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Journal of Chromatography B, 844 (2006) 213–216
JOURNAL OF CHROMATOGRAPHY B
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A hemagglutinin with mitogenic activity from dark red kidney beans

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Received 22 March 2006; accepted 9 July 2006
Available online 1 September 2006

Abstract

A 67-kDa hemagglutinin composed of two identical subunits was purified from *Phaseolus vulgaris* cv. 'Dark Red Kidney Bean'. It was unadsorbed on DEAE-cellulose but adsorbed on Affi-gel blue gel. The hemagglutinin was highly purified after the two aforementioned chromatographic steps as revealed by a single peak in gel filtration on Superdex 75 and a single band in SDS-PAGE. The hemagglutinating activity was stable between 25 °C and 70 °C, and between pH 4 and pH 11, and in the presence of a variety of divalent metal chlorides at 500 mM concentration. The activity was reduced by 50% at 80 °C, and also when the pH was lowered to 3 or elevated to 12. The activity was reduced by 75% in the presence of 250 mM KCl or NaCl. A variety of sugars tested failed to inhibit the hemagglutinating activity of the hemagglutinin. Although the hemagglutinin exhibited mitogenic activity toward murine splenocytes, it had no effect on the activity of HIV-1 reverse transcriptase or mycelial growth in the fungi *Botrytis cinerea, Fusarium oxysporum* and *Mycosphaerella arachidicola*. It exerted an antiproliferative activity on leukemia L1210 cells.

Keywords: Dark red kidney bean; Isolation; Hemagglutinin

1. Introduction

Lectins reported from a multitude of organisms comprising animals, plants, bacteria, viruses and fungi have captured the attention of a large number of researchers on account of their exploitable biological activities encompassing mitogenic [1], immunomodulatory [2], antiproliferative [1], antitumor [3], anti-insect [4], antifungal [5], antiviral [6] and anti-HIV-1 reverse transcriptase [1] activities. Lectins are involved in the symbiotic relationship between leguminous plants and nitrogen-fixing bacteria [7]. In animals, lectins are implicated in phagocytosis of micro-organisms [8], differentiation and organ formation [9], lymphocyte migration from blood into lymphoid organs [10], and metastasis of cancer cells [11].

The seeds of leguminous plants represent a rich source of biologically active proteins including lectins [12], antifungal proteins [13], protease inhibitors [14], α-amylase inhibitors [15], ribosome inactivating proteins [16], and peroxidases [17]. Previously a hemagglutinin with antifungal activity has been reported from the red kidney bean variety of *Phaseolus vulgaris* [12]. The purpose of the present study was to purify and characterize in greater detail a lectin from the dark red kidney bean variety of *P. vulgaris*.

2. Materials and methods

2.1. Purification protocol

P. vulgaris cv. 'Dark Red Kidney Bean' (100 g) from the U.S.A. were smashed and homogenized (3 ml/g) in 10 mM Tris–HCl buffer (pH 7.4). The homogenate was centrifuged (12000 g, 30 min, 4 °C), and the supernatant was saved for ion exchange chromatography on a 5 × 20 cm of DEAE-cellulose (Sigma Chemical Co.) in 10 mM Tris–HCl buffer (pH 7.4). The flowthrough fraction (DD1) was collected before adsorbed proteins were eluted with 10 mM Tris–HCl buffer containing 1 M NaCl and collected as fraction DD2. Fraction DD1 was subsequently subjected to affinity chromatography on a 2.5 cm × 20 cm column of Affi-gel blue gel (Bio-Rad) in 10 mM Tris–HCl buffer (pH 7.4). After the flowthrough fraction (DB1) had all been collected, adsorbed proteins were desorbed with the starting buffer containing 1.5 M NaCl and collected as fraction DB2. Fraction DB2 was further purified by fast protein liquid chromatography on a Superdex 75 HR 10/30

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doi:10.1016/j.jchromb.2006.07.042

gel filtration column (Amersham Biosciences) using an AKTA Purifier System (Amersham Biosciences).

