Journal of Peptide Science
J. Peptide Sci. **9**: 442–449 (2003)
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.460

JOURNAL OF PEPTIDE SCIENCE

Isolation of a Napin-like Polypeptide with Potent Translation-Inhibitory Activity from Chinese Cabbage (*Brassica parachinensis* cv green-stalked) Seeds

PATRICK H. K. NGAI and T. B. NG*

Department of Biochemistry, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China

Received 7 January 2003
Accepted 23 January 2003

Abstract: A heterodimeric napin-like polypeptide was isolated from *Brassica parachinensis* seeds with a procedure involving ion exchange chromatography on DEAE-cellulose, affinity chromatography on Affi-gel blue gel, FPLC-ion exchange chromatography on Mono S and FPLC-gel filtration on Superdex 75. The *N*-terminal sequence of the 5 kDa subunit of the polypeptide (PAGPFRIPKKRKKEE) showed high homology with other 2S storage proteins like napins and albumins. The polypeptide potently inhibited translation in a cell free system with an IC_{50} of 6.2 nM. The translation-inhibiting activity of the polypeptide was relatively stable in the pH range 6–11 and in the temperature range 10–50 °C. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: napin; cabbage; *Brassica parachinensis*; seeds; translation-inhibitory activity

INTRODUCTION

Seeds produce a number of interesting and important peptides and proteins including protease inhibitors [1], arginine- and glutamate-rich peptides [2], antifungal proteins and peptides [3–5], ribonucleases [6], ribosome inactivating proteins [7], napins [8], lectins [9], arcelins [10] and α-amylase inhibitors [11]. Some of these proteins and peptides display potentially exploitable biological activities including antifungal, HIV-1 reverse transcriptase inhibiting [12], immunomodulatory [5,13] and antiproliferative [14] activities.

Brassica parachinensis is a very popular vegetable in the southern part of China that is usually eaten with pork. The isolation of a napin-like polypeptide from the seeds of *B. parachinensis* is reported. The polypeptide is made up of a larger peptide and a smaller peptide with molecular mass similar to napins of other *Brassica* species. The polypeptide inhibits translation in the cell-free rabbit reticulocyte lysate system with a potency like those of ribosome inactivating proteins and peptides [15–18].

MATERIALS AND METHODS

The seeds of green-stalked Chinese cabbage (*Brassica parachinensis* cv green-stalked) were purchased from a local seed shop. The seeds were soaked and then homogenized in distilled water using a Waring blender. Tris-HCl buffer (pH 7.4) was added to the supernatant, obtained by centrifuging the homogenate, until the molarity of the Tris-HCl buffer attained 10 mM. The supernatant was then applied to a column of DEAE-cellulose (Sigma) (5 × 25 cm) which had previously been equilibrated with and was then eluted with 10 mM Tris-HCl.

* Correspondence to: Dr T. B. Ng, Department of Biochemistry, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China; e-mail: biochemistry@cuhk.edu.hk
Contract/grant sponsor: Research Grants Council of Hong Kong.

The unadsorbed fraction containing translation-inhibitory activity was collected while the adsorbed fraction eluted with 10 mM Tris-HCl buffer containing 1 M NaCl and devoid of translation-inhibiting activity was discarded. The unadsorbed fraction was directly chromatographed on an Affi-gel blue gel (Bio-Rad) column (2.5 × 15 cm) which was eluted with 10 mM Tris-HCl buffer (pH 7.4). After removal of unadsorbed proteins devoid of translation-inhibitory activity, adsorbed proteins were eluted using a linear NaCl concentration (0–2 M) gradient in the same buffer. The second adsorbed peak containing translation-inhibitory activity was then dialysed before ion exchange chromatography on a 1 ml Mono S column (Amersham Biosciences) using a fast protein liquid chromatography (FPLC) Akta Purifier system (Amersham Biosciences). The column was washed with 10 mM NH_4OAc (pH 5.5) to remove unadsorbed proteins devoid of translation-inhibitory activity. Adsorbed proteins were eluted with two consecutive linear concentration gradients (0–0.3 M NaCl and 0.3–1 M NaCl). The peak eluted with the first salt gradient, which contained translation-inhibitory activity, was further purified by FPLC-gel filtration on a Superdex 75 HR 10/30 column (Amersham Biosciences) in 20 mM NH_4HCO_3 buffer (pH 9.4). The first eluted peak represented purified napin-like polypeptide.

