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Purification of a Lectin from Fruit Bodies of *Lactarius pergamenus* (Fr.) Fr. and Studies of Its Properties

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Abstract—A lectin was purified from fruit bodies of the milk mushroom *Lactarius pergamenus* (Fr.) Fr. by a combination of ethanol precipitation, affinity chromatography on copolymer of polyvinyl alcohol and human blood B-group-specific substance, and ion-exchange chromatography on DEAE-Toyopearl. The lectin yield was 3 mg/kg of fresh mushrooms. Considerable loss of primary activity was observed during its purification, which, presumably, could be explained by disintegration of the lectin molecule, which consisted of six subunits, first to two molecules of three subunits, and then to individual subunits. There was a reverse tendency to aggregation during concentration of lectin solutions. Similar processes can take place in nature because of considerable individual variations of the lectin activity during growth of mushroom fruit bodies. The lectin weakly interacts with DGalNAc, while DGalβ1-3DGalNAc and DGalβ1-3DGlcNAc are the most probable candidates for ligands, with which the *L. pergamenus* lectin interacts at disaccharides level. The purified lectin may find application in histochemical research.

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Lectins are proteins and/or glycoproteins of non-immune origin that are able to bind carbohydrates without causing chemical transformations in the latter. Lectins that possess high selectivity to carbohydrate determinants of living human and animal tissues are of the most interest. This allows using them both for identification of carbohydrate determinants and for affinity purification of glycoproteins that contain such determinants. Lectins can also be used for purposeful delivery of drugs [1], early detection of apoptosis [2], mitogenetic stimulation of lymphocytes [3] and other aims described in a number of monographs [4-6].

Lectins from higher fungi as well as lectins from plants are widely applied in medico-biological studies. For example, lectins from fruit bodies of higher mushrooms *Aleuria aurantia* [7], *Marasmius oreades* [8], and *Polyporus squamosus* [9] are used in histochemical research for the exposure of specific carbohydrate residues on the surface of cells and tissues in animals. A lectin from *Agaricus campestris* possesses a potent antiproliferative effect without cytotoxicity that can find application in prevention of formation of scars in surgical operations on the eyeball [10]. Thus, the search for novel lectins possessing properties important for practical application is now very important. For example, L-fucose-specific lectin previously isolated by us from fruit bodies of *Peziza badia* [11], as well as a lectin possessing high affinity to alkaline phosphatase from fruit bodies of *Mycena pura* [12].

However, the vast mushroom family Russulaceae, in spite of considerable variety of species and being widespread in nature, is studied insufficiently. Obtaining lectins only from fruit bodies of *Lactarius lignyotus* [13], *L. deliciosus* [14], *L. deterrimus* [15], and *L. rufus* [16] is described in the literature. Some of their physical and chemical properties and carbohydrate specificity were studied, as well as their decisive role in mycorrhiza formation between mushroom hyphae and conifers was suggested [15].

A lectin from fruit bodies of *Russula foetens* (Russulaceae family) was obtained by us. Although purification was carried out under soft conditions and the mass

Abbreviations: DGalNAc, N-acetyl-D-galactopyranoside; DGalβ1-3DGalNAc, β-D-galactosyl(1-3)N-acetyl-D-galactopyranoside; DGalβ1-3DGlcNAc, β-D-galactosyl(1-3)N-acetyl-D-glucosaminopyranoside, isolactosamine.

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loss could not exceed several percent, we discovered that the purified product after a few stages of purification possessed less than 0.2% of hemagglutinating activity from the primary one [17]. When investigating *Russula* genus mushrooms, we detected variability of hemagglutinating activity of mushroom extracts, high sensitivity of extracts to changes in pH, to freezing of fresh mushrooms at −18°C, and precipitation of protein fraction with ammonium sulfate and ethanol solutions [17]. Study of influence of the same factors on lectins in the extracts of other mushrooms related to families of Agaricaceae, Amanitaceae, and Tricholomataceae showed their significantly greater stability in comparison with the representatives of the *Russula* genus [17]. When studying hemagglutinating activity in saps from fruit bodies of *Lactarius* genus mushrooms, also belonging to the Russulaceae family, we detected that high hemagglutinating activity was peculiar to *Lactarius pergamenus*. In the literature this lectin is not described although this mushroom represents a substantial raw materials base in the Ukrainian part of the Carpathians. The lectin may possess the same carbohydrate specificity as *Lactarius deterrimus* lectin. Thus, it can be specific to disaccharide DGalβ1-3DGalNAc, which occurs in oligosaccharide chains in complex glucoconjugates that may be of interest in histochemical studies.

The purpose of the present study was to develop methods for purification of the lectin from fruit bodies of *L. pergamenus*, as well as to study the physical and chemical characteristics of the purified lectin, its carbohydrate specificity, and possible reasons for instability of hemagglutinating activity during purification of the lectin from the raw material.

MATERIALS AND METHODS

Purification of the lectin. Fruit bodies of *L. pergamenus* were collected in July in the mixed forest near Skole in the Lviv Region (Carpathians). Fresh mushroom fruit bodies (7.5 kg) were ground using a meat chopper. The resulting mass was processed to sap using a press. The remaining pomace was dried in a desiccator at 52°C and was used for the isolation of other biologically active substances.

Sap (4.2 liters) was cleared by centrifugation at 1500-2000*g* for 10 min. The supernatant was cooled in a refrigerator to 4°C, and then two volumes of 96% ethanol (8.4 liters), preliminarily cooled to −18°C, were added to it. The formed precipitate was collected by centrifugation using a refrigerated centrifuge (10 min, 2000*g*). To dissolve the lectin, 1 M NaCl (~350 ml) was added to the precipitate. The precipitate was mixed for 15 min. The undissolved precipitate was removed by centrifugation, once again 1 M NaCl (~130 ml) was added, and after centrifugation the supernatants were combined. The combined liquid, which contained the lectin, was cooled again to 4°C and once again two volumes of a 96% ethanol cooled to −18°C were added to it. The precipitate was collected by centrifugation using the refrigerated centrifuge (10 min, 2000*g*) and then dissolved in 1 M NaCl (~120 ml). The undissolved precipitate was removed by centrifugation. The supernatant was additionally filtered through a filtration paper.

