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Limenin, a defensin-like peptide with multiple exploitable activities from shelf beans

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Abstract: From the seeds of the shelf bean, an antifungal peptide with a molecular mass of 6.5 kDa was isolated. The isolation procedure comprised affinity chromatography on Affi-gel blue gel, ion exchange chromatography on Mono S, and gel filtration on Superdex 75. The peptide was adsorbed on Affi-gel blue gel and Mono S. It potently suppressed mycelial growth in *Botrytis cinerea*, *Fusarium oxysporum*, and *Mycosphaerella arachidicola* with an IC_{50} of 2.9, 2.1, and 0.34 μM, respectively. It exerted antibacterial activity toward several bacterial species with an IC_{50} approximating 100 μM. [Methyl-^{3}H]-thymidine incorporation into isolated mouse splenocytes was stimulated. [Methyl-^{3}H]-thymidine incorporation into M1 (myeloma) and L1210 (leukemia) cells was inhibited. The peptide reduced the activity of HIV-1 reverse transcriptase and also inhibited translation in a cell-free rabbit reticulocyte lysate system. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: limenin; antifungal peptide; shelf bean

INTRODUCTION

Fungi are ubiquitous; there are about 250 000 species widely distributed in essentially every ecosystem. Muller and Loeffler estimate that the weight of fungi on earth exceeds that of human beings [1]. Fungi are able to use almost any surface for their growth. In daily life, we are exposed to fungi from the moment of birth. Fortunately, in 250 000 species, only 200 or so species of fungi are pathogenic to mammals [2].

Beans are highly nutritious and abundant in proteins and vitamins [3], and are thus favorite foods for animals, insect, fungi, and even bacteria. Leguminous plants synthesize toxic compounds, proteins, or peptides in their seeds, beans, and peas to avoid predation by other organisms so that they can perpetuate their species. Legumes produce antifungal proteins/peptides including chitinases [4,5], ribosome-inactivating proteins (RIP) [6], cyclophilin-like proteins [7], defensins [8,9], protease inhibitors [10], lectins [11], peroxidases [12], and lysozymes [13].

In this study, an antifungal peptide was isolated from shelf beans, which are one kind of common table beans in China. This is the first investigation that demonstrates an antifungal peptide in shelf beans. Its characteristics are compared with those of previously isolated antifungal peptides/proteins.

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MATERIALS AND METHODS

Materials

Shelf beans (*Phaseolus limensis* cv. 'shelf bean') from China were used in the present study. Affi-gel blue gel was purchased from Bio-Rad, and Mono S and Superdex 75 columns were from Amersham Biosciences. Chemicals for sequence analysis were obtained from Hewlett Packard (Palo Alto, CA, USA). All other chemicals used were of reagent grade.

Purification of Antifungal Peptide

The beans were soaked in distilled water and then homogenized using a Waring blender. The homogenate was centrifuged (12 000 g, 30 min, 4 °C). The resulting supernatant was collected. Tris-HCl buffer (1 M, pH 7.4) was added to the supernatant until the concentration of Tris-HCl attained 10 mM. The supernatant was chromatographed on a 5 × 10 cm column of Affi-gel blue gel (Bio-Rad) in 10 mM Tris-HCl buffer (pH 7.4). Unadsorbed proteins were collected as fraction B1 before the adsorbed proteins were desorbed with 10 mM Tris-HCl buffer (pH 7.4) containing 1 M NaCl and collected as fraction B2. Fraction B2 was dialyzed before ion exchange chromatography on Mono S in 10 mM NH_4OAc buffer (pH 4.5). After removal of unadsorbed proteins as fraction S1, adsorbed proteins were desorbed using a linear NaCl concentration gradient (0–1 M) in 10 mM NH_4OAc buffer (pH 4.5) collected as fraction S3. Fraction S3 was subjected to final purification on a Superdex 75 HR 10/30 column in 10 mM NH_4HCO_3 buffer (pH 9.0). The purified peptide was designated as limenin.

