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Effects of Sucrose and Trehalose on Stability, Kinetic Properties, and Thermal Aggregation of Firefly Luciferase

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Abstract In this study, we used sugars as stabilizing additives to improve the thermostability and to inhibit aggregation of firefly luciferase. The combination of sucrose and trehalose has a strong stabilizing effect on firefly luciferase activity and prevents its thermoinactivation. These additives can also increase optimum temperature. It has been shown that the presence of both sucrose and trehalose can inhibit thermal aggregation of firefly luciferase and decrease bioluminescence decay rate. In order to understand the molecular mechanism of thermostabilization, we investigated the effects of sucrose and trehalose combination on the secondary structure of luciferase by Fourier transform infrared spectroscopy. Minor changes in content of secondary structure of firefly luciferase are observed upon treatment with additives.

Keywords Aggregation · Bioluminescence · Firefly luciferase · Thermostability

Introduction

In nature, there is a relatively large diversity of organisms that emit light [1]. For instance, firefly luciferase of North America (1.13.12.7) is a monooxygenase that emits flash of light in the presence of D-luciferin, adenosine triphosphate (ATP), Mg^{+2}, and oxygen [2–4]. Rapid relaxation of the excited state of the bioluminescence product, oxyluciferin, to the ground state is accompanied by the emission of green light ($\lambda_{max}560$ *nm*) [5, 6]. The crystal structure of North American firefly luciferase (*Photinus pyralis*) without substrate and from the Japanese firefly (*Lunularia cruciata*) with an intermediate analog has been determined

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by X-ray crystallography [7, 8]. Biological reactions (bioluminescence) have a wide range of biotechnological, clinical, and analytical applications [9–12]. For instance, they are used in bioluminescence immunoassay [13], protein blotting [14, 15], DNA sequencing [16], measuring of microbial contamination [17–19] and as reporter enzymes for studies of gene regulation [20, 21]. Moreover, firefly luciferase has been used to determine the amount of ATP in molecular and cell biology and many enzymes can be assayed by coupling with ATP production reaction; this is because of the high specificity of this protein for ATP [22, 23]. However, due to the low stability of enzyme in both in vivo and in vitro conditions, the firefly luciferase is not widely used [24–26].

Previously, thermostable mutants have been designed to improve the utilization and thermostability of firefly luciferase [27–31]. Moreover, osmolytes as general stabilizing additives have been used to increase the stability of firefly luciferase at higher temperatures [32–34]. Firefly luciferase is a thermosensitive protein that usually loses its activity at 37 °C in 3 min, but it keeps its activity in vivo for 4–5 h [30, 31].

In the present study, we monitored the enhancement of enzymatic activity in the presence of sucrose and trehalose at higher temperatures. We also measured the effects of these additives on bioluminescence decay rate and thermal aggregation. In addition, secondary structural changes of firefly luciferase in the presence or absence of these additives were investigated by Fourier transform infrared (FTIR) spectroscopy.

Materials and Methods

Materials

D(+)sucrose, D(+)trehalose, tricine, and $MgSO_4$ were purchased from Sigma Chemical Co. ATP was from Roche. D-luciferin potassium salt was obtained from Synchem Crop. The experiments carried in 50 mM Tricine–NaOH, pH 7.8 as assay buffer. Sucrose and trehalose were dissolved in the buffer to final concentrations. The concentrations of additives were 1.5 M sucrose, 1.2 M trehalose, 0.5 M sucrose, and 0.5 M trehalose. All the measurements were done in triplicate.

For the luciferase assay, substrate solution contained the following components: 2 mM luciferin, 10 mM $MgSO_4$, 4 mM ATP, 50 mM Tricine–NaOH, and pH 7.8. Standard substrate (100 μM) mixed by luciferase. Protein concentration was 1.5 mg/ml. Enzyme was diluted about ten times via buffer or different concentrations of additives. Light emission was recorded over 10 s. (Orion Microplate Luminometer, Berthold Detection System).

Purification

The firefly luciferase, *P. pyralis*, was produced in *Escherichia coli* cells, strain BL21 using pET expression system, and purified with Ni-NTA Sepharose (Qiagen, Inc.) column to homogeneity. The purity of the luciferase was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was determined by Bradford method [35].

