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The cytochrome P450 system is involved in the light emission of higher fungi

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Abstract

The present study reports experimental data that suggest the participation of the cytochrome P450 system in light emission by higher fungi. The luminous mycelia of the basidiomycetes *Neonothopanus nambi*, *Armillaria borealis*, *Panellus stipticus*, and *Mycena citricolor* were used to prepare "cold" extracts, which contained luminescent enzyme systems responsible for the *in vitro* luminescence of the extracts. A "hot" extract from the mycelium of the nonluminous higher fungus *Pholiota squarrosa* was used as a substrate for the luminescent reaction. The conditions for preparing "cold" extracts (treatment of mycelial biomass with ultrasound and clarification of homogenates by centrifugation at 40000*g*) indicate the presence of membrane structures in them, microsomes, in particular, which are formed during ultrasonic destruction of the endoplasmic reticulum. In samples of the extracts treated with sodium dithionite and CO, differential spectroscopy revealed two absorption peaks, at 410 and 450 nm, which indicates the presence of cytochromes b_5 and P450 in them. The luminescence of extracts is stimulated by reduced pyridine nucleotides, but it has been found that the addition of NADPH leads to a higher level of luminescence compared to NADH. The addition of NADPH results in the V_{max} values of light emission higher by a factor of almost 2 and the apparent K_m values lower by a factor of 2 compared to the addition of NADH. It has been shown that the addition of hydrogen peroxide substantially increases the luminescence intensity of extracts activated by NADPH and the reaction substrate. It has been found that fluconazole additives considerably inhibit the light emission of extracts when they are activated by NADPH and the reaction substrate. The results obtained in this study suggest that the mechanism of luminescence of higher fungi may involve the cytochrome P450 system associated with endoplasmic reticulum membranes, involving in the process the electron transport enzyme systems. In that case, cytochrome P450 carries out the hydroxylation of hispidin, the precursor of the substrate of the luminescence reaction, with the formation of luciferin, and catalyzes its oxidation in the presence of reactive oxygen species (hydrogen peroxide, in particular) with the emission of visible light quanta.

Keywords – luminous mycelium – luminescent system – mechanism of light emission – cytochrome P450 system – hispidin – reduced pyridine nucleotides – fluconazole – hydrogen peroxide

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Introduction

It is well known that many species of higher fungi exhibit bioluminescence – emission of light visible in darkness (Shimomura 2006, Desjardin et al. 2008, Bondar et al. 2012). Over 100 basidiomycetes that are able to glow in the dark have been discovered by now (Ke & Tsai 2022). Luminous higher fungi have been found in various regions of the world (North and South America, Europe, Asia, Australia, and Africa), especially in the subtropical and tropical zones – regions with the most favorable environmental conditions for their development (Desjardin et al. 2010, 2016, Chew et al. 2014, 2015, Mihail 2015, Puzyr et al. 2016).

Despite the considerable achievements in fungal bioluminescence research of the past decade (Bondar et al. 2014, Purtov et al. 2015, Oba et al. 2017, Kaskova et al. 2017, Puzyr et al. 2017, Kotlobay et al. 2018, Teranishi 2018, Puzyr et al. 2019, Garcia-Iriepa et al. 2020, Ronzhin et al. 2022), certain biochemical aspects of this phenomenon remain insufficiently clear and need to be studied. It has now been proven that hispidin is the luciferin precursor in luminous higher fungi. In the presence of oxygen and NADPH, NADPH-dependent hydroxylase transforms hispidin into luciferin (3-hydroxyhispidin), which is then oxidized by the insoluble luciferase in the presence of oxygen, generating a light quantum (Purtov et al. 2015). At the same time, it is still unclear what enzyme (or enzyme complex) functions as luciferase in luminous basidiomycetes, as it has not been isolated in pure form and characterized. An equally important question is whether the luciferin–luciferase mechanism is the only mechanism of fungal luminescence or the generation of visible light quanta in basidiomycetes occurs via different biochemical pathways involving different enzymes (or enzyme systems) and whether 3-hydroxyhispidin is the only substrate of the fungal luminescence reaction.

