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Isolation and characterization of a novel mung bean protease inhibitor with antipathogenic and anti-proliferative activities

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ABSTRACT

A novel protease inhibitor, designated mungoin, with both antifungal and antibacterial activities, and exhibiting a molecular mass of 10 kDa in SDS-polyacrylamide gel electrophoresis, was isolated from mung bean (*Phaseolus mungo*) seeds. The isolation procedure involved a combination of extraction, ammonium sulfate precipitation, ion exchange chromatography on CM-Sephadex, and high-performance liquid chromatography (HPLC) on SP-Toyopearl. Its isoelectric point was estimated to be 9.8 by isoelectric focusing. Its N-terminal amino acid sequence was determined to be EMPGKPACLDTDDFCYKP, demonstrating some resemblance to the C-terminal sequences of other protease inhibitors and inhibitor precursors from leguminous plants. It exerted a potent inhibitory action toward a variety of fungal species including *Physalospora piricola*, *Mycosphaerella arachidicola*, *Botrytis cinerea*, *Pythium aphanidermatum*, *Sclerotium rolfsii* and *Fusarium oxysporum*, as well as an antibacterial action against *Staphylococcus aureus*. In addition, this novel plant protease inhibitor displayed anti-proliferative activity toward tumor cells.

1. Introduction

Like other plants, leguminous plants do not contain an immune system and thus are vulnerable to pathogens. Fungal infection results in serious damage to crops. Antifungal proteins have captured the attention of a large number of investigators because of their economic implications. Antifungal proteins may contribute to both defence against predators such as insects [11] as well as pathogens such as fungi [1,31]. To date, antifungal proteins with a variety of amino acid sequences have been described. Some of these proteins and peptides have been classified, based on their structures and/or functions, into groups including thaumatin-like proteins [30], chitinases [13,16], glucanases [16], embryo-abundant proteins [17], miraculin-like proteins [31], cyclophilin-like proteins [27], allergen-like proteins [32], thionins [15], and lipid transfer proteins [20]. Some are well known proteins, e.g. protease inhibitors [29], peroxidases [5,25], lectins [4], deoxyribonucleases [18], and ribosome inactivating proteins [7], and novel proteins and peptides [18,19,28,31].

Sometimes a combination of antifungal proteins is found in a single leguminous species. For example, proteins and peptides such as cicerin and arietin, thaumatin-like proteins, chitinase I, chitinase II and glucanases have been isolated from chickpea, and all inhibit fungal growth [6,19,20]. The mung bean is also very popular in the Orient. It serves as a health food because of medicinal values such as producing a cooling effect on the human body in the summer. Proteins comprising a malate dehydrogenase [24], a lectin [2], a cyclophilin-like protein [26,27], a chitinase [21], a non-specific lipid transfer protein [22,23] and a lysozyme[20] have previously been reported from mung bean. The last four proteins all exhibit antifungal activity to varying degrees.

A 12-kDa trypsin inhibitor with an amino acid sequence deduced from the cDNA sequence has recently been reported from the mung bean *Vigna radiata* var. *radiata* [9], Another

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protease inhibitor, with a full sequence of 72 amino acid residues, was earlier extracted from the same variety of mung bean [8], although information pertaining to its biological significance is lacking. We present herein a novel protease inhibitor, designated mungoin, from mung bean (*Phaseolus mungo*) seeds. It exerts both antifungal activity against a range of fungal species as well as antibacterial activity. Additionally, this novel plant protease inhibitor demonstrates anti-tumor activity. Its N-terminal amino acid sequence bears some resemblance to those of protease inhibitors and inhibitor precursors from other leguminous plants, but has not previously been reported from *P. mungo*.

2. Materials and methods

2.1. Materials

Mung bean (*P. mungo*) seeds were purchased from a local supermarket. The fungi *Physalospora piricola*, *Mycosphaerella arachidicola*, *Botrytis cinerea*, and *Fusarium oxysporum* were kindly provided by Department of Biochemistry, The Chinese University of Hong Kong, Hong Kong, China. The fungi *Fusarium solani*, *Pythium aphanidermatum* and *Sclerotium rolfsii* were obtained from the Department of Plant Pathology, Fujian Agricultural University, Fujian, China. RPMI-1640 and fetal calf serum (FCS) were purchased from Hyclone Co., USA. Bel-7402, a human liver hepatoma cell line, was obtained from Shanghai Cell Institute, China Science Academy, China.

CM-Sephadex C-50 and SP-Toyopearl were purchased from Amersham Biosciences (Sweden) and TOSOH Co. (Japan), respectively. Standard proteins for molecular mass determination were purchased from Gibco-BRL (Life Tech., USA). All chemicals were of the highest purity available.

2.2. Sample preparation

Exactly 100 g of mung bean seeds were soaked in distilled water for several hours and homogenized in 0.2 M sodium acetate buffer, pH 5.4. The homogenate was centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was designated as the crude extract for further investigations.

2.3. Isolation and purification

2.3.1. Ammonium sulfate precipitation

The crude extract was first fractionated by ammonium sulfate precipitation, in which its solution in distilled water was treated with ammonium sulfate to 20% saturation. After centrifugation (12,000 rpm, 20 min) the resulting supernatant was then adjusted to 80% saturation by addition of ammonium sulfate. After centrifugation at 12,000 rpm for 20 min, the supernatant was discarded while the precipitate was collected and dissolved in 100 ml of 0.02 M sodium acetate buffer (pH 5.4).

