Lipid transfer proteins from *Brassica campestris* and mung bean surpass mung bean chitinase in exploitability

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Abstract: Antifungal peptides with a molecular mass of 9 kDa and an *N*-terminal sequence demonstrating remarkable similarity to those of nonspecific lipid transfer proteins (nsLTPs) were isolated from seeds of the vegetable *Brassica campestris* and the mung bean. The purified peptides exerted an inhibitory action on mycelial growth in various fungal species. The antifungal activity of *Brassica* and mung bean nsLTPs were thermostable, pH-stable, and stable after treatment with pepsin and trypsin. In contrast, the antifungal activity of mung bean chitinase was much less stable to changes in pH and temperature. *Brassica* LTP inhibited proliferation of hepatoma Hep G2 cells and breast cancer MCF 7 cells with an IC_{50} of 5.8 and 1.6 μM, respectively, and the activity of HIV-1 reverse transcriptase with an IC_{50} of 4 μM. However, mung bean LTP and chitinase were devoid of antiproliferative and HIV-1 reverse transcriptase inhibitory activities. In contrast to the mung bean LTP, which exhibited antibacterial activity, *Brassica* LTP was inactive. All three antifungal peptides lacked mitogenic activity toward splenocytes. These results indicate that the two LTPs have more desirable activities than the chitinase and that there is a dissociation between the antifungal and other activities of these antifungal proteins. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: lipid transfer proteins; antifungal proteins; mung bean; *Brassica compestris*

INTRODUCTION

Antifungal proteins are produced by leguminous as well as nonleguminous plants. From leguminous plants chitinases, glucanases, defensins, protease inhibitors, and lipid transfer proteins (LTPs) have been extracted and purified. From nonleguminous plants, embryo-abundant proteins, thaumatin-like proteins, and other antifungal proteins have been isolated. The aforementioned proteins exhibit antifungal activity [1].

Napins and napin-like polypeptides have been isolated from the seeds of *Brassica* species which are common vegetables [2]. However, no reports about the antifungal activity of *Brassica* seeds have appeared. The isolation and characterization of an LTP with remarkably stable antfiungal activity from *B. campestris* seeds are described here. This protein is then compared with its counterpart from mung bean and also with mung bean chitinase. A similar LTP from mung bean seeds [3] also exhibits spectacular stability despite drastic changes in ambient temperature and pH and upon exposure to proteases. By contrast, mung bean chitinase [4] is far less stable.

MATERIALS AND METHODS

Materials

Seeds of *Brassica campestris* L. var. purpurea Bailey and *Phaseolus mungo* were purchased from a local vendor. The fungi were provided by Department of Microbiology, China Agricultural University, China, and the Department of Plant Pathology, FuJian Agricultural University, China. POROS-HS was purchased from PerSeptive Biosystem Co. (USA). CM-Sephadex C-50, Q-Sepharose, Mono S and Superdex Peptide were from Amersham Biosciences (Sweden), and Affi-gel blue gel was from Bio-Rad (USA). Standard proteins for molecular mass determination were purchased from Gibco-BRL (Life Tech., USA). All chemicals were of the highest purity available.

Isolation of Antifungal Peptides

The crude extract of *B. campestris* seeds was subjected to ion exchange chromatography on a 5×20 cm column of Q-Sepharose (Amersham Biosciences) that had been equilibrated and was then eluted with 10 mM Tris–HCl buffer (pH 7.8). After the unadsorbed proteins (fraction Q1) had come off the column, the column was eluted with 10 mM Tris–HCl buffer (pH 7.8) containing 1 M NaCl to yield fraction Q2. Fraction Q1 was then chromatographed on a 2.5×20 cm column of Affi-gel blue gel (Bio-Rad) in 10 mM Tris–HCl buffer (pH 7.8). Unadsorbed proteins (fraction B1) were eluted with the same buffer, while adsorbed proteins (fraction B2) were eluted with 10 mM Tris–HCl buffer (pH 7.8) containing 1 M NaCl. Fraction B2 was taken for purification on a Mono S (Amersham Bioscience) column in 10 mM NH$_4$OAc buffer (pH 4.5). After elution of the unadsorbed proteins, the column

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was eluted with a linear 0–0.1 M NaCl gradient in the starting buffer to desorb the first adsorbed fraction S2, which was then subjected to final purification on a Superdex Peptide column (Amersham Bioscience). The main peak constituted the purified antifungal peptide.

