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BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

Finding the Light Emission Stimulator of *Neonothopanus nambi* Basidiomycete and Studying Its Properties

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Abstract—A stimulator of light emission of the fungus was found in an aqueous extract from mycelium of the luminous basidiomycete *Neonothopanus nambi* after its treatment with β-glucosidase. The addition of the extract to the luminous mycelium increases the level of light emission from several times to 1.5 orders of magnitude or more. The luminescence stimulator is a low-molecular-weight thermostable compound: it is detected in the permeate after filtering the extract through a 10-kDa cutoff membrane and it retains the stimulating effect after heat treatment at 100°C for 5 min. In the absorption spectrum of the aqueous sample of the stimulator, two main peaks are observed in the shortwave region (205 and 260 nm) and a shoulder in the range of 350–370 nm can be seen. The luminescence stimulator exhibits blue fluorescence with an emission maximum at 440 nm when excited at 360 nm. It was established that the luminescence-stimulating component is not a substrate (or its precursor) of the luminescent system of the *N. nambi* fungus.

Keywords: bioluminescence, *Neonothopanus nambi*, luminescent mycelium, light emission stimulator, fluorescence

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In recent studies, we used an original method of treating the mycelium of the luminous basidiomycete *Neonothopanus nambi* with β-glucosidase to isolate its extracellular enzymes with an oxidase function [1]. In additional experiments with the resulting aqueous extracts, we found an interesting effect. Additions of extract aliquots to *N. nambi* luminous mycelium samples significantly increased the intensity of their light emission. The extracts themselves had no recordable luminescence. This allowed us to assume that the extracts contain a component that stimulates the luminescence of the fungus.

The present study is devoted to the investigation of some properties of the discovered luminescence stimulator of the basidiomycete *N. nambi*.

We used the mycelium of the higher fungus *N. nambi* IBSO 2391 from the Collection of Microorganisms (CCIBSO 836) of the Institute of Biophysics of the Krasnoyarsk Scientific Center, Siberian Branch, Russian Academy of Sciences (Krasnoyarsk), which was previously provided by N.V. Psurtseva from the LE-BIN Collection of Basidiomycete Cultures of the Botanical Institute, Russian Academy of Sciences (St. Petersburg). Experiments were performed with spherical mycelium pellets 2–7 mm in diameter, which were obtained by deep cultivation of the fungus in a liquid potato-sucrose medium (HiMedia Laboratory, India) [1]. Biomass was cultivated for 8 days. After cultivation, the pellets were removed from the nutrient medium and washed many times with deionized (DI) water (Milli-Q system, Millipore, United States) to remove the remains of the nutrient medium and metabolites. An aqueous extract containing *N. nambi* luminescence stimulator was prepared as follows. Washed mycelium pellets were placed in a fresh volume of DI water containing sweet almond β-glucosidase (Serva, Germany) at a concentration of 0.5 IU/mL and incubated at 25°C for 24 h with slow stirring at a speed of 80 rpm on an OS-10 shaker (BIOSAN, Latvia). The stock solution of β-glucosidase was prepared in 10 mM phosphate buffer (pH 6.0). After incubation, the liquid part (water extract) was separated from the biomass by filtration through a paper filter. Separation of high- and low-molecular-weight components of the obtained extract was performed using its ultrafiltration through a membrane with a 10-kDa exclusion limit (EMD Millipore Amicon, Darmstadt, Germany). For a more complete separation of the low-molecular-weight compounds from the high-molecular-weight components during ultrafiltration of the extract, DI water was replaced in it

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three times, each time the submembrane filtrates were collected. After completion of dialysis, the supramembrane fraction containing the high-molecular-weight components of the extract (extracellular fungal proteins and enzymes) was collected and used in the studies. The submembrane filtrates containing low-molecular-weight components were combined and concentrated on a Rotavapor R-215 rotary evaporator (Buchi, Switzerland). The resulting concentrate was used in experiments.

Spectral studies of aqueous samples containing low-molecular-weight components (extract, concentrate, and samples after chromatography) were performed on a UV-1800 spectrophotometer (Shimadzu, Japan) and a Varian Cary Eclipse spectrofluorometer (Agilent Technologies, United States).

