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Preparation and Biological Properties of a Melibiose Binding Lectin from *Bauhinia variegata* Seeds

PENG LIN AND TZI BUN NG*

Department of Biochemistry, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China

A dimeric 64-kDa melibiose-binding lectin was isolated from the seeds of *Bauhinia variegata*. The isolation procedure comprised affinity chromatography on Affi-gel blue gel, ion exchange chromatography on Mono Q, and gel filtration on Superdex 75. The lectin was adsorbed on the first two chromatographic media. Its hemagglutinating activity was stable after 30-min exposure to temperatures up to 70 °C. Since lectins may demonstrate biological activities such as antiproliferative, immunomodulatory, antifungal, antiviral, and HIV-1 reverse transcriptase inhibitory activities, the isolated lectin was tested for these activities. It was found that the lectin inhibited proliferation in hepatoma HepG2 cells and breast cancer MCF7 cells with an IC_{50} of 1.4 μM and 0.18 μM, respectively. HIV-1 reverse transcriptase activity was inhibited with an IC_{50} of 1.02 μM. The lectin and concanavalin A (Con A) evoked maximal mitogenic response from mouse splenocytes at similar concentrations, but the maximal response to *B. variegata* lectin was only 1/5 of that induced by Con A in magnitude. *B. variegata* lectin was devoid of antifungal activity.

KEYWORDS: Bauhinia variegate; seeds; purification; lectin; biological properties.

INTRODUCTION

Lectins are carbohydrate-binding proteins possessing at least one noncatalytic domain, which binds reversibly to a specific saccharide (*1*). The binding of a lectin to a sugar residue is specific, albeit not as specific as antigenic recognition by antibodies or enzyme substrate specificity. Lectins can be divided into different groups according to their carbohydrate binding specificity, e.g., mannose-binding, glucose/mannose-binding, *N*-acetylglucosamine-binding, galactose-binding, *N*-acetylgalactosamine binding, fucose-binding, and sialic acid-binding. Lectins have been isolated from a diversity of organisms including flowering plants (*1*), animals (*2*) fungi (*3*), cycads (*4*), ferns (*5*) and algae (*6*). They display a variety of interesting biological activities such as blood group specificity (*7*), antitumor (*8*), immunomodulatory (*9*), antifungal (*10*), and anti-insect (*2*) activities.

Glycosylation is the most common post-translational modification of proteins. It changes immature proteins into biologically active proteins. Currently, there are some technologies available for glycan analysis, such as mass spectrometry, Western blotting, and chromatography, but they are time-consuming and require expertise, making it difficult for the average researcher to have access to and master the technique. Nowadays, a new technology, lectin microarray, has become a rapid and simple tool for analysis of protein glycosylation. So lectins also have immense value in glycomics study (*11*).

* Corresponding author. Tel: 852-2609 6872. Fax: 852-2603 5123. E-mail: b021770@mailserv.cuhk.edu.hk.

Bauhinia variegata belongs to the most primitive subfamily (Caesalpinoideae) of the Leguminosae family. Very few lectins have been isolated from this family (*12*−*15*), in contrast to the numerous reports on lectins from the subfamily Papilionoideae. The Caesalpinoidae lectins in the literature include *Griffonia simplicifolia* lectin, *Bauhinia purpurea* lectin (*12*−*14*) and *Bauhinia variegata var. candida* (common name: White Bauhinia) lectin (*15*). However, the biological activities of the isolated lectin other than hemagglutinating activity have not been examined. The intent of the present investigation was to fill this knowledge gap.

MATERIALS AND METHODS

Lectin Purification. Fresh *Bauhinia variegata* L. (common name: Camel's foot tree or orchid tree) seeds were collected on the campus of the Chinese University of Hong Kong. They were authenticated by Prof. Shiuying Hu, honorary Professor of Chinese Medicine, and Dr. David Lau, Institute of Chinese Medicine, The Chinese University of Hong Kong. The seeds were extracted by homogenizing in 1 L of distilled water, followed by centrifugation at 13000*g* at 4 °C. Tris-HCl buffer (pH 7.4) was added to the supernatant until the concentration of Tris attained 10 mM. The supernatant was then loaded on a 5 × 20 cm column of Affi-gel blue gel (Bio-Rad) in 10 mM Tris-HCl buffer (pH 7.8). Unadsorbed proteins (fraction BG1) were eluted with the same buffer while adsorbed proteins (fraction BG2) were eluted with 10 mM Tris-HCl buffer (pH 7.8) containing 1 M NaCl. The lectin-enriched fraction BG2 was dialyzed, lyophilized, dissolved (5 mg/mL) in 10 mM NH_4HCO_3 buffer (pH 9) and then applied on a 1-mL Mono Q (GE Healthcare) column in the same buffer. Following elution of unadsorbed materials, adsorbed proteins (fraction S1, S2, S3 and S4) were desorbed with three linear NaCl concentration gradients (0−0.1