Remaining Activity and Optimum Temperature

Enzymatic activity of the luciferase was assayed by incubating luciferase in the absence or presence of sucrose and trehalose at 30, 35, and 40 °C every 5 min for the duration of

40 min. Also, the thermosensitivity of *P. pyralis* in the absence or presence of two sugars was determined by incubating luciferase at 20–40 °C for 5 min in the assay buffer and then the activity was measured.

Bioluminescence Decay Rate and Thermal Aggregation

Ten microliters of diluted enzyme via buffer and different concentrations of additives mixed by 100 μl of substrate were used. The enzyme activity was measured in the absence or presence of sucrose and trehalose at 15-s intervals for about 5 min. Moreover, the effects of sugars on thermal aggregation at 50 °C and 420 nm were investigated by spectrophotometer. Ten microliters of aliquots of stock enzyme were added to assay buffer and two concentrations of additives.

FTIR Measurements

The effect of sucrose and trehalose on the secondary structure of firefly luciferase was indicated by FTIR. FTIR spectra were recorded on a Nexus 870 FTIR spectrophotometer with a deuterated triglycine sulfate detector. We added enzyme to the sugars solutions with two concentrations. The final concentration was 0.1 mg/ml. Typically, 0.1 mg/ml of protein mixture was injected into the cell and a spectrum was recorded. Second derivative spectra were calculated by a method reported earlier [36].

Results

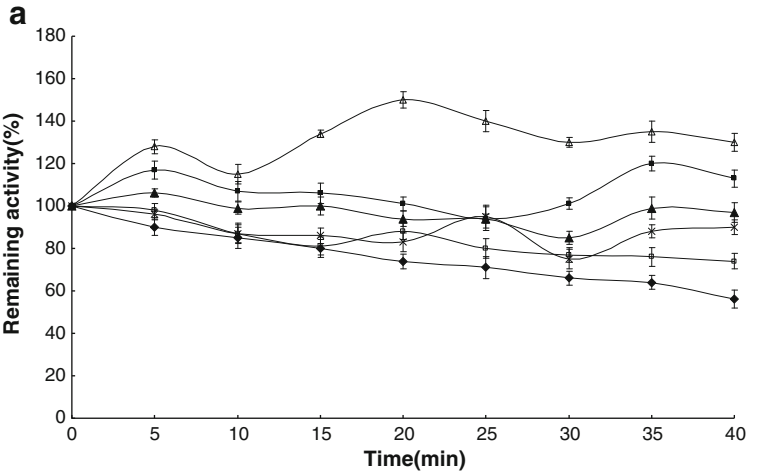
Purification

The luciferase protein was purified to more than 95% based on analyses by SDS-PAGE in which luciferase was present as a single band of about 62 kDa. Moreover, after adding the substrate to the purified luciferase, we could see the emitted light with naked eyes in a dark room.

Remaining Activity of Firefly Luciferase

According to our results, in the absence of additives, the enzyme activity was lost rapidly. On the other hand, adding two concentrations of sugars resulted in a more stable luciferase activity at higher temperatures. Among different tested concentrations, combination of 0.5 M sucrose and 0.5 M trehalose was the most effective procedure which was followed by 1.5 M sucrose and 1.2 M trehalose at all temperatures, respectively (Fig. 1a, b, and c). Our results show that using of 0.5 M sucrose and 0.5 M trehalose separately do not have a strong stabilizing effect. As it is obvious from Fig. 1, upon enzyme incubation with

