ISSN 0026-2617, Microbiology, 2007, Vol. 76, No. 4, pp. 429–434. © Pleiades Publishing, Ltd., 2007.
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EXPERIMENTAL ARTICLES

Isolation and Characterization of Lectin from the Surface of *Grifola frondosa* (Fr.) S.F.Gray Mycelium

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Recieved October 10, 2006

Abstract—From the surface of the dikaryotic mycelium of the xylotrophic basidiomycete *Grifola frondosa* 0917 a lectin has been isolated with a molecular mass of 68 ± 1 kDa, consisting of two subunits of 33–34 kDa each. The lectin is a hydrophilic glycoprotein with the protein : glycan ratio of 3 : 1. It exhibits high affinity to native rabbit erythrocytes and to human erythrocytes of the 0 blood group, but not to trypsin-treated ones. The hemagglutination (HA) caused by lectin was not blocked by any of the 25 tested mono-, di-, and amino sugars; it was also not blocked by some of glyco derivatives. Only 13.9 μg/ml of the homogeneous preparation of a polysaccharide, a linear D-rhamnan with the structure of the repeated component ⟶2)-β-*D*-Rha*p*-(1⟶3)-α-*D*-Rha*p*-(1⟶3)-α-*D*-Rha*p*-(1⟶2)-α-*D*-Rha*p*-(1⟶2)-α-s*D*-Rha*p*-(1⟶ blocked hemagglutination completely. The analysis of the amino acid composition of the lectin showed the greatest percentage of amino acids with positively charged R groups, arginine, lysine, and histidine, as well as the complete absence of sulfur-containing amino acids, cysteine, and methionine. D-glucose and D-glucosamine were detected in the carbohydrate part.

Key words: Grifola frondosa, mycelial lectin, xylotrophic basidiomycete, glycoprotein, carbohydrate specificity, D-rhamnan.

DOI: 10.1134/S0026261707040078

Interest in edible cultivated basidiomycetes as sources of biologically active materials, including lectins, most of which are glycoconjugants [1], is growing steadily.

The concept of the special role of protein glycosylation as an integral part of carbohydrate metabolism in various organisms has become widespread in the scientific community. This concept is based on contemporary glycobiological findings, which confirm that carbohydrates have not only an energetic function in metabolism, but a bioinformatic potential as well. Therefore, in any organism, including fungi, most of the proteins are glycosylated [2]. The functioning of some metabolic systems, particularly those with an extracellular direction which utilizes the bioinformation potential of carbohydrates, is possible due to the realization of a biospecific carbohydrate–protein interaction. These interactions are realized mainly via lectins, which can recognize specific carbohydrate determinants in the cellular structures (as, for example, in the metabolic scheme of the processes of adhesion of rhizobial cells to the tissues of leguminous roots and initiation of symbiosis, in which the lectins of macrosymbionts, leguminous plants, play the critical role [3]). Thus, if some glycoside structures of a living cell have a specific information potential, then its realization requires the participation of lectin substances [4, 5].

Studies of basidiomycete lectins are not numerous and are related mainly to the isolation of lectins from their fruit bodies, which are only short-term formations with strictly defined functions. Publications on the study of basidiomycete lectins at the stage of the dikaryotic mycelium are scarce [6, 7], although this is the main and most significant stage of growth of a fungal culture as a biological individual. The study of lectins as biospecific agents in the metabolism of a basidial xylotroph will undoubtedly increase the spectrum of fundamental knowledge about the existence and, possibly, the realization of carbohydrate bioinformation potential in these organisms. Furthermore, since lectins of various origin, including those isolated from some higher fungi [8], are produced as pharmacological preparations, widely used in biomedical studies [9], such studies have an applied value as well.

The basidiomycete polypore *G. frondosa*, a member of the family *Albatrellaceae,* belongs to the ecological group of phytopathogenic fungi (xylotrophs) [10]. It is a lignin-degrading organism, parasitic or extremely

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rarely saprotrophic, causing white corrosive rot of old or weakened trees. This fungus was chosen as a model because of its promise as a source of food biomass (one fruit body can reach 10–12 kg) and of efficient immunomodulating, antitumor glycans and glycoproteins (approximately 30 biologically active compounds have been isolated from its fruit body and mycelium) [11]. At the beginning of our studies, only one work was found in the available literature dealing with the isolation of lectin from the fruit bodies of this fungus [12]; no data was discovered concerning the studies of lectin activity at the stage of the dikaryotic mycelium.

