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PURIFICATION AND ANTIOXIDANT ACTIVITY OF *ALOE VERA* LEAF LECTIN

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SUMMARY

A new and rapid affinity chromatography method based on cyanogen bromide (CNBr)-activated Sepharose 4B bound-ovalbumin is presented for the purification of the main lectin present in *Aloe vera* (L.) Burm. fil. The lectin was purified 60 fold to apparent homogeneity in native polyacrylamide disc gel electrophoresis (PAGE) showing an apparent molecular weight of 45000 kDa. The fact that sodium dodecyl sulfate (SDS)-PAGE gave a subunit molecular weight of 14 400 kDa tends to propose that the lectin is composed of three subunits and thus is in agreement with Aloctin I previously partially purified and characterized by us. The lectin did not exhibit antioxidant effect as assessed by the $DPPH^{\cdot}$ radical-scavenging assay.

ÖZET

Aloe vera (L.) Burm. fil. lektinin saflaştırılması için, yeni ve hızlı bir kromatografi yöntemi olan, ovalbumin bağlı-siyanojen bromür (CNBr) ile aktive edilmiş Sefaroz 4B, afinite kromatografisi tanıtılmaktadır. Lektin, kromatografi sonucunda 60 kez saflaştırıldı. Poliakrilamid disk jel elektroforezi (PAGE) ile homojen olduğu gözlemlenen lektinin molekül ağırlığı bu yöntemle, 45 000 kD olarak saptandı. Sodyum dodesil sülfat (SDS)-PAGE ile altbirim molekül ağırlığı 14 400 kDa olarak belirlenen lektinin, üç alt birimden oluştuğu ve bunun da daha önce tarafımızdan kısmen saflaştırılmış ve karakterize edilmiş olan Aloctin I ile uyum içinde olduğu sonucuna varıldı. Saflaştırılan lektin, $DPPH^{\cdot}$ radikal süpürme tayini ile değerlendirildiğinde antioksidan etki göstermedi.

Key words: *Aloe vera*, lectin, purification, affinity chromatography, antioxidant activity.

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INTRODUCTION

Reffered in the ancient times as a miracoulous plant, *Aloe vera* (L.) Burm fil. (= *Aloe barbadensis* Miller; in Turkish "Sarisabir"), member of Aloaceae family (formerly member of Liliaceae), possesses many biological and pharmacological activities. The use of and research on this plant have been described in well-referenced reviews (1,2) and books (3,4).

The multiplicity of the biological activities of *A. vera* has been attributed to the variety of its chemical components including anthraquinones, glycoproteins, polysaccharides, vitamins and enzymes (5,6). The substances responsible for the biological and antioxidant activity of the plant are under investigation although some resarchers claim that the synergistic relationship between the elements could be useful for maintaining the integrity of the antioxidant status (7).

Many biological and pharmacological activities of *A. vera*, like antiinflammatory (8), mitogenic (9), burn healing (10) and immunomodulatory (11) have been attributed to the lectin.

As lectins are known to be proteins or glycoproteins binding to specific sugar moieties on the cell surfaces (12), affinity chromatography has been widely used for their purification from natural sources (13). For this purpose, hemmaglutination inhibition tests are usually undertaken in order to determine the specific binding sugar which can be a mono-, di-, or polysaccharide, a glycopeptide or a glycoprotein and this component is chosen as the coupling affinity ligand.

The first study reporting the isolation of two lectins from *Aloe arborescens* Miller, dates from 30 years (14). In the last 20 years some work has been done for the isolation of *Aloe* lectins (8,9,15) mainly from the gel portion of the leaves. The separation and partial purification of lectins from *A. vera* leaves devoid of the gel was undertaken in our laboratory (16). As the main lectin, named Aloctin I, did not show one band in polyacrylamide gel electrophoresis (PAGE), further investigation was undertaken to find an appropriate ligand in order to purify the lectin by affinity chromatography.

MATERIAL AND METHODS

Plant material: Specimens of *Aloe vera* (L.) Burm. fil. (in Turkish Sarisabir) were collected from Kale (Demre) in Antalya, identified by Prof.

Dr. Nurhayat Sütlüpinar (May 1993; a voucher specimen was deposited in the Herbarium of the Faculty of Pharmacy, ISTE No. 65118). The plant was cultivated in the greenhouse of the Istanbul University, Faculty of Sciences, Department of Botany since that time. The fresh leaves of this cultivated plant were used in the study.

