Asian Journal of Mycology 7(1): 86–98 (2024) ISSN 2651-1339
www.asianjournalofmycology.org

Article
Doi 10.5943/ajom/7/1/7

A stimulator of light emission of the luminous fungus *Neonothopanus nambi*

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Ronzhin NO, Posokhina ED, Mogilnaya OA, Bondar VS 2024 – A stimulator of light emission of the luminous fungus *Neonothopanus nambi*. Asian Journal of Mycology 7(1), 86–98, Doi 10.5943/ajom/7/1/7

Abstract

Biochemical mechanisms of bioluminescence of higher fungi remain poorly understood, and further research is needed. By now, in vitro experiments have shown that hispidin is a precursor of the substrate of basidiomycete luminescence reaction: NADPH-dependent hydroxylase transforms it into 3-hydroxyhispidin (luciferin), which is then oxidized by luciferase, generating light quanta. In the present study, the component stimulating luminescence of the fungus *Neonothopanus nambi* in vivo was detected in the aqueous extract after treatment of mycelium with β-glucosidase, and some of its properties were investigated. The addition of the extract to the luminous mycelium increased the level of its light emission from several times to 1.5 orders of magnitude or more. The light emission stimulator is a thermostable low-molecular-weight compound with an apparent molecular weight of 0.46 kDa. In the absorption spectrum of the aqueous sample of the stimulator, there are two major peaks in the short wavelength region at 205 and 260 nm and a shoulder in the 350–370 nm range. The stimulator is capable of blue fluorescence with the maximum light emission at 440 nm after excitation at 350–370 nm. The current study demonstrates that the component stimulating light emission is not the substrate (or substrate precursor) of the luminescence system of the fungus *N. nambi*. Having discovered the stimulator of fungal luminescence, we are faced with new research objectives to determine its structure and mechanism of stimulation.

Keywords – bioluminescence – basidiomycete *Neonothopanus nambi* – luminous mycelium – luminescence stimulator – fluorescence

Introduction

Visible light emission by higher fungi is a widespread phenomenon (Shimomura 2006, Desjardin et al. 2008). More than 100 species of basidiomycetes that are capable of emitting visible light in the dark have been discovered (Ke & Tsai 2022). Luminous species of higher fungi have been found throughout the world – mainly in the subtropical and tropical zones, which are their most favorable habitats (Desjardin et al. 2010, Chew et al. 2014, Chew et al. 2015, Mihail 2015, Desjardin et al. 2016, Puzyr et al. 2016). A large number of studies showed that basidiomycetes emit green light with a maximum wavelength of 520–530 nm at different stages in their life cycle (Endo et al. 1970, Lavelle et al. 1972, O'Kane et al. 1990, Shimomura 2006, Desjardin et al. 2008, Bondar et al. 2012, Kobzeva et al. 2014). In some species of higher fungi, light is emitted by the entire fruiting body (Brandl 2011, Vydryakova et al. 2012, Oliveira et al. 2015), while in others,

Submitted 10 October 2023, Accepted 4 April 2024, Published 30 May 2024
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only the cap or the stipe of the fruiting body is luminescent (Desjardin et al. 2007, Desjardin et al. 2008, Teranishi 2016a). In the *Armillaria*, visible luminescence only occurs in mycelium and rhizomorphs (Harvey 1952, Wassink 1978, Desjardin et al. 2008, Mihail 2013, Mihail 2015). Luminescent basidiomycetes are white-rot fungi capable of lignin degradation (Desjardin et al. 2008), and, as a rule, they are saprotrophic (or, more seldom, plant pathogenic) species (Shimomura 2006, Ke & Tsai 2022).

Although there have been considerable achievements in research of bioluminescence in higher fungi, especially in the last decade (Bondar et al. 2014, Purtov et al. 2015, Oba et al. 2017, Kaskova et al. 2017, Kotlobay et al. 2018, Puzyr et al. 2019, Garcia-Iriepa et al. 2020), some biochemical aspects of this phenomenon have not been sufficiently understood and should be studied further.

In the second half of the 20th century, Airth & McElroy (1959) first reported cell-free fungal luminescence *in vitro* in studies of "cold" and "hot" extracts of luminous fungi, such as *Collybia velutipes* and *Armillaria mellea* (Airth & McElroy 1959). Further studies by Airth & Foerster (1962) allowed the authors to propose a two-step mechanism for the luciferin/luciferase reaction responsible for the emission of light quanta. First, activation of luciferin by the "soluble enzyme" in the presence of the reduced pyridine nucleotide, and second, luciferin oxidation with "insoluble particles of luciferase" in the presence of oxygen (Airth & Foerster 1962, Airth & Foerster 1964). In the new millennium, the two-step luciferin/luciferase mechanism of cell-free fungal luminescence *in vitro* was demonstrated by other researchers through experiments with "cold" and "hot" extracts of various species of luminous fungi (Oliveira & Stevani 2009, Oliveira et al. 2012). Moreover, the cross-reactions of "hot" and "cold" extracts from different fungal species led the authors to conclude that all luminous fungi share the same bioluminescence system (Oliveira et al. 2012).

Bondar et al. (2014), for the first time, prepared a "cold" extract from the mycelium of the luminous basidiomycete *Neonothopanus nambi*, which contained functionally active luminescence system capable of sustaining detectable luminescence of the extract *in vitro* (Bondar et al. 2014). The authors identified this extracted luminescence system as a stable complex that includes protein and non-protein components and does not require exogenous reagents to sustain light emission. Additionally, their study showed that the addition of reduced pyridine nucleotide to the extract enhanced luminescence intensity, and the addition of hydrogen peroxide to the NADPH-activated luminescence system increased the level of light emission by several orders of magnitude. These data further supported the authors' previous hypothesis about the possible participation of ligninolytic peroxidases and the cytochrome P450 enzymes in the mechanism of fungal luminescence (Bondar et al. 2012, Bondar et al. 2013). Subsequently, "cold" extracts capable of luminescence and containing active luminescence systems were also prepared from the luminous mycelium of the basidiomycetes *Armillaria borealis* and *Mycena citricolor* (Puzyr et al. 2017).

