Spectroscopic and functional characterization of *Lampyris turkestanicus* luciferase: a comparative study

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Functional expression and spectroscopic analysis of luciferases from *Lampyris turkestanicus* and *Photinus pyralis* were carried out. cDNA encoding *L. turkestanicus* luciferase was isolated by reverse transcription-polymerase chain reaction, cloned, and functionally expressed in *Escherichia coli*. The luciferases were purified to homogeneity using Ni-nitrilotriacetic acid Sepharose, and kinetic properties of luciferase from *L. turkestanicus* were compared with that from *P. pyralis*. Amino acid differences in its primary structures in relation to *P. pyralis* luciferase brought about changes in the kinetic properties of the enzyme as evidenced by substantial lowering of $K_m$ for ATP, increased light decay time, and decreased thermostability. Luciferase from *L. turkestanicus* was used to carry out Michaelis-Menten kinetics with a $K_m$ of 95.5 μM for ATP and 20 μM for luciferin. Maximum activity was recorded at pH 8.5, so it might be a suitable reporter for microbial screening at alkaline pH. Tryptophan fluorescence for *P. pyralis* luciferase was higher than *L. turkestanicus* luciferase. Substitution of some residues in *L. turkestanicus* luciferase appears to change the kinetic properties by inducing a substantial tertiary structural change, without a large effect on secondary structural elements, as revealed by intrinsic and extrinsic fluorescence, Fourier transform infrared spectroscopy, and near-ultraviolet circular dichroism spectra.

Keywords firefly luciferase; enzyme activity; *Lampyris turkestanicus*; emission spectrum; decay rate

Many living organisms are capable of producing visible light, a phenomenon generally known as bioluminescence. The best-known bioluminescent organism is the North American firefly, *Photinus pyralis*, with flash-type luminescence reaction [1]. The enzyme responsible for the light emission by firefly is called luciferase, a monomeric enzyme of 62 kDa [2]. Firefly luciferase (EC 1.13.12.7) catalyzes the oxidation of substrate luciferin in the presence of ATP, Mg²⁺, and molecular oxygen [2,3]. The product, oxyluciferin, is generated in an excited state, then decays to the ground state with the emission of light. The color of the light emitted from luminous beetles ranges from green (540 nm) to red (620 nm), and is solely determined by the active site residues [4,5]. The luciferases have similar spectra to the color *in situ*. Each luminous beetle emits a distinctive flashing pattern that is recognized by the opposite sex in the same species [6]. The flash may last a few milliseconds, as with the adult *Photinus*, or may be a glow lasting several hours, as in the glow worm *Lampyris* [7].

The enzyme converts chemical energy, efficiently, into light with a quantum yield of 0.88 [8]. The sensitivity and convenience of the firefly luciferase assay has created considerable interest in luciferase-based biosensors, with a detection limit in the femtomole range. Light production of firefly luciferase is one of the most sensitive analytical tools in the ultrasensitive detection of ATP for measuring microbial contamination [9], genetic reporter assays in molecular biology [10], detection of phosphatase activity [11], use in DNA sequencing [12], and as a tool for monitoring *in vivo* protein folding and chaperonin activity [13].

Assays based on luciferase are preferred due to its high sensitivity, rapidity, and non-invasiveness. However, several factors limit further application and development of this technology, including a low turnover number, high $K_m$ for the substrate ATP, and low stability [14]. For the
practical use of luciferase like other enzymes, both genetic engineering methods such as DNA shuffling and looking for natural variants are important. Since the first cloning of *P. pyralis* luciferase, the luciferase genes have been isolated from several species of fireflies [15]. The cDNA encoding a glow worm luciferase from lantern mRNA of *Lampyris turkestanicus* was cloned and functionally expressed in *Escherichia coli* [16].

