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Protease inhibitors of *Acacia leucophloea* gum extracts

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Abstract: Plant protease inhibitors (PIs) are very important for their defensive function against plant pathogens and predators. In present work trypsin and chymotrypsin inhibitors (PIs) from gum of one tree about seven species, *Azadirachta indica, Acacia leucophloea, Acacia nilotica, Terminalia spp., Anogeissus latifolia, Mangifera indica* and *Moringa oleofera* are studied. Protease inhibitory activity in gum extract was detected by dot blot assay. PI bands were resolved on electrophoresis gel and detected by Gel X-ray film contact print technique (GXCP). PIs from gum extracts were purified by gel filtration (Sephadex G-75). Among all gum extracts studied, the gum extract from *Acacia leucophloea* showed highest number of trypsin and chymotrypsin inhibitors. One PI from the *Acacia leucophloea* of 97.00kDa was purified and characterized. Purified PI was not destroyed by heat treatments up to 70°C, but lost its activity when incubated at 80°C, showing moderate thermo stability.

Key words: *Acacia leucophloea*; GXCP; plant gum; protease inhibitors; BApNA; SAAPFpNA

Introduction

Protease inhibitors (PIs) are of very common occurrence. They have been isolated and Characterized from a large number of organisms, including plants, animals and microorganisms[1-6]. Plant PIs (PPIs) are generally small proteins that have mainly been described as occurring in storage tissues, such as tubers and seeds, but they have also been found in the aerial parts of plants[7]. They are natural, defense-related proteins often present in seeds and induced in certain plant tissues by herbivore or wounding[8]. PIs are induced in different plant species in response to attack by bacteria[9], viruses[10, 11] upon treatment with fungal elicitors[12,] and wounding[13, 9, 14].

In plants, these PIs act as anti-metabolic proteins, which interfere with the digestive process of the insect. The defensive capacities of plant PIs rely on inhibition of proteases present in insect guts or secreted by microorganisms, causing a reduction in the availability of amino acids necessary for their growth and development[15]. Some 11 families of inhibitors have been described, based on their amino acid sequence relationships and inhibitory properties[16]. Majority of proteinase inhibitors studied in plants kingdom originate from three main families namely Leguminosae, Solanaceae and Gramineae[17].

The term gum is commonly applied; it is water soluble and non-starch mucilaginous polysaccharides of commercial value. The gums are known to contain varying amounts of proteinaceous and non-proteinaceous materials[18]. Gum is a complex of different substances, mostly polysaccharides of diverse structure; the composition of gum polysaccharides varies from species to species and from cultivar to cultivar[19-21]. Gum ducts are formed naturally in healthy fruit trees. The formation of gum in plant is induced by environmental stresses such as infection, insect attack, flooding and mechanical or chemical injury[22]. All of the factors which stimulate gum exudation also promote ethylene production in plant tissues[23].

In this paper, we report the presence of trypsin and chymotrypsin inhibitors activity in *Acacia leucophloea* plant gum. Also in paper a novel protease inhibitor was purified and characterized. As per our knowledge this is the first report on the presence of PIs in gum.

Material and Methods

Materials

Synthetic substrate N-Alfa -Benzoyl -DL-Arginine P-nitroanilide (BApNA), N-Succinyl-Ala-Ala-Pro-Phe r-nitroanilide (SAAPFpNA), protein molecular weight markers were purchased from Merck chemicals, Germany. Bovine serum albumin (BSA) was purchased from Sigma, USA. Trypsin, Chymotrypsin and Casein were purchased from SRL, Mumbai, India. X-ray films were purchased from Agfa, India.

Collection of the plant gum

Gum from *Azadirechta Indica, Acacia leucophloea, Acacia nilotica, Terminalia spp., Anogeissus latifolia, Mangifera indica* and *Moringa oleofera* was collected from the campus of Dr. B. A. M. University Aurangabad (MS), India.

