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Hypotin, a Novel Antipathogenic and Antiproliferative Protein from Peanuts with a Sequence Similar to Those of Chitinase Precursors

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A protein designated *Hypotin,* with both antifungal and antibacterial activity, was isolated from peanut (*Arachis hypogaea*) seeds. The isolation procedure included extraction, ammonium sulfate precipitation, affinity chromatography on Affi-gel blue gel, ion chromatography, and gel filtration. The protein exhibited a molecular mass of 30.4 kDa in SDS–polyacrylamide gel electrophoresis under both reducing and nonreducing conditions, indicating that it is a monomeric protein. Its N-terminal sequence was highly homologous to those of chitinases and chitinase precursors from plants. It exerted potent antifungal action toward a variety of fungal species, including *Pythium aphanidermatum*, *Fusarium solani*, *Physalospora piricola*, *Alternaria alternata*, *Botrytis cinerea*, and *Fusarium oxysporum*. In addition, this novel protein exhibited antiproliferative activity against tumor cells. These findings further the progress in the research of leguminous plants.

KEYWORDS: Peanut; antifungal; antibacterial; antiproliferative

INTRODUCTION

Plants are an important and indispensable part of life. Floristic analyses indicate that approximately 250000–500000 plant species currently exist (*1*, *2*). Plants have evolved a variety of potent defense mechanisms because they are exposed to a large number of pathogenic organisms. Although they do not have an immune system, they synthesize low-molecular weight compounds, proteins, and peptides that have antifungal activity. As their names imply, antifungal proteins and peptides serve a protective function against fungal invasion and play an important role in the defense of crops against fungal attack, thus diminishing serious economic losses. Consequently, plants have been one of the hot research topics in the search for potent antifungals. Antifungal proteins have captured the attention of a large number of investigators because of their economic implications. Transgenic plants expressing antifungal proteins are known to have increased resistance to fungal diseases.

Antifungal proteins have been purified from a large number of leguminous species, and the seeds of leguminous plants produce an array of proteins and peptides that inhibit fungal growth. They are structurally diverse and comprise glucanases (*3*, *4*), chitinases (*4*), chitinase-like proteins (*5*, *6*), chitin-binding proteins (*7*–*9*), allergen-like peptides (*10*), cyclophilin-like proteins (*11*), miraculin-like proteins (*12*), lipid transfer protein-like proteins (*13*, *14*), thaumatin-like proteins (*15*, *16*), peroxidases (*17*), defensins and defensin-like proteins (*18*–*21*), lectins/hemagglutinins (*22*–*24*), ribosome-inactivating proteins (*25*, *26*), protease inhibitors (*27*–*30*), and novel proteins and peptides (*31*–*33*). Research continues to uncover novel antifungal plant-produced peptides and proteins. Moreover, novel ones with both antifungal activity and other significant bioactivities, such as antibacterial and antiproliferative activity, will make them potential candidate for agriculture, medicine, and food safety.

Peanuts are extensively cultivated worldwide and serve as a healthy food because of abundant proteins and essential amino acids. They are used in cakes and milk and as a functional porridge. Because leguminous plants may produce some proteins with functions apart from antifungal activity, we undertook this investigation to find new antifungal proteins demonstrating diverse bioactivity. Subsequently, we present a novel protein designated *Hypotin,* isolated from peanut (*Arachis hypogaea*) seeds. Its N-terminal sequence is highly homologous to those of chitinases and chitinase precursors from other plants. It both exerts antifungal activity against a range of fungal species and has antibacterial activity. Furthermore, it exhibited antiproliferative activity against tumor cells.

MATERIALS AND METHODS

Materials. Peanut (*A. hypogaea*) seeds were purchased from a local supermarket, which were planted in Zhaoyuan farm, Shandong Province, China, and ranked as the first market class. The fungi *Alternaria alternata*, *Botrytis cinerea*, and *Fusarium oxysporum* were kindly provided by the Department of Biochemistry of The Chinese University of Hong Kong (Hong Kong, China). The fungi *Fusarium solani*, *Pythium aphanidermatum*, and *Sclerotium rolfsii* and the bacteria *Staphylococcus aureus* and *Salmonella* were obtained from the Department of Plant Pathology of Fujian Agricultual University (Fujian, China). RPMI-1640 and fetal calf serum (FCS) were purchased from

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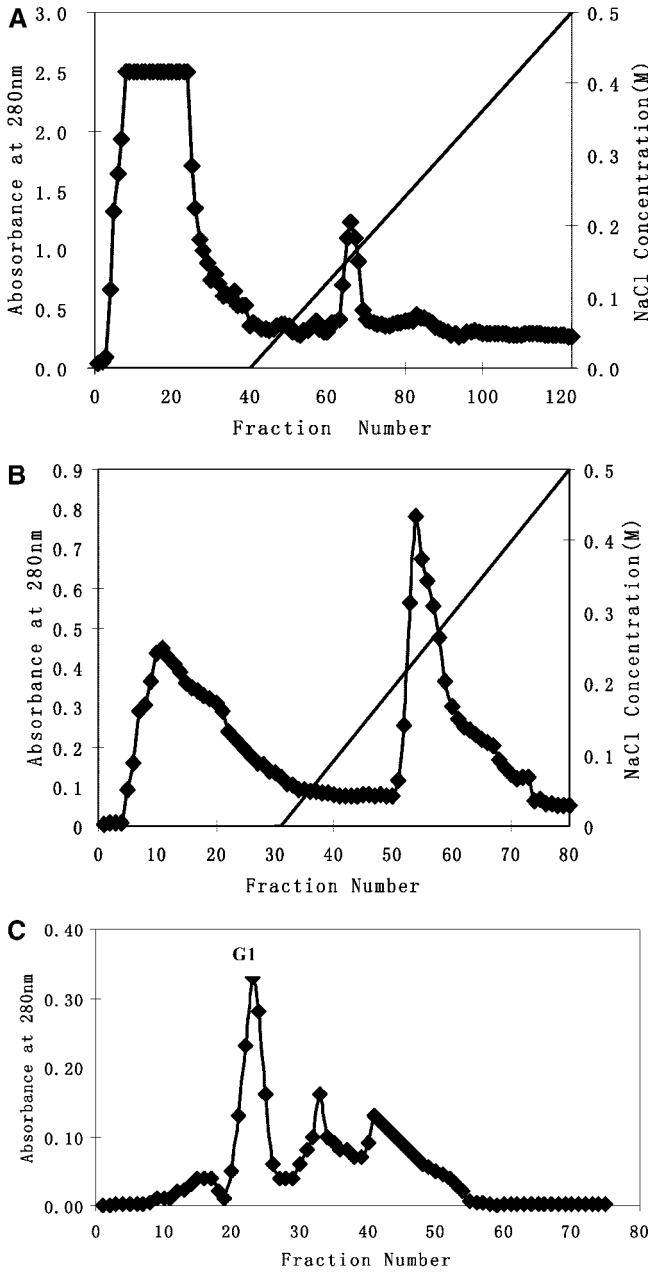

