ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Purification And Characterization Of Extracellular Lectin Extracted From Culture Liquid *Thermodesulfobacterium Mobile* ECM – 1128.

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ABSTRACT

A new, highly purified drug of the extracellular lectin sulfate-reducing bacteria Thermodesulfobacterium mobile ECM - 1128 is obtained. The cleaning of lectin from the related extracellular proteins was carried out by ion exchange and gel-chromatography. The purification degree of lectin increased 6.5 times as compared with the original protein fraction, the activity yield made 1.56%. The specific activity made 16,000 titer/mg. The electrophoresis of lectin in 12.5% PAAG within SDS confirmed the presence of one protein fraction, indicating the purity of the isolated lectin. The comparative characteristics of lectin with protein markers showed that the purified drug had the molecular weight of 60 ± 1 kDa. The obtained lectin has a high resistance to temperature and pH. It retains its hemagglutinating activity in a wide temperature range (40 - 80 ° C, the optimum temperature makes 60 °C) and the pH of the reaction mixture (4.0 - 8.0). Hemagglutinating activity of the obtained lectin is independent of metal ions. The inclusion of 14 different metal compounds to the reaction mixture in various concentrations showed the stability of lectin to most of them, especially at low concentrations (12-50 mM). There were the following exceptions: $CaCl_2$, $CdSO_4$, $FeSO_4$, •$9H_2O$, K_2SO_4, and $NiCl_2$, $MnCl_2$ at the concentration of 200mM.

Keywords: sulfate-reducing bacteria, lectins, protein purification, physical and chemical factors.

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ISSN: 0975-8585

INTRODUCTION

Nowadays, there is a growing demand for lectins in drugs that are widely used in various fields of national economy. These are lectins used in laboratory practice for the diagnosis of certain genetic diseases, in research as the matter for the identification of some species of microorganisms as the reagents sorbing certain complex substances including glycoproteins, hormones, sialoproteins, etc. [1]. With the ability to specific carbohydrate interactions, they expand the opportunities for valuable substance obyaining, as well as for the study of cell metabolism individual processes. However, the implementation of these objectives is only possible upon the receipt of individual proteins with a high degree of purification and characterized by major physical and chemical parameters. The effect of temperature, pH, the presence of metals are among these factors. These factors make a significant impact on the hemagglutinating activity of different lectins. For example, extracellular lectin of enteroaggregative Escherichia coli has an optimum temperature (around 10-25 ^{o}C), and pH (within the range of 7.0-8.0), where lectin activity is the highest one [5].

Earlier in our study, the ability of Thermodesulfobacterium mobile ECM - 1128 strain to the synthesis of the extracellular lectin was shown in our study. Its hemagglutinating activity was two-fold higher compared with other strains of sulfate-reducing bacteria. The aim of this work was the purification of this extracellular lectin Thermodesulfobacterium mobile ECM - 1128 to homogeneity and to characterize it according to the main physical and chemical parameters.

MATERIALS AND METHODS

The strain of sulfate-reducing bacteria Thermodesulfobacterium mobile ECM - 1128 was used previously received from the All-Union Collection of RAS microorganisms (Pushchino city) and stored in the Museum of Cultures, at Biochemistry and Biotechnology Department, the Institute of Basic Medicine and Biology, Kazan (Volga region) Federal University.

Bacteria were grown in Postgate medium B [9]. The cultivation was carried out without air access, in sealed vials of 500 ml. The culture temperature was 37 oC.

The ability of sulfate-reducing bacteria development under experimental conditions was judged by protein increase in the culture medium by analyzing the culture liquid using microspectrophotometer NanoDrop ™ 2000 / 2000c of the company Thermo Fisher Scientific. The experience was repeated three times.

During the studies the purity of sulfate-reducing bacteria was controlled using the microscope with a phase contrasting device (KF-4) and the inoculations on glucose-peptone medium for anaerobic and aerobic heterotrophic bacteria.

