ORIGINAL ARTICLE

Veterinary Research Forum. 2026; 17 (2): 119 - 125
doi: 10.30466/vrf.2025.2050523.4628
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Kinetic and thermostability modulation of *Aspergillus flavus* urate oxidase by proline and glycine osmolytes

Sima Jafari[1], Hossein Tayefi-Nasrabadi[1], Mehdi Imani[2]*

[1] Department of Basic Sciences, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran; [2] Department of Basic Sciences, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

Article Info

Article history:

Received: 13 January 2025
Accepted: 20 May 2025
Available online: 15 February 2026

Keywords:

Glycine
Osmolyte
Proline
Thermal stability
Urate oxidase

Abstract

Hyperuricemia, caused by impaired uric acid excretion, poses significant health risks. Urate oxidase (UOX) from *Aspergillus flavus* offers therapeutic potential by converting uric acid into soluble allantoin; however, its instability limits clinical applications. This study investigated the effects of osmolytes, including proline and glycine, on the kinetics and thermostability of recombinant *A. flavus* UOX. Following the expression of UOX coding sequence in *Escherichia coli* BL21, it was purified using Ni^{2+}-NTA agarose affinity chromatography and confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The enzyme maintained its activity up to 35.00 ˚ C and lost its activity at higher temperatures as it lost 70.00 % of its activity after 60 min at 40.00 ˚ C, and the enzyme with proline and glycine additives maintained 73.00% and 30.00% of the activity, respectively. The inactivation rate constant of enzyme (k_{in}) was decreased in the presence of proline, indicating that the enzyme was more stable with proline, but glycine had no effect on k_{in}. Half-life of enzyme was raised to 86 min in the presence of proline and the Michaelis constant (K_m) was decreased significantly by both osmolytes, as well. These results demonstrated that proline stabilized UOX by mitigating thermal denaturation, likely through preferential hydration and hydrophobic interactions, while glycine enhanced substrate binding. The stabilizing capacity of proline highlighted its utility for inclusion in biopharmaceutical formulations, offering a solution to the persistent challenge of UOX instability in therapeutic contexts. These findings yielded practical strategies for enhancing both structural integrity and catalytic performance of enzymes in pharmaceutical development.

Introduction

Urate oxidase (UOX; EC 1.7.3.3) is a therapeutic enzyme of the oxidoreductase family, catalyzing the oxidation of uric acid into a more soluble and easily excreted product namely 5-hydroxyisourate (allantoin).[1] Non-recombinant UOX obtained from *Aspergillus flavus* has been available since 1968 and it was first used in 1975 to prevent and treat hyperuricemia. The recombinant type of UOX (rasburicase) is produced in *Saccharomyces cerevisiae* and is a new strategy for the treatment of secondary hyperuricemia, acting by converting insoluble uric acid into a more soluble molecule that can be easily excreted.[2-4] The *A. flavus* UOX is a homotetramer protein with a molecular weight of 135 kDa and enzymatic activity on each sub-unit. It is not present in humans and higher primates due to the nonsense mutations over time during evolution.[5,6]

Since UOX is a therapeutic agent reducing the accumulation of toxic urate, it has been used to treat gout disorders, elevation of uric acid after chemotherapy in adults and children with lymphoma, leukemia, and solid tumors (tumor lysis syndrome). Moreover, the enzyme widely employed in laboratory analysis for the detection of uric acid in blood and biosensors, as well.[7-9]

Therapeutic enzymes are distinguished from other types of drugs by their high affinity and ability to convert large numbers of target molecules into products. These two properties make enzymes specific and powerful drugs that can perform biochemical reactions.[10]

Maintaining the natural structure of the protein or protein stabilization is essential for pharmaceutical industry. Many proteins, including pharmaceutical proteins, are structurally conformational but unstable in the face of multiple stresses, like temperature rise during production

