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An antifungal protein from flageolet beans

Lixin Xia [a], T.B. Ng [b,*]

[a] College of Life Science, Shenzheng University, Shenzhen, China
[b] Department of Biochemistry, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, Hong Kong, China

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Abstract

A protein with antifungal and hemagglutinating activities was isolated from dried flageolet beans (*Phaseolus vulgaris* cv. 'Flageolet Bean'). The protein was unadsorbed on DEAE-cellulose but adsorbed on Affi-gel blue gel and CM-cellulose. The protein demonstrated antifungal activity against *Mycophaerella arachidicola* with an IC_{50} of 9.8 μM, but was inactive toward *Fusarium oxysporum* and *Botrytis cinerea*. Its hemagglutinating activity could not be inhibited by a variety of the sugars tested. The activity was stable up to 60 °C. At 70 °C, 75% of the hemagglutinating activity remained while no activity was discernible at and above 100 °C. The hemagglutinating activity was stable in the presence of a variety of monovalent, divalent and trivalent chlorides, and also when the ambient pH changed from 3 to 12. It did not exert any mitogenic activity on mouse splenocytes in vitro. Neither did it inhibit HIV-1 reverse transcriptase. It inhibited [^{3}H-methyl]-thymidine incorporation into leukemia L1210 cells with an IC_{50} of about 4 μM.

Keywords: Antifungal protein; Flageolet bean; Purification

1. Introduction

Antifungal proteins are a family of structurally different proteins produced by animals [8,12,36], plants [1–7,9–11,13,15–17,18,20–22,35–46] and fungi [39,40]. Some of them resemble thaumatin [6,10,22,28,38,52], miraculin [54], cyclophilin [45,47], allergen [44], and embryo-abundant proteins [33] in N-terminal sequence. Some of them are classified according to their function, into chitinases [51,53,55], chitin binding proteins [2,11], glucanases [29], ribosome inactivating proteins [18,21,24], protease inhibitors [4,13,50], peroxidases [48], nucleases [20,32,35] and lipid transfer proteins [3] and lectins [9].

Lectins are widely distributed in living organisms including viruses, bacteria, fungi, plants and animals. Most plant lectins are storage proteins which acquire a potential role in defense against insects and fungi [49]. Some legume lectins mediate symbiosis between nitrogen-fixing bacteria and leguminous plants. Macrophage lectins specific for mannose and *N*-acetylglucosamine mediate binding and phagocytosis of microorganisms. Animal lectins with specificity toward β-galactoside are involved in regulating differentiation and organogenesis. Lectins also play a role in lymphocyte migration from the blood into lymphoid organs and in cancer metastasis [23,25,26]. Some lectins exhibit antitumor and immunomodulatory activities [23,25,26].

The aim of the present investigation was to isolate and characterize an antifungal protein with lectin activity from the flageolet bean, a cultivar of *Phaseolus vulgaris* which has not previously been examined. Hitherto there are only a few lectins found with antifungal activity.

2. Materials and methods

2.1. Isolation of antifungal protein

Flageolet beans (*P. vulgaris* cv. 'Flageolet Beans') from the UK (80 g) were cut up into pieces and then homogenized

* Corresponding author. Tel.: +852 26096872; fax: +852 26095123.
E-mail address: b021770@mailserv.cuhk.edu.hk (T.B. Ng).

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in distilled water (2 ml/g) using a Waring blender. Tris–HCl buffer (pH 7.4) was added to the supernatant obtained by centrifuging the homogenate, until the concentration of Tris attained 10 mM. The supernatant was then applied on a 5 cm × 20 cm column of DEAE-cellulose (Sigma) which was eluted with 10 mM Tris–HCl buffer (pH 7.4). After elution of the unbound fraction (D1) with antifungal and hemagglutinating activities, the column was eluted with the aforementioned Tris–HCl buffer to which IM NaCl had been added. Fraction D1 was chromatographed on a 2.5 cm × 20 cm column of Affi-gel blue gel (Bio-Rad). Following removal of the unbound fraction B1 with 10 mM Tris–HCl buffer (pH 7.4), the adsorbed material (B2) was desorbed with 10 mM Tris–HCl buffer containing 1.5 M NaCl. Fraction B2 was then dialyzed before ion exchange chromatography on a 2.5 cm × 20 cm column of CM-cellulose (Sigma) which had been equilibrated and was eluted with 10 mM NH_4OAc buffer (pH 4.6). After elution of unadsorbed material, adsorbed proteins were eluted first with the NH_4OAc buffer containing 0.2 M NaCl and next with the NH_4OAc buffer containing 1 M NaCl. The fraction eluted with 0.2 M NaCl (C2) represented purified antifungal protein with hemagglutinating activity.