The aim of this work was to characterize the luciferase from *L. turkestanicus* and compare its properties with a flash-emitter luciferase from *P. pyralis*. Both enzymes were purified to homogeneity; their enzymatic and structural properties were compared. A further objective was to determine whether the amino acid sequence or enzyme properties could account for the characteristics of the glow worm’s glow, in contrast with the flashing pattern of luciferase from *P. pyralis*.

**Materials and Methods**

**Chemicals**

Mg-ATP and D-luciferin (sodium salt), ATP, and isopropyl-β-D-thiogalactopyranoside were purchased from Sigma (Poole, UK). Ni-nitritolriacetic acid (NTA) Sepharose was from Novagen (Madison, USA).

**Plasmids and strains**

The cDNA of *L. turkestanicus* luciferase was previously cloned in pQE30 vector [16]. pET-luc (*P. pyralis*) was kindly provided by Prof. Laurence Tisi (University of Cambridge, Cambridge, UK). Both luciferase-containing vectors (pQE30-luc and pET-16b-luc) were transferred into *E. coli* strains XL1-Blue and BL21, respectively. The pQE30-luc and pET-16b-luc vectors used the T5 and T7 promoters, respectively, and encoded six histidine residues on the amino terminus of the protein in the multiple cloning sites that can be used for immobilized metal affinity chromatography. Transformed colonies by pQE30-luc and pET-16b-luc were screened by X-ray films. Bacteria containing the pQE30-luc and pET-16b-luc were grown overnight at 37 ºC on Luria-Bertani agar plates containing ampicillin. The master plates were sprayed with 200 µl solution containing 0.2 mM D-luciferin (in 0.1 M Tris acetate, pH 7.8) for 5 min and exposed to X-ray film for 4 h. After film development, the positive colonies (bioluminescent) were identified (they also could be observed by eye after dark-adaptation).

**Expression and purification of recombinant luciferase**

A 5 ml culture [2XYT (yeast extract, 10 g; tryptone, 16 g; NaCl, 5 g/L of medium) plus 50 µg/ml ampicillin] was inoculated with a single colony from a freshly streaked plate of XL1-Blue. The culture was incubated at 37 ºC for 6–8 h then used to inoculate a 200 ml fresh culture (2XYT plus 50 µg/ml ampicillin). The 200 ml culture was grown for approximately 5 h at 37 ºC (OD$_{600}$=0.7). The temperature was then lowered to 22 ºC and the expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside and 1 mM lactose. Following overnight induction, the cells were harvested by centrifugation (12,000 g for 15 min at 4 ºC). The cell pellets were washed with 10 ml Tris buffer (50 mM, pH 7.8) and repelleted. Cells were resuspended in 15 ml lysis buffer (10 mM imidazole, 50 mM NaH$_2$PO$_4$, 300 mM NaCl, pH 7.8) containing 1 mM phenylmethylsulfonyl fluoride (Sigma). The cells were lysed by ultrasonication. Sonication was carried out seven times with 10 s bursts with the cells on ice to prevent excessive heating of the lysate. The cell lysate was clarified by centrifugation (18,000 g for 25 min at 4 ºC). The clear lysate was applied to the Ni-NTA Sepharose column then washed with 20 mM imidazole in 50 mM NaH$_2$PO$_4$ and 300 mM NaCl (pH 7.8). The histidine-tagged *L. turkestanicus* luciferase was eluted with 120–250 mM imidazole, desalted using dialysis, and placed into storage buffer (20% glycerol). The desalted *L. turkestanicus* luciferase was concentrated with a Centriprep-10 concentrator (Amicon, Beverly, USA). The purity of the *L. turkestanicus* luciferase was more than 95% as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This process was also carried out to purify *P. pyralis* luciferase.

**Luciferase assay**

The activity levels were measured using a Sirius Single Tube Luminometer (Berthold Detection Systems, Pforzheim, Germany) by integration of total light emitted in 10 s. After the addition of 100 µl standard solution [1 mM ATP, 0.5 mM D-luciferin, and 10 mM MgSO$_4$ in 50 mM Tris-HCl buffer (pH 7.8)] to 100 µl luciferase-containing extracts or purified luciferase at 25 ºC, total light units were measured in 10 s.