***Correspondence:**

Mehdi Imani. PhD
Department of Basic Sciences, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
E-mail: m.imani@urmia.ac.ir

stages, such as purification, processing, and storage, limiting their commercial and industrial applications.[11] To solve this problem, various methods have been employed, including targeted mutagenesis, chemical modification, and the use of additives, including osmolytes.[12] Osmolytes are small natural molecules protecting the native tertiary fold of the protein under unfavorable denaturation conditions and helping cells cope with osmotic stress. Osmolytes were first observed in nature to prevent the unfolding of organic molecules, and then used by the industry.[13] Due to the difficulties of genetic manipulation, protein stabilization by osmolytes is an easy and useful way for industry.[14] In general, osmolytes are divided into three main categories, including polyols, such as glucose, sorbitol, sucrose, and certain methyl ammonium compounds, like trimethylamine N-oxide, stabilizing proteins. Second, osmolytes, such as amino acids and their derivatives, such as taurine, proline, glycine, and betaine, which on average modify the stability of proteins, and finally the third group of denaturing osmolytes, such as urea.[15] Glycine is one of the most widely used amino acids in biology being used as an agent for lyophilized products, bulking, and buffer agents.[13] Proline, glycine, betaine, and mannitol are examples of the many compatible solutes which can accumulate intracellularly without inhibiting enzymatic activities. These osmolytes are also known as non-perturbing or compatible solutes due to their compatibility with cell function at high cytoplasmic levels.[16] Proline has anti-aggregative effects on creatine phosphokinase of the rabbit skeletal muscle, chicken liver fat synthase, and chicken egg lysozyme. L-cystine, L-hydroxyproline, and glycine are the most commonly used amino acids for mammalian cell culture. Also, incorporating stress-specific osmolytes into pharmaceutical formulations could increase stress tolerance and improve drug effectiveness. The basic assumption of biological compatibility is that osmolytes stabilize proteins and other macro-molecules against stress but have little or no impact on the functional activity of these molecules.[17] Based on the established role of glycine and proline as protein-stabilizing osmolytes, this study aimed to evaluate effects of glycine and proline on the UOX thermal stability and kinetic properties.

Materials and Methods

Materials. Yeast extract and tryptone were obtained from Scharlau Co. (Barcelona, Spain). The Ni^{2+}-NTA agarose was purchased from Qiagen Co. (Hilden, Germany). Proline, glycine, lactose, isopropyl-b-D-thio-galactopyranoside, uric acids sodium salt, and all sodium dodecyl sulfate–polyacrylamide gel electro-phoresis chemicals were obtained from Merck (Rahway, USA). A low molecular weight protein ladder (10.00 - 170 kDa) was purchased from Sinaclon (Tehran, Iran). Kanamycin and other materials were purchased from Bio Basic Inc. (Markham, Canada). *Escherichia coli* Bl21 (Pasteur Institute of Iran, Tehran, Iran) and for UOX enzyme production, *pET-28a(+)* plasmid harboring *UOX* gene were also used.[18]

Induction and purification of recombinant UOX. Bacterial induction and expression of recombinant protein were performed according to the previously published method with a little modification. Briefly, a new bacterial colony containing *pET-28a(+)-UOX* was inoculated into 10.00 mL of Luria-Bertani (LB) medium containing 50.00 μg mL^{-1} of kanamycin and incubated at 37.00 ˚ C overnight. Next, 1.00 mL of overnight culture was inoculated into 200 mL of LB medium and incubated with strong shaking at 37.00 ˚ C until the optical density 600 culture reached 0.60 - 0.80. For protein induction, isopropyl β-D-1-thiogalactopyranoside 1.00 mM was added to the culture and incubated at 22.00 ˚ C for 12 hr. At the end of incubation, the cells were collected by centrifugation at 12,000 *g* for 20 min at 4.00 ˚ C, resuspended in lysis buffer (tris buffer with pH of 7.40, including 300 mM NaCl and 5.00 mM imidazole), and sonicated for 20 cycles of 20 sec pulses at 50.00% amplitude and 70.00 W (Hielscher, Teltow, Germany) on ice. The cellular debris was then centrifuged at 12,000 *g* for 20 min at 4.00 ˚ C and the protein extract was harvested as supernatant. Consequently, to purify the enzyme with 6 His-tag, the Ni^{2+}-NTA column was first washed with 9.00 mL of distilled water and then, equilibrated with 9.00 mL of lysis buffer (3-column volumes); afterwards, the supernatant was applied to the column. In the next step, the column was washed with 9.00 mL of wash buffer (tris buffer with pH of 7.40, containing 300 mM NaCl and 25.00 mM imidazole). Eventually, the recombinant UOX was eluted using elution buffer (tris buffer, containing 300 mM imidazole). Once the enzyme purified, it was dialyzed (Dialysis Tubing, Benzoylated; Sigma-Aldrich, St. Louis, USA) against 20.00 mM tris-HCl (pH: 7.50), and the purity and molecular weight of the enzyme were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis 12.00% stained with Coomassie Brilliant Blue. The concentration of the enzyme was determined by the Bradford method using bovine serum albumin as a protein standard.[19]