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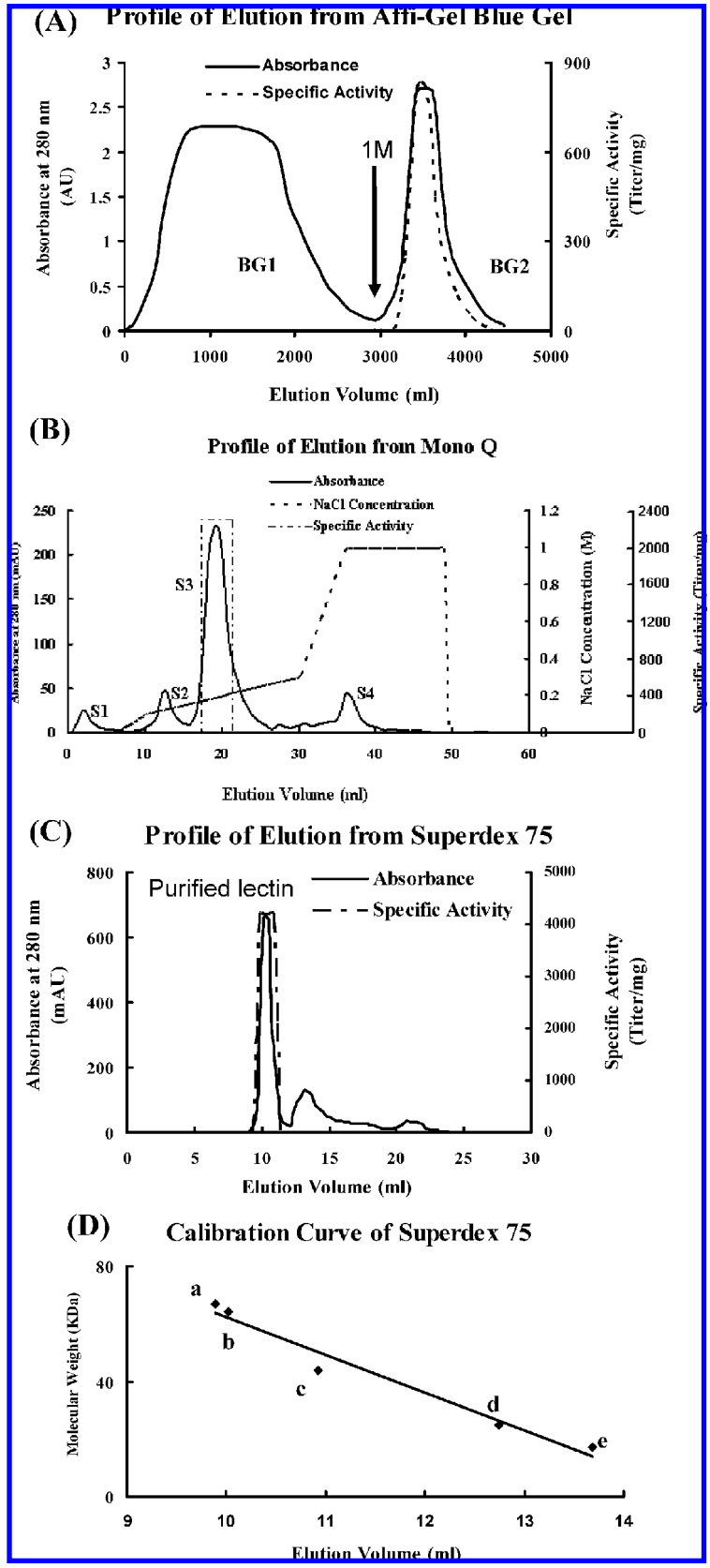

