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Isolation and Structural Characterization of *Rhizoctonia solani* Fungal Lectin.

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ABSTRACT

In this paper was isolated and purified the lectin of *Rhizoctonia solani* according to the scheme, comprising the following stages: obtaining protein extract, salting out proteins by the crystalline ammonium sulfate, dialysis, ion exchange chromatography using Bio-Scale ™ Mini Macro-Prep High Q and DEAE-Sepharose columns, gel filtration on the column with Sephadex G-50. The most complete lectin removal was observed at 65% saturation of fungal mycelium buffer extract by ammonium sulfate in 12 hours of salt acting. Ion exchange chromatography by using Mini Macro-Prep High Q and DEAE-Sepharose columns allowed to increase the degree of lectin purification in 26.5 and 41.8 times, respectively, relative to the initial fraction of the glycoprotein. The highest degree of purification was obtained after gel filtration of *R. Solani* lectin using column with Sephadex G-50. As a result of the experiments there was obtained lectin preparation with 107.54% degree of purification and specific activity of 1.0×10 5 U/mg. The output on the protein activity was 17.2%. By electrophoresis method in denaturing conditions and by gel filtration using Sephadex G-100 it was revealed that the lectin of *Rh. solani* is a low molecular weight glycoprotein comprising two subunits of molecular weight 18.0 ± 1.5 kDa. The total molecular weight of the native protein is 36.0 ± 2.0 kDa.

Keywords: lectin, Rhizoctonia solani, purification.

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INTRODUCTION

Lectins are glycoproteins with specific biological properties, which can recognize and bind sugars without causing any chemical transformations towards them [1, 2]. They are involved in interactions between cells, regulate the processes of mono-, di - and polysaccharides transportation, control the differentiation of cells into tissues [3, 4], play a key role as signaling molecules in the responsiveness of different organisms [5]. Lectins are used as antiviral drugs, for blood grouping, for allocation of lymphocytes immature forms in bone marrow transplantation, and they are important reagents in proteins chemistry [5, 6]. In addition, carbohydrate-binding proteins have attracted the attention of lectinologists as compounds that have antimicrobial, antitumor and immunostimulatory effects.

Lectins were first found and characterized in plants. It was further established that this group of proteins was found in various organisms including animals, bacteria and fungi [7]. It should be noted that in many biological objects it is frequently observed the formation of not only one lectin, but the group of proteins or glycoproteins having a certain structure and specificity. In this respect, the lower fungi are no exception, therefore, to obtain lectin with a certain structure and specificity is required the selection and implementation of appropriate conditions of its isolation and purification.

The objective of this research was isolation and characterization of *Rhizoctonia solani* lectin.

MATERIALS AND METHODS

As an object of the research were taken *Rhizoctonia solani* fungi from the Museum of fungal cultures at the Department of biochemistry and biotechnology of the Fundamental medicine and biology Institute, Kazan Federal University.

Strain cultivation and isolation of total lectin fraction from fungal extract was carried out according to previously published methods [8]. Lectins isolation from the fungal mycelium was carried out on the 8th day of cultivation.

The activity of lectins was determined by direct reaction of hemagglutination with native erythrocytes of human 1st blood group. Erythrocytes for hemagglutination reactions were obtained by the method of Lutsik et al. [9].

Concentration of lectin fractions was carried out by salting out the proteins of the crystalline ammonium sulphate from 20 mM Tris-HCl buffer extract of fungal mycelium, pH 7.8. Contained in the supernatant lectins were salted out at the gradual addition of (NH4)2SO4 to the supernatant to the saturation of 85%.

The removal of salt from the precipitated protein was performed in 20 mM Tris-HCl buffer solution (pH 7.8), using dialysis bags with a width of 25 mm and a pore diameter of 12-14 kDa (the production of Orange Scientific, Belgium) under stirring on a magnetic stirrer for 48 hours at 4°C.

Protein fractions separation of the total *Rh. solani* lectins preparation was carried out using a chromatographic system of low pressure BioLogic LP (Bio-Rad, USA) using column Bio-Scale™ Mini Macro-Prep High Q, DEAE-Sepharose, Sephadex G-50. Prior to depositing the sample on the column there was carried out the filtration of protein through a special sterile disposable nozzle membrane Millex (d 33 mm, d of pores 0.45 µm, polietilsulfon, Millipore).

