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The enzymatic nature of fungal bioluminescence†‡

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The uncertainty about the possible involvement of a luciferase in fungal bioluminescence has not only hindered the understanding of its biochemistry but also delayed the characterization of its constituents. The present report describes how *in vitro* light emission can be obtained enzymatically from the cold and hot extracts assay using different species of fungi, which also indicates a common mechanism for all these organisms. Kinetic data suggest a consecutive two-step enzymatic mechanism and corroborate the enzymatic proposal of Airth and Foerster. Finally, overlapping of light emission spectra from the fungal bioluminescence and the *in vitro* assay confirm that this reaction is the same one that occurs in live fungi.

Introduction

In the past five decades, the chemical and enzymatic pathways involved in light emission by several living organisms have been unveiled.[1–3] However, the biochemistry of many systems remains unsolved and some of the proposed mechanisms are still controversial. For example, whether a luciferase[4] is actually involved in fungal bioluminescence has not yet been established.[5]

Since the early 20th century, many researchers have attempted to reproduce with fungi the classical luciferin–luciferase test,[6] which consists of mixing hot (substrate) and cold (enzyme) water extracts.[5,7] The first successful experiment was reported by Airth and McElroy, who found that the addition of reduced pyridine nucleotide, NADH or NADPH [here abbreviated as NAD(P)H],[4] resulted in sustained light emission. In this respect the system resembled the then-recently discovered bacterial luciferase system (Scheme 1),[8] but differed in that neither reduced riboflavin phosphate ($FMNH_2$) nor long chain aliphatic aldehyde, known to be substrates in the bacterial reaction,[9,10] were active in the fungal system.[11]

In the early work, although no clue as to the nature of the luciferin (substrate) was obtained, it was shown that the protein (cold water extract) could be resolved into soluble and particulate fractions, both of which were required for light emission.[12,13] Experiments indicated that the former catalyzes a first step in which a factor, possibly luciferin, is reduced, while the second, associated with luciferase, reduced luciferin was oxidized to generate an excited product and then light emission (Scheme 1).

Fungal bioluminescence
- Airth and Foerster's proposal

$$L + NAD(P)H + H^+ \xrightarrow[(reductase)]{\text{soluble enzyme}} LH_2 + NAD(P)^+$$

$$LH_2 + O_2 \xrightarrow[(luciferase)]{\text{insoluble enzyme}} LO + H_2O + h\nu$$

L: luciferin, LH_2: reduced luciferin, LO: oxidized luciferin

Bacterial bioluminescence

$$FMN + NAD(P)H + H^+ \xrightarrow{\text{reductase}} FMNH_2 + NAD(P)^+$$

$$FMNH_2 + RCHO + O_2 \xrightarrow{\textit{luciferase}} FMN + RCO_2H + H_2O + h\nu$$

FMN: oxidized flavin mononucleotide (luciferin), $FMNH_2$: reduced FMN
RCHO: long-chain aliphatic aldehyde, RCO_2H: fatty acid

Scheme 1 Adapted from ref. 5.

Over the next two decades two groups, Kamzolkina *et al.* (1983 and 1984) and Kuwabara and Wassink (1966), confirmed the involvement of enzymes.[14–16] The latter authors further reported the purification and crystallization of luciferin from *Omphalia flavida*, which was active for bioluminescence with enzyme prepared by the Airth procedure. This luciferin also emitted a brief flash of light without enzyme upon the addition of hydrogen peroxide, thus a chemiluminescence, which could be used to track activity in the purification. The emission spectrum of the former peaked at 524 nm, close to the value previously reported for all fungal species examined, whereas the latter peaked at 542 nm. However, the chemical nature of the putative luciferin was not reported, and their work was not continued.

