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Study on bioluminescence and photoluminescence in the earthworm *Eisenia lucens*

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O. Pes [a], A. Midlik[a,b], J. Schlaghamersky[c], M. Zitnan[d] and P. Taborsky[b,e]

Eisenia lucens is an earthworm living in the organic soil layer of decomposing wood. When irritated, the worm expels coelomic fluid through pores in its body wall, exhibiting blue-green bioluminescence. The mechanism of the bioluminescence, which seems to be different to other bioluminescence systems of terrestrial animals, has been studied in this work. Many lines of evidence indicate that riboflavin stored in coelomycetes plays an important role in this glowing reaction.

Introduction

Bioluminescence has been reported from a considerable number of species of segmented worms (Annelida). Many belong to predominant marine polychaetes with several bioluminescent representatives of the taxon Clitellata, specifically of earthworms (Megadrili), from the families Lumbricidae and Megascolecidae, and of the related but substantially smaller potworms (Enchytraeidae).[1] Except of a few species of the megascolecid genus *Pontodrilus*[2, 3], all bioluminescent clitellates (at least 33 species) are terrestrial.[4] Bioluminescence in clitellates has been mostly studied in the earthworms *Diplocardia longa* [5, 6], *Microscolex phosphoreus* [4], and the enchytraeid *Fridericia heliota.*[7-11] *Diplocardia longa*, which has been studied in detail by Wampler and colleagues[5, 6, 12, 13], expels bioluminescent fluid from its pores with bioluminescence originating from discrete subcellular loci within free chloragogen cells suspended in the coelomic fluid.[12] Wampler *et al.* proved that *N*-isovaleryl-3-aminopropanal was the luciferin molecule.[4, 6] They also suggested that all bioluminescent earthworms used the *N*-isovaleryl-3-aminopropanal/hydrogen peroxide reaction, although they admitted that colour of bioluminescence varied among species (from 490 nm to over 600 nm).[4] The Siberian potworm *Fridericia heliota (*Enchytraeidae) exhibits blue bioluminiscence (maximum at 478 nm) with the light being emitted from epidermal cells. The bioluminescencent system included a luciferin combined of CompX, lysine, GABA, and oxalate. Apart from that, the reaction required atmospheric oxygen, ATP, Mg^{2+} and *Fridericia* luciferase.[7-11, 14]

Eisenia lucens (Waga, 1857) from Lumbricidae is an earthworm occurring in Central and Eastern Europe. Its bioluminescence has been firstly reported by the Polish scientist Antoni S. Waga in 1854, who described it and named it as *Lumbricus lucens*. Later, the species was re-discovered by F. Vejdovský and described as *Eisenia submontana,* which is now considered a junior synonym of *E. lucens*. The constitution and shape of an *Eisenia lucens* body is similar to other *Eisenia* species such as *Eisenia fetida* (erroneously given as *E. foetida*) and *Eisenia andrei*. Adults have 60-130 segments and reach 4.5-18 cm in length and 5-6.5 mm in width. The body coloration is conspicuous: each segment has a transverse band of red-brown to red-violet colour in its middle, whereas the parts towards the intersegmental groves are of lighter yellowish to greyish colour (this colour also prevails ventrally). The prostomium is epilobic, the clitellum saddle-shaped. Typical of montane forest habitats are in the Carpathian arch but might also be found in other parts of Europe, west to northern Spain; completely missing in Northern Europe. The earthworm commonly lives under the bark and in wet decomposing wood of downed tree trunks and stumps, but has been reported in the organic soil, in particular under moist to wet conditions. The bioluminescence reaction might be inspected after a mechanical, chemical, or electrical irritation of the earthworm when the coelomic fluid is expelled from its dorsal pores. An attempt to describe the bioluminescence reaction of *E. lucens* was done in the work of Bačkovský *et al* from 1939; however, the authors studied merely the photoluminescence reaction by comparing fluorescence spectra of riboflavin and *E. lucens* extract. Although they reported increased amount of riboflavin

[a] Department of Biochemistry, Faculty of Medicine, Masaryk University, Kamenice 753/5, Brno, , CZ 62500, Czech Rep.