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Protease inhibitors (PIs) extraction: Plant gum powder (1gm) was homogenized in 15ml of the Millie-Q water (1:15w/v) and allowed to stand for 24 hr at room temperature. These suspensions were then filtered through sintered glass funnel having pore size (40-90 and 5-15microns). The filtrate was sterilized by passing through 0.2 µm filters. The clean filtrate was acetone extracted using acetone, the 5ml of filtrate and 25ml chilled acetone (1:5v/v). The precipitate was collected and dried at room temperature. Finally dried precipitate was suspended in the 1% Polyvinylpyrrolidone (PVP) at a ratio (1:3w/v) and stored for the 24hr at 4ºC. The suspension was then centrifuged at 10,000 rpm for 10 min at 6ºC. The upper clear supernatant containing water soluble protein was used for the detection of the trypsin and chymotrypsin inhibitors. Gum proteins in crude extract were estimated using Lowry's method[24].

Detection of trypsin and chymotrypsin inhibitors activity by dot blot assay: Gel-X-ray film contact print method developed in our laboratory[25] was slightly modified for detection of proteinase inhibitors activity in plant gum extract. Gum extract was used as in source of PI the dot blot assay, the three varying volumes of enzymes (1.0mg/ml trypsin enzyme) and gum extract were mixed in the following ratio, 3:1 v/v (15µl enzymes: 5µl inhibitors), 1:1v/v (10µl enzymes: 10µl inhibitors) and 1:3v/v (5µl enzymes: 15µl inhibitors) and incubated for 30 min., then mixture was used for the spotting on undeveloped X-ray film. Appropriate controls which included enzymes alone and extract alone spots were loaded on X-ray film. The film with spots was again incubated for 7-8min., the film was washed with either tap or warm water and the hydrolysis of the gelatine was visually monitored.

Detection of proteinase inhibitor bands in *Acacia leucophloea* by GXCP: TI of gum extract of *Acacia leucophloea* plant was electrophoresed on a vertical slab gel using a discontinuous buffer system[26]. After electrophoresis the gel was processed for activity staining of PI by the gel X-ray film-contact print method[25]. The gel was incubated in 0.1M Tris-HCl buffer, pH 7.8, for 4-5min. followed by incubation in the trypsin (1.0mg/ml) for 7-8min at 37°C on a shaking water bath. The gel was then washed with the same buffer and placed on a piece of undeveloped X-ray film. The extent of gelatine hydrolysis was visually monitored. Depending on the extent of gelatine hydrolysis, the film was washed with either tap or warm water (45°C). To detect bands with less PI activity the same gel was overlaid three or more times with different pieces of X-ray film for 8, 12, and 20min, respectively. PI bands appeared as unhydrolyzed gelatine against the background of hydrolyzed gelatine. Same procedure was used for visualization of chymotrypsin inhibitors.

Protease inhibitors assay: Proteinase inhibitory activity was measured using a caseinolytic assay[27, 28]. As well as using synthetic substrate BApNA and SAAPFpNA as described[29]. PIs activity was determined by mixing 20µg of trypsin. Equivalent amount of the enzyme used in the study with sufficient amount of the *Acacia leucophoea* plant gum extract containing PIs. The incubation of enzyme with PIs was conducted at 37°C for 10min. The residual proteinase activity was measured using the assay. One unit of proteinase activity was defined as the amount of enzyme that caused an increase of one optical density at 410nm in the TCA-soluble products of substrate hydrolysis per minute [27, 28]. One PI unit was defined as the amount of inhibitor that inhibited one unit of proteinase activity.

Molecular weight determination of the *Acacia Leucophoea* plant gum extract inhibitors: Molecular weight of the PIs in the extract and the purified one was determined using Laemmli buffer system[30]. In 12% SDS-PAGE, *Acacia leucophoea* gum extract was mixed with sample loading buffer containing tris-HCl buffer 0.12M pH6.8, SDS 4%, 10% glycerol and bromophenol blue 0.02% and incubated in boiling water bath for one minute and loaded in well. Standard molecular weight markers were loaded in one well. After the electrophoresis the gel was washed for 10min with 2.5% triton X-100 in distilled water. After the visualization of proteins bands by staining gel in coomassie brilliant blue R-250, the proteins corresponding with protein activity bands were detected.

