of the enzyme thermostability. As a result, the half-life of Mutant336 activity was 120 min at 60 °C, which was 24-fold of ManTJ102, and the irreversible thermal denaturation constant of Mutant336 was only about 2/5 of ManTJ102, indicating that Mutant336 has better thermostability than ManTJ102. Furthermore, Mutant336 has much higher β-mannanase activity and specific activity than ManTJ102. Therefore, Mutant336 was more suitable to further research for applications.

1. Introduction

Enzymes are biomolecules with tertiary structures which are maintained by non-bond interaction such as hydrophobic interaction, electrostatic interaction, hydrogen bond and van der Waals force [1]. The free energy of these weak interaction forces is usually lower than 60 kJ·mol^{-1}, which indicates that the structure of most proteins is extremely unstable [2]. In the process of preparation, separation, purification, storage and application, the factors such as temperature, pH, salt concentration and stirring may change enzyme structure, resulting in the inactivation. Therefore, it is of great significance to enhance the enzyme stability.

According to the structural analysis of thermophilic enzymes, they were found to be more rigid [1,3]. This is mainly because there are more intensive covalent and non-covalent interactions in the conformation of thermophilic enzymes including hydrogen bonds, disulfide bonds, salt bridges, hydrophobic interactions, and pi-pi interactions [4]. On the contrary, psychrophilic enzymes are more flexible than mesophilic enzymes. Therefore, to improve the thermal stability of enzymes, firstly the flexible area could be inferred from analyzing the structure information of proteins [5]. Then the thermostability of the enzyme can be further improved through enhancement of the rigidity of the flexible area by various ways such as mutation (rational design [6,7]), random mutation and saturated mutation (semi-rational design).

β-Mannanase (β-1,4-D-mannanmannohydrolase, EC 3.2.1.78) is an important hemicellulase belonging to GH26 family, which is responsible for the cleavage of β-1,4-linked internal linkages randomly within the main chain of galactomannan, glucomannan, galactoglucomannan and mannan [8–11]. A majority of the identified β-mannanases are synthesized in bacteria and fungi [12,13], which have found applications in diverse industrial processes, such as biofuel production, oil exploitation, food and animal feed production, coffee production, textiles, and biological research, etc., and its market demands are increasing continuously [14–16]. Specially, thermophilic β-mannanases, derived from thermotolerant or thermophilic microorganisms, are of industrial interest including biofuel production, oil drilling, and coffee

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https://doi.org/10.1016/j.enzmictec.2018.07.005
Received 28 March 2018; Received in revised form 13 June 2018; Accepted 18 July 2018
Available online 19 July 2018
0141-0229/

production [17,18], as they exhibit enhanced robustness and are more suitable to withstand harsh process conditions [19,20].

In our previous report, a novel thermophilic β-mannanase-producing strain named *Bacillus subtilis* TJ-102 was identified which belongs to genus *Bacillus*. Then optimization of culture conditions for high-production of β-mannanase (ManTJ102) was investigated and the enzyme activity could achieve 205.3 U/mL under optimum conditions in a 7-L fermenter [21]. Furthermore, a simple enzymatic hydrolysis pretreatment for konjac powder to produce ManTJ102 by *Bacillus subtilis* TJ-102 was developed in a 7-L fermenter, which could avoid operation difficulties and high energy consumption problems of the traditional fermentation using konjac powder [22]. The new process was a practical and efficient fermentation process for industrial production of β-mannanase using konjac powder as substrate, providing the feasibility for industrial application. In view of the enzyme applications, the ManTJ102 exhibited enzymatic efficiency at 50 °C in the preparation of gluco-mannooligosaccharides (GMOS) by hydrolyzing konjac powder [21], and it was used as konjac gel breaking agent in petroleum mining industry over 60 °C showing many advantages such as controllable breaking time, full hydrolysis, high-permeability recovery and environment-friendly performance [23]. For further research about the enzyme, it's greatly important to improve its thermostability for more enzymatic efficiency at high temperature (higher than 50 °C) in applications of GMOS production and utilization as konjac gel breaking agent.

In this study, a rational design method for improvement of the ManTJ102 thermostability was developed as follows: Flexible areas in ManTJ102 were firstly selected from analysis of molecular dynamics simulation. Subsequently, amino-acid scanning mutagenesis on each residue of the flexible area by mutating each of them to proline was carried out and the energy effect of each mutation on the protein stability (mutation energy) was calculated. The single-point mutant with the lowest mutation energy was regarded as the most stable mutation. Then the dynamics transition temperature (T_{dtt}) of ManTJ102 and mutant was evaluated by simulated annealing and heating, and the mutant with higher T_{dtt} was implemented for experimental verification on the thermostability. If the mutant thermostability was lower than that of ManTJ102, we should choose another amino acid residue in the flexible region and repeat the upper steps until the satisfied results are achieved. This method can provide valuable information for rational design of other enzymes.

