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Concurrent isolation of a Kunitz-type trypsin inhibitor with antifungal activity and a novel lectin from *Pseudostellaria heterophylla* roots

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Abstract

A simple purification protocol, involving ion exchange chromatography on DEAE–cellulose and CM–cellulose and fast protein liquid chromatography-gel filtration on Superdex 75, was employed to isolate a Kunitz-type trypsin inhibitor with antifungal activity and a novel lectin from *Pseudostellaria heterophylla* roots. Both the trypsin inhibitor and the lectin were unadsorbed on DEAE–cellulose and adsorbed on CM–cellulose. They could be separated from one another by gel filtration on Superdex 75 in which the 36-kDa lectin appeared as the first peak and the 20.5-kDa trypsin inhibitor as the second peak. *P. heterophylla* trypsin inhibitor exhibited a trypsin inhibitory potency similar to that of soybean trypsin inhibitor. It also demonstrated antifungal activity toward *Fusarium oxysporum* like aprotinin and Kunitz-type trypsin inhibitors from soybeans and lima beans. *P. heterophylla* lectin was devoid of antifungal activity and exhibited low thermostability and also lability in the presence of acid and alkali. The novel aspects of the present report include demonstration of antifungal activity in Kunitz-type trypsin inhibitors and isolation of a novel lectin as well as a trypsin inhibitor from roots.

Keywords: Trypsin inhibitor; Lectin; Roots; *Pseudostellaria heterophylla*

Pseudostellaria heterophylla (Family Caryophyllaceae) is a Chinese medicinal herb the root of which is best used during recuperation. The cyclic peptides pseudostellarins A–C and D–F [1–4] and heterophyllins A and B, and pseudostellarins G and H [5–7] represent some of its constituents. Fraction PH-I, which is an alcohol-insoluble fraction from a hot water extract of *P. heterophylla* roots, has potent antitumor, mitogenic, and tumor necrosis factor (TNF)-eliciting activities [8]. Fraction PH-1C, derived from PH-1 by gel filtration, is highly potent in inducing TNF-α [9]. It demonstrates antitumor activity, potentiates phagocytic activity of peritoneal macrophages and cytotoxic activity of natural killer cells, induces tumor infiltrating lymphocytes in the tumor site, and boosts the levels of serum interferon-γ and interleukin-4 [10]. Fraction PH-1Cb, derived from PH-1C, is an acidic polysaccharide or proteoglycan with potent TNF-eliciting and antitumor activities [11]. Fraction PH-1Ba, derived from PH-1B, is a polysaccharide that stimulates the proliferation and differentiation of mouse bone marrow cells [12].

In view of the paucity of information about the protein constituents of *P. heterophylla*, the concurrent purification of a trypsin inhibitor with an antifungal activity and a novel lectin from *P. heterophylla* roots is described herein.

Materials and methods

Pseudostellaria heterophylla roots (500 g), obtained from a local vendor of traditional Chinese medicinal materials, were homogenized in water (3 ml/g) by means of a Waring blender. After centrifugation at 12,000*g* for 30 min, 1 M Tris–HCl buffer (pH 7.0), and distilled water were added to the resulting supernatant until the molarity of Tris reached 10 mM. The supernatant was subjected to ion exchange chromatography on a 1 × 20 cm column of DEAE–cellulose (Sigma) in 10 mM Tris–HCl buffer (pH 7.0). After unadsorbed proteins had been collected in the flowthrough fraction D1, adsorbed proteins were desorbed using 10 mM Tris–HCl buffer (pH 7.0) containing 1 M NaCl. Fraction D1 was dialyzed against 10

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mM NH_4OAc buffer (pH 4.5) before application on a 2.5×20 cm column of CM–Sepharose CL-6B (Amersham Biosciences) in the same buffer. Unadsorbed proteins were collected in the flowthrough fraction CM1. Adsorbed proteins were desorbed from the column using a linear 0–1 M concentration gradient of NaCl in the Tris–HCl buffer. The second adsorbed fraction CM3 was saved and further purified on a Superdex 75 HR 10/30 column in 0.2 M NH_4HCO_3 buffer (pH 8.5) using an AKTA Purifier (Amersham Biosciences). The first fraction collected represented purified lectin while the second fraction represented purified trypsin inhibitor.