The isolation of a nonspecific LTP and a chitinase from *P. mungo* seeds has been reported [3,4]. Briefly, the seeds were soaked and homogenized. The homogenate was centrifuged. The supernatant was designated as the crude extract. The crude extract was first fractionated by ammonium sulfate to 20% saturation. After centrifugation, ammonium sulfate was added to the supernatant obtained to 80% saturation. The mixture was then centrifuged. The supernatant was discarded, while the precipitate was collected and dissolved. The solution of ammonium sulfate precipitate was dialysed against 0.02 M sodium acetate buffer (pH 5.4) with several changes, and then applied to an open CM-Sephadex C-50 column (2.5×55 cm) previously equilibrated with the starting buffer. After removal of the unadsorbed proteins, the column was eluted with a linear gradient of NaCl (200–400 mM) in the same buffer. The first fraction (P1) was pooled, dialysed against 0.02 M phosphate-buffered saline (PBS), pH 6.0 at $4\,°C$ for 24 h, and subsequently chromatographed on a column of POROS-HS (0.75×7.5 cm) that had been equilibrated with 0.02 M PBS, pH 6.0. Following removal of a large quantity of unadsorbed materials, the column was eluted with a gradient of NaCl (0–0.7 M) in the same buffer to yield four peaks. The fourth eluted peak represented the purified mung bean LTP, while the first peak contained chitinase which could be further purified on Sephadex G15 to yield purified chitinase [3,4].

Protein Determination

Protein concentration was determined by the dye-binding method (Bio-Red) using bovine serum albumin as a standard.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was conducted according to the method of Laemmli and Favre [5]. After electrophoresis, the gel was stained with Coomassie Brilliant Blue. The molecular mass of the isolated antifungal peptide was determined by comparison of its electrophoretic mobility with those of the molecular mass marker proteins from Amersham Biosciences.

Mass Spectrometry

Mass spectrometry (MS) analysis of nsLTP was performed on a Finnigan LCQ-MS, an instrument that essentially consists of an atmospheric pressure electrospray positive-ion source attached to a triple-quadrupole mass analyzer. The purified peptide (100 pmol) was dissolved in water/methanol (50 : 50, v/v) containing 1% (v/v) acetic acid at a protein concentration of 5 μmol/l, and then subjected to MS analysis.

N-Terminal Amino Acid Sequence Analysis

The *N*-terminal amino acid sequence of the purified peptide was performed by Edman degradation using an amino acid sequencer.

Assay of Antifungal Activity

The assay for antifungal activity was carried out using 100×15 mm petri plates containing 10 ml of potato dextrose agar. After the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed around and at a distance of 1 cm away from the rim of mycelial colony. An aliquot (8 μl containing 60 μg or 300 μg) of the purified peptide in 20 mM PBS buffer (pH 6.0) was introduced to a disk. The plates were incubated at $23\,°C$ for 72 h until mycelial growth had enveloped peripheral disks containing the control (buffer) and had produced crescents of inhibition around disks containing samples with antifungal activity.

To determine the IC_{50} value for the antifungal activity of the isolated antifungal peptide, four doses of the peptide were added separately to four aliquots, each containing 4 ml potato dextrose agar at $45\,°C$, mixed rapidly, and poured into four separate small 6 mm petri dishes. After the agar had cooled down, a small amount of mycelia, the same amount to each plate, was added. The buffer alone without the antifungal peptide served as control. After incubation at $23\,°C$ for 72 h, the area of the mycelial colony was measured and the inhibition of fungal growth determined. The concentration of the isolated antifungal peptide that brought about 50% reduction in the area of mycelial colony is the IC_{50} [6].

To investigate thermal ($0–100\,°C$) stability and pH (pH 4–10) stability, the isolated antifungal peptide was pretreated accordingly and the antifungal assay was then conducted as mentioned above [6].

A solution of the isolated antifungal peptide (1 mg/ml) was incubated with an equal volume of trypsin or pepsin (1 mg/ml) at $37\,°C$ for 1 h. At the end of the incubation, the reaction mixture was examined for antifungal activity.

Assay of Lipid Binding

Binding of lysolecithin was conducted at $25\,°C$ with a Carywin-100 spectrofluorimeter (Varian Ltd, USA). The excitation wavelength was 229 nm, and emission spectra were recorded from 300 to 400 nm with 4-nm bandwidths and corrected for the buffer contribution. Small amounts of concentrated lyso-C_{12} (lyso-$α$-lauroyl-phosphatidylcholine) solution in water (5 mg/ml) were added stepwise to a cuvette containing 1 ml of an LTP solution (0.25 mg/ml) in 20 mM Tris–HCl buffer (pH 7.8). For each lipid–protein ratio, the maximum fluorescence intensity at 329 nm was used for constructing lipid titration curves. This maximum intensity was determined by averaging the intensity values obtained at 328, 329, and 330 nm [3].

