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Psc-AFP, an antifungal protein with trypsin inhibitor activity from *Psoralea corylifolia* seeds

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ABSTRACT

An antifungal protein designated as Psc-AFP, with an apparent molecular mass of 18 kDa, was isolated from a traditional Chinese herb, malaytea scurfpea (*Psoralea corylifolia* L.). The isolation procedure entailed extraction, cation exchange chromatography on CM FF, gel filtration chromatography on Superdex 75 and reversed-phase high performance liquid chromatography on SOURCE 5RPC column. Automated Edman degradation determined the partial N-terminal sequence of Psc-AFP to be NH_2-EWEPVQNGGSSYYMVPRIWA, which displayed homology with plant trypsin inhibitors. The protease inhibitor activity of Psc-AFP was then confirmed by the inhibition on trypsin. Psc-AFP at 10 μM inhibited the mycelial growth of *Alternari brassicae*, *Aspergillus niger*, *Fusarium oxysporum* and *Rhizoctonia cerealis*, suggesting that Psc-AFP has a role in the defense against pathogens.

1. Introduction

Plant seeds contain rich nutrients, such as proteins, oils and saccharides [4], which made them favorite foods for animals, insects and microbes. Accordingly, in the process of evolution, plant seeds have developed a set of measures, for example, physical barriers and antimicrobial compounds, to protect themselves from being destroyed by animals and pathogens [6,13]. During germination, only antimicrobial compounds are dependent on resisting microbial infections because physical barriers are not available at this point.

Among various compounds, antimicrobial proteins, which inhibit the growth of bacterial and fungal pathogens, have been considered very important in plant defense [13]. Up to now, a large number of antimicrobial proteins have been characterized. Structurally they can be divided into many types that comprise thaumatin-like proteins [15,22], chitinases and β-1,3-glucanases [21,26], thionins [10,11], plant defensins [27,32], ribosome-inactivating proteins [3,23], non-specific lipid transfer proteins (nsLTPs) [13,30] and protease inhibitor-like proteins [6,19].

Malaytea scurfpea, named Buguzhi in Chinese, the dry fruit of leguminous plant *Psoralea corylifolia* L., has been used to treat a wide range of diseases and conditions in traditional Chinese medicine. The seed extract of malaytea scurfpea was found to exert anti-oxidative, antimicrobial, anti-inflammatory, anti-tumor, antimutagenic and insect hormonal activities [2,16–18]. Recently, its constituents, lactone, flavone as well as

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terpenoid, e.g. psoralen, isopsoralen, psoralidin and bavachalcone, have been investigated [8,24]. However, antimicrobial proteins components in malaytea scurfpea are rarely questioned. In our endeavors to screen potential antimicrobial proteins from various plant seeds, we found that the extract of malaytea scurfpea seeds was able to inhibit the growth of several fungal pathogens. Here, we described the isolation and characterization of the antifungal protein (Psc-AFP) from the seeds of malaytea scurfpea.

2. Materials and methods

2.1. Plant materials

Malaytea scurfpea, *P. corylifolia* L. (Leguminosae), were obtained from a local medicine herb market in Chongqing, China.

2.2. Antifungal assays

Alternari brassicae, *Aspergillus niger*, *Fusarium oxysporum* and *Rhizoctonia cerealis* were employed for the assay of antifungal activity. All fungi were grown in potato dextrose agar (Difco). In vitro, antifungal activity assay was performed as described by Flores et al. [12] with minor modifications. In brief, the assay was executed using petri dishes 60 mm in diameter containing 10 ml of potato dextrose agar at quarter strength. The mycelia were initially grown on the plates at 28 °C to obtain colonies with size of 30–40 mm in diameter. The potential antifungal samples dissolved in 20 mM phosphate buffer (pH 6.5) were then loaded onto the filter paper discs which rested at a distance of 5 mm away from the rim of the fungal colonies. The plates were incubated in the dark at 28 °C and the zones of fungal inhibition around the discs were checked daily.

