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Araştırma Makalesi - Research Article

Purification and Characterization of a Lectin from Bulbs of *Cyclamen Mirabile*

Cyclamen Mirabile Soğanından Lektin Saflaştırılması ve Karakterizasyonu

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ABSTRACT

Lectin from the bulbs of *Cyclamen mirabile* which is an endemic species of Turkey was successfully isolated by affinity precipitation with alginate in one step. The purified protein produced two bands showing a dimeric structure in SDS-PAGE (13.5 and 14.8 kDa). *C. mirabile* lectin showed activity and stability in a broad pH scale and kept its haemagglutination activity in the temperature range of 4-40°C. $MgCl_2$ and $HgCl_2$ inhibited the haemagglutination activity of the lectin. In this study, a practical and efficient purification procedure was carried out for *C. mirabile* lectin by using affinity precipitation with alginate.

Keywords- Lectin, Protein Purification, Cyclamen Mirabile

ÖZ

Bu çalışmada, Türkiye'nin endemik bir türü olan *Cyclamen mirabile* soğanından tek adımda lektin saflaştırıldı. Yöntem olarak, amonyum sülfat ve ardından kalsiyum aljinat presipitasyonu kullanıldı. Saflaştırılan lektin SDS PAGE ile analiz edildiğinde 13.5 ve 14.8 kDa molekül kütlelerine sahip iki protein bandı gözlendi. Hemaglütinasyon inhibisyon yöntemi ile *C. mirabile* soğanından elde edilen lektinin mannoz spesifik lektin olduğu belirlendi. *C. mirabile* lektini geniş bir pH aralığında ve 4-40°C arasında hemaglütinasyon aktivitesini korudu. $MgCl_2$ ve $HgCl_2$ metallerinin lektinin hemaglütinasyon aktivitesini inhibe ettiği görüldü.

Anahtar Kelimeler- Lektin, Protein Saflaştırma, Cyclamen Mirabile

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I. INTRODUCTION

Lectins are naturally found proteins that can bind carbohydrates with characteristic specificities. Lectins are significant proteins for unraveling biological processes and enlightening of protein and carbohydrate structures [1]. The first identified plant lectin was named ricin which was purified from the castor beans. The term of hemagglutinin arose after the discovery of the agglutination of red blood cells by ricin. Next, it was found that some hemagglutinins can agglutinate selectively human erythrocytes according to their ABO blood group type [2]. Lectin molecule has carbohydrate-binding sites more than two which led to attaching sugars on the surface of the cell causing cross-linking of cells and subsequent precipitation. Lectins appear in most organisms, such as viruses, bacteria, animals, and plants [3]. Mannose-specific lectins prevail among higher plants and defense the plants by recognizing the high-mannose-type glycans of plant predators or pathogens [4,5]. Lectins are precious tools for glycobiology and biomedical research and can be used as diagnostic probes, antitumor cytotoxins, tumor-specific surface markers, immunotoxins, and adhesion molecules. Many human pathogens initiate infection by using the cell surface glycans as either receptors or ligands [6-10]. Mostly, affinity chromatography is used to purify lectins besides recombinant DNA techniques [3]. In this study, a plant lectin was purified by using a simple affinity technique from *Cyclamen mirabile*, which is an endemic species of Turkey.

II. MATERIALS AND METHODS

A. Materials

Coomassie brilliant blue G250 (Fluka, Buchs, Switzerland), ammonium sulfate, bovine serum albumin, molecular weight marker (14-66 kDa), D-glucuronic acid, fructose, glucose, sucrose, galactose, mannose, N-acetyl D-glucosamine, lactose, D-galacturonic acid, D-galactosamine hydrochloride, D(-) lyxose, sodium alginate, sulphuric acid, phenol (Sigma Chemical Co.,St. Louis, Mo., U.S.A). Sheep blood was collected from the waste of the local abattoir.