Making use of the peroxide-triggered chemiluminescence to measure activity, Shimomura and colleagues undertook studies directed at the determination of the luciferin structure and identified a new sesquiterpene from *Panellus stipticus*, which they named panal, as a possible precursor of fungal luciferin.[17,18] In further studies, substances capable of chemiluminescence were found to be present in *P. stipticus* and five other luminous species studied, and in non-luminous species as well.[3,19] These were assumed to be the functional luciferin in the luminous species, and it was proposed that an active oxygen species, such as superoxide radical anion, is involved in the luminescent reaction of luminous fungi.[19] At the same time Shimomura's efforts to demonstrate the presence of protein acting as a catalyst in the light emitting

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‡ Electronic supplementary information (ESI) available: Effect of the cold and hot concentrations on the decay rate constants, the rise rate constant and the relative light intensity integrals (Table 1S); *in vivo* BL spectra obtained from fruiting bodies (Fig. 1S); BL emission of *G. viridilucens* dried cultivated mycelium in the absence and in the presence of water, and of powdered dried mycelium of the same species in water (Fig. 2S). See DOI: 10.1039/b908982a

reaction were not successful, so it was suggested that in at least some species, fungal light emission does not involve a luciferase.

More than sixty species of bioluminescent fungi belonging to three distinct evolutionary lineages have been described worldwide, many in Brazil, and we ourselves have discovered nine new Brazilian species.[5,20,21] We have prepared cell-free extracts from several such species and obtained convincing evidence for the participation of enzymes in fungal bioluminescence. Cross reactions of the hot and cold water extracts from different species result in light emission, indicating that the components involved in the bioluminescence are functionally similar in different species. The peak wavelength of emission from the *in vitro* reaction is at about 530 nm, the same as that of the living fungus (Fig. 1S, ESI†), and similar to that reported for many different species, also suggesting, as earlier authors have,[3] that the luciferin and light emitter are the same in different species.

Experimental

Fungal species

Four species were used in these studies. Fruiting bodies of *Gerronema viridilucens*,[20] *Mycena lucentipes*[21] and *Mycena luxaeterna* nom. prov. were collected in the Brazilian Atlantic Forest in the municipality of Iporanga, SP, and kept frozen in liquid nitrogen until use. *"Pleurotus" gardneri* (Berk.) Sacc. fruiting bodies were collected in the Brazilian Cerrado (savannah-like vegetation) located in the municipality of Gilbués, PI, dried in a vacuum desiccator with $CaCl_2$ (Merck) and stored at room temperature until use.

Cultures

Cells of *G. viridilucens* were isolated from fruiting bodies and the mycelium grown on Petri dishes (100 mm diameter) with a medium of 1.0% (w/v) sugar cane molasses (82.2°Bx, Pol 56%) and 0.10% (w/v) yeast extract (Oxoid) in 2.0% (w/v) agar (Oxoid) at pH 6.0 (non-buffered).[22] From the Petri dishes the mycelium was inoculated and grown in static liquid cultures in 250 mL Erlenmeyer flasks in 50 mL of the same medium, but with no agar. Cultures were harvested after 14 days at 25 °C; during this time, the mycelium developed island-shaped colonies, emitting visually observable light only from the surface in contact with air. The harvested mycelium was then filtered, washed with deionized water and lyophilized (Micro Modulyo - Thermo Savant equipment). Afterwards, the dried mycelium was ground with a mortar and pestle under argon atmosphere inside a glove box (Labmaster 130, mBraun). 20 mg portions of the fine powder obtained were sealed under argon in 5 mL pharmaceutical vials and stored at –20 °C until use. Fruiting bodies of different fungal species were subjected to the same procedure. Exposure of lyophilized material to air led to a decrease of the light intensity in extracts (data not shown).

Extracts

Hot water extracts were prepared from lyophilized mycelium or fruiting bodies as follows: 20 mg were mixed with 1.5 mL of extraction buffer [100 mM phosphate pH 7.5, containing 1 mM 2-mercaptoethanol and 5 mM EDTA (Sigma)] in a 5 mL vial for 1 min in a water bath at 80 °C and then cooled in an ice bath. This extract was centrifuged at $5000 \times g$ for 5 min and the supernatant was reserved. The process was carried out under a pure nitrogen atmosphere whenever possible.