Sequential stage protein purification was performed using High Q column (Bio-Rad, USA) column and a column with diethylaminoethyl Sepharose, pre-balanced with a 20 mM Tris-HCl buffer, pH 7.8. The elution was conducted with a linear gradient with increase of the NaCl concentration from 0 to 1M.

Obtained after chromatography lectin fractions were concentrated on a rotary evaporator and desalted on a gel-filtration column Bio-Scale™ Mini Bio-Gel® P-6 (Bio-Rad, USA). The protein elution from column was performed with a 20 mm Tris-HCl buffer solution (pH 7.8), with a flow rate of 1 ml/min.

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Further purification of the obtained lectin *Rh. solani* fractions was carried out using Sephadex G-50 (Sigma, Sweden) column. Elution of lectin at this stage was performed with a 20 mM Tris-HCl buffer, pH 7.8, at a flow rate of 0.5 ml/min. Haemagglutinating lectin fractions were collected, concentrated and stored at -18 ° C.

The degree of lectin *Rh. solani* sample purity was determined by electrophoresis in 12.5% PAGE by Laemmli [10]. Sample separation was performed in the cell for vertical electrophoresis Mini-PROTEAN ® Tetra Vertical Electrophoresis Cell (Bio-Rad, USA) in Tris-glycine buffer system (pH 8.3). Fixation of the gels was carried out for 30 minutes in a 10% acetic acid solution containing 45% ethanol. Staining of samples was performed using 0.6% of the dye Coomassie Brilliant Blue R-250 (0.25% of Coomassie, 10% glacial acetic acid, 50% ethanol) or 0.4% silver nitrate solution (0.4% AgNO3, 0.09% NaOH and 0.2% ammonia). Scanning, visualization and analysis of protein gel bands were carried out on the device ChemiDoc™ MP Imaging System (Bio-Rad, USA) using the software package Image Lab™ Software.

Lectin's molecular weight determination was carried out by the method of SDS-electrophoresis in denaturing conditions (SDS-PAGE) and by the method of gel filtration using Sephadex G-100.

Electrophoretic determination of lectin's molecular weight was carried out in 12.5% PAGE in the presence of SDS. As protein markers were used PageRuler Pre/Unstained Protein Ladder commercial kits with a molecular weight in the range from 10 to 200 kDa and from 10 to 170 kDa.

Lectin's molecular weight determination was performed using Econo - Column (1.5 x 50 cm, Bio-Rad, USA) with Sephadex G-100 (average, particle diameter 40-120 µm), with a balanced Tris-HCl buffer solution (pH 7.8). As the protein markers were used lysozyme (14 kDa), α-chymotrypsin (26 kDa), ovalbumin (34 kDa), and bovine serum albumin (70 kDa). Samples were applied to the column in a volume of 1 ml (2 mg/ml) and eluted with the same buffer at a flow rate of 0.5 ml/min Determination of protein fractions concentration was carried out spectrophotometrically using NanoDrop ND-2000 (Thermo Fisher Scientific Inc., USA).

RESULTS AND DISCUSSION

Currently there are no universal methods to isolate and purify lectins. The main cause of the difficulty and the complexity of obtaining homogeneous drug were the lectins' structural heterogeneity, as well as a range of difficulties due, primarily, to the instability of the lectins and their activity under the influence of external conditions [11]. That is why in most cases for these purposes are used powerful combinations of various types of chromatography, such as gel filtration, ion-exchange chromatography, hydrophobic chromatography, affinity chromatography, as well as dialysis, proteins precipitation with organic solvents and salts.