Urate oxidase activity assay. Urate oxidase catalyzes uric acid into hydrogen peroxide and allantoin, and only uric acid has light absorption at 293 nm. Therefore, it is feasible to consider reduction of uric acid as the UOX activity. Enzymatic assay was performed at 25.00 ˚ C and in a reaction mixture containing 850 μL of 20.00 mM boric acid buffer (pH: 8.50), 30.00 μL of 0.10 mM uric acid, and 50.00 μL of the purified enzyme (0.25 mg mL^{-1}). After 6 min, the reaction was stopped by adding 70.00 μL KOH 20.00% (w/v) and the decrease in absorption of the mixture was measured at 293 nm. One U of UOX enzyme is

defined as the amount of the enzyme catalyzing the conversion of 1.00 µmol of uric acid into allantoin *per* min under the specified condition. Enzyme activity was determined based on the following equation:

$$Activity\ (U\ mL^{-1}) = (\Delta A293/min\ test - \Delta A293/min\ blank) \times (df)/12.60 \times 0.05$$

where, ΔA is absorbance change, df is dilution factor, 12.60 is mM extinction coefficient of uric acid at 293 nm, and 0.05 is the volume of enzyme in mL.[12]

Optimum temperature and pH, and osmolytes concentration. The optimum temperature for UOX activity was obtained by measurement of enzyme activity at different temperatures. For this purpose, the reaction mixture was placed on a thermomixer at a temperature range of 10.00 to 60.00 ˚C with intervals of 5.00 ˚C. After the reaction mixture was reached the desired temperature, 50.00 µL of the enzyme was added to the buffer mixture and immediately the activity of the enzyme was measured at that temperature for 6 min. To detect the optimum pH, first, a set of different pH ranges from 3.00 to 10.00 was prepared from a mixed buffer containing 100 mM acetic acid, 50.00 mM tris/HCL, and 100 mM citrate. Enzyme activity was determined at each pH by adding 30.00 µL of uric acid and 50.00 µL of the enzyme to 850 µL of mixed buffer and optical density 293 was measured after 6 min. To determine the appropriate concentration of additives for enzyme activity, different concentrations (0.00, 0.12, 0.25, 0.50, 0.75, and 1.00 M) of proline and glycine with the enzyme were incubated, followed by measurement of enzyme activity. It should be noted that to test the effect of optimum additives concentration on the optimum temperature and pH, enzymatic assay was done as mentioned above in "UOX activity assay" section.

Thermal inactivation and stability. To examine the thermal inactivation, the purified enzyme was placed on a thermomixer for 10 min at 0.00 to 60.00 ˚C range with a temperature interval of 5.00 ˚C. Later, the enzyme was immediately transferred to the ice to restore its proper structure. After 20 min of placing the enzyme on ice, the enzyme reaction was carried out by adding uric acid for 6 min at 25.00 ˚C. Finally, the sample absorbance was measured at 293 nm. For thermal stability, a sufficient quantity of the purified enzyme was incubated at 40.00 ˚C for 60 min. Then, 50.00 µL of the enzyme was withdrawn at 5 min intervals and incubated on the ice for 20 min to restore and reestablish its native folding. After that, the enzyme residual activity was measured. The activity of the enzymatic solution without osmolyte has been considered 100% control. To detect the effect of osmolytes on UOX activity, enzyme assays were carried out with proline and glycine.

Kinetic parameters. *Lineweaver–Burk* plot was used to calculate the maximum rate of reaction (V_{max}) and Michaelis constant (K_m) parameters of the UOX enzyme. To calculate the constant rate of enzyme inactivation (k_{in}), the natural logarithm of the enzyme activity was plotted against different times and the slope was determined which was equal to k_{in}. The half-life ($t_{1/2}$) of enzyme which was a measure of 50.00% loss of enzyme initial activity was calculated by the equation of $t_{1/2} = 0.693/k_{in}$.

