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A New Peptidic Protease Inhibitor from *Vicia faba* Seeds Exhibits Antifungal, HIV-1 Reverse Transcriptase Inhibiting and Mitogenic Activities

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Abstract: A new trypsin–chymotrypsin inhibitor, with an *N*-terminal sequence showing some differences from the previously reported trypsin–chymotrypsin inhibitor, was isolated from the broad bean *Vicia faba*. The inhibitor was a peptide with a molecular mass of 13 kDa. It was adsorbed on Affi-gel blue gel and CM-Sepharose. It exerted antifungal activity toward *Mycosphaerella arachidicola* and *Physalospora piricola*. In addition, the trypsin–chymotrypsin inhibitor elicited a mitogenic response from mouse splenocytes and inhibited the activity of human immunodeficiency virus-1 reverse transcriptase. Copyright © 2002 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: protease inhibitor; broad beans; antifungal; trypsin-chymotrypsin inhibitor; HIV-1 reverse transcriptase inhibitor

INTRODUCTION

Antifungal compounds including antifungal proteins and peptides are produced by plants [1–15] and animals [16] to combat fungal attack. Antifungal proteins and peptides can be categorized, on the basis of their structures and/or functions, into many different types such as chitinases [2,8,9,15,17], glucanases [9], ribosome inactivating proteins [3,4], cyclophilin-like proteins [11], miraculin-like proteins [14], thaumatin-like proteins [13], embryo-abundant proteins [10], protease inhibitors [6,18,19] and others [5,20,21].

Some of the aforementioned antifungal proteins and peptides have been purified from leguminous species [11–15,17]. Different antifungal proteins and peptides may vary in the specificity and/or potency of their antifungal action [5,10–15,17,22]. Novel antifungal compounds may be disclosed by examining different leguminous species [11,12,14,15]. The present study was thus undertaken on broad bean (*Vicia faba*) seeds. It revealed the presence of a trypsin–chymotrypsin inhibitor with an *N*-terminal sequence manifesting some variations from the trypsin–chymotrypsin inhibitor previously reported from the same species [19]. This new protease inhibitor displayed a suppressive action on mycelial growth in two fungi, a mitogenic activity toward murine splenocytes and an inhibitory effect on the activity of HIV-1 reverse transcriptase.

MATERIALS AND METHODS

Isolation Procedure

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

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(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 × 18 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 50 mM acetate buffer (pH 4.5) to remove unbound material and subsequently with a linear gradient of 0–0.5 M NaCl to desorb bound material. Fraction BP2 was further purified by FPLC on a Superdex 75 (Amersham Biosciences) column in 200 mM NH_4HCO_3 buffer (pH 8.2). The purity and molecular mass of the fraction with antifungal activity were assessed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli and Favre [23] using 15% gel. *N*-terminal sequencing of the purified 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. The fungi studied comprised *Mycosphaerella arachidicola* and *Physalospora piricola* [2,19,22]. The purified peptide is stable at the incubation temperature.

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.* [24] using a nonradioactive ELISA kit. The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hydrid poly(A) · oligo (dT) 15. In place of radio-labelled nucleotides, digoxigenin- and biotin-labelled 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-labelled DNA binds to the surface of microtitre 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-labelled DNA. In the final step, the peroxidase substrate is added. The peroxidase enzyme catalyses the cleavage of the substrate, producing a coloured reaction product. The absorbance of the samples at 405 nm can be determined using a microtitre plate (ELISA) reader and is directly correlated to the level of RT activity. A fixed amount of (4–6 ng) recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of the peptide was calculated as the percent inhibition compared with a control without the peptide.

Assay for Mitogenic Activity

The peptide was assayed for mitogenic activity in mouse splenocytes as detailed by Wang *et al.* [22,25]. 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 the peptide (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%

Table 1 Comparison of *N*-terminal Sequence of Broad Bean Trypsin Inhibitor Isolated in the Present Study with that from the Literature [24]

Broad bean protease inhibitor (this study)	GDKVKSACCDTTLLKKKEHPPPRR
Broad bean protease inhibitor (literature)	GDDVKSACCDTCLCTKSEPPTCPC

Identical amino acid residues are underlined.

CO_2 for 24 h, 10 µl methyl [^{3}H]-thymidine (0.25 µCi, Amersham Biosciences) 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 [22,25].

Measurement of Trypsin and Chymotrypsin Inhibitory Activities

A portion of the peptide 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 15 min incubation. The reaction mixture was centrifuged for 20 min at 10 000 rpm. The absorbance of the clear supernatant was determined at 280 nm [19].

