Biochemical and Biophysical Research Communications **289,** 91–96 (2001)
doi:10.1006/bbrc.2001.5965, available online at http://www.idealibrary.com on IDEAL®

A Bowman–Birk-Type Trypsin-Chymotrypsin Inhibitor from Broad Beans

X. Y. Ye,* T. B. Ng,* and P. F. Rao†

**Department of Biochemistry, Faculty of Medicine, Chinese University of Hong Kong, Shatin, Hong Kong, China; and †College of Light Industry, Institute of Biotechnology, Fuzhou University, China*

Received October 18, 2001

An isolation procedure comprising affinity chromatography on Affi-gel blue gel, ion exchange chromatography on SP-Toyopearl, and fast protein liquid chromatography on Mono S was used to purify a peptide from broad beans which manifested antifungal activity toward *Mycosphaerella arachidicola, Fusarium oxysporum,* and *Botrytis cinerea.* The peptide demonstrated a molecular mass of 7.5 kDa. N-terminal sequence analysis disclosed the identity of the antifungal peptide to be a trypsin-chymotrypsin inhibitor. The trypsin-chymotrypsin inhibitor also exerted an inhibitory action on chymotrypsin activity and HIV-1 reverse transcriptase activity. Proliferation of murine splenocytes was stimulated in the presence of the trypsin-chymotrypsin inhibitor. This report constitutes the first observation of antifungal activity of a leguminous peptidic protease inhibitor. © 2001 Academic Press

Key Words: **broad bean; trypsin inhibitor; antifungal; mitogenic.**

Antifungal proteins have captured the attention of a large number of researchers on account of the economic implications associated with the protection of crops against fungal attack. To date a diversity of antifungal proteins with different amino acid sequences has been isolated. Based on their structures or activities antifungal proteins have been classified into chitinases (1–6), glucanases (2), thaumatin-like proteins (7, 8), miraculin-like proteins (9), cyclophilin-like proteins (10), allergen-like proteins (11), embryo-abundant protein-like proteins (12), ribosome inactivating proteins (13, 14), and antifungal peptides (15).

Leguminous plants elaborate a range of antifungal proteins. Multiple antifungal proteins may be produced by a single leguminous species, for instance, the chickpea which produces a glucanase, a chitinase, and a thaumatin-like protein (2), all with dissimilar sequences. The cowpea also produces a chitinase in addition to a novel antifungal protein (5). We were thus prompted to look for antifungal proteins in the broad bean. The chromatographic fractions derived from the broad bean extract were monitored for antifungal activity. The active principle thus isolated was found to be a trypsin-chymotrypsin inhibitor by sequencing. The present report constitutes the first demonstration of the antifungal, anti-human immunodeficiency virus reverse transcriptase, and mitogenic activities of a leguminous trypsin-chymotrypsin inhibitor. These activities add to the well-known anti-carcinogenic activity of trypsin-chymotrypsin inhibitors (16).

MATERIALS AND METHODS

Broad beans (*Vicia faba*) were obtained from a local vendor. They were first soaked in distilled water for a few hours before homogenization. To the supernatant obtained after centrifugation, Tris–HCl buffer (pH 7.2) was added until the final concentration attained 10 mM. The supernatant was then chromatographed on a column of Affi-gel blue gel (2.5 × 10 cm) in 10 mM Tris–HCl buffer (pH 7.2). After elution of unadsorbed protein, adsorbed protein was desorbed with a linear gradient of 0–0.5 M NaCl in the starting buffer. The adsorbed peak A was then fractionated on a column of SP-Toyopearl (1.5 × 18 cm). The column was eluted initially with 10 mM Tris–HCl buffer to remove unbound material and subsequently with a linear gradient of 0–0.5 M NaCl to desorb bound material. Fraction P2 was further purified by FPLC on a Mono S column (1 ml) in 10 mM Tris–HCl buffer (pH 7.2). After elution of unadsorbed material, the column was eluted with a linear concentration gradient of 0–0.5 M NaCl in the Tris–HCl buffer to elute adsorbed material. The purity and molecular weight of the fraction with antifungal activity were assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli and Favre using 15% gel (17). N-terminal sequencing of the purified antifungal peptide was conducted using a Hewlett–Packard G-1000A Edman degradation unit and an HP 1000 HPLC system.

