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Article

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Stabilization of Recombinant Uricase Using L-Serine–*myo*-Inositol Deep Eutectic Systems: Purification, Operational Optimization, and Thermal Inactivation Kinetics

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Running Title: DES-Mediated Stabilization of Uricase

Abstract

Recombinant therapeutic urate oxidase (uricase) is prone to structural fragility and thermal denaturation, limiting its biopharmaceutical and biocatalytic utility. In this study, novel green deep eutectic systems (DESs) formulated with L-serine and *myo*-inositol at 1:1 and 1:2 molar ratios were evaluated as protective liquid media for recombinant uricase. The enzyme was successfully expressed and purified via Ni-NTA affinity chromatography, where step elution with 200 mM imidazole yielded a highly pure monomeric subunit migrating at ~35 kDa on SDS-PAGE. Operational assays established that uricase formulated in both DES systems retained robust catalytic activity across 0–25 min, achieving an optimal performance plateau within a 15–20 min incubation window. Thermal activity profiles revealed an expanded operational window centered at 28–30 °C, markedly outperforming the free enzyme control which suffered rapid denaturation. Thermal inactivation kinetics followed a first-order model (R^2 up to 0.991). Under thermal stress, the unformulated free enzyme exhibited rapid decay with an inactivation rate constant (k_{inact}) of 0.7052 min^{-1} and a half-life ($t_{1/2}$) of 0.9829 min, whereas the serine–*myo*-inositol eutectic matrices substantially mitigated activity loss (R^2=0.8034). These findings demonstrate that zwitterionic amino acid–polyol eutectic media provide an efficient, biocompatible microenvironment that shields uricase against thermal inactivation, offering a viable platform for therapeutic enzyme stabilization.

Keywords:

Deep eutectic systems; Recombinant uricase; Enzyme stabilization; *myo*-Inositol; L-Serine; Thermal inactivation kinetics

1. Introduction

Gout is a prevalent inflammatory joint disease characterized by chronic hyperuricemia and the pathological deposition of monosodium urate (MSU) crystals, with therapeutic urate oxidase (uricase, UOx) serving as a premier enzyme treatment to catalyze uric acid oxidation [1, 2]. Although recombinant uricase formulations effectively lower uric acid, unformulated enzymes suffer from intrinsic structural fragility, rapid thermal denaturation, and activity decay during bioprocessing and storage. To overcome these limitations, recent advanced nanobiotechnological strategies, such as magnetically responsive nanohybrid systems [2] and bioinspired dual-enzyme cascade platforms [1], have been explored to modulate in vivo catalytic activity. However, developing simple, biocompatible, and protective fluid media like deep eutectic systems (DESs) provides a compelling alternative to shield therapeutic enzymes against thermal unfolding without complex chemical modifications.

The exploration of green solvent engineering has evolved from traditional organic solvents, supercritical fluids, and ionic liquids to the recent advent of Deep Eutectic Solvents (DESs) [3]. First introduced by Abbott and coworkers as low-melting mixtures formed via hydrogen-bonding between hydrogen bond acceptors (HBAs) and hydrogen bond donors (HBDs) [4], DESs have garnered extensive attention since 2008 when Gorke and colleagues demonstrated that various enzymes could retain high catalytic activity in these media despite the presence of denaturing species like urea [3].

DESs typically comprise four primary classifications depending on their constituent metal salts and hydrogen-donating components, with Type III DESs, most commonly utilizing cheap, biodegradable, and

non-toxic quaternary ammonium salts such as choline chloride paired with polyols, amides, or amino acids, being the most extensively investigated for enzymatic reactions [4]. Unlike ionic liquids, DESs offer superior biocompatibility, low volatility, negligible vapor pressure, and wide tunability through the precise selection of HBAs, HBDs, and molar ratios [5, 6].