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

This was conducted according to the method of Laemmli and Favre [19] using 15% gel for napin-like polypeptide. After electrophoresis the gel was stained with Coomassie Brilliant Blue. The molecular mass of napin-like polypeptide was determined by comparison of electrophoretic mobility with those of molecular mass marker proteins from Amersham Biosciences.

Amino Acid Sequence Analysis

The *N*-terminal amino acid sequence of napin-like polypeptide was analysed by means of automated Edman degradation. Microsequencing was carried out using a Hewlett Packard 1000A protein sequencer equipped with an HPLC system.

Assay for Cell-free Translation-inhibitory Activity

Rabbit reticulocyte lysate was prepared from the blood of rabbits rendered anaemic by phenylhydrazine injections. An assay based on the rabbit reticulocyte lysate system was used. Napin-like polypeptide (10 µl) was added to 10 µl of radioactive 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 hydrolysate in a 96-well plate to precipitate radioactively labelled protein. The precipitate was collected on a glass fibre 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 [15].

RESULTS

The fraction of the extract of *Brassica parachinensis* seeds unadsorbed on DEAE-cellulose was fractionated on Affi-gel blue gel, using a linear NaCl concentration gradient, into three adjacent peaks. Peak B2 exhibited translation-inhibiting activity (Figure 1). Peak B2 was resolved by FPLC on Mono S into an unadsorbed peak, and two major adsorbed peaks MS1 and MS2. Translation-inhibiting activity resided in MS1 (Figure 2). MS1 was separated by FPLC on Superdex 75 into two peaks FP1 and FP2. Translation-inhibiting activity resided in FP1. FP1 exhibited a molecular mass of 13.5 kDa in gel filtration (Figure 3). FP1 appeared in SDS-PAGE as two bands, one with a molecular mass of 5 kDa and another with a molecular mass of 8.8 kDa (Figure 4). The 5 kDa subunit of the purified polypeptide demonstrated remarkable resemblance to storage proteins and napins in *N*-terminal sequence (Table 1). The protein yields and translation-inhibiting activities of the various chromatographic fractions throughout the purification procedure are shown in Table 2. The polypeptide inhibited translation in the rabbit reticulocyte lysate system with an IC_{50} of 6.2 nM (Figure 5). The translation-inhibitory activity of the polypeptide was stable in the pH range 7–10 and in the temperature range 10°–40 °C. There was a slight decline in activity at pH and pH 11 and at 60 °C. Activity was minimal at pH 3 and at 70 °C. At pH lower than 3 and at and above 13, and at temperatures at and above 80 °C, activity was undetectable (Figures 6 and 7).

