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Physicochemical properties of micromycetes *Alternaria alternate* 4 lectin

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Abstract

The physicochemical properties of the lectin of the lower fungus, micromycetes *Alternaria alternate* 4 have been studied. It was found that the lectin of the micromycetes *Alternaria alternate* 4 is stable over a wide range of temperatures and pH values (5-70°C and pH 6.0-9.0). The greatest activity of lectin was observed at a temperature of 5-50 °C and a pH of 6.5-8.5. The increased activity of *Alternaria alternate* 4 lectin was facilitated by the presence of divalent Ca^{2+}, Mn^{2+}, and Zn^{2+} ions in the reaction mixture, which indicates that *Alternaria alternate* 4 lectin is a metal-dependent glycoprotein. *Alternaria alternate* 4 lectin had the ability to bind simple carbohydrates. The activity of *Alternaria alternate* 4 lectin was inhibited to a greater extent by galactose, which indicates that *Alternaria alternate* 4 lectin belongs to the group of galactose-binding lectins.

Keywords: Lectins; Micromycetes; *Alternaria alternate*; Physicochemical properties

1. Introduction

Over the past few decades, lectins have been actively studied due to their diverse biological properties, including antibacterial, antifungal, insecticidal, and antiviral activity [1-3]. Previously, most of the research was focused on plant and animal lectins, whereas over the past few years there has been an increased interest in fungal lectins, due to their significant potential in the field of medicine and biology. Micromycetes lectins have been described as chemotherapeutic agents, mitogens, and drug delivery vehicles to necessary targets [4-7]. The advantage of lower fungi in this area is their genetic diversity, rapid growth, and virtually unlimited biosynthetic activity, including the active synthesis of lectins with diverse physicochemical properties. It has been established that micromycetes lectins can function in a wide range of temperatures and pH values, have different dependence on metal ions, and have specificity for interaction with various carbohydrates, however, these indicators differ significantly depending on the genus and species of micromycetes. Thus, *Fusarium solani* and *Rhizopus stolonifer* fungal lectins maintained their activity at temperatures of 70-80°C, while *Aspergillus sparsus* lectins maintained their activity up to 100°C. At the same time, *Aspergillus sparsus* lectin had high stability at pH values from 1.5-12.5 [8]. However, there are micromycetes lectins of the same genus, for example, *Aspergillus nidulans*, which were very sensitive to the temperature and pH of the reaction mixture [9]. Micromycetes lectins react differently to the presence of metals in the reaction medium. Many of them do not require metal ions for their activity, while others show their activity only in their presence [10]. A significant difference in micromycetes lectins is also related to their interaction with carbohydrates. They are capable of interacting with both simple carbohydrates and glycoproteins [11]. Based on the property of lectins by interaction with mono- carbohydrates,

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their classification is proposed [12]. Despite the interest of researchers in micromycetes lectins, there is a small amount of work on the study of their physicochemical properties in the literature. The purpose of this work was to study the physicochemical properties of a new lectin isolated from the micromycetes *Alternaria alternate* 4.

2. Materials and methods

The object of the study was lectin from the mycelium extract of the fungus *Alternaria alternate* 4, purified according to the method proposed for micromycetes lectins of the genus *Fusarium* [13]. The molecular weight of the purified *Alternaria alternate* 4 lectin was 38.0±2.0 kDa.

The homogeneity of the *Alternaria alternate* 4 lectin preparation was controlled by SDS electrophoresis in 12.5% polyacrylamide gel under denaturing conditions [14]. The activity of lectin was determined using a hemagglutination reaction using a 2% suspension of native erythrocytes of blood group 1. Erythrocytes for the hemagglutination reaction were obtained by the method of Lucik et al. [15].

The determination of the temperature optimum and pH optimum, the stability of the lectin, the effect of metal ions on its activity, as well as the carbohydrate specificity of the *Alternaria alternate* 4 lectin were determined using the method proposed in the work of Sinha and co-authors [16].