In biocatalysis, the catalytic properties, conformation, and operational stability of enzymes are intricately governed by solvent-enzyme interactions within the DES microenvironment. Structural alterations of enzymes in diverse DESs are not uniform; they are largely dictated by the native enzyme architecture, DES composition, and the specific hydrogen-bonding topology established at the protein-solvent interface [3, 4]. Furthermore, water plays a pivotal regulatory role in DES systems. A controlled, stoichiometric amount of water can integrate seamlessly into the supramolecular hydrogen-bonding network of the DES, reinforcing local structural integrity and preserving the conformational flexibility required for catalysis, whereas excessive water disrupts the cohesive eutectic network and impairs enzyme efficiency [3].

For fragile therapeutic enzymes, such as multimeric oxidoreductases and urate oxidases, designing customized zwitterionic or polyol-based DES formulations (e.g., combining amino acids like L-serine and cyclic polyols like *myo*-inositol) provides a tailored hydration boundary layer. This matrix effectively elevates the thermal unfolding threshold, prevents time-dependent activity decay, and suppresses aggregation without requiring covalent modification or rigid immobilization [5, 7]. Building upon these physicochemical principles, the present study comprehensively investigates the purification, operational optimization, and thermal inactivation kinetics of recombinant uricase formulated in novel L-serine–*myo*-inositol deep eutectic systems. By elucidating the underlying thermodynamic parameters, activation energies, and half-life behaviors, this work aims to establish a robust, non-covalent stabilization framework for advanced enzymatic biotherapy and industrial bioprocessing.

2. Materials and Methods

2.1. Materials

Analytical-grade uric acid (>=99% purity; CAS 69-93-2) and L-serine (CAS 56-45-1) were purchased from Sigma-Aldrich. *myo*-Inositol (CAS 87-89-8), potassium dihydrogen phosphate (KH2PO4), dipotassium hydrogen phosphate (K2HPO4), imidazole, sodium chloride (NaCl), and sodium hydroxide (NaOH) were obtained from Merck (Darmstadt, Germany). Ni-NTA agarose affinity resin was acquired from Qiagen (Hilden, Germany). All solutions and buffers were prepared using double-distilled deionized water (ddH2O).

2.2. Synthesis and Preparation of Serine–*myo*-Inositol Eutectic Media

The deep eutectic systems (DESs) were synthesized by combining L-serine (hydrogen bond acceptor/donor) and *myo*-inositol (hydrogen bond donor) at designated molar ratios of 1:1 and 1:2. The components were blended with a regulated stoichiometric volume of deionized water to facilitate eutectic liquid formation. The mixture was continuously stirred at 60–70 °C until clear, homogeneous, and stable liquid eutectic media were achieved. The prepared formulations were sealed and equilibrated at room temperature prior to enzymatic incorporation.

2.3. Expression and Ni-NTA Affinity Purification of Uricase

Recombinant urate oxidase was overexpressed and isolated via immobilized metal affinity chromatography (IMAC) using Ni-NTA resin. Cell lysates were clarified by centrifugation and loaded onto equilibrated Ni-NTA columns. Following stringency washes to eliminate non-specifically bound host cell proteins, the target enzyme was eluted using stepwise imidazole gradients (100 mM and 200 mM) in 50 mM potassium phosphate buffer (pH 7.4, 300 mM NaCl). Protein purity and monomeric subunit molecular weight were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized using Coomassie Brilliant Blue R-250 staining against a pre-stained protein molecular weight ladder.

2.4. Uricase Enzymatic Activity Assay

Uricase catalytic activity was monitored spectrophotometrically by tracking the enzymatic oxidation and consumption of uric acid at 293 nm (molar extinction coefficient epsilon = 12.6 mM^-1 cm^-1) using a UV-Vis spectrophotometer. The standard reaction mixture contained potassium phosphate buffer (50 mM, pH 8.0) and uric acid (100 uM) in the presence of formulated or free uricase. One unit (U) of uricase activity was defined as the amount of enzyme catalyzing the oxidation of 1 umol of uric acid per minute under the designated assay conditions.

2.5. Operational Optimization: Incubation Period and Temperature

Incubation Time Profile: The temporal stability of uricase formulated within the serine–*myo*-inositol DES matrices (1:1 and 1:2 molar ratios) was assessed across an incubation duration from 0 to 25 min at 5-min intervals.