2.3.2. Cation-exchange chromatography

The solution of ammonium sulfate precipitate was dialyzed against 0.02 M sodium acetate buffer (pH 5.4) with several changes, and then applied to an open column of CM-Sephadex C-50 column (2.5 cm × 55 cm) previously equilibrated with the starting buffer (0.02 M sodium acetate buffer, pH 5.4). Following removal of a large amount of unadsorbed proteins, the column was eluted with a linear gradient of NaCl (200–400 mM) in the same buffer. The flow rate was 0.5 ml/min, 10 min/tube. The absorbance of the eluate was monitored at 280 nm. The second fraction (P2) was dialyzed against 0.02 M Tris–HCl buffer (pH 7.2) for 24 h, and subsequently chromatographed on a column of SP-Toyopearl (0.75 cm × 7.5 cm) which had been equilibrated with 0.02 M Tris–HCl buffer (pH 7.2). Chromatography was carried out on a BioCAD 700E perfusion chromatography workstation from PerSeptive Biosystem Co. (USA) at room temperature with a flow rate of 1 ml/min. The absorbances of all fractions were monitored at 280 nm. After elution of a sizeable quantity of unadsorbed materials, the column was eluted with a concentration gradient of NaCl (0–0.5 M) in the same buffer to yield four peaks. The last eluted peak represents mungoin, the purified mung bean protease inhibitor.

2.3.3. Capillary liquid chromatography

The purified mungoin was chromatographed on a C18 capillary reverse-phase high-performance liquid chromatography (CLC) column using an analyzer (Applied Biosystems Model ABI 140D, Perkin-Elmer Co., MA, USA).

2.4. Characterization of the purified protein

2.4.1. SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (20% T, 4% C) was performed according to the method of Laemmli and Favre [6]. Gels were stained in 0.1% (w/v) coomassie blue–30% (v/v) methanol–10% (v/v) acetic acid in water, followed by destaining using 30% (v/v) methanol–10% (v/v) acetic acid in water.

2.4.2. Protein determination

Protein concentrations were determined by the method of Lowry et al. [10] using bovine serum albumin as a standard.

2.4.3. N-terminal amino acid sequence analysis

The N-terminal amino acid sequence of the purified peptide was performed by Edman degradation using a protein sequencer (Applied Biosystems Model 476A, Perkin-Elmer Co., MA, USA). Phenylthiohydantoin derivatives were separated and identified by capillary reverse-phase high-performance liquid chromatography in a C18 column with an analyzer (Applied Biosystems Model ABI 140D, Perkin-Elmer Co., MA, USA).

2.4.4. Measurement of trypsin and chymotrypsin inhibitory activities

Each of the vials containing 0.25, 50, 75, 100, 150, 200, 250, 350 and 450 μg of the inhibitor respectively was incubated with 25 μg trypsin or chymotrypsin in 100 μl 50 mM Tris–HCl buffer (pH 8.0) containing 200 mM $CaCl_2$ for 5 min at 25 °C. After the incubation residual trypsin or chymotrypsin activity was determined by adding 300 μl 1% casein substrate at 25 °C. After 15 min incubation the reaction was terminated by adding 1 ml

cold 5% trichloroacetic acid. The reaction mixture was centrifuged for 20 min at 10,000 rpm. The absorbance of the clear supernatant was determined at 280 nm.

2.5. Assay for antibacterial activity

The assay for antibacterial activity toward *Staphylococcus aureus* and *Salmonella typhimurium* was conducted using sterile petri dishes (100 mm × 15 mm) containing 10 ml LB agar (1.5% agar). Three milliliters of warm nutrient agar (0.7%) containing the bacteria were poured into the plates. A sterile blank paper disk (0.625 cm in diameter) was placed on the agar. Then a solution of mungoin (containing a 2^0 to 2^{-10}gradient of 15 μM) in 20 mM Tris–HCl buffer (pH 7.2) was introduced to a disk. The plates were incubated at 37 °C for 12–20 h. A transparent ring around the paper disk signifies antibacterial activity.

2.6. Assay for antifungal activity

The assay for antifungal activity was executed using 100 mm × 15 mm petri plates containing 10 ml of potato dextrose agar. Around and at a distance of 1 cm away from the rim of the mycelial colony were placed sterile blank paper disks of the same size (0.625 cm in diameter). An aliquot (8 μl containing 60 or 300 μg) of mungoin in 20 mM Tris–HCl buffer (pH 7.2) was introduced to a disk. The plates were incubated at 23 °C for 72 h until mycelial growth had enveloped peripheral disks containing the control (buffer) and had produced crescents of inhibition around disks containing samples with antifungal activity. The fungal species tested included *P. piricola*, *M. arachidicola*, *B. cinerea*, *F. oxysporum*, *F. solani*, *P. aphanidermatum* and *S. rolfsii* [27].