[b] Department of Chemistry, Faculty of Science, Masaryk University, Kamenice 753/5, Brno, CZ 62500, Czech Rep.

[c] Department of Botany and Zoology, Faculty of Science, Masaryk University, Kamenice 753/5, Brno, CZ 62500, Czech Rep.

[d] Material Research Centre, Faculty of Chemistry, Brno Technical University of Technology, Purkyňova 464/118, Brno, CZ 61200, Czech Rep.

[e] Central European Institute of Technology (CEITEC), Masaryk University, Czech Republic, Kamenice 753/5, Brno, CZ 62500, Czech Rep.

in the earthworm, they did not examined the chemical form of the emitting species (riboflavin, FAD, FMN), and concluded that riboflavin acted as a luciferase.[15]

In this paper, we attempted to characterize the bioluminescence system using a combination of techniques such as fluorescence microscopy, fluorescence lifetime imaging (FLIM), luminescence spectrometry, and mass spectrometry (LC MS).

Materials and methods

Riboflavin, FMN, FAD, palmitic acid, cis-11-hexadecenal, spectroscopy ethanol, ammonium formate (LC MS), methanol (MeOH) (LC MS) and acetonitrile (ACN) (LC MS) were purchased from Sigma Aldrich (Czech Republic). Specimens of *Eisenia lucens* were collected in two areas within the Czech Republic: the Bohemian-Moravian Highlands (49°39'18.9"N 15°59'34.2"E) and the Moravian-Silesian Beskids (49°32'14.0"N 18°39'13.4"E). Earthworms were stored in plastic containers filled with decaying wood and soil at a constant temperature of 20 °C. Earthworms were chemically stimulated by 50% ethanol. The expelled fluid was collected, centrifuged, diluted 10 times with water and filtered through a 0.22-µm PTFE syringe filter.

Luminescence

All spectra (photoluminescence and bioluminescence) were measured on a spectroluminometer Aminco-Bowman Series 2 (Thermospectronic, USA), equipped with a Xe lamp (for excitation between 200-900 nm). An Olympus BX60 fluorescence microscope (blue light excitation) with a digital image analysis system (Stream Motion 1.9.2) was employed in a study of native photoluminescence. FLIM (Fluorescence LifeTime Imaging) measurements were done using the confocal microscope Leica TCS SP8 X, equipped with a tuneable pulse white laser. Pulse laser 405 nm was used as an excitation source.

Mass spectrometry

The LC MS method was developed using a Dionex Ultimate 3000RS (Thermo Scientific, CA) module. Compound separation was achieved with a 3.0×100 mm, 2.6 µm Kinetex C18 (Phenomenex, CA) column equipped with a guard column at 23 °C, and a flow rate of 0.3 mL/min. The binary mobile phase system consisted of 0.1% ammonium formate (pH = 6.2) and MeOH or ACN. The injection volume was 10 µl. For separation of isoalloxazines (method A), MeOH was linearly increased from 15% to 75% over 20 min, kept constant at 75% over the next 2 min and followed by equilibration at the initial conditions for 3.0 min. For separation of fatty compounds (method B), ACN was held at initial 50% for 2 min and then linearly increased to 95% over 10 min, kept constant at 95% over the next 10 min and followed by equilibration at the initial conditions for 3.0 min. A complete LC run in each method took 25 min. The isoalloxazine ring was detected by monitoring absorbance at 448 nm. The HPLC system was connected to a MicrOTOF-QII (Bruker, Germany) mass spectrometer operated in positive electrospray (ESI+) mode with method A, negative electrospray (ESI–) mode and positive atmospheric pressure chemical ionization (APCI+) mode with method B. The ionization conditions were set by the software according to the mode used; nebulizing and desolvation gas was nitrogen. ESI+: capillary voltage +4500 V, end plate offset –500 V, source temperature 250 °C, desolvation gas flow 5 L/min, nebulizer pressure 2 bar, and collision cell voltage 35 eV. ESI–: capillary voltage –4500 V, end plate offset +500 V, source temperature 250 °C, desolvation gas flow 5 L/min, nebulizer pressure 2 bar, and collision cell voltage 35 eV. APCI+: capillary voltage +4500 V, end plate offset –500 V, corona needle 4000 nA, source temperature 210 °C, vaporizer temperature 250 °C, desolvation gas flow 2 L/min, nebulizer pressure 1 bar, and collision cell voltage 35 eV. A base-peak chromatogram (BPC) was acquired in the MS mode by monitoring a range of 50 to 2000 m/z with a spectra sample time of 1 s. Identification of target compounds relied on a combination of accurate m/z, isotopic pattern, MS/MS and retention behaviour. High-resolution MS and MS/MS spectra were first investigated to obtain the elemental formula of each compound. A compound was unambiguously identified if the fragmentation patterns of the unknown and the standard compound were identical. Due to the lack of fragmentation spectra of non-polar compounds obtained by method B, compounds were identified by comparing accurate m/z and retention time with those of a standard compound.

