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Research Article

PROTEASE INHIBITORS FROM *COCCINIA GRANDIS* (L.) VOIGT. LEAVES: PURIFICATION, CHARACTERIZATION AND KINETIC PROPERTIES

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ABSTRACT

Cucurbits are rich source of protease inhibitors. A protein of 14.3 kDa designated as *Coccinia grandis* protease inhibitors (CGPI) was isolated from leaves of *Coccinia grandis* and purified to homogeneity by ammonium sulfate precipitation, Sephadex G-75 column, DEAE Sepharose column and Trypsin-Sepharose affinity chromatography. The purity was checked by reversed phase chromatography. The molecular mass estimated by size exclusion chromatography was agreed well with the SDS-PAGE results. Both Native-PAGE as well as isoelectric focusing showed four isoinhibitors (pI values of 4.5, 5.0, 5.4, and 6.0). Inhibitory activity of CGPI remained unchanged over a broad range of temperatures (0 – 80 ºC) and pH amplitude (2–12). CGPI exhibited a non-competitive-type inhibitory activity against both bovine pancreatic trypsin (*K*i of 322.5 nM) and chymotrypsin (*K*i of 11.6 µM). The protein inhibited trypsin in the stoichiometric molar ratio of 1:2, but lacked similar stoichiometry against chymotrypsin. The temperature-induced conformational changes in secondary structure are reversed when CGPI was cooled from 90 to 25ºC. Further, upon reduction with dithiothreitol, CGPI lost both its inhibitory activity as well as secondary structural conformation. Lysine residue(s) present in the reactive site of CGPI are essential for the action of inhibiting the trypsin activity.

Keywords: *Coccinia grandis*, Kinetic studies, Protease inhibitors and Purification.

INTRODUCTION

Protease inhibitors are widely distributed in plants and are the most studied class of inhibitors. The molecular mass of these inhibitors can vary from 4 to 85 kDa, with majority in the range of 8-20 kDa[1]. In higher plants, several gene families of these protease inhibitors have been characterized. The plant serine protease inhibitors, particularly trypsin inhibitors, have been extensively studied and have been isolated from different parts of plants ranging from leaves to seeds. In biological systems, proteases are inactivated either by proteolytic degradation or by interaction with inhibitors that act as pseudo-substrates displaying variable degrees of affinity to the enzyme catalytic sites. These inhibitors have been described as endogenous regulators of proteolytic activity and as storage proteins. Interest in understanding the physiological roles of protease inhibitors has increased due to their importance in regulating diverse processes that involve proteases, such as intracellular protein breakdown, transcription, cell cycle, cell invasion and apoptosis[3]. Although serine protease inhibitors participate in reactions controlling proteinase activities involved in different physiological processes, their functions in the organism where they are found are not yet fully understood. However, plant protease inhibitors have been described as protective agents against pest attack[4]. Moreover, the serine protease inhibitor complex has been studied as a model of protein–protein recognition[5]. Until now serine proteases are extensively used in the food and medicine industry. However, their activities are affected by oxidation or metal ions, and by reducing or chelating agents. In contrast, various serine proteases including plant proteases have no requirement for any co-factors. Hence, it is highly desirable to identify and characterize novel protease inhibitors for their multipurpose values from plant resources. *Coccinia grandis* (L.) Voigt. belonging to Cucurbitaceae., a climbing perennial herb with ovoid fruits is used as vegetable when green, and eaten fresh when ripe. This paper describes the purification, characterization and inhibitory properties of protease inhibitor from the leaves of *C. grandis* (CGPI).

MATERIALS AND METHODS

Plant material

Fresh leaves from *Coccinia grandis* were collected from the department garden, University College, Trivandrum, Kerala and stored at -20°C.

Isolation and purification of CGPI

All the purification steps were carried out at 4°C as described in Macedo et al., [6]. A crude inhibitor preparation was obtained by extraction of fresh leaves from *C. grandis* with 100 mM sodium phosphate buffer (pH 7.6) (1:10, w/v) for 2 h with subsequent centrifugation at 7500g for 30minutes 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, 000g for 20 min, redissolved in 10 mM sodium phosphate buffer (pH 7.6) and dialyzed against distilled water for 24 h and lyophilized. Lyophilized protease inhibitor 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 450 mM NaCl showing highest protease inhibitor activity was applied to a Trypsin-Sepharose affinity column equilibrated with 100 mM phosphate buffer (pH 7.6) containing 100 mM NaCl. The adsorbed CGPI was eluted with 100 mM HCl and the resulted CGPI fractions were rechromatographed by reversed phase HPLC (RP-HPLC) equipped Symmetry C18 column. In the HPLC protocol, after the sample has been separated by Trypsin-Sepharose affinity column chromatography, the CGPI extract is loaded onto the HPLC column. Although for protein separation a 300Å pore column is sometimes recommended, the frequent experimentation shows that 100Å pore columns are also effective for CGPI purification. In the protocols used in our laboratory, purifications were performed by the use of a 300 Å C18 Symmetry column. The mobile phase consisted of (i) acetonitrile and (ii) HPLC-grade water containing 0.1% trifluoroacetic acid (TFA). The sample was loaded on the C18 column and separated by 80% acetonitrile over 60 min at a flow rate of 1.0 ml/min with 100% solvent A (0.1% trifluoroacetic acid (TFA) in water) for 10 minutes and a linear gradient (0–100%) of solvent B (0.08% TFA in 80% acetonitrile) over 60 minutes. Proteins were detected by monitoring the absorbance at 280 nm. Apparent molecular mass was obtained by Sephadex G-75 gel filtration column (100 mM phosphate buffer, pH 7.6) calibrated with proteins of known molecular mass.