To evaluate the protein in detail for its antifungal effect, *S. rolfsii* was taken as an example. The inhibition of hyphal growth of fungus by the purified protein was observed under a light microscope. Cell and spore suspensions (10^5 or 10^6 in 1 ml) of the fungus were prepared, and then 50 μl of cell and spore suspensions treated with the purified protein (300 μg) were added to a tube containing 1 ml medium. After incubation at 27 °C for 48 h, cells were separated from the growth medium by centrifugation, and plated for observation using an optical microscope at 100× magnification.

For a quantitative assay to determine the IC_{50} of antifungal activity, three different doses of the protein were added separately to three aliquots each containing 4 ml potato dextrose agar at 45 °C, mixed rapidly and poured into three separate small petri dishes. After the agar had cooled down, the same amount of mycelia was inoculated onto each plate. Buffer only without the protein served as a negative control. After incubation at 27 °C for 72 h, the area of the mycelial colony was measured and the inhibition of fungal growth and hence the IC_{50} value (i.e. concentration producing 50% reduction in area of mycelial colony) was determined [17].

2.7. Assay for anti-proliferative activity

The anti-proliferative activity of mungoin was carried out by testing its inhibition on the growth of the human hepatoma cell line Bel-7402. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were cultured in the flask and trypsinized for counting using a Thoma's hemoacytometer. The cell number was adjusted to 2×10^5 cells/ml by addition of the medium. The cells (7×10^5 cells/150 μl/well) were seeded into a 96-well culture plate and serial dilutions of a solution of mungoin (containing 300 μg in the first well) in 150 μl medium were added. After incubation of the cells at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h, the cells were harvested and stained with MTT. The absorbances of the samples at 590 nm were determined using a microtiter plate (ELISA) reader and were directly correlated to the level of its anti-tumor activity. The inhibitory activity of mungoin was

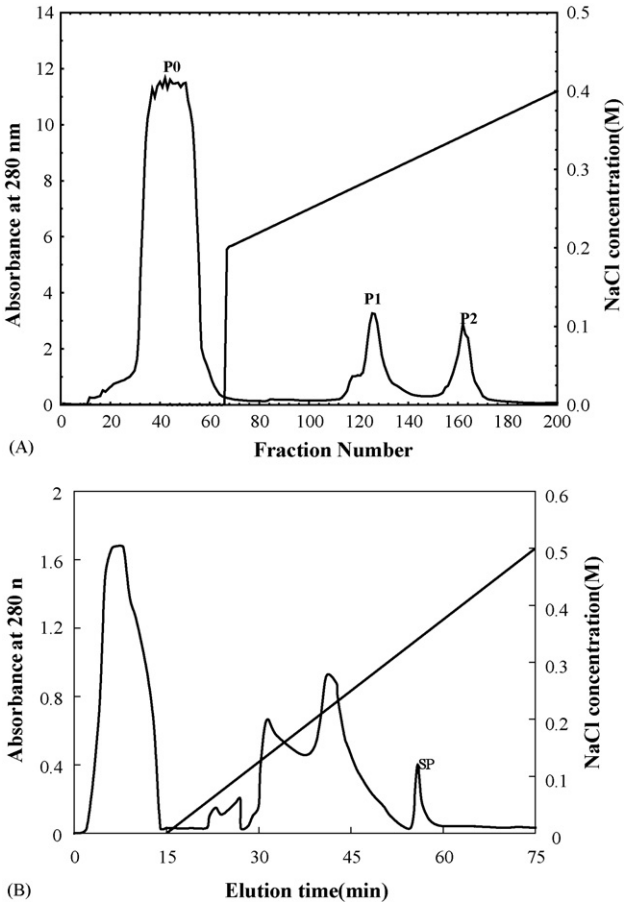

Fig. 1 – (A) Fractionation of a solution of the $(NH_4)_2SO_4$ precipitate of mung bean extract on a CM-Sephadex column equilibrated with the binding buffer (0.02 M sodium acetate buffer, pH 5.4). The gel was washed with the binding buffer and eluted with a linear gradient of 0.2–0.4 M NaCl in the same buffer. (B) Elution profile of fraction P2 from SP-Toyopearl column. The adsorbed fraction P2 from the CM-Sephadex column (P2) was pooled, dialyzed and then applied to a SP-Toyopearl column in 0.02 M Tris–HCl buffer, pH 7.2. The column was then washed with the binding buffer. Adsorbed proteins were eluted with a linear gradient of NaCl from 0 to 0.5 M in the same buffer.

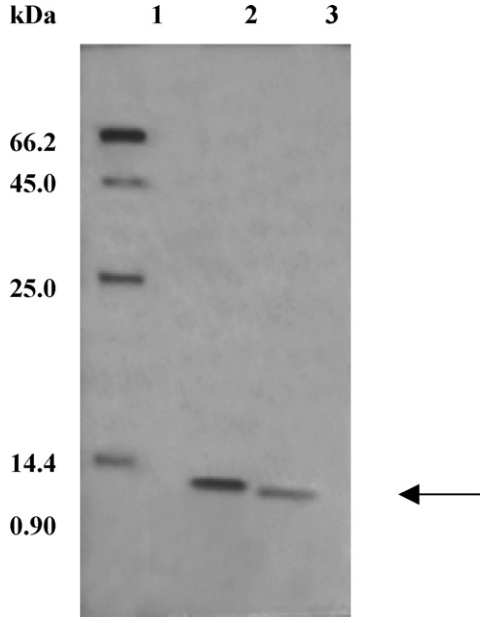

Fig. 2 – SDS-polyacrylamide gel electrophoresis of mung bean mungoin. Lanes 1 and 3 were molecular mass standards; lane 2 (sample) was loaded with 15 μg mungoin. The arrow indicates the position of mungoin band corresponding to a molecular mass of 10 kDa.

