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Short Communication

New insights on expression and purification of a recombinant luciferase protein from the bioluminescence marine dinoflagellate *Pyrocystis lunula*

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ABSTRACT. Bioluminescence is interesting, among other reasons, for the various technological applications that have been derived from it. Among these applications, developing visualization techniques to record the expression of one or more genes simultaneously in real-time are particularly useful. With this in mind, this study aimed to generate a recombinant *Pyrocystis lunula* luciferase protein (Luci D2-3 partial CDS). As the main results, i) a fragment of 1467 bp of the luciferase (LCFb) mRNA of the dinoflagellate *P. lunula*, containing part of domain 2 and all of the domain 3, was cloned in the pET28a vector; ii) the constructed vector was used to transform *Escherichia coli* to express the recombinant protein and subsequently purify it through an affinity chromatography procedure using a His-Tag; and iii) the purified protein (~50 kDa) was further analyzed by mass spectrometry to confirm its identity. Despite being unable to perform activity tests with the luciferin substrate, the evidence from previous studies indicates that the recombinant protein obtained in this case is enzymatically active. Due to the limited number of currently available luciferases, synthesizing this recombinant protein represents a useful tool, especially in designing expression assays coupled to multiple reporter genes, thus expanding the palette of proteins available for developing this type of biotechnological advances.

Keywords: *Pyrocystis lunula*; bioluminescence; dinoflagellate; protein expression; protein purification; reporter genes; gene cloning

Dinoflagellate luciferase (LCF) catalyzes the oxidation of the substrate luciferin, producing an electronically excited oxyluciferin that emits blue light at a λmax of 474 nm. In *Pyrocystis lunula* LCF (137 kDa), there are three catalytic domains and a single LCF/LBP (Luciferin-Binding Protein) N terminal domain, preceded by helicase bundle domains. Each catalytic domain is enzymatically active (Valiadi & Iglesias-Rodriguez 2013, Fajardo et al. 2020, Shih et al. 2022). The coding sequence of *P. lunula* LCF is organized in different copies arranged in tandem within the genome (Morishita et al. 2002). In this species, there are three variants of the LCF mRNA (LCFa GenBank AF394059.1; LCFb GenBank AF394060.1; LCFc

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GenBank AF394061.1), and their primary structure is highly conserved (Okamoto et al. 2001, Valiadi & Iglesias-Rodriguez 2013). Furthermore, luciferin isolated from *P. lunula* can cross-react with the LCFs of other bioluminescent dinoflagellate species. It can even cross-react with the bioluminescent system of *Euphausia superba* (krill) (Schmitter et al. 1976).

Most of the attention on *P. lunula* is due to various technological applications derived from the bioluminescence of this species (Perin et al. 2022, Espinosa-Rodríguez et al. 2023). For example, the bioluminescence capacity of *P. lunula* can be quantified by spectrophotometry due to an inverse relationship between the normal bioluminescent output, which declines in the presence of increasing concentrations of toxicants in the water (Craig et al. 2003, Perin et al. 2022). Following this finding, the QwikLite™ 200 bioassay was developed, a portable test kit that measures the toxicity of different pollutants (ammonia, copper, diuron, tributyltin) in the marine environment. Additionally, a more recent bioassay is used to assess potential instances of groundwater pollution related to fracking and natural gas extraction (Stauber et al. 2008, Hildenbrand et al. 2015).

Furthermore, a wide spectrum of *in vitro* and *in vivo* analytical techniques have been developed based on various bioluminescent systems, including environmental monitoring, tests for different analytes, drug screening, immunoassays, bio-imaging, as well as gene expression assays, among others (Kotlobay et al. 2020, Love & Prescher 2020). With the development of luminescent reporters, new possibilities for additional applications are promising. Following this paradigm, it has been possible to develop a new bioluminescence system with more efficient light emission (Hall et al. 2012).