Protein quantification

Protein concentrations were determined by the dye-binding Bradford method[7], with bovine serum albumin as the standard.

Assay of inhibitory activity

Trypsin inhibitory activity was determined by measuring the residual hydrolytic activity of trypsin and chymotrypsin towards the substrates BAPNA (N-benzoyl-L-arginine-p-nitroanilide) and BTPNA (N-benzoyl-L-tyrosyl-p-nitroanilide), respectively, at pH 8.0 after pre-incubation for 45 min with inhibitor[6]. One inhibitor unit was defined as the amount of inhibitor required to inhibit 50% of the corresponding enzyme activity.

SDS-PAGE and isoelectric focusing

The molecular mass and homogeneity of CGPI was determined by SDS–PAGE. Native-PAGE was done as described by Laemmli[8] excluding SDS in gel buffer, electrode buffer and sample buffer. Trypsin or chymotrypsin inhibitor bands were visualized using gelatin-SDS–PAGE. In 2-D electrophoresis, IEF was performed with immobiline dry strips pH 3–11 following the manufacturer's instructions (GE Healthcare). Second dimension was performed by SDS–PAGE as mentioned above. Proteins were stained with either Coomassie Brilliant Blue (CBB) R250 (0.1%) or silver nitrate method. The proteins used as molecular weight standards were phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and lysozyme (14.3 kDa).

Inhibition constant (*Ki*) determination

Inhibition constants of CGPI against both trypsin and chymotrypsin were determined by pre-incubating the respective enzyme with increasing concentrations of CGPI for 15minutes followed by 45minutes incubation at 37°C with different concentrations of (0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6. 1.8 and 2 mM) BAPNA or BTPNA, respectively. The *K*i value was estimated by using SigmaPlot 11.0, Enzyme Kinetics Module 1.3 (Systat Software Inc., San Jose, California, USA).

Effect of temperature

The inhibitor solution was heated for 30 min in a water bath at various temperatures (10°–100°C), and then cooled before testing for residual trypsin and chymotrypsin inhibitory activity.

Effect of pH

To measure the pH stability, the inhibitor solution (500 µg/ml) was diluted with an equal volume of various buffers (100 mM): sodium citrate (pH 2–3), sodium acetate (pH 4– 5), sodium phosphate (pH 6–7), Tris–HCl (pH 7–8) and sodium bicarbonate (pH 9–12). After incubation in each buffer for 1 h at 37°C, the inhibitory activity on trypsin and chymotrypsin were assayed as described above.

Effect of DTT

The CGPI was incubated with the reducing agent dithiothreitol (DTT) at different concentrations (0.25–5.0 mM) in 25 mM NH_4HCO_3

Circular dichroism spectra were recorded on a J-810 Jasco spectropolarimeter, equipped with a Peltier-based computer-driven temperature control, and analyzed by means of Jasco software. The cell path was 1 cm for measurements above 250 nm (Near- UV), and 0.1 cm for measurements in the 200–250 nm region (Far-UV). Time progressive heating of protein solutions was carried out at 0.5 ºC / min from 20 to 90ºC, while monitoring continuously ellipticity changes. The samples were then cooled back to 20ºC at 5º C / min and spectra recorded to assess the reversibility of temperature- induced spectral modifications. The protein was dissolved in 75 mM Tris-HCl, 5 mM $CaCl_2$, pH 8.0, which is the same buffer used for the kinetics of inhibition studies. Similarly, the effect of pH on the secondary structure of CGPI was monitored after incubation of CGPI at pH 3.0 and pH 8.0 for 1 h and the far-UV spectrum at each pH was recorded. The effect of disulfide bonds reduction and alkylation on the secondary structure of CGPI was determined in presence of 1 mM DTT and 2 mM iodoacetamide. CGPI was incubated with DTT for 1 h at 37°C followed by iodoacetamide for 45 min in dark at room temperature and spectra at 195–250 nm were recorded.

Chemical modification of amino acid residues

Arginine residues were modified using 1,2- cyclohexanedione (CHD) as described by Abe et al.,[9]. CGPI in 50 mM borate buffer, pH 9.0 was incubated with 15-fold molar excess of CHD and the reaction tube was flushed with nitrogen and kept at 37°C for 2 h. The reaction was terminated by the addition of 5% acetic acid. Lysine residues were modified using sodium trinitrobenzene sulphonate (TNBS)[10]. CGPI in 50 mM phosphate buffer, pH 7.6 was incubated with 10-fold molar excess of TNBS at 40°C for 2 h. The reaction was stopped by adding 10% SDS followed by 0.2 ml of 1 N HCl. Tyrosine residues were modified using N-acetylimidazole (NAI). CGPI in50 mM Tris–HCl, pH 7.5 was incubated with 60-fold molar excess of NAI at 37°C for 2 h. The reaction was terminated by adding excess NAI and dialyzed for 5 h at 4°C against 50 mM Tris–HCl, pH7.5. Modification of tryptophan residues was performed using N-bromosuccinimide (NBS). CGPI in 50 mM sodium acetate buffer, pH 4.0 was incubated with 60-fold molar excess of NBS at 37°C for 2 h. The residual inhibitory activity against trypsin and chymotrypsin was estimated as described earlier. The activity staining gels were done by gelatin-SDS–PAGE (12.5%). SDS-polyacrylamide slab gels containing copolymerized substrate gelatin (0.1%). 25 µl samples were applied by a micro syringe. When the SDS-PAGE electrophoresis was completed; gels were cut into two parts**.** One part was removed and washed with gentle stirring in 500 ml of washing solution with three consecutive changes at 40 to 45 min for each to remove SDS. The gels were then washed for 45 min in distilled water and incubated in 450 ml of assay buffer containing (14.2 µg /ml) trypsin enzyme (1645U/mg) at 37 °C for 30 min to 5 h depending on the concentration of samples and then washed with distilled water and stained by immersion in coomassie staining solution for 45 min and then destained with destaining solution. Another part was stained directly with Coomassie staining solution. Then the gels were destained in destaining solution.