RESULTS

Affinity chromatography of the crude extract of broad beans on Affi-gel blue gel yielded a large unadsorbed peak and two adsorbed peaks A and B (Figure 1a). Ion exchange chromatography of peak B on SP-Toyopearl gave rise to a large unadsorbed peak, a tiny adsorbed peak BP1 and a much larger adsorbed peak BP2 (Figure 1b). FPLC-gel filtration of peak BP2 on Superdex 75 produced a large peak and a small peak. The latter peak designated SP2 (Figure 1c) represents purified protease inhibitor with a molecular mass of 13 kDa in SDS-PAGE (Figure 2). The *N*-terminal sequence of the purified protease inhibitor is presented in Table 1. Its yield was 8 mg/kg seeds (Table 2). It inhibits HIV-1 reverse transcriptase with an IC_{50} of 32 µM (Table 3). It stimulates the proliferation of mouse splenocytes. An approximately 10-fold stimulation was achieved at 6 µM broad bean protease inhibitor while a 6-fold stimulation was brought about by 0.12 µM Con A (Table 4). The protease inhibitor inhibited both trypsin and chymotrypsin (Figure 3). It inhibited mycelial growth in both *Mycosphaerella arachidicola* and *Physalospora piricola* (Figure 4).

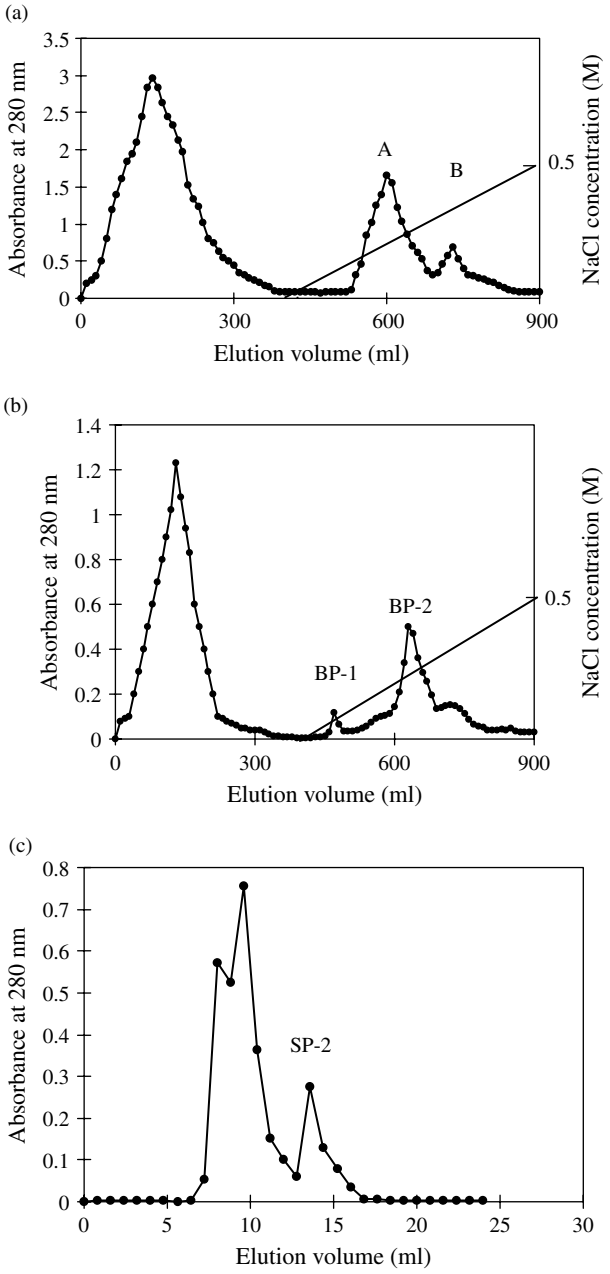


Figure 1 (a) Affinity chromatography of crude extract of broad beans on Affi-gel blue gel. (b) Ion exchange chromatography of the first adsorbed peak from Affi-gel blue gel on SP-Toyopearl. (c) Fast protein liquid chromatography of peak BP2 on Mono S.