The isolation of the extracellular proteins was performed from the culture fluid on the 3rd day of the strain growth after the separation of the cells by centrifugation at 15,000 g for 30 minutes. The resulting supernatant was further filtered through a 0.45µm Durapore filter with the membrane made of polivinilin fluoride (Merck Millipore, Ltd.) to remove non-relevant impurities. In order to increase the concentration of protein it was placed in a Pro Purification system with the bandwidth of 3kDa by the firm Amicon®, and was centrifuged at 7000 for 30 minutes.

The separation of protein fractions and the purification of lectin was performed in the chromatography system BioLogic ™ LP. The column Bio-Scale ™ Mini Macro-Prep High Q, equilibrated with 20 mM Tris-HCl buffer, pH 8.0 was used for ion-exchange chromatography. The solution of NaCl with a linear gradient increase from 0 to 1M was used as eluate. The activity of lectins was established in hemagglutination reactions with human erythrocytes of the first blood group [13].

The gel filtration of the separated lectin fraction was performed on a column with the sorbent Bio-Gel® P -100v 20 mM Tris-HCl buffer, pH 8.0.

The purity level of the samples and the molecular weight was determined by electrophoresis in 12.5% PAAG in the presence of SDS according to Laemmli method [8].

ISSN: 0975-8585

The effect of temperature on the lectin hemagglutinating activity was analyzed by protein incubation for 60 minutes at different temperatures ranging from 0 °C to 100 °C.

Determination of pH reaction pH impact on the activity of lectins was performed in buffers with different pH values: acetate (1,0 - 5,0 pH), Tris-HCl (6,0-8,0 pH) and NaOH - glycine (9.0 - 12,0 pH).

The action of metal ions on the activity of lectins was assessed by the changes in hemagglutinating activity of lectins after the following ion inclusion:

Ca2 +, Cd2 +, Fe2 +, Al3 +, K +, Mg2 +, Cu2 +, Ni2 +, Co2 +, Mn2 + the concentrations of which made 12,5mM, 25mM, 50mM, 100mM, 200mM,

RESULTS

In order to study the properties of lectin isolated from the culture medium Thermodesulfobacterium mobile ECM - 1128, the homogeneous preparation was obtained. For this purpose the ion exchange chromatography was performed on the column of Bio-Scale™ Mini Macro-Prep High Q (Table 1). After the performed experiment from lectin fraction a considerable amount of ballast proteins was removed, and the specific activity of the lectin fraction increased 2.6-2.7 times. The obtained preparation had a sufficiently high purification degree as compared with the initial fraction of lectin, the activity yield was 3.12%.
In order to increase the degree of protein purification, the resulting lectin fraction was subjected to an additional gel cchromatography on the column with the sorbent Bio-Gel® P-100. Thus, the specific activity of the fraction increased by the order and made 16,000 titer/mg. The degree of lectin purification increased by 6.5 - 6.6 times, compared with the original protein fraction, the activity yield made 1.56%.

Table 1: The general cleaning profile for the extracellular lectin of thermophilic anaerobic sulfate-reducing bacteria Thermodesulfobacterium mobile ECM - 1128.

Stages of purification	Volume (ml)	Protein concentration (mg/ml)	GA activity (titre)	Specific activity (titre/mg)	Purification stage	Activity yield %
Cultural liquid	1	2,1 ±0,2	256	2438,1	1,0 ± 0,1	100
Ion Exchange Chromatography (Bio-Scale ™ Mini Macro-Prep High Q column)	1	0,025 ±0,002	8	6400	2,62 ± 0,28	3,12
Gel chromatography (column with BioGel® P-100 sorbent)	1	0,005 ± 0,0001	4	16000	6,56 ± 0,51	1,56

The homogeneity of the obtained lectin was established by electrophoresis in 12.5% PAAG, which showed the presence of a single polypeptide with a molecular mass of 60 ± 1 kDa (Figure 1).

