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Luminescence of cold extracts from mycelium of luminous basidiomycetes during long–term storage

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Abstract

Cold extracts with high activities of enzymes of luminescent reaction were prepared from mycelia of luminous fungi *Armillaria borealis* IBSO 2328, *Mycena citricolor* IBSO 2331, and *Neonothopanus nambi* IBSO 2391. The authors describe techniques of preparing cold extracts with high levels of luminescence from mycelial biomass of different species of luminous basidiomycetes. The investigation of cold extracts showed that in experiments with freezing and thawing of the samples as well as in experiments with lyophilization followed by dissolution of the dry samples, the levels of enzyme activity were high, with *in vitro* luminescence exhibited after addition of NADPH and the hot extract containing the substrate. High activity levels of the enzymes of luminescent reaction were measured in lyophilized cold extracts stored over three years. In lyophilized preparations, the enzymes of luminescent reaction had high thermostability, even when dry preparations of cold extracts were exposed to a temperature of 100°C for 60 minutes.

Key words – *Armillaria borealis* **–** *Mycena citricolor* **–** *Neonothopanus nambi* **–** kinetics of luminescence – lyophilic preparations

Introduction

Research of luminous living organisms (bacteria, flagellates, coelenterates, worms, insects, crustaceans, mollusks, fish) (Shimomura 2006) resulted in isolation and investigation of enzymes (luciferases) and substrates (luciferins). Findings obtained from this research have many practical applications (Morin 1974, Blinks et al. 1976, 1982, Ugarova & Brovko 1981, Gitelson et al. 1984, Ugarova et al. 1987, 1993, Illarionov et al. 2000; Shimomura 2006; Girotti et al. 2008, Medvedeva et al. 2009, Fernandez–Pinas et al. 2014, Markova & Vysotski 2015).

The long history of research on luminous basidiomycetes knows several cases of successful isolation of luminescent systems that show *in vitro* activity from fungal biomass, by using the classical luciferin/luciferase test, which consists of mixing hot (substrate) and cold (enzymes) water extracts (Airth & McElroy 1959, Airth & Foerster 1962, Kamzolkina et al. 1983, 1984, Oliveira & Stevani 2009, Oliveira et al. 2012). Those studies, however, demonstrated that the isolated light emitting systems had rather low levels of luminescent activity and were unstable during storage. A possible reason for this suggested by the authors was instability of luciferin and luciferase or low

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concentrations thereof in the extracts. Thus, the luminescent system of luminous basidiomycetes remains less comprehensively understood than the luminescent systems of other living organisms. Only mycelia of various luminous basidiomycetes (*Armillaria mellea*, *Mycena citricolor*, *Gerronema viridilucens*) have been used in bioassays (Hollis et al. 2000, Weitz et al. 2002, Horswell et al. 2005, Mendes & Stevani 2010, Vydryakova et al. 2011).

Bondar et al. (2014) reported that the cold extract from the biomass of *Neonothopanus nambi* mycelium stored frozen for three months preserved its enzymatic activity and produced detectable light emission, and the substrate of luminescent reaction was thermostable, retaining its properties after heat treatment at 100°C. Another previous study showed that lyophilized powder of the cold extract dissolved in water had enzymatic activity (Puzyr et al. 2016). However, no data have been reported on changes in the luminescent activity of the cold extract after lyophilization and long-term storage of the dry preparation.

In the present paper, we report an approach to the extraction and storage of catalytic components required for cell–free fungal mycelial bioluminescence, which retain enzymatic activity over a long period of time.

Materials & Methods

In this study, we used the biomass of luminous mycelium of *Armillaria borealis* IBSO 2328, *Mycena citricolor* IBSO 2331, and *Neonothopanus nambi* IBSO 2391 from the Collection of the CCIBSO 836 of the Institute of Biophysics SB RAS (Krasnoyarsk, Russia).

Biomass of the luminous mycelium of *A. borealis* and *M. citricolor* was prepared by growing the fungi in submerged culture. Biomass of the luminous mycelium of *N. nambi* was prepared by growing the fungus on the surface of liquid nutrient medium. Finely crushed mycelium, which had been grown on solid medium in Petri–dishes, was used as the inoculum.