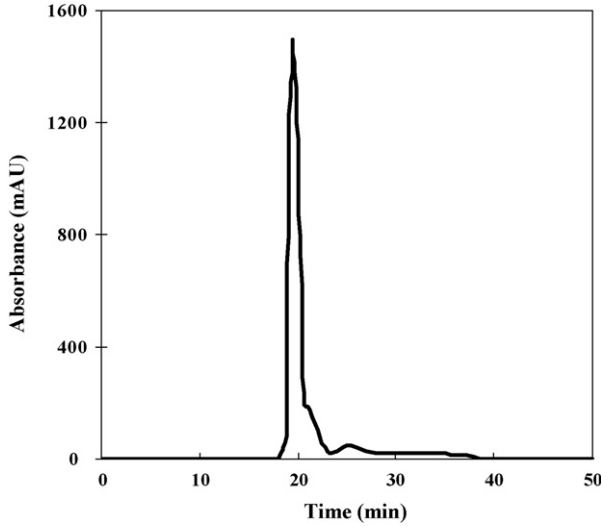

Fig. 3 – Capillary reverse-phase high-performance liquid chromatography of the purified mungoin using a C18 column.

calculated as percent inhibition compared to a control without the sample. All reported values are the means of triplicate samples.

3. Results

3.1. *Purification of mungoin*

The solution of the ammonium sulfate precipitate was applied to an open column of CM-Sephadex. Following removal of a large amount of unadsorbed proteins, the second adsorbed fraction (P2) exhibiting antifungal and antibacterial activities was desorbed from the column with a linear NaCl concentration gradient (Fig. 1A). The active peak was pooled, dialyzed against 0.02 M Tris–HCl buffer (pH 7.2) for 24 h, and chromatographed on an SP-Toyopearl column. The last adsorbed peak (SP peak) from the column possessed both antifungal activity and antibacterial activities (Fig. 1B). Its SDS-PAGE pattern is shown in Fig. 2. The purified protein designated as mungoin was shown by capillary reverse-phase high-performance liquid chromatography (Fig. 3) to be of high purity.

The protein yields at each purification step are presented in Table 1. Six milligrams of purified mungoin were obtained from 100 g mung bean seeds.

3.2. *N-terminal amino acid sequence*

The N-terminal amino acid sequence of the purified mung bean mungoin was determined to be EMPGKPACLDTDDF-CYKP. This demonstrated some resemblance (between 45% and 83%) to protease inhibitors and inhibitor precursors from other leguminous plants (Table 2).

3.3. *Properties of mungoin*

3.3.1. *Molecular mass and isoelectric point*

The molecular mass of mungoin was estimated by SDS-polyacrylamide gel electrophoresis to be 10 kDa. Its isoelectric point (p*I*), which was calculated to be 9.8 by isoelectric focusing, demonstrated it to be a typical basic protein (Fig. 4). This in turn supported its efficient purification through cation-exchange chromatography in two buffer systems at pH 5.4 and pH 7.2, respectively.

3.4. *Antibacterial activity*

The mung bean mungoin exerted antibacterial activity toward the Gram-positive bacterium *S. aureus* (Table 3). Antibacterial activity was demonstrated over the concentration gradient $2^0 \times 15$ to $2^{-6} \times 15$ μM; the MIC to *S. aureus* was 0.23 μM. However, it had no effect on Gram-negative bacteria such as *Salmonella* (data not shown).

Table 1 – Summary of purification of mungoin from *Phaseolus mungo*

Fraction	Protein (mg) from 100 g *P. mungo* seeds
Crude extract	4176.9
Ammonium sulfate precipitation	903.5
CM-Sephadex	246.0
SP-Toyopearl	6.0

Table 2 – Comparison of N-terminal sequence of mungoin with protease inhibitors from different beans

Source	Name	Residue number	N-terminal sequence	Residue number	Identity (%)	Length
P. mungo	Mungoin	1	EMPGKPACLDTDDFCYKP	18	100	
Vigna radiata var. *radiata*	Trypsin inhibitor	80	SMPGKCRCLDTDDFCYKP	97	83	106
V. radiata var. *radiata*	Protease inhibitor	48	SMPGKCRCLDTDDFCYKP	65	83	72
Glycine max	Protease inhibitor	57	SMPGQCRCLDTTDFCYKP	74	72	83
Glycine max	Protease inhibitor	81	RSQPGQCRCLDTNDFCYKP	96	61	102
Vigna unguiculata	Trypsin inhibitor	83	RSTPGKCRCLDIADFCYKP	98	56	99
Canavalia lineata	Protease inhibitor	51	LSFPAKCRCVDTTDFCYKP	66	45	75

Residue number 1 and residue number 18 for mung bean mungoin refer to E and P being the 1st and 18th amino acid residue in mungoin, respectively. Underlined characters and indicate identical amino acids with the purified mungoin from the mung bean (*P. mungo*). Length = total number of amino acids in protein.

