Biochemical and Biophysical Research Communications **279,** 74–80 (2000)
doi:10.1006/bbrc.2000.3821, available online at http://www.idealibrary.com on IDEAL®

A Robust Cysteine-Deficient Chitinase-like Antifungal Protein from Inner Shoots of the Edible Chive *Allium tuberosum*

Y. W. Lam,* H. X. Wang,† and T. B. Ng*
**Department of Biochemistry, Faculty of Medicine, Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China; and †Department of Microbiology, China Agricultural University, Beijing, China*

Received October 2, 2000

From the inner shoots of the chive *Allium tuberosum*, a single-chained protein with a molecular weight of 36 kDa and an N-terminal sequence manifesting resemblance to chitinases but lacking in cysteine residues characteristic of a cysteine-rich domain present in chitinases of other *Allium* species, was purified. The isolation procedure entailed affinity chromatography on Affi-gel blue gel, ion-exchange chromatography on DEAE-cellulose and Mono S, and gel filtration on Superdex 75. The protein was unadsorbed on DEAE-cellulose and adsorbed on Affi-gel blue gel and Mono S. It exhibited antifungal activity against *Rhizoctonia solani, Fusarium oxysporum, Coprinus comatus, Mycosphaerella arachidicola,* and *Botrytis cinerea.* The IC_{50} for its antifungal effect against *Botrytis cinerea* was 0.2 μM. The antifungal activity was stable after 1 h at pH 1.6 and 12.3, and up to 60°C for 5 min. Incubation of the protein with trypsin or chymotrypsin at an enzyme:substrate ratio of 1:100 and pH 7.6 up to 150 min did not affect its antifungal activity. The protein did not exhibit antibacterial activity. The protein inhibited cell-free translation in a rabbit reticulocyte system with an IC_{50} of 0.8 μM, but did not affect the proliferation of mouse splenocytes. It exerted some cytotoxic effect on breast cancer cells and was inhibitory toward HIV-1 reverse transcriptase. © 2000 Academic Press

Key Words: **antifungal protein; chive; chitinase.**

Several types of proteins play a defensive role in plants and counteract assaults from pathogens. They comprise antifungal proteins (1), ribosome-inactivating proteins (2), lectins (3) and protease inhibitors (4). The first three types of proteins may manifest antifungal activity (4, 5). Ribosome-inactivating proteins and lectins may display antiviral activity (6, 7). Lectins may evince an anti-insect function (8).

Antifungal proteins consist of chitinases (9), glucanases (10), miraculin-like protein (11), thaumatin-like proteins (12), cyclophilin-like proteins (13) and others. These proteins differ in amino acid sequence but have in common an antifungal attribute.

We report herein the purification of an antifungal protein, with structural analogy to chitinases albeit with some distinctive features, from inner shoots of the chive *Allium tuberosum*.

MATERIALS AND METHODS

Isolation of antifungal protein. Fresh inner shoots of chive (*Allium tuberosum*) were obtained from a local market. The cylindrical inner shoots are more tender compared with the flattened outer shoots. The extract was applied on a column of Affi-gel blue gel which had previously been equilibrated with 10 mM Tris-HCl buffer (pH 7). After elution of the unadsorbed proteins, the column was washed with 1.5 M NaCl in the starting buffer. The adsorbed peak BG2 was dialyzed and then applied on a column of DEAE-cellulose in 10 mM Tris-HCl buffer (pH 7). The unadsorbed material (DE1) was collected and chromatographed on a Mono S column in 10 mM NH_4OAc (pH 5.2) by FPLC. After elution of the unadsorbed peak named MS1, the column was eluted with a 0–80 mM NaCl gradient in the starting buffer to yield two peaks MS2 and MS3 which were eluted by 30 and 50 mM NaCl, respectively, and subsequently with a 80–1000 mM NaCl gradient to yield peak MS4 which was eluted by 600 mM NaCl. MS3 represented the purified antifungal protein. Its homogeneity and molecular weight were estimated by FPLC-gel filtration on a Superdex 75 column which had been calibrated with molecular weight markers from Amersham Pharmacia Biotech including phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and cytochrome *c* (12.4 kDa). N-terminal sequencing was carried out using a Hewlett-Packard (HP) Edman degradation unit and an HP HPLC System. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was conducted according to the method of Laemmli and Favre (14).

0006-291X/00 $35.00
Copyright © 2000 by Academic Press
All rights of reproduction in any form reserved.

