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A Lysozyme with Antifungal Activity from *Pithecellobium dulce* Seeds

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Summary

A protein of an apparent molecular mass of 14.4 kDa with antifungal activity was isolated from the seeds of *Pithecellobium dulce* using extraction with 100 mM Tris-HCl buffer (pH=8.0), precipitation with 80 % ammonium sulfate, and bioassay purification *via* Resource Q anion exchange chromatography and Superdex 200 gel filtration chromatography. The purified protein was putatively identified by tandem mass spectrometry with Mascot database searching, with the partial amino acid sequences showing a high degree of similarity to chicken egg white lysozyme. This putative plant lysozyme expressed antifungal activity with a rather high thermal stability of up to 80 °C for 15 min (at pH=8.0). It exerted an antifungal action towards *Macrophomina phaseolina* but displayed no antifungal activity against two other isolates, *Phymatotrichopsis omnivora* and *Fusarium avenaceum*.

Key words: *Pithecellobium dulce*, seed protein, lysozyme, antifungal activity

Introduction

Legume seeds are valuable agricultural and commercial crops that serve as important nutrient sources for both humans and animals (*1–3*). However, they also contain antinutritional compounds, including some protease inhibitors (*4–7*). In general, protease inhibitors have been shown to possess different biological effects ranging from antinutritional to beneficial (*4,8–10*). Pea seeds contain protease inhibitors with antifungal activities that are secreted during germination, indicating a potential protective role of these inhibitors (*11*). *Pithecellobium dulce* Benth. is a woody legume native to the northwestern regions of Mexico (*12*), but which is now introduced into and widely distributed throughout India, Southeast Asia (Thailand, Malaysia and Indonesia) and can also be found in South Africa and Australia (*2*). This tree species has been used for fencing, tanning, feed and food (*2,12*). Due to their high protein, dietary fiber and unsaturated fatty acid contents, seeds of *Pithecellobium* species are traditionally used worldwide for food and feedstuff (*4–6*). Inhibitory activity of proteins has been registered in the seeds of several *Pithecellobium* species, where, for example, the inhibitory activity against trypsin in *P. keyense* was higher than that described for soybean (*13,14*). The inhibitory activity identified in the extracts of *P. dulce* seeds was against trypsin, chymotrypsin and papain. A protease inhibitor of the Kunitz family was isolated from *P. dulce* seeds and characterized (*15*), whilst a Kunitz-type protease inhibitor was isolated from *P. dumosum* seeds and found to have insecticidal properties (*16*). Herein a lysozyme-like protein in *P. dulce* seeds with an antifungal activity is reported.

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Materials and Methods

Plant material

Pods of *Pithecellobium dulce* were obtained from Khlong Toei market (Bangkok, Thailand). A voucher specimen (BK48622) is deposited at the Bangkok Herbarium (BK) of the Plant Variety Protection Division, Department of Agriculture (Bangkok, Thailand). Fresh spiral reddish-brown pods were peeled and the seed pulp within was removed. The black seeds were manually separated and stored at –80 °C until use.

Protein extraction

P. dulce seeds (100 g) were soaked in 250 mL of deionized water and homogenized in 500 mL of 100 mM Tris-HCl buffer (pH=8.0) in a blender, followed by stirring overnight at 4 °C. The extracts were filtered through a cheesecloth and centrifuged at 5000×*g* for 30 min at 4 °C. The supernatant was recovered, brought to 80 % saturation with ammonium sulfate at 4 °C and then centrifuged at 13 000×*g* for 60 min at 4 °C. The precipitate was dissolved in 100 mL of deionized water and dialyzed overnight (4 °C) in SnakeSkin dialysis tubing (molecular mass cut-off of 3.5 kDa; Pierce Biotechnology, Rockford, IL, USA) against 2.5 L of deionized water with several changes of the water. The crude protein preparation was then freeze-dried and kept at –80 °C for further purification.