Figure 1. Purification of *Bauhinia variegata* lectin by chromatography on (**A**) Affi-Gel Blue gel, (**B**) Mono Q and (**C**) Superdex 75. In (**A**), the *Bauhinia variegata* seed extract was applied on an Affi-Gel Blue gel column (5 × 20 cm). Unadsorbed proteins (fraction BG1) were eluted with the same buffer while adsorbed proteins (fraction BG2) were eluted with 10 mM Tris-HCl buffer (pH 7.8) containing 1 M NaCl as indicated by the arrows. In (**B**), fraction BG2 from the Affi-Gel Blue gel column was dialyzed and applied on a 1-ml Mono Q column. Following elution of unadsorbed proteins with 10 mM NH_4HCO_3 buffer (pH 9), adsorbed proteins were eluted sequentially, first with a 0−0.1 M NaCl gradient and then with a 0.1−0.3 M and 0.3−1 M NaCl gradient. In (**C**), fraction S3 from the Mono Q column was subjected to gel filtration on a Superdex 75 HR 10/30 column in 10 mM NH_4HCO_3 buffer (pH 9.4) containing 200 mM galactose. In (**D**), calibration curve of Superdex 75 column is shown. The markers included (**a**) bovine serine albumin (67 kDa), (**b**) *B. variegata* lectin, (**c**) ovablumin (44 kDa), (**d**) chymotrypsinogen A (25 kDa) and (**e**) myoglobin (17 kDa).

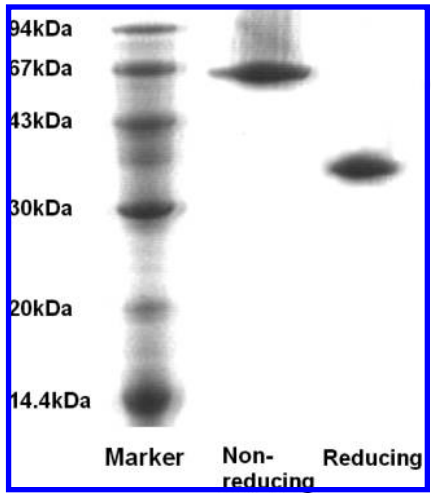

Figure 2. SDS−PAGE results. Left lane: molecular mass (MW) marker proteins from GE Healthcare including phosphorylase b (MW 94 kDa), bovine serum albumin (MW 67 kDa), ovalbumin (MW 43 kDa), carbonic anhydrase (MW 30 kDa), soybean trypsin inhibitor (MW 20 kDa), and α-lactalbumin (MW 14.4 kDa). Middle lane: purified *Bauhinia variegata* lectin under nonreducing condition. Right lane: purified *Bauhinia variegata* lectin under reducing condition. Molecular mass of the purified monomeric lectin is 64 kDa.

M, 0.1−0.3 M and 0.3−1 M) in 10 mM NH_4HCO_3 buffer (pH 9). The lectin-enriched fraction S3 was dialyzed, lyophilized and subjected to gel filtration on a Superdex 75 HR 10/30 column (GE Healthcare) using an AKTA Purifier (GE Healthcare) in 10 mM NH_4HCO_3 buffer (pH 9.4) containing 200 mM galactose. The first eluted absorbance peak constituted purified lectin.

Molecular Mass Determination by Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis, FPLC-Gel Filtration, and Mass Spectrometry. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out in accordance with the procedure of Nielsen and Reynolds (*16*), using a 12% resolving gel and a 5% stacking gel, under reducing as well as nonreducing conditions. At the end of electrophoresis, the gel was stained with Commassie brilliant blue.

FPLC-gel filtration was carried out using a Superdex 200 HR 10/30 column that had been calibrated with molecular-mass standards (GE Healthcare), in the presence of galactose to inhibit interactions, if any, of the lectin with Superdex 75. An AKTA Purifier (GE Healthcare) was used for this purpose.

Mass spectrometric analysis of the lectin was performed on a Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). An Applied Biosystems 4700 Proteomics Analyzer, an instrument that essentially consists of an atmospheric pressure electrospray positive-ion source attached to a triple-quadrupole mass analyzer, was used. The purified lectin (100 pmol) was dissolved in water/methanol (50:50, v/v) containing 1% (v/v) acetic acid at a protein concentration of 5 μmol/L, and then applied on the MS instrument.