Luminescence quantification, using LCF to catalyze the oxidation of luciferin and produce light, is widely used in biological phenomena assays to investigate the location of molecules of interest and for reporter gene assays. Indeed, LCFs from *Photinus pyralis* (firefly), *Renilla reniformis* (sea pansy), *Gaussia princeps* (copepod), *Pyrophorus noctilucus* (beetle), and even aequorin from *Aequorea victoria* (jellyfish), have been used in this type of analysis. Luminescence detection procedures are simple: live cell washing and substrate (luciferin) addition, allowing a higher throughput (Kotlobay et al. 2020, Love & Prescher 2020).

During the past decade, applications have focused on cell tracking and gene expression assays, but now new research is pushing the limits of what can be visualized. LCF-luciferin reactions can be linked to light-activatable proteins, thus triggering signal transduction and other downstream events. Nowadays, novel-engineered LCFs and luciferins are expanding the range of optical imaging and are used to control downstream biological processes (Williams & Prescher 2019, Love & Prescher 2020). With this perspective, our main goal was the synthesis of a recombinant *P. lunula* LCFb (Luci D2-3 partial CDS) to use this recombinant enzyme in processes of biotechnological interest.

Culture conditions

P. lunula (strain CCAP 1131/1) was cultured in F2 medium (Guillard & Ryther 1962), supplemented with vitamins, at a temperature of 18-20°C, the salinity of 31 g L^{-1}, and under a 12:12 h light-dark photoperiod (Fajardo et al. 2019). Cells were sampled (5 mL) for RNA analysis during the lunate coccoid stage.

Cloning

RNA isolation, cDNA synthesis, and PCR amplification

Total RNA was isolated following the instructions of the NucleoSpin RNA® kit (Macherey-Nagel); the RNA quality was verified using the Agilent RNA 6000 Nano® kit. The cDNA was synthesized with the SuperScript® First-Strand Synthesis System for RT-PCR kit (Invitrogen), following the manufacturer's instruction. PCR amplification was done using the OneTaq® DNA Polymerase (New England Biolabs) kit. The reaction conditions were the following: 10 µL of 5x OneTaq Standard Buffer, 1 µL of 10 mM dNTPs, 1 µL each of 10 µM of Luci D2-3 F: 5'-GGC ATA TGT GGG AGA TGG AGT CTG GAA AGT-3', and Luci D2-3 R: 5'-GGG TCG ACT CAT GCT TTG AAG CTT GTG G-3', including the cutting site for *Nde*I and *Sal*I (underlined sequences), respectively, 0.5 µL of OneTaq DNA polymerase (2.5 U), and 1 µL of cDNA (70 ng μL^{-1}), for a total reaction volume of 50 µL. With the primers Luci D2-3 F and Luci D2-3 R, a fragment of 1467 bp (partial mRNA of LCFb of *P. lunula*, corresponding to a part of 2nd domain and complete 3rd domain, GenBank AF394060.1) were obtained (Fig. 1). The thermocycling program was as follows: initial denaturation at 94°C for 30 s, followed by 20 cycles at 94°C for 30 s, 68°C for 30 s, and 68°C for 4 min, with a final extension cycle at 68°C for 5 min. The amplified products were analyzed by electrophoresis (1% agarose, 90 V, 1 h, Gel-Red, 100 bp Plus DNA Ladder 02002-500 AccuRuler). The isolation of the fragment was carried out following the instructions of the GeneJET Gel Extraction kit (Thermo Scientific), and

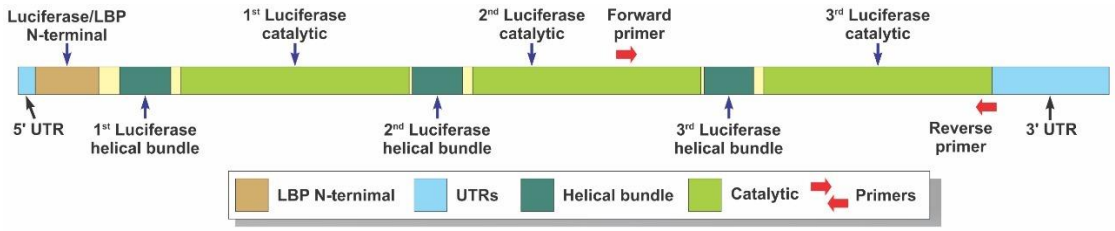

Figure 1. Structure of *P. lunula* luciferase mRNA (GenBank AF394060.1) showing the three catalytic domains (shown in green) and the fragment amplified by the designed primers Luci D2-3 F and Luci D2-3 R (shown by red arrows).

its purity and quantity were verified by electrophoresis (1% agarose, 90 V, 1 h, Gel-Red, 100 bp Plus DNA Ladder 02002-500 AccuRuler) and using a spectrophotometer. The fragment was also sequenced using the Sanger method by Big-Dye termination at Macrogen (South Korea) to verify the sequence of the amplified fragment.