Assay of Antibacterial Activity

Bacteria were incubated in 10 ml of nutrient broth in a thermal shaker for 12 h at $37\,°C$, and then 5 ml of this bacterial suspension was transferred to 50 ml of nutrient broth and incubated for another 3–6 h (the exact duration depending on the bacterial species) in order to shift the bacterial growth to the mid-logarithmic phase. The bacterial suspension was then centrifuged at 2000 g for 10 min, and the bacterial pellet was washed and resuspended in normal saline. A total of 10^5 or 10^7 bacteria per milliliter was obtained by dilution guided by the optical density at 595 nm. In the experiment, every condition was prepared in triplicate; one aliquot of bacterial

suspension was mixed with the isolated antifungal protein at 0.5, 0.25, and 0.125 mg/ml, and one aliquot was mixed with only bacteria in saline as control. The samples were then incubated in a shaker and aliquots were obtained at four time points (0, 3, 6 and 12 h), serially diluted with nutrient broth and spread on agar plates. After incubation at 37 °C for 24 h, the colonies were counted. The number of bacteria for each condition and dilution was determined from the average colony counts for three plates [7].

Assay of Mitogenic Activity

Four C57BL/6 mice (20–25 g) were killed by cervical dislocation and the spleens 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 isolated antifungal peptide 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 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 [7].

Assay of Antiproliferative Activity on Tumor Cell Lines

Breast cancer MCF-7 cell line and liver cancer HepG2 cell line were suspended in RPMI medium and adjusted to a cell density of 2×10^4 cells/ml. A 100 µl aliquot of this cell suspension was seeded to a well of a 96-well plate followed by incubation for 24 h. Different concentrations of the antifungal protein in 100 µl complete RPMI medium were then added to the wells and incubated for 72 h. After 72 h, 20 µl of 5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) in phosphate-buffered saline was spiked into each well and the plates were incubated for 4 h. The plates were then centrifuged at 324 *g* for 5 min. The supernatant was carefully removed and 150 µl of dimethyl sulfoxide was added to each well to dissolve the MTT-formazan at the bottom of the wells. After 10 min, the absorbance at 590 nm was measured using a microplate reader [7].

Assay for HIV-1 Reverse Transcriptase Inhibitory Activity and Integrase Inhibitory Activity

The assay for HIV reverse transcriptase inhibitory activity was carried out according to instructions supplied with the assay kit from Boehringer Mannhein (Germany). HIV integrase inhibitory activity was performed as previously described [8].

RESULTS

When the crude *B. campestris* seed extract was chromatographed on Q-Sepharose, antifungal activity resided in the broad unadsorbed fraction Q1 and not in the sharp adsorbed fraction Q2. Fraction Q1 was resolved on Affi-gel blue gel into a broad unadsorbed fraction B1 devoid of antifungal activity and a smaller adsorbed fraction B2 with antifungal activity. Fraction B2 was fractionated on Mono S into a number of fractions. Antifungal activity was found in fractions S2, S3, and S4. Final purification of fraction S2 was achieved by gel filtration on a Superdex Peptide column. Antifungal activity was detected in the main absorbance peak designated as P1. The yields of the crude extract, fractions Q1, B2, S2, and P1, from 400 g of seeds were 19 430, 7310, 850, 130, and 70 mg, respectively. Fraction P1 representing the purified peptide displayed a molecular mass below 14.4 kDa in SDS-PAGE, 94 kDa in gel filtration in Superdex peptide, and 9414 kDa in mass spectrometry. Its *N*-terminal sequence closely resembled those of nsLTPs from seeds and leaves of other species including that of *P. mungo* (Table 1). It inhibited mycelial growth in *Fusarium oxysporum, Helminthosporium staivum, Mycosphaerella arachidicola, Sclerotinia sclerotiorum* and *Verticivium albotarum* (Figure 1). The IC_{50} values of its antifungal activity toward *F. oxysporum* and *M. arachidicola* were 8.3 µM and 4.5 µM, respectively. The antifungal activity was stable at various temperatures between 20 and 100 °C (Figure 2(A)), and at various pH values between 0 and 4, and between 9 and 14 (Figure 2(B)), and after treatment with either trypsin or pepsin for 1–4 h (Figure 3). Mung bean nsLTP yielded similar thermostability, pH stability, and protease resistance data (data not shown). The antifungal activity of mung bean chitinase underwent an abrupt decline when the pH was raised to 8 and when the temperature was increased to 80 °C (data not shown). Mung bean nsLTP and chitinase inhibited mycelial growth in *F. oxysporum* with an IC_{50} of 17.6 µM and 20 µM, respectively. The *Brassica* nsLTP resembles mung bean nsLTP in lipid binding activity (Figure 4). The *Brassica* nsLTP inhibited proliferation of HepG2 cells and MCF7 cells with IC_{50} values of 5.8 µM and 1.6 µM, respectively (Figure 5(A)), and HIV-1 reverse transcriptase with an IC_{50} of 4 µM (Figure 5(B)). It did not inhibit HIV-1 integrase and SARS virus proteinase, however (data not shown). By contrast, mung bean nsLTP and chitinase were inactive toward the tumor cells and HIV-1 reverse transcriptase (data not shown). The antibacterial activity of mung bean nsLTP had previously been reported [3] but such activity as well as mitogenic activity was lacking in *B. campestris* nsLTP.

