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Short communication

A homotetrameric agglutinin with antiproliferative and mitogenic activities from haricot beans

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Abstract

A homotetrameric agglutinin with a molecular mass of 130 kDa was isolated from seeds of the haricot bean. The agglutinin was isolated using a procedure that involved ion exchange chromatography on DEAE-cellulose, affinity chromatography on Affi-gel blue gel and gel filtration by fast protein liquid chromatography on Superdex 200. Haricot bean agglutinin was adsorbed on DEAE-cellulose and Affi-gel blue gel. The hemagglutinating activity of the agglutinin was stable up to 40 °C. It underwent a 40% decline when the temperature was raised to 50 °C and a complete loss when the temperature was further increased to 80 °C. The hemagglutinating activity exhibited a time-dependent loss in activity when the agglutinin was incubated at 100 °C for different durations. No activity was discernible when the agglutinin was left at 100 °C for 1 min. The activity also underwent a decline in the presence of 500 mM $FeCl_3$ and $CaCl_2$. Haricot bean agglutinin manifested a weaker mitogenic activity than concanavalin A toward mouse splenocytes. It exhibited antiproliferative activity toward the tumor cell lines M1 [leukemia], HepG2 [hepatoma] and L1210 [leukemia] cells.
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Keywords: Haricot bean; Isolation; Agglutinin

1. Introduction

Leguminous plants produce a large variety of proteins including lectins [1,2] antifungal proteins [3], ribosome inactivating proteins [4], peroxidases [5,6], protease inhibitors [7,8] and amylase inhibitors [9]. Among these various proteins, lectins represent a group of extensively studied proteins. There are many lectins reported from *Phaseolus* species. Two lectins, GNL-1 and 2, isolated from extracts of Great Northern bean (*Phaseolus vulgaris*), had molecular masses of 175 and 145 kDa, respectively. In the assay of mitogenic activity, GNL-1 showed a strong mitogenic activity, but GNL-2 was devoid of the activity [10]. In tepary beans (*Phaseolus acutifolius*), a toxic and tetrametic lectin was found [11]. Black gram (*Phaseolus mungo*) seeds were shown to contain a monomeric lectin with a molecular mass 58 kDa [12]. A tetrameric 84 kDa lectin from *Phaseolus acutifolius* was reported to have mitogenic and immunosuppressive activities [13]. A 135 kDa lectin was purified from the leaves of Great Northern bean, *Phaseolus vulgaris* L [14]. A homotetrameric kidney bean (*Phaseolus vulgaris*) hemagglutinin has been reported [15]. The purification methods for isolation of bean lectins included affinity chromatography on a human alpha 1-acid glycoprotein Sepharose 4B column and ion-exchange chromatography on a Mono S column for red kidney bean lectin [16], ion-exchange chromatographies on CM- and DEAE-cellulose and gel filtration on Sephacryl S-300 HR for lectin from pods of the Great Northern beans [17]. Recombinant kidney bean lectin was expressed and secreted by the methylotrophic yeast *Pichia pastoris*. The recombinant lectin had a primary amino acid sequence identical to that of the native protein and an agglutination activity similar to that of native kidney bean lectin [18].

Research on haricot beans is restricted to only an early report on isolation of haricot bean agglutinin [19]. Haricot bean/agglutinin is a glycoprotein composed of four subunits each with a molecular mass of 30 kDa. It was purified with a procedure that entailed $[NH_4]_2SO_4$ precipitation and chromatography in DEAE-cellulose and Sephadex G200. However, no sequence information is available. The isolated agglutinin has not been examined with regard to biological activities such as mitogenic, antiproliferative and HIV-1 reverse transcriptase

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inhibitory activities which are characteristic of some lectins [19]. The objective of the present study was to purify and partially characterize haricot bean agglutinin.