Purification of *Acacia leucophloea* gum extract by sephadex G-75 column (Gel filtration column): Protein purification was carried out by gel filtration chromatography using sephadex G-75. A 25 x 1 cm column was used for gel filtration. The column was packed with sephadex G-75, preparative matrix. The matrix was initially washed with 2.5% alcohol and degassed for 60 min under suction. It was then prewashed with distilled water to remove the traces of alcohol and equilibrated with 0.1M tris-HCl buffer, pH 7.8, then it was loaded onto the column so as to fill 2/3rd of its length. The column was then washed with 5 bed volume of 0.1M tris-HCl buffer pH 7.8 before loading with sample materials. 2.0ml water soluble extract of the ammonium sulphate fraction loaded and eluted using 0.1M tris-HCl buffer, pH 7.8. 2.0ml fractions were collected using a fraction collector. The fractions were analyzed at 280nm using PC-based spectrophotometer (JASCO 500 series) and the graph was plotted. The fractions containing

proteins were identified and subjected to dot blot assay, electrophoresis and assay in solution.

Results and Discussion

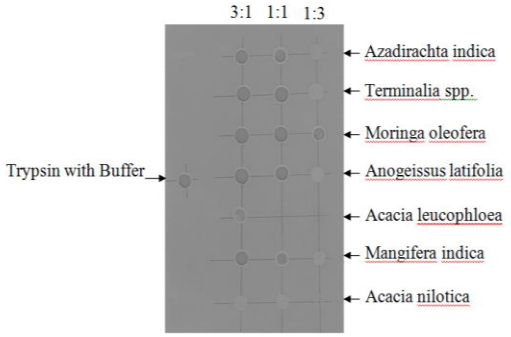
Dot blot assay of trypsin and chymotrypsin inhibitors: Different ratios of enzyme and gum extracts (PIs) gave different patterns of gelatine hydrolysis on the X-ray film depending on the potency of PIs which can be observed visually. Only *Acacia leucophloea* gum PIs strongly inhibited both trypsin and chymotrypsin activity (Figure.1A & 1B). Gum extracts of all other plant gums *Azadirechta Indica, Acacia nilotica, Terminalia spp., Anogeissus latifolia, Mangifera indica* and *Moringa oleofera* species did not show PIs activity, as seen by the clearance of gelatine on X- ray film. Also confirmed *Acacia leucophloea* gum PIs strongly inhibited both the trypsin and chymotrypsin activity by enzymatic assay (Table.1).

Table. 1: Trypsin and chymotrypsin inhibitors Potentials from crude extract of plants gum.

Sr.no	Name of Plant	Total protein	Total Trypsin	Specific activity	Total Chymotrypsin	Specific activity
		used in mg/ml	Inhibitor units	U/mg	Inhibitor units	U/mg
1	*Acacia leucophloea*	7.5±0.14	5.51±0.09	6.2±0.01	5.54±0.06	6.8±0.07
2	*Azadirechta indica*	7.5±0.19	1.83±0.12	0.89±0.21	1.08±0.31	1.75±0.18
3	*Terminalia spp.*	7.5±0.12	2.14±0.24	0.92±0.31	1.02±0.18	0.31±0.21
4	*Moringa oleofera*	7.5±0.2	3.18±0.31	2.36±0.09	3.7±0.21	2.09±0.4
5	*Anogeissus latifolia*	7.5±0.15	2.57±0.21	2.19±0.12	5.09±0.09	2.5±0.15
6	*Acacia nilotica*	7.5±0.17	1.53±0.19	1.35±0.14	1.13±0.1	1.41±0.21
7	*Mangifera indica*	7.5± 0.21	3.21±0.11	1.92±0.08	2.4±0.03	1.81±0.25

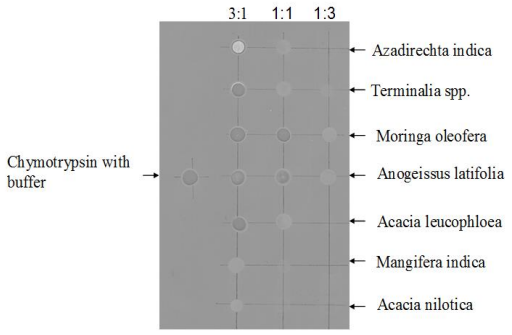
Each value is an average of three replicates ± SEM.

Figure 1A: Dot blot assay of trypsin inhibitors of the different gum extracts (PIs).



Different ratio of gum extracts and trypsin solution were incubated and loaded on X-ray film as described in material and methods.