**Optimum temperature**

Thermal sensitivity of the luciferases from *L. turkestanicus* and *P. pyralis* were compared. This was carried out by incubating each luciferase at 10 ºC–45 ºC in 50 mM Tricine-NaOH buffer (pH 7.8) containing 5% glycerol and 10 mM β-mercaptoethanol. Activity was measured at different temperatures.

**Optimum pH**
The pH optima of the luciferases were determined and compared by preparation of a mix buffer (100 mM glycine, 100 mM succinic acid, and 50 mM morpholinopropane sulfonic acid). The pH level of the mix buffer was set at 0.5 increments from 5.0 to 12.5. The reaction was initiated by injecting 15 μl diluted luciferase into 170 μl mix buffer at each pH, then 15 μl substrate was immediately added and initial activity was measured.

**Thermal stability**

Thermal stability of the luciferases from *L. turkestanicus* and *P. pyralis* were compared. This was carried out by incubating enzyme solutions in Eppendorf tubes in a circulating water bath at different temperatures (20 °C–45 °C) for 5 min [in 50 mM Tricine-NaOH buffer (pH 7.8) containing 5% glycerol and 10 mM β-mercaptoethanol]. After heating at different temperatures, the samples were cooled in ice water and remaining activities were assayed immediately at 25 °C.

**Determination of kinetic parameters**

Luciferase activity was assayed by measuring the peak light emission with a tube luminometer. The reaction was initiated by injecting 100 μl assay reagent into 100 μl of diluted luciferase. The assay reagent for estimation of $K_m$ and $V_{max}$ for ATP contained 10 mM MgSO$_4$ and 1 mM luciferin plus 0.1, 0.2, 0.4, 0.8, 1.6, 2.5, 3.5, or 4.5 mM (final concentration) ATP in 50 mM Tricine-NaOH buffer (pH 7.8). The assay reagent for luciferin $K_m$ and $V_{max}$ estimation contained 10 mM MgSO$_4$ and 2 mM ATP plus 0.015, 0.025, 0.05, 0.1, 0.2, 0.5, 1.0, or 2.0 mM luciferin in 50 mM Tricine-NaOH buffer (pH 7.8). The peak light emissions were taken as a measure of the initial velocity and expressed as relative light units per second. The data were collected and shown by Michaelis-Menten plots. The $K_m$ and $V_{max}$ values for ATP and luciferin were calculated from Lineweaver-Burk plots. Kinetic experiments were carried out three times and were reproducible within ±5%.

**Decay rate**

The time-course of the light emitted was measured with a luminometer. The assay was carried out by addition of 50 μl substrate [1 mM luciferin, 50 mM Tris buffer, 2 mM ATP, and 10 mM MgSO$_4$ (pH 7.8)] into a 50 μl purified luciferase solution from *P. pyralis* or *L. turkestanicus*. The estimated mixing time was 1.0 s, then data was acquired.

**Measurement of bioluminescence emission spectra**

Bioluminescence emission spectra of the luciferases from *P. pyralis* and *L. turkestanicus* were measured with an LS 50B luminescence spectrophotometer (PerkinElmer Optoelectronics, Fremont, USA). A volume of 2 ml substrate mixture consisting of 1 mM luciferin, 50 mM Tris buffer (pH 7.8), 2 mM ATP, and 10 mM MgSO$_4$ was added to 50 μl purified luciferase solution in a quartz cell. Data were collected over the wavelength range 400–700 nm. The spectra were automatically corrected for the photosensitivity of the equipment.