Previously, the idea was expressed about the participation of reactive oxygen species (ROS) and enzymes with oxidase function in the mechanisms of fungal bioluminescence (Shimomura 1991, 1992, 2006). Results of our studies also suggest the involvement of ROS and oxidase enzymes in the mechanism of fungal luminescence (Bondar et al. 2011, 2012, 2013, Medvedeva et al. 2014, Kobzeva et al. 2014, Mogilnaya et al. 2016, 2017, 2018). In addition, results of our research suggest the interaction between the fungal light-emitting system and the membrane structures and are in good agreement with the data reported by other researchers (Airth & McElroy 1959, Airth & Foerster 1962, 1964). Based on these experimental facts, we put forward the hypothesis that light emission by higher fungi may involve the following enzyme systems associated with the membrane structures: oxidases (peroxidases, in particular) of the ligninolytic complex, the cytochrome P450 system, and mitochondrial respiratory chain enzymes. These systems can all produce ROS, and two of them (oxidases of the ligninolytic complex and the cytochrome P450 system) can also catalyze, involving ROS, oxidation of organic substrates (including fungal luciferin), generating visible light quanta (Bondar et al. 2011, 2012, 2013).

The present work reports the results of the study of the luminescent systems from different species of basidiomycetes, which suggest the involvement of the cytochrome P450 system in the reaction of light emission by higher fungi.

Materials & Methods

The study was conducted using the luminous mycelium of the higher fungi *Neonothopanus nambi* (strain IBSO 2391), *Armillaria borealis* (strain IBSO 2328), *Mycena citricolor* (strain IBSO 2331), and *Panellus stipticus* (strain IBSO 2301) maintained at the Collection of Microorganisms CCIBSO 836 at the Institute of Biophysics of the Federal Research Center "Krasnoyarsk Scientific Center", Siberian Branch of the Russian Academy of Sciences (Krasnoyarsk). Experiments were conducted with the biomass of mycelial pellets, which had been produced by submerged cultivation of the fungi in the PDB nutrient medium (HiMedia Laboratory, India) (Puzyr et al. 2017, Mogilnaya et al. 2017, 2018). Below is a brief description of how "cold" extracts containing the luminescent enzyme systems of the fungi were prepared. The mycelial pellets grown in a submerged culture of basidiomycetes were taken out of the nutrient medium and washed in deionized (DI) water (Milli-Q system, Millipore, U.S.A.) several times to remove nutrient medium

and metabolites. After that, the pellets were placed into a large volume of DI water and incubated at 22–24 °C for 24 h to remove residual nutrient medium and metabolites. In addition, a number of studies showed that luminous fungi washed with water several times or incubated in water over a long time period exhibited increased light emission (Mendes & Stevani 2010, Mori et al. 2011, Bondar et al. 2013, Mogilnaya et al. 2015, 2016). The luminescent enzyme system was extracted at a temperature of 0–4 °C. After incubation, the mycelial pellets were taken out of the water and rubbed through a metal sieve with a pore size of 1 mm. The crushed biomass was transferred to a beaker placed in an ice bath, and a chilled 0.1 M phosphate buffer (pH 7.0) containing 1% BSA (Serva, Germany) was added at a ratio of 1:5 (biomass volume: buffer volume). The resulting suspension was treated with ultrasound using the Volna USTD-0.63/22 device (U-Sonic, Russia). The sonication was performed thrice, each time for 10 s with a 1 min interval. Then the homogenate was centrifuged at 40000*g* for 30 min at 4 °C using an Avanti® J-E centrifuge (Beckman-Coulter, U.S.A.). The sediment was discarded, and the supernatant ("cold" extract), containing components of the luminescence reaction, was collected and used in experiments.

A "hot" extract from the mycelial pellets of the nonluminous higher fungus *Pholiota squarrosa* (strain IBSO 2376, Collection of Microorganisms CCIBSO 836) was used as a substrate for the luminescent reaction. The biomass of this basidiomycete contains a large amount of hispidin – a precursor of luciferin of higher fungi (Purtov et al. 2015). The *P. squarrosa* mycelial pellets prepared by submerged cultivation of the fungus in the PDB nutrient medium were extracted from the nutrient medium and washed in DI water to remove the nutrient medium and metabolites. After washing, the pellets were rubbed through a metal sieve, as described above. DI water was added to the crushed biomass at a ratio of 1:2 (volume of biomass: volume of water). The sample was mixed to produce a homogeneous suspension, placed into a MW 712BR microwave oven (Samsung, Malaysia), and heated at 800 W until it boiled. Then, the sample was cooled in an ice bath and centrifuged at 16000*g* for 10 min at 4 °C using a 5415R centrifuge (Eppendorf, Germany). The supernatant ("hot" extract) was collected and used in experiments.

