Antimicrobial activity of protease inhibitor from leaves of Coccinia grandis (L.) Voigt.

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Antimicrobial activity of protease inhibitor isolated from Coccinia grandis (L.) Voigt. has been reported. A 14.3 kDa protease inhibitor (PI) was isolated and purified to homogeneity by ammonium sulfate precipitation (20-85% saturation), sephadex G-75, DEAE sepharose column and trypsin-sepharose affinity chromatography from the leaves of C. grandis. The purity was checked by reverse phase high performance liquid chromatography. PI exhibited marked growth inhibitory effects on colon cell lines in a dose-dependent manner. PI was thermostable and showed antimicrobial activity without hemolytic activity. PI strongly inhibited pathogenic microbial strains, including Staphylococcus aureus, Klebsiella pneumoniae, Proteus vulgaris, Escherchia coli, Bacillus subtilis and pathogenic fungus Candida albicans, Mucor indicus, Penicillium notatum, Aspergillus flavus and Cryptococcus neoformans. Examination by bright field microscopy showed inhibition of mycelial growth and sporulation. Morphologically, PI treated fungus showed a significant shrinkage of hyphal tips. Reduced PI completely lost its activity indicating that disulfide bridge is essential for its protease inhibitory and antifungal activity. Results reported in this study suggested that PI may be an excellent candidate for development of novel oral or other anti-infective agents.

Keywords: Antimicrobial activity, Chromatography, Coccinia grandis, Cytotoxicity, Protease inhibitors, Purification.

Protease inhibitors (PIs) play essential role in biological systems regulating proteolytic processes and in defense mechanisms against insects, and other pathogenic microorganisms. In recent years appearance of new mutant strains of microorganisms resistant to commonly used antibiotics have stimulated a systematic analysis of natural products for bactericidal and fungicidal properties having therapeutic applications. Several studies on PIs were published with the aim of investigating enzyme mechanisms of controlling disease and pathological processes using genes encoding protease inhibitors. Recent studies have revealed that protease inhibitors as new drugs in highly active antiretroviral combination therapy, increasing life expectancy in HIV-positive patients. Antimicrobial peptides (AMPs) are important effector molecules of innate immunity. Such peptides provide protection against bacteria, fungi and viruses by acting on cell membranes of pathogens. Recently, several antimicrobial plant proteins and peptides that inhibit growth of agronomically important pathogens have been isolated from various plant sources.

Antimicrobial peptides and proteins with a spectacular diversity of structures, includes thaumatin-like proteins, embryo-abundant proteins, allergen like peptides, cyclophilin-like proteins, cysteine-rich small proteins, arginine and glutamate rich proteins, lectins, thionins, peroxidases, ribosome inactivating proteins, lipid-transfer proteins, chitin binding proteins, ribonucleases, deoxyribonucleases and protease inhibitors. These proteins can inhibit a wide variety of phytopathogens, but display different potencies according to target microorganisms.

Coccinia grandis (L.) Voigt. is a wild cucurbitaceous medicinal plant with many pharmaceutical applications. As an ethnic tribal plant, it has potential therapeutic values as anti-diabetic, anti-ulcer, anti-inflammatory, antioxidant and antitumor properties. In this study, antimicrobial activity of a protease inhibitor (PI) purified from leaves of C. grandis has been reported.

Materials and Methods

Materials— Fresh leaves from Coccinia grandis (L.)Voigt. were collected from the department garden, University College, Trivandrum, Kerala and stored at -20°C.