Figure 1. (**A**) Fractionation of a solution of the $(NH_4)_2SO_4$ precipitate extract on an Affi-gel blue gel column equilibrated with the binding buffer [0.01 M Tris-HCl buffer (pH 7.2)]. The gel was washed with the binding buffer and eluted with a linear gradient from 0 to 0.5 M NaCl in the same buffer. (**B**) Elution profile of the fraction from the CM-Sephadex column. The adsorbed fraction from the Affi-gel blue gel column was pooled, dialyzed, and applied to the CM-Sephadex column in 0.01 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. (**C**) The adsorbed fraction with antifungal activity from CM-Sephadex column chromatography was pooled, concentrated, and applied to a Sephadex G-75 column. Protein elution was carried out with 0.01 M Tris-HCl buffer (pH 7.2).

Hyclone Co. The Bel-7402 cell line, a human liver hepatoma cell line, was obtained from the Shanghai Cell Institute of the China Science Academy (Shanghai, China).

Affi-gel blue gel, CM-Sephadex C-50, and Sephadex G-75 were purchased from Bio-Rad, Amersham Biosciences (Uppsala, Sweden), and TOSOH Co., respectively. Standard proteins for molecular mass determination were purchased from Gibco-BRL. All chemicals were of the highest available purity.

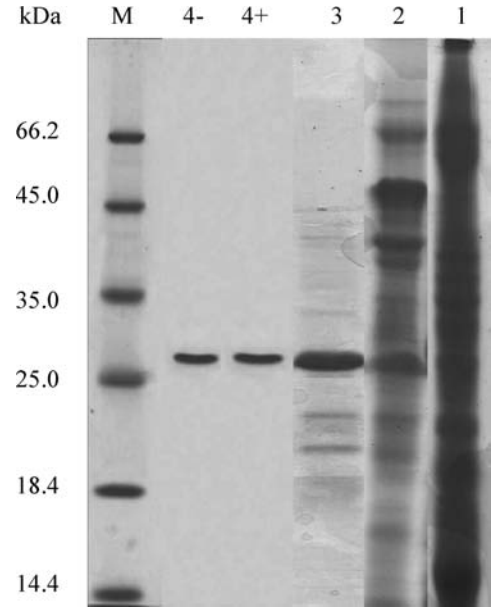

Figure 2. SDS–polyacrylamide gel electrophoresis of *Hypotin*: lane M, molecular mass standards; lane 4–, 12 μg of *Hypotin* from the Sephadex G-75 column under nonreducing conditions (without addition of dithiothreitol); lane 4+, 12 μg of *Hypotin* under reducing conditions (with dithiothreitol added); lane 3, 18 μg adsorbed peak on CM-Sepheadex; lane 2, 30 μg adsorbed peak on Affi-gel blue gel; lane 1, 46 μg of $(NH_4)_2SO_4$ precipitate extract.

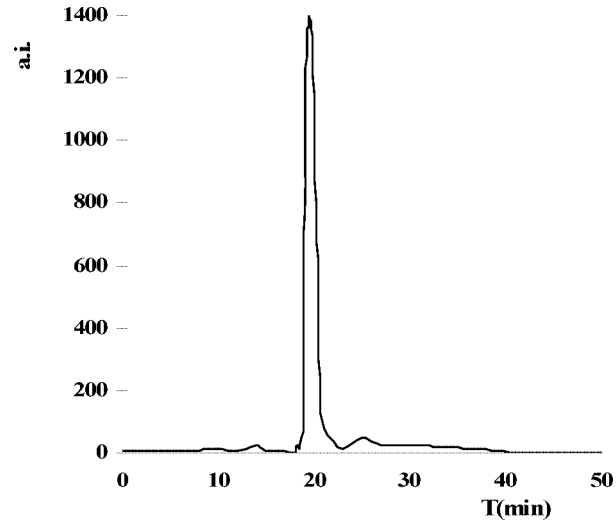

Figure 3. Capillary reversed-phase high-performance liquid chromatography of purified *Hypotin* using a C18 column.

Table 1. Summary of Purification of *Hypotin* from *A. hypogaea*

fraction	protein[a] (mg)
crude extract	16350.3
ammonium sulfate precipitation	4986.6
Affi-gel blue gel	200.5
CM-Sephadex	30.3
Sephadex G-75	5.9

[a] Protein obtained from 100 g of *A. hypogaea* seeds.

Sample Preparation. Exactly 100 g of peanut seeds was soaked in 800 mL of distilled water for several hours and homogenized in 1000 mL of 0.1 M Tris-HCl buffer (pH 7.2) for 30 min using an electronic homogenizer (Philips Corp., Shanghai, China). The homogenate was centrifuged at 12000 rpm for 20 min at 4 °C. The supernatant was designated as the crude extract for the further investigations.