The intensity and dynamics of the luminescence of "cold" extracts were measured using a Glomax® 20/20 luminometer (Promega, U.S.A.). For that, 100 µl of the extract was poured into MCT-200-C microtubes (Axygen Scientific, Inc., U.S.A.), which were then placed into the luminometer. Luminescence was monitored at a rate of one measurement per sec; light emission intensity was expressed as relative units. To assess the effect of different components on the activity of isolated luminescent systems, 5 µl of a solution of the studied component was added to 100 µl of the extract, and the level of light emission and the kinetics of the light signal were measured. The following materials were used in the experiments: NADPH and NADH (Serva, Germany), initial solutions of the reagents with a concentration of 10 mM were prepared *in situ* in DI water; a "hot" extract of *P. squarrosa*; a hydrogen peroxide solution (GalenoPharm™, Russia) – initial concentration 88 mM; a fluconazole solution (Dr. Reddy's Laboratories Ltd., India) – initial concentration 2 mg/ml. When testing the luminescence of "cold" extracts, the concentrations of NADPH, NADH, and fluconazole were varied to study the dependencies of the activity of the luminescent enzyme systems.

The presence of the cytochrome P450 enzymes in the "cold" extracts was determined using the method of differential spectroscopy (Omura & Sato 1964, Peisach et al. 1973). The method is based on the appearance of the characteristic spectral shift in the absorption spectrum due to carbon monoxide binding to a reduced atom of heme iron in the active site of the enzyme. This method is used to reveal the presence of the functionally active form of cytochrome P450 in complex biological systems without enzyme isolation (Moskaleva & Zgoda 2012). The differential spectrum was measured using a double-beam UV/VIS spectrophotometer UVIKON 943 (Kontron Instruments, Italy). The measurement was performed as follows. To reduce cytochrome P450, 5 mg of dry sodium dithionite (Serva, Germany) was added to 1.5 ml of the "cold" extract and after sodium dithionite was dissolved, the sample was incubated at a temperature of 23 °C for 5 minutes. Then, two 650-µl subsamples were taken from the sample for preparing control and test samples. In the first case, 100 µl of DI water was added to the 650-µl control sample. In the second case, DI

water was saturated with carbon monoxide by bubbling it with CO gas for 5 hours at 4 °C, and 100 µl of that water was added to the 650-µl test sample. After mixing, the control and test samples were incubated at a temperature of 23 °C for 10 minutes. After that, the samples were transferred to quartz cuvettes (optical path length 1 cm), which were simultaneously placed into a spectrophotometer. The differential spectrum was measured in the 300–700 nm wavelength range.

Results & Discussion

Our experiments showed that submerged cultivation of the studied basidiomycetes produced fungal mycelium in the shape of spherical pellets with a large number of surface filaments – hyphae. Images of mycelial pellets of the *N. nambi* and *A. borealis* basidiomycetes are presented in Fig. 1. The mycelial pellets that had been washed and incubated in DI water for 24 h to remove nutrient medium and metabolites exhibited detectable luminescence. The pellets clearly varied in size, number of surface filaments, and luminescence intensity (Fig. 1), which is consistent with the data obtained in our previous studies on submerged cultivation of basidiomycetes (Mogilnaya et al. 2017, 2021).

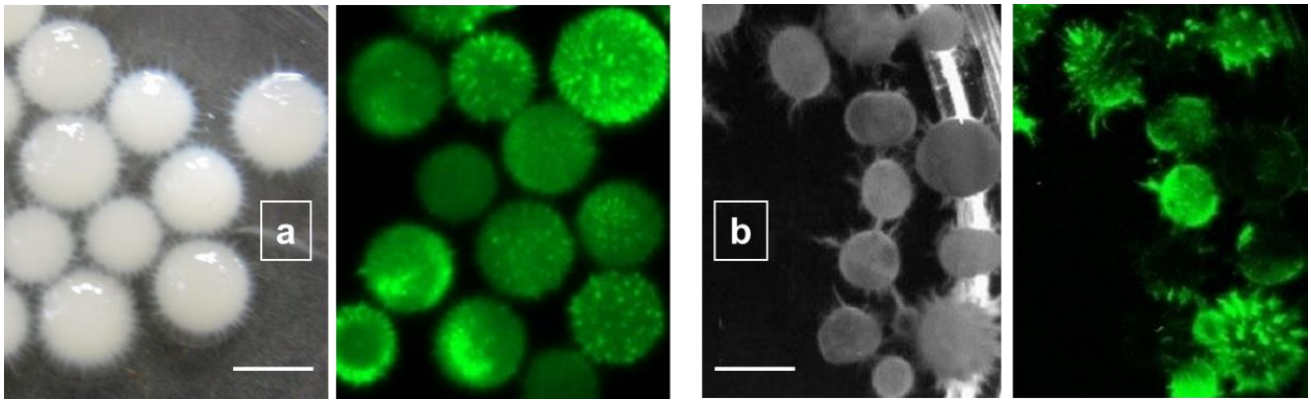

Fig. 1 – The appearance (left) and luminescence (right) of mycelial pellets of the *N. nambi* (a) and *A. borealis* (b) basidiomycetes produced by submerged cultivation and incubated in DI water. Images prepared using a ChemiDoc™ XRS System (Bio-Rad, U.S.A.). Bars – 5 mm.