Assay for antifungal activity. The antifungal activity of the purified peptide was assayed using sterile petri plates (100 × 15 mm) containing 10 ml potato dextrose agar. After the mycelial colony had developed, sterile paper disks (0.625 cm in diameter) were placed at a distance of 1 cm from the rim of the mycelial colony. The test sample was added to a disk, and the plate was incubated at 23°C until mycelial growth had enveloped disks containing the control (buffer) and had formed crescents of inhibition around disks with samples expressing antifungal activity.

0006-291X/01 $35.00

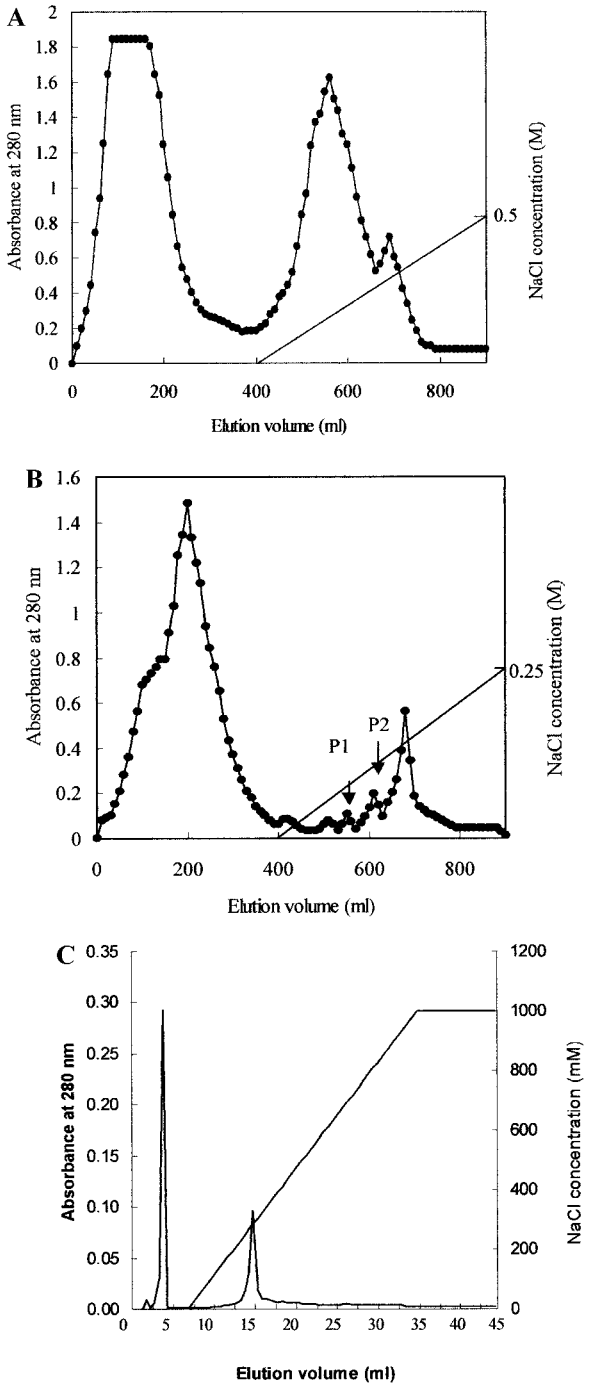

FIG. 1. (A) Affinity chromatography of crude extract of broad beans on Affi-gel blue gel. (B) Ion exchange chromatography of the first adsorbed peak from Affi-gel blue gel on SP-Toyopearl. (C) Fast protein liquid chromatography of peak P2 on Mono S.

The fungi studied comprised *Mycosphaerella arachidicola, Fusarium oxysporum,* and *Botrytis cinerea* (6). The IC_{50} value of antifungal activity was also determined (6). The purified peptide is stable at the incubation temperature.