Purtov et al. (2015) found 3-hydroxyhispidin as the luciferin of luminous basidiomycetes (Purtov et al. 2015). This luciferin is synthesized from the well-known fungal secondary metabolite hispidin when treated with NADPH-dependent hydroxylase in the presence of oxygen and NADPH. Then, in the presence of oxygen, 3-hydroxyhispidin is oxidized by insoluble luciferase, generating visible light quanta (Purtov et al. 2015, Oba et al. 2017, Kaskova et al. 2017, Kotlobay et al. 2018, Garcia-Iriepa et al. 2020).

Despite the aforementioned findings, it is still unclear what enzyme or group of enzymes function as luciferase in higher fungi, as it has not yet been isolated and characterized. Additionally, several equally important questions persist. Firstly, whether the luciferin/luciferase mechanism represents the only mechanism of light emission by basidiomycetes remains uncertain. Secondly, though 3-hydroxyhispidin has been identified as a substrate for fungal luminescence reactions, it is unclear whether it is the only substrate utilized in this process. Therefore, clarification of these questions requires further additional studies.

In 2016 and 2017, Teranishi conducted experiments with living pileus gills of the luminous fungus *Mycena chlorophos* and discovered two bioluminescence-activating components in their

extracts: trans-4-hydroxycinnamic acid (*p*-coumaric acid) and trans-3,4-dihydroxycinnamic acid (caffeic acid), with the latter synthesized from the former. The addition of these components to the living pileus gills resulted in a quick and structure-specific increase in their bioluminescence intensity *in vivo* (Teranishi 2016b, 2017). It is well-known that hispidin is biosynthesized from caffeic acid (Kotlobay et al. 2018); however, ^{13}C- and ^{18}O-labelling studies investigating the biotransformation of these bioluminescence-activating components in the gills of the fungus showed that caffeic acid was synthesized from *p*-coumaric acid, but caffeic acid did not produce hispidin (Teranishi 2017). Moreover, the addition of hispidin to the gills did not activate bioluminescence.

Puzyr et al. (2019), using the *A. borealis* luminescence system, discovered two forms of substrate for luminescence reaction in the culture broths after cultivation of the mycelia of the higher fungi such as *Inonotus obliquus*, *Pholiota* sp., and *A. borealis* (Puzyr et al. 2019). The authors found that one form of the substrate readily interacted with the enzymes of the luminescence system, while the other form became available for light emission reaction only after undergoing heat treatment. Based on their findings, the authors linked this phenomenon with hispidin precursors, which do not participate in the luminescence reaction in their original state but become the substrate for this reaction after the heat treatment.

In our recent study, we isolated extracellular enzymes of the luminous fungus *N. nambi* using an original method of treating the living pellets of fungal mycelium with β-glucosidase (Mogilnaya et al. 2018). An interesting effect was revealed through experiments with the extracts prepared by this method: the addition of aliquots of the extract to pellets of the *N. nambi* luminescent mycelium resulted in enhanced light emission intensity. At the same time, the extracts alone did not emit any detectable light. Therefore, we assumed that the extracts contained a component that induced light emission by the fungus *in vivo*. Thus, in the present work, we study some of the properties of the discovered compound that stimulates the luminescence of the basidiomycete *N. nambi*.

Materials & Methods

Instruments

To study the effect of the stimulator on light emission by *N. nambi* mycelium, luminescence intensity and dynamics were measured using a Glomax® 20/20 luminometer (Promega, U.S.A.) in the mode of one measurement per second.

The spectral study of the aqueous samples containing the luminescence stimulator (the extract, the concentrate of the low-molecular-weight components of the extract, and fractions after chromatography of the extract) was conducted using a UV-1800 spectrophotometer (Shimadzu, Japan) and a Varian Cary Eclipse spectrofluorometer (Agilent Technologies, U.S.A.).

Materials

The strain of the luminous higher fungus *Neonothopanus nambi* IBSO 2391 was used. This strain is maintained in 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 3–7 mm diameter spherical mycelial pellets produced by submerged fungus cultivation in the liquid nutrient medium (Mogilnaya et al. 2018).

The ready-to-use nutrient medium PDB (potato extract – 200 g/L, dextrose – 20 g/L), purchased from HiMedia Laboratory (India), was used to cultivate fungal mycelium. The prepared liquid medium was autoclaved at 120 °C for 15 min before use.

Enzyme β-glucosidase from sweet almond (Serva, Germany) (EC 3.2.1.21) dissolved in 10 mM phosphate buffer (pH 6.0) was used for enzymatic treatment of hyphal cell envelope.

Highly purified hispidin (≥ 98%, HPLC) (Sigma-Aldrich, U.S.A.), NADH (Serva, Germany), and NADPH (Sigma-Aldrich, U.S.A.) were used in the comparative study to investigate fluorescence and absorption spectra of the stimulator. The NAD, ATP, and ADP (Serva, Germany)

were used as marker compounds to estimate the molecular weight of the stimulator. A 20 mM solution of hispidin was prepared in methanol (Serva, Germany) and stored at a temperature of -20 °C until use. The 33 μM hispidin solution for experiments was prepared *in situ* by successive dilution of the stock solution with deionized (DI) water. The DI water was produced using a Milli-Q system (Millipore, U.S.A.). The aqueous solutions of the other reagents mentioned above were also prepared *in situ* using DI water.