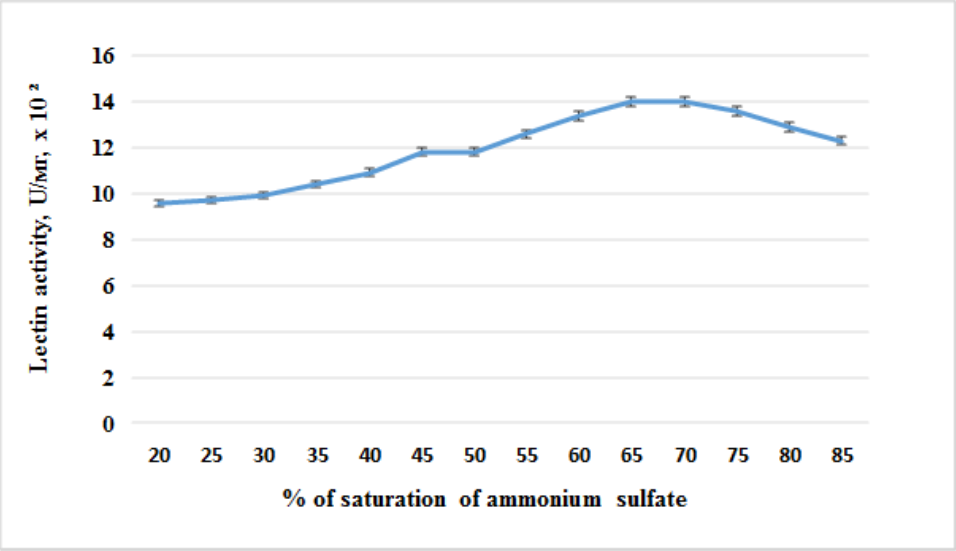

Figure 1: Lectin activity of *R. solani* dialysate under protein precipitation by ammonium sulfate at various concentrations.

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In our experiments there was developed and standardized the scheme of lectin preparation isolation and purification for *Rh. solani* fungi. Lectin preparation purification was carried out according to the six-stage scheme at 0-4°C, which included obtaining a protein extract, proteins salting out with a crystalline (NH4)2SO4, dialysis followed by filtration using Millex syringe filters, ion-exchange chromatography using column Bio-Scale™ Mini Macro-Prep High Q, DEAE-Sepharose, gel filtration using the column with Sephadex G-50.

Initial isolation of lectin *R. solani* was performed by protein precipitation with ammonium sulfate in 20 mM Tris-HCl of buffer fungal mycelium extract, pH 7.8, followed by selected fractions dialysis. In our variant of the experiment, the taken percentage of the solution (NH4) 2SO4 (20%) saturation precipitated only a part of the protein, therefore, were conducted the experiments on the effect of solution (NH4) 2SO4 saturation % on the degree of product extraction according to the specific activity (Figure 1).

The results of the research showed that the highest specific activity of lectin producer was observed at 65-70 % saturation of ammonium sulphate solution. If the original extract had a specific lectins activity - 0.95 x 10 3 ± 5.14 U/mg, at protein concentration - 17.2 ± 0.1 mg/ml, then at 65-70 % solution saturation by ammonium sulphate the output of lectin amounted to 103.5 %, protein's specific activity increased in 1.47 times and amounted to 1.4×10 4 ± 5.9 U/mg. In other experiment's variants the values of lectin extraction degree were significantly lower. The time required for the studied lectin's precipitation was 12 hours.

Further purification of *Rh. solani* lectin preparation was carried out by using two successive ion exchange chromatographies. At the first stage ion exchange chromatography using Bio-Scale ™ Mini Macro-Prep High Q column was performed.

The lectin elution with a buffer solution containing the gradient of NaCl allowed separating proteins into individual fractions. The study of their hemagglutinine activity showed that only fractions 7 and 8 could be attributed to the lectins (Fig. 2). Fractions having hemagglutinine activity were combined and examined for protein content and total hemagglutinins activity (Table 1).

The results of the research showed that with the help of chromatography using Bio-Scale™ Mini Macro-Prep High Q column we managed to remove a significant amount of ballast proteins. Received preparation had a sufficiently higher purification degree compared to the initial lectin's fraction; specific activity increased in 39 times, the total yield of lectins' protein activity was 25.9%.

The number of authors used a column with DEAE-Sepharose [12, 13] for a deep lectins' cleaning in their works. In this embodiment, lectin's fraction chromatography using this column allowed to increase the lectin fraction purification degree in 61.45 times (Figure 2; Table 1). The overall yield of lectins' protein activity remained at the same level.