Table 2. Comparison of the N-Terminal Sequence of Peanut Antifungal Protein *Hypotin* with Those of Chitinase Precursors from Other Plants[a]

Source	Name	Residue number		Residue number	% Identity
Arachis hypogaea	*Hypotin*	1	CDVGSVISASLFEALQKHRN	20	100
Canavalia ensiformis	chitinase precursor	28	DDVGSVIDASLFDQLLKHRN	47	75
Canavalia ensiformis	Chitinase Chain A	1	DVGSVIDASLFDQLLKHRND	20	75
Musa acuminata	putative chitinase	74	GSVGSIISSSLFEQMLKHRN	93	65
Castanea sativa	chitinase Ib	75	GDVGSLISASLFDQMLKYRN	94	65
petroselinum crispum	chitinase precursor	24	QDVGSLISKAMFEDMLKHRN	43	60
Humulus lupulus	endochitinase precursor	74	GDVSSVISSALFEEMLKHRN	93	60
Nicotiana tabacum	basic chitinase	77	CDLGSIISSSMFDQMLKHRN	96	60
Solanum tuberosum	endochitinase 4 precursor	76	GDIGSVISNSMFDQMLKHRN	95	60
Solanum dulcamara	chitinase-like thermal hysteresis protein	15	ADLGSVISNSMFDQMLKHRN	34	60
Nicotiana tabacum	basic chitinase	77	CDLGSIISSSMFDQMLKHRN	96	60
Nicotiana tabacum	endochitinase B precursor	74	GDLGSIISSSMFDQMLKHRN	92	55
Nicotiana tabacum	endochitinase A precursor	78	GDLGSIISSSMFDQMLKHRN	97	55
Nicotiana tabacum	endochitinase 3 precursor	83	GDISNIISSSMFDQMLKHRN	102	45

[a] Residue number 1 and residue number 20 for peanut *Hypotin* refer to C and N being the first and twentieth amino acid residue in *Hypotin*, respectively. Underlined characters are amino acids that are identical with those of the purified *Hypotin* from peanut (*A. hypogaea*).

Isolation and Purification. *Ammonium Sulfate Precipitation.* The crude sample was first fractionated by ammonium sulfate precipitation, in which the crude solution was treated with ammonium sulfate to 20% saturation. After centrifugation at 12000 rpm for 20 min, the resulting supernatant was then adjusted to 80% saturated ammonium sulfate. The supernatant from the same centrifugation was discarded while the precipitate was collected and dissolved in 100 mL of 0.01 M Tris-HCl buffer (pH 7.2).

Affinity Chromatography. The crude solution was dialyzed against distilled water, and then Tris-HCl buffer (pH 7.2) was added until the final concentration of Tris-HCl in the crude extract was 0.01 M. The crude extract was applied to an open column of Affi-gel blue gel (2.5 cm × 10 cm) previously equilibrated with the starting buffer, 0.01 M Tris-HCl buffer (pH 7.2). Following removal of a large amount of unadsorbed proteins, the column was eluted with a linear gradient of NaCl (from 0 to 0.5 M) in the same buffer. The flow rate was 1.0 mL/min (5 min/tube), and the eluate was monitored at 280 nm.

Cation-Exchange Chromatography. The adsorbed fraction demonstrating antifungal activity was pooled, dialyzed against 0.01 M Tris-HCl buffer (pH 7. 2) at 4 °C for 24 h with several changes, and subsequently applied to an open column of CM-Sephadex C-50 (2.5 cm × 55 cm) previously equilibrated with the starting buffer [0.01 M Tris-HCl (pH 7.2)]. The flow rate was 0.5 mL/min (10 min/tube). After elution of a sizeable quantity of unadsorbed material, the column was eluted with a gradient of NaCl (from 0 to 0.5 M) in the same buffer.

Gel Filtration. The adsorbed fraction from the CM-Sephadex C-50 column was pooled, dialyzed against 0. 01 M Tris-HCl buffer (pH 7. 2) at 4 °C for 24 h with several changes, concentrated using a vacuum concentration machine (Shanghai Machinery Co.) at a low temperature for 2 h, and then applied to the Sephadex G-75 column (2 cm × 100 cm) previously equilibrated with the starting buffer. The flow rate was 0.3 mL/min, and the eluate was monitored at 280 nm. The antifungal activity of each fraction was determined. Fraction G1 represents *Hypotin*, the purified peanut antifungal protein.

Capillary Liquid Chromatography. Purified *Hypotin* was chromatographed on a C18 capillary reversed-phase high-performance liquid chromatography (CLC) column using an analyzer (Applied Biosystems model ABI 140D, Perkin Elmer Co.).