Molecular Mass Determination

Molecular mass determination was conducted according to the method of Nielsen and Reynolds [14]. After electrophoresis, the gel was stained with Coomassie Brilliant Blue. The molecular mass of limenin was determined by comparison

of its electrophoretic mobility with those of molecular mass marker proteins from Amersham Biosciences. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was also employed to determine the molecular mass of the purified ribonuclease using an Applied Biosystems 4700 Proteomics Analyzer.

Amino Acid Sequence Analysis

The *N*-terminal amino acid sequence of limenin was analyzed by means of automated Edman degradation using a Hewlett Packard 1000A protein sequencer equipped with an HPLC system [6].

Assay of Antifungal Activity

The assay of limenin for antifungal activity toward *Mycosphaerella arachidicola*, *Botrytis cinerea*, and *Fusarium oxysporum* was carried out in 90 × 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 a distance of 0.5 cm away from the rim of the mycelial colony. An aliquot of a solution of limenin was added to a disk. The plates were incubated at 25 °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 [8].

To determine the IC_{50} value for the antifungal activity of limenin, four doses of the peptide were added separately to four aliquots each containing 4 ml potato dextrose agar at 45 °C, mixed rapidly, and poured into four separate small petri dishes. After the agar had cooled down, a small amount of mycelia, the same amount for each plate, was added. The buffer without any antifungal peptide served as a control. After incubation at 23 °C for 72 h, the area of the mycelial colony was measured and the inhibition of fungal growth was determined. The concentration of limenin that brought about 50% reduction in the area of mycelial colony is its IC_{50}.

To investigate the thermal stability, pH stability, and effects of ions, limenin was pretreated accordingly, and the antifungal assay was then conducted as mentioned above.

Assay of Antibacterial Activity

Bacteria were incubated in 10 ml of nutrient broth in a thermal shaker for 12 h at 37 °C, and then 5 ml of this bacterial suspension was transferred to 50 ml of nutrient broth and incubated for another 3–6 h (the exact duration depends on the bacterial species) in order to shift bacterial growth to the midlogarithmic phase. The bacterial suspension was then centrifuged at 2000 × g for 10 min, and the bacterial pellet was washed and resuspended in normal saline. A total of 10^5 or 10^7 bacteria per ml were obtained by dilution guided by the optical density at 595 nm. In the experiment, every condition was prepared in triplicate; the first aliquot of bacteria was mixed with limenin at 0.5, 0.25 and 0.125 mg/ml, respectively; the second aliquot was mixed with different concentrations of limenin in 5 mM $CaCl_2$; the third aliquot was mixed with different concentrations of limenin in 5 mM $MgCl_2$; and the fourth aliquot was mixed with only bacteria in saline as a control. The samples were then incubated in a shaker and aliquots were obtained at four time points (0, 3, 6, and 12 h), serially diluted with nutrient broth and spread on agar plates. After incubation at 37 °C for 24 h, the colonies were counted. The number of bacteria for each condition and dilution was determined from the average colony counts for three plates [15].

Assay for HIV Reverse Transcriptase Inhibitory Activity

The ability of limenin to inhibit HIV-1 reverse transcriptase was assessed by using an ELISA kit from Boehringer Mannheim (Germany) as described by Collins *et al.* [16].

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 μg 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 limenin 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 [17].

Assay of Antiproliferative Activity on Tumor Cell Lines

Myeloma (M1) cell line and leukemia (L1210) cell line were each suspended in RPMI 1640 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 limenin 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 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) in phosphate-buffered saline was spiked into each well, and the plates were incubated for 4 h. The plates were then centrifuged at 324 × g 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 then measured by using a microplate reader.

Assay of Cell-free Translation-inhibitory Activity

In this assay, the rabbit reticulocyte lysate system was used. A 10-μl aliquot of the isolated peptide was added to 10 μl of a radioactive mixture (500 mM KCl, 5 mM $MgCl_2$, 130 mM creatine phosphate, and 1 μCi [4,5-^{3}H]leucine) and 30-μl working rabbit reticulocyte lysate containing 0.1 mM hemin and 5 μl creatine kinase. The mixture was incubated at 37 °C for 30 min, and 330 μl of 1 M NaOH containing 1.2% H_2O_2 was added. Further incubation for 10 min allowed decolorization and tRNA digestion. An equal volume of the reaction mixture was then added to 40% trichloroacetic acid

with 2% casein hydrolyzate in a 96-well plate to precipitate radioactively labeled protein. The precipitate was harvested with an automated cell harvester onto a glass filter, and the radioactivity was measured with a Packard Tri-Carb 2900TR low-activity liquid scintillation counter. Agrostin, a type 1 RIP isolated from the seeds of *Agrostemma githago*, was used as a positive control [6].