DISCUSSION

The chromatographic behavior of the nsLTP isolated from *Brassica* seeds resembles that of other antifungal proteins, being unadsorbed on Q-Sepharose and
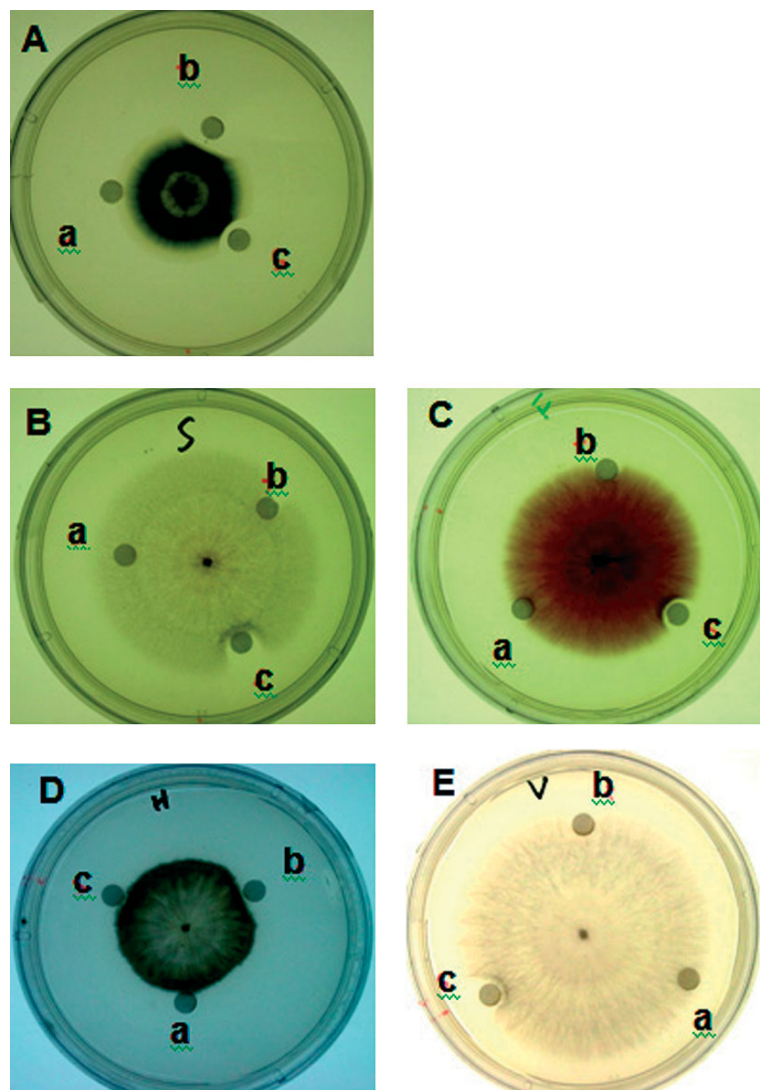
Figure 1 Antifungal activity of *B*. campestris nsLTP-like antifungal peptide. The fungi tested were: plate (A) *Mycosphaerella arachidicola*; plate (B) *Sclerotinia sclerotiorum*; plate (C) *Fusarium oxysporum*; plate (D) *Helminthosporium staivum*; and plate (E) *Verticillium albotarum.* The samples applied to the paper disks were as follows: (a) 10 mM Tris–HCl buffer (pH 7.3) as control; (b) 100 µg of thaumatin-like protein from chestnut as positive control; (c) 15 µg of nsLTP-like antifungal peptide.

Table 1 N-terminal sequence of *B. campestris* nsLTP-like antifungal peptide in comparison with those of *P. mungo* and other nsLTPs

nsLTP source	N-terminal sequence[a]	% Identity
B. campestris	[1]ALS**CG**T**V**S**GNLA**A**C**A**CAG**YV[18]	100
P. mungo	[1]MT**CG**Q**V**Q**GNLA**Q**CIG**FL[17]	50
Thellungiella halophila	[8]AIS**CG**T**V**ASS**LA**P**C**A**G**Y[24]	40
Brassica rapa subsp. pekinensis	[10]ALS**CG**T**V**S**G**YV**AP**C**IG**Y[26]	42
Brassica oleracea	[9]ALT**CG**T**V**NSNV**AP**C**IG**YI[26]	43
Prunus avium	[9]ALT**CG**Q**V**SSNL**AP**C**IAYV[26]	43
Lens culinaris	[13]AVS**CG**T**V**TGDL**APC**[26]	39
Triticum aestivum	[13]ALS**CG**Q**V**DSKL**APC**VAYV[30]	47

[a] Identical residues are shown in bold face.

adsorbed on Mono S and Affi-gel blue gel [7,9–14]. The finding that mung bean nsLTP is adsorbed on the cationic exchange chromatographic media CM-Sephadex and POROS HS-20 [4] is in line with reports on other antifungal proteins. Mung bean chitinase is, however, unadsorbed on POROS-HS [4].

