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AI-Guided Protein Engineering Unlocks DON-degrading Enzyme Thermal Stability via a Loop-to- β -Sheet Conformational Switch

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ABSTRACT: Deoxynivalenol (DON) is a prevalent mycotoxin that poses a serious threat to human and animal health. Although enzymatic biodegradation offers an effective detoxification strategy, the poor thermal stability of current DON-degrading enzymes severely limits their industrial application. In this study, the key residue associated with thermal stability of the artificial DON-degrading enzyme (ADDE) was identified through the integration of the protein language model SaProt and three-dimensional structural analysis. Site-saturation mutations of proline (Pro) at position 103 to any of the other 19 amino acids significantly enhanced thermal stability to varying degrees while maintaining catalytic activity. The best-performing mutant, P103H, exhibited a half-life of 185.78 ± 5.85 min at 50 °C, which is 5.22 times that of the wild-type. Further three-dimensional structural simulation and molecular dynamics analysis revealed that the unique five-membered ring structure of proline at position 103 eliminates potential hydrogen bond donors, thereby restricting the formation of local hydrogen bond networks. In contrast, substitution with other amino acids introduces new hydrogen bonds or enhances hydrophobic interactions, which collectively drive the conformational transition of the L94-L105 region from a flexible loop into two antiparallel β -sheets. This structural rearrangement significantly increases the overall rigidity of the protein, ultimately leading to enhanced thermal stability. This study not only provides a reliable computational framework for enzyme protein engineering but also represents the first successful enhancement of the thermal stability of a DON-degrading enzyme using a protein language model, thereby opening a new pathway for the development of industrial-grade biocatalysts.

Keywords: DON-degrading enzymes; Thermal stability; AI-guided; Molecular dynamics simulation; Structure analysis

1. Introduction

Mycotoxins are a diverse class of toxic secondary metabolites produced by fungi during growth and reproduction, frequently contaminating cereals and animal feed worldwide^[1, 2]. Among the most prevalent mycotoxins are DON, aflatoxin B1 (AFB1), and zearalenone (ZEN). In particular, DON poses a major food safety concern due to its high occurrence in staple crops such as wheat and maize, as well as its strong resistance to conventional processing methods. Exposure to DON has been associated with a range of toxic effects, including immunotoxicity, hepatotoxicity, intestinal damage, and neurotoxicity in both humans and

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animals^[3, 4]. Therefore, controlling DON contamination in food and feed is crucial for ensuring food safety and the healthy development of animal husbandry.

Enzymatic detoxification is currently regarded as a promising approach in food processing and safety, owing to its high efficiency, strong specificity, and environmental compatibility^[5]. The enzymatic degradation pathways of DON primarily involve the de-epoxidation of the epoxy groups at C12 and C13, as well as the oxidation, isomerization, acetylation, glycosylation of the hydroxyl group at C3, and hydroxylation at C16^[4, 6]. Among these, the oxidation and isomerization of the C3 hydroxyl group represent the most thoroughly studied detoxification mechanism, characterized by high specificity and irreversibility. Enzymes catalyzing these reactions have been identified from diverse microorganisms^[7-9]; however, most exhibit poor thermal stability and rapidly lose activity after exposure to 50 °C for 30-60 min. The stability of DON-degrading enzymes at moderate to high temperatures is crucial, as they are required to function during food and feed processing steps conducted under elevated temperatures, such as extrusion and palletization^[10]. However, the instability of most reported DON-degrading enzymes significantly limits their practical application. Therefore, it is essential to enhance the stability of these enzymes through molecular modification.

Currently, common enzyme engineering strategies mainly include rational design, semi-rational design, directed evolution, and artificial intelligence (AI)-assisted design^[11]. While rational design is well-targeted, it heavily relies on an in-depth understanding of enzyme structure and mechanism, and its prediction accuracy is limited^[12]. Semi-rational design requires the integration of partial structural and evolutionary information, yet still involves significant screening efforts. Directed evolution, although independent of prior structural knowledge, is time-consuming, labor-intensive, and exhibits a certain degree of randomness in mutation scope^[12]. To date, several studies have employed traditional protein engineering strategies to modify DON-degrading enzymes and achieved progress. For instance, molecular docking has been used to enhance catalytic performance and stability of sorbose dehydrogenase (SDH)^[9], while structure-guided sequence alignment improved the activity of DON-degrading enzymes derived from *Acidobacteriota*^[8]. Other approaches, including steric hindrance engineering, computer-aided design, and consensus design, have also yielded mutants with enhanced catalytic efficiency or thermal stability^[13-15]. However, studies on improving the catalytic performance of DON-degrading enzymes remain limited. Meanwhile, AI-assisted protein design has emerged as a promising strategy for enzyme stability engineering, enabling efficient identification of key mutation sites and construction of focused mutant libraries, thereby reducing screening costs and development time^[16, 17]. However, its application to enhance the thermal stability of DON-degrading enzymes has not yet been reported.

Protein language models (PLMs) are a class of artificial intelligence-based computational models that have rapidly advanced in the fields of computational biology and protein engineering in recent years^[18]. Typically pretrained in a self-supervised manner on large-scale protein sequences, often with structural information, PLMs can capture evolutionary, structural, and functional patterns. These models can be broadly categorized into sequence-based, structure-aware, and task-specific models for property prediction^[19-22].

Among them, SaProt represents a structure-aware PLM that integrates three-dimensional structural information into sequence representations through a specialized “structure-aware vocabulary”^[21]. This framework enables the model to effectively learn the intrinsic relationships between sequence and structure during pretraining. SaProt has been successfully applied to various tasks, including protein function prediction, mutation effect analysis, and structure prediction, and has assisted in the design of enzymes such as L-methionine γ -lyase and P450, significantly enhancing their catalytic activity^[23, 24]. Notably, its structure-aware framework provides enhanced capability in identifying stabilizing mutations, particularly in structurally constrained regions. However, to date, no studies have been reported on the use of SaProt to enhance enzyme thermal stability.

In previous studies, ADDE capable of efficiently degrading DON was constructed; however, its practical application was limited by insufficient thermal stability^[25]. To enhance the industrial applicability of this enzyme, the present study aimed to improve its thermal stability. The key amino acid residues related to thermal stability were identified by integrating the protein language model SaProt with three-dimensional structural analysis. Site-directed mutagenesis and site-saturation mutagenesis were then employed, through which a series of mutants with significantly enhanced thermal stability were screened. Finally, the molecular mechanism underlying the substantial improvement in thermal stability was further elucidated through three-dimensional structural simulation analysis and molecular dynamics simulation analysis, providing a theoretical basis for the rational design of DON-degrading enzymes.

2. Materials and methods

2.1. Strains and Chemicals

The expression vector of ADDE was constructed by linking the gene sequences of AKR13B3 and DADH using a peptide linker and cloning the fused sequence into the pET-28a(+) plasmid. The resulting recombinant plasmid was then transformed into *Escherichia coli* for protein expression. *E. coli* BL21(DE3) Competent Cells, the gene expression host, was purchased from Sangon Biotech Co., Ltd (Shanghai, China). The 2 × Phanta Max Master Mix for PCR was obtained from Vazyme Biotech Co., Ltd (Nanjing, China), and QuickCut™ DpnI, used for plasmid template digestion, was purchased from Takara Biomedical Technology Co., Ltd (Beijing, China). Gel Extraction Kit, One Step Seamless Cloning Kit, and PurePlasmid Mini Kit were all sourced from Jiangsu CoWin Biotech Co., Ltd (Taizhou, China). All constructed plasmids were sequenced at Jiangsu CoWin Biotech Co., Ltd (Taizhou, China). For protein purification, High Affinity Ni-Charged Resin FF was purchased from Nanjing GeneScript Biotech Co., Ltd (Nanjing, China). Protein standard markers were also purchased from Jiangsu CoWin Biotech Co., Ltd (Taizhou, China). PQQ and NADPH were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd (Shanghai, China), and DON were purchased from TripleBond Co., Ltd (Guelph, Canada). HPLC-grade acetonitrile, used for DON detection, was purchased from Anhui Tedia High Purity Solvents Co., Ltd (Anqing, China). All Other chemicals used in this study were of analytical grade and commercially available.