2. Materials and methods

2.1. Materials

Dried seeds of the haricot bean (*Phaseolus vulgaris*) were purchased from a local supermarket. The three species of phytopathogenic fungi examined in this study, namely *Rhizoctonia solani*, *Fusarium oxysporum* and *Coprinus comatus* were obtained from Department of Microbiology, China Agricultural University, Beijing, China. The HIV-1 reverse transcriptase ELISA kit was from Boehringer Mannheim, Mannheim, Germany. The L1210, M1 and HepG2 cell lines were purchased from American Type Culture Collection [ATCC], Manassas, USA. Superdex 200 column and [methyl-^{3}H]-thymidine and molecular-mass marker were purchased from Amersham Biosciences, Hong Kong, China. Affi-gel blue gel was from Bio-Rad, California, USA. DEAE-cellulose and Con A were purchased from Sigma, St. Louis, Missouri, USA. All other chemicals used were of analytical grade.

2.2. Purification protocol

The haricot beans were soaked in distilled water [3 ml/g] for 8 h in order to soften them up and were then blended using a Waring blender. To the supernatant obtained after centrifugation [15,000 × g, 30 min] of the homogenate, Tris–HCl buffer [pH 7.6] was added to the supernatant obtained after centrifugation until a concentration of 10 mM Tris was attained. The supernatant was then applied to a column [5 cm × 20 cm] of DEAE-cellulose which had previously been equilibrated and was then eluted with 10 mM Tris–HCl buffer [pH 7.6] at a flow rate of 5 ml/min. After the first peak of absorbance [D1] had come off the column, the column was eluted stepwise with 200, 500 and 1000 mM NaCl in the Tris–HCl buffer. The second peak of absorbance [D2] was dialyzed against 10 mM Tris–HCl buffer [pH 7.6] and then applied to a column of Affi-gel blue gel [2.5 cm × 20 cm] in the same buffer at a flow rate of 2 ml/min. After the first peak of absorbance had flowed through the column, the column was eluted by inclusion of 1 M NaCl in 10 mM Tris–HCl buffer [pH 7.6]. The second peak of absorbance B2 was then subjected to gel filtration by fast protein liquid chromatography on a Superdex 200 HR 10/30 column (effective separation range 10–600 kDa) at a flow rate of 0.5 ml/min. The first eluted peak S1 constituted purified haricot bean agglutinin.

2.3. Determination of molecular mass using sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] and gel filtration

The purified haricot bean agglutinin was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] for molecular mass determination in accordance with the procedure of Laemmli and Favre [20]. Gel filtration on an FPLC-Superdex 200 column, which had been calibrated with molecular mass markers [Amersham Biosciences, Hong Kong, China], was conducted to determine the molecular mass of the agglutinin.

2.4. Determination of N-terminal amino acid sequence

The N-terminal sequence of the haricot bean agglutinin was determined by using a Hewlett-Packard [HP] G1000A automated protein sequencer. This sequencer includes an automated Edman degradation unit and an HPLC on a reverse-phase C-18 column. For sample preparation, the target protein band in the SDS-PAGE gel was cut out, and then incubated in sequencing buffer [0.1 M CH_3COONa, 0.1 SDS, pH 8.5] overnight at room temperature. The solution was centrifuged at 14,000 rpm for 10 min in order to get rid of the particles of SDS-PAGE gel, and the supernatant was subjected to sequencer.

2.5. Assay of inhibitory activity toward human immunodeficiency virus [HIV-1] reverse transcriptase

The assay for HIV-1 reverse transcriptase inhibitory activity was measured by ELISA as described by Collins et al. [21] using a non-radioactive kit from Boehringer Mannheim [Germany]. The inhibitory activity of haricot bean agglutinin was calculated as percent inhibition as compared to a control without the protein.

2.6. Assay of hemagglutinating activity

In the assay for lectin [hemagglutinating] activity, a serial two-fold dilution of the haricot bean agglutinin 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 room temperature. The results were read after about 1 h when the blank had fully sedimented. 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 [22].

2.7. Test of inhibition of lectin-induced hemagglutination by various carbohydrates

Test of inhibition of lectin-induced hemagglutination by various carbohydrates was carried out as follows. A solution of haricot bean agglutinin [25 μl] with four hemagglutination units was mixed with an equal volume of a serial two-fold dilution of the carbohydrate sample to be tested. After incubation at room temperature for 30 min, the mixture was mixed with a 2% suspension of rabbit erythrocytes. The minimal concentration of the carbohydrate in the final reaction mixture capable of completely inhibiting four hemagglutination units of the haricot bean agglutinin was calculated from the results [22].