Thermal Activity Profile: The operational temperature optima for free uricase and DES-formulated enzymes (1:1 and 1:2) were determined across a temperature span from 15 °C to 55 °C under identical substrate concentrations. Residual relative activities were plotted against temperature.

2.6. Thermal Inactivation Kinetics and Thermodynamic Transition State Parameters

Thermal stability and unfolding kinetics were investigated by incubating free and formulated uricase under elevated thermal stress conditions over a 60-min period, with sampling at regular intervals. Residual activity (A_t / A_0) was fitted to the first-order kinetic model:

$$\ln(A_t / A_0) = -k_{inact} * t$$

where A_0 is the initial enzymatic activity, A_t is the residual activity at time t, and k_{inact} is the first-order thermal inactivation rate constant (min^{-1}) derived from the slope. The enzymatic half-life ($t_{1/2}$) was calculated as:

$$t_{1/2} = \ln(2) / k_{inact} = 0.693 / k_{inact}$$

Thermodynamic parameters of inactivation, including activation enthalpy ($\Delta H‡$), Gibbs free energy of activation ($\Delta G‡$), and activation entropy ($\Delta S‡$) for thermal unfolding, were determined based on transition state theory:

$$\Delta H‡ = E_a - R * T$$

$$\Delta G‡ = -R * T * \ln((k_{inact} * h) / (k_B * T))$$

$$\Delta S‡ = (\Delta H‡ - \Delta G‡) / T$$

where h is Planck's constant (6.626×10^{-34} J s), k_B is Boltzmann's constant (1.381×10^{-23} J K^{-1}), R is the universal gas constant (8.314 J mol^{-1} K^{-1}), and T is the absolute temperature in Kelvin.

3. Results and Discussion

3.1. Recombinant Uricase Expression, Ni-NTA Purification, and SDS-PAGE Verification

The successful heterologous overexpression and recovery of recombinant urate oxidase (uricase) were confirmed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (**Fig. 1 and Figure S1**). The electrophoretic pattern displays a distinct and prominent protein band migrating at approximately 35 kDa, which is in excellent agreement with the theoretical monomeric molecular weight of the uricase subunit.

To establish optimal downstream recovery parameters, immobilized metal affinity chromatography (IMAC) via Ni-NTA resin was evaluated using 100 mM and 200 mM imidazole elution steps. The elution profile demonstrated that 200 mM imidazole successfully recovered the target enzyme with superior purity, yielding a sharp, well-resolved single band with negligible co-eluting host cell protein contaminants. This confirms the high efficiency of the purification strategy and provides structurally intact, high-purity uricase for subsequent deep eutectic formulation studies.

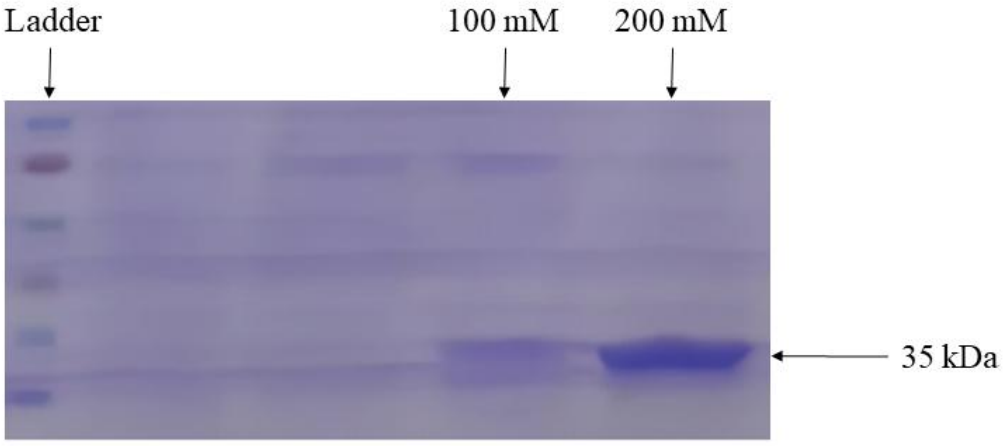

Fig. 1. SDS-PAGE verification of purified recombinant uricase. Lane 1: Protein molecular weight ladder; Lanes 2–3: Intermediate wash fractions; Lane 4: Elution fraction at 100 mM imidazole; Lane 5: Elution fraction at 200 mM imidazole, revealing a dominant single band at ~35 kDa.

