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Two forms of substrate for the bioluminescent reaction in three species of basidiomycetes

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ABSTRACT

The luminescent response of the enzymatic system of *Armillaria borealis* on the cold and hot extracts from cell-free culture liquids of *Inonotus obliquus, Pholiota* sp. and *A. borealis* was examined. The greatest influence on the light emission produced by the luminescent system of *A. borealis* was provided by the temperature at which the probes were prepared for assay. Boiling a culture liquid on water bath for a few minutes promoted a multifold increase in the luminescence. The results of luminescence assay suggest that the substance involved in the bioluminescent reaction in higher fungi is presented in culture liquids and mycelia in two forms. In one form, it is ready to interact with the enzymatic system and in the second form, it becomes accessible for the reaction after heat treatment. The pool of thermoactivated substance was found to be much large than the amount of the ready accessible one. We suggest that predecessors of hispidin, which is fungal luciferin precursor, are responsible for this phenomenon. They are not involved in bioluminescence at their original state and are converted into the substrate under the influence of high temperature.

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Cold and hot extracts; culture liquid; enzymatic system; hispidin; luminous fungi; substrate of luminescent reaction

Introduction

In the second half of the nineteenth century, Raphael Dubois (Dubois 1985, 1987) when studying the luminescence systems of the bivalve mollusk *Pholas dactylus* and Indian click-beetle *Pyrophorus* sp. developed a method that allowed him to demonstrate luminescence *in vitro*. He obtained "hot" and "cold" extracts from the biomass of luminous organisms and mixed them together, recording luminescent signals. Based on the results of these studies, the author introduced two key terms – "luciferase" and "luciferin". The former, he gave to a protein component (enzyme) that was present in the cold extract and catalysed the light-emission reaction, the latter to a low molecular weight, thermostable component contained in the hot extract, which was the substrate of the reaction. Currently, the luciferin–luciferase concept is generally accepted, the researchers widely use it in studies on luminescent systems of various living organisms. For many of them, the enzymes (luciferases) catalysing luminescent reactions and their substrates (luciferins) were isolated and characterised that provides a key insight into the mechanisms for light emission (Shimomura 2006). Despite the fact that over 80 species of luminous basidiomycetes have been found throughout the world, the fungal bioluminescence has been studied to a much lesser extent (Teranishi 2016).

The classical method for studying the luminescence of basidiomycetes *in vitro* consists of recording light emission produced upon mixing together the hot (substrate) and cold (enzymes) water extracts (Airth and McElroy 1959; Airth and Foerster 1962; Kamzolkina et al. 1983, 1984; Oliveira and Stevani 2009; Oliveira et al. 2012). The cold extracts from biomass of luminous objects (fruiting bodies or mycelium) are isolated at low temperatures (usually at 4°C). This allows to retain activity of the enzyme (or enzymatic system) that catalyses the light-emission reaction. In the process of cold extracting or further storage, the substrate of the reaction is usually totally consumed by the enzyme and is not present in the cold extracts. The hot extracts are obtained by heat treatment of homogenised fungal biomass at high temperatures (80–100°C), which leads to denaturation of luciferase and other enzymes involved in luminescence while preserving the substrate for luminescent reaction – luciferin. Interestingly, the hot extracts prepared from nonluminous fungi can also

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stimulate the light emission, thus implying that the substrate or its precursor is not specific for luminous species (Purtov et al. 2015; Puzyr et al. 2016, 2017).

There are known several successful attempts that demonstrated the fungal bioluminescence *in vitro* using the classical luciferin/luciferase test (Airth and McElroy 1959; Airth and Foerster 1962; Kamzolkina et al. 1983, 1984; Oliveira and Stevani 2009; Oliveira et al. 2012; Oba et al. 2017). Cross-reactions of the cold and hot extracts obtained from different fungal species indicated a common mechanism of bioluminescence in higher fungi (Oliveira and Stevani 2009). Using extracts from the fruiting bodies of luminous *Mycena chlorophos, Omphalotus japonicus* and *Neonothopanus gardneri*, it was shown that hispidin, a secondary metabolite found in many fungi, is at least one of the luciferin precursors (preluciferin) involved in the light-emission reaction in higher fungi (Oba et al. 2017). Earlier, we obtained cold extracts from three species of luminous basidiomycetes (*Neonothopanus nambi, Armillaria borealis* and *Mycena citricolor*), containing the fungal luminescence systems that exhibited enzymatic activity (Bondar et al. 2014; Puzyr et al. 2017).