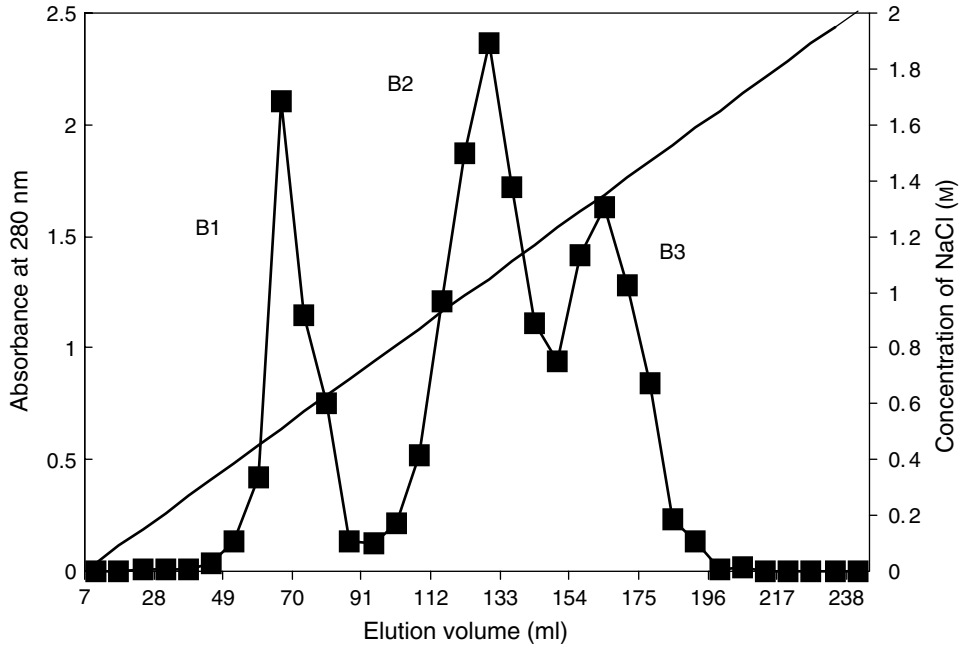

Figure 1 Affinity chromatography of DEAE-unadsorbed fraction on an Affi-gel blue gel column equilibrated with 10 mM Tris-HCl buffer (pH 7.4). The gel was washed with the buffer to remove unadsorbed proteins and eluted with a linear gradient of 0–2 M NaCl in the same buffer to desorb adsorbed proteins. Peak B2 contained napin-like polypeptide.

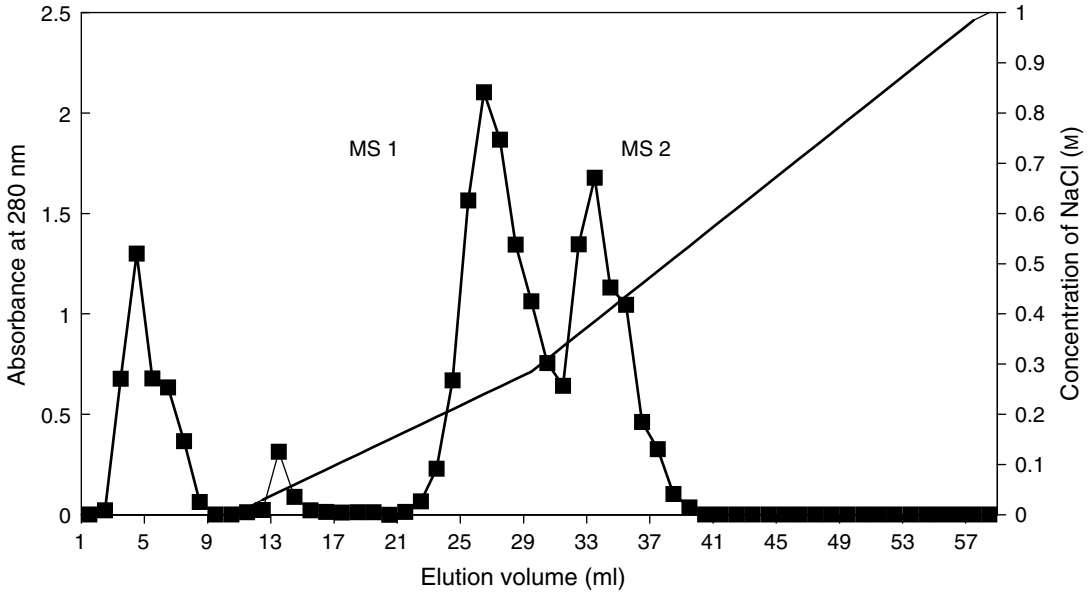

Figure 2 Fast protein liquid chromatography (FPLC) of peak B2 on a 1 ml Mono S column equilibrated with 10 mM NH_4OAc buffer (pH 5.5) at a flow rate of 1 ml/min. Peak MS1 contained napin-like polypeptide.

DISCUSSION

Napins are 1 : 1 disulfide-linked complexes of a 4.5 kDa small subunit and a 10 kDa large subunit [20]. They are storage proteins although other functions have been suggested [21]. The most extensively studied napins are those from the oilseed rape *Brassica napus* [21].