2.2 Three-dimensional structural simulations of ADDE

The amino acid sequences of ADDE and its mutants were submitted to the AlphaFold3 online server (<https://alphafoldserver.com/welcome>) for three-dimensional structural simulations, and the model with the highest confidence score was selected for three-dimensional structural analyses^[20].

2.3. Selection of the key residues for enhancing thermal stability of ADDE

The protein structure predicted by AlphaFold3 was submitted to ColabSaProt (the Colab implementation of SaProt; https://colab.research.google.com/github/westlake-repl/SaprotHub/blob/main/colab/SaprotHub_v2.ipynb?hl=en) to predict mutational effects based on the structural model^[26]. The resulting mutation scores were ranked in descending order, and the top 30 mutations were selected as candidate sites. Subsequently, based on the three-dimensional structural simulations, mutation sites located on α -helices and β -sheets were excluded, while those situated in flexible loop regions were retained for further mutant construction.

2.4 Construction of Site-Directed Mutagenesis

Site-directed mutagenesis was performed on the selected mutation sites. The primers used in this study are listed in Supplementary Table S1. The PCR reaction system had a total volume of 25 μ L, consisting of 2 ng of template DNA, 0.4 μ M of both upstream and downstream primers, 12.5 μ L of 2 $\times$ Phanta Max Master Mix, with the remaining volume made up with ddH₂O. The PCR cycling conditions were as follows: initial denaturation at 95 °C for 5 min; denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 4 min, for a total of 30 cycles; followed by a final extension at 72 °C for 5 min. The amplification product containing the original template plasmid was digested with QuickCut™ DpnI to avoid false-positive transformants. 20 μ L of PCR product was mixed with 1 μ L of QuickCut™ DpnI and incubated at 37 °C for 1 h. After purifying the target fragment using the Gel Extraction Kit, the linear DNA was circularized using One Step Seamless Cloning Kit and incubated at 50 °C for 20 min. Finally, transformation and sequencing were performed to complete the construction of the mutant.

2.5 Heterologous Expression and Purification of ADDE and its mutants

The constructed plasmid was retransformed into *E. coli* BL21(DE3) Competent Cells. *E. coli* strains carrying ADDE and mutant gene expression vectors were inoculated into 5 mL of LB medium containing 50 μ g/mL kanamycin and cultured overnight at 37 °C with shaking at 200 rpm. The next day, 1 mL of the bacterial culture was transferred into 100 mL of LB medium and incubated at 37 °C with shaking at 200 rpm. When the OD₆₀₀ reached 0.6-0.8, 0.2 mM IPTG was added, and the culture was incubated at 16 °C with shaking at 200 rpm for 16 h.

The fermented bacterial culture was then centrifuged at 8000 rpm for 3 min, and the supernatant was discarded. 5 mL of disruption buffer (20 mM Tris-HCl, 200 mM NaCl, pH 8.0) was added, and the cells were sonicated for 5 min at 500 W. The resulting cell lysate was centrifuged at 8000 rpm for 20 min, and the supernatant was collected. This was filtered through a 0.22 μ m filter and subsequently purified using the High Affinity Ni-Charged Resin FF.

For protein purification, a 1 mL Ni-charged resin was equilibrated with 10 mL of disruption buffer (20 mM Tris-HCl, 200 mM NaCl, pH 8.0). The supernatant was applied to the Ni-charged resin twice, followed by elution with elution buffer A (20 mM Tris-HCl, 300 mM NaCl, 50 mM imidazole, pH 8.0) to remove contaminating proteins. The target protein was then eluted using elution buffer B (20 mM Tris-HCl, 300 mM NaCl, 150 mM imidazole, pH 8.0). Finally, the protein's molecular weight and purity were confirmed by polyacrylamide gel electrophoresis.

2.6 Enzyme Activity Assays of ADDE and its mutants

The catalytic activity of ADDE and its mutants was measured by the degradation rate of DON, with slight modifications to the previously described method. Briefly, the reaction system consisted of 50 µg of protein, 1 mM Ca^{2+} , 50 µM PQQ, 50 µM DON, and 50 mM Tris-HCl (pH 7.5), and was incubated at 37 °C for 2 h. After incubation, the reaction was terminated by heating in a 95 °C water bath for 10 min, and residual DON was quantified using high-performance liquid chromatography (HPLC). The chromatographic column was a ZORBAX Eclipse Plus C18. Elution was carried out for 20 min with a mobile phase of 95% water and 5% acetonitrile (v/v) at a flow rate of 0.2 mL/min, and the detection wavelength was 218 nm. The specific activity of wild-type was accepted as 100%^[13].

2.7 Evaluation of half-life of ADDE and its mutants

To evaluate the half-life of ADDE and its mutants, purified proteins were diluted to a final concentration of 0.5 µg/mL, and 100 µL aliquots were transferred into 1.5 mL microcentrifuge tubes. The samples were first equilibrated in an ice-water bath for 30 min and then incubated in a 50 °C water bath for 0, 30, 60, 90, and 120 min. After incubated, the samples were immediately transferred back to an ice-water bath and cooled for 30 min. The specific activity of untreated wild-type and its mutants was defined as 100%. The $t_{1/2}$ (half-life) was calculated using the equation $t_{1/2} = \ln 2/k_d$, where k_d represents the rate constant obtained from linear regression according to the equation $\ln A = -k_d \times t + C$, with A denoting residual activity and t the incubation time.

2.8 Enzymatic characterization of ADDE and its mutants

The enzymatic properties of the purified enzyme and its mutants were characterized as follows. The optimum reaction temperature was determined by measuring enzymatic activity over a temperature range of 15–50 °C in Tris buffer (20 mM Tris-HCl, 200 mM NaCl, pH 7.5) under saturating substrate conditions, with the highest observed activity defined as 100%. The optimum pH was determined at the optimum temperature using a series of buffer systems, including 50 mM phosphate-citrate buffer (pH 5.0–7.0) and Tris buffer (pH 7.0–8.5), with activities expressed relative to the maximum value. Thermal stability was evaluated by incubating the enzyme at different temperatures ranging from 50 °C to 70 °C for varying durations. After incubation, the samples were returned to the optimum temperature, and the residual enzymatic activity was measured.

2.9 Amino acid interaction analysis

The interaction of the key amino acid were calculated using the LigPlot software (<https://www.ebi.ac.uk/thornton-srv/software/LigPlus/>). The three-dimensional structural simulation obtained from AlphaFold3 was input into LigPlot, where the residue at position 103 was selected to analyze its interactions with other amino acid residues. The resulting interaction data were then visualized by importing the three-dimensional structural simulations into Pymol (<https://pymol.org/>). Using the Wizard-Measurement tool in Pymol, hydrogen bonds between interacting atoms were displayed by connecting the relevant atoms, and hydrophobic interactions were visualized by displaying the corresponding amino acid residues.

2.10 Molecular Dynamics Simulation

All-atom molecular dynamics (MD) simulations were performed using protein structure obtained from AlphaFold3 predictions as the initial structures, employing the AMBER 24 software package^[27]. Prior to the simulations, AM1-BCC partial charges for the small molecules were calculated using the antechamber module. The proteins were parameterized using the GAFF2 force field and the ff14SB protein force field, respectively^[28, 29]. Hydrogen atoms were added to all systems using the LEaP module, after which each system was solvated in a truncated octahedral box of TIP3P water with a minimum distance of 10 Å from the solute^[30]. Sodium and chloride ions were added to neutralize the system and balance the overall charge. Finally, topology and parameter files were generated for subsequent simulations.

Energy minimization was first carried out using 2500 steps of steepest descent followed by 2500 steps of the conjugate gradient method. After minimization, the systems were gradually heated from 0 K to 298.15 K over 200 ps under constant volume conditions. Subsequently, a 500 ps NVT (constant number of particles, volume, and temperature) simulation was performed at 298.15 K to allow further equilibration of the solvent molecules. This was followed by a 500 ps equilibration under NPT (constant number of particles, pressure, and temperature) conditions. Finally, a 50 ns production MD simulation was conducted for each protein under NPT conditions with periodic boundary conditions.