RESULTS AND DISCUSSION

C. grandis protease inhibitor (CGPI) 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 fractions eluted from DEAE Sepharose column exhibiting protease inhibitor activity were pooled and loaded onto an affinity column yielding only one peak (CGPI) with antitryptic activity. It proved to be a convenient step for isolating CGPI, 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 named as CGPI (*Coccinia grandis* protease inhibitor).

Thus, the protocol yielded a purified CGPI 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* [11] and *Brassica campestris* [1].

Table 1: Purification profile of *Coccinia grandis* PI[a]

[a] One unit is defined as 1 µmol of substrate hydrolyzed/ min of reaction. One inhibition unit is defined as unit of enzyme inhibited.

Purification steps	Total activity U	Yield %	Total protein mg	Specific activity U	Fold purification
Crude extract	4359	100	1316	3.3	0
Ammonium sulfate 80%	2763	63.4	388	7.1	2.2
Sephadex G-75	1425	32.7	13	109.6	33.2
DEAE-Sepharose	874	20	3.2	273	82.7
Trypsin-Sepharose	529	12	1.4	377.9	114.5

The protein was further subjected to RP-HPLC where it showed a single peak with a retention time of 8.2minutes 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 and it showed a single thick polypeptide band with a molecular mass of approximately 14.3 kDa (Fig. 1A) which is not in agreement with other cucumisin-like protease of squash family and PI of legumes[12, 13, 10, 14]. The molecular mass estimated by size exclusion chromatography (14.3 kDa) was agreed fairly well with the SDS-PAGE results. CGPI appeared to exist as five different isoinhibitors as evident through native-PAGE (Fig. 1B) and two-dimensional (2- D) electrophoresis (Fig. 1C). These isoinhibitors had pI values of 4.3, 4.4, 5.1, 5.4 and 6.1, respectively. The presence of multiple genes and the possibility of hydrolysis implicated for the existence of a large number of isoinhibitors [15].

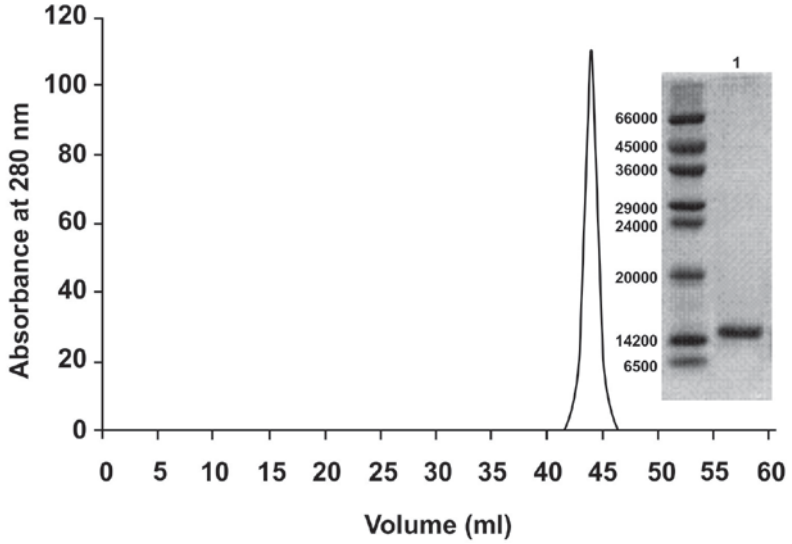

Fig. 1(A): Chromatogram and SDS-PAGE analysis of purified CGPI. C-18 reversed phase chromatography (RP-HPLC) elution, acetonitrile gradient (0±80%) in 0.1% TFA, flow rate: 1 ml/min. Lanes 1 protein from trypsin Sepharose affinity column fraction. Protein bands were stained with Coomassie blue R-250