Fractionation of low-molecular-weight components and a preliminary assessment of the molecular weight of the studied luminescence stimulator were performed using gel filtration chromatography of the concentrate on a column (0.8 × 20 cm) packed with Bio-Gel P2 (Bio-Rad, United States) equilibrated with DI water. The concentrate (0.5 mL) was applied onto the column. Chromatography was performed at a flow rate of 0.2 mL/min using DI water as eluent; 1-mL samples were collected. The presence and distribution of the stimulator in the chromatographic samples was determined by the intensity of their fluorescence recorded at a wavelength of 440 nm (Varian Cary Eclipse) after excitation at a wavelength of 360 nm. To assess the molecular weight of the stimulating component, chromatography of marker compounds, solutions of which were prepared in DI water, was performed under the above conditions (column size, sample volume, eluent, and flow rate). Riboflavin and FMN (Sigma, United States) and NAD (Serva, Germany) with molecular weights of 0.38, 0.46, and 0.66 kDa, respectively, were used as markers.

The effect of the stimulator on the luminescence of the fungus *N. nambi* was evaluated using mycelium pellets grown as described above and incubated in DI water for 12 h to more completely remove the remains of the nutrient medium and metabolites. Luminescence intensity and dynamics were recorded using a Glomax® 20/20 luminometer (Promega BioSystems Sunnyvale, Inc., United States) in the mode of one measurement per second. Individual mycelium pellets were placed in 1.5-mL transparent plastic tubes (Eppendorf, Germany) filled with 500 μL of DI water. The test tubes were placed in the measuring chamber of the luminometer, and the initial level of pellet luminescence was recorded. Then, 5 μL of the tested sample of the stimulator was carefully (without mixing) added to the samples, and the intensity and dynamics of the light signal of the pellets were recorded once again.

To study the thermal stability of the studied stimulator, samples of the concentrate of low-molecular-weight compounds (volume 200 μL) were placed in plastic test tubes and incubated at 100°C (TB-85 Thermo Batch thermostat, Shimadzu, Japan) for 1–5 min. Then, the samples were cooled in an ice bath, and the effect of the heat-treated stimulator on *N. nambi* mycelium luminescence was evaluated as described above.

To test the applicability of the discovered stimulator as a substrate for the light-emitting system of the *N. nambi* basidiomycete, we used a luminescent system isolated from the mycelium of the fungus by the method described earlier [2]. The functional activity of the resulting system was tested as follows. Aliquots of the sample of the isolated system (volume 200 μL) were added to plastic tubes, which were placed in a luminometer (Glomax® 20/20). A luminescent reaction was initiated by adding 5 μL of 10 mM NADPH solution and 5 μL of 33 μM hispidin solution, and the intensity and dynamics of light emission was recorded. It was established that hispidin is a precursor of the light-emission reaction substrate of higher fungi, which is converted by NAD(P)H-dependent hydroxylase into 3-hydroxyhispidin (luciferin) and then oxidized by luciferase with the emission of visible light quanta [3–8]. This is consistent with the previously stated hypothesis about the two-stage process of luminescence of basidiomycetes [9, 10]. We used NADPH (Serva, Germany) and hispidin (Sigma-Aldrich, United States). A stock solution of hispidin at a concentration of 20 mM was prepared in methanol (Sigma-Aldrich) and stored at –20°C. For experiments, an aqueous solution of hispidin with a concentration of 33 μM was prepared in situ by successive dilutions of the stock solution with DI water. The applicability of the stimulator as a substrate (or its precursor) for a light-emitting reaction was assessed using an isolated luminescent system under the above experimental conditions. In this case, 5 μL of an aqueous sample of the stimulator was added to the reaction mixture instead of hispidin solution.

