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Molecular basis for the blue bioluminescence of the Australian glow-worm *Arachnocampa richardsae* (Diptera: Keroplatidae)

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ABSTRACT

Bioluminescence is the emission of visible light by living organisms. Here we describe the isolation and characterisation of a cDNA encoding a MW ≈ 59,000 Da luciferase from the Australian glow-worm, *Arachnocampa richardsae*. The enzyme is a member of the acyl-CoA ligase superfamily and produces blue light on addition of D-luciferin. These results are contrary to earlier reports (Lee, J., *Photochem Photobiol* 24, 279–285 (1976), Viviani, V. R., Hastings, J. W. & Wilson, T., *Photochem Photobiol* 75, 22–27 (2002)), which suggested glow-worm luciferase has MW ≈ 36,000 Da and is unreactive with beetle luciferin. There are more than 2000 species of firefly, which all produce emissions from D-luciferin in the green to red regions of the electromagnetic spectrum. Although blue-emitting luciferases are known from marine organisms, they belong to different structural families and use a different substrate. The observation of blue emission from a D-luciferin-using enzyme is therefore unprecedented.

1. Introduction

Bioluminescence occurs in many groups of organisms, most of which live in the sea [1,2]. On land, the best known bioluminescent organisms are fireflies (Lampyridae) and related beetles [3]. It is generally believed that the firefly luminescence acts variously as a warning to predators and as a signal to attract mates. There are more than 2000 species of luminescent beetle and the majority of them glow in the yellow-green (490–590 nm) part of the electromagnetic spectrum. Beetle bioluminescence involves the oxidative adenylation of D-luciferin (D-(−)-2-(6′-hydroxybenzothiazolyl)-2-thiazoline-4-carboxylic acid, HBTTCA) substrate by the luciferase enzyme, a member of the acyl-coA ligase gene superfamily. The luminescent reaction results from the two-step enzyme-catalysed reaction of D-luciferin, with magnesium-adenosine triphosphate (Mg-ATP) to form an adenylated intermediate. The intermediate is oxidised by molecular oxygen to give a high-energy dioxetanone, which decomposes releasing carbon dioxide and a photon (Fig. 1a).

Firefly luciferase (E.C. 1.13.12.7) was originally isolated from the North American firefly, *Photinus pyralis* [4]. Luciferases have subsequently been cloned, sequenced and expressed from a number of other beetles, including other Lampyridae [5–7] (fireflies), Phengodidae [8] (railroadworms) and Elateridae [9] (luminous click beetles). Most of these enzymes generate a greenish-yellow luminescent reaction, although the range of emission maxima is 536–632 nm [10] and includes enzymes that luminesce orange [9] or red [8].

Some species of fly also bioluminesce, most notably the Keroplatidae of Australasia [11], whose larvae are known as glow-worms. Glow-worm bioluminescence differs from that of fireflies in a number of respects, including its blue colour, function in attracting prey and observed biochemical differences [12,13] but its molecular basis remains unknown. Their luminescence also requires ATP and molecular oxygen but has an emission spectrum with a peak in the blue (λ_{max} ≈ 490 nm [12,14]). Although the molecular identities of beetle luciferin [15,16] and luciferases [4,10,17] are known, neither a luciferase nor a luciferin [9] has previously been isolated from a glow-worm.

Here we describe the isolation and characterisation of a cDNA encoding a MW ≈ 59,000 Da luciferase from the Australian glow-worm *Arachnocampa richardsae*. We also demonstrate the presence

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of D-luciferin but not L-luciferin in glow-worm light organs. Finally, we reconstitute bioluminescence *in vitro*, by adding synthetic D-luciferin to microbially expressed *A. richardsae* luciferase.

2. Methods and materials

2.1. Glow-worm collection

One hundred larval *Arachnocampa richardsae* were collected from the Newnes Railway Tunnel, New South Wales, Australia. The larvae were transferred to the laboratory and light organs were dissected from the rest of the carcass under a microscope.

2.2. RNA isolation

Total RNA was isolated from either *Arachnocampa richardsae* carcass or approximately 10 light organs using the RNAqueous™-Micro Kit (Ambion) according to the manufacturer's instructions, except that the tissue was ground in 300 μl Lysis Solution.

2.3. cDNA library

cDNA libraries representative of the carcass and light organ were constructed using the Creator™ SMART™ cDNA Library Construction Kit (Clontech). A glycerol stock was prepared for each cDNA library and stored frozen at −80 °C.

