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EXPERIMENTAL ARTICLES

Hemagglutinating Activity of the Fungus *Lentinus edodes* (Berk.) Sing [*Lentinus edodes* (Berk.) Pegler]

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Abstract—The hemagglutinating (HA) activity of the submerged mycelium and the culture liquid (CL) of four strains of *Lentinus edodes* was studied. The HA activity of the CLs proved to be much higher than that of mycelia. The carbohydrate specificity of fungal agglutinating factors was determined. HA activity was investigated as a function of the inoculum size, cultivation temperature, and culture age. The agglutinating activity of different morphogenetic structures of *L. edodes* F-249, including mycelium, brown mycelial mat (MM), primordia, and fruiting bodies, was studied. MM was found to possess the maximum HA activity, which can be explained by the possible involvement of agglutinins in the formation of MM, which is composed of glued hyphae.

Key words: hemagglutinating activity, agglutinins of higher fungi, submerged mycelium, *Lentinus edodes*, cultivation conditions, fruiting bodies.

The hemagglutinating (HA) activity of cells indicates the presence of lectins on their surface. Among the different definitions of lectins [1–4], one given by Kocourek and Horejsi defines most precisely the nature of lectins as nonimmune proteins recognizing and reversibly binding to the carbohydrate moiety of glycoconjugants without disturbing the covalent structure of any of the recognizable glycosyl ligands [4]. Lectins are found in virtually all living organisms occurring at different stages of evolutionary development. The best studied plant lectins have found a wide variety of practical applications in biology and medicine.

The agglutinating activity of higher fungi is poorly studied, although it is believed that fungal agglutinins are more abundant than plant lectins [5]. Investigation of the lectins of higher fungi is usually reduced to their detection in extracts of fruiting bodies for ecological and taxonomic purposes, while only few works deal with the physiological and developmental aspects of fungal lectins.

To the best of our knowledge, there are no published data on the presence and activity of lectins in *Lentinus edodes* (shiitake or black forest mushroom), except a single work devoted to the isolation of a lectin from the fruiting bodies of this fungus [6].

The present work was undertaken to assay hemagglutinating activity in the culture liquid and extracts of the submerged mycelium of *L. edodes*; to determine the carbohydrate specificity of agglutinating factors; to investigate the dependence of HA activity on the inoculum size, culture age, and cultivation temperature; and to study the agglutinating activity of *L. edodes* F-249 occurring at different stages of its life cycle.

MATERIALS AND METHODS

Four strains of the fungus *Lentinus edodes*, NY, F-249, 2T, and 0779, used in this work were obtained from the collection of basidiomycetes of the Department of Mycology and Algology of the Moscow State University. The strains were maintained on wort agar at 4°C.

The submerged mycelium of *L. edodes* was obtained by growing this fungus in a chemically defined medium containing (g/l) glucose, 10; asparagine, 1; KH_2PO_4, 5; $MgSO_4 \cdot 7H_2O$, 2.5; $FeSO_4 \cdot 7H_2O$, 0.03; and inositol, 0.003. Medium components were weighed to an accuracy of 2×10^{-4} g.

The growth medium was inoculated with the 14-day-old mycelium of *L. edodes* grown on 9-cm 4°B wort agar plates at 26°C. To attain equal inoculum sizes, 100 ml of the growth medium was inoculated with 5-mm-diameter agar disks with mycelium, which

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were aseptically cut from one wort agar plate using a special instrument [8–10].

Cultivations were carried out on a shaker at different temperatures: 26°C (optimum growth temperature for the fungus under study), 16°C (minimum growth temperature), and 37°C (maximum growth temperature) [11–13].

Aqueous extracts of mycelia were prepared by a method modified from Banerjee *et al.* [14]: mycelium was separated from the growth medium, washed with distilled water, dried at 30°C to a constant weight, crushed, and extracted with water for 24 h. The extract was lyophilized, and the dry material was dissolved in a phosphate-buffered saline solution to give a concentration corresponding to 1 mg dry mycelium/ml.

Fruiting bodies of *L. edodes* F-249 were obtained from wort agar plates incubated for 50–60 days [15].