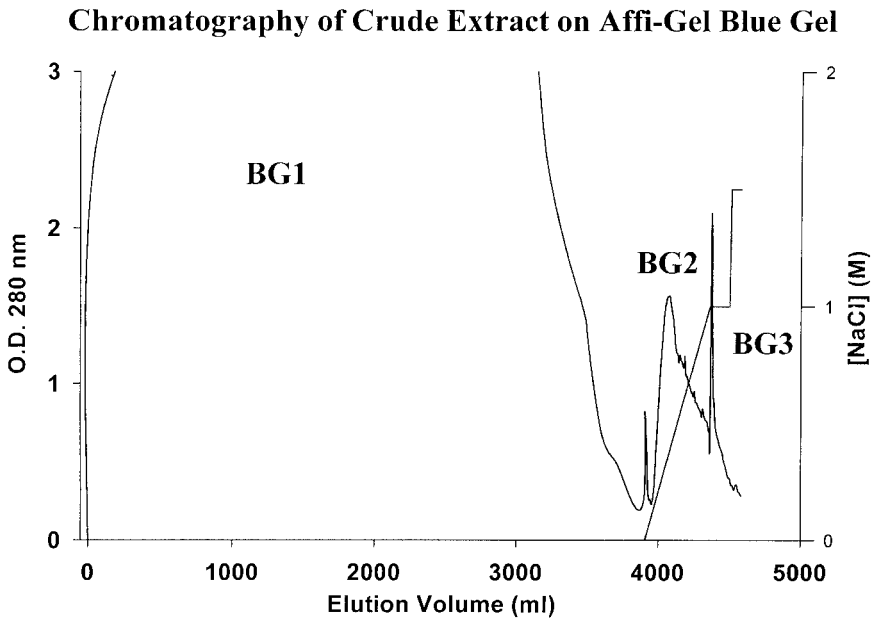

FIG. 1. Affinity chromatography on an Affi-gel Blue Gel column (2.5 × 16 cm). Crude powder of *Allium tuberosum* shoots was applied to the column. Unadsorbed material BG1 was eluted from the column in 10 mM Tris–HCl (pH 7.2). Changes of buffer by addition of 1 and 1.5 M NaCl in the starting buffer to elute adsorbed materials BG2 and BG3, respectively, are indicated.

Assay of antifungal activity. The assay for antifungal activity toward *Rhizoctonia solani* and other fungal species was carried out in 100 × 15 mm petri plates containing 10 ml of potato dextrose agar. At a distance of 0.5 cm away from the rim of the mycelial colony were placed sterile blank paper disks (0.625 cm in diameter). An aliquot (6 μl) of the test sample was added to a disk. The plates were incubated at 23°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 (15).

For a quantitative assay, three doses of the antifungal protein (0.1 μM, 0.66 μM and 2.4 μM) were added separately to three aliquots each containing 4 ml potato dextrose agar at 45°C, mixed rapidly, and poured into 3 separate 6-cm petri dishes. After the agar had cooled down, a small amount of mycelia, the same amount to each plate, was inoculated. Buffer only without antifungal protein served as a negative control. After incubation at 23°C for 72 h, the area of the mycelial colony was measured and the inhibition of fungal growth determined.

Assay of antibacterial activity. The assay for antibacterial activity was conducted using sterile petri plates (90 × 15 mm) containing 10 ml LB agar (1.5% agar). Three milliters of warm nutrient agar (0.7%) containing the bacterium was poured into the plates. A sterile blank paper disk (0.625 cm in diameter) was placed on the agar. The *Allium tuberosum* antifungal protein (10 μl of a 15 mg/ml solution) in 10 mM Tris-HCl buffer (pH 7.4) was added to one of the disks. Only Tris-HCl buffer was added to the control disk. The plate was incubated at 30°C for 20–24 h. A transparent ring around the paper disk signifies antibacterial activity. Penicillin (5 mg/ml) served as a positive control. The bacteria tested included *Staphylococcus aureus, Pseudomonas aeruginosa, Proteus vulgaris, Aeromonas hydrophila, Bacillus subtilis,* and *Escherichia coli.*

Assay of HIV reverse transcriptase inhibitory activity. Reverse transcriptase activity was measured by ELISA as described by Collins *et al.* (16) using a nonradioactive kit from Boehringer Mannheim (Germany). The inhibition assay was performed as described in the protocol included with the kit.