2.2. Electrophoresis, molecular mass determination, and N-terminal sequence analysis

The purified protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for molecular mass determination in accordance with the procedure of Laemmli and Favre [14]. 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 of the protein. The N-terminal sequence of the protein was determined by using a Hewlett-Packard HP G1000A Edman degradation unit and an HP 1000 HPLC System [16].

2.3. Assay of antifungal activity

The assay of the purified protein for antifungal activity toward *Botrytis cinerea*, *Mycosphaerella arachidicola* and *Fusarium oxysporum* was carried out in 100 mm × 15 mm Petri plates containing 10 ml of potato dextrose agar. After the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed at distance of 0.5 cm away from the rim of the mycelial colony. An aliquot of a solution of the purified protein was added to a disk. The plates were incubated at 23 °C for 72 h until mycelial growth had enveloped disks containing the control and had formed crescents of inhibition around disks containing samples with antifungal activity [44].

2.4. Assay for hemagglutinating activity

In the assay for lectin (hemagglutinating) activity, a serial two-fold dilution of the protein 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 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 [27,30].

The hemagglutinating inhibition tests to investigate inhibition of lectin-induced hemagglutination by various carbohydrates were performed in a manner analogous to the hemagglutination test. Serial two-fold 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 lectin with 16 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 16 hemagglutination units of the agglutinin preparation was calculated [30].

The effects of temperature, NaOH solution, HCl solution and solutions of metallic chlorides on hemagglutinating activity of the agglutinin were examined as previously described [31].

2.5. Assay for antiproliferative activity toward leukemia cells

The antiproliferative activity of the purified protein was determined as follows. The cell line L1210 (leukemia) was purchased from American Tissue Culture Collection. The cell line was maintained in Dulbecco Modified Eagles' Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) 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 lectin. Incubation was carried out for another 48 h. Radioactive precursor, 1 μCi, ([^{3}H-methyl]-thymidine, from Amersham Biosciences) was then 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. Assay for mitogenic activity toward splenocytes

The assay of mitogenic activity was performed as described by Wang et al. [4]. Splenocytes were isolated from BALB/c mice. The cells were diluted with RPMI medium containing 10% fetal bovine serum and then seeded (2×10^6 cells/0.2 ml/well) in 96-well microplates. The protein was then added at various concentrations. Cells cultured in the absence of the protein 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).

2.7. Assay for HIV-1 reverse transcriptase inhibitory activity

The assay for HIV reverse transcriptase inhibitory activity was carried out according to instructions supplied with the assay kit from Boehringer Mannhein (Germany). The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hybrid poly(A) oligo (dT) 15. The digoxigenin- and biotin-labeled nucleotides in an optimized ratio are incorporated into one of the same DNA molecule, which is freshly synthesized by the reverse transcriptase (RT). The detection and quantification of synthesized DNA as a parameter for RT activity follows a sandwich ELISA protocol. Biotin-labeled DNA binds to the surface of microtiter plate modules that have been precoated with strepatavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase, binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrates is added. The peroxidase enzymes catalyzes the cleavage of the substrate, producing a colored reaction product. The absorbance of the samples at 405 nm can be determined using microtiter plate (ELISA) reader and is directly correlated to the level of RT activity. A fixed amount (4–6 ng) of recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of the protein was calculated as percent inhibition as compared to a control without the protein [46].

3. Results

3.1. Isolation of antifungal protein

When the crude extract of 80 g of flageolet beans (*P. vulgaris* cv. 'Flageolet Bean') was loaded on a DEAE-cellulose column, two fractions were obtained. PD1, which was unadsorbed on the DEAE-cellulose column, exhibited antifungal and hemagglutinating activities (Fig. 1). Two fractions, a larger unadsorbed fraction PB1 and a smaller adsorbed fraction PB2, were produced when PD1 was directly subjected to affinity chromatography on an Affi-gel blue gel column (Fig. 2). Only PB2 exhibited antifungal and hemagglutinating activities. This fraction was fractionated on CM-cellulose to yield two fractions of approximately equal size, a slightly larger unadsorbed fraction PC1 and a slightly smaller adsorbed fraction PC2 (Fig. 3). Only the adsorbed fraction, PC2, exhibited antifungal and hemagglutinating activities. PC2 yielded a single peak upon gel filtration on a Superdex 75 HR 10/30 column (Fig. 4), and a single band with a molecular mass of 33 kDa in SDS-PAGE (Fig. 5).

