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Purification and characterization of a novel thermostable luciferase from *Benthosema pterotum*

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ABSTRACT

A novel luciferase from *Benthosema pterotum*, collected from Port of Jask, close to Persian Gulf, was purified for the first time, using Q-Sepharose anion exchange chromatography. The molecular mass of the novel enzyme, measured by SDS–PAGE technique, was about 27 kDa and its K_m value is 0.4 μM; both values are similar to those of other coelenterazine luciferases. *B. pterotum* (BP) luciferase showed maximum intensity of emitted light at 40 °C, in 20 mM Tris buffer, pH 9 and 20 mM magnesium concentration. Experimental measurements indicated that BP luciferase is a relatively thermostable enzyme; furthermore it shows a high residual activity at extreme pH values. Its biological activity is strongly inhibited by 1 mM Cu^{2+}, Zn^{2+} and Ni^{2+}, while calcium and mainly magnesium ions strongly increase BP luciferase activity. The *B. pterotum* luciferase generated blue light with a maximum emission wavelength at 475 nm and showed some similarity with other luciferases, while other parameters appeared quite different, in this way, confirming that a novel protein has been purified.

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1. Introduction

Bioluminescence is referred to the light emission by a living organism due to a specific biochemical reaction. This interesting feature of the organisms could highly influences behavioral and ecosystem dynamics [1]. Luminescence, mostly observed in marine species, is generally higher in deep-living genera than in benthic or shallow organisms. However, among creatures living in land, fireflies, beetles, springtails and fungi have shown some bioluminescent activities [1,2]. *Myctophids* are mesopelagic fishes from family of *Myctophidae*, represented by about 250 species in 33 genera. They are called "*Lanternfishes*", a family of the largest fish species inhabit in deep sea and oceans but not in the Arctic [3–5]. Although a significant number of these species are identified, biochemical knowledge about their bioluminescence is limited and studies of the photochemical systems, of unidentified species are of scientific importance [6–8]. *Benthosema pterotum* species from this family are among the most abundant and wide spread fishes in deep oceans. Their average length is usually 15 mm, ranging from 2 to 30 mm, and their weight from 2 to 6 g. They have a specific big and shiny head with oval eyes and small body; color varies

from greenish silver in shore species to dark brown for deep sea species [3,5]. The most specific feature that discriminates them from all similar organisms in the sea is the presence of luminescence photophores on their head and body. *B. pterotum* shows the presence of non-bacterial bioluminescent organs known as "Photophores", which are ventrally arranged and species-specific (Fig. 1). These are complex structures consisting of modified cup-like (lens) scales, containing photogenic tissue [9–11]. Expanded chemical knowledge on their luminescence is rather meager at present. Clearly, some further study is needed to identify the luminescence system of this species. Discussing the type of luminescence systems involved, they might utilize a luciferin–luciferase system, a photoprotein system, or other type of luminescence system that is not yet known. In this research, the photosystems of *B. pterotum* has been studied for the first time and a novel luciferase, the enzyme catalyzing the reaction responsible of the emission of blue light, was purified and characterized. Considering the fact that *B. pterotum* is exclusively found in Persian Gulf and the luciferase responsible for its unique bioluminescence behavior it not studied so far, we aimed to extract, purify, identify and characterize this novel luciferase. The specific information obtained from this research has now opened the way to further characterize the enzyme and design for its various ecological, industrial and medical applications.

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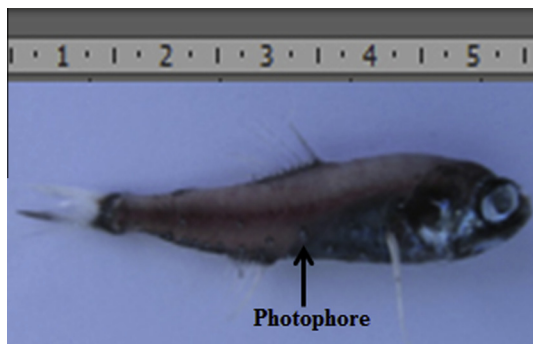E-mail address: khajeh@modares.ac.ir (K. Khajeh).

Fig. 1. *Benthosema pterotum* from Jask port, Persian Gulf; position of luminescence photophores is indicated.