A. borealis and *M. citricolor* mycelial pellets were grown in PDB (potato extract – 200 g/L, dextrose – 20 g/L). The fungi were cultivated in 300 ml flasks containing 100 ml of nutrient medium. Cultivation was conducted for 16–20 days at 24°C under constant agitation at 140 rpm, using a Max Q 4000 incubating shaker (Thermo Scientific, U.S.).

N. nambi mycelial mats were grown on the surface of YMB (malt extract – 3 g/L, yeast extract – 3 g/L, dextrose – 10 g/L, peptone – 5 g/L). The fungus was cultivated in Petri–dishes, 90 mm in diameter, containing 20–25 ml of nutrient medium. Cultivation was conducted for 7–10 days at 27°C without agitation, using TCO-1/80 and SPU thermostatic incubators (Russia).

PDB and YMB were purchased from HiMedia Laboratory (India).

The biomass of *A. borealis* and *M. citricolor* mycelial pellets or mycelial mats of *N. nambi* was taken out of the nutrient medium and rinsed in deionized water to remove residual nutrient medium and metabolites. Deionized water was produced in a Milli–Q system (Millipore, U.S.). The rinsed pellets were transferred into a larger volume of deionized water and incubated for 24 hours with the air constantly bubbling through the water. Mycelial mats were transferred onto the surface of deionized water of larger volume and incubated for 24 hours without air bubbling through the water. It is well known (Mendes & Stevani 2010, Mori et al. 2011, Bondar et al. 2013, Mogilnaya et al. 2015, 2016) that incubation of the biomass of luminous fungi in water increases their luminescence level.

The cold extracts were prepared from the fungal biomass (mycelial pellets or mycelial mats) at a temperature of 0–4°C. The procedure was as follows. After incubation, mycelium was taken out of the water, placed in the cooled 0.1M phosphate buffer solution (pH 7.0) that contained 1% BSA (Serva, Germany) at a 1:5 ratio (biomass weight : buffer volume), cut into pieces using scissors and homogenized ultrasonically (with a Volna UZTA system 0.63/22–0, Russia), using an ice bath. The ultrasonic treatment was performed three times, each lasting 10 seconds, with 1 minute. The homogenate was centrifuged at a temperature of 4°C, at 40000*g* for 30 minutes (Avanti® J–E centrifuge, Beckman–Coulter, U.S.). The sediment was discarded, and the supernatant (cold extract), containing components of the luminescence system, was placed into MCT-150-C microtubes (Axygen Scientific, Inc., U.S.) and frozen at a temperature of –30°C in a

Sanyo Biomedical Freezer, model MDF – U333 (SANYO Electric Co., Ltd., Japan). Some of the microtubes with the frozen cold extract were freeze dried (in an LS–500 freeze drier, Russia). All samples were stored at a temperature of -30°C in a Sanyo Biomedical Freezer until use.

To estimate thermostability of the enzymes of luminescent reaction contained in the lyophilized cold extract, the dry powder stored for three years was heat-treated at 100°C. An MCT–150°C microtube containing the lyophilized powder was placed into the water bath boiling at 100°C. The sample was incubated in water bath for 60 minutes. Then we cooled it, added deionized water and measured luminescence.

Hot extracts (containing the substrate of luminescence reaction) were prepared from the biomass of luminous mycelium. As a substrate analog of the luminescence reaction, we also used the hot extract of the fruiting bodies of the nonluminous *Pholiota* sp. (collected in the forest in the vicinity of Krasnoyarsk in September 2016). Hot extracts from the fruiting bodies of *Pholiota* fungi are known to increase the luminescence of the cold extracts from some of the luminous fungi in the presence of NADPH (Purtov et al. 2015, Puzyr et al. 2016). The procedure of preparing hot extracts from the fungal biomass (mycelium and fruiting bodies) was described in detail elsewhere (Puzyr et al. 2016).

Samples of cold and hot extracts were mixed, and the levels of light emission were measured to determine the presence of enzymes and substrates of the luminescent reaction in the extracts. The amplitude and dynamics of the light signal were measured using a Glomax® 20/20 luminometer (Promega BioSystems Sunnyvale, Inc., U.S.). The luminescence measurements were conducted as follows. MCT–150°C microtubes containing the extracts were taken out of the freezer. The lyophilic powders were dissolved in deionized water, and the frozen samples were thawed at room temperature (23–25°C). Then, the samples were placed into a glass of ice, where they were kept throughout the study. A sample of cold extract (100 µl) was poured into MCT–150°C microtubes, which were placed in a luminometer; then it was supplemented with 5 µl 10 mM NADPH and 5 µl hot extract. Luminescence was monitored at a rate of one measurement per sec at room temperature (23–25°C). Light emission intensity was expressed as relative light units (RLU) per sec.