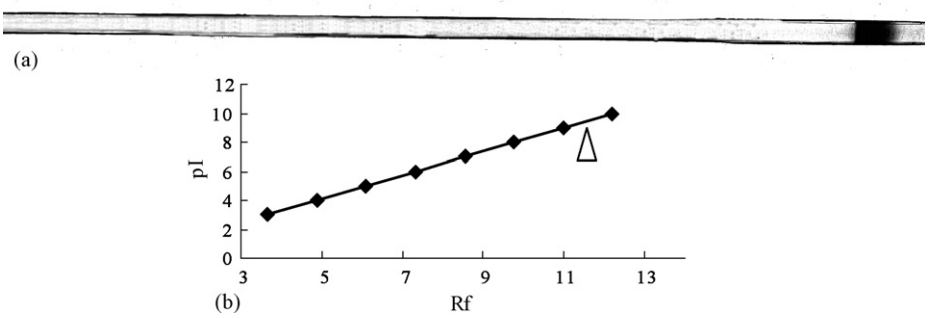

Fig. 4 – (A) Isoelectric focusing results of mung bean mungoin. (B) A plot of isoelectric point of standard proteins versus migration rates. (Triangle represents mungoin migration rate.)

3.5. *Antifungal activity*

The antifungal activity of mung bean mungoin against five fungal species is illustrated in Fig. 5I–VI. It can be seen that the protein showed strong antifungal activity toward *P. piricola* (Fig. 5I), *M. arachidicola* (Fig. 5II), *B. cinerea* (Fig. 5III) and *P. aphanidermatum* (Fig. 5IV), and obvious antifungal activity toward *S. rolfsii* (Fig. 5V) and *F. oxysporum* (Fig. 5VI). The IC_{50} value of the antifungal activity toward *S. rolfsii* was calculated to be 6.2 μM (Fig. 6). However, it had hardly any antifungal effect on *F. solani* (data not shown).

Light microscopic examination disclosed mungoin-induced distortion of the fungi. Photographs revealed morphological alterations in hyphae and scanty hyphae in those fungi growing in the presence of mungoin (Fig. 7B) as compared with growth in control medium showing normal hyphal development (Fig. 7A). It could be surmised that the antipathogenic activity of purified protein was due to its corrosion of the cellular wall of the pathogen resulting in cytoplasmic leakage.

Table 3 – The antibacterial gradient of mungoin to *Staphylococcus aureus*

Sample concentration gradient (×15 μM)	Antibacterial effect
2^{0}	+
2^{-1}	+
2^{-2}	+
2^{-3}	+
2^{-4}	+
2^{-5}	+
2^{-6}	+
2^{-7}	–
2^{-8}	–
2^{-9}	–
2^{-10}	–

(+) present; (–) lacking.

3.6. *Anti-proliferative activity*

The anti-proliferative activity of mungoin on human hepatoma cells Bel-7402 was calculated as percent inhibition compared to a control without mungoin. All reported values are the means of triplicate samples. The results are shown in Fig. 8. The IC_{50} value toward Bel-7402 cells was calculated to be 176 μM.

3.7. *Trypsin or chymotrypsin inhibitory activities*

Mungoin inhibited trypsin or chymotrypsin activity. When the molar ratio of inhibitor to chymotrypsin attained 3, the enzyme activity was inhibited by slightly more than 50%. However, the inhibition of trypsin was somewhat strong. When the molar ratio of mungoin to trypsin was 10, enzyme activity was indiscernible.

