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Protease Inhibitors of Botanical Origin

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ABSTRACT

The intent of this article is to review recent literature on plant protease inhibitors which can be divided into different types comprising Kunitz, Bowman-Birk, squash, serine protease and potato inhibitor types. Some of them specifically inhibit trypsin. Others also inhibit chymotrypsin and other proteases. The mode of inhibition can be either competitive or noncompetitive. Some of them are characterized by remarkable thermostability and pH stability. In addition to protease inhibitory activity, antifungal, anti-insect, anticancer and/or HIV-1 reverse transcriptase inhibitory activity are present in some plant protease inhibitors.

Keywords: antibacterial; anticancer; antifungal; Bowman-Birk trypsin inhibitor; chymotrypsin inhibitor; Kunitz-type trypsin inhibitor
Abbreviations: BAEE, N-α-benzoyl-L-arginine ethyl ester; **BTEE**, N-benzoyl-L-tyrosine ethyl ester; **CM FF**, carboxymethyl fast flow; **DEAE**, diethylaminoethyl; **DTT**, dithiothreitol; **FPLC**, fast protein liquid chromatography; **HIV-RT**, human immunodeficiency virus reverse transcriptase; **HPLC**, high-performance liquid chromatography; **IC_{50}**, half maximal inhibitory concentration; **K_i**, inhibitor constant; **pI**, isoelectric point; **RPC**, reverse phase chromatography; **SP-Sepharose**, sulfopropyl_sepharose

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Invited Review

INTRODUCTION

Plants are exposed to insects and fungi in their environment. In order to survive they have to devise ways to combat or protect themselves from insect predators and fungal pathogens. Protease inhibitors (PIs) enable plants to inhibit proteases in the insect gut and as a consequence insects that feed on the plants may succumb. There is a voluminous literature on plant PIs. One of the reasons why these inhibitors have prompted the investigations was that it has been reported that there is a reduced incidence of malignant diseases in human populations with a high dietary intake of beans that are rich in PIs. In this review, recently published reports on plant PIs have been reviewed.

Abortiporus biennis (Family Meruliaceae)

Thermostable trypsin inhibitors (TI) with a molecular mass of 20 and 21.5 kDa respectively from the white rot fungus *A. biennis* inhibited trypsin most strongly, and chymotrypsin, proteinase K and subilisin to a lesser extent. Heat treatment, ion exchange chromatography and gel filtration were used to purify the inhibitors (Zuchowski *et al.* 2009).

Acacia plumosa (Family Fabaceae)

Lopes *et al*. (2009) purified and characterized 20-kDa Kunitz-type isoinhibitors from saline extracts of *A. plumosa* seeds. Gel filtration on Superdex 75 and ion exchange chromatography on Mono S were used for purification. Antifungal activity of the isoinhibitors against *Aspergillus niger*, *Colletotrichum* sp. P10 and *Thielaviopsis paradoxa* was observed. The isoinhibitors had acidic pIs, and were composed of two chains connected by a disulfide bridge. Surface plasmon resonance studies yielded dissociation constants of 0.38 nM, 0.56 nM and 0.56 M with trypsin and 7.5 nM, 6.9 nM and 3.5 nM with chymotrypsin. Trypsin, chymotrypsin and kallikrein were inhibited with a K_i of 1.8, 10.3 and 0.58 nM, respectively. There was a predominance of disordered and β-strands in the secondary structure.

Acacia victoriae (Family Fabaceae)

A 13-kDa Kunitz-type TI which existed in three isoforms was reported from seeds of the prickly wattle, *A. victoriae* (Ee *et al*. 2009). The pI values of 4.27, 4.76 and 5.13 were noted for the isoinhibitors. They were composed of two chains connected by at least one S-S bridge. Their chymotrypsin inhibiting activity was meager compared with their trypsin inhibiting activity.

Albizzia kalkora (Family Fabaceae)

A heterodimeric Kunitz-type TI from *A keelkora s*eeds, composed of a 15.5 kDa subunit and a 4.5-kDa subunit, inhibited trypsin with a K_i of 2.5×10^{-7} M. Its trypsin inhibitory activity was preserved after exposure to an ambient pH of 2 to 12 for 24 h, but underwent a decline when exposed to a temperature of 80°C for 10 min. Partial amino acid sequencing disclosed pronounced resemblance to other Kunitz-type inhibitors. The inhibitor also inhibited trypsin-like proteases from insects including *Helicoverpa amiger*a, *Pieris rapie* and *Spodoptera exigua* (Zhou *et al*. 2008).

Anredera cordifolia (Family Basellaceae)

The 23-kDa TI from *A. cordifolia* (also called *Boussingaultia baselloides* and *B. gracilis* var. *pseudobaselloidos*, or Madeira vine) rhizomes, designated as ancordin, manifested an N-terminal amino acid sequence with striking homology to those of soybean inhibitor, sporamin, and *Medicago truncatula* PI, It was isolated by affinity chromatography on trypsin-Sepharose. The recovery was 15.52%. Ancordin dose-dependently stimulated nitric oxide production in RAW264.7 cells without significant cytotoxicity, Polymyxin B eliminated the effects of lipopolysaccharide but not ancordin on nitric oxide production by RAW264.7 cells (Chuang *et al*. 2007).

Apios americana (Family Fabaceae)

The Bowman-Birk TI from *A. americana* tubers exhibited a molecular weight of 6437 Da. It inhibited trypsin and chymotrypsin with a K_i of 3×10^{-9} and 10^{-6} M, respectively. The inhibitor was thermostable and pH-stable. Its activity was untarnished after exposure to 80°C for 120 min and a wide pH range (pH 1-12). Lys 62 and Arg88 are two possible trypsin-reactive sites. DEAE-cellulofine and Sephadex G-50 were used in the isolation protocol with 10.5% recovery and 27.2-fold purification (Zhang *et al*. 2008).

Archidendron ellipticum (Family Fabaceae)

A protocol, encompassing fractionation using acetone and $(NH_4)_2SO_4$, ion exchange chromatography, gel filtration and reverse-phase chromatography, was adopted to purify a heterodimeric Kunitz-type trypsin/chymotrypsin inhibitor from seeds of *A. ellipticum*, a mimosoid plant (Bhattacharyya *et al*. 2006). The inhibitor was composed of a 15-kDa chain and a 5-kDa chain. Four isoforms with a pI value of 4.1, 4.55, 5.27 and 5.65 were revealed by isoelectric focusing. The inhibitor inhibited trypsin in a 1:1 molar ratio and with a K_i of 2.46×10^{-10} M. Its K_i against chymotrypsin was 0.5×10^{-10} M. Its protease inhibitory activity was preserved from pH1 to pH 10 and from 0 to 60°C. About 70% of the activity remained after storage at -20°C for over a year. Its complex with trypsin was stable in the presence of the detergent sodium dodecyl sulfate, in contrast to its complex with chymotrypsin. The inhibitor exhibited anti-insect activity and it can be used as a potential insect anti-feedant. The anti-insect activity is actually the test for the activity of TI against the proteinase from the midguts of 5th instar larvae of *Spodoptera litura* using tosyl-arginyl-methyl ester hydrochloridethe as the substrate. The TI elicited a 25% increase in the midgut trypsin inhibitory activity as compared to that of the well-known soybean Kunitz-type TI. Besides, it also showed a 1.5-fold increase in the suppression of bovine trypsin as compared to soybean Kunitz-type TI.

Bauhinia variegata var. variegata (Family Fabaceae)

The Kunitz-type TI from *B. variegata* (camel's foot tree) possessed an N-terminal amino acid sequence similar to protease inhibtors of other *Bauhinia* species. The TI was purified by ion exchange chromatography on SP-Sepharose and Mono S column, followed by size exclusion chromatography on Superdex 75. Nevertheless its trypsin inhibiting activity ($K_i = 1 \times 10^{-10}$ M) was the highest among them. The trypsin inhibitory activity was stable up to 50°C and in the pH range 4-12. Furthermore, it was able to withstand reducing conditions (1 mM DTT for 0.5 h). The anti-HIV-1-RT activity assay was performed using an HIV-1-RT ELISA kit. The assay is based on the mechanism that HIV-1-RT can synthesize DNA, commencing from the template/primer hybrid poly(A) oligo (dT). The inhibitory activity of the TI was calculated as percentage inhibition. The results showed that the TI could inhibit the activity of HIV-1-RT with an IC_{50} value of 6.4 μM. The antiproliferative activity of the TI was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. It is a colorimetric assay for measuring the activity of enzymes that reduces MTT to formazan dyes, giving a purple color. This activity is reflected by proliferation of cells. Cancer cell lines were incubated with TI and the inhibition of cell growth was determined by MTT assay, while apoptosis was observed by chromatin staining with Hoechst 33342. The TI exhibited HIV-1 reverse transcriptase inhibitory activity and antiproliferative activity toward CNE-1 nasopharyngeal cancer cells. The latter activity may be partially contributed by induction of cytokines and fomation of apoptotic bodies (Fang *et al*. 2010). There was no significant effect on other tumor cells and a normal nasopharyngeal cell line.

