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Isolation of Unguilin, a Cyclophilin-Like Protein with Anti-Mitogenic, Antiviral, and Antifungal Activities, from Black-Eyed Pea

X. Y. Ye[1] and T. B. Ng[1]

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A protein designated unguilin was isolated from seeds of the black-eyed pea *(Vigna unguiculata).* It possesses a molecular weight of 18 kDa and an N-terminal sequence resembling that of cyclophilins and the cyclophilin-like antifungal protein from mung beans, and was adsorbed on Affi-gel blue gel and CM-Sepharose. Unguilin exerted an antifungal effect toward fungi including *Coprinus comatus, Mycosphaerella arachidicola,* and *Botrytis cinerea.* In addition, unguilin was capable of inhibiting human immunodeficiency virus-1 reverse transcriptase and the glycohydrolases α- and β-glucosidases which are involved in HIV infection. Unguilin was devoid of lectin and ribonuclease activities. It inhibited methyl-^{3}H-thymidine uptake by mouse splenocytes and it weakly inhibited translation in a rabbit reticulocyte lysate system. Unguilin resembles mungin in some aspects, but differs from it in others.

KEY WORDS: Cyclophilin-like protein; antifungal; antiviral; peas.

1. INTRODUCTION

A variety of proteins have been purified from leguminous plants, including lectins with antitumor, immunomodulatory, and other activities (Kamemura *et al.,* 1993), trypsin inhibitors with antitumor activity (Birk, 1985), antifungal proteins with different structures including thaumatin-like proteins (Ye *et al.,* 1999), miraculin-like protein (Ye *et al.,* 2000*b*), chitinases (Benhamou *et al.,* 1993; Vogelsang and Barz, 1993; Ye *et al.,* 2000*a*), and ribosome-inactivating proteins (Leah *et al.,* 1991), in addition to a host of other proteins. Leguminous plants have drawn the attention of many investigators. Since different leguminous plants may elaborate different kinds of antifungal proteins (Ye *et al.,* 1999, 2000*a, b*), the present investigation was undertaken to ascertain if the black-eyed pea produces a new antifungal protein. In the present report the finding of an antifungal protein carrying an N-terminal sequence displaying remarkable resembance to that of cyclophilins, which have been described as a superfamily of ubiquitous folding catalysts (Gothel and Marahiel, 1999), is presented. The antifungal protein is an inhibitor of HIV-1 reverse transcriptase.[2]

A cyclophilin-like antifungal protein was first reported from the mung bean (Ye and Ng, 2000). The present study provides evidence for the presence of this new class of antifungal proteins in another leguminous species, the black-eyed pea, and thus corroborates the defensive role played by the cyclophilin-like proteins.

2. MATERIALS AND METHODS

2.1. Isolation of Antifungal Protein

Black-eyed pea *(Vigna unguiculata)* seeds purchased locally were soaked in distilled water and homogenized, and the supernatant that was collected after centrifugation

[1] Department of Biochemistry, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong, China.

[2] Abbreviations: CM-Sepharose, carboxymethyl-Sepharose; HIV, human immunodeficiency virus; ELISA, enzyme-linked immunosorbent assay; MES, 2-[*N*-morpholine] ethanesulfonic acid; Tris, Tris [hydroxymethyl] amino methane.

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of the homogenate was dialyzed against distilled water. Tris–HCl buffer (pH 7.2) was added until its final concentration reached 10 mM. The crude extract was applied to a column of Affi-gel blue gel (Bio-Rad) (2.5 × 10 cm) which had been equilibrated and eluted with 10 mM Tris–HCl buffer (pH 7.2). After elution of the unadsorbed proteins, adsorbed proteins were eluted by NaCl gradient (0–500 mM) in the Tris–HCl buffer. Following dialysis against 10 mM Tris–HCl buffer (pH 7.2), the adsorbed proteins were loaded on a column of CM-Sepharose (1.5 × 18 cm). The column was eluted with the same buffer to remove unadsorbed materials. Adsorbed proteins were desorbed by NaCl gradient (0–500 mM) in 10 mM Tris–HCl buffer (pH 7.2).