The present study showed that "cold" extracts of the mycelia of the basidiomycetes *N. nambi* IBSO 2391, *A. borealis* IBSO 2328, *M. citricolor* IBSO 2331, and *P. stipticus* IBSO 2301 contain active luminescent fungal systems, which enable *in vitro* luminescence of the extracts. The data on luminescence of the "cold" extract from the *N. nambi* mycelium are given as an example (Fig. 2). These data demonstrate that the extract alone, without any reagents added to it, emits detectable light. Thus, the extract contains all components necessary for the luminescent reaction, and this is consistent with the results of our previous research (Bondar et al. 2014). The additions of reduced pyridine nucleotides (NADPH or NADH) and the reaction substrate (the "hot" extract of *P. squarrosa*) considerably increase the intensity of light emission by the *N. nambi* "cold" extract (Fig. 2).

The conditions that we used to prepare "cold" extracts (ultrasonic treatment of mechanically destroyed biomass and centrifugation of homogenates at 40000*g*) suggested the presence of membrane structures in the extracts, microsomes, in particular, which are formed when the endoplasmic reticulum is destroyed by ultrasound. The positive Tyndall effect observed in clarified extracts was indicative of their optical heterogeneity and the presence of insoluble particles. In eukaryotic cells, the cytochrome P450 enzyme system is known to be associated with endoplasmic reticulum membranes (e.g., Maddy 1976). Therefore, membrane structures of the endoplasmic reticulum (microsomes and their fragments) containing the cytochrome P450 enzyme system are isolated using ultrasonic treatment of mechanically disintegrated biomass, followed by centrifugation of homogenates at accelerations of more than 15000*g* to remove large fragments of

cell membranes, nuclei, and mitochondria. A study by Maddy (1976) showed that mechanical destruction of biomass is not sufficient for the effective extraction of enzymes of the cytochrome P450 system. In this regard, an important point should be noted. Previously, we established that one of the necessary conditions for obtaining "cold" extracts with a high level of activity of luminescent systems is the treatment of disintegrated mycelial biomass with ultrasound (Puzyr et al. 2017). The extracts obtained after mechanical destruction of the mycelium either did not glow with the addition of NAD(P)H and the reaction substrate or had an extremely low level of luminescence.

In the current study, the presence of the enzymes of the cytochrome P450 system in the "cold" extracts was supported by the data obtained by differential spectral analysis. A differential absorption spectrum of the extract from the *N. nambi* mycelium treated with sodium dithionite and CO is shown in Fig. 3 as an example. The spectrum has two absorption peaks with λ_{max} at 410 nm and 450 nm, which correspond to the absorption maxima of cytochromes b_5 and P450, respectively (Omura & Sato 1964, Peisach et al. 1973), and indicate the presence of these enzymes in the extract.