Assay of mitogenic activity (17). 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 concentrations of *Allium tuberosum* chitinase-like antifungal protein and Con A 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, Pharmacia Biotech) 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.

Assay of antiproliferative activity (18). Human breast carcinoma (MDA-MB-231) cells were seeded at a density of 2×10^4 cells/100 μl in 96-well plates for 24 h, at 37°C, in an atmosphere of 5% CO_2. Serials concentrations of *Allium tuberosum* chitinase-like antifungal protein (100 μl/well) were added before further incubation for 48 h. At the end of the incubation, the medium was removed and the plate was washed with phosphate-buffered saline. MTT (3-[4,5-Dimethylthyazol-2-yl]-2,5-diphenyltetrazolium bromide) solution (100 μl/well) was then added followed by further incubation for an hour. Absorbance at 540 nm was measured after incubation with dimethyl sulfoxide (100 μl/well) for 30 min. Reagent and control were included with the absence of cells or *Allium tuberosum* chitinase-like antifungal protein respectively (18).

Viability (%) of tumor cells

$$= OD_{540\ nm}\ (\text{sample})/OD_{540\ nm}\ (\text{control}) \times 100\%.$$

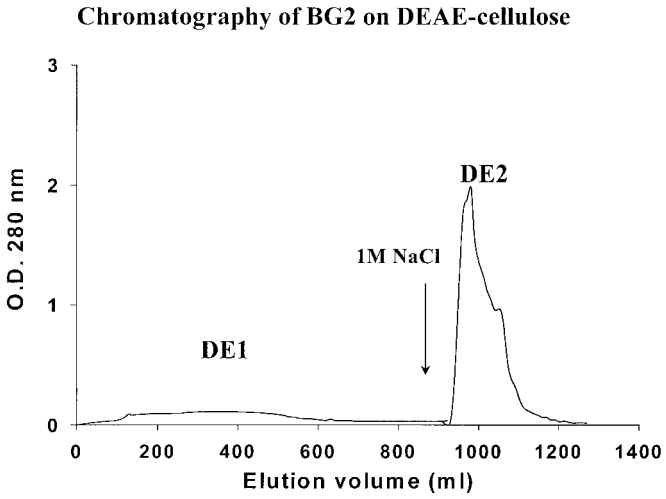

FIG. 2. Ion exchange chromatography of BG2 on a DEAE-cellulose column (2.5 × 36 cm). Unadsorbed material DE1 containing antifungal activity was eluted from the column in 10 mM Tris–HCl (pH 7.2). Change of buffer by addition of 1 M NaCl in the starting buffer to elute adsorbed material DE2 is indicated by an arrow.

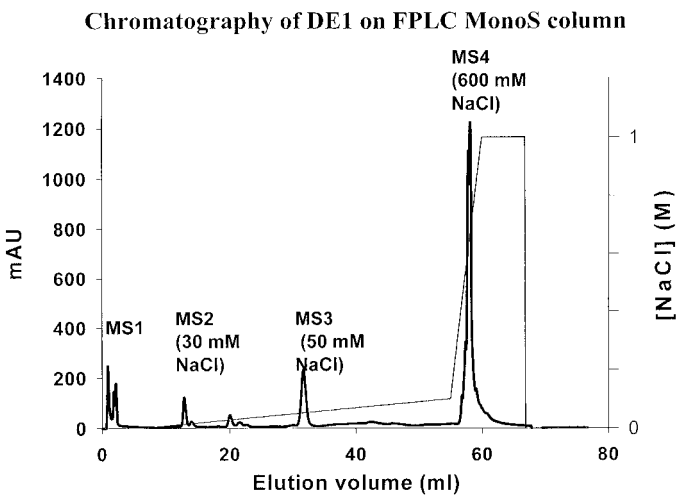

FIG. 3. Ion exchange chromatography of peak DE2 derived from DEAE-cellulose on Mono S HR 5/5 column (1 ml). The column was preequilibrated with 10 mM NH_4OAc (pH 5.2). Two peaks eluted with a 0–80 mM sodium chloride gradient were named MS2 and MS3, respectively, and the peak eluted with a 0.08–1 M sodium chloride gradient was named MS4 (600 mM). MS2, MS3, and MS4 were eluted by 30, 50, and 600 mM NaCl, respectively.