Antifungal activity is undetectable in the seed extracts of several *Brassica* species from which napin-like polypeptides have been isolated [15–17]. It is thus noteworthy that an antifungal nsLTP could be isolated from *B. campestris* and also from mung bean seeds. The nsLTPs have remarkable pH stability, thermostability, and resistance to pepsin and trypsin. This, together with the fact that they are peptides and that *B. campestris* nsLTP have HIV-1 reverse transcriptase inhibitory and antiproliferative activities, suggests that the two nsLTPs, especially *B. campestris* nsLTP, have potential applications.

(A) Thermostability
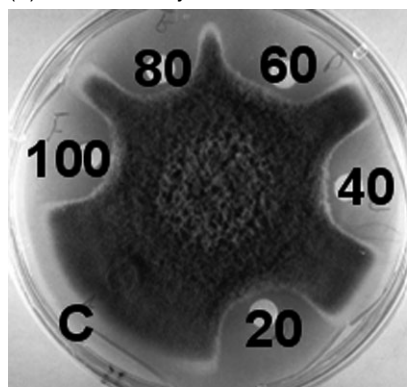
(B) pH stability
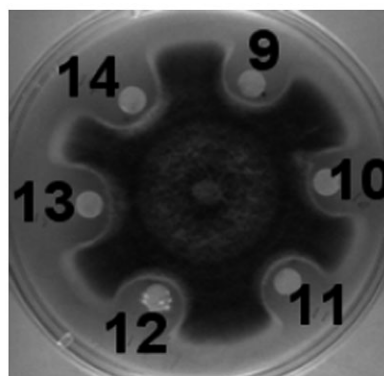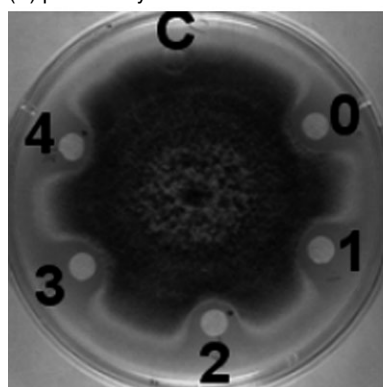
Figure 2 (A) Thermostability and (B) pH stability of antifungal activity of *B. campestris* nsLTP. The same amount (15 µg) of peptide was added to each paper disk (except the control disk labeled as (C). The numbers in panel (A) (20–100) and in panel (B) (0–4 and 9–14) near the paper disks represent the various temperatures (panel A) and pH's (panel B) at which the antifungal peptide introduced to the disk had been pretreated for 10 and 30 min, respectively. The antifungal activity of the peptide was found to be stable after incubation at $100\,°C$ for 10 min and also at various pH values. Mung bean nsLTP yielded similar data.

Some, but not all, LTP have been demonstrated to have antifungal activity [13,18–35]. It is interesting that *Brassica* and mung bean LTPs inhibit mycelial growth in a number of fungal species. Their high antifungal potency on *F. oxysporum* and *M. arachidicola*, a banana pathogen and a peanut pathogen, respectively, suggests their potential usefulness in the prevention of these diseases. In addition, they exert antifungal activity on a number of other fungal species. The two nsLTPs have similar lipid binding activity. Whether they have any of the functions/attributes reported for other nsLTPs such as allergenicity, surface wax biosynthesis, calmodulin binding, cryoprotection, and peroxisomal fatty acyl CoA binding remains to be elucidated [19–23]. Mung bean chitinase also inhibits a broad spectrum of fungal species [4].

It is worth mentioning that some antifungal proteins are capable of inhibiting only one out of the several fungal species tested [13] and that the IC_{50} values of the antifungal activity may well exceed 10 µM [13]. The range of pH values and temperatures over which some other antifungal proteins can withstand without loss of antifungal activity may be narrower than what has

been observed for *Brassica* and mungbean nsLTPs, like in the case of mung bean chitinase. The thermostability and pH stability of the chitinase activity [4] and antifungal activity of mung bean chitinase are similar, suggesting that the same part of the chitinase molecule is responsible for both activities.