The further purification was carried out by affinity chromatography. A clean transparent liquid was applied to a column of affinity sorbent (3 × 9.5 cm) (copolymer of polyvinyl alcohol and human blood B-group-specific substance was obtained as described in [18]) that was previously equilibrated with 0.05 M phosphate buffer, pH 7.0, supplemented with 1 M NaCl. After passing through the column of the applied extract from *L. pergamenus*, the column was washed with the same buffer until the absorbance at 280 nm of the protein in eluate was less than 0.1. The lectin adsorbed on the column was eluted with 1 M NaCl and heated to 65°C. The lectin yield from the column was monitored by determination of the protein content and also by titer of hemagglutination. Fractions that contained the active lectin were combined, and the protein was precipitated with ammonium sulfate at 90% saturation. After centrifugation the precipitate was dissolved in a small volume of distilled water and dialyzed against 0.05 M phosphate buffer. After thrice-repeated replacements of the dialysis solution, the resulting lectin was applied onto a column filled with DEAE-Toyopearl (5.0 × 0.8 cm), which was equilibrated with the same buffer solution. The lectin passed through the column without retention, while most other materials were retarded on the column. On increase of ionic strength of the buffer solution to 0.1 M, more of the lectin passed through the column, but it was more contaminated other materials.

Electrophoretic studies and determination of molecular mass. Determination of purity and analysis of fractional composition of the resulting preparation of lectin were performed by disc-electrophoresis in 7.5% polyacrylamide gel in acidic (β-alanine acetate, pH 4.3) and alkaline buffer systems (Tris-glycine buffer, pH 8.6) [19].

Molecular mass of polypeptide chains of the lectin was determined by electrophoresis in 15% polyacrylamide gel supplemented with 0.1% SDS [19]. A mixture of proteins with known molecular mass (Fermentas, Lithuania) was used as standards.

Total molecular mass was determined on a Toyopearl HW-55 column (39 × 1.5 cm) using as protein markers egg lysozyme (14.3 kDa), wheat germ agglutinin (36 kDa), pea seed lectin (49 kDa), human blood serum albumin (69 kDa), edible snail lectin (79 kDa), and golden chain bark lectin (100 kDa). The protein was eluted in 0.1 M phosphate buffer (pH 7.4) with flow rate 2 ml/min.

Study of hemagglutinating activity and carbohydrate specificity of the lectin. The hemagglutinating activity of the lectin in saps from mushroom fruit bodies and solu-

tions containing the lectin at stages of purification was determined using a method described previously [20]. For this purpose, 10-15 drops of human or animal blood were placed in a test tube containing 10 ml of phosphate buffered saline (PBS), mixed, and washed three times to remove the blood plasma. Then the content of erythrocytes in the suspension was determined by means of hematocrit and diluted with PBS to yield 2% suspension, which was used to carry out the reaction of hemagglutination. A series of consecutive double dilutions of the lectin solution were prepared in micro test tubes and then an equal volume of 2% suspension of erythrocytes was added to each tube. After 10 min the tubes were centrifuged for 30 sec at 500*g*. The last test tube in which after shaking hemagglutination was observed by sight was noted. The result of the reaction was expressed by the titer of hemagglutination (dilution in the last test tube where agglutination was still observed) or calculating concentration of the lectin in this test tube.

The erythrocytes were treated with trypsin using a method described previously [20]. A 0.5-ml sample of washed erythrocytes was added to an equal volume of 1% trypsin solution and incubated at 37°C for 30 min. Then the erythrocytes were washed three times with PBS, and 2% suspension of erythrocytes in PBS was prepared.

Carbohydrate specificity of the lectin was determined by the reaction of hemagglutination inhibition by carbohydrates and glycoproteins, determining concentration of 50% inhibition of lectin activity (titer 1 : 1024, protein concentration ~10 mg/ml). For this purpose 15 μl of inhibitor solution was added to a series of 10 microtubes. The lectin solution at the same concentration of an inhibitor was added to the first tube. A series of twofold dilutions in solution of inhibitor of known concentration was prepared from the solution in the first tube. Thereafter 15 μl of 2% suspension of erythrocytes in PBS was added to each tube. After 10 min incubation the tubes were centrifuged for 30 sec at 500*g* and the result of the reaction was observed. The concentration of the inhibitor that suppressed the hemagglutination by 50% was calculated from a diagram graphed for three different concentrations of the inhibitor [20].

To perform the reaction of hemagglutination inhibition of the lectin with human erythrocytes, the following reagents were used: D-glucose, D-galactose, and lactose (Soyuzkhimreaktiv, Russia); α- and β-methyl-D-galactopyranoside, L-rhamnose, and N-acetyl-D-galactosamine (Chemapol, Czech Republic); N-acetyl-D-glucosamine and N-acetyl-D-lactosamine (Fluka, Switzerland); D-mannose (Bratislava Chemical Institute, Slovakia); 4-nitrophenyl-β-D-galactopyranoside, 4-nitrophenyl-β-D-glucosamine, α-phenyl-N-acetyl-D-glucosaminopyranoside, and 4-nitrophenyl-α-D-mannopyranoside (Serva, Germany); L-fucose (Koch Light, United Kingdom). 4-Nitrophenyl-β-D-glucopyranoside was obtained as described by Kabat and Mayer [21].

To determine the interaction with glycoproteins and polysaccharides, we used water-soluble starch, pig liver glycogen, ovomucoid, thrice re-crystallized ovalbumin (Biolar, Latvia), gum arabic (Loba Feinchemie, Austria), alkaline phosphatase of calf intestine (Serva), inulin [22], yeast mannan [23], and bovine thyroglobulin [24]. Blood group substances H, A, and B were obtained from cystic liquid that was taken after operations on ovaries at patients with the proper blood types. They were purified by a previously described method [25]. The resulting group-specific substance preparations contained 27-32% protein (determined by the Lowry method) and about 70% carbohydrate (identified with 5% phenol and concentrated sulfuric acid). Human immunoglobulin G, fetuin of fetal calf serum, sheep and bovine submaxillary mucin, orosomucoid, and human transferrin were purified by Prof. Maxim Lutsik-Kordovsky, and we therefore express sincere gratitude. According to the data of electrophoresis in 15% polyacrylamide gel this preparations contained not less than 95% of the base material.