The goal of this work was the isolation and characterization of lectin from the surface of the dikaryotic mycelium of the basidium xylotroph *G. frondosa* 0917.

MATERIALS AND METHODS

Reagents used in the work: Sephadex G-75 (Sigma, United States), anion-exchange carrier DEAE–Toyopearl 650M (Tosoh, Japan), carbohydrate kit (Merck, Germany), and reagents for electrophoresis, including marker proteins (Amresco, United States).

Culture of a xylotrophic basidiomycete *G. frondosa* 0917 was obtained previously from the basidiomycete collection of the Komarov Botanical Institute (St. Petersburg, Russia), thanks to Dr. N. V. Psurtseva. In the course of experiments, the culture growth was maintained on plates with the agarized (18 g/l) medium based on Balling 4° wort. The 18-day dikaryotic mycelium was used for the investigation.

The extract of surface mycelium was obtained by washing the mycelial biomass with 0.15 M phosphate–buffer saline (PBS: NaCl, 8.5 g/l; $Na_2HPO_4 \cdot 12H_2O$, 2.70 g/l; NaH_2PO_4, 0.39 g/l; pH 7.2–7.4). For this purpose, the mycelium was placed into a flask with the buffer (200–250 mg of native biomass per 10 ml of buffer) and incubated at room temperature with shaking for 4 h.

Gel filtration. The extract was filtered and fractionated on a column (1.5 × 40 cm) with Sephadex G-75 using the Uvicord SII detector (LKB, Sweden) at $\lambda = 280$ nm; the same buffer was used as an eluent. The hemagglutinating fractions were collected, concentrated on a rotary evaporator, and dialyzed against distilled water in dialysis tubes with the 6 kDa limit of exclusion (Sigma-Aldrich, United States).

Ion exchange chromatography. The aqueous dialyzate was dialyzed against 0.02 M PBS (pH 7.9; per 100 ml of the buffer: NaCl, 0.114 g (1.14 g/l); 93 ml of separately prepared $Na_2HPO_4 \cdot 12H_2O$ (0.72 g/l); and 7 ml of separately prepared NaH_2PO_4 (0.24 g/l)). It was then chromatographed on the column (1 × 10 cm) with DEAE–Toyopearl 650M, equilibrated with the same buffer. The carrier and buffer conditions were selected so that the compound of interest was adsorbed, but not strongly bound to the column. Lectin was eluted with 0.25 M NaCl. Lectin fractions were dialyzed twice against distilled water and concentrated by passive evaporation and lyophilic drying.

Thus, the average yield of lectin from 200 mg of native biomass was 50–80 μg of lectin. The lectin preparation was accumulated for subsequent study in cycles; each cycle included several gel filtration processes and an ion exchange one.

The hemagglutination reaction was carried out by the semi-quantitative method of two-fold dilutions of lectin preparations (or of the tested fractions in the process of isolation) in 0.15 M PBS on standard multiwell plates for immunological reactions [13] with native rabbit erythrocytes. The titer of hemagglutination was taken as the highest dilution of the preparation, which resulted in a visible agglutination of erythrocytes, compared to the control well.

Lectin specificity was determined by the method of inhibition of the hemagglutination reaction by various carbohydrates [13]. The following carbohydrates and glycoderivatives were tested thoroughly as inhibitors: D-galactose, D-glucose, D-mannose, D-fructose, L-rhamnose, L-fucose, L-arabinose, D-lyxose, D-xylose, L-talose, D-lactose, D-maltose, D-cellobiose, D-melibiose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, N-acetyl-D-mannosamine, N, N-diacetyl-chitobiose, D-galacturonic acid, 2-deoxy-D-galactose, D-galactosamine, D-glucosamine, phenyl-β-D-galactopyranoside, and phenyl-β-D-glucopyranoside. The previously obtained preparations of carbohydrate polymers were also used (O-specific polysaccharides of the membrane lipopolysaccharides of gram-negative diazotrophic bacteria *Azospirillum brasilense* Sp245, *A. irakense* KBC1, and *A. lipoferum* Sp59b). The structure of the repeated carbohydrate links of these polysaccharides (mono-carbohydrate composition, nature of glucoside bonds, and absolute configuration) has been previously investigated [14–16]; the selection of these compounds as test inhibitors was therefore substantiated. The preparations were kindly provided by the researchers of the laboratory of biochemistry, Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences.

