Available online at www.sciencedirect.com
SCIENCE DIRECT®
Peptides 24 (2003) 963–968
PEPTIDES
www.elsevier.com/locate/peptides

Gymnin, a potent defensin-like antifungal peptide from the Yunnan bean (*Gymnocladus chinensis* Baill)

Jack H. Wong, T.B. Ng*

Department of Biochemistry, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China

Received 3 April 2003; accepted 22 May 2003

Abstract

From the seeds of the Yunnan bean, we purified an antifungal peptide using affinity chromatography on Affi-gel blue gel, FPLC-ion exchange chromatography on Mono S, and FPLC-gel filtration on Superdex 75. The antifungal peptide was adsorbed on Affi-gel blue gel at pH 7.8 and Mono S at pH 4.5. It exhibited a molecular mass of 6.5 kDa in both gel filtration and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Its N-terminal sequence closely resembled defensin-related peptides. The peptide exerted antifungal activity toward the fungal species *Fusarium oxysporum* and *Mycosphaerella arachidicola*, with an IC_{50} of 2 μM for the former fungus and 10 μM for the latter. It manifested a weaker mitogenic activity toward murine splenocytes than Concanavalin A. It also displayed antiproliferative activity on a murine leukemia (L1210), a hepatoma (HepG2), and a murine leukemia (M1) cell line. It inhibited human immunodeficiency virus-1 reverse transcriptase with an IC_{50} of 200 μM.

Keywords: Antifungal peptides; Isolation; Bean; *Gymnocladus chinensis*

1. Introduction

Antifungal protein and peptides are produced by a variety of phylogenetically unrelated organisms to protect themselves from the damaging effects of fungi. The organisms include flowering plants [1–3,5–8,10,12,14,15,17,18,22–31], mushrooms [12], and insects [5,9]. Interest in the isolation of antifungal proteins and peptides has continued in view of the great economic losses incurred due to devastation of crops by fungi.

Large structural differences exist among antifungal proteins and peptides synthesized by different organisms. The same organism may elaborate more than one type of antifungal protein or peptide [21,34,38]. Leguminous plants alone produce many types of antifungal proteins and peptides including thaumatin-like proteins [35], lectins [2], ribonucleases [17,23], cyclophilin-like proteins [27,29], ribosome inactivating proteins [14], miraculin-like proteins [37], peroxidase-like proteins [30], chitinases [1,6,8,12,21,36,38], embryo-abundant proteins [24], protease inhibitors [10], and novel proteins and peptides [3,20–28].

The Yunnan bean is a product of Yunnan Province in China that has not been investigated previously. It was thus examined for the presence of antifungal proteins or peptides.

2. Materials and methods

2.1. Materials

Dried Yunnan beans (*Gymnocladus chinensis*) from Mainland China were used. Affi-gel blue gel was purchased from Bio-Rad, and Mono S and Superdex 75 columns were from Amersham Biosciences. Chemicals for sequence analysis were obtained from Hewlett Packard (Palo Alto, CA, USA). All other chemicals used were of reagent grade.

2.2. Purification of antifungal peptide

The seeds were soaked in distilled water prior to extraction for 5 min with distilled water using a Waring blender. Tris–HCl buffer (pH 7.8) was added to the supernatant obtained after centrifugation until a concentration of 10 mM was attained. The supernatant was applied to a column (2.5 cm × 10 cm) of Affi-gel blue gel (Bio-Rad) in 10 mM Tris–HCl buffer (pH 7.8). After removal of unadsorbed material (B1), adsorbed proteins were desorbed with 1 M

* Corresponding author. Tel.: +86-852-2609-6872; fax: +86-852-2603-5123.
E-mail address: biochemistry@cuhk.edu.hk (T.B. Ng).