The polypeptide isolated from *B. parachinensis* seeds is a napin-like polypeptide since it is also

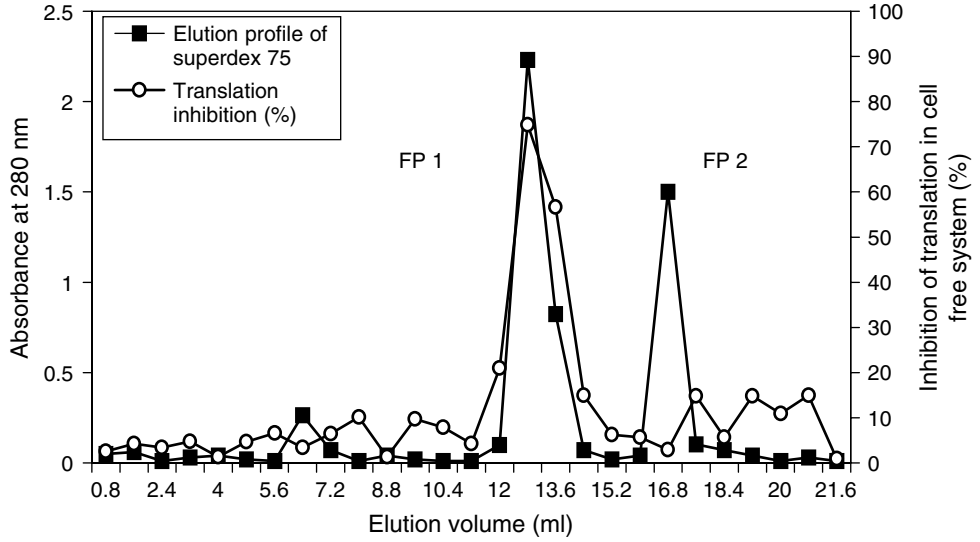

Figure 3 Gel filtration of peak MS1 on Superdex 75 FPLC column in 20 mM NH_4HCO_3 buffer (pH 9.4) at a flow rate of 0.4 ml/min. Peak FP1 contained napin-like polypeptide.

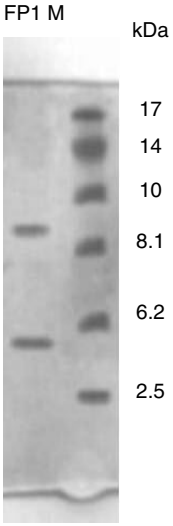

Figure 4 SDS-polyacrylamide gel electrophoresis of peak FP1 from FPLC Superdex 75 column representing *B. parachinensis* napin-like polypeptide. M: molecular mass marker (horse myoglobin peptides).

composed of a small (5 kDa) subunit and a large (8.8 kDa) subunit. The subunits exhibit only small differences in molecular mass from those of rapeseed napin. The 5 kDa subunit of *B. parachinensis* napin-like polypeptide manifests striking resemblance in *N*-terminal sequence to those of napins from various species. The *N*-terminal sequences of the large and small subunits of napin are similar in *Sinapis alba* napin [22] but different in *B. napus* napin [23,24].

Table 1 Comparison of *N*-terminal Sequence of the 5 kDa Subunit of *B. parachinensis* Napin with Related Proteins

	Residue no.	Sequence	Residue no.
B. parachinensis napin-like polypeptide	1	PAGPFRIPKKRKKEE	15
2S storage protein (Field mustard)	38	PAGPFRIPKCRK	49
2S storage protein (*B. oleracea*)	38	PAGPFRIPKCRK	49
2S storage protein (*B. juncea*)	39	PAGPFRIPKCRK	49
2S albumin (rape seed)	1	PAGPFRIPK	9
Napin (*B. napus*)	38	PAGPFRIPKCRK	49
Napin (Swedish turnip)	1	PAGPFRIPKCRK	12
Napin precursor (*Raphanus sativus*)	17	PAGPFRIPRRRK	28
Antifungal 2S storage albumin	1	PAGPFRIP	8
Trypsin inhibitor (TISA)	1	PAGPFRIP**RCRKE	13

The nitrogen storage function of napin is reflected in its high content of amides and arginine residues [25–28]. Napin exhibits trypsin-inhibiting activity

Table 2 Outline of the Purification of Napin

Purification step	Total protein (mg)[a]	IC_{50}[b]
Crude extract	6400	276
B2	562	31
MS1	234	13.5
FP1	55	6.2

[a] Starting material: 600 g *B. parachinensis* seeds.
[b] IC_{50} is expressed as the concentration of protein (nM) required to inhibit protein synthesis in the rabbit reticulocyte lysate system by 50%.

but its subunits are ineffective [20,23,24]. However, both napin and its subunits can function as calmodulin antagonists and substrates for plant calcium-dependent protein kinases since calmodulin and its small subunit possess similar α-helix-hinge-α-helix motifs [23,24]. Napin can inhibit calmodulin-dependent myosin light-chain kinase [23,24]. Napin may also elicit an antifungal action [21].