Analysis of N-Terminal Amino Acid Sequence. Amino acid sequence analysis was carried out using an HP G1000A Edman degradation unit and an HP 1000 HPLC system (*17*).

Assay of Hemagglutinating Activity. A serial 2-fold dilution of the lectin 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 recorded after about 1 h when the blank containing only red cells had fully sedimented and appeared as a dot at the bottom of the well. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, is defined as one hemagglutination unit. Specific activity is the number of hemagglutination units per mg of protein (*17*).

Inhibition of Lectin-Induced Hemagglutination by Carbohydrates. The hemagglutinating inhibition tests to investigate inhibition of lectin-induced hemagglutination after exposure to various carbohydrates at room temperature for 30 min were performed in a manner analogous to the hemagglutination test (*17*).

Effect of Temperature and pH on Lectin-Induced Hemagglutination. The effects of temperature and pH on hemagglutinating activity of the lectin were examined as previously described (*17*).

Assay of Mitogenic Activity. Incubation of splenocytes (5×10^5 cells/100 μL/well) from BALB/C mice (20−25 g) was carried out at 37 °C in a humidified atmosphere of 5% CO_2 in the presence or absence of the lectin for 72 h in a 96-well culture plate. [Methyl-^{3}H] thymidine (GE Healthcare) was then added (0.25 μCi/10 μL/well) and incubation of the splenocytes was continued for another 6 h under the same conditions. The splenocytes were then harvested before onto a glass fiber filter, and the radioactivity was measured (*18*).

Assay of Antiproliferative Activity on Tumor Cell Lines. Breast cancer cells (MCF7) and hepatoma cells (HepG2) (5×10^4 cells/100 μL/well), in their exponential growth phase, were seeded into a 96-well culture plate, and incubated for 4 h before addition of the lectin. Incubation was carried out for another 72 h. Radioactive precursor, 1 μCi ([methyl-^{3}H] thymidine (GE Healthcare), was then added to each well and incubated for 6 h. The cultures were then harvested. The incorporated radioactivity was determined (*17*).

Assay of HIV Reverse Transcriptase Inhibitory Activity. The assay was carried out by using an enzyme-linked immunosorbent assay kit as described by Collins et al. (*19*). The inhibitory activity of the lectin was calculated as percent inhibition as compared to a control without the lectin (*17, 19*).

Assay of Antifungal Activity. The assay was performed as described in (*18*) using the fungal species *Botrytis cinerea*, *Mycosphaerella archidicola* and *Fusarium oxysporum*.

Assay of HIV-1 Integrase Inhibitory and SARS Proteinase Inhibitory Activities. The assays were conducted as described in refs *20, 21*.

RESULTS AND DISCUSSION

The *Bauhinia variegata* seed extract was fractionated on Affi-Gel Blue gel into an unadsorbed fraction (BG1) devoid of hemagglutinating activity and the adsorbed fraction (BG2) obtained by elution with 1 M NaCl in the Tris-HCl buffer (**Figure 1A**). Hemagglutinating activity was confined to fraction BG2. This fraction was subjected to ion exchange chromatography on FPLC-Mono Q column into a small inactive unadsorbed fraction, several minor adsorbed fractions, and a major adsorbed fraction S3 (**Figure 1B**). Hemagglutinating activity was detected only in fraction S3. Final purification was achieved by FPLC-gel filtration on Superdex 75. Purified lectin was found in the first and the largest fraction (**Figure 1C**). In SDS−PAGE it appeared as a single band with a molecular mass of 32 kDa under reducing conditions and a 64 kDa band under nonreducing conditions (**Figure 2**). It exhibited a molecular mass of 64 kDa in gel filtration using buffer containing 200 mM galactose (**Figure 1D**). The mass spectrum of the lectin revealed a 64 kDa peak (Data not shown). The yields of the chromatographic fractions with hemagglutinating activity are presented in **Table 1**. It shared some similarity with previously reported *Bauhinia variegata* Candida (BvcL) lectin in N-terminal sequence (**Table 2**). The hemagglutinating activity of the lectin was stable up to 70 °C for 30 min. At 80 °C only half of the activity remained. At 90 °C the activity completely disappeared. Exposure to 100 °C for 30 s and longer led to complete abrogation of activity. Like Con A, the hemagglutinating activity of *Bauhinia variegata* lectin remained unaltered at various time intervals (0, 0.5, 1, 2, and 3 h) after treatment with trypsin (1:1, w/w). Melibiose most potently inhibited the hemagglutinating activity of the lectin, followed by galactose, glucuronic acid, lactose, raffinose, arabinose and xylose (**Table 3**). *Bauhinia variegata* lectin maintained its hemagglutinating activity in the range of pH 3−12 (data not shown). The lectin inhibited proliferation of HepG2 cells and MCF7 cells with an IC_{50} of 1.4 μM and 0.18 μM, respectively (**Figure 3**) and the activity of HIV-1 reverse

