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Original Research Article

Thermal Stability and Kinetic Characterization of Insect-Derived Glycosidases Using the Arrhenius Model

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ABSTRACT

Enzymes derived from insects are increasingly recognized as promising alternatives to conventional microbial or plant-derived enzymes, owing to their adaptation to diverse ecological niches and their potential for unique biochemical properties. Among these, glycosidases play a central role in biomass conversion and related industrial processes, where stability under varying environmental conditions is essential. In this study, we investigated the thermal stability and inactivation kinetics of two insect-derived glycosidases, β-glucosidase (Rpbglu) and β-galactosidase (Rpbgal), isolated from the digestive tract of *Rhynchophorus palmarum* larvae. The primary objective was to evaluate their kinetic and thermodynamic behaviors during heat treatment using the Arrhenius model, to explore their potential for industrial and biotechnological applications. Experimental results demonstrated that both enzymes exhibited a progressive loss of activity in a temperature- and time-dependent manner, following first-order kinetics. Inactivation rate constants (k) increased with rising temperatures. The calculated activation energies (E_a) were 62.78 kJ·mol^{-1} for β-glucosidase and 54.45 kJ·mol^{-1} for *β*-galactosidase. Thermodynamic analyses revealed average activation enthalpies ($\Delta H^{\#}$) of 60.01 and 51.64 kJ·mol^{-1}, respectively, and positive Gibbs free energies of activation ($\Delta G^{\#}$), indicating that inactivation is a non-spontaneous process requiring external energy input. The negative activation entropies ($\Delta S^{\#}$) further suggested a transition to a more ordered molecular state during inactivation. These findings highlight the notable thermal resilience of insect-derived glycosidases and provide fundamental insights into their kinetic properties. Overall, this study underscores their suitability as potential candidates for future biotechnological and industrial applications, particularly in sectors where catalytic efficiency and stability under heat stress are critical.

***Keywords*:** β-glucosidase, *β*-galactosidase, Thermal inactivation, Arrhenius model.

Introduction

Enzymes are biological catalysts that accelerate chemical reactions under mild conditions of temperature, pressure, and pH.[1] Among their various functions, a major role is the hydrolysis of complex molecules into simpler ones, such as the breakdown of carbohydrates into monosaccharides.[2] Given their remarkable catalytic potential, biocatalysts have been developed to enhance enzyme performance and improve the economic efficiency of industrial processes.[3] Improving techniques that increase the stability of biocatalysts is essential for large-scale production and industrial viability.[4, 5] Among the enzymes widely used in biotechnological applications, particularly in the food and pharmaceutical industries, are glycosidases or glycoside hydrolases.[6] These enzymes, which are ubiquitous in nature, found in plants, microorganisms, and animal tissues,[7] catalyze the hydrolysis of glycosidic bonds.

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In animals and insects, glycosidases play a crucial role in digestion.[8] Because of their significant roles in biotechnological processes, glycosidases have attracted considerable and ongoing scientific interest.[9–12] As a result, the characterization of glycosidases from various sources remains an expanding field of research.[13–15, 12] The main objective of these studies is to identify new enzyme sources with properties tailored to diverse technological and economic requirements. Despite growing interest in glycosidases, their large-scale industrial use is still constrained by high production costs, which hinders their performance in industrial processes and by their limited thermal stability.[16] Consequently, exploring new sources of thermostable glycosidases represents a promising strategy for various biotechnological and industrial applications.[17] One alternative to reduce production costs is to investigate glycosidases from insect sources. Insects possess a remarkably diverse enzymatic arsenal, and their glycosidases are essential for digesting complex polysaccharides, suggesting inherent efficiency and robustness.[18] Among these insects is *Rhynchophorus palmarum* L. (Coleoptera: Curculionidae), a widely distributed pest species.[19, 20] The larvae of *R. palmarum* are abundant, edible, and possess significant nutritional value.[21] In this context, two glycosidases, β-glucosidase (referred to as Rpbglu) and *β*-galactosidase (referred to as Rpbgal) extracted from the digestive tract of this insect's larvae are the focus of the present study. These enzymes were subjected to thermal inactivation analysis with the aim of determining the thermodynamic parameters required for their efficient industrial application.