Purification of antifungal protein (G4)

The crude protein from *P. dulce* seeds was dissolved in 20 mM Tris-HCl (pH=8.0) to 50 mg/mL and then 65 mL were loaded onto a 6-mL Resource Q anion exchange column (Amersham Biosciences, Piscataway, NJ, USA) previously equilibrated with the same buffer. Following the collection of unadsorbed proteins, the adsorbed proteins were differentially eluted with a linear gradient of 0–0.5 M NaCl in the same buffer (120 mL) at 4 °C and with a flow rate of 6 mL/min. The absorbance of the eluate was monitored at 280 nm to estimate protein-containing fractions, and protein-positive fractions were screened for antifungal activity as detailed below. The only protein fractions positive for antifungal activity were from the unbound fractions (Q1), which were pooled, lyophilized and then rehydrated in 20 mM Tris-HCl (pH=8.0) to 100 mg/mL, and 1.2 mL were loaded into a Superdex 200 gel filtration chromatography column (16×60 cm; Amersham Biosciences, Piscataway, NJ, USA). The column was previously equilibrated and proteins eluted with 20 mM Tris-HCl (pH=8.0) at 4 °C and flow rate of 1 mL/min. Fractions were analyzed for proteins by absorbance measurement (280 nm) and for antifungal activity as described below. The active fractions, corresponding to the fourth eluted peak (G4), were collected and lyophilized.

Reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Reducing SDS-PAGE (12.5 % T, 4 % C; T is the total amount of acrylamide; C is total amount of cross-linker, which is *N,N'*-methylene bisacrylamide) was performed according to the method of Laemmli and Favre (*17*) using 13.3×8.7 cm vertical gels. Briefly, samples were resuspended in sample buffer (62.5 mM Tris-HCl (pH=6.8), 10 % (by volume) glycerol, 2 % (by mass per volume) SDS, 5 % (by volume) 2-mercaptoethanol) and then heated at 100 °C for 3 min. After resolution, the gels were stained for 1 h in 250 mL of an aqueous Coomasie solution (0.1 % (by mass per volume) Coomassie blue, 30 % (by volume) methanol, 10 % (by volume) acetic acid) and then treated three times with 250 mL of aqueous destaining solution (30 % (by volume) methanol, 10 % (by volume) acetic acid).

Protein determination

Protein concentration was determined by the Bradford method (*18*) using a commercial dye reagent from Bio-Rad (Hercules, CA, USA) and bovine serum albumin as the standard.

In-gel digestion

The desired destained protein band was manually excised from the gel and cut into 1.5-mm diameter gel plugs. Then, 25 μL of a 1:1 (by volume) of 50 mM ammonium bicarbonate:acetonitrile solution were added and incubated at room temperature for 15 min. This process was repeated until the gel band was completely destained. The protein band was then dehydrated with 25 μL of acetonitrile for 15 min at room temperature, the excess acetonitrile was removed, and the gel band was dried under vacuum and rehydrated for 20 min in 20 μL of sequencing grade modified bovine trypsin (10 ng/μL; Roche Diagnostics, Basel, Switzerland) in 25 mM ammonium bicarbonate. The excess of trypsin solution was removed, and 15 μL of 25 mM ammonium bicarbonate were added to each well to prevent dehydration during incubation. Proteolysis was allowed to continue overnight at 37 °C and stopped by adding 15 μL of 10 % (by volume) formic acid. The supernatant was recovered, and the gel band was extracted twice with 25 μL of a 1:1 (by volume) solution of acetonitrile and 25 mM ammonium bicarbonate, and then once more with 25 μL of acetonitrile. The extracts were then combined and concentrated under vacuum to a final volume of 25 μL.

Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS)

Separation of the protein digests was achieved using a nanoscale HPLC system (LC Packings/Dionex Corp., Sunnyvale, CA, USA) consisting of an autosampler (Famos, LC Packings/Dionex Corp.), a precolumn switching device (Switchos, LC Packings/Dionex Corp.) and an HPLC pump system (Ultimate, LC Packings/Dionex Corp.). Samples (5 μL) were loaded onto a C_{18} precolumn (1.0×0.3 mm i.d., 100 Å, PepMap C_{18}, LC Packings) for desalting and concentrating at a flow rate of 50 μL/min using 5 % (by volume) acetonitrile and 95 % (by volume) water containing 0.1 % (by volume) formic acid as the mobile phase A. Peptides were then eluted from the precolumn and separated on a nanoanalytical C_{18} column (15 cm×75 μm i.d., 100 Å, PepMap C_{18}, LC Packings) at a flow rate of 200 nL/min. Peptides were eluted with a linear gradient of 5–40 % mobile phase B (95 % (by vol-