For each of the two light-organ cDNA libraries (fraction 10 library and fraction 9 library), plasmid DNA was prepared from 96 randomly picked clones and sequenced.

2.3.1. Dot blotting for screening of clones

Clones from the glow-worm light organ cDNA library glycerol stocks were spotted onto Hybond-XL™ membranes (Amersham). Membranes were then denatured, neutralised and fixed according to the manufacturer's instructions.

Dot blots were probed with a 796 bp α^{32}P-dATP-labelled PCR probe prepared using the forward primer 5′- GATGA-TAATGCACCAGAAAAG -3′ directed to nucleotides 142–162 of clone 1E1 and the reverse primer 5′- TTATAATATCCAGCATCACCA −3′ directed to nucleotides 938-918 of clone 1EBlots were hybridised and washed according to the membrane manufacturer's instructions, then exposed to X-ray film. Plasmid DNA was purified from clones that hybridised to 1E1 and the insert was sequenced.

2.4. Construction of full length cDNA encoding luciferase of *A. richardsae*

Glow-worm luciferase (GWLuc) was constructed using the 5′ region of clone 8F5 and the 3′ region of clone 4F12. Clone 8F5 is a full length cDNA isolated from the glow-worm light organ cDNA library. The 3′ end of clone 8F5 between the BamHI site and the XhoI site in the MCS of pDNR-LIB was removed and the corresponding fragment from clone 4F12 was spliced to clone 8F5.

2.4.1. Multiple sequence alignments

Multiple sequence alignments and phylogenetic trees were derived using the ClustalW2 (EMBL-EBI).

2.5. Construction of pETDuet-1:GWLuc and pETDuet-1:FFLuc

The full length cDNA of GWLuc was amplified and restriction clones into the *NcoI* and *AvrII* restriction sites of Novagen pET-Duet1 vector (EMD/Merck Biosciences, San Diego/Darmstadt). The resulting plasmid was designated pETDuet-1:GWLuc. Standard molecular biology techniques were used and the insert was sequences to confirm integrity. A full length construct of the *P. pyralis* luciferase (FFLuc) gene was inserted into the pETDuet-1 vector to give pETDuet-1:FFLuc as described for pETDuet-1:GWLuc.

2.6. Expression

Constructs were transformed into electrocompetent BL21 (DE3) cells (Novagen). Cultures were induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.4 mM and grown at 20 °C (150 rpm for 48 h)). Following expression cell pellets were resuspended in phosphate buffer (pH 7.4, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4) and analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Proteins were diluted in 1 × sample loading buffer (Invitrogen, Australia) for SDS-gel electrophoresis (NuPAGE system: 12% Bis-Tris gel with MOPS running buffer (Invitrogen, Australia)). Bands were visualised following staining with Fast stain ™ (Fisher Scientific, Australia).

2.8. Preparation of lysates

Cell aliquots were mixed with lysis mix (1 × luciferase cell culture lysis reagent (CCLR, Promega)), 1.25 mg/mL lysozyme (Sigma), 2.5 mg/mL bovine serum albumin (BSA) (Sigma) (CCLR, Promega) and incubated at room temperature for 10 min.

2.9. Preparation of Arachnochampa luciferin extract

An *Arachnochampa* luciferin (hereinafter “GW Luciferin”) containing fraction was prepared from light organs of *Arachnocampa* according to Viviani et al. [13]. Five light organs from *A. richardsae* were homogenized in 50 μl ethanol.

2.10. D-luciferin assay

Synthetic D-luciferin (Sigma) was assayed using the cell lysates (above). Ten μL aliquots of the cell lysate were mixed with 90 μL of 25 mM Tris-acetate buffer (pH 7.75) containing, 2 mM ATP, 4 mM magnesium acetate and 0.4 mM D-luciferin (Sigma). Total light output was measured using the Wallac1420 Victor 2 luminometer (Perkin-Elmer) and spectra recorded with a Cary Eclipse fluorescence spectrophotometer using the bioluminescence wavelength scan mode.