Assay for cell-free translation-inhibiting activity. The purified antifungal peptide was assayed for cell-free translation-inhibiting activity in a rabbit reticulocyte lysate system as described by Lam *et al.* (18). Rabbit reticulocyte lysate was prepared from the blood of rabbits rendered anemic by phenylhydrazine injections. The antifungal peptide (10 μl) was added to 10 μl of hot mixture (500 mM KCl, 5 mM $MgCl_2$, 130 mM phosphocreatine, and 1 μCi[4,5-^{3}H] leucine) and 30 μl working rabbit reticulocyte lysate containing 0.1 μM hemin and 5 μl creatine kinase. Incubation proceeded at 37°C for 30 min before addition of 330 μl 1 M NaOH and 1.2% H_2O_2. Further incubation for 10 min allowed decolorization and tRNA digestion. An equal volume of the reaction mixture was then added to 40% trichloroacetic acid with 2% casein hydrolyzate in a 96-well plate to precipitate radioactively labeled protein. The precipitate was collected on a glass fiber Whatman GF/A filter, washed and dried with absolute alcohol passing through a cell harvester attached to a vacuum pump. The filter was suspended in scintillant and counted in an LS6500 Beckman liquid scintillation counter.

Assay for anti-HIV reverse transcriptase activity. The assay for ability to inhibit human immunodeficiency virus (HIV) reverse transcriptase activity was carried out as detailed by Collins *et al.* (19) using a nonradioactive ELISA kit. The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hybrid poly(A) · oligo (dT) 15. In place of radiolabeled nucleotides, digoxigenin-, and biotin-labeled nucleotides in an optimized ratio are incorporated into one and the same DNA molecule, which is freshly synthesized by the reverse transcriptase (RT). The detection and quantification of synthesized DNA as a parameter for RT activity follows a sandwich ELISA protocol: biotin-labeled DNA binds to the surface of microtiter plate modules that have been precoated with streptavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase (anti-DIG-POD), binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrate is added. The peroxidase enzyme catalyzes the cleavage of the substrate, producing a colored reaction product. The absorbance of the samples at 405 nm can be determined using a microtiter plate (ELISA) reader and is directly correlated to the level of RT activity. A fixed amount (4–6 ng) recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of the antifungal peptide was calculated as percent inhibition compared to a control without the antifungal peptide.

Assay for mitogenic activity. The antifungal peptide was assayed for mitogenic activity in mouse splenocytes as detailed by Wang *et al.* (20). Four C57BL/6 mice (20–25 g) were killed by cervical dislocation and the spleens were aseptically removed. Spleen cells were isolated by pressing the tissue through a sterilized 100-mesh stainless steel sieve and resuspended to 5×10^6 cells/ml in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 100 units penicillin/ml, and 100 μg streptomycin/ml. The cells (7×10^5 cells/100 μl/well) were seeded into a 96-well culture plate and serial dilutions of a solution of (containing 100 μg in the first well) 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 uCi, 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 (20).

Measurement of trypsin and chymotrypsin inhibitory activities. A portion of the inhibitor was incubated with 25 μg trypsin or chymotrypsin in 100 μl of 50 mM Tris–HCl buffer (pH 8.0) containing 200 mM $CaCl_2$ for 5 min at 25°C. Residual trypsin or chymotrypsin activity was determined by adding 300 μl of 1% casein substrate at 25°C. The reaction was terminated by adding 1 ml of cold 5% trichloroacetic acid after a 15 min incubation. The reactions mixture was centrifuged for 20 min at 10,000 rpm. The absorbance of the clear supernatant was determined at 280 nm.

TABLE 1

Summary of Protein Yields at Various Stages of Purification of Trypsin-Chymotrypsin Inhibitor from Broad Bean

Purification step	Proteins (mg[a])
Crude extract	33200
Affi-Gel Blue Gel (adsorbed fraction A)	296.6
SP-Toyopearl (fraction P2)	32.9
Mono S (adsorbed fraction)	16.5

[a] Protein obtained from 150 g broad beans.