**Fluorescence measurements**

Tryptophan fluorescence was measured on the LS 50B luminescence spectrophotometer. The excitation wavelength was set at 295 nm and the emission spectra were obtained. Extrinsic fluorescence studies were carried out as previously described [17] using 8-anilino-1-naphthalenesulfonic acid (ANS) as a fluorescence probe. Measurements were taken on the same spectrofluorometer as used for intrinsic fluorescence studies. All experiments were carried out at 25 °C with ANS and protein concentrations of 30 μM and 1 μM, respectively, in 50 mM phosphate buffer. An excitation wavelength of 350 nm was used.

**Infrared (IR) spectroscopy**

Fourier transforms (FT)-IR spectra were recorded on a Nexus 870 FT-IR spectrophotometer (Thermo Fisher Scientific, Waltham, USA) with a deuterated triglycine sulfate (DTGS) detector. The samples (1 μM) were placed in a cell. Typically, 1 μM protein mixture was injected into the cell and a spectrum recorded. The cell was then drained and the protein deposited on the crystal was dried. Second-derivative spectra were calculated by a method reported previously for bacterial luciferase [18].

**Circular dichroism (CD) measurements**

CD spectra were recorded on a Jasco J-715 spectropolarimeter (Tokyo, Japan) using solutions with protein concentrations of approximately 0.2 and 1.5 mg/ml for far- and near-ultraviolet (UV) regions, respectively. Results are expressed as molar ellipticity, [θ] (deg·cm$^2$·dmol$^{-1}$), based on a mean amino acid residue weight (MRW) of 115 for luciferase. The molar ellipticity was determined as [θ] = (θ×100MRW)/(cI), where c is the protein concentration in mg/ml, I is the light path length in centimeters, and θ is the measured ellipticity in degrees at a wavelength θ. The instrument was calibrated with (+)-10-camphorsulfonic acid, assuming [θ]$_{290}$=7820 deg·cm$^2$·dmol$^{-1}$, and with Jasco standard non-hydroscopic ammonium (+)-10-camphorsulphonate, assuming [θ]$_{290}$=7910 deg·cm$^2$·dmol$^{-1}$. 

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**Characterization of Lampyris turkestanicus luciferase**
The data were smoothed using the fast FT noise reduction routine that allows enhancement of most noisy spectra without distorting their peak shapes.

**Calculation of essential residues pKa**

The data of crystal structure of Japanese firefly (*Luciola cruciata*) luciferase were downloaded from Protein Data Bank (2D1Q; [http://www.pdb.org/pdb/home/home.do](http://www.pdb.org/pdb/home/home.do)). The crystal structure of Japanese thermostable firefly luciferase was used as a template to make a suitable model for *L. turkestanicus* and *P. pyralis*. We used the software package MacroDox (Northrup, Cookeville, USA) for our analysis [19]. The modeling data were then used to calculate pKa of critical residues involved in enzyme catalysis or substrate binding.

**Results**

**Purification of the polyhistidine-tagged luciferases**

The cDNA encoding both luciferases containing a polyhistidine tag (6×His) at the amino terminus of the protein were used to express luciferases. Cells were lysed and the clarified cytoplasmic extracts applied on a column. After washing with low imidazole (20 mM), unbound and weakly bound proteins were removed. The polyhistidine-tagged luciferase was finally eluted from the column by increasing the imidazole concentration gradient up to 250 mM. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis and Coomassie Brilliant Blue staining of the eluted fractions showed that the polyhistidine-tagged enzymes were efficiently bound to the column and that the corresponding fractions contained highly purified protein (more than 95%). Determination of protein contents in each fraction was carried out using the Bradford method. These results clearly showed that the enzymes were expressed at relatively high levels (approximately 10 mg per liter culture), and were efficiently purified, and that the enzymes had retained their biological activity [22].

**Decay rate**

The time-course of the light emitted by both luciferases was also measured for glow worm and firefly luciferases (Fig. 1). The decay in light emission was different for each enzyme under the same conditions. However, the rate of luminescence decay for *P. pyralis* firefly luciferase was faster, suggesting lower stability of the intermediate structure of the luciferase reaction (Table 1).