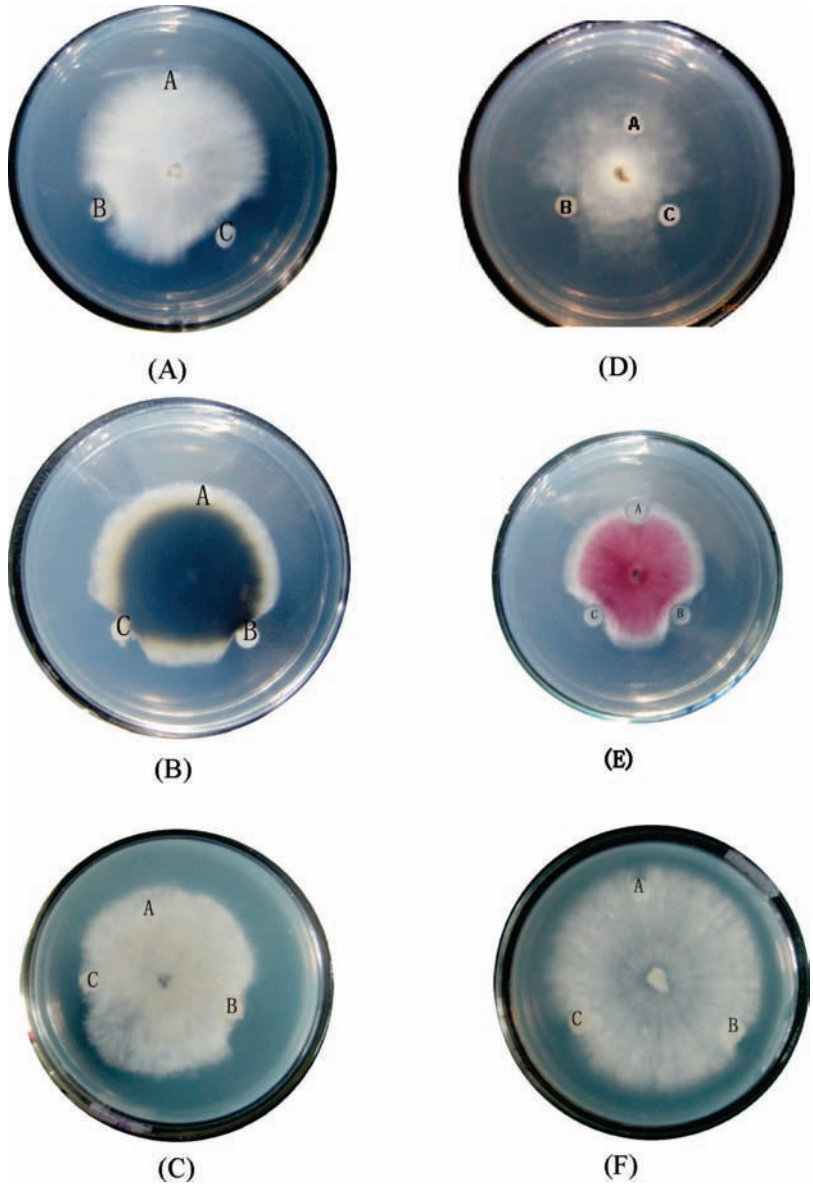

Figure 4. (**A–F**) Inhibitory activity of peanut *Hypotin* against *B. cinerea*, *A. alternata*, *Sc. rolfsii*, *F. solani*, *F. oxysporum*, and *P. aphanidermatum*, respectively: (spot A) 0.01 M Tris-HCl buffer (pH 7.2), (spot B) 60 μg of peanut *Hypotin*, and (spot C) 300 μg of peanut *Hypotin*.

Characterization of the Purified Protein. *SDS–PAGE.* Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (12.5% T, 4% C) was performed according to the method of Laemmli and Favre (*34*). Gels were stained in 0.1% (w/v) Coomassie blue, 30% (v/v) methanol, and 10% (v/v) acetic acid in water. The destaining solution was 30% (v/v) methanol and 10% (v/v) acetic acid in water.

Protein Determination. Protein concentrations were determined by the method of Lowry et al. (*35*) using bovine serum albumin as a standard.

N-Terminal Amino Acid Sequence Analysis. The N-terminal amino acid sequence of the purified peptide was determined by Edman degradation using a protein sequencer (Applied Biosystems model 476A, Perkin Elmer Co.). Phenylthiohydantoin derivatives were separated and identified by capillary reversed-phase high-performance liquid chromatography on a C18 column with an analyzer (Applied Biosystems model ABI 140D, Perkin Elmer Co.).

Assay for Antibacterial Activity. The assay for antibacterial activity against *S. aureus* and *Salmonella* was conducted using sterile Petri dishes (100 mm $\times$ 15 mm) each containing 10 mL of LB agar (1.5% agar). Three milliliters of warm nutrient agar (0.7%) containing the bacteria was poured into each plate. A sterile blank paper disk (0.625 cm in diameter) was placed on the agar. Then a solution of *Hypotin* (containing a 2^0 to 2^{-10} gradient of 15 μM) in 10 mM Tris-HCl buffer (pH 7.2) was applied to individual disks. The plates were incubated at 37 °C for 12–20 h. A clear zone around the paper disk signified antibacterial activity.

Assay for Antifungal Activity. The assay for antifungal activity was executed using 100 mm $\times$ 15 mm Petri plates containing 10 mL of potato dextrose agar. Around and at a distance of 1 cm from the central disk (0.625 cm in diameter) were placed sterile blank paper disks of the same size. An aliquot (8 μL containing 60 or 300 μg) of *Hypotin* in 10 mM Tris-HCl buffer (pH 7.2) was introduced onto a disk. The plates were incubated at 23 °C for 72 h until mycelial growth from the central disk 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 are listed in this section.

To evaluate the *Hypotin* in detail for its antifungal effect, *P. aphanidermatum* was used as an example. The inhibition of hyphal growth of fungus by purified protein was observed under a light microscope. Cell and spore suspensions (10^5 or 10^6 in 1 mL) of the fungus were developed, and then 50 μL of cell and spore suspensions treated with the purified protein (300 μg) was dropped into a tube with 1 mL of medium. After incubation at 27 °C for 48 h, cells were separated from the growth medium by centrifugation and plated for observation under an optical microscope at a magnification of 100$\times$.

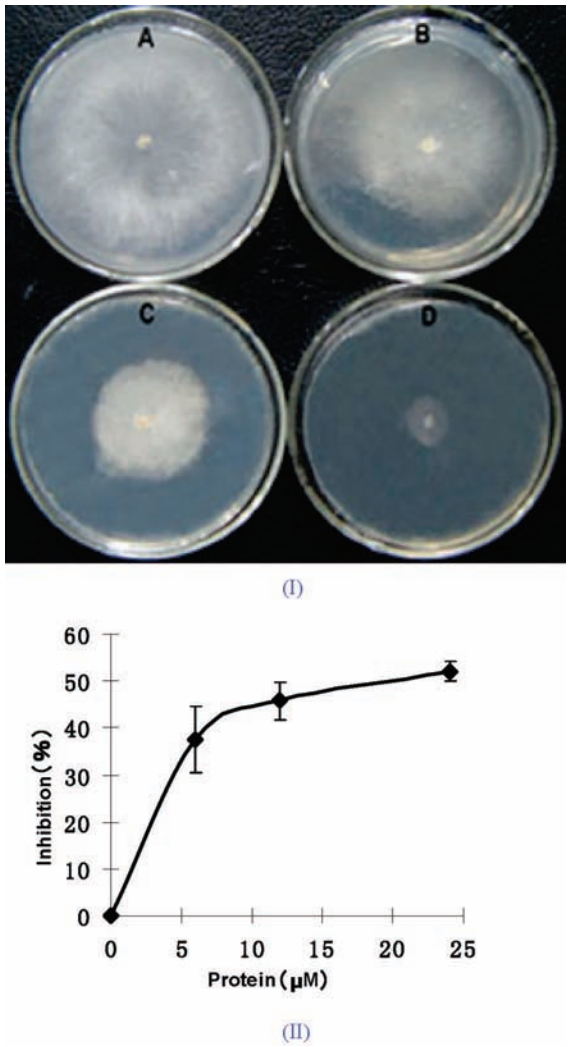

Figure 5. (**I**) Quantitatively antifungal activity of *Hypotin* against *P. aphanidermatum*. Plate **A** represents the control, and plates **B–D** represent treatments with 6, 18, and 30 μM *Hypotin*, respectively. (**II**) Determination of the IC_{50} value of the antifungal activity of *Hypotin*. Each data point represents the mean $\pm$ standard deviation of the triplicate inhibition radius of mycelia on plates (**I**). The IC_{50} value was calculated to be 18.9 μM.