2.11. Chiral high performance liquid chromatography (HPLC)

One μL of 150 pmoles of D- or L-luciferin, or a mixture of both in water:ethanol (1:1 v/v), were injected into a HPLC system (Alliance HPLC System with a 2996 Photodiode Array Detector (Waters)). Linear gradient elution (15–40% acetonitrile/water with 0.1% trifluoroacetic acid (TFA), 20 min, 1.0 ml/min) was implemented for the separation with a chiral fused silica column (CHIRAL-CEL OD-RH, 4.6 × 150 mm, Daicel Chemical Industry, Tokyo, Japan). D- and L-luciferin were detected at 330 nm. Samples were collected at the respective retention times for D- and L-luciferin peaks and fluorescence spectra recorded using a Cary Eclipse fluorescence fluorimeter using the wavelength scan mode. GW Luciferin extract was prepared as described above by homogenizing 10 light organs in ethanol. Following centrifugation, 1 μL of a mixture of GW Luciferin extract in ethanol:water (1:1 v/v) was injected into the HPLC column.

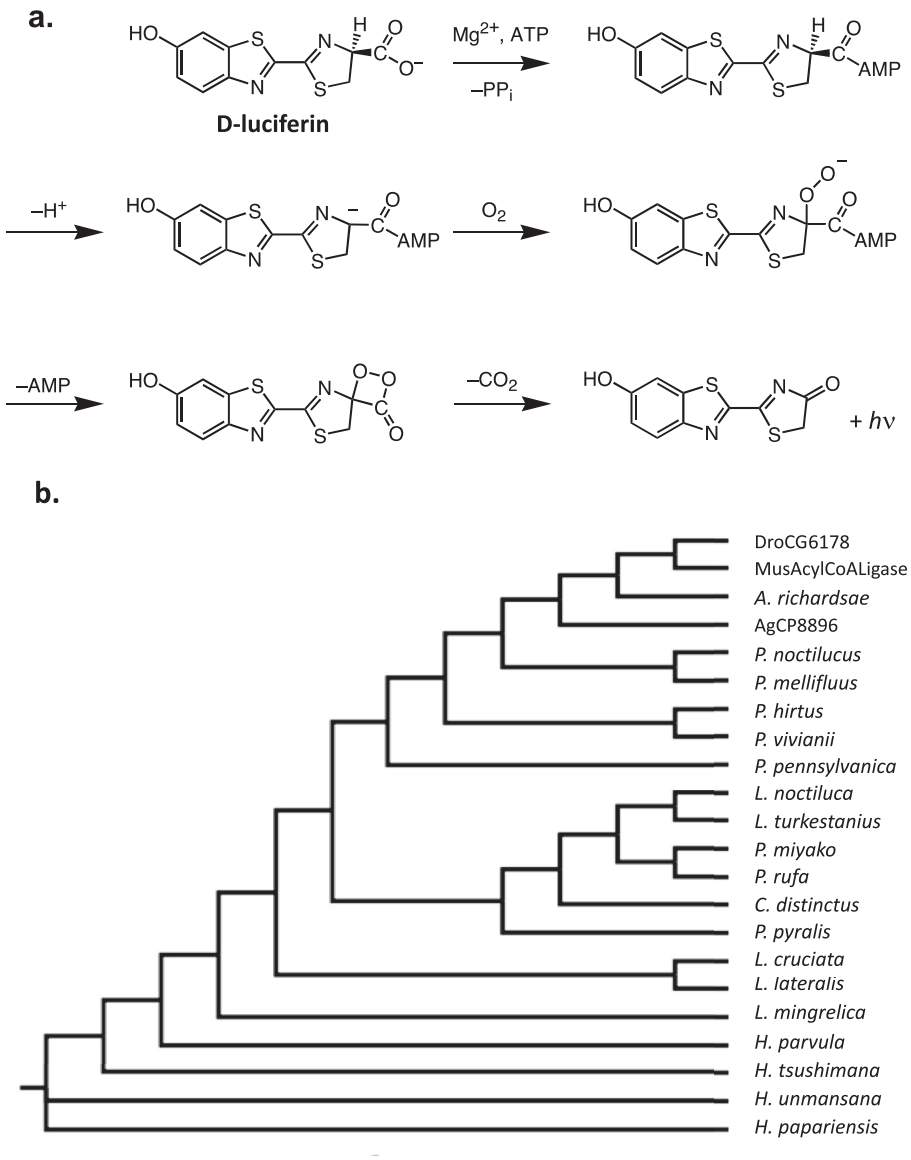

Fig. 1. a. Scheme for the enzymatic conversion of D-luciferin (1) to oxyluciferin with emission of a photon. **b**. Sequence conservation between *A. richardsae* luciferase and other luciferases and luciferase-like proteins. Dendrogram indicating that glow-worm luciferase has greater affinity with non-luminescent dipteran luciferases DroCG6178 and AgCP8896 than with a representative selection of luminescent beetle luciferases.