Recently, we have experimentally shown the applicability of the luminescent system isolated from the luminous *A. borealis* for quantitative detection of hispidin in aqueous solutions (Puzyr et al. 2018). A linear dose dependence of luminescence intensity has been demonstrated for the analyte concentration range of 5.4×10^{-5}–1.4×10^{-2} μM.

In this work, we examined the luminescent response of the enzymatic system of *A. borealis* on the hot extracts (substrate) performing the classical luciferin/luciferase test and the cold (substrate) extracts from cell-free culture liquids of *Inonotus obliquus, Pholiota* sp. and *A. borealis*. The reasoning behind the choice of the species was the reported presence of hispidin and its analogies in fruiting bodies and mycelia of these fungi.

A medicinal fungus *I. obliquus* belonging to the Hymenochaetaceae family of Basidiomycetes has been used for a long time as traditional medicines in Asian countries for the treatment of various human diseases. It is believed that a high pharmacological activity of *I. obliquus* is associated with polyphenols consisted mainly of hispidin analogues and melanins, which are dominant in the sclerotia. However, only trace elements of these compounds were detected in submerged cultures of *I. obliquus* (Zheng et al. 2008). The content of hispidin isolated from the dried fruiting bodies of *I. obliquus* using the molecularly imprinted polymer as an adsorbent was found to be 2.9 μg/g (Li et al. 2015).

Pholiota is a widespread genus in the family Strophariaceae. There are about 150 known species included in this genus (Kirk et al. 2008). The bisnoryangonin and hispidin are common among fungi of the *Pholiota* genus (Velisek and Cejpek 2011). Their presence in the fruiting bodies of *Pholiota squarrosa* as *in vitro* bioluminescence activating substances was recently confirmed (Purtov et al. 2015; Puzyr et al. 2016, 2017). However, none of the *Pholiota* fungi is luminous (Chew et al. 2015).

The genus *Armillaria* in the family Physalacriaceae contains about 40 species distributed throughout the world and is most known among bioluminescent fungi (Watling et al. 1991). Only mycelia and rhizomorphs of *Armillaria* species are known to exhibit luminescence. There is opinion that the mycelium of most or even all *Armillaria* species is luminescent (Desjardin et al. 2008). There were no data available in the open literature that any of *Armillaria* members contain hispidin (or its analogues) until this substance was detected in *A. borealis* as a precursor of luciferin (Purtov et al. 2015).

The studies were also aimed at revealing luminescence stimulating substances, which could be secreted by the mycelium into the culture broth during cultivation. It is well known that culture liquids of basidiomycetes grown using submerged cultivation are a rich source of various bioactive metabolites (Elisashvili 2012). In this work, we consider the culture liquids as aqueous extracts. To our knowledge, the culture liquids were used for the first time in fungal bioluminescence assays. Comparison of the light emission induced by using classical and non-classical tests allowed to identify two forms of the substrate for the bioluminescent reaction – accessible and activated by heat treatment.

Materials and methods

The strains of *A. borealis* (Marxm. & Korhonen), IBSO 2328 and *Pholiota* sp. were obtained from Collection CCIBSO 836 of the Institute of Biophysics SB RAS (Krasnoyarsk, Russia). Culture of *I. obliquus* (Ach. Ex Pers.) Pil. was provided by the laboratory of forest cultures, mycology and phytopathology at the Forest Institute of the SB RAS (Krasnoyarsk, Russia).

The finely crushed mycelium grown on solid medium in Petri dishes was used as the inoculum. The mycelia were cultivated in 300 ml Erlenmeyer flasks containing 100 ml of a liquid potato dextrose broth (PDB) nutrient medium (potato extract – 200 g/l, dextrose – 20 g/l) for 16–20 days at 24 ± 1°C under constant agitation at 140 rpm using a Max Q 4000 incubating shaker (Thermo Scientific, US). In case of *I. obliquus* malt-extract medium (ME) containing malt-extract (20 g/l), glucose (2 g/l) and peptone (1 g/l) were also used as a nutrient source.