The determination of the temperature range at which the maximum titer of *Alternaria alternate* 4 lectin activity was observed was studied after incubation of lectin samples at temperatures from 10 to 90 °C for 20 minutes. The control was a lectin preparation incubated in a 20 mM Tris-HCl buffer, pH 7.2, at a temperature of 22 ° C.

The thermal stability of *Alternaria alternate* 4 lectin was determined by a decrease in lectin activity over time at different temperatures (from 10°C to 90°C). Lectin samples in 20 mM Tris-HCl buffer pH 7.2 were kept at a certain temperature (from 10°C to 90°C) for 1 hour, aliquots were taken every 5-10 minutes, then cooled to 22°C and the residual hemagglutinating activity was determined.

The range of optimal pH values for maintaining the maximum activity of *Alternaria alternate* 4 lectin was determined by incubation of lectin samples in various buffer solutions, pH from 5.0 to 10.0, at a temperature of 22°C for 1 hour. The buffer solutions used were: 20 mM acetate buffer, pH 4.0-6.0, 20 mM phosphate buffer, pH 6.0-7.0, 20 mM Tris-HCl buffer, pH 7.0-9.0, and 20 mM glycine-NaOH buffer, pH 9.0-11.0. The control was a lectin preparation incubated in a 20 mM Tris-HCl buffer, pH 7.2.

To determine the pH stability, lectin samples were kept by dialysis in a buffer solution with different pH values (from 5.0 to 10.0). The incubation time of the samples in buffer solutions was 24 hours at a temperature of 22°C, after which the pH was adjusted to 7.2 and the residual hemagglutinating activity was analyzed.

The activity of lectins was expressed as a percentage of relative activity to the control. The time of semi-inactivation was considered as the time and temperature at which 50% lectin activity was maintained.

The effect of metal ions on lectin activity was determined by direct hemagglutination reaction in immunological U-shaped plates using a 2% suspension of native human erythrocytes of blood group 1 and solutions of metal salts. Solutions of $CaCl_2$, $MgCl_2$, $ZnCl_2$, $FeSO_4$, $CuSO_4$, $MnCl_2$, KCl were prepared on the basis of 20 mM Tris-HCl buffer pH 7.2, in concentrations from 1.25mM to 20.0 mM. The initial reaction mixture contained 0.025 ml of a solution of purified lectin and 0.025 ml of solutions of metal salts of various concentrations. The incubation of the reaction mixture was carried out at a temperature of 22^0C for 60 minutes. Then red blood cells were added and the hemagglutinating activity of lectin was determined [16].

The carbohydrate specificity of the drug was determined by the method of inhibiting the hemagglutination reaction after the interaction of lectin with various carbohydrates [17]. The results on the carbohydrate specificity of lectin were expressed in terms of MIC (minimum inhibitory concentration) values, which was defined as the lowest concentration of carbohydrate capable of completely inhibiting the hemagglutination reaction.

All experiments were carried out in three recurrences, the results of the analysis were expressed as the average value and the standard deviation error of the experiments performed.

3. Result and Discussion

In the technology of obtaining and using protein preparations, important indicators are their resistance to temperature and pH values.

In our experiments, we determined several such indicators: optimal values of temperature and pH, at which the maximum titer of lectin activity is observed, as well as their thermal and pH stability when these values change in the reaction medium.

The determination of the temperature optimum showed that the titer of the activity of the micromycetes lectin *Alternaria alternate* 4 was observed in a wide temperature range of 5-70°C (Fig. 1). The maximum activity of the lectin *Alternaria alternate* 4 was in the range from 5°C to 50°C. These temperature values had no effect on the hemagglutination reaction of lectin and the lectin activity titer values were at the highest level. A 2-fold decrease in the titer of lectin activity was observed at a temperature of 60°C. At a temperature of 75°C and incubation of lectin for 20 minutes, no hemagglutination reaction was observed, which is probably due to a violation of its structure.