RESULTS

Affinity chromatography of the broad bean extract on Affi-gel blue gel yielded a large unadsorbed peak and two adsorbed peaks, a large one and a small one (Fig. 1A). The large adsorbed peak was in turn applied onto a SP-Toyopearl column to produce a big unadsorbed peak and several small adsorbed peaks including P2 (Fig. 1B). P2 was next chromatographed on an FPLC-Mono S column to give a large unadsorbed peak and a small adsorbed peak (Fig. 1C). The protein yields at various chromatographic steps are presented in Table 1. The small adsorbed peak contained a purified peptide that exhibited a molecular mass of 7.5 kDa in SDS–polyacrylamide gel electrophoresis (Fig. 2) and an N-terminal sequence GDDVKSACCDTCLCTK-SEPPT identical to that of the broad bean trypsin inhibitor. The trypsin-chymotrypsin inhibitor inhibited trypsin as well as chymotrypsin (Fig. 3).

The broad bean trypsin-chymotrypsin inhibitor exerted a prominent suppressive action on mycelial growth in *Fusarium oxysporum* and *Mycosphaerella arachidicola* and a less notable inhibitory effect on *Botrytis cinerea* (Fig. 4). The IC_{50} value of the antifungal activity of the trypsin-chymotrypsin inhibitor toward *B. cinerea* was 51 μM (Fig. 5) HIV-1 reverse transcriptase activity was also curtailed (Table 2). A strong stimulatory influence on proliferation of mouse spleen cells was observed (Table 3).

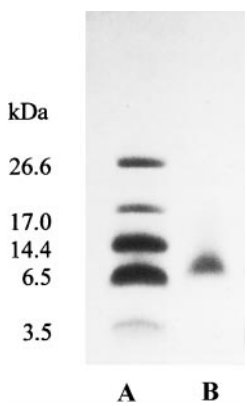

FIG. 2. SDS–polyacrylamide gel electrophoresis. Lane A, molecular weight markers. Lane B, broad bean trypsin-chymotrypsin inhibitor.

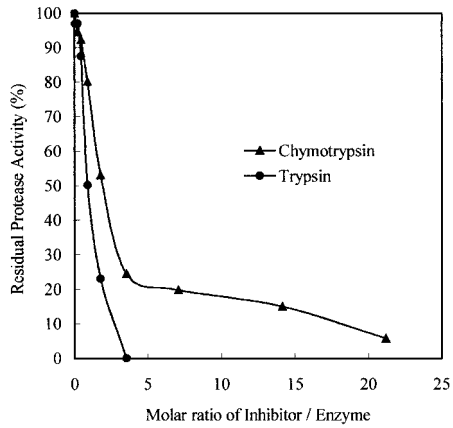

FIG. 3. Inhibitory activity of broad bean trypsin-chymotrypsin inhibitor on trypsin and chymotrypsin.

DISCUSSION

The potent antifungal peptide isolated from broad beans is a trypsin-chymotrypsin inhibitor. Evidence has accumulated showing that some trypsin inhibitors express antifungal activity. A 2S-albumin-like trypsin inhibitor from barley seeds had some antifungal activity (21). It acted synergistically with thionins to permeabilize fungal membranes (22). Trypsin and chymotrypsin inhibitors from cabbage leaves induced leakage of intracellular contents from susceptible fungal species (23). A corn 14-kDa trypsin inhibitor retarded the growth of *Aspergillus flavus*. The inhibitor was present at high concentrations in *Aspergillus*-resistant genotypes, and either detected at low levels or absent altogether in *Aspergillus*-susceptible genotypes (24). The results of the study of Chen *et al.* (25) revealed that the resistance of certain corn genotypes to fungal infection might be related to the action of trypsin inhibitor in lowering the production of fungal α-amylase and its activity and consequently reducing the availability of simple sugars for fungal growth. A trypsin inhibitor from wheat kernel also elicited a potent antifungal

TABLE 2

Inhibition of HIV-Reverse Transcriptase Caused by Broad Bean Trypsin-Chymotrypsin Inhibitor

	Concentration (μM)	Inhibition (%) (mean ± SD, n = 3)
Trypsin-chymotrypsin inhibitor	196.1	100 ± 1.2
	98.0	87.8 ± 3.4
	49.0	58.2 ± 2.1
	24.5	29.6 ± 1.1