For a quantitative assay to determine the IC_{50} of antifungal activity, three doses of the antifungal protein were added separately to three aliquots each containing 4 mL of potato dextrose agar at 45 °C; the samples were mixed rapidly and poured into three separate small Petri dishes. Here IC_{50} is defined as the half-maximal inhibitory concentration, representing the concentration of purified protein that is required for 50% inhibition of fungal growth. After the agar had cooled, the same small amount of mycelia was inoculated onto each plate. Buffer only without antifungal protein served as a negative control. After incubation at 27 °C for 72 h, the area of the mycelial colony was measured and the level of inhibition of fungal growth and the IC_{50} value were determined.

Assay for Antiproliferative Activity. The antiproliferative activity of *Hypotin* was determined by testing its growth inhibition on human liver hepatoma cells (Bel-7402). The cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum, in a humidified atmosphere of 5% CO_2 at 37 °C for 24 h. The cells (3×10^5 cells per 150 μL per well) were seeded on a 96-well culture plate, and serial dilutions of a solution of antifungal protein (containing 300 μg in the first well) in 150 μL of medium were added. After incubation, cells were harvested and dyed with MTT. The absorbance of the samples at 590 nm was determined using a microtiter plate (ELISA) reader and was directly correlated to the level of its antitumor activity. The inhibitory activity of *Hypotin* was calculated as the percent inhibition compared to a control without the sample. All reported values are the means of triplicate samples. Reversed microscopic observation was conducted for growth inhibition in Bel-7402 tumor cells. After the cells had grown for 24 h in the presence of 300 μg of *Hypotin*, the morphology of Bel-7402 cells was observed at a magnification of 10×.

RESULTS AND DISCUSSION

Purification of *Hypotin*. The solution of the ammonium sulfate precipitate was applied to an open column of Affi-gel blue gel, and the adsorbed fraction exhibiting antifungal activity was desorbed from the column with a linear NaCl concentration gradient (**Figure 1A**). The adsorbed peak was pooled and dialyzed, and chromatography on a CM-Sephadex column was carried out (**Figure 1B**). Subsequently, the Sephadex G-75 column was used for further purification (**Figure 1C**). The only main peak, which is designated G1, displayed both antifungal activity and antibacterial activity. Its SDS–PAGE pattern is shown in **Figure 2**. The protein exhibited a molecular mass of 30.4 kDa in SDS–polyacrylamide gel electrophoresis, under both reducing and nonreducing conditions (with or without dithiothreitol), indicating it is a monomeric protein. The purified *Hypotin* was shown by capillary reversed-phase high-performance liquid chromatography (**Figure 3**) to be highly pure. The *Y*-axis (a.i.) of **Figure 3** signifies the detection signal. If a single signal peak was evident, it demonstrated the purified protein was highly pure (*41*).

The protein yields at each purification step are listed in **Table 1**. A total of 5.9 mg of purified *Hypotin* was obtained via the Sephadex G-75 column from 100 g of peanuts.

Some antifungal proteins are considered hydrophobic proteins, because they have a conserved cysteine pattern which forms disulfide bonds (*14*, *40*). Also, they show a basic isoelectric point (*14*, *36*). The affinity chromatography media and the ion-exchange media at low ionic strength and neutral pH were extensively used in isolating antifungal proteins. Affi-gel blue affinity gel is a beaded, cross-linked agarose gel with covalently attached Cibacron Blue F3GA dye. Its highly effective affinity matrix has biospecific affinity for the protein with hydrophobic groups. *Hypotin* was adsorbed on Affi-gel blue gel and CM-Sephdex. This finding is analogous to similar observations with other antifungal proteins (*10–12*, *17*, *36*, *37*).

A chitinase-like protein, with a molecular mass of 28 kDa, has also been isolated from cowpea (*Vigna unguiculata*) seeds (*6*), pinto bean (*Phaseolus vulgaris* cv. *pinto*) seeds (*36*), and rice bean (*Delandia umbellata*) seeds (*37*). The newly reported chitinase-like protein from peanuts (*A. hypogaea*) shows a molecular mass slightly higher than those of the counterparts mentioned above. Nevertheless, they all fall into the regular range of molecular masses (between 25 and 35 kDa) reported for chitinase-like proteins (*5*, *37*). All these chitinase-like proteins exert potent antifungal activity and similarity to plant chitinase precursors (*5*, *6*, *36*, *37*).

N-Terminal Amino Acid Sequence. The N-terminal 20-amino acid sequence of the purified peanut *Hypotin* was determined to be C-D-V-G-S-V-I-S-A-S-L-F-E-A-L-Q-K-H-R-N. This demonstrated some resemblance (between 45 and 75%) to those chitinases and chitinase precursors from other plants according to the results of a BLAST Search of the NCBI database (**Table 2**).

