Postprint: Structure-Based Rational Design and Site-Directed Mutagenesis for Enhanced Enzyme Thermal Stability

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Abstract

Poor thermal stability of enzymes is one of the most critical factors limiting their practical applications; therefore, engineering enzyme thermostability holds significant theoretical and practical importance for expanding their industrial applications[1]. Recently, enhancing enzyme thermostability through molecular engineering via semi-rational and rational design has garnered widespread attention among researchers due to its reduced workload and high efficiency. The general strategy involves replacing unstable amino acids in the target enzyme with more stable ones to enhance structural stability; consequently, selecting appropriate mutation sites is crucial, which is typically achieved based on a profound understanding of the structure-function relationship of the enzyme. Currently, commonly employed methods reported in the literature include homologous sequence alignment[2], B-factor analysis[3], and modification of highly flexible loops , among others. However, accurately and efficiently identifying potential mutation sites remains a significant challenge. This study focuses on -glucuronidase from a fungal source (PGUS) previously screened by our research group. Building upon the previously determined crystal structure of recombinant -glucuronidase expressed in E. coli (PGUS-E), key amino acid residues in the catalytic domain affecting PGUS-E stability were identified through homologous sequence alignment, and site-directed mutagenesis was employed to significantly enhance its thermostability. Three mutants were obtained: F292L/T293K, S35P, and R304L, all exhibiting significant improvements in both kinetic and thermodynamic stability compared to the wild type. Notably, the F292L/T293K mutant displayed a 5-fold increase in half-life at 65 °C, a 3.2 °C increase in Tm value, and a catalytic efficiency 6.4 times that of the wild type. Finally, molecular dynamics simulations were conducted to elucidate the molecular mechanisms underlying the enhanced thermostability. The results demonstrated that the improved thermostability of the three mutants could be

attributed to C-terminal immobilization effects, proline effects, and hydrophobic interactions .

Full Text

Preamble

Title: Improving Enzyme Thermostability through Rational Design of Site-Directed Mutagenesis Based on Structural Analysis

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Poor thermostability is one of the most critical factors limiting the practical application of enzymes. Consequently, engineering enzymes for enhanced thermostability holds significant theoretical and practical importance for expanding their industrial utility. Recently, molecular modification through semi-rational and rational design has garnered considerable attention due to its high efficiency and reduced workload compared to traditional approaches. The general strategy involves replacing unstable amino acid residues in the target enzyme with more stable alternatives to enhance structural rigidity. Therefore, selecting appropriate mutation sites is paramount and typically requires a deep understanding of the relationship between enzyme structure and function. Commonly reported methods for identifying potential mutation sites include homologous sequence alignment [2], B-factor analysis [3], and engineering of highly flexible loops [4]. However, accurately and efficiently pinpointing these sites remains a significant challenge.

In this study, we focused on a fungal -glucuronidase (PGUS) previously identified by our research group. Building upon our prior determination of the crystal structure of recombinant -glucuronidase expressed in *E. coli* (PGUS-E), we employed homologous sequence alignment to identify key amino acid residues in the catalytic domain that influence PGUS-E stability. Through site-directed mutagenesis, we successfully generated three mutants—F292L/T293K, S35P, and R304L—that exhibited substantial improvements in both kinetic and thermodynamic stability compared to the wild-type enzyme. The F292L/T293K mutant, in particular, demonstrated a fivefold increase in half-life at 65°C, a 3.2°C elevation in Tm value, and 6.4-fold higher catalytic efficiency. Finally, molecular dynamics simulations elucidated the molecular mechanisms underlying these thermostability enhancements, revealing that the improvements stemmed from C-terminal fixation effects, proline effects, and hydrophobic interactions.

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Note: Figure translations are in progress. See original paper for figures.