DISCUSSION

The protease inhibitor isolated in this study from broad bean seeds differs from the previously reported broad bean trypsin inhibitor [19] from the same source, at various positions along the *N*-terminal sequence, although a gross similarity is discernible. The phenomenon of dual/multiple protease inhibitors or antifungal proteins/peptides

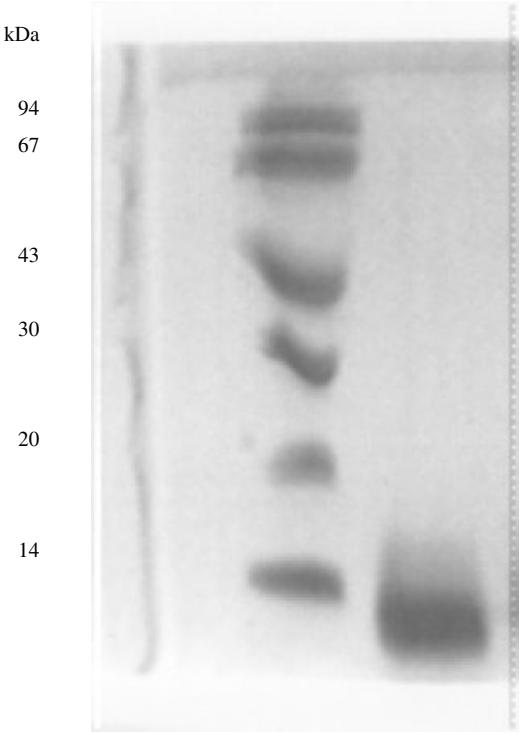


Figure 2 SDS-polyacrylamide gel electrophoresis. Lane a: molecular weight markers. Lane b: broad bean trypsin–chymotrypsin inhibitor.

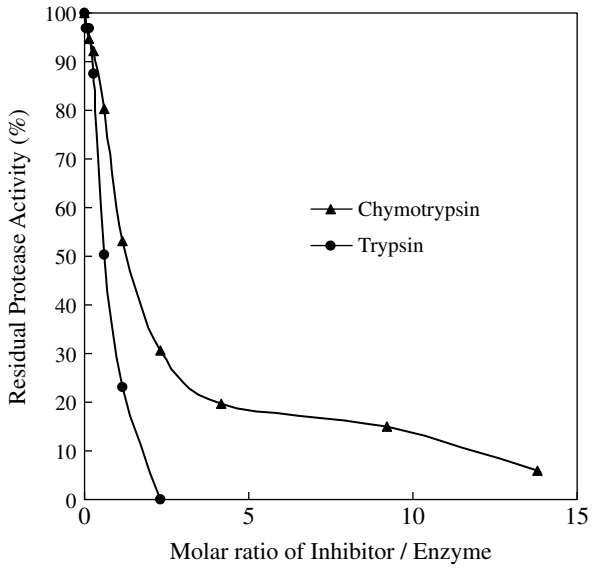
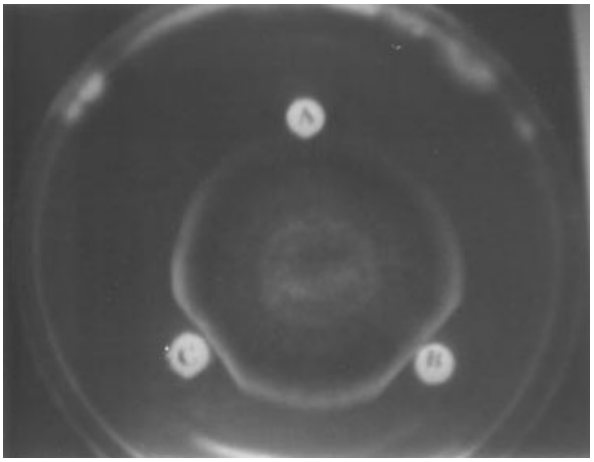


Figure 3 Inhibitory activity of broad bean trypsin–chymotrypsin inhibitor on trypsin and chymotrypsin.



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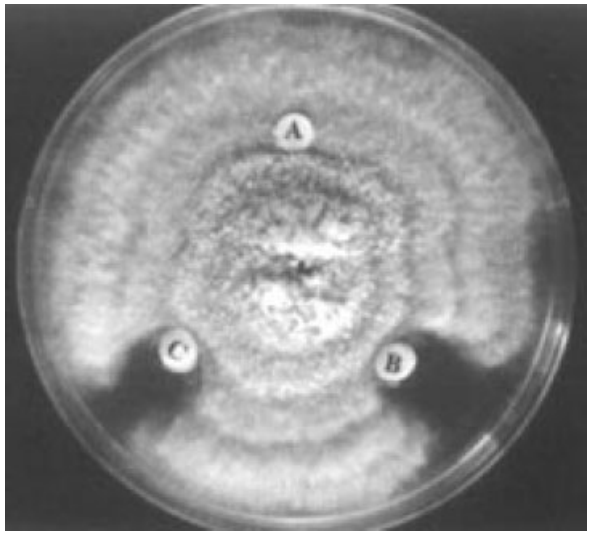

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Figure 4 Antifungal activity of broad bean trypsin–chymotrypsin inhibitor on (M) *Mycosphaerella arachidicola* and (P) *Physalospora piricola*. (A) control (B) 300 μg inhibitor (C) 60 μg inhibitor.

from the same source has been observed previously [4,9,15].