![Decay of luminescence of *Photinus pyralis* (■) and *Lampyris turkestanicus* (▲) luciferases](image)

**Bioluminescence emission spectra**

The color of the light emitted by the *L. turkestanicus* luciferase was green as that of the *P. pyralis* luciferase. Fig. 2 compares the bioluminescence emission spectra of these luciferases in the presence of luciferin and ATP at pH 7.8. Emission spectra of luciferases from *L. turkestanicus* and *P. pyralis* showed a high similarity without changing the λmax (Fig. 2) and only a minor broadening of

**Table 1 Steady-state kinetic constants for overall bioluminescence reactions of luciferase enzymes at pH 7.8**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (μM)</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>Specific activity † (RLU·s&lt;sup&gt;−1&lt;/sup&gt;·mg&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>Decay rate ‡ (s&lt;sup&gt;−1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Photinus pyralis</em></td>
<td>30 140</td>
<td>561</td>
<td>1.6×10&lt;sup&gt;12&lt;/sup&gt;</td>
<td>2.32</td>
</tr>
<tr>
<td><em>Lampyris turkestanicus</em></td>
<td>20 95.5</td>
<td>561</td>
<td>2.1×10&lt;sup&gt;13&lt;/sup&gt;</td>
<td>1.25</td>
</tr>
</tbody>
</table>

† Specific activity measurements were made at pH 7.8 with D-luciferin and Mg-ATP. ‡ Decay rate obtained from attainment of maximum flash height to 20% of the maximum emission intensity. RLU, relative light units.
Characterization of Lampyris turkestanicus luciferase

the spectrum for *P. pyralis* luciferase was observed.

**Optimum temperature of luciferase**

The thermostability of the *P. pyralis* and *L. turkestanicus* luciferases was compared. The activity of both luciferases reached a maximum at 25 °C and started to decrease at higher temperatures (Fig. 3). When the temperature increased above the thermal transition temperature (approximately 45 °C), both luciferases lost almost all of their activity.

**Thermostability of luciferase**

The thermostability of the *P. pyralis* and *L. turkestanicus* luciferases was also compared. The remaining activity of luciferase from *P. pyralis* reached its maximum at 25 °C, whereas the activity of luciferase from *L. turkestanicus* started to decrease at this temperature (Fig. 4). When the temperature increased above the thermal transition temperature (approximately 45 °C), both luciferases lost almost all of their activity. The thermostability of these enzymes, however, showed that luciferase from *L. turkestanicus* has lower temperature stability than luciferase from *P. pyralis*.

**pH optimums**

The pH optimum of these luciferases was determined and compared. The pH optimum for luciferase from *P. pyralis* was 8.0, whereas the pH optimum for luciferase from *L. turkestanicus* showed a maximum at 8.5 (Fig. 5). Therefore, comparative study of these enzymes confirmed a higher optimum pH for *L. turkestanicus* when compared to *P. pyralis*.

**Kinetic properties of purified luciferases**

Table 1 compares some properties of *L. turkestanicus* and *P. pyralis* luciferases. The bioluminescent activity of luciferase is directly proportional to the concentration of their activity.

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Fig. 2 Emission spectra of *Photinus pyralis* (1) and *Lampyris turkestanicus* (2) luciferases, measured with a spectrofluorometer
Emission spectra were measured by adding 50 μl purified luciferase solution into 2 ml substrate mixture.

Fig. 3 Optimum temperatures of *Photinus pyralis* (●) and *Lampyris turkestanicus* (▲) luciferases
Each luciferase was incubated at 10 °C–45 °C in 50 mM Tricine-NaOH buffer (pH 7.8) containing 5% glycerol and 10 mM β-mercaptoethanol. Activity was measured at different temperatures.

Fig. 4 Remaining activity of *Photinus pyralis* (▲) and *Lampyris turkestanicus* (◆) luciferases
Luciferase activity was assayed after 5 min incubation at each temperature.