2.11 Statistical Analysis

All experiments were performed with at least three independent replicates. Data are presented as the mean $\pm$ standard deviation (SD). Figures were prepared and layout using Origin 2022.

3. Result

3.1 Selection of the key residues for enhancing thermal stability of ADDE

SaProt is a structure-aware protein language model capable of predicting the effects of protein mutations and generating a score that reflects the potential impact of these mutations on the protein's functional stability or activity^[21]. The score produced by SaProt is typically a continuous value, quantifying the “harmfulness” or “stability change” of an amino acid mutation relative to the wild-type sequence. A lower score generally indicates a more disruptive effect on protein structure and function, while a near-zero score suggests that the mutation may be neutral or have minimal impact. Based the three-dimensional structural model of ADDE, SaProt produced a residue-level mutation score table (Figure S1). Similar to the report by Fan et al., most

candidate mutations had low scores, with only a few exhibiting potential beneficial effects^[24]. Mutants with a score greater than 4.0 were selected as candidate mutations (29 in total), corresponding to 14 distinct mutation sites (including D83, P103, V147, F188, H232, E297, M319, E387, W447, N516, L578, E609, Q656, E673) (Table S2). As rigidifying flexible regions of a protein is one of the effective strategies for enhancing thermal stability^[31], six mutation sites located in α -helices and β -sheets (V147, H232, E297, E387, N516, Q656) were excluded. This left amino acid sites located in flexible loop regions as key candidates (D83, P103, F188, M319, W447, L578, E609, E673) (Figure 1A). Therefore, the final list of mutation candidates was narrowed down to include D83P, P103A, P103E, P103G, P103Q, P103S, P103T, P103V, F188L, M319P, W447A, W447G, W447I, W447L, W447P, W447S, W447T, W447V, L578N, E609D, and E673Q, which are likely to be key sites and amino acids influencing the thermal stability of ADDE.

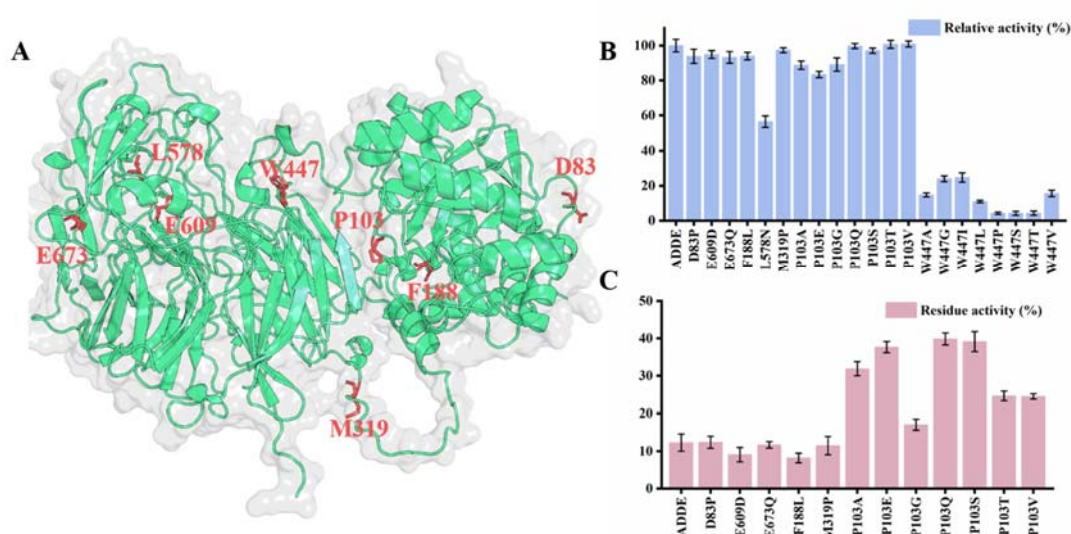


Fig. 1 (A) Three-dimensional structural simulations of ADDE. (B) Catalytic and residual activities of the wild-type and (C) its mutants after incubated at 50 °C.

3.2. Effect of key amino acid residues on the thermal stability of ADDE

To evaluate whether the designed mutations could enhance the catalytic performance of ADDE, 21 mutants were successfully constructed, expressed, and purified together with the wild-type enzyme. SDS-PAGE analysis confirmed that all proteins exhibited comparable purity and molecular weight (Figure S2). The effects of mutation were then assessed by measuring relative catalytic activity and residual activity after incubated at 50 °C for 120 min. Relative activity measurements showed that most mutants retained catalytic efficiency comparable to the wild-type. Mutants D83P, P103A, P103E, P103G, P103Q, P103S, P103T, P103V, F188L, M319P, E609D, and E673Q exhibited 80-105% activity relative to wild type, indicating that substitutions at these positions did not significantly disrupt catalysis. In contrast, L578N showed reduced activity ($56.41 \pm 3.28\%$). Notably, all substitutions at residue W447 dramatically impaired enzyme function, reducing activity to 5-30% of wild-type (Figure 1B), highlighting its essential catalytic role.

To balance catalytic competence with thermal tolerance, mutants retaining more than 80% relative activity were further subjected to thermal stability analysis. After incubation at 50 °C for 120 min, mutants D83P, E609D, E673Q, F188L, and M319P displayed residual activities of $12.39 \pm 1.61\%$, $9.07 \pm 0.93\%$,

11.69 ± 0.85%, 8.19 ± 1.27%, and 11.48 ± 2.43%, respectively, all lower than or not significantly higher than that of the wild-type (12.27 ± 2.30%). In contrast, all substitutions at residue P103 markedly improved thermal tolerance. Mutants P103A, P103E, P103G, P103Q, P103S, P103T, and P103V retained residual activities of 31.88 ± 1.89%, 37.65 ± 1.48%, 17.06 ± 1.45%, 39.85 ± 1.61%, 39.15 ± 2.67%, 24.63 ± 1.29%, and 24.50 ± 0.72%, respectively, all higher than that of the wild type. Collectively, these results demonstrate that residue W447 is critical for catalytic activity, whereas residue P103 is a key determinant governing the thermal stability of ADDE.

To further explore the contribution of residue 103 to enzyme thermal stability, site-saturation mutagenesis was performed at this position. The wild-type enzyme and all 19 mutants were successfully constructed, expressed, and purified (Figure S3). Among them, P103W, P103N, and P103H retained 51.81 ± 2.77%, 43.39 ± 2.00%, and 64.08 ± 4.86% activity, respectively, all markedly higher than the wild type. To directly compare thermal inactivation kinetics, residual activities were further monitored over increasing incubation times. The wild-type enzyme showed the fastest loss of activity, whereas all variants inactivated more slowly, indicating enhanced thermal stability relative to the wild type. Consistently, P103H exhibited the smallest color change in the heat map, reflecting the slowest inactivation rate (Figure 2B). To quantitatively compare the inactivation kinetics, the corresponding first-order reaction rate constants (k_d) were calculated for the wild-type enzyme and all key mutants (Table S3). The wild-type enzyme showed a relatively high k_d value of 0.0195 ± 0.0011 min⁻¹, whereas most mutants exhibited markedly reduced inactivation rates, with k_d values ranging from 0.0037 to 0.0150 min⁻¹. Notably, the P103H variant displayed the lowest k_d value (0.0037 ± 0.0001 min⁻¹), followed by P103W (0.0056 ± 0.0004 min⁻¹) and P103Y (0.0064 ± 0.0002 min⁻¹), indicating significantly enhanced thermal stability. Half-life values at 50 °C were subsequently calculated (Figure 2C). The wild-type enzyme displayed a half-life values of 35.56 ± 2.04 min, whereas the half-life values of mutants ranged 46-185 min. Notably, P103H reached a half-life values of 185.78 ± 5.85 min, which is 5.22 times that of the wild-type enzyme. Collectively, these results confirm that residue P103 is a key determinant governing the thermal stability of ADDE.

