Concurrent purification of two defense proteins from French bean seeds: a defensin-like antifungal peptide and a hemagglutinin

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Abstract: A purification protocol is described herein for concurrent isolation of two defense proteins including a 6-kDa defensin-like antifungal peptide and a 60-kDa dimeric hemagglutinin from seeds of the French bean (Phaseolus vulgaris). It involved ion-exchange chromatography on SP-Sepharose, affinity chromatography on Affi-gel blue gel, ion-exchange chromatography on Q-Sepharose, and gel filtration on Superdex Peptide (for defensin-like antifungal peptide) or Superdex 200 (for hemagglutinin). Both antifungal and hemagglutinating activities were adsorbed on SP-Sepharose and then on Affi-gel blue gel. Hemagglutinin was subsequently unadsorbed and defensin-like antifungal peptide adsorbed on Q-Sepharose. The antifungal activity of the antifungal peptide was stable in the temperature range of 0–90 °C for 20 min, in the pH range of 4–10, and after exposure to trypsin (1 mg/ml) at 37 °C for 1 h. The hemagglutinin was stable from 10 to 80 °C, from pH 1 to 12, and after treatment with trypsin at 37 °C for 2 h. It inhibited [methyl-3H]thymidine incorporation into breast cancer (MCF-7), leukemia (L1210), hepatoma (HepG2) and human embryonic liver (WRL68) cells with an IC50 of 6.6, 7, 13 and 15 µM, respectively, and elicited maximal mitogenic response from mouse splenocytes at 1 µM concentration. It curtailed HIV-1 reverse transcriptase activity with an IC50 of 1.9 µM, but was devoid of antifungal activity. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: defensin-like antifungal peptide; hemagglutinin; isolation; French bean

INTRODUCTION

Leguminous plants have tremendous economic importance. Their products including phytoestrogens [1] and protease inhibitors [2] may have some bearing on human health. Other products include antifungal proteins that may have value in protecting crops from fungal destruction [3–5], α-amylase inhibitors that may have anti-insect activity [6], lectins/hemagglutinins that may have mitogenic and antiproliferative activities [3,7–9], and ribosome inactivating proteins with translation-inhibitory, antifungal, antiviral and antitumor activities [10]. Thus, there is a voluminous amount of literature on leguminous products, especially proteins, many of which have a function of defense.

Antifungal proteins and lectins have been isolated from diverse organisms. They represent intensively studied proteins on account of their potentially exploitable activities including anticancer/antiproliferative, immunoenhancing and antiviral [8,9,11–13] activities. However, to date, antifungal proteins and lectins have not been reported from the French bean, a common vegetable.

Phaseolus vulgaris is a leguminous species with different cultivars. The objective of the present study was to isolate, for the first time, a defensin-like antifungal peptide and a hemagglutinin/lectin from the French bean cultivar of P. vulgaris, and to compare its characteristics and activities with its counterparts from other cultivars.

MATERIALS AND METHODS

Purification Protocol

Dried seeds of P. vulgaris cv. French bean cultivar number 12 from Mainland China (25 g) were soaked in distilled water overnight prior to homogenization and then centrifugation. To the resulting supernatant, NH4OAc buffer (pH 4.5) was added until a final concentration of 10 mM was attained. Cation-exchange chromatography on a 2.5 × 16 cm column of SP-Sepharose (Amersham Biosciences) was carried out. After removal of unadsorbed proteins (fraction SP1), adsorbed proteins were eluted sequentially with 0.2 M NaCl and 1 M NaCl in 10 mM NH4OAc buffer (pH 4.5) into fractions SP2 and SP3, respectively. Fraction SP3 was dialyzed extensively against distilled water. Tris-HCl buffer (pH 7.4) was added till a final concentration of 10 mM Tris was reached. SP3 was then applied on a 2.5 × 16 cm column of Affi-gel blue gel (Bio-Rad) that had previously been equilibrated with and was eluted with 10 mM Tris-HCl buffer (pH 7.4). After unadsorbed proteins (fraction BG1) had come off the column, adsorbed proteins (fraction BG2) were desorbed with 10 mM Tris-HCl buffer (pH 7.4) containing 1 M NaCl. Fraction BG2 was dialyzed against distilled water before addition of concentrated NH4HCO3 solution to produce a final concentration of 10 mM NH4HCO3. Fraction BG2 was then loaded on a 2.5 × 16 cm
column of Q-Sepharose (Amersham Biosciences) in 10 mM 
NH$_4$HCO$_3$ buffer (pH 9.4). The trace amount of proteins, 
unadsorbed on Q-Sepharose, was subjected to gel filtration 
on Superdex Peptide (Amersham Biosciences). The single peak 
eluted represented defensin-like antifungal peptide. The Q-
Sepharose column was eluted with 10 mM NH$_4$HCO$_3$ buffer 
(pH 9.4) containing 1 M NaCl. The fraction desorbed was then 
chromatographed on Superdex 200 in 10 mM NH$_4$HCO$_3$ (pH 
9.4). The single peak eluted represented purified French bean 
haemagglutinin.