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Isolation and purification of PI—All the purification steps were carried out at 4°C as described by Macedo et al.⁶ A crude inhibitor preparation was obtained by extraction of fresh leaves from *C. grandis* with 100 mM phosphate buffer, pH 7.6 (1:10, w/v) for 2 h with subsequent centrifugation at 7500 g for 30 min and the supernatant was collected. The crude inhibitor was fractionated with solid ammonium sulfate (20–85% saturation) and the precipitate was recovered by centrifugation at 10,000 × g for 20 min, redissolved in 0.01 M phosphate buffer (pH 7.6) and dialyzed against distilled water for 24 h and lyophilized. Lyophilized PI was dissolved in 100 mM phosphate buffer, pH 7.6, containing 100 mM NaCl, and applied to a Sephadex G-75 column equilibrated with the same buffer. The fraction with inhibitory activity was further fractionated by ion-exchange chromatography on a DEAE-Sepharose column equilibrated with 50 mM Tris–HCl buffer, pH 8.0, and eluted with a linear gradient of NaCl (0–1 M) in the same buffer. The fraction eluted with 0.4 to 0.5 M NaCl showing highest PI activity was applied to a trypsin-Sepharose affinity column equilibrated with 100 mM phosphate buffer, pH 7.6, containing 100 mM NaCl. The adsorbed PI was eluted with 0.01 N HCl. The purity was further checked by reverse phase HPLC (C18 column) at a flow rate of 1.0 ml/min with 100% solvent A (0.1% trifluoroacetic acid (TFA) in water) for 10 min and a linear gradient (0–100%) of solvent B (0.08% TFA in 80% acetonitrile) over 55 min. Proteins were detected by monitoring the absorbance at 280 nm.

Apparent molecular mass was obtained by Sephadex G-75 gel filtration column (0.1 M phosphate buffer, pH 7.6) calibrated with proteins of known molecular mass. The molecular mass and homogeneity of PI was determined by SDS–PAGE as described by Schagger and von Jagow⁷.

**Assay of trypsin and chymotrypsin inhibitory activity**—Trypsin or chymotrypsin inhibitory activity was determined by using an appropriate volume of purified PI as per the methodology of Macedo et al.⁶ that results in a 40–60% decrease in corresponding enzyme activity. Assay mixture consists of inhibitor in assay buffer 50 mM Tris–HCl containing 20 mM CaCl₂ either at pH 8.2 for trypsin or pH 7.8 for chymotrypsin. Trypsin (10 µg) or chymotrypsin (80 µg) was added to the assay mixture and incubated for 15 min at 37°C. Residual trypsin or chymotrypsin activity in the above assay mixture was determined after incubating for 45 min at 37°C with synthetic substrates: 1 mM BAPNA (N-benzoyl-L-arginine-p-nitroanilide) or 1 mM BTPNA (N-benzoyl-L-tyrosyl-p-nitroanilide)⁵, respectively. The reaction was terminated by adding 30% acetic acid. The resulting absorbance at 410 nm was recorded. One inhibitor unit was defined as the amount of inhibitor required to inhibit 50% of the corresponding enzyme activity per unit time.

**Test microorganisms**—Selected pathogenic bacteria; *Staphylococcus aureus* (MTCC 740), *Klebsiella pneumoniae* (MTCC109), *Escherichia coli* (MTCC 42), *Bacillus subtilis* (MTCC 47), *Proteus vulgaris* (MTCC 426) and fungi like *Cryptococcus neoformans*, *Candida albicans*, *Mucor indicus*, *Penicillium notatum* and *Aspergillus flavus* were obtained from culture collection of the Department of Applied Botany and Biotechnology, University of Mysore, Mysore, India. All the test bacterial stock cultures were maintained at 4°C on nutrient medium (HiMedia). Active cultures for experiments were prepared by transferring a loopful of culture to 10 ml of nutrient broth (HiMedia) and incubated at 37°C for bacterial proliferation. After 24 h incubation, the bacterial suspension was centrifuged at 10,000 g for 15 min. The pellet was resuspended in sterile distilled water and the concentration was adjusted to 1 × 10⁶ CFU/ml by reading the OD of the solution (A₆₁₀ nm) and used for further studies. The fungal colonies were inoculated into Sabauroud Dextrose Agar (SDA) and incubated at 35 ± 2°C for 4 h. The turbidity of the resulting suspensions was diluted with SBCB to obtain a transmittance of 25.0 % at 580 nm. That percentage was found spectrophotometrically comparable to McFarland turbidity standard. This level of turbidity is equivalent to approximately 1 × 10⁸ CFU/ml.