B. Preparation of Crude Extract with Ammonium Sulfate Precipitation

200 g of *C. mirabile* bulbs were peeled, cut into small pieces, and homogenized in 400 mL 25% ammonium sulfate using a blender. The crude extract was filtered from cheesecloth and centrifuged for 30 min at 9000 g. The supernatant was precipitated by 70% ammonium sulfate and left overnight under the stirring condition for complete precipitation. The pellet was centrifuged at 9000 g for 30 min and dissolved in deionized water. Dialysis performed against deionized water to remove ammonium sulfate and the precipitate was kept at -80°C until further use.

C. Protein and Carbohydrate Analysis

Bradford assay was used to estimate the protein concentrations with some modifications. As the standard known concentrations of bovine serum albumin were used [11]. Phenol sulphuric acid method was used to estimate total carbohydrate content, with dilutions of a known concentration of D-glucose [12].

D. Affinity Precipitation of Lectin with Alginate

50 mM of sodium acetate buffer at pH 4.5 was used to prepare alginate solution (2% v/v). Necessary dilutions were made before use. The *C. mirabile* lectin was mixed with 0.5 mL of alginate solution and made up to 5.0 mL with acetate buffer. The lectin-alginate complex was precipitated with the addition of 0.4 mL of 1 M $CaCl_2$ solution and incubated for 20 min at 25ºC. The suspension was centrifuged for 10 min at 9000 g. The precipitate was washed using pH 7.4 100 mM phosphate buffer containing 0.074 M $CaCl_2$ [13]. 3 mL of acetate buffer was used for dissolving the lectin-alginate complex, containing 1 M mannose, and incubated at 4°C for 18h. The alginate was precipitated and the eluate was dialyzed against distilled water (24h at 4°C). The eluate was checked for lectin activity.

E. Haemagglutinating Activity

Haemagglutination activity (HA) was measured in a U bottom microtiter plate according to Sureshkumar and Priya, using erythrocytes from sheep blood [14]. The definition of the haemagglutination unit (titer) was the highest dilution of the sample showing haemagglutination.

F. Haemagglutination-Inhibition Assay

Agglutination of the sheep red blood cells by the *C.mirabile* lectin was estimated according to Peng [15]. The sugar inhibition assay was performed using different concentrations of sugars such as fructose, sucrose, glucose, D-glucuronic acid, galactose, mannose, lactose, D-galacturonic acid, D-galactosamine hydrochloride, N-acetyl D-glucosamine, and D(-) lyxose.

G. pH Effect on Haemagglutination Activity and Stability

The effect of pH on the activity of the *C.mirabile* lectin was investigated by incubating the lectin in the stated buffers of 0.05 M at different pH values: glycine-HCl (pH 2.0-3.0), sodium acetate-acetic acid (pH 4.0-5.0), potassium phosphate (pH 6.0-7.0), Tris-HCl (pH 8.0-9.0), sodium carbonate (pH 10.0-11.0) at room temperature for 24 h. The agglutination titer of the lectin in PBS was used as the control [16].

H. Temperature Effect on Haemagglutination Activity and Stability

The effect of temperature on the lectin activity was investigated with the standard assay procedure with PBS as the control within a temperature range from 4 to 60°C. Thermostability was monitored by incubation of crude extract at ph 7.4 at temperatures ranging from 4-60°C for 30 min and the relative lectin activities were assayed with the standard assay conditions.

İ. Effect of Metal Ions

The *C. mirabile* lectin (50 µL) was incubated for 24 h with metal ions (Mg^{2+},Ba^{2+},Ni^{2+},Hg^{2+},Na^{+} and Ca^{2+}) at various concentrations with constant shaking. After that, each sample was mixed with 50 µL of erythrocyte suspension (2% v/v) and the hemagglutination activities were measured.

J. SDS PAGE Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) was carried out using the discontinuous buffer system described by Laemmli using a 4-12% mini gel [17]. After electrophoresis, the gel was stained with Coomassie brilliant blue in 10% acetic acid for one hour and destained with distilled water by heating in a microwave oven in short periods and replacing of distilled water until protein bands are clearly defined.

III. RESULTS AND DISCUSSIONS

A. Purification of C. mirabile Lectin

The *C. mirabile* lectin was purified by affinity precipitation with alginate and separated on 12% polyacrylamide gel. Figure 1 reveals that *C. mirabile* lectin is a dimeric protein, two proteins with molecular sizes of 13.5 and 14.8 kDa.