3.2. Operational Incubation Time Optimization in Eutectic Media

The temporal stability and operational durability of formulated uricase were evaluated within the L-serine–*myo*-inositol deep eutectic systems (DESs) across 1:1 and 1:2 molar ratios over a 0 to 25-minute incubation period **(Figs. 2 and 3).**

Quantitative analysis revealed that enzymatic activity remained remarkably stable across all tested intervals for both formulations, showing no signs of time-dependent denaturation or aggregation. Specifically, relative activity values reached an optimal and sustained plateau within the 15 to 20-minute incubation window. This outcome indicates that the zwitterionic network of L-serine combined with the polyhydroxy structure of *myo*-inositol establishes a protective hydration shell around the enzyme, preserving its native conformation during prolonged operational exposure.

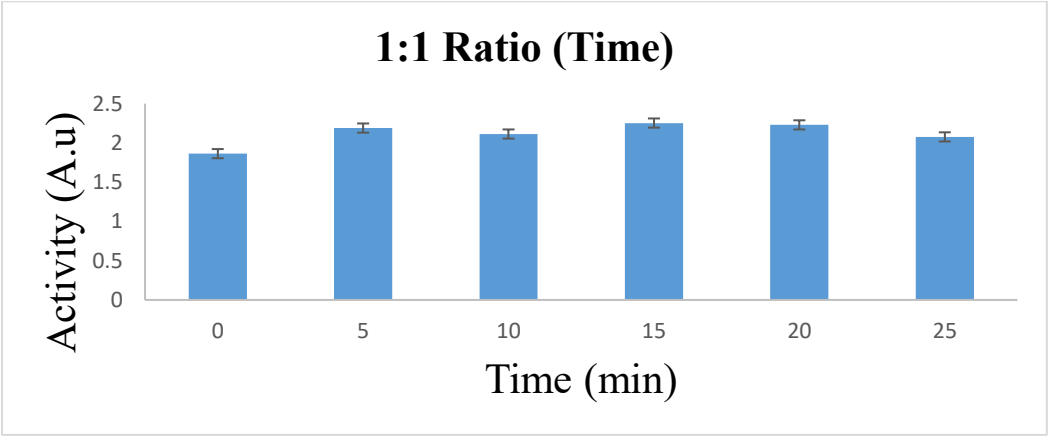

Fig. 2. Effect of incubation time on uricase catalytic activity in the serine–myo-inositol eutectic formulation at a 1:1 molar ratio.

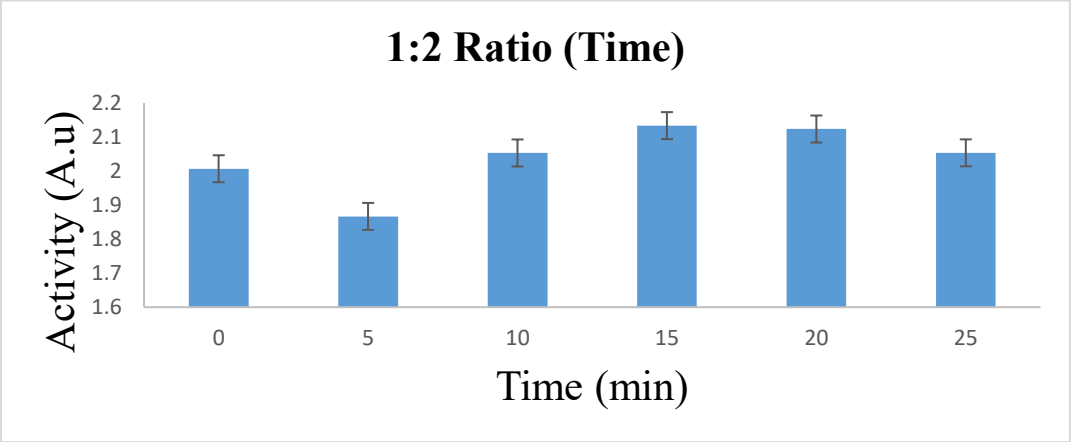

Fig. 3. Effect of incubation time on uricase catalytic activity in the serine–myo-inositol eutectic formulation at a 1:2 molar ratio.