Results

Luminescence of cold extracts from A. borealis IBSO 2328 mycelium

Typical luminescent signals measured in the experiments with the fresh cold extract from *A. borealis* mycelium, with the frozen and thawed extract, and with the lyophilized sample diluted to its initial volume with deionized water are shown in Fig. 1.

Luminescence of cold extracts from mycelia of different species of fungi after long-term storage of lyophilized preparations

Fig. 2 shows typical luminescent signals measured in the experiments with water solutions of lyophilized cold extracts from mycelia of *A. borealis*, *M. citricolor*, and *N. nambi* after three years storage at a temperature of –30°C.

Luminescence of the cold extract from Armillaria borealis IBSO 2328 mycelium after incubation of the lyophilized preparation at 100°C

Fig. 3 shows intensities of luminescence of lyophilized cold extract from *A. borealis* mycelium without heat treatment and after incubation of the dry sample at 100°C for 1 h.

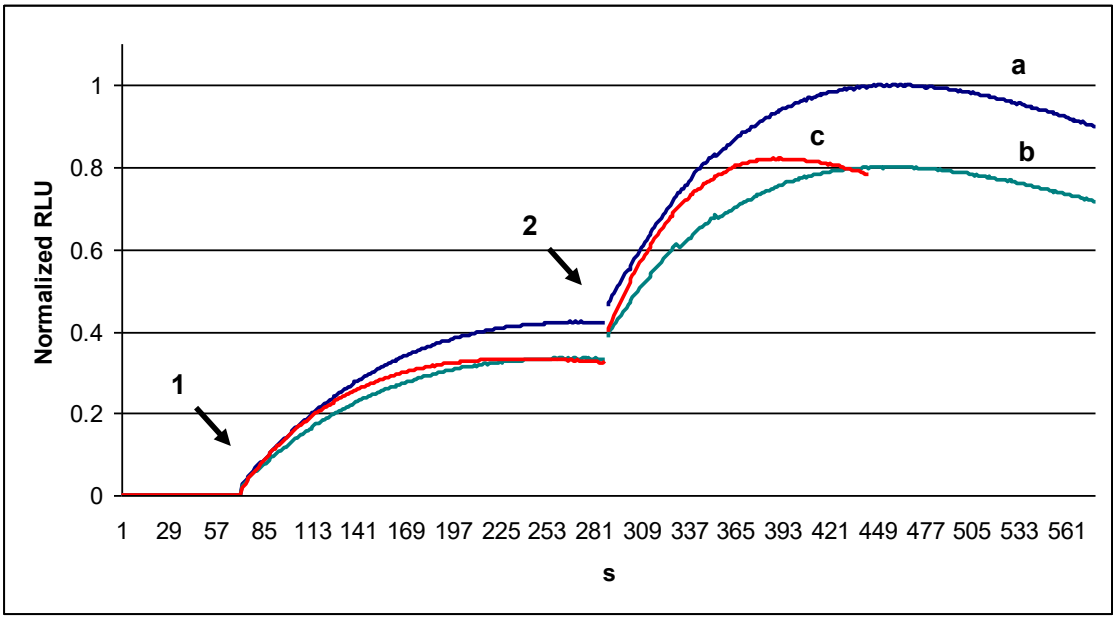

Fig. 1 – Intensity and dynamics of luminescence of the cold extract from *Armillaria borealis* mycelium, with NADPH (arrow 1) and the hot extract from the mycelium of this fungus (arrow 2) added consecutively: a. fresh extract, b. frozen and thawed extract, c. lyophilized extract dissolved in water. The data are normalized to the value of the maximal luminescence of the fresh cold extract (URL/URL_{max}).