RESULTS

Affinity chromatography on Affi-gel blue gel resolved the extract into a large unadsorbed fraction B1 and a small adsorbed fraction B2 (Figure 1). Antifungal activity was confined to fraction B2. Fraction B2 yielded, upon ion exchange chromatography on Mono S, an unadsorbed fraction S1 and two adsorbed fractions S2 and S3 (Figure 2). Only fraction S3 manifested antifungal activity. Fraction S3 was separated on Superdex 75 into two fractions of different sizes, F1 and F2 (Figure 3). Only the small fraction F2 exhibited antifungal activity. Fraction F2 manifested a single band with a molecular mass of 7 kDa in SDS-PAGE (Figure 4) and the same molecular mass in gel filtration on a Superdex peptide column (data not shown). In MALDI-TOF MS study, it exhibited a molecular mass of 6509.4 Da (Figure 5). Its amino acid sequence showed considerable similarity to defensins (Table 1). Its antifungal potencies toward several fungal species are summarized in Table 2. It inhibited mycelial growth in *B. cinerea*, *F. oxysporum*, and *M. arachidicola* with an IC_{50} of 2.9 ± 0.06 μM, 2.1 ± 0.05 μM, and 0.34 ± 0.02 μM, respectively. The growth of several bacterial species was also inhibited by limenin (Figure 6 and Table 3). Limenin increased the incorporation of [methyl-^{3}H]-thymidine into mouse splenocytes (Table 4), but reduced MTT uptake into M1 and L1210 cells (Figure 7). The activity of HIV-1 reverse transcriptase was attenuated by the antifungal peptide with an IC_{50} of 106 μM. It also possessed a cell-free translation-inhibitory activity in the rabbit reticulocyte lysate system ($IC_{50} = 20$ μM).

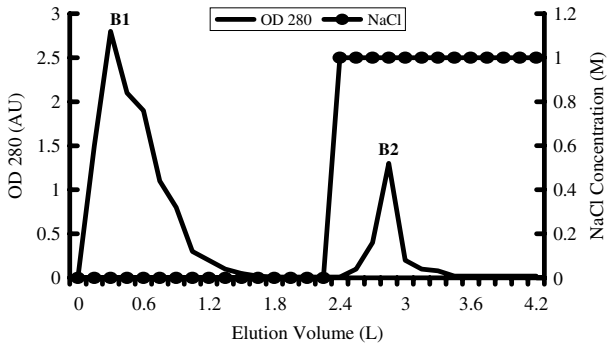

Figure 1 Fractionation of the crude extract of shelf beans on an Affi-gel blue gel column equilibrated with the binding buffer (10 mM Tris-HCl, pH 7.4). The column was washed with the binding buffer to remove unadsorbed fraction B1 and then eluted with 1000 mM NaCl in the same buffer to desorb B2 which contained antifungal activity.

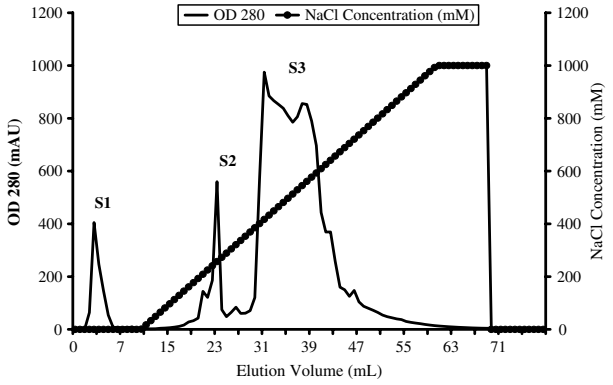

Figure 2 After chromatography on Affi-gel blue gel, the adsorbed fraction B2 was dialyzed and then applied to the FPLC-Mono S column in 20 mM NH_4OAc buffer (pH 4.5) at a flow rate of 1 ml/min. The column was then washed with the same buffer to remove unadsorbed fraction S1. Adsorbed fractions (S2–S3) were eluted with a linear concentration gradient of NaCl in the same buffer. S3 contained antifungal activity.