Table 1. Summary of Purification of *Bauhinia variegata* Lectin from 100 g of Seeds with Hemagglutinating Activity

chromatographic fraction with hemagglutinating act.	total protein (mg)	purifn fold	total act.[c] (titer × 10^3)	sp act.[b] (titer[a]/mg)	recovery of act. (%)
crude extract	19240	1	6138	319 ± 4	100
BG2 (after Affi-Gel Blue Gel)	5824	2.3	4269	733 ± 2	69
MQ3 (after Mono Q)	1050	7.5	2513	2393 ± 2	41
purified lectin (After Superdex 75)	337	13	1398	4147 ± 3	23

[a] Titer = the reciprocal of the highest dilution of lectin solution exhibiting hemagglutination. [b] Specific activity = the number of hemagglutination titers per mg protein. [c] Total activity = specific activity × total protein.

Table 2. N-Terminal Sequence of *Bauhinia variegata* Lectin in Comparison with Lectin from *Bauhinia variegata* Candida (BvcL) Seeds (Results of BLAST Search)

	N-terminal sequence	% identity
Lectin (*Bauhinia variegate* L.)	^{1}QRNRLTSFTFPNNRS^{15}	100
BvcL (*Bauhinia variegata*)	^{1}TSSTLTSFTFPNFWS^{15}	60
Lectin (*Bauhinia purpurea*)	^{1}TSSTLTGFTFPNFWS^{15}	53
Lectin (*Bauhinia ungulata*)	^{1}TNSTLTGFTFPNFWS^{15}	53

Table 3. Inhibition of *Bauhinia variegata* Lectin-Induced Hemagglutination by Carbohydrates[a]

sugar	MIC[b] of sugar (mM)
melibiose	7.8
galactose	15.6
glucuronic acid	15.6
lactose	62.5
arabinose	125
raffinose	125
xylose	500

[a] Glucose, fucose, rhamnose, mannose, polygalacturonic acid, glucosamine, galactosamine and mannosamine were all inactive when tested in 500 mM. [b] MIC = minimum inhibitory concentration.