β-glucosidases (EC 3.2.1.21) are enzymes that catalyze the hydrolysis of *β*-1,4-glycosidic bonds, releasing non-reducing terminal glucosyl residues from glycosides and oligosaccharides.[22–27] They are found across all domains of life, from bacteria to mammals, and perform

various biological functions.[24, 28–31] Moreover, *β*-glucosidases are involved in numerous biotechnological processes, such as glycoside synthesis, ethanol production and the saccharification of cellulosic materials.[32-35, 29] Due to their potential industrial applications, β-glucosidases have received significant and ongoing attention.[36]

β-galactosidase, commonly known as lactase, is responsible for the hydrolysis of lactose into glucose and galactose and has promising applications in the food industry, particularly in dairy products.[37, 38] The importance of lactose hydrolysis in dairy products and derivatives, such as milk and whey, is related to several factors.[3] Lactose easily crystallizes and has low sweetness, leading to issues such as poor solubility and lack of taste. Moreover, a large portion of the global population is lactose intolerant and requires enzyme supplementation to digest products like cheese, milk, and their derivatives. Therefore, lactose hydrolysis not only allows consumption by lactose-intolerant individuals but also helps address environmental concerns related to whey disposal.[37, 38] In previous studies, these two enzymes have been characterized, but no thermodynamic parameters have been determined.[15, 14]

More recently, thermodynamic and kinetic parameters of these glycosidases using the equilibrium model,[39] have been determined.[40, 41] The parameters ΔG_{inact} (Gibbs free enthalpy of the thermal inactivation process), ΔH_{eq} (enthalpic change associated with the reversible, temperature-driven interconversion of an enzyme between its active and inactive state or enthalpy of the equilibrium), T_{eq} (the temperature at the mid-point of transition between active and inactive forms of the enzyme or equilibrium temperature) and ΔS_{eq} (Equilibrium entropy deduced from T_{eq} and ΔH_{eq}) were provided.

However, the present study highlights the application of the Arrhenius model to analyze the thermal inactivation kinetics of both biocatalysts, offering a comparative dataset with the equilibrium model for β-glucosidase and β-galactosidase from *Rhynchophorus palmarum*. The Arrhenius approach is particularly relevant here as it provides a robust framework to evaluate temperature-dependent reaction rates and derive thermodynamic parameters (e.g., activation energy, enthalpy, and entropy),[42, 43] which are critical for assessing enzyme stability under industrial conditions [44]. In this context, the objective of this work was to analyze the thermal stability of these two glycosidases at different inactivation temperatures and to determine the temperature dependence and thermodynamic parameters of their thermal inactivation.

Materials and Methods

Chemicals

The substrates, *o*-nitrophenyl-β-D-galactopyranoside (oNPG) (purity > 98 %) and p-nitrophenyl-*β*-D-glucopyranoside (*p*NPG) ((purity = 99 %) are purchased respectively from Merck KGaA®, Germany and Shaanxi Dideu Medichem Co. LTD, China. All other chemicals are analytical grade and purchased from Merck KGaA® (Darmstadt, Germany).

Biological material

Rhynchophorus palmarum larvae were harvested from unearthed or slaughtered palms in the process of putrefaction in Abidjan (Côte d'Ivoire) (5°27′32.9"N3°59′41.1"W).

Enzyme sample.

Extraction, purification and biochemical characterization of *β*-glucosidase and *β*-galactosidase from the digestive juice of *Rhynchophorus palmarum* larvae, have already been performed.[40,15]

Enzymatic Assay

Enzyme activity is determined using a UV-visible spectrophotometer (Pioway Medical Lab. Equipment Co., Ltd. 5100, China). The reaction mixture contains 75 µL of 5 mM *p*-nitrophenyl-*β*-D-glucopyranoside (pNPG) or *o*-nitrophenyl-*β*-D-galactopyranoside (oNPG) as substrates, dissolved in 150 µL of sodium acetate buffer (100 mM, pH = 5.6), and 50 µL of enzyme solution. *Rpbglu* and *Rpbgal* concentration in the reaction mixture is respectively thus 50.17 and 9.09 nM (in protein). After incubation (310.15 K for 10 min) using a thermostatically controlled water bath (Thermomix BM-S, B. Braun Biotech International, Melsungen, Germany), the reaction is stopped by adding 3 mL of 1 M sodium carbonate, and the absorbance is measured at 410 nm.