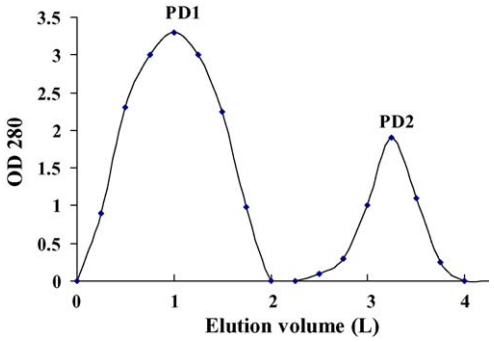

Fig. 1. Ion exchange chromatography on a DEAE-cellulose column (5 cm × 20 cm). The crude extract of 80 g of flageolet beans was loaded on the column. The column was first eluted with 10 mM Tris–HCl buffer (pH 7.4). The column was then eluted with 1.0 M NaCl in the same buffer after fraction PD1 had all been eluted. Antifungal and hemagglutinating activities resided only in the unadsorbed fraction PD1. Flow rate: 4 ml/min.

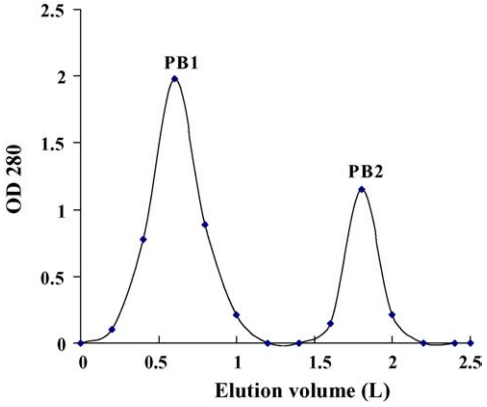

Fig. 2. Affinity chromatography on an Affi-gel blue gel column (2.5 cm × 20 cm). Fraction PD1 was applied to the column. The column was eluted initially with 10 mM Tris–HCl buffer (pH 7.4). The column was then washed with 1.5 M NaCl in the Tris–HCl buffer after the unadsorbed fraction PB1 had all been eluted. Only the fraction eluted with 1.5 M NaCl, PB2, contained antifungal and hemagglutinating activities. Flow rate: 4 ml/min.

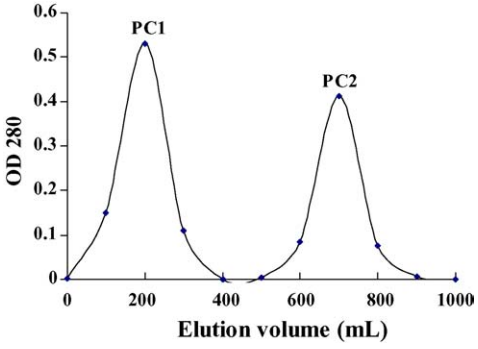

Fig. 3. Ion exchange chromatography on a CM-cellulose column (2.5 cm × 20 cm). Fraction PB2 was applied to the column. The column was eluted first with 10 mM NH_4OAc buffer (pH 4.6). After fraction PC1 had all been eluted, the column was then washed with 0.2 M NaCl in 10 mM NH_4OAc buffer (pH 4.6). The fraction eluted with 0.2 M NaCl buffer, PC2, contained antifungal and hemagglutinating activities. Flow rate: 3 ml/min.

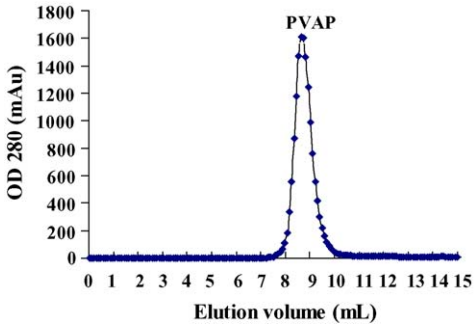

Fig. 4. Gel filtration of fraction PC2 on an FPLC Superdex 75 HR 10/30 column. Fraction PC2 was dialyzed and lyophilized and then loaded into the column. The column was eluted with 20 mM NH_4HCO_3 buffer (pH 9.0). The resulting fraction PVAP exhibited antifungal and hemagglutinating activities. Flow rate: 0.5 ml/min. mAu = milli-absorbance units.