2. Materials and methods

2.1. Materials

Coelenterazine hcp was purchased from Sigma (St. Louis, MO, USA) and Q-Sepharose from Pharmacia (Uppsala, Sweden). All other chemicals were reagent grade and purchased from Merck (Darmstadt, Germany).

2.2. Specimen collection

B. pterotum (*Lantern fish*), caught from Port of Jask, was immediately frozen in liquid nitrogen and transported to our enzymology laboratory in Tehran. The frozen samples were then stored at −80 °C until used.

2.3. Selective extraction and purification of luciferase from B. pterotum photogenic organ

The yellowish photogenic organ was washed with distilled water and scraped with scalpel from luminescent photophores. Photogenic tissue was re-suspended in 10 ml of lysis buffer (20 mM Tris–HCl, pH 7.8, 0.5 mM NaCl, 50 mM EDTA and 1 mM PMSF). The suspension was subjected to sonic disruption at 0 °C (10 s on, 45 s off for 15 min) and the cell debris were discarded by centrifugation at 15,000*g* for 20 min. The supernatant (cell free extract) was subjected to ammonium sulfate precipitation (85% saturation). The protein was then dissolved in minimal amount of 20 mM Tris–HCl buffer, pH 7.8 and dialyzed against the same buffer for 24 h at 4 °C, changing dialysis buffer every 8 h. The dialyzed sample was loaded onto Q-Sepharose column equilibrated with Tris buffer. After washing the column, adsorbed proteins were eluted with a linear 0–2 M NaCl gradient in 20 mM Tris–HCl buffer (pH 7.8). The flow rate was 3 ml/min and 2 ml fractions were collected. Fractions exhibiting luciferase activity were pooled and concentrated by an Amicon 8050 ultrafiltration system equipped with a 10 kDa cut-off membrane.

The proteins were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) using a Mini-PROTEAN electrophoretic system (BioRad). Electrophoresis was carried out at a constant current of 80 mA [12] and the gels were stained with Coomassie brilliant blue R-250 [13].

2.4. Spectral measurements, determination of bioluminescence activity and protein concentration

Bioluminescence emission spectra were obtained using a Biotek Synergy H4 Multi-Mode Plate Reader with its excitation lamp turned off; operating at room temperature in the range 400–700 nm in a plate reader .The spectra were automatically corrected for the spectral photosensitivity of the equipment with an internal program identified as M-correct on the instrument by the manufacture. The bioluminescence spectra were measured in 20 mM Tris–HCl, pH 7.8, and initiated by injection of MgCl$_2$ solution in the same buffer. The concentration of free magnesium was around 30 mM in order to provide an approximately constant light level during the spectral scan. In cases where a substantial change in bioluminescence intensity took place during the spectral scan, the data points were also corrected for bioluminescence decay. The emission slit was 17 nm, emission step was 10 nm, delay after play movement was 100 ms, and the scan rate equal to 170 nm/s.

Luciferase activity was measured by a Sirius tube luminometer, Berthold Detection System, Germany. In a typical experiment, 25 µl of the enzyme solution was mixed with coelenterazine hcp (luciferin) as substrate, in 20 mM Tris–HCl buffer, pH 7.8, and the light emitted immediately recorded and integrated at 0.2 s intervals. K_m and V_{max} values were determined by Lineweaver–Burk plots. All experiments were carried out in triplicate at least. Protein concentration was estimated by the Bradford method, using bovine serum albumin as standard [14].

2.5. Effect of pH and temperature on enzyme activity and stability

Luciferase activity versus pH was measured at room temperature in the range 2–12 pH values, using a mixed buffer containing 20 mM acetate, phosphate and glycine, according to the assay conditions.

In order to verify the activity dependence with temperature, enzyme activity were carried out in the range of 10–65 °C, in 20 mM Tris–HCl buffer pH 7.8, as described above.

For determination of optimal reaction pH and temperature, the maximum activities obtained under the conditions tested were taken as 100%.

Enzyme stability to pH was checked incubating the enzyme at room temperature in 20 mM of mixed buffer at pH 3 and 12 for different intervals of time; then pH value was adjusted to 7.4 and the residual activity measured.