Ovomucoid and bovine mucin submandibular gland was desialyzed by treatment with 0.1 N H_2SO_4 at 80°C for 30 min [26].

For study of possible effect of bivalent metal ions on the lectin, we conducted the following experiment. The titer of hemagglutination was measured in the sap from the milk mushroom after its clarification by centrifugation (5000*g* for 10 min). Then it was divided into three portions. The first portion was dialyzed against distilled water, the second against 0.15 M acetate buffer containing 1 mM $CaCl_2$ and $MgCl_2$, and the third against 1% water solution of EDTA (disodium salt). A 1% solution of concanavalin A in 1% solution of NaCl was prepared for control. After measuring the titer of hemagglutination and volume of the solution, the last was also divided into three portions and dialyzed against the same solutions, whereupon total volume and titer of hemagglutination were measured in each sample.

To obtain substances that pass through a dialysis membrane and that probably influence the activity of *L. pergamenus* lectin, the liquid obtained by dialysis against distilled water was dried in a desiccator at 52°C to obtain a dark brown solid mass. It was analyzed for the presence of carbohydrates (using the reaction with 5% phenol and concentrated sulfuric acid), and also probable occurrence of organic acids and their salts by means of precipitation with lead acetate.

To study the influence of brief freezing of mushroom fruit bodies on hemagglutinating activity of the lectins, the fruit body of *L. pergamenus* was cut into two pieces; from one piece the sap was squeezed out and the titer of hemagglutination was measured, and other piece was placed in a refrigerated chamber at −18°C for 12 h. The next day the mushroom material was thawed and the sap was squeezed out. Further the squeezed sap was divided into two portions. The first portion was subjected to dial-

ysis against distilled water with threefold complete replacement of water every 8 h, the second portion was concentrated in a dialysis sack by exposing it to warm air using a ventilator.

RESULTS

Lactarius pergamenus mushrooms are rather thermophilic and drought-resistant. Their mass appearance is typical for July-August (if the weather is hot enough and moist).

It was found that activity of lectins in mushrooms varied greatly even within one mycelium. Significant variability in titers of hemagglutination in sap of individual mushrooms even within one mycelium is characteristic for mushrooms of the *Lactarius* genus. The results of investigation of hemagglutinating activity in sap from *L. pergamenus* mushrooms from one mycelium collected on July 23, 2006 are represented in Fig. 1. From this figure one can see that the titers of hemagglutination differ widely: from 1 : 8 (mushrooms Nos. 1 and 11) to 1 : 2048 (mushroom No. 8), which is more than in 250 times! Although clear correlation between size of mushroom hats and the titer of hemagglutination was not found, smaller in size (junior) mushrooms had higher hemagglutinating activity. A difference in the titers of hemagglutination was more expressed in periods that promoted growth of mushrooms and less in periods that stunted their growth.

The hemagglutinating activity of the mushroom extract or sap greatly depends on external influences. For example, 8-h dialysis of the mushroom saps against distilled water leads to 4-8-fold decrease in activity of the lectin. The same decrease in the lectin activity is observed by freezing of mushroom fruit bodies with their subsequent thawing, or precipitation of a protein fraction with ammonium sulfate or ethanol (see Table 1). However, storage of the sap from mushroom fruit bodies even for a