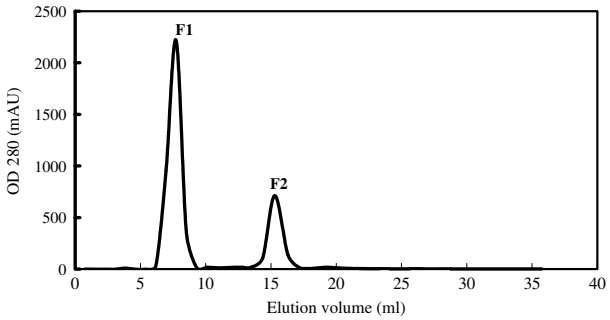

Figure 3 Gel filtration of peak S3 on a Superdex 75 HR 10/30 FPLC column in 10 mM NH_4HCO_3 buffer (pH 9.0) at a flow rate of 0.5 ml/min. The peak labeled F2 exhibited antifungal activity.

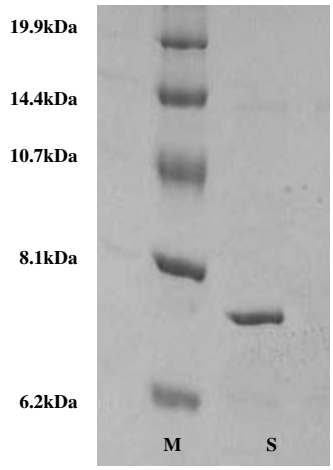

Figure 4 SDS-PAGE results. Lane S: limenin. Lane M: molecular mass marker.

Table 1 Comparison of amino acid sequence of limenin with those of relatedproteins

Species (protein)	Sequence																				
Phaseolus limensis (limenin)	1	K	T	C	E	N	L	A	D	T	Y	K	G	P	C	F	T	T	G	G	C 20
Vigna radiata (PDF1)	29	K	T	C	E	N	L	A	N	T	Y	R	G	P	C	F	T	T	G	S	C 48
Pachyrhizus erosus (defensin)	1	K	T	C	E	N	L	A	D	T	F	R	G	P	C	F	T	D	G	S	C 20
Medicago truncatula (defensin)	29	-	T	C	E	N	L	A	D	T	Y	R	G	P	C	F	T	E	G	S	C 47
Cajanus cajan (antifungal protein)	28	K	T	C	E	N	L	A	D	K	Y	R	G	P	C	F	S	-	-	G	C 45
Medicago sativa (antifungal protein precursor)	-	29	T	C	E	N	L	A	D	K	Y	R	G	P	C	F	S	-	-	G	C 45
Pisum sativum (pI39)	-	30	T	C	E	H	L	A	D	T	Y	R	G	V	C	F	T	N	A	S	C 48

Species (protein)	Sequence																								
Phaseolus limensis (limenin)	21	D	D	H	C	K	N	K	E	H	L	L	S	G	R	C	R	D	D	F	R	C	W	C	T	R	N	C 47
Vigna radiata (PDF1)	49	D	D	H	C	K	N	K	E	H	L	R	S	G	R	C	R	D	D	F	R	C	W	C	T	R	N	C 75
Pachyrhizus erosus (defensin)	21	D	D	H	C	K	N	K	E	H	L	I	K	G	R	C	R	D	D	F	R	C	W	C	T	R	N	C 47
Medicago truncatula (defensin)	48	D	D	H	C	K	N	K	A	H	L	I	S	G	T	C	H	-	N	F	Q	C	F	C	J	Q	N	C 73
Cajanus cajan (antifungal protein)	46	D	T	H	C	T	T	K	E	H	A	V	S	G	R	C	R	D	D	F	R	C	W	C	T	K	R	C 72
Medicago sativa (antifungal protein precursor)	46	D	T	H	C	T	T	K	E	N	A	V	S	G	R	C	R	D	D	F	R	C	W	C	T	K	R	C 72
Pisum sativum (pI39)	49	D	D	H	C	K	N	K	A	H	L	I	S	G	T	C	H	-	D	W	K	C	F	C	T	Q	N	C 74

* ^{1}K and C^{47} indicate that the 1st amino acid is K and the 47th amino acid is C.