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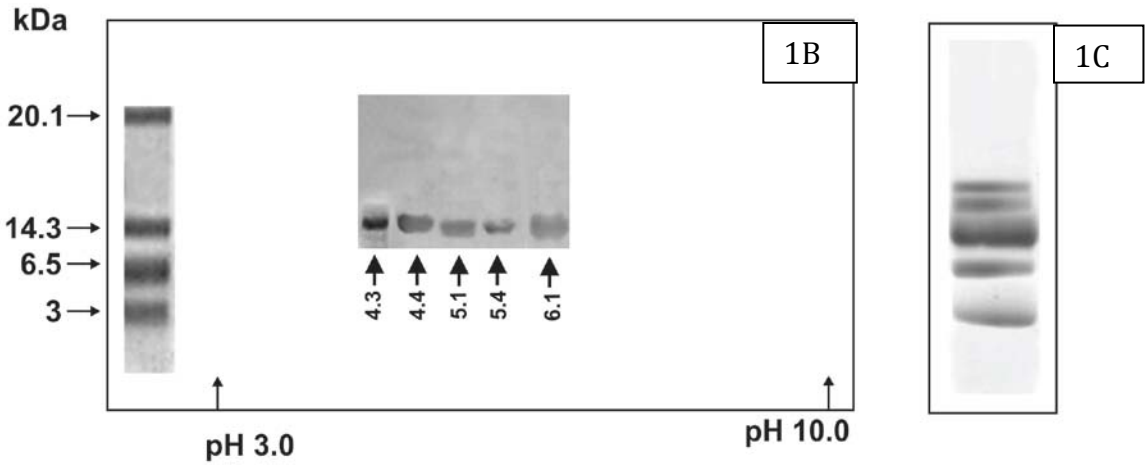

Fig. 1(B): 2-D gel showing the isoinhibitors of CGPI. The proteins were separated in IEF using 3.0–11.0 pH non-linear strips having maximum resolution at pH 4.0–7.0.; Fig. 1(C): Five isoinhibitors of CGPI separated on native-PAGE (12.5%). The second dimension was performed in SDS–PAGE (18%)

Existence of multiple inhibitor isoforms in pea was attributed to the post-translational modification of the primary gene product and which occurs during the desiccation phase of seed development[16]. Harsulkar et al. [17] proposed that host plants evolved isoinhibitors as a defense strategy to combat against insect proteinases. Bowman-Birk Inhibitors isolated from various legumes also show isoinbitors [18-20].

CGPI showed inhibitory activity against trypsin and chymotrypsin, a characteristic feature of BBI type PIs since they possess two different reactive sites[21]. Stoichiometry inhibitory activity of CGPI was more pronounced against trypsin, when compared with chymotrypsin. A linear extrapolation to obtain 100% inhibition indicated that CGPI bound to trypsin in a 1:2 molar ratio i.e., one molecule of CGPI will exhibit TI activity by binding with two molecules of trypsin [22], whereas there was no obvious stoichiometry with chymotrypsin from the titration pattern of the inhibitory activity (Fig. 2) similar to the CGPI isolated from *Peltophorum dubium* seeds [23] and *Apios americana* tubers[24]. The mode of CGPI inhibition was evaluated from the double reciprocal plots of trypsin/chymotrypsin titrated with different concentrations of their respective substrates. The results indicate that CGPI is a non-competitive inhibitor of both trypsin and chymotrypsin.

The apparent *K*i for trypsin and chymotrypsin inhibition are 312.8 ± 15.8 and 11660 ± 2.4 nM, respectively (Figs. 3A and 3 B). BBI type PIs isolated from different plants possessed K_i values for trypsin in the range between 0.1 and 52,000 nM[22, 25]. The lower K_i value observed for trypsin, compared with chymotrypsin indicates that CGPI had ~28-fold higher affinity for trypsin than chymotrypsin. Such differences in the K_i of trypsin and chymotrypsin were also evident in BBIs isolated from *A. americana*[24].

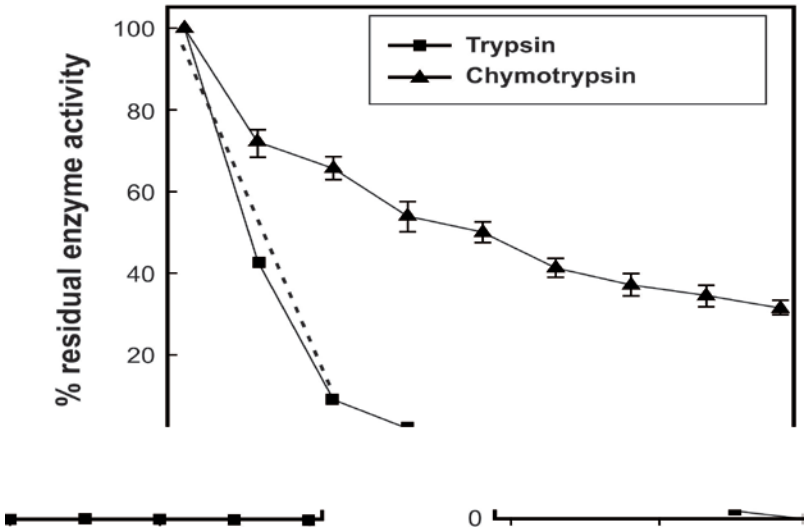

Fig. 2: Titration curves of trypsin and chymotrypsin inhibition by CGPI

Increasing concentrations of CGPI was added to a fixed concentration of enzyme. The concentration of trypsin or chymotrypsin in the reaction mixture was 1 µM. After incubating the reaction mixture for 15 min, residual trypsin or chymotrypsin activity was determined as described in methodology. The molar ratio of the inhibitor to the trypsin or chymotrypsin was the intercept of x-coordinate, when the tangent was extrapolated to the zero activity. The points are the mean ±S.E. of six assays