**Antimicrobial assay**—Antimicrobial assay was performed in 96 well, sterile, flat bottom microtiter plates, based on broth microdilution assay, which is an automated colorimetric method, uses the absorbance (optical density) of cultures in a microtiter plate⁸. Each well of microtiterplates was filled with 50 µl of test organism and 200 µl different concentrations PI extracts. For bacteria and fungi the microtiterplates were incubated at 37±2°C and 30±2°C for 24 h and 48 h respectively. After the incubation period the plates were read at 620 nm using ELISA reader. Minimum inhibitory concentration (MIC) was determined as the lowest
concentration of PI inhibiting the growth of the organism, based on the readings.

To determine minimum killing concentration, 50 µl broth was taken from each well and inoculated in 200 µl nutrient broth for 24 h at 37° C for bacteria and 48 h at 30°C for the fungal cells. The minimum killing concentration is defined as the lowest concentration of the PI at which inoculated microorganism was completely killed. Each test was performed in triplicate and repeated twice. Levofoxacin was used as a positive control.

Preparation of human red blood cells and assay of hemolytic activity—Human red blood cells were centrifuged and washed three times with phosphate-buffered saline (PBS; 35 mM phosphate buffer with 0.15M NaCl, pH 7.0). The hemolytic activity of the potamin-1 and melittin (positive control) were evaluated by measuring the release of hemoglobin from fresh human erythrocytes. Aliquots (100 µl) of an 8% suspension of red blood cells were transferred to 96-well plates, and hemolysis was determined by measuring the absorbance at 414 nm using the Emax plate reader. No hemolysis (0%) and full hemolysis (100%) were determined in the presence of PBS and 0.1% Triton X-100, respectively. The percent hemolysis was calculated using the following equation: % hemolysis = [(A414 nm with TPI solution - A414 nm in PBS)/(A414 nm with 0.1% Triton X-100 - A414 nm in PBS) × 100]%.

MTT assay—This assay detects the reduction of MTT [3-(4, 5-dimethylthiazoly)-2,5-diphenyl-tetrazolium bromide] by mitochondrial dehydrogenase, to blue formazan product, which reflects the normal functioning of mitochondrial and cell viability. HeLa cells were seeded in 96 well plates at a density of 1 × 10⁴ followed by incubation for 24 h. Different concentrations of the PI were then added. After 72 h, 20 µl of a 5-mg/ml solution of MTT was spiked into each well. After 4 h, the plates were centrifuged at 324 g for 5 min. The supernatant was removed and 150 µl of dimethyl sulfoxide was added to dissolve the MTT-formazan at the bottom of the wells. After 10 min, the absorbance at 570 nm was measured using a microplate ELISA reader.

Statistical analysis—All the experiments were designed with six replications. The data were statistically analyzed for a student t-test.

Results C. grandis protease inhibitor (PI) was purified to homogeneity in four steps by ammonium sulfate precipitation (20-85% saturation), sephadex G-75, DEAE sepharose column and trypsin-sepharose affinity chromatography. The crude PI was fractionated with (NH₄)₂SO₄ and dialyzed. The fractions showing maximum PI activity was applied to gel-filtration column (Sephadex G-75) and eluted with 100 mM phosphate buffer (pH 7.6) containing 100 mM NaCl. The active fractions with PI activity (fraction number 66 to 70) were pooled, dialyzed and loaded to a DEAE-sepharose column, pre-equilibrated with 50 mM Tris–HCl, pH 8.0. The bound PI was eluted with a gradient of irrigating buffer (0 to 1.0 M NaCl in the same buffer) at a flow rate of 30 ml/h. The fractions showing PI activity (0.4 to 0.5 M NaCl elution) were pooled, dialyzed against 50 mM Tris–HCl buffer (pH 8.0). The pooled PI fractions were loaded onto a Tryptsin sepharose affinity column, pre-equilibrated with 100 mM Tris–HCl, pH 7.6. The bound protein was eluted with 0.01 N HCl at a flow rate of 30 ml/h and subsequently the fractions (1.0 ml) were neutralized with 2 M Tris. The fractions with PI activity were pooled and concentrated using Amicon filters for further use. It proved to be a convenient step for isolating PI, although the possibility of limited digestion of the inhibitor by the immobilized trypsin during purification cannot be excluded. After affinity purification high inhibitory activity and purification fold observed in the present study suggests its functional integrity was retained even after binding with trypsin. The fractions showing PI activity were pooled and subjected to further analysis.