Results and Discussion

After irradiation by violet or blue light, several parts of the earthworm bodies showed bright green photoluminescence (occasionally reported as autofluorescence). Main centres of photoluminescence emission were rows of bristles protruding through the body wall and granules inside eleocytes (Fig 1). Eleocytes, a type of coelomocytes (cells floating in the coelomic fluid), are detached chloragocytes, i.e. cells that originally covered the outer wall of the intestine forming the chloragogen tissue. Similar fluorescent coelomocytes were observed also in other, non-bioluminescent species of earthworms e.g., *E. fetida*.[16-18]

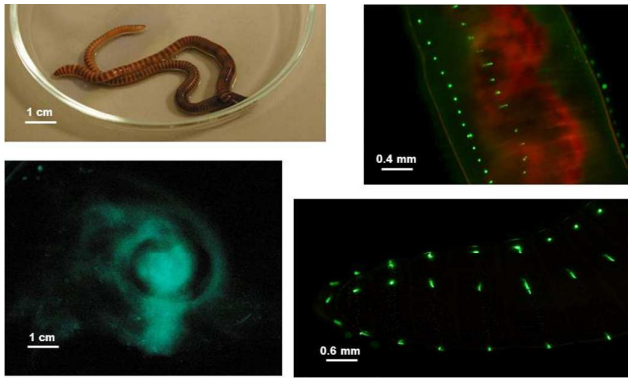

Fig 1. Visualisation of the earthworm Eisenia lucens by photography – under daylight (upper-left) and in the dark after irritation (lower-left), and by fluorescence microscopy – native green photoluminescence of bristles (lower-right) and detached chloragocytes (upper-right). The red fluorescence originates inside of the intestinal tract.

To obtain coelomic fluid a few earthworms were irritated with a 50% solution of ethanol on a glass plate. The expelled fluid, when collected and diluted by re-distilled water, showed two

bands (370 and 440 nm) in the luminescence excitation and absorption spectrum, which were typical for tricyclic isoalloxazine rings. If excited at 370 nm the sample emitted green fluorescence with a maximum at 523 nm. By comparing such spectra with those of biogenic isoalloxazines, riboflavin and its derivatives flavinadenindinucleotide (FAD) and flavinmononucletide (FMN) were found as the best match. In order to identify which of the three compounds is the emitting species in the earthworm an LC analysis with UV-Vis and MS detection was performed (Fig 2). By comparing chromatograms of a standard mixture of riboflavin, FMN, and FAD with a coelomic fluid sample, we noticed that a single compound of riboflavin appears in the system. To confirm this, a peak (m/z 377.1612) at 9.4 min was subjected to MS/MS analysis and fragmentation to lumichrome (m/z 243.0977), as in pure riboflavin, was observed. This unambiguously confirmed the presence of riboflavin in the earthworm, however; there were no signs of FMN or FAD. Other peaks in the base peak chromatogram (BPC) were not characterized in detail, nevertheless; they might be attributable to phospholipids, peptides and other low-molecular compounds commonly found in living organisms. UV-Vis detection with external standard calibration provided the content of riboflavin in the expelled coelomic fluid to equal a concentration of 23×10^{-6} mol.l^{-1}.

