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LC–MS and microscale NMR analysis of luciferin-related compounds from the bioluminescent earthworm *Fridericia heliota*

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ABSTRACT

This paper presents the main results of RP-HPLC–MS and microscale NMR analysis performed on Accompanying similar to Luciferin (AsLn(x)), compounds present in extracts of the bioluminescent earthworm *Fridericia heliota* that display similarities with *Fridericia*'s luciferin, the substrate of the bioluminescent reaction. Three isomers of AsLn were discovered, AsLn(1), AsLn(2) and AsLn(3), all of which present a molecular weight of 529 Da. Their UV–Vis absorption spectra show maxima at 235 nm for AsLn(1), 238 and 295 nm for AsLn(2) and 241 and 295 nm for AsLn(3). MS^n fragmentation patterns suggest the existence of carboxylic acid and hydroxyl moieties, and possibly chemical groups found in other luciferins like pterin or benzothiazole. The major isomer, AsLn(2), presents an aromatic ring and alkene and alkyl moieties. These luciferin-like compounds can be used as models that could give further insights into the structure of this newly discovered luciferin.

1. Introduction

Bioluminescence refers to a process in which an enzyme, luciferase, catalyses the oxidation of its substrate, luciferin, generating photons of visible light [1,2]. The most studied bioluminescent system is that of the North American firefly *Photinus pyralis* but other systems are known, like those of bacteria and coelenterates, which have important applications in basic and applied research [1,2].

Recently a new bioluminescent Siberian earthworm was described, *Fridericia heliota* (*Annelida*: *Clitellata*: *Oligochaeta*: *Enchytraeidae*) [3]. Besides the basic components, luciferase, luciferin and oxygen, its bioluminescent reaction requires the co-factors ATP and Mg^{2+}, similarly to fireflies, emitting a blue–green light with a maximum at 478 nm [4]. The presence of anions, cations of divalent metals, detergents and certain lipids, as well as changes in pH and temperature, may alter the emission profile *in vitro* [5].

Little is known about the structures of luciferase and luciferin, as well as the whole bioluminescent mechanism. Preliminary results on gel-filtration chromatography have assigned a molecular weight (MW) close to 70,000 and 500 Da to luciferase and luciferin, respectively [6]. A major drawback in the study of *F. heliota*'s bioluminescent system is the extreme difficulty in obtaining extracts of luciferase and luciferin. *F. heliota* has a reduced size (no more than 2 cm each worm) and a reduced luciferin content (0.5–0.7 μg/g of biomass, approximately 500 worms) [5]. Furthermore, the collection process is laborious, demanding the picking up of the earthworms one by one from soil by hand, in the dark and only during summer (2–3 months per year in Siberia). As a result it is common to get only 30–50 g of wet clean worms per season. Since many extraction and purification steps need to be performed, the luciferin loss is significant. Taking these facts into account it is useful to find model molecules, similar to luciferin in terms of structure and chemical properties but present in higher concentration than luciferin in extracts, and such was the purpose of the present work.

2. Materials and methods

2.1. Preparation of F. heliota extracts

The separation and purification of different fractions were achieved according to Scheme 1. Briefly, to obtain a crude total luciferin fraction 70 mL of crude total extract (cell-free extract) of *F. heliota*, prepared from 9.0 g of wet worms, was loaded onto a column (16 mm × 200 mm) packed with diethylaminoethyl (DEAE) Sepharose™ Fast Flow (Pharmacia Biotech, Uppsala, Sweden) coupled to the BioLogic™ LP chromatography system (BIO-RAD Laboratories, Hercules, USA). The column was equilibrated with tris(hydroxymethyl)-aminomethane-hydrochloric acid (Tris-HCl) buffer 10 mM, pH 8.1 (Serva Electrophoresis, Heidelberg, Germany). Elution was done by a linear gradient elution program of sodium chloride from 0 to 1 M. Before the second chromatographic step,

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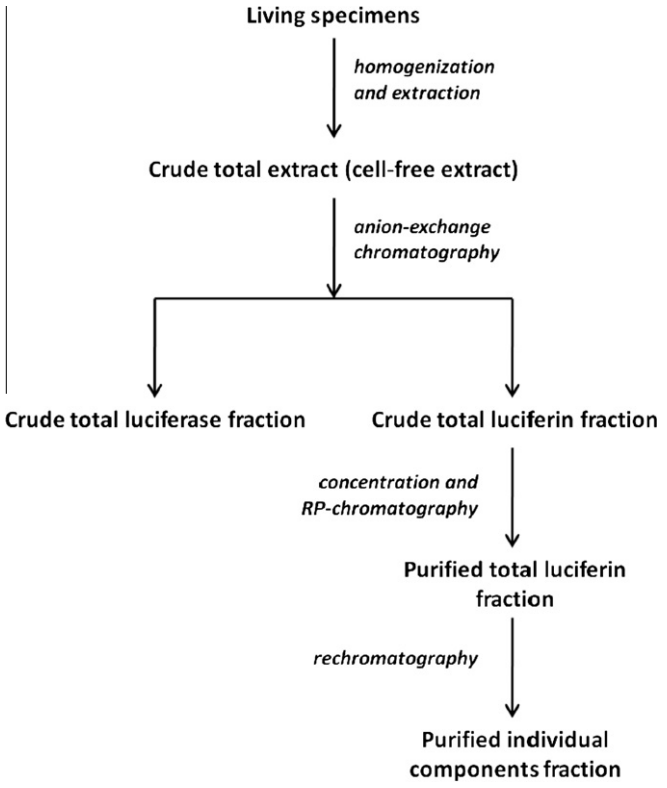

Scheme 1. Sequential chromatographic steps to obtain total and individual fractions from *Fridericia heliota*.