Figure 1B: Dot blot assay of chymotrypsin inhibitors of the different gum extracts (PIs).



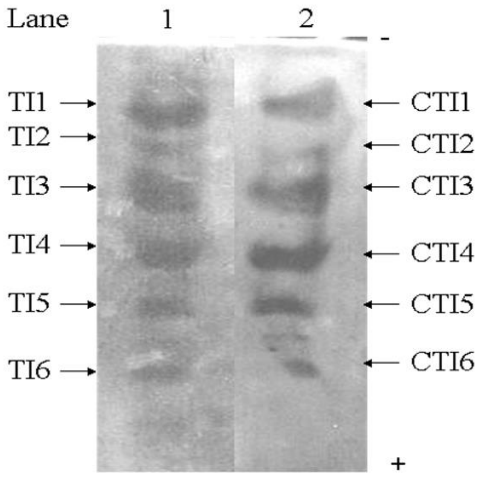
Different ratio of gum extracts and chymotrypsin solution were incubated and loaded on X-ray film as described in material and methods.

Detection of proteinase inhibitors in crude *Acacia leucophloea* gum extracts: The *Acacia leucophloea* plant gum extracts contain at least six major trypsin and six chymotrypsin inhibitors (Figure.2). All higher inhibitory activity bands appear much thicker and broader. They are distinct, band pattern is stable. It is evident that the inhibitory activity is distributed among the different isoforms in the *Acacia leucophloea* gum extracts. TI and CTI isoforms are exactly identical. This indicates that *Acacia leucophloea* plant gum extract PIs may be double-headed or single headed inhibitor might have been interacting with both trypsin and chymotrypsin. As TI and CTI pattern is similar and identical, we used a broder term PI, in steads of TI and CTI.

Earlier studies have reported double headed PIs. Proteinase inhibitors capable of acting on trypsin and chymotrypsin and possessing distinct binding sites for the two enzymes have been reported[31]. They are capable of simultaneously inhibiting two molecules of enzymes per molecules of inhibitor[32]. Qi et al., [33] have reported the presence of double headed Bowman- Birk inhibitors (BBIs) in dicots with 8kDa molecular weight having two reactive sites. These inhibitors interact simultaneously and independently with two (not necessarily identical) molecules of protease. The first reactive site of most dicot inhibitors is more specific and inhibits, trypsin, whereas the second one inhibits trypsin, chymotryspin and elastase[33]. Tamhane et al., [34]have reported diverse forms of Pin-II family proteinase inhibitors from *Capsicum annuum* (CanPIs) having both trypsin and chymotrypsin sites. Similarly, Haq and Khan[35] characterized PIs from pigeonpea and reported

the kinetic properties of pigeonpea PIs. Single headed inhibitors can interact with both trypsin and chymotrypsins have been reported in potato and in pepper[36]. There are several reported methods of visualization of protease inhibitors[37]. The method we have described here requires neither ampholytes nor synthetic substrates, so it is cheaper and more convenient. One unique advantage of this method is to detect inhibitors of low activity or low abundance; the gel can be exposed to X- ray film several times at increasing time intervals. Initially only strong TI activity bands appear on the X- ray film while for the same gel exposed to another film for longer time the weaker activity bands appear.

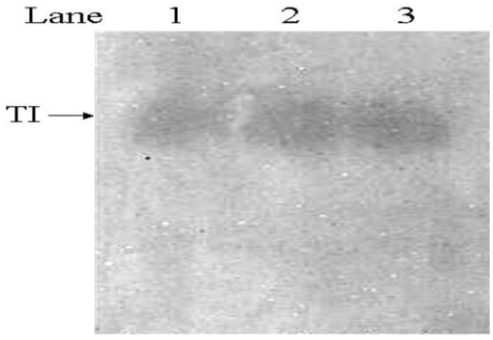
Figure 2: Gel X-ray film contact print of protease inhibitors (PIs) in *Acacia leucophloea*.



Electrophoresis was carried out on 10% SDS PAGE. Protease inhibitor (PI) activity bands were visualized using gel X-ray film contact print technique as described in Materials and Methods. Approx. 25µl of protein was loaded in each lane. Lane 1- Trypsin inhibitors and lane 2- Chymotrypsin inhibitors.