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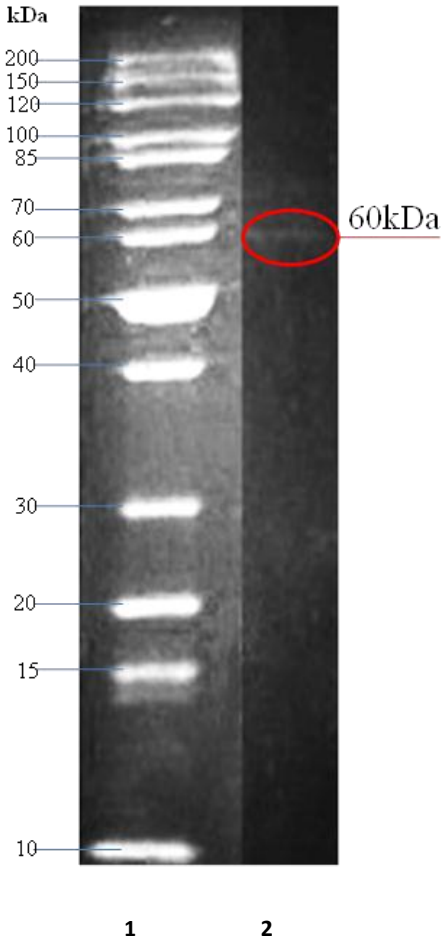

Figure 1: Electrophoresis of lectin in 12.5% PAAG with SDS:

1 - protein markers PageRuler ™ (10-200 kDa), 2 - the protein fraction after chromatography on Bio-Scale ™ Mini Macro-Prep High Q column and subsequent gel filtration on the column with Bio-Gel® P -100 sorbent.

Thermodesulfobacterium mobile ECM - 1128 strain is characterized by relatively high resistance to temperature (up to 85 °C) [6], [7], [13]. Therefore it was interesting to find out the thermal stability of its synthesized extracellular lectin. The work used the temperature in the range of 0-100 °C (Fig. 1).

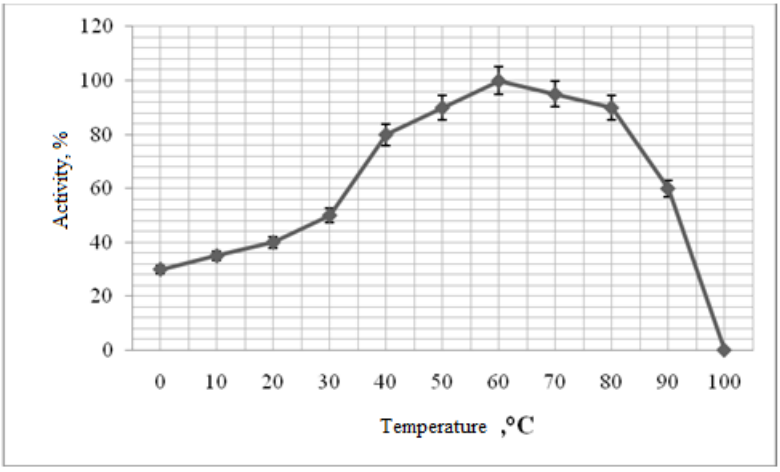

Figure 2: Effect of temperature on the lectin activity of Thermodesulfobacterium mobile ECM -1128.

ISSN: 0975-8585

The obtained results show that the purified sample of homogeneous protein appeared to be thermally stable within a wide temperature range of 0 - 90 °C. The most hemagglutinating activity of lectin was observed in the range of 40 - 80 ° C, with the maximum activity at 60 °C. The temperature increase during the incubation of protein up to 90 -100 °C led to a drastic loss of its activity, presumably due to the coagulation or destruction of the protein molecule. The lectin incubation temperature reduction starting from 30 °C and below also caused the lectin activity decrease, but it maintained even at 0 °C. These results are consistent with the literature about the dependence on the hemagglutinating activity of lectins on the temperature [17].

The activity of any protein depends on the pH of the reaction mixture. In our case the pH range from 1.0 to 12.0 was studied (Figure 3).