**Assay of Antifungal Activity**

The assay of antifungal activity toward *Mycosphaerella 
arachidicola*, *Rhizoctonia solani* and *Valsa mali* was carried 
out in plates containing potato dextrose agar. After the 
mycelial colony had developed, sterile blank paper disks were 
placed around the rim of the mycelial colony. The plates 
were incubated at 23 °C for 72 h until mycelial growth had 
enveloped the disks containing the control and had formed 
crescents of inhibition around disks and containing samples 
with antifungal activity [14]. The IC$_{50}$-value of antifungal 
activity was determined as described in [14].

**Assay of Hemagglutinating Activity**

In the assay for lectin (hemagglutinating) activity, a serial 
dilution of the haemagglutinin solution was mixed with rabbit 
red blood cells. The results were read after about 1 h [8].

The tests to investigate inhibition of haemagglutinin-
induced haemagglutination by various carbohydrates including 
D-mannose, D-fructose, D-xylene, L-arabinose, raffinose, 
L-rhamnose, D-melezitose, D-melibiose, cellobiose, D-ribose, 
inositol, D-glucose, sucrose, D-galactose, galactitol, 
O-nitropheryl-β-D-galactopyranoside and 4-O-β-D-galactopy-
ranosyl-D-glucose were performed as described in [8]. The 
effects of temperature, NaOH, HCl and metallic chloride 
solutions on haemagglutinating activity of the haemagglutinin 
were examined, as previously described [8].

**Molecular Mass Determination and N-Terminal 
Sequence Determination**

For molecular mass determination, the purified antifungal 
peptide and haemagglutinin were subjected to sodium dodecyl 
sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) [15] 
and gel filtration on an FPLC-Superdex 200 or Superdex 
peptide column, which had been calibrated with molecular 
mass markers. The N-terminal sequences were determined by 
using a Hewlett-Packard HP G1000A Edman degradation unit 
and an HP 1000 HPLC System.

**Assay of Mitogenic Activity**

The assay of mitogenic activity was performed as described in 
[9] using splenocytes, isolated from BALB/c mice. The cellular 
uptake of $[^3]$H-methyl-thymidine was used as an index of the 
proliferative (mitogenic) response.

**Assay of Nitric Oxide Inducing Activity**

Assay of nitric oxide production by murine peritoneal 
macrophages. The assay was conducted as described by

Wong and Ng [16]. Lipopolysaccharide was used as a positive 
control in this assay.

**Assay of Antiproliferative Activity**

The cell lines L121 (leukemia), MCF-7 (mammary tumor), 
HepG2 (hepatoma) and WR168 (embryonic liver) were pur-
chased from American Type Culture Collection were incubated 
for 3 h before addition of the hemagglutinin. Incubation was 
carried out for another 48 h. A MTT solution was added to 
each well and incubated for 4 h. The medium was removed 
and DMSO was added to dissolve MTT-formazan formed [9].

**Assay for Ability to Inhibit Human Immunodeficiency 
Virus Type 1 (HIV-1) Reverse Transcriptase Activity**

The assay for ability to inhibit human immunodeficiency virus 
(HIV) reverse transcriptase activity was carried out as detailed 
in [8] using a nonradioactive ELISA kit.

**Assay for Ability to Inhibit HIV-1 Integrase**

The plasmid that expressed His-tagged wild-type HIV-1 
integrase, pT7-7-His(YTX)-HIV-1-IN, was a generous gift from 
Dr S.A. Chow (School of Medicine, UCLA). The expression 
and purification of the protein were carried out as previously 
described [17]. A nonradioactive ELISA-based HIV-1 integrase 
assay was performed according to the method of DNA coated 
plates [17].