ume) acetonitrile and 5 % (by volume) water containing 0.08 % (by volume) formic acid) for over 40 min. The separated peptides were directly analyzed with an ABI QSTAR Pulsar *i* hybrid Quadrupole time-of-flight (Q-TOF) mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a nano-electrospray ionization source (Protana, Toronto, Canada). The nano-electrospray was generated using a PicoTip 10-μm inner diameter needle (New Objectives, Woburn, MA, USA) maintained at a voltage of 2400 V. TOF-MS and tandem mass spectral data were acquired using information-dependent acquisition (IDA) with the following settings: charge state selection from 2 to 5, an intensity threshold of 10 counts/s for the tandem experiment, and a collision energy setting automatically determined by the IDA, based on the *m/z* values of each precursor ion. Following information-dependent data acquisition, precursor ions were excluded for 90 s using a window of 6 atomic mass units (amu) to minimize the redundancy in tandem mass spectra.

Data analysis and Mascot searching

The acquired mass spectrometry data were queried against the NCBInr protein database using the Mascot (*19*) (v. 2.2, Matrix Science Ltd, London, UK) search engine with a mass tolerance of 150 ppm, one trypsin miscleavage allowance and two variable amino acid modifications: methionine oxidation and cysteine carbamidomethylation.

Antifungal activity assays

Antifungal activity was performed using sterile Petri plates containing potato dextrose agar (PDA). After the mycelial colony had started to develop outwards from the application zone, sterile paper disks (0.625 cm in diameter) were placed at a distance of 0.5 cm from the rim of the mycelial colony. An aliquot (40 μL) of the fraction under investigation was added to the disk and the plates were incubated at 28 °C for 72 h until mycelial growth from the central region had enveloped the peripheral disks containing the control and formed crescents of inhibition (positive) or enveloped (negative) the disks with test samples for antifungal activity (*20*). Three fungi, obtained from the Plant Biology Division, The Samuel Roberts Noble Foundation, Ardmore, Oklahoma, USA, *Macrophomina phaseolina* (charcoal rot), *Phymatotrichopsis omnivora* (cotton rot) and *Fusarium avenaceum*, were used in this assay.

Thermal stability

The thermal stability of the antifungal activity of the purified protein against *M. phaseolina* was conducted by using the disc diffusion growth inhibition assay on PDA plates as mentioned above. The purified antifungal protein solution (20 mM Tris-HCl buffer containing 0.5 M NaCl, pH=8.0) was exposed to various temperatures from 20 to 100 °C for 15 min and then subjected to the antifungal activity assay as mentioned above.

Results

The crude protein extract from the seeds of *P. dulce*, obtained after the precipitation with 80 % ammonium sulfate and dialysis, was evaluated for antifungal growth activity (fungistatic and/or fungicidal) against three phytopathogenic fungi. The crude protein showed good antifungal activity, in terms of growth inhibition, against *Macrophomina phaseolina* at 0.5 mg mass, but showed little inhibition against the growth of either *Phymatotrichopsis omnivora* or *Fusarium avenaceum* at 1 mg mass (data not shown). Ion exchange chromatography of *P. dulce* seed extract on Resource Q produced an unadsorbed fraction (Q1) and four adsorbed fractions (Q2, Q3, Q4, and Q5) (Fig. 1). The unbound fraction (Q1) was found to correspond to the bulk of the antifungal activity against *M. phaseolina*, with no significant activity (data not shown) being detected in the bound fractions (Q2–5). The active

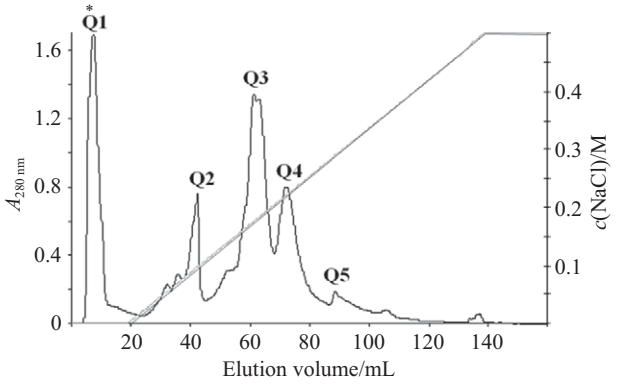

Fig. 1. Resource Q anion exchange chromatography of the precipitate with 80 % ammonium sulfate from the Tris-HCl extract of *Pithecellobium dulce* seeds. Fraction with antifungal activity is indicated by asterisk (*)

fraction was separated on a Superdex 200 column, yielding four (three distinct) peaks (Fig. 2). However, only one of the fractions (G4) revealed antifungal activity against *M. phaseolina* at 9.6 μg of the purified antifungal protein