2.3. Protein extraction and isolation

Mature malaytea scurfpea dry seeds (200 g) were ground into a fine powder in a laboratory blender. The resulting flour was suspended in five volumes (w/v) of buffer A (20 mM phosphate buffer, pH 6.5) containing 2 mM EDTA, 1 mM PMSF, 1 mM aprotinin and 5 mM thiourea. The suspension was then kept at 4 °C overnight with constant stir. The slurry was filtered through two layers of gauze and subsequently centrifuged for 15 min at 10,000 × *g* at 4 °C. The supernatant was loaded into a CM cation exchange column (HiPrep 16/10 CM FF column from Amersham Biosciences, Sweden) pre-equilibrated with buffer A. After the column was washed with the same buffer to remove unabsorbed proteins, the adsorbed proteins were eluted with a NaCl gradient (0–1.0 M) in buffer A at a flow rate of 2.0 mL min^{-1}. The antifungal fractions were then pooled, concentrated and then applied into HiLoad 26/60 Superdex 75 column (Amersham Biosciences) in buffer A at a flow rate of 0.5 mL min^{-1}. The antifungal fraction was further purified by reversed-phase high performance liquid chromatography (RP-HPLC) on a SOURCE 5RPC column (4.6 × 100 mm, 5 μm; Amersham Biosciences) equilibrated with ultra-pure water. Separation of the fraction was performed with water over 5 min, and a linear gradient of 0–50% acetonitrile (v/v) over 45 min at a flow rate of 1.0 mL min^{-1}. Individual peaks were collected, then condensed in 2 kDa cutoff dialysis tubing (Sigma, USA). After dialysis against ultra-purified water, the samples were used for bioassays.

The CM cation exchange and gel filtration chromatography were performed on an ÄKTA prime system (Amersham Biosciences). The last HPLC purification step was carried out on ÄKTAexplorer 10S (Amersham Biosciences). All chromatography procedures were carried out at room temperature unless otherwise stated.

2.4. Electrophoresis

SDS-PAGE was carried out on 0.75-mm-thick slab gels containing a 12.5% (w/v) polyacrylamide separating gel with a 4% (w/v) stacking gel using an electrophoresis cell (Amersham Biosciences) according to the manufacturer's instructions. Low molecular mass markers were run simultaneously in the gel. The protein bands were visualized by Coomassie blue staining (Amersham Biosciences). Protein concentration was measured by the Bradford method [5] using bovine serum albumin (BSA) as a standard.

2.5. Amino acid sequencing

The purified sample was subjected to SDS-PAGE and then electroblotted to a PVDF membrane (Bio-Rad, USA). Protein bands were visualized with Coomassie blue and the target band was excised for sequencing. The N-terminal amino acid sequence determination was performed by the Laboratory for Protein Chemistry of Hunan Normal University (Changsha, China) on a protein sequencer (model 491, Applied Biosystems, USA).

2.6. Measurement of trypsin inhibitory activity

Trypsin activity was measured using α-*N*-benzoyl-L-phenylalanine-L-valine DL-arginine-*p*-nitroanilide (Bz-Phe-Val-Arg-

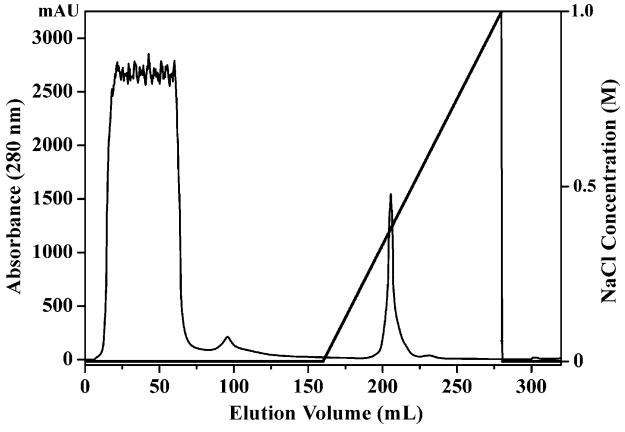

Fig. 1 – Fractionation of the crude extract from the malaytea scurfpea on CM FF cation exchange column equilibrated with 20 mM phosphate buffer (pH 6.5). The column was washed with the phosphate buffer to remove unadsorbed fraction, and then eluted with NaCl gradient (0–1.0 M) in the same buffer at 2.0 mL min^{-1} to desorb the adsorbed fraction which contained antifungal activity. mAU: milli-absorbance unit.