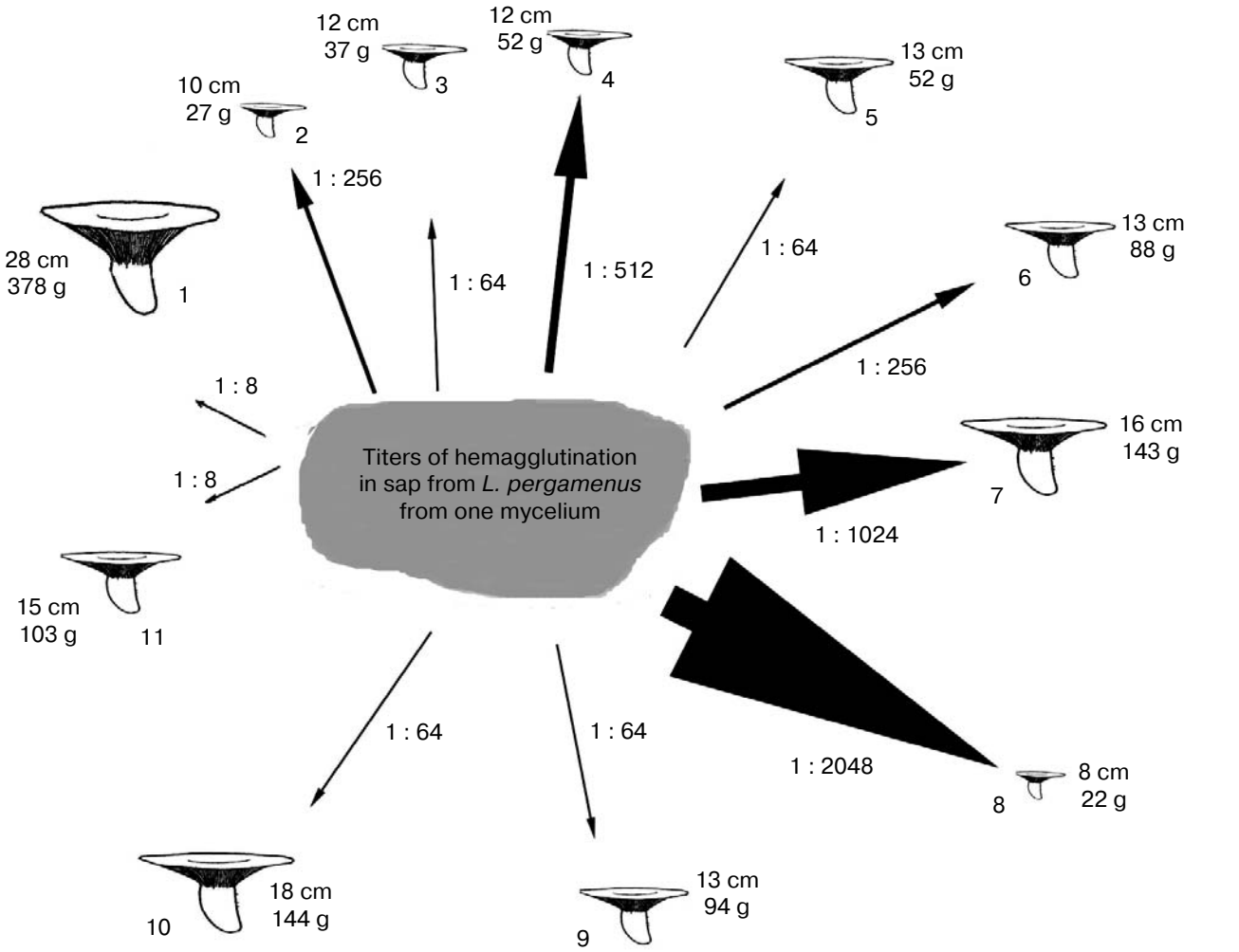


Fig. 1. Hemagglutinating activity in sap from *L. pergamenus* from one mycelium. The thickness of arrows is proportional to the titer of hemagglutination.

Table 1. Effects of storage conditions and other factors on the lectin activity in the fruit bodies of *L. pergamenus*

Percentage of remaining lectin activity				
drying at 52°C	freezing at −18°C	precipitation of protein fraction with ammonium sulfate	precipitation of protein fraction with 96% ethanol at 4°C	10-day storage of the sap at 4°C (in the presence of a preserving agent)
12.5 ± 3%	12.5 ± 3%	25 ± 6%	85 ± 12.5%	100%

Note: Hemagglutinating activity of the lectin was calculated by multiplying the titer of hemagglutination by the volume of solution containing the lectin. In experiments the human erythrocytes of A(II) group were used.

Table 2. Influence of dialysis and concentration of *L. pergamenus* sap on lectin titer of hemagglutination (human erythrocytes of A(II) group)

	Before experiment			After experiment		
	volume of solution	titer of hemagglutination	activity, %	volume of solution	titer of hemagglutination	activity, %
Dialysis	2.1	1 : 64	100	3.8	1 : 16	45
Drying	3.6	1 : 64	100	1.6	1 : 512	356

Table 3. Characteristic of extracted fractions of *L. pergamenus* lectin

	Stage	V, ml	C, mg/ml	Titer of hemagglutination	Yield, %	
					activity	protein
1	Sap of mushrooms	4240	0.8	1 : 32	100	100
2	1st precipitation with ethanol	510	3.76	1 : 256	96.2	56.5
3	2nd precipitation with ethanol	145	6.74	1 : 512	54.7	28.8
4	Affinity chromatography: – absorbed lectin	66	0.37	1 : 256	12.5	0.72
4a	– lectin that passed through the column without retention	161	5.82	1 : 64	7.6	27.7
5	After dialysis against 0.05 M phosphate buffer	12	1.92	1 : 256	2.26	0.68
6	Chromatography on DEAE-Toyopearl	4	5.4	1 : 512	1.5	0.64

Note: The primary activity of the lectin in the sap (4240 ml × titer 32 = 135,680 activity units) and the primary protein content (3392 mg) were taken as 100%. All subsequent numbers in the table were calculated similarly. For example, the solution obtained after affinity chromatography (stage 4) contained 16,896 activity units or 12.45% of the primary activity units. C, concentration of protein in solution; V, volume of solutions.

year in the presence of 0.1% sodium azide as a preserving agent does not lead to the noticeable decrease in activity of the lectin.

The results indicate a possible intramolecular regrouping of the lectin molecule or the loss of some constituent of its molecule. Lectins that require bivalent metal ions for activity, more often calcium and magnesium, are described in literature. Concanavalin A is one of the lectins that is most often used in laboratory works [27]. That is why we have chosen it as a positive control.

After performing the dialysis total volume and the titer of hemagglutination were measured in each sample. In all cases the volume of solution changed by no more than by 25%. In three cases after dialysis of samples of the

sap from the milk mushroom 8-fold decrease in activity of the lectin was observed. A sample of concanavalin A after dialysis against water solution of 1% EDTA-disodium salt lost its activity, while noticeable changes of hemagglutinating activity of concanavalin A by dialysis against water and salt solution were not observed.

The low molecular weight substances that are passing through a dialysis membrane include carbohydrates (presumably mannitol), the amount of which constituted 24% of the original mass, and 43% of the material obtained by dialysis was precipitated by lead acetate, which is peculiar to organic acids or their salts. However, dissolution of these substances in the solution obtained by dialysis and containing the lectin did not result in significant increase of the titer of hemagglutination.

The experiments conducted by freezing of mushroom fruit bodies showed that even brief freezing of mushroom fruit bodies lead to 4-fold decrease in hemagglutinating activity in the mushroom sap.

Results of study of the influence of dialysis and concentration of the lectin solutions are given in Table 2. By dialysis the dialysate lost low molecular weight substances and its volume was slightly increased; in the case of concentration of the solution the total volume was decreased due to evaporation of water and the increase of concentration of both high and low molecular weight substances was observed. Concentration of the lectin solution by drying led to disproportional increase in hemagglutinating activity.

We monitored the efficiency of the lectin purification by determination of protein concentration (Lowry method) and by hemagglutinating activity. Results of these measurements are given in Table 3.

We pay attention to a very low yield in activity of the lectin. A usually used scheme of purification led to 30-70% yield of activity. We obtained only 1.5%. The main loss of activity was observed on stages (dialysis, ion-exchange and affinity chromatography) where lectins usually lost their activity insignificantly. This can only be because of high lability of the lectin molecule.

The obtained lectin preparation showed one diffuse band by disc-electrophoresis in 7.5% polyacrylamide gel in acidic and alkaline buffer solutions and revealed the presence of a band with molecular mass ~16 kDa by electrophoresis in 15% polyacrylamide gel supplemented with 0.1% SDS (Fig. 2).