3.3. Thermal Profile and Operating Temperature Optimization

The thermal behavior and operational temperature ranges of both free and formulated uricase were examined across a thermal gradient from 15 °C to 55 °C **(Figs. 4, 5, and 6).**

- Free Enzyme Control: The aqueous unformulated uricase displayed a classic bell-shaped activity profile, characterized by sharp thermal vulnerability and rapid denaturation as temperatures exceeded physiological thresholds **(Fig. 4).**
- DES-Formulated Uricase: In contrast, the serine–*myo*-inositol formulations at both 1:1 **(Fig. 5)** and 1:2 **(Fig. 6)** molar ratios demonstrated significantly enhanced thermal resilience. Both formulations exhibited an optimal operational window centered around 28–30 °C, while effectively dampening the rate of thermal decline at elevated stress temperatures. This highlights the ability of the deep eutectic environment to restrict thermal flexibility fluctuations and stabilize the tertiary structure of the enzyme against heat-induced unfolding.

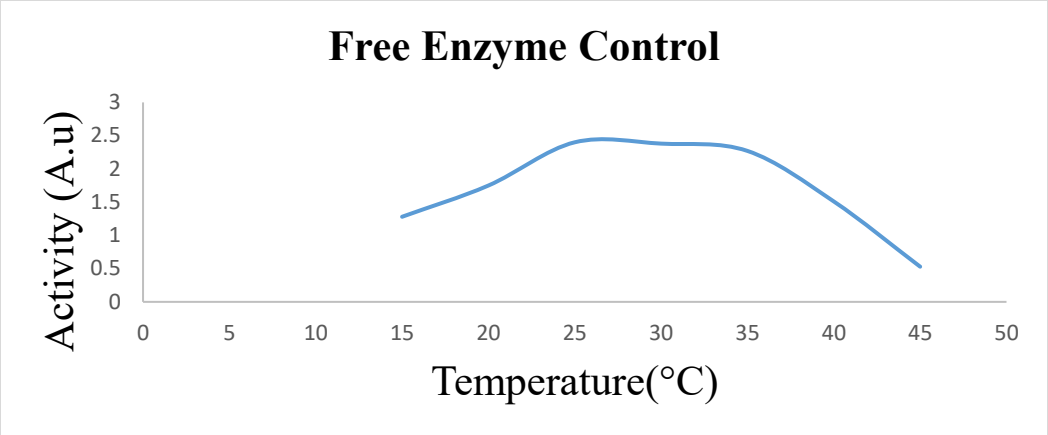

Fig. 4. Temperature-dependent activity profile of the free recombinant uricase control in aqueous solution.

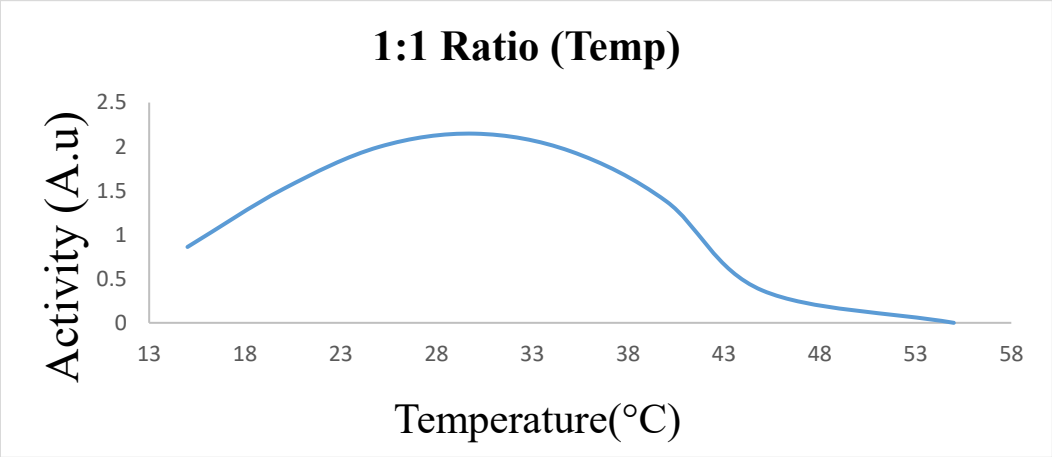

Fig. 5. Temperature optimization profile of uricase formulated in the serine–myo-inositol eutectic system at a 1:1 molar ratio.

