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EXPERIMENTAL ARTICLES

Immunochemical Properties and Localization of Lectin from the Basidiomycete *Grifola frondosa* (Fr.) S.F. Gray

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Abstract—The complex of immunochemical methods was applied to study the ability of the lectin from the fungus *Grifola frondosa* (Fr.) S.F. Gray to interact with homologous and non-homologous rabbit and human polyclonal antibodies. The results of immunodot assay with the fragments of proteolytically cleaved antibodies demonstrated the binding of the lectin only with the *Fab* fragments (antigen-binding center) of homologous antibodies, which is evidence of specific "antigen–antibody" interaction. The revealed interaction of the lectin with non-homologous antibodies (rabbit antibodies to bacterial O-antigens and the commercial preparation of human g-globulin) is most likely accomplished due to the contact of the carbohydrate-binding region of the lectin with the carbohydrate moiety of the antibodies ("lectin–carbohydrate"). Immunofluorescence microscopy with homologous antibodies revealed that lectin was diffusely and unevenly distributed over the surface of the hyphae, forming agglomerates in the region of buckles and young shoots.

Key words: Grifola frondosa, lectin, surface localization, homologous and non-homologous antibodies, binding specificity.

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The higher cultivated basidiomycetes attract attention of the scientific community as sources of a number of biologically active substances. Representatives of the order of *Aphyllophorales*, or polypores, belonging to the ecological group of wood destructors, hold a particular position. The pioneering works of Japanese researchers [1] have shown that aphyllophore basidial xylotrophs are sources of active antitumor substances. For example, about 30 biologically active substances, mostly polysaccharides with immunomodulating and antitumor properties, have been isolated by now from the fruit bodies and mycelium of the polypore *Grifola frondosa* (Fr.) S.F. Gray, or maitake mushrooms, [2, 3]. The protein fractions of the biomass of this fungus also contain substances with a high functional activity towards human cells and tissues. It has been established that the hydrophilic proteins of *G. frondosa* fruit bodies include a lectin with the specificity to *N*-acetyl-D-galactosamine [4]. Our works have shown that the mycelium of this fungus also has a lectin activity [5]. The hemagglutinating agent present in the passive water–saline washout from the surface of *G. frondosa* mycelium was isolated and characterized [6]. Mycelial lectin (a hydrophilic dimeric glycoprotein of about 68 kDa) differed from the described lectin from the fruit bodies [4] in molecular mass and showed no specificity to free carbohydrates (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 were tested). Its activity was blocked by the linear D-rhamnan [6]. According to the work [7], the affinity to certain carbohydrate sequences made it possible to define the mycelial agglutinin of *G. frondosa* as an endolectine. The mentioned D-rhamnan is one of the two O-specific polysaccharide components (O-PS) of the lipopolysaccharide (LPS or O-antigen) of the gram-negative soil bacterium *Azospirillum brasilense* Sp245 [8]. It should be noted that, according to the results of chemical analysis, the two O-PS from *A. brasilense* Sp245 do not differ in structure and are homopolymers of D-rhamnan [8], whereas immunochemical analysis showed the difference between these O-PS in the structure of their antigen determinants [9, 10] correlating with different abilities for the interaction with mycelial lectin of the fungus *G. frondosa*. The above information suggests a very fine carbohydrate-binding specificity of this lectin.

The stated characteristics suggest that the lectin under study is associated with the surface of hyphae

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cells and is probably involved in biorecognition at a contact with foreign organisms.

The goal of this work was to obtain specific antibodies, to study the immunochemical properties of mycelial lectin of *G. frondosa*, and to determine the peculiar features of lectin localization on mycelium surface.

MATERIALS AND METHODS

The culture of *Grifola frondosa* (Fr.) S.F. Gray, strain 0917, has been provided previously by the Collection of Basidiomycetous Cultures of the Komarov Institute of Botany (St.-Petersburg, Russia).

The preparation of lectin from *G. frondosa* was obtained as described [6].

Homologous (specific) polyclonal rabbit antibodies to the lectin of *G. frondosa* 0917 were obtained by threefold injections (with 2-week intervals) of 0.5, 1.0, and 1.5 mg of lectin into popliteal lymph nodes of rabbits; the lectin was mixed with the complete Freund's adjuvant (1 : 1) at the first immunization and with the incomplete adjuvant at subsequent immunizations. Blood (50–70 ml) from the edge of the auricular vein was taken one week after the last immunization. The fractions of immunoglobulins G (IgG) were obtained from the antisera by precipitation with ammonium sulfate [11]. IgG concentration in the solutions was determined by spectroscopy at $\lambda = 280$ nm, assuming the optical density of an IgG solution in a 1 cm cuvette at a protein concentration of 1 mg/ml to be 1.4 [12].