2.2. Determination of Molecular Weight

For assessing the molecular weight of unguilin, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out in accordance with the procedure of Laemmli and Farve (1973). The N-terminal sequences of unguilin were elucidated by employing a Hewlett-Packard HP G1000A Edman degradation unit and an HP 1000 HPLC System.

2.3. Assay of Antifungal Activity

In the assay for antifungal activity, sterile petri plates (100 × 15 mm) containing 10 ml of potato dextrose agar were used. After the fungal colony had developed, paper disks (0.625 cm in diameter) were placed 0.5 cm from the rim of the colony. A 6-μl aliquot of the test sample in 10 mM Tris–HCl buffer (pH 7.2) was applied to each disk. Incubation of the petri plate was carried out at 23°C for 72 h until mycelial growth had enveloped peripheral disks containing the control and had generated crescents of inhibition around disks with antifungal samples. Three fungal species, *Coprinus comatus, Mycosphaerella arachidicola,* and *Botrytis cinerea,* were examined in the assay (Ye *et al.,* 1999).

2.4. Assay of HIV-1 Reverse Transcriptase Inhibitory Activity

HIV-1 reverse transcriptase inhibitory activity was determined by ELISA following the method of Collins *et al.* (1997*a*), using a nonradioactive kit from Boehringer Mannheim (Germany). The inhibition assay was carried out as detailed in the procedure included with the kit, except that each well contained 2 ng of recombinant HIV-1 reverse transcriptase in a total reaction volume of 60 μl.

The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hybrid poly(A) · oligo(dT)15. In place of radiolabeled nucleotides, digoxigenin- and biotin-labeled nucleotides in an optimized ratio are incorporated into 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 streptavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase (anti-DIG-POD), binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrate is added. The peroxidase enzyme catalyzes the cleavage of the substrate, producing a colored reaction product. The absorbance of the samples at 405 nm can be determined using a 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 unguilin was calculated as percentage inhibition compared to a control without the protein.

2.5. Assay of Glycohydrolase-Inhibitory Activity

The glycohydrolases α- and β-glucosidase are found in the Golgi complex and are associated with the processing of viral proteins. α-Glucosidase in particular is related to HIV-1 gp 120 glycosylation. Thus glycohydrolase-inhibitory activity is related to anti-HIV activity. In the assay for glycohydrolase-inhibitory activity, α-glucosidase, β-glucosidase, and β-glucuronidase and their corresponding β-nitrophenyl glucoside substrates were used to set up the enzymatic reaction in 10 mM MES buffer (pH 6.4) in a 96-well microplate. Solutions of substrates and enzymes were made in 50 mM buffer appropriate for each enzyme; MES-NaOH (pH 6.5) for α-glucuronidase, sodium acetate (pH 5.5) for β-glucosidase, and sodium acetate (pH 5.6) for β-glucuronidase. Each well of the microplate contained 2 mM substrate, 40 mM buffer, and enough enzyme to produce a measurable change in absorbance at 405 nm (0.2 unit/well for α-glucosidase, 0.01 unit/well for β-glucosidase, and 100 units/well for β-glucuronidase). Unguilin was allowed to interact with the enzyme for 5 min before the enzymatic reaction was initiated by addition of substrate. The total reaction volume was 0.2 ml. After incubation at room temperature for 15 min the reaction was brought

to an end by addition of 60 μl of 2 M glycine-NaOH, pH 10. The plate was then read on a BioRad microplate reader at 405 nm (Collins *et al.,* 1997*b*).