Luciferase thermal stability was measured incubating the enzyme in 20 mM Tris–HCl buffer pH 7.4, at 55, 65 and 90 °C for different intervals of time; the solution was then cooled on ice and the residual activity determined under the assay conditions.

Control measurements were carried out determining the activity of the same enzyme solutions kept on ice for the thermal stability and in the buffer at pH 7.8 for the pH stability experiment.

2.6. Effect of metal ions on BP luciferase activity

Enzyme activity was measured at the usual experimental conditions in the presence of various chloride metal ions in the concentration range of 0–40 mM.

2.7. Calculation of thermodynamic parameters

The rate constants of luciferatic reaction (k_{cat}) were used to calculate the activation energy according to the Arrhenius equation [15].

$$k = Ae^{-Ea/RT} \tag{1}$$

where k (s^{-1}) is the rate constant at temperature T (K), A is a pre-exponential factor related to steric effects and the molecular collision frequency, R is the gas constant (8.314 J mol^{-1} K^{-1}), and E_a the activation energy of the reaction. Hence, a plot of ln k as a function of $1/T$ gives a curve with slope of $-Ea/R$. The thermodynamic parameters of activation were determined as follows:

$$\Delta G^{\#} = RT \ln(K_B T/h) - RT \ln k_{cat} \tag{2}$$
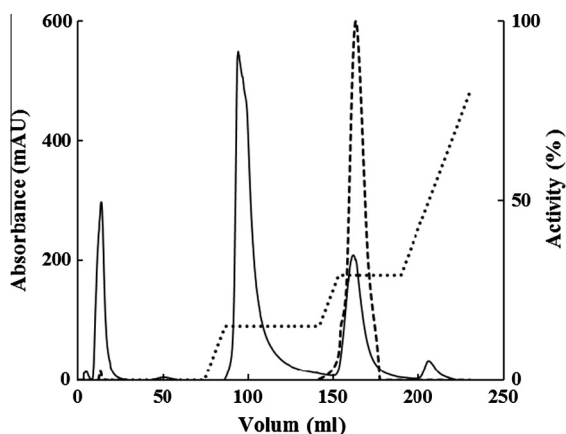
Fig. 2. Anion-exchange chromatography on a Q-Sepharose column. The extracts (in 20 mM Tris–HCl buffer, pH 7.8) were applied to the column which was equilibrated with the same buffer. The BP luciferase was eluted with a linear 0–2 M NaCl gradient at a flow rate of 3 ml/min. Chromatographic profile of luciferase activity (dotted line), protein absorbance (solid line) in Q-Sepharose eluate and salt concentrations (dash dot line).

Table 1
Purification procedures of BP Luciferase from *Benthosema pterotum*.

Steps of purification	Total protein (mg)	Total activity ($\times 10^5$ RLU)	Specific activity ($\times 10^5$ RLU mg^{-1})	Yield (%)	Purification fold
Cell extract	123	161 ± 12	1.3 ± 0.1	100	1
(NH$_4$)$_2$SO$_4$ precipitation	33	135 ± 9	4.1 ± 0.1	83.8	3.2
Q-Sepharose	12	118 ± 7	10 ± 0.2	73.3	7.7