Cold water extracts were prepared by extraction of 80 mg of lyophilized mycelium/fruiting bodies in 4.0 mL of cold extraction buffer with a Potter-Elvehjem homogenizer. The resulting homogenate was then centrifuged at $10\,000 \times g$ for 10 min at 4 °C and the pellet discarded. The supernatant was used as source of enzymes and kept on ice until used. Deaeration is not required during the preparation of cold extract as the oxidation of luciferin is desirable to diminish its concentration in solution. The main intention in the preparation of cold extract is to preserve the enzymes not the substrate.

Assays

Assays were carried out in test tubes at 23 ± 1 °C. Light intensities were measured using a Berthold DS Sirius tube luminometer set with an integration time of 1 s. Light intensities were measured in relative light units (RLU) and converted to einsteins per second (einstein s^{-1}), using the luminol standard in alkaline aqueous solution in the following conditions:[23,24] standard assay tube, final volume 350 μL, and 2.72×10^{-13} mol luminol.

The standard assay contained 200 μL of cold extract, 50 μL of 1 g L^{-1} bovine serum albumin (BSA, Sigma), and 50 μL of hot extract, and the reaction initiated by the addition of 50 μL of NADPH (Sigma) solution (0.70 mM in extraction buffer, final concentration in assay is 100 μM). NADH (Sigma) can replace NADPH, although the light yield is normally a bit lower.

The supernatant (4 mL) of the cold extract was resolved into two fractions by centrifugation at $194\,000 \times g$ for 60 min at 4 °C (Hitachi RP50T ultracentrifuge, P50AT2–716 rotor). The supernatant (soluble fraction) was removed with a syringe and the pellet (insoluble fraction) re-suspended in 3 mL of cold extraction buffer containing 250 mM sucrose (Sigma). Assays were performed by the following procedure: (i) light intensity acquisition was initially started ($t = 0$ s) with the luminometer assay tube containing 50 μL of the hot extract, 50 μL of 1 g L^{-1} BSA, and 50 μL of NADPH solution (0.70 mM); (ii) 100 μL of soluble fraction was then added after 60 s; and (iii) 100 μL of the re-suspended pellet was added after 20 s ($t = 80$ s), triggering the light emission. The experiment was also performed in reverse order, first adding the insoluble fraction ($t = 60$ s), and then the soluble fraction ($t = 80$ s). The assay shows good repeatability with the same batch of mycelium/fruiting bodies, as attested by the variation of less than 10% in maximum light intensity of triplicates obtained in all the experiments performed with cold and hot extracts.

Kinetics studies

The effect of concentration of both hot and cold water extracts on reaction kinetics was determined by varying their volumes systematically and independently in the standard assay, maintaining a final volume of 350 μL. Kinetic curves were fitted by a triexponential function model yielding three rate constants, two of them associated with the light intensity decay (k_{obs1} and k_{obs2}) and one associated with the intensity rise (k_{obs3}). The k_{obs2} term is necessary in the expression to fit the experimental points obtained

with the variation of hot extract, but only at higher concentrations of cold extract (Table 1S, ESI†). When studying the dependence of the BL emission on the cold extract concentration, the observation time did not ensure complete decay to zero (900 s). Hence, fitting functions determined for extract concentrations (Table 1S, ESI†) were used to plot the intensity *vs.* time curves traced during 6000 s. The resulting plots were integrated using Microcal Origin® 6.0.

Luciferin extraction

Ten grams of *P. gardneri* dried fruiting bodies were ground in a blender (Tosco Equipamentos) and then mixed with 5 g of Celite 545 (particle size 0.02–0.1 mm, Merck). The fine powder obtained was then loaded onto the 66 mL stainless steel extraction cell of a Dionex ASE 300 accelerated solvent extractor, which had a cellulose disk at the bottom previously filled with a 5 g layer of Celite 545, used to clean up the extract. The extraction was carried out using optimized conditions described as follows: (i) solvent: aqueous solution containing 0.025% of formic acid (Merck), 20 mM of 2-mercaptoethanol, pH 3; (ii) pressure: 1500 psi; (ii) temperature: 80 °C; (iii) heat time: 5 min; (iv) static time: 15 min; (v) flush volume: 60%; (vi) purge time: 120 s; and (vii) static cycles: 1. 100 mL of final extract was partitioned in 200 mL glass vials previously purged with argon.