Fig. 5 pH optimums of *Photinus pyralis* (●) and *Lampyris turkestanicus* (▲) luciferases
Characterization of *Lampyris turkestanicus* luciferase

ATP present in the reaction mixture [9,20]. Therefore, extensive washing procedures are necessary to eliminate ATP and other components originating from the host organism [21]. For *L. turkestanicus* luciferase the $K_m$ for luciferin was 20 μM; for *P. pyralis* luciferase the $K_m$ for luciferin was 30 μM. However, for *L. turkestanicus* luciferase the $K_m$ for ATP was 95.5 μM, whereas for *P. pyralis* luciferase it was 140 μM. The $V_{max}$ values for different concentrations of ATP were similar for both luciferases. It should be noted that subcloning of the luciferases in the same vector (pET-28a) did not change their kinetic properties.

### Spectra of luciferases

CD spectra of both luciferases obtained in Tricine buffer (50 mM, pH 7.8) are shown in Fig. 6. As indicated in Fig. 6(A), the far-UV CD spectra of luciferase showed a small decrease in negative ellipticity at 208 nm and 222 nm in *L. turkestanicus* luciferase. The near-UV CD spectra indicated that the defined tertiary structure of the *L. turkestanicus* luciferase was decreased compared to *P. pyralis* luciferase [Fig. 6(B)].

### Fluorescence measurements

Intrinsic and ANS fluorescence spectroscopy were used to characterize the microenvironments of Trp and also hydrophobic clusters of luciferases. As indicated in Fig. 7, an increase

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Fig. 5 Effect of pH level on *Photinus pyralis* (■) and *Lampyris turkestanicus* (▲) luciferase activities  Luciferase activity was assayed by injection of diluted luciferase into a mix buffer at each pH level in 0.5 increments from 5.0 to 12.5.

Fig. 6 Far-ultraviolet (A) and near-ultraviolet (B) circular dichroism spectra of *Lampyris turkestanicus* (1) and *Photinus pyralis* (2) luciferases in 50 mM Tricine buffer (pH 7.8) Spectra were taken at 25 °C in 50 mM phosphate buffer (pH 7.8). The concentration of proteins was 1 μM. The excitation wavelength was 295 nm. a.u., arbitrary unit.

Fig. 7 Intrinsic fluorescence spectra of *Lampyris turkestanicus* (1) and *Photinus pyralis* (2) luciferases Spectra were taken at 25 °C in 50 mM phosphate buffer (pH 7.8). The concentration of proteins was 1 μM. The excitation wavelength was 295 nm. a.u., arbitrary unit.
of fluorescence intensity was observed for *P. pyralis* luciferase. Loss of hydrophobic patches on the *L. turkestanicus* protein surface was confirmed by fluorescence measurements using ANS (Fig. 8). ANS fluorescence was clearly enhanced on interaction with native luciferase, as observed earlier [22].

**IR spectra**

Fig. 9 compares the second-derivative spectrum of luciferases at pH 7.8. In γH₂O, the amide I band showed a maximum at 1652 cm⁻¹ for both luciferases. The bands at 1658, 1666, and 1672 cm⁻¹ have been assigned to α-structures, β-turns, and β-sheets, respectively. Changes in luciferase structure seemed to cause a small modification in the amount of α-structures and β-turns. The band at 1658 cm⁻¹ had been previously assigned to the C=O stretching vibration of the side chain amide groups. The most obvious difference was the intensity of the band around 1658 cm⁻¹, which was visibly a shift to longer wave numbers in *L. turkestanicus* luciferase.