2.6. Assay of Lectin Activity

The assay for lectin (hemagglutinating) activity was performed as follows. A serial twofold dilution of the unguilin solution in microtiter U-plates (50 μl) was mixed with 50 μl of a 2% suspension of rabbit erythrocytes in phosphate-buffered saline (pH 7.2) at room temperature. The results were recorded after about 1 hr when the blank had fully sedimented. The hemagglutinating titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was taken as one hemagglutination unit. Specific activity is the number of hemagglutinating units per milligram of protein (Wang *et al.,* 1996).

2.7. Assay of Ribonuclease Activity

In the assay for ribonuclease (RNase) activity toward yeast tRNA, unguilin was incubated with 200 μg of tRNA in 150 μl of 100 mM Tris–HCl buffer (pH 7.5) at 37°C for 15 min. Ice-cold 3.4% perchloric acid (350 μl) was added to terminate the reaction. The reaction mixture was centrifuged (15,000 *g,* 15 min at 4°C) to collect the supernatant after it had been allowed to stand on ice for 15 min. The absorbance of the supernatant was measured at 260 nm after suitable dilution. One unit of RNase activity is defined as the amount of enzyme which produces an absorbance increase of one per minute in the acid-soluble supernatant per milliliter of reaction mixture under the specified conditions (Mock *et al.,* 1996).

2.8. Assay of Translation-Inhibiting Activity

The assay of unguilin for the ability to inhibit synthesis from ^{3}H-leucine in a cell-free rabbit reticulocyte lysate system was conducted as previously described (Pelham and Jackson, 1976; Lam *et al.,* 1998). Rabbit reticulocyte lysate was prepared from the blood of rabbits rendered anemic by phenylhydrazine injections. The test sample (10 μl) was added to 10 μl of hot mixture (500 mM KCl, 5 mM $MgCl_2$, 130 mM phosphocreatine, and 1 μCi of [4,5-^{3}H]leucine) and 30 μl of working rabbit reticulocyte lysate containing 0.1 μM hemin and 5 μl of creatine kinase. Incubation proceeded at 37°C for 30 min before addition of 330 μl of 1 M NaOH and 1.2% H_2O_2. 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 collected on a glass fiber Whatman GF/A filter, washed, and dried with absolute alcohol passing through a cell harvester attached to a vacuum pump. The filter was suspended in scintillant and counted in an LS6500 Beckman liquid scintillation counter.

2.9. Assay of Antimitogenic Activity

The assay for the effect of unguilin on methyl ^{3}H-thymidine uptake by mouse splenocytes was carried out as detailed by Wang *et al.* (1995). 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 of penicillin/ml, and 100 μg of streptomycin/ml. The cells (7×10^5 cells/100 μl/well) were seeded into a 96-well culture plate and serial concentrations of unguilin in 100 μl of medium were added. After incubation of the cells at 37°C in a humidified atmosphere of 5% CO_2 for 24 hr, 10 μl of methyl ^{3}H-thymidine (0.25 μCi, Amersham, Pharmacia Biotech) was added, and the cells were incubated for a further 6 hr 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.

3. RESULTS

Fractionation of the black-eyed pea extract on Affi-gel blue gel produced a large unadsorbed peak (Fig. 1). An adsorbed peak of smaller size was obtained and it was fractionated on CM-Sepharose to yield a large unadsorbed peak and three adsorbed peaks (Fig. 2). The first adsorbed peak represents the antifungal protein designated unguilin. The yields throughout the various stages of purification from 100 g of seeds for crude extract were 8.8 g; for fraction absorbed on Affi-gel blue gel, 130.4 mg; and for unguilin, 8.0 mg. The molecular weight of the protein unguilin was 18 kDa according to SDS–PAGE (Fig. 3). The N-terminal sequence of the unguilin is presented in Table I. It demonstrated substantial homology to cyclophilins from a variety of organisms.