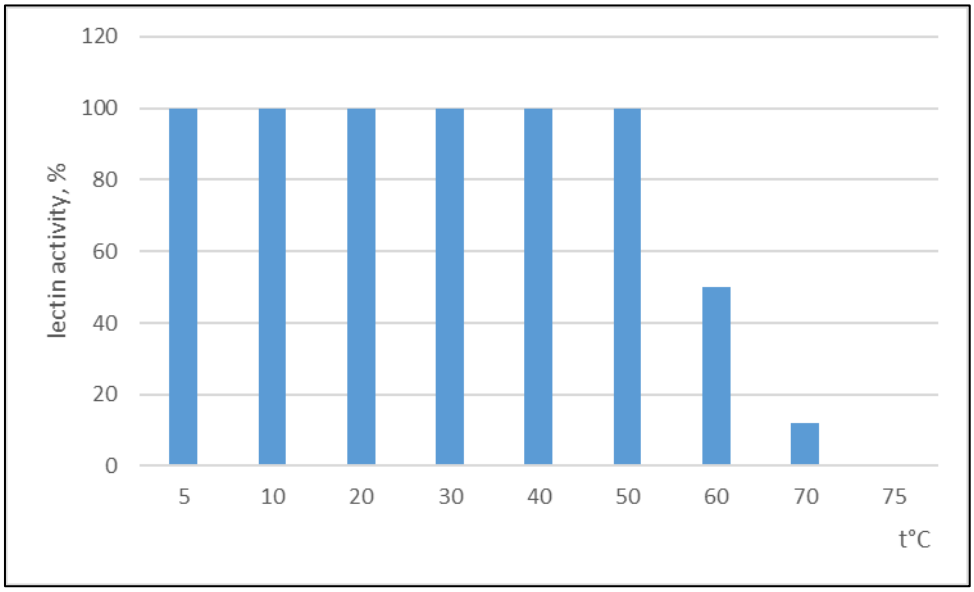

Figure 1 Temperature optimum of *Alternaria alternate* 4 lectin

A study of the temperature stability of the *Alternaria alternate* 4 micromycetes lectin preparation showed that it lies in the range of 5-70 °C (Table 1). This range of lectin stability depended on the time of temperature action: at 5-50°C, lectin activity remained fully maintained for 60 minutes, at 60°C, lectin activity decreased by 50% after 30 minutes of incubation. A further increase in temperature to 65°C and 70°C led to a loss of lectin activity after 15 minutes and 10 minutes, respectively. At 75 °C, lectin completely lost its biological activity.

Table 1 Effect of temperature on the activity of *Alternaria alternate* 4 lectin

	T – optimum (T 50%)	**Half-inactivation time, min**					
		5-50°C	**55°C**	**60°C**	**65°C**	**70°C**	**75°C**
Lectin	5-50 (5-70)	stable	50	30	15	10	unstable

T50% is the range of temperature values at which hemagglutinating activity is higher than 50% of the maximum value of the lectin activity titer; stable protein is stable during incubation for 60 minutes; unstable protein is unstable and inactivated in 5 minutes.

The results of studies to determine the pH-optimal values at which the highest titer of lectin activity was observed showed that the activity of the micromycetes *Alternaria alternate* 4 lectin was highest in the pH range 6.5-8.5 (Fig.2). Lectin activity decreased by 50% at pH 6.0 and 9.0, and at pH 5.0 and 10.0 by 90%.