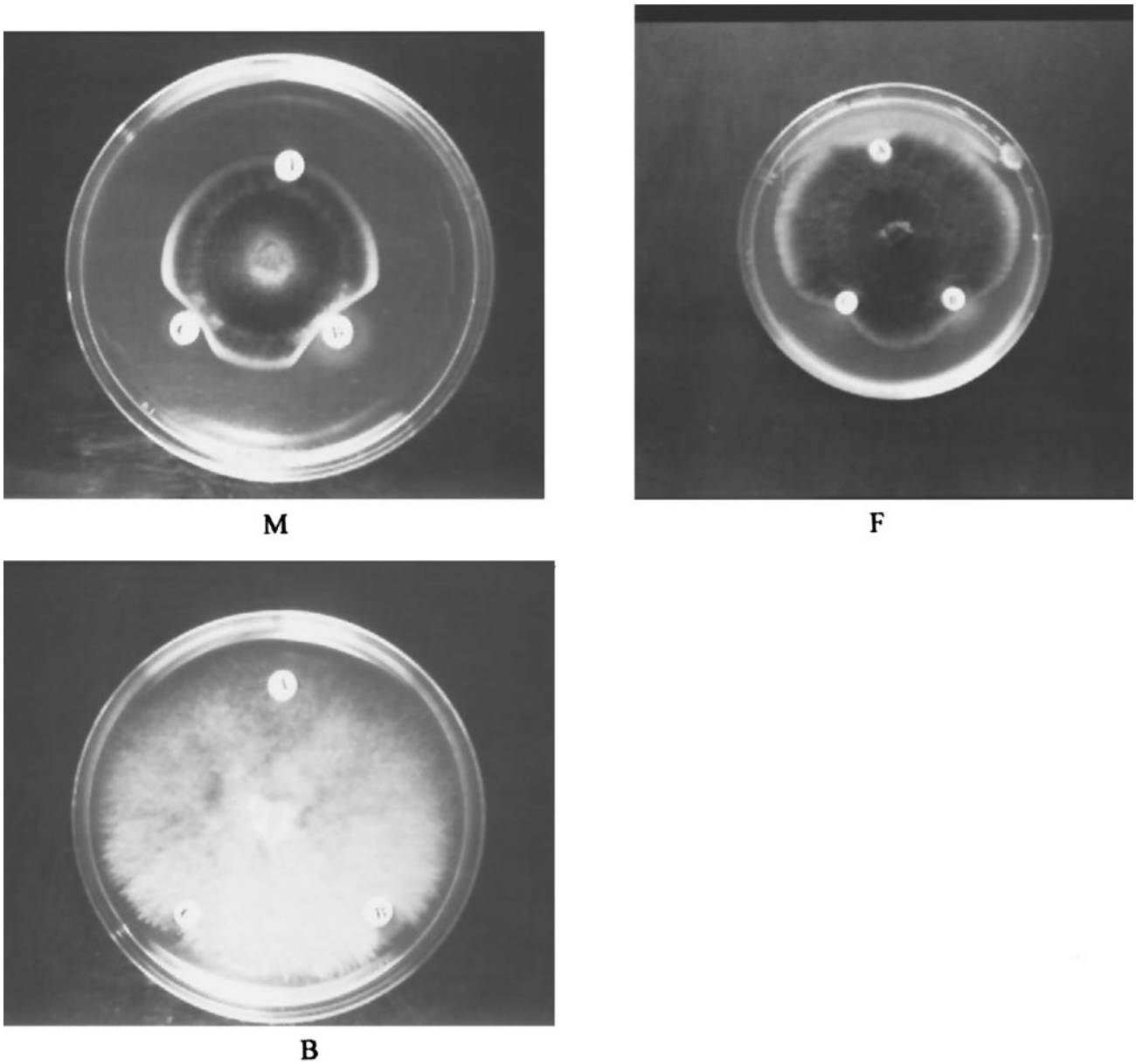

FIG. 4. Antifungal activity of broad bean trypsin-chymotrypsin inhibitor on (M) *Mycosphaerella arachidicola*, (F) *Fusarium oxysporum*, and (B) *Botrytis cinerea*. (A) control, (B) 300 μg inhibitor, (C) 60 μg inhibitor.

effect (26). A 28-kDa alkaline protease inhibitor from *Streptomyces* (27), a 24-kDa cysteine protease inhibitor from pearl millet (28), and the HIV-1 protease inhibitors (29) exhibited antifungal activity. Whether the HIV-1 protease inhibitors are useful for treatment of mucosal candidiasis resistant to current antimycotics in HIV-1 infected subjects remains to be tested (29). Antileukoprotease, a protease inhibitor or mucosal surfaces including those of the respiratory and genital tracts, exerts antifungal activity (30) and may present a new therapeutic option (31). The present investigation furnished evidence for the antifungal activity of a leguminous trypsin inhibitor.