Fig. 1 Percentage of remaining activity of firefly luciferase at 30 °C (**a**), 35 °C (**b**), 40 °C (**c**) at different time intervals. In the absence (*black diamond suit*) or presence of 1.5 M sucrose (*black square*), 1.2 M trehalose (*black up-pointing triangle*), 0.5 M sucrose (*white square*), 0.5 M trehalose (*multiplication sign*) and 0.5 M sucrose+0.5 M trehalose (*white up-pointing triangle*). Protein concentration was 1.5 mg/ml. Enzyme was diluted (ten times) via assay buffer (50 mM Tricine–NaOH, pH 7.8) or different concentrations of additives. For all solutions, at zero time the activity was supposed 100%. For other times, the activity was measured on the basis of 100%. For further details, see Materials and Methods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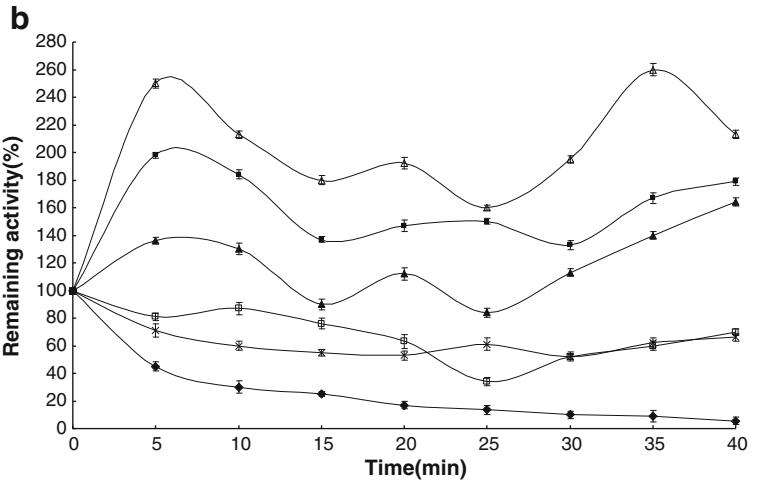
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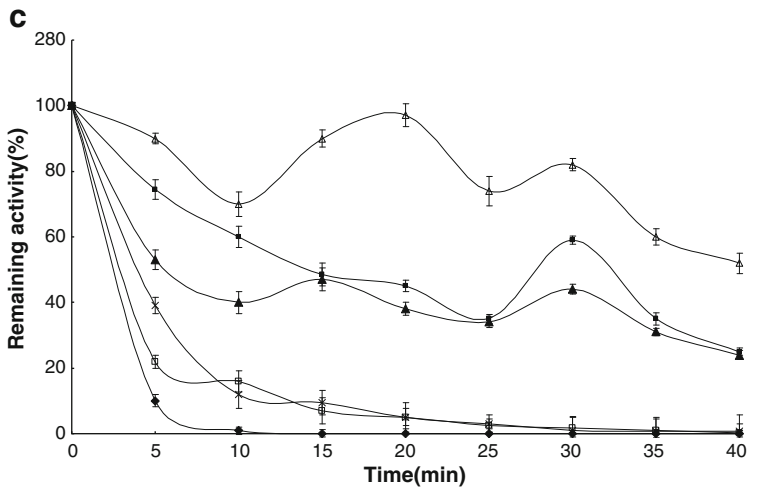
c
Remaining activity(%)
Time(min)
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40
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osmolytes apparent enzyme activation observed. In fact, upon enzyme incubation at each temperature, luciferase starts inactivation. At the same time, in the presence of osmolytes rate of enzyme inactivation decreased and therefore, an apparent activation is observed.

Optimum Temperature of Luciferase

We compared the optimum temperature of luciferase in the absence or presence of two concentrations of sugars (Fig. 2). Using the combination of 0.5 M sucrose and 0.5 M trehalose, we observed an increase in the optimum temperature of enzyme from 25 to 30 °C. However the optimum temperature of luciferase was not affected when we used 0.5 M sucrose and 0.5 M trehalose separately (Fig. 2).

Decay Rate of the Luciferase

The time of light decay of *P. pyralis* was measured in presence or absence of the additives and results are shown in (Fig. 3). The rate of bioluminescence light decay in presence of 1.5 M sucrose and 1.2 M trehalose decreased clearly, where 1.5 M sucrose was most effective. Our results indicate that in lower concentrations of sugars (0.5 M sucrose and 0.5 M trehalose) the rate of light decay did not show any obvious shift.

