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EXPERIMENTAL ARTICLES

Characterization of an Extracellular Glycolipid from *Lentinus edodes* (Berk.) Sing [*Lentinula edodes* (Berk.) Pegler]

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Abstract—Submerged mycelium of a xylotrophic basidiomycete *Lentinus edodes* produces an extracellular glycolipid, *S*3, associated with a lectin. Galactose glycan residue, as well as the lipid pool composition, which includes nonhydroxylated short-chain fatty acids, is uncommon for basidiomycetes. The glycolipid consists of *D*-galactopyranose (15% of *S*3 contains galactose sulfate) acylated by octadecanoic and nonadecanoic fatty acid residues (28 and 72%, respectively). The glycolipid structure and composition are confirmed by physicochemical analysis. The glycolipid is assumed to be a regulator of lectin activity.

Key words: Lentinus edodes, glycolipid, higher fungi lectins, extracellular glycoproteins.

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The foremost role of various glycolipids secreted by yeastlike fungi is generally believed to be that of biosurfactants, which promote solubilization of organic hydrophobic substances and their uptake from the media by microbial cells [1]. In recent years these glycolipids have been found to display biological activity as well.

Secreted glycolipids were found in yeast species *Pseudozyma fusiformata* [2], *P. flocculosa* [3], and *Cryptococcus humicola* [4]. The glycolipids possess an antifungal activity against a wide variety of yeastlike fungi, including a number of species of medical importance (*Candida albicans, C. glabrata, C. viswanathii, Filobasidiella neoformans*, and *Clavispora lusitaniae*). The extracellular cellobiosolipid containing 2,15,16-trihydroxyhexadecanoic acid was shown to be a fungicidal agent of *Sympodiomycopsis paphiopedili* [5]. In a publication of 2007, *P. fusiformata* cellobiosolipid, similar in structure to the one from *C. humicola*, with the exception of hydroxyhexanoic acid as an O-substituent and an extra 15-OH-group in the hyrdocarbon side chain of the former glycolipid, was reported to suppress growth of some filamentous fungi more effectively than the *C. humicola* cellobiosolipid [1]. Thus, the latest experimental data prove the importance of details in exoglycolipids structure for their biological activity.

In 1991, the first phenol-containing fungal glycolipids were discovered [6]. Along with fatty acids and a carbohydrate residue, glycolipid from the lipid fractions of the zygomycete *Blakeslea trispora* was found to contain a phenol structure. There is probably little point in comparison of this intracellular glycolipid with extracellular ones. However, we found intriguing that galactose was the carbohydrate component of the glycolipid described by Konova et al. [6], as in *S*3. The same authors noted that *B. trispora* glycolipid synthesis, and in particular the synthesis of the phenol-containing galactosolipid, was stimulated by lack of phosphates in the medium. This observation suggests that synthesis of a phenol-containing galactosolipid, different from the previously known glycolipids, is an adaptive reaction of *B. trispora* cells under conditions of phosphate limitation [6].

There are few data on the lectin content in *Lentinus edodes* (Berk.) Sing [*Lentinula edodes* (Berk.) Pegler] (shiitake) [7, 8], and nothing is known about preparations of extracellular lectins from xylotrophic basidiomycetes. On discovery, isolation, and purification of *L. edodes* exolectins [9–11], the endogenous regulators of its biological activity were to be revealed. The research was not aimed at glycolipids initially, since there is no literature data on glycolipid regulatory activity towards lectins. Success was achieved upon finding that lectin activity changed in the course of purification.

The aim of the present work was isolation and physicochemical characterization of the *L. edodes* extracellular glycolipid, as well as investigation of its ability to affect the activity of the fungus extracellular lectins preparations.

MATERIALS AND METHODS

In this study, we used *L. edodes* strain F-249 from the collection of higher basidial fungi (Department of Mycology and Algology, Moscow State University). Fungal cultures were maintained on wort agar slants.

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The media for submerged cultivation were inoculated with 14-day *L. edodes* mycelium.

Liquid-phase batch cultivation was performed on synthetic medium containing *D*-glucose (50 mM) and *L*-asparagine (mM) at a C : N ratio of 15 : 1 [12].