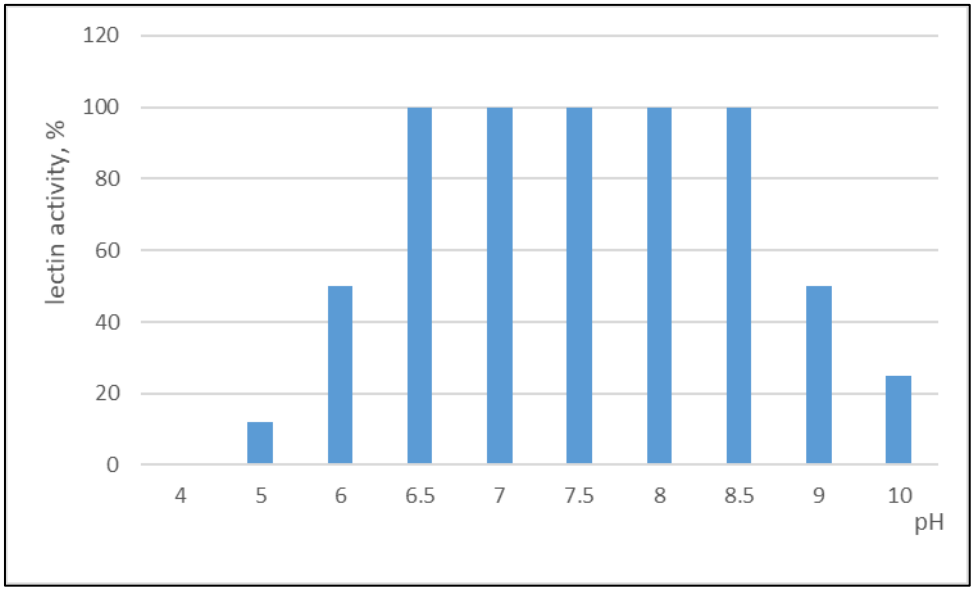

Figure 2 pH optimum of *Alternaria alternate* 4 lectin activity

A study of the pH stability of *Alternaria alternate* 4 micromycetes lectin showed that lectin maintained its activity within the pH range from 6.5 to 8.5 for an hour (Table 2). A 50% decrease in lectin activity was observed during incubation of lectin in a buffer with pH 6.0 and pH 9.0. When the lectin was kept in a buffer with a pH value of 5.0 or pH 10.0, the protein was unstable and completely inactivated.

Table 2 Effect of pH on the activity of *Alternaria alternate* 4 lectin

Lectin	**pH – optimum (pH 50%)**	**Half-inactivation time, min**								
		5	**6**	**6,5**	**7**	**7,5**	**8**	**8,5**	**9**	**10**
	6,5-8,5 (6,0-9,0)	unstable	1	stable	Stable	stable	stable	stable	1	unstable

pH 50% is the range of pH values at which hemagglutinating activity is higher than 50% of the highest value; protein is stable during incubation for 24 hours; protein is unstable and inactivated in 1 hour.

The research results showed that *Alternaria alternate* 4 lectin showed activity and stability over a wide temperature range (5-50°C), but was sensitive to pH changes. The range of pH optimum and pH stability was in the region of neutral or slightly alkaline values (pH 6.5-8.5 and 6.5-8.5).

The data obtained are consistent with the results of studies on the characterization of micromycetes lectins of other genera, which noted the ability of lectins to function in a wide range of temperatures and pH of the medium [8,9].

One of the factors influencing the activity of lectins is the presence of metal ions in the reaction medium [10].

The effect of metal ions on the activity of *Alternaria alternate* 4 lectin was determined by their interaction with various metals. Solutions of $FeSO_4$, $ZnSO_4$, $CuSO_4$, $CaCl_2$, $MgCl_2$, $MnCl_2$, and KCl based on 20 mM Tris-HCl buffer, pH 7.2, were used as metal salts. The control was the titer of lectin activity under standard conditions without adding metal ions to the reaction medium (Table 3).