to obtain purified total luciferin fractions, the main luciferin fractions were acidified to pH 3 by adding hydrochloric acid and concentrated in a 3 mL disposable C_{16} extraction cartridge (Diapack-C_{16}, BioChemMak S&T, Moscow, Russia) which was previously equilibrated using hydrochloric acid 10 mM with acetonitrile 3%. The cartridge was rinsed with 15 mL of the equilibration solution, luciferin was washed off with 3 mL of acetonitrile 75% and then concentrated to 0.8 mL by vacuum (Vac-Rotor Concentrator Type 350P, Unipan Scientific Instruments, Warsaw, Poland). Reversed-phase liquid chromatography (RP-LC) was performed using a Mili-Chrom A-02 chromatograph (EcoNova, Novosibirsk, Russia) with a 2 mm × 75 mm column packed with ProntoSil® 120-5-C_{18} (EcoNova Novosibirsk, Russia). Elution was performed using a gradient elution program. Eluent A was a solution of ammonium formate 0.1%, pH 5, obtained by adding the corresponding acid to deionized water and dropping ammonium hydroxide to the desired pH. Eluent B was acetonitrile. The standard gradient program was 5–40% B for 28 min. Both the column and solvents were maintained at 40 °C, with a flow rate of 0.1 mL/min. Absorbance was monitored at 210, 230, 250, 270, 290, 310, 330 and 360 nm. To obtain purified individual component fractions, fractions of each luciferin-like component obtained from identical chromatograms were collected, evaporated to minimal volume under low pressure and subjected to rechromatography under conditions previously described.

2.2. Luciferase activity measurement

The reaction mixture to locate luciferin in chromatograms of purified total luciferin fractions by measuring luciferase activity (in arbitrary units) was composed of adenosine 5′-triphosphate 10 mM (ATP, 2.5 μL), magnesium chloride 100 mM (2.5 μL), Triton® X-100 10% (10 μL) (Sigma–Aldrich, St. Louis, USA), Tris-HCl buffer 20 mM, pH 8.1 (180 μL) and anion-exchange purified luciferase extract (10 μL). The reaction was initiated by adding luciferin fractions (1 μL) and was performed on a custom-made luminometer (Oberon, Krasnoyarsk, Russia). Each time before the addition of luciferin fractions the background luminescence was measured for 20s and subtracted from the total measurement.

2.3. RP-HPLC analysis

The characterization of purified individual AsLn fractions employed a chromatographic system composed of a HPLC pump (Finnigan™ Surveyor™ LC Pump Plus), an autosampler (Finnigan™ Surveyor™ Autosampler Plus) and a photodiode array detector equipped with a LightPipe™ flowcell (Finnigan™ Surveyor™ PDA Plus Detector) (all instrumentation from Thermo Electron Corporation, Waltham, USA), together with a silica-based C_{18} reversed-phase column (Hypersil™ GOLD Column 2.1 mm × 150 mm, particle size 5.0 μm, pore diameter 175 Å, Thermo Scientific, Waltham, USA). In each run 10 μL of samples were injected. The mobile phase consisted of (A) LC–MS grade deionized water with formic acid 0.1% and (B) LC–MS grade acetonitrile. Elutions were performed at a constant flow rate of 0.3 mL/min under a gradient elution program: 0–14 min, 5% B; 14–38 min, 40% B; 38–39 min, 80% B; 39–50 min, 100% B, and 51–60 min, 5% B, and absorbance was monitored at a total scan mode from 220 to 750 nm.

2.4. MS analysis

The mass spectrometer was a Finnigan™ LCQ™ Deca XP Max (Thermo Electron Corporation, Waltham, USA) coupled to the HPLC system. This device was equipped with an electrospray interface as ionization source and a quadrupole ion trap for MS^n experiments, and was operated both in positive and negative ion modes with the following conditions: spray voltage, 5 kV; capillary voltage, −15 V or 15 V in negative and positive ion modes, respectively; capillary temperature, 300 °C. Full-scan spectra were acquired over a mass range from 250 to 1500 Da in MS mode, from 135 to 540 Da in MS^2 mode and from 120 to 495 Da in MS^3 mode, all in negative ion mode; from 250 to 1500 Da in MS mode, from 135 to 545 Da in MS^2 mode and from 115 to 480 Da or 85 to 360 Da in MS^3 mode for AsLn(1) or AsLn(2), respectively, all in positive ion mode. The system was controlled by Xcalibur™ version 1.4 SR1 and data was treated using QualBrowser version 1.4 SR1 (both Thermo Electron Corporation, Waltham, USA). Assignment of some of the main fragments generated during MS^n experiences was done using the open database for mass spectrometry MassBank [7].

2.5. NMR analysis

Nuclear magnetic resonance measurements were performed at RIAIDT (Rede de Infraestruturas de Apoio à Investigação e ao Desenvolvimento Tecnológico), Unity of Magnetic Resonance, Section of Nuclear Magnetic Resonance, CACTUS Building at University of Santiago de Compostela (Spain) on a Bruker Avance DRX-500 NMR spectrometer (Bruker Corporation, Billerica, USA) operating at 500 MHz. Purified individual AsLn fractions (see subsection 2.1.) were lyophilized and ^1H and COSY data was recorded in hexadeuterated dimethyl sulfoxide (DMSO-d6, CD_3SOCD_3) with a 1 mm microprobe. Chemical shifts are given in parts per million (ppm) and were referenced to the solvent signal at 2.50 ppm. Data was treated using MestReNova Lite version 6.1.1-6384 (Mestrelab Research, Santiago de Compostela, Spain).

3. Results

Previous results have shown the presence of at least two compounds in total luciferin fractions, luciferin itself and a