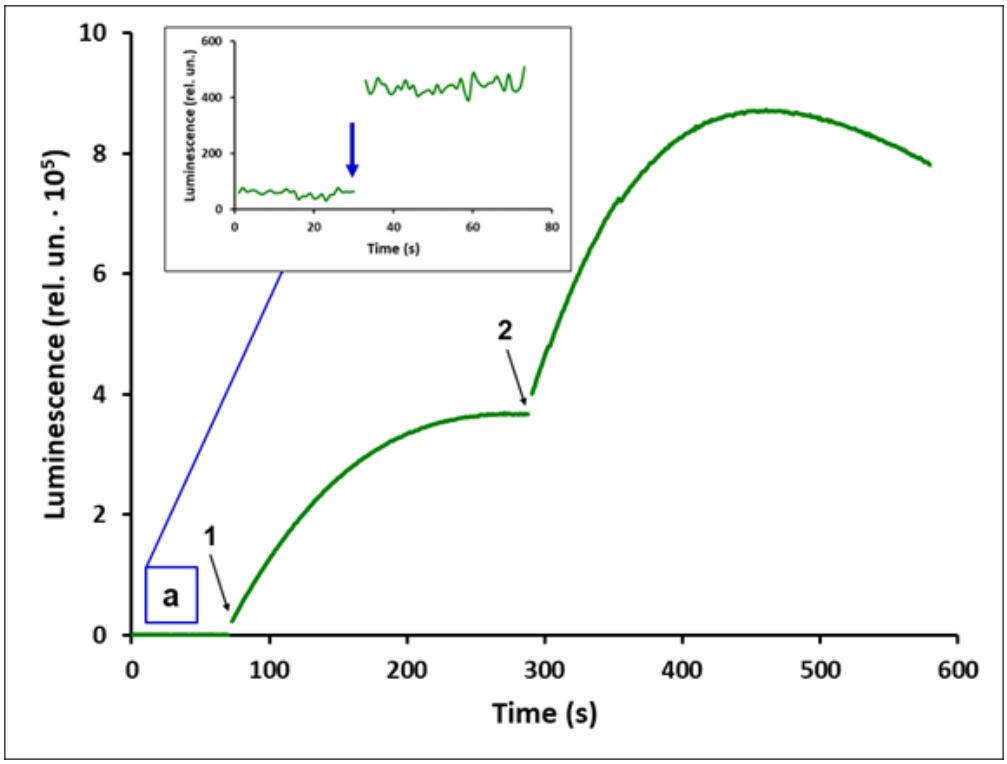

Fig. 2 – The initial luminescence level of the "cold" extract from *N. nambi* mycelium (a) and luminescence activation by successive additions of NADPH (1) and the "hot" extract from the mycelium of *P. squarrosa* (2). The arrows indicate the moments of adding reagents to the extract. The inset shows fragment (a): the level of the background signal of the measuring system and the initial level of luminescence of the tested extract; the arrow shows the moment of placing the test tube with the extract into the luminometer.

The present study demonstrated that the luminescence intensity of "cold" extracts from the mycelium of the study basidiomycetes, activated by the addition of NADPH and the reaction substrate, increases substantially (from several times to 1–2 orders of magnitude) after the addition of hydrogen peroxide (Fig. 4). The level of luminescence of extracts after the addition of NAD(P)H, the reaction substrate, and H_2O_2 can be so high that it is visually observed in the dark and recorded by the ChemiDoc™ XRS System (Bio-Rad, U.S.A.) operating in dark mode (Fig. 5).

