Biochemistry and Biophysics Reports 37 (2024) 101617

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Uncovering the role of sorbitol in *Renilla* luciferase kinetics: Insights from spectroscopic and molecular dynamics studies

Golnoosh Khoshnevisan [a], Rahman Emamzadeh [b,*], Mahboobeh Nazari [c,d], Mina Oliayi [b], Reyhaneh Sariri [a]

[a] Department of Biology, University of Guilan, Rasht, Iran
[b] Department of Cell and Molecular Biology and Microbiology, Faculty of Biological Science and Technology, University of Isfahan, Isfahan, Iran
[c] Endocrine Research Center, Institute of Endocrinology and Metabolism, Iran University of Medical Sciences, Tehran, Iran
[d] Nanobiotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran

ARTICLE INFO

Keywords:
Renilla luciferase
Sorbitol
Enzyme kinetics
Molecular dynamics
Catalytic efficiency

ABSTRACT

Renilla luciferase catalyzes the oxidation of coelenterazine to coelenteramide, resulting in the emission of a photon of light. This study investigated the impact of sorbitol on the structural and kinetic properties of *Renilla* luciferase using circular dichroism, fluorescence spectroscopy, and molecular dynamics simulations. Our investigation, carried out using circular dichroism and fluorescence analyses, as well as a thermal stability assay, has revealed that sorbitol induces conformational changes in the enzyme but does not improve its thermal stability. Moreover, through kinetic studies, it has been demonstrated that at a concentration of 0.4 M, sorbitol enhances the catalytic efficiency of *Renilla* luciferase. However, at higher concentrations, sorbitol results in a decrease in catalytic efficiency. Additionally, molecular dynamics simulations have shown that sorbitol increases the presence of hydrophobic pockets on the enzyme's surface. These simulations have also provided evidence that at a concentration of 0.4 M, sorbitol facilitates substrate access to the active site of the enzyme. Nevertheless, at higher concentrations, sorbitol obstructs substrate trafficking, most likely due to its impact on the gateway to the active site. This study may provide insights into the kinetic changes observed in enzymes with buried active sites, such as those with α/β hydrolase fold.

1. Introduction

One of the widely used bioluminescent enzymes is *Renilla* Luciferase which has extracted from *Renilla reniformis* [1]. This single subunit enzyme contains 311 amino acids with 36 kDa molecular weight which emits blue light (480 nm) in the presence of molecular oxygen and coelenterazine as a substrate [2,3]. The structure of *Renilla* luciferase has been revealed through crystallographic studies conducted by Loening et al. [4]. This structure exhibits a globular fold and is comprised of two domains: a main domain and a cap domain. The main domain consists of eight β-strands (β1 to β8) surrounded by six α-helices (named A to F), and adopts the conformation of an α/β hydrolase domain. Additionally, three key residues (D120, E144, and H285) essential for the catalytic reaction are situated within the main domain. Moreover, the main domain contains a gateway structure formed by the folding of the C-terminal of the beta strands alongside the alpha helices. Woo et al. have shown that this gateway is covered by hydrophobic amino acids, which likely facilitate the binding of the substrate to the enzyme as demonstrated through mutagenesis studies [5]. Furthermore, the gateway can adopt both open and closed conformations. The cap domain, adjacent to the gateway, consists of alpha helix structures and flexible loops.

Relying on some advantages like the relatively small size and non-use ATP feature, this enzyme has been applied in certain applications case in point imaging and biosensing [6–8]. In recent years, several studies have been conducted to address the limitations of *Renilla* luciferase, including issues such as blue bioluminescence, momentary emission, and unstable structure, as well as the behavior of the protein under different conditions. Super *Renilla* luciferase [9], *Renilla* luciferase 8 [10], and super *Renilla* luciferase 8 [11] mutant super *Renilla* luciferase 8 [12], an ancestral *Renilla* luciferase [13] and a new and interesting *Renilla* luciferase [14] are mutated variants of *Renilla* luciferase with distinct features from native *Renilla* luciferase. Although significant progress has been made in enhancing the stability of the *Renilla* luciferase, it is still

* Corresponding author.
E-mail address: r.emamzadeh@sci.ui.ac.ir (R. Emamzadeh).

https://doi.org/10.1016/j.bbrep.2023.101617
Received 26 July 2023; Received in revised form 10 December 2023; Accepted 12 December 2023
Available online 25 December 2023

worthwhile to investigate the impact of additive agents on its stability. Such investigations can also provide valuable insights into the kinetic behavior of this enzyme.

Osmolytes are produced by an organism under stressful conditions. They are found to stabilize and protect macromolecule integrity in organisms or cells against denaturing stresses. These low molecular weight compounds are included sugars, polyols, amino acids, polyhydric alcohols, and methylamines [15]. One of the most commonly used osmolytes due to protecting organisms from freezing and osmotic pressure and also preventing protein aggregation are polyols [16]. Polyol molecule sorbitol is widely known as a protein stabilizer that different mechanisms have been ascribed to stabilizing impacts of this osmolyte. The mechanisms include preferential hydration, preferential exclusion from the unfolded protein, and coating impact [17–19], even so, sorbitol molecular level effects are not predictable. A previous study aimed to enhance the stability of *Renilla* luciferase by investigating the impact of glycerol on the enzyme's structure and function. Surprisingly, the study found that the stabilizing effect of glycerol on *Renilla* luciferase at 30 °C is negligible. Instead, glycerol has a parabolic obstructive effect on the enzyme by entering its main tunnel [20]. However, due to glycerol's small size, an important question arises: What is the impact of sorbitol, which is twice the size of glycerol, on the kinetics and stability of *Renilla* luciferase? Therefore, in this study, we tried to explore the effect of sorbitol on the function and structure of *Renilla* luciferase by focusing on spectroscopy methods, molecular dynamics (MD) simulations and kinetic features.

2. Materials and methods

2.1. Materials

The sorbitol (catalog number 85529) and coelenterazine (catalog number C3230) were obtained from Sigma Aldrich. Tris[hydroxymethyl]aminomethane (tris) (catalog number TB0195), sodium chloride (NaCl) (catalog number DB0483), ethylenediaminetetraacetic acid (EDTA) (catalog number EB0185) and isopropyl β-D-1-thiogalactopyranoside (IPTG) (catalog number IB0168) were purchased from BioBasic (Canada). The HisPurTM Ni-NTA Resin (catalog number 88225) was purified from Thermos Scientific (the United States). Deionized water at a conductivity of 18.2 MΩ cm was purified using a system manufactured by zolalan (Iran).

2.2. Expression and purification

The expression and purification of *Renilla* luciferase was similar to previous studies [20,21]. Briefly, The *Renilla* luciferase coding sequence was inserted into pET-21a (+) plasmid and transformed into *Escherichia coli* BL21 (DE3) PLYS strain (Novagen, USA). The transformed cells were cultured in 10 mL Luria-Broth (LB) supplemented with ampicillin (100 μg mL^{-1}), induced with 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG, Sigma-Aldrich), and incubated at 30 °C for 16 h to express *Renilla* luciferase. The cells were lysed in lysis buffer (50 mM Tris-HCl and Triton 0.01 %, pH 8.0) via sonication and the lysate was centrifuged at 8000 rpm at 4 °C for 8 min to obtain the supernatant. The protein was purified using HisPurTM Ni-NTA Resin (Thermos scientific, USA).

2.3. Evaluation of Renilla luciferase kinetic parameter

In previous research, the enzyme activity of *Renilla* luciferase has been evaluated within a range of substrate concentrations from 56.2 to 0.8 μM and the Km value was determined to be 2.9 ± 1. To investigate the impact of sorbitol concentration on the kinetic parameters of *Renilla* luciferase, a Tris-NaCl buffer (50 mM, pH 7.5) NaCl (50 mM), and EDTA (0.5 mM) was prepared, containing varying concentrations of sorbitol (0.2, 0.4, 0.6, 0.8, and 1 M). Subsequently, 50 μL of coelenterazine with final concentrations of 56.25, 28.125, 14.06, 7.03, 3.51, 1.75, and 0.87 μM, along with 1 μg mL^{-1} of the enzyme, were combined with the sorbitol-containing buffer. The emission light was then measured using a luminometer (Sirius-single tube Luminometer, Berthold Detection System, GmbH) at 25 °C, and the results were reported in relative light units per second (RLU.Sec^{-1}). The V_{max} and K_m of *Renilla* luciferase were determined in the presence of different concentrations of sorbitol. Moreover, to study the effect of sorbitol on the thermal stability of the enzyme, the enzyme was treated with the aforementioned concentrations of sorbitol at 30 °C and the then emission luminescence was recorded every 5 min. Each experiment was repeated at least three times.

2.4. Secondary structure assay

The purified *Renilla* luciferase was dialyzed in a buffer containing Tris-HCl (10 mM, pH 7.5), NaCl (50 mM), and EDTA (0.5 mM) at 4 °C. Subsequently, the far-UV circular dichroism (CD) spectra of the purified and dialyzed *Renilla* luciferase (final concentration 2.7 μM) were recorded using a JASCO J-715 spectropolarimeter equipped with a quartz cuvette at wavelengths ranging from 200 to 250 nm, in accordance with the description given by Hashemi-Shahraki et al. [22].

The CD spectra were recorded in the presence and absence of sorbitol at various concentrations (0, 0.4, and 0.6 M) and corrected against buffer blank. The molar ellipticity, [θ], was calculated based on the mean amino acid residue weight and the protein concentration. The instrument was calibrated using (+)-10-camphorsulfonic acid and JASCO standard non-hygroscopic ammonium (+)-10-camphorsulfonate. Data noise was reduced using JASCO J-715 software, including the fast Fourier transform noise reduction routine.

2.5. Fluorescence spectroscopy studies

Fluorescence spectroscopy studies of *Renilla* luciferase was studied in the presence of various final concentrations of sorbitol (0, 0.4 and 0.6 M) using a Cary-Eclipse fluorescence spectrophotometer equipped with a quartz cuvette.

Intrinsic fluorescence *of Renilla* luciferase (final concentration 5.9 μM) was determined with an excitation wavelength of 290 nm and emission spectra were recorded between 295 and 440 nm at 25 °C, similar to the description provided by Raeessi-Babaheydari et al. [23]. The excitation and emission slit widths of samples were kept at 5 nm. Additionally, the fluorescence spectra of 8-anilino-1-naphthalenesulfonic acid (ANS) were analyzed in the presence of various final concentrations of sorbitol (0, 0.4 and 0.8 M) using a Cary Eclipse fluorescence spectrophotometer. The concentration of *Renilla* luciferase used in the experiment was 6.7 μM, and the molar ratio of ANS to enzyme was 1:50. The ANS emission was observed within the range of 380–700 nm, while the excitation wavelength was set to 350 nm.

2.6. Molecular dynamic simulation

A homology model of *Renilla* luciferase was developed using the Swiss model server (http://swissmodel.expasy.org), with the crystal structure of *Renilla* luciferase 8 (2PSF) serving as the template. Molecular dynamics (MD) simulations were undertaken to investigate the interactions between *Renilla* luciferase and sorbitol in aqueous solutions with varying concentrations (0.0, 0.4, and 0.6 mol dm^{-3}). The simulations were conducted using GROMACS 5.1.2, a molecular dynamics simulation package, along with the CHARMM27 all-atom force field [24]. The initial protein and sorbitol configurations for each simulation were generated using the Swissparam server [25]. The simulation boxes were solvated by including TIP3P water molecules through the SOL command. For the sorbitol systems, the number of sorbitol molecules was determined based on the number of sorbitol molecules present in the water box, considering the Avogadro's number for each molecule. The systems were built using the Packmol software [26]. An

isothermal-isobaric ensemble was utilized, with a pressure and temperature set at 105 Pa (1 bar) and 298.15 K, respectively. The protein underwent steepest descent energy minimization in NpT for 50000 steps, followed by an additional 50000 steps in NvT (100 ps for each minimization). The minimization procedures were continued until the maximum force decreased below 50 kJ mol^{-1} nm^{-1}. Subsequently, a production run of 100 ns was carried out for further analysis, followed by an energy minimization of the entire system. The structural features of *Renilla* luciferase were analyzed using the Visual Molecular Dynamics (VMD) tools (version 1.9.2) for the Linux [27].

3. Results

3.1. Protein expression and purification

Renilla luciferase was expressed in the *E. coli* system (BL21) by pET21a plasmid and was purified via Ni-NTA column chromatography, and then was evaluated using SDS-PAGE (Fig. 1). The purified protein had a molecular weight of 37 kDa which was consistent with *Renilla* luciferase molecular weight. Additionally, the protein purity was determined to be more than 95 % based on ImageJ analysis. Using Bradford assay the concentration of purified protein was calculated at 0.3 mg mL^{-1} via standard graph with $R^2 = 0.97$.

3.2. Kinetics of Renilla luciferase

Taking into account the estimation of kinetic parameters (Vmax and K_m) of *Renilla* luciferase in the absence of sorbitol, we measured the activity of *Renilla* luciferase in different substrate concentrations (Fig. 2A, Table 1). In the result, the values of V_{max} and K_m were calculated at $3.2 \pm 0.28 \times 10^6$ RLU.Sec^{-1} and 1.64 ± 0.096 μM, respectively, by Lineweaver-Burk Plot, which they showed excellent accordance with previous studies [10,21].

To evaluate *Renilla* luciferase kinetic parameter in the presence of Sorbitol, the enzyme activity was measured in the various amounts of sorbitol accompanied with different concentrations of substrate. Based on the Lineweaver-Burk equation, the V_{max} and K_m values were accounted for sorbitol attendance as illustrated in Fig. 2A and Table 1. The k_{cat} plot showed sorbitol caused a dramatic increase in k_{cat} at 0.4 M and a mild increase at 0.2 M, while 0.6 M and more concentrations displayed a reduction in k_{cat} in comparison with an aqueous solution (Fig. 2B). The observed change in k_{cat} in the presence of sorbitol can be attributed to a decrease in K_m and a simultaneous increase in V_{max} at 0.4 M sorbitol. It is worth noting that while V_{max} remains relatively stable across sorbitol concentrations of 0.6–1 M, the reduction in K_m leads to a decrease in k_{cat}, indicating a correlation with substrate binding affinity.

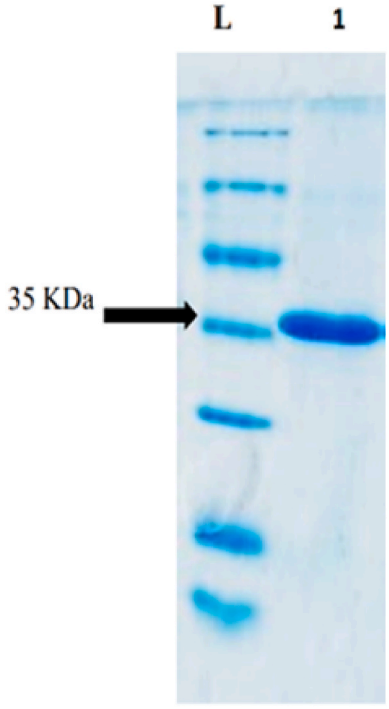

Fig. 1. SDS-PAGE was performed to analyze the purity of *Renilla* luciferase after purification. Lane L shows the protein marker and lane 1 shows the purified enzyme.

3.3. Renilla luciferase thermal stability assay

In order to investigate the thermostabilizing effect of sorbitol on *Renilla* luciferase, the enzyme activity was measured at 30 °C with 5-min intervals after treatment with various concentrations of sorbitol. The percentage of relative activity over time of incubation in various concentrations of sorbitol is presented in Fig. 3. As shown, the enzyme activity did not exhibit a significant improvement with the addition of sorbitol.

3.4. Spectroscopy studies

Peptide bonds have an absorbance spectrum in the UV range, making UV CD a useful tool for investigating the secondary structure of proteins. In this study, the CD spectrum of *Renilla* luciferase was analyzed at 0.0, 0.4, and 0.6 M sorbitol concentrations. The results showed that the secondary structure of the protein in aqueous solution was similar at different concentrations of sorbitol (Fig. 4A).

Tryptophan fluorescence emission spectrum varies depending on the microenvironment, resulting in an intensified and shifted emission spectrum towards shorter wavelengths in the hydrophobic internal environment of the protein. Therefore, the intrinsic fluorescence spectroscopy of *Renilla* luciferase was measured to evaluate the effect of 0.4 and 0.6 M sorbitol on the tertiary structure. The data indicated that the structure of the enzyme had been locally changed with increasing concentrations of sorbitol (Fig. 4B).

Using ANS, the conformational alteration of *Renilla* luciferase affected by sorbitol was studied, and the results showed that higher concentrations of sorbitol led to an increase in ANS fluorescence (Fig. 4C).

3.5. MD simulation study

The MD was carried out using NAMD software to investigate the effect of sorbitol on *Renilla* luciferase structure. The simulation was performed in the absence and presence of sorbitol, and the output files were evaluated. The results showed the interaction of sorbitol with the gateway of the main channel (Fig. 5). In 0.4 M of Sorbitol, sorbitol molecules were observed to be dispersed around the gateway (Fig. 5B), while in 0.6 M of Sorbitol, they were found to be gathered in close vicinity of the gateway with no entry into the channel (Fig. 5C).

Molecular dynamics studies have revealed that sorbitol molecules engage in the formation of hydrogen bonds with amino acid residues, particularly in secondary structures in close proximity to the cap domain (Table 2). Additionally, Molecular dynamics studies revealed that the presence of sorbitol causes a significant conformational change in the enzyme. This change occurs due to sorbitol binding to a flexible loop near the entrance of the enzyme, leading to improved accessibility to the enzyme's active site (Fig. 6).

Furthermore, the structural changes of *Renilla* luciferase were investigated in the presence of 0.4 and 0.6 M sorbitol. The solvent accessible surface plot of the whole protein revealed that sorbitol increased the accessible surface in a concentration-dependent manner (Fig. 7A). The plots of hydrogen bonds between water and sorbitol

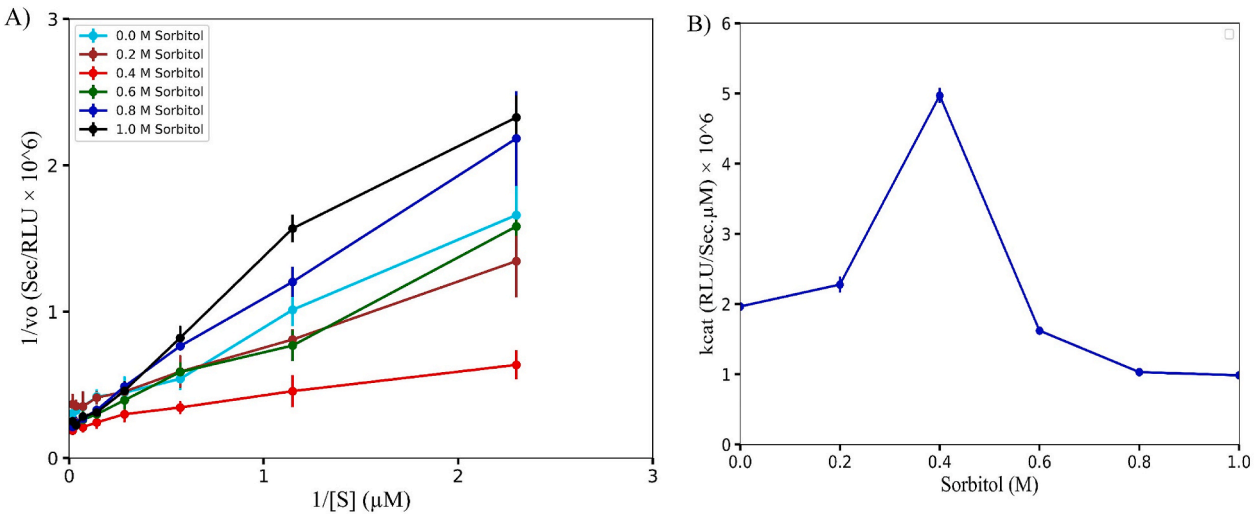

Fig. 2. A) The Lineweaver-Burk plot of *Renilla* luciferase in the presence of different concentrations of sorbitol (0, 0.2, 0.4, 0.6, 0.8, and 1 M). The x-axis represents 1/[s] (μM^{-1}) and the y-axis represents $1/v_0$ (RLU^{-1}.Sec). B) A graph illustrating the catalytic efficiency of Renilla luciferase at different concentrations of sorbitol. The x-axis shows the catalytic efficiency and the y-axis represents different concentrations of sorbitol (0, 0.2 0.4, 0.6, 0.8, and 1 M).

Table 1
Kinetic parameter of *Renilla* luciferase calculated based on different concentration of Sorbitol.

Sorbitol (M)	K_m (μM)	V_{max} (RLU.Sec $^{-1}$) × 10^6	kcat (RLU.Sec^{-1}.μM^{-1}) × 10^6
0.0	**1.64 ± 0.096**	**3.2 ± 0.28**	**1.95 ± 0.025**
0.2	***1.31* ± 0.038**	**3.0 ± 0.28**	**2.28 ± 0.010**
0.4	**0.98 ± 0.077**	**4.8 ± 0.56**	**4.95 ± 0.010**
0.6	**2.96 ± 0.111**	**4.7 ± 0.47**	**1.60 ± 0.014**
0.8	**4.93 ± 0.151**	**5.0 ± 0.26**	**1.01 ± 0.026**
1.0	**5.14 ± 0.169**	**4.9 ± 0.76**	**0.968 ± 0.005**

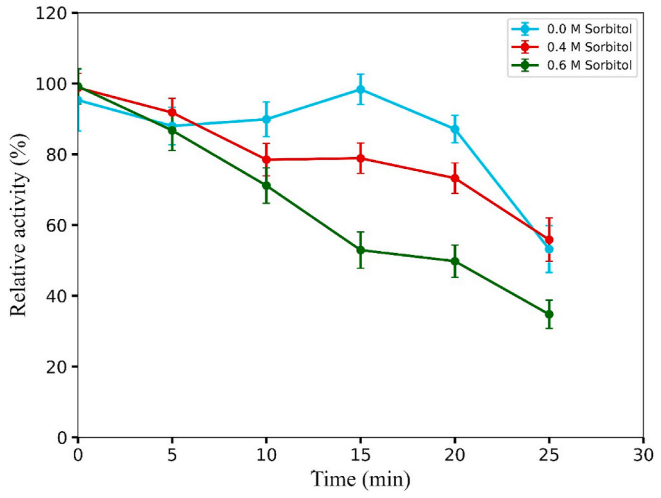

Fig. 3. The thermal stability of *Renilla* luciferase was evaluated at 30 °C for 0–20 min in the presence of sorbitol. The blue represents 0 M sorbitol, the red line represents 0.4 M sorbitol, and the green line represents 0.6 M sorbitol.

molecules with the protein surface were also analyzed (Fig. 7B and C, respectively). The results indicated that increasing the concentration of sorbitol caused an elevation in the hydrogen bonds between sorbitol and the protein surface, while it led to a decline in the hydrogen bonds between water and the protein surface. Based on these results, it can be concluded that increasing the concentration of sorbitol promotes the substitution of water molecules with sorbitol molecules around the protein.

4. Discussion

Renilla luciferase is a protein consisting of a single polypeptide chain, with a molecular weight of 37 kDa and 311 amino acids. It has been employed in various biological and biotechnological applications [6–8]. In this study, we utilized experimental and computational research to gain a molecular-level understanding of the impact of sorbitol, an osmolyte, on the kinetics and stability of *Renilla* luciferase as a protein model.

The kinetics of *Renilla* luciferase in the presence of varying concentrations of sorbitol demonstrated that the enzyme's kinetic parameters, including K_m and V_{max}, underwent changes. Specifically, the K_m value of the enzyme decreased as the sorbitol concentration increased from 0 to 0.4 M, but then increased as the concentration rose from 0.6 to 1 M. Furthermore, the V_{max} increased in the presence of sorbitol. These results were in line with the findings of Dehghanpoor et al. regarding the effects of sorbitol on the kinetic parameters of recombinant Lepidium draba peroxidase [28]. It may be proposed that sorbitol could enhance the thermal stability of the enzyme and preserve its complete activity, leading to an increase in the Vmax of *Renilla* luciferase, as indicated by the rise in the Vmax value. However, kinetic stability study conducted at sorbitol concentrations of 0.4 and 0.8 M showed no significant effect on the thermal stability of the enzyme compared to the control. Thus, it was hypothesized that the changes in *Renilla* luciferase kinetics observed in the presence of sorbitol are likely due to structural alterations induced by sorbitol, rather than an increase in the population of active enzymes facilitated by the protective mechanism of sorbitol.

The effect of sorbitol on the structure of *Renilla* luciferase was examined using CD and fluorescence spectroscopy. The impact of sorbitol on the secondary structure of various proteins cannot be accurately predicted as it varies. While some studies [28,29] have reported no significant changes in the secondary structure, others [30,31] have found that sorbitol can affect it. In this study, the reference spectrum for *Renilla* luciferase, in the absence of sorbitol, exhibited two peaks

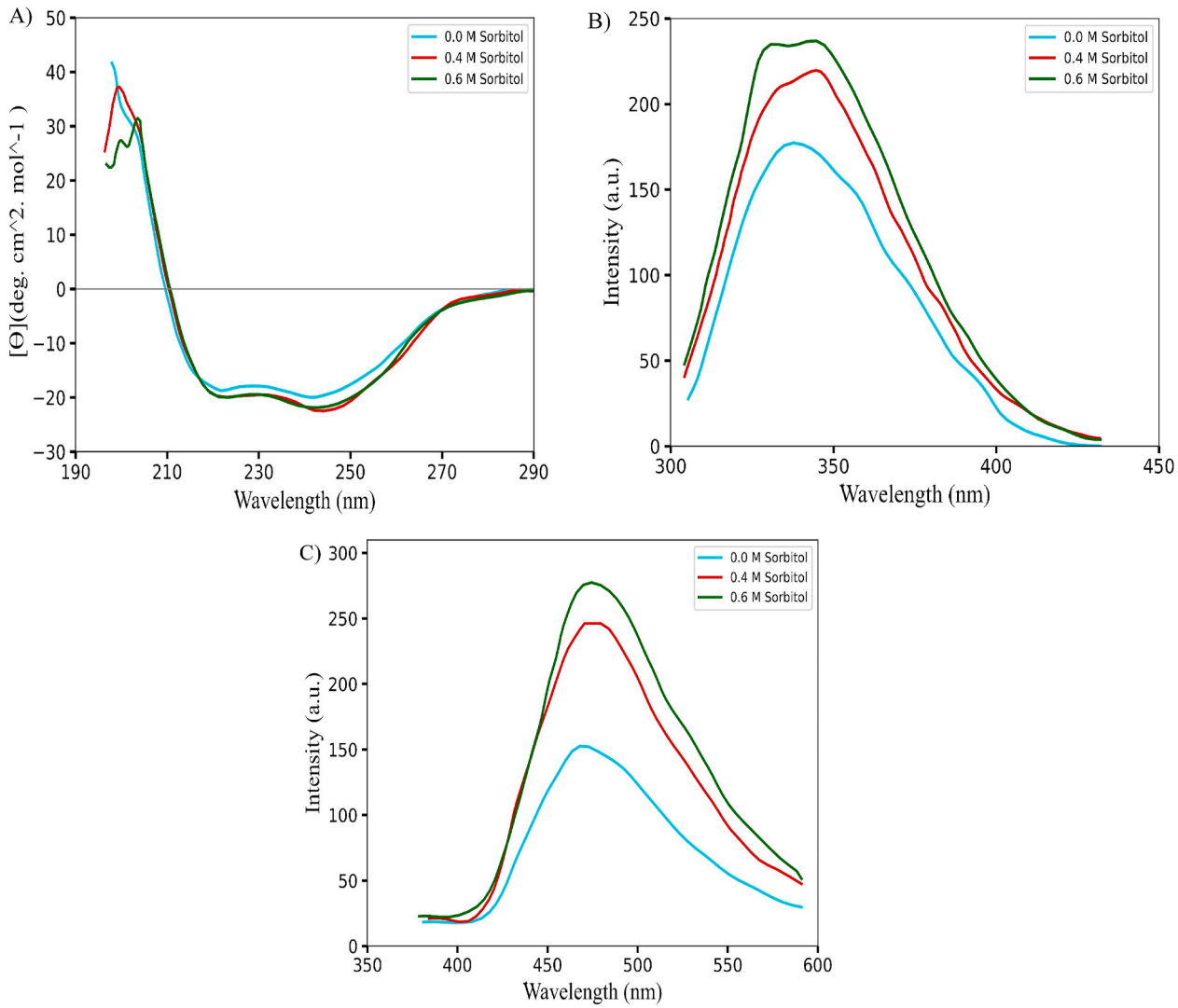

Fig. 4. A) The circular dichroism spectrum of the *Renilla* luciferase was measured at wavelengths of 190–250 nm in the presence of different concentrations of sorbitol. The blue color represents 0 M sorbitol, the red color represents 0.4 M sorbitol, and the green color represents 0.6 M sorbitol. No significant changes in the secondary structure of *Renilla* luciferase were observed in the presence of sorbitol. B) The intrinsic fluorescence spectrum of *Renilla* luciferase was measured at wavelengths of 300–400 nm in the presence of different concentrations of sorbitol. The blue color represents 0 M sorbitol, the red color represents 0.4 M sorbitol, and the green color represents 0.6 M sorbitol. The microenvironment surrounding chromophore groups in the structure of *Renilla* luciferase showed changes in the presence of sorbitol. C) The fluorescence spectrum of ANS (8-anilino-1-naphthalenesulfonic acid) of *Renilla* luciferase was measured at wavelengths of 500–700 nm in the presence of different concentrations of sorbitol. The blue color represents 0 M sorbitol, the red color represents 0.4 M sorbitol, and the green color represents 0.6 M sorbitol. The surface hydrophobicity of *Renilla* luciferase increased with increasing sorbitol concentration.

associated with alpha and beta substructures, with *Renilla* luciferase comprising 40 % alpha-helix and 17 % beta-sheet. It was also observed that there were no significant changes in the percentage of alpha-helix in the secondary structure in the presence of sorbitol. By conducting the tryptophan intrinsic fluorescence assay, we investigated conformational changes in *Renilla* luciferase and observed an increase in intrinsic fluorescence with rising sorbitol concentration. This increase indicates alterations in the microenvironment of certain chromophores. Therefore, findings are consistent with some previous studies [28,29]. Moreover, the analysis of the ANS fluorescence emission spectrum demonstrated that the hydrophobic pockets on the surface of *Renilla* luciferase increased with increasing sorbitol concentration. Based on structural studies, our understanding of the kinetic changes in *Renilla* luciferase observed in the presence of sorbitol was strengthened by evidence of structural alterations in the enzyme.

We employed MD to simulate the impact of sorbitol on *Renilla* luciferase. Prior to interpreting the results, the MD data was compared with fluorescence studies. MD simulations revealed changes in the RMSD value (data was not shown) in the presence of sorbitol at 0.4 and 0.8 M compared to 0 M sorbitol. Moreover, the enzyme's surface accessibility increased with increasing sorbitol concentration. These outcomes were consistent with alterations observed in intrinsic fluorescence spectra and ANS spectra. Consequently, we concluded that the results obtained from molecular dynamics are appropriate for modeling the effect of sorbitol on *Renilla* luciferase.

At the outset of this study, our initial proposal was that the addition of sorbitol to *Renilla* luciferase could potentially enhance enzyme stability through preferential hydration. However, molecular dynamics (MD) investigations demonstrated that sorbitol not only fails to enhance preferential hydration around *Renilla* luciferase, but also creates a covering effect around the enzyme, as reported by McClements et al. [16]. This covering effect reduces the number of hydrogen bonds around luciferase as the sorbitol concentration increases. The reduction of hydrogen bonds weakens the protein's hydrophobicity and leads to conformational changes that result in the growth of hydrophobic pockets on the surface of *Renilla* luciferase, which can be observed in MD

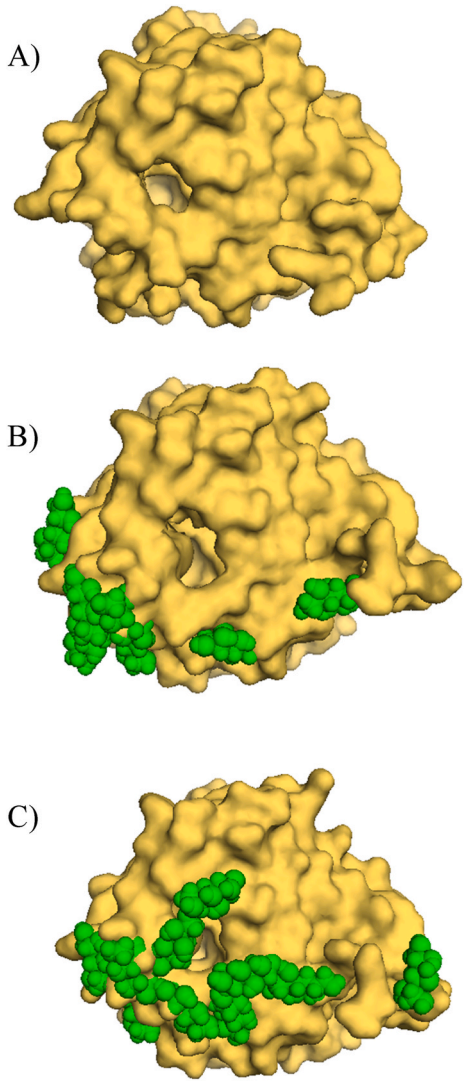

Fig. 5. The gateway of the *Renilla* luciferase was showed in the presence of different concentrations of sorbitol. A represents 0 M sorbitol, B represents 0.4 M sorbitol, and C represents 0.6 M sorbitol. The gateway was more open in the presence of 0.4 M sorbitol and greater access to the active site was observed. However, in the presence of 0.6 M sorbitol, the accumulation of sorbitol molecules at the gateway reduced access to the active site.

Table 2
The amino acids that are involved in hydrogen bonding in the N-terminus loop of the cap domain.

0.4 M	0.6 M
Serine 145	
Aspartic acid148	Aspartic acid 148
	Isoleucine 150
Glutamic acid 151	Glutamic acid 151
	Aspartic acid 152
Aspartic acid 154	Aspartic acid 154
Glutamic acid 155	Glutamic acid 155
	Proline 157
	Aspartic acid 158

and ANS spectroscopic studies. Ultimately, the thermal stability of the enzyme does not improve in the presence of sorbitol.

Based on the kinetic results, the presence of sorbitol induced structural changes that enhanced *Renilla* luciferase performance at a concentration of 0.4 M sorbitol, whereas higher concentrations had a

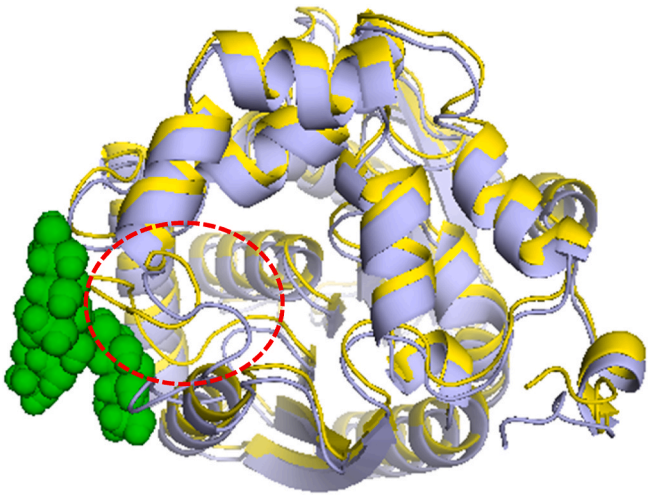

Fig. 6. Superimposed *Renilla* luciferase structures in the absence of glycerol (purple) and in the presence of 0.4 M glycerol (yellow) are shown. The N-terminus loop of the cap domain is marked with a red dotted line and the nearby molecules of sorbitol are shown in green. In the presence of glycerol, conformational changes occur in the loop.

detrimental effect on enzyme performance. The active site of the *Renilla* luciferase is structured as a bowl-like arrangement within the main domain. The cap domain, which comprises of helices and flexible loops, is linked to the beta 6 and alpha helix D of the main domain via two flexible loops located at its N-terminus and C-terminus respectively. In the three-dimensional structure of the enzyme, the N-terminus loop of the cap domain is located near the gateway. At a concentration of 0.4 sorbitol, the binding of sorbitol molecules to the N-terminus loop through hydrogen bonds induces a conformational change. This leads to an increase in the size of the gateway. Consequently, it can be argued that the concentration of 0.4 M sorbitol has increased substrate access to the active site, which has resulted in a decrease in the Km of the enzyme. Moreover, due to conformational changes in the active site, the Vmax of the enzyme has increased.

The molecular dynamics (MD) studies revealed that, as the concentration increased from 0.4 to 0.6 M, the number of sorbitol molecules around the gateway of the main channel increased, leading to the accumulation of sorbitol molecules in the gateway and reducing substrate access to the active site. Therefore, the observed decrease in catalytic efficiency may be attributed to an issue in substrate trafficking.

Previous research demonstrated that the small molecule glycerol hinders the access of coelenterazine to the active site of the enzyme upon entering [20]. This obstruction results in a 10-fold increase in the Km of *Renilla* luciferase. In our current study, we examined the effects of sorbitol, a molecule twice the size of glycerol, on the enzyme's active site. While sorbitol cannot penetrate the active site itself, the accumulation of sorbitol molecules at the entrance has a comparable impact to glycerol, obstructing the active site and increasing the enzyme's Km. However, the increase in Km observed in the presence of sorbitol is only half as substantial compared to glycerol. This study proposes a mechanism for understanding the influence of sorbitol on the active site's entrance channel. It sheds light on kinetic changes in enzymes with buried active sites, such as those with α/β hydrolase fold, and provides insight into why higher concentrations of osmolytes may hinder the kinetic properties of enzymes despite the benefits of lower concentrations.

Biopharmaceuticals frequently use osmolytes to enhance protein stability. These osmolytes have proven to be effective in numerous biological formulas, which have been approved by the US Food and Drug Administration (FDA) [32]. For example, sorbitol has been used as an additive in commercial drugs such as Skyrizi and Crysvita [33]. Li et al. demonstrated that osmolytes affect the molecular hydrogen bonding

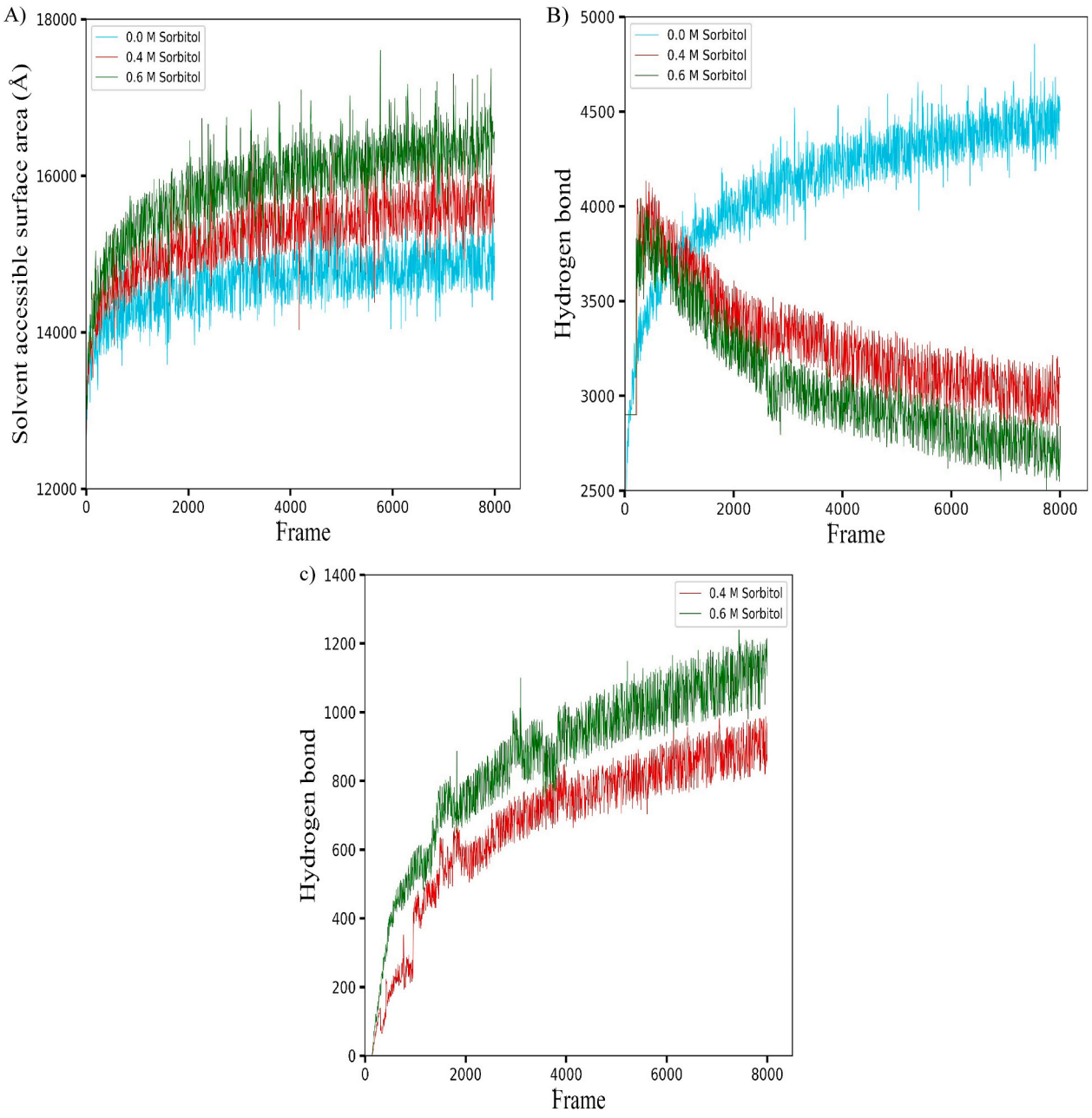

Fig. 7. A) The surface accessibility of *Renilla* luciferase was measured in the presence of different concentrations of sorbitol. The blue color represents 0 M sorbitol, the red color represents 0.4 M sorbitol, and the green color represents 0.6 M sorbitol. The surface accessibility of *Renilla* luciferase increased with increasing sorbitol concentration. B) The graph shows the number of hydrogen bonds in water molecules surrounding the *Renilla* luciferase in the presence of different concentrations of sorbitol. The blue color represents 0 M sorbitol, the red color represents 0.4 M sorbitol, and the green color represents 0.6 M sorbitol. The number of hydrogen bonds in water molecules surrounding the *Renilla* luciferase decreased with increasing sorbitol concentration. C) The graph shows the number of hydrogen bonds between glycerol molecules and *Renilla* luciferase in the presence of different concentrations of sorbitol. The red color represents 0.4 M sorbitol, and the green color represents 0.6 M sorbitol. The number of hydrogen between glycerol molecules and *Renilla* luciferase increased with increasing sorbitol concentration.

pattern through the mechanism of "hydrogen bond competition" and can have an impact on the conformation [34]. Our study aligns with Li et al. and shows that changes in hydrogen bonding can improve enzyme performance at specific concentrations, such as the effect of 0.4 M sorbitol on *Renilla* luciferase. Furthermore, the effects of osmolytes on protein function may not be limited to conformational changes alone. Higher concentrations, such as 0.6 M sorbitol on *Renilla* luciferase, may interfere with enzyme activity by blocking active sites, binding sites, and allosteric positions. Therefore, this study may provide a perspective for formulating protein-based drugs in the presence of sorbitol as an additive.

5. Conclusion

Our objective in measuring *Renilla* luciferase activity in the presence of sorbitol was to enhance its stability. However, we discovered that sorbitol had no impact on the thermal stability of the enzyme. Instead, the unpredictable behavior of sorbitol at a concentration of 0.4 M drew our attention to the kinetic changes induced by its presence. Our study demonstrates that sorbitol at a concentration of 0.4 M enhances substrate access to the enzyme's active site by opening the gateway. Nonetheless, the accumulation of sorbitol at the enzyme's gateway significantly increases the enzyme's K_m at this concentration, resulting

in a reduction in the catalytic efficiency of the luciferase.

CRediT authorship contribution statement

Golnoosh Khoshnevisan: Data curation, Formal analysis, Investigation, Software, Writing - original draft. **Rahman Emamzadeh:** Conceptualization, Supervision, Writing - review & editing. **Mahboobeh Nazari:** Supervision, Writing - review & editing. **Mina Oliayi:** Validation, Writing - original draft. **Reyhaneh Sariri:** Supervision.

Declaration of competing interest

The authors declare no conflicts of interest related to manuscript entitled "**Uncovering the Role of Sorbitol in *Renilla* Luciferase Kinetics: Insights from Spectroscopic and Molecular Dynamics Studies**". The research was conducted in an unbiased manner, and the authors were not influenced by any financial or personal relationships that could have affected the interpretation of the results. All relevant sources of funding for this study have been disclosed, and the authors have no financial relationships with any organizations that could be perceived as having biased the research reported in this manuscript.

Acknowledgements

We would like to express our gratitude to the Iran National Science Foundation (INSF) for their support of our project.

References

[1] W.W. Lorenz, R.O. McCann, M. Longiaru, M.J. Cormier, Isolation and expression of a CDNA encoding *Renilla* reniformis luciferase, Proc. Natl. Acad. Sci. USA 88 (10) (1991) 4438–4442.
[2] J.C. Matthews, K. Hori, M.J. Cormier, Substrate and substrate analogue binding properties of *Renilla* luciferase, Biochemistry 16 (24) (1977) 5217–5220.
[3] Y. Ohmiya, T. Hirano, Shining the light: the mechanism of the bioluminescence reaction of calcium-binding photoproteins, Chem. Biol. 3 (5) (1996) 337–347.
[4] A.M. Loening, T.D. Fenn, S.S. Gambhir, Crystal structures of the luciferase and green fluorescent protein from *Renilla* reniformis, J. Mol. Biol. 374 (4) (2007) 1017.
[5] J. Woo, M.H. Howell, A.G. von Arnim, Structure-function studies on the active site of the coelenterazine-dependent luciferase from Renilla, Protein Sci. 17 (4) (2008) 725–735.
[6] B.R. Branchini, T.L. Southworth, D.M. Fontaine, D. Kohrt, C.M. Florentine, M. J. Grossel, A firefly luciferase dual color bioluminescence reporter assay using two substrates to simultaneously monitor two gene expression events, Sci. Rep. 8 (1) (2018) 5990.
[7] M. Kenda, J. Vegelj, B. Herlah, A. Perdih, P. Mladėnka, M. Sollner Dolenc, Evaluation of firefly and *Renilla* luciferase inhibition in reporter-gene assays: a case of isoflavonoids, Int. J. Mol. Sci. 22 (13) (2021) 6927.
[8] Z.G. Varnosfaderani, R. Emamzadeh, M. Nazari, M. Zarean, Detection of a prostate cancer cell line using a bioluminescent affiprobe: an attempt to develop a new molecular probe for ex vivo studies, Int. J. Biol. Macromol. 138 (2019) 755–763.
[9] J. Woo, A.G. von Arnim, Mutational optimization of the coelenterazine-dependent luciferase from Renilla, Plant Methods 4 (1) (2008) 23.
[10] A.M. Loening, T.D. Fenn, A.M. Wu, S.S. Gambhir, Consensus guided mutagenesis of *Renilla* luciferase yields enhanced stability and light output, Protein Eng. Des. Sel. 19 (9) (2006) 391–400.
[11] S. Rahnama, B. Saffar, Z.F. Kahrani, M. Nazari, R. Emamzadeh, Super RLuc8: a novel engineered *Renilla* luciferase with a red-shifted spectrum and stable light emission, Enzym. Microb. Technol. 96 (2017) 60–66.
[12] Z.F. Kahrani, R. Emamzadeh, M. Nazari, S.M.M. Rasa, Molecular basis of thermostability enhancement of *Renilla* luciferase at higher temperatures by insertion of a disulfide bridge into the structure, Biochim. Biophys. Acta Protein Proteonomics 1865 (1) (2017) 69–78.
[13] A. Schenkmayerova, G.P. Pinto, M. Toul, M. Marek, L. Hernychova, J. Planas-Iglesias, V.D. Liskova, D. Pluskal, M. Vasina, S. Emond, M. Dörr, R. Chaloupkova, D. Bednar, Z. Prokop, F. Hollfelder, U.T. Bornscheuer, J. Damborsky, Engineering the protein dynamics of an ancestral luciferase, Nat. Commun. 12 (2021) 3616.
[14] A.H.W. Yeh, C. Norn, Y. Kipnis, D. Tischer, S.J. Pellock, D. Evans, M. Pengchen, G. R. Lee, J.Z. Zhang, I. Anishchenko, B. Coventry, L. Cao, J. Dauparas, S. Halabiya, M. DeWitt, L. Carter, K.N. Houk, D. Baker, De novo design of luciferases using deep learning, Nature 614 (7949) (2023) 774–780.
[15] G.S. Sharma, S. Krishna, S. Khan, T.A. Dar, K.A. Khan, L.R. Singh, Protecting thermodynamic stability of protein: the basic paradigm against stress and unfolded protein response by osmolytes, Int. J. Biol. Macromol. 177 (2021) 229–240.
[16] S.R. Wlodarczyk, D. Custódio, A. Pessoa, G. Monteiro, Influence and effect of osmolytes in biopharmaceutical formulations, Eur. J. Pharm. Biopharm. 131 (2018) 92–98.
[17] S.N. Timasheff, Control of protein stability and reactions by weakly interacting cosolvents: the simplicity of the complicated, Adv. Protein Chem. 51 (1998) 355–432.
[18] F. Mehrnejad, M.M. Ghahremanpour, M. Khadem-Maaref, F. Doustdar, Effects of osmolytes on the helical conformation of model peptide: molecular dynamics simulation, J. Chem. Phys. 134 (3) (2011), 01B620.
[19] D.J. McClements, Modulation of globular protein functionality by weakly interacting cosolvents, Crit. Rev. Food Sci. Nutr. 42 (5) (2002) 417–471.
[20] G. Khoshnevisan, R. Emamzadeh, M. Nazari, S.M.M. Rasa, R. Sariri, L. Hassani, Kinetics, structure, and dynamics of *Renilla* luciferase solvated in binary mixtures of glycerol and water and the mechanism by which glycerol obstructs the enzyme emitter site, Int. J. Biol. Macromol. 117 (2018) 617–624.
[21] F. Salehi, R. Emamzadeh, M. Nazari, S.M.M. Rasa, Probing the emitter site of *Renilla* luciferase using small organic molecules; an attempt to understand the molecular architecture of the emitter site, Int. J. Biol. Macromol. 93 (Pt A) (2016) 1253–1260.
[22] F. Hashemi-Shahraki, B. Shareghi, S. Farhadian, Characterizing the binding affinity and molecular interplay between quinoline yellow and pepsin, J. Mol. Liq. 341 (11) (2021), 117317.
[23] E. Raeessi-Babaheydari, S. Farhadian, B. Shareghi, Comparative studies on the interaction of ascorbic acid with gastric enzyme using multispectroscopic and docking methods, J. Mol. Struct. 1245 (2021), 131270.
[24] GROMACS groningen machine for chemical simulations USER MANUAL, n.d. https://ftp.gromacs.org/pub/manual/manual-5.0.4.pdf.
[25] V. Zoete, M.A. Cuendet, A. Grosdidier, O. Michielin, SwissParam: a fast force field generation tool for small organic molecules, J. Comput. Chem. 32 (11) (2011) 2359–2368.
[26] L. Martínez, R. Andrade, E.G. Birgin, J.M. Martínez, Packmol: a package for building initial configurations for molecular dynamics simulations, J. Comput. Chem. 30 (13) (2009) 2157–2164.
[27] W. Humphrey, A. Dalke, K. Schulten, VMD: Visual molecular dynamics, J. Mol. Graph. 14 (1) (1996) 33–38.
[28] E. Sarvandi-Dehghanpoor, A. Riahi-Madvar, S. Lotfi, M. Torkzadeh-Mahani, Improvement of kinetic properties and thermostability of recombinant Lepidium draba peroxidase (LDP) upon exposed to osmolytes, Int. J. Biol. Macromol. 119 (2018) 1036–1041.
[29] L. Hassani, B. Ranjbar, K. Khajeh, H. Naderi-Manesh, M. Naderi-Manesh, M. Sadeghi, Horseradish peroxidase thermostabilization: the combinatorial effects of the surface modification and the polyols, Enzym. Microb. Technol. 38 (1) (2006) 118–125.
[30] A. Azizi, B. Ranjbar, K. Khajeh, T. Ghodselahi, S. Hoornam, H. Mobasheri, M. R. Ganjalikhany, Effects of trehalose and sorbitol on the activity and structure of Pseudomonas cepacia lipase: spectroscopic insight, Int. J. Biol. Macromol. 49 (4) (2011) 652–656.
[31] M. Mohammadi, B. Shareghi, S. Farhadian, A.A. Saboury, Investigating the effect of osmolytes on the structure and thermodynamics of human carbonic anhydrase II: a molecular dynamics simulation study, J. Biomol. Struct. Dyn. 35 (2) (2017) 374–384.
[32] S.R. Wlodarczyk, D. Custodio, A. Pessoa, G. Monteiro, Influence and effect of osmolytes in biopharmaceutical formulations, Eur. J. Pharmaceut. Sci. 131 (2018) 92–98.
[33] Y. Zhang, R.O. Williams III, H.O. Tucker, Formulation strategies in immunotherapeutic pharmaceutical products, World J. Clin. Oncol. 11 (5) (2020) 275–282.
[34] J. Li, J. Chen, L. An, X. Yuan, L. Yao, Polyol and sugar osmolytes can shorten protein hydrogen bonds to modulate function, Commun. Biol. 3 (1) (2020) 528.