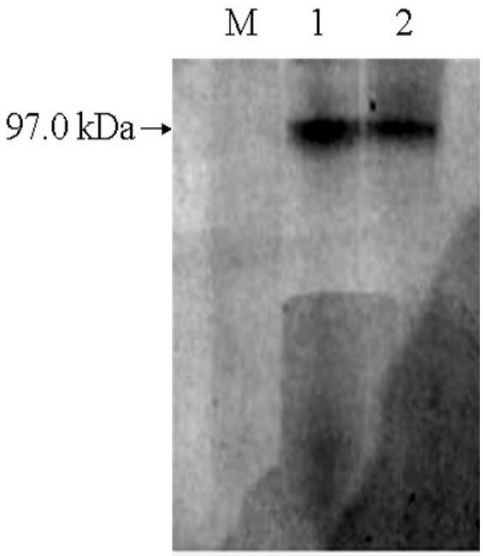
Purification and characterization of *Acacia leucophloea* gum PIs: Yield of purified *Acacia leucophloea* fractions were trypsin and chymotrypsin specific activity and purification fold (Table 2 and 3). The eluted *Acacia leucophloea* protein fractions were analyzed for PIs activity on X-ray film as explained in material and method. The major peaks were containing the inhibitory activity. One step purification method used here was satisfactory since the purified protein exhibited a single band in SDS-PAGE analysis. It was a single high activity band detected on X-ray film (Figure.3). The eluted fraction exhibiting highest PI activity was loaded on electrophoresis gel and after electrophoresis; a single PI band detected on GXCP confirmed the separation of single PI. SDS PAGE analysis followed by staining gel with CBB of same fraction revealed a single band which confirmed purification. Plant PIs are quite stable, molecules and are often resistant to heat[16, 32, 38, 39]. An effect of heat on *Acacia leucophloea* purified inhibitor was studied by incubating at various temperatures ranging from 20-80°C. Trypsin inhibitor activity was lost at 80°C. Heat treated PIs of *Acacia leucophloea* gum extracts were not destroyed up to 70°C (Figure.5). Trypsin inhibitor activity was detected over the range 30-70°C temperatures. Heat treatment at 100°C for 5 min resulted in the complete loss of enzyme activity in purified fraction of Neem gum (*Azadirachta indica*) [37][40].

Figure 3: Gel X-ray film contact print of purified trypsin inhibitors (TI) from *Acacia leucophloea* gum extract.



Electrophoresis was carried out on 10% SDS PAGE. Trypsin inhibitor (TI) activity band was visualized using gel X-ray film contact print technique as described in Materials and Methods. Lane1-5µl. Lane 2-10µl and Lane 3-15 µl of purified protein was loaded.

Figure 4: Molecular weight determination of purified TI from gum extract without addition of a reducing agent.



Purified extract was separated on 12% SDS PAGE as described in Material and Methods. Lane M-molecular weight markers, lane 1and 2-10µl and 15µl of purified *Acacia leucophloea* gum extracts, respectively.

In general, protease inhibitors from legumes are quite stable up to 80°C but lose activity above this temperature[41-43]. There was appreciable loss in TI activity only when the fraction was exposed to highly acidic conditions (data not shown). Gum

samples, stored at room temperature for 6 months did not show any appreciable loss of TI activity. The molecular weight markers (Range between the 97.4kDa to 14.3kDa) were used to determine the molecular weight. The molecular weight of *Acacia leucophloea* purified inhibitor determined on SDS-PAGE, in the absence of reducing agent. Approximate molecular weight of purified inhibitor was found to be 97kDa (Figure.4).

Besides disulfide bridges, many non-covalent interactions also contribute to the stability of inhibitors. Their molecular weights range from 4kDa-60kDa. Generally speaking, plants PIs vary from 4 to 85kDa, with the majority in the range of 8kDa-20kDa[44]. The cereal trypsin/-amylase inhibitors consist of a single polypeptide chain containing five disulfide bonds with a molecular mass of about 13kDa[45].Kunitz-type protease inhibitors having different molecular masses than that of the chickpea trypsin inhibitor have been reported in soybean (19kDa), mustard seeds (20kDa) and *Cajanus cajan* (14kDa)[46]. High Molecular Weight kininogens (HMW) with a molecular mass of 120kDa and Low Molecular Weight kininogens (LMW) with molecular mass ranging between 60 and 80kDa are known. A third type T kininogens with molecular mass of 68kDa has also been reported. These proteins contain tandem domains that result from gene duplication of the family-2 cystatins. These proteins are also secreted and play key roles in blood coagulation[47].