2.2. Assay of hemagglutinating activity

In the assay for lectin (hemagglutinating) activity, a serial two-fold dilution of the hemagglutinin solution in microtiter U-plates (50 μl) was mixed with 50 μl of a 2% suspension of rabbit red blood cells in phosphate buffered saline (pH 7.2) at 20 °C. The results were read after about 1 h when erythrocytes in the blank had fully sedimented and formed a dot at the bottom of the well. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was reckoned as one hemagglutination unit. Specific activity is the number of hemagglutination units per mg protein [1].

The tests to investigate inhibition of hemagglutinin-induced hemagglutination by various carbohydrates including D-mannose, D-fructose, D-xylose, L-arabinose, raffinose, L-rhamnose, D-melezitose, D-melibiose, cellobiose, D-ribose, inositol, D-glucose, sucrose, D-galactose, galactitol, O-nitrophenyl-β-D-galactopyranoside and 4-O-β-D-galactopyranosyl-D-glucose were performed in a manner analogous to the hemagglutination test. Serial twofold dilutions of sugar samples were prepared in phosphate buffered saline. All of the dilutions were mixed with an equal volume (25 μl) of a solution of the hemagglutinin with 4 hemagglutination units. The mixture was allowed to stand for 30 min at room temperature and then mixed with 50 μl of a 2% rabbit erythrocyte suspension. The minimum concentration of the sugar in the final reaction mixture which completely inhibited 4 hemagglutination units of the hemagglutinin preparation was calculated [1].

The effects of temperature, NaOH solution, HCl solution and solutions of metallic chlorides on hemagglutinating activity of the hemagglutinin were examined as previously described [1].

2.3. Molecular mass determination and N-terminal sequence determination

The purified hemagglutinin was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for molecular mass determination in accordance with the procedure of Nielsen and Reynolds [18]. Gel filtration on an FPLC-Superdex 75 column, which had been calibrated with molecular mass markers (Amersham Biosciences), was conducted to determine the molecular mass. The N-terminal sequence of the hemagglutinin was determined by using a Hewlett-Packard HP G1000A Edman degradation unit and an HP 1000 HPLC System.

2.4. Assay of mitogenic activity

The assay of mitogenic activity was performed as described by Wong and Ng [1]. Splenocytes were isolated from BALB/c mice. The cells were diluted with RPMI medium containing 10% fetal bovine serum (FBS) and then seeded (2×10^6 cells/0.2 ml/well) in 96-well microplates. The hemagglutinin was then added at various concentrations. Cells cultured in the absence of the hemagglutinin served as control. The cells were incubated at 37 °C in a humidified atmosphere of 5% carbon dioxide for 24 h. The cells were viable after 24 h. During the last 6 h, the cells in one well were pulsed with 0.5 μCi of [^{3}H-methyl]-thymidine (specific activity 5 μCi/mmol, Amersham Biosciences) in 10 μl and were then harvested on to a glass fiber filter using a cell harvester. The radioactivity was determined using a Beckman scintillation counter. The proliferative (mitogenic) response was expressed as mean counts per min (cpm).

2.5. Assay of antiproliferative activity

The antiproliferative activity of the purified hemagglutinin was determined as follows [1]. The cell line L121 (leukemia) was purchased from American Type Culture Collection. The various cell lines were maintained in Dulbecco Modified Eagles' Medium (DMEM) supplemented with 10% fetal bovine serum and 100 mg/l streptomycin and 100 IU/ml penicillin at 37 °C in a humidified atmosphere of 5% CO_2. Cells (1×10^4) in their exponential growth phase were seeded into each well of a 96-well culture plate (Nunc, Denmark) and incubated for 3 h before addition of the hemagglutinin. Incubation was carried out for another 48 h. Radioactive precursor, 1 μCi ([^{3}H-methyl]-thymidine, from Amersham Biosciences) was added to each well and incubated for 6 h. The cultures were then harvested by a cell harvester. The incorporated radioactivity was determined by liquid scintillation counting.