Statistical analysis. Assays were repeated three times at each temperature and sample (n = 3). To analyze the significance of difference between the results, the ANOVA and Tukey tests were implemented using GraphPad Prism Software (version 9.0; GraphPad, San Diego, USA). All data with *p* value less than 0.05 were typically judged to be statistically significant.

Results

Purification of UOX. After induction and expression of recombinant UOX and bacterial sonication, the recombinant enzyme was purified by employing Ni^{2+}-NTA agarose column. Then, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to ensure the purity of the UOX enzyme. As shown in Figure 1A, the presence of a single band on the gel with a relative molecular weight of 35.00 kDa indicated suitable purity of UOX.

Enzyme activity. To evaluate the activity of the recombinant UOX enzyme, we monitored the absorbance of uric acid at 293 nm. The significant reduction of uric acid absorbance over 6 min compared to the initial absorbance at 0 min indicated that the purified enzyme was catalytically active. On the other hand, as shown in Figure 1B, the decline in absorbance from 0.45 to 0.14 was the indicative of UOX function.

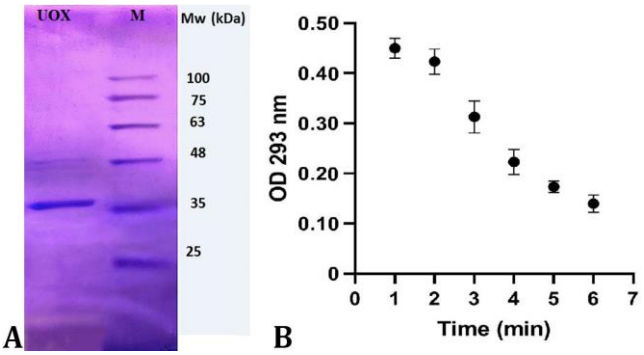

Fig. 1. A) Confirmation of urate oxidase (UOX) purity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis. The gel is stained with Coomassie Brilliant Blue R250. M: Protein marker; UOX: Purified UOX; **B)** Reduction of uric acid concentration against time at 293 nm. For UOX assay, the reaction mixture contained 850 µL of 20.00 mM boric acid (pH: 8.50), 30.00 µL of 0.10 mM uric acid, and 50.00 µL of the purified enzyme. The UOX catalysis was spectrophotometrically read every min and the reduction of substrate was plotted against time.

Optimum pH and temperature. The optimum pH and temperature of the UOX enzyme without and in the presence of 0.25 M proline and glycine are listed in Table 1. First, the activity of UOX was assessed at different concentrations of osmolytes which resulted in the 0.25 M of additives (data not shown). Thereafter, the optimal temperature and pH of UOX were measured at 0.25 M of glycine and proline. As summarized in Table 1, the optimum temperature of the UOX enzyme with proline was 30.00 ˚ C which was 5.00 ˚ C higher than that of the pure UOX, while presence of glycine did not affect the optimum temperature of UOX. Moreover, optimum pH for UOX in the presence of proline and glycine was 8.00 and 8.50, respectively.

Table 1. Optimal pH and temperature of the urate oxidase (UOX) enzyme in the presence and absence of 0.25 M proline and glycine additives.

Enzymes	pH	Temperature (˚ C)
UOX	8.50	25.00
UOX + proline	8.00	30.00
UOX + glycine	8.50	25.00

Thermal inactivation and stability. For thermal inactivation, the purified UOX was placed on a thermomixer at 0.00 to 60.00 ˚ C for 10 min. The results showed that the UOX was active up to 35.00 ˚ C and lost its activity at higher temperatures, while its remaining activity was increased with proline additive at lower temperatures (Fig. 2A). Furthermore, in order to assess the thermal stability of UOX, following incubation of enzyme at different times at 40.00 ˚ C, the activity was monitored by determining the percentage of activity and plotting against different times. The time course of thermal stability of the enzyme showed that the pure UOX lost 70.00% of its original activity at 40.00 ˚ C after 60 min, while the enzyme with additive proline maintained almost 70.00% of its activity during the same period. Additionally, glycine did not affect the thermal stability (Fig. 2B).