To isolate the luminescence system, the *A. borealis* mycelial pellets were rinsed with deionised water (Milli-*Q* system, Millipore, US) to remove traces of broth and metabolites. The pellets were immersed into a large amount of water and incubated for 24 h with constant air bubbling. Then the mycelial biomass was rubbed through a stainless sieve with a mesh diameter of 0.5 mm and ultrasonically homogenised using the Volna USTD-0.63/22 device (U-Sonic, Russia) in ice-cooled 0.1 M phosphate buffer solution (pH 7.0) containing 1% bovine serum albumin (BSA) . The sonication was performed thrice, each time for 10 s with 1 min interval. Then the homogenate was centrifuged at 40,000 *g* for 30 min at 4°C using an Avanti® J-E centrifuge (Beckman-Coulter, US). The sediment was discarded, and the supernatant (cold enzymatic extract) containing components of the luminescence reaction was frozen at −80°C (−86°C Ultralow Freezer, NuAire, Inc., US), freeze dried in the LS-500 lyophiliser (Prointex, Russia) and stored at a temperature of −20° C until use.

Five hundred microliters of culture liquid were retrieved from the flask containing fungal biomass and centrifuged at 16,100 *g* for 10 min. Two 100 µl cell-free aliquots of culture liquid were transferred to MCT-150-C microtubes (Axygen Scientific Inc., US). One aliquot (cold extract) was maintained at 4°C before use. The second aliquot (hot extract) was boiled on water bath for 5 min, cooled to 4°C and then centrifuged at 16,100 *g* for 20 s to precipitate any condensate from the tube walls. The similar procedure with varied time of heat treatment (2, 4 and 6 min) was performed with the cold enzymatic extract of *A. borealis* to obtain the corresponding hot extracts.

Bioluminescence assays were performed in MCT-150-C microtubes at room temperature. The amplitude and kinetics of light intensities were measured using a Glomax® 20/20 luminometer (Promega, US) at data acquisition rate of 1/s. Luminescence values are expressed in relative light units. The integrated luminescence is calculated as the total amount of light emitted in the assay during the test time. The measurements were conducted as follows: luminescence acquisition commenced with the cold enzymatic extract (50 µl) poured into the luminometer test tube; after about 100 s, it was supplemented with 2.5 µl 10 mM nicotinamide adenine dinucleotide phosphate (NADPH); later when light emission declined to a relatively low and steady level, 2.5 or 5 µl of cold or hot extracts were added to the tube.

Results

Stimulation of bioluminescence by I. obliquus *culture liquids*

As it was described above, the mycelial pellets of *I. obliquus* were simultaneously grown in submerged culture on PDB and ME nutrient media under the same cultivation conditions. The dissimilar composition of the media resulted in different yield and appearance of the mycelial pellets (Figure 1). Figure 2 shows the intensity and kinetics of luminescence recorded when the cold and hot liquids from submerged cultures were added to the enzymatic system of *A. borealis*. Irrespective of the medium composition, the addition of cold culture liquids resulted in no activation of luminescence. However, a significant enhancement of luminescence intensity was observed when the enzymatic system was supplemented with the same amount of culture liquids after heat treatment. The luminescence activation yielded by the hot culture liquid from *I. obliquus* grown on PDB medium was more than two times higher than that one obtained when the hot culture liquid from *I. obliquus* grown on ME medium was used.

Stimulation of bioluminescence by Pholiota *sp. culture liquids*

The luminescent response of the enzymatic system of *A. borealis* on addition of the cold and hot culture liquids of *Pholiota* sp. differed from that one observed on the *I. obliquus* samples. As shown in Figure 3, the luminescence intensity was enhanced upon the addition to the luminescence assay of both the cold and hot culture