Thermal inactivation study

Experimental

Thermal inactivation of enzymes is studied using pNPG and oNPG as substrates. Enzyme and substrate are incubated for 60 minutes with a sampling frequency of 5 minutes, and at temperature intervals of 5°C between 50 and 75°C, using a temperature-controlled water bath (Thermomix BM-S, B. Braun Biotech International, Melsungen, Germany). Every 5 minutes, an aliquot is withdrawn and immediately cooled on ice. The specific activities are then determined.

Thermodynamic and kinetic study

Arrhenius equation (equation 1) is the most common mathematical expression to describe the temperature effect on the inactivation rate constants and the dependence is given by the activation energy (equation 1).[45]

$$lnk = lnk_0 - \left(\frac{E_a}{R}\right).\frac{1}{T} \quad (1)$$

k_o is the Arrhenius pre-exponential factor, E_a is the activation energy, R (8.31 J·mol^{-1}·K^{-1}) is the universal gas constant, and T is the absolute temperature. The value of E_a was estimated from the slope and intercept of the graph representing ln(k) versus 1/T. The times required for each enzyme to lose half of its activity ($t_{1/2}$) and 90% of its activity (t_R) at a specific temperature were calculated using equations 2 and 3, respectively:

$$t_{1/2} = \frac{\ln 2}{k} \quad (2)$$

$$t_R = \frac{\ln 10}{k} \quad (3)$$

The activation enthalpy ($\Delta H^{\#}$), the free energy of inactivation ($\Delta G^{\#}$), and the activation entropy ($\Delta S^{\#}$) can be calculated using the following expressions:

$$\Delta H = E_a - R.T \quad (4)$$

$$\Delta G = -R.T ln\left(\frac{k.h}{K_B.T}\right) \quad (5)$$

$$\Delta S = \frac{\Delta H - \Delta G}{T} \quad (6)$$

where h (6.6262×10^{-34} J·s) is Planck's constant and K_B (1.3806×10^{-23} J·K^{-1}) is Boltzmann's constant.

Data processing

Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA) was used to plot the inactivation curves and the Arrhenius plots for activation energy determination. Kinetic parameters (rate constant *k*) were obtained with SigmaPlot version 15.0 (Systat Software Inc., San Jose, CA, USA; released 2020) by fitting the data to the exponential decay model $a = \exp(-kt)$.

Results and Discussion

Thermal inactivation

The thermal inactivation profiles of Rpbglu and Rpbgal (Figures 1-4) clearly show a time and temperature-dependent decline in residual activity, well described by a first-order kinetic model, characterized by an exponential decay of residual activity. These results confirm that thermal denaturation is the primary mechanism of enzymatic inactivation, with the rate of activity loss accelerating as temperature increases. At 50°C, Rpbglu retained more than 80% of its initial activity after 60 minutes highlighting good stability at moderate temperature. However, at T ≥ 55°C, inactivation was accelerated: Rpbglu lost 80% of its activity within 5 min at 70°C, while Rpbgal lost nearly all activity within 20 minutes at 75°C.

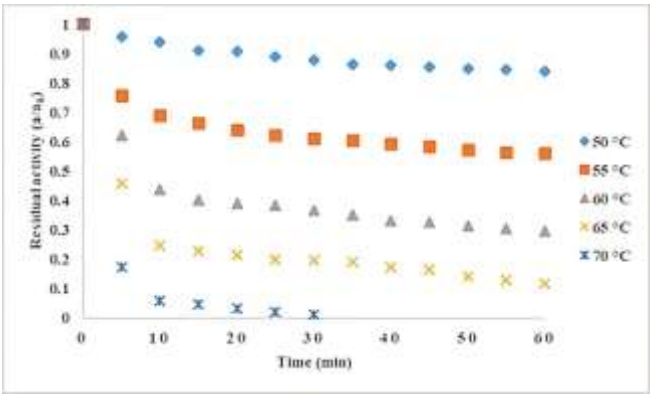

Figure 1: Thermal inactivation of Rpbglu at different temperatures. Enzyme activity was measured in sodium acetate buffer (100 mM, pH = 5.6) using pNPG as substrate.