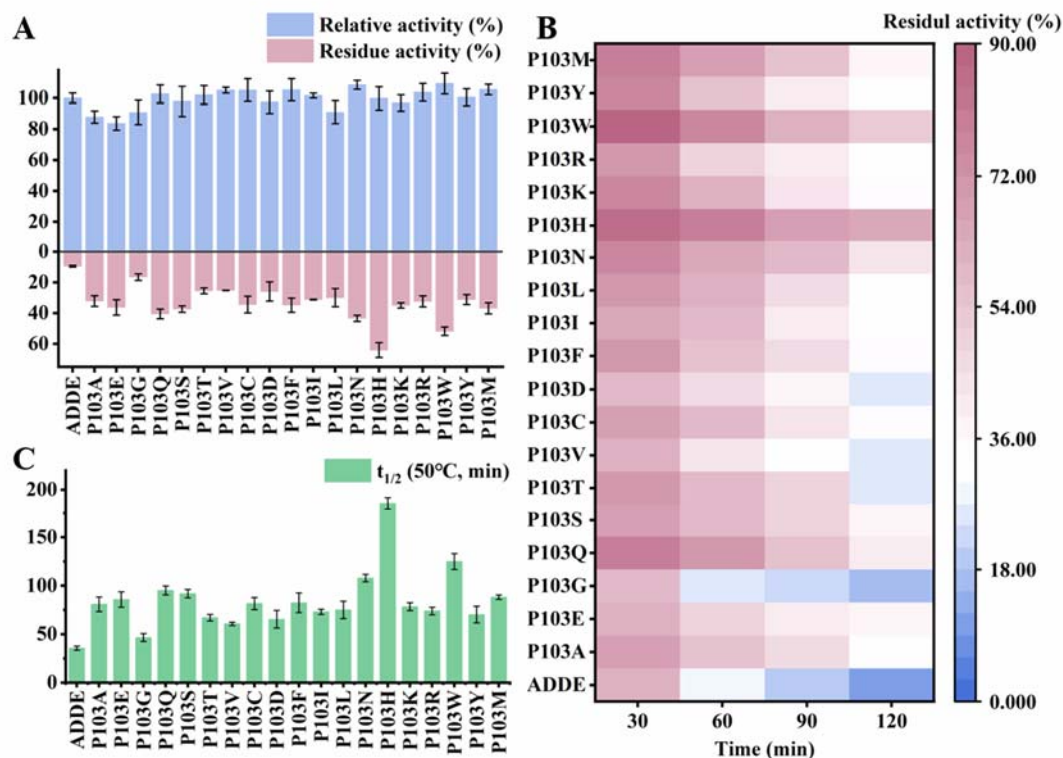


Fig. 2 (A) Initial catalytic activity and residual activity after incubation at 50 °C of the wild-type and its mutants; (B) Time-dependent loss of activity for the wild-type and its mutants during incubation at 50 °C; (C) Half-lives of the wild-type and its mutants at 50 °C

3.3 Characterization of the enzymatic properties of wild-type and mutant P103H

To further determine whether the mutation affected enzymatic properties, the optimum reaction pH, optimum reaction temperature, thermal stability at different temperatures, and half-life ($t_{1/2}$) of the wild-type and mutant P103H were characterized. Both enzymes displayed similar pH-activity profiles (Figure 3A). Catalytic activity increased progressively within pH 5.0–7.5, reached a maximum at pH 7.5, and declined at higher pH values, indicating that the optimum reaction pH remained unchanged upon mutation. The optimum reaction temperature of both the wild-type and mutant P103H was 35 °C (Figure 3B). Within 15–35 °C, catalytic activity increased with temperature, reflecting enhanced molecular motion and reaction kinetics. Above 35 °C, activity decreased sharply, likely due to thermal destabilization and partial unfolding. Notably, at mild temperatures (≤ 35 °C), no significant difference in catalytic activity was observed between the two enzymes, indicating that the mutant P103H substitution does not alter catalytic performance under permissive conditions. In contrast, clear differences emerged at elevated temperatures. When the temperature exceeded 40 °C, the mutant P103H consistently exhibited higher activity than the wild-type enzyme. At 50 °C, the mutant P103H retained $55.79 \pm 6.89\%$ relative activity, whereas the wild type retained only $21.50 \pm 4.73\%$. These results demonstrate that the P103H mutation enhances thermal tolerance without affecting optimum pH or temperature.

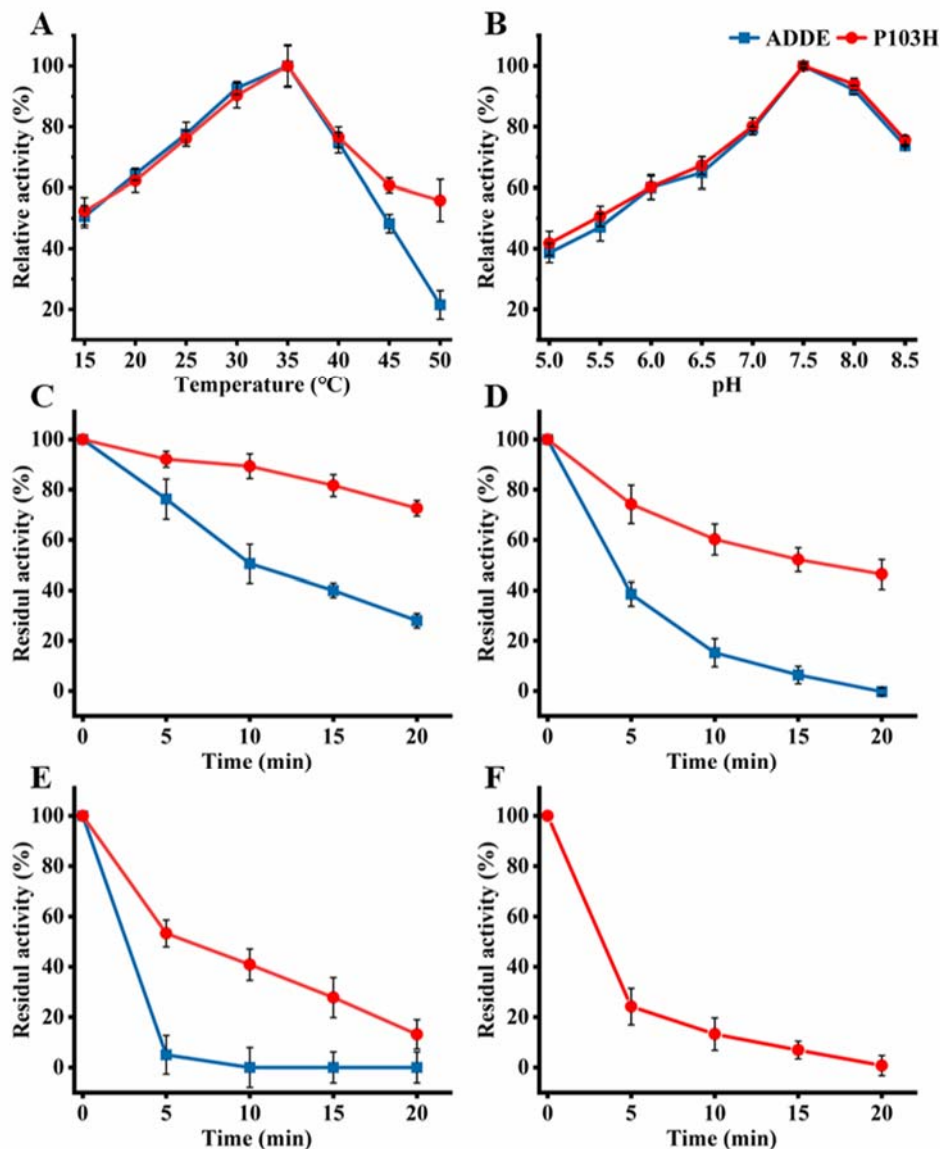


Fig. 3 Enzymatic characterization of wild-type and mutant P103H. (A) Optimum reaction pH.(B) Optimum reaction temperature.(C-F) Thermal stability of wild-type and mutant P103H after incubation at 55, 60, 65, and 70 °C, respectively.