Assay of ribonuclease activity (19). Yeast tRNA was used as substrate. *Allium tuberosum* chitinase-like antifungal protein was incubated with 200 μg tRNA in 150 μl 100 mM Hepes buffer (pH 7) before 350 μl ice-cold 3.4% perchloric acid was added to terminate the reaction. After standing on ice for 15 min, the reaction mixture was centrifuged and the absorbance of the supernatant was measured after suitable dilution. One unit of ribonuclease activity is defined as the amount of enzyme which produces an absorbance increase of one per minute in the acid-soluble supernatant per ml of reaction mixture under specified conditions.

Assay of cell-free translation inhibiting activity was based on the ability to inhibit protein synthesis from [^{3}H]leucine in a cell-free rabbit reticulocyte lysate system. It has been described previously (20, 21).

RESULTS

Affinity chromatography of the crude extract of *Allium tuberosum* shoots on Affi-gel Blue Gel yielded a large unadsorbed peak BG1 containing the bulk of proteins without antifungal activity. Adsorbed proteins were desorbed with 1 M NaCl and then with 1.5 M NaCl in 10 mM Tris-HCl (pH 7.2) to yield fractions BG2 and BG3 respectively (Fig. 1). BG2 with antifungal activity was then fractionated on DEAE-cellulose in 10 mM Tris–HCl (pH 7.2) into an unadsorbed DE1 peak containing antifungal activity and an adsorbed DE2 peak devoid of activity (Fig. 2). When DE1 was chromatographed by FPLC on a Mono S column, a small unadsorbed inactive peak (MS1) appeared, followed by two small adsorbed peaks (MS2 and MS3) and a large adsorbed peak MS4 which were eluted by 30 mM, 50 mM and 600 mM NaCl in 10 mM NH_4OAc (pH 5.2) respectively (Fig. 3). Only MS3 possessed antifungal activity, and it appeared as a single band with a molecular weight of 36.8 kDa in SDS-PAGE (Fig. 4). Hence it represented purified antifungal protein. The yield of the purified antifungal protein was about 0.65 mg/kg fresh inner shoots (Table 1). N-terminal sequence analysis revealed that it was a chitinase-like protein although it was lacking in cysteine residues present

FIG. 4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of purified *Allium tuberosum* chitinase (MS3). Lane 1, Pharmacia molecular weight markers, from top downward: phosphorylase *b* (94 kDa), bovine serum albumin (MW 67 kDa), ovalbumin (MW 43 kDa), carbonic anhydrase (MW 30 kDa), and lactalbumin (MW 14.4 kDa); lane 2, purified *Allium tuberosum* chitinase.

TABLE 1

Protein Yields of Different Fractions at Different Stages of Purification of *Allium tuberosum* Chitinase-like Antifungal Protein

Chromatographic fraction	Yield[a] (mg)
Crude extract	7247.5
BG1	487.5
BG2	164.6
BG3	11.7
DE1	19.3
DE2	99.1
MS1	1.1
MS2	0.5
MS3	1.1
MS4	6.1

[a] Protein obtained from 1.7 kg starting material. MS3 was the purified antifungal protein.

in other chitinases (Table 2). The antifungal activity of the chitinase-like protein against *Botrytis cinerea, Coprinus comatus* and *Rhizoctonia solani* is shown in Fig. 5. Antifungal activity was also observed against *Fusarium oxysporum* and *Mycosphaerella arachidicola* (data not shown). The protein inhibited mycelial growth of *Botrytis cinerea* with an IC_{50} of 0.2 μM (Fig. 6). The antifungal activity was unaffected after treatment with 0.025 M HCl (at pH 1.6) or 0.025 M NaOH (at pH 12.3) for 1 h. Incubation of the antifungal protein at 20°C, 40°C and 60°C for 5 min had no effect on its antifungal activity while incubation at 70°C, 80°C and 100°C for 5 min abolished the activity. Incubation of the antifungal protein with either trypsin or chymotrypsin at an enzyme: substrate ratio of 1:100 for 1 h or 150 min in 0.1 M Hepes buffer (pH 7.6) did not affect its antifungal activity against *Botrytis cinerea* (Fig. 7). The antifungal protein did not inhibit any of the bacteria tested (data not shown).