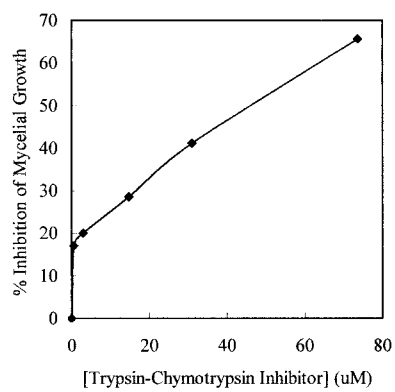

FIG. 5. Determination of IC_{50} for antifungal activity of broad bean trypsin-chymotrypsin inhibitor toward Botrytis cinerea.

TABLE 3

Stimulatory Activity of Broad Bean Trypsin-Chymotrypsin Inhibitor on the Proliferation of Mouse Splenocytes in Vitro

Trypsin-chymotrypsin inhibitor		Con A	
Dose (μM)	Methyl[^{3}H]-thymidine uptake (mean ± SD, n = 3)	Dose (μM)	Methyl[^{3}H]-thymidine uptake (mean ± SD, n = 3)
147.00	4402.0 ± 25	7.35	1779.0 ± 34
73.50	5266.7 ± 37	3.67	2328.0 ± 13
36.80	6778.0 ± 26	1.84	2458.8 ± 83
18.40	13122.0 ± 92	0.91	3834.3 ± 102
9.20	15656.0 ± 39	0.46	6837.3 ± 34
4.60	12077.0 ± 81	0.23	7754.7 ± 81
2.30	3081.0 ± 12	0.12	8466.9 ± 54
1.15	2879.0 ± 53	0.06	5857.0 ± 93
0.58	2477.0 ± 16	0.03	3464.3 ± 24
0.29	1770.0 ± 43	0.02	1946.0 ± 14
0	1466.0 ± 23	0	1442.0 ± 31

The majority of leguminous antifungal proteins previously tested including dolichin, French bean thaumatin-like protein and mungin did not exert a striking antifungal effect on *Fusarium oxysporum* (1, 7, 10). In contradistinction, broad bean trypsin-chymotrypsin inhibitor evoked a strong antifungal action against *F. oxysporum*. It was also potent against *Mycosphaerella arachidicola,* producing a similar extent of inhibition to that caused by ginkbilobin (12). However, the antifungal effect of broad bean trypsin-chymotrypsin inhibitor toward *Botrytis cinerea* was attenuated in comparison with other leguminous antifungal proteins including ginkbilobin (12).

Apart from its high antifungal potency, broad bean trypsin-chymotrypsin inhibitor manifested a strong stimulatory effect on proliferation of murine splenocytes as indicated by incorporation of methyl [^{3}H] thymidine. It deserves mention that the cyclophilin-like antifungal protein from mung bean suppressed (10) while the antifungal protein from garland chrysanthemum seeds stimulated (32) splenocyte proliferation.

The mitogenic effect of broad bean trypsin-chymotrypsin inhibitor was less potent than that of Con A, but the maximal mitogenic response attained in the former case was greater than and approximately double that of the latter.

A variety of antifungal proteins were inhibitory to the activity of HIV-1 reverse transcriptase (1, 5, 6). Similarly broad bean trypsin-chymotrypsin inhibitor inhibited the HIV enzyme, and it did so with a potency similar to those of some natural products (33).

Trypsin inhibitors may possess an anti-cancer property (16). The aforementioned activities shown by broad bean trypsin-chymotrypsin inhibitor provide further evidence for the therapeutic potential of this class of protease inhibitors.

ACKNOWLEDGMENTS

We thank the Research Committee, Chinese University of Hong Kong, for award of a direct grant, and Miss Fion Yung for excellent secretarial assistance.

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