The HA activity of mycelial extracts was assayed with a 2% suspension of trypsinized rabbit erythrocytes as described by Lutsik *et al.* [16] and expressed as the highest dilutions of extracts to cause an appreciable agglutination of the erythrocytes.

The carbohydrate specificity of agglutinating factors was studied by inhibiting the agglutination reaction with carbohydrates. Seventeen different carbohydrates purchased from Serva (Germany) were used in these experiments.

The growth rate of submerged *L. edodes* cultures was determined from changes in the dry weight m of mycelium, as described in the handbook [7].

The results were statistically processed to calculate the standard deviations of arithmetic means and confidence limits at a confidence level of 0.95 [17].

RESULTS AND DISCUSSION

The HA activity of the culture liquid and the mycelial extracts of *L. edodes* strains was assessed on the 21st day of their growth in synthetic medium at 26°C. As is evident from Table 1, strains F-249 and 2T exhibited the maximum HA activity, whereas the HA activity of strain NY was at a minimum. The HA activity of CLs was four and more times higher than that of mycelial extracts (MEs), especially in the case of strain 0779, for which the HA activity of CL was as high as 32 times that of the mycelial extracts.

These data indicated that the agglutinins of *L. edodes* were extracellular and accumulated in the medium during cultivation. To elucidate whether the different growth rates of *L. edodes* strains can be responsible for their different HA activities, we estimated the growth rates of these strains. The results presented in Table 2 show that, at a cultivation temperature of 26°C, the growth rates of strains F-249, 2T, and NY were virtually the same and differed by only 1.2 times from the growth rate of strain 0779. Small differences in the growth rates of *L. edodes* strains evidently could not explain the large differences in their HA activities (Tables 1, 2).

Table 1. Hemagglutinating activity titers of the culture liquids and mycelial extracts of different *L. edodes* strains

F-249		2T		0779		NY	
CL	ME	CL	ME	CL	ME	CL	ME
256	32	128	32	512	16	16	4

Table 2. Effect of the cultivation temperature on the growth rate of *L. edodes* strains expressed in mg dry mycelium/day

Temperature, °C	Strain			
	F-249	2T	0779	NY
16	5.24 ± 0.69	5.73 ± 0.43	6.64 ± 0.41	6.19 ± 0.46
26	6.14 ± 0.45	6.25 ± 0.39	7.57 ± 0.46	6.41 ± 0.62
37	4.44 ± 0.46	4.41 ± 0.43	4.55 ± 0.36	4.63 ± 0.40

Determination of the carbohydrate specificity of agglutinating factors in CLs and MEs through the inhibition of the agglutination reaction with carbohydrates showed that, for each of the strains studied, the range of carbohydrates inhibitory to CL agglutinins was wider than the range of carbohydrates inhibitory to ME agglutinins (Table 3). Agglutinins of all the strains studied showed a high affinity for galactose (except strain NY), lactose, and, to a lesser degree, for maltose. The CL agglutinins of strain NY were characterized by a high affinity for *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine. Therefore, the affinity of *L. edodes* agglutinins was maximum with respect to carbohydrates containing galactosyl and glucosyl units in their structure.

The dependence of HA activity on the inoculum size was studied in experiments in which growth media were inoculated with identical agar disks cut from wort agar plates covered with *L. edodes* mycelium. Such a method of inoculation has been described in the literature [8–10] and, as shown in our previous studies, provides for the successful reproducibility of HA activity.

These experiments were carried out at an optimum cultivation temperature of 26°C with the number of mycelial disks S equal to 1 and 5. As can be seen from Fig. 1, the HA activity of CL in the case of $S = 5$ was two orders of magnitude higher than in the case of $S = 1$. More detailed studies showed that the maximum HA activity of the CL of strain F-249 was in the case of inoculation with $S = 8$ (Table 4), showing no further rise with increasing S. Therefore, the dependence of HA activity on the number of inoculation disks was characterized by a steep initial rise with a maximum observed at a certain inoculation dose.