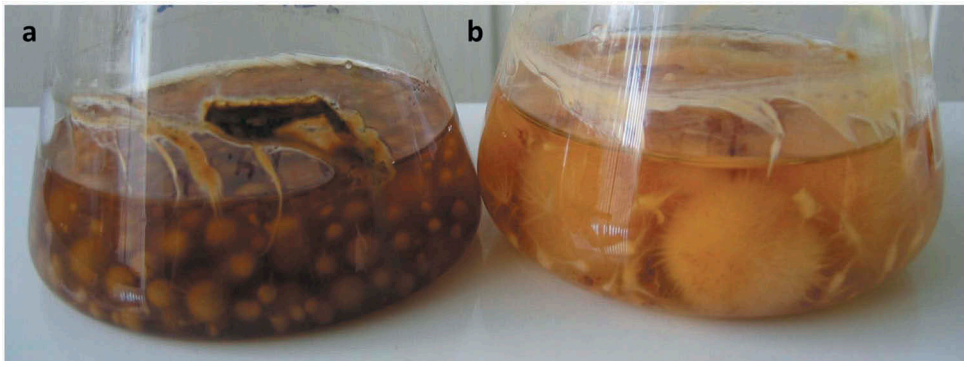

Figure 1. Mycelial pellets of *I. obliquus* grown: (a) in PDB liquid medium and (b) in ME liquid medium.

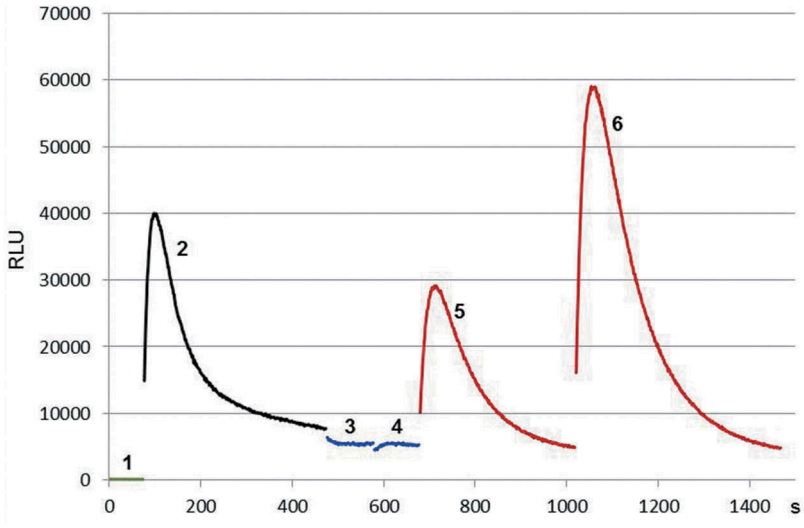

Figure 2. The intensity and kinetics of luminescence upon addition of culture liquids from submerged cultures of *I. obliquus* to the enzymatic system of *A. borealis*: (1) the cold enzymatic extract (50 µl) of *A. borealis*, (2) 2.5 µl NADPH, (3) and (4) 2.5 µl cold culture liquids from *I. obliquus* grown on ME and on PDB media, (5) and (6) 2.5 µl hot culture liquids from *I. obliquus* grown on ME and PDB media.

liquids indicating the presence of the reaction substrate. However, the substrate content in the hot sample was significantly higher, which gave a more than ninefold increase in the peak value of luminescence intensity, compared with the use of its cold analogue. It should also note a strong light emission produced by the enzymatic system with the hot culture liquid of *Pholiota* sp., which was the largest observed in all experiments.

Stimulation of bioluminescence by A. borealis *culture liquids*

Light emission profiles obtained with the assay upon the addition of the hot and cold culture liquids of *A. borealis* to the enzymatic system of the same fungus are shown in Figure 4. In order to check if the probe sequence affects the luminescent response, we intentionally added the cold culture liquid followed by the heat treated sample. Both the hot and cold culture liquids stimulated the luminescence although with different intensities. As in previous experiments with *I. obliquus* and *Pholiota* sp., the hot culture liquid exhibited a higher content of substrate than the cold one, judging from the light emission profiles. The integrated luminescence measured in the former case was five times higher than in the latter.