Urate oxidase kinetic parameters. The kinetic parameters of the UOX are given in Table 2. As presented in Table 2, the K_m of recombinant UOX was decreased in the presence of proline and glycine from 70.00 to 28.00 and 25.00 µM, respectively. Evaluation of the V_{max} exhibited a reduction in V_{max} for both osmolytes. The k_{in} was decreased in the presence of proline to 0.008 min^{-1}. In fact, the enzyme was more stable with proline, and glycine did not influence the enzyme k_{in}. Estimation of $t_{1/2}$ using $t_{1/2} = 0.693/k_{in}$ showed no change with glycine additive, but significant elevation with proline from 53.30 min to 86.60 min was observed.

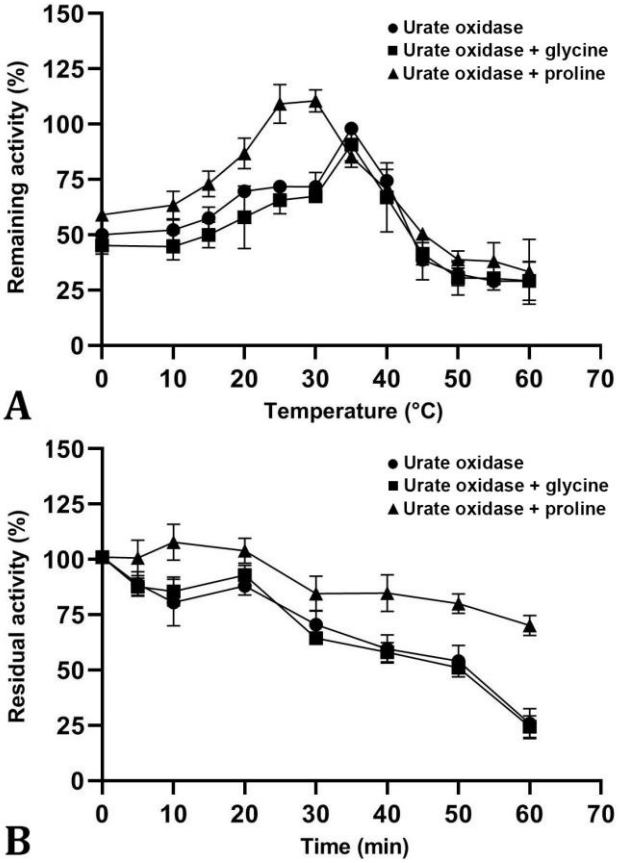

Fig. 2. A) Thermal inactivation of urate oxidase (UOX) in the presence of glycine and proline. The remaining activity was expressed as a percentage of UOX maximum activity; **B)** Thermal stability of UOX in the presence of glycine and proline. The error bars are based on the standard of the mean (n = 3).

Discussion

Pharmaceutical proteins, such as enzymes and antibodies, have the properties of many ideal medicines and are very specific as they impose less side effects. Urate oxidase is one of the protein drugs that can be used as a drug-based protein. A recombinant form of this enzyme being Food and Drug Administration-approved and produced under the name of rasburicase is a fascinating example of the enzyme therapy.[20] Among the various

Table 2. Kinetic and thermodynamic parameters of recombinant urate oxidase (UOX) with and without proline and glycine additives. Data are presented as mean ± standard error of the mean.

Enzymes	k_{in} (1 *per* min)	Half-life (min)	K_m (µM)	V_{max} (U)
UOX	0.013[a]	53.30[a]	75.00 ± 80.00[a]	7.19 ± 0.15[a]
UOX + glycine	0.013[a]	53.30[a]	28.40 ± 60.00[b]	4.50 ± 0.17[b]
UOX + proline	0.008[b]	86.62[b]	25.60 ± 30.00[b]	4.20 ± 0.12[b]

V_{max}: Maximum rate of reaction; K_m: Michaelis constant; k_{in}: Constant rate of enzyme inactivation.
[ab] Different letters in each column indicate a significant difference at the level of $p < 0.05$.