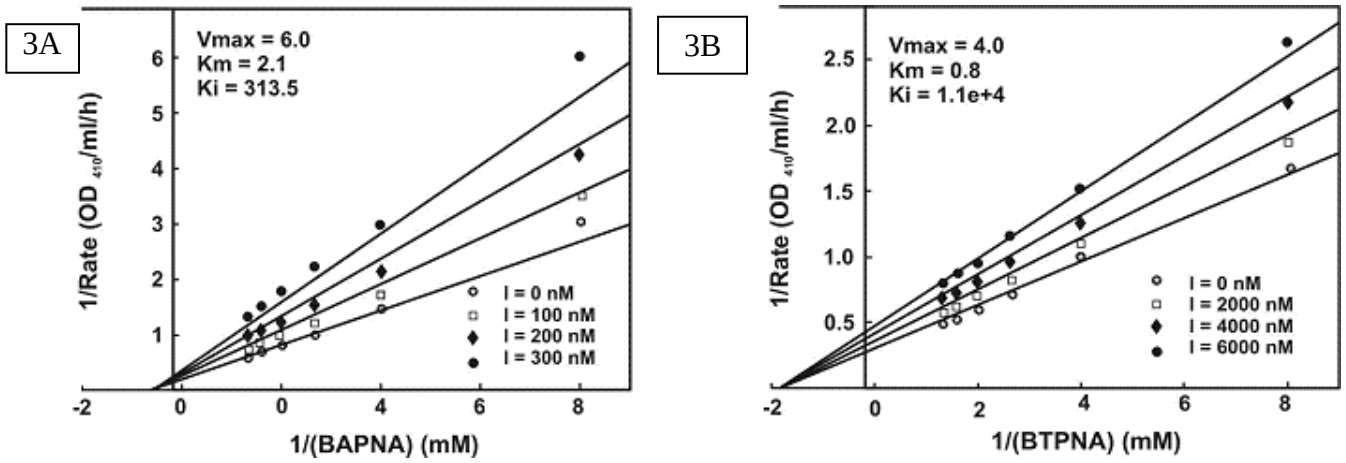

Fig. 3(A): Double reciprocal plot showing non-competitive nature of trypsin inhibition by CGPI at various concentrations of BAPNA (0.125, 0.250, 0.375, 0.500, 0.625 and 0.750 mM); Fig. 3(B) Non-competitive nature of chymotrypsin inhibition by CGPI at various concentrations of BTPNA (0.125, 0.250, 0.375, 0.500, 0.625 and 0.750 mM)

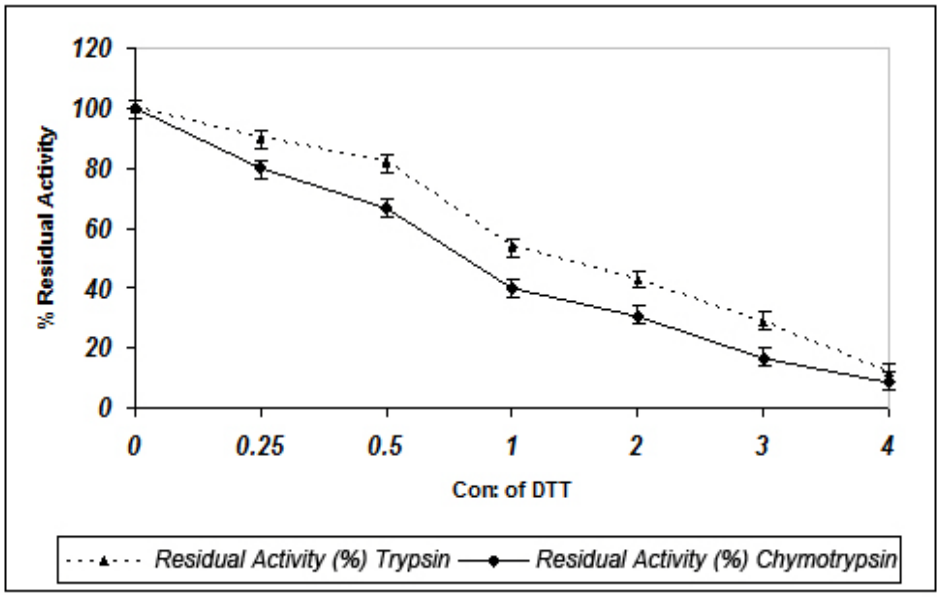

Fig. 4: Effect of DTT reduction (0.25 mM to 4 mM) on trypsin and chymotrypsin inhibitory activity of CGPI. The residual inhibitory activity against trypsin and chymotrypsin was determined as described in methodology

To examine the changes accorded by CGPI at a structural level following DTT exposure, far-UV CD measurements were performed. Noticeably, marked differences in the far-UV spectrum of native and reduced CGPI were recorded which included a loss of secondary structure (Fig. 5). However, when the reduction of CGPI with DTT was followed by alkylation with iodoacetamide, the ellipticity at 203 nm was increased, which might have been due to the chirality of the cysteine bonds. These results suggest that loss in inhibitory activity of CGPI could be attributed to the loss of reactive site loop conformation due to disulfide bond disassociation. Similar results have been reported earlier in BBI type PI from *Vigna mungo* [26]. It is presumed that the intramolecular disulfide bonds and their distance from the reactive site residues determine the functional stability of these inhibitors in presence of denaturing agents[3]. However, studies on the susceptible (single polypeptide) soybean trypsin inhibitor[27] or stable *Bauhinia* species inhibitors (devoid of any disulfide linkages), indicated that intramolecular disulfide bonds in Kunitztype PIs may be essential, but not exclusive to the stabilization of the reactive site loop.