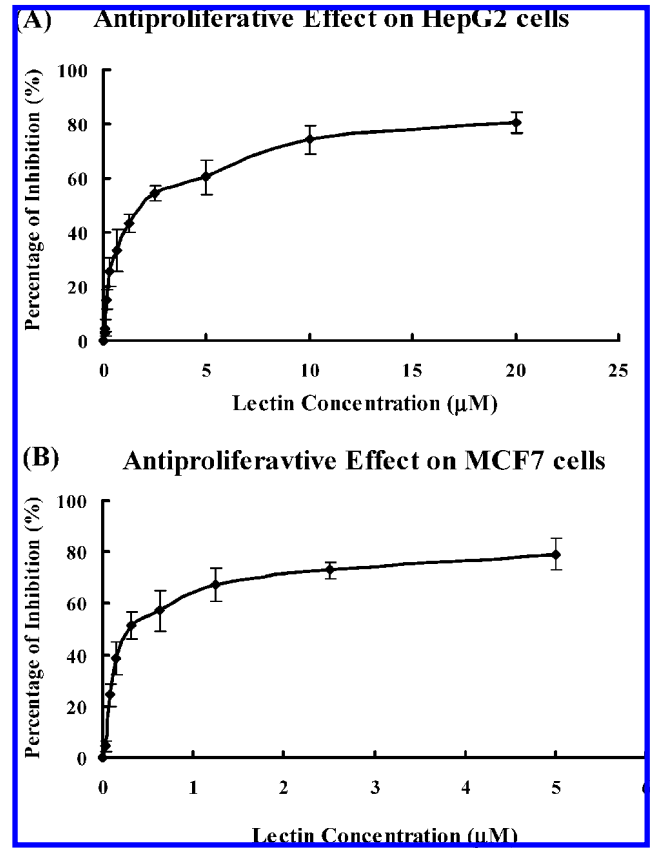

Figure 3. Antiproliferative activity of *Bauhinia variegata* lectin on (**A**) Hep G2 hepatoma cells and (**B**) MCF7 breast cancer cells after 72 h of treatment at 37 °C in an atmosphere of 95% CO_2−5% CO_2. Results are presented as mean ± SD (n = 3). IC_{50} values for Hep G2 cells and MCF cells were 1.4 μM and 0.18 μM, respectively.

transcriptase with an IC_{50} of 1.02 μM (**Figure 4**). The mitogenic activity of the lectin on mouse splenocytes is shown in **Figure 5**. It started to evoke a mitogenic response at a lower concentration than Con A although the maximal response was much smaller in magnitude. Both the lectin and Con A stimulated maximal mitogenic response at similar concentrations. However, the maximal response achieved by *Bauhinia variegata* lectin was weaker than that evoked by Con A (**Figure 5**). *Bauhinia variegata* lectin did not inhibit HIV integrase or SARS proteinase (data not shown). There was no antifungal activity (data not shown).

The protocol employed in the present investigation for purifying *B. variegata* lectin entailed successive ion exchange chromatography on Affi-Gel Blue gel, Mono Q and gel filtration on Superdex 75. The procedure used by Silva et al. (*15*) for isolations *B. variegata* lectin was different. It comprised gel filtration on Sephadex 75, affinity chromatography on immobilized D-lactose, and reversed-phase HPLC on a μ-Bondapack C 18 column. The lectin yield in the present study was 337 mg/100 g of seeds, slightly smaller than the yield of 350−400 mg/100 g of seeds reported by Silva et al. (*15*). The specific hemagglutinating activity of the lectin preparation of Silva et al. (*15*) is not available. The lectin preparation from Silva et al. (*15*) is similar to our lectin preparation in sugar binding specificity (galactose > lactose > raffinose) although in the former case the effect of melibiose has not been tested. The molecular mass of our preparation (64 kDa) is lower than that of Silva's preparation (100 kDa). The subunit molecular mass of our preparation (32 kDa) is identical to that of Silva's preparation (32 kDa). Discrepancies in molecular mass and N-terminal amino acid sequence between the two lectin preparations may be due to cultivar difference. The stability of *B. variegata* lectin when exposed to trypsin or a temperature up to 70 °C, together with the potentially activities of the lectin including mitogenic, anticancer and HIV-1 reverse transcriptase activities, make it worthy of future investigation.

The procedure used for isolating *B. purpurea* lectin used by Irimura and Osawa (*29*) involved $(NH_4)_2SO_4$ fractionation and affinity chromatography on Sepharose 4B, and that used by Young et al. (*14*) included affinity chromatography on immobilized *N*-acetyl-D-galactosamine. They are both different from the protocol utilized for purifying *B. variegata* lectin in the present study. *B. purpurea* lectin is a tetramer composed of 32 kDa subunits. Thus it appears to be different from our *B. variegata* lectin preparation. *B. purpurea* lectin is highly specific for *N*-acetyl-D-galactosamine and galactose, similar to *B. variegata* lectin. The former lectin exerts antiviral activity

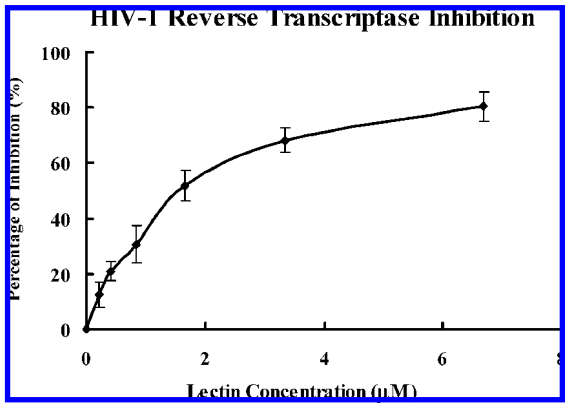

Figure 4. HIV-1 reverse transcriptase inhibition by *Bauhinia variegata* lectin. Results are presented as mean ± SD ($n = 3$). IC_{50} was 1.02 μM.