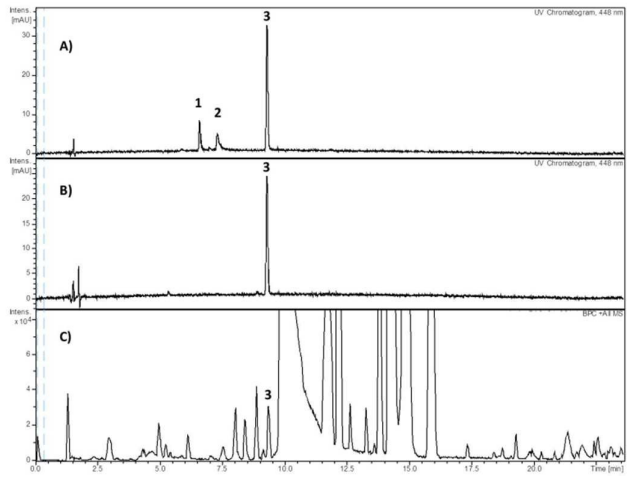

Fig 2. LC with detection at 448 nm of A) a model solution containing 1) FAD (6.6 min), 2) FMN (7.3 min), and 3) riboflavin (9.4 min), and expelled coelomic fluid of *Eisenia lucens* detected B) at 448 nm and C) by MS of a base peak (BPC) in positive ESI. Injection volume was 10 µL and the concentration of each analyte was 3 µg/mL except for riboflavin (10 µg/mL). Retention time of riboflavin in BPC is slightly increased (~3.6 s) due to the sequential design of used detectors.

Photoluminescence of *Eisenia lucens* can be assigned to the presence of such a high quantity of riboflavin. This essential vitamin plays a key role in immune potency of animals including earthworms and its presence at high concentrations was described for other earthworms as well e.g., the non-luminescent *E. andrei* and *E. fetida*.[19, 20]

While the expelled coelomic fluid containing coelomocytes was of a yellow colour in daylight, blue-green bioluminescence was observed when earthworms were irritated in the dark (see Fig 1 and a video in the Supplementary Information). When a freshly expelled portion of the coelomic fluid was subsequently placed into a cuvette and bioluminescent emission spectra were recorded, the emission maximum observed immediately after irritation was 493 nm, and the dependence of intensity at 493 nm on time was found to be exponential with bioluminescence half-life of about 6 s (Fig. 3). The measured bioluminescent emission spectra of *Eisenia lucens* are clearly different to those obtained when excited by light. On the other hand, they are in a close match to bioluminescence of bacteria from the genera Photobacterium, Beneckea, Vibrio, and Xenorhabdus.[21, 22] Bacterial luminescence is generally based on a high molecular weight flavoprotein (luciferase) catalyzing the oxidation of $FMNH_2$ by oxygen in the presence of a long-chain aldehyde.[23] Emitted light maximum is ~495 nm, which indicates the FMN to be the emitting species. LC MS analysis confirmed that although the expelled coelomic fluid of *Eisenia lucens* did not contain any FMN/FAD, a precursor of FMN – riboflavin was present in great excess. This may indicate that the bioluminescence mechanisms of bacteria and *E. lucens* are similar (Fig 3) except for riboflavin being the emitting species. We suggest that riboflavin is *in vivo* converted to the reduced, highly-energetic, non-aromatic form by NAD(P)H as in the case of FAD to $FADH_2$ conversion in higher organisms. Such oxidation of flavines (including the oxidation of the reduced riboflavin), was simulated *in vitro* by several groups. Zeng *et al*. prepared an experiment where riboflavin was converted to the reduced form by Fe^{2+} ions in a pyrophosphate buffer.[24] Chemiluminescence with emission maximum at ~490 nm was induced by an addition of hydrogen peroxide. Similarly, Towner *et al.* demonstrated in his report (Towner, Technical manuscript, 1969) another experiment. Os(III) was used instead of Fe(II) to reduce riboflavin in a phosphate or borate buffer. All these experiments showed that riboflavin chemiluminescence/bioluminescence reaction is plausible.