2.8. Effect of metal ions on hemagglutinating activity

The effects of NaCl, KCl, $CaCl_2$, $MgCl_2$, $MnCl_2$, $CuCl_2$ and $FeCl_2$, all at 500 mM concentration, were studied. A solution of

haricot bean agglutinin [25 μl] with 16 hemagglutination units was two-fold diluted with buffer containing the various ions. Incubation was carried out at room temperature for 1 h. The assay of hemagglutinating activity was then carried out.

2.9. Effect of heat treatment on hemagglutinating activity

A solution of haricot agglutinin was incubated at various temperatures for 30 min: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 °C. The tubes were then put on ice and assay of hemagglutinating activity was then carried out.

2.10. Assay of mitogenic activity

Four C57BL/6 mice [20–25 g] were killed by cervical dislocation and the spleens were aseptically removed. Spleen cells were isolated by pressing the tissue through a sterilized 100-mesh stainless steel sieve and resuspended to 5×10^6 cells/ml in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 100 units penicillin/ml, and 100 ug streptomycin/ml. The cells [7×10^5 cells/100 μl/well] were seeded into a 96-well culture plate and serial dilutions of a solution of haricot bean agglutinin in 100 μl medium were added. After incubation of the cells at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h, 10 μl [methyl ^{3}H]-thymidine [0.25 μCi, Amersham Biosciences] was added, and the cells were incubated for a further 6 h under the same conditions. The cells were then harvested with an automated cell harvester onto a glass filter, and the radioactivity was measured with a Beckman model LS 6000SC scintillation counter. All reported values are the means of triplicate samples [23].

2.11. Assay of antiproliferative activity on tumor lines

Leukemia L1210 cell line, leukemia M1 cell line or hepatomas HepG2 cell line was suspended in RPMI medium and adjusted to a cell density of 2×10^4 cells/ml. A 100 μl aliquot of this cell suspension was seeded to a well of a 96-well plate followed by incubation for 24 h. Different concentrations of agglutinin in 100 μl complete RPMI medium were then added to the wells and incubated for 72 h. After 72 h, 20 μl of 5 mg/ml MTT in phosphate buffered saline was spiked into each well and the plates were incubated for 4 h. The plates were then centrifuged at 2500 rpm for 5 min. The supernatant was carefully removed and 150 μl of dimethyl sulfoxide was added in each well to dissolve the [MTT] formazan at the bottom of the wells. After 10 min, the absorbance at 590 nm was measured with a microplate reader.

3. Results

Ion exchange chromatography of the crude extract of haricot beans on DEAE-cellulose yielded a small unadsorbed peak D1, two large adsorbed peaks D2 and D3 eluted with 200 and 500 mM NaCl respectively, and a small peak D4 eluted with 1000 mM NaCl (Fig. 1). Hemagglutinating activity resided in peak D2. Affinity chromatography of peak D2 on Affi-gel blue

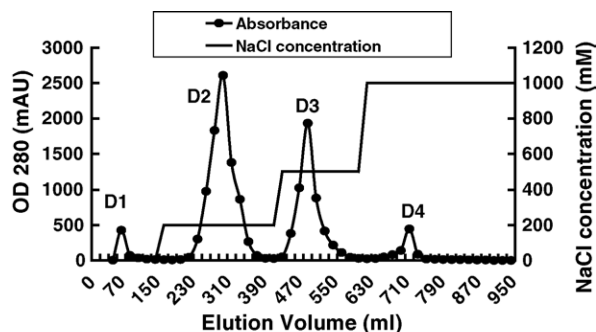

Fig. 1. Fractionation of the crude extract of haricot beans on a DEAE-cellulose column equilibrated with the binding buffer [10 mM Tris–HCl, pH 7.6]. The column was washed with the binding buffer to remove D1 and then eluted with stepwise gradients of 200, 500 and 1000 mM NaCl in the same buffer to desorb fractions D2–D4, respectively. Hemagglutinating activity was found in D2.