Native erythrocytes were obtained from 1.0–1.5 ml of rabbit blood taken from the marginal ear vein. The blood was immediately treated in a small vial with glass beads and buffer (0.5 ml) until fibrin clots formed (10–15 min), then it was filtered through a multilayered gauze filter. It was then centrifuged 3–5 times in 0.15 M PBS for 4 min at 1500 g; each time, the supernatant was removed and replaced with fresh buffer. For the hemagglutination reaction, a 2% suspension of erythrocytes was prepared in 0.15 M PBS.

Gel electrophoresis was carried out in a vertical cell (Helicon, Russia) using 15% polyacrylamide gel with 0.1% sodium dodecyl sulfate. The gels were stained with silver nitrate.

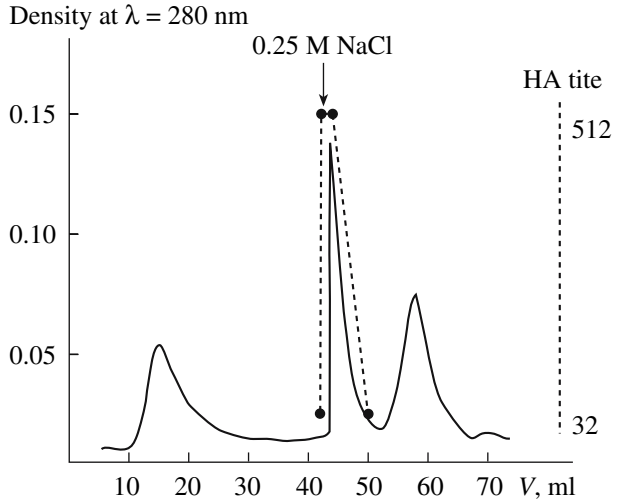

Fig. 1. Elution profile of the hemagglutinating fractions in the course of ion exchange chromatography on DEAE–Toyopearl 650M.

Amino acid analysis was performed using an AAA 339 analyzer (Czechoslovakia). The sample was hydrolyzed with 6 N HC1 at 105°C for 24 h.

Protein concentration was determined by the Bradford method [17].

Carbohydrate concentration in lectin solutions was determined by the phenol– sulfuric acid reaction [18]. After hydrolysis with 4N trifluoroacetic acid for 4 h, the qualitative composition of the samples was determined by thin–layer chromatography in the system pyridine : ethyl acetate : water : acetic acid in the ratio 5 : 5 : 3 : 1.

Reliability of the results: the t value at n = 5, from 2.61 to 3.28; $0.05 \geq P$ 0.03.

RESULTS AND DISCUSSION

We have previously studied the dynamics of lectin activity of the *G. frondosa* 0917 dikaryotic mycelium grown on certain solid and liquid media. The highest titer of lectin activity against the native human and rabbit erythrocytes of the 0 blood group was detected in the washings from the surface of a dikaryotic mycelium cultured on agarized media [19]. The present work is the next stage in the study of the lectin activity of the *G. frondosa* 0917dikaryotic mycelium.

The isolation of the hemagglutinating protein from the extracts of the surface mycelium was accomplished by a two-stage chromatographic process. During the first stage, mycelial extracts were fractionated by gel filtration on Sephadex G-75. The fractions with hemagglutinating properties were combined for subsequent study by ion exchange chromatography (the second stage of the isolation process). Fig. 1 illustrates sequential elution of the separated substances present in the agglutinating fractions, from the column with DEAE-Toyopearl 650M, performed in a stepwise salt gradient. It can be seen that the hemagglutinin was eluted with 0.25 M NaCl after the unadsorbed fraction and the working buffer.