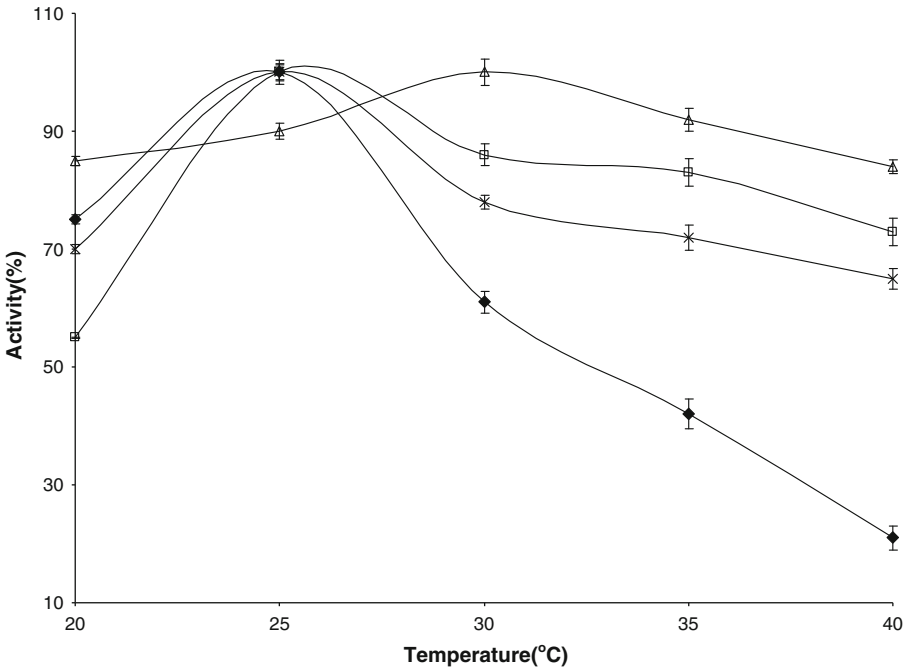


Fig. 2 Optimum temperature of *P. pyralis* luciferase in the absence (*black diamond suit*) or presence of 0.5 M sucrose (*white square*), 0.5 M trehalose (*multiplication sign*) and 0.5 M sucrose+0.5 M trehalose (*white up-pointing triangle*). Luciferase activity was assayed after 5 min incubation at each temperature. Protein concentration was 1.5 mg/ml. Enzyme was diluted (ten times) via assay buffer (50 mM Tricine–NaOH, pH 7.8) or different concentrations of additives. For further details, see Materials and Methods