The *A. tuberosum* chitinase-like antifungal protein inhibited translation in rabbit reticulocyte lysate with an IC_{50} of 850 nM and it exerted a low RNase activity of only 1.63 U/mg toward yeast tRNA (data not shown). It displayed cytotoxic effect against breast cancer (MDA-MB-231) cells. Cell viability was 87.54 $\pm$ 5.67 and 44.20 $\pm$ 3.08 percent (n = 3, mean $\pm$ SD) at a dosage of 15 μg and 30 μg antifungal protein respectively per culture well. In contrast to Con A, it did not demonstrate mitogenic activity. The protein was inhibitory toward HIV-1 reverse transcriptase. The % enzyme inhibition achieved at 0.5 and 5 mg protein/ml was respectively 32.59 $\pm$ 2.32 and 84.97 $\pm$ 3.53 (n = 2, mean $\pm$ SD).

DISCUSSION

The antifungal protein isolated in this study was a chitinase-like protein as judged by its N-terminal sequence. Its cell-free translation-inhibiting and RNase activities were higher than those of previously isolated antifungal proteins including sativin, the miraculin-like antifungal protein from *Pisum sativum var. macrocarpon* (11), dolichin, the chitinase-like protein from the field bean *Dolichos lablab* (21), and mungin, the cyclophilin-like antifungal protein from mung beans (13). The IC_{50} value for the cell-free translation-inhibiting activity of the *A. tuberosum* chitinase-like antifungal protein was slightly under 1 μM whereas those for the other aforementioned antifungal proteins lay in the range 10–25 μM. RNase activity was detectable in the *A. tuberosum* chitinase-like antifungal protein but not so in the other cases. The inhibitory activity of *A. tuberosum* chitinase-like antifungal protein toward HIV-1 reverse transcriptase was more potent than those of dolichin (22) and mungin (13). The anti-mitogenic activity of mungin (13) and the lack of mitogenic

TABLE 2

N-Terminal Sequence Comparison between *Allium tuberosum* Chitinase-like Antifungal Protein and Other Chitinases

	Residue	Sequence	% identity
ATC	1	EQHGSQAGGALHPGXLHYSKYGGYGGTTPDYYGDGQQ	100
BLC	27	EQCGRQAGGALCPGGNCCSQFGWCSGYTTDYCGPGCQ	56
GP	1	EQCGTQAGGALCPGGLCCSQFGWCSGTI · DKGPGCQEYCGDG	53
APC-Dr	1	EQCGRQAGGALCPGGLC	70
APC-D	1	EQCGRQA	70
APC-F	1	EQCGRQAGGAL	81
CHITAS1	1	QQCGSDAGGALCSNRLCCSKFGYCGSTDPYCGTGCQS	46
CHITAS2	1	QQCGSDGSGALCSNGLCCSQYGYCGNGGPYCGTGCQS	40

Note. Residues identical those of *Allium tuberosum* chitinase are underlined. ATC, *Allium tuberosum* chitinase; APC-Dr, *Allium porrum* chitinase isoform Dr; APC, *Allium porrum* chitinase isoform D; CHITAS1, *Allium sativum* chitinase 1; CHITAS2, *Allium sativum* chitinase 2; BLC, bean PCH18 chitinase; GP, pea A2 chitinase. The sequences of APC-Dr and APC-D are taken from reference 23, those of CHITAS1 and CHITAS2 from Ref. (24), and those of BLC and GP from Ref. (25).