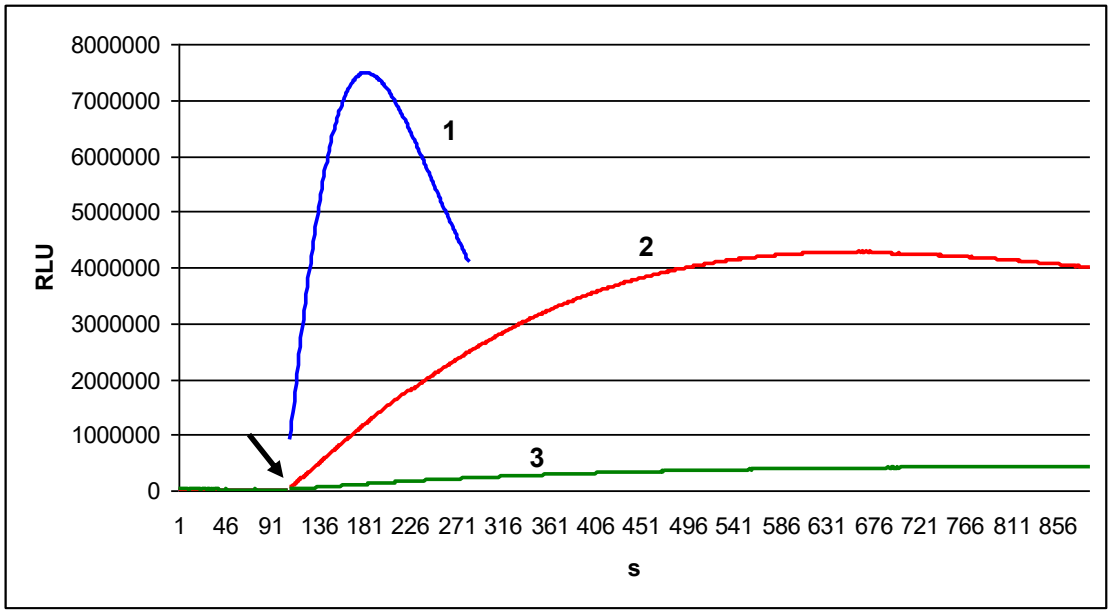

Fig. 2 – Intensity and dynamics of luminescence of the cold extracts from mycelia of: 1. *Armillaria borealis*, 2. *Mycena citricolor*, 3. *Neonothopanus nambi* after storing the lyophilized samples over three years. The arrow shows the addition of NADPH and the hot extract from the fruiting bodies of nonluminous *Pholiota* sp. to the extracts.

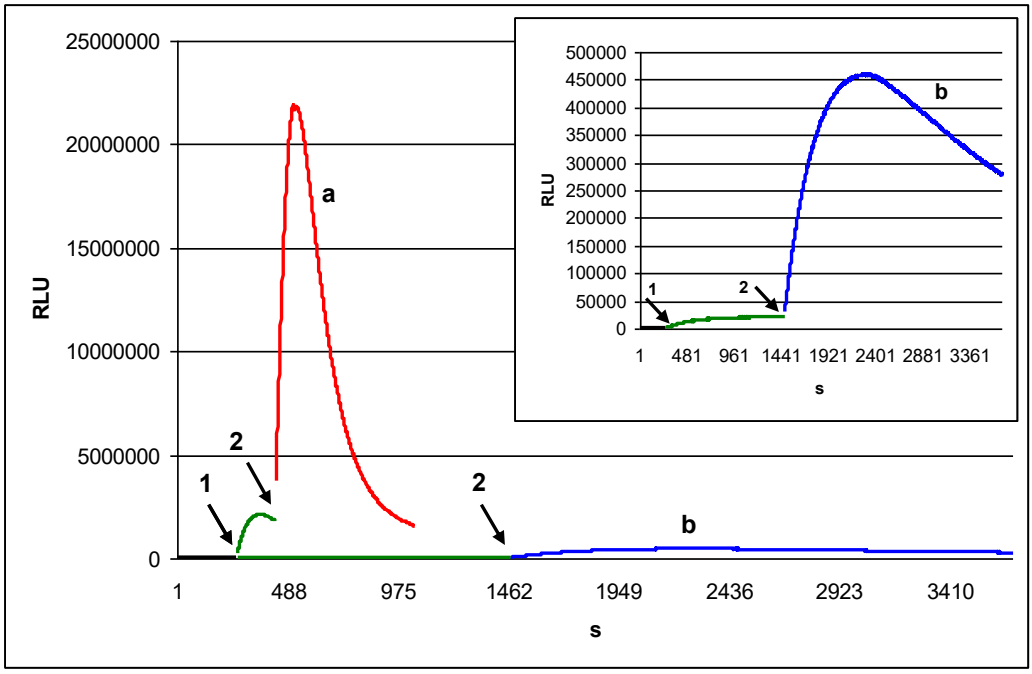

Fig. 3 – Intensity and dynamics of luminescence of the cold extract from *Armillaria borealis* mycelium after the storage of the lyophilized extract over three years and subsequent dissolution of the sample in water: a. without heat treatment of the dry sample. b. after incubation of the dry sample at 100°C for 1 h (1. after addition of 5 µl of NADPH. 2. After addition of 5 µl of the hot extract from the fruiting bodies of *Pholiota* sp.).