To further evaluate thermal stability, heat-inactivation assays were performed at multiple temperatures (Figure 3C-3F). After incubation at 55 °C for 20 min, the wild-type retained only $27.98 \pm 2.87\%$ residual activity, whereas the mutant P103H retained $72.55 \pm 3.10\%$. At 60 °C for 10 min, residual activities were $15.22 \pm 5.63\%$ and $60.15 \pm 6.16\%$, respectively. At 65 °C for 10 min, the wild-type was completely inactivated, while the mutant P103H still retained $40.87 \pm 6.22\%$ activity. Notably, even after treatment at 70 °C for 5 min, the mutant P103H maintained $24.21 \pm 7.28\%$ residual activity, whereas the wild-type was nearly inactive. These results further confirm the substantial improvement in thermal stability conferred by the P103H substitution. Half-life is a fundamental kinetic parameter defined as the time required for the reactant concentration to decrease to half of its initial value. It directly quantifies the rate at which an enzyme loses activity at elevated temperatures and is therefore a key kinetic indicator for evaluating protein thermal stability^[32]. By determining half-lives at different temperatures (Table 1), the results showed that at 55 °C the half-life of the wild type was only 10.69 ± 0.58 min and decreased sharply to 3.65 ± 0.68 min at 60 °C. In

contrast, the half-lives of the mutant P103H were markedly extended to 45.94 ± 6.86 min at 55 °C and 18.47 ± 2.61 min at 60 °C, respectively. Notably, the half-life of the mutant P103H at 65 °C was 7.30 ± 1.50 min, whereas that of the wild type could not be determined at this temperature due to rapid inactivation. Collectively, these results demonstrate that the P103H mutation substantially enhances the thermal robustness of the wild-type enzyme across a wide temperature range.

Table 1. Half-lives of wild-type and mutant P103H in different temperature

Enzymes	$t_{1/2}$ (50 °C, min)	$t_{1/2}$ (55 °C, min)	$t_{1/2}$ (60 °C, min)	$t_{1/2}$ (65 °C, min)
Wild-type	35.56 ± 2.04	10.69 ± 0.58	3.65 ± 0.68	ND
P103H	185.78 ± 5.85	45.94 ± 6.86	18.47 ± 2.61	7.30 ± 1.50

3.4. Molecular mechanisms of increased thermal stability of mutant enzymes

3.4.1. Sequence analysis of wild-type and mutant P103H

In the ProtScale tool, average flexibility is a sequence-based parameter used to predict the local flexibility of proteins^[33]. The value reflects the structural mobility of the region surrounding each amino acid residue: higher values indicate greater flexibility (less propensity to form stable structures), whereas lower values indicate increased rigidity (greater tendency to form stable secondary structures such as α -helices or β -sheets). To investigate the molecular basis underlying the improved thermal stability, the mutant P103H, which exhibited markedly enhanced thermal stability, was selected for analysis. The wild-type and mutant P103H were submitted to the online ProtScale server for flexibility prediction. For the G98-G106 region, the flexibility range for the wild-type was from 0.454 to 0.486, with an average of 0.470 ± 0.009 . For the P103H mutant, the flexibility range was from 0.433 to 0.464, with an average of 0.449 ± 0.009 . Compared to the wild-type enzyme, the P103H mutation showed a slight decrease in flexibility, indicating that the mutation reduces flexibility in the G98-G106 region overall. Therefore, the enhanced thermal stability observed in the mutant P103H can be attributed to decreased local flexibility (Figure S4).

3.4.2 3D Structural analysis of wild-type and mutant enzymes

(1) The loop-to- β -sheet conformational transition contributes to the thermal stability of the mutants

To further investigate the molecular mechanism underlying the enhanced thermal stability, three-dimensional structural models of the wild type and its mutants were generated using AlphaFold3. Given the notable improvement in thermal stability caused by the P103H substitution, structural changes resulting from the replacement of proline with histidine were examined in detail. Structural alignment of the wild type and the P103H mutant yielded an RMSD of 0.855 Å, indicating that the substitution of a single amino acid residue did not result in significant global conformational changes, except in the vicinity of the mutation site (Figure 4A). Interestingly, mutation of Pro103 to His converted the loop region (L94-L105) into two antiparallel β -sheets, consistent with the results of the average flexibility analysis. Further three-dimensional structural simulations of additional mutants showed that their overall protein architectures also remained unchanged; however, the same loop region (L94-L105) was consistently transformed into two antiparallel

β -sheets (Figure 4B, 4C). Thus, the enhanced thermal stability of the mutants can be attributed to the loop-to- β -sheet transition, which decreases flexibility and increases structural rigidity.

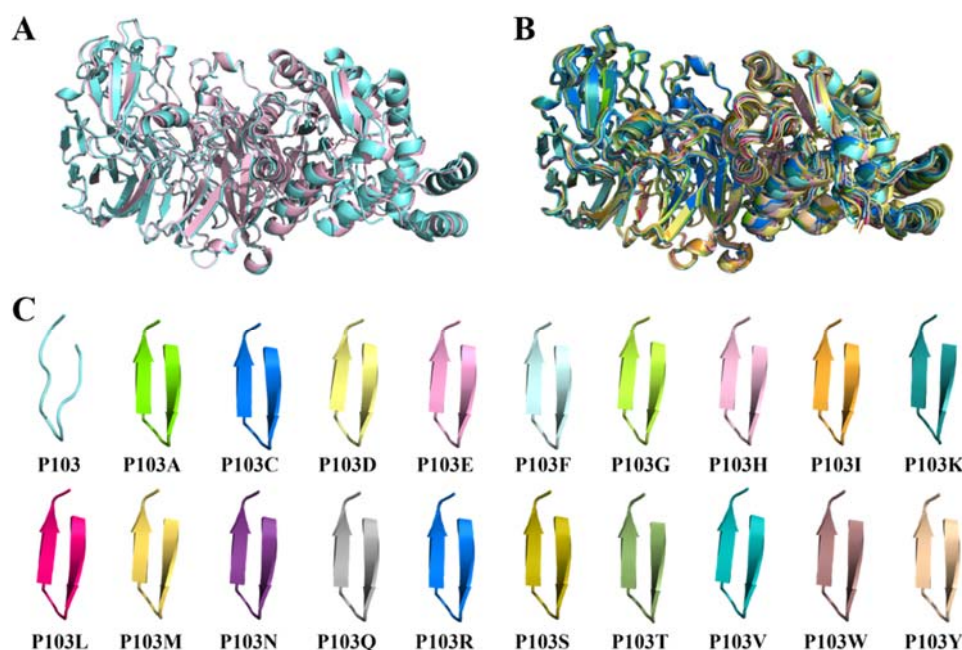


Fig. 4 (A) Structural alignment of the wild type and mutant P103H; (B) Structural alignment of the wild type and saturation mutagenesis; (C) Secondary structure of the local region L94-L105.

(2) Enhanced interactions drive loop-to- β -sheet transition and thermal stability enhancement

To elucidate the molecular basis for β -sheet formation, hydrogen-bonding interactions among residues within the L94-L105 region were analyzed for both the wild-type and the mutant P103H (Figure 5). In the wild type, P103 formed 2 hydrogen bonds with T95 (2.90 Å; 3.16 Å), R96 formed one hydrogen bond with Q101 and one with G98, S97 formed one hydrogen bond with Q101, and G98 formed one hydrogen bond with Q101, giving a total of six hydrogen bonds in this region. Upon substitution of Pro with His at position 103, most of the original interactions are largely retained, while H103 forms a new hydrogen bond with T95 (3.00 Å) and an additional hydrogen bond with S97 (2.82 Å). Consequently, the total number of hydrogen bonds in this region increased from 6 in the wild type to 8 in the mutant P103H, primarily due to the substitution at position 103. In addition, distance measurements between residue 103 and the C α atom of T95 revealed a reduction from 4.7 Å in the wild type to 4.3 Å in P103H, indicating a closer spatial proximity between these residues. Combined with local conformational analysis, these results suggest that altered interactions between the mutation site and neighboring key residues likely promote the loop-to- β -sheet transition in this region.