Some of the previously reported antifungal proteins, as well as *Brassica* nsLTP, inhibit HIV-1 reverse transcriptase, probably by protein–protein interaction such as that between the retroviral enzyme and its homologous protease. The *Brassica* protein with an IC_{50} of 4 µM is fairly potent in this regard compared with anti-HIV products [36]. In contrast to other antifungal proteins [8], it does not inhibit HIV-1 integrase. There is no effect on the SARS virus proteinase either. The *Brassica* protein also manifests antiproliferative activity toward hepatoma and breast cancer cells with a fairly high potency. Some of the antifungal proteins such as the chive antifungal protein [14] and the leguminous defensin-like peptide [7] exhibit similar activity.

Brassica and mung bean nsLTPs are similar in antifungal activity but differ in other activities including antibacterial, HIV-1 reverse transcriptase inhibitory,
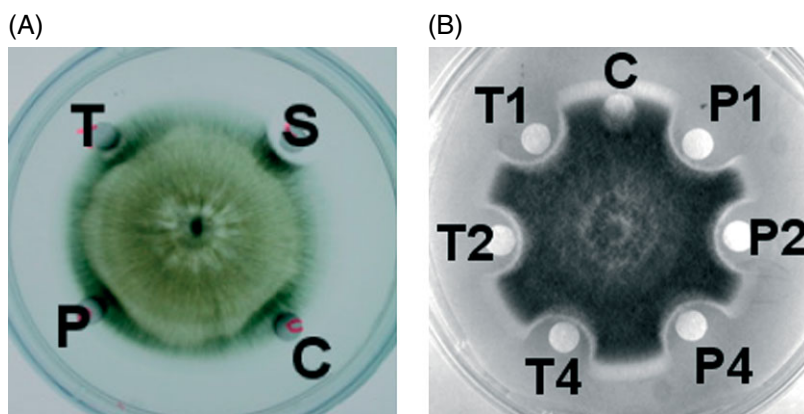
Figure 3 Digestion of *B. campestris* nsLTP with two different proteases, trypsin and pepsin, separately. The digests were applied to paper disks on the agar plate. The label T on the paper disk represents trypsin (10 µg), P represents pepsin (10 µg), S represents antifungal peptide (15 µg) and C represents Tris–HCl buffer (pH 7.3) as negative control. T1–T4 and P1–P4 represent antifungal peptides pretreated for 1–4 h with trypsin and pepsin, respectively. Panel (A): Proteases (T and P) at the concentration used in the digestion were devoid of antifungal activity. Panel (B) The antifungal peptide was resistant to the digestion of trypsin (T1, T2 and T4) and pepsin (P1, P2, and P4). Mung bean nsLTP yielded similar data.
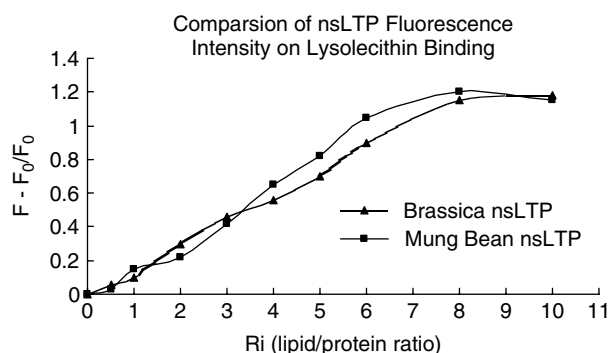

Figure 4 Relative increase of nsLTP fluorescence intensity at 329 nm on lysolecithin binding. $F - F_0$ represents the fluorescence intensity of nsLTP. F is the intensity obtained at each lipid–protein ratio.

and antiproliferative activities. Brassica nsLTP is devoid of any antibacterial activity but exhibits HIV reverse transcriptase inhibitory activity. On the other hand, mung bean nsLTP is antibacterial, but does not possess the other activities. Nevertheless, both manifest potent and extremely stable antifungal activity. The discrepancy in some of the activities is probably due to differences in their amino acid sequence, and different portions of the same protein may contribute to antifungal and the other activities.

In conclusion, nsLTPs purified from *B. campestris* seeds and mung bean seeds are highly stable and exhibit exploitable activities.

Acknowledgements
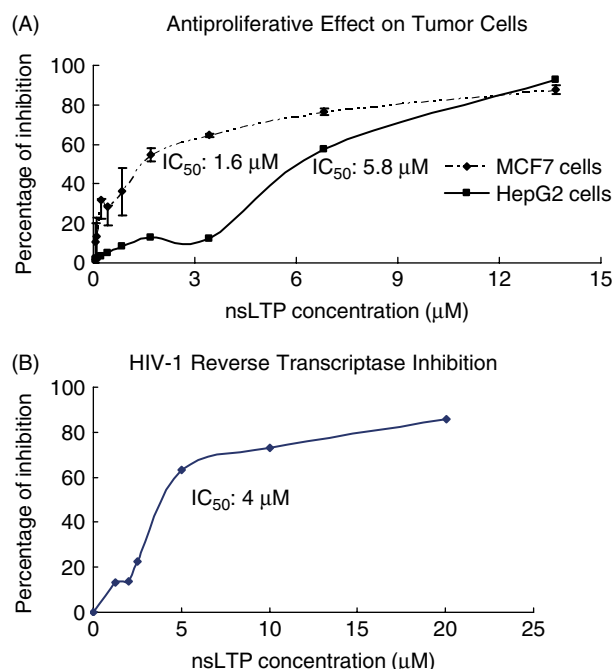
Figure 5 (A) Antiproliferative activity of *B. campestris* nsLTP on HepG2 cells and MCF7 cells. (B) HIV-1 reverse transcriptase inhibitory activity of *B. campestris* nsLTP. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