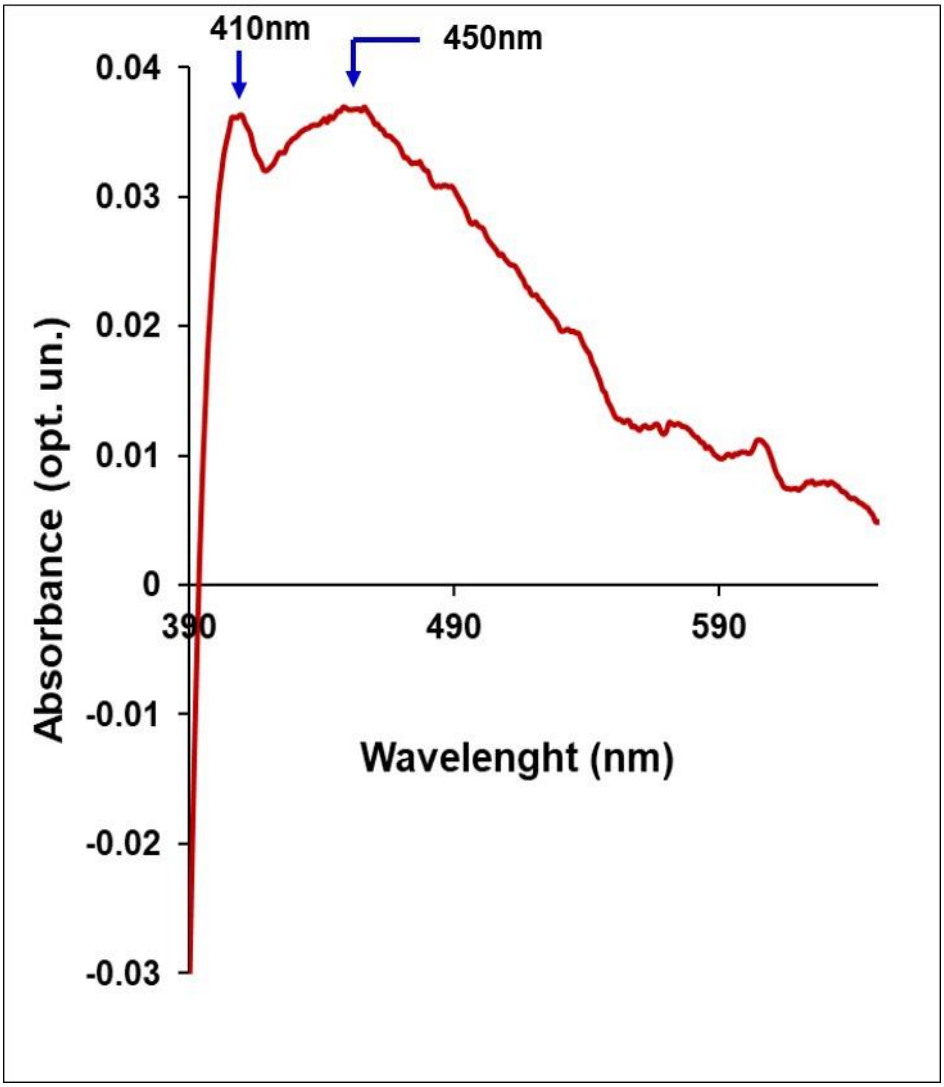

Fig. 3 – A differential absorption spectrum of the "cold" extract from *N. nambi* mycelium: control (dithionite) / test (dithionite + CO).

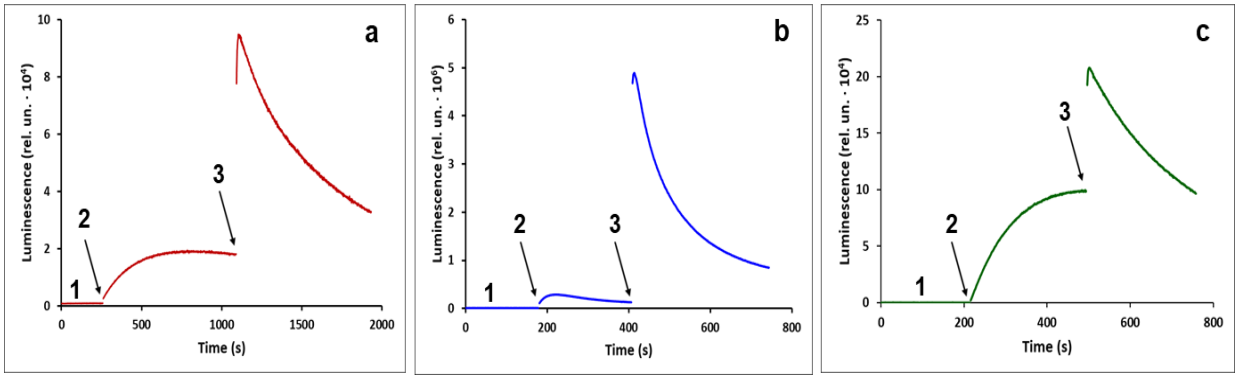

Fig. 4 – Stimulation of luminescence of "cold" extracts from the mycelia of *N. nambi* (a), *M. citricolor* (b), and *P. stipticus* (c) by hydrogen peroxide: 1 – the initial level of luminescence of extracts, 2 – activation of luminescence by adding NADPH and the reaction substrate, 3 – the level of light emission after addition of H_2O_2. Arrows indicate the moments of adding reagents to extracts.

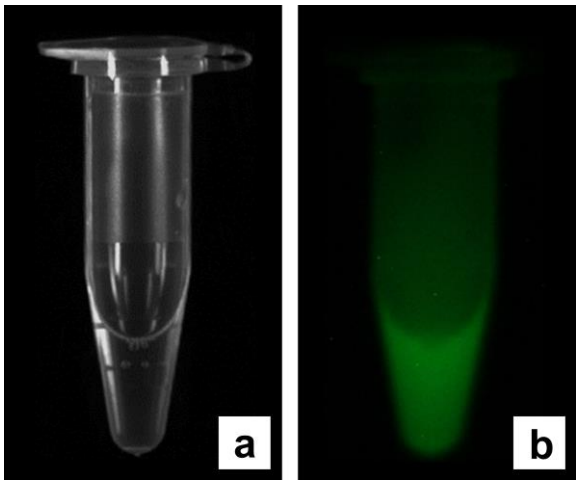

Fig. 5 – The appearance (a) and luminescence (b) of the "cold" extract from *N. nambi* after additions of NADPH, the reaction substrate, and H_2O_2. Images were prepared using a ChemiDoc™ XRS System (Bio-Rad, U.S.A.).