Cultivation and processing

The fungus was cultivated in 300 ml flasks containing 100 ml PDB nutrient medium at a temperature of 27 °C and continuous agitation at 160–180 rpm using an Environmental Shaker-Incubator ES-20 (Biosan, Latvia). Mycelium, which had been grown in Petri dishes in PDA medium for 8–10 days, was crushed and used as inoculum for submerged cultivation of the fungus. The volume of the inoculum was 2–5% of the broth volume. The biomass was grown for eight days. Then, the pellets were taken out of the nutrient medium and washed in DI water several times to remove the nutrient medium and metabolites. The aqueous extract containing the stimulator of luminescence of the basidiomycete *N. nambi* was prepared using the technique we previously employed to isolate the extracellular enzymes of the fungus (Mogilnaya et al. 2018). The washed mycelial pellets were placed into fresh DI water containing β-glucosidase at a concentration of 0.5 IU/ml and incubated at 25 °C for 24 h under continuous stirring at 80 rpm on an OS-10 shaker (Biosan, Latvia). After incubation, the liquid (aqueous extract) was separated from the biomass by allowing it to pass through the filter paper. The separation of the high- and low-molecular-weight components of the extract was performed by ultrafiltration through a membrane with a molecular weight cutoff of 10 kDa (Merk Millipore, Germany). To separate low-molecular-weight compounds from the high-molecular-weight components more effectively, the DI water in the extract was replaced three times during ultrafiltration, and each time permeates were collected. After dialysis, the retentate, which contained high-molecular-weight components of the extract (extracellular proteins and enzymes of the fungus), was collected and used in experiments. The permeates containing low-molecular-weight components were combined and concentrated using a Rotavapor R-215 rotary evaporator (Buchi, Switzerland). The resulting concentrate was used in experiments.

Gel-filtration

Fractionation of low-molecular-weight components and preliminary assessment of the molecular weight of the luminescence stimulator were performed by gel-filtration chromatography of the concentrate using a column (0.8 × 20 cm) with Bio-Gel P2 (Bio-Rad, U.S.A.) equilibrated with DI water. The concentrate (0.5 ml) was applied to the column. Chromatography was conducted at a flow rate of 0.2 ml/min, using DI water as the eluent; 1 ml samples were collected. The presence of the stimulator and its distribution in the samples was determined by the fluorescence intensity of the samples recorded at a 440 nm wavelength (Varian Cary Eclipse) after excitation at a 360 nm wavelength. To assess the molecular weight of the stimulator, chromatography of the marker compounds and the solutions prepared in DI water were carried out under the conditions listed above (column size, volume of the sample applied, eluent, and flow rate). The markers were NAD, ATP, and ADP of molecular weights 0.66, 0.51, and 0.43 kDa, respectively.

Stimulating effect *in vivo*

The effect of the stimulator on light emission by *N. nambi* was assessed using mycelial pellets grown as described above and incubated in DI water for 12 h to remove nutrient medium and metabolites altogether. Individual mycelial pellets were placed into 1.5-ml transparent plastic tubes (Eppendorf, Germany), each containing 500 μl of DI water. The test tubes were placed into the measuring chamber of the luminometer, and the initial level of light emission by pellets was measured. Then, 5 μl of the stimulator sample was added carefully (without mixing) to the samples,

and the intensity and dynamics of the light signal from the pellets were measured again. The luminescence level was expressed in relative units.

Testing the application of the stimulator as the substrate for the fungal luminescence system

To test whether the discovered stimulator of luminescence can be a substrate of the light-emitting system of the basidiomycete *N. nambi*, we used "cold" extract containing the enzyme luminescent system of the fungus, which was produced from mycelium by the method described in Bondar et al. (2014). The functional activity of the isolated system was tested as follows: The 200-µl aliquots of the "cold" extract were poured into the plastic test tubes, which were placed in the luminometer (Glomax® 20/20), and a luminescence reaction was initiated by the addition of 5 µl of the 10 mM NADPH solution and 5 µl of the 33 µM hispidin solution; light emission intensity and dynamics were measured. As noted above, hispidin is the precursor of the substrate for the luminescence reaction of higher fungi, which is transformed into 3-hydroxyhispidin (luciferin) by NADPH-dependent hydroxylase and is then oxidized by luciferase, generating visible light quanta (Purtov et al. 2015, Oba et al. 2017, Kaskova et al. 2017, Kotlobay et al. 2018, Garcia-Iriepa et al. 2020). The potential use of the stimulator as the substrate (or its precursor) of the luminescence reaction was assessed using the isolated luminescence system under the conditions described above. In that experiment, after the addition of 5 µl of the 10 mM NADPH solution, 5 µl of the aqueous sample of the stimulator was added to the reaction mixture instead of the hispidin solution.

Evaluation of thermal stability of the stimulator

To study the thermal stability of the stimulator, 200 µl samples of the concentrated low-molecular-weight compounds were placed into plastic test tubes and incubated for 1–5 min at 100 °C in the TB-85 Thermo Bath (Shimadzu, Japan). After that, the samples were cooled in the ice bath, and the effect of the thermally treated stimulator on light emission by *N. nambi* mycelium was assessed as described above.

Results & Discussion

In a previous study, we isolated extracellular enzymes with the oxidase function from the luminous basidiomycete *N. nambi* using an original method of treating the fungal mycelium with β-glucosidase (Mogilnaya et al. 2018). In further experiments with the aqueous extracts prepared by that method, we noticed an interesting effect. The addition of aliquots of the extract to the *N. nambi* luminescent mycelium enhanced its light emission intensity. At the same time, the extracts alone did not emit detectable light. Thus, we assumed that the extracts contained a component that stimulated light emission by the fungus.