Unguilin was able to inhibit HIV-1 reverse transcriptase, α-glucosidase and β-glucosidase, while there

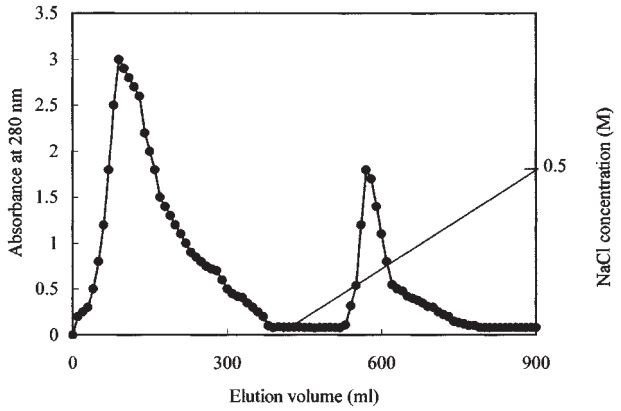


Fig. 1. Affinity chromatography of the crude extract of black-eyed peas on an Affi-gel blue gel column equilibrated with 10 mM Tris–HCl buffer (pH 7.2). The gel was washed with the buffer and eluted with a linear gradient of 0–500 mM NaCl in the same buffer.

was no suppressive action on β-glucuronidase (Table II). It inhibited cell-free translation with an IC_{50} of about 22 μM (Table 3), but did not possess RNase or lectin activity (data not shown). It inhibited methyl ^{3}H-thymidine incorporation by mouse splenocytes in a dose-dependent manner (Fig. 4).

Unguilin demonstrated antifungal activity against *Mycosphaerella arachidicola*, *Coprinus comatus*, and *Botrytis cinerea* (Fig. 5).

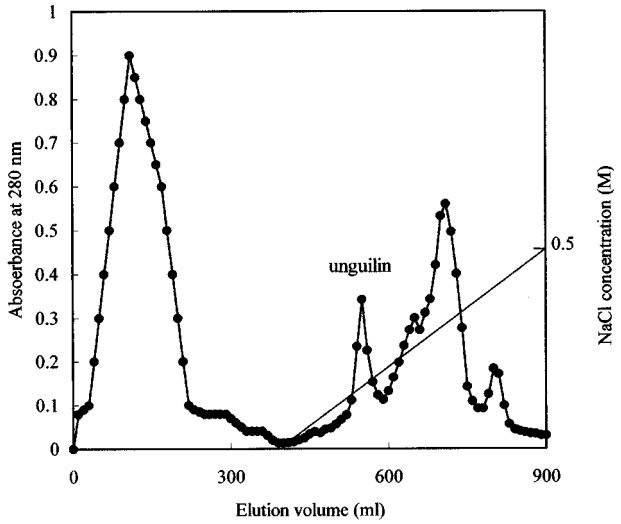


Fig. 2. Ion exchange chromatography on CM-Sepharose. Following chromatography on Affi-gel blue gel, the adsorbed fraction was dialyzed and applied to a CM-Sepharose column in 10 mM Tris–HCl buffer (pH 7.2). The column was eluted with the buffer to remove unadsorbed proteins. Adsorbed proteins were eluted with a linear concentration of NaCl from 0 to 500 mM in 10 mM Tris–HCl buffer (pH 7.2). Unguilin was eluted in the first adsorbed peak.

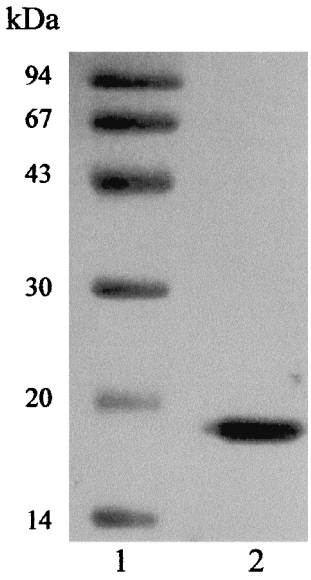


Fig. 3. SDS–polyacrylamide gel electrophoresis of unguilin. Lane 1, Pharmacia molecular weight standards (from top downward, phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibior, 20 kDa and lactalbumin, 14 kDa). Lane 2, unguilin.