gel gave rise to a small peak B1 devoid of hemagglutinating activity and a large peak B2 in which hemagglutinating activity resided (Fig. 2). B2 was fractionated on Superdex 200 into a large peak S1 with hemagglutinating activity and a small peak S2 without activity. The molecular mass of S1 was 130 kDa (Fig. 3). In SDS-PAGE, peak S1 appeared as a single band with a molecular mass of 33 kDa (Fig. 4). The hemagglutinating activity of haricot bean agglutinin was stable up to 40 °C. At 50, 60, 70 and 80 °C, approximately 60, 45, 20 and 0%, respectively of the activity remained. About 50% of the activity remained after treatment at 100 °C for 30 s. The mitogenic activity of haricot bean agglutinin on mouse splenocytes was weaker than that of Con A. The concentration of haricot bean agglutinin required for optimal activity was 65 μM (Fig. 5A). The antiproliferative activity of haricot bean agglutinin toward three tumor cell lines, L1210, Hep G2 and M1, is shown in Fig. 5B.

The hemagglutinating activity of haricot bean agglutinin could not be inhibited by the following sugars when they were tested at a concentration of 500 mM. L[−]-Fucose, D[+]-glucose, D[+]-glucosamine, D[−]-galactosamine, D[+]-galactose, D[+]-glucose, D[+]-lactose, D[+]-melibiose, D[−]-mannosamine, L[−]-mannose, D[+]-mannose, D[+]-raffinose, L-

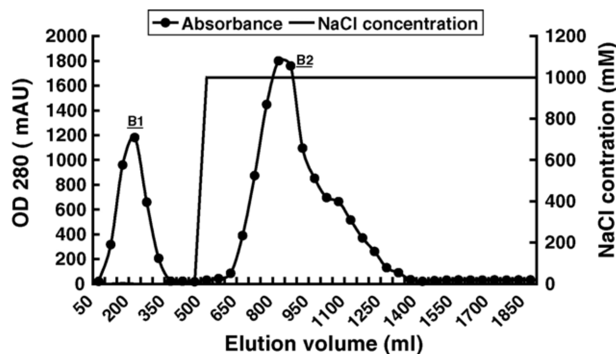

Fig. 2. Elution profile from the Affi-gel blue gel column. After chromatography on DEAE-celllulose, fraction D2 was dialyzed and then applied to an Affi-gel blue gel column in 10 mM Tris–HCl buffer [pH 7.6]. The column was then washed with the binding buffer to remove fraction B1. Adsorbed proteins were eluted as fraction B2 with 1000 mM NaCl in the Tris–HCl buffer. Hemagglutinating activity was found in fraction B2.

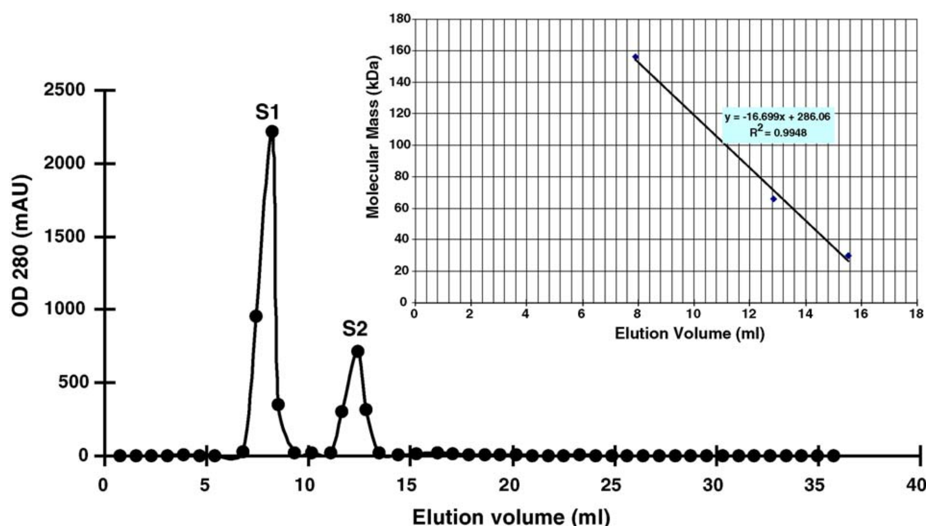

Fig. 3. FPLC-gel filtration of fraction B2 from the Affi-gel blue gel column on a Superdex 200 HR 10/30 column in 200 mM NH_4HCO_3 buffer [pH 9.6]. Fraction size: 1 ml. Hemagglutinating activity was located in peak S1 (elution volume = 8.3 mL). The calibration curve is shown at the top right corner. Three standard proteins shown on the calibration curve are aldolase (156 kDa), BSA (66 kDa), and carbonic anhydrase (30 kDa).