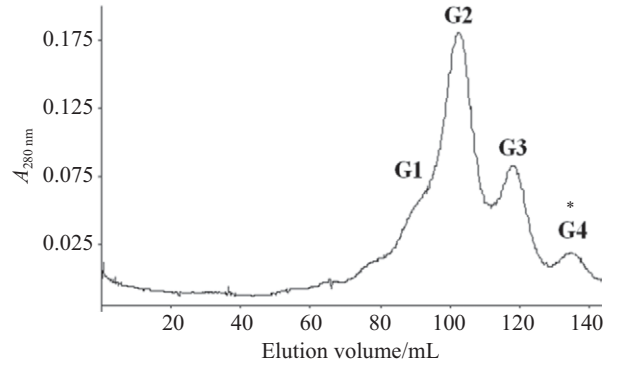

Fig. 2. Gel filtration chromatography of fraction Q1 using a Superdex 200 column. Fraction with antifungal activity is indicated by asterisk (*)

(Figs. 2 and 3). Reducing SDS–PAGE suggested that fraction G4 was purified to near homogeneity and comprised a single protein of ~14.4 kDa, which thus likely represents the purified antifungal protein from *P. dulce* seeds (Fig. 4). The purification yield of the antifungal protein

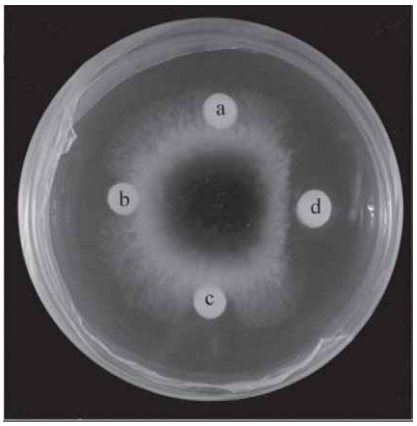

Fig. 3. Inhibitory activity of the purified antifungal protein (G4) against *Macrophomina phaseolina*: a) 20 mM Tris-HCl, pH=8.0, containing 0.5 M NaCl; b) 0.5 and c) 1 mg of crude protein obtained by precipitation with 80 % ammonium sulfate; and d) 9.6 μg of the purified antifungal protein (G4). The plate shown is representative of at least 3 replicated assays

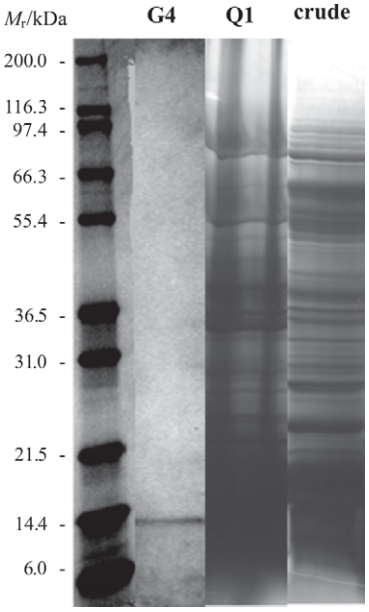

Fig. 4. Reducing SDS-PAGE of the purified antifungal protein from *P. dulce* seeds (fraction G4 from Fig. 2). From left to right: molecular mass standards; the purified antifungal protein (fraction G4 from Superdex 200 column); fraction Q1 from the Resource Q column chromatography; crude protein extract obtained by precipitation with 80 % ammonium sulfate

from *P. dulce* seeds at each stage is summarized in Table 1, where the final G4 protein extract was ~2.4 % of the starting crude extract and 0.079 % of the total seed mass.

Table 1. Purification yields of the antifungal protein from *P. dulce* seeds (100 g)

Purification step	*m*(total protein)/mg
crude extract from ammonium sulfate precipitation (80 % saturation)	3260
Resource Q (first peak, Q1)	814
Superdex 200 (fourth peak, G4)	79

The antifungal activity assay revealed that inhibition of *M. phaseolina* growth was detectable with the G4 protein fraction at the lowest tested dose of 0.06 mg/mL (Fig. 5A). Moreover, this G4 protein fraction retained significant antifungal activity at pH=8.0 when incubated at up to 80 °C for 15 min, but was inactivated at 100 °C for 15 min (Fig. 5B). To identify this antifungal protein, the purified protein was further characterized by peptide mass fingerprinting using a nano LC-ESI-MS/MS, and the MS/MS results of the tryptic peptides were queried against the NCBInr database *via* the Mascot program. The antifungal protein G4 was found to match the chicken (*Gallus gallus*) egg white lysozyme (NCBI accession no. 630460A), with a Mascot molecular weight search (MOWSE) score of 144, derived from a 21 % amino acid sequence cover-