After the separation of the high- and low-molecular-weight components of the extract by ultrafiltration through a membrane with a molecular weight cutoff of 10 kDa, we identified the stimulator of *N. nambi* luminescence as a low-molecular-weight compound. The addition of 5 µl of the concentrated low-molecular-weight components to the luminous mycelial pellets induced a rapid (within a few seconds) increase in the level of their light emission, ranging from several-folds to 1.5 orders of magnitude or higher, relative to the initial level (Fig. 1). After the light signal reached its maximum, it declined in a few minutes. Further addition of the concentrate stimulated luminescence increase again (Fig. 1). Multiple (4 or more) sequential additions of the concentrated low-molecular-weight components to the same pellets resulted in the stimulation of light emission by the mycelium. On the other hand, the addition of 5 µl of the concentrated high-molecular-weight compounds (the retentate after ultrafiltration of the extract) to the luminous pellets did not cause any noticeable changes in their light emission levels. At the same time, the concentrates of high- and low-molecular-weight components used in the experiment did not emit detectable light. The levels of luminescence of both concentrates were the same as the background signal of the luminometer.

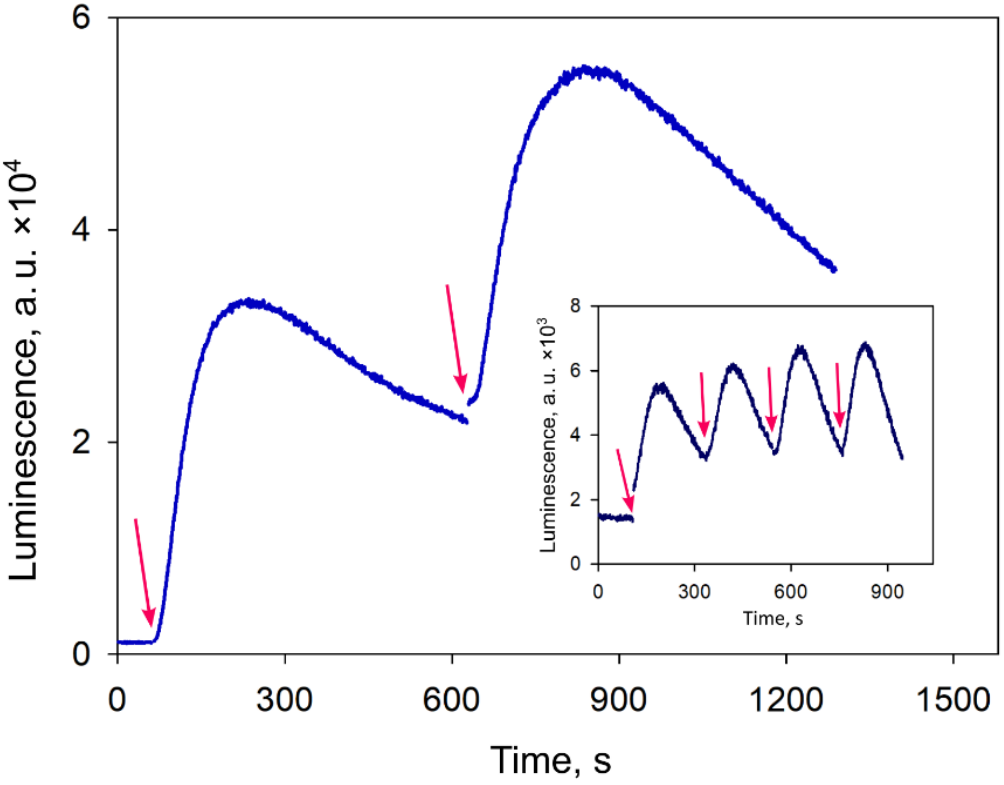

Fig. 1 – Stimulation of light emission of individual *N. nambi* mycelial pellets by adding aliquots of the concentrate of low-molecular-weight components. Arrows indicate time points when the concentrate (5 µl) was added to pellets.

As noted above, hispidin and reduced pyridine nucleotides (NADPH and NADH) are necessary components for the function of the fungal luminescence system, which is responsible for the emission of visible light quanta *in vitro*. Therefore, we could not rule out the possibility of one of those components being the stimulator of luminescence of the fungus *N. nambi in vivo*. To test that assumption, we studied the effects of hispidin and NAD(P)H on the level of light emission from mycelial pellets *in vivo*. Those experiments did not reveal any perceptible changes in the light emission intensity of mycelial pellets. In any case, we did not observe any changes after adding 5 µl of the 33 µM hispidin aqueous solution or 5 µl of 1 mM aqueous solutions of NADPH and NADH. Thus, we can now say with greater confidence that the stimulator of *N. nambi* luminescence that we have discovered is not hispidin nor the NAD(P)H. In addition, the fact that hispidin did not stimulate the luminescence of *N. nambi* mycelial pellets was in good agreement with the data previously reported by other authors (Teranishi 2016b), which were obtained in experiments with hispidin and living pileus gills of the basidiomycete *M. chlorophos*.

We carried out additional experiments to compare the absorption and fluorescence spectra of hispidin, NAD(P)H, and concentrate of low-molecular-weight components containing the stimulator of *N. nambi* luminescence.