microbial sources available for uric acid degradation, the UOX enzyme from *A. flavus* has gained main advantage in terms of activity and stability, making it applicable for therapeutic purposes.[21,22] Therefore, stabilization of UOX as a medicinal enzyme can be very important in biotechnology. Chemical chaperones are small molecules having a positive effect on the folding and stability of a protein. Chaperones which are divided into the osmolytes and hydrophobic molecules, do this by affecting the rate of folding response. Osmolytes or additives stabilize the natural state of proteins by increasing the free energy of the unfolding state, thus, shifting the equilibrium of the structure towards natural folded structures. They stabilize the protein by increasing surface tension and preferential hydration; so that, increased surface tension overcomes the effects of heat and causes the enzyme to be stable against heat.[23,24] The advantages of using additives compared to the other stabilization methods include low cost and no change in the structure of enzymes.[25] Osmolytes protecting against water stress without affecting protein function are known to be compatible solvents. Examples of amino acids which are compatible osmolytes include glycine, proline, alanine, taurine, and beta-alanine.[17,26] Several researches have shown that many osmolytes, such as sucrose, sorbitol, glycerol, trimethyl N-amine, sarcosine, betaine, proline, and glycine are effective in preventing protein inactivation.[23,27] In the current research, the impacts of two naturally-occurring osmolytes, proline and glycine, were evaluated on the UOX activity. The optimal pH and temperature of UOX in the absence and presence of proline and glycine were determined. As shown in Table 1, the appropriate pH and temperature for UOX were calculated 8.50 and 25.00 ˚ C, respectively, being in agreement with other studies.[28] Addition of osmolytes to the UOX only changed the value of optimal temperature from 25.00 to 30.00 ˚ C in the case of proline but glycine did not influence it. The K_m is defined as a concentration of substrate in which the enzyme reaches to half V_{max}. On the other hand, it is an inverse measure of affinity (the lower the k_m, the higher the affinity). In the current study, the K_m was significantly reduced by proline and glycine which means that the enzyme affinity for the substrate was increased. The reason behind this might be the influence of osmolytes on the active site micro-environment of UOX either by direct interaction or by disturbing the water molecules around active site.

Enzyme inactivation is usually defined as a loss of activity which is the result of various structural alterations involving aggregation, sub-units separation, or denaturation. However, by measuring enzyme activity one can obtain basic information regarding enzyme structural changes. Examination of the thermal inactivation of the enzyme indicated that UOX maintained its highest enzymatic activity up to 35.00 ˚ C, but exceeding temperature declined its activity; so that, at the three temperatures of 40.00, 45.00, and 55.00 ˚ C, almost 50.00, 32.00, and 25.00% of UOX activity was conserved, respectively. It is suggested that UOX is irreversibly denatured and inactivated at 40.00 ˚ C, and it is considered the denaturation temperature of UOX. Evaluation of the glycine and proline effects showed no significant protection against heat except for proline at lower temperatures. It is suggested that proline may have protected the integrity of the enzyme structure and maintained enzyme activity. Studies have shown that proline in aqueous media leads to the formation of hydrophobic colloids with the hydrophobic structure of the interacting protein. Unlike other osmolytes, proline stabilizes enzymes not only by causing preferential hydration of proteins but also by inducing hydrophobic interactions of its structure with available hydrophobic regions of the enzyme solvent.[29] Measuring the thermal stability of the UOX revealed that proline significantly preserved UOX activity for longer times. Calculation of $t_{1/2}$ at 40.00 ˚ C did not show any significant change in the presence of glycine, but in the presence of proline the UOX $t_{1/2}$ was remarkably increased. Moreover, the k_{in} of UOX was reduced in the presence of proline, indicating that the enzyme was mostly stabilized in the presence of proline rather than glycine. In addition to structural parameters, kinetic parameters, including K_m and V_{max}, were assessed, as well.

Furthermore, the effects of polyol additives, such as sucrose, glucose, glycerol, trehalose, and sorbitol, were evaluated and the results exhibited that glucose and sucrose had a greater effect on thermal stability of the enzyme than glycerol and sorbitol.[12,30,31] It has been reported that the mechanism by which glucose and sucrose exert the stabilizing effect is increasing the surface hydration of proteins by increasing the surface tension of water.[32,33] Additionally, theoretical simulations illustrated that switching secondary structure of UOX to more stable motifs, such as conversion of random coil to regular structures, including α-helix and β-sheets, might be another explanation for its resistance against heat.[30] Moreover, research on the refolding of egg-white lysozyme has shown that proline prevents lysozyme aggregation during protein refolding.[34] It is postulated that proline might exert its stabilizing influence by the similar manner. Moreover, the solubilizing ability of proline is reported from *in vitro* experiments, as well. In the equilibrium of protein folding reaction (unfolded ↔ native), protecting osmolytes push the equilibrium toward native; whereas, denaturing osmolytes push the equilibrium toward unfolded. As yet, there is no universal molecular theory that can explain the mechanism by which osmolytes interact with the protein to affect protein stability.[16] Destabilizing unfolded state of protein pushes the folding equilibrium toward native state; hence, proteins gain protection against stressful conditions.