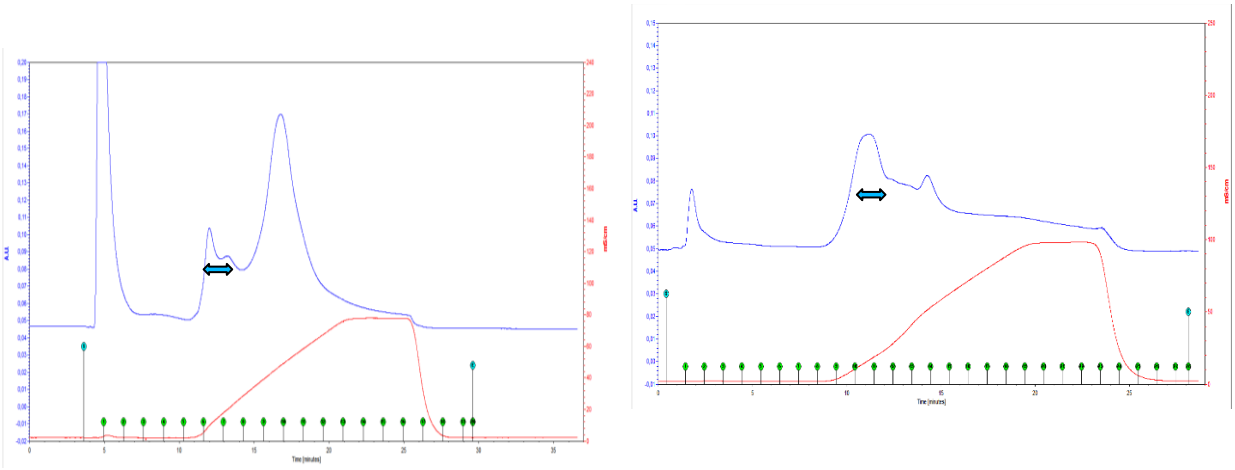

Figure 2: The chromatogram of *R. solani* fungal lectin preparation after ion exchange chromatography using Bio-Scale™ Mini Macro-Prep High Q column (on the right) and DEAE-Sepharose (left), balanced by Tris-HCl buffer. Arrows indicate fractions possessing hemagglutinine activity.

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Given that the used methods and techniques allowed to only partially purify the protein, in further studies for *Rh. solani* lectin preparation purification was applied fractionation using gel - filtration column of Sephadex G - 50.

As a result of the gel-filtration using column with Sephadex G - 50 was able to completely get rid of the accompanying proteins and increase the activity of *Rh. solani* lectins in 107.54 times relative to the lectins activity in the crude fungal extract (Table 1).

Table 1: Isolation and purification of lectin from *Rhizoctonia solani* crude extract.

Purification	Volume, ml	Protein, mg/ml	Total protein, mg	HA titer	Total HA[1], U	Specific HA[2], U/mg	Purification degree	Output, %
Crude extract	5.8	17.2	99.8	16384	95027	0.95×10^3	1	100
Fractionation of $(NH_4)_2SO_4$ and dialysis	3	23.4	70.2	32768	98304	1.4×10^3	1.47	103.5
Bio-Scale™ Mini Macro-Prep High Q	3	0.22	0.66	8192	24576	3.7×10^4	39.1	25.9
DEAE-Sepharose	3	0.14	0.42	8192	24576	5.9×10^4	61.45	25.9
Sephadex G-50	1	0.16	0.16	16384	16384	1.0×10^5	107.54	17.2

[1]Total hemagglutinating activity × HA = volume (ml).
[2]Specific hemagglutinating activity = U / total protein (mg).

Thus, used techniques and methods of protein purification allowed us to obtain a lectin preparation with a specific activity of 1.0×10 5 U/mg.