In the assay for lectin (hemagglutinating) activity, a serial twofold dilution of the lectin solution in microtiter U-plates (50 μl) was mixed with 50 μl of a 2% suspension of rabbit red blood cells in phosphate buffered saline (pH 7.2) at 20 °C. The results were read after about 1 h when the blank had fully sedimented. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was reckoned as one hemagglutination unit. Specific activity is the number of hemagglutination units per milligram protein [13].

The hemagglutinating inhibition tests to examine inhibition of lectin-induced hemagglutination by various sugars were conducted largely like the hemagglutination test. Serial twofold dilutions of sugar samples in phosphate buffered saline were incubated with an equal volume (25 μl) of a solution of the *P. heterophylla* lectin containing eight hemagglutination units for 30 min at room temperature before addition of 50 μl of a 2% rabbit erythrocyte suspension. The minimum concentration of the sugar in the final reaction mixture for complete inhibition of eight hemagglutination units of the *P. heterophylla* lectin preparation was calculated [13,20].

The tests for thermostability and pH stability of the *P. heterophylla* lectin were conducted as previously described for the mushroom (*Tricholoma mongolicum*) lectin [13,14].

The molecular masses of the purified *P. hereophylla* lectin and trypsin inhibitor were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as described by Laemmli and Favre [15]. Gel filtration on an FPLC-Superdex 75 column, which had been calibrated with molecular mass markers (Amersham Biosciences), was also carried out to the estimated molecular mass of the lectin. N-terminal sequencing of the lectin and trypsin inhibitor was carried out by using a Hewlett-Packard HP G1000A Edman degradation unit and an HP 1000 HPLC System [18].

The assay for trypsin inhibitory activity was carried out by addition of test sample (20 μl) to 160 μl of a 1% casein solution in 0.1 M Tris–HCl buffer (pH 7.4). Trypsin (20 μl of a 0.5 mg/ml solution) was then added and the mixture was incubated at 37 °C for 15 min before 0.4 ml 5% trichloroacetic acid was added to terminate the reaction. After centrifugation the absorbance of the supernatant, which reflects the amount of casein fragments, was measured at 280 nm.

The assay of the purified *P. heterophylla* lectin and trypsin inhibitor for antifungal activity was performed using sterile-petri plates (100×15 mm) containing 10 ml potato dextrose agar. At a distance of 1 cm from the rim of the mycelial colony were placed paper disks of the same size (0.625 cm in diameter). A 6-μl aliquot of *P. heterophylla* lectin in 10 mM sodium acetate buffer (pH 5.5) containing 0.13 M NaCl was added to a disk. Incubation of the petri plate was carried out at 23 °C for 72 h until mycelial growth had enveloped disks containing the control and had formed crescents of inhibition around disks with antifungal samples. Three fungal species, *Fusarium oxysporum*, *Rhizoctonia solani*, and *Botrytis cinerea* were examined in the assay [16].

The assay for HIV reverse transcriptase inhibitory activity was performed by ELISA as described by Collins et al. [17] using a non-radioactive kit from Boehringer Mannheim (Germany).

Results

Both trypsin inhibitory and hemagglutinating activities were located in fraction D1 whereas fraction D2 was devoid of either activity. Upon ion exchange chromatography on CM–Sepharose, both activities were recovered only in fraction CM3. The activities were separated by gel filtration on Superdex 75. Hemagglutinating activity was present in the first fraction SU1 and trypsin inhibitory activity resided in the second fraction SU2 (Fig. 1). Fraction SU1 appeared as a single band with a molecular mass of 36 kDa in SDS–PAGE. Fraction SU2 showed up as a single band with a molecular mass of 20.5 kDa in SDS–PAGE (Fig. 2). A summary of the purification is provided in Table 1. The N-terminal sequence of SU2 (purified trypsin inhibitor) was to some extent similar to that of a Kunitz-type trypsin inhibitor from *Leucaena leucephala* trypsin inhibitor [28]. The N-terminal sequence of SU1 (purified lectin) was slightly similar to those of thaumatin-like protein but with a large stretch missing in the former. There was no similarity to previously reported lectin (Table 2). The trypsin inhibitory activity of *P. heterophylla* trypsin inhibitor (fraction SU2) is presented in Table 3 in comparison with trypsin inhibitors from soybean trypsin inhibitor, lima bean trypsin inhibitor, and aprotinin. *P. heterophylla* trypsin inhibitor exhibited a potency similar to that of soybean trypsin inhibitor but lower than that of lima bean trypsin inhibitor. The four aforementioned

