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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-^{3}H]thymidine incorporation into breast cancer (MCF-7), leukemia (L1210), hepatoma (HepG2) and human embryonic liver (WRL68) cells with an IC_{50} 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 IC_{50} 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.

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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, NH_4OAc 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 NH_4OAc 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 NH_4HCO_3 solution to produce a final concentration of 10 mM NH_4HCO_3. Fraction BG2 was then loaded on a 2.5 × 16 cm

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column of Q-Sepharose (Amersham Biosciences) in 10 mM NH_4HCO_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_4HCO_3 buffer (pH 9.4) containing 1 M NaCl. The fraction desorbed was then chromatographed on Superdex 200 in 10 mm NH_4HCO_3 (pH 9.4). The single peak eluted represented purified French bean hemagglutinin.

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 hemagglutinin solution was mixed with rabbit red blood cells. The results were read after about 1h [8].

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 as described in [8].

The effects of temperature, NaOH, HCl and metallic chloride solutions on hemagglutinating activity of the hemagglutinin were examined, as previously described [8].

Molecular Mass Determination and N-Terminal Sequence Determination

For molecular mass determination, the purified antifungal peptide and hemagglutinin 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 WRL68 (embryonic liver) were purchased 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(Y|TX)-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 mM Tris-HCl (pH 7.5), 20 mM NaCl and 10 mM 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

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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 lectins/hemagglutinins from other *P. vulgaris* cultivars and *Phaseolus* species in *N*-terminal sequence (Table 2). The defensin-like antifungal peptide which exhibited a molecular mass of 6 kDa (Figure 2) inhibited mycelial growth in *M. arachidicola*. The peptide also exerted antifungal activity toward *V. mali* and *R. solani* (Figure 3) with an IC_{50} of 3 and 4.5 μM, respectively. The antifungal peptide lacked inhibitory activity toward HIV-1 integrase and SARS proteinase. Only a single amino acid residue was detected in each sequencing cycle for both French bean hemagglutinin and defensin-like antifungal peptide. The antifungal activity was stable in the pH 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 retained its hemagglutinating activity after exposure for 30 min to various temperatures from 0 to 80 °C and various pH values from 1 to 12. However, there was a complete loss of activity at and above 90 °C, and at pH 13 and 14 and pH <1 (data not shown). Full retention of activity after exposure to trypsin (1 mg/ml) for 15, 30, 60 and 120 min at 37 °C was noted (data not shown). The activity could not be inhibited by any of the following sugars at 800 mM concentration: arabinose, fructose, fucose, galactose, galacturonic acid, glucosamine, glucose, mannose, melibiose, raffinose, rhamnose, sorbitol, sucrose and xylose. The hemagglutinin evoked mitogenic response from mouse splenocytes with the maximal response hemagglutinin-stimulated (uptake of 4000 cpm of radiolabeled thymidine compared with 1500 cpm of basal uptake) observed at a hemagglutinin concentration of 1 μM. The subsequent decline in mitogenic activity was similar to previous observations on other lectins. However, the hemagglutinin did not affect nitric oxide production by mouse macrophages. It inhibited proliferation of various tumor cell lines including L1210, HepG2 and MCF-7 and embryonic liver cell line WRL-68 with an IC_{50} of 6.6, 7, 13 and

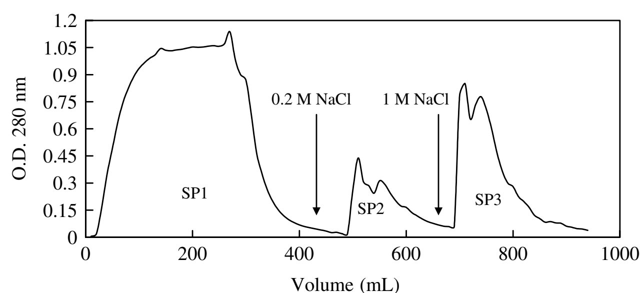

Figure 1 Cation-exchange chromatography of French bean extract on SP-Sepharose column starting buffer: 10 mM NH_4OAc buffer (pH 4.5). The third fraction (SP3) eluted with 1 M NaCl was the fraction enriched in antifungal and hemagglutinating activities.