In vivo/*vitro* bioluminescence spectra

Bioluminescence emission spectra were obtained in field from freshly collected fruiting bodies of *G. viridilucens*, *M. lucentipes*, *Mycena asterina* and *Mycena fera* (black variation) with a portable fiber optic spectrofluorometer (USB 2000, Ocean Optics; Fig. 1S, ESI†). The *in vitro* spectrum was acquired using a Hitachi F4500 spectrofluorometer in luminescence mode (not corrected) with a scan rate of 2400 nm min^{-1}, 20 nm slit width and a PMT voltage of 950 V. Light intensities were measured in arbitrary units per second and converted to einstein per second (einstein s^{-1}) using the luminol standard in alkaline aqueous solution in the following conditions:[23,24] 1.0 mL cuvette with 10 mm optical path, final volume 1050 μL, and 2.30×10^{-12} mol luminol. The assay was performed using 600 μL of cold extract of *M. luxaeterna*, 150 μL BSA, 150 μL of luciferin extracted with ASE 300 equipment, and 150 μL of 0.70 mM NADH (final concentration, 100 μM), final volume 1050 μL. The reaction was triggered upon addition of NADH solution.

Results and discussion

In this study cold and hot water extracts of both fruiting bodies and cultured mycelia of four different Brazilian species were prepared (*G. viridilucens*, *"Pleurotus" gardneri* and two species of *Mycena*). All extracts were active for light emission upon the addition of either reduced pyridine nucleotide NADPH or NADH, thus confirming the results of Airth and colleagues. In some reports (*e.g.* Airth and Foerster, 1964;[25] *P. stipticus*), activity was detected in cold but not in hot water extracts. In the present study, the addition of NAD(P)H to cold water extracts alone rarely led to light emission. This result may have been related to the amount of luciferin present in the fungal material used in the extraction. Light emission was usually detected only in the presence of cold and hot extracts and NAD(P)H.

It is uncertain from the literature whether the fungal material should be used fresh or lyophilized. Experiments with integral and dried mycelium of *G. viridilucens* confirmed that the lyophilization did not lead to total degradation of components associated with the fungal bioluminescence, as it was possible to observe light emission upon simple rehydration of the mycelium (Fig. 2Sa, ESI†). Moreover, water added to powdered and dried mycelium also yields light, but with a higher intensity and faster decay (Fig. 2Sb, compared to Fig. 2Sa†). We recommend preparing the hot extract using dried and powdered mycelium/fruiting bodies under anaerobic and reducing conditions.

All four species were tested for the ability of hot and cold water extracts to cross react. The positive results for two are shown in Fig. 1; these indicate that the substrates involved are the same or similar, and that the enzymes are similar. Extracts can be prepared from either the mycelium cultivated in laboratory or fruiting bodies harvested from the field and from either a single species or from the combination of two bioluminescent ones. Addition of bovine serum albumin (BSA) to the assay increases the light emission 4-fold, as observed in the purification process of luciferases from *Photinus pyralis* and *Vibrio fischeri*.[26] It is also known that addition of BSA or reducing 2-mercaptoethanol at low concentrations to crude homogenates prevents luciferase denaturation.[27]