Table 2: Purification of trypsin inhibitor from *Acacia leucophloea* plant gum.

Purification steps	Total Protein (mg)	Inhibitor Units (TI)	Specific activity	Purification fold
Crude extract	2300±0.25	12000±0.12	20.3±0.06	1±0.1
Ammonium sulphate fraction	890±0.28	7000±0.12	67.4±0.24	3.32±0.32
Sephadex G-75	60±0.18	2500±0.35	412±0.11	20.29±0.13

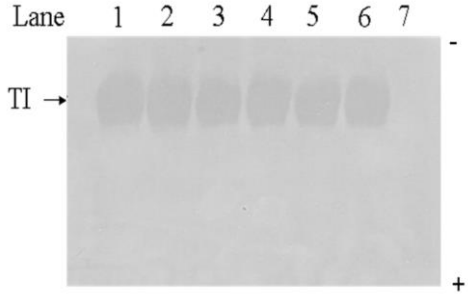
Each value is an average of three replicates ± SEM.

Table 3: Purification of chymotrypsin inhibitor from *Acacia leucophloea* plant gum.

Purification steps	Total Protein (mg)	Inhibitor Units (CTI)	Specific activity	Purification fold
Crude extract	2300±0.31	10000±0.12	16.12±0.21	1±0.18
Ammonium sulphate fraction	890±0.35	63000±0.18	81.2±0.24	5.037±0.19
Sephadex G-75	60±0.27	21000±0.24	390±0.18	24.19±0.1

Each value is an average of three replicates ± SEM.

Figure 5: Effect of the temperature on purified *Acacia leucophloea* plant gum extract



PIs. Lane 1-Purified PI, and Lane 2- Purified PI incubated at 30ºC. Lane 3- Purified PI incubated at 40ºC. Lane 4- Purified PI incubated at 50ºC. Lane 5- Purified PI incubated at 60ºC. Lane 6- Purified PI incubated at 70ºC and Lane 7-Purified PI incubated at 80ºC, before loading in the gel.

Conclusion

In the present research report, a novel protease inhibitor was purified and characterized. *Acacia leucophloea* plant gum is rich source of serine protease inhibitors. These protease inhibitors are moderately heat tolearent with broad range of the pH (4-10). We conclude that these inhibitors are biologically active protein molecules. These investigations establish for the first time the presence of protease inhibitor in *Acacia leucophloea* gum extract. Also this research paper investigates optimum extraction steps which result in large amount of extracted proteins. This method could also be used in extraction of other proteins from any other different sources. The observed trypsin inhibitor activity of this plant gum may be useful for therapeutics. Detection of amylase inhibitors, elastase inhibitors and microbial activity however further work is needed in this area.

References

1. Valueva TA, Mosolov VV, Role of Inhibitors of Proteolytic Enzymes in Plant Defense against Phytopathogenic Microorganisms. Biochemistry, 2004, 69 (11), 1305-1309.

2. Christell er JT, Evolutionary mechanisms acting on proteinase inhibitor variability. FEBS Journal, 2005, 272, 5710-5722.

3. Haq SK, Atif SM, Khan RH, Protein proteinase inhibitor genes in combat against insects, pests, and pathogens: natural and engineered phytoprotection. Arch Biochem Biophys, 2004, 43(1), 145-159.

4. Supuran CT, Scozzafava A, Clare BW, Bacterial protease inhibitors, Med Res Rev, 2002, 22(4), 329-372.

5. Mosolov VV, Valueva TA, Proteinase inhibitors and their function in plants: a review, Prikl Biokhim Mikrobiol, 2005, 41(3), 261-282.

6. Mosolov VV, Grigoreva LI, Valueva TA, Plant proteinase inhibitors as polyfunctional proteins (a review), Prikl Biokhem Mikrobiol, 2001, 37(6), 643-650.