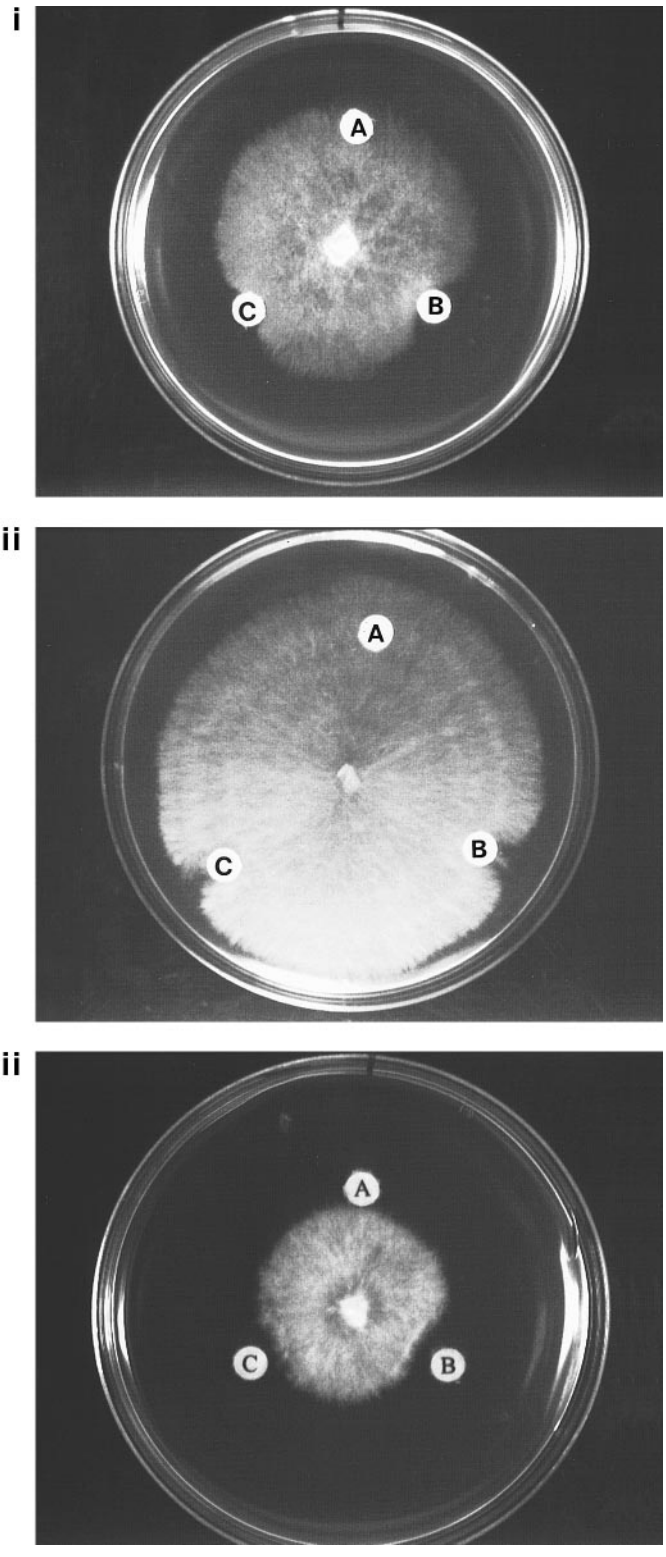

FIG. 5. Inhibitory activity of *Allium tuberosum* chitinase-like antifungal protein toward (i) *Botrytis cinerea,* (ii) *Rhizoctonia solani,* and (iii) *Coprinus comatus.* (A) control (0.1 M Hepes, pH 7.6), (B) 200 μg *Allium tuberosum* chitinase, and (C) 60 μg *Allium tuberosum* chitinase.

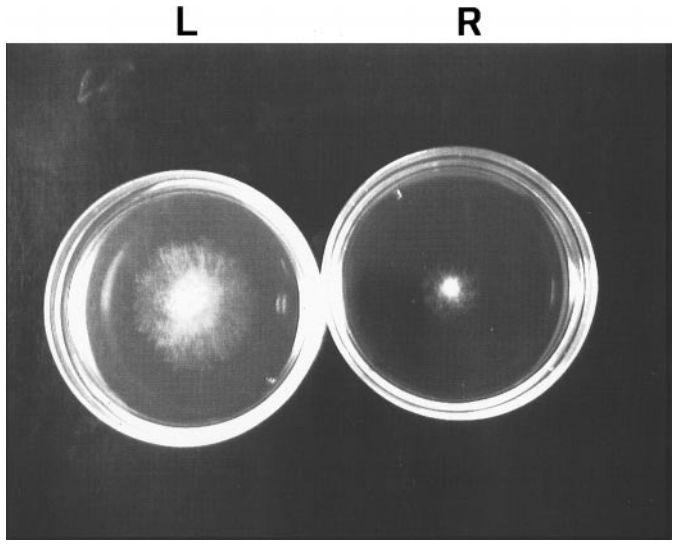

FIG. 6. Determination of IC_{50} value of antifungal activity of *Allium tuberosum* chitinase-like antifungal protein toward *Botrytis cinerea.* Left (L): control. Right (R): 2.4 μM *Allium tuberosum* chitinase-like antifungal protein. Responses to 0.1 μM and 0.66 μM *Allium tuberosum* chitinase-like antifungal protein are not shown here. IC_{50} was estimated to be 0.2 μM.

activity of *A. tuberosum* chitinase-like antifungal protein suggest that these antifungal proteins may not be stimulatory toward the immune cells. The cytotoxic activity of *A. tuberosum* chitinase-like antifungal protein on breast cancer cells was not very high in potency. It did not possess antibacterial activity.