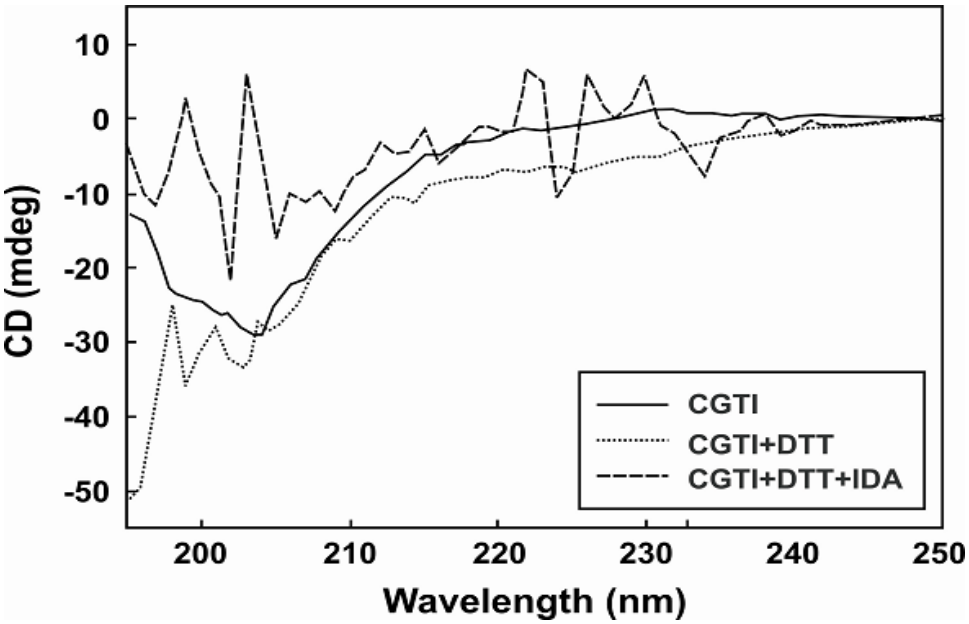

Fig. 5: Far-UV CD spectra of native CGPI, reduced CGPI with 1 mM DTT, and reduced CGPI alkylated with 2 mM iodoacetamide (IDA)

Pre-incubation of CGPI for 60 min in the pH range of 2.0–12.0 had less effect on the trypsin and chymotrypsin inhibitor activity suggesting that pH amplitude did not perturb the overall folding of the protein. Further 90% of the inhibitor activity was retained at all pH studied (Fig. 6A). The possible occurrence of many cysteine residues forming disulfide bonds may account for this striking stability in structural conformation and inhibitory properties of CGPI [26] (Fig. 7A). These properties of CGPI corroborate well with those exhibited by BBI type PIs isolated from *Dolichos biflorus*[20], *Cratylia mollis* [18], *Phaseolus. Coccineus* [28] and L*upinus albus*[4].

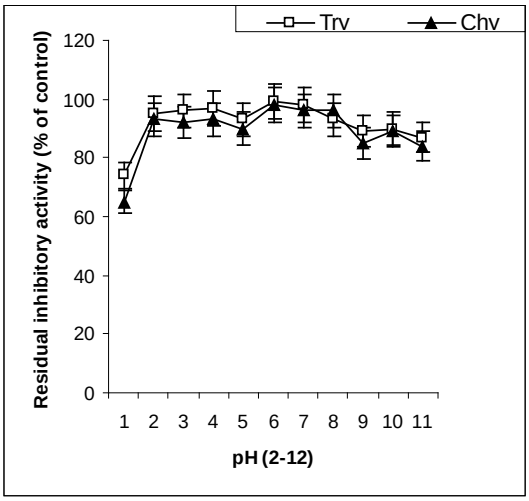

Fig. 6 (A) : Stability of CGPI after incubation at various pH ranging from 2.0 to 12.0 for 1 h

Analysis of the temperature effects on the CGPI against trypsin and chymotrypsin revealed the stability of the protein up to 80°C for 30 min (Fig. 6B). However, at lower (10°-20°C) and higher temperatures (90°C - 100°C), CGPI showed a decrease in the activity (35-49 %) after 30 min. These results are in agreement to those reported for other plant PIs. Secondary and tertiary structures have been assessed by circular dichroism (CD) spectral analysis. Although the buffer system used to dissolve the protein did not allowed to record spectra below 200 nm[22], it was however possible to get enough information to evaluate the conformational changes of the protein under the tested conditions. Far-UV CD spectra (Fig. 7B and C) of the protein showed that the protein secondary structure appeared almost completely restored after cooling. As far as tertiary structure modifications are concerned, near-UV CD spectra indicated that the heat treatment induced only modest not completely reversible structural changes.