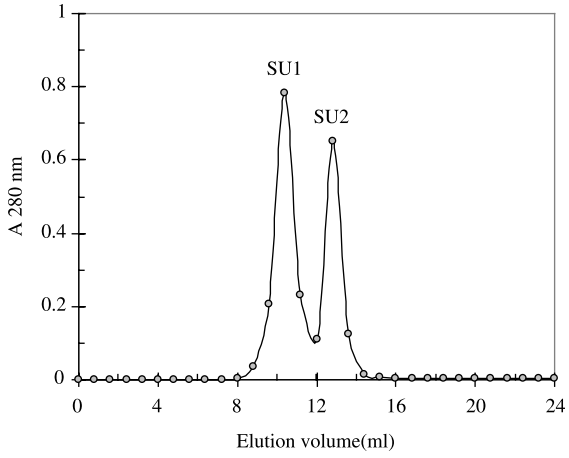

Fig. 1. Gel filtration on Superdex 75 HR 10/30 column. Sample: fraction of *P. heterophylla* root extract unadsorbed on DEAE–cellulose and then adsorbed on CM–cellulose. Buffer: 0.2 M NH_4HCO_3 buffer (pH 8.5). Flow rate: 0.4 ml/min.

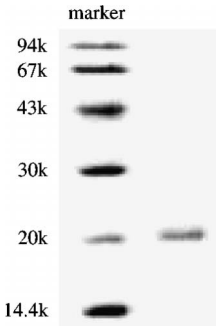

Fig. 2. SDS–PAGE results. Left lane: molecular weight markers from Amersham Biosciences. Right lane: *P. heterophylla* trypsin inhibitor (Fraction SU2).

Table 1
Hemagglutinating activities of *Pseudostellaria heterophylla* chromatographic fractions (from 500 g dried roots) against rabbit red blood cells

	Yield (mg/500 g)	Specific hemagglutinating activity (titer/mg)	Recovery of hemagglutinating activity (%)	Trypsin inhibitory activity (IC_{50} in μg/ml)
D1	217	2626	100	220
D2	493	<10	—	—
CM1	15	<10	—	—
CM2	67	80	0.95	—
CM3	70	6780	83.8	80
SU1	25.8	15800	72.0	—
SU2	23.9	<10	—	35

UD, undetectable.

Table 2
NH_2-terminal amino acid sequences of *Pseudostellaria heterophylla* trypsin inhibitor (PHTI) and lectin (PHL) in comparison with other proteins

PHTI:	-FVVDLDGDPLRNGGEYYYFVRVRTAGAELEYY
LlTI:	QVLVDLDGDPLYNGMSYYILRVARGKGGGLELG
PHL:	KAQFEVLNAKD----------GRRLI
TTLP:	--ATFEVNNCPYTVWAASTRIGGRRL
FBTLP:	--ANFEIVNNCPYTVWAAASPGGGRRLD

LlTI, *Leucaena leucocephala* trypsin inhibitor; TTLP, tomato thaumatin-like protein; FBTLP, French bean thaumatin-like protein. Identical corresponding amino acid residues and underlined.

trypsin inhibitors demonstrated similar antifungal activity toward *F. oxysporum* (Fig. 3). None of the four trypsin inhibitors inhibited mycelial growth in *R. solani*. Neither lima bean trypsin inhibitor nor aprotinin was inhibitory to the fungus *B. cinerea*. Only soybean trypsin inhibitor was inhibitory to *B. cinerea* (data not shown).