0196-9781/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0196-9781(03)00192-X

NaCl in the same buffer. The adsorbed fraction (B2) was dialyzed and then applied on an FPLC Mono S column (1 ml, Amersham Biosciences) in 20 mM NH_4OAc buffer (pH 4.5). Unadsorbed proteins were washed off the column with the starting buffer. Adsorbed proteins were eluted with two linear NaCl concentration gradients (0–0.3 and 0.3–1 M) in the starting buffer. Fraction M2, in which antifungal activity resided, was then subjected to gel filtration by FPLC on a Superdex 75 HR 10/30 column (Amersham Biosciences) in 200 mM NH_4HCO_3 (pH 9.6). The last peak S3 represented purified antifungal peptide which was designated gymnin.

2.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was conducted according to the method of Laemmli and Favre using an 18% (w/v) gel [11]. After electrophoresis the gel was stained with Coomassie Brilliant Blue. The molecular mass of gymnin was determined by comparison of its electrophoretic mobility with those of molecular mass marker proteins from Amersham Biosciences.

2.4. Amino acid sequence analysis

The N-terminal amino acid sequence of gymnin was analyzed by means of automated Edman degradation using a Hewlett-Packard 1000A protein sequencer equipped with an HPLC system [14].

2.5. Assay of antifungal activity

The assay of gymnin for antifungal activity toward *Mycosphaerella arachidicola* and *Fusarium oxysporum* was carried out in 100 mm × 15 mm petri dishes containing 10 ml of potato dextrose agar. After the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed at a distance of 0.5 cm away from the rim of the mycelial colony. An aliquot of a solution of gymnin was added to a disk. The dishes 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 [28].

To determine the IC_{50} value for the antifungal activity of gymnin, three doses of the peptide were added separately to three aliquots each containing 4 ml potato dextrose agar at 45 °C, mixed rapidly and poured into three separate small petri dishes. After the agar had cooled down, a small amount of mycelia, the same amount to each plate, was added. Buffer only without antifungal peptide served as a control. After incubation at 23 °C for 72 h, the area of the mycelial colony was measured and the inhibition of fungal growth determined [28].

2.6. Assay for HIV reverse transcriptase inhibitory activity

The ability of gymnin to inhibit HIV-1 reverse transcriptase was assessed by using an ELISA kit from Boehringer Mannheim (Germany) as described by Collins et al. [4].

2.7. Assay of mitogenic activity

Four male 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

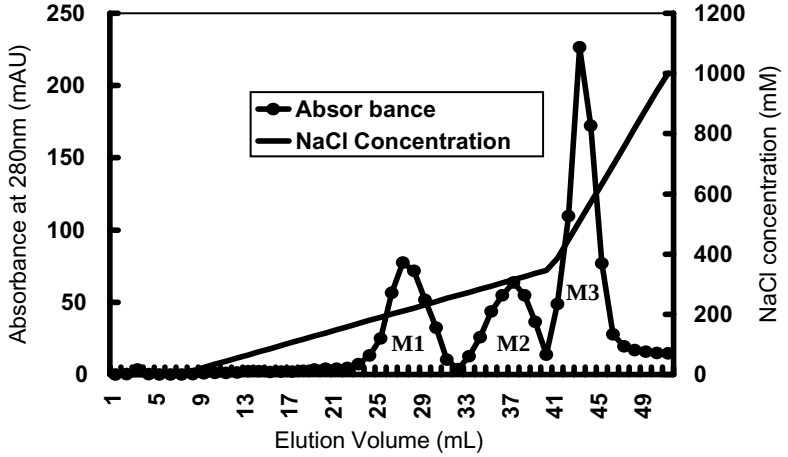

Fig. 1. Ion exchange chromatography of fraction B2 (adsorbed on Affi-gel blue gel) on a 1 ml Mono S column. After chromatography on Affi-gel blue gel, the adsorbed fraction B2 obtained was dialysed and then applied to a Mono S column by fast protein liquid chromatography (FPLC) using an AKTA Purifier. The column was washed with the binding buffer (20 mM NH_4OAc, pH 4.5). Adsorbed proteins were eluted first with a linear gradient of NaCl from 0 to 0.3 M in the binding buffer followed by another linear gradient of 0.3–1 M NaCl in the binding buffer. Antifungal activity was found in fraction M2.

with 10% fetal bovine serum, 100 units penicillin/ml, and 100 μg streptomycin/ml. The cells (7×10^5 cells/100 μl per well) were seeded into a 96-well culture plate and serial dilutions of a solution of gymnin 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 [^{3}H-methyl]-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 [25].