Content of bioluminescence substrate in culture liquids

Earlier (Puzyr et al. 2018), we have demonstrated the application of enzymatic system isolated from

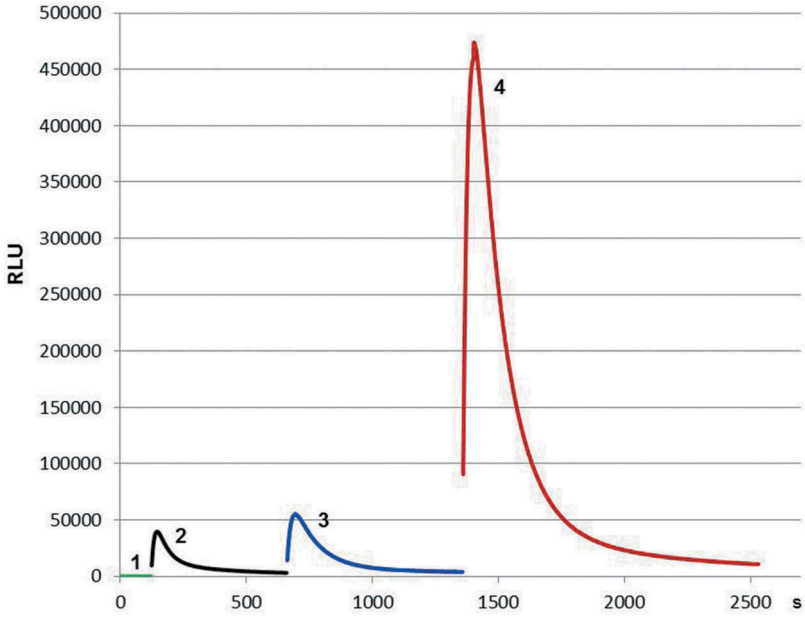

Figure 3. The intensity and kinetics of luminescence upon addition of culture liquids from submerged culture of *Pholiota* sp. to the enzymatic system of *A. borealis*: (1) the cold enzymatic extract (50 µl) of *A. borealis*, (2) 2.5 µl NADPH, (3) 2.5 µl cold culture liquid from *Pholiota* sp. and (4) 2.5 µl hot culture liquid from *Pholiota* sp.

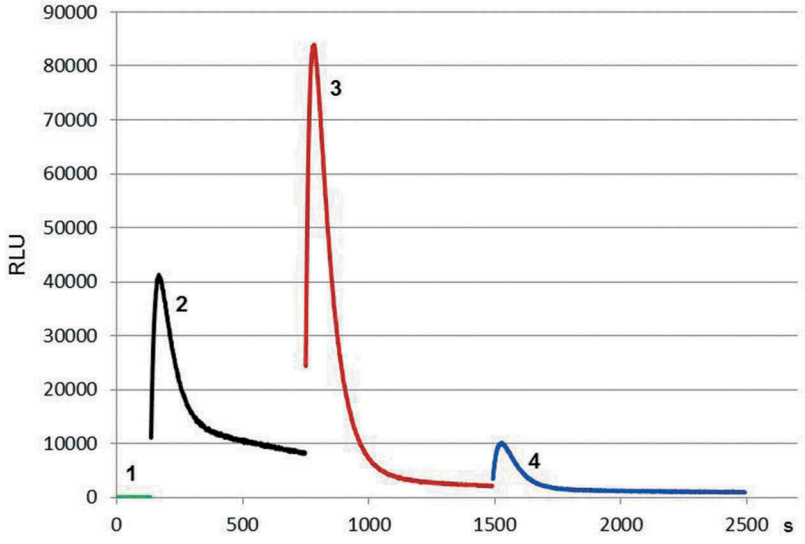

Figure 4. The intensity and kinetics of luminescence upon addition of culture liquids from submerged culture of *A. borealis* to the enzymatic system of *A. borealis*: (1) the cold enzymatic extract (50 µl) of *A. borealis*, (2) 2.5 µl NADPH, (3) 5 µl hot culture liquid from *A. borealis* and (4) 5 µl cold culture liquid from *A. borealis*.

A. borealis for a quantitative detection of hispidin in aqueous solutions. Based on this method, the amount of substrate (in equivalent units of hispidin, as in the experiments we used a pure hispidin) in the culture liquids was calculated and results are presented in Table 1.