4. DISCUSSION

The antifungal protein designated unguilin, isolated from seeds of the black-eyed pea, was characterized by an N-terminal sequence with resemblance to cyclophilin and the possession of inhibitory activities against HIV reverse transcriptase and glycohydrolases including α- and β-glucosidase, which are involved in HIV infection. These inhibitory activities were more potent than those of mungin (Ye and Ng, 2000) and either comparable to or stronger than those observed in other leguminous antifungal proteins (Ye *et al.*, 2000*b*). It demonstrated fungistatic action against a number of fungi including *Mycosphaerella arachidicola*, *Coprinus comatus*, and *Botrytis cinerea.*

Cyclophilin, also known as immunophilin, peptidyl-prolyl *cis–trans* isomerase, and cyclosporin A-binding protein, has been sequenced from plants, yeast, fruitflies, parasites, and the rat (Galat, 1999). Sequence homology exists among cyclophilins from these diverse organisms (Table I). Cyclophilins catalyze *cis–trans* isomerization of imide bonds in peptides and proteins. Cyclophilins may be involved in protein folding and in distant interaction between cells (Pliyev and Gurvits, 1999). The prolyl isomerase activity of cyclophilins is not essential for their immunosuppressive activity (Gothel and Marahiel, 1999). High-molecular-weight cyclophilins bind and ac-

Table I. Comparison of N-Terminal Sequence of Unguilin with Those of Cyclophilin-Like Antifungal Proteins from *Phaseolus mungo* (Mungin) and Cyclophilins[a]

	Residue		Residue	% Identity
Unguilin	1	FDMTAGPQPAGRIVFEGFADMVGRTAVN	28	100
Phaseolus mungo	7	FDMTIGGQRAKRIVFELFADTTPRTAEN	34	71
Phaseolus vulgaris	8	FDMTIGGQPAGRIVFELYADVTPRTAEN	35	71
Vicia faba	8	FDMTVGGQNAGRIIFELFADVTPRTAEN	35	67
Catharanthus roseus	8	FDMSVGGQPAGRIVMELFADTTPRTAEN	35	67
Digitalis lanata	8	FDMTIGGQPCGRIVMELYADVVPKTAEN	35	64
Trypanosoma congolense	18	FDITIGGTPAGRITFELFADAVPKTAEN	45	64
Solanum commersonii	8	FDLTIGGAPAGRVVMELFADTTPKTAEN	35	57
Mus musculus	8	FDITADDEPLGRVSFELFADKVPRTAEN	35	57
Rattus norvegicus	12	FDIAINNQPRAGRVVFELFSDVCPKTCEN	39	50
Arabidopsis thaliana	8	FDMTIDGQPAGRIVMELYTDKTPRTAEN	35	60
Saccharomyces cerevisiae	8	FDISIGGKPQGRIVFELYNDIVPKTAEN	35	53
Entamoeba histolytica	8	FDITIGGEKAGRIVMELFNDIVPKTAEN	35	57
Drosophila melanogaster	9	FDMTADNEPLGRIVMELRSDVVPKTAEN	36	57
Homo sapiens	12	FDIAINNQPAGRVVFELFSDVCPKTCEN	39	50

[a] Results of a Blast search. Residues in various cyclophilins identical to corresponding residues in unguilin are underlined.

tivate steroid receptors (Cunningham, 1999; Silverstein *et al.,* 1999). Cyclophilins promote the assembly of multiprotein complexes that often include a protein kinase or a phosphoprotein phosphatase or both (Cunningham, 1999). Gothel and Marahiel (1999) suggested the involvement of cyclophilins in protein folding, signal transduction, trafficking, assembly, and cell cycle regulation. Marivet *et al.* (1994) suggested that cyclophilins might be stress-related proteins. The demonstration of antifungal and antiviral activities in the cyclophilin-like protein from the black-eyed pea is important. Fungal and viral invasions may constitute stresses. The antimitogenic activity of unguilin may be attributed to its N-terminal sequence similarity to cyclophilins, which exhibit immunosuppressive activity (Gothel and Marahiel, 1999). It is worth nothing that ribosome-inactivating proteins displayed antimitogenic activity at similar concentrations (Wang and Ng, 2001).