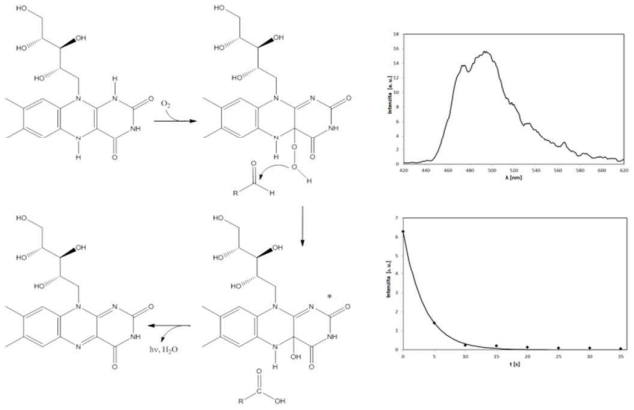

Fig 3. A suggested mechanism of *Eisenia lucens* bioluminescence, maximum emission (493 nm) and bioluminescence half-life (6.2 s).

When the coelomic fluid was studied by fluorescence microscopy, it contained glowing granules inside the eleocytes, which were identified as the apparent source of

bioluminescence (Fig 4). FLIM revealed a uniform distribution of fluorescence lifetimes (~2 ns) along the entire cell strengthening the fact that riboflavin is the only emitting species in the earthworm. As a small organic molecule, its lifetime is accepted to be in units of nanoseconds. Prior to any bioluminescence, an oxidation step is required. Generally, two oxidation agents – oxygen or hydrogen peroxide are associated with a glowing reaction. A simple experiment confirmed that bioluminescence of Eisenia lucens is caused by atmospheric oxygen: A specimen, irritated in N_2 atmosphere, was expelling coelomic fluid in the flask. The expelled fluid was not bioluminescent until the flask was opened and aerial oxygen allowed for the reaction. By combining this observation with microscopy, we suggest that riboflavin molecules from the expelled eleocytes are oxidized by aerial oxygen.

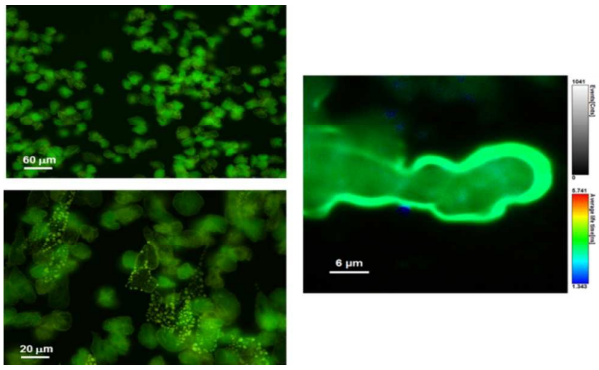

Fig 4. *Eisenia lucens* coelomocytes (eleocytes) subjected to fluorescence microscopy (left) and FLIM (right) – green colour here represents a lifetime of about 2 ns.