3.2. Antifungal activity of purified protein

At 10 and 30 μg it demonstrated strong antifungal activity toward fungus *M. arachidicola*. It retained its antifungal activity after treatment at 80 °C for 15 min but lost the activity when temperature was raised to 100 °C (Fig. 6). The antifungal activity could be inactivated by incubation with trypsin (1:1, w/w) at 37 °C for 30 min (Fig. 7). The protein inhibited mycelial growth in *M. arachidicola* with an IC_{50} of 9.8 μM (Fig. 8). However, it was inactive toward *Botrytis cinerea* and *Fusarium oysporum*.

3.3. Hemagglutinating activity of purified protein

The hemagglutinating activity of the purified antifungal protein was not affected in the presence of any of the

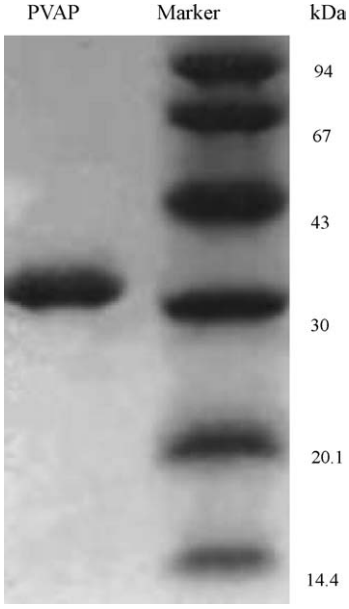

Fig. 5. SDS-PAGE results. Left lane: fraction PVAP (representing purified flageolet bean antifungal protein, 1-0 μg. Right lane: molecular mass markers from Amersham Biosciences.

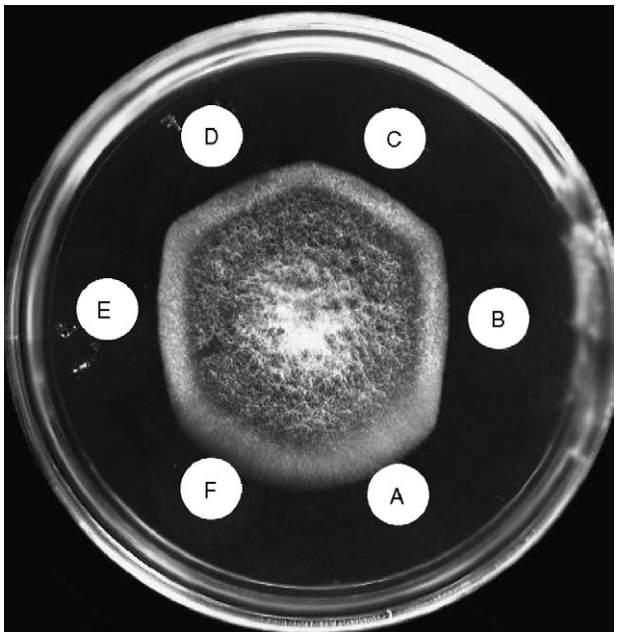

Fig. 6. Effect of heat treatment (15 min) on antifungal activity of flageolet bean antifungal protein toward *Mycosphaerella arachidicola*. (30 μg flageolet bean antifungal protein applied to each paper disk in 10 mM Tris–HCl buffer, pH 7.4; (A) no antifungal protein added, serving as control; (B) flageolet bean antifungal protein previously exposed to 20 °C; (C) 40 °C; (D) 60 °C; (E) 80 °C; (F) 100 °C.).

following carbohydrates at 500 mM concentration, including *N*-acetyl-D-galactosamine, 3-*N*-acetylneuraminic-lactose, L(+)arabinose, D-galacturonic acid, L(−)fuose, α-L(−)fucose, D(+)galactosamine, D(+)galactose, *N*-glycoly-