2.8. Assay of inhibitory activity on tumor lines

Leukemia L1210 cell line, leukemia M1 cell line or hepatoma hep G2 cell line was suspended in RPMI medium and adjusted to a cell density of 2×10^4 cells/ml. One hundred microliters of this cell suspension was seeded to a well of a 96-well plate followed by incubation for 24 h. Different amounts of gymnin 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 MTT in phosphate buffered saline was spiked into each well and the plates were incubated for 4 h. The well plates were then centrifuged at 2500 rpm for 5 min. The supernatant was carefully removed and 150 μl of dimethyl sulfoxide was added in each well to dissolve the (MTT) formazan at the bottom of the wells. After 10 min, the absorbance at 590 nm was then measured by a microplate reader. The inhibitory activity reflects a summation of inhibitory effects on proliferation and viability of tumor cells.

3. Results

Affinity chromatography of an extract of Yunnan bean seeds on Affi-gel blue gel revealed that there was more adsorbed material (B2) than unadsorbed material (B1). Antifungal activity resided in the adsorbed fraction.

The adsorbed fraction (B2) was fractionated by FPLC on Mono S into an unadsorbed peak, two small adsorbed peaks M1 and M2 and a large strongly adsorbed peak M3 (Fig. 1). Antifungal activity was concentrated in the large peak M2.

Peak M2 was separated on Superdex 75 into a large sharp peak S1 and two small peaks S2 and S3 (Fig. 2). The last peak (S3) represented purified antifungal peptide designated gymnin. From 150 g Yunnan beans, 2.9 g B1, 0.7 g M2, and 20 mg S3 (gymnin) were obtained. Gymnin demonstrated a molecular mass of 6.5 kDa in SDS–PAGE (Fig. 3).

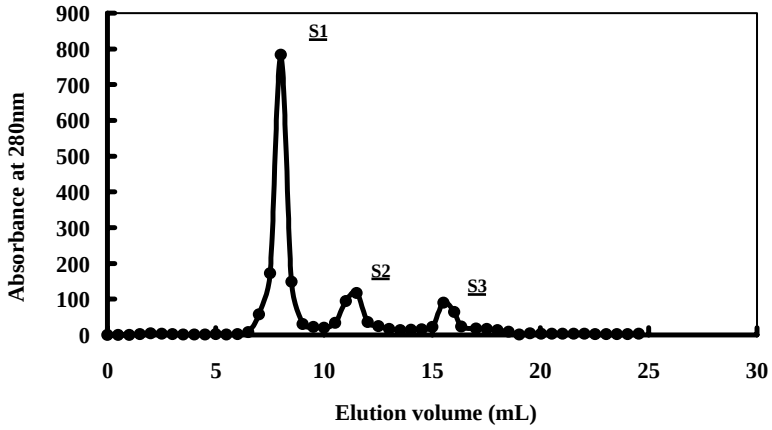

Fig. 2. Ion exchange chromatography of fraction M2 on a Superdex 75 HR 10/30 column by FPLC using an AKTA Purifier. Buffer: 200 mM NH_4HCO_3 (pH 9.6). Antifungal activity was found in fraction S3.