A. tuberosum chitinase-like antifungal protein resembled sativin (11), dolichin (22), mungin (13) and the thaumatin-like antifungal protein from *Phaseolus vulgaris* (15) in that they were adsorbed on Affi-gel Blue Gel and CM-Sepharose/Mono S. The molecular weight of *A. tuberosum* chitinase-like antifungal protein was close to that of sativin (11) and those of other chitinases including chitinases from the leek *Allium porrum* (23) and the garlic *Allium sativum* (24), but larger than those of dolichin (22), mungin (13) and the thaumatin-like protein from *P. vulgaris* (15).

Antifungal proteins have been isolated from monocot species related to *A. tuberosum* including the leek *A. porrum* (23) and the garlic *A. sativum* (24). The leek antifungal protein exists in several isoforms. Some of these isoforms have a blocked N-terminus while the remainder has the same N-terminal sequence. The garlic chitinase also exists in two forms (24). The N-terminal sequences in leek and garlic chitinases are strikingly similar to that of *A. tuberosum* chitinase-like antifungal protein. The latter is distinctive in the lack of cysteine residues which are conserved in leek, garlic and other chitinases. The

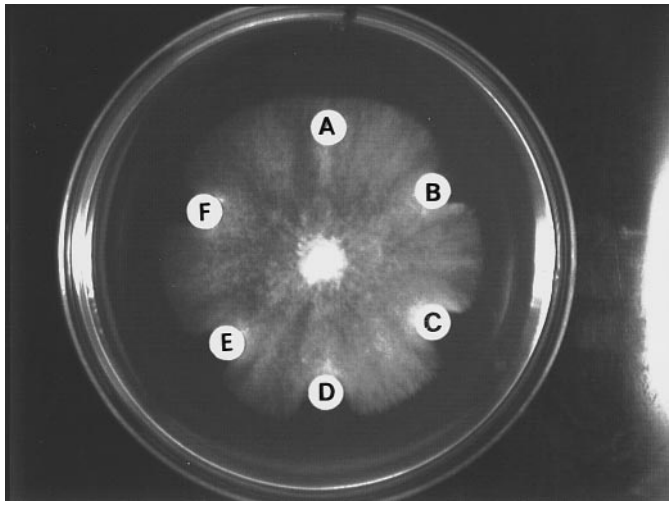

FIG. 7. Effect of protease digestion on antifungal activity of *Allium tuberosum* chitinase-like antifungal protein against *Botrytis cinerea:* (A) control (0.1 M Hepes, pH 7.6), (B) chitinase (160 μg) pretreated with chymotrypsin (enzyme:substrate ratio = 1:100) for 1 h, (C) chitinase pretreated with chymotrypsin for 150 min, (D) untreated chitinase (160 μg), (E) chitinase (160 μg) pretreated with trypsin (enzyme:substrate ratio = 1:100) for 1 h, (F) chitinase pretreated with trypsin for 150 min.

purification of leek and garlic chitinase isoforms depended on the use of a chitin column (23, 24), a mannose-Sepharose column (24), a phenyl-Sepharose column (24) and a Mono Q column (23) or a Mono S column (24). A different isolation procedure was adopted for purifying chive antifungal protein in the present investigation. It is perhaps noteworthy that leek chitinases were adsorbed on Mono Q in 20 mM ethanolamine (pH 9.7) (23), garlic chitinases were adsorbed on Mono S in 20 mM sodium formate (pH 3.8) (24) while chive chitinase-like protein was adsorbed on Mono S in 10 mM NH_4OAc (pH 5.3). The roots and outer leaf sheaths of salicylate-treated leek were used for the isolation of leek antifungal protein (23), the bulbs and leaves were employed for preparing garlic chitinase (24) while the inner shoots of untreated chive were utilized for the purification of chive antifungal protein. The yield of the chive antifungal protein was 0.65 mg/kg, lower than the value of 10 mg/kg for the garlic leaf chitinase (24).

The garlic chitinases were characterized with regard to their endochitinase activity but not with reference to their antifungal activity (24). The chive antifungal protein was more potent than leek chitinase (23) in antifungal activity. *Fusarium oxysporum* and *Botrytis cinerea*, which were not affected by leek chitinases (23), were inhibited by the chive antifungal protein. The chive antifungal protein was a fairly hardy protein because it was able to withstand relatively high temperatures, extreme pH and protease digestion. On the other hand, the activity leek chitinase isoforms dwindled as pH was increased or reduced from the optimum at pH 5–6 (23).