Content of bioluminescence substrate in extracts from mycelium of A. borealis

To check whether such temperature dependence is specific only for the culture liquids, the luminescence assay was performed with the hot extracts

Table 1. Concentration of substrate in culture liquids, ng/µl.[a]

Species	Cold	Heat treated
I. obliquus (PDB)	n/d[b]	0.62 ± 0.062
I. obliquus (ME)	n/d	0.29 ± 0.027
Pholiota sp.	1.78 ± 0.192	17.21 ± 1.107
A. borealis	0.05 ± 0.007	0.30 ± 0.043

[a]The values are mean ± SD (n = 3).
[b]Not detected.

Table 2. Integrated luminescence obtained in experiment with cold and hot extracts from mycelium of *A. borealis*.

Sample volume (µl)	Heat treatment time (min)	Integrated luminescence (RLU)	Specific luminescence (RLU/µl)	Relative-specific luminescence
50	0	9401,996	188,040	1
2.5	2	3566,513	1426,605	7.6
2.5	4	3791,989	1516,796	8.0
2.5	6	3768,546	1507,418	8.0

RLU: Relative light units.

obtained by boiling on water bath the cold enzymatic extract of *A. borealis* for 2, 4 and 6 min. After the endogenous substrate was almost depleted in the luminescence reaction activated by NADPH (Figure 5, curve 2), subsequent adding of the hot extracts promptly increased the light emission (Figure 5, curves 3–5). The light emission of the enzymatic system on the addition of extract heated for 2 min was slightly less than that obtained with samples treated for 4 and 6 min, which showed almost identical results (inset in Figure 5). Given that the volume of hot extracts was significantly less than that of cold extracts, it can be seen that the hot extracts contained a comparably higher amount of bioluminescence activating substance.

Since the only difference between cold and hot extracts from mycelium of *A. borealis* is the heat treatment of the latter, we can estimate the relative content of bioluminescence activating substances by comparing the values of integrated luminescence obtained in the luminescence assay. From the data given in Table 2, one can see that the light emitted with the hot extracts was significantly higher than the emission from the cold extract. As with the use of culture liquids as a source of substrate for the luminescent reaction, the heat treatment of the mycelial extract led to a multifold increase in luminescence.

Discussion

The results presented above evidence that submerged cultures of *I. obliquus, Pholiota* sp. and *A. borealis* produce and secrete the bioluminescence

