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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.

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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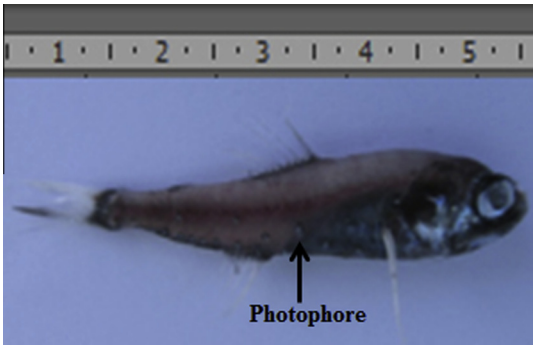

Fig. 1. *Benthosema pterotum* from Jask port, Persian Gulf; position of luminescence photophores is indicated.

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].

$$k = Ae^{-Ea/RT} \quad (1)$$

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:

$$\Delta G^{\#} = RT\ln(K_B T/h) - RT\ln k_{cat} \quad (2)$$