2.12. Fourier transform ion-cyclotron resonance mass spectroscopy (FT-ICR-MS)

GW Luciferin crude extracts were prepared in ethanol as described above and the mass spectrum of GW Luciferin extracts recorded using a FT-ICR mass spectrometer (Bruker Apex IV). D-luciferin was dissolved in ethyl acetate (1% (w/v) and further diluted in methanol (1 μL/200 μL)) prior to analysis. The mass spectrum of the GW Luciferin extract was recorded before that of D-luciferin to avoid contamination.

For full experimental details, see supplementary materials and methods (Supplementary Material).

3. Results and discussion

3.1. A. richardsae luciferase (GWLuc)

We isolated glow-worm luciferase (GWLuc) using a cloning strategy that made no assumptions about its sequence. The light organ of *A. richardsae* larvae comprises the fused tips of the malphigian tubules backed by a reflector comprising a large number of fine tracheoles [18] (Fig. S1). A size-fractionated cDNA library was prepared from the light organs of ten larval *A. richardsae*. Ninety-two cDNA clones were picked randomly and sequenced. Five of the clones were 5′-truncated partial cDNAs representing the same open reading frame and all five showed homology to luciferases from *Phrixothrix vivianii* and *Phrixothrix hirtus*. The longest of the clones, 1E1, was used as a probe to screen 960 cDNA clones from the same library, with 29 (3%) screening positive. The same probe was used to screen 1000 clones from an equivalent size-fractioned library prepared from glow-worm carcasses from which light organs had been removed, yielding no positives.

The sequence of the complete open reading frame of *A. richardsae* luciferase was deduced by sequencing multiple independent amplicons representing the five clones initially isolated and three cDNA clones isolated by additional screening. A cDNA

Table 1
Thin Layer Chromatography. Characterisation of luciferin in *Arachnocampa* crude extracts by thin layer chromatography in four different solvent systems and comparison with retention factor (R_f) values cited in the literature for synthetic D-luciferin and extracts prepared from 13 different species of elateridae (* data from literature [24]) and *Arachnocampa flava* (** data from literature [13]).

Solvent system	R_F values			
	Synthetic D-luciferin		Crude extract	
	Experimental	Literature*	*Arachnocampa*	*Elateridae**
Ethylacetate/butanol/ethanol/water (3:2:2:3)	0.73	0.71	0.74	0.71
Ethylacetate/ethanol/acetone (8:1:1)	0.17	0.12	0.18	0.12
Ethanol/Water (3:1)	0.84	N/A	0.85	N/A
Ethylacetate/ethanol/water (5:2:3)	0.67	0.80	0.65/0.68**	0.77

(GWLuc) comprising the consensus sequence of the entire open reading frame of the putative *A. richardsae* luciferase was reconstructed by splicing the 5′ region of one clone (8F5) to the 3′ region of a second clone (4F12). GWLuc contains a single long open reading frame in one translation frame. The ORF commences with an AUG, in the correct AXXAUGG context for ribosome initiation [19,20] and ends with a UAA termination codon at nucleotide position 1621. The ORF codes for a protein 530 amino acids long, a similar length to other insect luciferases and luciferase-like proteins (Supplementary Figs. S2 and S3).

The encoded luciferase has a calculated molecular weight of 58,955 Da. It is therefore marginally smaller than the luciferase of

