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Luciferase of the Japanese syllid polychaete *Odontosyllis umdecimdonta*

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ABSTRACT

Odontosyllis undecimdonta is a marine syllid polychaete that produces bright internal and exuded bioluminescence. Despite over fifty years of biochemical investigation into *Odontosyllis* bioluminescence, the light-emitting small molecule substrate and catalyzing luciferase protein have remained a mystery. Here we describe the discovery of a bioluminescent protein fraction from *O. undecimdonta,* the identification of the luciferase using peptide and RNA sequencing, and the *in vitro* reconstruction of the bioluminescence reaction using highly purified *O. undecimdonta* luciferin and recombinant luciferase. Lastly, we found no identifiably homologous proteins in publicly available datasets. This suggests that the syllid polychaetes contain an evolutionarily unique luciferase among all characterized luminous taxa.

1. Introduction

Odontosyllis is a widely distributed genus of marine syllid polychaete worms that are noted for their striking bioluminescent courtship displays [1–5]. The bioluminescence (BL) of *Odontosyllis* is a luciferin-luciferase system [6], but the structure of the luciferin and the luciferase protein remain unknown despite several biochemical studies following the first in 1931 by Harvey [6–11]. More broadly, to date the enzyme sequences and luciferin structures remain a mystery for all polychaete species in the thirteen families containing luminous species [12].

Previous studies of the *Odontosyllis* bioluminescence system generated conflicting results regarding whether the system is a soluble oxygen-dependent luciferin-luciferase reaction [7,8], or is a photoprotein system in which the light-emitting small molecule substrate is covalently bound to the enzyme [10]. The above studies used a different *Odontosyllis* species, and the different colors of aqueous extracts identified from those species make it unclear whether there are multiple bioluminescent chemistries within *Odontosyllis*. However, both species have the same behavior of secreting luminescence during mating [1,4], so both species presumably share a homologous bioluminescent system.

Odontosyllis undecimdonta is a species found in Toyama Bay, Japan which engages in bioluminescent surface courtship displays around the first new moon in October [13]. Recently a protein-coding sequence from *O. undecimdonta* was patented that produces a recombinant protein with luminescence activity similar to that of crude worm extract mixed with crude luciferin isolate (WO2017155036A1). Here, we describe the identification, cloning, and characterization of the *O. undecimdonta* luciferase. In addition, our results suggest that the *O. undecimdonta* luminescence system is a luciferase-luciferin type without requisite cofactors, despite reports of magnesium ions as a necessary cofactor [14].

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2. Materials and methods

2.1. Specimen collection

Professor S. Inoue provided lyophilized *O. undecimdonta* worms collected in 1993 to develop the protein purification strategy [15]. The final specimens used in this study for protein purification, MS transcript identification, and nucleic acid purification were collected on October 06, 2016 in Toyama Prefecture Japan, Namerikawa City, at the coordinates 36° 46′ 40.3032" N 137° 20′ 42.378" E. At dusk, *Odontosyllis* worms were attracted to a handheld light at the surface and collected with a hand dip net. Worms were individually preserved in Invitrogen RNAlater or lyophilized for later analysis.

2.2. Molecular biology methods

2.2.1. DNA and RNA isolation

Methods for DNA and RNA isolation, as well as construction of the RNA-seq and genomic DNA libraries are as described in the Supplementary Information. Briefly, the *O. undecimdonta* transcriptome was assembled using 32, 457, 166 Illumina 2 × 150 read pairs and 343,752 Oxford Nanopore long reads using the Trinity assembler [16]. DNA from a single *O. undecimdonta* specimen was used to prepare both a 10X Genomics chromium library [17] and a PCR-free library. All sequencing reads are available to download from the European Nucleotide Archive under project PRJEB26709. Individual luciferase transcripts are available at NCBI accession numvers MH350412 and MH350413.

2.3. Protein extraction from biomaterial

Five ml of phosphate buffer (5 mM sodium phosphate buffer, pH 7.4) was added to 150 mg of lyophilized worms. Then this mixture was dropped in to the liquid nitrogen, using a 1 ml pipette, to create small drops of frozen material. These small ice drops were ground in a mortar. Frozen powder was added to 10 ml of phosphate buffer (5 mM sodium phosphate buffer, pH 7.4) and incubated 40 min on an ice bath with stirring. After incubation this solution was centrifuged at 40000 g (4 °C) for 40 min. The supernatant, containing luciferase, was then collected and used for further purification by anion exchange chromatography.