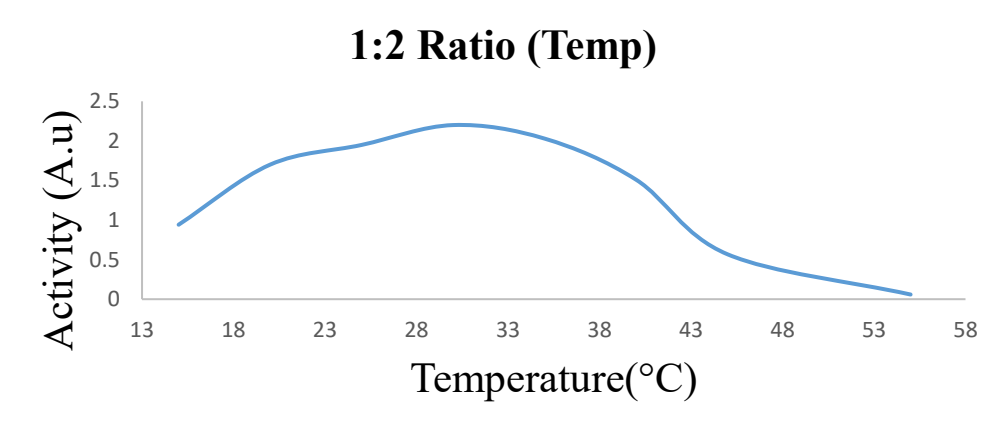

Fig. 6. Temperature optimization profile of uricase formulated in the serine–myo-inositol eutectic system at a 1:2 molar ratio.

3.4. Thermal Inactivation Kinetics

To quantitatively evaluate the stabilizing efficacy of the engineered deep eutectic media, thermal inactivation kinetics were monitored by subjecting the enzyme systems to elevated stress temperatures over a 60-minute duration **(Fig. 7).**

The semi-logarithmic plots of residual activity (At/A0) versus heating time demonstrated strong linearity, confirming that the thermal inactivation process follows a pseudo-first-order kinetic model. High correlation coefficients (R^2 up to 0.991) were achieved across the regression models. Under the investigated stress conditions, the free enzyme and control formulations underwent rapid inactivation with a rate constant (k_{inact}) of 0.7052 min^-1 and a corresponding short half-life ($t_{1/2}$) of 0.9829 min. The introduction of the serine–*myo*-inositol eutectic environment markedly retarded this inactivation rate (yielding improved regression fits, e.g., R^2=0.8034 for the stabilized matrix), substantiating its robust protective capacity against thermal degradation.