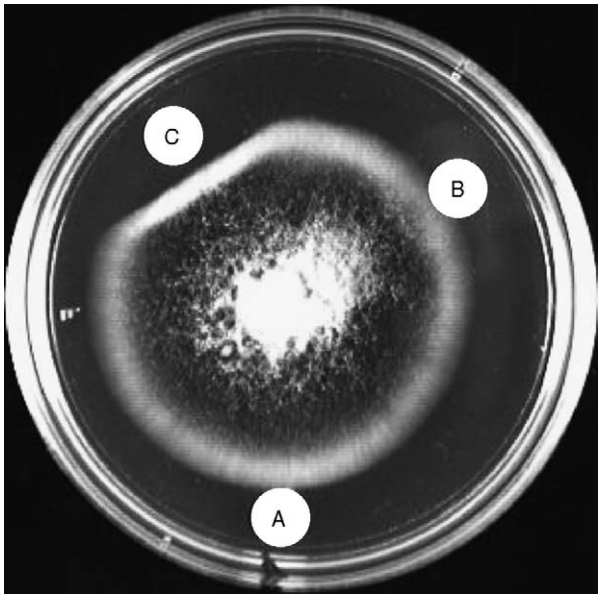

Fig. 7. Effect of trypsin treatment on antifungal activity of flageolet bean antifungal protein against *Mycosphaerella arachidicola*. ((A) 10 mM Tris–HCl (pH 7.4); (C) 30 μg flageolet bean antifungal protein in the buffer; (B) 30 μg flageolet bean antifungal protein in the buffer treated with trypsin (1:1, w/w) for 30 min at 37 °C.).

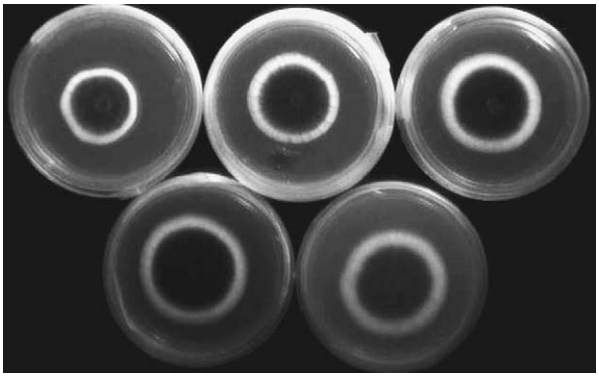

Fig. 8. Determination of IC_{50} value of antifungal activity of flageolet bean antifungal protein (PVAP) toward *Mycosphaerella arachidicola* (upper left: 10 μM flageolet bean antifungal protein in 10 mM Tris–HCl (pH 7.4); upper middle: 5 μM flageolet bean antifungal protein in 10 mM Tris–HCl (pH 7.4); upper right: 2 μM flageolet bean antifungal protein in 10 mM Tris–HCl (pH 7.4); lower left: 1 μM flageolet bean antifungal protein in 10 mM Tris–HCl (pH 7.4); lower right: control without PVAP).

lneuraminic acid, D-glucuronic acid, α-lactose, α-D(+)melibiose, L(−)mannose, D-mannosamine, α-methyl-D-glucoside, polygalacturonic acid, rhamnose and sucrose. The yields at each purification step and specific hemagglutinating activity are displayed in Table 1. The sequence of the first ten N-terminal residues of the protein is shown in Table 2. The N-terminal sequence shared an identity of 80% with a hemagglutinin of tenuifolius beans (*Phaseolus acutifolius* cv. 'Tenuifolius Bean'). The protein was purified 70-fold and 43.9 mg purified protein was obtained from 80 g flageolet beans.

The hemagglutinating activity of flageolet bean antifungal protein was stable in the temperature range of 25–80 °C. There was a reduction in its hemagglutinating activity when the temperature was elevated to 90 °C and complete abolition of activity occurred at 100 °C. Change of pH from pH 3–12 did not affect the hemagglutinating activity. Exposure to a number of monovalent, divalent and trivalent metal chlorides including $CaCl_2$, $CuCl_2$, $FcCl_2$, $FeCl_3$, KCl, $MgCl_2$, $MnCl_2$, NaCl and $ZnCl_2$ (250 mM at 25 °C for 30 min) did not bring about changes in the hemagglutinating activity either.

3.4. Other activities of purified protein

There was no mitogenic or anti-mitogenic activity on mouse splenocytes from 14.6 nM to 3.73 μM. There was no inhibitory effect on HIV-1 reverse transcriptase when tested from 77 nM to 4.93 μM. The protein exerted an antiproliferative activity on L1210 cells with an IC_{50} of 3.6 μM (Fig. 9). At a concentration of 3.6 μM flageolet bean antifungal protein brought about (46.23 ± 0.35)% (mean ± S.D., $n = 3$) and undetectable inhibition of [^{3}H-methyl]-thymidine uptake by leukemia L1210 cells, after heat treatment of the protein at 80 and 100 °C for 15 min, respectively.