Restriction enzyme digestion

In order to linearize the vector pET28a (+) (Novagen), the following reaction was carried out: pET28a 7 µL (269.2 ng μL^{-1}), 10x Buffer D 10 µL, *Nde*I (10 U μL^{-1}) 2 µL, *Sal*I (10 U μL^{-1}) 2 µL, and water 79 µL, for a total reaction volume of 100 µL. The reaction was incubated for 4 h at 37°C, and subsequently, 2 µL of alkaline phosphatase (1 U μL^{-1}) and 11 µL of FastAp 10x buffer were added. The mixture was additionally incubated for 1 h at 37°C. In the case of the amplified fragment, the following reaction was carried out: 20 µL of isolated DNA (Luci D2-3 partial CDS: 35 ng μL^{-1}), 10 µL of 10x Buffer D, 2 µL of each enzyme: *Nde*I (10 U μL^{-1}), *Sal*I (10 U μL^{-1}) and 66 µL of sterile Milli-Q water, for a total reaction volume of 100 µL. Following this, the mixture was incubated at 37°C during 4 h. Both vector and amplified fragment were purified following the instructions of the GeneJET Gel Extraction kit (Thermo Scientific), and the resulting fragments were analyzed by electrophoresis (1% agarose, 90 V, 1 h, Gel-Red, 100 bp Plus DNA Ladder 02002-500 AccuRuler).

Ligation

Subsequently, 2 µL of the linearized vector (pET28a +; 5,369 bp; 13.4 ng μL^{-1}) was mixed with 4 µL of the fragment (Luci D2-3 partial CDS: 17.2 ng μL^{-1}) and incubated for 5 min at 70°C, before being cooled on ice for 15 min. Later, 13 µL of nuclease-free water, 1 µL of T4 DNA ligase enzyme (5 U μL^{-1}) (Thermo Scientific), and 5 µL of 5x Rapid Ligation Buffer were added for a total reaction volume of 25 µL. Then, the mixture was incubated at 22°C for 1 h.

Transformation

Frozen chemically competent *E. coli* TOP-10 cells were used for the transformation process. The transformation was carried out according to the following protocol: 50 µL of the competent cells were mixed with 5 µL of the ligation mixture and incubated on ice for 20 min. Subsequently, a thermal shock at 42°C was applied, lasting 45 s, followed by immediate incubation on ice for 5 min. Later, 200 µL of LB medium was added, and the cells were incubated for 1 h at 37°C with shaking (190 rpm). The reaction mixture was spread on solid medium plates (LB/kanamycin, 50 µg mL^{-1}) and incubated overnight at 37°C.

Colony PCR

The following protocol was used to verify the insertion of the partial LCFb gene: three colonies were selected for individual PCR amplification by mixing cells with 12.5 µL of 2x GoTaq® Green Master Mix (Promega), 1.25 µL of primer Luci D2-3 F (10 µM), 1.25 µL of primer Luci D2-3 R (10 µM), and 10 µL of water for a total reaction volume of 25 µL. The thermocycler program was as follows: initial denaturation at 95°C for 5 min, followed by 20 cycles at 95°C for 30 s, 72°C for 30 s, and 72°C for 4 min, with a final extension cycle at 72°C for 5 min. The amplified products were visualized by electrophoresis (1% agarose, 90 V, 1 h, Gel-Red, 100 bp Plus DNA Ladder 02002-500 AccuRuler). The amplified fragment of 1467 bp (partial mRNA D2-3 of LCFb from *P. lunula*, GenBank AF394060.1) was expected.