The effect of the growth temperature on the HA activity of CLs and MEs was studied at three temperatures,

Table 3. Minimal concentrations of carbohydrates (mM) inhibitory to the hemagglutinating activity of *L. edodes*

Carbohydrate	Strain							
	F-249		2T		0779		NY	
	CL	ME	CL	ME	CL	ME	CL	ME
L-Rhamnose	11.1	–	–	–	–	–	33.3	–
L-Arabinose	66.7	–	–	–	–	–	33.3	–
D-Arabinose	–	–	–	–	–	–	–	–
L-Fucose	–	–	–	–	–	–	33.3	–
D-Mannose	–	–	–	–	66.7	–	–	–
D-Galactose	2.78	33.3	16.7	16.7	5.60	33.3	–	–
D-Glucose	–	–	–	–	–	–	–	–
D-Fructose	–	–	–	–	–	–	–	–
D-Lactose	2.08	–	8.33	16.7	2.78	33.3	2.78	33.3
D-Maltose	16.7	–	16.7	–	16.7	–	33.3	–
α-Methyl-glucoside	–	–	–	–	–	–	–	–
2-Deoxy-D-glucose	–	–	–	–	–	–	–	–
D-Glucosamine	66.7	66.7	–	–	–	33.3	–	–
D-Galactosamine	66.7	66.7	–	–	66.7	33.3	–	–
N-Acetyl-D-glucosamine	–	–	–	–	66.7	–	16.7	–
N-Acetyl-D-galactosamine	–	–	–	–	–	–	4.17	–
N-Acetyl-D-mannosamine	66.7	66.7	–	–	–	–	–	–

Note: Symbol "–" stands for the absence of inhibition by a given carbohydrate taken at concentrations of up to 100 mM.

Table 4. Effect of inoculum dose *S* on the hemagglutinating activity titer of *L. edodes* F-249

S	Cultivation time, days				
	5	14	17	22	29
1	8	8	8	8	8
3	32	32	32	64	64
5	3.20×10^3	256	256	256	256
7	1.28×10^5	1.64×10^3	2.05×10^3	1.64×10^4	4.10×10^3
8	2.56×10^5	1.64×10^4	3.20×10^5	1.32×10^5	1.64×10^4
9	1.64×10^4	3.20×10^3	4.10×10^3	1.64×10^4	4.10×10^3
11	8.20×10^3	1.02×10^3	8.20×10^3	8.20×10^3	4.10×10^3
13	4.10×10^3	4.10×10^3	2.05×10^3	8.20×10^3	4.10×10^3
15	2.05×10^3	2.05×10^3	2.05×10^3	8.20×10^3	4.10×10^3

optimal (26°C), suboptimal (16°C), and supraoptimal (37°C). As is evident from data presented in Table 5, the HA activity of mycelial extracts of *L. edodes* was higher at suboptimal and supraoptimal cultivation temperatures, except strain 0779, whose HA titer (16) was the same at optimal and suboptimal temperatures.

Similarly, the HA activity of culture liquids was maximum when the fungus was grown at suboptimal and supraoptimal temperatures (Table 6).

Investigation of the effect of the cultivation temperature on the HA activity of the CLs of different strains in the course of their growth showed that the relatively small strain differences in the HA activity of CLs observed at 16°C (Fig. 2b) became greater when cultivation was performed at 26°C (Fig. 2a) and were maximum at 37°C (Fig. 3c). In the latter case, the HA activity of strain NY was low and barely changed in the course of cultivation, whereas the HA activities of other strains were considerably higher and tended to increase with the time of cultivation either monotonically (strain F-249) or with some extrema (strains 2T and 0779).

A comparison of data presented in Figs. 1 and 2 with the dynamics of the specific growth rates of strains

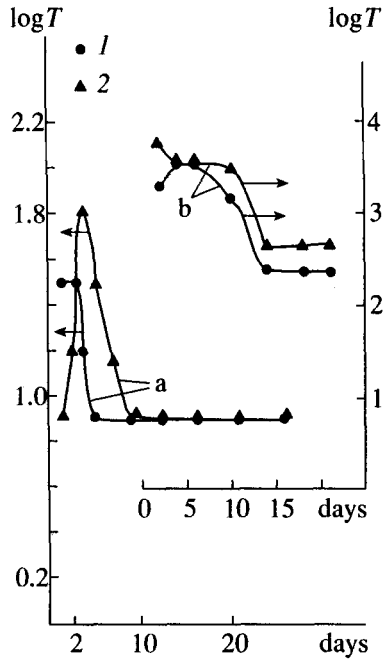

Fig. 1. Effect of the cultivation time on the HA activity of the culture liquid of *L. edodes* grown at an optimal temperature of 26°C and inoculum dose *S* equal to (a) 1 and (b) 5 agar disks with mycelium.