7. De Leo F, Volpicella M, Licciulli F, Liuni S, Gallerani R, Ceci LR, PLANT-PIs: a database for plant protease inhibitors and their genes, Nucleic, Acids Res, 2002, 30 (1), 347-348.

8. Padul MV, Tak RD, Kachole MS, Protease inhibitor (PI) mediated defense in leaves and flowers of pigeonpea (Protease inhibitor mediated defense in pigeonpea) Plant, Physiology and Biochemistry, 2012, 52, 77-82.

9. Pautot V, Holzer FM, Walling LL, Differential expression of tomato proteinase inhibitor I and II genes during bacterial pathogen invasion and wounding, Mol Plant Microbe Interact, 1991, 4, 284-292.

10. Geoffroy P, Legrand M, Fritig B, Isolation and characterization of a proteinaceous inhibitor of microbial proteinases induced during the hypersensitive reaction of tobacco to tobacco Mosaic Virus, Mol Plant Microbe Interact, 1990, 3, 327-333.

11. Linthorst HJM, Brederode FT, Vander Does C, Bol JF, Tobacco proteinase inhibitor I genes are locally, but not systemically induced by stress, Plant Mol Biol, 1993, 21, 285-992.

12. Rickauer M, Fournier J, Esquerre-Tugaye MT, Induction of proteinase inhibitors in tobacco cell suspension culture by elicitors of *Phytophthora parasitica* var. *nicotaniae*. Plant Physiol, 1989, 90, 1065-1070.

13. De Bowles JB, Defense related proteins in higher plants, Annu Rev Biochem, 1990, 9, 873-907.

14. Eckelkamp C, Ehmann B, Shopfer P, Wound-induced systemic accumulation of a transcript coding for a Bowman- Birk trypsin inhibitor-related protein in maize (*Zea mays* L.) seedlings, FEBS Lett, 1993, 323, 73-76.

15. Leo F, Volpicolla M, Liociulli F, Liuni S, Gallerani R, Ced LR, Plant PIs: a database for plant protease inhibitors and their genes, Nucleic acids, 2002, 30(1), 347-348.

16. Richardson M, Seed storage proteins. The enzyme inhibitors, Plant Biochem, 1991, 5, 259-305.

17. Richardson MJ, Seed storage proteins; the enzyme inhibitors in: Richardson, M.J.ed method in plant biochemistry, New York, Academic. press, 1999. pp. 259-305.

18. Anderson DMW, Hendrie A, Munro AC, The amino acid and amino sugar composition of some plant gum, Photochemistry, 1972, 11, 733.

19. Saniewski M, Veda J, Horbowicz M, Miyamoto K, Puchalskit J, Gum in apricot (prunus armeniaca L), shoots induced by methyl jasmonate, Acta Agro Bot, 2002, 54, 27-34.

20. Saniewski M, Miyamoto K, Veda J, Carbohydrate composition of gum, anthocyanin accumulation and leaf abscission in peach shoot induced by methyl jasmonate and for ethylene. Zesz. Prob. Post. Navk. Roln., 2004a, 496, 619-626.

21. Saniewski M, Miyamoto K, Veda J, Gum induction by methyl jasmonate in fruits, stems and petioles of prenus domestica L. Acta Hort, 2004b, 636, 151-158.

22. Morrison JC, Polito VS, Gum duct development in almond fruit, *Prunus dulcis* (Mill.). D.A. Webb. Bot. Gaz. 1985, 146, 15-25.

23. Boothby D, Gummosis of stone fruit trees and their fruits. Journal of Sci Food Agric, 1983, 34, 1-7.

24. Lowry OH, Rosebrough NJ, Farr A L, Randall RJ, Protein measurement with the folin phenol reagent, Journal of Biol Chem, 1951, 193, 265-275.

25. Pichare MM, Kachole MS, Detection of electrophoretically separated proteinase inhibitors using X-ray film, Biochem Biophys Method, 1994, 28, 215-224.

26. Davis BJ, Disc electrophoresis ii, methods and application to human serum, Ann. N. Y. Acad. Sci, 1964, 121, 404-427.

27. Giri AP, Harsulkar AM, Deshpande VV, Sainani MN, Gupta VS, Ranjekar PK. (1998) Chickpea defensive proteinase inhibitors can be inactivated by podborer gut proteinases, Plant Physiology, 1998, 116, 393-401.

