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EXPERIMENTAL ARTICLES

Intracellular Lectins of *Lentinus edodes* at Various Developmental Stages of the Fungus

E. P. Vetchinkina[1], O. I. Sokolov, and V. E. Nikitina

Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, pr. Entuziastov 13, Saratov, 410049 Russia

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Abstract—A number of lectins varying in polypeptide composition and carbohydrate specificity were isolated from *Lentinus edodes* at different stages of its morphogenesis: nonpigmented mycelium, brown mycelium film, and fruiting body. Three lectins were identified at the nonpigmented mycelium stage, two of them being dimers consisting of 16 and 45 kDa and 16 and 42 kDa subunits; the third is a tetramer of 16, 39, 42, and 45 kDa subunits. The fractions with lectin activity obtained at the brown mycelium film stage contained polypeptides of 24, 30, and 38 kDa, characteristic of this morphological structure. The fruiting body was shown to contain two lectins of 43 and 55 kDa. All of the isolated lectins expressed the highest affinity towards L,D-melibiose, D-lactose, and D-galactose.

Key words: Lentinus edodes, morphogenesis, lectins, anion exchange chromatography, hemagglutination activity, carbohydrate specificity.

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Lectins exert a wide spectrum of biological activity important for functioning of the cell and the whole organism. They are essential participants in the processes of intercellular binding and signal transduction in biological systems, and perform various functions depending upon cellular type, organism stage of development, and environmental conditions [1, 2].

The role of lectins in growth and development of higher basidiomycetes is poorly studied except for three works [3–5], in which the regulatory role of lectins in morphogenesis was suggested.

The shiitake mushroom, or forest black mushroom (*Lentinus edodes* Berk. Sing or *Lentinula edodes* Berk. Pegler), is a representative of xylotrophic basidiomycetes. It is second place in world mushroom production after the champignons (*Agaricus bisporus*) [6], and is valued as a high quality edible and medicinal mushroom [7, 8]. During the past few years, extracellular lectins of *L. edodes* have been intensively studied [9–11]. Nevertheless, there are only occasional reports on the intracellular lectins obtained from the fruiting bodies of the shiitake mushroom [12, 13].

The aim of the present research was to isolate and characterize intracellular lectins of basidiomycete *L. edodes* at various developmental stages.

[1] Corresponding author; e-mail: elenavetrus@yandex.ru

MATERIALS AND METHODS

Subject of research. Experiments were performed with *L. edodes* strain F-249 from the collection of higher basidiomycetes, Chair of Mycology and Algology, Moscow State University. The cultures were grown on wort agar (4° Balling) at 26°C, which is the optimum temperature for mycelium growth of this species [14]. We used *L. edodes* nonpigmented mycelium formed on the 14th day of cultivation, brown mycelium film on the 50th day, and fruiting bodies 60–70 days after inoculation [15].

Protein extracts preparation. Mycelium extracts were obtained by the following procedure: separation from the growth medium, washing with distilled water, drying at 25°C, mechanical disintegration, extraction for 2 h with 10 ml of extraction solution per 100 mg of dry mycelium, centrifugation to remove the supernatant, and filtration. Proteins were precipitated by adding ammonium sulfate to the final concentration equivalent to 85% of the saturated solution. The suspension obtained was incubated for 12 h at 4°C and then centrifuged. The precipitate was resuspended and dialyzed against water. Protein concentrations in solutions were determined by the Bradford method [16].

Gel filtration. Protein samples were applied to a Sephadex G-75 column (Sigma–Aldrich, United States) and eluted with 20 mM *Tris*-HCl buffer solution, pH 8.0 at 0.5 ml/min flow rate. The eluate was

monitored at 280 nm using a Uvicord S-II detector (LKB, Sweden).

Anion exchange chromatography. Hemagglutinating protein fractions were concentrated by precipitation with ammonium sulfate, dialyzed against 20 mM *Tris*-HCl buffer, pH 8.0, and applied to a Mono Q anion exchange column (FPLC, Sweden). Proteins were eluted in a NaCl gradient (0 to 1 M).

Hemagglutination activity. The hemagglutination activity of the lectins was determined in the hemagglutination reaction with 2% suspensions of trypsin-treated and intact animal and human erythrocytes. Hemagglutination titers were expressed as the maximum lectin dilution at which erythrocyte agglutination was still recorded [17].