The present report represents the first on the translation-inhibiting activity of a napin. This activity may be related to the proposed antifungal activity of napins in view of the observation that antifungal proteins and peptides in general possess the ability to inhibit translation in the cell-free rabbit reticulocyte lysate system. However, they do so with only a low potency [5,29–33]. On the other hand, ribosome inactivating proteins, which may represent storage proteins in some seeds, display a highly potent translation-inhibiting activity [7,15] similar in magnitude to that of *B. parachinensis* napin-like polypeptide. Lectins, another type of seed storage proteins, however, lack translation-inhibiting activity unless they have antifungal activity [13]. Proteases have an effect equivalent to translation inhibition on account of their proteolytic action. It may thus seem paradoxical that a protease inhibitor should exhibit translation-inhibitory activity, but the sporamin-type trypsin inhibitor from wampee seeds indeed inhibits translation-inhibitory activity probably due to its antifungal activity [34]. There may be a relationship between the translation-inhibitory activity of a polypeptide like napin and its proposed defensive role. The translation inhibiting activity of *B. parachinensis* napin is stable over half of the pH scale, from pH 6 to pH 11 but is stable only up to 50 °C.

In sum, a napin-like polypeptide has been isolated from a member of the Brassicaceae family that has not been studied before. The ability to inhibit translation in a cell-free rabbit reticulocyte lysate system is demonstrated herein for a napin-like polypeptide. Kohlrabi seeds [23,24], radish seeds [35] and *Arabidopsis thaliana* [36] produce multiple napins. It is noteworthy that only one napin-like polypeptide can be purified from *B. parachinensis* seeds.

Acknowledgement

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

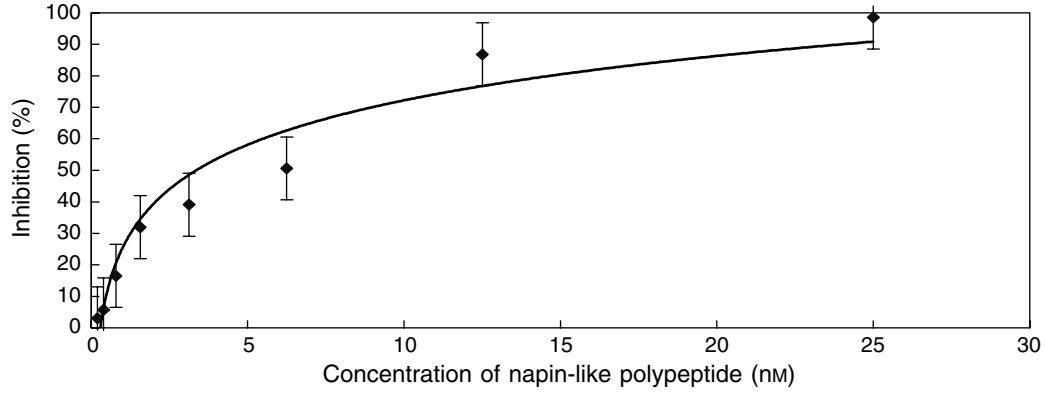

Figure 5 Inhibition of cell-free translation in rabbit reticulocyte lysate by *B. parachinensis* napin-like polypeptide (data represent mean ± SD, $n = 3$).