28. Harsulkar AM, Giri AP, Patankar AG, Gupta VS, Sainani MN, Ranjekar PK, Deshpande VV, Successive use of non-host plant proteinase inhibitors required for effective inhibition of gut proteinases and larval growth of *Helicoverpa armigera*, Plant Physiology, 1999, 121, 497–506.

29. Erlanger BF, Kokowksy N, Cohen withers preparation and properties of two new chromogenic substrates of trypsin, Arch Biochem Biophys, 1961, 95, 271-281.

30. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature, 1970, *227*, 680-685.

31. Shivaraj B, Pattabiraman TN, Characterization of an unusual a-amylase/trypsin inhibitor from ragi (Eleusine coracana Geartn.), Journal of Biochem, 1981, 193, 29-36.

32. Ryan CA, Proteinase inhibitors. In:Marcus A. the Biochemistry of plant. New York. Academic, Press, 1981, 6, 351-370.

33. Qi RF, Song ZW, Chi CW, Structural features and molecular evolution of Bowman-birk protease inhibitors and theirbpotential application, Acta Biochimica Biophysica Sinica, 2005, 37(5), 283-292.

34. Tamhane VA, Chougule NP, Giri AP, Dixit AR, Sainani MN, Gupta VS, In vitro and vivo effects of *Capsicum annum* proteinase inhibitors on *Helicoverpa armigera* gut proteinases, Biochim Biophys Acta, 2005, 1722, 155-167.

35. Haq SK,Khan RH, Characterization of a proteinase inhibitor from *Cajanus cajan* (L.), Journal of protein chem, 2003, 22(6), 541-554.

36. Moura DS,Ryan CA, Wound- inducible proteinase inhibitors in pepper, differential regulation upon wounding, systemin, and methyl jasmonate, Plant Physiol, 2001, 126, 289-298.

37. Filho JX, De Azevedo Moreira R, Visualization of proteinase inhibitors in SDS- polyacrylamide gels, Anal Biochem, 1978, 84, 296-303.

38. Sohonie K, Bhandarkar AP, Trypsin inhibitors in Indian foodstuffs; Part I- Inhibitors in vegetables, J Sci Ind Res, 1954, 500 (13B).

39. Sohonie K, Bhandarkar AP, Trypsin inhibitors in Indian foodstuffs; Part II- Inhibitors in pulses, J Sci Ind Res, 1955, 100 (14C).

40. Nayak BR, Rao NM, Pattsbiraman TN, Studies on plant gums. Proteases in neem (*Azadirachta indica*) gum, Journal of Biosci, 1979, 4 (1), 393-400.

41. Hajela N, Sharma AH, Sharma S, Rao DN, Hajela K, Studies on a double headed protease inhibitor from *Phaseolus mungo.* J Plant Biochem Biotech, 1999, 8, 57-60

42. Maggo S, Malhotra SP, Dhawan K, Singh R, Purification and characterization of protease inhibitor from rice bean (*Vigna umbellate*), J Plant Biochem Biotech, 1999, 8, 61-64.

43. Gupta P, Dhawan K, Malhotra SP, Singh R, Purification and characterization of trypsin inhibitor from seeds of faba bean (*Vicia faba* L.), Acta Physiol Plant, 2000, 22, 433-438.

44. Hung CH, Huang CC, Tsai WS, Wang HL, Chen YL, Purification and Characterization of a Trypsin Inhibitor from *Brassica campestris* Seeds, J Yuanpei Univ Sci Tech, 2003, 10, 13-22.

45. Christeller J, Liang W, Plant serine protease inhibitors, Protein Peptide Letters, 2005, 12, 439-447.

46. Mandal S, Kundu P, Roy B, Manda RK, Precursor of the inactive 2S seed storageprotein from the Indian mustard *Brassica juncea* is a novel trypsin inhibitor. Characterization, post-translational processing studies, and transgenic expression to develop insect-resistant plants, J Biol Chem, 2002, 277, 37161-37168.

47. Otto HH, Schirmeister T, Cysteine Proteases and Their Inhibitors, Chem Rev, 1997, 97, 133-171

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