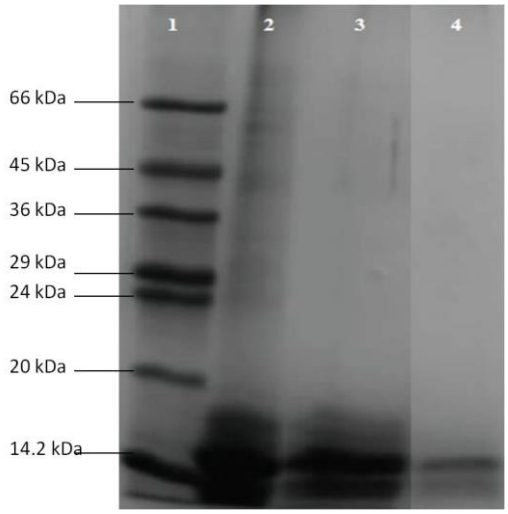

Figure 1. SDS PAGE gel image. Protein standards (1), Crude extract (2), 70% Supernatant (3), Purified lectin (4)

B. Protein, Carbohydrate and Haemagglutination Units for Lectin

Protein concentration, carbohydrate content, and agglutination activity for each step of purification were shown in Table 1. Specific activity increased with purification steps. Pure lectin had the most specific activity.

Table 1. Protein concentration, carbohydrate content and agglutinate activity

	Volume (mL)	Protein (mg mL^{-1})	Total Protein (mg)	Activity (HAU)	Spesific Activity (HAU mg^{-1} protein)	Carbohydrate (mg)
Homogenate	470	0.132	62.04	1024	16.51	3236
25% Supernatant	460	0.038	17.48	256	14.65	-
25% Pellet	9	1.279	11.51	2048	117.93	113.9
70% Supernatant	450	0.015	6.75	16	2.37	-
70% Pellet	32	0.453	14.5	16384	1130	719.36
Pure Lectin	1.5	15	0.0225	128	5688	-

C. pH Effect on Haemagglutination Activity and Stability

The purified lectin was able to maintain its stability in a wide pH range which is between pH 5.0 and 11.0 (Figure 2). The optimum pH for purified *C.mirabile* lectin to agglutinate sheep blood erythrocytes was 5.0-8.0 (Figure 3). Agglutination capacity decreased below pH 5.0 and above pH 9.0.

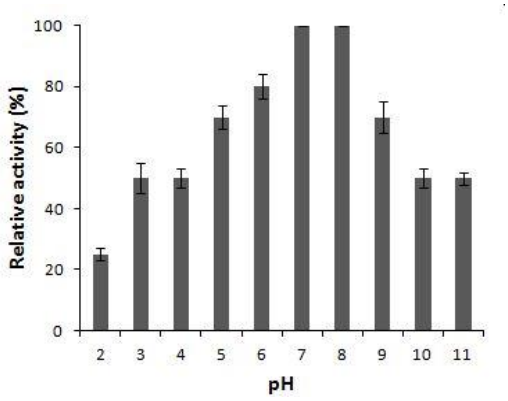

Figure 2. Alteration of lectin haemagglutination stability depending on pH

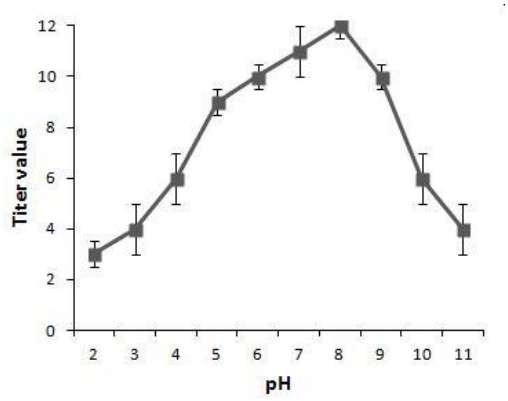

Figure 3. Alteration of lectin haemagglutination activity depending on pH

D. Temperature Effect on Haemagglutination Activity and Stability

Agglutination stability was studied within the temperature range of 0-60°C for pure lectin which is most stable at 37ºC and 40ºC. The results are shown in Figure 4. Purified *C. mirabile* lectin retained the full agglutinating activity in the temperature range of 4-40°C, but there was a dramatic decrease between 40 and 55°C, and at 55°C the activity was completely abolished showing thermal inactivation. The results are shown in Figure 5.