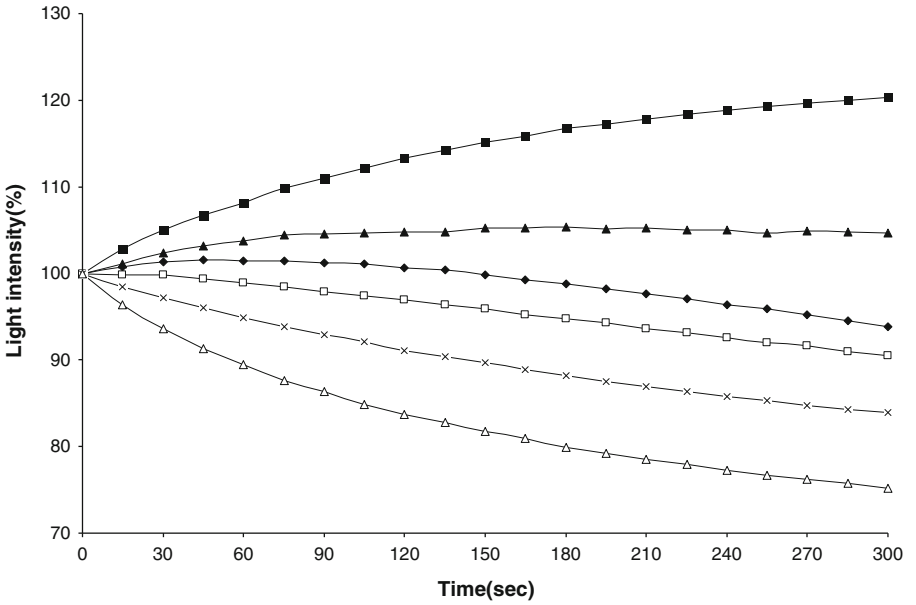


Fig. 3 Comparison of decay times in the absence (*black diamond suit*) or presence of 1.5 M sucrose (*black square*), 1.2 M trehalose (*black up-pointing triangle*), 0.5 M sucrose (*white square*), 0.5 M trehalose (*multiplication sign*) and 0.5 M sucrose+0.5 M trehalose (*white up-pointing triangle*). Samples were assayed at 25 °C. Protein concentration was 1.5 mg/ml. Enzyme was diluted (ten times) via assay buffer (50 mM Tricine–NaOH, pH 7.8) or different concentrations of additives. For all solutions, at zero time the activity was supposed 100%. For other times, the activity was measured on the basis of 100%. For further details, see Materials and Methods

Thermal Aggregation of Enzyme

As indicated in (Fig. 4), a reduction of enzyme thermal aggregation in the presence of two concentrations of sucrose and trehalose, as recorded by absorbance changes at 420 nm and 50 °C. The highest aggregation inhibition occurred at 1.5 M sucrose and the lowest inhibitory effect was observed at 0.5 M trehalose. The inhibitory effect on enzyme thermal aggregation upon the combination of 0.5 M sucrose and trehalose and in the presence of only 0.5 M sucrose was nearly identical (Fig. 4). It should be noted; visible aggregation of luciferase start at 40 °C, but it takes longer time for their appearance.

Infrared Spectroscopy

Figure 5a and b compares the second derivative spectrum of native luciferase at pH 7.0 with those obtained in the presence of additives at the same pH. The bands at 1,658, 1,666, and 1,620 cm^{-1} have been assigned to α-structures, β-turns, and β-sheets, respectively [37–41]. Stabilization of enzyme by combination of 0.5 M sucrose and 0.5 M trehalose seemed to cause a very small change of amount of α-structures. A small shift of 1,658 band showed a change in α-structures content and a shift of 1,666 band showed a change in β-turns content (Fig. 5a). For other concentrations of additives, the most obvious difference was the

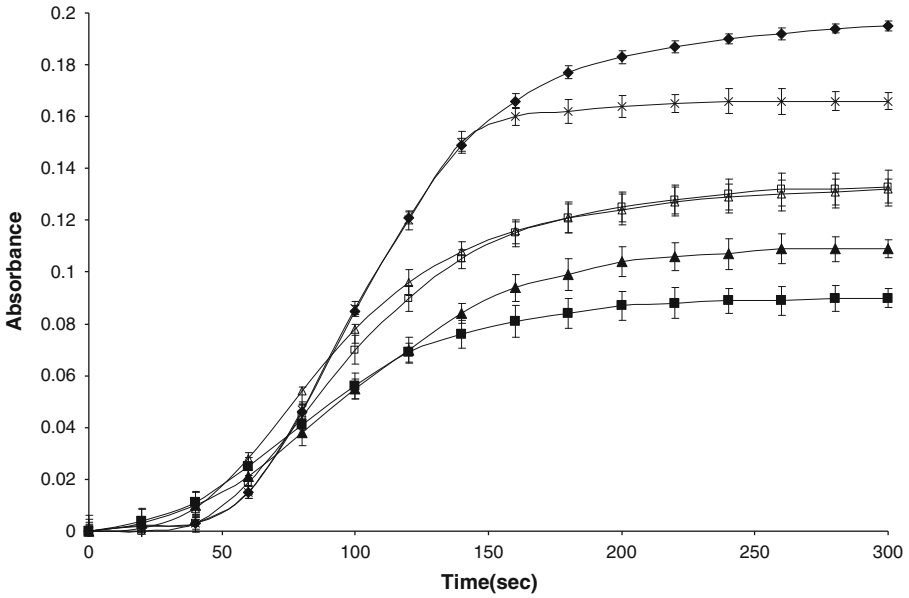


Fig. 4 Thermal aggregation of firefly luciferase at 50 °C and 420 nm in the absence (*black diamond suit*) or presence of 1.5 M sucrose (*black square*), 1.2 M trehalose (*black up-pointing triangle*), 0.5 M sucrose (*white square*), 0.5 M trehalose (*multiplication sign*) and 0.5 M sucrose+0.5 M trehalose (*white up-pointing triangle*)