The hemagglutinating activity of the lectin remained unaltered in the presence of the following sugars tested at 0.2 M concentration: D-fructose, L-arabinose, D-arabinose, L-rhamnose, D-xylose, L-sorbose, inositol, lactose, D-galactose, inulin, inositol, cellobiose, glucose, D-mannose, raffinose, turanose, D-melibiose, D-galactosamine, α-methyl-D-galactopyranoside, and α-methyl-D-glucopyranoside.

After treatment with 0.025 M HCl, slightly over 50% of hemagglutinating activity of the lectin was left but minimal activity remained in the presence of 0.05 M NaOH. Lectin activity was retained in the temperature range of 10–30 °C. At 40 °C only 50% of the activity remained. Between 40 and 70 °C, every 10 °C rise in temperature led to a reduction of lectin activity by 50%. At 80 °C only slight lectin activity was recovered. Above 80 °C virtually all lectin activity disappeared.

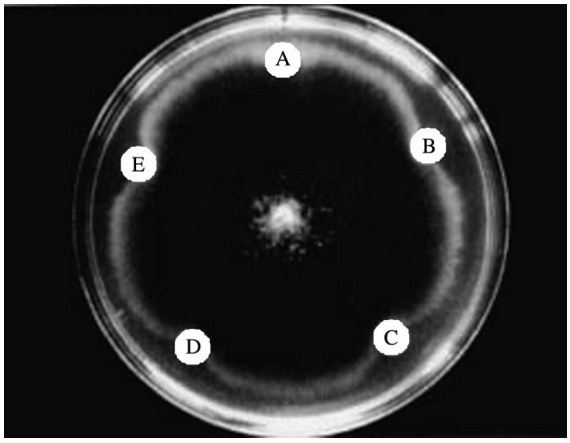

Fig. 3. Inhibitory activity against mycelial growth in *F. oxysporum*. (A) 0.1 M NH_4OAc buffer (pH 5.5). (B) Soybean trypsin inhibitor (60 μg) in 0.1 M NH_4OAc buffer (pH 5.5). (C) Lima bean trypsin inhibitor (60 μg) in 0.1 M NH_4OAc buffer (pH 5.5). (D) Aprotinin (60 μg) in 0.1 M NH_4OAc buffer (pH 5.5). (E) *P. heterophylla* tryspin inhibitor (60 μg) in 0.1 M NH_4OAc buffer (pH 5.5).

Pseudostellaria heterophylla lectin could not inhibit HIV-1 reverse transcriptase. The kidney bean (*Phaseolus vulgaris*) lectin from Sigma Chemical exhibited anti-HIV reverse transcriptase activity (95.4 ± 7.8% inhibition at 5 mg/ml). *P. heterophylla* lectin did not retard mycelial growth in any of the three fungal species tested (data not shown).

Discussion

A simple procedure is described herein for simultaneous purification of a trypsin inhibitor and a lectin from *P. hete-*

Table 3
Trypsin inhibitory activities of various trypsin inhibitors

	% inhibition of trypsin (Mean ± SD, $n = 3$)				
	120 μg/ml	60 μg/ml	30 μg/ml	15 μg/ml	7.5 μg/ml
Trypsin inhibitor from soybean	98.3 ± 4.7	92.6 ± 6.1	53.1 ± 4.7	26.5 ± 3.2	6.3 ± 2.0
Trypsin inhibitor from lima bean	96.3 ± 5.6	90.2 ± 5.8	81.5 ± 6.4	63.8 ± 4.1	25.6 ± 3.2
Aprotinin	92.8 ± 4.4	88.0 ± 6.2	80.4 ± 5.9	68.6 ± 4.9	17.3 ± 2.4
P. heterophylla trypsin inhibitor (PHTI)	94.9 ± 5.2	79.8 ± 3.4	45.9 ± 4.3	24.3 ± 2.2	3.5 ± 1.8

IC_{50} of trypsin inhibitory activity of PHTI = 1.7 μM.

rophylla roots. The procedure comprised only three steps ion exchange chromatography on DEAE–cellulose and then on CM–Sepharose followed by gel filtration on Superdex 75. The two proteins are co-eluted in the first two steps but can be separated due to difference in molecular mass in the last step.