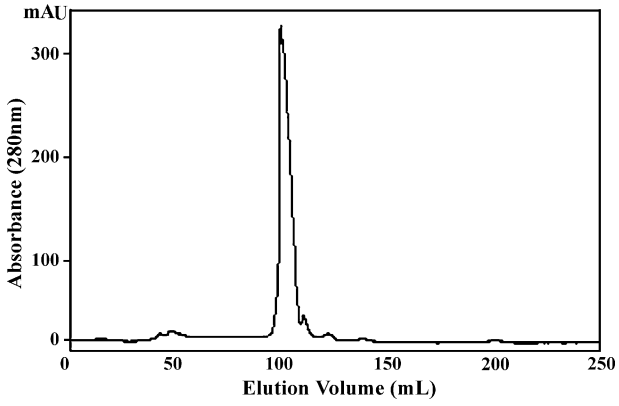

Fig. 2 – Gel filtration profiles of the fraction with antifungal activity from CM FF cation exchange chromatography on HiLoad 26/60 Superdex 75 column in 20 mM phosphate buffer (pH 6.5) at 0.5 mL min^{-1}.

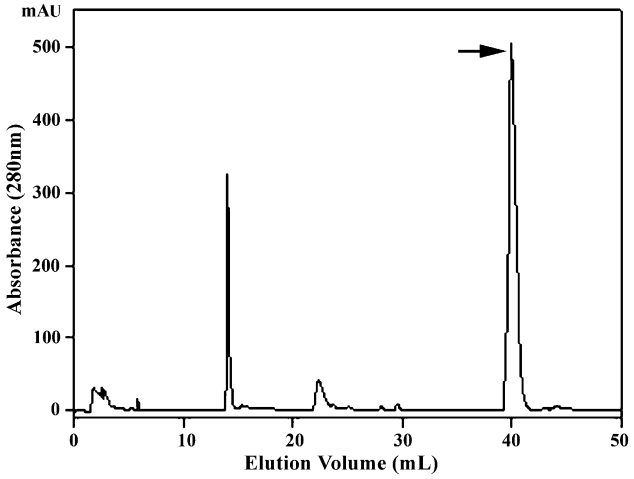

Fig. 3 – RP-HPLC profiles of the fraction with antifungal activity from gel filtration chromatography on a SOURCE 5RPC analytical reversed-phase column (4.6 mm × 150 mm; Amersham Biosciences). The column was eluted at 1 mL min^{-1} with water over 5 min, and a linear gradient of 0–50% acetonitrile over 45 min.

NA; Sigma) as a substrate [28]. Purified Psc-AFP was pre-incubated with trypsin (Sigma) at room temperature for 1 h. Then the substrate (Bz-Phe-Val-Arg-NA) was added into the mixture and the residual activity was measured by the method previously described [33].

3. Results

3.1. *Purification of Psc-AFP*

From the cation exchange chromatogram (Fig. 1), most proteins appeared in the unbound peak that had no antifungal activity, while the fraction collected from a small peak that was eluted between 0.33 and 0.37 M NaCl showed markedly inhibition against *A. niger*. The pooled antifungal fraction was further purified by a gel filtration chromatography, resulting in a single peak (Fig. 2) with inhibition to the growth of *A. niger*. After the collected antifungal fraction was applied to RP-HPLC (Fig. 3), an antifungal protein, designated as Psc-AFP, was purified to homogeneity as indicated by SDS-PAGE (Fig. 4). From 200 g malaytea scurfpea dry seeds, 12.4 mg purified Psc-AFP was obtained (Table 1).

3.2. *Characterization of Psc-AFP*

Purified Psc-AFP showed an apparent molecular mass of 18 kDa calibrated with low molecular mass markers on SDS-PAGE (Fig. 4). The N-terminal amino acid sequence of Psc-AFP was determined to be NH_2-EPILDVNGGSSYYMVPRIWA which displayed high similarity to several plant trypsin inhibitors (Table 2), indicating that Psc-AFP could be a member of the plant trypsin inhibitor family.