The absorption and fluorescence spectra of the aqueous sample of the concentrate containing luminescence stimulator and the aqueous solution of hispidin differed considerably (Fig. 2). In the absorption spectrum of the concentrate, we detected two major peaks in the short wavelength region at 205 and 260 nm and a shoulder in the 350–370-nm range (Fig. 2a). In the spectrum of the hispidin aqueous solution, there were two major absorption peaks at 221 and 360 nm, a minor peak at 245 nm, and a shoulder in the 280–310-nm range. The experiments showed that excitation of the aqueous sample of the concentrate at 350–370 nm was accompanied by the blue fluorescence of the sample with the maximum of light emission at 440 nm (Fig. 2b). On the other hand, excitation of the hispidin aqueous solution at the same wavelengths was accompanied by fluorescence of the solution with the emission maximum at about 500 nm (Fig. 2b).

The current study also showed that the fluorescence and absorption spectra of the NADPH and NADH aqueous solutions differed from the corresponding spectra of the luminescence stimulator (Figs. 3a, 4a). The maxima of NAD(P)H fluorescence were in the longer wavelength region compared to the maximum of stimulator fluorescence (Fig. 3a). Excitation of the aqueous solutions of reduced pyridine nucleotides at 335 nm (nicotinamide absorption) was accompanied by fluorescence of the solutions with the maximum of light emission at 465 nm, which was consistent with the literature (Patterson et al. 2000). Excitation at 360 nm (absorption of the stimulator) did not change the NADH and NADPH fluorescence spectra, but decreased levels of emission in the maxima, which was caused by the non-optimal absorption wavelength of nicotinamide. In additional experiments, we investigated fluorescence parameters of NADPH, NADH, and luminescence stimulator after incubation of their aqueous solutions at 100 °C for 5 min. After the heat treatment, the NADH and NADPH fluorescence spectra did not change, but the levels of emission in the maxima were decreased by 20–25% and 60–65%, respectively (Fig. 3c, d). That was in agreement with the data indicating that only the reduced pyridine nucleotides are the fluorescent forms while their oxidized counterparts, NAD+ and NADP+, do not fluoresce (Blacker & Duchen 2016). In the present study, considerable changes in the absorption spectra of the heat-treated NAD(P)H aqueous solutions suggested the emergence of pyridine nucleotide molecules in the oxidized state (Fig. 4c, d). By contrast, we did not note any perceptible differences in the absorption and fluorescence spectra and emission maximum of the luminescence stimulator before and after heat treatment (Figs. 3b, 4b). Moreover, the stimulator of luminescence of the fungus *N. nambi* retained its stimulating effect after the 5-min heat treatment of the concentrate of low-molecular-weight components at 100 °C. Thus, the results presented above (Figs. 2–4) suggest that the discovered stimulator of fungal luminescence differs essentially from hispidin and pyridine nucleotides, NADPH and NADH.

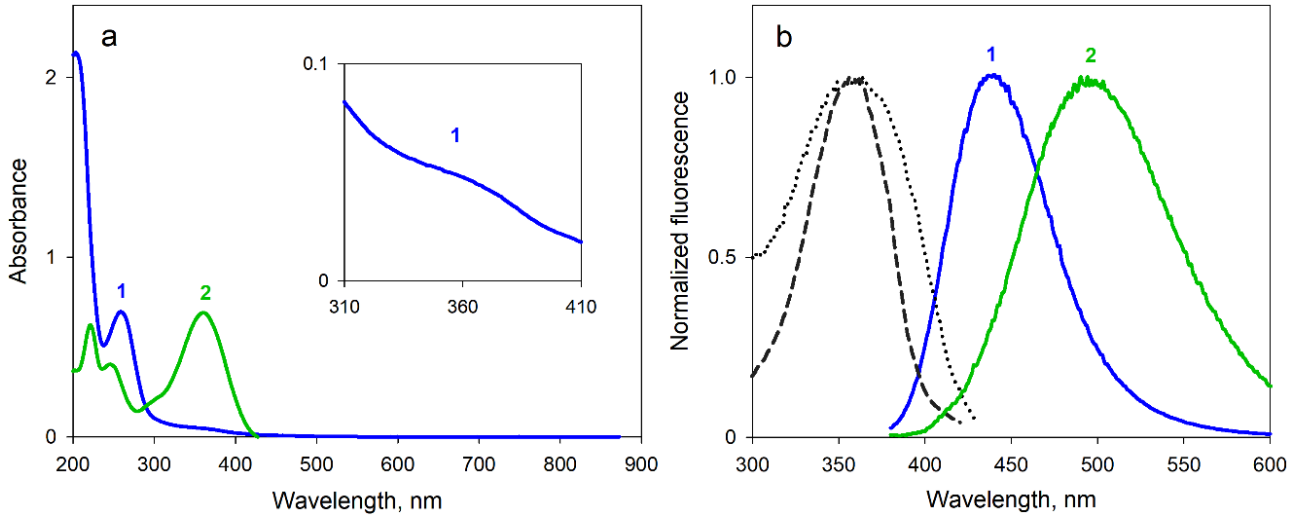

Fig. 2 – Absorption (a) and fluorescence (b) spectra of the aqueous solutions of the concentrate of low-molecular-weight components containing luminescence stimulator (1) and hispidin (2). The dashed and dotted lines correspond to the excitation spectra of the concentrate and hispidin samples, respectively. In the excitation and fluorescence spectra, the values are normalized to the maximal levels of light emission.