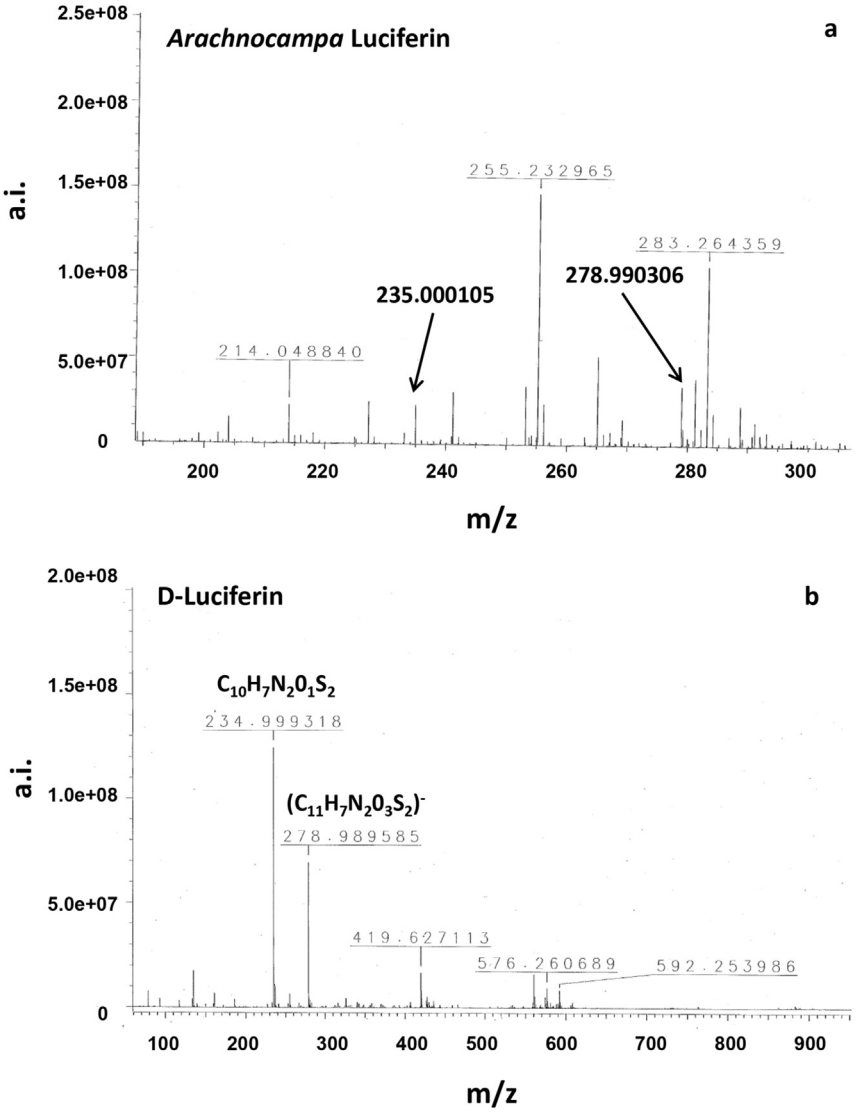

Fig. 2. Fourier Transform Ion-Cyclotron resonance mass spectroscopy (FT-ICR-MS) traces. **a**. Analysis of GW Luciferin ethanol extract by FT-ICR-MS showed the presence of two peaks with mass defects typical of S_2-species at *m/z* 278.990 and 235.001. **b**. Characteristic parent negative ion (278.9895) and decarboxylated ion (234.9996) of synthetic D-luciferin. a.i = arbitrary intensity.