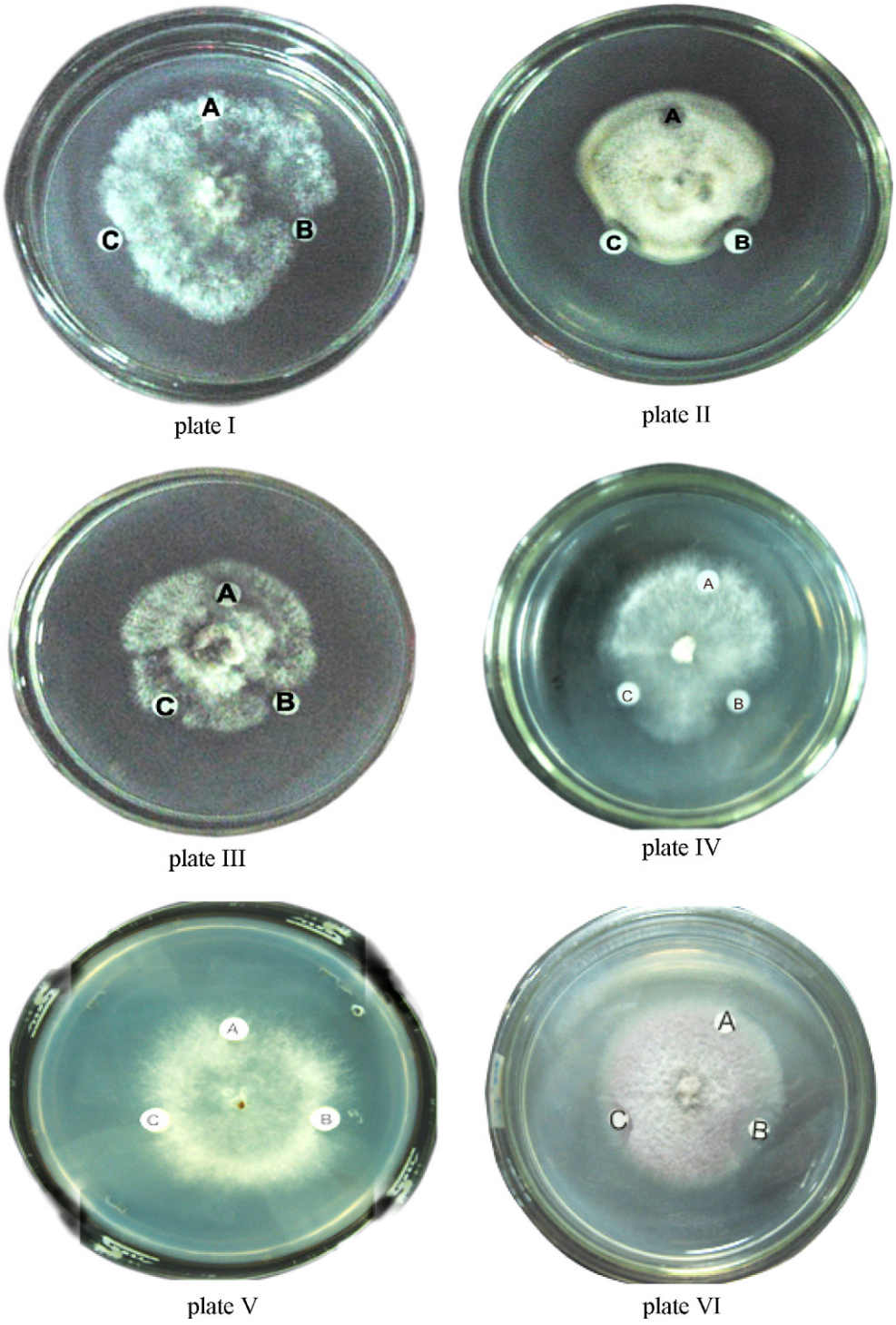

Fig. 5 – (I–VI) Inhibitory activity of mung bean mungoin toward plate (I) *Physalospora piricola*, plate (II) *Mycosphaerella arachidicola*, plate (III) *Botrytis cinerea*, plate (IV) *Pythium aphanidermatum*, plate (V) *Sclerotium rolfsii*, and plate (VI) *Fusarium oxysporum*. Disk (A) 0.02 M Tris–HCl buffer, pH 7.2, disk (B) 300 μg mung bean mungoin, and disk (C) 60 μg mung bean mungoin.

4. Discussion

A novel protease inhibitor, designated mungoin, has been isolated from mung bean seeds. The observation that the purified protein showed antifungal activity toward different fungal species suggests an important role of this purified protein in constitutive host defense mechanisms against microbial pathogens. This may contribute to the development of biological control of fungal pathogens harmful to crops. Interestingly, a lysozyme [20], a non-specific lipid

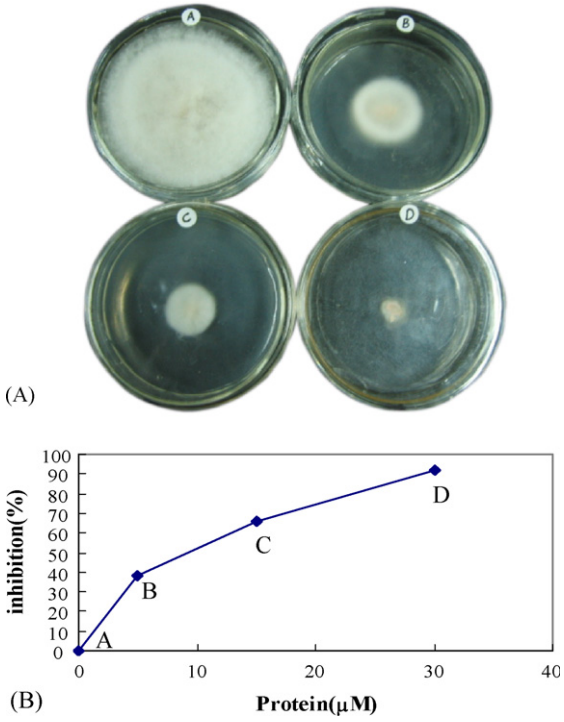

Fig. 6 – Determination of the IC_{50} value of the antifungal activity of mungoin toward *S. rolfsii*. Plate A represents the control and plates B–D represent treatments with 5, 15, and 30 μM mungoin, respectively. The IC_{50} was calculated to be 6.2 μM.