Non-homologous (nonspecific) antibodies. The commercial preparation of human γ-globulin and the antibodies to the O-antigens of bacteria *A. brasilense* Sp245, S17, and *Sinorhizobium meliloti* P221 obtained as described [13, 14] were used in the work. Bacterial strains *A. brasilense* Sp245 and S17 possessed different O-specific antigen determinants and formed different serological groups [14].

The conjugate of *G. frondosa* lectin and homologous antibodies with colloid gold (CG) particles of 15 nm in diameter was obtained as in [15]. The conjugates were stored at 2–4°C.

Immunodot assay. Nitrocellulose Millipore membranes (United States) were used for the immunodot assay of the interaction of the lectin–CG conjugate with specific and nonspecific polyclonal rabbit antibodies and the commercial human γ-globulin preparation and the interaction of the protein A–CG conjugate with the fragments of antibodies. Solutions of antibodies (2 μl) from the series of double dilutions were applied to the membrane and fixed in a drying chamber at 50°C for 10 min. For prevention of nonspecific sorption of the label on the membrane sites free from the samples, the dried membrane was placed into 0.15 M phosphate buffered saline (PBS; pH 7.2) containing 0.02% Tween-20 and 0.1% polyethylene glycol (PEG-20000) and incubated in this solution for 30 min under stirring. The membrane was then washed in PBS for 10 min and placed into a solution of the lectin–CG conjugate (or protein A–CG conjugate, in the case of antibody fragments) for 10–20 min, where the presence or absence of interaction was observed. The membrane was washed with PBS and dried in filter paper.

Obtaining the *Fab* and *Fc* fragments of antibodies. The antibodies to lectin and to the commercial human γ-globulin preparation were subjected to cleavage by the procedure proposed in [16] with certain modifications. Papain and cysteine-H (0.5 mg each) were introduced into 1 ml of the solutions of antibodies and human γ-globulin in 0.1 M phosphate buffer (PB). The mixtures were incubated for 4 h at 37°C and then placed into a freezer at –20°C to stop the reaction. The defrosted mixture was dialyzed against 0.01 M PB and then applied to a column with the DEAE-Toyopearl 650M ion-exchange carrier. The gradient elution with 0.01–0.3 PB after separation of each mixture by the Uvicord SII detector (LKB, Sweden) was used to obtain two fractions: (1) *Fab* fragments and (2) *Fc* fragments with probable admixture of uncleaved antibody molecules. The dot assay of the interaction of antibody fragments with the protein A–CG conjugate was used for identification of *Fc* fragments.

Immunoenzyme assay of the interaction between the lectin and nonspecific antibodies. Solid-phase immunoenzyme assay (IEA) was performed in polystyrene 96-well plates [17]. Lectin, hapten O-PS, and the complex of these compounds in different concentrations were used as antigen samples. *ortho*-Phenylenediamine with hydrogen peroxide was used as a substrate reagent. The optical density of the samples was measured at 490 nm in an AIF-Ts-01C immunoassay analyzer (ILIP, St.-Petersburg, Russia). Concentrations of the preparations of *G. frondosa* 0917 lectin (50 μg/ml) and O-PS (200 μg/ml) were chosen in accordance with the proportion of these substances established previously in the specific reaction of "lectin–carbohydrate hapten".

Localization of the *G. frondosa* lectin was determined by the immunofluorescence microscopic technique proposed in the works [18, 19]. The samples of 5-day fungal mycelium culture grown on a petri dish with agarized wort (4° Balling) were placed for 1 h into the blocking buffer (NaCl, 8.5 g/l; $Na_2HPO_4 \cdot 12H_2O$, 2.70 g/l; NaH_2PO_4, 0.39 g/l; bovine serum albumin (BSA), 1%; Tween-20, 0.01%; pH 7.2–7.4). Then the samples were treated with the solution of antibodies to lectin for 1 h. After secondary washing with the blocking buffer for 30 min, the samples were treated by the commercial preparation of antirabbit (asinine) antibodies conjugated with fluorescein isothiocyanate (FITC) (Gamaleya Institute of Epidemiology and Microbiology, Russian Academy of Medical Sciences) in a 1 : 100 dilution. After washing with PBS, the samples were analyzed in a Leica DMLB microscope (Germany) with 400° magnification.