Table 1 – Summary of purification of Psc-AFP from *P. corylifolia*

Step	Amount of protein (mg)	Recovery (%)
Crude extraction	1356.0	100
CM cation exchange	117.8	8.7
Superdex 75 gel filtration	50.1	3.7
RP-HPLC (Psc-AFP)	12.4	0.9

3.3. *Antifungal and trypsin inhibitory activities of Psc-AFP*

The antifungal activity of purified Psc-AFP was further tested against *A. niger*, *A. brassicae*, *F. oxysporum* and *R. cerealis*. As shown in Fig. 5, Psc-AFP had a noticeable inhibition to the four fungi at 10 μM. It was also found that Psc-AFP had weaker

Table 2 – Comparison of N-terminal sequence of Psc-AFP with those of related proteins

Species (protein)	Sequence	Accession no.
Psoralea corylifolia (Psc-AFP)	^{1}EPILDVNGGSSYYMVPRIWA^{20}	
Erythrina latissima (trypsin inhibitor)	^{5}GNGEVVQNGGT-YYLLPQVWA^{24}	P68171
Psophocarpus tetragonolobus (chymotrypsin inhibitor)	^{31}AEGNLVENGGT-YYLLPHIWA^{50}	P10822
Glycine max (trypsin inhibitor)	^{31}TDDDPLQNGGT-YYMLPVMRG^{50}	P25273
Erythrina caffra (trypsin inhibitor)	^{5}GNGEVVQNGGT-YYLLPQVWA^{24}	Q09943
Erythrina variegate (trypsin inhibitor)	^{30}VEGNLVENGGT-YYLLPHIWA^{49}	P81366

^{5}G and A^{24} indicate that the fifth N-terminal amino acid is G and 24th N-terminal amino acid is A.

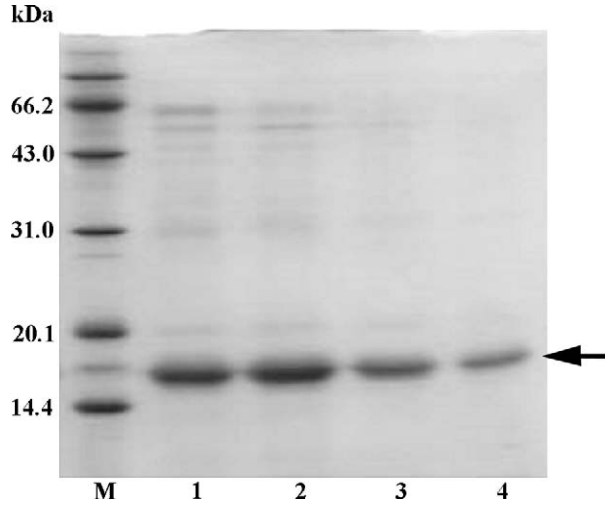

Fig. 4 – SDS-polyacrylamide gel electrophoresis of Psc-AFP. Psc-AFP was analyzed by SDS-polyacrylamide gel electrophoresis on 12.5% separating gel with a 4% stacking gel stained with silver. Lane M, protein low molecular mass markers; lanes 1 and 2, the sample from CM cation exchange column; lane 3, the sample from gel filtration column; lane 4, the sample from RP-HPLC.

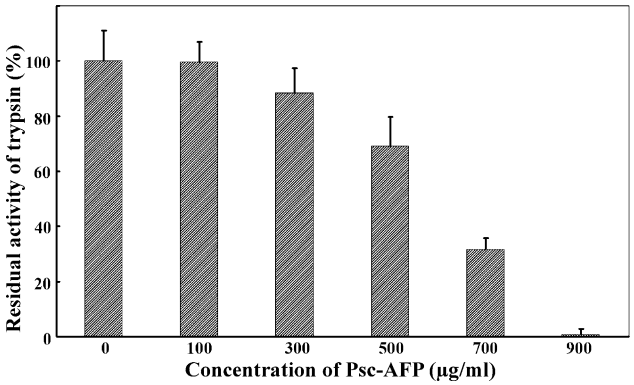

Fig. 6 – The inhibition of purified Psc-AFP against trypsin. The assay for the inhibition of Psc-AFP was carried out using 1 μg soybean trypsin. The results are shown as the percentage of trypsin activity in the absence of Psc-AFP. Results are mean ± S.D., $n = 3$.

inhibition to *F. oxysporum* than to *R. cerealis*, *A. brassicae* and *A. niger* (Fig. 5).