REFERENCES

1. Ng TB. Peptides and proteins from fungi. *Peptides* 2004; **25**: 1055–1073.
2. Ngai PH, Ng TB. A napin-like polypeptide with translation-inhibitory, trypsin-inhibitory, antiproliferative and antibacterial activities from kale seeds. *J. Pept. Res.* 2004; **64**: 202–208.
3. Wang SY, Wu JH, Ng TB, Ye XY, Rao PF. A non-specific lipid transfer protein with antifungal and antibacterial activities from the mung bean. *Peptides* 2004; **25**: 1235–1242.

4. Wang S, Wu J, Rao P, Ng TB, Ye XY. A chitinase with antifungal activities from mungbean. *Protein Express. Purif.* 2005; **40**: 230–236.

5. Laemmli UK, Favre M. Gel electrophoresis of proteins. *J. Mol. Biol.* 1973; **80**: 575–599.

6. Wang HX, Ng TB. Ginkbilobin, a novel antifungal protein from *Ginkgo biloba* seeds with sequence similarity to embryo-abundant protein. *Biochem. Biophys. Res. Commun.* 2000; **279**: 407–411.

7. Wong JH, Ng TB. Limenin, a defensin-like peptide with multiple exploitable activities from shelf beans. *J. Pept. Sci.* 2006; **12**: 341–346.

8. Ye XY, Wang HX, Ng TB. Structurally dissimilar proteins with antiviral and antifungal potency from cowpea (*Vigna unguiculata*) seeds. *Life Sci.* 2000; **67**: 3199–3207.

9. TB Ng, TK Au, TL Lam, XY Ye, DCC Wan, Inhibitory effects of antifungal proteins on human immunodeficiency virus type 1 reverse transcriptase, protease and integrase. *Life Sci.* 2002; **70**: 927–935.

10. Ye XY, Ng TB. Mungin, a novel cyclophilin-like antifungal protein from the mung bean. *Biochem. Biophys. Res. Commun.* 2000; **273**: 1111–1115.

11. Ye XY, Wang HX, Ng TB. First chromatographic isolation of an antifungal thaumatin-like protein from french bean legumes and demonstration of its antifungal activity. *Biochem. Biophys. Res. Commun.* 1999; **263**: 130–134.

12. Ye XY, Wang HX, Ng TB. Sativin, a novel antifungal miraculin-like protein isolated from legumes of the sugar snap. *Pisum sativum var. macrocarpon. Life Sci.* 2000; **67**: 775–781.

13. Wang HX, Ng TB. Ascalin, a new anti-fungal peptide with human immunodeficiency virus type 1 reverse transcriptase-inhibiting activity from shallot bulbs. *Peptides* 2002; **23**: 1025–1029.

14. Lam YW, Wang HX, Ng TB. 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.

15. Ngai PH, Ng TB. A napin-like polypeptide from dwarf chinese white cabbage seeds with translation-inhibitory, trypsin-inhibitory, and antibacterial activities. *Peptides* 2004; **25**: 171–176.

16. Ng TB, Ngai PH. The trypsin-inhibitory, immunostimulatory and antiproliferative activities of a napin-like polypeptide from chinese cabbage seeds. *J. Pept. Sci.* 2004; **10**: 103–108.

17. Ngai PH, Ng TB. Isolation of a napin-like polypeptide with potent translation-inhibitory activity from chinese cabbage (*Brassica parachinensis* cv green-stalked) seeds. *J. Pept. Sci.* 2003; **9**: 442–449.

18. Yang X, Li J, Li X, She R, Pei Y. Isolation and characterization of a novel thermostable non-specific lipid transfer protein-like antimicrobial protein from motherwort (*Leonurus japonicus* Houtt) seeds. *Peptides* 2006; **27**: 322–328.

19. Pyee J, Yu H, Kolattukudy PE. Identification of a lipid transfer protein as the major protein in the surface wax of broccoli (*Brassica oleracea*) leaves. *Arch. Biochem. Biophys.* 1994; **311**: 460–468.

20. Palacin A, Cumplido J, Figueroa J, Ahrazem O, Sanchez-Monge R, Carrillo T, Salcedo G, Blanco C. Cabbage lipid transfer protein Bra o 3 is a major allergen responsible for cross-reactivity between plant foods and pollens. *J. Allergy Clin. Immunol.* 2006; **117**: 1423–1429.