In this study a chitinase-like protein from the inner shoots of the chive was isolated. Although it possessed a sequence grossly similar to those of two related monocot species, the garlic *Allium sativum* and the leek *Allium pornum*, its sequence was unique in the sense that it was deficient in cysteine residues in the N-terminal region which is cysteine-rich in other chitinases. The chive protein retarded growth in fungal species against which leek chitinases were ineffectual. Its antifungal activity was acid-stable, alkali-stable, protease-resistant and fairly thermostable.

ACKNOWLEDGMENTS

We thank the Research Grants Council for award of an earmarked grant and Ms. Fion Yung for excellent secretarial assistance.

REFERENCES

1. Benhamou, N., Broglie, K., Broglie, R., and Chet, I. (1993) *Can. J. Microbiol.* **39,** 318–328.
2. Roberts, W. K., and Selitrennikoff, C. P. (1986) *Biochim. Biophys. Acta* **880,** 161–170.
3. Kamemura, K., Furuichi, Y., Umekawa, H., and Takahashi, H. C. (1993) *Biochim. Biophys. Acta* **1158,** 181–188.
4. Birk, Y. (1985) *Int. J. Peptide Protein Res.* **25,** 113–131.
5. Leah, R., Tommerup, H., Svendsen, J., and Mundy, J. (1991) *J. Biol. Chem.* **246,** 1564–1573.
6. Balzarini, J., Schols, D., Neyts, J., Van Damme, E., Peumans, W., and De Clercq, E. (1991) *Antimicrob. Agents Chemother.* **35,** 410–416.
7. Ng, T. B., Huang, B., Fong, W. P., and Yeung, H. W. (1997) *Life Sciences* **61,** 933–949.
8. Hilder, V. A., Powell, K. S., Gatehouse, A. M. R., Gatehouse, J. A., Gatehouse, L. N., Shi, Y., Hamilton, W. D. O., Merryweather, A., Newall, C. A. (1995) *Transgenic Res.* **4,** 18–25.
9. Vogelsang, R., and Barz, C. (1993) *Planta* **189,** 60–69.
10. Zhu, Q., Maher, E. A., Masoud, S., Dixon, R. A., and Lamb, C. (1994) *Bio/Technology* **12,** 807–812.
11. Ye, X. Y., Wang, H. X., and Ng, T. B. (2000) *Life Sci.* **67,** 775–781.
12. Graham, J. S., Burkhart, W., Xiong, J., and Gillikin, J. W. (1992) *Plant Physiol.* **98,** 163–165.
13. Ye, X. Y., and Ng, T. B. (2000) *Biochem. Biophys. Res. Commun.* **273,** 1111–1115.
14. Laemmli, U. K., and Favre, M. (1973) *J. Mol. Biol.* **80,** 575–599.
15. Ye, X. Y., Wang, H. X., and Ng, T. B. (1999) *Biochem. Biophys. Res. Commun.* **263,** 130–134.
16. Collins, R. A., Ng, T. B., Fong, W. P., Wan, C. C., and Yeung, H. W. (1997) *Life Sci.* **69,** 933–949.
17. Wang, H. X., Liu, W. K., Ng, T. B., Ooi, V. E. C., and Chang, S. T. (1995) *Life Sci.* **57,** 269–281.
18. Ooi, L. S. M., Ng, T. B., Geng, Y., and Ooi, V. E. C. (2000) *Biochem. Cell Biol.*, in press.
19. Wang, H. X., and Ng, T. B. (1999) *Biochem. Biophys. Res. Commun.* **264,** 714–718.

20. Pelham, R. B., and Jackson, R. J. (1976) *Eur. J. Biochem*. **67,** 247–256.

21. Dong, T. X., Ng, T. B., Wong, R. N. S., Yeung, H. W., and Xu, G. J. (1993) *Int. J. Biochem*. **25,** 415–419.

22. Ye, X. Y., Wang, H. X., and Ng, T. B. (2000) *Biochem. Biophys. Res. Commun*. **269,** 155–159.

23. Vergawen, R., Van Leuven, F., and Van Laere, A. (1998) *Physiol. Plant*. **104,** 175–182.

24. Van Damme, E. J. M., Willems, P., Torrekans, S., Van Leuven, F., and Peumans, W. J. (1993) *Physiol. Plant*. **87,** 177–186.

25. Graham, L. S., and Sticklen, M. B. (1994) *Can. J. Bot*. **72,** 1057–1083.