Isolation of plasmid DNA, clone screening by PCR, and restriction analyses

According to the manufacturer's instructions, plasmid DNA was isolated from three colonies grown in the plates with kanamycin using the GeneJET Miniprep kit (Thermo Scientific). Clone screening was carried out by PCR as follows: 1 µL of the plasmid DNA extracted from each colony (concentrations between 54.4-104.4

ng μL^{-1}) was mixed with 12.5 µL of 2x GoTaq® Green Master Mix (Promega), 1.25 µL of primer T7-F: 5'-AAT ACG ACT CAC TAT AG-3' (10 µM), 1.25 µL of primer Luci D2-3 R (10 µM), and 9 µL of water for a total reaction volume of 25 µL. The thermocycler program was as follows: initial denaturation at 95°C for 5 min, followed by 20 cycles at 95°C for 30 s, 56°C for 30 s, and 72°C for 4 min, with a final extension cycle at 72°C for 5 min. The amplified products were visualized by electrophoresis (1% agarose, 100 V, 45 min, Gel-Red, 100 bp Plus DNA Ladder 02002-500 AccuRuler). The following reaction to screen clones was carried out using restriction analysis with positive clones: 10 µL of DNA (Luci D2-3 partial CDS: 85.2-104.1 ng μL^{-1}), 1 µL of *Nde*I (10 U μL^{-1}), 1 µL of *Sal*I (10 U μL^{-1}), 10 µL of 10x Buffer D, and 78 µL of water for a total reaction volume of 100 µL. The mixture was incubated for 4 h at 37°C. The amplified products were visualized by electrophoresis (1% agarose, 100 V, 45 min, Gel-Red, 100 bp Plus DNA Ladder 02002-500 AccuRuler).

Induction of expression

Once the plasmid integrity was analyzed, the plasmid pET28-LuciD2-3 (Luci D2-3 partial CDS) extracted in the previous section was used to transform *Escherichia coli* Rosetta gami (DE3), as described above. Subsequently, fresh LB-kanamycin medium (50 µg mL^{-1}) (1:50 dilution) was inoculated and incubated at 23°C with shaking (200 rpm) until reaching an optical density, at wavelength of 600 nm (OD_{600}), between 0.2-0.3. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and incubated at 23°C with shaking (200 rpm) for 12 h to induce protein expression. Grown cells were centrifuged and resuspended in 250 µL of phosphate buffer (pH = 7.5). The cells were lysed by successive freeze-thaw cycles (-80 to 37°C) and centrifuged at 12,000 g for 5 min to precipitate the cell debris and keep the supernatant. The protein was visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%, 200 V, 45 min, Kaleidoscope Precision Plus Protein Standard (BioRad) and stained with Coomassie blue).

Purification of the recombinant LCF protein

To carry out the purification of the recombinant protein (Luci D2-3 partial CDS), instructions in the commercial His Spin Trap kit (GE Healthcare) were followed according to the purification protocol under native conditions (binding buffer: 20 mM sodium phosphate dihydrate, 500 mM NaCl, 20 mM imidazole, pH 7.4; elution buffer: 20 mM sodium phosphate dihydrate, 500 mM NaCl, 500 mM imidazole, pH 7.4). For this purpose, a volume of 800 mL of *E. coli* Rosetta gami (DE3) was cultured, which was induced in the way previously explained. The result was visualized by SDS-PAGE (10%, 200 V, 45 min, Kaleidoscope Precision Plus Protein Standard (BioRad), and stained with Coomassie blue).