Brassica juncea (Family Brassicaceae)

The *B. juncea* (mustard) TI was expressed in *E. coli* and isolated by ion exchange chromatography on Mono Q column and gel filtration on Superose 12 column. The K_i value of the recombinant TI was 1.57×10^{-9} M (Stefan *et al*. 2009).

Caesalpinia bonduc (Family Fabaceae)

A 16-kDa chymotrypsin-specific inhibitor and two 20-kDa trypsin/chymotrypsin isoinhibitors were isolated from *C. bonduc* seeds. Ammonium sulfate precipitation, ion exchange chromatography, gel filtration, and affinity chromatography on immobilized trypsin were used for purification. The trypsin/chymotrypsin inhibitor inhibited trypsin and chymotrypsin with a K_i of 275×10^{-10} M and 0.95×10^{-10} M, respectively. The chymotrypsin inhibitor, characterized by fair themostability (0°C to 60°C) and pH stability (pH 1 to 12), displayed anti-insect activity (Bhattacharyya and Babu 2007). The anti-insect activity assay was the same assay used in study on *Archidendron ellipticum* TI. The enzyme was isolated from the midguts of 5th instar larvae of *Spodoptera litura.* Dosage of the TI required for inhibition of gut proteolytic activity was 1.5-fold lower than that of soybean Kunitz-type TI.

Cajanus cajan (Family Fabaceae)

Inhibitory activity against midgut trypsin-like enzyme of the lepidopteran insects *Achaea janata* and *Spodoptera litura*, as well as trypsin and chymotrypsin from bovine pancreas, was detected in the seeds of eight wild types and 14 cultivars of the pigeon pea/red gram (*C. cajan*) (Prasad *et al*. 2009). The cultivars showed identical protease inhibitory activity and activity profile in gelatin-polyacrylamide gel electrophoresis. However, the wild types displayed differences among them. In comparison with bovine trypsin, TIs from both wild types and cultivars were more potent toward *A. janata* and less potent toward *S. litura.*

A 8.5-kDa Bowman-Birk PI from *C. cajan* seeds was reported by the same group of investigators (Prasad *et al*. 2010b). A procedure involving $(NH_4)_2\ SO_4$ precipitation, ion exchange chromatography, affinity chromatography and gel filtration was employed. The presence of isoinhibitors with different pI values (5.59, 6.25, 6.5, 6.9 and 7.05) was revealed by two-dimensional gel electrophoresis. The mode of inhibition was competitive with a K_i of 292 nM for bovine trypsin and 2265 nM for bovine chymotrypsin. DDT or beta-mercaptoethanol treatment abolished the inhibitory activity. Addition of the pigeon pea inhibitor to the diet reduced the trypsin-like activity in the midgut of the insect *Maduca sexta* and also larval growth and development.

Calliandra selloi (Family Fabaceae)

A heterodimeric Kunitz-type TI composed of a 16-kDa subunit and a 6-kDa subunit was isolated from *C. selloi* seeds (Yoshizake *et al*. 2007).

Trypsin, chymotrypsin and kallikrein were inhibited with a K_i of 2.21×10^{-7} M, 4.95×10^{-7} M, and 4.2×10^{-7} M. Elastase was not affected. The inhibitor was stable up to 80°C and in a pH range from 2-11. Reduction with DDT induced very little alterations in the ultraviolet-circular dichroism spectrum. Drastic disruption of secondary structure ensued after carbamidomethylation.

Cicer arietinum (Family Fabaceae)

An 18- kDa Kunitz-type TI protein from chickpea (*C. arietinum*) seeds inhibited trypsin with an IC_{50} value of 2.5 μg. Its specific trypsin inhibitory activity was 114 trypsin inhibitory units per mg protein, high compared with other legume Kunitz-type TIs. It crystallized in three different orthorhombic crystal forms: $P2_12_12$ form A, $P2_12_12$ form B and $P2_12_12_1$. The crystals of $P2_12_12$ form A, with unit-cell parameters a = 37.2, b = 41.2, c = 104.6 Å, diffracted to 2.0 Å resolution at the home source and to 1.4 Å on beamline BM14 at the ESRF. The Matthew's coefficient for crystals grown in the presence of iodine was 2.37 $Å^3\ Da^{-1}$, corresponding to a solvent content of 42%. The other two crystal forms ($P2_12_12$ form B and $P2_12_12_1$) diffracted relatively poorly (Sharma and Suresh 2011).

Coccinia grandis (Family Cucurbitaceae)

A thermostable 14.3-kDa PI from *C. grandis* was devoid of hemolytic activity but demonstrated a pronounced growth-inhibitory action on colon cell lines. It strongly inhibited pathogenic bacteria including *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Staphylococcus aureus*, and pathogenic fungi including *Aspergillus flavus*, *Candida albicans*, *Cryptococcus neoformans*, *Mucor indicus*, and *Penicillium notatum.* Mycelial growth was inhibited with shrinkage of hyphal tips. Sporulation was also suppressed. An intact disulfide bridge is crucial for its protease inhibitory and antifungal activities (Satheesh and Murugan 2011).

Crotalaria pallida (Family Fabaceae)

A 32.5-kDa heterodimeric TI was reported from *C. pallida* seeds by Gomes *et al*. (2005). It was composed of a 27.7-kDa subunit and a 5.6-kDa subunit connected by disulfide bonds. Ammonium sulfate precipitation, affinity chromatography on trypsin-Sepharose and trichloroacetic acid precipitation were used sequentially to purify the inhibitor. Besides inhibiting trypsin, the inhibitor was also capable of inhibiting chymotrypsin (36.2 ± 7.9% inhibition), elastase (15.9 ± 7.4% inhibition) and papain, (43.9 ± 8.6% inhibition). Its trypsin inhibitory activity was stable from pH 2 to pH 12, and approximately 60% of the activity remained at 100°C.

Digestive enzymes from the insect guts that were inhibited by the inhibitor included those of *Alabama argillacea*

(97.9 ± 17.2% inhibition), *Anthonomus grandis* (52.1 ± 10.1% inhibition), *Plodia interpunctella* (66.7 ± 7.4% inhibition), *Spodoptara frugiperda* (100 ± 18.4% inhibition), and *Zabrotes subfasciatus* (36.5 ± 13.7% inhibition). *Callosobruchus maculatus* and *Ceratitis capitata* enzymes were also inhibited with the latter being more susceptible (74.4 ± 15.8% inhibition for the former and 97.9 ± 17.2% inhibition for the latter). The reverse was observed when the larvae of both species were fed a diet containing the inhibitor at the following concentrations: 0.5, 1, 2 and 4% (w/w).

Cyclanthera pedata (Family Cucurbitaceae)

Kowalska *et al.* (2006) reported the purification of seven squash-type serine PIs from *C. pedata* seeds. The purification scheme adopted comprised affinity chromatography on immobilized chymotrypsin in the presence of 5 M NaCl and subsequently preparative native polyacrylamide gel electrophoresis. The recovery was around 2%. The inhibitors, composed of 28-30 amino acid residues with molecular weights from 3031 to 3367 Da inhibited bovine beta-trypsin and bovine alpha-chymotrypsin with association constant of approximately 10^{11} M^{-1} and 10^{4}-10^{6} M^{-1}, respectively.

Derris trifoliata (Family Fabaceae)

Three 20-kDa isoinhibitors, with N-terminal sequence homology to Kunitz-type inhibitors of the Papilionoideae family, were reported from the tropical legume liana, *D. trifoliata* (Bhattachargya and Babu 2009). $(NH_4)_2SO_4$ precipitation, ion exchange chromatography, and gel filtration were used in the purification scheme. The isoinhibitors demonstrated a pI value of 4.55, 5.43 and 5.72, respectively. Trypsin and chymotrypsin were competitively inhibited in a 1:1 molar ratio, with a K_i of 1.7×10^{-10} and 1.25×10^{-10} M, respectively. The inhibition was attenuated in the presence of DTT. The inhibitor also expressed antimalarial activity which was determined by measuring the decrease in the pLDH (parasite lactate dehydrogenase) activity against the two *Plasmodium falciparum* lines, 3D7 and FCR3. The IC_{50} values obtained for the TI for *P. falciparum* 3D7 and FCR3 were 9 and 16.35 μM, respectively. This may be due to an effective blocking of the essential serine proteolytic activity on some major surface antigens of the parasite. A high content of β-sheets and intramolecular disulfide bonds confer conformational stability to its active site.

Diplotaxis muralis, *D. tenufolia* (Family Brassicaceae)

Analysis of the genomes of the two *Diplotaxia* species belonging to the *Brassicaceae* family by polymerase chain reaction revealed several genes encoding PIs belonging to the mustard inhibitor family. This is the first PI characterized in *Brassicaceae*. Two recombinant TIs were expressed in *Pichia pastoris*. The K_i values for bovine β-trypsin of the *Diplotaxis muralis* and *Diplotaxis tenuifolia*, TIs were found to be 1.2 ± 0.29 and 0.59 ± 0.027 nM, respectively. When compared with mustard TI MTI-2, the *Diplotaxis* inhibitors exhibited a less potent activity against bovine trypsin, but similar activity against trypsin-like enzymes in the guts of the larvae of *Helicoverpa zea* (Volpicella *et al*. 2009).