21. Liu H, Xue L, Li C, Zhang R, Ling Q. Calmodulin-binding protein BP-10, a probable new member of plant nonspecific lipid transfer protein superfamily. *Biochem. Biophys. Res. Commun.* 2001; **285**: 633–638.

22. Hincha DK, Neukamm B, Sror HA, Sieg F, Weckwarth W, Ruckels M, Lullien-Pellerin V, Schroder W, Schmitt JM. Cabbage cryoprotectin is a member of the nonspecific plant lipid transfer protein gene family. *Plant Physiol.* 2001; **125**: 835–846.

23. Wirtz KW, Wouters FS, Bastiaens PH, Wanders RJ, Seedorf U, Jovin TM. The non-specific lipid transfer protein (sterol carrier protein 2) acts as a peroxisomal fatty acyl-CoA binding protein. *Biochem. Soc. Trans.* 1998; **26**: 374–378.

24. Thoma S, Kaneko Y, Somerville C. A non-specific lipid transfer protein from *Arabidopsis* is a cell wall protein. *Plant J.* 1993; **3**: 427–436.

25. Tomassen MM, Barrett DM, van der Valk HC, Woltering EJ. Isolation and characterization of a tomato non-specific lipid transfer protein involved in polygalacturonase-mediated pectin degradation. *J. Exp. Bot.* 2007; **58**: 1151–1160.

26. Boutrot F, Meynard D, Guiderdoni E, Joudrier P, Gautier MF. The *Triticum aestivum* non-specific lipid transfer protein (TaLtp) gene family: comparative promoter activity of six TaLtp genes in transgenic rice. *Planta* 2007; **225**: 843–862.

27. Capocchi A, Fontanini D, Muccilli V, Cunsolo V, Saviozzi F, Saletti R, Lorenzi R, Foti S, Galleschi L. NsLTP1 and NsLTP2 isoforms in soft wheat (*Triticum aestivum Cv. Centauro*) and farro (*Triticum dicoccon Schrank*) bran. *J. Agric. Food Chem.* 2005; **53**: 7976–7984.

28. Liu K, Jiang H, Moore SL, Watkins CB, Jahn MM. Isolation and characterization of a lipid transfer protein expressed in ripening fruit of *Capsicum chinense. Planta* 2006; **223**: 672–683.

29. Castro MS, Gerhardt IR, Orru S, Pucci P, Bloch C Jr. Purification and characterization of a small (7.3 kDa) putative lipid transfer protein from maize seeds. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2003; **794**: 109–114.

30. Liu YJ, Samuel D, Lin CH, Lyu PC. Purification and characterization of a novel 7-kDa non-specific lipid transfer protein-2 from rice (*Oryza sativa*). *Biochem. Biophys. Res. Commun.* 2002; **294**: 535–540.

31. Kristensen AK, Brunstedt J, Nielsen KK, Roepstorff P, Mikkelsen JD. Characterization of a new antifungal non-specific lipid transfer protein (nsLTP) from sugar beet leaves. *Plant Sci.* 2000; **155**: 31–40.

32. Pastorello EA, D'Ambrosio FP, Pravettoni V, Farioli L, Giuffrida G, Monza M, Ansaloni R, Fortunato D, Scibola E, Rivolta F, Incorvaia C, Bengtsson A, Conti A, Ortolani C. Evidence for a lipid transfer protein as the major allergen of apricot. *J. Allergy Clin. Immunol.* 2000; **105**: 371–377.

33. Pastorello EA, Farioli L, Pravettoni V, Ortolani C, Ispano M, Monza M, Baroglio C, Scibola E, Ansaloni R, Incorvaia C, Conti A. The major allergen of peach (*Prunus persica*) is a lipid transfer protein. *J. Allergy Clin. Immunol.* 1999; **103**: 520–526.

34. Kotilainen M, Helariutta Y, Elomaa P, Paulin L, Teeri TH. A corolla- and carpel-abundant, non-specific lipid transfer protein gene is expressed in the epidermis and parenchyma of *Gerbera hybrida* var. Regina (Compositae). *Plant Mol. Biol.* 1994; **26**: 971–978.

35. Meijer EA, de Vries SC, Sterk P, Gadella DW Jr, Wirtz KW, Hendriks T. Characterization of the non-specific lipid transfer protein EP2 from carrot (*Daucus carota L.*). *Mol. Cell. Biochem.* 1993; **123**: 159–166.

36. Ng TB, Huang B, Fong WP, Yeung HW. Anti-human immunodeficiency virus (anti-HIV) natural products with special emphasis on HIV reverse transcriptase inhibitors. *Life Sci.* 1997; **61**: 933–949.