According to the sequence comparison, Psc-AFP is a potential plant trypsin inhibitor, so we tested the ability of Psc-AFP to inhibit trypsin. The result showed that Psc-AFP had a dose-dependent inhibition against trypsin from soybean (Fig. 6).

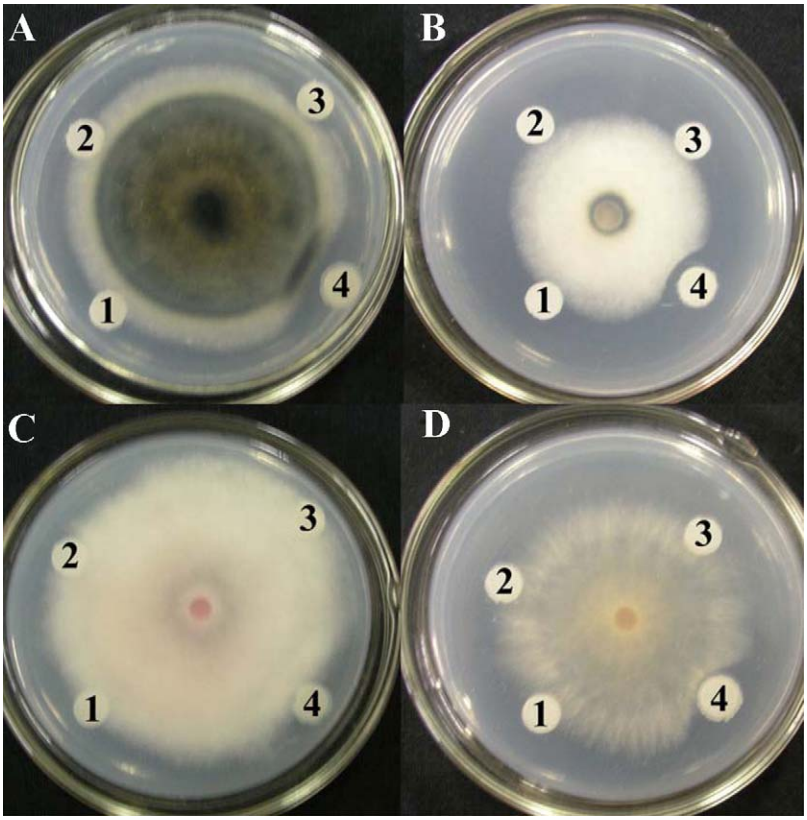

Fig. 5 – The effect on growth of hyphae for phytopathogenic fungi of the purified Psc-AFP. (1) 20 mM phosphate buffer (pH 6.5); (2) 10 μM BSA in 20 mM phosphate buffer (pH 6.5); (3) 5 μM Psc-AFP in 20 mM phosphate buffer (pH 6.5); (4) 10 μM Psc-AFP in 20 mM phosphate buffer (pH 6.5). (A) *A. brassicae*; **(B)** *A. niger*; **(C)** *F. oxysporum*; **(D)** *R. cerealis*.

4. Discussion

We purified an antifungal protein with trypsin inhibitor activity, named Psc-AFP, from malaytea scurfpea seeds. It was indicated that Psc-AFP is a member of the plant trypsin inhibitors by comparing the N-terminal amino acid homology. The trypsin inhibitor activity of Psc-AFP was then confirmed by the inhibition on trypsin from soybean. Up to date, some antifungal proteins with an associated activity of protease inhibitor have been isolated and characterized from plants, animals and microorganisms [1,7,9,14,19,25,29,34,35]. Protease inhibitors are generally found in plant storage and vegetative tissues where they are believed to play a defense role against herbivores by the inhibition of their proteases [20]. Further studies have shown that some protease inhibitors also inhibit fungal growth [6,19]. It has been suggested that protease inhibitors may be part of defense-related proteins that offer a barrier against fungal infection.

