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Protein engineering of *Bacillus acidopullulyticus* pullulanase for enhanced thermostability using *in silico* data driven rational design methods

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ABSTRACT

Thermostability has been considered as a requirement in the starch processing industry to maintain high catalytic activity of pullulanase under high temperatures. Four data driven rational design methods (B-FITTER, proline theory, PoPMuSiC-2.1, and sequence consensus approach) were adopted to identify the key residue potential links with thermostability, and 39 residues of *Bacillus acidopullulyticus* pullulanase were chosen as mutagenesis targets. Single mutagenesis followed by combined mutagenesis resulted in the best mutant E518I-S662R-Q706P, which exhibited an 11-fold half-life improvement at 60 °C and a 9.5 °C increase in T_m. The optimum temperature of the mutant increased from 60 to 65 °C. Fluorescence spectroscopy results demonstrated that the tertiary structure of the mutant enzyme was more compact than that of the wild-type (WT) enzyme. Structural change analysis revealed that the increase in thermostability was most probably caused by a combination of lower stability free-energy and higher hydrophobicity of E518I, more hydrogen bonds of S662R, and higher rigidity of Q706P compared with the WT. The findings demonstrated the effectiveness of combined data-driven rational design approaches in engineering an industrial enzyme to improve thermostability.

1. Introduction

Pullulanase (pullulan-6-glucanohydrolase EC 3.2.1.41) is a debranching enzyme that specifically hydrolyzes α-1,6 glycosidic linkages in pullulan, amylopectin, and other related polymers. Pullulanase belongs to the α-amylase (GH-13) family [1,2], and is generally used together with other amylolytic enzymes in the starch processing industry, where enzymatic processing usually comprises two hydrolysis processes: liquefaction and saccharification [3]. Currently, saccharification is typically performed at 60 °C and at a pH of below 4.5, e.g., pH 3.8, for 24–72 h. Smaller branched oligosaccharides are converted into dextrose by adding glucoamylase and pullulanase during saccharification [4].

Bacillus acidopullulyticus pullulanase is the most practical debranching enzyme because the optimum temperature (60 °C) and pH stability (≥90% residual activity at pH 4.0 for more than 24 h) of this enzyme are suitable for the saccharification process. However, this enzyme has poor thermostability (55% residual activity at 60 °C for 30 min in this study), which is far from the optimal range for the saccharification process. Performing the overall liquefaction and saccharification processes under one temperature, e.g., 95 °C, is ideal as long as the saccharifying enzymes are sufficiently thermostable at this temperature. This single temperature process would simplify the overall process and reduce energy consumption. One way to overcome the limitations of the native enzyme is to increase the amount of enzyme, but this technique inevitably incurs higher costs. Another strategy is to select or develop thermostable pullulanases.

Various strategies have been proposed to obtain thermostable pullulanases. One of the most common and effective methods is to screen wild-type (WT) strains from nature, but this strategy is both laborious and time consuming. Since, the first discovery in 1966

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by Wallenfels et al., several thermostable pullulanases have been discovered worldwide [5–7]. However, none of these thermostable pullulanases have been successfully used in industrial production for having poor enzymatic properties which cannot be used in saccharification. An alternative approach is to reconstruct existing mesophilic enzymes *via* protein engineering for better stability at higher temperatures. Duan et al. have successfully employed site-directed mutagenesis to improve the thermostability of *Bacillus deramificans* pullulanase [3].

Enzyme thermostability can be enhanced through the random mutagenesis approach. However, this approach requires large libraries and many mutagenesis cycles to find improved mutants. Thus, extensive time and effort are required to identify a potential target *via* this approach. By contrast, the rational design approach is useful to create thermostable mutants because this approach leads to smaller, smarter libraries, thereby minimizing the screening effort and shortening the time required to achieve the desired goal [8,9]. The main drawbacks of this approach are the lack of structural data in many cases and the limited ability to predict the influence of amino acid alterations on the enzyme activity and stability [10].

The most recent phase of protein engineering is the data-driven rational design, which aims to build the smallest possible library that usually contains less than 100 possible mutants [11]. Many computational tools, such as B-FITTER [12], SCHEMA [13], RONN [3], Rosetta design [8], and molecular dynamics simulations [14], have been examined to analyze enzyme tertiary structures or homology models and guide data-driven rational designs. These tools have been successfully adopted to explore "hot spots" that are closely correlated with thermostability [8,12,13,15]. Some of these tools, such as Rosetta design, can predict the optimal amino acid substitution that would theoretically improve thermostability. The strategies generally used to improve thermostability include the following: introduction of increased rigidity in flexible regions, cavity-filling with amino acids with bulkier side chains, altering charge distribution, introduction of specific stabilizing interactions, and metal-chelating sites [9,16,17]. Although, applying of these strategies to particular enzymes has yielded improved variants, protein engineering is still conducted depending on the case [18,19]. Predicting the appropriate sites and the optimal amino acid substitution remains a challenge because of the limited understanding of structural features, the individual and interacting contributions of these features to function, and the currently limited understanding of protein dynamics.