Chemicals: CNBr-activated Sepharose 4B was purchased from Amersham Bioscience AB (Uppsala, Sweeden). 4-Nitrophenyl-α-D-glucopyranoside, 4-Nitrophenyl-β-D-glucopyranoside, 4-Nitrophenyl-α-D-galactopyranoside, 4-Nitrophenyl-β-D-galactopyranoside, lactose, N-acetyl-D-galactosamine and ovalbumin were obtained from Fluka Chemical Co. (Bushs, Switzerland). Pharmacia Electrophoresis Calibration kits for low and high molecular weight proteins were used as standard reference proteins in polyacrylamide gel electrophoresis. For the assesement of antioxidant activity, 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of *A. vera* leaf extract: Freshly collected *A. vera* leaves (2168.9 g) were washed and cut open from the middle, the inner gel was scraped and separated. The remaining outer leaves (499.96 g) were cut in small pieces, homogenized with 800 ml PBS (phosphate-buffered saline: 0.06 mM sodium phosphate buffer containing 0.15 M NaCl, pH 7.4) in a Waring blender, extracted with PBS, at 4°C overnight, filtered through cloth and then centrifuged at 6000 rpm for 30 min. The precipitate was discarded and the clear yellow supernatant (450 ml) was named *A. vera* leaf *crude extract*.

Ammonium sulphate was added to the *crude extract* to 50 % saturation. After 6 h at 4°C, this material was centrifuged at 6000 rpm for 30 min and the precipitate was resuspended in PBS. After centrifugation at 6000 for 10 min to remove insoluble material the supernatant named *50% $(NH_4)_2SO_4$ fraction* (0.84 mg/ml protein), was applied to affinity chromatography.

Coupling of the ligand: 1.25 g ovalbumin, dissolved in 25 ml coupling buffer (100 mM $NaHCO_3$, containing 0.5 M NaCl, pH 8.3), was incubated with 5 g of CNBr-activated Sepharose 4B (preswollen in 500 ml 1 mM HCl for 1 h and equilibrated in coupling buffer) and allowed to react, by gently stirring, overnight at 4°C. The conjugated resin was first washed with 100 ml of coupling buffer and residual amine binding sites were blocked by incubation in 100 mM Tris-HCl, pH 8.0 for 2 h at 4°C. For washing, the column was incubated with 100 mM sodium acetate, containing 0.5 M

NaCl (pH 4.5) and 100 mM Tris-HCl, containing 0.5 M NaCl (pH 8.0) and this procedure was repeated three times. Finally the affinity matrix was washed with PBS.

Affinity chromatography: The *50% $(NH_4)_2SO_4$ fraction* was incubated with the CNBr-activated Sepharose 4B coupled-ovalbumin overnight, then loaded on a column of 1.5 x 16 cm. After extensive washing with PBS, the ovalbumin-bound material was eluted with 50 mM glycine-HCl, containing 0.15 M NaCl, pH 2.9. The pH of the eluted material was immediately readjusted to 7.0 by addition of M Tris-HCl buffer, pH 9.0. The protein content of the produced fractions was monitored at 280 nm and the protein fractions were combined (30 ml) and examined for hemagglutinating activity. The resulting lectin preparation was dialyzed against PBS, concentrated in an ultrafiltration cell device (Millipore Corporation, Bedford, MA 01730 U.S.A.) to 3 ml (0.28mg/ml protein) through an Amicon PM 10 membrane.

Hemagglutination tests: These tests were performed by making serial dilutions of samples in PBS using microplates (in 50 µl). To each well 25 µl of 4% rabbit erythrocyte suspension prepared from rabbit blood was then added. Hemagglutination was determined visually and microscopically after standing at room temperature for 1 h. Hemagglutination titer was expressed as the reciprocal of the dilution showing detectable agglutination and the specific activity (HU/mg) was calculated according to Lis and Sharon (17).

Hemagglutination inhibition tests: These tests were carried out in order to determine the effects of saccharides (4-Nitrophenyl-α-D-glucopyranoside, 4-Nitrophenyl-β-D-glucopyranoside, 4-Nitrophenyl-α-D-galactopyranoside, 4-Nitrophenyl-β-D galactopyranoside, lactose, N-acetyl-D-galactosamine) or glycoproteins (ovalbumin) on hemagglutination and thus find the specific carbohydrate of *A. vera* lectin. Appropriate concentrations of saccharide or glycoprotein in 0.15 M NaCl were added to the wells in 50 µl aliquots, replacing the equivalent volume of saline. Hemagglutination titers were determined after 1 h.

Protein determination: The protein content of the samples obtained during the purification process was determined by the $E_{280/}E_{260}$ method (18) and the method of Lowry *et al.* (19) using bovine serum albumin as standard.

Polyacrylamide gel electrophoresis (PAGE): Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE*)* was carried out in 12.5%

polyacrylamide gel slabs at pH 8.3 by using M Tris-glycine buffer containing 0.1% (w/v) SDS. Subunit molar mass was analysed under reduced conditions. The purified lectin sample was prepared by heating a protein solution in a sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.02% bromphenol, 5% 2-mercaptoethanol) at 100°C for 5 min. A low molecular weight calibration mixture was used as standard.