Mass-spectrometry analysis

The confirmation of the expression of the recombinant protein (Luci D2-3 partial CDS) was carried out by mass spectrometry (MS) analysis of the samples extracted from the bands (~50 kDa) of the polyacrylamide gels. The nano-scale liquid chromatographic-mass spectrometry (nLC-MS) proteomics procedure was applied, as described in Fajardo et al. (2019). Briefly, the samples were cleaned up by 1-D electrophoresis, digested with trypsin on a polyacrylamide gel to remove possible contaminants, and finally dried in SpeedVac. Peptides were diluted in 20 µL of 2% ACN, 0.05% TFA, and 5 µL of each band were injected for analysis. The nLC-MS method was performed on a Dionex Ultimate 3000 nLC (Thermo Scientific) with a flow of 300 nL min^{-1} and an ACN gradient from 3 to 40% in 60 min in 0.1% FA. Peptides were first trapped in a 5 mm × 300 µm Acclaim Pepmap precolumn and subsequently separated on a 50 cm × 75 µm Acclaim Pepmap nano-column (Thermo Scientific) with a 2 µm particle size. Eluted peptides were analyzed on an Orbitrap Fusion mass spectrometer (Thermo Scientific) equipped with a nanoelectrospray source. Data Dependent Acquisition method was applied, first detecting the peptides in the Orbitrap detector at 120,000 resolution and subsequent CID fragmentation in the ion trap to obtain the MS2 spectra. Raw data were processed using Proteome Discoverer (version 2.1.0.81, Thermo Scientific). MS2 spectra were searched with the SEQUEST engine against a database of Uniprot_Dinoflagellate_Jun2017 (71,524 sequences, www.uniprot.org). Peptides were generated from tryptic digestion with up to one missed cleavage, with carbamidomethylation of cysteine as fixed modifications and methionine oxidation as variable modifications. Precursor mass tolerance was 10 ppm, and product ions were searched with a tolerance of 0.2 Da. Peptide spectral matches (PSM) were validated using a percolator based on *q*-values at a 1% false discovery rate (FDR). With proteome Discoverer, peptide identifications were grouped into proteins according to the law of parsimony and filtered to 1% FDR (Fajardo et al. 2019).

The main result of this study was the generation of a recombinant *P. lunula* LCFb protein (Luci D2-3 partial CDS). RNA extraction from the cultured *P. lunula* cells produced high-quality RNA. From that, cDNA was synthesized to produce the Luci D2-3 partial CDS fragment by RT-PCR of 1467 bp. This fragment was subsequently purified, and the results were corroborated by electrophoresis analysis. Digestion with restriction enzymes was carried out to linearize the expression plasmid pET28 and on the amplified fragment to generate the matching overhangs for constructing the expression vector. The result of the restriction analysis was visualized by electrophoresis.

After the competent *E. coli* TOP-10 cells were transformed, three isolated colonies were used for plasmid extraction, as described previously. PCR detected the presence of the Luci D2-3 partial CDS fragment to verify the correct insertion of the fragment in the expression plasmid pET28. Additionally, sequencing of the amplified fragment, restriction analysis, and electrophoresis of the constructed vector were conducted to corroborate the results.

Once the correct construction of the recombinant expression plasmid was verified, one of the clones was selected for the induction of the expression of the protein. For this, the clone was grown in an LB-kanamycin medium for about 12 h under the abovementioned conditions. Then, IPTG was added to the medium for 12 h to induce the expression. The cell lysate was used for protein electrophoresis. It showed a band in the induced cells of the recombinant protein with a size somewhat larger than 50 kDa (Fig. 2). The affinity chromatography columns were highly efficient in purifying the recombinant LCF protein from the cell lysate (Fig. 3). Finally, the identity of purified protein was verified by MS analysis.

On the other hand, and from a broad perspective, one of the main factors limiting the implementation of this approach, with prospects for the development of expression assays coupled with multiple bioluminescent reporters, is the lack of a simple and inexpensive method to isolate the substrate of the bioluminescent reaction, in this case, the luciferin of *P. lunula* (Dunlap & Hastings 1981). Dinoflagellate luciferin is a tetrapyrrole-type molecule very similar to chlorophyll-a and *E. superba* luciferin (Nakamura et al. 1989, Topalov & Kishi 2001, Tsarkova 2021) and is characterized by extreme sensitivity to oxidation and degradation (Dunlap & Hastings 1981); thus, its isolation and purification are complex. Importantly, it is considered "universal" in dinoflagellates since luciferin from any bioluminescent species of dinoflagellates can be used as a substrate to produce light (Fajardo et al. 2020). Nonetheless, *P. lunula* presents the highest concentration of luciferin concerning all other bioluminescent dinoflagellates currently on record (Wang & Liu 2017), even 100 times more than *L. polyedra* for instance (Knaust et al. 1998), so future research could be focused on optimization processes in the methodology applied for the isolation of luciferin from *P. lunula*.