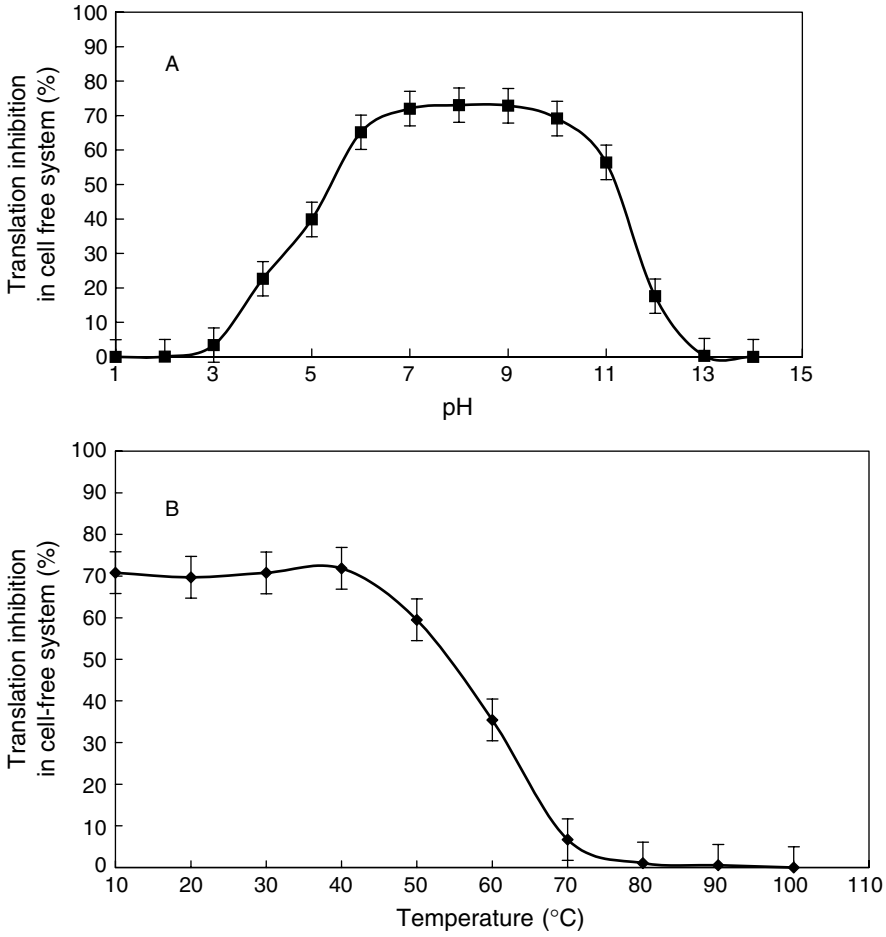

Figure 6 Effect of (A) pH and (B) temperature on translation-inhibitory activity of *B. parachinensis* napin-like polypeptide. The napin-like polypeptide was subjected to various pH and temperatures for 15 min prior to assay for translation-inhibiting activity. Data represent mean ± SD, $n = 3$.

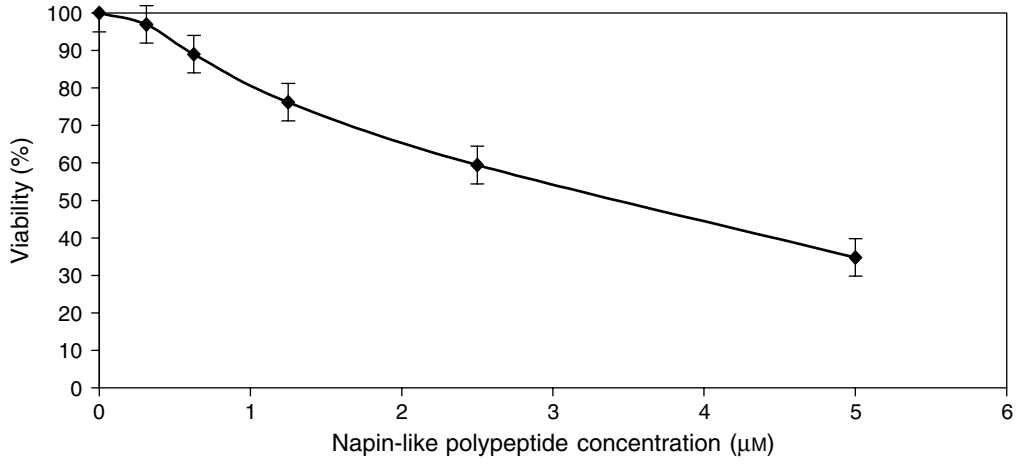

Figure 7 Effect of *B. parachinensis* napin-like on viability of leukaemia cells (L1210). (data represent mean ± SD, $n = 3$).