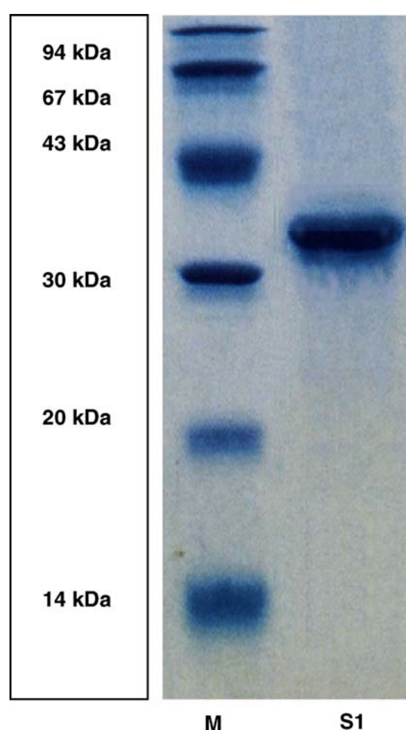

Fig. 4. SDS-PAGE of haricot bean agglutinin represented by fraction S1. This agglutinin is a tetramer. The native protein is around 130 kDa in molecular mass, and each subunit is around 33 kDa in molecular mass.

Table 2
N-terminal sequence of haricot bean agglutinin in comparison with lectins of various *Phaseolus* species

Sample name	Sequence	% Identity
Haricot bean agglutinin	1ASESYFNFQRFEETN15—	100
Great Northern beans [*Phaseolus vulgaris*]	1ANEIYFNFQRFXETN15—	80
Phytohemagglutinin-L	25YFNFQRFNETN35—	90
leucoagglutinin of kidney beans [*Phaseolus vulgaris*]	22YFNFQRFNETN32—	90
Phaseolus acutifolius	28YFNFQRFNETN38—	90
lectin [*Phaseolus vulgaris*]	12ASETSFSFQRFNETN27—	73
phytohemagglutinin [*Phaseolus coccineus*]	24ASETSFSFQRFNETN39—	73
phytohemagglutinin prepeptide [*Phaseolus vulgaris*]	22ASQTFFSFDRFNETN37—	66

rhamnose and D[+]-xylose. Of the various salts tested, only the chlorides of Fe[II] and Ca[II] inhibited its hemagglutinating activity [inhitially eight wells showing hemagglutination reduced to two wells]. The other chlorides had no effect.

The protein yield and specific hemagglutination activities of the chromatographic fractions obtained at each purification step are shown in Table 1. The N-terminal sequence of haricot bean agglutinin is presented in Table 2. It showed similarity to lectins from other *Phaseolus* species.

Table 1
Yields and specific hemagglutinating activities of chromatographic fractions obtained at different stages of purification of haricot bean agglutinin

	Specific activity (titre/mg)	Purification fold	Total protein (mg)	Total activity (titre/mg × 10^3)	Recovery (%)
Crude extract	512	1	10980	5621	100
D2 (after DEAE-cellulose)	2048	4	1535	3142	55
B2 (after Affi-gel blue gel)	3150	6.15	736	2318	41
S1 (after Superdex 200)	4096	8	522	2138	38