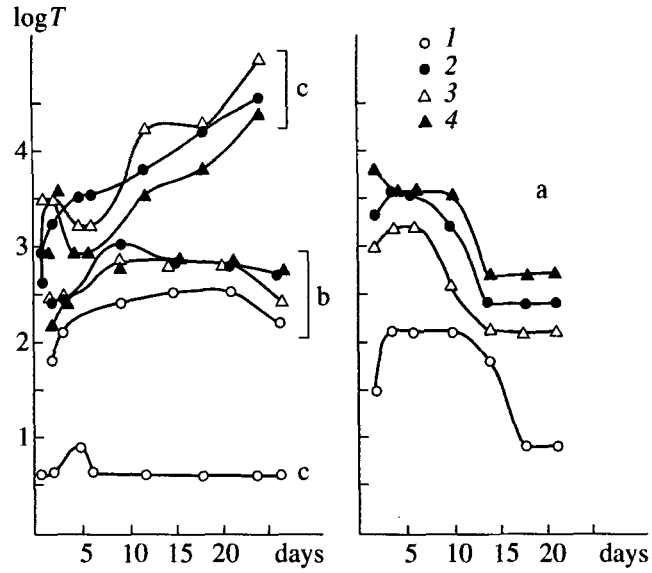

Fig. 2. Effect of the cultivation time on the HA activity of the culture liquids of *L. edodes* strains grown at (a) an optimal temperature (26°C), (b) a suboptimal temperature (16°C), and (c) a supraoptimal temperature (37°C). The inoculum dose *S* was 5 agar disks with mycelium. Strains: (*1*) NY; (*2*) F-249; (*3*) 2T; and (*4*) 0779.

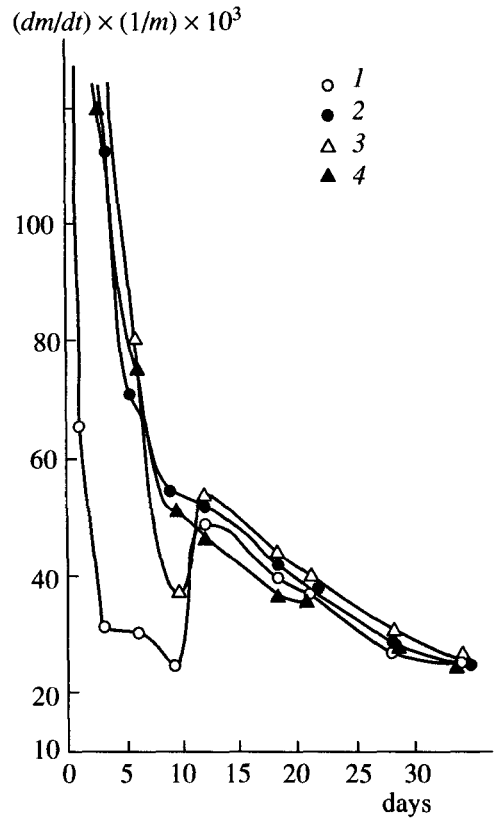

Fig. 3. Dynamics of the growth rate of *L. edodes* strains in the course of cultivation at an optimal temperature (26°C). Strains: (*1*) NY, (*2*) F-249, (*3*) 2T, and (*4*) 0779.