Eleusine coracana (Family Poaceae)

A bifunctional protease/amylase inhibitor with a molecular weight of 14 kDa was isolated from *E. coracana* (*ragi* or millet) by $(NH_4)_2SO_4$ precipitation and affinity chromatography, gave 6.59-fold of purification purity with 81.48% recovery yield. Purification by $(NH_4)_2SO_4$ precipitation and ion exchange chromatography resulted in 4.28-fold purification and 75.95% recovery yield. When the ion exchange fraction was subjected to gel filtration the inhibitor was purified to 6.67-fold with 67.36% yield (Saxena *et al*. 2010). The high recovery yield makes *ragi* a cheap and rich source of amylase/protease inhibitor. This gives us further scope for exploring other such natural and cheap sources for the development of bioaffinity ligands, which can be used in purification of enzymes and therapeutic proteins. The inhibitor could be employed as an affinity ligand for purification of a commercial pancreatic amylase preparation.

Entada scandens (Family Fabaceae)

From the seeds of another mimosoid plant, *E. scandens* (sword bean), a 19766-Da Kunitz-type TI with a disnifide bond and a K_i of 4.9×10^{-9} M toward bovine trypsin was isolated (Lingaraja and Gowda 2008). It inhibited trypsin with 1: 1 molar ratio and suppressed the midgut protease of rice moth (*Corcyra cephalonica*) larvae with an IC_{50} of 26.4 nM. It manifested a pI of 7.43. The scheme used for isolation comprised $(NH_4)_2SO_4$ precipitation, affinity chromatography on trypsin-Sepharose, and ion exchange chromatography on DEAE-Sephacel leading to 2.3-fold of purification and 28.8% yield.

Fagopyrum tataricum (Family Polygonaceae)

A TI from tartary buckwheat (*F. tataricum*) seeds, a member of the PI I family, possessed two disulfide bonds linking Cys(8) to Cys(65) and Cys(49) to Cys(58). Its active site contained an Asp(66)-Arg(67) bond. It competitively inhibited trypsin with an inhibition constant of 1.6 nM. It strongly inhibited phytopathogenic fungi. There were two isoforms with a molecular mass of 11.487 and 13.838 kDa, respectively (Ruan *et al*. 2011).

Glycine spp. (Family Fabaceae)

Soybean (*Glycine* spp.) Kunitz-type TI induced apoptosis in human Jurkat cells (Troncoso *et al*. 2007). The large black soybean from Hokkaido, Japan produces a thermostable Bowman-Birk TI. Its trypsin inhibitory activity was completely retained after exposure to the pH range 2-13 and to temperatures up to 100°C for 30 min. It inhibited the activity of HIV-1 reverse transcriptase and proliferation of HepG2 hepatoma cells and MCF-7 breast cancer cells with an IC_{50} of 38, 140 and 35 μM, respectively. However, there was no antifungal activity toward *Fusarium oxysporum* and *Mycosphaerella arachidicola*. The inhibitor was isolated by using a procedure that involved ion exchange chromatography on SP-Sepharose, DEAE-cellulose and Mono Q. It did not adsorb on the first but adsorbed on the remaining two ion exchangers (Ho and Ng 2008).

The wild soybean *Glycine soja* produces Kunitz-type TI with several polymorphic types controlled by co-dominant multiple alleles at a single locus. Tia and Tib are the main types. New variants including Tiaa1, Tiaa2, and Tiab1 with the same electrophoretic mobility as Tia were found by gene sequence analysis. Differences in amino acid were found between Tia and Tib. Tiab 1 is a transitional intermediate between Tia and Tib. The variant Tig exhibited a slightly lower electrophoretic mobility. The gene sequence of Tiaa 1 differed from that of Tia by a G to A mutation at position +376 and Tiaa 2 differed from Tia by a T to C mutation at position +340 (Wang *et al*. 2008).

A19-kDa trypsin/chymotrypsin inhibitor was isolated from small glossy black soybean cultivated in China (Ye and Ng 2009). A chromatographic procedure that involved adsorption on the ion exchangers Q-Sepharose, SP-Sepharose and DEAE-cellulose was used for isolation. The trypsin inhibitory activity of the protein was preserved from 0 to 60°C, and from pH 3-13. Trypsin and chymotrypsin were inhibited by the inhibitor with an IC_{50} of 19 and 14.3 μM, respectively. Proliferation of HepG2 hepatoma cells and MCF-7 breast cancer cells and activity of HIV-1 reverse transcriptase were inhibited with an IC_{50} of 25, 4.3 and 0.16 μM, respectively.

Zhang *et al.* (2009) used affinity chromatography to

isolate TIs from wild soybean and domesticated soybean (*Glycine max*). For the domesticated soybean TI and wild soybean TI 718- and 279-fold of purification, with the activity recovery of 62 and 59%, respectively, were achieved. The two TIs suppressed the growth of the fungus *Aspergillus flavus* with an IC_{50} of 1 and 1.6 μM, respectively. The antifungal activity was probably a consequence of the inhibition of α-amylase in *A. flavus* by the inhibitors. Germination and growth o*f A. flavus* on peanut were inhibited.

Gymnocladus chinensis (Family Fabaceae)

A 20-kDa Kunitz-type TI from *G. chinensis* (Yunnan beans) seeds inhibited trypsin with an IC_{50} value of 0.4 μM. DTT reduced its trypsin inhibitory activity, suggesting the pivotal role played by an intact disulfide bond. It inhibited [methyl-^{3}H] thymidine incorporation by leukemia L1210 cells and lymphoma MBL2 cells with an IC_{50} value of 4.7 and 9.4 μM, respectively (Zhu *et al*. 2011).

Hevea brasiliensis (Family Euphorbiaceae)

Three PIs, all composed of 69 amino acid residues, but with different molecular weights due to post-translational modification, have been purified from the C-serum of the rubber tree (*H. brasiliensis*) latex (Sritanyara *et al.* 2006). They exhibited a molecular weight of 14893, 7757 and 7565, respectively, and all possessed a blocked N-terminal. Amino acid sequence analysis revealed some (50-74%) similarity to the potato PI 1 family. They were weak inhibitors of trypsin but were devoid of inhibitory activity toward chymotrysin. Potent inhibition of subtilisin, however, was observed. Gln 45 and Asp 46 were present in the reactive site P1 and P1', different from other members of potato PI 1 family. Their K_i values toward subtilisin A were estimated to be 0.21, 0.08, and 0.10 nM, respectively, which is approximately 40-fold higher than that toward trypsin.

Inga laurina (Family Fabaceae)

A 20-kDa Kunitz-type TI for the mimosoid plant *I. laurima* specifically inhibited bovine trypsin with a K_i of 6×10^{-9} M. There was no effect on chymotrypsin, α-amylase and papain. The inhibitor also exhibited sequence resemblance to sporamin and mirachlin. The purification scheme included $(NH_4)_2SO_4$ precipitation, ion exchange chromatography and HiTrap Q ion exchanger which gave 24-fold of purification and 22.4% recovery. The inhibitor lost 20% of its activity when incubated at 80°C for 30 min. When heated to 100°C, a greater decrease in activity was observed. However, the inhibitory activity was not sensitive to pH over the range 2.0–10.0 (Macedo *et al*. 2007).

Ipomoea batatas (Family Convolvulaceae)

A 23-kDa inhibitor was isolated by Jaw *et al*. (2007) from sweet potato in a single step by employing an aqueous two-phase system of polyethylene glycol 6000 (11% w/v), phosphate (16.5%, w/c), KCl (9% w/v). This procedure resulted in 3.7-fold purification, and a recovery of 95%.

Lathyrus sativus (Family Fabaceae)

Four Bowman-Birk inhibitors from *L. sativus* seeds, named LSI-1, LSI-2, LSI-3 and LSI-4, possessed relative molecular weights of 7914.41, 6867.67, 7341.24, and 7460.01 Da. N-terminal sequences (up to 30 residues) of the inhibitors were identical except for amino acid residue number 21, 27 and 28 and highly homologous to those of other leguminous Bowman-Birk inhibitors. Inhibitors LSI-1/4 were active towards trypsin and α-chymotrypsin. Peptide mapping of the LSI-1 sequence revealed an Ala residue in the second reactive site, thus accounting for the weaker anti-chymotrypsin activity of the inhibitor. LSI-1 inhibited human leukocyte elastase (Rocco *et al*. 2011).

Lens culinaris (Family Fabaceae)

Cheung and Ng (2007) isolated a 16-kDa Bowman-Birk type trypsin/chymotrypsin inhibitor from green lentil (*L. culinaris*) seeds. Affi-gel blue gel, Q-Sepharose, Mono Q, Mono S and Superdex 75 were used in succession to purify the inhibitor. This procedure resulted in 36-fold purification and 3% recovery. The inhibitor was devoid of antifungal activity toward *Botrytis cinerea*, *Fusarium oxysporum* and *Mycosphaerella arachidicola.* It showed no antiproliferative activity and exhibited weak HIV-1 reverse transcriptase inhibitory activity with an IC_{50} of 30 mM. The trypsin inhibitory activity of the inhibitor was sensitive to reducing agents such as DTT. It was completely abrogated after treatment with 10 mM DDT for 20 min.