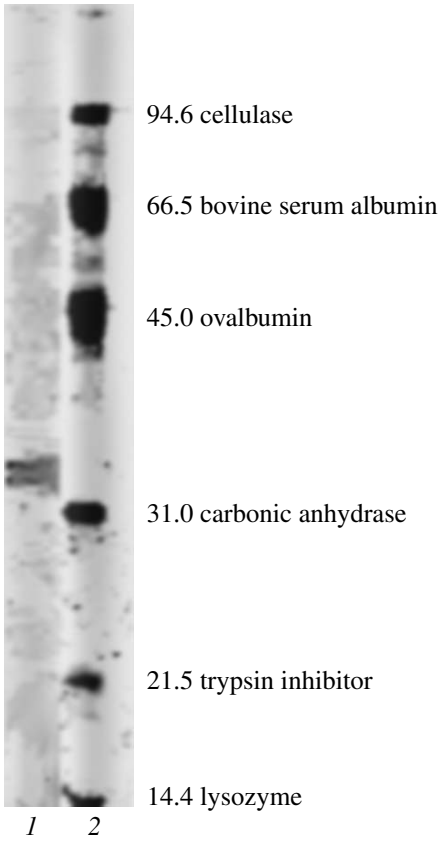

Fig. 2. Electrophoregram of lectin: lane *1*, *G. frondosa* 0917 lectin; lane *2*, marker proteins, kDa.

The subunit structure of the substance was determined by gel electrophoresis. Fig. 2 demonstrates two bands at the level of 33–34 kDa. According to the literature data, this subunit structure is characteristic of the overwhelming majority of lectins of various origin [20, 21]. Dimers consisting of small (tens of kDa) subunits constitute a substantial fraction of the investigated basidiomycete lectins [22, 23]. A much smaller number of lectins have monomeric [24] or tetrameric [25] structure.

Determination of the molecular mass of the native substance by gel filtration on Sephadex, in comparison with the model proteins, revealed that the time and the volume of lectin elution from the column coincide with these values for the same concentration of bovine albumin with a molecular mass of 67 kDa.

The ratio of the glycan component was determined in the solutions of the isolated substance at different concentrations. According to the results of all the experiments, the glycan : protein ratio was 1 : 3 (Table 1). Thin-layer chromatography revealed the presence of D-glucose and D-glucosamine in the carbo-

Table 1. Protein and carbohydrate content at various concentrations of solutions of the homogeneous preparation of *G. frondosa* 0917 lectin

Protein concentration, μg/ml	Carbohydrate concentration, μg/ml
13.6 ± 0.24	4.2 ± 0.07
28.2 ± 0.73	8.7 ± 0.11
54.8 ± 1.64	17.8 ± 0.29
110.4 ± 2.76	36.2 ± 0.97
218 ± 3.73	71.9 ± 1.12

Table 2. Amino acid composition of the *G. frondosa* mycelium 0917 lectin

Amino acid	% of total amino acids	Amino acid	% of total amino acids
Asp	8.2	Met	–
Thr	5.4	Ile	4.0
Ser	3.5	Leu	6.4
Glu	4.0	Tyr	5.7
Pro	5.7	Phe	5.4
Gly	7.1	His	7.5
Ala	6.8	Trp	4.3
Cys	–	Lys	7.5
Val	5.0	Arg	8.5

hydrate part. According to the available data, the overwhelming majority of lectins isolated from various organisms are glycoproteins; in higher fungi, only one lectin has been found which is pure protein (in the ascomycete *Aleuria aurantia* [26]). As was thoroughly described in the case of certain plant lectins, glycosylation occurs in strictly defined peptide regions of the molecules in the course of post-translational modification [27]. According to [28], the role of the glycan part in the processing of the investigated plant lectins consists in the monitoring of the correct folding of their molecules with the formation of a tertiary structure, as well as in holding the protein subunits together.