A cysteine protease inhibitor from pearl millet seeds [26] and an alkaline protease inhibitor from *Streptomyces* [11] species exert an antifungal action. Protease inhibitors from corn [18,27], wheat [28] and cabbage [29] are also known to elicit an antifungal effect. The presence of the antifungal protease inhibitor in fungus-resistant genotypes and its absence in fungus-susceptible genotypes add to the physiological significance of protease inhibitors as molecules of defence against pathogens [18].

Table 2 Summary of Protein Yields at Various Stages of Purification of Trypsin–Chymotrypsin Inhibitor from Broad Bean

Purification step	Protein yield (mg[a])
Crude extract	33 200
Affi-gel blue gel (adsorbed fraction B)	87.1
SP-Toyopearl (fraction BP2)	43.5
Superdex 75 (fraction SP-2)	1.2

[a] From 150 g broad beans.

Table 3 Inhibition of HIV-1 Reverse Transcriptase Caused by Broad Bean Trypsin–Chymotrypsin Inhibitor

Concentration (μM)	Inhibition (%) (mean ± SD, $n = 3$)
128.2	100 ± 1.4
64.1	83.8 ± 2.7
32.0	51.2 ± 1.1
16.0	30.5 ± 2.1

Recently the antifungal activity of HIV-1 protease inhibitors has been demonstrated and these anti-HIV drugs may be useful in the treatment of fungal disease in HIV-infected patients [30]. Antileukoprotease is a protease inhibitor present on mucosal surfaces including those of respiratory and genital tracts. It exhibits antifungal activity [20,31]. The bulk of the aforementioned experimental observations constitutes evidence for the antifungal activity of different classes of protease inhibitors. The broad bean protease inhibitor isolated in the present investigation belongs to the Bowman-Birk type of inhibitors. The present findings would support the addition of Bowman-Birk trypsin inhibitors to the list of protease inhibitors with antifungal activity.

The broad bean protease inhibitor isolated in this study exhibited potent mitogenic activity toward mouse splenocytes. The observation is noteworthy because only some of the antifungal proteins examined, such as chrysancorin from garland chrysanthemum seeds [22], demonstrate mitogenic activity. Others like mungin from mung beans possess antimitogenic activity [11], and the chitinase-like antifungal protein from chive is devoid of any influence on the proliferation of mouse splenocytes [2].

The trypsin-chymotrypsin inhibitor from broad bean was capable of reducing the activity of HIV-1 reverse transcriptase, an enzyme crucial to the life cycle of the pathogenic retrovirus, with a potency comparable to other anti-HIV natural products [32]. This activity is also an attribute of many antifungal proteins previously examined [15,17]. It is well established that leguminous protease inhibitors

Table 4 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, $n = 3$)
96.1	3400.6 ± 15	7.35	1779.0 ± 34
48.1	4237.1 ± 25	3.67	2328.0 ± 13
24.1	5706.8 ± 31	1.84	2458.8 ± 83
12.0	11032.5 ± 82	0.91	3834.3 ± 102
6.1	14389.1 ± 26	0.46	6837.3 ± 34
3.0	12354.9 ± 61	0.23	7754.7 ± 81
1.5	6345.7 ± 31	0.12	8466.9 ± 54
0.8	3896.5 ± 24	0.06	5857.0 ± 93
0.4	2105.8 ± 26	0.03	3464.3 ± 24
0.2	1632.4 ± 23	0.02	1946.0 ± 14
0	1466.0 ± 23	0	1442.0 ± 31

The results were reproducible in two subsequent similar experiments.

have anti-insect and antitumour [33] activities. The antifungal, mitogenic and anti-HIV reverse transcriptase activities of the broad bean protease inhibitor uncovered in the present study are additional potentially exploitable properties.

The protease inhibitor isolated in the present study differed from that reported earlier [19] in possessing a larger molecular mass (13 kDa versus 7.5 kDa), slightly more potent mitogenic and HIV-1 reverse transcriptase activities, and it was isolated in lower yield (8 mg/kg seeds compared with 110 mg/kg seeds). Their trypsin-chymotrypsin inhibitory activities are, however, similar. Both bring about similar extents of inhibition of mycelial growth in *Mycosphaerella arachidicola*.

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