Lilium brownii (Family Liliaceae)

The 17-kDa *Lilium brownii* bulb TI inhibited bovine trypsin with an IC_{50} of 1.3 μM, stimulated nitric oxide production by murine macrophages, but neither inhibited HIV-1 reverse transcriptase nor suppressed proliferation of L1210 leukemia cells and MBL2 lymphoma cells. Its N-terminal sequence resembled partial sequences of sporamin B from sweet potatoes, TI from *Populus tremula* and a putative TI from *Arabidopsis thaliana*. It possessed a molecular weight of 17 kDa, and was adsorbed on DEAE-cellulose, unadsorbed on Affi-gel blue gel, and adsorbed on SP-Sepharose (Zhang *et al*. 2008). There was a stimulatory effect on macrophage production of nitric oxide. However, it did not inhibit HIV-1 reverse transcriptase activity and [methyl-^{3}H]thymidine incorporation by leukemia L1210 cells and MBL2 cells when tested up to 100 μM.

Lupinus albus (Family Fabaceae)

A 6858-kDa Bowman-Birk inhibitor, capable of simultaneously inhibiting two trypsin molecules with a Kd of 4.2 nM, was isolated from seeds of *Lupinus albus.* DEAE-cellulose column and trypsin affinity cellulose column were used to purify the inhibitor. The procedure resulted in 411.1-fold purification and 0.022% recovery (Scarafori *et al*. 2008). The inhibitor retained its inhibitory activity at pH 3-8 and 20-90°C, respectively. It was devoid of chymotrypsin inhibiting activity.

Mirabilis jalapa (Family Nyctaginaceae) and *Spinacia oleracea* (Family Amaranthaceae)

Five serine proteinase inhibitors (*Mirabilis jalapa* (garden four o'clock) TIs, MJTI I and II and *Spinacia oleracea* (spinach) TIs, SOTI I, II, and III) were isolated from seeds of both plants. The inhibitors were composed of 35-37 amino acid residues connected by three disulfide bonds. Interestingly, their sequences resembled antimicrobial peptides but not TIs. They showed an association constant of about 10^7 M^{-1} with bovine trypsin. The isolation protocol involved affinity chromatography on immobilized methylchymotrypsin in the presence of 5 M NaCl, ion exchange chromatography, preparative electrophoresis, and reverse phase HPLC on a C18 column. Chemical modification of guanidyl groups of arginine residues in SOTI I with 1,2-cyclohexanedione (CHD) led to complete loss of activity of all inhibitors. The antitryptic activity was not affected by acetylation of free amino groups with acetic anhydride. Taking into account that all the inhibitors isolated from spinach seeds contain only one arginine residue in the C-terminal part of molecules, the Arg–Ile peptide bond forms the reactive site in these inhibitors. The position of reactive site in MJTI molecules was established on the basis of primary structure homology with SOTIs. The synthesized peptides showed a correct molecular weight assayed by mass spectrometry using MALDI-TOF. HPLC analysis confirmed that the synthetic inhibitors are identical to those isolated from plants. The synthetic inhibitors coinjected with the cor-

responding standards isolated from the natural source displayed single major peaks in HPLC. This indicates that the disulfide bridge topology in synthetic and wild-type inhibitors is the same. Both synthesized peptides displayed trypsin inhibitory activity similar to inhibitors isolated from natural sources. Inhibitors were digested by thermolysin and proteinase K and then analyzed by MALDI-TOF. The results suggested that disulfide bridge pattern in both peptides fits well in the knottin disulfide pattern which is: Cys1–Cys4, Cys2–Cys5, Cys3–Cys6 (Kowalska *et al*. 2007).

Opuntia streptacantha (Family Cactaceae)

A 4.19-kDa serine PI characterized by remarkable thermostability (120°C for 1 h under a pressure of 1 kg/cm^2) was isolated from *O. streptacantha* (prickly pear) seeds (Totrres-Castillo *et al*. 2009). It possesses a blocked N-terminal.

Peltophorum dubium (Family Fabaceae)

A trypsin inhibitor (PDTI) was isolated from *P. dubium* seeds by affinity chromatography on a thyroglobulin-agarose or a trypsin-agarose column. In both cases, SDS-PAGE showed a 20 kDa band and a 22 kDa band, which could not be resolved. Their amino-terminal sequences were identical and similar to that of Kunitz-type soybean trypsin inhibitor (SBTI). Mass spectrometry analysis of tryptic digests of both bands showed 16 coincident peaks, suggesting that they are closely related proteins. The K_is for trypsin and chymotrypsin inhibitory activity of PDTI were 1.6×10^{-7} and 1.3×10^{-5} M, respectively. Lectin-like activity of PDTI and SBTI, detected by hemagglutination of rabbit erythrocytes, was inhibited by sialic acid-containing compounds. PDTI and SBTI caused apoptosis of Nb2 rat lymphoma cells, demonstrated by decrease of viability, DNA hypodiploidy, DNA fragmentation, and caspase-3-like activity. They had no effect on normal mouse splenocytes or lymphocytes, whereas they caused apoptosis of concanavalin A-stimulated mouse lymphocytes (Troncoso *et al*. 2003).

The TI from *P. dubium* seeds, as well as the Kunitz-type TI from soybeans, induced apoptosis in human leukemia Jurkat cells. Caspase-3 and caspase 8, but not capase 9, were activated. Jurkat cells were treated with PDTI and SBTI were pre-incubated with pan caspase inhibitor (Z-VAD-FMK). The inhibitor effectively prevented apoptosis of Jurkat cells as measured by DNA hypodiploidy. Similar results were obtained with the caspase-8 inhibitor (Z-IETD-FMK).However, in the presence of caspase-9 inhibitor (Z-LEHD-FMK), it had no effect on PDTI- and SBTI-induced apoptosis. The results suggested that these TIs activated caspases-3 and -8 while they did not significantly activate caspase-9. The participation of Fas-associated death domain (FADD) was revealed by its recruitment to the cell membrane. In this study the translocation of FADD from the cytosol to the cell membrane of Jurkat cell treated with PDTI or SBTI, as well as the activation of caspase-8 were demonstrated. These events were usually related to the death receptor pathway, although it cannot be ruled out that FADD functions in a receptor-independent manner, as in the case of cycloheximide-induced cell death in Jurkat cells. The intrinsic mitochondrial pathway did not play a role as there was no significant enhancement of mitochondrial release of cytochrome C. Besides, the viability of human peripheral lymphocytes, either stimulated with phytohemagglutinin or unstimulated, decreased in response to these inhibitors (Troncoso *et al*. 2007).

Phaseolus spp. (Family Fabaceae)

A 132-kDa navy bean (*Phaseolus vulgaris*) TI and a 118-kDa red kidney bean (*P. vulgaris*) TI were isolated by Wati *et al.* (2010) using a procedure that entailed incubation at 70°C for 10 min and precipitation with 60-80% saturated $(NH_4)_2SO_4$.

Two TIs with a molecular weight of 16 kDa were isolated from white cloud beans (*P. vulgaris*) by Sun *et al*. (2010). A procedure involving chromatography on the anion exchanger DEAE-cellulose, the affinity media Affi-gel blue gel, the cation exchanger SP-Sepharose and the gel filtration media Superdex 75 was employed. The inhibitors inhibited trypsin with an IC_{50} of approximately 0.6 μM. The importance of the S-S bond to the trypsin inhibitory activity was disclosed by the ability of the reducing agent DTT to reduce the activity. The inhibitor was devoid of inhibitory activity toward fungal growth, proliferation of lymphoma MBL2 cells and HIV-1 reverse transcriptase, but exerted antiproliferative activity toward L1210 leukemia cells.

Pithecellobium dumosum (Family Fabaceae)

A 19.7-kDa Kunitz-type PI, which competitively inhibited trypsin and noncompetitively inhibited papain with a K_i of 3.56×10^{-8} nM and 7.61×10^{-7} M, respectively, was isolated from *P. dumosum* seeds. Its trypsin inhbitory activity was stable at 50°C and from pH 2-10. It was capable of inhibiting digestive proteases from insects including *Alabama argillaceae*, *Callosobrachus mauilatus*, *Cetatitis capitata*, *Plodia interpunctella* and *Zabrotes subfasciatus*. The inhibitor was isolated using a protocol that entailed trichloroacetic acid precipitation, affinity chromatography on trypsin-Sepharose and reverse-phase HPLC, with 217-fold of purification (Oliveira *et al*. 2007).

Plathymenia foliolosa (Family Fabaceae)

The inhibitor was purified by gel filtration on Sephadex G-100, ion exchange chromatography on DEAE-Sepharose, and affinity chromatography on trypsin-Sepharose. Ramos *et al*. (2009) demonstrated the toxic effects of a TI from *P. foliolosa* seeds on growth and development of the larvae of the Mediterranean flour moth *Anagasta huehniella*, when fed a diet containing the TI at a concentration of 0.7% (w/w), the larvae excreted more trypsin in the feces. This was accompanied by a fall in the midgut trypsin activity, a decrease in the efficiency of conversion of ingested food and digested food, and rise in approximate digestibility and metabolic cost. A decrement in body mass and survival rate was also noted.