Thus, the protocol yielded a purified PI with specific activity 377.9 U/mg; with a low protein content of 1.4 mg. Overall, the specific activity increased about 114.5-fold with 12% yield of activity Table 1. This is significantly higher than that obtained for Arachidendron ellipticum and Brassica campestris.

The purified PI was further subjected to RP-HPLC showed a single peak with a retention time of 8.2 min in 50 mM Tris–HCl buffer, pH 8.0 and coincided with the protein peak. The purity of the protein was analyzed by SDS-PAGE showed a single thick polypeptide band with a molecular mass of approximately 14.3 kDa (Fig. 1), which is lower than those reported for other cucumisin-like proteases and PI of legumes. The molecular mass estimated by size elution chromatography (14.3 kDa) was agreed fairly well with the SDS-PAGE results.
Antimicrobial activity—Antimicrobial assay of the purified PI was examined against various bacterial and fungal cells by assessing the minimum inhibition concentration and minimum killing concentration. As shown in Table 2, PI had exhibited different degree of growth inhibition against tested bacterial and fungal strains such as *S. aureus*, *K. pneumoniae*, *P. vulgaris*, *E. coli*, *B. subtilis*, *C. albicans*, *M. indicus*, *P. notatum*, *A. flavus* and *C. neoformans*. The microbicidal effect of PI was further visualized as inhibition zone by treating the pathogens with PI and then spreading the cells on agar plates. Among the all pathogens tested *K. pneumoniae* and *A. flavus* were the most sensitive and *S. aureus*, *B. subtilis*, *C. albicans* and *C. neoformans* were the most resistant strains.

The MTT assay was used to examine the effects of PI on HeLa cells and their proliferation. HeLa cells were incubated with varying concentrations of PI (5-50 μM) for 72 h, the colour intensity was measured using an ELISA plate reader at 570 nm. Half maximal inhibitory concentration (IC₅₀) determined by dose-response curve was 25 μM (*P* < 0.01). The results of the apoptotic cell death indicate that PI is toxic to HeLa cells (Fig. 2). In this aspect it resembles Buckwheat antifungal peptide suppresses proliferation in a variety of tumor cells, including hepatoma, leukemia, and breast cancer cells.\(^\text{14}\)
Cytotoxicity of PI was checked against mammalian cells by measuring the lysis of human erythrocytes. PI had no hemolytic activity; melittin was used as a positive control showing strong hemolytic activity (Table 3). These results demonstrated that PI has a remarkable antimicrobial activity against various microbial cells but no hemolytic activity.

PI showed inhibitory activity against trypsin and chymotrypsin, a characteristic feature of Bowman-Birk inhibitors type (BBI) PIs. This inhibitory activity of PI was more pronounced against trypsin, when compared with chymotrypsin. Most of the BBIs were known to inhibit both trypsin and chymotrypsin due to the presence of two different reactive sites. However, with higher affinity towards trypsin compared with chymotrypsin. The stoichiometry of PI with trypsin was found to be 1:2, i.e., one molecule of PI will exhibit TI activity by binding with two molecules of trypsin (Fig. 3). On the other hand, PI showed no obvious stoichiometry with chymotrypsin, which is evident from the titration pattern of its inhibitory activity (Fig. 3). While the PIs isolated from *Apios americana* tubers showed a similar pattern of stoichiometry to that shown by PI with respect to trypsin, PIs isolated from *Peltophorum dubium* seeds and *A. americana* tubers showed a similar pattern of stoichiometry to that of chymotrypsin.