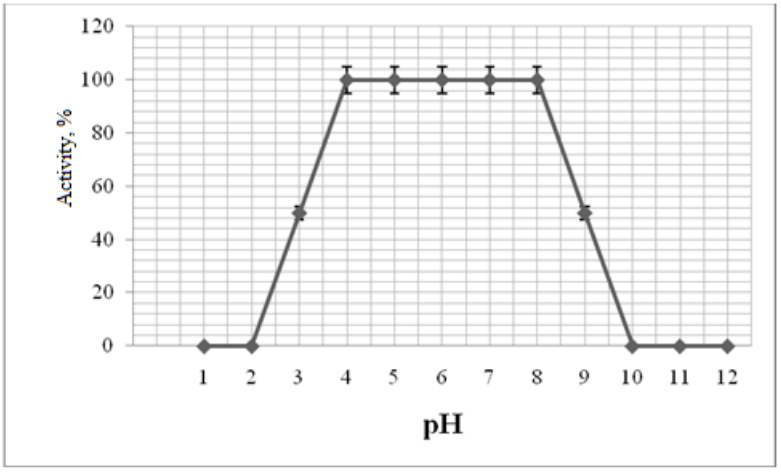

Figure 3: The effect of pH on the lectin activity of Thermodesulfobacterium mobile ECM - 1128.

The results showed that the purified lectin was stable in the pH range of 4.0-8.0. With further increase of pH, above 8.0 there was a gradual decrease of lectin activity, and at pH above 9.5 lectin lost the ability to agglutination. The ability to agglutination was by lectin also at low (pH 2.5) values of the reaction mixture.

The calculation of semiinactivation period for lectins at an optimal pH and at the temperature above 80 °C suggests that the protein is stable up to 90 °C, although it loses the part of its activity. During the heating above 95 °C, the protein is completely inactivated after 60 minutes (Table 2).

Table 2: Effect of temperature and pH on hemagglutinating ability of extracellular lectin.

pH-optimal pH(50%)	T-optimal T(50%)	Semiinactivation period, min				
		80°C	85°C	90°C	95°C	100°C
4,0 - 8,0 (3,0-9,0)	40°C - 80°C (30°C - 90°C)	CT	CT	CT	HC	HC

pH of 50%, T50% - the range of temperature and pH values at which the hemagglutinating activity is more than 50% of the highest value;
CT - protein is not destroyed and is stable at the incubation for 60 min.
HC - protein is unstable and breaks down after 60 minutes of incubation.

It is known from the literature that the active center of proteins, including lectins, often have metal ions, which play a significant role in the reactions of protein and receptor interaction [16]. On the other hand, metal ions, particularly heavy metals, may reduce or completely inactivate the activity of proteins [14].

In our experiments, the effect of metal ions on the hemagglutinating activity of the homogeneous lectin was studied (Table 3).

ISSN: 0975-8585

The study results showed that the cobalt, copper, aluminum, and magnesium salts do not influence the lectin activity. The potassium chloride salt also does not influence on lectin activity. However, potassium sulfate salt even at the concentration of 12,5-25 mM decreases the lectin activity two times, and then with the salt concentration increase the activity decreases 4 - 8 times. On the other hand, the salt of nickel sulfate and low concentrations of chloride nickel salts did not affect the lectin activity. However, higher concentrations of nickel chloride caused the decrease of lectin activity, particularly at the concentrations of 200 mM - 32 times.

Table 3: Effect of metal ions on the hemagglutinating ability of extracellular lectin Thermodesulfobacterium mobile ECM -1128.