The *P. heterophylla* root extract had been examined for the possible existence of ribosome inactivating protein by assay for inhibitory activity on cell-free translation in a rabbit reticulocyte lysate system [18], for the possible existence of ribonucleolytic activity toward yeast transfer RNA [19], and for the possible existence of antifungal protein by assay of antifungal activity [16]. Although the extract was devoid of the first two activities, it demonstrated hemagglutinating, antifungal and trypsin inhibitory activities. The lectin could be isolated with a simple procedure that employed ion exchange chromatography on DEAE–cellulose and SP–Sepharose and then gel filtration by FPLC on Superose 12. Affinity chromatography on an immobilized sugar column was not employed since the hemagglutinating activity of the lectin could not be inhibited by any of the simple sugars tested, like lectin from the straw mushroom *Volvariella volvacea* [20] which was also purified without employing affinity chromatography. Instead, successive chromatography on DEAE–cellulose, CM–cellulose and FPLC on Mono S were employed [20].

The hemagglutinating activity of *P. heterophylla* lectin was reduced under very acidic and alkaline conditions: a drastic decline in activity was observed in the presence of 0.05 M NaOH or HCl. It was also thermolabile: at 40 °C only half of the activity retained while at 50 °C only 25% remained. Thus its pH stability and thermostability were intermediate. It is perhaps worth mentioning that the mushroom (*T. mongolicum*) lectin was by comparison less sensitive to changes in temperature and pH than *P. heterophylla* lectin [13,14].

Mannose-specific lectins from *Cymbidium hybrid*, *Epiactis helleborine*, and *Listeria ovata* and the *N*-acetylglucosamine-specific *Urtica dioica* lectin inhibited HIV-1 and HIV-2 in MT-4 cells. Lectins with other sugar specificities lacked similar activities. The lectins did not affect HIV-1 adsorption to MT4 cells but presumably interacted at the level of virion fusion with the target cell [21]. In contrast, *P. heterophylla* lectin did not inhibit HIV-1 reverse transcriptase.

Potato tuber lectin, *Klaxveromyces bulgaricus* lectin, a lectin-like protein from *Amaranthus caudatus* and a chitinase-like lectin from *U. dioica* exhibited antifungal activity [22–24]. In contrast, *P. heterophylla* lectin did not display antifungal activity notwithstanding some resemblance of its N-terminal sequence to antifungal thaumatin-like proteins. French bean thaumatin-like protein manifested antifungal activity against the same three fungal species [16] toward which *P. heterophylla* lectin had no effect. The absence from *P. heterophylla* lectin of the sequence CPYTVWAASP, which is highly conserved among thaumatin-like proteins, probably explains its lack of antifungal activity. The N-terminal sequence of the lectin is not found in the sequence of previously reported lectins, indicating that it is a novel lectin.

Some protease inhibitors, e.g., *Vicia faba* Bowman–Birk trypsin inhibitor [25] and cysteine protease inhibitor from pearl millet [26], exhibited antifungal activity. In contrast, potato 1 family chymotrypsin inhibitor from *Momordica cochinchinensis* seeds was devoid of antifungal activity [27]. Thus it deserves attention that Kunitz-type trypsin inhibitors from *P. heterophylla*, soybean, and lima bean as well as aprotinin exerted a species-specific antifungal action. Protease inhibitors and lectins have often been isolated from seeds [25–27]. Thus their isolation from the roots of a Chinese medicinal plant is a notable finding. The demonstration of antifungal activity in Kunitz-type trypsin inhibitors and the isolation of a novel root lectin constitute the novel aspects of the present report.

In summary, an efficient procedure was used for concurrently isolating a lectin, and a Kunitz-type trypsin inhibitor, from the roots of a Chinese medicinal herb, *P. heterophylla*. The possession by the lectin of an N-terminal sequence with some similarity to antifungal thaumatin-like proteins does not confer on it antifungal activity, probably due to absence of a segment highly conserved among thaumatin-like proteins. However, the lectin is characterized by a high hemagglutinating activity and intermediate pH stability and thermostability. Unlike a lot of lectins which are composed of subunits, *P. heterophylla* lectin consists only of a single chain. The trypsin inhibitor has an N-terminal sequence similar to Kunitz-type trypsin inhibitors and trypsin-inhibitory potency similar to that of soybean trypsin inhibitor.