Four chitinase genes have been cloned from *A. hypogaea* (*38*); however, the N-terminal amino acid sequence of the new protein in this study is vastly incomparable with known chitinase genes. Moreover, information pertaining to their biological significance

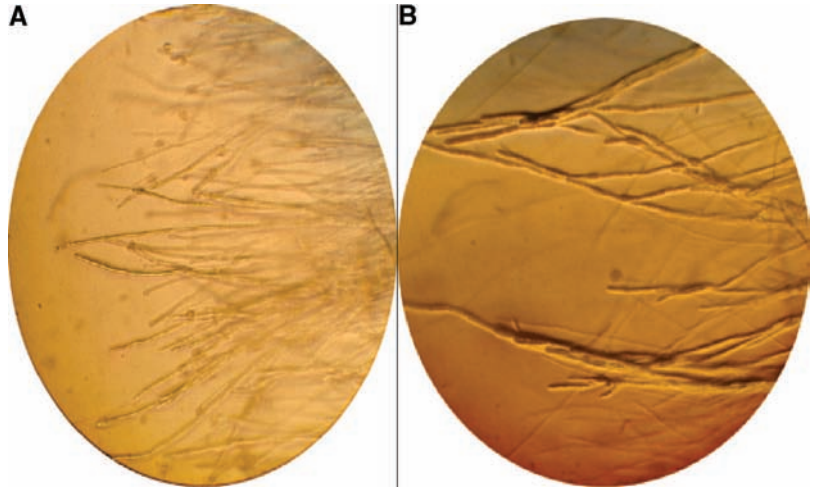

Figure 6. Light microscopic observation of inhibition of hyphal growth in *P. aphanidermatum* by purified peanut *Hypotin*. (**A**) Mycelia of fungus after growth for 48 h in the absence of *Hypotin*. (**B**) Mycelia of fungus after growth for 48 h, in the presence of 300 μg of *Hypotin*.

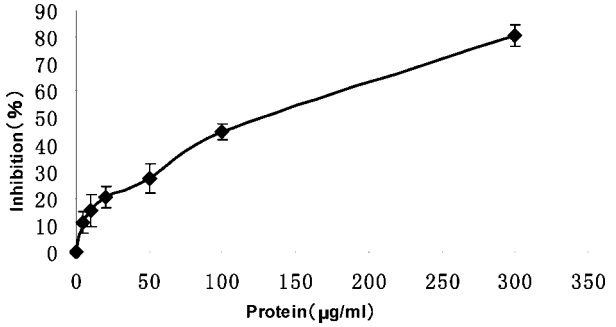

Figure 7. Inhibitory activity of peanut *Hypotin* against tumor cell Bel-7402. Each data point represents the mean ± the standard deviation of triplicate determinations.

was lacking. We herein report, for the first time, not only the protein's purification but also its bioactivity.

It is noteworthy that the N-terminal sequence of *Hypotin* resembles the middle sequence of other compared chitinases and chitinase precursors (**Table 2**), probably due to the different varieties or cultivars used in different studies. This is reminiscent of a similar finding for straw mushroom lectin compared with a previously reported straw mushroom lectin (*22*), in which the N-terminal sequence of the former lectin was similar to the C-terminal sequence of latter lectin isolated earlier.

Antibacterial and Antifungal Activity. The peanut *Hypotin* exerted antibacterial activity toward the Gram-positive bacterium *S. aureus*. Antibacterial activity was demonstrated over a concentration gradient from $2^0 \times 15$ to $2^{-6} \times 15$ μM; the MIC to *S. aureus* was 0.33 μM. However, it had no effect on Gram-negative bacteria such as *Salmonella* (data not shown).

The antifungal activity of peanut *Hypotin* against six fungal species is illustrated in **Figure 4A–F**. One can see that the protein showed strong antifungal activity toward *B. cinerea* (**Figure 4A**), *A. alternata* (**Figure 4B**), *Sc. rolfsii* (**Figure 4C**), *F. solani* (**Figure 4D**), *F. oxysporum* (**Figure 4E**), and *P. aphanidermatum* (**Figure 4F**). The IC_{50} value of the antifungal activity toward *P. aphanidermatum* was calculated to be 18.9 μM (**Figure 5**). Light microscopic examination disclosed *Hypotin*-induced distortion of the fungi. Photographs show hyphal morphological alterations and poor hyphal quantity in those fungi growing in the presence of *Hypotin* (**Figure 6B**) as compared with the growth on control medium showing normal hyphal development (**Figure 6A**).

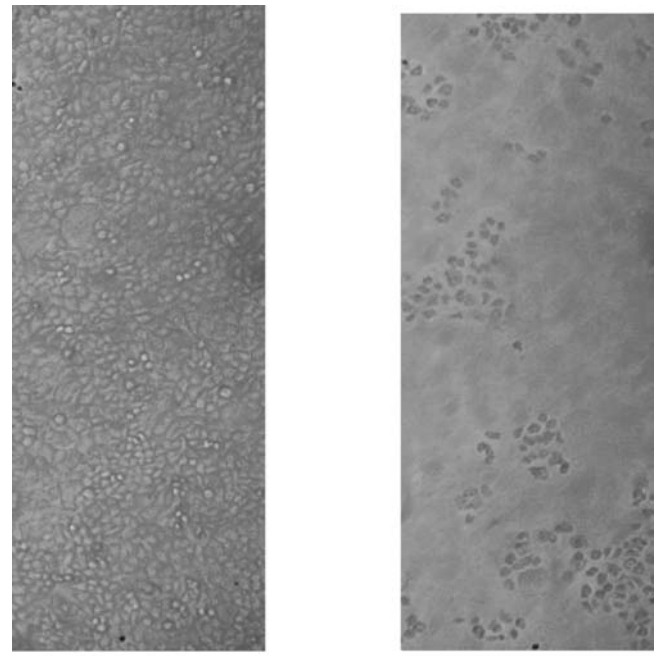

Figure 8. Reversed microscopic observation of inhibition of growth in Bel-7402 tumor cells by purified peanut *Hypotin*. (**A**) Tumor cell after growth for 24 h in the absence of peanut *Hypotin*. (**B**) Tumor cell after growth for 24 h in the presence of 300 μg of peanut *Hypotin*. Magnification: (**A**) 10× and (**B**) 12×.

Phytopathogenic fungi must overcome complex defenses. These defenses include a cell wall, composed of compounds such as lignin, tannins, phenols, and cellulose, which presents a physical barrier to inhibit microbial infections (*39*). Though plants lack an immune system, they do produce constitutive and (or) induced proteins in response to fungal infection (*39*, *40*). From the results depicted in **Figure 6**, it could be surmised that the antipathogenic activity of purified protein was due to its corrosion of the cellular wall of the pathogen which allowed the cytoplasm to leak out.

The observation that *Hypotin* showed antifungal activity toward broad fungal species suggested an important role for this purified protein in constitutive host defense mechanisms

against microbial pathogens. This may contribute to the development of the biological control of fungal pathogens typical of the plant.