Discussion

Luminescence of fresh cold extracts from Armillaria borealis IBSO 2328 mycelium

It is commonly known that to stabilize the properties of components of biological preparations stored over long periods of time, two main approaches are used: samples are either frozen or freeze-dried. Drying appears to be the more advantageous strategy for storing biological preparations. Experiments showed that a single freezing of the fresh cold extract from *A. borealis* mycelium caused a 20% decrease in the luminescent signal (Fig. 1). A similar (approximately 20%) decrease in luminescence intensity relative to the level of luminescence of the fresh cold extract was detected in the sample that had been lyophilized and then dissolved in deionized water. These data suggest that neither freezing nor lyophilization of cold extracts results in a considerable decrease in the level of their luminescent activity.

Addition of NADPH to cold extracts (Fig. 1, arrow 1) enhanced luminescent signal, suggesting that the cold extract from *A. borealis* mycelium prepared by using the technique described in this study contained the substrate of luminescent reaction, which was still present in the frozen and lyophilized preparations. Subsequent addition of the hot extract from *A. borealis* mycelium (Fig. 1, arrow 2) further enhanced the luminescent signal, suggesting the presence of the substrate of luminescent reaction in the hot extract.

Pre-storage freezing of biological preparations is clearly not the best approach if they are to be stored over extended periods of time. Certain difficulties may arise if they are transported over long distances, especially in the regions with warm climate. Thus, lyophilization of biological preparations that must remain active has obvious advantages, as low temperature is not a strict requirement for long-term storage or shipment of lyophilized preparations. Immediately after the fresh cold extract from *A. borealis* mycelium was lyophilized and the dry preparation dissolved, it did not show any considerable decrease in the level of light emission relative to the activity of the frozen and thawed sample (Fig. 1). Thus, in principle, lyophilization of cold extracts can produce preparations with the level of luminescent activity comparable to that of the frozen preparations.

Luminescence of cold extracts from mycelia of different species of fungi after long-term storage of lyophilized preparations

To estimate stability of the enzymes of luminescent reaction during long-term storage of lyophilized cold extracts from *A. borealis*, *M. citricolor*, and *N. nambi* mycelia, we investigated their luminescent activity after three years of storage. The hot extract from the fruiting bodies of the nonluminous *Pholiota* sp. was used as the substrate of bioluminescent reaction. Thus, we were able to add equal amounts of the substrate analog of the luminescent reaction while comparing the activities of the enzymes of luminescent reaction from different species of basidiomycetes.

After NADPH and the hot extract were added to 100 µl of the cold extract, its luminescence intensity varied between 400 000 and 7 500 000 RLU, depending on the species of the fungus, suggesting that lyophilized preparations of luminous fungi can retain the high level of luminescent activity during long storage. As the intensity and kinetics of luminescence of the extracts from mycelia of different fungal species varied substantially, we assumed that under the same measurement conditions (the amount of the cold extract, NADPH and substrate concentrations), luminescence intensity and kinetics were determined by the concentration of the enzymes of luminescent reaction. The data in Fig. 3 show that heat treatment resulted in partial inactivation of the enzymes. At the same time, cold extracts from mycelia of different species of basidiomycetes might have certain individual properties such as different sets of biochemical components that may influence the enzymes and/or the mechanism of luminescent reaction.

Luminescence of the cold extract from Armillaria borealis IBSO 2328 mycelium after incubation of the lyophilized preparation at 100°C

Our study showed (Fig. 3) that after the lyophilized preparation of the cold extract from *A. borealis* mycelium that had been stored for three years was allowed to incubate for 1 h at a temperature of 100°C, the amplitude of its luminescent signal was reduced by approximately a factor of 50 compared to the level of light emission of the initial dry preparation (4.6×10^5 RLU/sec and 2.2×10^7 RLU/sec, respectively). However, even after extreme heat treatment, the level of luminescence of the lyophilized preparation remained high enough to be measured (the inset in Fig 3).