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

	Protein yield (mg)	Specific ha activity (units/mg)	Total ha activity (units)	Recovery of ha activity (%)	Purification fold of ha activities	IC_{50}[a]
Crude extract	69	2.23×10^4	1.54×10^6	100	1	251
Fraction SP3 from SP-Sepharose	12	7.83×10^4	9.4×10^5	61.3	3.52	72
Fraction BG2 from Affi-gel blue gel	5.15	1.49×10^5	7.68×10^5	50	6.71	55
Fraction adsorbed on Q-Sepharose	3.41	1.80×10^5	6.14×10^5	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	2.32×10^5	2.78×10^5	18.1	10.41	—

[a] IC_{50} 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	ASETSFSFQRFVETNLILQR	[9]
Red kidney bean hemagglutinin	ANQTSFNFQRFDETNLILQR	[3]
Haricot bean hemagglutinin	ASESYFNFQRFEETN	[14]
Phaseolus acutifolius lectin (25–44)	ANDISFNFQRFNETNLILQG	[7]
Phaseolus coccineus lectin (22–41)	ASETSFSFDRFNETNLILQU	BLAST search

Identical amino acid residues are underscored.

15 µM, respectively. It inhibited HIV-1 reverse transcriptase with an IC_{50} 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-I integrase and SARS proteinase.

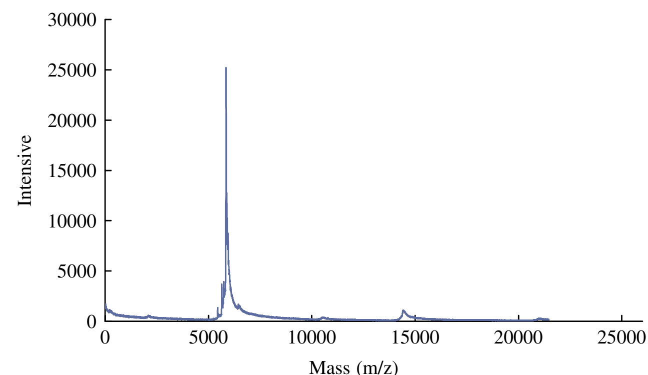

Figure 2 Molecular mass determination of French bean defensin-like antifungal peptide by MALDI-TOF MS. This figure is available in colour online at www.interscience.wiley.com /journal/jpepsci.

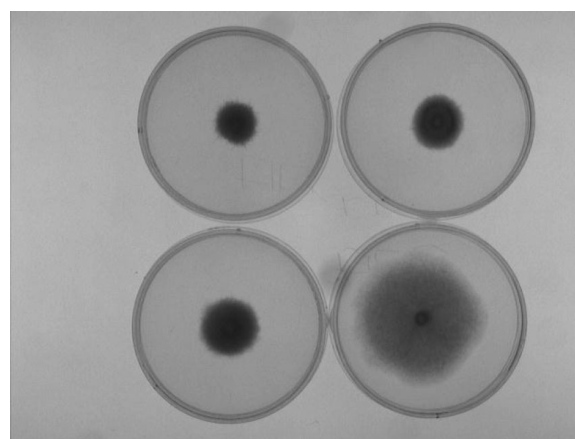

Figure 3 Determination of IC_{50}-value of antifungal activity of French bean defensin-like antifungal peptide toward *Rhizoctonia solani*. Lower right: 0 µM defensin. Lower left: 1.78 µM defensin. Upper right: 7.1 µM defensin. Upper left: 35.6 µM defensin.

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

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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 ($IC_{50} = 1.9$ μM) which is slightly more potent than that of pinto bean lectin ($IC_{50} = 3$ μM). 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 been 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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