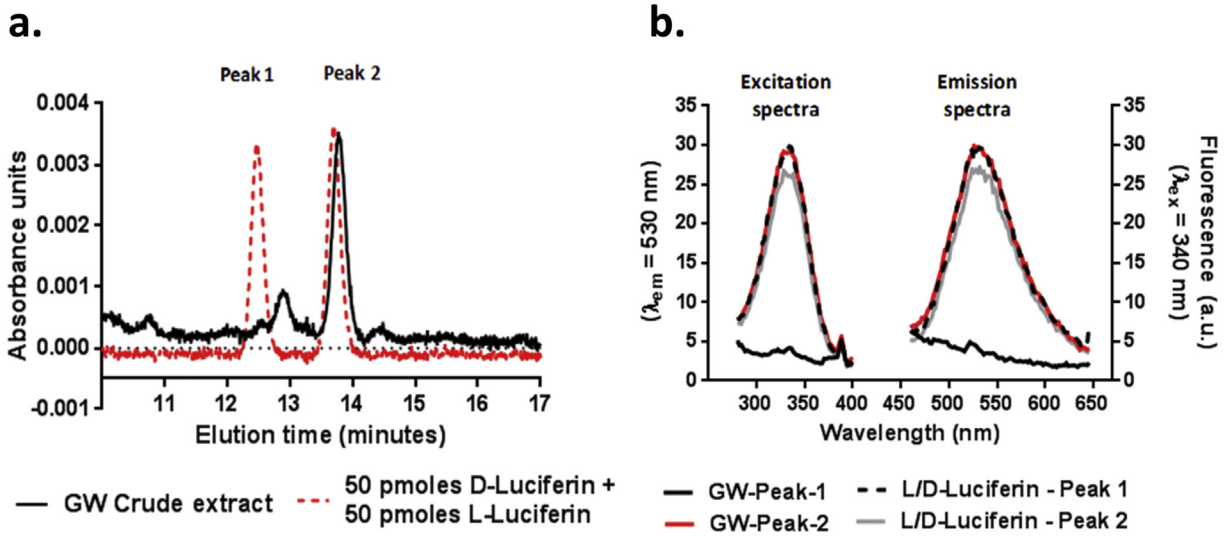

Fig. 3. Isolated glow-worm light organs contain approximately 7 pmoles of D-luciferin but no detectable L-luciferin. **a**. Chiral HPLC of an extract of glow-worm light organs compared with authentic L- and D-luciferins. Red trace: Extract of ~7.5 glow-worm light organs in 75 μL of 50% ethanol (v/v in water). Black trace: A mixture containing approximately 50 pmoles each of synthetic D- and L-luciferin in 50% ethanol (v/v in water) was separated using the same chiral HPLC system. The elution positions of D- and L-luciferin were established by separate injections (not shown). Baseline separation was achieved for the two enantiomers. **b**. Fluorescence excitation and emission spectra of putative luciferin enantiomers isolated from light organs by chiral HPLC. Solid black trace, 12.9 min peak (peak 1) and solid red trace, 13.8 min peak (peak 2) from the chiral HPLC separation shown in panel 'a'. The black dashed trace and grey trace correspond respectively to the 12.1–12.9 and 13.4–14.1 min cuts from an identical chiral separation of 50 pmoles of a mixture of authentic L and D-luciferin (not shown). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

P. pyralis and a number of other beetle luciferases [21]. The calculated molecular weight of the *Arachnocampa* luciferase differs substantially from the value previously estimated by gel filtration, 36,000 Da [13].

3.2. Sequence analysis

The GWLuc has 38.8% amino acid sequence identity with the luciferase-like protein encoded by the *Drosophila melanogaster* CG6178 gene [22]. The most closely related functional luciferase from existing known luciferases, with 34.9% amino acid sequence identity, is from *Phrixothrix viviani* (Supplementary Fig. S4). The putative luciferase of *A. richardsae* belongs to the same acyl-CoA ligase gene superfamily as the luciferases of beetles but appears to be only distantly related. Molecular phylogenetic analysis clusters *A. richardsae* luciferase closer to non-luminescent members of the acyl-CoA ligases of *D. melanogaster* and *Anopheles gambiae* than to any of the known beetle luciferase sequences (Fig. 1 b). These results suggest *A. richardsae* luciferase may have fatty acyl-CoA ligase activity, as is the case with the firefly luciferase [23].

3.3. Arachnocampa luciferin

3.3.1. Identifying GW luciferin

The presence of a compound with the structure of D-luciferin (**1**) in *A. richardsae* was established by chromatography and mass spectrometry. Thin layer chromatography of the clear supernatant in four solvent systems [24] showed in each case a single fluorescent spot with a retention factor (R_f) value similar to that of synthetic D-luciferin (**1**) (Table 1). These R_f values were also similar to those reported in the literature for D-luciferin and extracts prepared from elateridae [24] and *Arachnocampa flava* [13]. Analysis of the GW Luciferin ethanol extract by negative ion FT-ICR-MS showed the presence of two ions with mass defects typical of S_2-species at *m*/*z* 278.9903 and 235.0001 (Fig. 2a and Supplementary Fig. S5). These masses correspond to the compositions $C_{11}H_7N_2O_3S_2^-$ (278.9895) and $C_{10}H_7N_2OS_2^-$ (234.9996) (Fig. 2b), the characteristic parent negative ion and decarboxylated daughter ion of D-luciferin (**1**). The same luciferin was also detected by TLC in larval haemolymph of *A. richardsae,* and by TLC and FTICR-MS in the carcass remaining after removal of light organs and haemolymph (results not shown).

3.3.2. Stereochemistry of GW luciferin

The stereochemistry of the luciferin in *A. richardsae* light organs was determined by HPLC on a chiral column as described by Niwa et al. [25], enabling direct comparison to be made between the glow-worm and firefly bioluminescence systems. Authentic D- and L-luciferin eluted between 13.4-14.1 min and 12.1–12.9 min, respectively (Fig. 3a). An ethanol extract (75 μl) prepared from ~7.5 glow-worm light organs revealed two minor peaks within the retention windows of the authentic luciferins, the stronger eluting at 13.8 min with a much weaker second peak at 12.9 min (Fig. 3a). The peaks were collected and compared with authentic D- and L-luciferins by fluorescence spectroscopy (Fig. 3b). The glow-worm peak eluting at 13.8 min displayed identical excitation and emission spectra to the luciferin enantiomers [26]. The lesser component eluting at 12.9 min showed insignificant fluorescence excitation or emission at wavelengths characteristic of luciferins, and is unlikely to be structurally related. We conclude that *A. richardsae* light organs contain D-luciferin, with no evidence for the presence of the L-enantiomer. Calibration of the integrated peak absorption of the extract against known concentrations of standard indicated that each light organ contained at least 7 pmol of D-luciferin, potentially equivalent to ~4×10^{12} light quanta. This would be sufficient to maintain the highest measured *in vivo* light outputs [12] of 10^{11}-10^{12} quanta s^{-1} for between 4 and 40 s.