Antiproliferative Activity. The antiproliferative activity of *Hypotin* to the human liver hepatoma Bel-7402 cells was calculated as percent inhibition compared to a control without the sample. All reported values are the means of triplicate samples. According to the inhibition result (**Figure 7**), the IC_{50} value toward Bel-7402 was calculated to be 296 μg/mL. Reversed microscopic observation of inhibition of growth in Bel-7402 tumor cells by purified peanut *Hypotin* is shown in **Figure 8**.

Hypotin possesses antiproliferative and antibacterial activities. However, the previous reports of antifungal proteins focused on their antifungal activity, although some antifungal proteins also demonstrate in addition antibacterial activities (*13*, *30*, *41*). The chitinase-like proteins from cowpea and pinto bean and the rice bean protein exerted both potent antifungal activity and some inhibitory activity against HIV-1 reverse transcriptase (*6*, *36*, *37*). The newly reported chitinase-like protein from peanuts shows antipathogenic and antiproliferative activities with respect to tumor cells compared with those of the other counterparts. Plant defense proteins could interact selectively with specific structures in the fungal membrane, such as phosphorylinositol containing sphingolipids or glycosylceramides (*42*, *43*), and then trigger the death of fungal cells. This specific binding would enable proteins to kill cancer cells. The binding of defense proteins with target cancer cell membranes is the first important event in tumor lysis (*44*). The tumor inhibiting activity and HIV inhibiting activity may be related to the antifungal activity.

Conclusion. To summarize, we are the first to report the isolation and characterization of a novel antifungal protein, designated *Hypotin*, from peanut seeds. It is noted that *Hypotin* manifests antibacterial activity against certain bacterial species and antiproliferative activity toward tumor cells. These activities, together with antifungal activity, make *Hypotin* a desirable defense molecule for potential application in medicine, agriculture, and food science.

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LITERATURE CITED

(1) Borris, R. P. Natural products research: Perspectives from a major pharmaceutical company. *J. Ethnopharmacol.* **1996**, *51*, 29–38.
(2) Tivy, J. *Biogeography, a study of plants in the ecosphere*, 3rd ed.; Longman House: Burnt Mill, Harlow, Essex, U.K., 1993.
(3) Grenier, J.; Potvin, C.; Asselin, A. Some fungi express β-1,3-glucanases similar to thaumatin-like proteins. *Mycologia* **2000**, *92*, 841–848.
(4) 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*, 60–69.
(5) Lam, Y. W.; Wang, H. X.; Ng, T. B. A robust cysteine-deficient chitinase-like antifungal protein from inner shoots of the edible chive *Allium tuberosum*. *Biochem. Biophys. Res. Commun.* **2000**, *279*, 74–80.
(6) Ye, X. Y.; Wang, H. X.; Ng, T. B. Structurally dissimilar proteins with antiviral and antifungal potency from cowpea (*Vigna unguiculata*) seeds. *Life Sci.* **2000**, *67*, 3199–3207.
(7) Huang, X.; Xie, W. J.; Gong, Z. Z. Characterization and antifungal activity of a chitin binding protein from Ginkgo biloba. *FEBS Lett.* **2000**, *478*, 123–126.
(8) Van Den Bergh, K. P.; Rouge, P.; Proost, P.; Coosemans, J.; Krouglova, T.; Engelborghs, Y.; Peumans, W. J.; Van Damme, E. J. Synergistic antifungal activity of two chitin-binding proteins from spindle tree (*Euonymus europaeus* L.). *Planta* **2004**, *219*, 221–232.
(9) Vergauwen, R.; Van Leuven, F.; Van Laere, A. Purification and characterization of strongly chitin-binding chitinase from salicylic acid-treated leek (*Allium porrum*). *Physiol. Planta* **1998**, *104*, 175–182.
(10) Ye, X. Y.; Ng, T. B. Hypogin, a novel antifungal peptide from peanuts with sequence similarity to peanut allergen. *J. Pept. Res.* **2000**, *57*, 330–336.
(11) Ye, X. Y.; Ng, T. B. Isolation of a new cyclophilin-like protein from chickpeas with mitogenic, antifungal and anti-HIV-1 reverse transcriptase activities. *Life Sci.* **2002**, *70*, 1129–1138.
(12) Ye, X. Y.; Wang, H. X.; Ng, T. B. Sativin, a novel antifungal miraculin-like protein isolated from the legumes of the sugar snap *Pisum sativum* var. macrocarpon. *Life Sci.* **2000**, *67*, 775–781.
(13) Wang, S. Y.; Wu, J. H.; Ng, T. B.; Ye, X. Y.; Rao, P. F. A non-specific lipid transfer protein with antifungal and antibacterial activities from the mung bean. *Peptides* **2004**, *25*, 1235–1242.
(14) Wang, S. Y.; Zhou, K. J.; Ye, X. Y.; Wu, J. H.; Xu, Z. B.; Rao, P. F. Crystallization and preliminary X-ray crystallographic analysis of a non-specific lipid transfer protein with antipathogenic activity from *Phaseolus mungo*. *Acta Crystallogr.* **2004**, *D6*, 2391–2393.
(15) Chu, K. T.; Ng, T. B. Isolation of a large thaumatin-like antifungal protein from seeds of the Kweilin chestnut *Castanopsis chinensis*. *Biochem. Biophys. Res. Commun.* **2003**, *301*, 364–370.
(16) Wong, J. H.; Ng, T. B. Isolation and characterization of a glucose/mannose/rhamnose-specific lectin from the knife bean *Canavalia gladiata*. *Arch. Biochem. Biophys.* **2005**, *439*, 91–98.
(17) Ye, X. Y.; Ng, T. B. Isolation of a novel peroxidase from French bean legumes and first demonstration of antifungal activity of a non-milk peroxidase. *Life Sci.* **2002**, *71*, 1667–1680.
(18) Lichtenstein, A.; Ganz, T.; Selsted, M. E.; Lehrer, R. I. In vitro tumor cell cytolysis mediated by peptide defensins of human and rabbit granulocytes. *Blood* **1986**, *68*, 1407–1410.
(19) Ngai, P. H.; Ng, T. B. Coccinin, an antifungal peptide with antiproliferative and HIV-1 reverse transcriptase inhibitory activities from large scarlet runner beans. *Peptides* **2004**, *25*, 2063–2068.
(20) Raj, P. A.; Dentino, A. R. Current status of defensins and their role in innate and adaptive immunity. *FEMS Microbiol. Lett.* **2002**, *266*, 9–18.
(21) Wong, J. H.; Ng, T. B. Gymnin, a potent defensin-like antifungal peptides from the Yunnan bean (*Gymnocladus chinensis* Baill). *Peptides* **2003**, *24*, 963–968.
(22) She, Q. B.; Ng, T. B.; Liu, W. K. 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*, 106–111.
(23) Does, M. P.; Houterman, P. M.; Dekker, H. L.; Cornelissen, B. J. Processing, targeting and antifungal activity of stinging nettle agglutinin in transgenic tobacco. *Plant Physiol.* **1999**, *120*, 421–422.
(24) Gozia, O.; Ciopraga, J.; Bentia, T.; Lungu, M.; Zamfirescu, I.; Tuder, R.; Roseanu, A.; Nitu, F. Antifungal properties of lectin and new chitinase from potato tuber. *FEBS Lett.* **1995**, *370*, 245–249.
(25) Leah, R.; Tommerup, H.; Svendsen, I.; Mundy, J. Biochemical and molecular characterization of three barley seed proteins with antifungal properties. *J. Biol. Chem.* **1991**, *246*, 1564–1573.
(26) Vigers, A. J.; Roberts, W. K.; Selitrennikoff, C. P. A new family of plant antifungal proteins. *Mol. Plant–Microbe Interact.* **1991**, *4*, 315–323.
(27) Chilosi, G.; Caruso, C.; Caporale, C.; Leonardi, L.; Bertini, L.; Buzi, A.; Nobile, M. Antifungal activity of a Bowman-Birk type