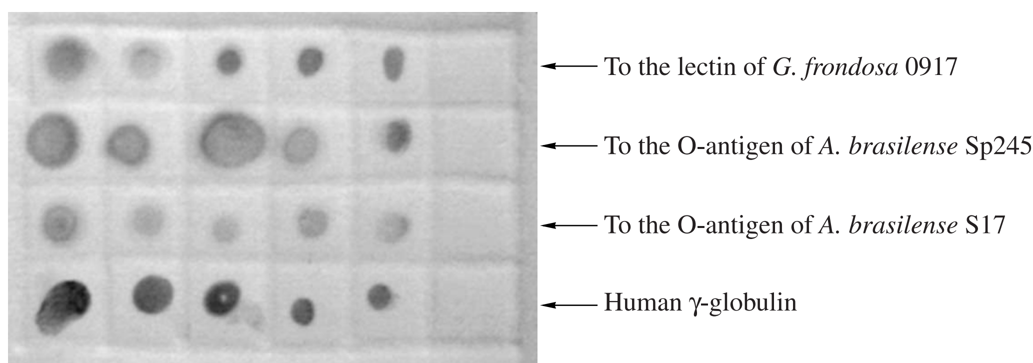

Fig. 1. Interaction of the lectin and CG conjugate with specific and nonspecific antibodies.

RESULTS AND DISCUSSION

The immunochemical approach is very informative for the study of surface structures of various biological objects, because specific antibodies are unique probes, in particular, for assessment of the peculiarities of localization and the level of production of analyzed compounds, as well as the effect of external factors on their qualitative and quantitative characteristics. Lectins as a special class of biologically active substances recognizing certain carbohydrate structures are often mentioned in the literature as immunomodulating agents, including lectins of the higher fungi [20]. However, few works describe the application of homologous antibodies to investigate the lectins of different origin [21]. This is partially due to the laborious obtaining of pure lectin preparations. Besides, due to the known ability of these substances to bind the carbohydrate moiety of immunoglobulins isolated from the blood of experimental animals and humans [22], the immunochemical specificity of the obtained antibodies to lectins requires special evidence, the absence of which may impede the interpretation of the results.

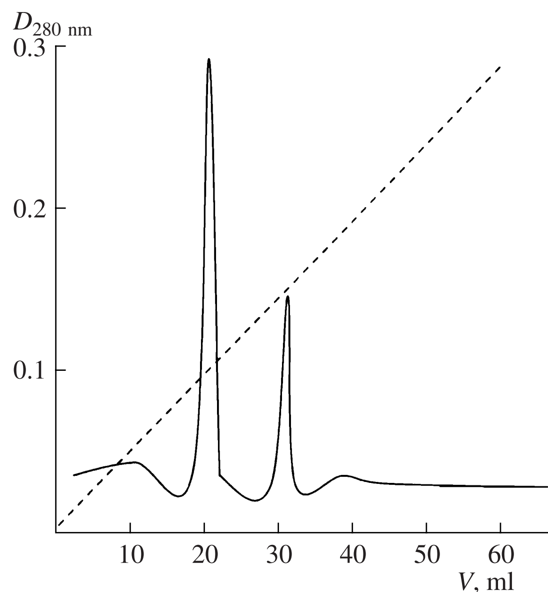

Fig. 2. Elution profile from the column with DEAE-Toyopearl 650M for the antibodies specific to the *G. frondosa* lectin after the treatment with proteolytic enzyme papain. Dotted line is the gradient of PB 0.01–0.3 M.

We have obtained rabbit polyclonal antibodies to the homogenous preparation of mycelial lectin of the fungus *G. frondosa* (Fr.) S.F. Gray and demonstrated the immunochemical specificity of their interaction with the antigen preparation. Since available literature contains very little information about lectins as immunogens, we applied a procedure when an antigen preparation is introduced directly into the lymph nodes of immunized animals. The interaction of the obtained antibodies with the antigen (lectin) was confirmed by immunodot assay; however, the immunodiffusion test showed no specific interaction. The latter fact was evidence of the low titer of specific antibodies in the serum which, in turn, indicated very low immunogenicity of the *G. frondosa* mycelial lectin. At the same time, the antibodies to the O-antigens of *A. brasilense* Sp245 and S17 and to the commercial preparation of human γ-globulin used in the dot assay as a negative control also exhibited a cross reaction with the studied lectin conjugated with CG particles (Fig. 1).