The hemagglutinating activity of the extracts was determined in the hemagglutination reaction with free erythrocyte sedimentation using 2% suspensions of trypsin-treated rabbit erythrocytes in a series of lectin dilutions. Hemagglutination titers were expressed as the highest dilution at which erythrocyte agglutination was still recorded [13].

For isolation of *L. edodes* F-249 extracellular lectins, the culture liquid was used as a crude protein extract. Tenfold concentrated culture filtrate was treated with two volumes of acetone at 4°C. After separation of the precipitate, acetone was removed from the supernatant. The remainder was dried at 30–32°C and dissolved in water to get unpurified lectin *L*2 solution [11]. The *L*2 water solution was applied to a Sephadex-G25 column (1.7 × 9 cm) and eluted with water. Protein in the fractions was detected by absorbance monitoring at 280 nm using a Uvicord S-II (LKB) device. The fraction with Hemagglutination activity was purified by gel filtration on a Sephadex G-75 column (1.5 × 43 cm) equilibrated with 0.1 M NaCl.

For further purification of lectins *L*1 and *L*2, ion exchange chromatography was used. Active hemagglutinating fractions obtained by gel-exclusion chromatography were applied to a cation exchange Toyopearl CM-650M column (1.0 × 16 cm). Most impurities were bound to the resin while the lectins were eluted in the void volume of the column. Anion exchange chromatography of the fractions containing mainly *L*1 and *L*2 on a Toyopearl DEAE-650M column (1.0 × 16 cm) resulted in complete separation of these lectins, as they were eluted by eluent of significantly different ionic strength.

After purification on Sephadex G-75 column, desalinated water solution of *S*3, contaminated with some *L*2, was dried at 30–32°C. The dry substance was treated with methanol–water–chloroform in the ratio of 1 : 2 : 3 according to a modified lipid extraction procedure [14] and then dried in nitrogen or argon flow.

To determine fatty acid (FA) content, the dry remainder was extracted with hexane. Fatty acids were analyzed in the form of methyl esters by gas–liquid chromatography [15]. Methyl esters of FA were separated on a Biochrom-1 gas–liquid chromatograph on a quartz capillary column (25 m long, internal diameter 0.2 mm) with SE-54 stationary phase. The column thermostat temperature was programmed to increase 4°C/min from 130 to 270°C. The temperatures of evaporator and detector were 150 and 270°C, respectively. The carrier gas was helium at a flow rate 1.6 ml/min.

Fatty acids were identified according to the retention times of their methyl esters. Bacterial Fatty Acid Methyl Esters CP Mix (Supelco) was used as standard for FA methyl esters, along with some individual FA methyl esters (Sigma).

Composition of the carbohydrate fraction of dry samples was determined by capillary gas chromatography on SE-54 stationary phase in helium flow, temperature programmed from 150 to 280°C. Prior to chromatographic analysis, trimethylsilyl esters of the samples and the standards were obtained using hexamethyldisilazane and trimethylchlorosilane in pyridine [16].

Infrared spectra were recorded on an FSM 1201 IR Fourier transform spectrometer in a thin layer of paraffin oil with hexachlorobutadiene.