3.3.3. In vitro studies

The presence of a putative luciferase and luciferin in glow-worm is necessary but not sufficient to conclude that these molecules are responsible for glow-worm bioluminescence. We therefore expressed GWLuc in bacterial and eukaryotic systems and reconstituted the expressed product with synthetic D-luciferin and Mg-

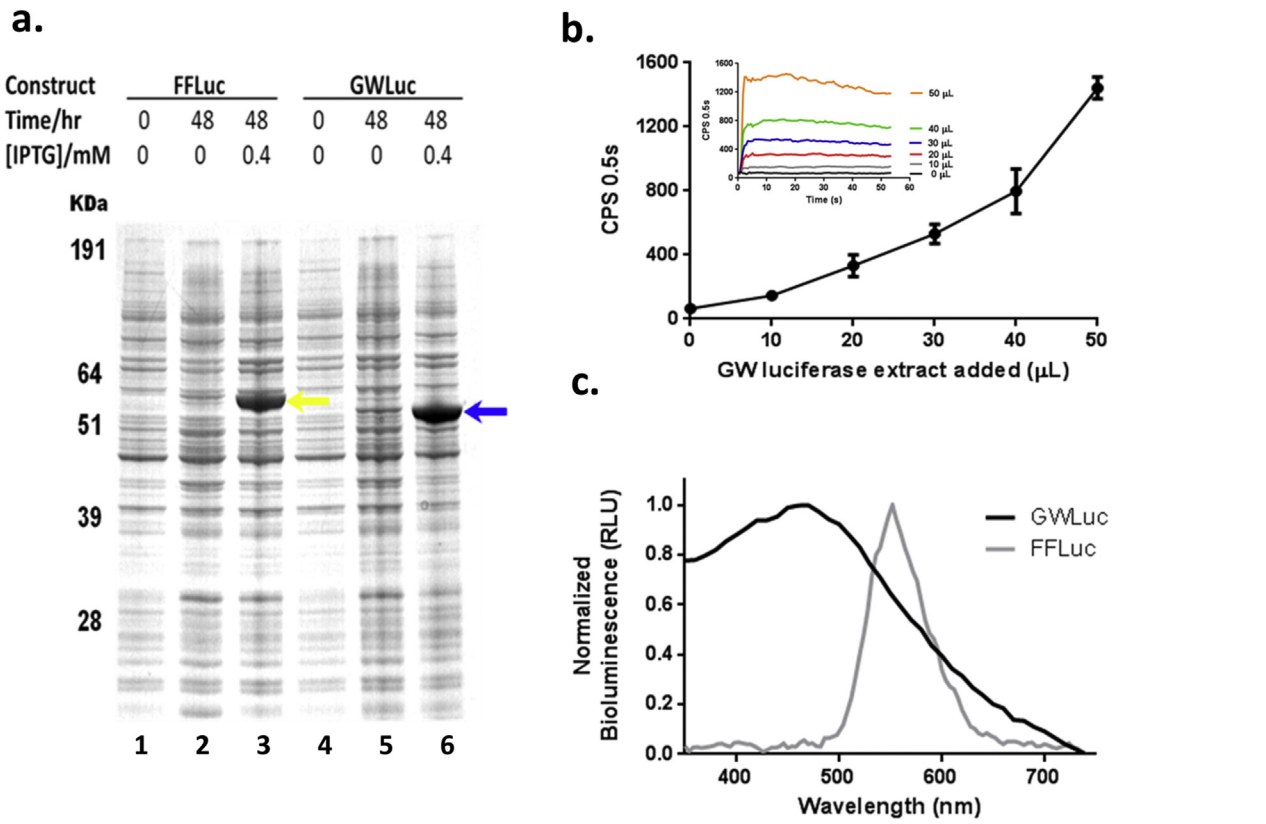

Fig. 4. Functional expression of *A. richardsae luciferase* **a**. SDS-polyacrylamide gel analysis following bacterial expression of GWLuc cDNA. cDNAs encoding *A. richardsae* (GWLuc) and *Photinus pyralis* luciferases (FFluc). Lanes Lane 2 (no isopropyl β-D-1-thiogalactopyranoside (IPTG)) and Lane 3 (0.4 mM IPTG) are from cultures transformed with FFLuc and incubated for 48 h. Lane 5 (no IPTG) and Lane 6 (0.4 mM IPTG) are from cultures containing the GWLuc construct and incubated for 48 h. Lane 1 and 4 are zero time point controls containing the FFLuc and GWLuc construct and prepared for electrophoresis before incubation. The yellow and blue arrows indicate the presence of the *P. pyralis* and *A. richardsae* luciferase proteins. Comparison between lanes 1 and 2 and lanes 4 and 5 indicates there is leaky expression even in the absence of IPTG. **b**. Dependence of luminescence on the quantity of bacterially expressed GWLuc extract. Induced bacterial cells expressing GWLuc, were lysed and aliquots (10–50 µL) were assayed in a total volume 100 µL in 25 mM Tris-acetate buffer (pH 7.75), containing 2 mM ATP, 4 mM magnesium acetate and 0.4 mM D-luciferin. Light output was measured using a Wallac1420 Victor 2 luminometer (Perkin-Elmer) in flash kinetics mode. The integration time was set to 0.5 s and the number of repeats set as 100. 10 µL of a control lysate containing no luciferase gene gave a luminescence signal approximately equivalent to no added extract not shown (cps = counts per second). **c**. Normalised emission spectra of GWLuc crude extract and FFLuc crude extract. Spectra were normalised by intensities at 470 nm and 552 nm for GWLuc and FFLuc spectra, respectively. The GWLuc spectrum was smoothed (0^{th} order smoothing, 4 neighbours) using the Savitzky-Golay method [33]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ATP. Bacterially expressed GWLuc cDNA and firefly luciferase each yielded a single prominent band on a gel with apparent MW = 64,000 Da for glow-worm and MW = 67,600 Da for firefly (Fig. 4a).