**Discussion**

The firefly luciferase gene, *luc*, is frequently used as a reporter of genetic function, or for producing of recombinant luciferase [23]. To simplify the purification process, luciferases with six histidine residues on their N-terminus were used. The luciferase bound to Ni-NTA Sepharose through the strong chelating interaction between the His tag and Ni²⁺ directly from the cell lysate. A high purity recombinant luciferase with a yield of 95% was achieved. The result reported earlier showed that the luciferase responsible for the light emitting reaction in *L. turkestanicus*, although three residues shorter, has 85% sequence similarity with that of the firefly *P. pyralis* [16]. Both enzymes have a similar C-terminus with the same key residues.

**Kinetic differences**

According to its kinetic properties, *L. turkestanicus* luciferase could be considered a suitable indicator for ATP detection. In fact, the *Kₘ* of *L. turkestanicus* luciferase for ATP was lower than *P. pyralis* luciferase, whereas the *Kₘ* towards luciferin was almost identical (Table 1). Using ProSite [24], putative interacting residues with AMP in both luciferases have been identified as 195-IMNSSGSTGLPK-206 [16]. The crystal structure of *P. pyralis* luciferase shows that invariant residues Arg218, His245, Phe247, Ala348, and Lys529 appear to interact with luciferin in luciferase binding site [25]. Furthermore, the residues Asn197, Ser199, Thr343, Tyr340, Ala317, and Gly339 in the *P. pyralis* luciferase sequence are believed to interact with ATP [26]. Using homology modeling, the same residues have been found to interact with ATP in *L. turkestanicus* luciferase (data not shown). Therefore, it could be suggested that the higher affinity of *L. turkestanicus* luciferase for ATP might be due to specific orientation of these residues in the active site of the enzyme or conformational changes.

The time-course of the light decay for both luciferases was also compared (Fig. 1 and Table 1). The rate of light decay for *L. turkestanicus*, similar to another glow worm.
luciferase, was slower than *P. pyralis* [27]. However, *P. pyralis* firefly luciferase produced a reproducibly faster decay, suggesting faster decomposition of the intermediate state of the luciferase reaction or more sensitivity to product inhibition [27].

Similar light emission spectra of the *L. turkestanicus* and *P. pyralis* luciferases (Fig. 2) also suggests similarities in critical residues involved in the formation of substrate intermediate structures. One of the main reasons for differences in the luciferase bioluminescence color is the property of the emitter microenvironment localized in the enzyme active site [28]. The variety of bioluminescence property of the emitter microenvironment localized in the intermediate structures. One of the main reasons for differences in the luciferase bioluminescence color is the property of the emitter microenvironment localized in the enzyme active site [28].

Another intriguing result is that the shape of the bioluminescence spectrum for *L. turkestanicus* luciferase under acidic conditions was slightly changed [30]. In contrast to most firefly (*Lampyridae*) luciferases, luciferase from *L. turkestanicus* showed a minor shift in its emission peak under this condition, similar to that of *P. pyralis* luciferase (click beetles and railroad worms). Although firefly luciferases have high amino acid similarity, multiple sequence alignment of *L. turkestanicus* luciferase with other firefly luciferases revealed certain amino acid changes in *L. turkestanicus* that are essentially sufficient for it to have a pH-independent luminescence spectra profile. Among them is Phe268, a conserved residue in firefly luciferases, which has been substituted by Cys in *L. turkestanicus* [31].

The optimum temperature of both luciferases was similar (Fig. 3). However, the increased temperature sensitivity of the glow worm luciferase (Fig. 4) might make it a slightly better reporter for the study of gene control elements for high turnover mRNAs and unstable proteins in live cells [32]. The precise mechanism responsible for the low thermostability of luciferase from *L. turkestanicus* is not fully understood. The lower thermostability of *L. turkestanicus* luciferase compared to *P. pyralis* luciferase was similar to another glow worm luciferase, as reported earlier [27].

Another property that should be considered in the application of *L. turkestanicus* luciferase is its optimum pH. As indicated in Fig. 5, its optimum pH is 0.5 higher than *P. pyralis* luciferase. Calculation of the pKₐ of essential residues showed that most of the critical residues involved in binding of substrates or catalysis had a higher pKₐ in *L. turkestanicus* compared to *P. pyralis* (Table 2). Therefore, it could be suggested that the higher optimum pH of *L. turkestanicus* luciferase could arise from the higher pKₐ of some critical-reported residues.