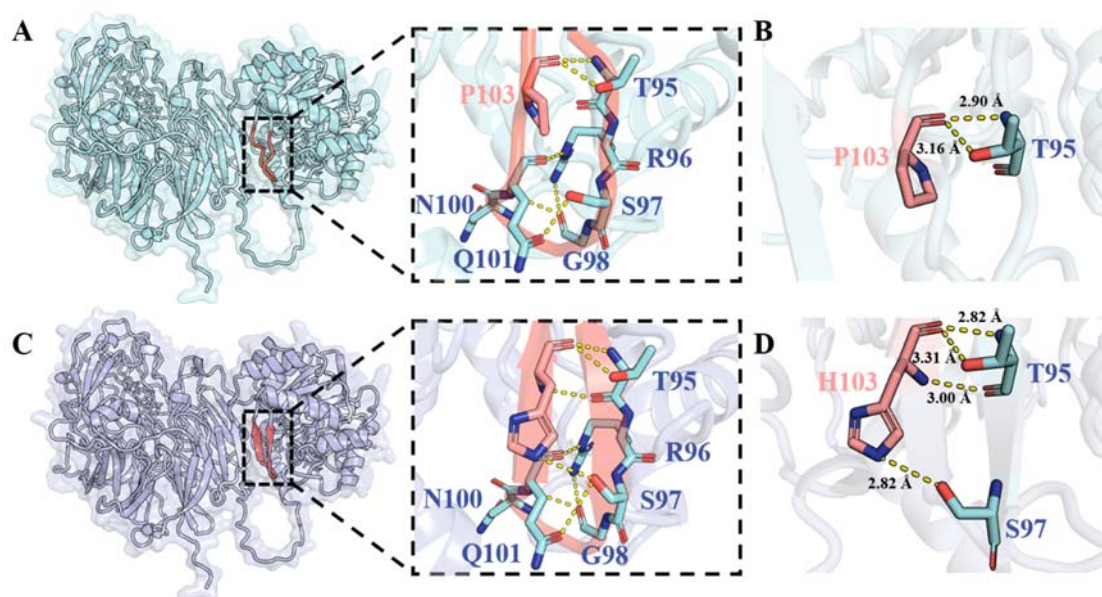


Fig. 5 (A) Overall structure of the wild-type and the local hydrogen-bonding interactions involving residue L94-L105; (B) Hydrogen-bonding interactions between P103 and neighboring residues in wild-type; (C) Overall structure of mutant P103 and the local hydrogen-bonding interactions involving residue L94-L105; (D) Hydrogen-bonding interactions between H103 and neighboring residues in mutant P103. Hydrogen bonds are represented by yellow dashed lines, and hydrogen bond distances are labeled.

To verify this hypothesis, the remaining mutants were systematically analyzed by examining changes in hydrogen bonding and hydrophobic interactions between the mutation site and surrounding key residues (Figure 6). In the wild-type, proline's unique cyclic structure causes it to lack a backbone amide hydrogen, preventing it from acting as a hydrogen-bond donor at the amide position. However, proline can still serve as a hydrogen-bond acceptor through its carbonyl oxygen. As a result, P103 forms only 2 hydrogen bonds with T95 and hydrophobic interactions with L94 and Q101. In most mutants (P103A/E/G/K/L/Q/R/S/T), substitution at position 103 results in the formation of 3 hydrogen bonds with T95, with typical distances in the range of 2.71–3.28 Å, along with hydrophobic interactions with L94. Similarly, in mutants such as P103C/D/I/V, 3 hydrogen bonds with T95 are maintained (2.81–3.32 Å), accompanied by additional hydrophobic contacts with L94 and S97 (or L105). A comparable interaction pattern is observed in P103M, P103F/Y, and related variants, where 3 hydrogen bonds with T95 (approximately 2.81–3.29 Å) are combined with expanded hydrophobic interactions involving residues such as L94, S97, L105, and, in some cases, Q101. Notably, in mutants P103H, P103N, and P103W, 3 hydrogen bonds with T95 (2.79–3.31 Å) are further supplemented by an additional hydrogen bond with S97 (2.81–3.09 Å), together with hydrophobic interactions with L94 and L105. Overall, within the L94-L105 region of this enzyme, the wild-type primarily maintains a flexible loop conformation through 2 hydrogen bonds with T95 and hydrophobic contacts with L94 and Q101. After substitution with other amino acids, most mutants establish 3 hydrogen bonds with T95 and additional or stronger hydrophobic contacts with residues such as L94, S97, and L105. These enhanced local interactions markedly increase structural order and rigidity in this region, driving the originally flexible loop to rearrange into two antiparallel β -sheets and thereby producing a conformational transition from flexibility to rigidity.

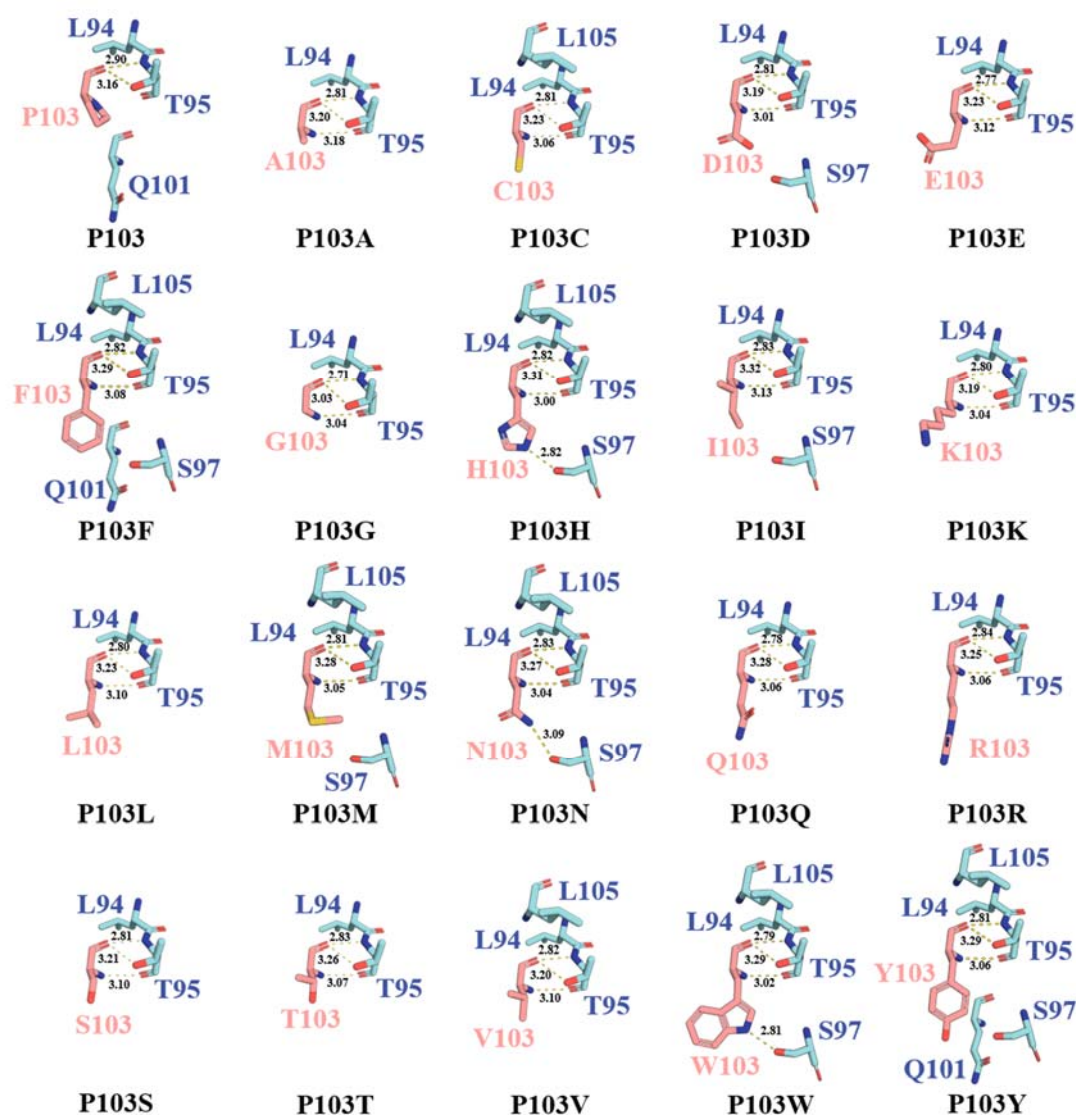


Fig. 6 Interaction analysis of set-saturation mutants at residue 103. Hydrogen bonds are represented by yellow dashed lines, with lengths labeled in Å.