Table 1
Comparison of N-terminal sequences of gymnin with those of related peptides and proteins

	Residue no.		Residue no.
Gymnin	1	KTCENLADDY	10
Defense-related peptide 1 (Defensin 1)	1	KTCEHLADTY	10
Defense-related peptide 2 (Defensin 2)	1	KTCENLSGTF	10
Disease resistance response protein 39 precursor	30	-TCEHLADTY	38
Disease resistance response protein 230 precursor	29	-TCENLAGSY	37
Antifungal protein precursor (*Medicago sativa*)	29	-TCENLADKY	37
Antimicrobial defensin peptide DRR230-c	22	-TCEHLADTY	30
PDF1 (*Vigna radiata*)	29	KTCENLANTY	38

Residues nos. 1 and 10 for gymnin refer to K being its 1st and Y being its 10th residues.

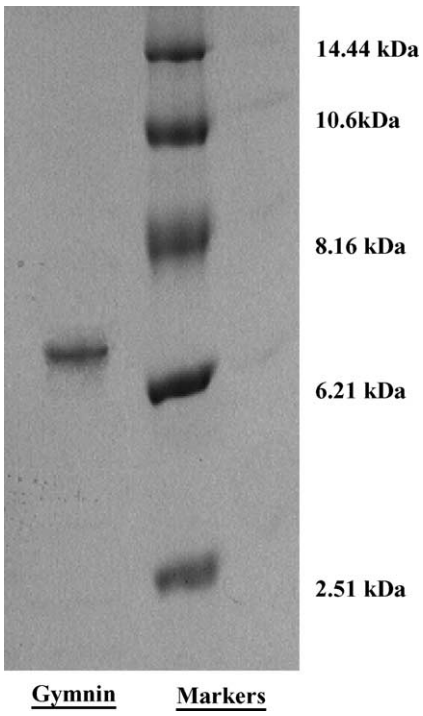

Fig. 3. SDS–PAGE of gymnin. Molecular mass was estimated to be 6.5 kDa.

Its N-terminal sequence exhibited marked resemblance to defensin-related peptides (Table 1).

The antifungal activity of the antifungal peptide against *M. arachidicola* is illustrated in Fig. 4. It was active against both *F. oxysporum* and *M. arachidicola* even at 5 μg. The IC_{50} values of its antifungal activity against *F. oxysporum* and *M. arachidicola* were 2 and 10 μM, respectively.

Gymnin had a lower mitogenic activity than Concanavalin A (Con A) toward mouse splenocytes, eliciting a lower maximal response (50,508 cpm versus 108,811 cpm) (Table 2). Its inhibitory effects toward the growth of leukemia and hepatoma cell lines are shown in Table 3. Gymnin inhibited HIV-1 reverse transcriptase with an IC_{50} of 200 μM.

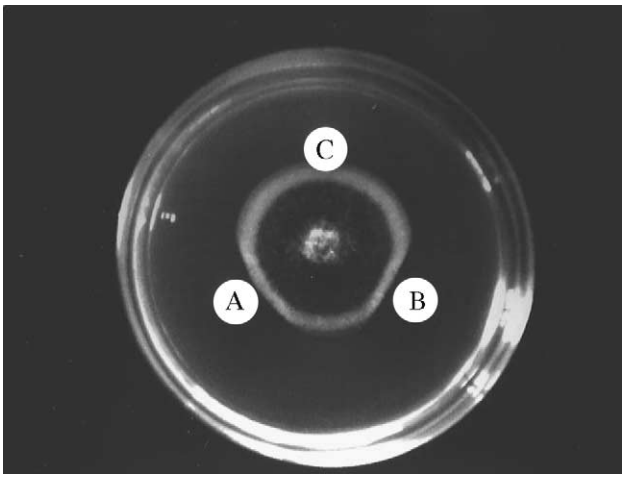

Fig. 4. Inhibitory activity of gymnin toward *M. arachidicola*: (A) 10 μg gymnin, (B) 5 μg gymnin, and (C) control (buffer).