$$\Delta H^{\#} = E_a - RT \tag{3}$$

$$\Delta S^{\#} = (\Delta H^{\#} - \Delta G^{\#})/T \tag{4}$$

where k_B is the Boltzman constant (1.3805×10^{-23} J K^{-1}), h the Planck's constant (6.6256×10^{-34} J s), and k_{cat}(s^{-1}) is the rate constant at temperature T(K).
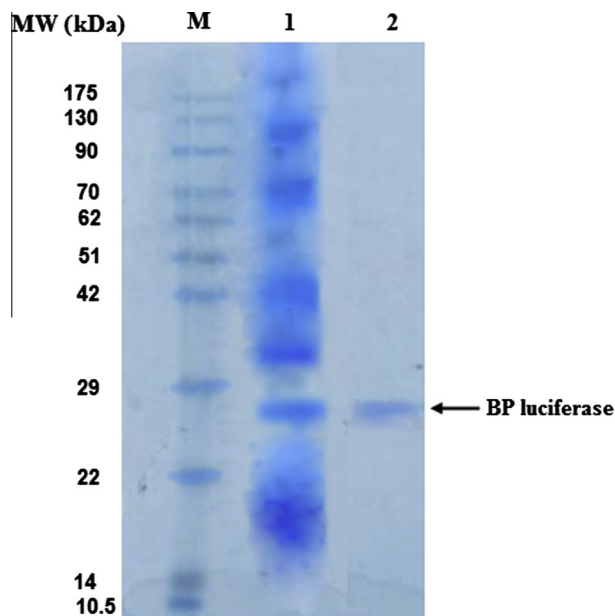
Fig. 3. SDS–PAGE of BP luciferase after purification by chromatography. Lane 1: cell extract; lane 2: purified luciferase. First lane is the molecular mass marker.

All of the results are taken as the mean value obtained from at least three repeated experiments in a typical run to confirm reproducibility.

3. Results and discussion

3.1. Extraction, purification and molecular mass determination of the enzyme

The BP luciferase was purified from *B. pterotum* using Q-Sepharose chromatography. The supernatant obtained from cell free extract was concentrated by precipitation with ammonium sulfate. The resulting protein solution was then loaded into a Q-Sepharose column. BP luciferase was eluted from the third peak with 0.29 M NaCl (Fig. 2). The overall purification procedure is summarized in Table 1.

Fractions exhibiting luciferase activity were pooled out and concentrated using Amicon 8050 ultrafiltration system equipped with a 10 kDa membrane cut-off. SDS–PAGE course showed electrophoretic purity grade for the enzyme. The molecular mass of BP luciferase was about 27 kDa (Fig. 3), a value in the range of that of other coelenterazine luciferases, with the exception of *Oplophorus* luciferase (*gracilirostris*) which shows a protein molecular mass value about four times higher [16–20].

The specific activity of BP luciferase was 1.3×10^5 RLU mg^{-1} and 4.1×10^5 RLU mg^{-1} after cell extraction and ammonium sulfate precipitation, respectively. In the Q-Sepharose anion exchange chromatography, the BP luciferase was separated from most of extra cellular proteins. It was shown that luciferatic specific activity was 10×10^5 RLU mg^{-1}.

3.2. BP luciferase apparent kinetic constants and bioluminescence emission spectra

BP luciferase activity was assayed at different coelenterazine concentrations in the range 0.1–3.3 μM substrate. In Table 2 columns 1–4, the values of K_m, V_{max}, k_{cat} and k_{cat}/K_m, are reported. K_m value of BP luciferase is in good agreement, varying of about ±3 times, with that of several other luciferases reported in literature [20–22], and it appears very low, indicating high affinity for the substrate. Like the most bioluminescence sea animals, BP luciferases generated blue light with a maximum emission wavelength at 475 nm (Fig. 4). Coelenterazine luciferases have been isolated from about 10 kinds of organisms, including the anthozoans *Renilla* and *Ptilosarcus*, the scyphozoan jellyfish *Periphylla*, three kinds of decapod shrimps, and two copepod species; some of them have been cloned. All of the luciferases cause the emission of a bluish light when they catalyze the oxidation of coelenterazine [20].

3.3. Temperature and pH effect on enzyme activity and stability

From Fig. 5a, where the behavior of BP luciferase activity with temperature in the range of 10–65 °C is reported, it appears that temperature of maximum activity is 40 °C, a value similar of that found for Decapod shrimp, but higher than that referred to *Ptilosarcus* luciferase (23 °C) and *Renilla* luciferase (32 °C) [20]. Furthermore, residual activity is high, more than 50% of the maximum, in a wide temperature range (from about 20 to 55 °C) if compared with other coelenterazine luciferases. Moreover, it appears that about 30% of its maximum bioluminescence activity is retained at low (10 °C) and very high (65 °C) temperatures. In the bioluminescence systems of *Cypridina*, *Latia*, *Chaetopterus*, the relative activity decrease steeply when the temperature is raised, and become almost zero at a temperature higher than 40 °C [23].

Table 2
The apparent kinetic constants of BP luciferase. An enzyme concentration of 0.08 mg/mL for all kinetic measurements was used.