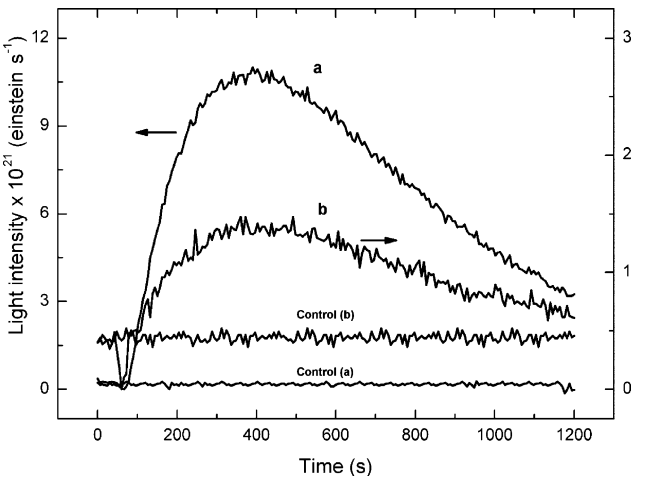

Fig. 1 Cross-reactions between extracts of two species of bioluminescent fungi. Light intensity time courses obtained using the standard assay prepared with: (a) *M. lucentipes* fruiting bodies hot extract and *G. viridilucens* cold extract; (b) *G. viridilucens* for hot extract and *M. lucentipes* for cold extract. Reactions were initiated by the addition of NADPH. [NADPH] = 100 μM, [BSA] = 140 mg L^{-1}, hot extracts: 50 μL, cold extracts: 200 μL, final volume: 350 μL. Controls: all components except NADPH.

In an enzymatic reaction, because of turnover, the apparent rate constant is dependent on the enzyme concentration, whereas it is independent of substrate concentration, which only affects the amount of product.[28] The concentrations of hot and cold water extracts of *G. viridilucens*, representing substrate and enzyme, respectively, were varied systematically (Table 1S,† Fig. 2). The total light is invariant with the amount of cold water extract (enzyme), but proportional to substrate (hot water extract). The results are consistent with the conclusion that the light-emitting reaction is enzymatic. The light emission curves obtained with

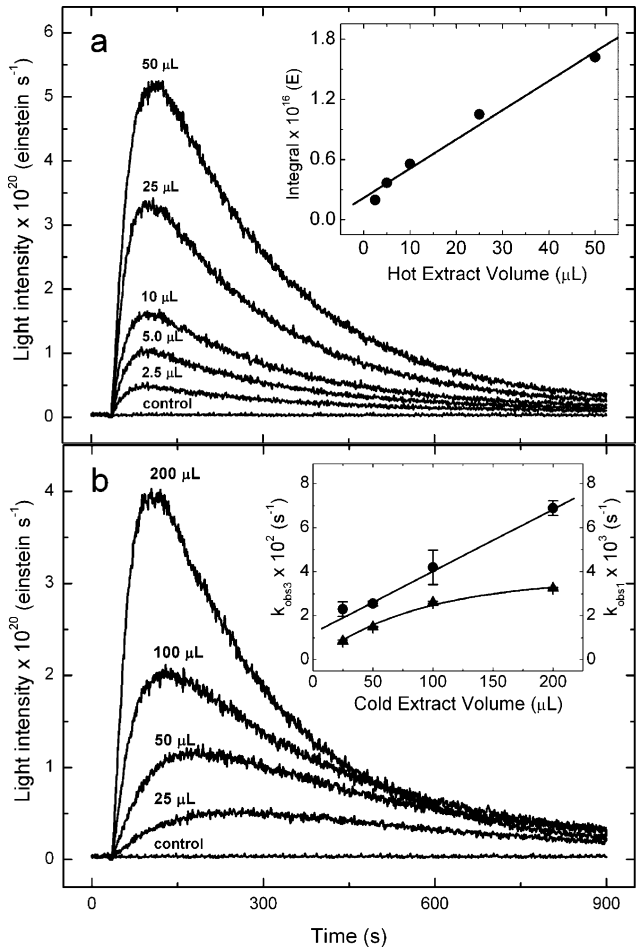

Fig. 2 Light emission kinetics of *in vitro* reactions using hot and cold water extracts of lyophilized powders prepared from the cultivated mycelium of *G. viridilucens.* (a) Different concentrations of the hot water (substrate) extract: [NADPH] = 100 µM; [BSA] = 140 mg L^{-1}; cold extract, 200 µL. Inset: light emission integral *vs.* amount of hot extract. (b) Different concentrations of the cold water (enzymes) extract: [NADPH] = 100 µM, [BSA] = 140 mg L^{-1}, hot extract: 50 µL, inset: dependence of rate constants k_{obs1} (●) and k_{obs3} (▲) on the cold extract concentration. The final volume was 350 µL in all experiments. Controls: all components except NADPH.