**Screening for Inhibitory Effect on SARS Coronavirus 
(CoV) Proteinase**

The activity of severe acute respiratory syndrome (SARS) 
coronavirus(CoV) protease was indicated by a designed 
substrate composed of two proteins linked by a cleavage 
site for SARS CoV proteinase. The reaction was performed 
in a mixture containing 5 μM SARS CoV proteinase, 5 μM test 
sample, 20 μM substrate and buffer (20 μM Tris-HCl (pH 7.5), 
20 μM NaCl and 10 μM beta-mercaptoethanol) for 40 min at 
37 °C. After 40 min, the reaction was stopped by heating at 
100 °C for 2 min. Then the reaction mixture was analyzed by 
SDS-PAGE. If SARS CoV proteinase is inhibited by the test 
sample, there is only one band, which represents the intact 
substrate, revealed by SDS-PAGE.

**RESULTS**

**Isolation of French Bean Defensin-Like Antifungal 
Peptide and Hemagglutinin**

Ion-exchange chromatography of French bean seed 
extract on SP-Sepharose yielded a large unadsorbed 
fraction SP1 and a small adsorbed fraction SP2. both 
devoid of antifungal and hemagglutinating activities 
(Figure 1). Fraction SP3 eluted with 1 M NaCl 
was largely adsorbed on Affi-gel blue gel. The antifungal 
and hemagglutinating activities in fraction SP3 
were recovered in fraction BG2 adsorbed on Affi-gel blue gel. The traces of unadsorbed materials in fraction BG1
lacked antifungal and hemagglutinating activities (data not shown). Upon chromatography on Q-Sepharose fraction, BG2 appeared as a very small unadsorbed peak and a single adsorbed peak that was eluted by 1 M NaCl in the starting buffer (data not shown). The tiny unadsorbed peak from Q-Sepharose was chromatographed on Superdex Peptide to yield a single 6-kDa peak that represented purified defensin-like antifungal peptide (data not shown). The adsorbed peak on Q-Sepharose, which yielded a single 60-kDa peak after gel filtration on Superdex 200 (data not shown) and appeared as a single band with a molecular mass of 30 kDa in SDS-PAGE (data not shown), represented purified hemagglutinin. The purification of French bean defensin-like antifungal peptide and hemagglutinin is summarized in Table 1.

Characterization of French Bean Defensin-Like Antifungal Peptide and Hemagglutinin

The N-terminal sequence of defensin-like antifungal peptide was homologous to plant defensins (Table 2). French bean hemagglutinin closely resembled

![Cation-exchange chromatography of French bean extract on SP-Sepharose column starting buffer: 10 mM NH₄OAc buffer (pH 4.5). The third fraction (SP3) eluted with 1 M NaCl was the fraction enriched in antifungal and hemagglutinating activities.](image)

**Table 1** Yields and specific hemagglutinating (ha) and antifungal activities of different chromatographic fractions derived from French bean seed extract (25 g)

| Fraction                  | Protein yield (mg) | Specific ha activity (units/mg) | Total ha activity (units) | Recovery of ha activity (%) | Purification fold of ha activities | IC₅₀° |
|---------------------------|--------------------|--------------------------------|--------------------------|-----------------------------|-----------------------------------|------|
| Crude extract             |                    |                                |                          |                             |                                   |      |
| Fraction SP3 from SP-Sepharose | 12                | 7.83 × 10⁴                     | 9.4 × 10⁶                 | 61.3                        | 3.52                              | 72   |
| Fraction BG2 from Affi-gel blue gel | 5.15            | 1.49 × 10⁵                     | 7.68 × 10⁵                 | 50                          | 6.71                              | 55   |
| Fraction adsorbed on Q-Sepharose | 3.41         | 1.80 × 10⁵                     | 6.14 × 10⁵                 | 40                          | 8.10                              | —    |
| Fraction unadsorbed on Q-Sepharose (= purified defensin-like antifungal peptide) | 0.28          | —                              | —                         | —                           | —                                 | 27   |
| Fraction SU from Superdex 200 (= purified hemagglutinin) | 1.2            | 2.32 × 10⁵                     | 2.78 × 10⁵                 | 18.1                        | 10.41                             | —    |

°IC₅₀ of antifungal activity toward *Rhizoctonia solani* in microgram/ml.

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Table 2  Comparison of N-terminal sequences of French bean defensin-like antifungal peptide and hemagglutinin with other defensin-like antifungal peptides and hemagglutinins/lectins

| N-terminal sequence | Reference |
|---------------------|-----------|
| French bean defensin-like antifungal peptide (1–10) | KTCENLADTY | This study |
| Vulgarinin (1–10) | KTCENLADTY | [14] |
| *Medicago trunculata* defensin (29–37) | TCENLADTY | BLAST search |
| *Pachyrhizus erosus* defensin (1–10) | KTCENLADTY | BLAST search |
| French bean hemagglutinin | ATETYFNFQRFCETNIFIQR | This study |
| Pinto bean lectin | ASETSFSEFQFVEVTLNQL QR | [9] |
| Red kidney bean hemagglutinin | ANQTSFNFQRDFETNLNIQR | [3] |
| Haricot bean hemagglutinin | ASESYFNFQRFEETN | [14] |
| *Phaseolus acutifolius* lectin (25–44) | ANDISFNQRFNETNLNLQG | [7] |
| *Phaseolus coccineus* lectin (22–41) | ASETSFSEFQFNETNLNLQ | BLAST search |

Identical amino acid residues are underscored.