As the effect of light emission stimulation and the fluorescence parameters of the concentrate of low-molecular-weight components did not change after heat treatment, we hypothesized that the thermostable stimulator of *N. nambi* luminescence is a fluorescent compound. The hypothesis was tested experimentally as follows: First, we separated low-molecular-weight components of the concentrate using Bio-Gel P2 column chromatography and measured fluorescence levels in the chromatography fractions. The screening of chromatography fractions revealed one fluorescence

peak (Fig. 5). Thus, we then combined the fractions on the rise, at the maximum, and at the decline of the fluorescence peak to form three separate samples – 1, 2, and 3, respectively, which were then concentrated to equal volumes using a rotary concentrator (Concentrator 5301, Eppendorf, Germany). The stimulating effect of the concentrated samples on light emission by *N. nambi* mycelial pellets was estimated as described above by adding equal amounts of the concentrates (5 µl) to the tested samples.

Experiments demonstrated that concentrated sample 2 produced much stronger stimulation of light emission of *N. nambi* mycelium than samples 1 and 3 (Fig. 6). This finding was based on the analysis of the maximal level of light emission from mycelial pellets stimulated by the addition of the samples and the area under the curves of the recorded luminescence signals, which corresponds to the total quantum yield of the reaction (Fig. 6). Calculation of the areas under the curves of luminescent signals in experiments with *N. nambi* pellet luminescence stimulated by samples 1, 2, and 3 showed that quantum yield ratio was 1.5: 8.4: 1, respectively. Based on these data, we can say quite confidently that the luminescence stimulator we discovered is a fluorescent compound.

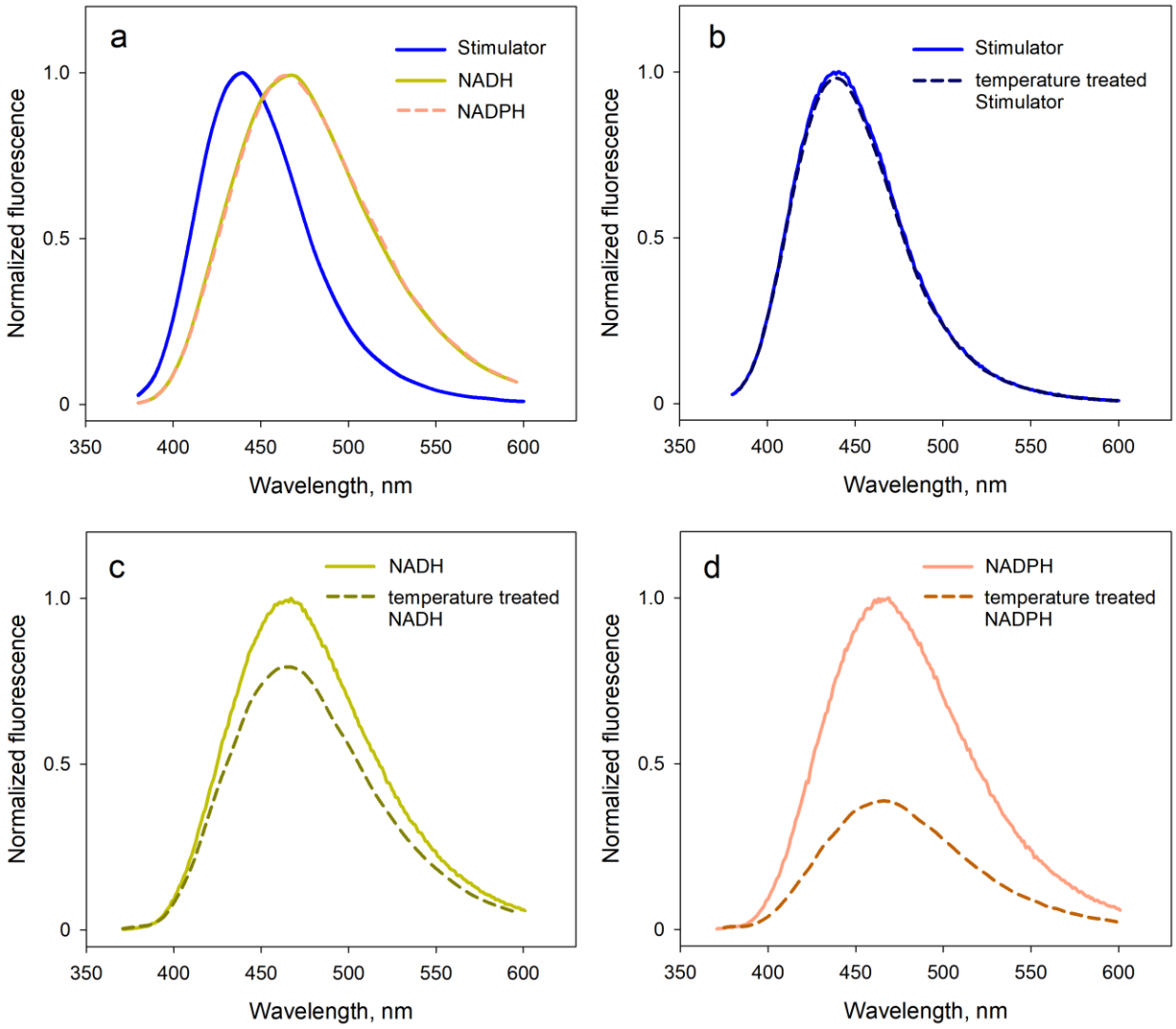

Fig. 3 – Fluorescence spectra of the aqueous solutions of the concentrate containing luminescence stimulator and NADH and NADPH before (a) and after heat treatment at 100 °C for 5 min (b, c, d). In the spectra, the values are normalized to the corresponding maximal levels of light emission.