The latter circumstance made it necessary to test the immunochemical specificity of the binding of the fungal lectin to homologous antibodies. The treatment with the proteolytic enzyme papain and ion exchange chromatography were used to obtain antigen-binding fragments (*Fab* fragments) of the antibodies to mycelial lectin and human γ-globulins used as a control. Fig. 2 shows sequential elution of the products of cleavage of the antibodies to lectin in the course of ion exchange chromatography. As was stated by the authors of the method [16], the first peak was a fraction of *Fab* fragments. Identification of the fragments was confirmed by the interaction with CG-labeled protein A, specifically recognizing the *Fc* fragments of immunoglobulin molecules [23] (Fig. 3a). The antigen-binding fractions (*Fab* fragments not involved in the contact

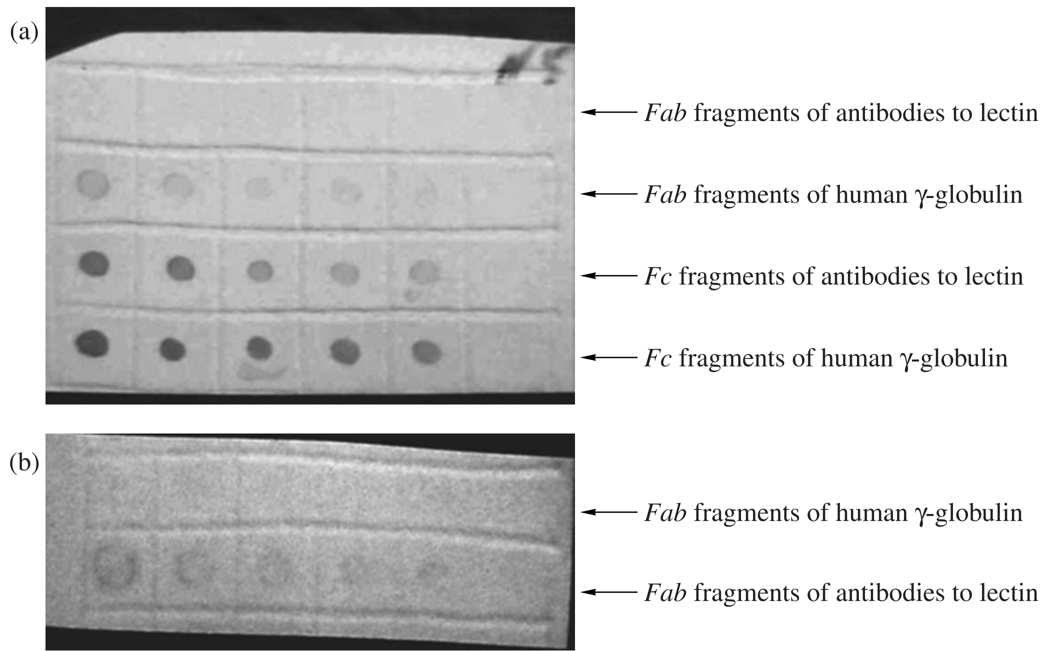

Fig. 3. Interaction of the *Fab* and *Fc* fragments of antibodies with the conjugate of protein A and CG (a) and *Fab* fragments of antibodies with the conjugate of *G. frondosa* lectin and CG (b).

with protein A) of the antibodies were tested for the interaction with the antigen under study, and a positive reaction was observed only in the *Fab* fragments of the antibodies to mycelial lectin (Fig. 3b).

In view of the absence of binding of the *Fab* fragments of human γ-globulins with lectin, it can be considered proven that the antigen-binding center of γ-globulin molecules does not participate in their interaction with lectin. It is evident that the interaction of *Fc* fragments of nonspecific immunoglobulins with the studied fungal lectin is based on lectin–carbohydrate interactions, when certain oligosaccharides from the carbohydrate moiety of the antibodies interact with the competent region of the fungal lectin. The main limiting factor for such an interaction is the spatial complementarity of the carbohydrate-binding region of a lectin, changing at the formation of a complex with a haptenic polysaccharide. As was mentioned above, such interactions are mentioned by a number of researchers and underlie the lectin–enzyme analysis commonly used for the study of variations in the composition of the carbohydrate moiety of immunoglobulins of different classes [22, 24]. If the interaction of lectin with nonspecific antibodies occurs through the contact of its carbohydrate-binding region with oligosaccharides of the antibodies, then the blocking of this region by a haptenic polysaccharide should reduce the efficiency of interaction with the antibodies. Using immune-enzyme analysis, we obtained semiquantitative characteristics of the reactions of the lectin under study and its complex with haptenic O-PS (D-rhamnan) with non-homologous antibodies (Fig. 4). The latter were the antibodies to O-antigens of the bacterium *S. meliloti* P221. The interaction of lectin with the antibodies was characterized by linear dependence. The absorption curve characterizing the dynamics of this interaction under decreasing lectin concentrations reached the minimum value already in the titer 1 : 16 of the initial antigen concentration and then passed close to the control curve. The curve of the interactions of the lectin complex with hapten was notable for lower absorption values not depending on dilution. Hence, it was concluded that reduction of the degree of binding of the "lectin–hapten" complex with non-homologous antibodies is associated with the blocking of its carbohydrate-binding capacity which, in turn, is indicative of specific "lectin–