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References

[1] H. Morita, T. Kayashita, H. Kobata, A. Gonda, K. Takeya, H. Itokawa, Pseudostellarins A–C, new tyrosinase inhibitory cyclic peptides from *Pseudostellaria heterophylla*, Tetrahedron 50 (1994) 6797–6804.

[2] H. Morita, T. Kayashita, H. Kobata, A. Gonda, K. Takeya, H. Itokawa, Pseudostellarins D–F, new tyrosinase inhibitory cyclic peptides from *Pseudostellaria heterophylla*, Tetrahedron 50 (1994) 9975–9982.

[3] H. Morita, T. Kayashita, K. Takeya, H. Itokawa, M. Shiro, Crystal and solution forms of a cyclic heptapeptide, pseudostellarin D, Tetrahedron 51 (1995) 12539–12548.

[4] H. Morita, T. Kayashita, K. Takeya, H. Itokawa, Conformational analysis of a cyclic heptapeptide, pseudostellarin D by molecular dynamics and Monte Carlo simulations, Chem. Pharmaceut. Bull. (Tokyo) 44 (1996) 477–480.

[5] N.H. Tan, J. Zou, C.X. Chen, S.X. Zhao, Cyclopeptides from the roots of *Pseudostellaria heterophylla*, Phytochemistry 32 (1993) 1327–1330.

[6] H. Morita, H. Kobata, K. Taekya, H. Itokawa, Pseudostellarin G, a new tyrosinase inhibitory cyclic octapeptide from *Pseudostellaria heterophylla*, Tetrahedron Lett. 32 (1994) 3563–3564.