The *L. pergamenus* lectin on the Toyopearl HW-55 column emerged in the range of molecular masses from

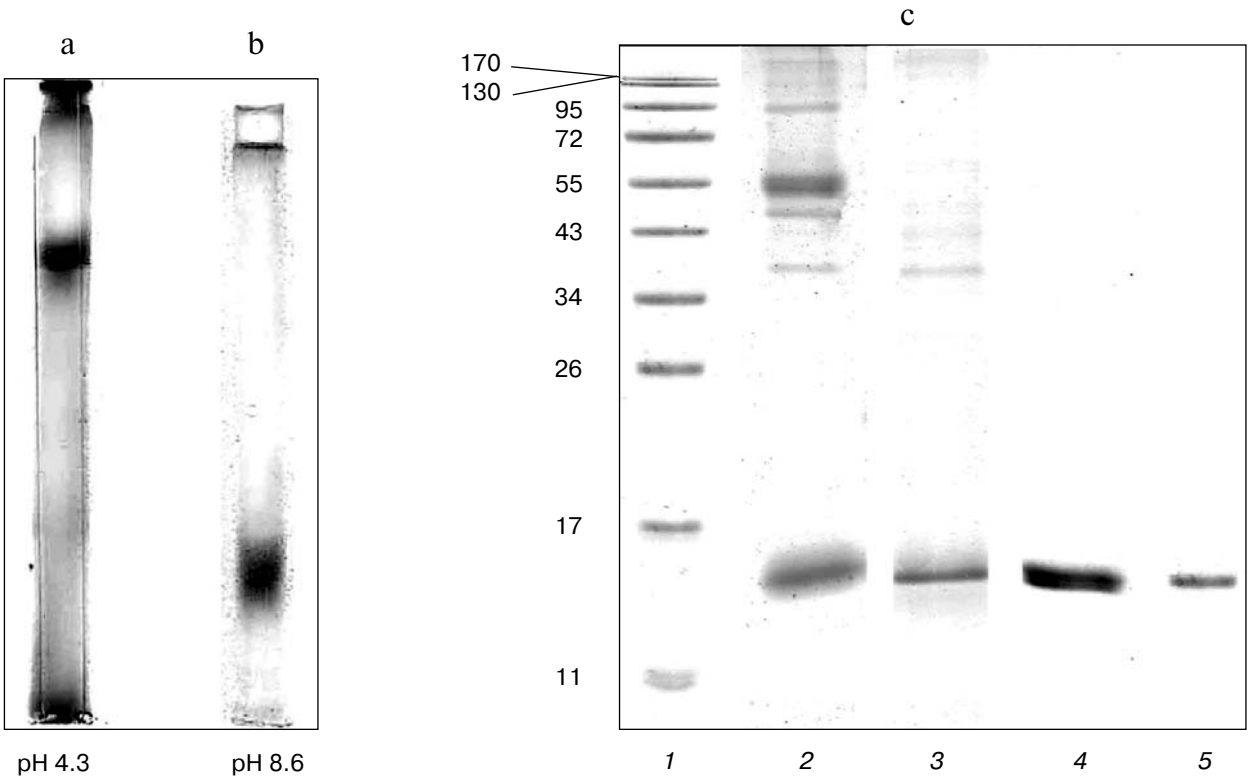


Fig. 2. Electrophoresis of lectin preparation from fruit bodies of *L. pergamenus*. a) Disc-electrophoresis of the lectin preparation in acidic buffer system (pH 4.3); b) disc-electrophoresis of the lectin preparation in alkaline buffer system (pH 8.6); c) electrophoresis of the lectin preparation in 15% polyacrylamide gel supplemented with 0.1% SDS. *1*) Protein markers with known molecular mass; *2*) sap from a *L. pergamenus* mushroom; *3*) the purified *L. pergamenus* lectin after twofold precipitation with ethanol; *4*) the *L. pergamenus* lectin purified by affinity chromatography, without β-mercaptoethanol; *5*) the *L. pergamenus* lectin purified by affinity chromatography in presence of 1% β-mercaptoethanol.