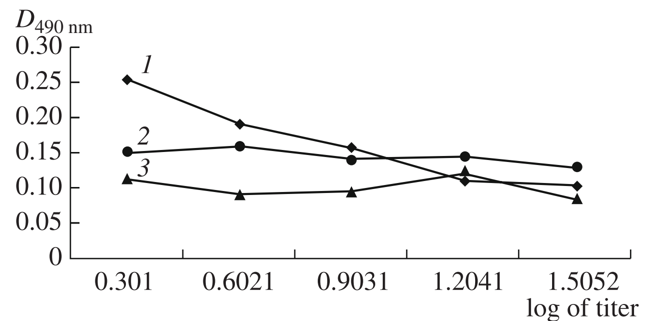

Fig. 4. Interaction of lectin, haptene O-PS, and "lectin–haptene" complex with the antibodies to O-antigen of *S. meliloti* P221: lectin (*1*); complex (*2*); O-PS (control) (*3*).

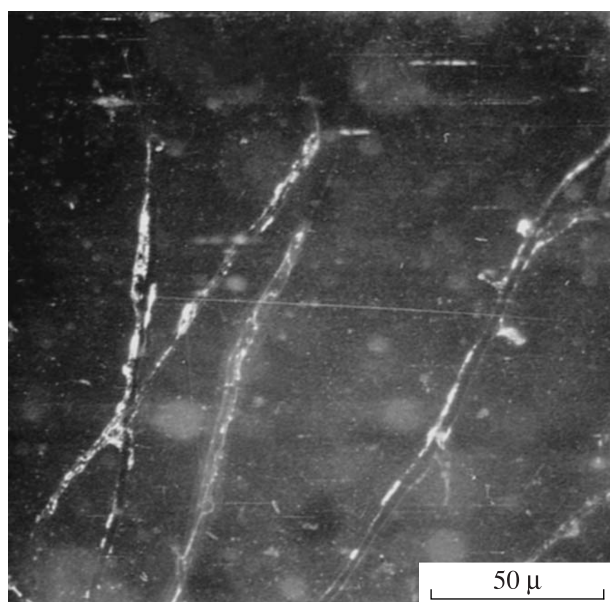

Fig. 5. Localization of the lectin on the surface of *G. frondosa* mycelium.

carbohydrate" interaction at a contact of lectin with non-homologous antibodies.

Assessment of localization of the *G. frondos*a lectin using antibodies. Immunofluorescence microscopy with specific antibodies showed diffuse distribution of the lectin over the surface of hyphae (Fig. 5). Such localization has been described previously for some bacterial lectins [25]. The authors emphasized that the described lectins were not connected with any particular bacterial filaments but were evenly distributed over the surface of the microbial cell. However, as follows from Fig. 5, distribution of the lectin over the fungal hyphae is nonuniform; lectin agglomerates are typical of the buckles formed in the course of dikaryotization and of the apical parts of hyphae. As is known, development of any morphological structures requires energy inputs, which provokes the inflow of considerable amounts of carbohydrates to the region of active growth. This process is all the more relevant for the apical ends of hyphae characterized by continuous growth under changing environmental conditions. The latter circumstance compels the expenditure of some portion of energy for adaptation, which is manifested primarily by the change of the functioning of certain enzyme systems. As is stated by the authors [26], lectins are most probably involved in these processes as a controlling factor either through direct binding with free carbohydrates or indirectly through the binding to the carbohydrate determinant within the enzymes.

Thus, the mycelial lectin, diffusely distributed over the hyphae surface, was characterized as a substance with low immunogenicity. The totality of these findings demonstrates that the ability of the lectin to bind with homologous antibodies is conditioned by a specific interaction with the antigen-binding center, whereas the revealed interaction with non-homologous antibodies is realized due to the binding of the lectin to the carbohydrate moiety of antibodies.

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