[7] H. Morita, T. Kayashita, K. Takeya, H. Itokawa, Cyclic peptides from higher plants: part 15. Pseudostellarin H, a new cyclic octapeptide from *Pseudostellaria heterophylla*, J. Nat. Prod. 58 (1995) 943–947.
[8] C.K. Wong, K.N. Leung, M.C. Fung, K.P. Fung, Y.M. Choy, The induction of cytokine gene expression in murine peritoneal maerophages by *Pseudostellaria heterophylla*, Immunopharmacol. Immunotoxicol. 16 (1994) 347–357.
[9] C.K. Wong, K.N. Leung, K.P. Fung, R.K.T. Pang, Y.M. Choy, Mitogenic and tumor necrosis factor producing activities of *Pseudostellaria heterophylla*, Int. J. Immunopharmacol. 14 (1992) 1315–1320.
[10] C.K. Wong, K.N. Leung, K.P. Fung, Y.M. Choy, The immunostimulating activities of anti-tumor polysaccharides from *Pseudostellaria heterophylla*, Immunopharmacology 28 (1994) 47–54.
[11] C.K. Wong, K.N. Leung, K.P. Fung, K.J. Pang, Y.M. Choy, Tumor neurosis factor eliciting fractions separated from *Pseudostellaria heterophylla*, Int. J. Immunopharmacol. 16 (1994) 271–277.
[12] C.K. Wong, K.N. Leung, K.P. Fung, Y.M. Choy, Effects of *Pseudostellaria heterophylla* on proliferation and differentiation of murine bone marrow cells, Immunopharmacol. Immunotoxicol. 16 (1994) 71–84.
[13] H.X. Wang, T.B. Ng, W.K. Liu, V.E.C. Ooi, S.T. Chang, Isolation and characterization of two distinct lectins with antiproliferative activity from the cultured mycelia of the mushroom *Tricholoma mongolicum*, Int. J. Pept. Protein Res. 46 (1996) 508–513.
[14] H.X. Wang, W.K. Liu, T.B. Ng, V.E.C. Ooi, S.T. Chang, The immunomodulatory and antitumor activities of lectins from the mushroom *Tricholoma mongolicum*, Immunopharmacology 31 (1996) 205–211.
[15] U.K. Leammli, M. Favre, Maturation of the head of bacteriophage T4. I. DNA packaging events, J. Mol. Biol. 80 (1973) 575–599.
[16] X.Y. Ye, H.X. Wang, T.B. Ng, First chromatographic isolation of an antifungal thaumatin-like protein from French bean legumes and demonstration of its antifungal activity, Biochem. Biophys. Res. Commun. 263 (1999) 130–134.
[17] R.A. Collins, T.B. Ng, W.P. Fong, C.C. Wan, H.W. Yeung, A comparison of human immunodeficiency virus type 1 inhibition by partially purified aqueous extracts of Chinese medicinal herbs, Life Sci. 60 (1997) PL345–PL351.
[18] S.S.L. Lam, H. Wang, T.B. Ng, Purification and characterization of novel ribosome inactivating proteins, alpha- and beta-pisavins, from seeds of the garden pea *Pisum Sativum*, Biochem. Biophys. Res. Commun. 253 (1998) 135–142.
[19] M. Kunitz, A spectrophotometric method for the measurement of ribonuclease activity, J. Biol. Chem. 164 (1946) 563–568.
[20] Q.B. She, T.B. Ng, W.K. Liu, A novel lectin with potent immunomodulatory activity isolated from both fruiting bodies and cultured mycelia of the edible mushroom *Volvariella volvacea*, Biochem. Biophys. Res. Commu. 247 (1998) 106–111.
[21] J. Balzarini, J. Neytes, D. Schols, M. Hosoya, E. Van Damme, W. Peumans, E. Declercq, The mannose-specific plant lectins from *Cymbidium hybrid* and *Epipactis helleborine* and the (*N*-acetylglucosamine)$_n$-specific plant lectin from *Urtica dioica* are potent and selective inhibitors of human immunodeficiency virus and cytomegalovirus replication in vitro, Antiviral Res. 18 (1992) 191–207.
[22] O. Gozia, J. Ciopraga, T. Bentia, M. Lunga, I. Zamfirescu, R. Tudor, A. Roseanu, F. Nitu, Antifungal properties of lectin and new chitinases from potato tubers, FEBS Lett. 370 (1993) 245–249.
[23] R. Verheyden, J. Pletickx, D. Maes, H.A.M. Peperman, L. Wyns, R. Willem, J.C. Martins, ^{1}H NMR study of the interaction of *N*,*N*',*N*''-triacetyl chitotriose with Ac-AMP2, sugar binding antimicrobial protein isolated from *Amaranthus caudatus*, C.R. Acad. Sci. Ser. III 316 (1995) 788–792.
[24] W.F. Broekaert, J. Van Parijs, F. Leyn, H. Joos, W. Peumans, A chitin-binding lectin from stinging nettle rhizomes with antifungal properties, Science 245 (1989) 1100–1102.
[25] X.Y. Ye, T.B. Ng, P.F. Rao, A Bowman–Birk-type trypsin chymotrypsin inhibitor from broad beans, Biochem. Biophys. Res. Commun. 289 (2001) 91–96.
[26] B.N. Joshi, M.N. Sainani, K.B. Bastawade, V.S. Gupta, P.K. Ranjekar, Cysteine protease inhibitor from pearl millet: a new class of antifungal protein, Biochem. Biophys. Res. Commun. 246 (1998) 382–387.
[27] A.Y. Tsoi, R.C. Wong, T.B. Ng, W.P. Fong, First report on a potato I family chymotrypsin inhibitor from the seeds of a Cucurbitaceous plant, *Momordica cochinchinensis*, Biol. Chem. 385 (2004) 185–189.
[28] M.L. Oliva, J.C. Souza-Pinot, I.F. Batista, M.S. Araujo, V.F. Silveira, E.A. Auerswald, R. Montele, C. Edcerskorn, M.U. Sampaio, C.A. Sampaio, *Leucaena leucocephala* serine proteinase inhibitor: primary structure and action on blood coagulation. Kinin release and rat paw edema, Biochim. Biophys. Acta 1477 (2000) 64–74.