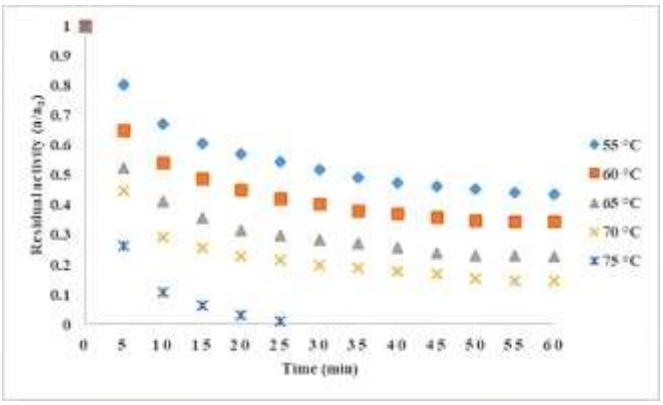

Figure 2: Thermal inactivation of Rpbgal at different temperatures. Enzyme activity was measured in sodium acetate buffer (100 mM, pH 5.6) using oNPG as substrate.

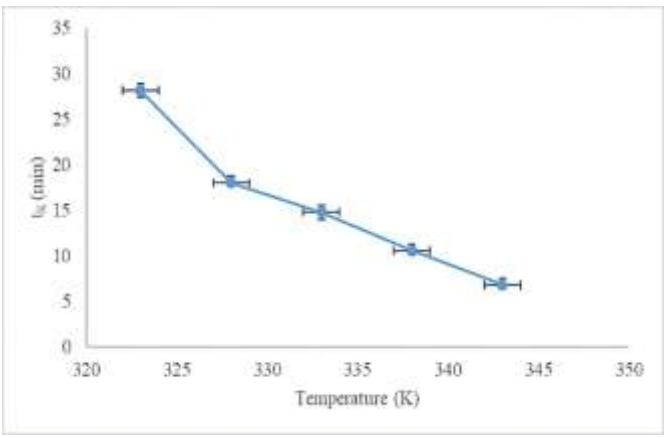

Figure 3: Evolution of the necessary time to Rpbglu activity decays 90% of its initial activity (t_R) with temperature**.** Values are expressed as mean ± SD of three independent determinations (n = 3).

The t_R (time required for 90% loss of activity) provided a quantitative comparison: 7 minutes at 70°C for Rpbglu versus 9 minutes at 75°C for Rpbgal. These findings suggest that both enzymes are relatively stable at mild temperatures but rapidly inactivate at higher ones, a typical behavior of mesophilic glycosidases, with implications for their

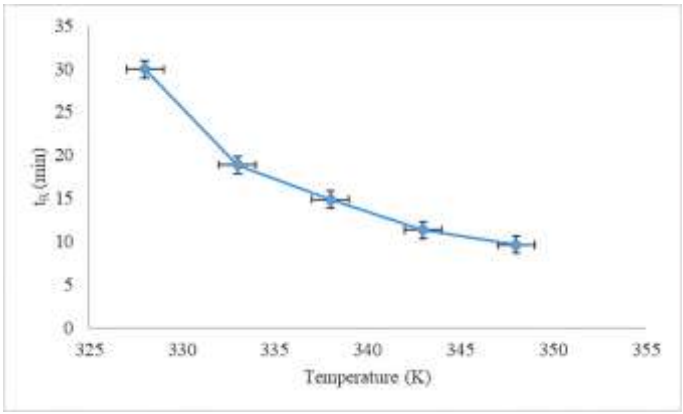

Figure 4: Evolution of the necessary time to Rpbgal activity decays 90% of its initial activity (t_R) with temperature. Values are expressed as mean ± SD of three independent determinations (n = 3).

potential applications in processes requiring controlled thermal conditions. Our results are consistent with earlier studies on *Rhynchophorus palmarum* glycosidases, which showed complete loss of activity after 100 minutes (β-galactosidase) and 120 minutes (β-glucosidase) at 55°C in acetate buffer.[15, 14] Similarly, the β-glucosidase from *Periplaneta americana* was stable for 210 min at 37°C but retained only 60% of its activity after 120 minutes at 55°C .[13] The recombinant β-glucosidase from *Bombyx mori* was reported to be stable at 50°C for 10 minutes.[46] These comparisons indicate that Rpbglu and Rpbgal behave within the expected thermal stability range for insect glycosidases.