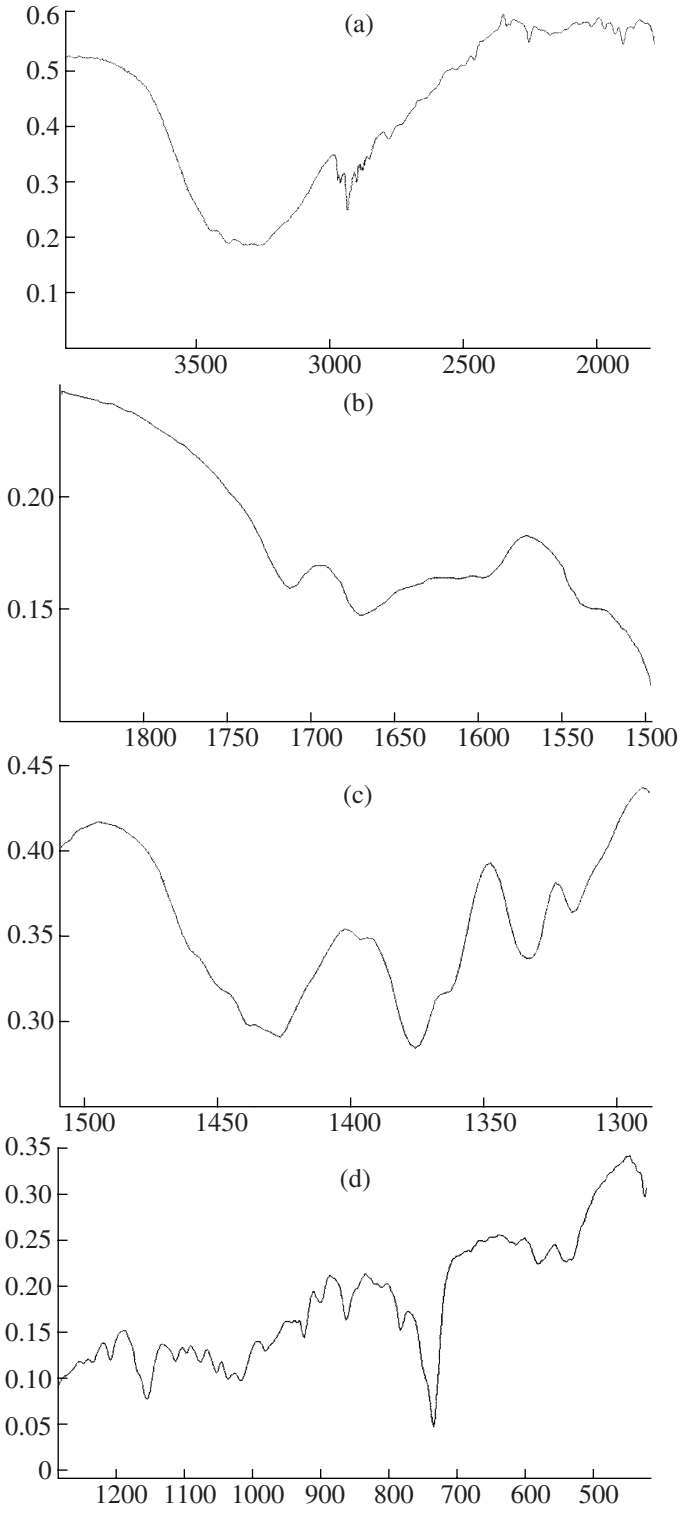
RESULTS AND DISCUSSION

Using *L. edodes* lectin *L*2 extraction in the course of purification, we have obtained a preparation (designated as *S*3) containing galactose (0.19 % yield) and two FA (0.17 % yield), $C_{8:0}$ and $C_{9:0}$ (28 and 72 % of the sum, respectively).

Accumulation of short chain FA in the galactolipid of shiitake mycelium takes place only during the brown mycelium film formation stage and correlates with a sharp increase in extracellular lectins activity in mycelium washouts. Neither $C_{8:0}$ and $C_{9:0}$ FA accumulation nor increase in lectin activity were observed during nondifferentiated fruiting bodies formation on white mycelium. A more detailed description of the changes in FA content in mycelium in relation with cultural lectin activity in the course of morphogenesis is given in an earlier publication [17].

We assume *S*3 to be one of the regulators of the extracellular lectin activity. In addition to the correlation between *S*3 component accumulation and the increase in lectin activity, other observations contribute to the idea of the glycolipid possible participation in the regulation of this activity. In the mixtures of preparations *S*3 + *L*1 and *S*3 + *L*2 with equal mass concentration of the components, hemagglutination titer increases 8 and 16 times, respectively (no hemagglutinating activity was registered for *S*3 alone). Furthermore, in the course of purification of *L*2 preparation, its specific activity reached a peak at the stage when *L*2 is bound to *S*3 and then decreases significantly with further purification.