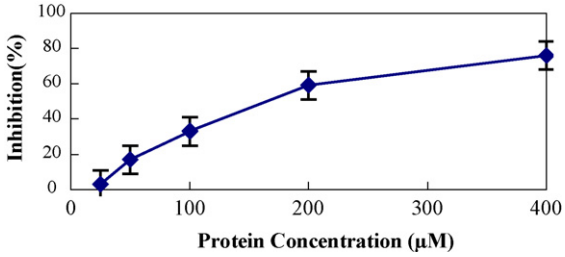

Fig. 8 – Inhibitory activity of mung bean mungoin toward BEL-7402 tumor cells. Each data point represents mean ± S.D. of triplicate determinations.

transfer protein [22] and a chitinase [21] have previously been reported from mung bean, and all exhibited antifungal effects on *F. solani* but there was no effect on *P. piricola*. On the contrary, mungoin exhibited its own characteristic antifungal spectrum, with particularly strong antifungal activity toward *P. piricola* as well as *M. arachidicola*, *B. cinerea* and *P. aphanidermatum*. It may be reasonable to deduce that a combination of antifungal proteins is present in the mung bean, and that they work together to defend against attack from pathogens such as fungi. Although plants do not have an immune system, plants have evolved a variety of potent defense mechanisms, including the synthesis of low-molecular-weight compounds, proteins, and peptides that have antifungal activity.

Mungoin possessed anti-proliferative and antibacterial activities. It is known that some antifungal proteins demonstrate in addition antibacterial activities [20,22]. However, protease inhibitors may display antitumor [3,12] and anti-proliferative activities, but rarely antibacterial activity. The aforementioned activities shown by mungoin, a protease inhibitor, provide further evidence for the therapeutic potential of this class of antifungal proteins.

It is noteworthy that the N-terminal sequence of mungoin resembles the C-terminal sequence of other protease inhibitors, probably due to the different varieties/cultivars used in different studies. This is reminiscent of a similar finding on straw mushroom lectin compared with a previously reported straw mushroom lectin [14], in which the N-terminal sequence of the former lectin was similar to the C-terminal sequence of latter lectin isolated earlier.

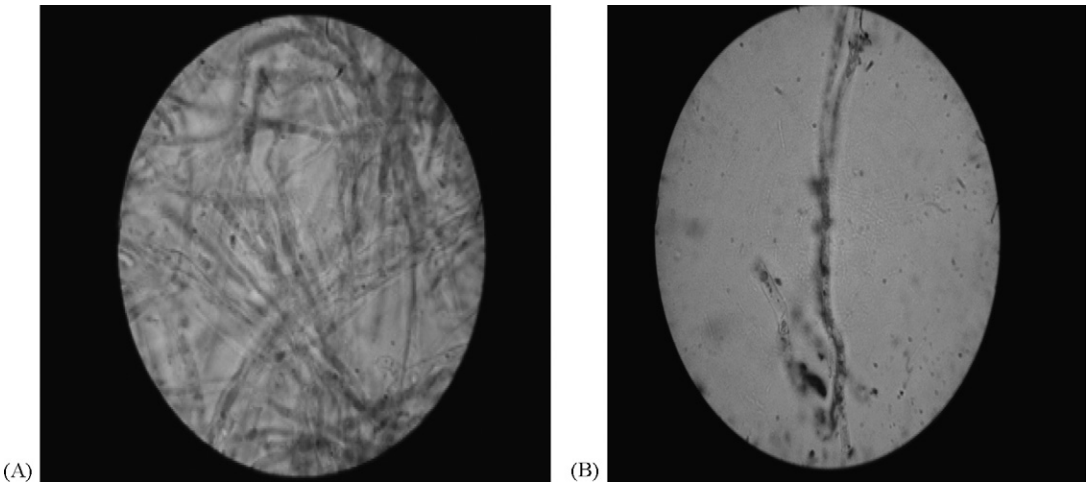

Fig. 7 – Light microscopic observation on inhibition of hyphal growth in *S. rolfsii* by purified mung bean mungoin. (A) Mycelia of fungus after 48 h of growth in the absence of mung bean mungoin and (B) mycelia of fungus after 48 h of growth, in the presence of 300 μg mung bean mungoin.