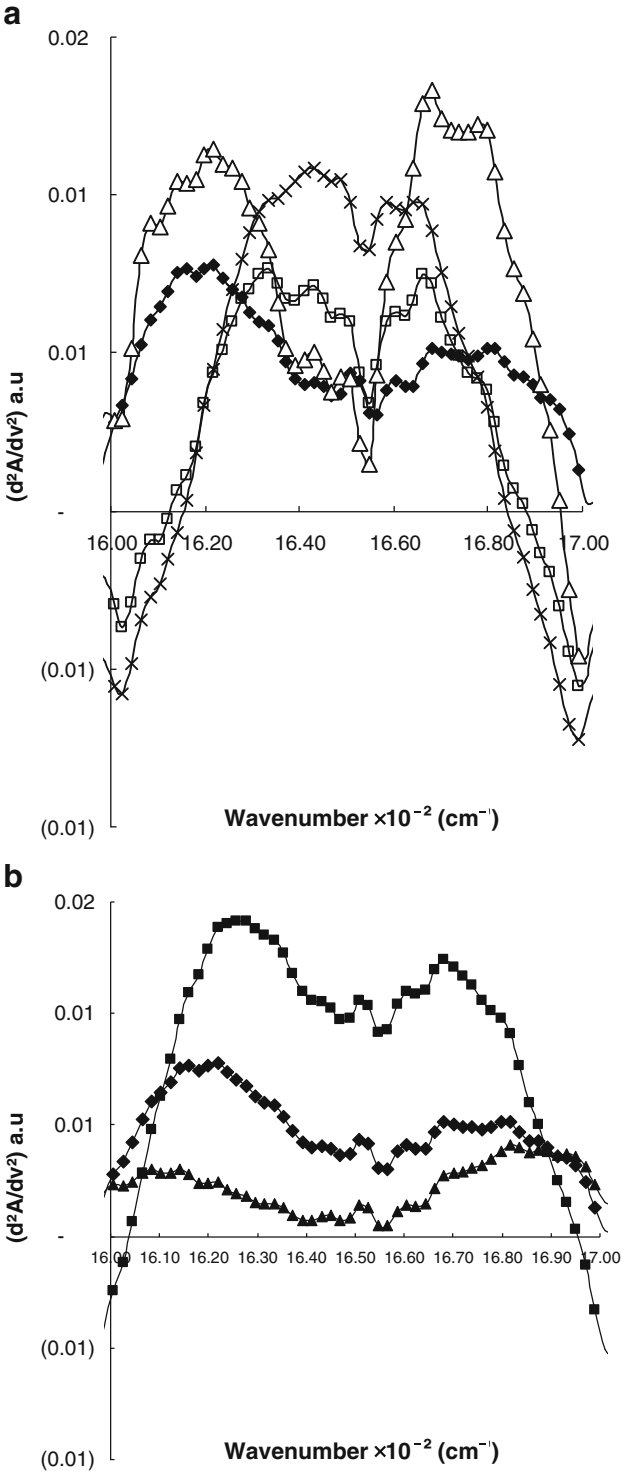
intensity of the bands. As there is no any shift, we suppose that this was likely to be due to changes in the extinction coefficient (Fig. 5).

Discussion

Using the firefly luciferase is the basis of a wide range of detective techniques in different fields such as: nutrition, medical purposes, sanitation, and also cell and molecular biology [9–15]. However, this enzyme is unstable and loses its activity very fast. Due to extensive utilization of this enzyme, the first concern about luciferase is its stability at high temperature. In the present study, the effect of two sugars, namely sucrose and trehalose, was probed on enzyme thermal stabilization, both separately and synchronously. Also, the effects of these two sugars were studied on kinetic properties and enzyme structure.