In summary, the enhanced thermal stability observed in all mutants originates from a common structural transition: the L94-L105 region converts from a flexible loop into two antiparallel β -sheets. This transition is driven by a pronounced strengthening of the local interaction network, which manifests in two key ways. Qualitative and quantitative enhancement of the hydrogen-bond network: In all mutants, residue 103 forms 3 hydrogen bonds with T95, compared with only two in the wild type. This increase is not merely numerical; it likely reflects an optimized hydrogen-bond geometry and enhanced network cooperativity, providing a rigid scaffold that supports the nascent β -sheets; Expansion and reshaping of the hydrophobic core: Compared with the wild type, which only engages L94 and Q101, all mutants incorporate S97 and/or Leu105 to varying extents into the local hydrophobic cluster formed by the residue at position 103. This expansion and reinforcement of hydrophobic contacts effectively fill the inter-strand space of the new β -sheet, reduce local conformational entropy, and serve as a key driving force for structural rigidification (i.e., β -sheet formation) and enhanced thermal stability. The synergistic effect of these reinforced hydrogen bonds and hydrophobic interactions likely elevates the folding-state energy of this local structure, stabilizing it and directly translating

into a longer thermal inactivation half-life. Consistent with the half-life data, mutants P103H, P103N, and P103W exhibit significantly longer half-lives (107–185 min) than other mutants (46–95 min). The key distinction is that these three mutants all form an additional hydrogen bond with S97. This hydrogen bond effectively “stitches” the two forming β -sheets more tightly together, substantially strengthening and stabilizing the core hydrogen-bond network of the nascent β -sheet. Other mutants (e.g., P103A, P103M, P103F) either form only hydrophobic contacts or no interaction with S97, resulting in comparatively weaker β -sheet cohesion. Moreover, the additional hydrogen bond likely contributes to a higher unfolding activation energy, as disrupting it requires breaking more cooperative polar interactions, thereby significantly extending the half-life of P103H/N/W.

Furthermore, among mutants sharing this “3+1 hydrogen-bond network,” differences in thermal stability ($H > W > N$) may stem from subtle variations in side-chain physicochemical properties that fine-tune the local energy landscape. In P103H, the imidazole ring of histidine serves as an excellent hydrogen-bond donor, forming the strongest and geometrically optimal hydrogen-bond network. Its partial aromaticity and moderate size allow tight, strain-free π -alkyl hydrophobic stacking, while possible protonation near physiological pH introduces additional electrostatic stabilization. These factors maximize local rigidity while maintaining compatibility with the global structure, conferring the highest thermal stability. In P103W, the large indole ring of tryptophan provides strong rigidity and hydrophobicity. However, the bulky side chain may induce minor backbone or side-chain rearrangements to accommodate it, introducing conformational strain that may partially counteract the stability gains provided by its hydrophobic and hydrogen-bonding interactions. Consequently, its overall stabilizing effect is reduced, resulting in slightly lower thermal stability than that of mutant P103H. In the mutant P103N, the smaller, more flexible asparagine side chain can form hydrogen bonds but with weaker strength and geometric stability compared with histidine; its hydrophobic contribution is minimal, and higher side-chain flexibility increases local conformational entropy, making it less resistant to thermal perturbation. Therefore, it provides a “baseline” level of stabilization but does not reach the optimized effect seen in H and W. These findings indicate that engineering protein thermal stability is a stepwise optimization process: first, mutations induce global structural transitions (loop to β -sheet) to achieve baseline stability; next, the introduction of critical cooperative interactions (hydrogen bond with S97) provides selective advantage; finally, fine-tuning of side-chain physicochemical properties maximizes stability. P103H represents a perfect example of achieving optimal configuration at all three levels.

3.4.3 Molecular dynamics analysis of wild-type and mutant P103H

To further elucidate the molecular basis for the markedly enhanced thermal stability of mutant P103H, full-atom molecular dynamics (MD) simulations were performed at 298.15 K for 50 ns for both the wild-type and mutant P103H. The root-mean-square deviation (RMSD) reflects the average deviation of protein structures from their initial conformations over time and is an important parameter for assessing thermal fluctuations; RMSD values are negatively correlated with protein rigidity and thermal stability^[31, 32]. During the simulation, the wild-type enzyme showed a gradual increase in RMSD, reaching 2.0–4.0 Å, indicating

large structural fluctuations and high flexibility, and suggesting that the wild-type is highly unstable at this temperature (Figure 7A). In contrast, the mutant P103H rapidly reached equilibrium within the first 10 ns and remained stable thereafter, with RMSD converging around 2.0-2.5 Å, indicating smaller structural fluctuations. In addition, the average RMSD of the mutant P103H was 2.251 Å, lower than that of the wild-type (2.764 Å), indicating that the mutant P103H is more rigid and overall structurally more stable. The root-mean-square fluctuation (RMSF) reflects the mobility of individual atoms or residues during simulation; higher RMSF values indicate more flexible regions that may participate in functional or flexible segments, while lower RMSF values suggest structural stability, often corresponding to the protein core or domains^[34, 35]. Over the 50 ns simulation, RMSF analysis (Figure 7B) revealed that the mutant P103H exhibits lower fluctuations across the entire protein, with a substantial reduction in thermally mobile regions, indicating decreased flexibility and enhanced thermal stability.

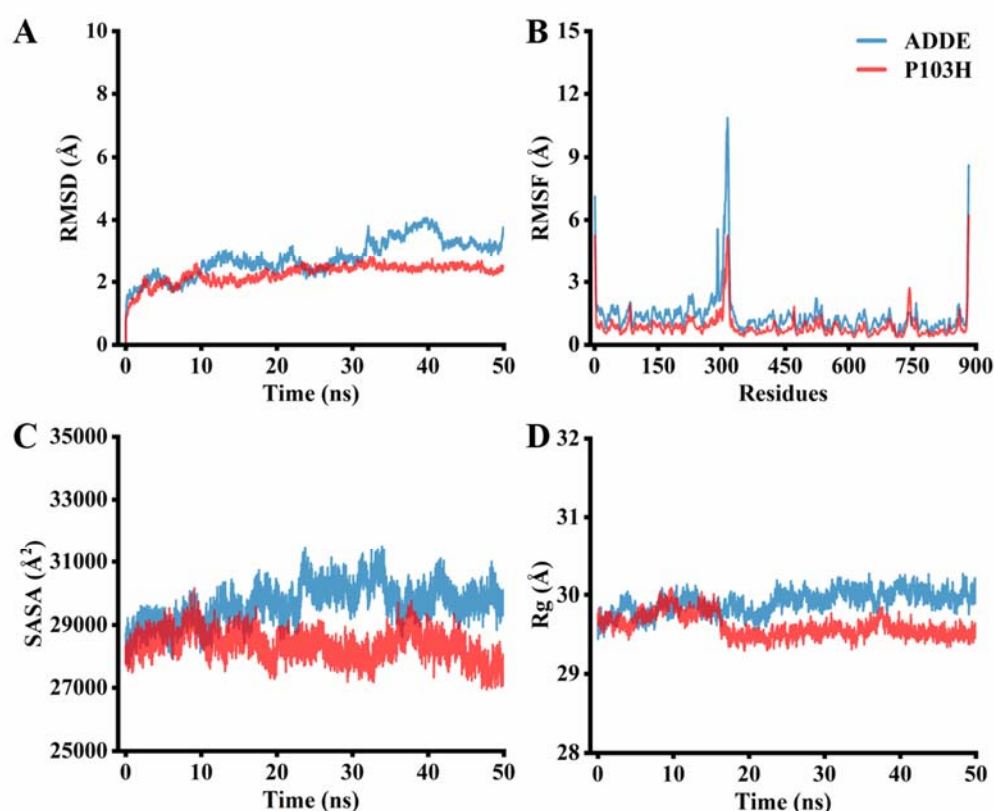


Fig. 7 Comparison of dynamic properties between wild-type and mutant P103H. (A) RMSF; (B) RMSD; (C) SASA; (D) Rg

Solvent-accessible surface area (SASA) measures the surface area of a protein exposed to solvent^[36]. Higher SASA values indicate more exposed hydrophobic residues, which may correlate with increased flexibility or structural perturbation. The wild-type maintained relatively large SASA values (29,500-31,000 Å²) throughout the simulation (Figure 7C), whereas mutant P103H exhibited lower and more stable SASA values (27,500-28,500 Å²), indicating reduced exposure of hydrophobic regions and increased structural compactness. The radius of gyration (Rg) reflects overall structural compactness. An increase in Rg generally indicates a more expanded or loosened structure, whereas stable Rg values reflect maintained compactness.