Table 2
Mitogenic effects of Concanavalin A (Con A) and gymnin on mouse splenocytes

Con A/gymnin concentration (μM)	[^{3}H-Methyl]-thymidine incorporation by splenocytes (cpm)	
	Con A	Gymnin
102.4	1527 ± 520	2777 ± 544
51.2	2107 ± 908	3665 ± 640
25.6	2307 ± 217	8320 ± 1135
12.8	32221 ± 2570	24608 ± 3107
6.4	108811 ± 8728	30898 ± 4392
3.2	31289 ± 5226	50508 ± 2584
1.6	26660 ± 2136	37197 ± 5013
0.8	12643 ± 1476	31056 ± 3898
0.4	7794 ± 1053	17464 ± 2076
0.2	2842 ± 758	8226 ± 991
0.1	5819 ± 898	8561 ± 1369
0	1729 ± 628	2218 ± 668

Data are presented as means ± S.D. ($n = 3$).

Table 3
Effect of gymnin on viability of tumor cell lines

Gymnin concentration (μM)	% Reduction in viability		
	M1	HepG2	L1210
50.0	49 ± 3	37 ± 4	40 ± 3
25.0	49 ± 2	22 ± 2	26 ± 3
12.5	45 ± 5	19 ± 3	24 ± 3
6.25	32 ± 6	18 ± 2	20 ± 1
3.13	9 ± 2	16 ± 2	17 ± 1
1.56	10 ± 3	5 ± 2	6 ± 3

Data are presented as means ± S.D. ($n = 3$).

4. Discussion

The antifungal peptide isolated in the present study from the Yunnan bean structurally resembles the 10-kDa cowpea precursor, the antifungal peptides from the pinto bean and the red bean [28], and defensin-related peptides [20] in N-terminal sequence. Employing the same antifungal assay system as that previously used for detecting the antifungal activity of pinto bean and red bean antifungal peptides, it can be demonstrated that gymnin exhibits more potent antifungal activity than its structurally related counterparts in pinto bean and red bean. The difference in molecular mass (6.5 kDa for gymnin and 5 kDa for the other two) probably accounts for this variation in antifungal activity.

The antifungal peptides from pinto bean and red bean have not been examined for antiproliferative activity against tumor cell lines [28]. Gymnin demonsrates a suppressive action on the uptake of MTT by three tumor cell lines, hepatoma (HepG2), leukemia (L1210), and leukemia (M1). It deserves mention that the antifungal chitinase-like protein

from the chive *Allium tuberosum* exerts an antiproliferative action against breast cancer cells [14].

The mitogenic response of mouse splenocytes elicited by gymnin is similar to that induced by the pinto bean antifungal peptide [25]. Different antifungal proteins and peptides exert different actions on mouse splenocytes. Mitogenic [28,29] as well as and anti-mitogenic [27] activities have been observed. It is known that a variety of antifungal proteins can inhibit HIV-1 reverse transcriptase, protease, and integrase [15]. The antifungal peptides from pinto bean, red bean [28], and Yunnan bean inhibit HIV-1 reverse transcriptase with an IC_{50} of 65, 280, and 200 μM, respectively.

Gymnin is adsorbed on Affi-gel blue gel and Mono S. This observation is in line with the finding that a number of previously isolated antifungal proteins and peptides are unadsorbed on DEAE-cellulose but adsorbed on Affi-gel blue gel and CM-Sepharose/SP-Sepharose/Mono S [12,13,17,23,24,26–28].

In the pinto bean [34], red bean [32], and rice bean [33], the antifungal peptides resembling gymnin co-exist with antifungal proteins such as chitinases and other antifungal peptides. In contradistinction, gymnin is the only antifungal compound of proteinaceous nature isolated from the Yunnan beans.