Same purification scheme was applied as described above (Ramos *et al*. 2008). The 19-kDa inhibitor inhibited trypsin-like proteases in the midguts of the larvae of *A. kuehniella* and *D. sauharalis* and also retarded larval development. Bovine trypsin and chymotrypsin were inhibited with a K_i of 4×10^{-8} M and 4×10^{-6} M, respectively. The inhibitor was themolabile with substantial (80%) and total destruction of activity at 60 and 70°C, respectively.

Psoralea corylifolia (Family Fabaceae)

A 18-kDa TI with antifungal activity was purified from the seeds of *P. corylifolia* (malaytea scurfpea), a traditional Chinese medicinal herb. It displayed N-terminal sequence homology to plant PIs. It demonstrated antifungal activity toward various pathogenic fungi including *Rhizoctonia cerealis*, *Fusarium oxysporum*, *Aspergillus niger* and *Alternari brassicae.* The isolation procedure comprised ion exchange chromatography on CMFF, gel filtration on Superdex 75, and reverse-phase HPLC on SOURCE 5 RPC column. It resulted in 0.9% recovery (Yang *et al*. 2006).

Putranjiva roxburghii (Family Putranjivaceae)

The 34-kDa single-chain TI form *P. roxburghii* seeds competitively inhibited trypsin with a K_i of 1.4×10^{-11} M. The inhibitor was relatively stable and retained activity in the pH range 2-12, the temperature range 20-80°C, and in the presence of DTT up to 100 mM. However, its activity was totally abrogated at the temperatures > 80°C. The sequence of first two N-terminal amino acid residues showed dis-

similarity from known Kunitz-type inhibitors (Chauhary *et al*. 2008).

Sapindus saponaria (Family Sapindaceae)

The TI from *S. saponaria* seeds, which inhibited bovine trypsin at a 1: 1 M ratio, was composed of two polypeptide chains, with a molecular mass of about 15 and 3 kDa, respectively. It competitively inhibited trypsin with an equilibrium dissociation constant of 10^{-9} M. It was stable in the presence of denaturing agents. It inhibited trypsin-like proteases in the midguts of *Anagasta kuehniella*, *Anticarsia gemmatalis*, *Corcyra cephalonica*, and *Diatreae saccharalis* larvae (Macedo *et al*. 2011).

Sechium edule (Family Cucurbitaceae)

Squash-type inhibitors were reported from *S. edules* seeds by Laure *et al.* (2006). Acetone fractionation, gel filtration, affinity chromatography and reverse-phase HPLC were used in the isolation procedure and gave a 0.05% recovery. They were composed of about 30 amino acid residues. Two of them have been shown to complex with trypsin with a 1: 1 stoichiometric ratio and displayed dissociation constants of 5.4×10^{-11} M and 1.1×10^{-9} M, respectively. Two of the inhibitors, SETI-IIa and SETI-IIb, closely resembled each other in sequence. SETI-IV manifested a smaller extent of sequence homology.

Solanum nigrum (Family Solanaceae)

Hartl *et al*. (2011) found four *S. nigrum* serine PIs that differed in accumulation patterns, substrate specificity, and effect against different natural herbivorous insects in field- and glasshouse experiments. The differences suggest that these serine PIs have at least partially diversified to provide protection against different attackers (Hartl *et al*. 2011).

Solanum tuberosum (Family Solanaceae)

Potide-G is a 5579-1 a themostable serine PI with antifungal and antibacterial activities. The fungi *Rhizoctonia solani* and *Candida albicans*, and the bacteria *Clavibacter michiganense* subsp, michiganensis, *E. coli*, *Listeria monocytogenes* and *Staphylococcus aureus* were inhibited. It exerted highly potent inhibitory activity against trypsin, chymotrypsin and papain. Its N-terminal sequence exhibited identity to that of potato PI. The purification scheme involved ion exchange chromatography of DEAE-cellulose and reverse-phase HPLC on a C18 column and it accounted for a recovery of 0.05%. Potide-G was stable up to 70°C and showed no hemolytic activity (Kim *et al*. 2006).

A 22-kDa TI, with N-terminal sequence similarity to potato Kunitz-type PIs of group B, inhibited trypsin strongly but chymotrypsin with a lower potency. There was no reactivity toward subtilisin. Ion exchange chromatography on CM-Sepharose was used to isolate the inhibitor (Revina *et al*. 2010).

Kunitz-type chymotrypsin inhibitor (PKCI-23) from potato tubers (*S. tuberosum* L., Zhukov's Jubilee breed) inhibited both chymotrypsin and trypsin with equal efficacy and formed equimolar complexes with the two proteases. However, it exhibited only a weak inhibitory effect toward Carlsberg subtilisin. It inhibited the growth and development of the pathogenic microorganisms *Fusarium culmorum* Sm. and *Phytophtora infestans* which infect potato (Revina *et al*. 2011).

Triticum aestivum (Family Poaceae)

A themostable noncompetitive inhibitor of trypsin and α-amylase was purified from whole wheat grains by using trypsin-Sepharose. In the presence of sulfhydryl group reducing agents its pronounced themostability vanished. The inhibitor-trypsin complex and the inhibitor-αamylase complex exhibited α-amylase inhibiting activity and trypsin inhibiting activity, respectively (Islamov and Furusov 2009).

Veronica hederifolia (Family Plantaginaceae)

The TI from *V. hederifolia* seeds possessed a helix-turn-helix motif and two alpha helices tightly connected by two disulfide bridges. The crystallized complex existed as a stabilized acyl-enzyme intermediate with the scissile bond of the inhibitor cleaved and the resulting N-terminal portion of the inhibitor still connected to the trypsin catalytic serine 195 by an ester bond. It inhibited trypsin with a submicromolar K_i (Conners *et al*. 2007).

Vicia faba (Family Fabaceae)

A broad or faba bean (*V. faba*) trypsin/chymotrypsin inhibitor with antifungal, mitogenic and HIV-I reverse transcriptase inhibitory activity has been reported. It was adsorbed on Affi-gel blue gel and CM-Sepharose and exerted antifungal activity toward *Mycosphaerella arachidicola* and *Physalospora piricola.* This purification scheme gave 0.004% recovery. Besides, the trypsin-chymotrypsin inhibitor elicited mitogenic response from mouse splenocytes and inhibited the activity of human immunodeficiency virus-1 reverse transcriptase with an IC_{50} of 32 μM (Ye and Ng 2002).

A 15-kDa Bowman-Birk type TI from the seeds of faba bean (*Vica faba* cv. 'Giza 843') exhibited significant antiproteolytic activity against trypsin (5761 BAEE units/mg, K_i 20.4×10^{-9} M) which was inhibited by the reducing agent DTT in a dose-dependent manner, indicating the significance of intact disulfide bonds to the activity. The TI showed only slight chymotrypsin inhibitory activity (< 10 BTEE units/mg) It inhibited HIV-1 reverse transcriptase activity with an IC_{50} of about 0.76 μM and induced chromatin condensation and apoptosis in HepG2 hepatoma cells (Fang *et al*. 2011).

Vigna spp. (Family Fabaceae)

A 13.6-kDa helix-rich protein with inhibitory activity against trypsin, chymotrypsin, and α-amylase was isolated from the mung bean *Vigna radiata* (syn. *Phaseolus aureus*). A procedure involving acetic acid precipitation, salt fractionation, ion exchange chromatography on DEAE-cellulose, and affinity chromatography on trypsin-Sepharose was utilized which gave 12.5-fold of purification and 1.07% recovery. The protein contained 2 Cys, 1 Trp and 8 Tyr residues. It was markedly thermostable and relatively pH-stable. At extreme temperatures and pH values, the protein exhibited some changes in the near and far-ultraviolet spectra. Its protease inhibiting activity was retained but its amylase inhibitory activity underwent a slight decline after incubation with trypsin. Its N-terminal sequence was distinct from those of known inhibitors (Haq *et al*. 2005).

Three 16-kDa trypsin/chymotrypsin inhibitors (BGTI1, BGTI2 and BGTI3) were isolated from the black gram *Vigna mungo* (Cheung *et al*. 2009). One of them was unadsorbed while the remaining two were adsorbed on SP-Sepharose. All three were adsorbed on Q-Sepharose, Mono Q, and Mono S. FPLC-gel filtration on Superdex 75 was employed as the final step in the purification scheme. It gave 18.8-20.9-fold of purification and the recovery was 1-2%. In the presence of the reducing agent DTT, the trypsin inhibiting activity of the inhibitors was reduced. The trypsin inhibitory activity of BGTI2 was unaffected after exposure to 100 mM DTT for 2 h. 87.5% of trypsin inhibitory activity of BGTI1 was retained after treatment with 10 mM DTT for 2-h, and the activity dwindled to 12.5% after treatment with 100 mM DTT for 2 h. BGTI3 was also unstable upon DTT treatment. In the presence of 10 mM DTT, trypsin inhibitory activity of BGTI3 dropped to 57.1% after 1 h and became undetectable after 2 h, while treatment of 100 mM DTT for 1 h produced the same effect. Similar results were obtained for BGTI 2. The inhibitors did not exhibit

antiproliferation activity on hepatoma (Hep G2) cells and breast cancer (MCF-7) cells or antifungal action toward *Botrytis cinerea*, *Fusarium oxysporum* and *Mycosphaerella arachidicola.* They possessed very weak HIV-1 reverse transcriptase inhibitory activity in the mM range.