Kinetics parameters				Activation			
K_m (μM)	V_{max} (RLU)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	E_a (kcal/mol)	$\Delta H^{\#}$ (kcal/mol)	$\Delta G^{\#}$ (kcal/mol)	$\Delta S^{\#}$ (cal/mol K)
0.4 ± 0.01	2 ± 0.1 × 10^6	13.7 ± 0.3	34.3 ± 0.8	6.3 ± 0.8	5.7 ± 0.3	16.7 ± 0.5	−35 ± 0.7
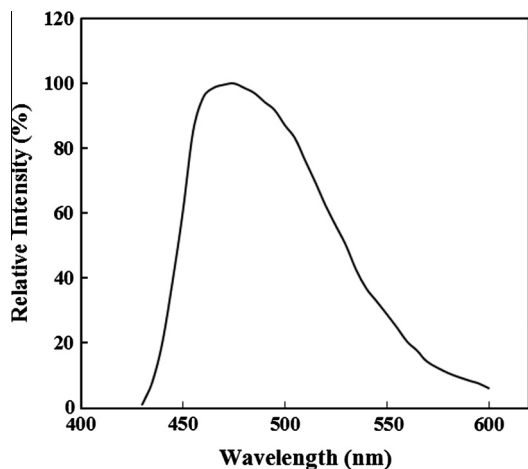
Fig. 4. Bioluminescence spectrum of coelentrazine catalyzed by BP luciferase in 20 mM Tris–HCl, pH 7.8. Light intensity is expressed in RLU; the wavelength is in nanometers; and the maximum emission was at 475 nm. The initial concentration of magnesium was 30 mM.
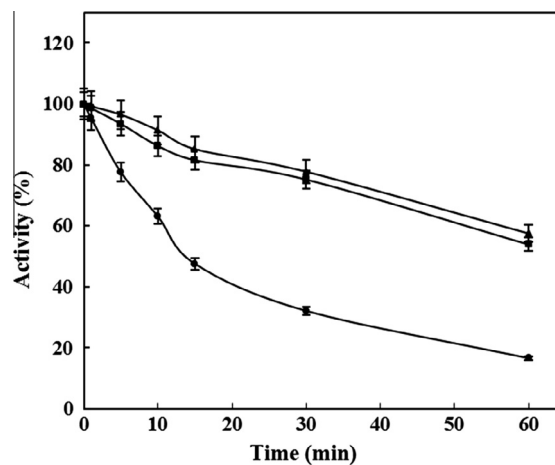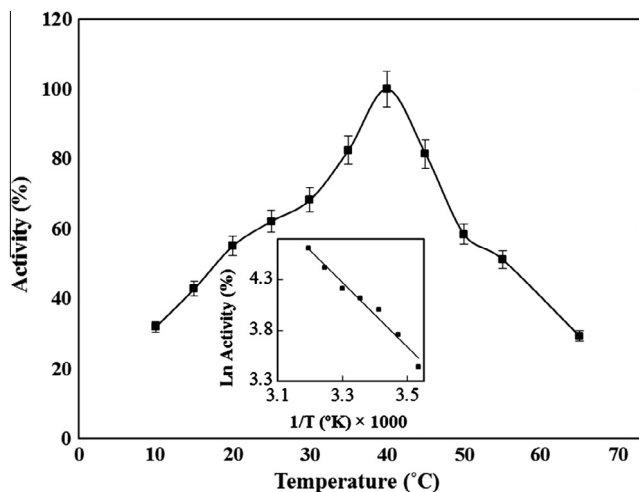

Fig. 5b. Irreversible inactivation of BP luciferase after incubation at different times at 55 (filled triangles), 65 (filled squares) and 90 °C (filled circles). The activity of the same enzyme solution, kept on ice, was considered as the control (100%).

From the Arrhenius plots (inset of Fig. 5a), graphed utilizing BP luciferase activity values in the temperature range of 10–40 °C, a value of activation energy of the catalytic reaction of 6.3 kcal mol^{-1} K^{-1} was obtained. In Table 2, columns 6–8, values of activation free energy ($\Delta G^{\#}$), activation enthalpy ($\Delta H^{\#}$) and activation entropy ($\Delta S^{\#}$) for the catalytic reaction are reported. The negative value of activation entropy ($\Delta S^{\#}$) indicates that the structure of enzyme–substrate at transition state is characterized by very few degrees of freedom [24].