The wild type exhibited higher and more variable Rg values (30.0–30.5 Å), while mutant P103H maintained lower and more stable Rg values (29.5–30.0 Å), suggesting a more compact folded state (Figure 7D). Collectively, these analyses indicate that the mutant P103H mutation enhances enzyme thermal stability through the following molecular mechanisms: reduction of local structural flexibility, stabilization of overall conformation, formation of a more compact tertiary structure, and reinforcement of the stabilizing interaction network centered on hydrogen bonds and hydrophobic contacts.

4. Discussion

Poor thermal stability remains a key bottleneck preventing DON-degrading enzymes from practical application^[37, 38]. Research in this area is still limited and has mainly relied on traditional protein-engineering strategies, namely directed evolution and consensus design^[15]. Directed evolution depends on random screening of large mutant libraries, but suffers from severe throughput constraints and inherent inefficiency^[12]. Although consensus design possesses a certain rational basis, its stabilizing effect is often modest and strongly dependent on the quality and representativeness of homologous sequences, making breakthrough improvements difficult^[39]. To overcome these limitations, this study developed a novel structure-aware computationally driven strategy that integrates high-accuracy AlphaFold3 structural modeling, the SaProt protein language model, and three-dimensional structural analysis to systematically engineer the artificial DON-degrading enzyme ADDE for improved thermal stability. This approach precisely identified residue P103 as the key determinant from 8 candidate sites and successfully obtained an outstanding mutant, P103H, exhibiting a 5.22-fold increase in thermal stability. This work not only provides a new methodological framework for molecular engineering of DON-degrading enzymes, but more importantly establishes a structure-guided design paradigm that combines advanced computational biology tools. The strategy is broadly transferable to stability engineering of other industrial enzymes, particularly those lacking abundant structural information or homologous sequences, thereby opening a new technological avenue for rational protein design.

The industrial application of DON-degrading enzymes is primarily aimed at high-temperature processes such as feed manufacturing. During pelleting and extrusion, transient temperatures of 70–90 °C frequently occur, and prolonged exposure above 55–60 °C is unavoidable^[40–42]. However, most currently reported DON-degrading enzymes exhibit markedly insufficient stability under industrially relevant temperatures. For instance, the mutant M526E from *Pelagibacterium nitratreducens* ZH15 and DepA from *Devosia* mutans 17-2-E-8 are completely inactivated after 1 h at 50 °C^[14, 43]. Likewise, DADH from *Devosia* sp. A6-243 and YoDDH from *Youhaiella tibetensis* retain only about 20% of their degradation activity after 1 h at 60 °C^[7, 13]. In contrast, the rationally designed mutant P103H obtained in this study exhibits substantially enhanced thermal robustness: its half-life reaches 18.47 ± 2.61 min at 60 °C and 7.30 ± 1.50 min at 65 °C, and it still preserves $24.21 \pm 7.28\%$ residual activity after 5 min at 70 °C. These results demonstrate that mutant P103H possesses excellent thermal stability across a broad temperature range and shows the best performance

reported to date within critical industrial temperature windows, thereby providing a solid enzymological foundation for practical application in high-temperature feed-processing environments.

Structural analysis revealed that the enhanced thermal stability originates from local conformational rearrangements. In the wild-type enzyme, residues L94–L105 form a flexible surface loop, a structural motif typically characterized by high conformational entropy and considered a potential nucleation site for heat-induced unfolding^[44]. Notably, recent studies have highlighted that ordering of flexible loop regions is an effective strategy to enhance protein thermal stability^[45]. In this study, introduction of a mutation at position 103 induced stable reconstruction of this region into two antiparallel β -sheets, while the overall protein fold remained unchanged. Formation of the β -sheets significantly reduced backbone dynamics and increased local structural order, thereby effectively delaying the nucleation of unfolding. Unlike previous strategies that rely on multiple mutations to globally reinforce protein structure, the single substitution at P103 precisely stabilizes this structurally weak region. This suggests that targeted local conformational optimization of specific “unfolding hotspots” represents a highly efficient approach to achieve thermal stability, with loop-to- β -sheet transitions serving as a particularly effective mechanism for local rigidification.

The driving force behind this conformational transition arises from systematic strengthening of the local interaction network. In the wild-type, residue 103 participates in only a few hydrogen bonds, resulting in a loose and dynamic loop conformation. Upon mutation, newly formed hydrogen bonds not only increase in number but, more importantly, enhance cooperative interactions between residues, consistent with previous observations^[46, 47]. The reinforced hydrogen-bond network effectively stabilizes the nascent β -sheets and significantly raises the energetic barrier for unfolding^[48]. Notably, the P103H mutant, which forms an additional hydrogen bond with S97, exhibits the longest half-life, further supporting the critical role of cooperative hydrogen-bond networks in maintaining structural stability rather than mere bond quantity. Mechanistically, these findings indicate that the improved thermal stability is fundamentally due to the formation of a highly cooperative hydrogen-bond lattice, which locks adjacent β -sheets and suppresses thermal fluctuations, ultimately preserving enzyme function at elevated temperatures.

Furthermore, this study demonstrates that replacing the structurally rigid proline residue can substantially enhance the thermal stability of the DON-degrading enzyme, revealing an unconventional stabilization mechanism. Proline is widely recognized as the most conformationally restricted amino acid in proteins, and its unique structural characteristics profoundly influence protein folding and stability^[49]. Its cyclic pyrrolidine ring constrains the backbone conformation and favors turn structures; however, this rigidity eliminates the backbone amide hydrogen, thereby limiting the formation of ordered hydrogen-bond networks^[50, 51]. In the wild-type enzyme, this proline blocks critical intramolecular hydrogen bonding, forcing the surrounding region to remain a flexible and partially disordered loop, thereby compromising thermal robustness. Upon substitution of proline with alternative amino acids, the backbone nitrogen is released, enabling the establishment of new hydrogen bonds that stabilize local secondary structure elements and reduce conformational flexibility. Consistent with the findings reported by Iyer et al., proline, as a secondary structure

breaker, can significantly destabilize those structural regions that rely on H-bonding^[52]. Importantly, this finding challenges the prevailing paradigm in protein engineering, where proline is often introduced at β -turns to improve thermal stability by reducing conformational entropy in the unfolded state^[49, 53]. In contrast, our results indicate that, in this enzyme, proline represents a structural bottleneck rather than a stabilizing element. Its removal alleviates constraints on hydrogen-bond formation and allows the protein to adopt a more stable and ordered conformation.

5. Conclusion

In summary, for the first time, key sites regulating the thermal stability of DON-degrading enzymes were successfully identified through the integration of the protein language model SaProt and three-dimensional structural simulations. It was found via site-saturation mutagenesis that substituting the P103 site with any of the other 19 amino acids could enhance the thermal stability of DON-degrading enzymes to varying degrees, with the mutant P103H exhibiting a half-life at 50 °C that was 5.22 times that of the wild-type. Molecular dynamics and structural simulations further revealed that the enhancement of protein thermal stability is a stepwise optimization process: first, global conformational transition (loop-to- β -sheet) is induced by mutation to achieve basic stability enhancement; second, selective advantage is gained through key cooperative interactions, such as hydrogen bonding with S97; and finally, the optimal stability level is reached through fine-tuning of the physicochemical properties of the side chains. This study not only establishes an artificial intelligence-guided structure-based strategy for enzyme thermal stability engineering but also provides mechanistic insights into the role of proline in the regulation of interaction networks, offering valuable references for the rational design of other DON-degrading enzymes.

Conflicts of Interest

The authors declare no competing financial interest.

Acknowledgments

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