An 8-kDa Bowman-Birk trypsin/chymotrypsin inhibitor was isolated from *V. mungo* cv. TAU-1 seeds using $(NH_4)_2SO_4$ fractionation, ion exchange chromatography, affinity chromatography, and gel filtration. It non-competitively inhibited bovine pancreatic trypsin and chymotrypsin with a K_i of 309.8 nM and 10.7 μM, respectively. The inhibitor displayed relative thermostability (up to 80°C) and pH stability (pH 2-12). Lysine residues at the reactive site of the inhibitor were crucial to the trypsin inhibiting activity. Reduction with DTT also destroyed the trypsin inhibitory activity and the secondary structure conformation. When the ambient temperature was reduced from 90 to 25°C, the conformational changes in secondary structure could be reversed (Prasad *et al*. 2010a).

The Bowman-Birk trypsin/chymotrypsin inhibitor from black-eyed pea (*Vigna unguiculata*) exerted antiproliferative and apoptotic effects on MCF-7 breast cancer cells. It brought about arrest of the cells in S and G2/M phase, morphological changes in the nucleus, fragmentation of plasma membrane, disorganization of the cytoplasm, swelling of mitochondrial, lysosomal enlargement, fragmentation of DNA, increase in number of annexin V (+) cells, decrease of mitochondinal membrane potential, and acidification of cytoplasm. Thus, the inhibitor may be useful for the treatment of breast cancer (Joanitti *et al*. 2010).

A 13-kDa TI was purified from adzuki beans (*Vigna angularis)* by incubation at 70°C for 10 min and precipitation with 60-80% saturated $(NH_4)_2SO_4$. The precipitated fraction effectively prevented degradation of tilapia muscle in a concentration-dependent manner. The myosin heavy chain increased as the concentration of the inhibitor fraction increased, especially at the highest level of addition. The result indicated that the precipitated fraction has potential for use as a PI in fishery-related product (Wati *et al*. 2010).

Xanthosoma blandum (Family Araceae)

Lima *et al*. (2011) screened corms of 15 *Xanthosoma* species for the presence of antibacterial proteins. A 24-kDa Kunitz-type serine proteinase inhibitor with antibacterial activity was isolated from *X. blandum* corms. It was purified by RP-HPLC and gave a recovery of 21.9% and 20.6-fold of purification. It showed antimicrobial activity toward *Salmonella typhimurium*.

DISCUSSION

The plant PIs reviewed in this article mostly belong to Kunitz-type inhibitors, Bowman-Birk inhibitors and squash-type inhibitors with different amino acid sequences (**Table 1**) and a molecular weight of approximately 20, 8 and 3 kDa, respectively (**Table 2**). Kunitz-type type inhibitors are those from small glossy black Chinese soybean (Ye and Ng 2009) and *Inga laurina* (Maccdo *et al*. 2007). Examples of the second type are those from large Hokkaido black soybean (Ho and Ng 2008) and pigeon pea (Prasad *et al*. 2010b). Examples of the third type include those from *Sechium edule* (Laure *et al*. 2006).

A variety of chromatographic techniques were used to isolate plant PIs including affinity chromatography on immobilized trypsin, ion exchange chromatography and gel filtration (Bhattacharyya *et al.* 2006; Sun *et al*. 2010). Affinity chromatography on immobilized trypsin has been used alone or in combination with ion exchange chromatography and gel filtration for isolation of TIs. The most widely used method is a combination of ion exchange chromatography and gel filtration. The yield and recovery vary depending on the plant species.

Many plant PIs manifest relatively high thermostability (up to 80°C) and pH stability (pH 3-12) (Haq *et al*. 2005; Gomes *et al.* 2005, 2008). Very often the protease inhibitory activity was reduced in the presence of the reducing agent DTT, signifying the pivotal role played by the disulfide bond (Cheung *et al*. 2009; Prasad *et al*. 2010a; Sun *et al*. 2010).

The mode of protease inhibition may be competitive as in the case of *C. cajan* inhibitor (Prasad *et al*. 2010b) or noncompetitive like *Triticum aestivum* inhibitor (Islamov and Fusarov 2007).

Some of the plant PIs are active toward only trypsin (Mace do *et al*. 2007). Others act on both trypsin and chymotrypsin (Cheung *et al*. 2009; Prasad *et al*. 2010a; Joanitti *et al*. 2010) and also other enzymes like subtilisin (Sritanyarat *et al*. 2006) and kallikrein (Yohizaki *et al*. 2007) (**Table 2**).

Isoinhibitors have been reported for *A. plumosa* (Lopes *et al*. 2009), *P. vulgaris* cv. White cloud bean (Sun *et al*. 2010), *V. mungo* (Cheung *et al.* 2009), *D. trifoliata* (Bhattacharyya and Babu 2009) and *E. scandens* (Lingaraja and Gowda 2008).

Other biological activities have been reported for some of the plant PIs. Antifungal activity has been observed in soybean inhibitor (Zhang *et al*. 2009) and potato inhibitor (Kim *et al*. 2006). Anti-insect activity is detected in *C. cajans* inhibitor (Prasad *et al*. 2009). HIV-1 reverse transcriptase inhibitory activity is found in *B. variegata* inhibitor (Fang *et al*. 2010) and black soybean inhibitor (Ye and Ng 2009). Antiproliferative activity toward cancer cells has been noted in inhibitors from *B. variegata* (Fang *et al.* 2010) black soybean (Ho and Ng 2008; Ye and Ng 2009), *V. unguiculata* (Joanitti *et al.* 2010), and *P. dubium* (Troncoso *et al*. 2007). Others are devoid of these activities (Cheng *et al*. 2009; Zhang *et al*. 2009).

Some of the plant PIs exist in the form of heterodimers. *A. kalkora* inhibitor is composed of a 15.5-kDa subunit and a 4.5-kDa subnit (Zhou *et al*. 2008). *C. selloi* inhibitor consists of a 16-kDa subunit and a 6-kDa subunit (Yoshizaki *et al*. 2007).

In summary, plant PIs are defense proteins that protect plants from phytophagous insects and pathogenic fungi. Human populations consuming a diet abundant in plant PIs may have a lowered risk of contracting cancer due to the anticancer activity of these inhibitors.

Table 1 N-terminal sequences of selected plant inhibitors.

Protease inhibitor	N-terminal sequence
Trypsin inhibitor from *Psoralea corylifolea* seeds (Yang *et al*. 2006)	EDRKCPKILMRCKRDSDCLAKCTCQESGYCG
SETI-IIa	EDRKCPKILMRCKRDSDCLAKCTCQESGYCG
SETI-IIb	EEDRKCPKILMRCKRDSDCLAKCTCQEGGYCG
SETI-IV from *Sechium edules* seeds (Laure *et al*. 2006)	CPRILMKCKLDTDCFPTCTCRPSGFCG
TI from *Anredera cordifolia* rhizomes (Chuang *et al*. 2007)	KDDLLVLDGGNPVV
Cajanus cajan trypsin inhibitor (Prasad *et al*. 2010)	DOHHSSKACC
GCTI from *Gymnocladus chinensis* (Zhu *et al.* 2011)	KSGHRHESTDEPSESKKADDDHCACTK SIPPQQ
FtTI from *Fagopyrum tataricum seeds* (Ruan *et al.* 2011)	LIYAKVKCLITGVRTYVGKQSWPELVGTKG
XB-KTI from *Xanthosoma blandum* (Lima *et al.* 2011)	PVVDTTGNNPLQQQEEYYV
SSTI from *Sapindus saponaria seeds* (Macedo *et al.* 2011)	KTQVLDANGNIRNGGTYYVLPDSFALQGGFELAATR

Table 2 Biochemical characteristics of selected plant protease inhibitors.