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REFERENCES

[1] Caruso C, Capovale C, Chilosi G, Vacca F, Bertini L, Magro P. Structural and antifungal properties of a pathogenesis-related protein from wheat kernel. J Protein Chem 1996;15:35–344.
[2] Del Campillo E, Shannon LM, Hankins CN. Molecular properties of the enzymic phytohemagglutinin of mung bean. J Biol Chem 1981;256:7177–80.
[3] Kennedy AR, Bilings PC, Maki PA, Newberne P. Effects of various preparations of dietary protease inhibitors on oral carcinogenesis in hamsters induced by DMBA. Nutr Cancer 1993;19:191–200.
[4] Kortt AA, Caldwell JB. Isolation and properties of the lectins from tuberous roots of winged bean *Psophocarpus tetragonolobus* (L) DC. J Sci Food Agric 1987;39:47–57.
[5] Kristensen BK, Bloch H, Rasmuseen SK. Barley coleoptile peroxidases. Purification, molecular cloning and induction by pathogens. Plant Physiol 1999;120:501–12.
[6] Laemmli UK, Favre M. Gel electrophoresis of proteins. J Mol Biol 1973;80:575–99.
[7] Lam SSL, Wang HX, Ng TB. Purification and characterization of novel ribosome inactivating proteins, alpha- and beta-pisavins, from seeds of the garden pea. *Pisum sativum*. Biochem Biophys Res Commun 1998;253:135–42.
[8] Li Y, Huang Q, Zhang S, Liu S, Chi C, Tang Y. Studies on an artificial trypsin inhibitor peptide derived from the mung bean trypsin inhibitor: chemical synthesis, refolding, and crystallographic analysis of its complex with trypsin. J Biochem 1994;116:18–25.
[9] Liu Z, Fei H, Chi C. cDNA of mung bean (*Vigna radiata* var. *radiata*) proteinase inhibitor. Swiss-Prot entry accession no. AAP40784.1; 2004.
[10] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[11] Murdock LL, Huesing JE, Nielsen SS, Prat RC, Shade RE. Biological effects of plant lectins on the cowpea weevil. Phytochemistry 1990;29:85–8.
[12] Sammon AM. Protease inhibitors and carcinoma of the esophagus. Cancer 1998;83:405–8.
[13] Schlumbaum A, Mauch F, Vogeli U. Plant chitinases are potent inhibitors of fungal growth. Nature 1986;324:355–6.
[14] She QB, Ng TB, Liu WK. A novel lectin with potent immunomodulatory activity isolated from both fruiting bodies and cultured mycelia of the edible mushroom *Volvariella volvacea*. Biochem Biophys Res Commun 1998;247:106–11.
[15] Terras FRG, Schoofs HME, Thevissen K, Osborn RW, Vanderleyden J, Cammue BRA, et al. Synergistic enhancement of the antifungal activity of wheat thionins by radish and oilseed rape 2S albumins and by barly trypsin inhibitor. Plant Pathol 1993;103:1311–9.
[16] Vogelsang R, Barz W. Purification, characterization and differential hormonal regulation of a β-1,3-glucanase and two chitinases from chickpea (*Cicer arietinum* L.). Planta 1993;189:60–9.
[17] Wang HX, Ng TB. Ginkbilobin, a novel antifungal protein from *Ginkgo biloba* seeds with sequence similarity to embryo-abundant protein. Biochem Biophys Res Commun 2000;279:407–11.
[18] Wang HX, Ng TB. Isolation of a novel deoxyribonuclease with antifungal activity from *Asparagus officinalis* seeds. Biochem Biophys Res Commun 2001;289:120–4.
[19] Wang HX, Ng TB. Purification of chrysancorin, a novel antifungal protein with mitogenic activity from garland chrysanthemum seeds. Biol Chem 2001;382:947–51.
[20] Wang SY, Ng TB, Chen T, Lin DUJH, Rao PF, Ye XY. First report of a novel plant lysozyme with both antifungal and antibacterial activities from *Phaseolus mungo*. Biochem Biophys Res Commun 2005;327:820–7.
[21] Wang SY, Wu JH, Rao PF, Ye XY, Ng TB. A chitinase with antifungal activity from the mung bean. Protein Exp Purif 2005;40:230–6.
[22] Wang SY, Wu JH, Ye XY, Ng TB, Rao PF. A non-specific lipid transfer protein with antifungal and antibacterial activities from the mung bean. Peptides 2004;25:1235–42.
[23] Wang SY, Wu JH, Ye XY, Ng TB, Rao PF. Crystallization and preliminary X-ray crystallographic analysis of a non-specific lipid-transfer protein with antipathogenic activity from *Phaseolus mungo*. Acta Crystallogr Section D Biol Crystallogr 2004;60:2391–3.
[24] Wang SY, Xu ZB, Ye XY, Rao PF. Purification and characterization of a malate dehydrogenase from *Phaseolus mungo*. J Food Biochem 2005;29:117–31.
[25] Ye XY, Ng TB. Isolation of a novel peroxidase from French bean legumes and first demonstration of antifungal activity of a non-milk peroxidase. Life Sci 2002;71:1667–80.
[26] Ye XY, Ng TB. Isolation of a new cyclophilin-like protein from chickpeas with mitogenic, antifungal and anti-HIV-1 reverse transcriptase activities. Life Sci 2002;70:1129–38.
[27] Ye XY, Ng TB. Mungin, a novel cyclophilin-like antifungal protein from the mung bean. Biochem Biophys Res Commun 2000;273:1111–5.
[28] Ye XY, Ng TB, Rao PF. Cicerin and arietin, novel antifungal proteins from chickpea. Peptides 2002;23:817–22.
[29] Ye XY, Ng TB, Rao PF. A Bowman-Birk-type trypsin-chymotrypsin inhibitor from broad beans. Biochem Biophys Res Commun 2001;289:91–6.
[30] Ye XY, Wang HX, Ng TB. First chromatographic isolation of an antifungal thaumatin-like protein from French bean legumes and demonstration of its antifungal activity. Biochem Biophys Res Commun 1999;263:130–4.
[31] Ye XY, Wang HX, Ng TB. Sativin, a novel antifungal miraculin-like protein isolated from legumes of the sugar snap *Pisum sativum* var. *macrocarpon*. Life Sci 2000;67:775–81.
[32] Ye XY, Ng TB. Hypogin, a novel antifungal peptide from peanuts with sequence similarity to peanut allergen. J Peptide Res 2000;57:330–6.