trypsin inhibitor from wheat kernel. *J. Phytopathol.* **2000**, *148*, 477–481.
(28) Huynh, Q. K.; Bergmeyer, J. R.; Zobel, J. F. Isolation and characterization of a 22-kDa protein with antifungal properties from maize seeds. *Biochem. Biophys. Res. Commun.* **1992**, *182*, 1–5.
(29) Joshi, B. N.; Sainani, M. N.; Bastawade, K. B.; Gupta, V. S.; Ranjekar, P. K. Cysteine protease inhibitor from pearl millet: A new class of antifungal protein. *Biochem. Biophys. Res. Commun.* **1998**, *246*, 382–387.
(30) Wang, S. Y.; Lin, J.; Ye, M. Y.; Ng, T. B.; Rao, P. F.; Ye, X. Y. Isolation and characterization of a novel mung bean protease inhibitor with antipathogenic and anti-proliferative activities. *Peptides* **2006**, *27*, 3129–3136.
(31) Chu, K. T.; Xia, L.; Ng, T. B. Pleurostrin, an antifungal peptide from the oyster mushroom. *Peptides* **2005**, *26*, 2098–2103.
(32) Ngai, P. H.; Zhao, Z.; Ng, T. B. Agrocybin, an antifungal peptide from the edible mushroom *Agrocybe cylindracea*. *Peptides* **2005**, *26*, 191–196.
(33) Terras, F. R. G.; Eggermont, K.; Kovaleva, V.; Raikhel, N. V.; Osborn, R. W.; Kester, A.; Rees, S. B.; Torrekens, S.; Van Leuven, F.; Vanderleyden, J. Small cysteine-rich antifungal proteins from radish: Their role in host defense. *Plant Cell.* **1995**, *7*, 573–588.
(34) Laemmli, U. K.; Favre, M. Gel electrophoresis of proteins. *J. Mol. Biol.* **1973**, *80*, 575–599.
(35) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin Phenol Reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.
(36) Ye, X. Y.; Ng, T. B. Peptides from pinto bean and red bean with sequence homology to cowpea 10-kDa protein precursor exhibit antifungal, mitogenic, and HIV-1 reverse transcriptase-inhibitory activities. *Biochem. Biophys. Res. Commun.* **2001**, *285*, 424–429.
(37) Ye, X. Y.; Ng, T. B. Delandin, a chitinase-like protein with antifungal, HIV-1 revere transcriptase inhibitory and mitogenic activities from the rice bean *Delandia umbellata*. *Protein Expression Purif.* **2002**, *24*, 524–529.
(38) Herget, T.; Schell, J.; Schreier, P. H. Elicitor-specific induction of one member of the chitinase gene family in *Arachis hypogaea*. *Mol. Gen. Genet.* **1990**, *224* (3), 469–476.
(39) Linthorst, H. J. Pathogen-related proteins of plants. *Crit. Rev. Plant Sci.* **1991**, *10*, 123–150.
(40) Selitrennikoff, C. Antifungal proteins. *Appl. Environ. Microbiol.* **2001**, *67*, 2883–2894.
(41) Wang, S. Y.; Ng, T. B.; Chen, T.; Lin, D. Y.; Rao, P. F.; Ye, X. Y. First report of a novel plant lysozyme with both antifungal and antibacterial activities from *Phaseolus mungo*. *Biochem. Biophys. Res. Commun.* **2005**, *327*, 820–827.
(42) Thevissen, K.; Ferket, K. K.; Francois, I. E.; Cammue, B. P. Interactions of antifungal plant defensins with fungal membrane components. *Peptides* **2003**, *24*, 1705–1712.
(43) Thevissen, K.; Francois, I. E. J. A.; Takemoto, J. Y.; Ferket, K. K. A.; Meert, E. M. K.; Cammue, B. P. A. DmAMP1, an antifungal plant defensin from dahlia (*Dahlia merckii*), interacts with sphingolipids from *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* **2003**, *226*, 169–173.
(44) Lichtenstein, A. K.; Ganz, T.; Nguyen, T. M.; Selsted, M. E.; Lehrer, R. I. Mechanism of target cytolysis by peptide defensins. *J. Immunol.* **1988**, *140*, 2686–2694.

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