In our studies, we found that the stimulator of luminescence of the basidiomycete *N. nambi* found by us is a low-molecular-weight compound. When aliquots of the concentrate of low-molecular-weight components were added to the luminous mycelium pellets, a rapid (several seconds) increase in their light emission level was observed, which could exceed the initial level from several times to 1.5 orders of magnitude or more (Fig. 1). It can be seen from the presented data that, after the light signal reaches its maximum, its decrease is observed for several minutes. Repeated additions of the concentrate again stimulated an increase in luminescence with a light signal amplitude comparable to or greater than that of the first signal (Fig. 1). We observed the stimulation of the luminescence of the *N. nambi* mycelium after successive multiple (4–6 times) additions of the concentrate of low-molecular-weight components to the same pellet samples. At the same time, the addition of 5 μL of

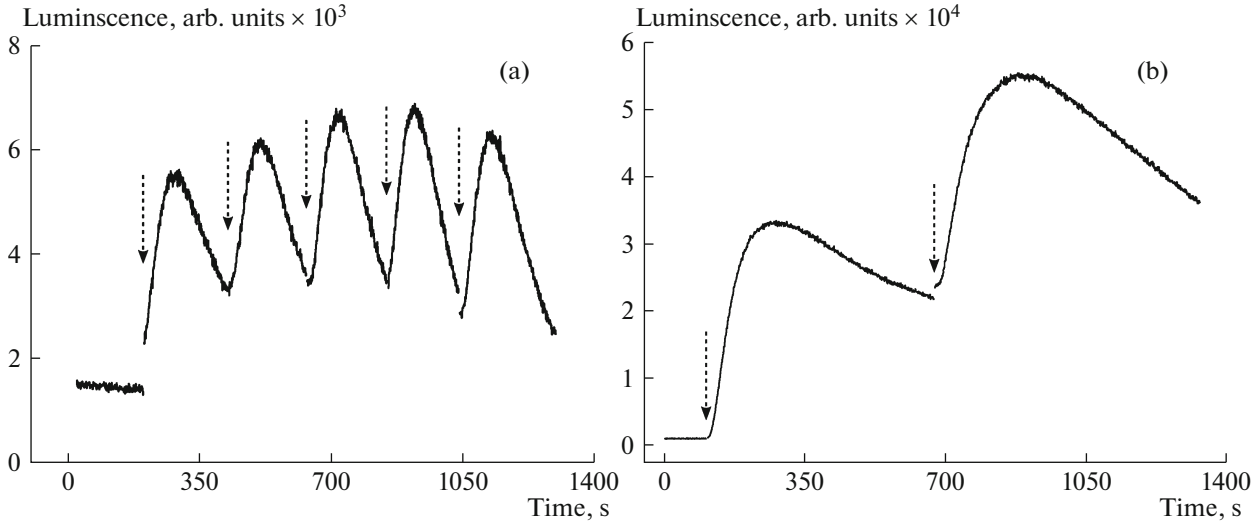

Fig. 1. Effect of increasing the luminescence intensity of *N. nambi* mycelium pellets from several times (a) to 1.5 orders of magnitude (b) after successive additions of the concentrate of low-molecular-weight components. Arrows show the moments of adding the concentrate (5 μL) to the sample.

the concentrate of high-molecular-weight compounds to the luminous pellets (the supramembrane fraction after ultrafiltration of the extract) did not cause any noticeable changes in the levels of their light emission. It is important to note that the concentrates of high- and low-molecular-weight components of the extract from *N. nambi* mycelium pellets used by us did not have a detectable luminescence. When testing both samples, their luminescence levels did not differ from the baseline signal of the measuring device (luminometer Glomax® 20/20).

In this work, we found that the stimulator of luminescence of the fungus *N. nambi* found by us is a thermostable compound that retains its stimulating effect after heat treatment of the concentrate of low-molecular-weight components at 100°C for 5 min.

In the absorption spectrum of the concentrate containing the luminescence stimulator, we revealed the presence of two major peaks in the shortwave region at 205 and 260 nm and a shoulder in the region of 350–370 nm (Fig. 2). It was found in experiments that the excitation of aqueous samples of the concentrate at wavelengths of 350–370 nm is accompanied by their fluorescence with a maximum of light emission at 440 nm (Fig. 2). It is important to note that we did not reveal any changes in the fluorescence spectrum and in the value of the light emission maximum after heat treatment of the concentrate containing the luminescence stimulator for 5 min at 100°C. This allowed us to assume that the heat-resistant luminescence stimulator from *N. nambi* discovered by us is a fluorescent compound.