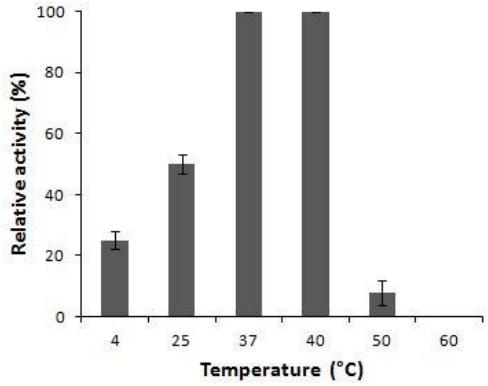

Figure 4. Alteration of lectin stability depending on temperature

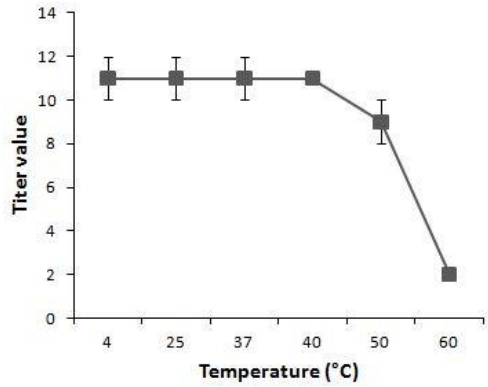

Figure 5. Alteration of lectin haemagglutination activity depending on temperature

E. Haemagglutination-Inhibition Assay

Fructose, glucose, galactose, mannose, lactose, sucrose, D-galactosamine hydrochloride, N-acetyl glucosamine, D-galacturonic acid, D(-)lyxose, and D-glucuronic acid used for haemagglutination-inhibition assay of purified *C. mirabile* lectin to agglutinate sheep erythrocytes. Lectin bound mannose with the most affinity, indicating that purified lectin is mannose-specific. The results are shown in Table 2.

Table 2. The sugar inhibition assay for pure lectin

Carbohydrate	**mM**
Mannose	0.156
D-galacturonic acid	0.625
Lactose	1.2500
D(-) lyxose	2.500m
Glucose	-
N-Acetyl-D-Glucosamine	-
Xylose	-
Galactose	-
Sucrose	-
Fructose	-
D-Galactosamine hydrochloride	-
D-Glucuronic acid	-

F. Inhibition of Hemagglutination by Metal Ions

Various metal ions were used to investigate the effect of metal ions on the hemagglutination activity. Results showed that lectin has the most affinity for magnesium and mercury while giving negative results for other metal ions (Table 3).

Table 3. Lectin affinity for metal ions

Metal ion	**Concentration (mM)**
$CaCl_2$	-
NaCl	-
$HgCl_2$	5.0
Ni(II)	-
$MgCl_2$	2.5
$BaCl_2$	-

IV. CONCLUSION

A mannose-specific, dimeric *C. mirabile* lectin with the molecular weights of 13.5 and 14.8 kDa was isolated by affinity precipitation with alginate. Haemagglutination activity of the crude extract increased with purification steps and purified lectin maintained its activity and stability in a broad pH scale. Nevertheless, it showed maximum activity at pH 8. Although haemagglutination activity and lectin stability were observed between 4-40°C, there was a drastic decrease with ascending temperature. Also, it was seen that only mercury and magnesium were able to inhibit haemagglutination activity among other metal ions. Purified lectin showed the most affinity to mannose among other sugars which reveals *C. mirabile* lectin is a mannose-specific lectin. In this study, the *C. mirabile* lectin was purified and characterized for the first time. Further work will be necessary to study the bioactivity of lectin, such as anticancer and antimicrobial activity.

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