Carbohydrate specificity. Carbohydrate specificity was determined by inhibition of hemagglutination reaction using 2% suspensions of trypsin-treated rabbit erythrocytes and a standard carbohydrates kit. The following carbohydrates were tested as inhibitors (0.3 M): D-arabinose, L-arabinose, D-galactose, D-glucose, D-mannose, D-fructose, L-rhamnose, L-fucose, D-ribose, D-sorbite, D-mannite, D-fructose, sucrose, D-lyxose, D-xylose, L-talose, D-lactose, D-maltose, D-cellobiose, D-melibiose, D-galactosamine, N-acetyl-D-galactosamine, D-glucosamine, N-acetyl-D-glucosamine, N-acetyl-D-mannosamine, N,N-diacetylchitobiose, D-galacturonic acid, 2-deoxy-D-galactose, D-mesoinositol, phenyl-β-D-galactopyranoside, and phenyl-β-D-glucopyranoside (Sigma, USA).

Electrophoresis. To investigate the lectin composition, denaturing electrophoresis by Laemmli technique of the samples (40 μl per pit) in 15% polyacrylamide gel (pH 8.3) was used [18]. Amresco protein markers panel (United States) was used as molecular mass standard: Phosphorylase B (97 kDa), Albumin (66.2 kDa), Ovalbumin (45 kDa), Carbonic anhydrase (31 kDa), Trypsin inhibitor (21.5 kDa), and Lysozyme (14.4 kDa). The gels were stained with silver nitrate [19]. The Weber and Osborn techniques were used to calculate lectin molecular mass [20].

RESULTS AND DISCUSSION

Three stages, characteristic of *L. edodes* morphogenesis, viz. nonpigmented mycelium, brown mycelium film, and fruiting body, were chosen to study lectins of the species through the life cycle. To be able to detect the full spectrum of lectins, we set up a search for the most efficient extracting system. For this purpose extraction at each morphogenesis stage was performed with a number of solutions: distilled water; 20 mM *Tris*-HCl buffer (pH 8.0), 20 mM *Tris*-HCl buffer supplemented with 1 M NaCl, 3 M NaCl; 2% SDS, or 10% glycerol.

The highest level of protein extraction from the nonpigmented mycelium and fruiting body was obtained with 20 mM *Tris*-HCl buffer and with water, and from the brown mycelium film, with 1 M NaCl. An increase in NaCl concentration in the buffer up to 3 M, as well as the use of buffer containing 2% SDS and 10% glycerol, didn't result in significant increase of the protein yield. Thus, the most efficient extracting system for the brown mycelium film stage was found to be 1 M NaCl, and for nonpigmented mycelium and fruiting body, *Tris*-HCl buffer solution.

The hemagglutination reaction is the most common and appropriate test system for detection and determination of lectin activity. To identify the full repertoire of lectins, we used trypsin-treated and intact rabbit, ram, bovine, and equine erythrocytes, as well as four human blood types. Trypsinized rabbit and, to a lesser extent, human erythrocytes were found to be the most sensitive test subject for *L. edodes* lectins. Bovine, ram, and equine erythrocytes practically didn't interact with lectins.

Our experiments revealed hemagglutination activity in mycelial extracts obtained at all morphogenetic stages, although the titers varied depending on the stage. Nonpigmented mycelium proteins showed high titers of hemagglutination; however, it increased further with pigmentation, and the highest titer was observed at the brown mycelium film stage. Hemagglutination titer of the fruiting body proteins was the lowest.

Nonpigmented mycelium proteins separation on Mono Q anion exchange column resulted in three hemagglutinating peaks, eluted in the NaCl concentration range from 0.3 to 0.4 M (Fig. 1). The hemagglutination titers of the proteins were 8192, 32768, and 16384, respectively. The study of their carbohydrate specificity revealed the highest affinity towards L-D-melibiose at a carbohydrate minimal inhibiting concentration of 3 mM, as well as towards D-lactose (12 mM), and D-galactose (12 mM).

A similar procedure was applied to lectin proteins of the brown mycelium film stage, which precedes fruiting body formation. The protein fractions eluted from the column with 0.25 to 0.55 M NaCl, showed hemagglutination titers in the range from 256 to 65536 units. The proteins showed specificity towards L-D-melibiose (3 mM) as in the case of nonpigmented mycelium, and also towards D-galactosamine (25 mM), N-acetyl-D-galactosamine (25 mM), and N-acetyl-D-glucosamine (25 mM).