Under unfavorable mycelium growth conditions (temperature, starvation), we have observed significant increase in lectin activity, which is probably due to stabilizing and adaptogenic activity of *L. edodes* lectins [18]. Nonspecific reactions of microbial cells towards various external stimuli have been discussed in the literature for a long time [19]. The general mechanism of adaptation to unfavorable conditions or stress includes excretion of regulatory compounds (stress factors) into the extracellular medium [19]. Uracil, phosphates, fatty acids ($C_{9:0}$ and $C_{10:0}$), and oligosaccharides were identified among the stress factor components. Stress factor



IR spectrum of *L. edodes* F-249 extracellular glycolipid *S*3 in the frequency ranges 4000–1800 cm^{-1} (a); 1800–1500 cm^{-1} (b); 1500–1300 cm^{-1} (c); and 1300–400 cm^{-1} (d). Ordinate axis is used to represent relative transmission.

is one of the first to be synthesized at a time when normal metabolic processes fade [20].

Microorganisms (a number of bacteria and the yeast *Saccharomyces cerevisiae*) under a constant electric field were found to excrete regulatory stress factor (EF-factor) into the medium, which provides increased resistance to various harmful conditions. EF-factor was found to have no strict species specificity. After a cell receives a signal of unfavorable changes in the environment, it produces a universal response and secretes a stress pro-factor, a certain precursor compound (or a complex of substances). Pro-factor molecules most likely consist of weakly associated subunits. In the culture medium, pro-factor molecules are exposed to the energetic effects accompanying the stress and dissociate to form stress factor subunits with enhanced regulatory activity. Acting upon cytoplasmic membranes, stress factor molecules transform the cells by slowing down the metabolic rate and transferring them into a low activity state, which is more stable towards external factors [19].

According to a broader view on participation of the compounds under discussion in the stress signal transduction [20], these substances refer to a group of secondary metabolites and are believed to modulate cell differentiation. Thus, secondary metabolites are considered to be a sort of transducer of intracellular signals.

We assume that an unfavorable change in environmental conditions (starvation, or transferring to cell differentiation because of nutrients exhaustion), induces, in addition to secondary metabolites excreted by *L. edodes*, secretion of lectin activity regulators, including *S*3.

The experimental mass ratio between galactose and alkane acids in *S*3 preparation was 0.19 : 0.17 or 53 mass % of galactose and 47 mass % of FA mixture ($C_{8:0} + C_{9:0}$), which corresponds to one FA molecule per one molecule of galactose. The result is in agreement with theoretical calculation within the experimental error. The molecular mass ratio between the carbohydrate (M_r(galactose) = 180) and FA, taking into account galactose OH group substitution and FA composition, is 52% : 48%.

Elemental analysis revealed the presence of sulfur (1.5%) in *S*3. Assuming sulfonate groups as substituents in the galactose ring as the most probable form of sulfur disposition, simple calculation results in 15% of galactolipid molecules to be sulfated.

Thus, according to the data of chemical and elemental analysis, the principal components of *S*3 are galactose (1-sulfogalactose) acylated by octadecanoic and nonadecanoic acids. The methods were complemented by IR spectrometry data (see figure).

In the IR spectrum of *S*3 there are bands that probably correspond to a sulfate group, specifically, antisymmetrical valence vibrations of S=O bonds in the sulfate group (a wide band 1465–1405 cm^{-1}, or a 1375 cm^{-1} band, or a 1331 cm^{-1} band) and symmetrical valence vibrations in the sulfate group (1209 and 1155 cm^{-1}). In

general, the indicated values fit the ranges of antisymmetrical (1440–1350 cm^{-1}) and symmetrical (1230–1150 cm^{-1}) valence vibrations of sulfate groups [21]. It is less likely that the bands 1032 or 1012 cm^{-1} (doublet) also correspond to symmetrical valence vibrations of S=O bond in sulfate groups. The deformation vibrations bands are to be situated in the region 700–600 cm^{-1}, which, in reality, contains so many deformation vibration bands of various groups that no band assignment is possible. The statement is partially true for the wide band with maximum at 852 cm^{-1}, although its form indicates the presence of both axial and equatorial sulfate groups. Thus, sulfate groups can occupy any position in galactose residues.