Table 3 The effect of metal ions on activity Alternaria alternate lectin 4

Metal compounds	Lectin activity, titer				
	20 мМ	**10 мМ**	**5 мМ**	**2,5 мМ**	**1,25 мМ**
$CaCl_2$	1024	1024	1024	1024	512
$MgCl_2$	512	512	512	512	512
$MnCl_2$	1024	1024	1024	512	512
KCl	512	512	512	512	512
$FeSO_4$	512	512	512	512	512
$ZnSO_4$	1024	1024	512	512	512
$CuSO_4$	512	512	512	512	512
control	512				

The research results showed that incubation of *Alternaria alternate* 4 lectin with buffered solutions of divalent metal ion salts: Ca^{2+} at a concentration of 2.5-20 mM, $MnCl_2$ ions at a concentration of 5-20 mm and Zn^{2+} ions at a concentration of 10-20 mM increased the hemagglutinating activity of lectins by 2 times, which indicates that lectin is a metal- a dependent protein. It can be assumed that these metal ions apparently participate in the organization of the lectin-carbohydrate binding site and also ensure the overall stability of the lectin structure. The presence of other metal salts in various concentrations in the reaction mixture had no effect on the change in the activity of *Alternaria alternate* 4 lectin.

A specific property of lectins is its selective ability to interact with certain carbohydrates. This property makes lectins unique when used in medicine and biology.

The carbohydrate specificity of *Alternaria alternate* 4 lectin was determined by inhibition of the hemagglutination reaction, lectin with erythrocytes, in the presence of various carbohydrates in concentrations from 300 mM for simple carbohydrates and from 5 mg/ml for complex carbohydrates.

Table 4 Inhibition of the hemagglutination reaction of *Alternaria alternate* 4 lectin with erythrocytes and various carbohydrates

Carbohydrates (inhibitors)	Hemagglutination reaction
D- mannose	no inhibition
D- glucose	no inhibition
D- galactose	> 4,69 мМ
D- lactose	> 18,75 мМ
D- fucose	no inhibition
L- rhamnose	> 9,38 мМ
D- maltose	> 18,75 мМ
D -sucrose	no inhibition
N-acetylglucosamine	no inhibition
N-acetylgalactosamine	> 9,38 мМ
dextran	no inhibition
amylum	no inhibition
xylan	no inhibition

Does not inhibit: D-arabinose, D-xylose, D-ribose, D-mannitol, D-fructose, D-raffinose.

Determination of the specificity of the interaction of *Alternaria alternate* 4 lectin with carbohydrates showed its affinity with simple carbohydrates: D-galactose, N-acetylgalactosamine, and rhamnose (at a minimum inhibitory concentration of 4.69 mM, 9.38 mM, and 18.75 mM, respectively); with disaccharides: D-lactose and D-maltose (with a MIC of 18.75 mM) (Table 4). The presence of the remaining carbohydrates in the reaction mixture did not affect the ability of the lectins of this micromycetes agglutinate with erythrocytes, which indicates the absence of a specific interaction of *Alternaria alternate* 4 lectin with these carbohydrates.

4. Conclusion

The physicochemical properties of lectin isolated from the mycelium of the micromycetes *Alternaria alternate* 4 have been determined. It has been established that the lectin of the micromycetes *Alternaria alternate* 4 is a thermostable, neutral protein resistant to slightly alkaline conditions. The temperature range (5-50 °C) and pH values of the reaction medium (pH 6.5-8.5) allow the micromycetes lectin *Alternaria alternate* 4 to exhibit the greatest activity (512 units). *Alternaria alternate* 4 lectin is a metal-dependent protein, since $Ca^{2+,}$ Mn^{2+} and Zn^{2+} ions in the reaction mixture increased it hemagglutinating activity. *Alternaria alternate* 4 lectin was inhibited to a greater extent by galactose (minimum inhibitory concentration of 4.69 mM) than by other carbohydrates, therefore it can be attributed to the group of galactose-binding lectins. A comparative analysis of the results obtained on the physicochemical characteristics of *Alternaria alternate* 4 lectin indicates that its parameters differ from those of other micromycetes, and the established properties allow it to be recommended for practical use.

Compliance with ethical standards

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Disclosure of conflict of interest

A statement on the absence of a conflict of interest is attached.

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