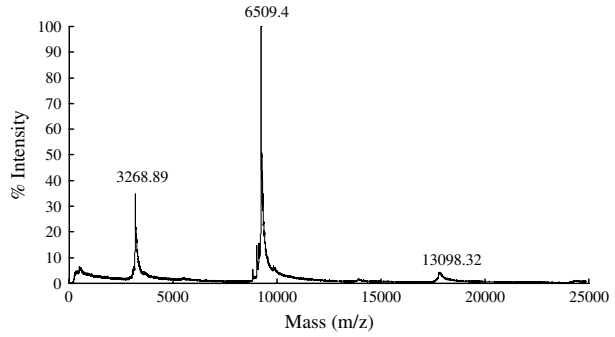

Figure 5 Molecular mass determination by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

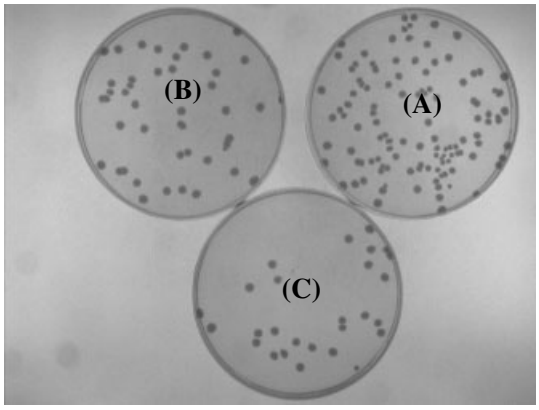

Figure 6 Determination of IC_{50} value of antibacterial activity of limenin toward *Mycobacterium phlei* (A: 35 μM limenin; B: 70 μM limenin; C: 140 μM limenin).

Table 2 Comparison of antifungal activity of limenin on various fungal species (data represent means ± SD, $n = 3$)

Fungal species	IC_{50} (μM)
Botrytis cinerea	2.9 ± 0.06
Fusarium oxysporum	2.1 ± 0.05
Mycosphaerella arachidicola	0.34 ± 0.02

DISCUSSION

A chromatographic procedure that has been proven effective for isolating antifungal proteins, entailing the use of the chromatographic media Affi-gel blue gel, Mono S, Superdex 75, and Superdex peptide [9,18,19], was used with success in the present study to prepare a defensin-like antifungal peptide from shelf beans.

Limenin exhibits antifungal potency, mitogenic activity toward mouse splenocytes, antiproliferative activity toward M1 and L1210 cells, HIV-1 reverse transcriptase inhibitory activity, and antibacterial activity. Its antifungal and antibacterial activities are in accordance with previous findings on defensins [20,21]. Its HIV-1 reverse transcriptase inhibitory activity, mitogenic activity toward mouse spleen cells, and antiproliferative activity toward tumor cells fall in line with earlier reports of similar activities in other antifungal proteins and peptides. Thus, it appears that the leguminous defensin-like peptide isolated in the present investigation is versatile and possesses a variety of potentially exploitable activities. It deserves mention that some antifungal proteins like mungin, a

Table 3 Comparison of antibacterial activity of limenin on various bacterial species (data represent means ± SD, $n = 3$)

Bacterial species	IC_{50} (μM)
Staphylococcus aureus	—
Mycobacterium phlei	96 ± 8
Bacillus cereus	—
Bacillus megaterium	102 ± 6
Bacillus subtilis	112 ± 2
Proteus vulgaris	81 ± 2
Escherichia coli	—
Enterobacter aerogenes	—
Pseudomonas aeruginosa	—
Pseudomonas fluorescens	—

– : no inhibition observed at a dose of 150-μM limenin.