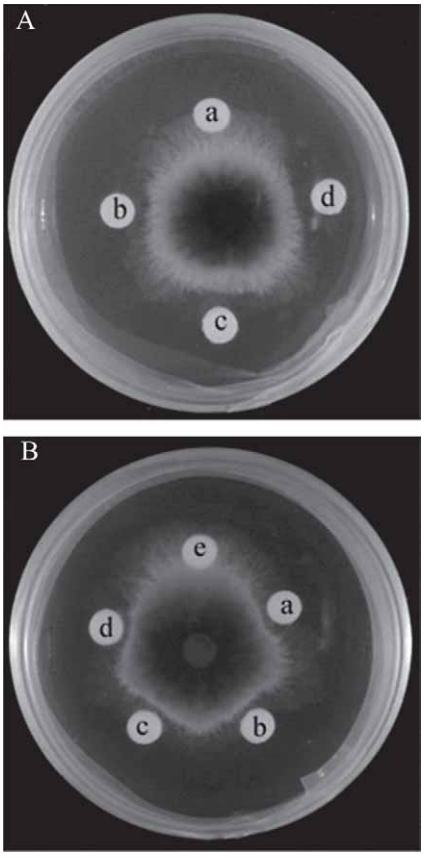

Fig. 5. Determination of the minimum concentration of the antifungal activity of purified antifungal protein (G4) against *M. phaseolina*: A: a) 20 mM Tris-HCl, pH=8.0 containing 0.5 M NaCl, b) 0.06, c) 0.12 and d) 0.18 μg/μL of purified antifungal protein (G4); B: effect of temperature on the antifungal activity of 30 μg of purified antifungal protein (G4) against *M. phaseolina*. Sample disks a, b, c, d and e represent protein previously exposed to 20, 40, 60, 80 and 100 °C for 15 min, respectively. The plates shown are representative of at least 3 replicated assays

age between the two matched peptides, which is significantly higher than the threshold score (52). The amino acid sequence of the purified antifungal protein (G4) compared to chicken egg white lysozyme is shown in Fig. 6 and it showed a matched amino acid sequence in 28 of the 129 chicken lysozyme amino acids, representing 21 % coverage, which is sufficient to specify that this anti-

GG **1** KVFGRCELAA AMKRHGLDNY RGYSLGNWVC AAKFESNFNT QATNRNTDGS

PD **FESNFNT QATNRNTDGS**

GG **51** TDYGILQINS RWWCNDGRTP GSRNLCNIPC SALLSSDITA SVNCAKKIVS

PD **TDYGILQINS R**

GG **101** DGDGMNAWVA WRNRCKGTDV QAWIRGCRL

Fig. 6. Amino acid sequences of the two tryptic peptide fragments of the purified lysozyme from *P. dulce* seeds (PD) compared with chicken (*Gallus gallus*) egg white lysozyme (GG) (identities are shown in bold)

fungal protein is highly likely to be a lysozyme. Moreover, this plant lysozyme has molecular mass of 14.4 kDa, which is close to the molecular mass of chicken egg white lysozyme, 14.3 kDa.

Discussion

Lysozymes, also known as muramidases or *N*-acetylmuramide glycanhydrolases, are a family of enzymes (EC 3.2.1.17) which degrade bacterial cell walls by catalyzing the hydrolysis of the 1,4-β-linkages between *N*-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycans, and between *N*-acetyl-D-glucosamine residues in chitodextrins. They are responsible for breaking down the polysaccharide walls of many kinds of bacteria and thus provide some degree of protection against infection. In biotechnological applications, the enzyme has been used for lysing *E. coli* and *Streptomycetes* sp. for extraction purposes (*21*), including extracting a group of specific antigens (*22*). Lysozymes are widely distributed in animals and plants. Moreover, large amounts of lysozyme can be found in chicken egg white. The plant lysozymes found in figs (*Ficus carica*) (*23*) and papaya (*Carica papaya*) latex (*24*) are chemically distinct from that in chicken egg white (*25,26*). Fig and papaya lysozymes appear to be homologous to each other, but unrelated to the egg white lysozyme. The two plant enzymes are similar in hydrodynamic properties and in amino acid composition. They have the same amino-terminal but different carboxyl-terminal residues (*23*).