Electrophoresis of native proteins was performed in the same PAGE gel system but in the abscence of SDS and without sample pretreatment.

Antioxidant activity assessement by the DPPH radical-scavenging activity assay: The DPPH free radical-scavenging activity of *A. vera* lectin was measured according to the procedure described by Brand-Williams *et al.,* (20). The lectin solution (0.1 ml) at different concentrations (0.02–0.28 mg/ml), ascorbic acid (0.008-0.25 mg/ml) or α-tocopherol (0.031-0.5 mg/ml) was added to 3.9 ml of 6 x 10^{-5} M methanolic solution of DPPH. The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 30 min. The decrease in absorbance of the resulting solution was then measured spectrophotometrically at 517 nm against methanol. Two controls were used for this test, a negative control (containing all reagents except the test sample) and positive controls (using the reference antioxidants: ascorbic acid and α-tocopherol) for comparison. The ability to scavenge $DPPH^{\cdot}$ radical was calculated by the following equation: DPPH radical-scavenging activity (%) = [1-(Absorbance of sample at 517 nm/ Absorbance of control at 517 nm)] x 100.

RESULTS AND DISCUSSION

The ability of lectins to agglutinate cells is a recogonized physiological effect that depends on their specificity and high binding affinity for a particular carbohydrate moiety on the cell surface (21). Carbohydrate-binding specificity of lectins is widely used for their purification by affinity chromatography. For this purpose hemagglutination inhibition assays are performed to determine the specific sugar, saccharide or glycoprotein of the lectin and then the given carbohydrate is bound to the affinity matrix.

Affinity columns containing Sepharose covalently coupled to a glycoprotein such as fetuin, thyroglobulin, bovine serum albumin or ovalbumin might be used as a single step procedure for the isolation of lectins that occur

naturally. The method once settled, can also be used in the other sense, *i.e.* for the purification of ovalbumin from a given source by *Aloe* lectin-affinity chromatography. Suzuki *et al*., (14) purified and characterized two lectins from *A. arborescens* for the first time. Further purification of one of these lectins, was achieved later through Sephadex G-200 gel filtration and their chemical, biological and pharmacological activities were defined (8). The purification and characterization of two lectins named Aloctin I and Aloctin II from *A. vera* leaves through a combination of hydroxylapatite column chromatography and gel filtration on Sephadex G-50 was carried out previously in our laboratory (16,22). There is only one report on the use of affinity chromatography for the purification of a lectin from *Aloe* leaves (15) using glucose as coupling ligand. As none of the 20 sugars tested, including glucose, inhibited hemagglutinating activity of Aloctin I (16), affinity chromatography couldn't be undertaken. In the present study, several disaccharides and ovalbumin were tested for hemagglutination inhibition and ovalbumin was chosen as the best inhibitor. Accordingly, a new one step CNBr-Sepharose-ovalbumin affinity chromatography method for the purification of the lectin from *A. vera* leaf to homogeneity, was achieved and 60 fold purification was obtained (Table 1 and Fig 1).

Table 1. Purification of *Aloe vera* leaf lectin by affinity chromatography on CNBr Sepharose-ovalbumin column (starting with 499.96 g fresh leaves).

Purification step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity (HU/mg)	Purification fold
Crude extract	450	4,22	1899	7,58	1
50 % $(NH_4)_2SO_4$ fraction	176	0,84	147,8	152,4	20,1
Affinity chromatography	3	0,28	0,84	457,1	60,3

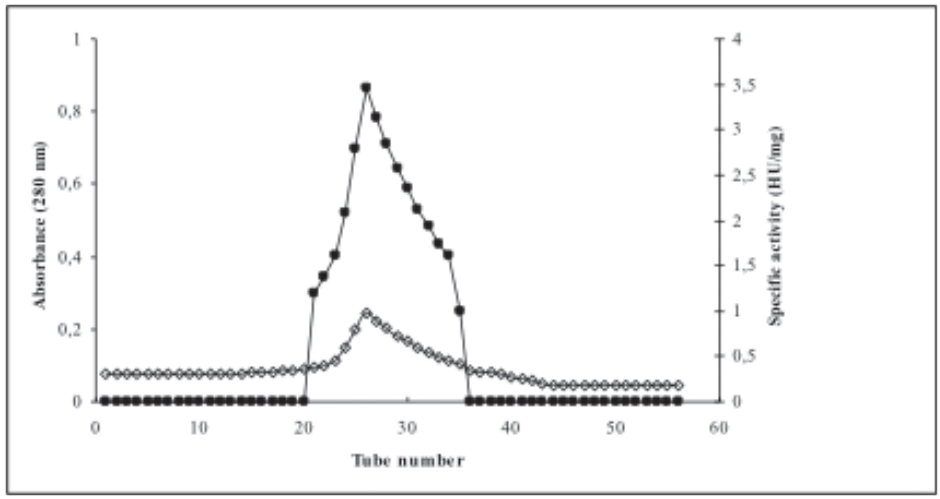

Fig. 1: Affinity chromatography elution profile of the fraction obtained by 50 % ammonium sulphate saturation of *Aloe vera* leaf crude extract.