Metal compounds	Concentration of metals				
	12,5mM	25mM	50mM	100mM	200mM
$CdSO_4$	128	128	128	128	128
$CoCl_2$	256	256	256	256	256
$CuCl_2 \cdot 2H_2O$	256	256	256	256	256
$CuSO_4 \cdot 5H_2O$	256	256	256	256	256
$FeSO_4 \cdot 9H_2O$	128	128	64	64	64
$NiCl_2$	256	256	256	128	8
$NiSO_4 \cdot 7H_2O$	256	256	256	256	256
$AlCl_3$	256	256	256	256	256
$Al_2(SO_4)_3 \cdot 18H_2O$	256	256	256	256	256
$MgSO_4 \cdot 7H_2O$	256	256	256	256	256
$MnCl_2 \cdot 4H_2O$	256	256	128	64	32
$CaCl_2$	64	64	32	16	4
KCl	256	256	256	256	256
K_2SO_4	128	128	64	64	32
CONTROL	256				

The concentration dependence of the lectin activity was set for manganese chloride and iron sulfate. Manganese salt at 12,5-25 mM did not affect the activity of lectin, and then a gradual decrease in the lectin activity was observed: 2 time, 4 time, 8 time decrease. Iron sulfate salt decreases the lectin activity at the concentration of 12,5-25 mM 2 times, and at the concentration of 50 mM and above - 4 times. Especially pronounced inhibiting effect on the activity of lectins was found for the calcium salt, when the lectin activity decreased 4-fold at the concentration of 12,5-25 mM, compared with the control, and then during the increase of salt concentration - 8 times, 16 times , 64 times, respectively.

An interesting fact is the lectin stability to cadmium salt, which reduces the lectin activity two times in comparison with the control, starting from the low concentrations (12,5mM), but it does not change depending on the concentration of an included metal.

CONCLUSIONS

The performed successive chromatography on the column Bio-Scale ™ Mini Macro-Prep High Q and gel filtration on the sorbent Bio-Gel® P - 100 allowed to remove any significant amount of ballast proteins and obtain a highly purified lectin with the specific activity of 16000 (titer/mg). The purity of the obtained preparation was confirmed especially clear by electrophoresis in PAAG. The comparison of obtained lectin molecular weight with the electrophoregrams of marker proteins showed that its molecular weight made 60 ± 1 kDa, i.e. this lectin may be attributed to low molecular proteins.

The biological activity of proteins, including lectins, depends on many physical and chemical factors, including temperature, pH of the reaction mixture, on metal ions and other factors. Thermodesulfobacterium mobile ECM -1128 strain refers to thermophilic microorganisms and so it could be assumed that its

synthesized lectin will be also thermostable. The experimental results confirm this assumption. Lectin retains its activity even at 90 °C, which is 5-6 °C higher than the allowable temperature for the bacteria growth [11] and [12]. The activity of proteins depends mainly on the pH of the reaction mixture. However, we were unable to establish one optimum pH for lectin. The activity of lectins was maintained over a wide pH range, including acid and alkaline values (pH 4.0-8.0), which is typical for microbial lectins [2]. It is known that the presence of their activity dependence on the divalent metal is peculiar for many lectins. Their removal often results in reversible loss of biological activity. Ca, Mg are among them. Zn, Mn is presented in less quantities. There are also lectins on which the metal ions do not make any impact [15]. In our case, it was shown that the metal ions are not a mandatory component of sulfate-reducing bacteria extracellular lectin and are not relevant for its biological activity. This attitude of lectins to metals is set for snail and wheat germ agglutinins [4], [10] i.e., the obtained lectin may be attributed to the group of agglutinins. On the other hand, a number of metal ions made an inhibiting impact on lectin activity. With respect to reduced activity of lectins these metals may be arranged in the following order: CaCl2> NiCl2> MnCl2> K2SO4> FeSO4> CdSO4. However, making the overall conclusion concerning the effect of metal ions on the lectin activity, we may say that the obtained lectin has a sufficiently high resistance to metal ions, including heavy metals.

SUMMARY

Thus, a new, highly purified preparation of extracellular lectin is obtained with a high resistance to a wide range of temperatures (40 - 80 °C), pH (4.0-8.0), metal ions, including heavy metals. According to its characteristics this lectin may be recommended for the study as a biological object of environmental biotechnology.

ACKNOWLEDGEMENT

The work is performed according to the Russian Government Program of Competitive Growth of Kazan Federal University.

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