Conclusion

Since the lyophilized preparation of the cold extract from *A. borealis* mycelium has higher amplitude of luminescence than the cold extracts of *M. citricolor* and *N. nambi*, we think that this preparation should be used in further studies. It is considerably more convenient to store lyophilic preparations than frozen ones. Moreover, a lyophilic sample dissolved in different volumes of water produces preparations with different intensities of the luminescent signal. For instance, when we decreased the volume of water by a factor of three, the intensity of the luminescent signal increased from 7474320 RLU (Fig. 2) to 21791168 RLU (Fig. 3).

The literature data suggest that researchers studying luminescent systems of basidiomycetes have experienced problems while trying to prepare extracts containing substrates and enzymes of luminescent reaction (Harvey 1952, Airth & McElroy 1959, Airth & Foerster 1962, Kamzolkina et al. 1983, 1984, Oliveira & Stevani 2009, Oliveira et al. 2012). Various approaches have been proposed to preparing cold and hot extracts. Airth & McElroy (1959) prepared cold and hot extracts by using mycelial mats dried over P_20_5 under reduced pressure and then extracted the acetone powder. Kamzolkina et al. (1983, 1984) prepared hot extract using mycelial powder. Oliveira et al. (2012) used dried powder of mycelia/fruiting bodies to prepare hot extract and lyophilized mycelia/fruiting bodies or fresh mycelia under argon atmosphere to prepare cold extract.

Based on results of this study, we think that we managed to prepare cold extracts with the high and stable activity level of enzymes of luminescent reaction because we employed the following approaches:

(i) Using the biomass of live mycelium as a source of components of the luminescent system. It seems obvious that drying of live mycelium to prepare the biomass powder and then using it as a source of the enzymes and substrate may diminish their properties.

(ii) Preliminary incubation of mycelium in deionized water. The mechanism of increase in the intensity of luminescence of mycelium associated with incubation in water is unknown. A possible explanation may be that rinsing of biomass removes not only residual nutrient broth but also metabolites that could inhibit the enzymes and oxidize substrates of the luminescent system. Another possible reason may be increased synthesis of the enzymes and substrate of the luminescent reaction under osmotic and starvation stresses. Extraction of components of the luminescent system should apparently be performed when the efficiency of the light emission of the system is at its maximum.

(iii) Using buffer solution containing BSA in the stage of preparing cold extract. A number of studies reported that BSA enhanced luminescence intensity. In those studies, however, BSA was added to the mixture of cold and hot extracts in the stage of luminescence measurement rather than in the stage of extraction of the enzymes of luminescent reaction (Airth & McElroy 1959, Kamzolkina et al. 1983, 1984, Oliveira & Stevani 2009). This protein could exert a protective effect during extraction of the luminescent system, mainly during extraction of the enzymes of the luminescent reaction, due to their stabilization through protein-protein interactions with BSA.

(iv) Ultrasonic treatment of biomass in production of cold extract. It has been assumed that the cytochrome P450 system may be involved in the mechanism of luminescence of higher fungi (Kamzolkina et al. 1983, 1984, Bondar et al. 2012, 2014). In eukaryotic cells, enzymes of the cytochrome P450 system are associated with endoplasmic reticulum membranes (e.g., Maddy 1976). Therefore, in order to extract the membrane structures of endoplasmic reticulum (microsomes or microsome fragments), mechanically disintegrated biomass is subjected to ultrasonic treatment. Mechanical disintegration of the biomass is not sufficient for effective extraction of the enzymes of the cytochrome P450 system. Our study showed (data not included) that cold extracts from the mechanically disintegrated mycelium biomass (without ultrasonic treatment) either had no luminescent activity at all or exhibited a very low luminescence level.

Thus, using the techniques listed above, we prepared cold extracts with high levels of luminescence from mycelia of three species of luminous basidiomycetes. Lyophilized samples retained 80% of the luminescent activity of the fresh cold extract. The level of luminescent activity of lyophilic preparations remained high for three years. The lyophilic material withstood heating to 100°C. Therefore, it can, for example, be transported to laboratories that study luminescence of living organisms but do not cultivate luminous basidiomycetes.

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