The question whether any luciferin molecule was involved in the reaction mechanism was again pursued using LC MS. It is worth noting that the term "luciferin" has never been strictly defined. Originally, it meant an essential ingredient needed for emission of bioluminescence. Now it seems appropriate to define it as the general term of an organic compound that exists in a luminous organism and provides the energy for light emission by being oxidized, normally in a presence of a specific luciferase.[21] We have included already known luciferin molecules, such as N-isovaleryl-3-aminopropanal suggested by Wampler in *Diplocardia longa* or long chain aldehydes (or related products of oxidation – carboxylic acids) typical for luminous bacteria, in the search. Under conditions of analysis of isoalloxazines, there were no signs of N-isovaleryl-3-aminopropanal or fatty compounds presence. In order to bring attention to more non-polar compounds, the elution strength of the mobile phase was increased and the MS was operated in negative ESI and positive APCI mode. This allowed detection of fatty acids or fatty aldehydes, respectively and palmitic and palmitoleic acid as well as three isomers of hexadecenal were found. However, no structural data on the location or conformation of the double bond were acquired. While all these 16-C compounds may naturally be present in living organisms and are required for specific purposes (fuel, signalling, building blocks, etc.) they might also be involved in the *E. lucens* bioluminescence reaction as a potential luciferin molecule. Bioluminescence reactions are also catalysed by compounds called luciferase enzymes. Identification of the luciferase proteins is considered in further research.

Use and adaptive value of bioluminescence in earthworms is still not well understood. It is often mentioned that it might deter predators when earthworms are attacked.[25] Bioluminescence is possibly a part of a complex defence strategy of earthworms. We observed that after irritation, the earthworms attempted to change their position quickly by convulsive movements, leaving a glowing spot in a place of their previous position, which should keep the predator confused about the worm true location. Similar strategies are used by some marine luminous organisms such as squids. Another approach to deflect predators is used by a few species of terrestrial earthworms e.g., *Megascolides australis* that expel non-luminescent coelomic fluid, which is believed to be toxic or repellent.[25] The obvious resemblance of the bioluminescence mechanism of luminous bacteria and earthworms could indicate a similar origin of this ability. However, the adaptive value of bacterial bioluminescence has not been yet uncovered.

Conclusions

The present work reports about novel findings in photoluminescence and bioluminescence of the earthworm *Eisenia lucens*. As its body contains a vast amount of riboflavin, there is a high probability that, among other functions in the organism, riboflavin stored in the eleocytes plays a key role in the bioluminescence of this species. We propose that the mechanism of bioluminescence in *E. lucens* is analogous to the mechanism described in bioluminescent bacteria except for the emitting species to be riboflavin molecules oxidized by atmospheric oxygen. The relationship is deepened by existence of possible luciferins/oxoluciferins as derivatives of the 16-C aldehyde/carboxylic acid redox pairs.

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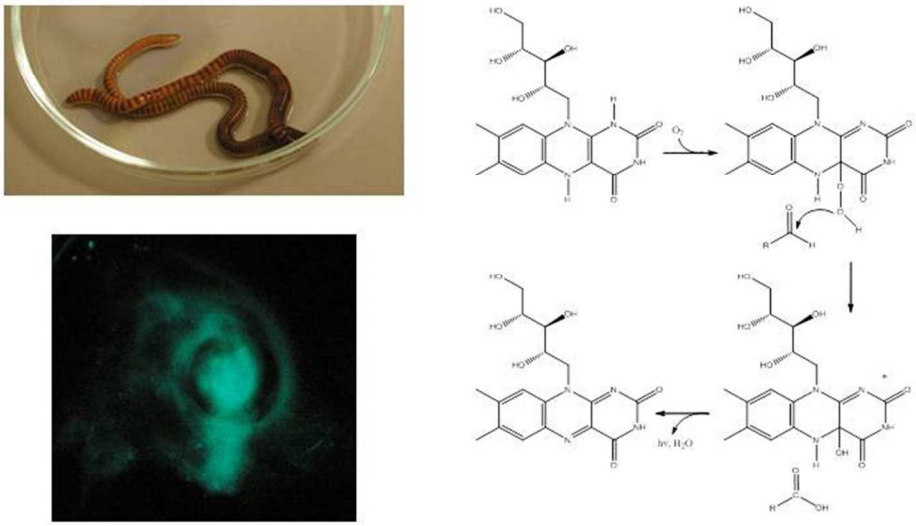

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