Besides experimental data, using molecular dynamics simulation, it seems that water molecules accumulated in the hydration shell around the lysozyme and proline itself were excluded from the protein surface, thus, favoring stabilization of the protein.[35]

Overall, it was found that proline as an osmolyte had a remarkable and greater effect than glycine. In other words, it was postulated that the addition of proline molecules to aqueous solutions of UOX possibly increased the order of water molecules around the enzyme, resulting in higher stability and protection against heat. Besides, since the therapeutic enzyme UOX is usually produced and supplied in liquid form for the treatment of hyper-uricemia patients, its shelf life and stability can be increased by using proline as an additive or a stabilizer. Therefore, we suggest proline in the formulation process of UOX, as well as other protein-based medicine for increasing and maintaining the stabilization.

Acknowledgments

The authors express their gratitude to the Research Council of the University of Tabriz, Tabriz, Iran, for financial support during this project.

Conflict of interest

The authors declare no conflict of financial interests.

References

1. Nelapati AK, Meena SK. An approach to increase the efficiency of uricase by computational mutagenesis. Phys Chem Res 2023; 11(3): 481-491.
2. Khalighi PR, Martens KL, White AA, et al. Utilization patterns and clinical outcomes of rasburicase administration according to tumor risk stratification. J Oncol Pharm Pract 2020; 26(3): 529-535.
3. Alakel N, Middeke JM, Schetelig J, et al. Prevention and treatment of tumor lysis syndrome, and the efficacy and role of rasburicase. Onco Targets Ther 2017; 10: 597-605.
4. Dean L, Kane M. Rasburicase therapy and G6PD and CYB5R genotype. 2020. In: Pratt VM, Scott SA, Pirmohamed M, Esquivel B, Kattman BL, Malheiro AJ, editors. Medical Genetics Summaries [Internet]. Bethesda (MD): National Center for Biotechnology Information (US); 2012.
5. Chiu YC, Hsu TS, Huang CY, et al. Structural and biochemical insights into a hyperthermostable urate oxidase from Thermobispora bispora for hyper-uricemia and gout therapy. Int J Biol Macromol 2021; 188: 914-923.
6. Kratzer JT, Lanaspa MA, Murphy MN, et al. Evolutionary history and metabolic insights of ancient mammalian uricases. Proc Natl Acad Sci U S A 2014; 111(10): 3763-3768.
7. Shaaban MI, Abdelmegeed E, Ali YM. Cloning, expression, and purification of recombinant uricase enzyme from *Pseudomonas aeruginosa* Ps43 using *Escherichia coli*. J Microbiol Biotechnol 2015; 25(6): 887-892.
8. Tan QY, Wang N, Yang H, et al. Characterization, stabilization and activity of uricase loaded in lipid vesicles. Int J Pharm 2010; 384(1-2): 165-172.
9. Wu J, Yang X, Wang D, et al. A numerical approach for kinetic analysis of the nonexponential thermoinactivation process of uricase. Protein J 2016; 35(4): 318-329.
10. Vellard M. The enzyme as drug: application of enzymes as pharmaceuticals. Curr Opin Biotechnol 2003; 14(4): 444-450.
11. Ohtake S, Kita Y, Arakawa T. Interactions of formulation excipients with proteins in solution and in the dried state. Adv Drug Deliv Rev 2011; 63(13): 1053-1073.
12. Mirzaeinia S, Pazhang M, Imani M, et al. Improving the stability of uricase from *Aspergillus flavus* by osmolytes: use of response surface methodology for optimization of the enzyme stability. Process Biochem 2020; 94: 86-98.
13. Wlodarczyk SR, Custódio D, Pessoa A Jr, et al. Influence and effect of osmolytes in biopharmaceutical formulations. Eur J Pharm Biopharm 2018; 131: 92-98.
14. Rariy RV, Klibanov AM. Correct protein folding in glycerol. Proc Natl Acad Sci U S A 1997; 94(25): 13520-13523.
15. Ferreira LA, Breydo L, Reichardt C, et al. Effects of osmolytes on solvent features of water in aqueous solutions. J Biomol Struct Dyn 2017; 35(5): 1055-1068.
16. Rabbani G, Choi I. Roles of osmolytes in protein folding and aggregation in cells and their biotechnological applications. Int J Biol Macromol 2018; 109: 483-491.
17. Wang A, Bolen DW. Effect of proline on lactate dehydrogenase activity: testing the generality and scope of the compatibility paradigm. Biophys J 1996; 71(4): 2117-2122.
18. Imani M, Shahmohamadnejad S. Recombinant production of *Aspergillus Flavus* uricase and investigation of its thermal stability in the presence of raffinose and lactose. 3 Biotech 2017; 7(3): 201. doi: 10.1007/s13205-017-0841-3.
19. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-254.
20. Ngo JS, Ho MHM. Evaluation of rasburicase use in the Fraser Health Authority: a retrospective review. Can J Hosp Pharm 2019; 72(4): 311-319.
21. Li J, Chen Z, Hou L, et al. High-level expression,