Under these conditions of expression, firefly luciferase partitioned to a 16,000 g supernatant but the glow-worm luciferase was found predominantly in the 16,000 g pellet. Nevertheless, a crude lysate of bacteria expressing glow-worm luciferase luminesced significantly above control in the presence of D-luciferin and Mg-ATP (Fig. 4b). Luminescence was not observed when D-luciferin was omitted or substituted with coelenterazine and was heat-sensitive and dependent on the volume of lysate added (Fig. 4b). Luminescence was not observed under these conditions with extracts of uninduced bacteria, nor with samples expressing the CG6178 gene product from *D. melanogaster*. Preliminary measurements of the reconstituted glow-worm bioluminescent system indicate a broad emission maximum with λ_{max} = 450–500 nm compared with firefly λ_{max} = 550 nm measured under the same conditions (Fig. 4c). These preliminary results are consistent with an emission maximum of 487 ± 5 nm reported by to Shimomura et al. [14] for *Arachnocampa Luminosa* and in close agreement with the corrected spectrum for *A. Richardsae* [12] and *A. Flava* at 484 nm [13].

Our sequence data suggest that the same ancestral gene family has been co-opted independently by beetles and flies as a starting point for the evolution of highly active luciferases. The fact that both species in both taxa utilise D-luciferin is consistent with the suggestion that evolution of luciferin biosynthesis is the limiting factor [27] in the evolution of insect bioluminescence. The discovery of D-luciferin in taxa other than the Coleoptera is novel and should provide new opportunities to determine its biosynthetic origins. There is no evidence for the presence of L-luciferin in the glow-worm light organ, although we cannot exclude its occurrence in other glow-worm tissues.

Many studies have explored the molecular basis of the emission spectra produced by beetle luciferases. Investigations have examined the sequence differences between naturally occurring colour variants of luciferase [8,9], the effects on the luciferase emission spectrum of specific targeted mutations [28], the effects of the environment on the fluorescence spectrum of D-luciferin or model substrates [29,30], and combined approaches [31]. Despite these studies and publication of the crystal structure of a red-shifted mutant of Genji firefly luciferase [32], there is no agreement on how the active site environment of luciferase influences the bioluminescence spectrum [10]. The observation that a D-luciferin-utilising luciferase emits with $\lambda_{max} \approx 490$ nm is unprecedented. Glow-worm luciferase will provide novel opportunities to test existing models of colour determination.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.07.081.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2016.07.081.

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