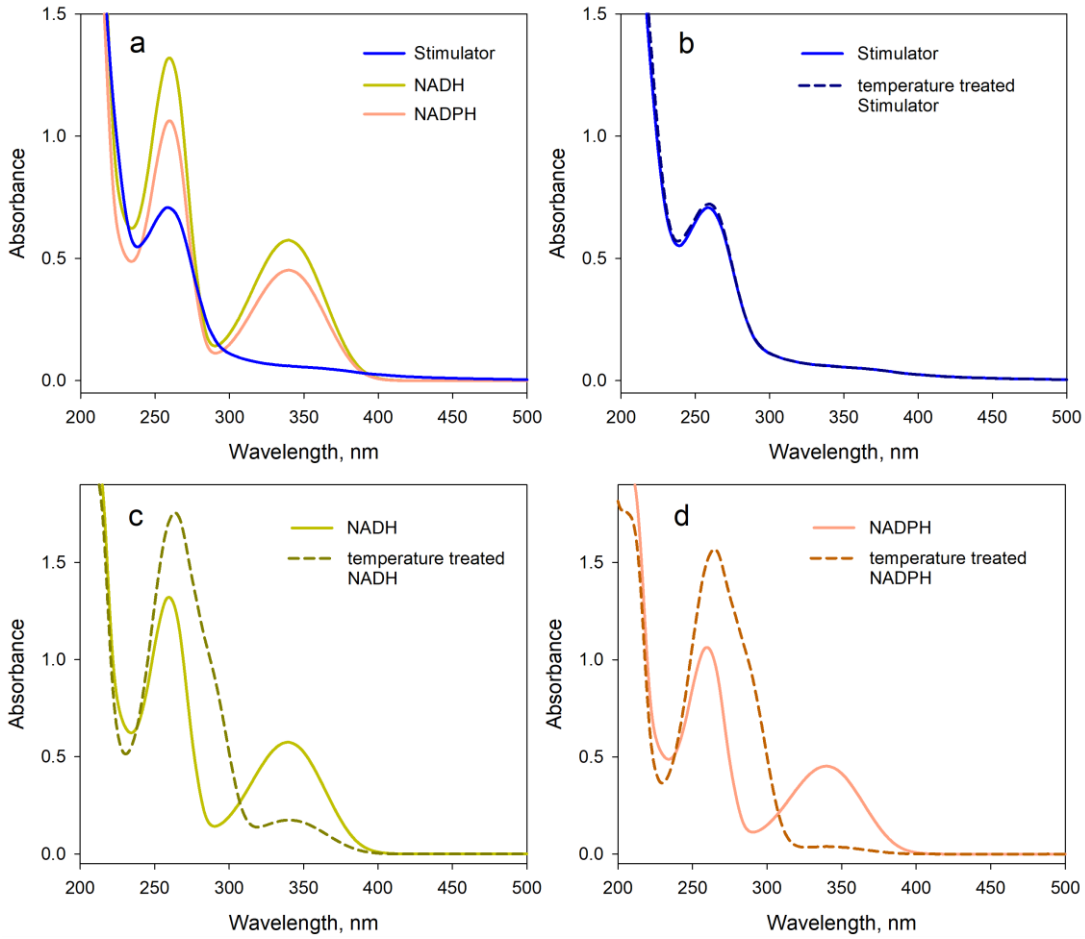

Fig. 4 – Absorption spectra of the aqueous solutions of the concentrate containing luminescence stimulator and NADH and NADPH before (a) and after heat treatment at 100 °C for 5 min (b, c, d).

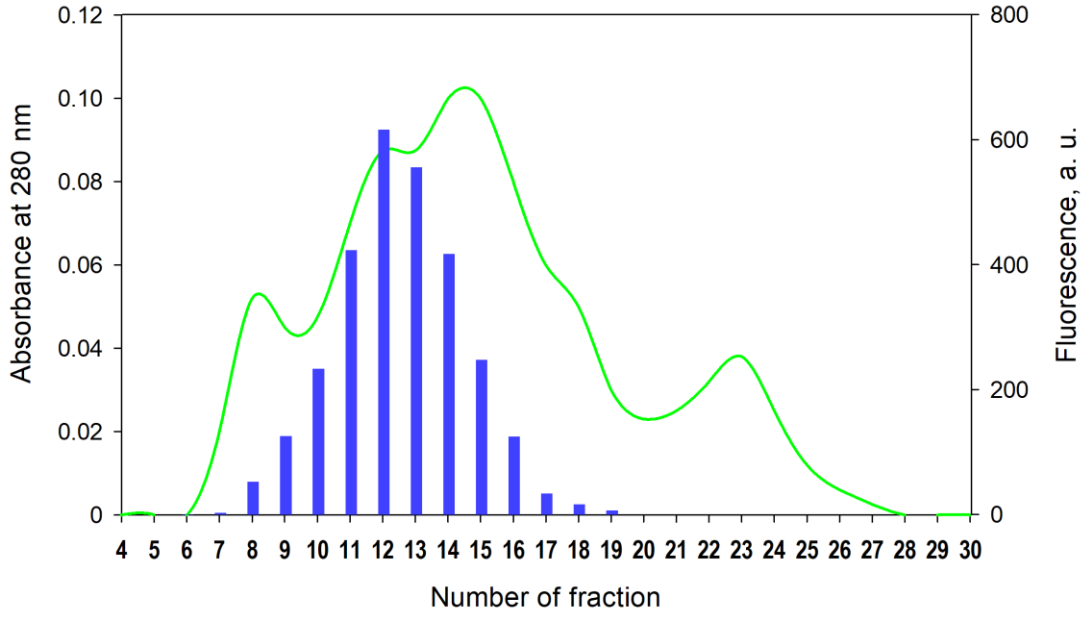

Fig. 5 – Elution profile (curve) and fluorescence level in chromatography fractions (histogram) obtained by gel-filtration Bio-Gel P2 column chromatography of the concentrate of low-molecular-weight components.