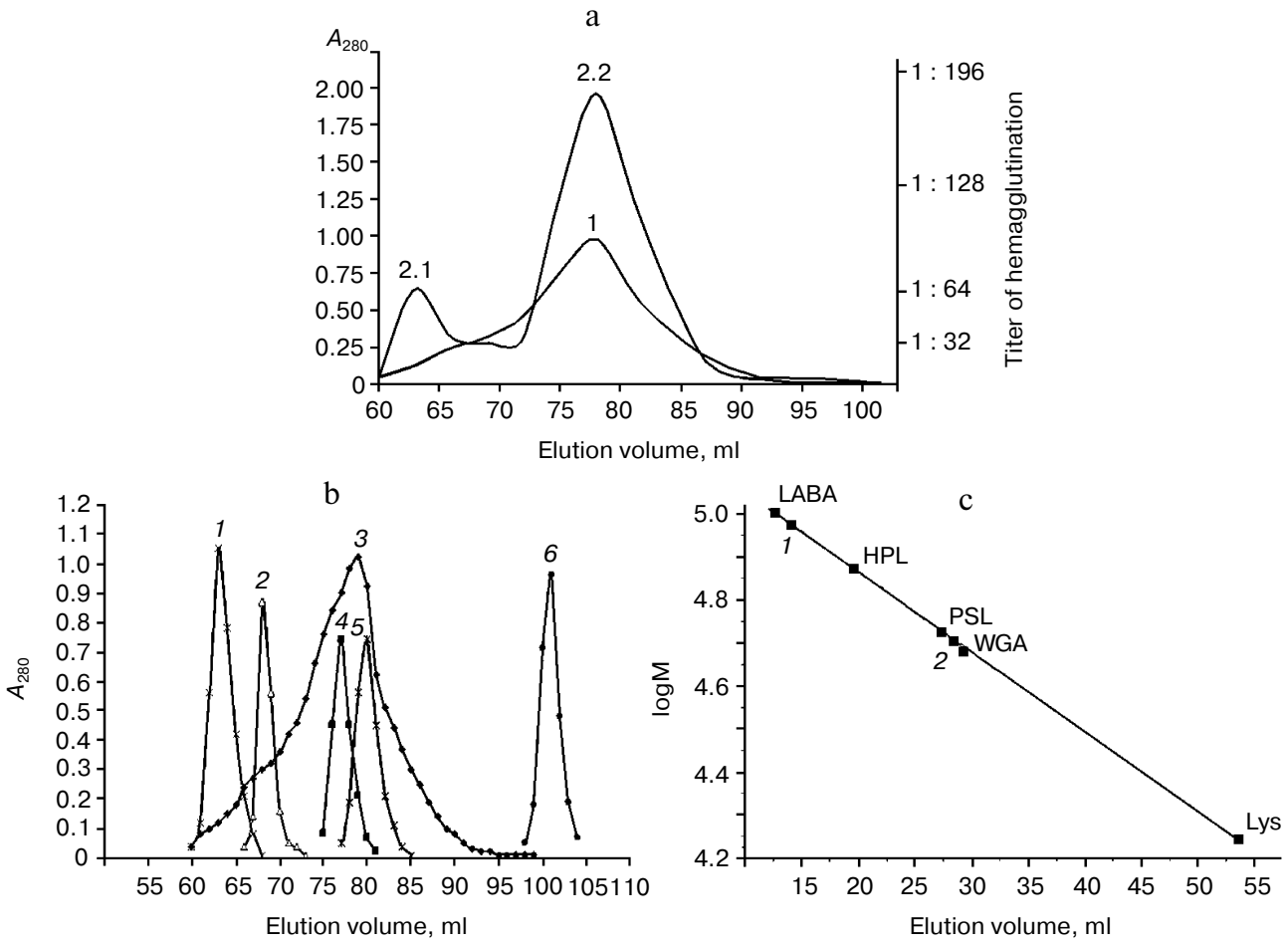


Fig. 3. Molecular mass determination of the *L. pergamenus* lectin by gel chromatography on the Toyopearl HW-55 column (39 × 1.5 cm; volume of the applied sample 1.5 ml; the protein eluted by 0.1 M phosphate buffer, pH 7.4, with flow rate 2 ml/min). a) Lectin yield monitored by optical density at 280 nm (*1*) and by hemagglutinating activity (2.1 and 2.2). b) Graph of *L. pergamenus* lectin elution (*3*) and protein markers on the same column: *1*) golden chain bark lectin (LABA, M_r 100 kDa); *2*) edible snail lectin (HPL, M_r 79 kDa); *3*) *L. pergamenus* lectin; *4*) pea seed lectin (PSL, M_r 48 kDa); *5*) wheat germ agglutinin (WGA, M_r 36 kDa); *6*) egg lysozyme (Lys, M_r 14.3 kDa). c) Graph of logM dependence on elution volume for protein markers and the *L. pergamenus* lectin ((*1*) for a maximum 2.1 and (*2*) for 2.2 accordingly the data in Fig. 3a).

16 to 96 kDa (Fig. 3, a and c) in contrast to most proteins, which on the same column emerged as a sharp peak (see Fig. 3b).

The purified lectin agglutinated human erythrocytes without expressed group specificity at minimal concentration 8-16 μg/ml, although it agglutinated O-type erythrocytes at lower concentrations. Trypsin-treated human erythrocytes were agglutinated at 2-4 times lower concentration of the lectin in comparison with native erythrocytes. The lectin agglutinated erythrocytes of rabbit, guinea pig, dog, and horse at approximately the same level. Erythrocytes of a ram agglutinated at concentration 128-196 μg/ml while erythrocytes of a cow and a goat did not agglutinate at lectin concentration 1 mg/ml.

Various mono- and disaccharides in concentration 100 mM did not inhibit the agglutination of B-type human erythrocytes by the *L. pergamenus* lectin. It should be noted there was only very weak interaction of the lectin with N-acetyl-D-galactosamine, that inhibited hemagglutination by up to 12.5%. Nevertheless, some glycoproteins were good inhibitors of the lectin activity (see Table 4). Among them fetuin of fetal calf serum and group-specific substances A, B, and H of human blood were the strongest. Desialyzation of bovine mucin submandibular gland increased its inhibitory activity by almost 13-fold.

DISCUSSION

When analyzing the results presented in Table 3, very low final yield in activity of the lectin should be noted.

Table 4. Influence of glycosides, monosaccharides, and glycoproteins on agglutination of human erythrocytes of B group by the lectin from fruit bodies of *Lactarius pergamenus*

No.	Inhibitor	Concentration causing 50% inhibition of hemagglutination
1	N-Acetyl-D-glucosamine	0% (100 mM)
2	N-Acetyl-D-galactosamine	12.5% (100 mM)
3	Lactose (DGalβ1→4Glc)	0% (100 mM)
4	N-Acetyl-D-lactosamine (DGalβ1→4GlcNAc)	0% (100 mM)
5	4-Nitrophenyl-β-D-galactopyranoside	12.5% (60 mM)
6	α-Phenyl-N-acetyl-D-glucosaminopyranoside	12.5% (100 mM)
7	Bovine thyroglobulin	11.5 mg/ml
8	Human transferrin	10 mg/ml
9	Orosomucoid (α-glycoprotein)	4 mg/ml
10	Alkaline phosphatase of calf intestine	2.5 mg/ml
11	Human immunoglobulin G	2 mg/ml
12	Sheep submaxillary mucin	1.5 mg/ml
13	Group-specific substance H	0.53 mg/ml
14	Group-specific substance A	0.24 mg/ml
15	Group-specific substance B	0.24 mg/ml
16	Bovine submaxillary mucin	1.5 mg/ml
17	Bovine submaxillary mucin desialyzed	0.12 mg/ml
18	Fetuin of fetal calf serum	0.1 mg/ml
19	Ovomucoid	0% (15 mg/ml)
20	Asialoovomucoid	0% (15 mg/ml)

Note: The following carbohydrates are not included in the table since they do not interact with the lectin of interest at designated concentrations: D-glucose, D-galactose, D-mannose, L-fucose, α-methyl-D-galactopyranoside, β-methyl-D-galactopyranoside, and L-rhamnose (all at concentration 100 mM) as well as 4-nitrophenyl-β-D-glucopyranoside and 4-nitrophenyl-α-D-mannopyranoside (both at concentration 20 mM). Polysaccharides (starch, inulin, yeast mannan, gum arabic) and glycoproteins (ovalbumin), which do not interact with the lectin at concentration 10 mg/ml, are not included in the table as well.