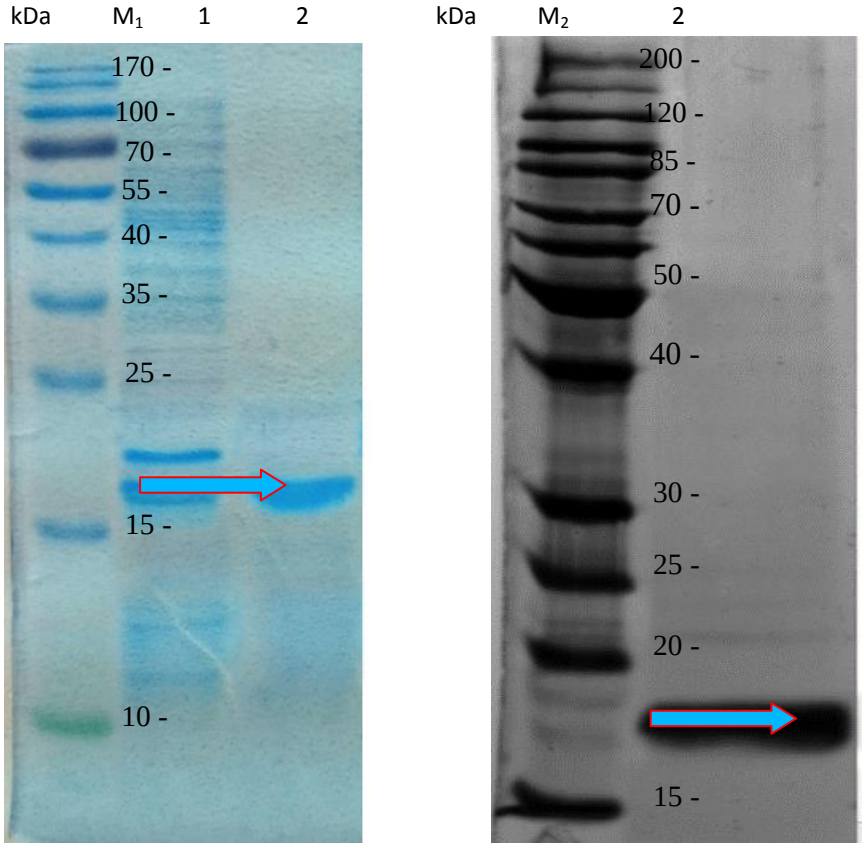

Figure 3: Electrophoregram of the purified *R. solani* lectin was shown by using Coomassie R – 250 dye (left) and silver nitrate (right): M1 – marker proteins of PageRuler™ Prestained Protein Ladder (Fermentas) with known molecular weight of 10 to 170 kDa; M2 - marker proteins of PageRuler™ Unstained Protein Ladder (Fermentas) with molecular

mass from 10 to 200 kDa; 1 – lectin preparation after ion-exchange chromatography (column Bio-Scale™ Mini Macro-Prep High Q); 2 - lectin preparation after ion-exchange chromatography (column Bio-Scale™ Mini Macro-Prep High Q and DEAE-Sepharose) and gel-filtration on Sephadex G-50. The arrow shows the lectin of *R. solani*.

The homogeneity of the obtained lectin preparation of *Rh. solani* and its molecular weight was determined by electrophoresis in a 12.5 % polyacrylamide gel in denaturing conditions. In order to obtain more precise and reliable information coloring was performed with two dyes (Coomassie R – 250 and silver nitrate solution). Proteins staining by silver nitrate provides higher sensitivity in 1-2 times, allowing to paint and reveal the impurities of proteins and proteins at low concentrations, however, this method of coloring is very sensitive to temperature, requires a high purity of the solutions and is more time-consuming [14].

Lectins electrophoresis in polyacrylamide gel and buffer with SDS system in the process of their purification using chromatographic techniques showed a gradual decrease in the number of protein bands. After two ion-exchange chromatographies using column Bio-Scale™ Mini Macro-Prep High Q, DEAE-Sepharose and gel-filtration using a column with Sephadex G-50 on the electrophoregram remains one bright band, characteristic of high purity protein (Fig. 3). The obtained lectin of *Rh. solani* had a molecular weight of 18 ± 1.5 kDa.

However, native lectin molecular weight determination by the method of gel filtration using Econo - Column (1.5 × 50 cm) with Sephadex G-100 revealed that the molecular weight of the lectin *R. solani* is within 36.0 kDa ± 2.0 (Figure 4). Comparison of the results obtained by electrophoresis in denaturing conditions and by gel filtration of the native protein indicates that *R. solani* lectin has a dimeric structure and consists of 2-identical subunits, each of them having a molecular weight of 18.0 ± 1.5 kDa, and 36 kDa in total. This lectin's molecular weight is close to the molecular weight *of Arthrobotrys oligospora, Macrophomina phaseolina, Sclerotina trifoliorum* lectins [7, 15]. Homodimer lectin structure was shown for lectins of *Sclerotina trifoliorum, Sclerotina miyabeana, Botrytis sinerea* [15].