2.6. Assays of antifungal and HIV-1 reverse transcriptase inhibitory activities

The assay for antifungal activity was conducted as described [13]. The assay for HIV-1 reverse transcriptase inhibitory activity was carried out as detailed in [1].

3. Results

The crude extract (containing 8343 mg protein, 4910 hemagglutination titers/mg) of 100 g of dark red kidney beans (*P. vulgaris* cv. 'Dark Red Kidney Bean') was fractionated into an unadsorbed fraction (DD1, 3983 mg) and a slightly smaller adsorbed fraction (DD2, 3477 mg) on a DEAE-cellulose column. DD1 contained hemagglutinating activity. DD2 yielded an unadsorbed fraction (DB1, 1554 mg) and a slightly smaller adsorbed fraction DB2 (140 mg, 58514 hemagglutination titers/mg) after it was subjected to affinity chromatography on Affi-gel blue gel. DB2, which contained the hemagglutination activity, yielded a single peak (DS1, 107 mg, 64810 hemagglutination titers/mg) when chromatographed on a Superdex 75 HR 10/30 column (Fig. 1). The molecular mass was estimated to be 67 kDa. In SDS-PAGE it only showed one band of about 33 kDa (Fig. 2). This demonstrated that this hemagglutinin is composed of two identical subunits each of about 33 kDa. There was 13 fold purification with 17% recovery of hemagglutinating activity. The N-terminal sequence of the hemagglutinin was identical with that of flageolet beans (*P. vulgaris* cv. 'Flageolet Bean')

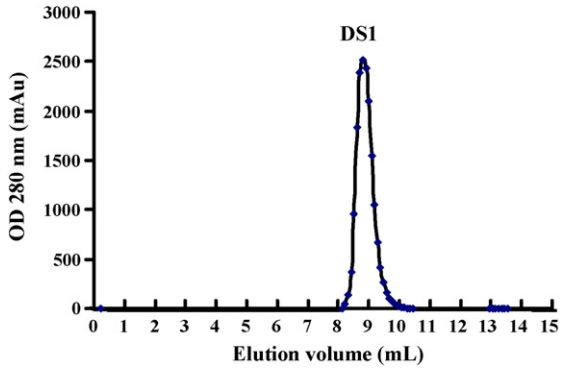

Fig. 1. Gel filtration of fraction DB2 on an FPLC Superdex 75 HR 10/30 column. Fraction DB2 was dialyzed, lyophilized, and then loaded on the column. The column was eluted with 20 mM NH_4HCO_3 buffer (pH 9.0). Fraction DS1 exhibited hemagglutinating activity. Its elution volume corresponded to a molecular mass of 67 kDa. Flow rate: 0.5 ml/min. Fraction size: 0.8 ml.

hemagglutinatinin and similar to that of a hemagglutinin from tenuifolius beans (*Phaseolus acutifolius* cv. Tenuifolius Beans) (Table 1).

The hemagglutinating activity was stable between 25 °C and 70 °C. Loss of 50% of the hemagglutinating activity took place when temperature was raised to 80 °C for 20 min. Activity was indiscernible when the temperature reached 90 °C and 100 °C. The activity was stable when exposed to pH values between pH 4 and pH 11 for 20 min but at pH 3 and pH 12 there was about 50% loss in activity. The activity of the hemagglutinin could not be inhibited by any of the sugars tested at 250 mM concentration. When monovalent, divalent and trivalent metal chlorides were tested for inhibitory effect on hemagglutinating activity, only chlorides of the monovalent metals NaCl and KCl (250 mM) proved inhibitory. Other chlorides including $CaCl_2$, $CnCl_2$, $FeCl_2$, $FeCl_3$, $MgCl_2$, $MnCl_2$ and $ZnCl_2$ did not have any effect. Treatment with trypsin (1:1, w/w; 37 °C for 30 min)