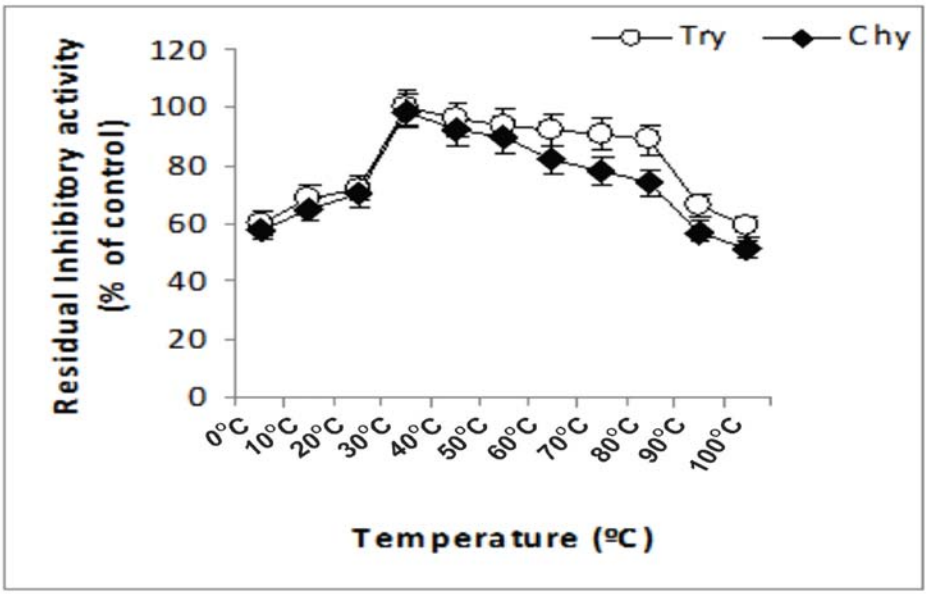

Fig. 6 (B): Thermal stability of CGPI after incubation at various temperatures ranging from 10 °C to 100° C for 30 min

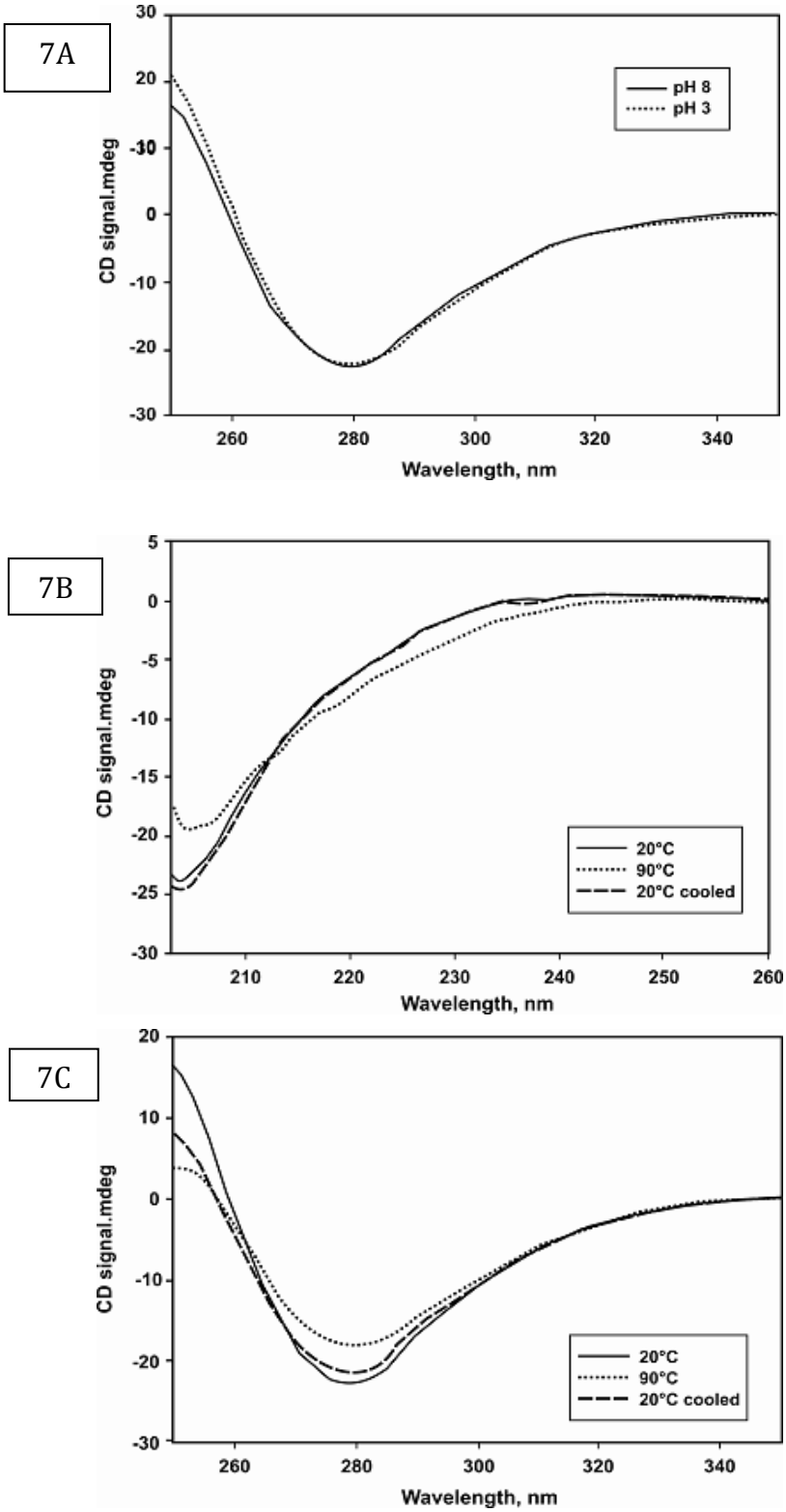

Fig. 7(A), (B), (C): CD spectra of CGPI. Temperature effects on the secondary and tertiary structures have been assessed by far-UV (A) and near-UV (B) spectral analyses, respectively. Stability at pH 3.0 was assessed in the near-UV (C). In the near-UV spectra (B and C) the protein concentration was 0.23 mg ml/1, whereas in the far- UV (A) experiment was 0.058 mg ml/1. The protein was dissolved in 75 mM Tris/ HCl, 5 mM $CaCl_2$, pH 8.0. Time progressive heating of protein solutions was carried out at 0.5°C/min from 20 to 90°C, while continuously monitoring ellipticity changes. The samples were then cooled back to 20°C at 5°C/min and the spectrum recorded

CGPI was inactivated by the lysine or tryptophan modifying agents TNBS and NBS, respectively. Figure 8A shows that modifications of amino acid residues in CGPI significantly affected its inhibitory activity against trypsin by 68% and 100%, respectively. On the other hand, modification of arginine or tyrosine residues of CGPI had no effect on its inhibitory activity against trypsin.