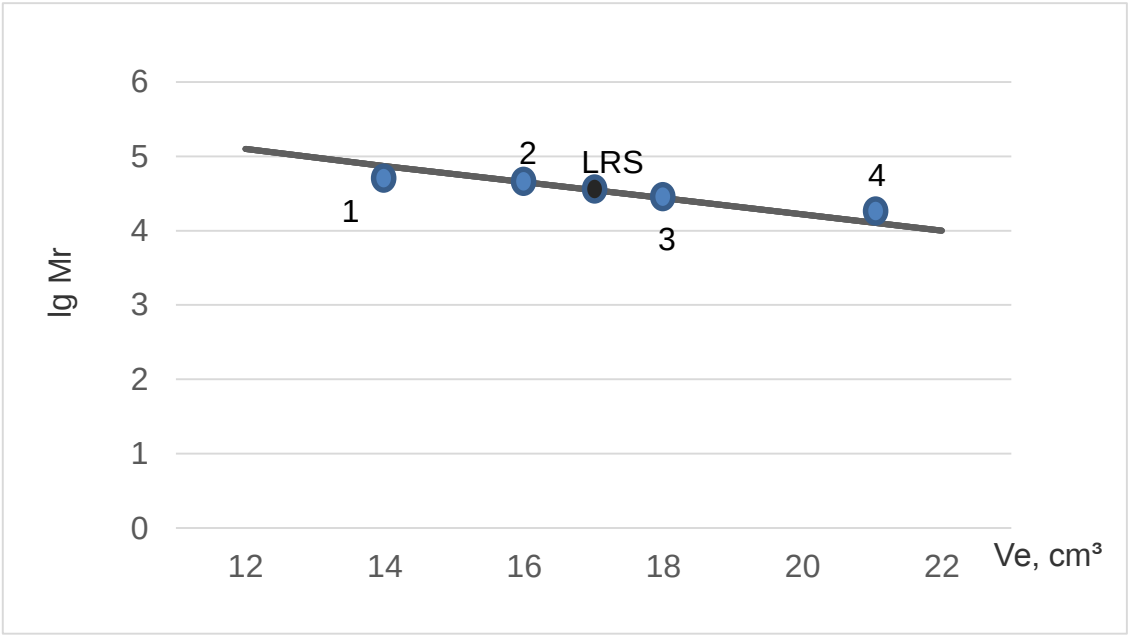

Figure 4: Determination of *R. solani lectin* molecular weight: 1 - bovine serum albumin (70 kDa), 2 ovalbumin (45 kDa), 3 - α-chymotrypsin (26 kDa), 4 - lysozyme (14 kDa). The calibration curve was constructed using a linear logarithm dependence of protein molecular weight (lg M) from the volume of its elution from the column (Ve).

SUMMARY

As a result of the conducted research there was received highly purified lectin of *Rh solani*.

The most complete lectin extraction from buffer solution of mycelial extract was observed when adding ammonium sulfate in a concentration of 65 %.

Ion-exchange chromatography on a column with Bio-Scale™ Mini Macro-Prep High Q and DEAE-Sepharose and gel filtration using Sephadex G-50 allowed to obtain active lectin with a high degree of purification.

A high purification degree of the selected *Rh. solani* lectin is confirmed by the presence of a single band on the electrophoregram.

By the method of SDS-electrophoresis in denaturing conditions and by gel filtration on Sephadex G-100 it was revealed that lectin *Rh. solani* is a low molecular glycoprotein with a molecular weight of 36.0 ± 2.0 kDa. By structure lectin *Rh. solani* is a dimer with subunits size of 18.0 ± 1.5 kDa. These data are consistent with foreign studies, where it was found that isolated from microscopic fungi lectins are low molecular weight glycoproteins with a molecular weight of 15-90 kDa, with a prevalence range in size 23-36 kDa [7].

CONCLUSION

Thus, the developed scheme of lectin isolation and purification from the mycelium of *Rh. solani*, incliding protein concentration from the buffer extract of the fungal mycelium with the subsequent step purification using Bio-Scale™ Mini Macro-Prep High Q, DEAE-Sepharose and Sephadex G-50, allowed to obtain a highly purified lectin preparation with a specific activity of 1.0×10 5 U/mg. This scheme of the preparation isolation and purification can be recommended for obtaining lectins of other micromycetes.

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