In comparison with vegetative mycelium, fruiting body lectins showed low agglutination activity. Two lectins were revealed at this stage. The first one was extracted with water and eluted from the anion exchange column with 0.25–0.35 M NaCl. The other was absent in the water extract, but was detected in the 20 mM *Tris*-HCl buffer (pH 8.0) extract and eluted with 0.43 M NaCl. The titers were 64 and 128, respectively. The first lectin was more specific towards L-D-melibiose (3 mM), D-lactose (6 mM), N-acetyl-D-galactosamine (25 mM), and N-acetyl-D-glucosamine (25 mM). The other lectin was specific towards L-D-

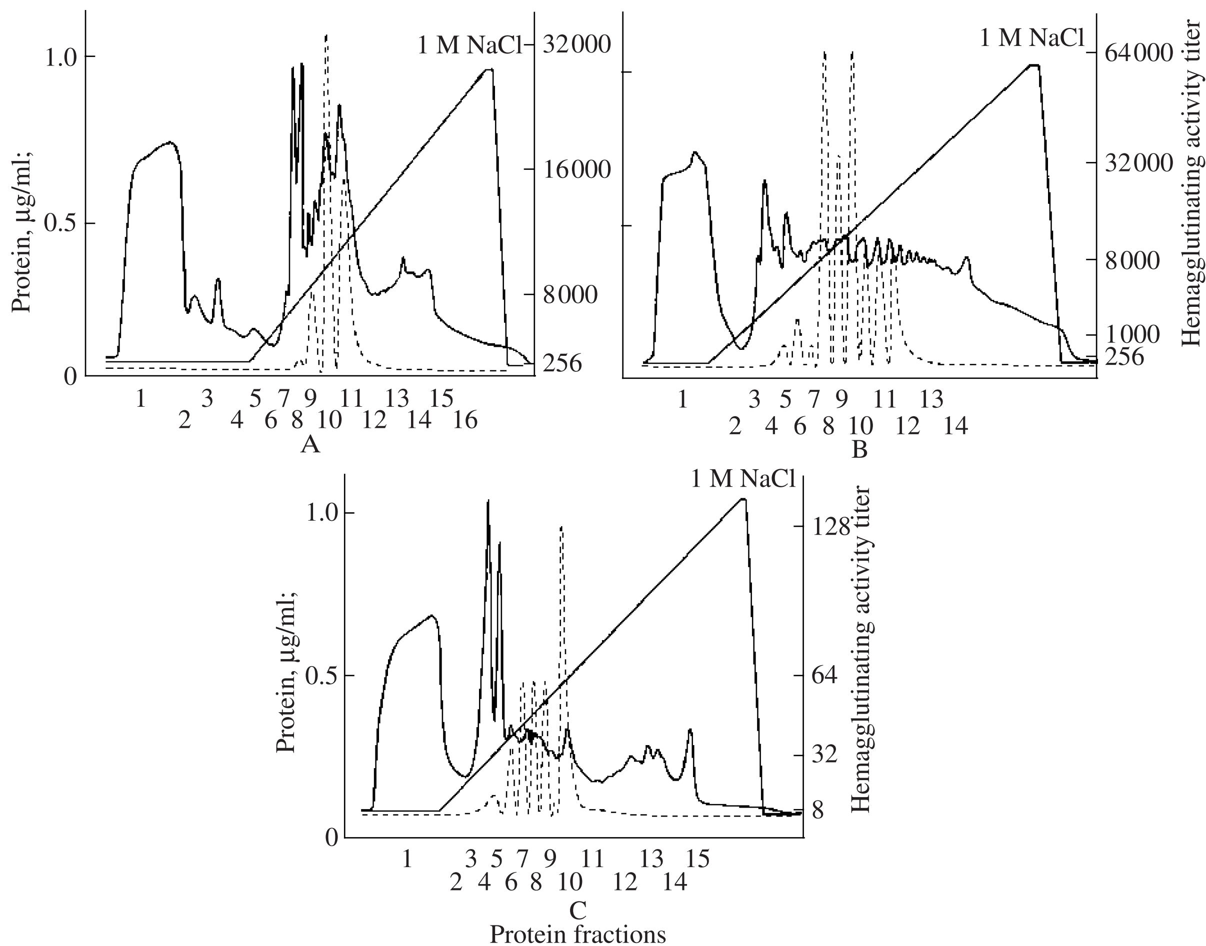

Fig. 1. *L. edodes* proteins anion exchange chromatography elution profiles. Morphogenesis stages: A, nonpigmented mycelium; B, brown mycelium film; C, fruiting body. 1–15 are the protein fractions obtained on a Mono Q anion exchange column.

melibiose (3 mM), D-lactose (6 mM), D-galactose, and D-galactosamine (12 mM).

The rest of the protein fractions isolated from nonpigmented mycelium, brown mycelium film, and fruiting body didn't display hemagglutinating activity. Consequently, these proteins either were not lectins, or did not exhibit lectin activity under the experimental conditions.