The above assumption was verified experimentally as follows. First, we separated the low-molecular-weight components of the concentrate using gel filtration chromatography on a Bio-Gel P2 column, followed by an assessment of the fluorescence level in the obtained chromatographic samples. When screening the chromatographic fractions, we revealed the presence of a single fluorescence peak (Fig. 3). Based on this fact, at the next stage we pooled the fractions that were on the rise, at the maximum, and at the decline of the fluorescent peak into three separate samples (1, 2, and 3, respectively), which were then concentrated on a rotary concentrator (Concentrator 5301, Eppendorf, Germany) to equal volumes. A comparative assessment of the effect of the obtained concentrates on the luminescence of *N. nambi* mycelium pellets was carried out as described above, adding equal volumes (5 μL) of concentrates to the tested samples.

In comparative experiments, we found that the concentrated sample 2 had a much greater stimulating effect on luminescence of the *N. nambi* mycelium as compared to samples 1 and 3 (Fig. 4). This follows from the analysis of the maximum level of pellet luminescence stimulated by the addition of the samples and the area under the curves of the recorded luminescent signals, which reflects the total quantum yield of the reaction (Fig. 4).

From the calculations of the areas under the curves of luminescent signals when the luminescence of *N. nambi* pellets was stimulated with samples 1, 2, and 3, the ratio of quantum yields was 2 : 11 : 0.5, respectively. Taken together, these data allowed us to assume with sufficient confidence that the luminescence stimulator discovered by us is a fluorescent compound.

According to the preliminary data of gel filtration chromatography on a column with Bio-Gel P2, the apparent molecular weight of the stimulator, calculated by us from the maximum of the fluorescence

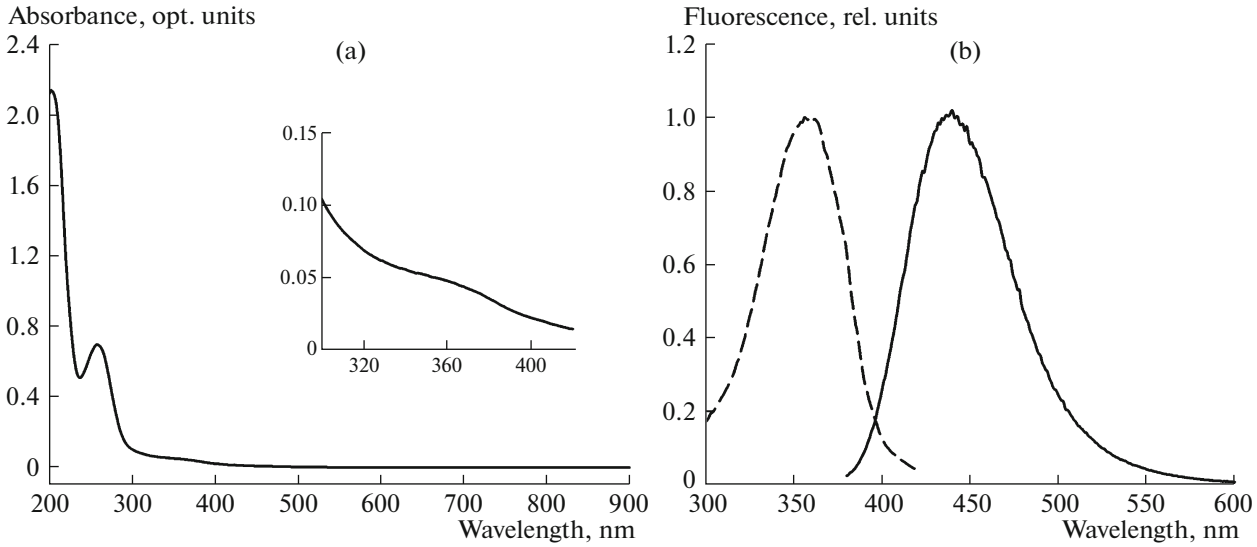

Fig. 2. Absorption spectrum (a) and excitation and fluorescence spectra (b) of the concentrate of low-molecular-weight compounds containing the stimulator of luminescence of the fungus *N. nambi*. The inset shows a fragment of the absorption spectrum in the range of 300–420 nm. In the excitation and fluorescence spectra, the values were normalized to the maximum levels of light emission.