The main loss of activity was observed on stages where its loss was unexpected. The amount of the lectin that passed through the column and absorbed on the column, measured by activity, is in total 20.1%, while the total amount of protein measured by the Lowry method is 98.7%. Activity of the lectin also decreased both after dialysis and ion-exchange chromatography. However, 1% solution of the purified lectin shows sufficient stability and possesses high hemagglutinating activity (1 : 1024, B-type human erythrocytes).

The logical question arises: do mushrooms with higher hemagglutinating activity contain proportionally higher amount of the lectin? The experimental data make this in doubt.

While comparing the results of molecular mass determination of polypeptide chains, obtained by electrophoresis in 15% polyacrylamide gel supplemented with SDS, with results of molecular mass determination by gel chromatography on the Toyopearl HW-55, we conclude that the lectin from *L. pergamenus* consists of a few subunits that are weakly linked with each other. Two maxima of hemagglutinating activity (Fig. 3a) conform to hexameric ($(16)_6$ = 96 kDa) and trimeric ($(16)_3$ = 48 kDa) structure of the lectin molecule. The hexamer possesses

higher specific hemagglutinating activity. If one divides the titer of hemagglutination by the value of optical density we get the number 266 for the first maximum (2.1 – 96 kDa) and 131 for the second (2.2 – 48 kDa), so the hexamer possesses two times higher specific activity. However, the lectin does not emerge from the column as two sharp peaks, but rather it stretches between them. This is probably caused by disintegration of the molecule in subunits even during the procedure of gel chromatography. The process does not result in formation of trimer but apparently goes on further, because the decrease of the lectin activity is observed at the further stages of purification. A separate subunit most probably has one binding site for carbohydrates and does not possess hemagglutinating activity. Graphically this process can be expressed by the following equilibrium:

$$(16)_6 \leftrightarrow 2(16)_3 \leftrightarrow 6(16).$$

These reasonings are confirmed by the results presented in Table 2. The titer decreased four times by dialysis of the mushroom sap against the expected twofold decrease (due to the increase of volume). And vice versa, after concentration of the solution by preliminary drying in a dialysis sack, the lectin activity disproportionately increased by 3.6-fold, that is possible only due to formation of the aggregated molecule with higher numbers of active centers. Thereby, it is possible to assert that activity of the *L. pergamenus* mushroom lectin may be regenerated under certain conditions.

Apparently, similar processes take place in vegetative mushrooms. Probably, intensively growing fruit bodies of *L. pergamenus* have higher content of hexameric form of the lectin. In mushrooms with slower growth both lower total content of the lectin and its hexameric form is observed.

Variations of lectin activities in plants were also observed by us before. For example, in spring, during motion of sap, activity of lectins in bark of arboreal and shrubby species is able to increase by 1000 and more times [28, 29]. It is possible that similar lectins are responsible for transport function in plants and mushrooms.

It should be noted that hexameric structure of a molecule is a phenomenon quite rare in lectins; no more than 10 such molecules are described (see [4-6]). The most known example of a lectin of similar type is a lectin from an edible snail (*Helix pomatia*). This lectin possesses very high hemagglutinating activity, but in contrast to the *L. pergamenus* lectin its molecule is stable. Lectins previously purified by us from *L. rufus* and *L. torminosus* [16, 30] had similar to *L. pergamenus* lectin molecular structure; it is quite possible that a similar molecular structure is typical for the majority of lectins from the *Lactarius* genus.

The absence of interaction of the lectin with a number of monosaccharides and high affinity of the *L. pergamenus* lectin to fetuin of fetal calf serum and to the group-specific substances of human blood are worth noting. The rather high affinity of the lectin to sheep and bovine mucin, immunoglobulin G of a human blood serum, and to alkaline phosphatase of calf intestine should be noted. However, transferrin and bovine thyroglobulin had relatively low affinity to the lectin. Therefore, the *L. pergamenus* lectin, like lectins from *L. rufus* and *L. torminosus*, has prolonged binding sites for carbohydrates, with maximal affinity to oligosaccharides of complex unknown structure. Ovomucoid and ovalbumin, which have terminal residues of N-acetyl-D-glucosamine that are linked to D-mannose, did not interact with the lectins at concentration 1%.

In the structure of glycoproteins with which the lectin weakly interacts there are disaccharide units DGalβ1-4DGlcNAc, affinity to which the lectin did not display (Table 4). However, the DGalβ1-3DGlcNAc subterminal disaccharides are in the structure of group-specific substances of human blood and fetuin of fetal calf serum (chain of type 1). According to literature data fetuin contains three three-antenna carbohydrate chains, where each for eight lactosamine units there is one isolactosamine unit. There are similar isolactosamine units in glycopeptides of alkaline phosphatase (see Table 5). The content of DGalβ1-3DGlcNAc sequences in bovine submaxillary mucin and thyroglobulin is lower, which is possibly the reason that the lectin interacts with them more weakly. At the same time, availability of low hemagglutinating activity at N-acetyl-D-galactosamine and 4-nitrophenyl-β-D-galactopyranoside (Table 4) suggests that ligands of the *L. pergamenus* lectin may be disaccharides that contain D-galactose and N-acetyl-D-galactosamine, as for instance DGalβ1-3DGalNAc. The *Lactarius deterrimus* lectin, which is described in literature and belongs to the same genus as *L. pergamenus* lectin, possesses similar specificity [15].