the cold and hot extracts assay (Table 1S,† Fig. 2) reveal a rapid increase followed by a slower decay of the emission intensity and can be fitted by a combination of three exponential functions (Table 1S†), yielding three observed rate constants, k_{obs1}, k_{obs2} and k_{obs3}. The lower constants k_{obs1} and k_{obs2} are contained mainly in the slow decay, whereas the highest constant k_{obs3} is contained mainly in the fast rise. The profile of the curves is typical for a chemiluminescent reaction with consecutive steps,[24,29] and supports the proposed involvement of two enzymes, as depicted in Scheme 1. Although the assignment of rate constants to the steps catalyzed by reductase and luciferase at this point may be merely speculative, preliminary results obtained by varying the concentration of NADPH (data not shown) suggest that only the rising rate constant (k_{obs3}) increases linearly with the [NADPH], thus justifying its assignment to the luciferin reduction step. Finally, k_{obs1} shows a linear dependence on the concentration of cold extract, whereas k_{obs3} seems to present a saturation curve profile (Fig. 2b, inset).

The resolution by centrifugation of the cold water (enzyme) extract[12] into particulate and soluble fractions was also confirmed. Addition of the soluble extract to the hot water extract and NADPH followed 20 s later by the addition of the particulate fraction resulted in a faster onset of luminescence than the converse order of addition (Fig. 3). Thus, light emission with the hot water extract requires both fractions, but they can function independently, such that the first can proceed in the absence of the second. This step, postulated to result in the reduction of the luciferin substrate, is attributed to the soluble component, while the particulate fraction is identified with the subsequent step, which catalyzes the light-emitting reaction with oxygen (Scheme 1).

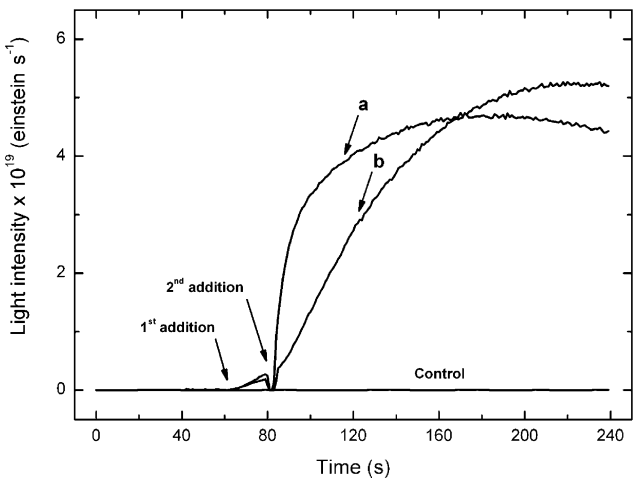

Fig. 3 Effect of the order of addition on the light emission kinetics using the fractions obtained by high-speed centrifugation of the cold water extract. (a) 100 µL of the supernatant (reductase) was first added to a cuvette containing 100 µM of NADPH, 140 mg L^{-1} BSA, and 50 µL of the hot extract, followed by the later addition of 100 µL of the pellet (luciferase); (b) the order of addition of supernatant and pellet was reversed. Controls: all components except NADPH.