### Structural differences

Characterization of *L. turkestanicus* luciferases containing an N-terminal His-tag by spectroscopic devices revealed differences with *P. pyralis* luciferase. It has been shown that the His-tag has very little effect on the structure, activity, or stability of firefly luciferases [33]. Differences in primary structure of *L. turkestanicus* compared to *P. pyralis* luciferase have changed the protein conformation, as confirmed by intrinsic fluorescence. For example, *L. turkestanicus* luciferase has only one Trp at position 419, whereas *P. pyralis* has two Trp residues. W419 can be used as a suitable reporter in the study of *L. turkestanicus* luciferase conformational changes under different conditions. Therefore, as shown in Fig. 7, differences in primary structure and the presence of an additional Trp caused a clear increase in intrinsic fluorescence of *P. pyralis* luciferase, suggesting that Trp(s) are located in a more hydrophobic environment. A minor red shift in the emission spectra was also seen, suggesting that other factors might be involved in fluorescence emission. A similar result has been observed in changes of fluorescence intensity in native and a mutant form of bacterial luciferase [18,34]. The hydrophathy profiles of these luciferases were similar, however, there were some regions that showed major differences. In particular, the regions from residue 140 to 200 and from 300 to 340, which indicates the additional hydrophobic residues in *P. pyralis* luciferase, showed major differences in hydrophobic pattern when compared to *L. turkestanicus* luciferase. The presence of some substitution in the *L. turkestanicus* luciferase primary structure is associated with decreased surface hydrophobicity.

<table>
<thead>
<tr>
<th>Residue (side chain)</th>
<th><em>L. turkestanicus</em></th>
<th><em>P. pyralis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys206</td>
<td>11.735</td>
<td>10.866</td>
</tr>
<tr>
<td>His245 (ND1)</td>
<td>6.667</td>
<td>6.487</td>
</tr>
<tr>
<td>His245 (NE1)</td>
<td>6.829</td>
<td>6.430</td>
</tr>
<tr>
<td>Glu344 (OE1)</td>
<td>6.063</td>
<td>5.719</td>
</tr>
<tr>
<td>Glu344 (OE2)</td>
<td>5.999</td>
<td>5.592</td>
</tr>
<tr>
<td>Asp422 (OD1)</td>
<td>5.335</td>
<td>5.738</td>
</tr>
<tr>
<td>Asp422 (OD2)</td>
<td>5.371</td>
<td>5.722</td>
</tr>
<tr>
<td>Arg437 (NH1)</td>
<td>12.785</td>
<td>11.878</td>
</tr>
<tr>
<td>Arg437 (NH2)</td>
<td>13.232</td>
<td>12.331</td>
</tr>
<tr>
<td>Lys529</td>
<td>11.784</td>
<td>10.962</td>
</tr>
</tbody>
</table>
compared to *P. pyralis* luciferase, as was also confirmed by ANS fluorescence spectroscopy (Fig. 8). Such a feature could be potentially involved with considerable conformational changes among these luciferases. It has been reported that such changes could affect the bioluminescence colors and other kinetic properties of luciferases [35]. The far-UV and near-UV CD spectra of both forms were taken, and they reflected substantial changes in the tertiary structure of the protein with small changes in its secondary structure (Fig. 6). The type of changes observed in the CD and IR spectra of luciferases suggests conformational differences.

In conclusion, results presented in this study show that *L. turkestanicus* luciferase, similar to *P. pyralis* luciferase, was successfully expressed in sufficient amounts in *E. coli*. Moreover, the kinetic properties of *L. turkestanicus*, including higher affinity to ATP, show it can be used as a suitable reporter in molecular biology.

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**References**

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