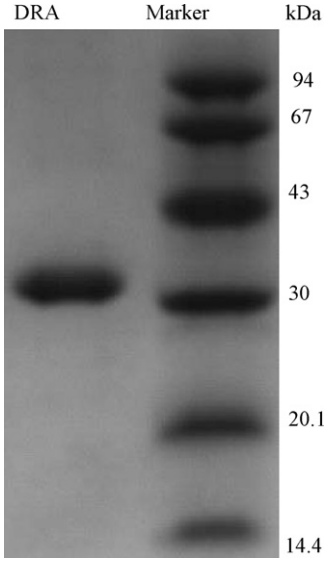

Fig. 2. SDS-PAGE results. Left lane: fraction DS1 (representing purified dark red kidney bean hemagglutinin DRA). Right lane: molecular mass markers from Amersham Biosciences.

Table 1
Comparison of N-terminal sequence of hemagglutinin from dark red kidney beans (*P. vulgaris* cv. 'Dark Red Kidney Bean') (DRA) with hemagglutinins from other sources (results of blast search)

Hemagglutinin	Residue no.	Sequence	Total no. of amino acid residues
DRA	1[a]	SNDIYFNFQR	~600
RKA	1	ANQTSFNFQR	~600
PCL	22[b]	ASETSFSFQR	273
PAL	25	ANDISFNFQR	276

Identical corresponding residues are underlined. DRA: hemagglutinin (SRA) from dark red kidney beans (*P. vulgaris* cv. 'Dark Red Kidney Bean'); RKA: hemagglutinin from red kidney beans (*P. vulgaris* cv. 'Red Kidney Bean'); PAL: hemagglutinin from tenuifolius beans (*Phaseolus acutifolius* cv. 'Tenuifolius Bean'); PCL: lectin from *Phaseolus coccineus L.* beans.
[a] Refers to the first residue of the hemagglutinin.
[b] Refers to the 22nd residue of the hemagglutinin.

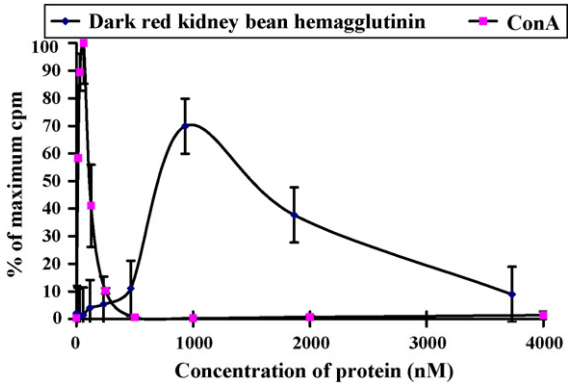

Fig. 3. The stimulatory effect of dark red kidney bean hemagglutinin on the mitogenic response of mouse splenocytes as reflected by uptake of [methyl-^{3}H] thymidine. Results represent mean ± S.D., $n = 3$.

boosted the hemagglutinating activity. There was no effect of the trypsin treatment on the electrophoretic mobility of the hemagglutinin in SDS-PAGE (data not shown). The hemagglutinin exerted a weaker mitogenic effect than Con A on mouse splenocytes. When compared with Con A, it took 10 times higher concentration of the hemagglutinin to achieve maximal mitogenic response, i.e. 100 nM for Con A and 1000 nM for the hemagglutinin. The magnitude of the maximal mitogenic response to

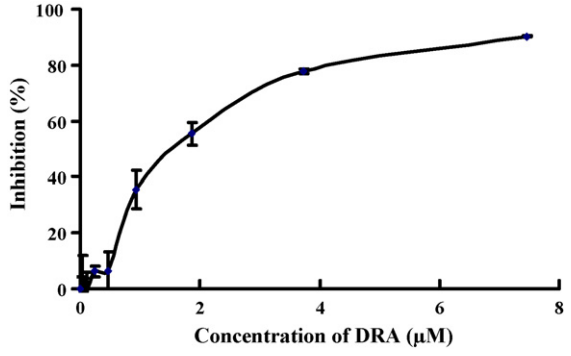

Fig. 4. Inhibitory effect of dark red kidney bean hemagglutinin (DRA) on the proliferation of L1210 cells as reflected by inhibition of [methyl-^{3}H] thymidine uptake (the IC_{50} is determined as 1.6 μM). Data represent mean ± S.D., $n = 3$.