4. Discussion

It is noteworthy that flageolet bean antifungal protein is similar to red kidney bean lectin [49] in N-terminal sequence and molecular mass. The chromatographic behavior of

Table 1
Yields (from 80 g flageolet beans) and specific hemagglutinating activities of chromatographic fractions at each purification step

Chromatographic fraction	Protein yield (mg)	Specific activity (Hemagglutination titer/mg)	% Recovery	Purification fold
Extract	7320	699	100	1
PD1	4312	1424	120	2
PD2	2247	–	–	–
PB1	1603	–	–	–
PB2	310	9910	60	14
PC1	75.2	–	–	–
PC2	76.7	32785	49	–
PVAP	43.9	48984	42	70

–: hemagglutinating activity undetectable; PVAP represents purified flageolet bean antifungal protein.

Table 2
Comparison of N-terminal sequence of antifungal protein (PVAP) from flageolet beans (*P. vulgaris* cv. 'Flageolet Bean') with hemagglutinins from other sources (Results of Blast search)

Hemagglutinin	Residue no.	Sequence										Total no. of amino acid residues
PVAP	1[a]	S	N	D	I	Y	F	N	F	Q	R	~600
RKA	1[a]	A	N	Q	F	S	F	N	F	Q	R	~600
PAL	25[b]	A	N	D	I	S	F	N	F	Q	R	276

Identical corresponding residues are underlined. RKA: hemagglutinin from red kidney beans (*P. vulgaris* cv. Red Kidney Bean); PVAP: antifungal protein from flageolet beans (*P. vulgaris* cv. 'Flageolet Bean'); PAL: hemagglutinin from tenuifolius beans (*P. acutifolius cv.* 'Tenuifolius Bean').

[a] Refers to the first amino acid residue of the protein.

[b] Refers to the 25th amino acid residue of the protein.

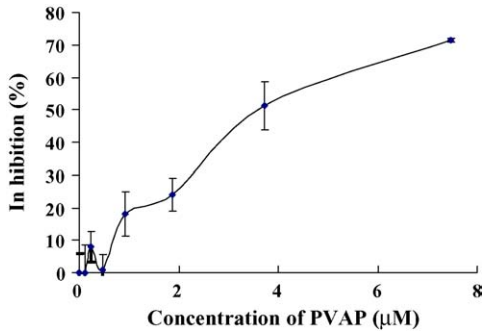

Fig. 9. Antiproliferative activity of flageolet bean *Phascolus vulgaris* antifungal protein (PVAP) toward leukemia L1210 cells as reflected by inhibition of [^{3}H-methyl]-thymidine uptake by the cells. Results are mean ± S.D., $n = 3$.

flageolet bean antifungal protein on Affi-gel blue gel, DEAE-cellulose and CM-cellulose is similar to that of previously published antifungal proteins [5,7,17,20,32,35–40] and red kidney bean lectin with antifungal activity [49].

Red kidney bean lectin has not been examined for antiproliferative activity toward tumor cells. Like some of the previously reported antifungal proteins and leguminous lectins [7,17,43], flageolet bean antifungal protein exhibits a suppressive effect on [methyl-^{3}H]-thymidine uptake by tumors cells. Red kidney bean lectin has not been tested for mitogenic and HIV-1 reverse transcriptase inhibitory activities but it is known that it displays antifungal activity [49]. Although flageolet bean antifungal protein lacks the first two activities, it exhibits specific antifungal activity against *M. arachidicola*. It is devoid of inhibitory activity toward two other fungal species, *B. cinerea* and *F. oxysporum*. This observation is reminiscent of prevous reports that shallot antifungal protein and asparagus DNase are active only against *B. cinerea* among the several fungal species examined [35,37]. In contrast, red kidney bean lectin has antifungal activity against *F. oxysporum*, *Rhizoctonia solani* and *Coprinus comatus* [51]. To date only several lectins including red kidney bean lectin have been reported with HIV-1 reverse transcriptase inhibitory [34,51], anti-HIV [19], and antifungal [9,49] activities. Some antifungal proteins lack HIV-1 reverse transcriptase inhibiting activitiy [45]. The lack of mitogenic activity in flageolet bean antifungal protein toward mouse splenocytes is in contrast to observations on leguminous lectins such as Con A [43] and some antifungal proteins [43,46,47,48].

Flageolet bean antifungal protein has fair stability. It is fairly thermostable. Its hemagglutinating activity is not deleteriously affected by pH changes and a variety of metal chlorides. Red kidney bean lectin has not been similarly studied [47]. The sensitivity of the antifungal activity of flageolet bean antifungal protein to trypsin is in contrast to chive chitinase-like antifungal protein [17].

In summary, the results of this study furnish evidence for antifungal activity of a hemagglutinin and represent an addition to the meager literature of antifungal lectins hemagglutinins.

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