The time dependence of the BP luciferase stability with temperature, measured after incubating the enzyme at 55, 65 and 90 °C, evidences a half life of more than 60 min at T ⩽ 65 °C and of about

15 min at 90 °C (Fig. 5b), values better than those referred to other luciferase, such as Gaussia luciferase, Cypridina noctiluca luciferase and Vargula hilgendorfii luciferase [25–27] which evidence negligible activity after 60 min incubation at 65 °C.

Activity vs pH behavior of BP luciferase in the pH range 2–12, shows wide asymmetrical bell-shaped dependence with a maximum value at about pH 9 (Fig. 6a) and a relative high residual activity at pH 12 (≅40% of the maximum). This profile appears similar to those of copepod Gaussia princeps luciferase, squid Watasenia scintillans and copepod Metridia luciferase [28–30]. In the case of some luciferase, photoproteins can be inactive at some pH values, without resulting in permanent inactivation. For example, the luminescence of euphausiids can be quenched at pH 6, that of

Fig. 5a. Behavior of BP luciferase activity with temperature in the range 10–65 °C. The activity at optimal temperature was taken as 100%. In the inset is the respective Arrhenius plots. Activation energies for BP luciferase was 6.3 kcal mol^{-1} K^{-1}.
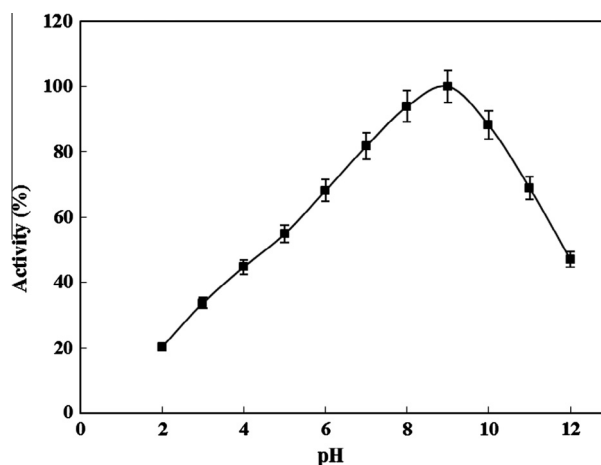
Fig. 6a. Behavior of BP Luciferase activity with pH in the range 2–12. The relative activity (%) refers to the percentage of the initial reaction rate obtained by the enzyme in the maximum pH compere to other pH conditions.
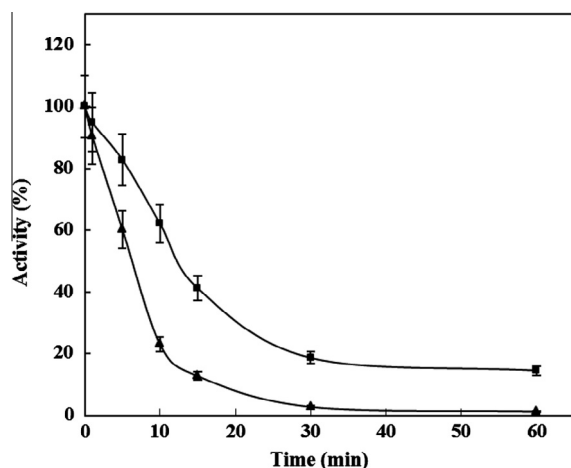
Fig. 6b. Irreversible inactivation of BP luciferase after incubation for different times at pH 3.0 (filled triangles) and 12.0 (filled squares). The activity of untreated enzyme was taken as 100%.
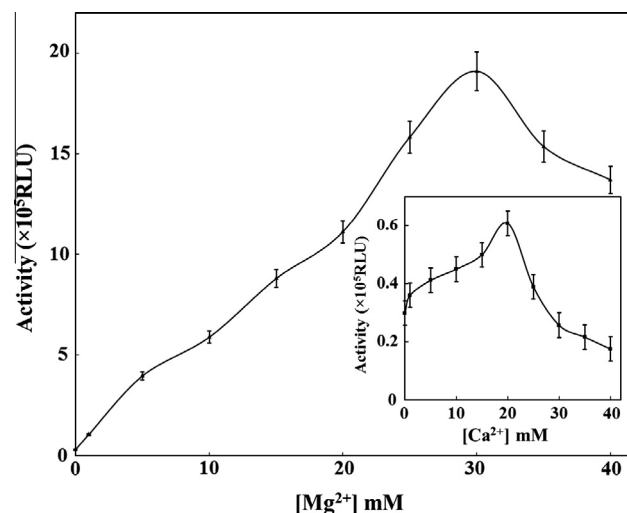