Recent studies on β-glucosidases and β-galactosidases from microbial and metagenomic sources report the discovery or engineering of significantly more thermostable variants, highlighting the potential for improvement of insect-derived enzymes through protein engineering or immobilization strategies.[47]

Kinetic and thermodynamic properties

Kinetic analysis

The temperature dependence of inactivation rate constants (k) and half-lives ($t_{1/2}$) (Tables 1 and 2) confirms that both enzymes are heat-labile, with $t_{1/2}$ decreasing sharply as temperature increases. The higher the activation energy (E_a), the greater the energy barrier required for the inactivation process, suggesting that the enzyme exhibits increased thermal stability.[48] The calculated activation energies (E_a), 62.78 kJ·mol^{-1} for Rpbglu and 54.45 kJ·mol^{-1} for Rpbgal fall within the ranges reported for insect glycosidases [34, 49–53] The higher E_a of Rpbglu indicates a greater energy barrier to inactivation and correlates with its better stability at moderate temperatures.

From an applied perspective, enzymes with higher E_a and longer $t_{1/2}$ at operational temperatures are preferred for industrial processes (e.g., food hydrolysis, biomass conversion). However, the rapid loss of activity at T ≥ 60°C limits direct application at elevated process temperatures unless stabilization strategies are implemented.

Thermodynamic analysis

Thermodynamic parameters (Tables 3 and 4) revealed average activation enthalpies ($\Delta H^{\#}$) of 60.01 kJ·mol^{-1} (Rpbglu) and 51.64 kJ·mol^{-1} (Rpbgal), with consistently positive Gibbs free energies of activation ($\Delta G^{\#}$) and negative entropies of activation ($\Delta S^{\#}$). The positive $\Delta H^{\#}$ indicates that inactivation requires thermal energy to disrupt stabilizing non-covalent interactions. The relatively small variation of $\Delta H^{\#}$ across the studied range suggests a conserved inactivation mechanism between 50 and 75°C.

The negative $\Delta S^{\#}$ values suggest a transition to a more ordered or aggregated state during inactivation, consistent with reports of protein aggregation driven by exposure of hydrophobic surfaces and water structuring effects.[54, 55]

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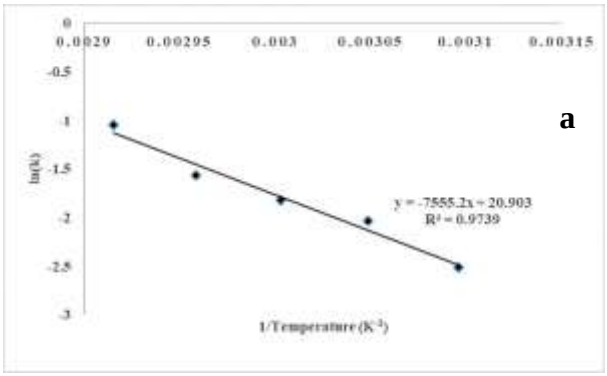
Table 1: Kinetic parameters for thermal inactivation of Rpbglu

T (°C)	k (min^{-1})	E_a (kJ.mol^{-1})	$t_{1/2}$ (min)
50	0.08		8.59
55	0.13		5.29
60	0.16	62.78	4.25
65	0.21		3.32
70	0.35		1.97

Table 2: Kinetic parameters for thermal inactivation of Rpbgal

T (°C)	k (min^{-1})	E_a (kJ.mol^{-1})	$t_{1/2}$ (min)
55	0.078		8.84
60	0.12		5.73
65	0.16	54.45	4.23
70	0.20		3.43
75	0.25		2.73

Compared with glycosidases from other origins,[56, 45] the $\Delta H^{\#}$ and $\Delta S^{\#}$ values of Rpbglu and Rpbgal are lower, underlining their mesophilic nature.