REFERENCES

1. Birk Y. The Bowman-Birk inhibitor. Trypsin- and chymotrypsin-inhibitor from soybeans. *Int. J. Protein Peptide Res.* 1985; **25**: 113–131.
2. Wang HX, Ng TB. Isolation of lilin, a novel arginine- and glutamate-rich protein with potent antifungal and mitogenic activities from lily bulbs. *Life Sci.* 2002; **70**: 1075–1084.
3. Terras TRG, Eggermont K, Kovaleva K, Baikhel NN, Osborn RW, Kester A, Rees SB, Torreskens S, Van Leuven F, Van Clenleyden J, Cammune BPA, Broekaert WF. Small cysteine-rich antifungal proteins from radish: their role in host defence. *Plant Cell* 1995; **7**: 571–588.
4. Wang HX, Ng TB. Novel antifungal peptides from Ceylon spinach seeds. *Biochem. Biophys. Res. Commun.* 2001; **288**: 765–770.
5. Wang HX, Ye XY, Ng TB. Purification of chrysancorin, a novel antifungal protein with mitogenic activity from garland chrysanthemum seeds. *Biol. Chem.* 2001; **382**: 947–951.
6. Fong WP, Mock WY, Ng TB. Intrinsic ribonuclease activities in ribonuclease and ribosome inactivating proteins from the seeds of bitter gourd. *Int. J. Biochem. Cell Biol.* 2000; **32**: 571–577.
7. Barbieri L, Battelli MG, Stirpe F. Ribosome inactivating proteins from plants. *Biochem. Biophys. Acta* 1993; **1154**: 237–282.
8. Scarafoni A, Carzaniga R, Harris N, Croy RRD. Manipulation of the napin primary structure alters its packaging and deposition in transgenic tobacco (*Nicotiana tabacum*) seeds. *Plant Mol. Biol.* 2001; **46**: 727–739.
9. Wang HX, Ng TB. Ribosome inactivating protein and lectin from bitter melon (*Momordica charantia*) seeds: sequence comparison with related proteins. *Biochem. Biophys. Res. Commun.* 1998; **253**: 143–146.
10. Fabre C, Causse H, Mourey L, Konin KJ, Riviere M, Hendricks H, Puzo G, Samama JP, Rouge R. Characterization and sugar-binding properties of arcelin-1, an insecticidal lectin-like protein isolated from kidney bean *(Phaseolus vulgaris)* seeds. *Biochem. J.* 1998; **329**: 551–560.
11. Le-Berre-Anton V, Bompard-Gilles C, Philo JS, Wen J, Ishimoto M, Yamaguchi H. Characterization and functional properties of the alpha-amylase inhibitor (alpha-AI) from kidney bean (*Phaseolus vulgaris)* seeds. *Biochim. Biophys. Acta* 1997; **1347**: 31–40.
12. Ng TB, Au TK, Lam TL, Ye XY, Wan DCC. Inhibitory effects of antifungal proteins on human immunodeficiency virus type 1 reverse transcriptase, protease and integrase. *Life Sci.* 2002; **70**: 927–936.
13. Ye XY, Ng TB, Tsang PW, Wang J. Isolation of a homodimeric lectin with antifungal and antiviral activities from red kidney bean (*Phaseolus vulgaris*) seeds. *J. Protein Chem.* 2001; **20**: 367–375.
14. Tsao SW, Ng TB, Yeung HW. Toxicities of trichosanthin and alpha-momorcharin, abortifacient proteins from Chinese medicinal plants, on cultured tumor cell lines. *Toxicon* 1990; **28**: 1183–1192.
15. Lam SSL, Wang HX, Ng TB. Purification and characterization of novel ribosome inactivating proteins, alpha- and beta-pisavins, from seeds of the garden pea *Pisum sativum*. *Biochem. Biophys. Res. Commun.* 1998; **253**: 135–142.
16. Parkash A, Ng TB, Tso WW. Isolation and characterization of luffacylin, a ribosome inactivating peptide with antifungal activity from sponge gourd (*Luffa cylindrica*) seeds. *Peptides* 2002; **23**: 1019–1024.
17. Parkash A, Ng TB, Tso WW. Purification and characterization of charantin, a napin-like ribosome-inactivating peptide from bitter gourd (*Momordica charantia*) seeds. *J. Peptide Res.* 2002; **59**: 197–202.
18. Wang HX, Ng TB. Luffangulin, a novel ribosome inactivating peptide from ridge gourd (*Luffa acutangula*) seeds. *Life Sci.* 2002; **70**: 899–906.
19. Laemmli UK, Favre M. Maturation of the head of bacterophage T4. 1. DNA packaging events. *J. Mol. Biol.* 1973; **80**: 575–599.
20. Gehrig PM, Biemann K. Assignment of the disulfide bonds in napin, a seed storage protein from *Brassica napus*, using matrix-assisted laser description ionization mass spectrometry. *Peptide Res.* 1996; **9**: 308–314.
21. Barciszewki J, Szymanski M, Haertle T. Minireview: Analysis of rape seed napin structure and potential roles of the storage protein. *J. Protein Chem.* 2000; **19**: 249–254.
22. Ericson L, Muren E, Gustavvson HO, Josefasson LG, Rask L. Analysis of the promoter region of napin genes from *Brassica napus* demonstrates binding of nuclear protein *in vitro* to a conserved sequence motif. *Eur. J. Biochem.* 1991; **197**: 741–746.
23. Neumann GM, Condron R, Thomas I, Polya GM. Purification and sequencing of multiple forms of *Brassica napus* seed napin large chains that are calmodulin antagonists and substrates for plant calcium dependent protein kinases. *Biochim. Biophys. Acta* 1996; **1295**: 23–33.
24. Neumann GM, Condron R, Thomas I, Polya GM. Purification and sequencing of multiple forms of *Brassica napus* seed napin small chains that are calmodulin antagonists and substrates for plant calcium dependent protein kinases. *Biochim. Biophys. Acta* 1996; **1295**: 34–43.
25. Frigerio L, de Virgillio M, Prada A, Fauro F, Vitale A. Sorting of phaseolin to the vacuole is saturable and requires a short *C*-terminal peptide. *Plant Cell* 1998; **10**: 1031–1042.
26. Muntz K. Deposition of storage proteins. *Plant Mol Biol.* 1998; **38**: 77–99.