First, we studied enzyme remaining activity in the presence of two concentrations of sucrose and trehalose at 30, 35, 40 °C (Fig. 1a, b, and c). We found that at each temperature, native enzyme lost its activity rapidly and the rate of losing the enzyme activity increased at higher temperatures. In the absence of additives, the rate of

Fig. 5 Second derivative spectrum of firefly luciferase in the absence (*black diamond suit*) or presence of (**a**) ▶ 0.5 M sucrose (*white square*), 0.5 M trehalose (*multiplication sign*) and 0.5 M sucrose+0.5 M trehalose (*white up-pointing triangle*) and (**b**) 1.5 M sucrose (*black square*) and 1.2 M trehalose (*black up-pointing triangle*). Spectra were taken at 25 °C in Tricine–NaOH buffer (50 mM, pH=7.8)


a
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-
(0.01)
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16.00
16.20
16.40
16.60
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17.00
(d²A/dv²) a.u
Wavenumber ×10⁻² (cm⁻¹)
b
0.02
0.01
0.01
-
(0.01)
(0.01)
16.00
16.10
16.20
16.30
16.40
16.50
16.60
16.70
16.80
16.90
17.00
(d²A/dv²) a.u
Wavenumber ×10⁻² (cm⁻¹)

denaturation was higher than the rate of inactivation. At all concentrations of sugars, the enzyme remaining activity increased and the highest efficacy was seen with the use of combination of sucrose and trehalose. For naked protein, the activity was decreased with time, but in the presence of additives it was initially increased and a lag in thermoinactivation was observed, presumably due to the protection of protein structure and increasing of flexibility. The optimum temperature of enzyme in the presence of the combination of 0.5 M sucrose and 0.5 M trehalose increased from 25 up to 30 °C (Fig. 2). In the presence of sugars, the enhancement of optimum temperature might be due to the extension of the surface tension as well as the protein rigidity. However, the presence of molar concentration of osmolytes may increase protein rigidity through more viscosity in media.

The enzyme activity versus time includes two phases; rise time and decay phase. Enzyme decay phase may be glow or fast. The fast decay rate of firefly luciferase is due to formation and inhibitory effect of side products of luminescence reaction [42–44]. Higher concentrations of sugars decreased the decay rate and sucrose was more effective than trehalose (Fig. 3). This might suggest that in the presence of these additives, the production rate of the main products was higher than side products. Alternatively, our findings prove that higher structural stability of firefly luciferase in presence of additives results in controlled release of light energy. Thus, the enzyme light emission was performed as glow form in comparison with native enzyme.

We also showed that in the presence of osmolytes the rise time of luminescence reaction increased. Therefore, suggesting that in the presence of protein stabilizers, the rise time of luminescence reaction can be increased.

The procedure that makes the luciferase inactive is irreversible denaturation in which one of the mechanisms is aggregation [34, 45]; this is why we focused on the effects of these additives on enzyme aggregation. As a result of enzyme aggregation, light can neither be absorbed nor be transmitted, it can only scatter. The absorbance which has been shown is not real absorbance and increases by addition in aggregation amount. Our results showed that in two concentrations of sugars, the aggregation process was inhibited and higher concentrations had more inhibitory effect (Fig. 4). In fact, in a few minutes, presence of luciferase at higher temperatures, luciferase inactivated due to formation of visible aggregated particles. This result confirms our previous data regarding the decay rate. In fact, dissimilar to other proteins, firefly luciferase aggregation starts very fast at low temperatures [46]. Actually, these concentrations decreased decay rate (Fig. 3). Moreover, 50% of aggregation is achieved after 2 min for naked enzyme while in presence of additive it was started earlier. Since irreversible denaturation is also inhibited, this could suggest that enzyme stability increases in the presence of additives because of the extent of aggregation inhibition. The effect of additives on the content of enzyme secondary structure was also investigated. The preliminary structural analysis by FTIR showed that the combination of 0.5 M sucrose and trehalose resulted in little changes in α-helix and β-turns amounts (Fig. 5a). Meanwhile, other concentrations of additives resulted in extinction coefficient change (Fig. 5b). However, it should be noted; change of extinction coefficient may be arisen from change in conformation in presence of additives.

- In conclusion, our work shows that the addition of some osmolytes (sugars) is responsible for increasing of luciferase thermostability and decreasing of light decay rate concomitant with aggregation inhibition as one of the main mechanism of irreversible thermoinactivation.

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