2.4. Protein purification

2.4.1. Anion exchange chromatography of water extract

An extract of *O. undecimdonta* was applied to a DEAE Sepharose (GE Healthcare, Uppsala, Sweden) HiTrap Fast Flow column (1.6 × 2.5 cm), equilibrated and washed with 5 mM sodium phosphate buffer, pH 7.4 at rate of 5 ml/min. The elution was done by linear NaCl gradient from 0 to 0.4 M (80 ml) and 5 ml fractions were collected. To minimize bioluminescent reactions, the solvent, fractions and column were maintained at 4 °C. Automatic fraction collection and solvent application was controlled with an Akta Prime chromatography system (GE Healthcare, Uppsala, Sweden). After elution, fractions containing luciferase and luciferin were detected by pairwise mixing all possible fraction combinations. The reaction was monitored with a custom-made luminometer Oberon-K (Krasnoyarsk, Russia).

2.4.2. Ultrafiltration and concentration

To discard additional proteins from the luciferase-containing fractions the ultrafiltration procedure was used. First, the active fraction was filtered on a 50 kDa Amicon® Ultra centrifugal filter unit (Merck Millipore, Germany). BL activity was measured for the concentrated retentate and the permeate. We found that only the permeate was bioluminescent. The bioluminescent permeate was then concentrated on 30 kDa Amicon® Ultra centrifugal filter unit (Merck Millipore, Germany). The resulting retentate possessed BL activity while the permeate did not. Thus this concentrated luciferase sample was used for size exclusion chromatography.

2.4.3. Size exclusion chromatography

The bioluminescent retentate from ultrafiltration was applied to a Superdex 200 column (Phenomenex, USA) on a Shimadzu chromatography system (Shimadzu, Japan). The loaded column was washed with 5 mM sodium phosphate buffer, 150 mM NaCl, pH 7.4 at rate of 0.4 ml/min. During separation 0.5 ml fractions were collected. The solvent, fractions, and column were maintained at 4 °C. BL-active fractions were used in the subsequent gel electrophoresis experiments.

2.5. Denaturing polyacrylamide gel electrophoresis and amino acid sequence analysis

SDS-PAGE of the BL-active fractions was performed using a 10–25% gradient gel according to [18]. Gel staining was done according to the silver staining protocol from [19], or using a standard Coomassie G250 staining protocol. Protein bands were excised from the gel and subjected to in-gel trypsinolysis [20]. LC-MS was performed on the Ultimate 3000 Nano LC System (Thermo Fisher Scientific), connected to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific). For data analysis, Mascot software (Matrix Science) with the *O. undecimdonta* transcriptome as a reference was used.

2.5.1. Molecular cloning

Four Odontosyllis luciferase candidate genes were codon-optimized for expression in mammalian cells, domesticated for compatibility with MoClo assembly [21] and ordered from a commercial supplier (Twist Biosciences, USA) as linear dsDNA fragments. Molecular cloning is described in detail in the Supplementary Materials.

2.5.2. Mammalian cell culture

HEK293NT cells were grown under standard conditions and transfected with FuGene 6 reagent (Promega, Fitchburg, WI, USA) in accordance to the manufacturer's protocol. For more details see Supplementary materials.

2.6. In vitro bioluminescence assay

The reaction was monitored with a custom-made luminometer Oberon-K (Krasnoyarsk, Russia) at room temperature. For each measurement 100 μl of reaction mix (10 mM sodium phosphate buffer, 150 mM NaCl, pH 7.4, 2 μl of luciferase fraction, 2 μl of highly purified luciferin [22] were used. In experiments with mammalian cells lysates, the same amount of cells was used for each clone in each bioluminescence analysis to make results comparable.

The involvement of additional cofactors in the *O. undecimdonta* bioluminescence reaction was tested using an *in vitro* assay with only purified luciferase and highly purified luciferin. Since previous studies suggest the involvement of Mg^{2+} in the *Odontosyllis* bioluminescence reaction (optimum conc is 30 mM; [14]), we also testing the *in vitro* bioluminescence assay with 30 mM–60 mM Mg^{2+} with cell lysate.

2.7. Protein structure and homology analysis

We used HMMER and the BLAST suite to predict structural domains and interspecies homology of transcripts that produced

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