We believe that the increase in the intensity of light emission of "cold" extracts caused by the addition of NAD(P)H, the reaction substrate, and hydrogen peroxide (Fig. 2 and Fig. 4) can be explained using well-known data on the functioning of the microsomal cytochrome P450 system. Enzymes of the cytochrome P450 family carry out oxidative transformation of a wide range of organic compounds, including catalysis of the hydroxylation reaction of cyclic compounds (Danielson 2002, Ortiz de Montellano 2005). The catalytic function of cytochrome P450 is performed with the obligatory participation of redox partners (NADPH-dependent reductase of cytochrome P450 and cytochrome b_5) and reduced pyridine nucleotides (NADPH and NADH) as electron donors (Archakov & Bachmanova 1990, Lewis 2001, Schenkman & Jansson 2003). The catalytic cycle of microsomal cytochromes P450 involves the formation of reactive oxygen species (superoxide radical, hydrogen peroxide, and hydroxyl radical) (Denisov et al. 2005, Hardwick 2015), which participate in oxidative reactions catalyzed by these enzymes (Rabe et al. 2010, Hrycay & Bandiera 2015, Munro et al. 2018). In our case, the combination of these facts allows us to assume that hispidin added with the "hot" extract (see above) can be transformed into luciferin (3-hydroxyhispidin) not only by NADPH-dependent hydroxylase (Purtov et al. 2015) but also due to the cytochrome P450-catalyzed hydroxylation reaction. Cytochrome P450 can then catalyze the oxidation of luciferin in the presence of ROS (hydrogen peroxide, in particular) with the emission of light quanta.

The present study showed that the luminescence of "cold" extracts from the mycelium of different species of basidiomycetes is activated by reduced pyridine nucleotides. At the same time, we found that a significantly higher level of light emission from extracts is observed with the addition of NADPH compared to NADH. Calculations of kinetic parameters based on the dependence of the luminescence level of extracts on the concentration of reduced pyridine nucleotides showed that the addition of NADPH leads to the V_{max} values of light emission higher by a factor of almost 2 and the apparent K_m values lower by a factor of 2, compared to the addition of NADH (Table 1). Assuming the participation of the P450 system in the light emission reaction, these differences can be explained as follows. In eukaryotic cells, the microsomal cytochrome P450 system functions with the participation of two-electron transport systems: NADPH-dependent reductase of cytochrome P450/ cytochrome P450 and NADH-dependent reductase of cytochrome b_5/ cytochrome b_5/ cytochrome P450 (Correia & Mannering 1973, Yamazaki et al 1996a, Yamazaki et al. 1996b, Vergeres & Waskell 1995, Severin 2006). NADPH-dependent reductase transfers an electron from NADPH directly to cytochrome P450. However, electron transfer from NADH occurs in two stages: by NADH-dependent reductase to cytochrome b_5, which then transfers the electron to cytochrome P450. At the same time, in microsomes, reduced cytochrome b_5 ensures the

transfer of reducing equivalents in two directions – to cytochrome P450 and microsomal desaturases involved in lipid biosynthesis (Vergeres & Waskell 1995, Severin 2006). Based on the above facts, a higher catalytic efficiency of cytochrome P450 should be observed when NADPH is used as an electron donor.

Table 1 – Values of kinetic parameters V_{max} and K_m calculated from the dependences of the light emission intensity of "cold" extracts from the mycelia of different types of basidiomycetes on the concentration of reduced pyridine nucleotides.

"Cold" extract	V_{max} NADPH (rel. un.)	V_{max} NADH (rel. un.)	K_m NADPH (μM)	K_m NADH (μM)
N. nambi	$7.21 \cdot 10^6$	$3.96 \cdot 10^6$	43	85
A. borealis	$2.44 \cdot 10^7$	$1.10 \cdot 10^7$	46	92
P. stipticus	$1.12 \cdot 10^7$	$5.83 \cdot 10^6$	44	83

In this study, we found that additions of fluconazole to "cold" extracts from different types of basidiomycetes followed by the additions of NAD(P)H and the reaction substrate significantly suppress light emission (Fig. 6), which also suggests the participation of the cytochrome P450 system in the light emission of higher fungi. It is well known that azole compounds (including fluconazole) are selective inhibitors of cytochrome P450s, blocking their active center due to the formation of a complex bond between the heme iron atom and one of the nitrogen atoms of the azole ring (Jackson et al. 2000, Kelly et al. 2001, Podust et al. 2001, Guardiola-Diaz et al. 2001, Jackson et al. 2003).