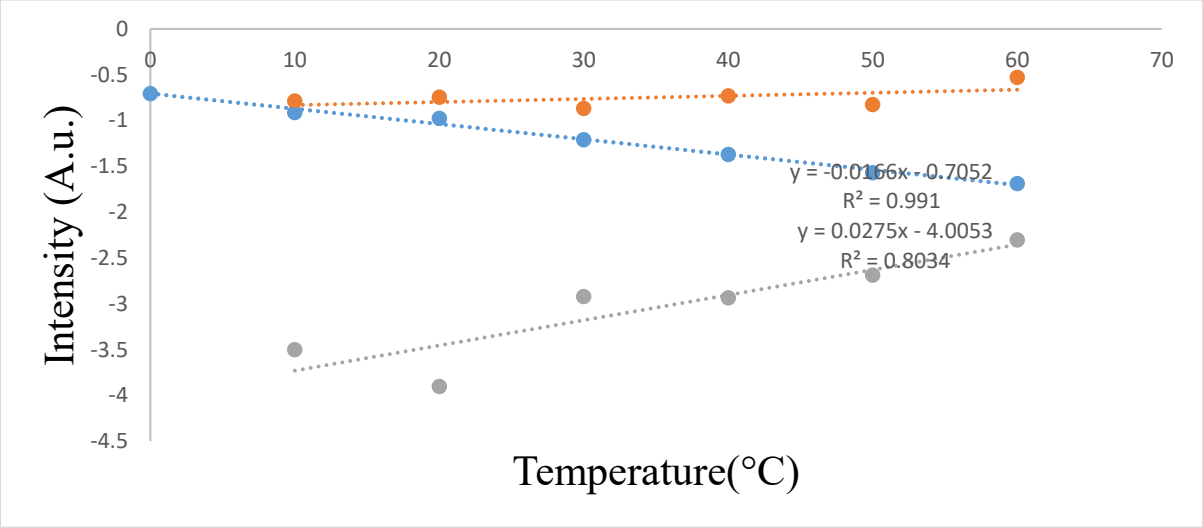

Fig. 7. First-order thermal inactivation kinetics and linear regression models of uricase under elevated thermal stress conditions, displaying correlation coefficients (R^2=0.991 and 0.8034).

Discussion

The successful heterologous overexpression and recovery of recombinant urate oxidase (uricase), confirmed by the prominent ~35 kDa band on SDS-PAGE, establish a reliable foundation for downstream bioprocess applications. The high purification efficiency achieved via 200 mM imidazole elution in Ni-NTA chromatography underscores the structural integrity and purity of the recovered enzyme, providing a clean baseline essential for subsequent deep eutectic formulation studies [8, 9]. Building upon this purified platform, the temporal stability observed within the L-serine–*myo*-inositol deep eutectic systems (DESs) across 1:1 and 1:2 molar ratios demonstrates that the formulated enzyme maintains remarkable durability over extended operational periods. Specifically, the sustained activity plateau achieved within the 15 to 20-minute incubation window highlights how the zwitterionic network of L-serine combined with the polyhydroxy structure of *myo*-inositol establishes a protective hydration shell around the enzyme. This prevents time-dependent denaturation and aggregation by fostering favorable hydrogen-bonding topologies between the DES components and protein surfaces, mirroring solvent-enzyme compatibility dynamics reported in contemporary biocatalytic systems [10-13].

Extending these insights into thermal performance, the sharp thermal vulnerability and rapid denaturation of the unformulated aqueous uricase contrast markedly with the enhanced thermal resilience of the serine–*myo*-inositol formulations. While the free enzyme exhibits classic thermal fragility beyond physiological thresholds, the formulated systems maintain an optimal operational window centered around 28–30 °C while effectively dampening thermal decline at elevated stress temperatures. This superior thermal stability indicates that the deep eutectic microenvironment successfully restricts thermal flexibility fluctuations and elevates the thermal unfolding threshold (T_m). The formation of extensive intermolecular hydrogen bonds between the DES components and the protein surface creates a rigid yet flexible matrix that prevents heat-induced structural collapse-consistent with established paradigms regarding polyol-, sugar-, and amino acid-based DES stabilization of oxidoreductase enzymes [9, 11, 14-16]. Furthermore, precise modulation of physicochemical parameters such as polarity, hydrogen-bond donor/acceptor ratios, and water activity plays a decisive role in preventing enzyme deactivation in non-conventional media [17-19].

The quantitative evaluation of thermal inactivation kinetics further substantiates the robust protective capacity of the engineered deep eutectic media. The linearity of the semi-logarithmic residual activity plots confirms that thermal inactivation conforms to a pseudo-first-order kinetic model. Under the investigated stress conditions, the rapid inactivation rate (k_{inact} = 0.7052 min^{-1}) and short half-life ($t_{1/2}$ = 0.9829 min) characteristic of the free enzyme are markedly retarded in the presence of the serine–*myo*-inositol eutectic environment. Thermodynamically, this stabilization arises because the protective DES hydration shell increases the activation energy barrier for thermal denaturation, thereby curtailing conformational mobility

and averting irreversible aggregation. These kinetic and thermodynamic findings corroborate recent molecular simulation and experimental insights demonstrating that well-designed eutectic solvents enhance structural rigidity, compactness, and operational longevity, thus offering a highly sustainable and advanced framework for therapeutic and industrial biocatalysis [8, 9, 20, 21].