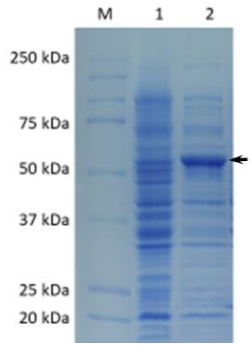

Figure 2. Polyacrylamide gel electrophoresis of the product of the cell lysis of the uninduced, transformed cells (1) and the transformed cells after induction (2) of the expression of the recombinant protein (LCFb D2-3), showing the recombinant protein of a little over 50 kDa (arrow). M: molecular weight marker.

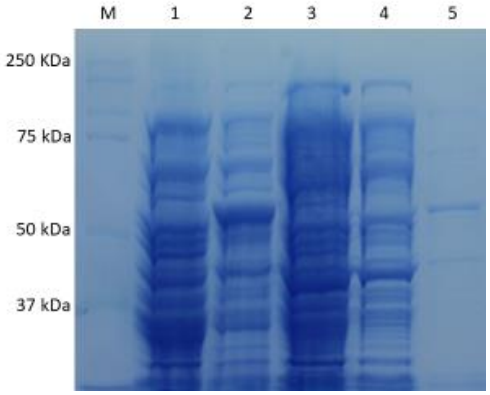

Figure 3. Polyacrylamide gel electrophoresis of the protein purification process under native conditions. M: molecular weight marker, 1: cell lysate of uninduced, transformed cells, 2: cell lysate of induced, transformed cells, 3: flow-through of the His-Spin-Trap column, 4: flow-through wash solution, 5: elution.

However, despite not having been able to carry out activity tests due to the lack of the luciferin substrate, it could be inferred, based on the results obtained by Morishita et al. (2002), that the recombinant protein obtained in this case (*P. lunula* LCFb D2-3) is enzymatically active, since previous reports state that

the three catalytic domains function independently (Li et al. 1997, Morishita et al. 2002). Regarding this subject, it is appropriate to point out that a fundamental characteristic that determines the patterns of enzymatic activity, especially in the case of the bioluminescent reaction of dinoflagellates, is the pH present in the reaction medium (Schultz et al. 2005). It has been reported that wild-type LCFs isolated from *Lingulodinium polyedrum* and *P. lunula* had a low activity at pH 8.0. On the other hand, active proteolytic fragments, as in this case, exhibited higher activities at the same level (pH 8.0) (Schmitter et al. 1976). Nonetheless, a pH- activity profile analysis conducted by Morishita et al. (2002) revealed that bioluminescence spectrum of a recombinant enzyme with the 3rd domain is very similar to that obtained with an extract from *P. lunula* cultured *in vitro*. Furthermore, the histidine in the central area of the 3rd domain could be particularly determinant for pH sensitivity in the case of the *P. lunula* LCF since this residue can react with the negatively charged amino acids located at the alkaline region (Li et al. 2001). This evidence suggests that the 3rd domain of *P. lunula* LCF is equivalent to an active proteolytic fragment (Morishita et al. 2002, Delroisse et al. 2021).

In consequence, the establishment of this detailed protocol for the cloning, expression, and purification of a recombinant LCF protein from *P. lunula* may constitute a useful tool for the development of new biotechnological applications, especially regarding the multiple reporter gene assays, thus expanding the palette of tools available in this particular field. Possible applications include using *P. lunula* LCF (Luci D2-3 partial CDS) coupled, for example, with *R. reniformis* or *P. pyralis* LCFs for multiple or dual reporter assays. These assays could simultaneously identify the transcriptional activities of two or more genes (Suzuki et al. 2005). Therefore, despite the current obstacle represented by the complexity of isolating the dinoflagellate luciferin, establishing a reliable and easily available supply of LCF and luciferin from *P. lunula* deserves more investigation efforts.

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