Psc-AFP from malaytea scurfpea inhibited mycelial growth of *A. brassicae*, *A. niger*, *F. oxysporum* and *R. cerealis* in vitro. Compared to a variety of antifungal proteins with protease inhibitor activity from plant tissues, Psc-AFP has a moderate antifungal activity. For instance, a corn TI inhibits *A. flavus* at concentration of 7.1 μM [6]; lunatusin from lima beans has an IC_{50} value of 1.9 μM against *F. oxysporum* [31]; potamin-1 and AFP-J from potato inhibit the growth of some plant pathogens with a MIC > 100 μM [19,25]; while Psc-AFP has antifungal activity against the four fungi tested at concentration of 10 μM (Fig. 5).

Very few bioactive proteins have been isolated from malaytea scurfpea (*P. corylifolia* L.). The isolation of an antifungal protein with trypsin inhibitor activity enhances the sparse literature on this traditional medicinal plant.

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REFERENCES

[1] Aguirre C, Valdes-Rodriguez S, Mendoza-Hernandez G, Rojo-Dominguez A, Blanco-Labra A. A novel 8.7 kDa protease inhibitor from chan seeds (*Hyptis suaveolens* L.) inhibits proteases from the larger grain borer *Prostephanus truncatus* (Coleoptera: Bostrichidae). Comp Biochem Physiol B 2004;138:81–9.

[2] Bapat K, Chintalwar GJ, Pandey U, Thakur VS, Sarma HD, Samuel G, et al. Preparation and in vitro evaluation of radioiodinated bakuchiol as an antitumor agent. Appl Radiat Isot 2005;62:389–93.

[3] Barbieri L, Stirpe F. Ribosome-inactivating proteins from plants. Biochim Biophys Acta 1993;1154:237–82.

[4] Bewley JD. Seed germination and dormancy. Plant Cell 1997;9:1055–66.

[5] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.

[6] Chen ZY, Brown RL, Russin JS, Lax AR, Cleveland TE. A corn trypsin inhibitor with antifungal act inhibits *Aspergillus flavus* alpha-amylase. Phytopathology 1999;89:902–7.

[7] Chilosi G, Caruso C, Caporale C, Leonardi L, Bertini L, Buzi A, et al. Antifungal activity of a Bowman–Birk-type trypsin inhibitor from wheat kernel. J Phytopathol Phytopathologische Z 2000;148:477–81.

[8] Editorial Committee of the Pharmacopoeia of People's Republic of China. The pharmacopoeia of People's Republic of China, part 1 Beijing: Chemical Industry Press; 2000

[9] Doumas S, Kolokotronis A, Stefanopoulos P. Anti-inflammatory and antimicrobial roles of secretory leukocyte protease inhibitor. Infect Immun 2005;73:1271–4.

[10] Epple P, Apel K, Bohlmann H. Overexpression of an endogenous thionin enhances resistance of *Arabidopsis* against *Fusarium oxysporum*. Plant Cell 1997;9:509–20.

[11] Florack DEA, Stiekema WJ. Thionins—properties, possible biological roles and mechanisms of action. Plant Mol Biol 1994;26:25–37.

[12] Flores T, Alape-Giron A, Flores-Diaz M, Flores HE. Ocatin. A novel tuber storage protein from the Andean tuber crop oca with antibacterial and antifungal activities. Plant Physiol 2002;128:1291–302.

[13] Garcia-Olmedo F, Molina A, Alamillo JM, Rodriguez-Palenzuela P. Plant defense peptides. Biopolymers 1998;47:479–91.

[14] Giudici AM, Regente MC, de la Canal L. A potent antifungal protein from *Helianthus annuus* flowers is a trypsin inhibitor. Plant Physiol Biochem 2000;38:881–8.

[15] Grenier J, Potvin C, Trudel J, Asselin A. Some thaumatin-like proteins hydrolyse polymeric beta-1,3-glucans. Plant J 1999;19:473–80.