purification, and characterization of non-tagged *Aspergillus flavus* urate oxidase in *Escherichia coli*. Protein Expr Purif 2006; 49(1): 55-59.
22. Fazel R, Zarei N, Ghaemi N, et al. Cloning and expression of *Aspergillus flavus* urate oxidase in *Pichia pastoris*. Springerplus 2014; 3: 395. doi: 10.1186/2193-1801-3-395.
23. Choudhary S, Save SN, Kishore N, et al. Synergistic inhibition of protein fibrillation by proline and sorbitol: biophysical investigations. PLoS One 2016; 11(11): e0166487. doi: 10.1371/journal.pone.0166487.
24. Lin TY, Timasheff SN. On the role of surface tension in the stabilization of globular proteins. Protein Sci 1996; 5(2): 372-381.
25. Jain S, Seechurn S, Gupta P, et al. Effects of osmolytes on the structural stability of bovine trypsin: a brief review. J Pharm Res 2015; 9(8): 500-508.
26. Stasiulewicz M, Panuszko A, Bruzdziak P, et al. Mechanism of osmolyte stabilization-destabilization of proteins: experimental evidence. J Phys Chem B 2022; 126(16): 2990-2999.
27. Salehian M, Emamzadeh R, Nazari M, et al. Glycine as a stabilizing osmolyte for *Renilla luciferase*: a kinetic and molecular dynamics analysis. JBCBT 2024; 43(1): 61-70.
28. Imani M, Pazhang M, Mirzaeinia S. Cloning and expression of therapeutic enzyme, *Aspergillus flavus* uricase in *E. coli*. J Adv Med Biomed Res 2016; 24(106): 109-121.
29. Rajendrakumar CS, Reddy BV, Reddy AR. Proline-protein interactions: protection of structural and functional integrity of M4 lactate dehydrogenase. Biochem Biophys Res Commun 1994; 201(2): 957-963.
30. Taherimehr Z, Zaboli M, Torkzadeh-Mahani M. New insight into the molecular mechanism of the trehalose effect on urate oxidase stability. J Biomol Struct Dyn 2022; 40(4): 1461-1471.
31. Shahmoradipour P, Zaboli M, Torkzadeh-Mahani M. Exploring the impact of taurine on the biochemical properties of urate oxidase: response surface methodology and molecular dynamics simulation. J Biol Eng 2024; 18(1): 10. doi: 10.1186/s13036-023-00397-x.
32. Kumar V, Chari R, Sharma VK, et al. Modulation of the thermodynamic stability of proteins by polyols: significance of polyol hydrophobicity and impact on the chemical potential of water. Int J Pharm 2011; 413(1-2): 19-28.
33. Pazhang M, Khajeh K, Ranjbar B, et al. Effects of water-miscible solvents and polyhydroxy compounds on the structure and enzymatic activity of thermolysin. J Biotechnol 2006; 127(1): 45-53.
34. Samuel D, Kumar TK, Ganesh G, et al. Proline inhibits aggregation during protein refolding. Protein Sci 2000; 9(2): 344-352.
35. Bozorgmehr MR, Monhemi H. How can a free amino acid stabilize a protein? Insights from molecular dynamics simulation. J Solution Chem 2015; 44: 45-53.