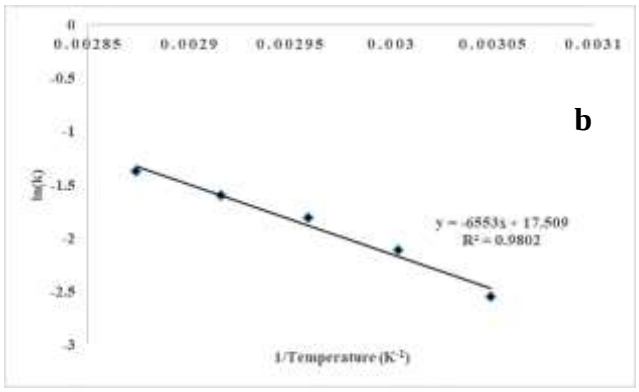


Figure 5: Arrhenius plot for thermal inactivation of Rpbglu (a) and Rpbgal (b) (pH = 5.6)

Table 3: Thermodynamic parameters for first-order inactivation of Rpbglu

T (°C)	T (K)	$\Delta H^{\#}$ (kJ.mol^{-1})	$\Delta G^{\#}$ (kJ.mol^{-1})	$\Delta S^{\#}$ (J.mol^{-1}.K^{-1})
50	323	60.09	86.06	-80.40
55	328	60.05	86.11	-79.45
60	333	60.01	86.89	-80.72
65	338	59.97	87.47	-81.36
70	343	59.93	87.35	-79.94
Mean value		**60.01**	**86.78**	**-80.37**

Table 4: Thermodynamic parameters for first-order inactivation of Rpbgal

T (°C)	T (K)	$\Delta H^{\#}$ (kJ.mol^{-1})	$\Delta G^{\#}$ (kJ.mol^{-1})	$\Delta S^{\#}$ (J.mol^{-1}.K^{-1})
55	328	51.72	87.50	-109.10
60	333	51.68	87.69	-108.14
65	338	51.64	88.24	-108.29
70	343	51.60	88.95	-108.89
75	348	51.56	89.64	-109.42
Mean value		**51.64**	**88.40**	**-108.77**

The thermodynamic signature, relatively low $\Delta H^{\#}$ but positive $\Delta G^{\#}$, resembles that of moderately thermostable enzymes, which resist denaturation to some degree but aggregate rapidly under high thermal stress. Based on their thermal behaviour, Rpbglu and Rpbgal appear suitable for biotechnological applications at mild to moderate temperatures (≤ 50–55°C), such as controlled substrate hydrolysis in food processing, aroma modification, or biocatalytic steps in agro-industrial settings. For high-temperature processes, their inherent stability is insufficient. Several strategies could be applied to enhance thermostability: (i) enzyme immobilization, which often increases resistance to thermal denaturation and allows reusability; (ii) additive formulations (e.g., sugars, polyols, salts) that stabilize protein structure; and (iii) protein engineering approaches, including site-directed mutagenesis, directed evolution, or consensus design.[57] Recent advances in deep-learning–guided protein engineering have enabled the design of glycosidases with markedly improved thermal tolerance without compromising activity, suggesting promising routes for Rpbglu and Rpbgal enhancement. While limited by the use of free enzymes, the absence of structural analyses and the unexplored isoform variability in *Rhynchophorus palmarum*, these factors nevertheless open promising avenues for future research. In this context, advancing the thermostability of insect-derived glycosidases will likely rely on the integration of immobilization techniques, structural characterization and AI-guided mutagenesis.[58]

Conclusion

The kinetic and thermodynamic study of *β*-glucosidase and *β*-galactosidase from *Rhynchophorus palmarum* provided valuable insights into their behavior under thermal inactivation. Both enzymes exhibited first-order kinetics, with inactivation strongly influenced by temperature. The estimated parameters (E_a, $\Delta H^{\#}$, $\Delta G^{\#}$, $\Delta S^{\#}$) confirmed that both glycosidases possess good thermal stability, particularly β-glucosidase. The positive $\Delta G^{\#}$ values and negative ΔS# values indicate a non-spontaneous inactivation process requiring an ordered molecular rearrangement. These thermostable glycosidases derived from *Rhynchophorus palmarum* larvae could be used in the formulation of lactose-free dairy products or in the enzymatic conversion of biomass into fermentable sugars, making them promising candidates for the food and biofuel industries. Future efforts integrating immobilization, structural analyses, and AI-guided mutagenesis could unlock their full biotechnological potential.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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