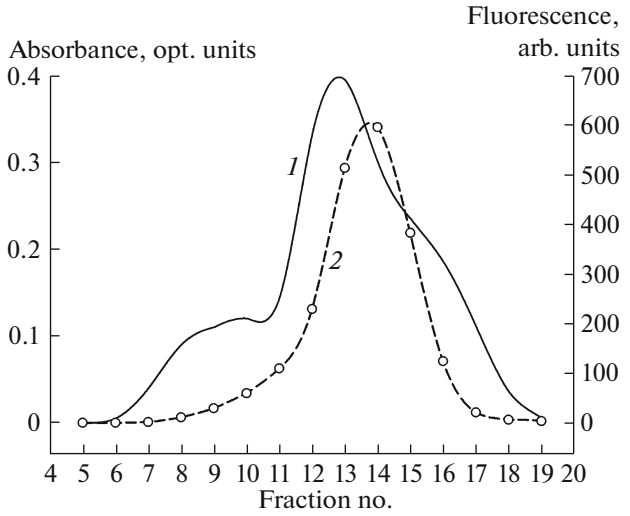

Fig. 3. Gel-filtration chromatography of the concentrate of low-molecular-weight components from *N. nambi* mycelium on a Bio-Gel P2 column: (*1*) elution profile, (*2*) fluorescence level of chromatographic fractions.

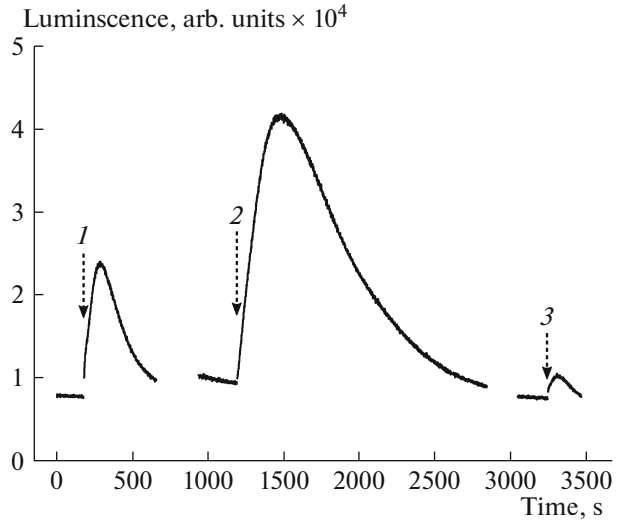

Fig. 4. Stimulation of luminescence of *N. nambi* mycelium pellets by the samples of pooled and concentrated chromatographic fractions collected on the rise (*1*), in the maximum (*2*), and on the decline (*3*) of the fluorescent peak (see Fig. 3). Arrows show the moments of addition of samples (5 μL) to the pellets.

peak (Fig. 3), according to the data of several experiments, is in the range of 0.4–0.6 kDa.

In our studies, we established that the component that stimulates the luminescence of *N. nambi* mycelium pellets is not a substrate (or its precursor) of the luminescent system isolated from this fungus. At least, after the additions of 5 μL of the concentrate of low-molecule-weight compounds containing the stimulator and sample 2 concentrated after its chromatography to the NADPH-activated luminescent system, we did not observe changes in the level of its light emission.

Thus, in extracts from the mycelium of the luminous fungus *Neonothopanus nambi* after its treatment with β-glucosidase, a fungal luminescence stimulator was found. It was established that the luminescence stimulator is a low-molecular-weight thermostable compound and that, when added to luminous mycelium pellets, increases the level of their light emission from several times to 1.5 orders of magnitude or more.

The results of experiments indicate that the discovered component is a fluorescent compound that, upon excitation at wavelengths of 350–370 nm, has blue fluorescence with a light emission maximum at 440 nm. It was established that the luminescence stimulator is not a substrate (or substrate precursor) of the luminescent system of the fungus *N. nambi*. The mechanism of luminescence stimulation by the detected component is still unclear and requires further studies. The immediate priority task for further research will be to isolate the detected component in its pure form to establish its structure and the mechanism of the luminescence-stimulating effect.

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