Unguilin inhibited translation in a cell-free rabbit reticulocyte lysate system with a low potency. In this regard unguilin was similar to other leguminous antifungal proteins (Ye *et al.,* 2000*a*, *b*), but unlike ribosome-inactivating proteins, which potently inhibit cell-free translation (Barbieri *et al.,* 1993), although the latter may also exhibit antifungal activity (Leah *et al.,* 1991). Some lectins have been reported to display antifungal activity as well (Broekaert *et al.,* 1989). However, unguilin was devoid of lectin activity. The data indicate that unguilin is neither a ribosome-inactivating protein nor a lectin. Like other leguminous antifungal proteins, unguilin did not possess ribonuclease activity.

On the other hand, unguilin resembled ribosome-inactivating proteins (Ng *et al.,* 1992; Fong *et al.,* 1996; Lam *et al.,* 1998), antifungal thaumatin-like proteins (Ye *et al.,* 1999), chitinases (Ye *et al.,* 2000*b*), and miraculin-like antifungal protein (Ye *et al.,* 2000*a*) in that they are adsorbed on Affi-gel blue gel and CM-Sepharose. The chromatographic procedure described herein is thus useful for isolating antifungal proteins and ribosome-inactivating proteins.

Recently a cyclophilin-like protein with antifungal activity designated mungin was isolated from mung

Table II. Inhibition of HIV-Reverse Transcriptase and Glycohydrolases Caused by Unguilin at 5 mg/ml[a]

	Percentage inhibition (mean ± SD, $n = 3$)			
	HIV RT	α-Glucosidase	β-Glucosidase	β-Glucorinidase
Unguilin	84.8 ± 2.7	76.9 ± 5.2	40.8 ± 0.9	NI

[a] Enzyme activity in the absence of unguilin was taken as 100%, and % inhibition was thus 0%. RT, Reverse transcriptase. NI, No inhibition.

Table III. Inhibition of Cell-Free Translation in Rabbit Reticulocyte Lysate by Unguilin[a]

Protein	Concentration (μM)	Inhibition (%)
Unguilin	111.1	85.9 ± 0.8
	22.2	49.5 ± 0.9
	4.4	36.0 ± 1.6
	0.9	10.1 ± 1.2
	0	0

[a] Data represent means ± SD (n = 3). Cell-free translation in the absence of unguilin was taken as 100%, and % inhibition was thus 0%.

beans using the same chromatographic procedure (Ye and Ng, 2000). Unguilin differed from mungin and other cyclophilins in the absence of the N-terminal sequence PNPKVF. The antifungal activity of mungin toward *Coprinus comatus* was stronger than that of unguilin. Compared to mungin, unguilin exhibited a stronger inhibitory activity toward α-glucosidase and β-glucosidase, and a considerably more potent suppressive activity on HIV-1 reverse transcriptase. The antimitogenic activity of mungin was stronger than that of unguilin. Unguilin and mungin were similar in cell-free translation-inhibitory activity and in antifungal activity against *Mycosphaerella arachidicola* and *Botrytis cinerea* (Ye and Ng, 2000). The differences in activity between unguilin and mungin may be attributed to absence of the sequence PNPKVF from the N-terminal of unguilin and perhaps to possible differences in the remaining parts of the protein molecules. The yield of unguilin from black-eyed peas was higher than that of mungin from mung beans (Ye and Ng, 2000).