Fig. 7. Activity of BP luciferase at different concentrations of magnesium. The inset is the activity profile of the enzyme in various concentrations of calcium.

Table 3
Effect of various metal ions and their concentrations on BP luciferase.[a]

Metal ions (mM)		Relative activity (%)
Control (no add)		100
Na$^+$	1	100
	5	96
	10	93
	20	87
K$^+$	1	80
	5	73
	10	65
	20	51
NH$_4^+$	1	97
	5	91
	10	82
	20	75
Ca^{2+}	1	120
	5	137
	10	150
	20	200
Mg^2	1	350
	5	1320
	10	1960
	20	3740
Cu^{2+}	1	0.09
	5	0.00
	10	0.00
	20	0.00
Zn^{2+}	5	7
	10	0.05
	20	0.00
	40	0.00
Ni^{2+}	1	17.3
	5	2.1
	10	0.00
	20	0.00

[a] All metal ions were added as chloride salts.

aequorin at pH 4.2–4.4, and the luciferase of the decapod shrimp *Oplophorus* becomes inactive at about pH 4. In the case of *Cypridina* the acidification of an extract to pH below 5 induces irreversible inactivation of the luciferase [20].

The stability of the BP luciferase was measured at two extreme pH, 3.0 and 12. As shown in Fig. 6b, the enzyme retains residual activity at alkaline pH values, indicatively about 15%, after 60 min of incubation; while a nearly complete inactivation of enzyme at pH 3 after 30 min was observed.

3.4. Effect of metal ions on luminescence activity

In Table 3, the results relative to the effect of metal ions at different concentration on luminescence activity are reported. Experimental data show that monovalent ions, such as Na$^+$, K$^+$, and NH$_4^+$, have little effect decreasing activity with concentration, probably owing to ionic strength effect. On the contrary, strong inhibition was observed in the presence of the heavy metal ions Cu^{2+}, Zn^{2+} and Ni^{2+}. At 1 mM ions concentration, residual luciferase activity was about 17% for nickel, 7% for zinc and < 1% for copper.

Among coelenterazine-type luciferase, Periphylla luciferases-O and luciferase-L were strongly inhibited by micromolar orders of Cu^{2+} [31] and BFP-aq was also strongly inhibited by 1 µM Cu^{2+} [29,32,33]. These results suggest that inhibition by Cu^{2+} of BP luciferase might occur in the same manner, but more extended research is needed in order to draw the exact mechanism.

Calcium and magnesium ions strongly increase BP luciferase activity, with a factor of about 2 in the case of Ca^{2+} and until 37 for Mg^{2+}, according to literature [34–36]. In fact these ions participate as cofactor in bioluminescence reaction for many calcium and magnesium sensitive photosystems. The dependence of activity with concentration of these ions in a more large concentration range (Fig. 7) shows direct correlation until about 20 mM in the case of calcium and 30 mM in the case of magnesium, followed, in both the cases by a high activity decrease. Maximum activity of enzyme in the presence of 20 mM calcium and 30 mM magnesium was 0.6 × 10^5 RLU and 20 × 10^5 RLU, respectively.

4. Conclusion

In the present study a new luciferase from the yellowish photogenic organ of *B. pterotum* was purified to homogeneity on SDS–PAGE by column chromatography. The new luciferase shows some similarity with other luciferases, while other parameters appear very different, in this way confirming the originality of the investigation. Biotechnology applications appear very interesting, in particular on the possibility to generate some structural variations of BP luciferase in order to improve its bioluminescence applications.

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