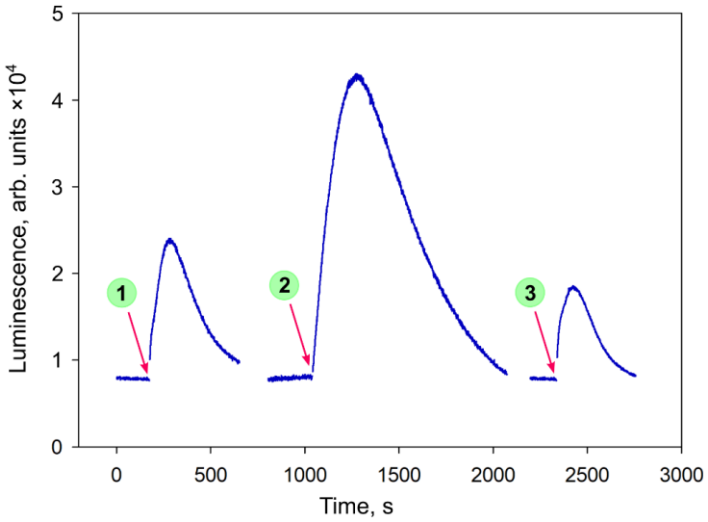

Fig. 6 – Stimulation of luminescence of *N. nambi* mycelial pellets by samples of combined and concentrated chromatography fractions on the rise (1), at the maximum (2), and the decline (3) of the fluorescence peak (see Fig. 5). Arrows indicate time points at which samples (5 µl) were added to pellets.

Based on the data obtained by gel-filtration Bio-Gel P2 column chromatography of the concentrate of low-molecular-weight components, apparent molecular weight of the luminescence stimulator was 0.46 kDa (Fig. 7). It was calculated using the fluorescence peak maximum (Fig. 5) and the absorption maxima obtained by chromatography of the markers (NAD, ATP, and ADP) of known molecular weights, which was performed under similar conditions. This finding suggested that the component stimulating the luminescence of *N. nambi* mycelium *in vivo* was not *p*-coumaric acid (molecular weight of 0.164 kDa) nor caffeic acid (molecular weight of 0.180 kDa). In our opinion, this finding is of fundamental importance because other researchers previously observed (in experiments with living pileus gills of the luminous fungus *Mycena chlorophos*) that *p*-coumaric acid and caffeic acid were able to stimulate fungal bioluminescence *in vivo* (Teranishi 2016b, Teranishi 2017).

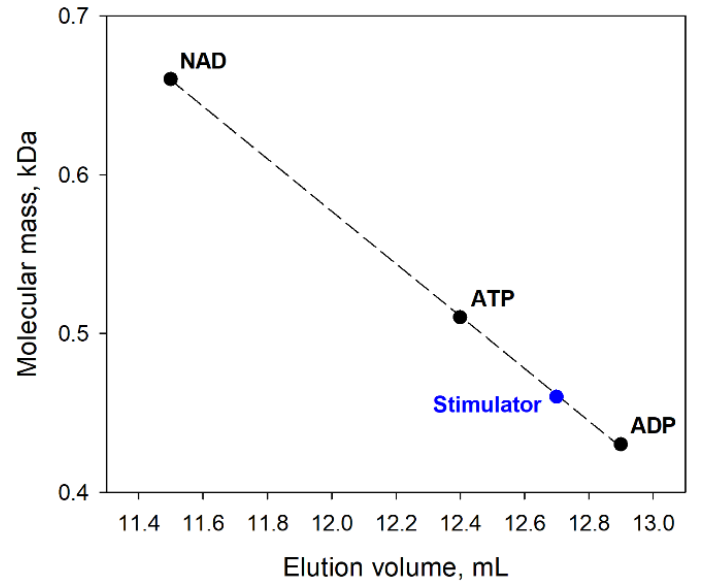

Fig. 7 – Elution volumes of low-molecular-weight marker compounds (NAD, ATP, ADP) and stimulator of luminescence of the fungus *N. nambi* obtained by gel-filtration Bio-Gel P2 column chromatography, as dependent on their molecular weights.

Our additional experiments *in vitro* showed that the component stimulating light emission by *N. nambi* mycelial pellets was not the substrate (or substrate precursor) of the enzymatic luminescence system isolated from this fungus. Aliquots of the concentrate of low-molecular-weight compounds and sample 2, concentrated after chromatography (see Fig. 6), added to the NADPH-activated luminescence system, did not cause any changes in its light emission intensity. These experimental results provide additional support for the conclusion that the stimulator discovered in this study is different from hispidin.

Conclusion

A component enhancing the level of *in vivo* bioluminescence of the luminous basidiomycete *Neonothopanus nambi* was discovered in the mycelial extracts of the fungus after the fungal biomass was treated with β-glucosidase. The study revealed that the stimulator of light emission was a low-molecular-weight, thermostable compound that, when added to the pellets of luminous mycelium, increased the level of their light emission from several times to 1.5 orders of magnitude or more. The results of the experiments indicate that the discovered component is a fluorescent compound, and its excitation at 350–370 nm in the sample containing it leads to blue fluorescence of the sample, with the maximum light emission at 440 nm. The present study showed that the luminescence stimulator was not the substrate (or substrate precursor) of the enzymatic luminescence system of the fungus *N. nambi*. However, the mechanism responsible for the stimulation of fungal luminescence by the discovered component remains unclear and requires further research. The main objective of our future study will be to isolate the discovered component in order to determine its structure and mechanism of luminescence stimulation.

Acknowledgements

The study was funded by the State Assignment of the Ministry of Science and Higher Education of the Russian Federation (project No. FWES-2024-0018).

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