Column: 16 x 1,5 cm., Elution buffer: 0.05 M glycine-HCl buffer containing 0.15 M NaCl; Flow rate: 75 ml/h.; Fractions: 2.5 ml; ●–●: Specific activity (HU/mg); <>-<>: Protein (mg/ml). Elution was carried out at 4°C.

Native PAGE gave a single band (Fig 2 A) and showed that the purified lectin has an apparent molecular weight of 45000 kDa. This value was close to 55000 kDa for the lectin purified by Chung and Park (15). The fact that SDS-PAGE gave a subunit molecular weight of 14400 kDa (Fig. 2 B and C), tends to propose that the purified lectin is composed of three subunits of equal molecular weight which is not in accordance with Chung and Park who have found two bands of 26 and 28 kDa in SDS-PAGE (15). Our results were in agreement with the molecular weights of Aloctin I purified by us (22), and that of Aloctin A purified from *Aloe arborescens* Miller (8).

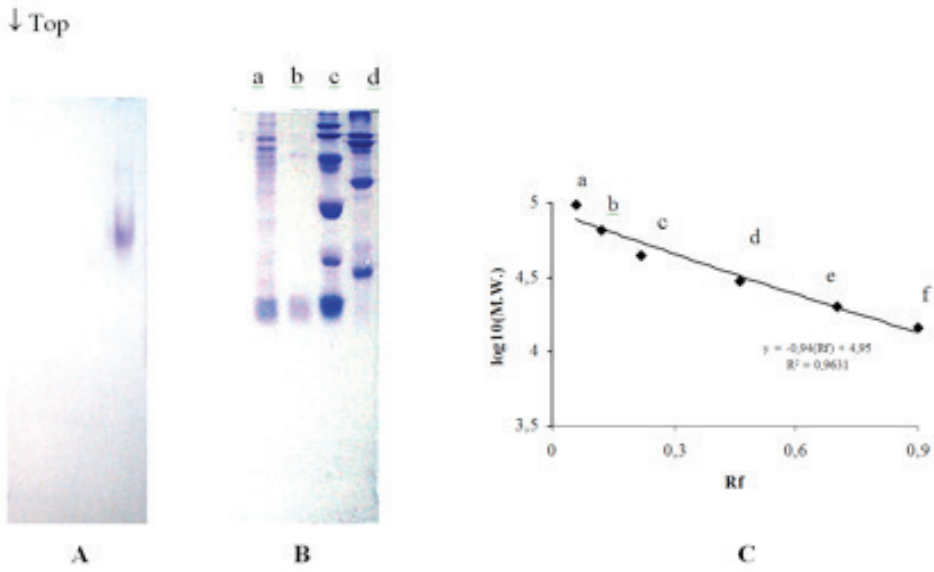

Fig. 2: Polyacrylamide gel electrophoresis (PAGE) of *Aloe vera* leaf crude extract and purified lectin.

A) Native PAGE showing purified lectin after affinity chromatography

B) SDS-PAGE. a – Crude extract. b – Purified lectin after affinity chromatography. c – Low molecular weight proteins. d – High molecular weight proteins

C) Molecular weight determination based on SDS- polyacrylamide gel electrophoresis. Standards used were a– phosphorylase b (M_r=97000), b– albumin (M_r=66000), c– ovalbumin (M_r=45000), d– carbonic anhydrase (M_r =30000), e– trypsin inhibitor (Mr=20000) and f– α-lactalbumin (M_r = 14400).

There is no report on the antioxidant activity of lectins in literature. The antioxidant activity of polysachharides (23-25) was reported. Our lectin is known to have a glycoprotein structure (16). In order to determine whether the antioxidant activity of *A. vera* reported in literature (26), comes from the phenolic compounds, vitamins, polysaccharides or from the glycoprotein lectin, the antioxidant activity of the purified lectin was assessed by the $DPPH^{\cdot}$ radical scavenging method and no antioxidant activity was found. The antitumour activity of the plants is commonly correlated with their antioxidant effect. In our previous studies we have reported that the tumour preventive effect of *A.vera* leaf extract was higher than that of the purified lectin (27), this finding is in agreement with the lack of antioxidant activity of the lectin.

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