The bands around 1460 and 1380 cm^{-1} are very typical of alkyl groups [21]. Overlapping bands of antisymmetrical and symmetrical C–H bond valence vibrations in CH_3 and CH_2 (wide band 2980–2870 cm^{-1}), antisymmetrical and symmetrical CH_3 group deformation vibrations (1427 and 1375 cm^{-1}, respectively), scissoring vibrations in CH_2 groups (1465–1405 cm^{-1}), hydrocarbon C–H in-plane deformation vibrations (1427 and 1315 cm^{-1}), and hydrocarbon C–H out-of-plane deformation vibrations (974 cm^{-1}) possibly indicate the presence of a hydrocarbon chain in the acyl residue. Polymethylene chain characteristic features are seen in the frequency range 720–770 cm^{-1}. The assignment of C–O bond vibration frequencies in carboxylic acids and esters (1155 and 1111 cm^{-1}) indicates the ester nature of the bond between FA and galactose residue. Other frequency assignments are in agreement with the presence of carbohydrate units and FA residues in the structure of *S*3.

It should be mentioned that *L. edodes* galactolipid *S*3 is not only homologous to the glycolipids of lower fungi mentioned above in major structural components, but also resembles widely known glycolipids of nonfungal origin. Lipid X (LX) is the structural precursor of lipid A, the major cytoplasmic membrane component in gram-negative bacteria. Taking into account variations between species, lipid A is known to consist of β-(1⟶6)-linked 2-amino-2-desoxy-*D*-glucopyranose disaccharide, phosphorylated at 1 and 4' positions and acylated with (*R*)-3-hydroxyalkane acids at the 2, 3, 2', and 3' positions [quoted from 22]. LX is a monosaccharide (glucosamine) precursor of lipid A also phosphorylated at carbon 1 and acylated by (*R*)-3-hydroxytetradecane acid residues (3-OH-14 : 0) at carbons 2 and 3.

In the work of Vorob'eva et al. [22], the isolation of lipid A of an unusual monosaccharide structure isolated from marine gram-negative *Chryseobacterium scophtalmum* was described. It consists of 1-phosphate-*D*-glucoseamine, acylated by (*R*)-3-hydroxy-15-methylhexadecanoic acid (ester bond with glucosamine) and (*R*)-3-hydroxy-13-methyltetradecanoic acid (amide bond with glucosamine) at the C2- and C3-atoms, respectively, and is tightly bound to other outer membrane components, probably, through chemical linkage. The authors [22] conclude that the unusual phosphoglycolipid is most likely not an LPS.

It is easy to see that the only difference between *Chr. scophtalmum* LA and lipid X structures is the *iso*-structure of acyl substituents with a hydrocarbon chain longer than in LX. In the LA molecule there are two substituents, (3-OH-*iso*-15 : 0) and (3-OH-*iso*-17 : 0), instead of two (3-OH-14 : 0) as in the LX molecule. Another glycolipid of a similar structure, LXLS, identified in 2006 by Pluzhnikov et al. [23], is not of microbial origin. It was isolated from the poison of the South American ant *Paraponera clavata*, and represents 3-myristoyl-2-acetamido-2-desoxy-α-*D*-glucopyranosylphosphate. Structural homology between the ant glycolipid and LX is not complete, as LXLS contains only one nonhydroxylated fatty acid residue per molecule of N-acetylglucosamine (albeit of the same acyl chain length), while LX contains two β-hydroxylated residues.

Therefore, *L*entinus *edodes* extracellular galactolipid *S*3 contains one nonhydroxylated fatty acid residue, similarly to LXLS; on the other hand, *S*3 is not a glucosamine derivative, as yeast cellobiosolipids; its glycan part is represented by galactose. Glycolipids producers are diverse in their biological origin. Glycolipids possess similar features, and, as revealed by modern investigations, functionally significant differences in their molecular structure [1, 3, 5, 22, 23]. It is no wonder, therefore, that *L. edodes* and, probably, other macrobasidiomycetes, are able to synthesize similar compounds. However, no information of the kind concerning this systematical group is available.

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