[16] Haraguchi H, Inoue J, Tamura Y, Mizutani K. Antioxidative components of *Psoralea corylifolia* (Leguminosae). Phytother Res 2002;16:539–44.

[17] Katsura H, Tsukiyama RI, Suzuki A, Kobayashi M. In vitro antimicrobial activities of bakuchiol against oral microorganisms. Antimicrob Agents Chemother 2001;45:3009–13.

[18] Khatune NA, Islam ME, Haque ME, Khondkar P, Rahman MM. Antibacterial compounds from the seeds of *Psoralea corylifolia*. Fitoterapia 2004;75:228–30.

[19] Kim JY, Park SC, Kim MH, Lim HT, Park Y, Hahm KS. Antimicrobial activity studies on a trypsin–chymotrypsin protease inhibitor obtained from potato. Biochem Biophys Res Commun 2005;330:921–7.

[20] Koiwa H, Bressan RA, Hasegawa PM. Regulation of protease inhibitors and plant defense. Trends Plant Sci 1997;2:379–84.

[21] Krishnaveni S, Liang GH, Muthukrishnan S, Manickam A. Purification and partial characterization of chitinases from sorghum seeds. Plant Sci 1999;144:1–7.

[22] Kuboyama T, Yoshida KT, Takeda G. An acidic 39-kDa protein secreted from stigmas of tobacco has an amino-terminal motif that is conserved among thaumatin-like proteins. Plant Cell Physiol 1997;38:91–5.

[23] Mishra V, Sharma RS, Paramasivam M, Bilgrami S, Yadav S, Srinivasan A, et al. cDNA cloning and characterization of a ribosome inactivating protein of a hemi-parasitic plant (*Viscum album* L.) from north-western Himalaya (India). Plant Sci 2005;168:615–25.

[24] Park EJ, Zhao YZ, Kim YC, Sohn DH. Protective effect of (s)-bakuchiol from *Psoralea corylifolia*

on rat liver injury in vitro and in vivo. Planta Med 2005;71:508–13.
[25] Park Y, Choi BH, Kwak J-S, Kang C-W, Lim H-T, Cheong H-S, et al. Kunitz-type serine protease inhibitor from potato (*Solanum tuberosum* L. cv. Jopung). J Agric Food Chem 2005;53:6491–6.
[26] Petruzzelli L, Muller K, Hermann K, Leubner-Metzger G. Distinct expression patterns of beta-1,3-glucanases and chitinases during the germination of *Solanaceous* seeds. Seed Sci Res 2003;13:139–53.
[27] Thomma B, Cammue BPA, Thevissen K. Plant defensins. Planta 2002;216:193–202.
[28] Tunlid A, Rosen S, Ek B, Rask L. Purification and characterization of an extracellular serine protease from the nematode-trapping fungus *Arthrobotrys oligospora*. Microbiology 1994;140:1687–95.
[29] Vernekar JV, Ghatge MS, Deshpande VV. Alkaline protease inhibitor: a novel class of antifungal proteins against phytopathogenic fungi. Biochem Biophys Res Commun 1999;262:702–7.
[30] 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;25:1235–42.
[31] Wong JH, Ng TB. Lunatusin, a trypsin-stable antimicrobial peptide from lima beans (*Phaseolus lunatus* L.). Peptides 2005;26:2086–92.
[32] Wong JH, Ng TB. Sesquin, a potent defensin-like antimicrobial peptide from ground beans with inhibitory activities toward tumor cells and HIV-1 reverse transcriptase. Peptides 2005;26:1120–6.
[33] Yang X-Y, Lu X-F, Pei Y. Characterization and purification of gut fibrinolytic protease from *Tabanus amaenus*. Entomol Sin 2001;8:343–52.
[34] Ye XY, Ng TB. A new peptidic protease inhibitor from *Vicia faba* seeds exhibits antifungal, HIV-1 reverse transcriptase inhibiting and mitogenic activities. J Pept Sci 2002;8:656–62.
[35] Zeeuwen PLJM. Epidermal differentiation: the role of proteases and their inhibitors. Eur J Cell Biol 2004;83:761–73.