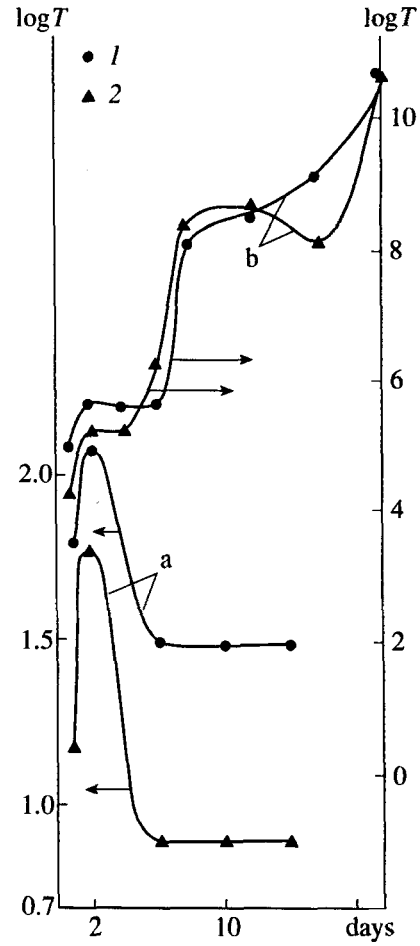

Fig. 4. Effect of the cultivation time on the HA activity of the culture liquid of *L. edodes* strains grown at a supraoptimal temperature (37°C) and inoculum dose *S* equal to (a) 1 and (b) 10 agar disks with mycelium. Strains: (*1*) F-249, and (*2*) 0779.

Table 5. Effect of cultivation temperature on the hemagglutinating activity titers of mycelial extracts of different *L. edodes* strains

Temperature, °C	Strain			
	F-249	2T	0779	NY
16	512	128	16	32
26	32	32	16	4
37	256	512	512	8

Table 6. Effect of cultivation temperature on the hemagglutinating activity titers of the culture liquids of different *L. edodes* strains

Temperature, °C	Strain			
	F-249	2T	0779	NY
16	640	640	640	320
26	256	128	512	16
37	16400	41000	12800	8

Table 7. Hemagglutinating activity titers of extracts of different morphogenetic structures of *L. edodes* F-249

Mycelium		MM	Primordia	Fruiting bodies	
nonpigmented	pigmented			stems	pilei
2048	2048	16400	4096	256	64

shown in Fig. 3 allows the inference that the dynamics of HA activity in the course of cultivation depends not only on changes in the specific growth rate but also on the cultivation temperature and the inoculum size. The most pronounced effect of cultivation time on the HA activity of CLs was observed for the case of growth at 37°C and the inoculum size $S = 10$ (Fig. 4).

Of much interest are data on the HA activity of *L. edodes* occurring at different stages of its life cycle, in particular, at the stage of the mycelial mat representing a network of pigmented thick-walled hyphae glued together [18]. In *L. edodes*, the stage of MM precedes the stages of formation of primordia and fruiting bodies [19] and, therefore, can be considered as a special morphogenetic stage of this fungus.

Investigation of the HA activity of mycelial extracts, brown MM, primordia, and fruiting bodies of strain F-249 showed that these morphogenetic structures are characterized by different HA activities (Table 7), which agrees with the relevant data obtained with other basidiomycetes [5, 20–23]. The HA activity of MM was maximum, and that of fruiting body's pilei was minimum. The pigmentation of mycelium did not affect its HA activity, while the formation of brown MM brought about a considerable increase in agglutinating activity. According to data available in the literature [24, 25], agglutinins can be involved in the formation of different morphogenetic structures of fungi, especially at the stage of formation of the mycelial mat when lectins provide for the mutual attachment of hyphae [23].

Analysis of carbohydrate haptens for the agglutinating factors of different morphogenetic structures of *L. edodes* showed that all agglutinating factors were specific to D-galactose and D-lactose. As shown above, agglutinins with such carbohydrate specificity are also present in CLs. It should be noted, however, that the carbohydrate specificity of agglutinins present in CLs is wider than the carbohydrate specificity of agglutinins present in mycelium, mycelial mat, primordia, and fruiting bodies.

To conclude, experimental data obtained in this work show the presence of agglutinating factors in the submerged mycelium and the culture liquid of *L. edodes*. Agglutinating activity varies in the course of the growth of this fungus, which is indicative of the possible involvement of agglutinins in the developmental changes of this organism. The high content of agglutinating factors in the culture liquid of *L. edodes* makes this fungus promising for the commercial production of agglutinins with an affinity for different carbohydrates.

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