Species name	Type of inhibitor	Molecular mass (kDa)	Ki (Protease)	Other activities
Vigna mungo cv. TAU-1 (Prasad *et al.* 2010a)	Bowman-Birk	8	309.8 nM (bovine trypsin) 10.7 uM (bovine chymotrypsin)	
Cajanus cajan (Prasad *et al.* 2010b)	Bowman-Birk	8.5	292 nM (bovine trypsin) 2265 nM (bovine chymotrypsin)	Inhibition of insect midgut trypsin and larval growth
Entada scandens (Lingaraja and Gowda 2008)	Kunitz	19.766	4.9 nM (bovine trypsin)	Inhibition of midgut protease of rice moth larvae
Derris trifoliate (Bhattachargya and Babu 2009)	Kunitz	20	0.17 nM (trypsin) 0.125 nM (chymotrypsin)	antimalarial
Bauhinia variegata var. variegata (Fang *et al.* 2010)	Kunitz	20	0.1 nM (trypsin)	HIV-1 reverse transcriptase inhibitory Antitumor
Caesalpinia bonduc (Bhattacharyya and Babu 2007)	Serine protease	16	0.095 nM (chymotrypsin) 27.5 nM (trypsin)	
Lupinus albus (Scarafori *et al.* 2008)	Booman-Birk	6.858	4.2 nM (trypsin) No effect on chymotrypsin	
Calliandra selloi (Yoshizaki *et al.* 2007)	Kunitz	One 16-kDa subunit + one 6-kDa subunit	221 nM (trypsin) 495 nM (chymotrypsin) 420 nM (kallikrein)	
Hevea brasiliensis (Sritanyara *et al.* 2006)	Potato protease inhibitor	14.89 7.75 7.56	Potent inhibition of subtilisin Weak inhibition of trypsin No inhibition of chymotrypsin	
Sechium edule (Laure *et al.* 2006)	Squash	3.4		
Vicia faba (Fang *et al.* 2011)	Bowman-Birk	15	20.4 nM (trypsin)	HIV-1 reverse transcriptase inhibitory Antitumor
Gymnocladus chinensis (Zhu *et al.* 2011)	Kunitz	20	0.4 μM (trypsin)	Antitumor
Fagopyrum tataricum seeds (Ruan *et al.* 2011)	Serine protease	14	1.6 nM (trypsin)	Antifungal
Cicer arietinum (Sharma and Suresh 2011)	Kunitz	18	0.14 nM (trypsin)	
Xanthosoma blandum (Lima *et al.* 2011)	Kunitz	24		Antibacterial
Sapindus saponaria (Mecado *et al.* 2011)	Kunitz	18	2.4 nM (bovine trypsin)	Inhibition of insect midgut trypsin and larval growth

CONCLUSIONS AND FUTURE PERSPECTIVES

Plant PIs manifest a variety of potentially exploitable activities. The most notable and commonly observed is anti-insect activity. The inhibitor from *A keelkora s*eeds also inhibited trypsin-like proteases from insects including *Helicoverpa amiger*a, *Pieris rapie* and *Spodoptera exigua* (Zhou *et al*. 2008) a heterodimeric Kunitz-type trypsin/chymotrypsin inhibitor from seeds of *A. ellipticum* inhibited the proteinase from the midgut of 5th instar larvae of *Spodoptera litura* (Bhattacharyya *et al*. 2006) The TI isolated from *Crotalaria pallida* seeds by Gomes *et al*. (2005) exhibited anti-insect activity against a number of insect species. Anti-fungal activity is found in only some plant PIs such as potato inhibitor (Kim *et al*. 2006). Antiproliferative activity toward cancer cells is a characteristic of some plant PIs such as sweet potato inhibitor (Huang *et al*. 2007). It suppressed proliferation of NB4 promyelocytic leukemia cells with an IC_{50} of 57.1 μg/mL. TI caused cell cycle arrest at the G1 phase and apoptosis. There was an elevation of P53 and Bax proteins, a decline in antiapoptotic Bcl-2, and a release of cytochrome c from the mitochondria into the cytosol. The induction of mitochondria-dependent apoptosis in NB4 cells was associated with the activation of caspase-3 and -8.

REFERENCES

Bhattacharyya A, Babu CR (2009) Purification and biochemical characterization of a serine proteinase inhibitor from *Derris trifoliata* Lour. seeds: Insight into structural and antimalarial features. *Phytochemistry* **70**, 703-712

Bhattacharyya A, Mazumdar S, Leighton SM, Babu CR (2006) A Kunitz proteinase inhibitor from *Archidendron ellipticum* seeds: Purification, characterization, and kinetic properties. *Phytochemistry* **67**, 232-241

Bhattacharyya A, Rai S, Babu CR (2007) A trypsin and chymotrypsin inhibitor from *Caesalpinia bonduc* seeds: Isolation, partial characterization and insecticidal properties. *Plant Physiology and Biochemistry* **45**, 169-177

Cheung AH, Ng TB (2007) Isolation and characterization of a trypsin-chymotrypsin inhibitor from the seeds of green lentil (*Lens culinaris*). *Protein and Peptide Letters* **14**, 859-864

Cheung AH, Wong JH, Ng TB (2009) Trypsin-chymotrypsin inhibitors from *Vigna mungo* seeds. *Protein and Peptide Letters* **16**, 277-284

Chuang MT, Lin YS, Hou WC (2007) Ancordin, the major rhizome protein of Madeira-vine, with trypsin inhibitory and stimulatory activities in nitric oxide productions. *Peptides* **28**, 1311-1316

Conners R, Konarev AV, Forsyth J, Lovegrove A, Marsh J, Joseph-Horne T, Shewry P, Brady RL (2007) An unusual helix-turn-helix protease inhibitory motif in a novel trypsin inhibitor from seeds of veronica (*Veronica hederifolia* L.). *The Journal of Biological Chemistry* **282**, 27760-27768

Ee KY, Zhao J, Rehman AU, Agboola SO (2009) Purification and characterization of a Kunitz-type trypsin inhibitor from *Acacia victoriae* Bentham seeds. *Journal of Agricultural and Food Chemistry* **12**, 7022-7029

Fang EF, Hassanien AA, Wong JH, Bah CS, Soliman SS, Ng TB (2011) Isolation of a new trypsin inhibitor from the Faba bean (*Vicia faba* cv. Giza 843) with potential medicinal applications. *Protein and Peptide Letters* **18**, 64-72

Fang EF, Wong JH, Bah CS, Lin P, Tsao SW, Ng TB (2010) *Bauhinia variegata* var. variegata trypsin inhibitor: From isolation to potential medicinal applications. *Biochemical and Biophysical Research Communications* **11**, 806-811

Gomes CE, Barbosa AE, Macedo LL, Pitanga JC, Moura FT, Oliveira AS, Moura RM, Queiroz AF, Macedo FP, Andrade LB, Vidal MS, Sales MP (2005) Effect of trypsin inhibitor from *Crotalaria pallida* seeds on *Callosobruchus maculatus* (cowpea weevil) and *Ceratitis capitata* (fruit fly). *Plant Physiology and Biochemistry* **43**, 1095-1102

Haq SK, Atif SM, Khan RH (2005) Biochemical characterization, stability studies and N-terminal sequence of a bi-functional inhibitor from *Phaseolus aureus* Roxb. (mung bean). *Biochimie* **87**, 1127-1136

Hartl M, Giri AP, Kaur H, Baldwin IT (2011) The multiple functions of plant serine protease inhibitors: Defense against herbivores and beyond. *Plant Signaling and Behavior* **6**, 1009-1011

Ho VS, Ng TB (2008) A Bowman-Birk trypsin inhibitor with antiproliferative activity from Hokkaido large black soybeans. *Journal of Peptide Science* **14**, 278-282

Huang GJ, Sheu MJ, Chen HJ, Chang YS, Lin YH (2007) Growth inhibition and induction of apoptosis in NB4 promyelocytic leukemia cells by trypsin inhibitor from sweet potato storage roots. *Journal of Agricultural and Food Chemistry* **55**, 2548-2553

Inokoshi J, Shiomi K, Masuma R, Tanaka H, Yamada H, Omura S (1999) Funalenone, a novel collagenase inhibitor produced by *Aspergillus niger*. *The Journal of Antibiotics (Tokyo)* **52**, 1095-1100

Islamov RA, Furusov OV (2007) Bifunctional inhibitor of alpha-amylase/trypsin from wheat grain. *Prikladnaya Biokhimiya i Mikrobiologiya* **43**, 419-423

Jaw KS, Chou LH, Chang SM, Duan KJ (2007) Purification of a trypsin in-

hibitor from sweet potato in an aqueous two phase system. *Biotechnology Letters* **29**, 137-140

Joanitti GA, Azevedo RB, Freitas SM (2010) Apoptosis and lysosome membrane permeabilization induction on breast cancer cells by an anticarcinogenic Bowman-Birk protease inhibitor from *Vigna unguiculata* seeds. *Cancer Letters* **1**, 73-81

Kim MH, Park SC, Kim JY, Lee SY, Lim HT, Cheong H, Hahm KS, Park Y (2006) Purification and characterization of a heat-stable serine protease inhibitor from the tubers of new potato variety "Golden Valley". *Biochemical and Biophysical Research Communications* **346**, 681-686

Kowalska J, Pszczoła K, Wilimowska-Pelc A, Lorenc-Kubis I, Zuziak E, Ługowski M, Łegowska A, Kwiatkowska A, Sleszyńska M, Lesner A, Walewska A, Zabłotna E, Rolka K, Wilusz T (2007) Trypsin inhibitors from the garden four o'clock (*Mirabilis jalapa*) and spinach (*Spinacia oleracea*) seeds: Isolation, characterization and chemical synthesis. *Phytochemistry* **68**, 1487-1496

Kowalska J, Zabłocka A, Wilusz T (2006) Isolation and primary structures of seven serine proteinase inhibitors from *Cyclanthera pedata* seeds. *Biochimica Biophysica Acta* **1760**, 1054-1063