In summary, a potent defensin-like antifungal peptide has been isolated from an exotic leguminous species, the Yunnan bean *G. chinensis*. Defensins have antimicrobial function in the respiratory system and the urinary tract [16] and have been shown to be present in human buccal epithelia with candidiasis [19]. They are non-toxic to plant cells and mammalian cells [20]. Thus gymnin may hold some promise as a therapeutic agent.

Acknowledgments

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

References

[1] Benhamou N, Broglie K, Broglie R, Chet I. Antifungal effect of bean endochitinase on *Rhizoctonia solani*: ultrastructural changes and cytochemical aspect of chitin breakdown. Can J Microbiol 1993;39:318–28.

[2] Broekaert WF, Van Parijs J, Leyns F, Joos H, Peumans WJ. A chitin-binding lectin from stinging nettle rhizomes with antifungal properties. Science 1989;245:1100–2.

[3] Cammue BPA, Thevissen K, Hendriks M, Eggermont K, Goderis IJ, Proost P, et al. A potent antimicrobiol protein from onion seeds showing sequence homology to plant lipid transfer protein. Plant Physiol 1995;109:445–55.

[4] Collins RA, Ng TB, Fong WP, Wan CC, Yeung HW. A comparison of human immunodeficiency virus type 1 inhibition by partially purified aqueous extracts of Chinese medicinal herbs. Life Sci 1997;60 PL:345–51.

[5] Fehlbaum P, Bulet P, Michaut L, Lagueux M, Broekaert WF, Hertu C, et al. Insect immunity. Septic injury of *Drosophila* induces the synthesis of a potent antifungal peptide with sequence similarity to plant antifungal peptides. J Biol Chem 1995;269:33159–67.

[6] Gozia O, Ciopraga J, Bentia T, Lungu M, Zamfirescu I, Tudor R, et al. Antifungal properties of lectin and new chitinases from potato tuber. FEBS Lett 1995;370:245–9.

[7] Hanselle T. Thesis. West Faelisehe Wilhelms-Universitaet Muenster, Institute for Biochemistry and Biotechnology of Plants; 1988.

[8] Huang X, Xie WJ, Gong ZZ. Characteristics and antifungal activity of a chitin binding protein from *Ginkgo biloba*. FEBS Lett 2000;478:123–6.

[9] Iijina R, Kurata S, Natori S. Purification, characterization and cDNA cloning of an antifungal protein from the hemolymph of *Sarcophaga peregrina* (flesh fly). J Biol Chem 1993;268:12055–62.

[10] Joshi BN, Sainani MN, Bastawade KB, Gupta VS, Ranjekar PK. Cysteine protease inhibitor from pearl millet: a new class of antifungal protein. Biochem Biophys Res Commun 1998;246:382–7.

[11] Laemmli UK, Favre M. Gel electrophoresis of proteins. J Mol Biol 1973;80:575–99.

[12] Lam SK, Ng TB. Isolation of a small chitinase-like antifungal protein from *Panax notoginseng* (sanchi ginseng) roots. Int J Biochem Cell Biol 2001;33:287–92.

[13] Lam SK, Ng TB. First stimultaneous isolation of a ribosome-inactivating protein and an antifungal protein from mushroom (*Lyophllum shimeiji*) together with evidence for synergism of their antifungal effects. Arch Biochem Biophys 2001;393:271–80.

[14] 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;253:135–42.

[15] Leah R, Tommerup H, Svendsen I, Mundy J. Biochemical and molecular characterization of three barley seed proteins with antifungal properties. J Biol Chem 1991;246:1564–673.

[16] Morrison G, Kilanowski F, Davidson D, Dorin J. Characterization of the mouse beta defensin 1, Defb1, mutant mouse model. Infect Immunol 2002;70:3053–60.

[17] Ng TB, Wang HX. Panaxagin, a new protein from Chinese ginseng possesses anti-fungal, anti-viral, translation-inhibiting and ribonuclease activities. Life Sci 2001;68:739–49.

[18] Roberts WK, Selitrennikoff CP. Isolation and partial characterization of two antifungal proteins from barley. Biochim Biophys Acta 1986;880:161–70.