15 µM, respectively. It inhibited HIV-1 reverse transcriptase with an IC₅₀ of 1.9 µM (detailed data not shown), but was incapable of inhibiting HIV-1 integrase, SARS proteinase and mycelial growth (data not shown).

**DISCUSSION**

The present paper represents the first report of a defensin-like antifungal peptide and a hemagglutinin from the French bean. Previously, a thaumatin-like antifungal protein, and a peroxidase-like antifungal protein from the French bean have been described [4,5]. French bean defensin-like antifungal peptide resembles previously isolated plant defensin-like peptides in N-terminal sequence, molecular mass, chromatographic behavior on Affi-gel blue gel and ion exchangers, stability to trypsin, pH stability and thermostability [14,18]. However, it lacks inhibitory activity toward HIV-1 integrase and SARS proteinase.

Hemagglutinins/lectins, produced by French bean and other cultivars of *P. vulgaris* including pinto bean, red kidney bean, haricot bean and flageolet bean possess highly homologous but not identical N-terminal sequences. Lectins from other *Phaseolus* species also demonstrate similar N-terminal sequences. Lectins/hemagglutinins from French bean, flageolet bean, red kidney bean and pinto bean are dimeric but haricot bean hemagglutinin is tetrameric although the subunit molecular mass for all of them is approximately 30 kDa. With regard to sugar specificity, the hemagglutinating activity of hemagglutinins from French bean and other *P. vulgaris* cultivars cannot be inhibited by simple sugars whereas pinto bean lectin is galactose-specific. French bean hemagglutinin exhibits antiproliferative activity toward tumor cells like hemagglutinins from red kidney bean, haricot bean, and flageolet bean [3,9,19]. However, pinto bean...
lectin is devoid of antiproliferative activity. Mitogenic activity toward mouse splenocytes is a common feature of all these lectins/hemagglutinins. Unlike banana lectin [16], French bean hemagglutinin is not capable of augmenting nitric oxide production by mouse macrophages. In contrast to findings about the antifungal activity of some lectins [3]. French bean hemagglutinin is destitute of antifungal activity just like its counterparts from pinto bean and haricot bean.

Some lectins inhibit HIV-1 replication. French bean hemagglutinin manifests HIV-1 reverse transcriptase inhibitory activity \( \text{IC}_{50} = 1.9 \, \mu M \) which is slightly more potent than that of pinto bean lectin \( \text{IC}_{50} = 3 \, \mu M \). However, it does not inhibit HIV-1 integrase, unlike some of the milk proteins, antifungal proteins and ribosome inactivating proteins tested. It is also inactive against SARS proteinase. French bean hemagglutinin is stable over the temperature range 0–80°C and over the pH range 1–12. Its hemagglutinating activity is preserved after exposure to trypsin for 2 h, indicating that it does not have accessible lysine or arginine residues where cleavage and inactivation can occur or it may have dibasic pairs that are not cleaved. It is slightly more stable than pinto bean lectin which retains its hemagglutinating activity over the pH range 3–12 and the temperature range 0–70°C.

In summary, a highly efficient chromatographic procedure using SP-Sepharose chromatography initially for isolating French bean hemagglutinin and defensin-like antifungal peptide has been described herein. It is demonstrated in this study that lectins/hemagglutinins from different cultivars of a species are not identical in amino acid sequence and biological potency. French bean hemagglutinin isolated in the present study is fairly stable and biologically potent and possesses potentially exploitable activities. In addition, a defensin-like antifungal peptide has been isolated from French bean seeds. Previously a thaumatin-like protein and a peroxidase-like antifungal protein have been purified from French bean legumes. The antifungal activity of defensin toward \( R. \ solani \) and \( V. \ mali \) is first demonstrated in this study. Transgenic plants expressing French bean defensin would be expected to have a stronger resistance to fungal pathogens. French bean hemagglutinin, which is relatively stable to trypsin and a variety of pH values and temperatures, and exhibit mitogenic activity toward splenocytes and antiproliferative activity, may have health-promoting effects in human.

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