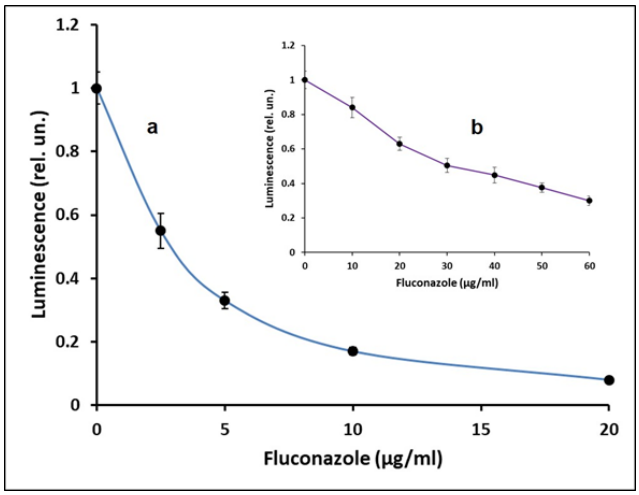

Fig. 6 – Fluconazole inhibition of luminescence of "cold" extracts from the mycelium of *N. nambi* (a) and *A. borealis* (b) when luminescence is activated by adding NADPH and the reaction substrate. The readings are normalized to the value of light emission from control samples of extracts (without the addition of fluconazole).

Based on the experimental data obtained in this study, we proposed a general scheme for the participation of the cytochrome P450 system associated with the endoplasmic reticulum membranes in the light emission of higher fungi (Fig. 7). The scheme suggests that with the participation of electron transport enzyme systems (NADPH-dependent reductase of cytochrome P450 / cytochrome P450 and NADH-dependent reductase of cytochrome b_5 / cytochrome b_5 / cytochrome P450), cytochrome P450 carries out the hydroxylation of hispidin with the formation of luciferin and then catalyzes its oxidation in the presence of ROS and the emission of visible light quanta. The proposed scheme (Fig. 7) seems quite reasonable since it is consistent with the ideas about the functioning of the microsomal cytochrome P450 system in eukaryotic cells. It is well

known that microsomal cytochromes P450 are involved in the oxidative transformation of a wide range of organic compounds, including catalysis of the hydroxylation reaction of cyclic compounds with the formation of ROS (hydrogen peroxide, in particular), which take part in oxidative reactions catalyzed by these enzymes (Archakov & Bachmanova 1990, Lewis 2001, Munro et al. 2018).

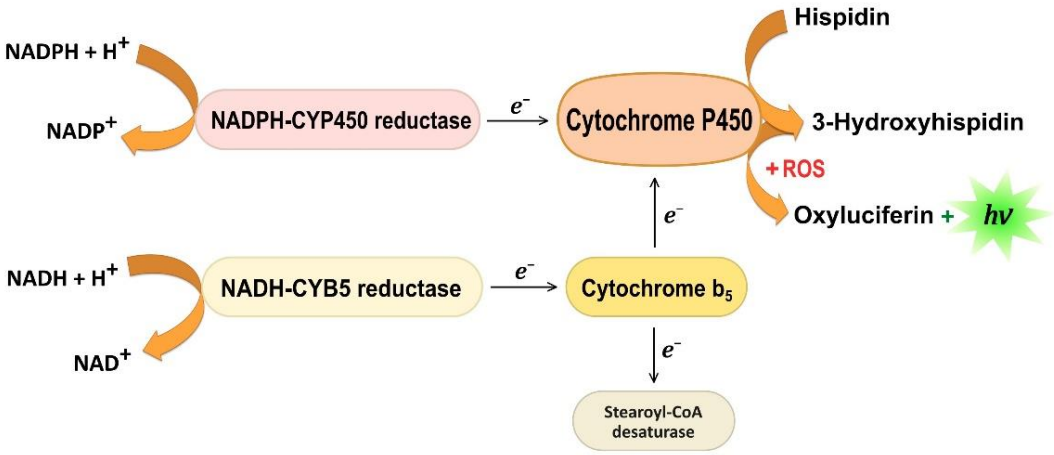

Fig. 7 – A scheme of participation of the cytochrome P450 system in the luminescence mechanism of higher fungi.

Conclusion

Thus, the results of the present study indicate the participation of the cytochrome P450 system in the bioluminescence of higher fungi, which suggests a new understanding of the mechanisms of their light emission. In turn, the data obtained show that the generation of visible light quanta in luminous basidiomycetes can be achieved through different biochemical pathways with the participation of different enzymes (or enzyme systems), due to the functioning of the NAD(P)H-dependent hydroxylase–luciferase system (Purtov et al. 2015) and the cytochrome P450 system, in particular.

Acknowledgements

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