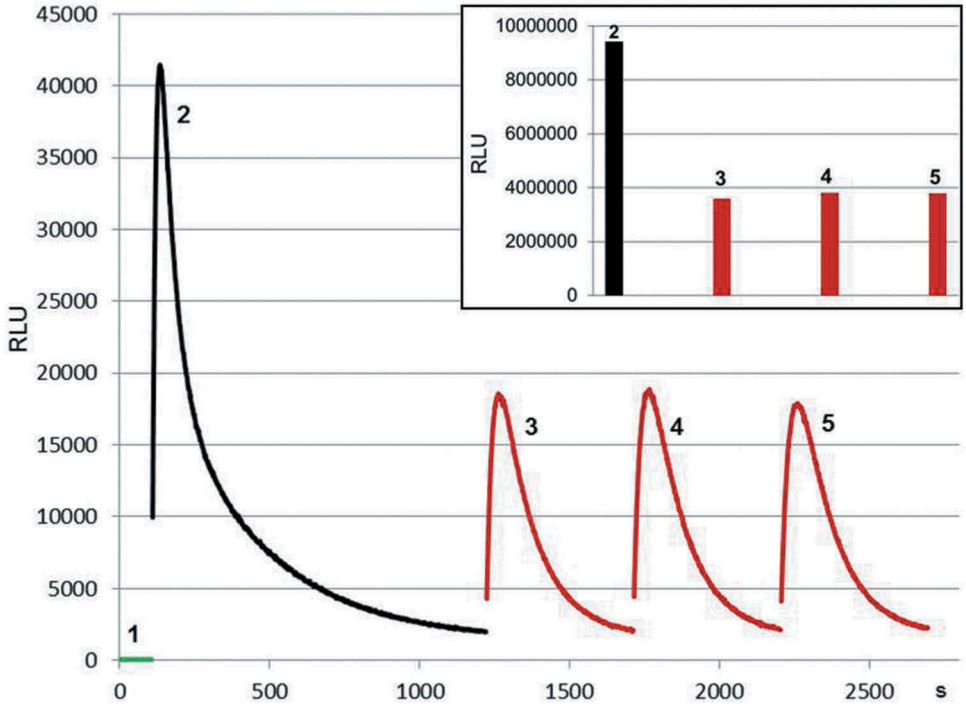

Figure 5. The effect of addition of NADPH and hot extract of *A. borealis* on the light emission produced by the enzymatic system of *A. borealis:* (1) the cold enzymatic extract (50 µl) of *A. borealis* and (2) 2.5 µl NADPH, (3–5) successive addition of 2.5 µl hot extracts of *A. borealis* obtained after boiling for 2, 4 and 6 min. The values of integrated luminescence intensity are presented on the inset.

activating substances (substrates) into the culture broth. The amount of substrate in the culture liquid depends on species and type of nutrient medium. Among all species studied, the culture liquids of *Pholiota* sp. were found to show the highest luminescence implying a large amount of substrates in this fungus. However, the greatest influence on the light emission produced by the luminescent system of *A. borealis* was provided by the temperature at which the probes were prepared for assay. Simple boiling on water bath of a culture liquid for a few minutes promoted a multifold increase in the luminescence.

There is some amount of an endogenous substrate in the cold enzymatic extracts from *A. borealis* prepared by the method used. The apparent reason for the preservation of the endogenous substrate in cold extracts of *A. borealis* is the absence of NADPH required for the luminescent reaction. The addition of NADPH to the extracts triggers the light emission which intensity sharply increases attaining a peak value and then gradually decreases with time reaching a relatively steady level as depicted in Figures 2–5. From this point, the luminescence slowly weakens for hours. During this period, a repeated NADPH addition to the assay does not influence the light emission. A subsequent addition of extracts (cold or hot) enhances the light intensity in a dose-dependent manner, thus evidence that (1) the light intensity is controlled by amount of substrate in the extract and (2) there is a possibility to perform multiple luminescence assays using the same enzymatic system isolated from *A. borealis*. Similar results were obtained using enzymatic systems of luminous fungi *Neonothopanus nambi* and *M. citricolor* (Bondar et al. 2014; Puzyr et al. 2017). It should be noted that after a certain time (*ca.* 8–10 h at room temperature), the loss of enzymatic activity resulting in no activation of luminescence upon the addition of NADPH and a substrate containing extract was observed.

It is very likely that the substrate present in the culture liquids exists in two forms. In one form, it is ready to interact with the enzymatic system and in the second form, it becomes accessible for the luminescent reaction after heat treatment. Regardless the species, the pool of thermoactivated substrate accumulated in culture liquids is found to be significantly larger than the pool of the ready accessible one. In case of *I. obliquus*, the latter was absent or accumulated in such a small amount that could not be detected in the assay we employed.

The different amount of hispidin depending on a method applied for its extraction from the dried fruiting bodies and fresh mycelium of luminous *N. gardneri* was reported by Oba et al. (2017). The authors argued that a 10-fold increase in the hispidin content was due to the extraction with liquid under pressure using an accelerated solvent extraction system (ASE procedure), which showed to be more efficient than the extraction procedure based on grinding and subsequent centrifugation. The grind/centrifuge method was carried out at 4°C, whereas the ASE procedure was employed at 80°C. Based on the results presented here, we suggest that this unexpectedly large difference in yield of hispidin was mainly attributed by the temperature at which extractions were performed. The close values of the integrated luminescence ratio obtained on the cold and heat treated extracts from *A. borealis* (Table 2) confirm this assumption.

The presence of hispidin predecessors produced by the fungi in culture liquids and mycelia can be a possible explanation of this phenomenon. The hispidin predecessors (e.g. hispidin analogues) are not involved in bioluminescence at their original state and are converted into the substrate under the influence of high temperature. In addition, the transformation of the substrate can take place through biochemical reactions. This can explain the sharp increase in luminescence of fungal mycelia when exposed to external stimuli, for example mechanical damage (Mogilnaya et al. 2015; Puzyr et al. 2016). The existence of another substance capable of bioluminescence other than hispidin, which is activated at high temperatures, should also not be excluded.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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