In summary, the present report furnishes corroborative evidence for the existence of a new class of antifungal

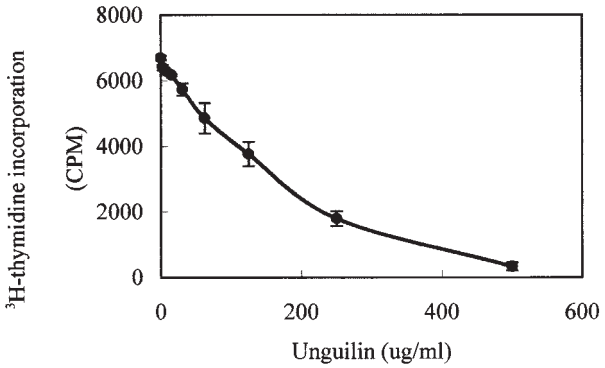
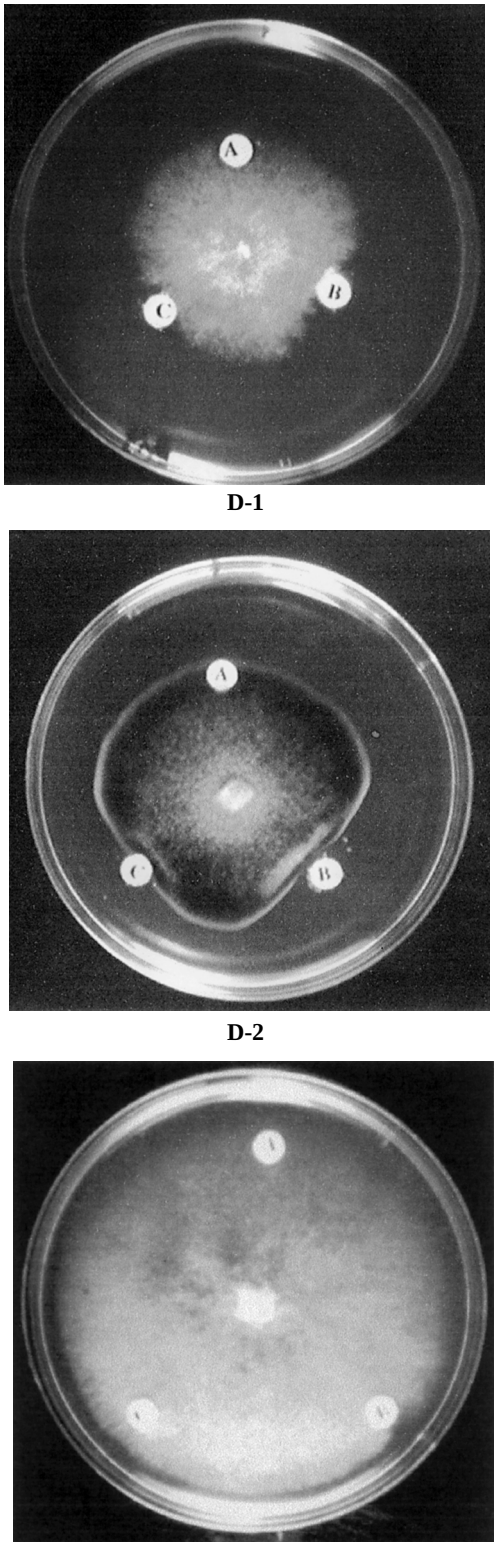


Fig. 4. Inhibitory activity of unguilin on methyl ^{3}H-thymidine incorporation by mouse splenocytes. Results represent means ± SEM of triplicate determinations.



D-1

D-2

D-3

Fig. 5. Inhibitory activity of unguilin toward (D-1) *Coprinus comatus,* (D-2) *Mycosphaerella arachidicola,* and (D-3) *Botrytis cinerea.* (A) 10 mM Tris–HCl buffer (pH 7.2) serving as negative control, (B) 300 μg of unguilin, and (C) 60 μg of unguilin.

proteins, cyclophilin-like antifungal proteins, in legume seeds. The differences in activity between cyclophilin-like proteins from mung beans and black-eyed peas may be related to structural differences at the N-terminals of the two proteins and/or the remaining parts of the protein molecules.

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