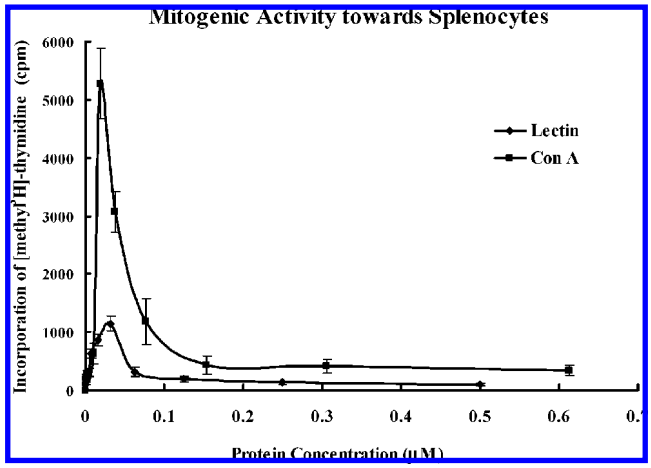

Figure 5. Mitogenic response of *Bauhinia variegata* lectin from mouse spleen cells.

toward *Herpes simplex* virus and rabies virus in vitro (*33*), in agreement with the HIV-reverse transciptase inhibitory activity of our *B. variegata* lectin preparation.

It is known that some lectins exhibit HIV-1 reverse transcriptase inhibitory activity (*17*). *B. variegata* lectin manifests potent HIV-1 reverse transcriptase activity with a low IC_{50} of 1.02 μM, which is much more potent than other anti-HIV molecules (*22*−*24*). The mechanism of inhibition is probably related to protein−protein interaction, similar to inhibition of the retroviral enzyme by the homologous protease. However, *B. variegata* lectin is devoid of HIV-1 integrase inhibitory and SARS proteinase inhibitory activity, just like French bean defensin (*20*). The antiproliferative/antitumor (*17*) activity of lectins is well documented. In fact, lectin-based immunotoxins have been used for cancer therapy (*25*). *B. variegata* lectin exerts potent antiproliferative activity toward HepG2 and MCF7 cells, with an IC_{50} of 1.4 μM and 0.18 μM, respectively. The lectin has manifestly weaker mitogenic activity than Con A in splenocytes. Other lectins also evoke a weaker mitogenic response from splenocytes than Con A (*26*). Moreover, it is noteworthy that the maximal mitogenic response is achieved by similar concentration of the lectin and Con A. Antifungal activity has been reported for several lectins (*10, 27, 28*). *B. variegata* lectin is inactive toward several fungal species tested. The demonstration of the various aforementioned biological activities of *B. variegata* lectin is an addition to the literature on this lectin because such information is presently lacking.

Two lectins closely related to *B. variegata* lectin are *B. purpurea* lectin (*14, 29, 30*) and *B. monandra* lectin (*31*). The second and third lectins have not been examined for mitogenic activity toward spleen cells, antiproliferative activity toward tumor cells, HIV-1 reverse transcriptase inhibitory, HIV-1 integrase inhibitory, SARS proteinase inhibitory and antifungal activities. *B. purpurea* lectin has been shown to have larvicidal activity on the European corn borer *Ostrinia nubilalis* (*32*), antiviral activity on *Herpes simplex* virus (*33*), and to bind to normal and cancerous thyroid glands (*34*). Only the insecticidal activity of *B. monandra* lectin against a lepidopteran and two coleopterans have been demonstrated (*31*).

In summary, the *B. variegata* lectin purified in this study differs from the lectin reported earlier by Silva et al. (*15*) in molecular mass and N-terminal sequence. The discrepancy has be attributed to the fact that the former lectin was prepared from seeds of the Camel's foot tree with pink flowers while the latter lectin was purified from seeds of the white Bauhinia with white flowers. The biological activities of the former lectin are disclosed in this investigation.

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