[19] Sawaki K, Mizukawa N, Yamaai , Fukunaga J, Suguhara I. Immunohistochemical study on expression of alpha-defensin and beta-defensin-2 in human buccal epithelia with candidiasis. Oral Dis 2002;8:37–41.

[20] Thomma BP, Cammue BP, Thevissen K. Plant defensin. Planta 2002;216:193–202.

[21] Vogelsang R, Barz W. Purification, characterization and differential hormonal regulation of a β-1,3-glucanase and two chitinases from chickpea (*Cicer arietinum* L.). Planta 1993;189:60–9.

[22] Vu L, Huynh QK. Isolation and characterization of a 27-kDa antifungal protein from the fruits of *Diospyros texana*. Biochem Biophys Res Commun 1994;202:666–72.

[23] Wang HX, Ng TB. Quinqueginsin, a novel protein with anti-human immunodeficiency virus, antifungal, ribonuclease and cell-free translation-inhibitory activities from American ginseng roots. Biochem Biophys Res Commun 2000;269:203–8.

[24] Wang H, Ng TB. Ginkbilobin, a novel antifungal protein from *Ginkgo biloba* seeds with sequence similarity to embryo-abundant protein. Biochem Biophys Res Commun 2000;279:407–11.

[25] Wang HX, Ng TB, Ooi VEC, Liu WK, Chang ST. A polysaccharide–peptide complex from cultured mycelia of the mushroom *Tricholoma mongolicum* with immunoenhancing and antitumor activities. Biochem Cell Biol 1996;74:95–100.

[26] Ye XY, Ng TB. Hypogin, a novel antifungal peptide from peanuts with sequence similarity to peanut allergen. J Pept Res 2001;57:330–6.
[27] Ye XY, Ng TB. Mungin, a novel cyclophilin-like antifungal protein from the mung bean. Biochem Biohys Res Commun 2000;273:1111–5.
[28] Ye XY, Ng TB. Peptides from pinto bean and red bean with sequence homology to cowpea 10-kDa protein precursor exhibit antifungal, mitogenic, and HIV-1 reverse transcriptase-inhibitory activities. Biochem Biophys Res Commun 2001;285:424–9.
[29] Ye XY, Ng TB. Isolation of a cyclophilin-like protein from chickpeas with mitogenic, antifungal and anti-HIV-1 reverse transcriptase activities. Life Sci 2002;70:1129–38.
[30] Ye XY, Ng TB. Isolation of a novel peroxidase from French bean legumes and first demonstration of antifungal activity of a non-milk peroxidase. Life Sci 2002;71:1667–80.
[31] Ye XY, Ng TB. A new antifungal peptide from rice beans. J Pept Res 2002;60:81–7.
[32] Ye XY, Ng TB. Purification of angularin, a novel antifungal peptie from adzuki beans. J Pept Sci 2002;8:101–6.
[33] Ye XY, Ng TB. Delandin, a chitinase-like protein with antifungal, HIV-1 reverse transcriptase inhibitory activities from the rice bean *Delandia umbellata*. Proteins Expr Purif 2002;24:524–9.
[34] Ye XY, Ng TB. A new antifungal protein and a chitinase with prominent macrophage-stimulating activity from seeds of *Phaseolus vulgaris* cv. pinto. Biochem Biophys Res Commun 2002;290:813–9.
[35] 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;263:130–4.
[36] Ye XY, Wang HX, Ng TB. Dolichin, a new chitinase-like antifungal protein isolated from field beans (*Dolichos lablab*). Biochem Biophys Res Commun 2000;269:155–9.
[37] 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;67:775–81.
[38] Ye XY, Wang HX, Ng TB. Structurally dissimilar proteins with antiviral and antifungal potency from cowpea (*Vigna unguiculata*) seeds. Life Sci 2000;67:3199–207.