Limitations

Despite these promising findings, several limitations should be acknowledged. First, the current investigation focused primarily on short-term operational stability and thermal inactivation kinetics; long-term storage stability over extended shelf-life periods remains to be evaluated. Second, while the 1:1 and 1:2 molar ratios of the L-serine–*myo*-inositol DES provided substantial protection, a broader screening of different hydrogen bond donor-to-acceptor ratios and alternative ternary eutectic compositions could potentially yield further optimized kinetic and thermodynamic parameters. Finally, in vivo efficacy, biocompatibility, immunogenicity, and pharmacokinetic profiles in physiological or pathological models (such as hyperuricemia and gout) were beyond the scope of this in vitro biophysical study and will require comprehensive preclinical validation in future work.

Conclusions

In this study, recombinant urate oxidase (uricase) was successfully purified via Ni-NTA affinity chromatography using 200 mM imidazole and formulated within novel L-serine–*myo*-inositol deep eutectic systems (DESs) at 1:1 and 1:2 molar ratios. The formulated enzyme exhibited remarkable operational stability, achieving a sustained activity plateau within a 15–20 minute incubation window and an optimal operating temperature range of 28–30 °C. Furthermore, thermal inactivation kinetics demonstrated that the engineered DES matrices significantly retard thermal degradation compared to the unformulated free enzyme (k_{inact} = 0.7052 min^{-1}, $t_{1/2}$ = 0.9829 min), elevating the activation energy barrier and preserving structural integrity through robust intermolecular hydrogen bonding and protective hydration shells. These findings highlight the potential of amino acid- and polyol-based deep eutectic systems as effective, green stabilizing media for therapeutic oxidoreductases.

Disclosure statement

The authors report there are no competing interests to declare.

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Declaration of generative AI use

The authors acknowledge the use of generative AI tools (such as ChatGPT) solely for language polishing and grammatical editing during the preparation of this manuscript.

Author Contributions: D.D. conceived and designed the study, performed the experiments, analyzed the kinetic and thermodynamic data, and wrote the original draft; M.T.M. supervised the research, validated the results, and critically reviewed and edited the manuscript; M.M. provided essential resources, assisted in data validation, and contributed to the final review and editing.

Data Availability

All datasets generated and/or analyzed during the current study are included in this published article. Additional raw data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supplementary:

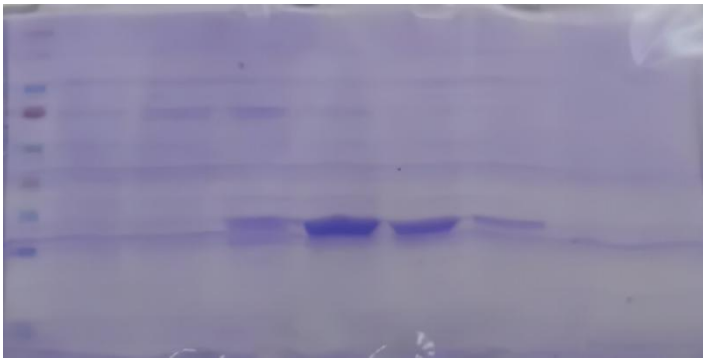

Figure S1. SDS-PAGE analysis of purified recombinant uricase (Uox). The main panel shows selected lanes cropped from the full-length gel to highlight the target fractions. Lanes correspond to wash and elution fractions obtained using increasing imidazole concentrations: 100 mM and 200 mM (derived from the original gel containing lanes A–F: 50, 100, 150, 200, 200, and 250 mM, respectively). A prominent protein band appears at approximately 35 kDa, consistent with the expected molecular weight of recombinant Uox,

confirming successful purification. The full-length, uncropped original gel image is presented in Supplementary Figure S1.