2. Materials and methods

2.1. Strains and media

Bacillus subtilis TJ-102 producing ManTJ102 (GenBank accession number: MH469235) was preserved in our laboratory and grew in solid media (beef extract 5 g/L, peptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L, agar 20 g/L, pH 7.0) at 30 °C [21]. *E. coli* DH5α (TransGen Biotech, Beijing, China) was grew at 37 °C for cloning and plasmid construction in LB media (10 g tryptone, 5 g yeast extract, and 10 g sodium chloride per 1 L). *E. coli* BL21 (DE3) (TransGen Biotech, Beijing, China) was used as the host for expression of β-mannanase gene (ManTJ102 or mutant) and was cultured at 37 °C in 2 × YT media with slight modification (50 g konjac powder, 16 g tryptone, 10 g yeast extract, and 5 g sodium chloride per 1 L).

2.2. Rational design for improvement of the ManTJ102 thermostability

A rational design method for improvement of the ManTJ102 thermostability was developed as follows: (1) 3-D structure of ManTJ102 with high resolution and no substrates was selected from the Protein Data Bank (PDB); (2) temperature factor (B-Factor) was extracted from ManTJ102 crystal structure for achievement of protein static flexible area, and the root mean square fluctuation was calculated in molecular dynamics simulation to obtain the dynamic flexible area; (3) the residues at the peak of both RMSF and B-Factor were regarded as a flexible region; (4) proline searching on the residues in the flexible region was carried out by the Calculate Mutation Energy/Stability protocol of Discovery Studio 2016, and the single-point mutant with the lowest mutation energy was regarded as the most stable mutation; (5) the simulated annealing and heating were performed to obtain the T_{dtt} of mutant and ManTJ102 [24]. If the T_{dtt} of mutant was higher than that of ManTJ102, then experimental verification was carried out. On the contrary, we should choose another amino acid in the flexible region to repeat the upper steps (4) – (5).

2.3. Molecular dynamics simulation

Same as the amino acid sequence of the chain A of a YdhT protein from *Bacillus subtilis* [19] (Protein Data Bank entry 3CBW; resolution, 1.269 Å), ManTJ102 (PDB number: 3CBW) was refined with molecular dynamics simulation using the Gromos 96.1(53A6) force field. Firstly, hydrogen atoms of the peptides were added using tLeaP [25]. Then the system was placed at the center of a cubic box and solvated with TIP3P water molecules. In addition, enough Na^+ ions were added to neutralize the negative charges in the system. The system was minimized using the steepest descent method of 5000 steps and following conjugate gradient method of 5000 steps. Then the system was heated to the target temperature of 300 K for a period of 20 ps in constant pressure and periodic boundary conditions (NPT), and equilibrated for 1 ns of constant pressure and temperature (NPT) with the integration time step of 1 fs. Subsequent production simulation was performed for 1 ns of constant pressure and temperature (NPT). A cutoff of 14 Å was used for non-bonded interactions and long-range electrostatic interactions were treated by means of the Particle Mesh Ewald (PME) method. Finally, the molecular dynamics (MD) simulation results were analyzed using the ptraj program in the Gromacs Tools package and PyMOL. The RMSF was analyzed by g_rmsf program.

2.4. Virtual mutation

Virtual mutation was utilized to select suitable mutation points to improve the thermostability of ManTJ02 in this study. The protein was initially pretreated and subjected to the CHARMM force field. The flexible area of residues 330–340 was selected from analysis of molecular dynamics simulation in the section of 3.1. Subsequently, proline searching on these residues was carried out by the Calculate Mutation Energy/Stability protocol of Discovery Studio 2016 in order to calculate mutation energy. It performed amino-acid scanning mutagenesis on each residue of the flexible area by mutating each of them to proline. The energy effect of each mutation on the protein stability (mutation energy) was calculated, as the difference of the free energy of folding between the mutated structure and the wild type protein. The single-point mutant with the lowest mutation energy was regarded as the most stable mutation.

2.5. Homology modeling

Homology modeling was utilized to construct the 3D structure of Mutant336. The chain A of a YdhT protein from *Bacillus subtilis* [19] had the same amino acid sequence of ManTJ102 and was used as a template for three-dimensional structure modeling of Mutant336. The protein sequence of Mutant336 was aligned with the 3CBW-A protein sequence using ClustalW. The homology modeling was performed by MODELLER 9.11 program package. The model with the lowest DOPE score was selected and evaluated according to the standard of VERIFY-3D [26]. The model with the lowest Probability Density Function Total Energy was selected for refinement and validation.