Laure HJ, Faça VM, Izumi C, Padovan JC, Greene LJ (2006) Low molecular weight squash trypsin inhibitors from *Sechium edule* seeds. *Phytochemistry* **67**, 362-370

Lima TB, Silva ON, Migliolo L, Souza-Filho CR, Gonçalves EG, Vasconcelos IM, Oliveira JT, Amaral AC, Franco OL (2011) A Kunitz proteinase inhibitor from corms of *Xanthosoma blandum* with bactericidal activity. *Journal of Natural Products* **74**, 969-975

Lingaraju MH, Gowda LR (2008) A Kunitz trypsin inhibitor of *Entada scandens* seeds: Another member with single disulfide bridge. *Biochimica Biophysica Acta*, **1784**, 850-855

Lopes JL, Valadares NF, Moraes DI, Rosa JC, Araújo HS, Beltramini LM (2009) Physico-chemical and antifungal properties of protease inhibitors from *Acacia plumosa. Phytochemistry* **70**, 871-879

Macedo ML, Diz Filho EB, Freire MG, Oliva ML, Sumikawa JT, Toyama MH, Marangoni S (2011) A trypsin inhibitor from *Sapindus saponaria* L. seeds: Purification, characterization, and activity towards pest insect digestive enzyme. *The Protein Journal* **30**, 9-19

Macedo ML, Garcia VA, Freire MG, Richardson M (2007) Characterization of a Kunitz trypsin inhibitor with a single disulfide bridge from seeds of *Inga laurina* (SW.) Willd. *Phytochemistry* **68**, 1104-1111

Okumura Y, Ogawa K, Nikai T (2004) Elastase and elastase inhibitor from *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus niger*. *Journal of Medical Microbiology* **53**, 351-354

Oliveira AS, Migliolo L, Aquino RO, Ribeiro JK, Macedo LL, Andrade LB, Bemquerer MP, Santos EA, Kiyota S, Sales MP (2007) Identification of a Kunitz-type proteinase inhibitor from *Pithecellobium dumosum* seeds with insecticidal properties and double activity. *Journal of Agricultural and Food Chemistry* **55**, 7342-7349

Prasad ER, Dutta-Gupta A, Padmasree K (2009) Inhibitors from pigeonpea active against lepidopteran gut proteinases. *Journal of Economic Entomology* **102**, 2343-2349

Prasad ER, Dutta-Gupta A, Padmasree K (2010a) Purification and characterization of a Bowman-Birk proteinase inhibitor from the seeds of black gram (*Vigna mungo*). *Phytochemistry* **71**, 363-372

Prasad ER, Merzendorfer H, Madhurarekha C, Dutta-Gupta A, Padmasree K (2010b) Bowman-Birk proteinase inhibitor from *Cajanus cajan* seeds: Purification, characterization, and insecticidal properties. *Journal of Agricultural and Food Chemistry* **10**, 2838-2847

Ramos V da S, Silva G de S, Freire MG, Machado OL, Parra JR, Macedo ML (2008) Purification and characterization of a trypsin inhibitor from *Plathymenia foliolosa* seeds. *Journal of Agricultural and Food Chemistry* **56**, 11348-11355

Revina TA, Kladnitskaya GV, Gerasimova NG, Gvozdeva EL, Valueva TA (2010) Protein trypsin inhibitor from potato tubers. *Biochemistry (Moscow)* **75**, 36-40

Revina TA, Parfenov IA, Gvozdeva EL, Gerasimova NG, Valueva TA (2011) Chymotrypsin and trypsin inhibitor isolated from potato tubers. *Prikladnaia Biokhimiia i Mikrobiologiia* **47**, 265-271

Rocco M, Malorni L, Chambery A, Poerio E, Parente A, Di Maro A (2011) A Bowman-Birk inhibitor with anti-elastase activity from *Lathyrus sativus* L. seeds. *Molecular Biosystems* **7**, 2500-2507

Ruan JJ, Chen H, Shao JR, Wu Q, Han XY (2011) An antifungal peptide from *Fagopyrum tataricum* seeds. *Peptides* **32**, 1151-1158

Satheesh LS, Murugan K (2011) Antimicrobial activity of protease inhibitor from leaves of *Coccinia grandis* (L.) Voigt. *Indian Journal of Experimental Biology* **49**, 366-374

Scarafoni A, Consonni A, Galbusera V, Negri A, Tedeschi G, Rasmussen P, Magni C, Duranti M (2008) Identification and characterization of a Bowman-Birk inhibitor active towards trypsin but not chymotrypsin in *Lupinus albus* seeds. *Phytochemistry* **69**, 1820-1825

Sharma U, Suresh CG (2011) Purification, crystallization and X-ray characterization of a Kunitz-type trypsin inhibitor protein from the seeds of chickpea (*Cicer arietinum*). *Acta Crystallographica Section F: Structural Biology and Crystallization Communications* **67**, 714-717

Stefan A, Ugolini L, Martelli E, Palmieri S, Hochkoeppler A (2009) Expression and purification of the recombinant mustard trypsin inhibitor 2 (MTI2) in *Escherichia coli*. *Journal of Bioscience and Bioengineering* **108**, 282-285

Sun J, Wang H, Ng TB (2010) Trypsin isoinhibitors with antiproliferative activity toward leukemia cells from *Phaseolus vulgaris* cv "White Cloud Bean". *Journal of Biomedicine and Biotechnology* **2010**, 219793

Torres-Castillo JA, Mondragón Jacobo C, Blanco-Labra A (2009) Characterization of a highly stable trypsin-like proteinase inhibitor from the seeds of *Opuntia streptacantha* (*O. streptacantha* Lemaire). *Phytochemistry* **70**, 1374-1381

Troncoso MF, Biron VA, Longhi SA, Retegui LA, **Wolfenstein-Todel C** (2007) *Peltophorum dubium* and soybean Kunitz-type trypsin inhibitors induce human Jurkat cell apoptosis. *International Immunopharmacology* **7**, 625-636

Troncoso FM, Zolezzi CP, Hellman U, Wolfenstein-Todel C (2003) A novel trypsin inhibitor from Peltophorum dubium seeds, with lectin-like properties, triggers rat lymphoma cell apoptosis. *Archives of Biochemistry and Biophysics* **411**, 93-104

Volpicella M, De Leo F, Sciancalepore M, Sonnante G, Pignone D, Gallerani R, Ceci LR (2009) Identification and characterization of protease inhibitors in *Diplotaxis* species. *Plant Physiology and Biochemistry* **47**, 175-180

Wang KJ, Takahata Y, Kono Y, Kaizuma N (2008) Allelic differentiation of Kunitz trypsin inhibitor in wild soybean (*Glycine soja*). *Theoretical and Applied Genetic*s **117**, 565-573

Wati RK, Theppakorn T, Benjakul S, Rawdkuen S (2010) Trypsin inhibitor from 3 legume seeds: Fractionation and proteolytic inhibition study. *Journal of Food Science* **75**, 223-228

Yang X, Li J, Wang X, Fang W, Bidochka MJ, She R, Xiao Y, Pei Y (2006) Psc-AFP, an antifungal protein with trypsin inhibitor activity from *Psoralea corylifolia* seeds. *Peptides* **27**, 1726-1731

Ye X, Ng TB (2009) A trypsin-chymotrypsin inhibitor with antiproliferative activity from small glossy black soybeans. *Planta Medica* **75**, 550-556

Yoshizaki L, Troncoso MF, Lopes JL, Hellman U, Beltramini LM, Wolfenstein-Todel C (2007) *Calliandra sello*i Macbride trypsin inhibitor: isolation, characterization, stability, spectroscopic analyses. *Phytochemistry* **68**, 2625-2634

Zhang B, Wang DF, Fan Y, Zhang L, Luo Y (2009) Affinity purification of trypsin inhibitor with anti-*Aspergillus flavus* activity from cultivated and wild soybean. *Mycopathologia* **167**, 163-171

Zhang X, Wang H, Ng TB (2008) Isolation and characterization of a novel trypsin inhibitor from fresh lily bulbs. *Planta Medica* **74**, 546-550

Zhang Y, Kouzuma Y, Miyaji T, Yonekura M (2008) Purification, characterization, and cDNA cloning of a Bowman-Birk type trypsin inhibitor from *Apios americana* Medikus tubers. *Bioscience Biotechnology and Biochemistry* **72**, 171-178

Zhou JY, Liao H, Zhang NH, Tang L, Xu Y, Chen F (2008) Identification of a Kunitz inhibitor from *Albizzia kalkora* and its inhibitory effect against pest midgut proteases. *Biotechnology Letters* **30**, 1495-1499

Zhu MJ, Zhang GQ, Wang HX, Ng TB (2011) Isolation and characterization of a Kunitz-type trypsin inhibitor with antiproliferative activity from *Gymnocladus chinensis* (Yunnan bean) seeds. *The Protein Journal* **30**, 240-246

Zuchowski J, Jaszek M, Grzywnowicz K (2009) Novel trypsin inhibitors from the white rot fungus *Abortiporus biennis* Partial purification and characterization. *Biochemistry (Moscow)* **74**, 226-230