These results provide strong evidence of the involvement of a NAD(P)H-dependent reductase and a luciferase in fungal bioluminescence, thus confirming Airth and Foerster's mechanistic proposal for these organisms. However, the authors did not match the *in vitro* bioluminescence spectrum with that emitted by living fungi,[12] which is crucial to differentiate bioluminescence and ultraweak chemiluminescence sustained by reactive oxygen species.[30] Several earlier studies have reported the *in vivo* emission of all species to be in the green, peaking at about 530 nm. Using *M. luxaeterna* and *P. gardneri* fruiting bodies as sources of enzymes and substrate, respectively, the spectrum of the *in vitro* reaction was found to be close to that from *G. viridilucens* fruiting bodies (Fig. 4). This indicates that the *in vitro* light emission is likely to be elicited by the same reaction as occurs in the live fungus. Interestingly, the highest intensity of light we were able to attain in the cold and hot extracts assay detected with the Hitachi F4500 spectrofluorometer, namely 10^{-14} einstein s^{-1} with the hot extract obtained from the accelerated solvent extractor, was one order of magnitude lower than the light emitted by a ten-day old culture of *G. viridilucens*, which can be visualized with dark-adapted eyes.

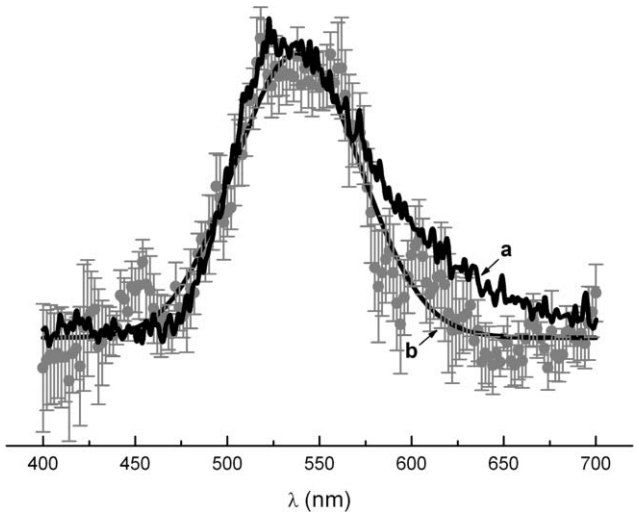

Fig. 4 (a) *In vivo* bioluminescence emission spectrum of *G. viridilucens* fruiting bodies, $\lambda_{max} = 533$ nm. (b) *In vitro* light emission observed using the standard assay procedure with the fungi *P. gardneri* (hot extract) and *M. luxaeterna* (cold extract), $\lambda_{max} = 538$ nm (maximum intensity 3.0×10^{-14} einstein s^{-1}). Errors bars represent standard deviation of triplicates for each experimental point ($n = 3$).

Although the present study, in confirmation of earlier work by several groups, demonstrates that a luciferase occurs in extracts of luminous fungi, and can be assumed to be responsible for the light emission *in vivo*, it will be interesting to determine what role, if any, chemiluminescence plays in the normal emission of live fungi. Measurements of the respective quantum yields of the enzymatic and non-enzymatic reactions could clarify this.

Conclusion

In accordance with the mechanism proposed by Airth and Foerster for fungal bioluminescence, our findings attest to the participation of a luciferin reduced by NAD(P)H and a luciferase in the fungal bioluminescent reaction. Additionally, we point out that the hot extract should be prepared from dried powdered mycelium or fruiting bodies (preferentially in the absence of oxygen) and the cold extract from fresh material, in order to increase the chances of detecting light emission using the standard assay. The enzymatic nature of fungal bioluminescence was also supported by further experiments with the cold extract, which it was heated or precipitated with ammonium sulfate or filtered using a 3 kDa Amicon® Ultra Centrifugal filter (Millipore). In all these experiments, the cold extract obtained did not lead to light emission upon its reaction with the hot extract and NADPH. Ultimately, the assay used in the present study simulates the physiological conditions of the fungus and produces light with the same characteristics as those of fungal bioluminescence. Work is in progress to identify the fungus luciferin and characterize the physicochemical properties and structures of the enzymes.

Acknowledgements

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