the hemagglutinin was 70% of that of Con A (Fig. 3). It did not inhibit HIV-1 reverse transcriptase when tested at concentrations from 77 nM to 4.93 μM. However, it inhibited proliferation of leukemia L1210 cells with an IC_{50} of 1.6 μM (Fig. 4). There was a lack of antifungal activity toward *Botrytis cinerea*, *Fusarium oxysporum* and *Mycosphaerella arachidicola* at a dosage of 300 μg.

4. Discussion

The dark red kidney bean hemagglutinin isolated in this study displays an N-terminal sequence that is not completely identical to that of red kidney bean hemagglutinin reported earlier [12]. Both hemagglutinins are composed of two 33-kDa subunits and their hemagglutinating activity, like that of *Acacia constricta* lectins [19], cannot be inhibited by a variety of simple sugars. Dark red kidney bean hemagglutinin is devoid of antifungal activity, unlike red kidney bean hemagglutinin. The results thus indicate that each of the two cultivars of *P. vulgaris*, dark red kidney bean and red kidney bean, produce a hemagglutinin that is not identical. In fact, only very few lectins have been reported with antifungal activity [12].

The pH stability, thermostability, trypsin stability, stability in the presence of metallic chlorides, mitogenic activity and antiproliferative activity of red kidney bean lectin have not been examined [12]. Dark red kidney bean hemagglutinin is fairly stable with respect to pH stability, thermostability, and metal ion inhibition. Its pH stability, thermostability and trypsin stability are similar to those of lectin from the knife bean *Canavalia gladiata* [20]. The latter lectin maintained its hemagglutinating activity in the range of pH 3–11. The hemagglutinating activity was stable up to 70 °C for 20 min, was reduced to 50% at 80 °C, and completely abolished at 90 °C. The activity remained unaltered at various time intervals (0, 0.5, 1, 2 and 3 h) after treatment with trypsin (1:1, w/w). Similar to knife bean lectin, dark red kidney bean hemagglutinin manifests mitogenic activity toward mouse splenocytes and antiproliferative activity toward leukemia cells, albeit with a lower potency. Other legume lectins also exhibit antiproliferative activity toward cancer cell lines [1]. Unlike knife bean lectin which inhibits HIV-1 reverse transcriptase with an IC_{50} of 35 μM [22] and red kidney bean lectin with similar activity [12], dark red kidney bean hemagglutinin is devoid of such activity. In fact very few lectins have been reported with such activity [22].

The chromatographic behavior of dark red kidney bean hemagglutinin on Affi-gel blue gel is similar to that of knife bean lectin [22] in that both are adsorbed. Only an ion exchange chromatographic step and an affinity chromatographic step are required to produce a pure preparation of dark red kidney bean hemagglutinin and a nearly pure preparation of knife bean lectin [22].

Affinity chromatography on immobilized sugars has been employed in some of the previous studies on isolation of plant lectins. Only two chromatographic steps were required to produce a purified hemagglutinin in this study. The isolated hemagglutinin has fair pH stability and thermostability compared with other lectins.

Lectins serve as recognition factors between symbiotic nitrogen fixing bacterias and host plants and as storage proteins in plants [21]. Lectins have been used in the biological control of microorganisms and thus reduction of crop loss [22]. They are used in blood typing and stimulation of cells for chromosome analysis and gene mapping, in cell separation, identification of complex glycoproteins and typing of bacteria [21]. Mitogenic lectins have a curative potential. They may provide protection and recovery from the immunosuppressive and mycelosuppressive effects of tumors and infections. They may also be used agsint malignancies [23]. At present, the significance of mitogenic activity of lectins to plants is not known. Hence, the mitogenic activity of dark red kidney beans may find applications just like the mitogenic phytohemagglutinin-L4 [24].

Acknowledgements

We thank Ms. Fion Yung for her excellent secretarial assistance.

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