Table 4 Mitogenic activity of limenin toward murine splenocytes as reflected in uptake of [methyl-^{3}H]-thymidine (data represent means ± SD, $n = 3$)

Con A		Limenin	
Concentration (mg/ml)	CPM	Concentration (mg/ml)	CPM
15.6	3221 ± 543	10	2584 ± 324
7.8	31 289 ± 2435	5	14 013 ± 2344
3.9	102 900 ± 5732	2.5	33 899 ± 2567
1.9	26 661 ± 2143	1.2	42 076 ± 3678
0.9	12 643 ± 1298	0.6	8991 ± 982
0.4	7794 ± 1036	0.3	1970 ± 365

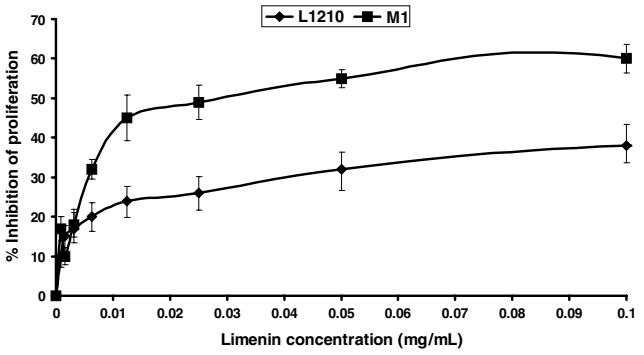

Figure 7 Inhibition of proliferation of leukemia cells L1210 and myeloma cells M1 by limenin (data represent means ± SD, $n = 3$).

cyclophilin-like protein from mung beans, lack HIV-1 reverse transcriptase inhibitory activity [7]. Some proteins such as the cyclophilin-like antifungal protein from mung bean exhibit antimitogenic activity. Others such as chive chitinase-like antifungal protein are devoid of antibacterial activity [22]. Some antifungal proteins are without retarding effects on proliferation of tumor cells [23]. The antifungal deoxyribonuclease from asparagus seeds [24] and antifungal protein from shallot bulbs [25] are active only against *B. cinerea* among the several fungi tested. The mitogenic activity of limenin toward tumor splenocytes and its antiproliferative activity toward tumor cells constitute a desirable combination. The potency of limenin in inhibiting translation in the cell-free reticulocyte system ($IC_{50} = 20$ μM) is similar to those of other antifungal proteins [26,27] and much lower than those of RIPs such as ricin with IC_{50} values in the nanomolar concentration [28]. Its HIV-1 reverse transcriptase inhibitory potency is fairly high, compared with other anti-HIV natural products with IC_{50} values exceeding 100 μM [29]. The multiplicity of biological activities of the leguminous defensin-like peptide is noteworthy.

Human beings are exposed to pathogenic fungi from the moment of birth. There are a limited number of antifungal drugs available for the treatment of an extending spectrum of pathogenic fungi. A lot of fungi have shown drug resistance to the traditional antifungal agents such as azoles [30], polyenes [31], and fluorinated pyrimidines [32]. Thus, it is imperative to develop new antifungal agents. We suggest that limenin would be a potential drug to treat fungal infections. Analysis of the *N*-terminal amino acid sequence of limenin reveals resemblance to those of other plant defensins, implying that the mechanism of antifungal action of limenin is similar to that of other plant defensins. Plant defensins could be used for the development of antifungal therapeutics because they could interact selectively with specific structures in fungal membranes such as phosphorylinositol-containing sphingolipids or glycosylceramides [33].

B. cinerea, *M. arachidicola*, and *F. oxysporum* are phytopathogenic fungi. *B. cinerea* can cause gray mold, which is an important postharvest disease that causes worldwide extensive damage to a wide range of economical crops such as apples, tomatoes, and grapes [34–37]. *M. arachidicola* is known as a fungal pathogen, which could form spots on leaves of the peanut plant. *F. oxysporum* is known as an agent of the Panama disease of banana plants [38]. It is feasible to incorporate, through genetic engineering, resistance against pathogens in transgenic plants. We suggest that limenin could be transferred into plants such as apples, tomatoes, grapes, peanuts, or bananas, which, as a result, would acquire reinforced antifungal activity against fungi.

In summary, limenin is a peptide with a diversity of exploitable activities.

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