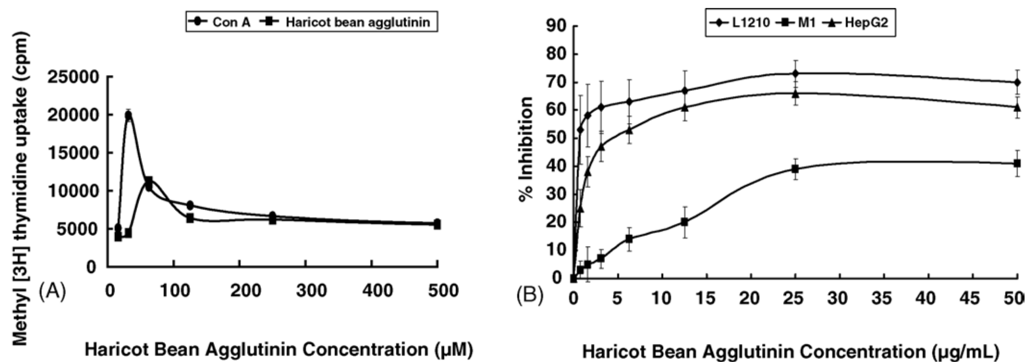

Fig. 5. (A) Mitogenic effect of Con A and haricot bean agglutinin on mouse splenocytes (data represents mean ± S.D., $n = 3$). (B) Inhibitory effect of haricot bean agglutinin on proliferation of cancer cell lines. Cell proliferation was determined by MTT assay (data represents mean ± S.D., $n = 3$).

4. Discussion

Haricot bean agglutinin was obtained in a purified state after successive chromatography on DEAE-cellulose, Affi-gel blue gel and Superdex 200. A significant amount of inactive materials can be eliminated from the starting materials in each of the first two chromatographic steps. This indicates that the purification protocol employed is an efficient one. An eight-fold increase in specific activity of the agglutinin [Table 1] was obtained. This compares favorably with the 5.7-fold increase in specific activity obtained by Andrews [19]. In the investigation of Andrews [19], DEAE-cellulose and Sephadex G200 are effective in removing inactive materials. The homotetrameric nature of haricot bean agglutinin is disclosed by the presence of a single 33 kDa band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, a single 130 kDa peak in gel filtration, and a single N-terminal sequence. The haricot bean agglutinin preparation of Andrews [19] exhibits a molecular mass of 114 kDa. The N-terminal sequence of haricot bean agglutinin is markedly similar to that of great Northern bean lectin and other *Phaseolus* species. Simple saccharides are without effect on hemagglutination induced by the agglutinins, in line with the observation of Andrews [19] on his own agglutinin preparation. Haricot bean agglutinin is thermolabile. The inhibitory effect of $CaCl_2$ and $FeCl_2$ on its hemagglutinating activity suggests that calcium and ferrous ions may bind to its active site and interfere with its ability to induce agglutination of red blood cells.

Lectins can be classified into mitogenic, non-mitogenic and anti-mitogenic according to their activity toward mouse splenocytes. Mitogenic lectins/agglutinins include Con A and phytohemagglutinin from *Phaseolus vulgaris*. Haricot bean agglutinin manifests a less potent mitogenic activity when compared with Con A. The maximal [methyl-^{3}H]thymidine incorporation induced by haricot bean agglutinin is of a smaller magnitude than that stimulated by Con A.

Like many lectins such as mushroom lectins, wheat germ agglutinin and others [24], haricot bean agglutinin exhibits an antiproliferative activity toward tumor cell lines. The hemagglutinating activitiy of haricot bean agglutinin is thermolabile. Beyond 40 °C there is a temperature-dependent decline in activity. Half of the activity disappears upon heating at 100 °C for 30 s.

Unlike red kidney bean agglutinin [25], haricot bean agglutinin is devoid of any inhibitory activity on HIV-1 reverse transcriptase and fungal growth, although haricot bean and red kidney bean are different cultivars of *Phaseolus vulgaris*. This is reminiscent of the finding of ribosome inactivating proteins in on variety of *Pisum sativum* [4] and antifungal protein in another variety of the same legume [26].

The isolation and partial characterization of haricot bean agglutinin has been accomplished in this investigation. The purification was achieved with the use of ion exchange chromatography on DEAE-cellulose, affinity chromatography on Affi-gel blue gel and gel filtration on Superdex 200. Affinity chromatography using immobilized mucin or other glycoproteins or sugar was not employed. Haricot bean agglutinin was shown to differ from red kidney bean agglutinin in the presence/absence of some biological activities, such as antifungal and HIV-1 reverse transcriptase inhibitory activities, although the two beans are but two different cultivars of the same species (*Phaseolus vulgaris*). Mitogenic activity toward splenocytes, antiproliferative activity tumor cells, and the effects of temperature, pH and salts on hemagglutining activity have not been reported all in a single study for a *Phaseolus* lectin, but are demonstrated in the present study.

Acknowledgments

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