27. Neuhaus JM, Rogers JC. Sorting of proteins to vacuoles in plant cells. *Plant Mol. Biol.* 1998; **38**: 127–144.
28. Vitale A, Raikhel NV. What do proteins need to reach different vacuoles? *Trends Plant Sci.* 1999; **4**: 149–155.
29. Ye XY, Ng TB. Mungin, a novel cyclophilin-like antifungal protein from the mungbean. *Biochem. Biophys. Res. Commun.* 2000; **273**: 1111–1115.
30. Ye XY, Ng TB. Delandin, a chitinase-like protein with antifungal, HIV-1 reverse transcriptase inhibitory and mitogenic activities from the rice bean *Delandia umbellata*. *Protein Expr. Purif.* 2002; **24**: 524–529.
31. 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.
32. Ye XY, Wang HX, Ng TB. Dolichin, a new chitinase-like antifungal protein isolated from field beans (*Dolichos lablab*). *Biochem. Biophys. Res. Commun.* 2000; **269**: 151–159.
33. 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.
34. Ng TB, Lam SK, Fong WP. A homodimeric sporamin-type trypsin inhibitor with antiproliferative, HIV reverse transcriptase-inhibitory and antifungal activities from wampee (*Clausena lansium*) seeds. *Biol. Chem.* 2003; **384**: In press.
35. Polya GM, Chandra S, Condron R. Purification and sequencing of radish seed calmodulin antagonists phosphorylated by calcium-dependent protein kinase. *Plant Physiol.* 1993; **101**: 545–551.
36. Krebbers E, Vandekerckhove J. Production of peptides in plant seeds. *Trends Biotechnol.* 1990; **8**: 1–3.