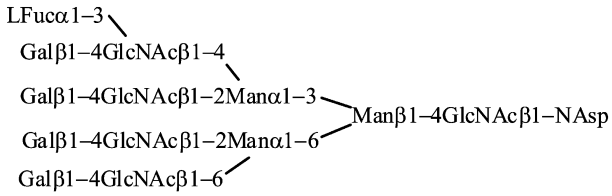
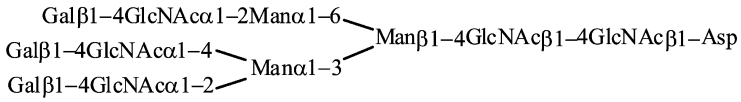
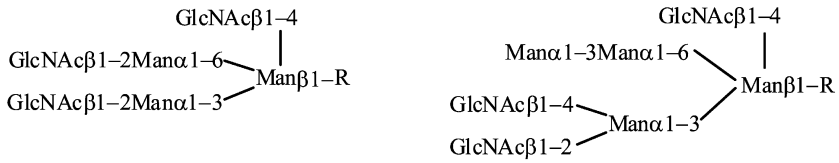
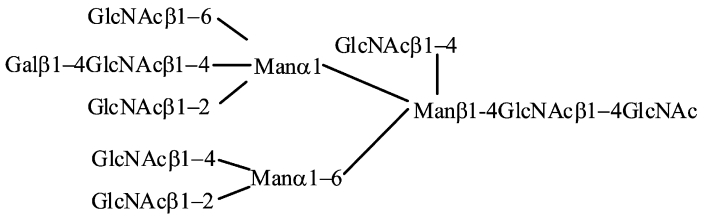
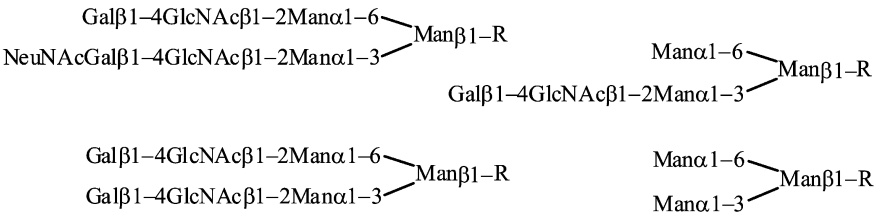
The second possible ligand with which the investigated lectin interacts may be Galβ1-3GlcNAc, as we did not find differences in properties of the lectin regarding residues of D-GalNAc or D-GlcNAc but paid attention to the advantage of β1-3 glycosidic linkage over β1-4 linkage. Similar cases are described in the literature (see [4, 5]). Also one can suppose possible strengthening influence of L-fucose or D-galactose residues in trisaccharide structures of glycans, which are available in group-specific substances, orosomucoid, and alkaline phosphatase. In spite of the above-mentioned studies the precise carbohydrate specificity of the lectin has not been determined yet and it still remains an outstanding problem.

Thus, as a result of our study a novel lectin from the fruit bodies of *L. pergamenus* was obtained. It was found that the lectin very easily loses its hemagglutinating activity by minor change of solution pH, by precipitation with ammonium sulfate and ethanol, freezing, and dialysis against water. The possible reason is that instability of quaternary structure of the lectin. It is possible that con-

Table 5. Structure and content (%) of carbohydrate part of glycoproteins

Glycoprotein carbohydrate (%)	Structure	References
1	2	3
Alkaline phosphatase of a calf intestine (see the note below) (8-17%)	A Galα1–3Galβ1–4GlcNAcβ1–2Manα1–3 Galα1–3Galβ1–4GlcNAcβ1–2Manα1–6 Manβ1–4GlcNAcβ1–4GlcNAcβ1-Asn GlcNAcβ1-4 B Galα1–3Galβ1–4GlcNAcβ1–2 Galα1–3Galβ1–4GlcNAcβ1–4 Manα1-3 Galα1–3Galβ1–4GlcNAcβ1–2 Galα1–3Galβ1–3,4GlcNAcβ1–6 Manα1-6 Manβ1–4GlcNAcβ1–4GlcNAcβ1-Asn GlcNAcβ1-4 Fucα1-6	[32]
Transferrin (5.5%)	NeuAcα2–6Galβ1–4GlcNAcβ1–2Manα1–3 NeuAcα2–6Galβ1–4GlcNAcβ1–2Manα1–6 Manβ1–4GlcNAcβ1–4GlcNAc-Asp	[33]
Bovine thyroglobulin (8.03%)	Neu-Gal(β)GlcNAc(β) Neu-Gal(β)GlcNAc(β) Gal(β)GlcNAc(β)Manα1–3 Manα1–6 Manβ1–4GlcNAcβ1–4GlcNAc-Asp	[24]
H-group-specific substance	Type 1 chain: LFucα1–2Galβ1–3DGlcNAcβ1–3R Type 2 chain: LFucα1–2Galβ1–4DGlcNAcβ1–6R	[34]
A-group-specific substance	Type 1 chain: LFucα1–2Galβ1–3DGlcNAcβ1–3R DGalNAcα1–3 Type 2 chain: LFucα1–2Galβ1–4DGlcNAcβ1–R DGalNAcα1–3	[34]
B-group-specific substance	LFucα1–2Galβ1–4DGlcNAcβ1–R Galα1–3	[34]
Bovine submaxillary mucin (75.5%)	NeuNAc 2-6 GalNAcα–O–Ser/Thr (Tn) DGalβ1–3GalNAc–α–O–Ser/Thr (T) LFucα1–2Galβ1–αGalNAc–O–Ser/Thr (H) LFucα1–2Galβ1–αGalNAc–O–Ser/Thr (A) DGalNAcα1–3	[35]

Table 5. (Contd.)

1	2	3
Orosomucoid		[36]
Fetuin (22%)		[37]
Ovalbumin (3.2%)		[38]
Ovomucoid (25%)		[39]
Immunoglobulin G (2.5%)		[38]

Note: A, a glycopeptide structure that is connected to Asp249 of alkaline phosphatase of calf intestine; B, a glycopeptide structure that is connected to Asp410 of alkaline phosphatase of calf intestine [32].

centration of solutions promotes the formation of higher molecular weight forms. Similar phenomena occur in nature during growth of not only *L. pergamenus*, but also other mushrooms of *Lactarius* and *Russula* genera.

However, covalent linkage with horseradish peroxidase stabilizes the conjugate molecule and allows using it as a histochemical reagent. Particularly, the *L. pergamenus* lectin labeled by us with horseradish peroxidase was used for the study of carbohydrates of rat kidney tissues. High affinity of the lectin to epitheliocytes of brush border of proximal tubules of nephrons from newborn rat kidney was detected. The renal structures of adult rats somewhat changed the expression of glycoproteins: reactivity of podocytes and basal membranes to the labeled lectin increased. The lectin selectively began to contour luminal surface of discharging tubules [31]. Therefore, the *L. pergamenus* lectin can be a valuable reagent in histochemical research.

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