Denaturing electrophoresis was used to determine molecular weights and subunit composition of the lectins at different developmental stages of *L. edodes.*

According to SDS-PAGE, three lectins are present in nonpigmented mycelium, two of them being dimers and the third a tetramer (Fig. 2). The molecular weights of the first lectin subunits are 16 and 45 kDa, and of the second one, 16 and 42 kDa; the tetramer, probably, consists of 16, 39, 42, and 45 kDa subunits.

Brown mycelium film fractions showing hemagglutination activity contain 24, 30, and 38 kDa polypeptide chains, characteristic of the given morphostructure and not present on electrophoregram of fractions obtained from nonpigmented mycelium or fruiting body.

Two lectins of 43 and 55 kDa were identified at the fruiting body stage.

Since lectin formation is closely related to transition from one morphogenetic stage to another, emergence of specific lectins in the succession of these stages is of great interest. Apart from basidiomycetes, some fruiting body-forming organisms (*Dictyostelium discoideum* [21] and *Myxococcus xantus* [22]) are known to produce lectins in the process of development. A number of studies have been devoted to isolation and characterization of the lectins obtained from basidiomycetes fruiting bodies [3–5, 12, 13]. Moreover, some authors note [3] that vegetative mycelium doesn't contain lectins, which, however, are formed during the generative developmental stages (primordia and fruiting bodies). These observations indicate that in a number of organisms, lectin production is coupled to fruiting body formation. The present research, however, gives evidence that both vegetative and generative mycelia contain lectins, different in polypeptide composition and in carbohydrate specificity. The logical conclusion is that lectins play a specific role at various stages of morphogenesis. It has been suggested that lectins may partici-

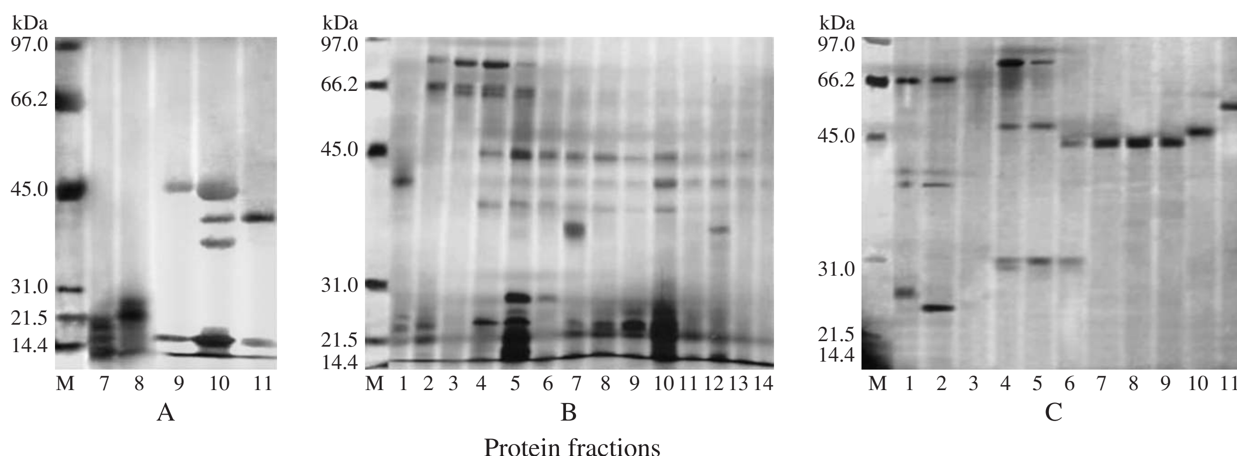

Fig. 2. Electrophoresis of the proteins extracted from *L. edodes* at different morphogenesis stages: A, nonpigmented mycelium; B, brown mycelium film; C, fruiting body. M indicates markers; 1–14 are the protein fractions obtained on a Mono Q anion exchange column.

pate in cell aggregation. Brown mycelium film is a structure formed in the fungal species under study prior to the fruiting body stage; it is a tight tangle of thick-walled pigmented hyphae [24]. Lectins arising at this stage are likely to be essential for hyphal aggregation. Further investigation is necessary to explain the functions of lectins in the process of fungal morphogenesis.

Therefore, lectins of different molecular mass and subunit composition, with the highest specificity towards L-D-melibiose, were isolated and purified from nonpigmented mycelium, brown mycelium film, and fruiting bodies. These proteins appear in the succession of developmental stages, are characteristic of these stages, and serve, therefore, as a kind of marker of morphogenesis.

Further investigation of the compounds is a promising approach towards analysis of morphogenetic processes regulation.

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