To study the importance of the disulfide bonds in PI, it was treated with the reducing agent, dithiothreitol (DTT) to visualize the fungal cell growth inhibition zone. The purified PI and reduced PI treated paper disk was tested on the spread fungal agar plate. As shown in Fig. 4, purified PI had strong antifungal activity, whereas reduced PI had no antifungal activity suggesting the presence of disulfide bonds in PIs functional integrity.

The synthesis of fungal cell wall at hyphal apex consists of a complex assembly sequence and it protects the organism against a hostile environment and relays signals for invasion and infection of a likely plant, animal or human host. Several classes of antimicrobial proteins involve inhibition of the synthesis of the fungal cell wall or disrupt cell wall structure and/or function; others perturb fungal membrane structure, resulting in fungal cell lysis. Examination by bright field microscopy showed inhibition of mycelial growth and sporulation. Morphologically, in PI treated fungus, a dramatic shrinkage of hyphal tips was observed (Figs 5 b, d, f, h, and j). Similar results were observed with Bavistin.

![Fig. 3—Titration curves of trypsin and chymotrypsin inhibition by PI [Values are mean ± SE of 3 assays]](image)

![Fig. 4— Antifungal activity of reduced PI [(a) purified PI + DTT (0.5 mM); (b) purified PI (100 µM); and (c) PI + DTT (1 mM)]](image)

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![Fig. 5—Differences in morphology of fungal hyphae (control treated with sterile Tris-HCl buffer; and test with CGPI). Shrinkage of hyphal tip (indicated by arrow) was observed in (a, b)-Candida albicans; (c, d)-Macor indicus; (e, f)-Aspergillus flavus; (g, h)-Penicillium notatum; (i, j)-Cryptococcus neoformans; and (k)-Candida albicans treated with bavistin, 40 x.](image)
at a concentration of 0.2% (W/V) (Fig. 5 k). In contrast, the hyphae growing under the influence of sterile Tris-HCl buffer did not show any obvious growth aberrations (Figs 5 a, c, e, g and i). Fungal growth was routinely checked microscopically to confirm the micro spectrophotometric data. Major morphological difference in the hyphae was apparent between fungi harvested from inhibited zone and those with control. PI produces the complete inhibition of K. pneumoniae and A. flavus spore germination at a concentration of 0.5 mg/ml while at 0.01 mg/ml, a reduction of hyphal growth is observed. S. aureus, B. subtilis, C. albicans and C. neoformans, were also inhibited but appears to be less sensitive than K. pneumoniae and A. flavus; at a concentration of 0.5 mg/ml only shortened hyphae could be observed (Figs 5). PIs can exert their activity by inhibiting the serine proteases involved in growth or differentiation of the microbial cells.

Discussion

PIs are ubiquitous in plants; generally act as storage proteins and wound-induced defense response of plants against herbivores and pathogens. Recently, protease inhibitors are reported as potent anticarcinogens in a wide variety of in vivo and in vitro systems. In addition, serine protease inhibitors can increase the level of cholecystokinin due to the inhibition of trypsin, they may be useful for reducing food intake in human. PIs for use in humans must be non-toxic and capable of inhibiting each of the major intestinal proteases, including pancreatic trypsin, α-chymotrypsin, and elastase. Several potentially non-toxic protease inhibitors, mostly of bacterial or plant origin, for example from barley seeds, cabbage leaves, and Streptomyces, have been purified and are commercially available for preventing protease-induced peri-anal dermatitis. Although the bactericidal mechanism of action of the PIs has not yet been elucidated in detail, the presented data confirm the in vitro antibacterial activity of PI against pathogenic bacteria. It has been proposed that the proteins with antibacterial action form a channel on cell membrane and the cell dies as a result of the out flowing of cellular contents, being this mechanism different from that of antibiotics. Cucurbit proteins, efficiently inhibit human fecal proteases, they could also be useful in the treatment of peri-anal dermatitis. The biological role of PIs may be considered antinutritional by decreasing the activity of proteases produced by the pathogens suggesting that the inhibitor might interfere in the development of fungi or bacteria by decreasing the endogenous protease activity.