The titer of agglutination by the homogeneous substance at the initial concentration of 50 μg/ml was 2048 for native rabbit erythrocytes and 64 for human erythrocytes of the 0 blood group. The absence of interaction with trypsinized erythrocytes established by us [19] distinguishes this lectin from the absolute majority of similar substances of basidiomycete origin, including the previously described lectin from *G. frondosa* fruit bodies [12]. According to the results of numerous studies, treating erythrocytes with some proteases increases their affinity to lectins. Therefore the affinity of the investigated substance to the surface of native erythrocytes certainly indicates that the receptors competent for the interaction with this lectin are different in nature from those for lectin from the fruit bodies. It was therefore possible to assume substantial differences in the carbohydrate specificity of the obtained lectin. Determination of the carbohydrate specificity of the lectin revealed that the mono-, di-, and amino sugars, as well as certain glyco derivatives tested in the experiment in 0.1 M concentration, did not inhibit the agglutination of native rabbit erythrocytes. According to the classification proposed in work [29], lectins, in accordance with their carbohydrate specificity, fall into two groups. The first group comprises the so-called exolectins, i.e., those that bind the specific external sugars of the nonreduced ends of complex saccharides; agglutination by these lectins can be inhibited by low concentrations of free sugars or of their methyl glycosides. The second group includes endolectins, which recognize only complex oligosaccharides; agglutination by these lectins can be inhibited only by specific carbohydrate sequences. Since no interaction with free sugars was discovered, homogeneous preparations of the abovementioned carbohydrate polymers were used as inhibitors. The O polysaccharide of *A. brasilense* Sp245, a linear D-rhamnan with the following structure of the repeated component: →2)-β-*D*-Rha*p*-(1→3)-α-*D*-Rha*p*-(1→3)-α-*D*-Rha*p*-(1→2)-α-*D*-Rha*p*-(1→2)-α-*D*-Rha*p*-(1→ in the concentration of 0.0139×10^{-3} mg/ml was found to inhibit agglutination of native rabbit erythrocytes in titer 4 of the lectin under investigation. Free rhamnose had no inhibitory effect; this can possibly be explained by the fact that pure rhamnose exists only in the form of an L epimer, whereas rhamnose in the pentasaccharide component of rhamnan is in D form. Since this configuration of rhamnose has been described only for complex polysaccharides of some microorganisms [14, 30], it can be supposed that the isolated substance which exhibited this specificity is a typical endolectin, sensitive to the conformational modifications of an affinity polysaccharide; such modifications are caused by the nature of glycoside bonds and by the epimerization of its component monosaccharides [29]. Conversely, the previously investigated lectin from the fruit bodies of *G. frondosa* proved to be a typical exolectin, since it exhibited high specificity to N-acetyl-*D*-galactosamine [12].

The study of the amino acid composition of the isolated lectin revealed the highest percentage of amino acids with positively charged by R groups of arginine, lysine, and histidine, as well as the comparable content of an amino acid with a negatively charged R group (aspartic acid). The sulfur-containing amino acids (cysteine and methionine) were completely absent (Table 2). The absence of the latter from the amino acid composition or their presence in trace quantities, together with the subunit structure, is also a character-

istic feature which has been noted by many researchers in their works on various lectins and agglutinins.

Thus, the study of the lectin isolated from the surface of the *G. frondosa* 0917 mycelium revealed features common to the lectins obtained from other basidial fungi (dimeric structure, the presence of a carbohydrate part, the absence of sulfur-containing amino acids). Only the carbohydrate specificity of the obtained lectin was unique: in contrast to the overwhelming majority, *G. frondosa* 0917 lectin had no affinity for free sugars and was blocked by small concentrations of a linear *D*-rhamnan.

The role of lectin from the surface of the dikaryotic mycelium of the studied fungus is presently under discussion. Considering the specificity to the *A. brasilense* Sp245 cell-wall O polysaccharide (also called O antigen), the role of this lectin in the recognition process in the case of contacts with bacterial cells and possibly in the provocation of protective reactions were suggested. Due to its carbohydrate specificity, the preparation of *G. frondosa* 0917 mycelial lectin can be recommended for the applications related to the study of the cell surfaces of gram-negative bacteria.

ACKNOWLEDGMENTS

The work was supported by the Russian Foundation for Basic Research (project no. 03-04-48129). We are thankful to Prof. S. A. Konnova and Dr. Yu. P. Fedonenko for the preparations of bacterial O polysaccharides.

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