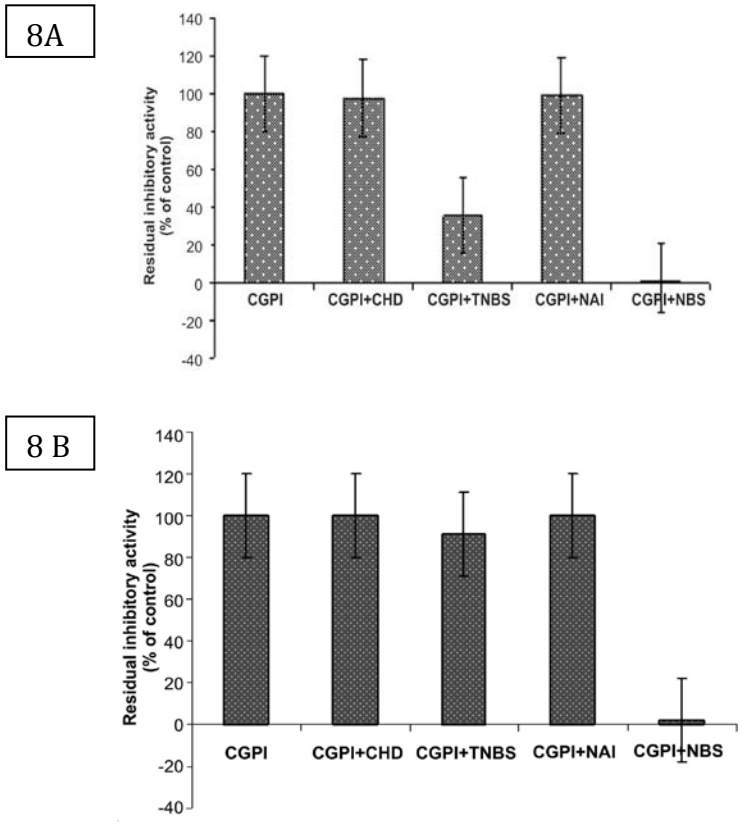

Fig. 8(A) and (B): Effect of amino acid residue modification on inhibitory activity of CGPI against trypsin and chymotrypsin. *CGPI lost complete inhibitory activity against trypsin and chymotrypsin after modifying tryptophan residues using NBS

Modification of tryptophan residues of CGPI also caused complete loss of inhibitory activity against chymotrypsin. However, modification of lysine, arginine or tyrosine residues of CGPI did not cause any serious effect (<11%) on inhibitory activity against chymotrypsin (Fig. 8B).

The importance of lysine or tryptophan residues of CGPI in forming a stable complex with trypsin/chymotrypsin proteases was further strengthened by the activity staining studies using gelatin- SDS–PAGE. The modification of lysine residues showed faint bands against trypsin but not chymotrypsin indicating the importance of lysine residue(s) at the reactive site for trypsin inhibition, which is a characteristic feature of BBI[19]. Corroborating with inhibitory activity studies, there was no change in trypsin or chymotrypsin inhibitor bands in gelatin-SDS–PAGE, when arginine or tyrosine residues of CGPI were modified. These results suggest that neither arginine nor tyrosine play a role in the inhibitory sites active against trypsin and chymotrypsin. In the present study, modification of tryptophan residues of CGPI resulted in complete disappearance of bands against trypsin and chymotrypsin, which emphasize the presence of tryptophan at both trypsin and chymotrypsin reactive sites of CGPI. This finding corroborates with *Vigna mungo* BBI type PIs.

The presence of amino acid residues lysine/arginine at P1 position of first reactive site (N-terminal) and aspartic acid/glutamic acid in the carboxy terminus were suggested to be responsible for self-association of monomers to form stable dimers and any deviation in these residues resulted in the existence of BBIs as monomers in solution[3]. Replacement of lysine/ arginine at P1 position by alanine in the first reactive site of BBI restricted the formation of oligomers in *G. soja* and BBI was retained as monomers in solution[29]. Therefore, future studies are warranted in this direction to completely elucidate the structural characteristic features of PI from *C. grandis*.

The present work unveils the presence of a protease inhibitor in the leaves of *Coccinia grandis*. Functionally, CGPI stoichiometrically inhibits trypsin in a molar ratio of 1:2. CGPI showed inhibitory activity against both trypsin and chymotrypsin, and CD spectral analysis revealed the predominance of secondary structure and random coils, which are characteristic features of BBI type PI. CGPI also exhibited a remarkable stability at temperatures up to 80°C and to a wide range of pH (pH 2.0–12.0). It completely lost its inhibitory activity against trypsin and chymotrypsin when incubated with DTT. Chemical modification studies indicated that lysine present in the reactive site of CGPI play a significant role in trypsin inhibition.

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