The growth inhibition of fungi cannot be fully explained by trypsin inhibition alone. In fact a later antifungal role has been reported for trypsin inhibitors, due to the ability of these proteins to interfere with the chitin biosynthetic process during fungal cell wall development by inhibiting the proteolytic activation of the chitin synthase zymogen. The trypsin inhibitors belonging to the Bowman Birk family are usually accumulating only in developing seeds. During germination these inhibitors are rapidly released from the seeds. Thus, the role of PI may be associated with the protection of seeds, which lack an active defense system, and during early germination, when the tissues are particularly exposed to potential attack by soil borne pathogens. It is, therefore, possible that PIs acts synergistically with other defense proteins expressed in plants contributing to limiting invasion by pathogens.

Various pathogenic fungi that are protease deficient have been characterized as non-pathogenic, suggesting a function for the proteases secreted during the process of infection, colonization, and pathogenesis of susceptible plants. Several studies confirmed that protease secretion by the fungus is a determining factor for pathogenicity, and its inhibition significantly reduces the fungal infection. Protease inhibitors are widely distributed within the plant kingdom. Some inhibitors are constitutively expressed in seeds and storage organs while others are induced on wounding in plants. The protease inhibitors play an important role in the protection of plant tissues from pest and pathogen attack by virtue of an antinutritional interaction. Thus, the PI can act directly on the proteases produced by pathogenic fungi and reduce their pathogenicity by decreasing their germination.

Molecular mechanism of PI related to antiproliferative activity in HeLa cells remains unclear but, probably may transinactivate epidermal growth factor receptor (EGFR) tyrosine kinase activity with marked reduction in phosphoryrosyl level of cellular proteins or may dampened the phosphorylation of focal adhesion kinase (FAK) and the secreted matrix metalloproteinase (MMP) that may lead to the suppression of invasive potential and cell migration in vitro.
The antiproliferative nature of PI on HeLa cell lines and no hemolytic nature may be because of their potent activity against a specific cell line, but they have not shown a wider range of action. Similar antitumor and antiproliferative actions are well documented in leguminous trypsin inhibitors. Some trypsin inhibitors manifest antifungal activity, e.g. those from the broad bean, lima bean, and the yellow soybean. However, trypsin inhibitor from Japanese large black soybean is devoid of antifungal activity but have HIV-1 reverse transcriptase inhibitory potency of this trypsin inhibitor than that reported for Kunitz-type trypsin inhibitor from soybean.

Amino acid composition, amphipathicity, charge and size of TPi allow them to attach to and insert into membrane bilayers to form pores by ‘barrel-stave’, ‘carpet’ or ‘toroidal-pore’ mechanisms. Although these models are helpful for defining mechanisms of antimicrobial peptide activity, their relevance to how peptides damage and kill microorganisms still need to be clarified. Brogden showed that transmembrane pore formation is not the only mechanism of microbial killing but also the translocated peptides can alter cytoplasmic membrane septum formation; inhibit cell-wall synthesis, nucleic-acid synthesis and protein synthesis or enzymatic activity.

The purification of a protease inhibitor by gel, ion exchange and affinity chromatography is described. The apparent molecular mass is 14.3 kDa. This PI has a potent antimicrobial activity as demonstrated by the growth inhibition (in vitro) of important pathogenic bacteria and fungi. These results indicate that future finding of PI applications obtained from medicinal plants can be of great importance for clinical microbiology and possible therapeutic applications.

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References