In this study, the plant lysozyme isolated from *P. dulce* seeds has a molecular mass of approx. 14.4 kDa and antifungal activity against *M. phaseolina*. This purified lysozyme was successfully identified through nano LC-MS/MS, and this is the second time that a lysozyme with antifungal activity has been purified from leguminous plants. Previously, a novel plant lysozyme of a similar mass (14.4 kDa) was reported from mung bean (*Phaseolus mungo*) seeds using a procedure that involved aqueous extraction, ammonium sulfate precipitation, ion exchange chromatography and high-performance liquid chromatography (*27*). This lysozyme, identified by N-terminal amino acid sequencing using the Edman degradation method, had 23 % identity in the N-terminal amino acid sequence with that of chicken egg white lysozyme. The mung bean lysozyme exhibited antifungal activity against diverse fungal isolates such as *Fusarium oxysporum*, *Fusarium solani*, *Pythium aphanidermatum*, *Sclerotium rolfsii* and *Botrytis cinerea*, as well as an antibacterial action against *Staphylococcus aureus*. In contrast, the lysozyme isolated here from *P. dulce* seeds seems more selective; it shows antifungal activity against *M. phaseolina* but not against *P. omnivora* and *F. avenaceum*. Moreover, lysozyme is well known as an antibacterial protein, with activity against Gram-positive and Gram-negative bacteria. Its bactericidal property is hypothesized to reside in its muramidase activity, leading to degradation of the murein layer and reduction of the mechanical strength of the bacterial cell wall, resulting in the killing of the bacteria by lysis (*28*). Thus, antibacterial activity and discrimination between fungistatic and fungicidal activities of this purified protein from *P. dulce* seed should be further studied.

Conclusion

A lysozyme has been isolated, purified and identified from *Pithecellobium dulce* seeds by using chromatography techniques and tandem mass spectrometry with Mascot database searching. This plant lysozyme has molecular mass of 14.4 kDa, which is close to the molecular mass of chicken egg white lysozyme (14.3 kDa), with which it also shares a high degree of partial amino acid sequence similarity. Moreover, plant lysozyme shows the antifungal ability against *Macrophomina phaseolina* with a rather high thermal stability at up to 80 °C for 15 min (at pH=8.0).

Acknowledgements

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References

1. V. Cervantes, V. Arriaga, J. Meave, J. Carabias, Growth analysis of nine multipurpose woody legumes native from southern Mexico, *Forest Ecol. Manag. 110* (1998) 329–341.
2. J.K. Candlish, L. Gourley, H.P. Lee, Dietary fiber and starch contents of some Southeast Asian vegetables, *J. Agric. Food Chem. 35* (1987) 319–321.