A high-resolution 3D structure of *B. acidopullulyticus* pullulanases (PDB code 2WAN) is available [2]. Thus, several *in silico* rational design methods combined with structural analysis are adopted to establish a "smart" library and a significantly smaller number of useful thermostable mutants. The results helped further explain the relationship between the structure and function of *B. acidopullulyticus* pullulanase.

2. Materials and methods

2.1. Materials

B. acidopullulyticus pullulanase gene (Accession No. Ax203843.1) was synthesized by Invitrogen (Shanghai, China). *Escherichia coli* BL21(DE3) (Novagen, USA) and pET-28a(+) (Novagen, USA) were used for protein expression. Q5 High-Fidelity DNA Polymerase and *Dpn*I were purchased from New England Biolabs (Ipswish, MA, USA). Medium for bacterial culture, kanamycin, isopropyl-β-D-thiogalactopyranoside (IPTG), imidazole, and SanPrep Column PCR Product Purification Kit were purchased from Sangon (Shanghai, China).

2.2. Site-directed and semi-saturation mutagenesis

Mutant libraries were generated by PCR using site-directed mutagenesis. Plasmid pET-28a(+)-pul was used as the template. Each PCR reaction contained (50 μL final volume): 5 × Q5 buffer (10 μL), dNTPs (10 mM, 1 μL), primers (forward and reverse 10 mM, 2.5 μL), template plasmid (1 μL, about 1 ng), Q5 High-Fidelity DNA Polymerase (0.5 μL), and ddH_2O (19.5 μL). PCR amplifications were carried out as follows: 98 °C for 30 s; 30 cycles of 98 °C for 10 s; 55–68 °C for 30 s; 72 °C for 4 min; and 72 °C for 2 min. The PCR products were purified using SanPrep Column PCR Product Purification Kit. The purified PCR products treated with DpnI (37 °C for 6 h) were transformed into BL21(DE3) competent cells. Semi-saturation mutagenesis was performed only at the residues selected by B-FITTER program using two degenerate primers. The mutation site was replaced with NDT (N: adenine/cytosine/guanine/thymine; D: adenine/guanine/thymine; T: thymine). A total of 46 colonies were selected for each mutation site during semi-saturation mutagenesis to ensure that every amino acid type has over 95% chance of presentation [20].

2.3. Protein expression

Transformed colonies were placed into 48-well deep plates (LIFEFENG Bio-Technology Limited, Hangzhou, China) containing 1 mL LB medium and 50 μg/mL kanamycin and incubated at 37 °C for 10 h with 200 rpm shaking. An aliquot (50 μL) of this seed culture was used to inoculate TB medium and was grown for 5 h at 37 °C in 48-well plates. Pullulanase expression was induced at 16 °C with the addition of IPTG to a final concentration of 0.1 mM for 36 h. The cells were harvested by centrifugation at 2000 × *g* for 10 min (Thermo, ST 16R, USA). The supernatant was removed, and the cells were resuspended in 1 mL buffer A (50 mM sodium acetate buffer, pH 5.0). Pullulanase was extracted twice by using a high-throughput cell lysis equipment (Bertin, precellys 24, France) at 6000 rpm for 30 s.

2.4. Screening for thermostable mutants

Enzyme solutions of cell lysis were prepared by diluting the supernatants in buffer A to adjust the pullulanase activity. Diluted supernatant (100 μL) was transferred into 96-well plates and then heated for 30 min at 60 °C. Diluted supernatant (100 μL) was transferred into 96-well plates without heat treatment to serve as the control. After cooling to 4 °C for 10 min (to stop the heat shock), the samples were kept at room temperature for 15 min. The enzyme activity was assayed as described below. The clones with residual activity higher than the WT were considered potentially positive. Positive clones were confirmed using the same steps described above with at least three samples running in parallel.

2.5. Enzyme activity assay

Pullulanase activity was measured by incubating the enzyme with 1% pullulan at 60 °C for 10 min. The activity was determined by assaying the release of reducing sugar by the 3,5-dinitrosalicylic acid method [21]. One unit of enzyme activity is defined as the amount of enzyme required to release 1.0 μmol of glucose reducing-sugar-equivalents per minute at 60 °C, pH 5.0.

2.6. Protein purification and quantification

The WT and the best mutant were purified with an AKTA purifier system (GE Healthcare, Sweden) for detailed analysis. The cell pellets of the overexpression product were re-suspended in binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM