Journal of Molecular Catalysis B: Enzymatic 62 (2010) 127–132

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Roles of trehalose and magnesium sulfate on structural and functional stability of firefly luciferase

Mohamad Reza Ganjalikhany[a], Bijan Ranjbar[a,*], Saman Hosseinkhani[b], Khosrow Khalifeh[a], Leila Hassani[a]

[a] Department of Biophysics, Faculty of Biological Science, Tarbiat Modares University, Ale-Ahmad Blv, P.O. Box 14115-175, Tehran, Iran
[b] Department of Biochemistry, Faculty of Biological Science, Tarbiat Modares University, P.O. Box 14115-175, Tehran, Iran

ARTICLE INFO

Article history:
Received 12 January 2009
Received in revised form 2 September 2009
Accepted 24 September 2009
Available online 1 October 2009

Keywords:
Firefly luciferase
Stabilization
Circular dichroism
Stopped-flow fluorescence
Additives
Bioluminescence

ABSTRACT

Firefly luciferase is widely used in many analytical techniques. However, the enzyme is unstable, so that its relative inactivation results in low sensitivity of those techniques. In this study, we have investigated the effects of $MgSO_4$ and trehalose on the structural stability and function of luciferase from *Photinus pyralis* using circular dichroism (CD), conventional and stopped-flow fluorescence spectroscopy and bioluminescence assay. The secondary structural content, compactness and its melting temperature are also studied, which showed that the stability of luciferase increased in the presence of additives. Measurements of refolding rate constants under conditions that favor folding, show that $MgSO_4$ accelerates the folding of enzyme, on the contrary, refolding rate constant decreases in the presence of trehalose which can be attributed to its high viscosity. Finally, combined with remaining activity assay we concluded that magnesium sulfate and trehalose can be used for short- and long-term stabilization, respectively.

1. Introduction

Firefly luciferase from *Photonis pyralis* is a single polypeptide chain of 550 amino acid residues and molecular weight of 62 kDa (E.C.1.13.12.7). This enzyme catalyses monooxygenase reaction in which D-luciferin is converted to oxyluciferin, using ATP, O_2 and also Mg^{2+} as cofactor. This is a two-step process that is accompanied by emission of yellow-green photon of light at 560 nm. First step is followed by the formation of enzyme luciferyl adenylate intermediate. In the second step luciferyl adenylate reacts with molecular oxygen, producing enzyme-bond excited state product. This excited state product decays to ground state by emitting light [1–3]. The crystal structures of firefly luciferases from *P. pyralis* and Japanese firefly (*Luciola cruciata*) have been obtained [1,3]. Luciferase from *P. pyralis* folds into two globular domains, a large N-terminal domain consisting of residues 4–435 and C-terminal domain consisting of residues 44–544. N-terminal can be further divided into three sub-domains, A–C. N-terminal domain comprising a β-barrel and two β-sheets, which arranged between three α-helices, forming αβαβα structure, C-terminal domain consists of three α-helices and five β-strands [1,2]. There is a linker between two domains, connecting residues 435 and 441 [2]. This enzyme is well known as a fundamental agent in several research areas. Some of its applications are: ATP-assay with high sensitivity in bacterial contamination, using as a reporter gene [4], pyrosequencing [5] and bioluminescence imaging [6].

Firefly luciferase is relatively unstable and its activity decreases at room temperature significantly. Also irreversible aggregation due to the exposure of its hydrophobic sites followed by structural changes, causes its further inactivation [7–9]. Obviously, this phenomenon can affect the sensitivity of analytical assays performing by luciferase. So, the structural stabilization of enzyme is essential for retaining its application.

It seems that site directed mutagenesis strategy is a good means for this purpose, however, there are reports that some mutant proteins with higher structural stability show lower activity relative to that of native protein and *vice versa* [10–13]. Therefore, structural stabilization of enzyme should be done to improve function at extreme environment. In other words, it was shown that sucrose; sorbitol and proline as additives can stabilize the structure and function of firefly luciferase against thermal stress [14]. In addition, the use of additives for stabilization of different kinds of enzymes has been recommended [15–17].

Surface free energy increase is the main proposed mechanism of the mentioned stabilizers. So, they can increase the surface free energy of protein in solvent. As the protein–solvent interface increases upon denaturation, the surface free energy would increase due to the surface increment which is unfavorable.

* Corresponding author. Tel.: +98 2188009730; fax: +98 2188009730.
E-mail address: ranjbarb@modares.ac.ir (B. Ranjbar).

1381-1177/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2009.09.015

Therefore the equilibrium of "Native ↔ Unfold" move toward the favorable energy, to the native form [18].

Trehalose is a non-reducing disaccharide, composed of two D-glucopyranose and is present in some organisms. It has interesting physico-chemical properties which draw researchers' attention [19]. It can be found in insects' hemolymph [20], some desert plants [21], yeast and fungi [20,22]. It has been shown that trehalose can increase the stability of folded state of proteins against conditions that favor denaturation [23–26]. Furthermore, it has cryoprotective property in some freeze-tolerant organisms [23,27]. It also can protect the structure of membrane structure in dry state [27].

$MgSO_4$ acts as a stabilizer in which its stabilization property determined by a competition phenomenon between two factors, "salt exclusion and salt binding effect". Increasing the surface tension of solvent results in salt exclusion of SO_4^{2-} (preferential hydration) whereas salt binding effect refers to Mg^{2+} affinity for ionic residues and peptide bonds. The extent of change in preferential hydration during denaturatrion of protein is determined by a delicate balance between these factors. In other words, as protein–solvent interaction increases during denaturation, these factors increase concomitantly, but dominant factor is the high exclusion of SO_4^{2-} ions, which yield in protein stabilization [18,28].

In present study, we have examined the effects of two different kinds of additives ($MgSO_4$ as a salt and trehalose as a sugar) on the structural stability and function of firefly luciferase using circular dichroism (CD), conventional and stopped-flow fluorescence spectroscopy and bioluminescence assay.

2. Materials and methods

2.1. Reagent preparation

2.1.1. Buffers and chemicals preparation

Affinity column of Ni-NTA resin for His_6-tagged proteins was purchased from Qiagen. Tris, KH_2PO_4 and K_2HPO_4 were purchased from CarloErba, glycerol, $MgSO_4$, trehalose, β-mercapto ethanol, ammonium sulfate, imidazole were purchased from Merck. Dialysis membrane, di-thio erytheritol (DTE) and guanidine hydrochloride (GdmCl) were purchased from Sigma. Phenyl methyl sulfonyl fluoride (PMSF) from Boehrienger and NaCl from Panreac. Buffers for affinity column were prepared according to the Qiagen manual: Washing buffer: NaH_2PO_4 50 mM, NaCl 300 mM, imidazole 20 mM, pH 8.0. Elution buffer: NaH_2PO_4 50 mM, NaCl 300 mM, imidazole 250 mM, pH 8.0. Lysis buffer: NaH_2PO_4 50 mM, NaCl 300 mM, imidazole 10 mM, pH 8.0. Dialysis buffer comprising: Tris–HCl buffer, pH 7.8, NaCl 150 mM, DTE 1 mM, β-mercapto ethanol 1 mM, ammonium sulfate 1 mM and glycerol 5% (w/v). Substrate solution: tricine–NaOH buffer 50 mM, ATP solution 4 mM, luciferine solution 2 mM, $MgSO_4$ solution 10 mM, pH 7.8 [7,14].

2.1.2. Additive preparation

Two additives dissolved in dialysis buffer at pH 7.8, as additive stocks: $MgSO_4$ at 2.4 M and trehalose at 2.0 M.

2.1.3. Enzyme preparation

Firefly luciferase from *P. pyralis* prepared in our laboratory. Its gene was cloned into pET-16b vector with a His_6-tagged and transfected to host bacterium *Escherichia coli* BL21. After over-expression, firefly luciferase was purified by affinity chromatography "Ni-NTA" resin [29]. All the steps in preparing and purification, handled in ice carefully. After addition of 20–30% glycerol to the protein solutions, a sample of 50–70 μl from each fraction, stored for further analysis, such as Bradford assay for protein concentration assay [30] and SDS-PAGE for its purity verification. Analysis showed that they had purity of more than 95% (data not shown). The enzymes were stored at −20 or −80 °C freezer.

2.1.4. Enzyme dialysis

Before all the experiments, luciferase should be dialyzed due to high concentration of glycerol and other salts present in elution buffer. One of the most unfavorable salts in the solution is imidazole, interrupting all the far-UV circular dichroism data, by producing noises through the spectra. Dialysis has done two times for 8–10 h at 4 °C in 1 l dialysis buffer and constant stirring 120–200 rpm.

2.2. Methods

2.2.1. Measurements of bioluminescent kinetics

2.2.1.1. Remaining activity measurements. Bioluminescence activities are measured by Sirius tube luminometer, connected to a PC, in which data were analyzed on its own software (Berthold Detecton System, Germany). Bioluminescence was measured by mixing of 100 μl enzyme solution (6 μg/ml) with 50 μl of substrate solution, reported in RUL/S (relative light unit per second). Remaining activities of luciferase were measured at different time intervals in time course of 60 min in the absence and presence of additives at different concentrations (0–1.2 M) at 35 °C. Luciferase was incubated with different concentrations of additives for 5 min, and then tubes were placed in water-bath circulation system in order to control the temperature easily. At regular intervals samples were removed and placed at 25 °C for 2 min then the remaining activity were measured. Remaining activities were calculated using percentages of Initial activity of enzyme at 25 °C as control (100% activity), as reported earlier [7,14,31].

2.2.1.2. Activation energy measurements. Activities of enzyme in the presence of additives at constant concentration of enzyme (8 μl/ml) and additives (0.7 M), pH 7.8, were measured. Temperature was varied from 20 to 45 °C. Luminometer cuvettes were pre-loaded with substrate solution, placed into water-bath for 5 min at the same degree for luciferase incubation, which means each assay measured at the same temperature for incubated luciferase. In order to obtain activation energy, natural logarithm of luciferase activity in RUL/S against 1/T, plotted in a graph, called Arrhenius plot [32,33]. Also, thermal sensitivity of the luciferase was determined by incubating luciferase at 20–45 °C and its activity was measured.

2.2.1.3. Circular dichroism measurements. Spectropolarimetry study of firefly luciferase has done at both far-UV and near-UV CD regions, for secondary and tertiary structural studies, respectively, by JASCO Spectropolarimeter J-715. Data analysis and smoothing performed on its own software J-715 using fast Fourier-transform enabling users to reduce and smooth the noisy spectra, preventing data distortion. The results were reported in molar ellipticity, $[\theta] = (\theta \times 100MRW/cl)$, where c is the sample concentration, l is the length of the cuvette cell, MRW "mean amino acid residue weight" calculated for firefly luciferase (113) and θ is the ellipticity measured by spectropolarimeter in degree at wavelength of λ. $[\theta]$ is reported in ($^\circ$ cm^2 dmol^{-1}) [34]. Thermal plots of luciferase in the presence and absence of additives obtained at 222 nm, temperature for thermal scanning, varied between 20 and 85 °C at rate of 2 °C/min. Far-UV and near-UV CD spectropolarimetry were performed at different concentrations of additives 0–1.2 M. Luciferase concentration was adjusted to 0.2 mg/ml for far-UV CD in 1 mm cuvette and 1.5 mg/ml for near-UV CD in 1 mm cuvette (0.2 mg/ml in 10 mm cuvette) at room temperature 25 °C (pH 7.8).

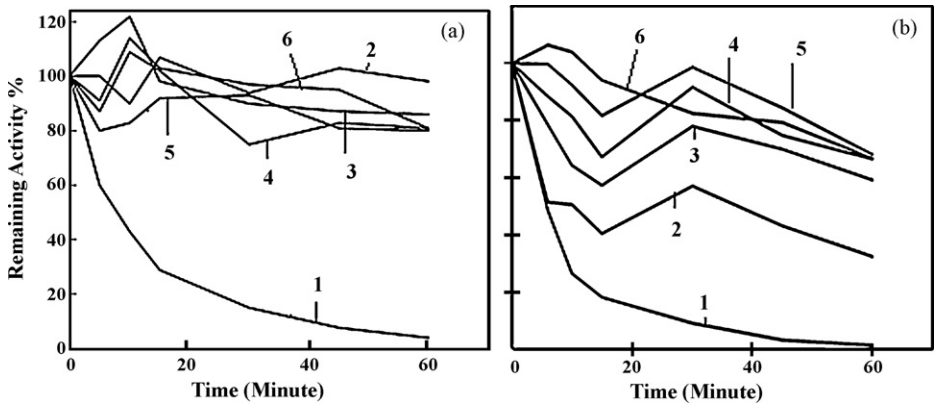

Fig. 1. Remaining activities of firefly luciferase versus time in the presence of $MgSO_4$ at different concentrations at 35 °C (a) and in the presence of trehalose at different concentrations at 35 °C (b). Labels: (1) 0 M, (2) 0.4 M, (3) 0.6 M, (4) 0.8 M, (5) 1.0 M and (6) 1.2 M.

2.2.1.4. Intrinsic fluorescence measurements. The fluorescence emission spectra of the enzyme were performed on a VARIANT fluorimeter. The spectra were measured in 50 mM Tris–HCl buffer pH 7.8, containing different concentrations of additives. The final concentration of luciferase was 0.05 mg/ml. The fluorescence emission was scanned between 300 and 400 nm with an excitation wavelength of 280 nm. The slits for excitation and emission were set to 2.5 and 10 nm, respectively.

2.2.1.5. Stopped-flow kinetic measurements. Stopped-flow fluorescence measurements were carried out with a BioLogic μ-SFM-20 using a 0.8 cm cuvette (FC-08) and the data were collected and analyzed with the Biokin analysis software. Enzyme was incubated at 50 mM Tris–HCl, pH 7.8, containing 3 M GdmCl, for 1 h. Refolding was initiated by rapidly diluting of 1 volume of unfolded protein, 2.5 mg/ml, in 20 mM Tris–HCl, pH 7.8, into 30 volume of 20 mM Tris–HCl, pH 7.8, containing 0.7 M of additives, resulted in final concentration of GdmCl of 0.1 M and that for protein 0.08 mg/ml. Refolding was followed by monitoring the changes in the intrinsic fluorescence of luciferase (excitation at 290 nm wavelength; emission wavelength was 320 nm). All experiments were performed at 25 °C.

2.2.1.6. Storage stability measurements. Storage stabilities of luciferase were measured at 4 °C at different concentrations of each additive after 3 months. Enzyme concentration was adjusted to 0.1 mg/ml and additives concentrations were adjusted 0–1.2 M.

3. Results

3.1. Remaining activity measurements

Remaining activities of luciferase in the presence and absence of additives are depicted in Fig. 1a and b. It can be seen that remaining activity is increased upon addition of additives to protein solution. Fig. 1a shows that remaining activity varies between 50% and 90% of initial activity depending on $MgSO_4$ concentration. However it was found that $MgSO_4$ is more effective even at concentration of 50 mM (data not shown). Fig. 1b indicates that trehalose increases remaining activity gradually as its concentration raised to 1.2 M.

3.2. Activation energy calculation

Activation energy of luciferase in the presence and absence of additives calculated from Arrhenius plot indicates that it is increased in the presence of trehalose compared to intact protein, whereas $MgSO_4$ decreased the activation energy of luciferase (Table 1). Optimum temperature of luciferase was obtained, indicating that its activity reached maximum at 25 °C and fully inactivated at 45 °C (Fig. 2).

Table 1
Activation energy of luciferase in the absence and the presence of additives.

Additives	Activation energy, E_a (kCal/mol)
No additive	1.958 ± 0.178
Trehalose	2.722 ± 0.188
$MgSO_4$	0.731 ± 0.0197

3.3. Circular dichroism measurements

Far-UV CD spectra of luciferase in the presence and absence of additives (Fig. 3a and b) show that molar ellipticity becomes relatively positive at certain concentrations. According to near-UV CD spectra (Fig. 3c and d) it was also found that molar ellipticity of enzyme is changed at certain concentrations of additives, indicating that rigidity of protein structure in regions containing aromatic residues is altered.

3.4. Thermal unfolding measurements

Thermal CD plots of luciferase at 222 nm in the presence and absence of additives are obtained (data not shown). Analysis of CD thermal plots (Table 2) indicates that luciferase has two melting phases in the absence of additives at 43.6 and 66.5 °C, respectively. Melting temperatures for both phases shifted to higher values upon addition of $MgSO_4$ and trehalose, although the effect of $MgSO_4$ is more considerable at the first thermal transition and that of trehalose is for the second one. Reversible luciferase denaturation

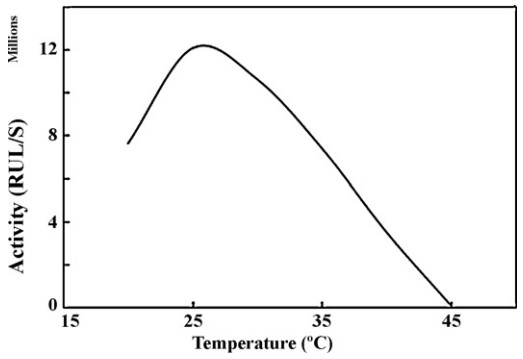

Fig. 2. Optimum temperature of luciferase activity in the absence of additives.

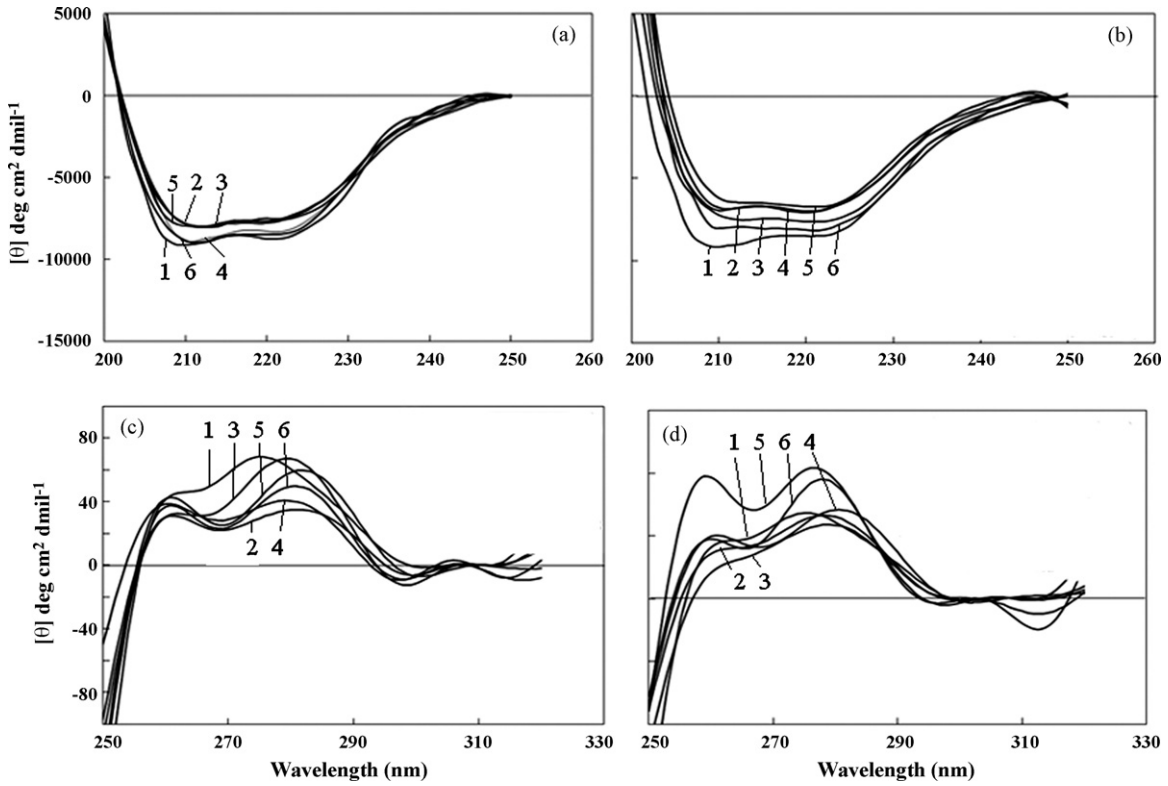

Fig. 3. Circular dichroism spectra of luciferase. Far-UV CD spectra of enzyme in the presence of $MgSO_4$ (a) and trehalose (b) at different concentrations. Near-UV CD spectra of luciferase in the presence of $MgSO_4$ (c) and trehalose (d) at different concentrations. Labels: (1) 0 M, (2) 0.4 M, (3) 0.6 M, (4) 0.8 M, (5) 1.0 M and (6) 1.2 M.

Table 2
ΔG° and melting temperature of luciferase in the presence of additives at constant concentration (0.7 M).

Additive	First T_m (°C)	First ΔG° at 25 °C (kCal/mol)	Second T_m (°C)	Second ΔG° at 25 °C (kCal/mol)
No additive	43.6 ± 0.5	5.05 ± 0.133	66.5 ± 0.5	15.5 ± 0.189
Trehalose	47.5 ± 0.5	7.652 ± 0.154	74.8 ± 0.5	32.544 ± 0.327
$MgSO_4$	49.0 ± 0.5	14.233 ± 0.168	73.2 ± 0.5	

showed that melting phases are reversible except for second phase in the presence of $MgSO_4$, so that aggregation was visible in this phase. It also found that the first $\Delta G^{\circ N\leftrightarrow U}$ at room temperature, increases in the same order of T_m.

3.5. Stopped-flow fluorescence measurements

Fig. 4 shows kinetic traces of refolding of luciferase. These spectra were fitted to an equation describing a single exponential differential equation. Analysis of kinetic traces shows that rate constant of refolding of luciferase increased upon addition of $MgSO_4$, but it was decreased in the presence of trehalose relative to that of intact protein.

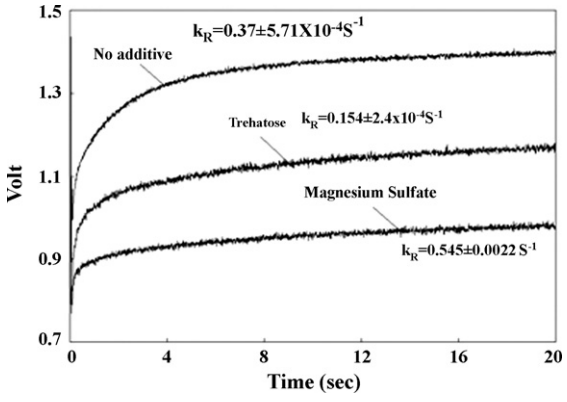

Fig. 4. Stopped-flow fluorescence spectra of luciferase refolding in the presence and absence of additives (0.7 M) at 3 M of GdmCl, at pH 7.8, in 20 s.

3.6. Storage stability measurements

Long-term stability of luciferase in the presence of additives showed that trehalose has positive effect on storage stability of enzyme at 4 °C, so that its activity remained intact even after 3 month, whereas, in contrast to its short-term effect, $MgSO_4$ could only save 3% of initial activity (Fig. 5).

3.7. Intrinsic fluorescence study

Intrinsic fluorescence spectra of luciferase have shown that trehalose increases fluorescence intensities proportional to its concentration; although red shift was observed (Fig. 6). $MgSO_4$ has no prominent effect on fluorescence spectra so that it is negligible compare to trehalose (data not shown).

4. Discussion

Inherent instability of Firefly luciferase has been considered as a compromising effect in luciferase-based assay. This inactivation and instability has deleterious effects on practical application of

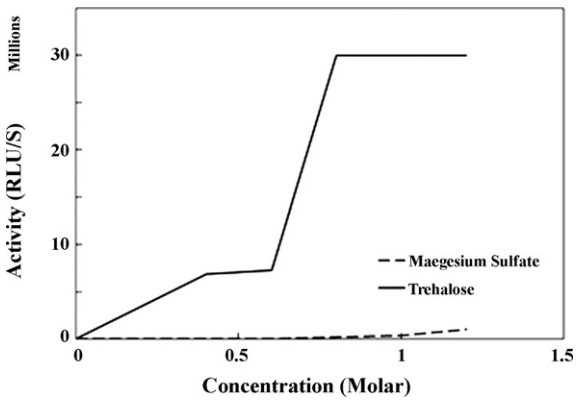

Fig. 5. Storage stability of luciferase in presence of trehalose and $MgSO_4$ after 3 months.

firefly luciferase [4,15]. In this work, the extent of stability and activity of luciferase upon addition of additives is examined.

According to CD data, it was shown that both melting temperatures are increased upon addition of additives. However, $MgSO_4$ can mainly affect the first T_m, whereas the second T_m is affected by trehalose. The presence of two melting phase shows that there are two main structural transitions upon heating of luciferase (Table 2). Furthermore, the results of enzyme assay measurements shows that enzyme become fully inactivated at first transition temperature (Fig. 2), indicating that the first T_m is somehow related to structural changes in active site. On the other hand, exact structural and functional examination of luciferase showed that one of the most probable positions for its active site is around the main cleft where the C-terminal and N-terminal domains facing each other. This region contains a linker which joins large N-terminal to C-terminal domain [2]. The second transition can be attributed to denaturation of other parts of protein rather than active site which are large and small domains.

As noted above, both T_m and functional stability of luciferase are increased upon addition of $MgSO_4$ to protein solution. It seems that the effect of SO_4^{2-}, which facilitate the hydration of enzyme due to preferential exclusion from its surface could take into account for its effect [18,28]. In contrast to $MgSO_4$, trehalose cannot dissociate into ion species, but it can increase the solvent viscosity, which in turn enhances the preferential hydration of enzyme and it can affect the structural dynamics and molecular collision of protein molecules [19].

It seems that at lower temperatures, preferential hydration of SO_4^{2-} as an ionic species plays an important role in the stability of folded state of protein, so that interaction of $MgSO_4$ with protein

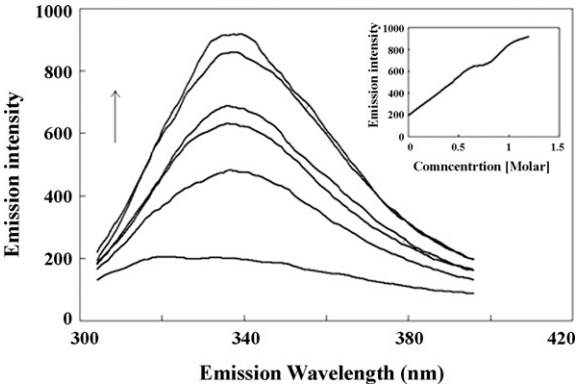

Fig. 6. Intrinsic fluorescence spectra of luciferase in presence of trehalose at different concentrations. Inset: emission intensity versus trehalose concentrations at λ_{max}.

is a dominant factor in determining the value of the first T_m. In microscopic point of view, a part of protein which is exposed to solvent can be regarded as a mosaic charge. This leads to a complex pattern of interactions between solvent components and regions of protein on its surface [35–39]. This surface gradually becomes bigger upon heating and denaturation of protein, especially during the second transition. It was proved that trehalose is more or less inert toward protein surface [16]. In contrast to trehalose, Mg^{2+} and SO_4^{2-} ions can bind to charged points on protein surface. In addition, inner-sphere binding of negatively charged asparatic or glutamic acid residues to metal dications on protein surface in the native state seems to be unlikely [40].

As temperature rises toward second T_m, heat capacity, as a thermodynamic factor become more important against thermal stress and trehalose with lower ΔC_p, relative to $MgSO_4$, could result in incomplete exposure of hydrophobic groups, results in increasing T_m relative to that of intact protein [16]. In addition, although the effect of preferential hydration is less important at higher temperatures, however this effect is relatively considerable in the case of compounds such as trehalose which can increase the viscosity and surface tension of solution [16]. Reversibility of melting phases in the presence of trehalose is a consequence of its effect on viscosity, so that molecular collisions are decreased. On the contrary, because of high frequency of molecular collisions in the presence of $MgSO_4$, aggregation occurred at higher temperatures, this is due to exposure of hydrophobic patches to solvent. Similar report has been published about the unfolding pathway (transition pathway) of human cyanomet myoglobin in the presence of $MgSO_4$ [41].

On the other hand, bioluminescence activation energy of luciferase was moved to a higher value upon addition of trehalose (Table 1) which may be resulted from a decrease in structural dynamics [29,40]. It becomes more convincing to know that luciferase bears conformational changes during the light emission [42].

According to stopped-flow fluorescence spectra data (Fig. 4), rate constant of refolding of luciferase is increased upon addition of $MgSO_4$, but it is decreased in the presence of trehalose. It seems that viscosity can also affect the internal dynamics of protein when it going from unfolded state to a folded one. Stopped-flow fluorescence spectra in the presence of trehalose indicate that it has an inhibitory effect on the refolding of protein. In this case, it is noticeable that there are two factors acting against each other. First one is preferential hydration as an enthalpy-driving factor, helping protein to gain its native structure [18]. The second one is viscosity as a dissipating factor, resists on dynamic movement of molecules and internal dynamics of unfolded protein molecules [32,43]. It seems that the second one dominated in the case of trehalose, but not about $MgSO_4$. As noted before about the mechanism of $MgSO_4$ stabilization, preferential hydration is due to the balance between Mg^{2+} interaction and SO_4^{2-} exclusion in denatured state, helps protein move toward the favorable energy state [18].

Luciferase from *P. pyralis* has two Trp residues that make them as suitable probes via fluorescence measurement. According to intrinsic fluorescence spectra of luciferase it can be seen that the fluorescence peaks undergone red shifts with an increase in their intensities upon addition of trehalose to protein solution (Fig. 6). These unusual phenomena have been reported for the Homeotric family proteins, which have few number of tryptophan residues. The red shift has been attributed to translocation of Trp from a non-polar to a polar environment followed by their interaction with polar molecules of solvent. Increasing the intrinsic fluorescence intensities is an indication of intrinsic quenching against Trp emission before structural alteration, which is eliminated upon dissociation of quenchers' coordination in native structure [44]. Table 3 lists potential interactions of Trp residues with some common native quenchers such as tyrosine, cysteine, histidine, lysine

Table 3
List of candidates for Trp-quencher residues, which may potentially have low quantum yield.

Tryptophan interaction		Distance (Å)
Lysine	Trp 417-Lys 372	4.07
	Trp 426-Lys 5	4.53
	Trp 426-Lys 8	3.55
Phenylalanin	Trp 426-Phe 432	3.74
Histidine	Trp 417-His 419	5.87
Tyrosine	Trp 426-Tyr 425	6.30
Cysteine	Trp 417-Cys	5.31

and phenylalanine [45]; this is done by surveying the PDB structure of luciferase for candidates that may be involved in quenching effect.

The results of the storage stability (long-term stability) differ from that of thermal stability (short-term stability). In thermal stability, magnesium sulfate is the better stabilizer; it significantly enhanced T_m of enzyme. On the contrary, it showed weak effect on storage stability. Trehalose shows maximum enhancement in long-term stability, so it will be the better long-term stabilizer relative to magnesium sulfate.

In conclusion, the summary of the results presented in this manuscript shows the stabilization effect of trehalose and $MgSO_4$ on structural and functional properties of firefly luciferase. Overall, our results indicate that both additives change structural and functional properties of intact luciferase. In addition, these additives can enhance the tolerance of the enzyme against both heat inactivation and denaturation.

Acknowledgments

Financial support for this work was provided by the Research Council of Tarbiat Modares University.

References

[1] T.O. Baldwin, Structure 4 (1996) 223–228.
[2] E. Conti, N.P. Franks, P. Brick, Structure 4 (1996) 287–298.
[3] T. Nakatsu, S. Ichiyama, J. Hiratake, A. Saldanha, N. Kobashi, Nature 440 (2006) 372–376.
[4] L.C. Tisi, P.J. White, D.J. Squirrell, M.J. Murphy, C.R. Lowe, J.A.H. Murray, Anal. Chim. Acta 457 (2002) 115–123.
[5] B. Gharizadeh, T. Nordstrom, A. Ahmadian, M. Ronaghi, P. Nyren, Anal. Biochem. 301 (2002) 82–90.
[6] I.C. Notting, J.T. Buijs, I. Que, R.E. Mintardjo, G. Van Der Horst, M. Karperien, G.S.O.A. Missotten, N.E. Schalij-Delfos, J.E.E. Keunen, G. Van Der Pluijm, Invest. Ophth. Vis. Sci. 46 (2005) 1581–1587.
[7] N.Kh. Tafreshi, S. Hosseinkhani, M. Sadeghizadeh, M. Sadeghi, B. Ranjbar, H. Naderi-Manesh, J. Biol. Chem. 282 (12) (2007) 8641–8647.
[8] Q. Xu, Z. Xie, J. Ding, S. Lin, G. Xu, Protein Sci. 13 (2004) 1851–1858.
[9] L.H. Chamberlain, R.D. Burgoyne, J. Biol. Chem. 272 (1997) 31420–31426.
[10] V.R. Viviani, Cell. Mol. Life Sci. 59 (2002) 1833–1850.
[11] E.M. Meiering, L. Serrano, A.R. Fresht, J. Mol. Biol. 225 (1992) 585–589.
[12] A. Riahi Madvar, S. Hosseinkhani, K. Khajeh, B. Ranjbar, A. Asoodeh, FEBS Lett. 579 (2005) 4701–4706.
[13] N. Hadizadeh Shirazy, B. Ranjbar, S. Hosseinkhani, K. Khalifeh, A. Riahi Madvar, H. Naderi-Manesh, J. Biochem. Mol. Biol. 40 (2007) 453–458.
[14] M. Mehrabi, S. Hosseinkhani, S. Ghobadi, Int. J. Biol. Macromol. 42 (2008) 75–80.
[15] J. Eriksson, T. Nordstorm, P. Nyren, Anal. Biochem. 314 (2003) 158–161.
[16] J.K. Kaushik, R. Bhat, J. Biol. Biochem. 278 (2003) 26458–26465.
[17] S. Matsue, T. Fuji, O. Miyawaki, Int. J. Biol. Macromol. 28 (2001) 343–349.
[18] T. Arakawa, R. Bhat, S.N. Timasheff, Biochemistry 29 (1990) 1924–1931.
[19] R.D. Lines, C.S. Pereira, H. Philip, Proteins 55 (2004) 177–186.
[20] G.R. Wyatt, G.F. Kalf, J. Gen. Physiol. 40 (1957) 833–847.
[21] R.P. Adams, E. Kendall, K.K. Kartha, Biochem. Syst. Ecol. 18 (1990) 107–110.
[22] A.D. Elbein, Adv. Carbohydr. Chem. Biochem. 30 (1974) 227–256.
[23] X. Guifu, S.N. Timasheff, Biophys. Chem. 64 (1997) 25–43.
[24] M. Nakagaki, H. Nagasse, H. Ueda, J. Mol. Sci. 73 (1992) 173–180.
[25] H. Takahashi, H. Ohmae, I. Hatta, Biophys. J. 73 (1997) 3030–3038.
[26] T. Chen, A. Fowler, M. Toner, Cryobiology 40 (2000) 277–282.
[27] G.M. Wang, A.D.J. Haymet, J. Phys. Chem. B 102 (1998) 5341–5347.
[28] T. Arakawa, S.N. Timasheff, Biochemistry 21 (1982) 6545–6552.
[29] L.M. Markillie, C.T. Lin, J.N. Adkins, D.L. Auberry, E.A. Hill, B.S. Hooker, P.A. Moore, R.J. Moore, L. Shi, H.S. Wiley, V. Kery, J. Proteome Res. 4 (2005) 268–274.
[30] M.M. Raman, Anal. Biochem. 72 (1976) 248–254.
[31] A. Moradi, S. Hosseinkhani, H. Naderi-Manesh, M. Sadeghizadeh, B.S. Alipour, Biochemistry 48 (2009) 575–582.
[32] J.G. Sampedro, R.A. Munoz-Clares, S. Uribe, J. Bacterial (2002) 4384–4391.
[33] L. Hassani, B. Ranjbar, K. Khajeh, H. Naderi-Manesh, M. Naderi-Manesh, M. Sadeghi, Enzyme Microb. Technol. 38 (2006) 118–125.
[34] D.W. Urray, Mod. Phys. Biochem. A (1985) 175–345.
[35] T.Y. Lin, S.N. Timasheff, Protein Sci. 5 (1996) 372–381.
[36] J.A. Schellman, Biophys. Chem. 37 (1990) 115–137.
[37] S.N. Timasheff, J. Am. Chem. Soc. 31 (1992) 9857–9864.
[38] E.P. Pittzi, S.N. Timasheff, Am. Chem. Soc. 17 (1978) 615–623.
[39] S.N. Timasheff, M.J. Kornman, Arch. Biochem. Biophys. 83 (1959) 60–75.
[40] T. Dudev, J. Cowan, C. Lim, J. Am. Chem. Soc. 121 (1999) 7665–7673.
[41] T. Konno, I. Morishima, Biophys. J. 65 (1993) 907–911.
[42] M. DeLuca, W.D. McElroy, Biochemistry 13 (1974) 921.
[43] J.G. Sampedro, S. Uribe, Mol. Cell. Biochem. 256/257 (2004) 319–327.
[44] V. Nada, L. Brand, Proteins 40 (2000) 112–125.
[45] Y. Chen, M. Barkely, Biochemistry 37 (1998) 9976–9982.