3. B.O. De Lumen, R. Becker, P.S. Reyes, Legumes and a cereal with high methionine/cysteine contents, *J. Agric. Food Chem. 34* (1986) 361–364.
4. J.J. Rackis, W.J. Wolf, E.C. Baker, Protease inhibitors in plant foods: Content and inactivation, *Adv. Exp. Med. Biol. 199* (1986) 299–347.
5. A. Sotelo, B. Lucas, L. Garza, F. Giral, Characteristics and fatty acids content of the fat of seeds of nine wild Mexican plants, *J. Agric. Food Chem. 38* (1990) 1503–1505.
6. S.F. Touré, B. Michalet-Doreau, E. Traoré, D. Friot, D. Richard, Occurrence of digestive interactions in tree forage-based diets for sheep, *Anim. Feed Sci. Technol. 74* (1998) 63–78.
7. P.R. Shewry: Enzyme Inhibitors of Seeds: Types and Properties. In: *Protease Inhibitors in Plant: Genes for Improving Defenses Against Insects and Pathogens*, P.R. Shewry, R. Casey (Eds.), Kluwer Academic, Dordrecht, the Netherlands (1999) pp. 587–615.
8. M. Al-Wesaly, N. Lambert, T. Welhman, C. Domoney, The influence of pea seed trypsin inhibitors on the *in vitro* digestibility of casein, *J. Sci. Food Agric. 68* (1995) 431–437.
9. N. Norioka, S. Hara, T. Ikenaka, J. Abe, Distribution of the Kunitz and the Bowman-Birk family proteinase inhibitors in leguminous seeds, *Agric. Biol. Chem. 52* (1988) 1245–1252.
10. F.H.M.G. Savelkoul, A.F.B. Van Der Poel, S. Tamminga, The presence and inactivation of trypsin inhibitors, tannins, lectins and amylase inhibitors in legume seeds during germination, *Plant Foods Hum. Nutr. 42* (1992) 71–85.
11. J.V. Vernekar, M.S. Ghatge, V.V. Deshpande, Alkaline protease inhibitor: A novel class of antifungal proteins against phytopathogenic fungi, *Biochem. Biophys. Res. Commun. 262* (1999) 702–707.
12. G. Misra, S.K. Nigam, *Pithecellobium dulce*: A tree of commercial importance, *Quart. J. Crude Drug Res. 16* (1978) 158–162.
13. A. Sotelo, E. Contreras, S. Flores, Nutritional value and content of antinutritional compounds and toxics in ten wild legumes of Yucatan Peninsula, *Plant Foods Hum. Nutr. 47* (1995) 115–123.
14. F.L. García-Carreño, M.A. Navarrete del Toro, M. Díaz-López, M.P. Hernández-Cortés, J.M. Ezquerra, Proteinase inhibition of fish muscle enzymes using legume seed extracts, *J. Food Prot. 59* (1996) 312–318.
15. F. Delgado-Vargas, H.E. López-Valdés, S. Valdés-Rodríguez, A. Blanco-Labra, A. Chagolla-López, E. de López-Valenzuela, Isolation and properties of a Kunitz-type protein inhibitor obtained from *Pithecellobium dulce* seeds, *J. Agric. Food Chem. 52* (2004) 6115–6121.
16. A.S. Oliveira, L. Migliolo, R.O. Aquino, J.K.C. Ribeiro, L.L.P. Macedo, L.B.S. Andrade *et al.*, Identification of a Kunitz-type proteinase inhibitor from *Pithecellobium dumosum* seeds with insecticidal properties and double activity, *J. Agric. Food Chem. 55* (2007) 7342–7349.
17. U.K. Laemmli, M. Favre, Gel electrophoresis of proteins, *J. Mol. Biol. 80* (1973) 575–599.
18. M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem. 72* (1976) 248–254.
19. D.N. Perkins, D.J. Pappin, D.M. Creasy, J.S. Cottrell, Probability-based protein identification by searching sequence databases using mass spectrometry data, *Electrophoresis*, *20* (1999) 3551–3567.
20. H. Wang, T.B. Ng, Isolation of cicadin, a novel and antifungal peptide from dried juvenile cicadas, *Peptides*, *23* (2002) 7–11.
21. M.J. Haas, J.E. Dowding, Aminoglycoside-modifying enzymes, *Methods Enzymol. 43* (1975) 611–628.
22. B.K. Watson, R.C. Moellering Jr, L.J. Kunz, Identification of streptococci: Use of lysozyme and *Streptomyces albus* filtrate in the preparation of extracts for Lancefield grouping, *J. Clin. Microbiol. 3* (1975) 274–278.
23. A.N. Glazer, A.O. Barel, J.B. Howard, D.M. Brown, Isolation and characterization of fig lysozyme, *J. Biol. Chem. 244* (1969) 3583–3589.
24. J.B. Howard, A.N. Glazer, Papaya lysozyme: Terminal sequences and enzymatic properties, *J. Biol. Chem. 244* (1969) 1399–1409.
25. M. Azarkan, A. Amrani, M. Nijs, A. Vandermeers, S. Zerhouni, N. Smolders, Y. Looze, *Carica papaya* latex is a rich source of a class II chitinase, *Phytochemistry*, *46* (1997) 1319–1325.
26. K. Meyer, E. Hahnel, A. Steinberg, Lysozyme of plant origin, *J. Biol. Chem. 163* (1946) 733–740.
27. S. Wang, T.B. Ng, T. Chen, D. Lin, J. Wu, P. Rao, X. Ye, First report of a novel plant lysozyme with both antifungal and antibacterial activities, *Biochem. Biophys. Res. Commun. 327* (2005) 820–827.
28. K. Düring, P. Porsch, A. Mahn, O. Brinkmann, W. Gieffers, The non-enzymatic microbicidal activity of lysozymes, *FEBS Lett. 449* (1999) 93–100.