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Purification, Characterization and Functional Analysis of a Serine Protease Inhibitor from the Pulps of *Cicer arietinum* L. (Chick Pea)

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Serine proteinase inhibitors (SPIs) are present in high amount in legume seeds where they play an important role in plant defence mechanism against pests. In the present study, a serine protease inhibitor has been identified from the seeds of *Cicer arietinum* (L.) and characterized for its inhibitory potency against trypsin and chymotrypsin. Ammonium sulphate fractionation was executed as an initial step to purify the inhibitor. The fraction which was obtained from 30-60% relative ammonium sulphate saturation exhibited the maximum trypsin inhibition activity against 0.2% casein using radial diffusion method. The 30–60% fraction was further subjected to ion exchange chromatography using 1 mL HiTrap Q HP column. The peak fractions were analyzed for the enzymatic activity and also characterized on 12% SDS PAGE. The results indicated that the flow through fraction has retained a significant proteolytic inhibition towards trypsin with a relative molecular mass of approximately 12-13 kDa. The kinetic results have demonstrated that the purified inhibitor from *Cicer arietinum* L. not only inhibited trypsin but also chymotrypsin. The Circular Dichroism spectrum analysis of the purified inhibitor has revealed that the secondary structure content is highly composed of random coils which were supported by the reports of other low molecular weight trypsin inhibitors. To conclude, a low molecular weight serine protease inhibitor possessing both trypsin and chymotrypsin inhibition from the seeds of *C. arietinum* has been purified, characterized and the results are reported.

Keywords: Circular dichroism spectrum, Competitive/non-competitive inhibition, Enzyme kinetics, Serine protease inhibitor, Trypsin/chymotrypsin inhibitor

In general, proteases especially from pathogens are often considered as an essential group of drug targets for developing antiprotozoal drugs as they play key roles in host-parasite interactions, parasite immune evasion, life cycle transition and pathogenesis of parasitic diseases[1]. Addition to this, owing to their role in life cycle and invasion many proteases have also been targeted for employing structure based drug designing against pathogens including bacteria and viruses[2]. The development of potent and selective serine protease inhibitors (SPIs) targeting secretory serine proteases could pave the way for developing suitable pest controls. By inhibiting larval gut proteases, reports of protease inhibitors conferring resistance against the invading insect pests in plants are available[3]. While the midgut proteases of *Pieris brassicae* larvae were tested by the cultivars of *Dolichos biflorus*, larval gut proteases of *Spodoptera littoralis* were efficiently inhibited by the seed extracts of developing and germinating seeds of HPK4 cultivars[3].

SPIs are present in high amount in legume seeds possessing strong antitrypsin and/or anti chymotrypsin activity, with poor or no activity towards other proteolytic enzymes. From the biochemical characterizations of SPIs, two families of inhibitors have been identified *viz*., Kunitz type and Bowman-Birk type[4-6]. Generally, Kunitz type inhibitors are of ~20 kDa single chain polypeptides consisting of two intra-chain disulfide bridges forming 1:1 complex with the target proteinase. The Bowman-Birk inhibitors (BBIs) are also single chain polypeptides with an apparent molecular mass of ~8-13 kDa possessing seven disulfide bridges and two active domains for trypsin and/or chymotrypsin. Their

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Abbreviations: *C. arietinum*, *Cicer arietinum*;CD Spectra, Circular dichroism spectra; CATIN, *Cicer arietinum* trypsin inhibitor; HGP, *Helicoverpa armigera* gut proteases; SPI, Serine proteinase inhibitor

direct applications in retarding growth and development of insect pests have been demonstrated[7-11] where the pests have been fed on diets incorporating PIs or on transgenic plants expressing PIs. However, it has been found that insect pests adapt to PIs by expressing inhibitor insensitive or inhibitor-degrading digestive proteinase(s)[12-14]. This response has prompted attempts to understand the total complement of digestive proteinases available to insect pest species and their regulation in response to dietary components[15-19].

Since plant seeds consist of the abundant amount of proteins for animal and human consumption, the presence of proteinase inhibitors from several legume seeds such as soya beans, lima beans and groundnuts have been well documented[20,21]. The inhibitory site of many of the trypsin inhibitors purified from plant sources were found to be 'double-headed', possessing separate sites for trypsin and chymotrypsin inhibition as found in the Bowman-Birk soya-bean inhibitor and the lima-bean inhibitor[21]. Pancreatic hypertrophy has been observed in a trypsin inhibitor isolated from soya bean seeds where they have been involved in governing the balance of biosynthesis and secretion of pancreatic enzymes[22-25]. Few low molecular weight (10 kDa) inhibitors of trypsin and chymotrypsin were isolated and purified from the crude extract of chick peas[26]. Among the purified inhibitors about four major inhibitors were reported to have similar amino acid compositions with cysteine as the predominant amino acid residue. Their mode of inhibition remained the same towards chymotrypsin but differed towards trypsin indicating that they are a combination of native and trypsin modified forms possessing separate sites for the two enzymes[26]. Further studies on the reactive site of many naturally occurring trypsin inhibitors have shown that they may possess a disulfide loop comprising a trypsin-accessible Arg - X or Lys - X bond. Eventually, this proposal has led to the identification of the reactive site for most of the trypsin inhibitors[27-30]. Furthermore, it has been found that in the trypsin-inhibitor complex, inhibitor remains inactive towards trypsin but remains fully active against chymotrypsin and *vice versa*[31]. Therefore, it has been concluded that the principal inhibitor in chickpea assumes a 'double-headed' type with separate reactive sites and thus inhibiting both enzymes simultaneously[31].

In view of their importance in various fields, it presents the isolation, purification and characterisation of an SPI from the seeds of Chickpea or *Cicer arietinum* L. (*C. arietinum*). In general, *C. arietinum* is an important self-pollinated diploid crop with genome sized approximately 931 Mbp[32]. India is the largest producer of chickpea, contributing with about 66% of the total global production, followed by Turkey, Pakistan and Mexico[33]. It is a major source of protein (12.4-31.5%), energy, fibre, vitamins, minerals and essential amino acids such as tryptophan and lysine[34].

Materials and Methods

Crude extraction from the *C. arietinum*

The chickpeas used in the present study are of small split desi chickpeas (brown gram) purchased from the local market (Chennai, India). They look similar to yellow split peas which were extensively washed with double distilled water and proceeded to crude extraction. Using a clean mixer grinder about 100 g of *C. arietinum* was smashed and made into dry powder. To this dry powder double the volume of ice-cold hexane (~300 mL) was poured and stirred at constant intervals. After an h, the hexane was discarded carefully and *C. arietinum* powder was allowed to air dry at room temperature. From the total content, about 75 g of defatted *C. arietinum* powder was mixed with ~200 mL of 100 mM Sodium phosphate buffer (pH 7.2). After infusing with the buffer for overnight at 20°C, the soluble form of *C. arietinum* aqueous extract was obtained.

Ammonium sulphate precipitation

Before proceeding for salt precipitation, aqueous extract of *C. arietinum* was subjected for heat treatment where the extract was placed in a boiling water bath set at 70°C for 15 min. Following this, the extract was spun down at 10000 rpm for 40 min. To the retained clear supernatant (about 100 mL), salt precipitation using ammonium sulphate was carried out[35]. The pulverized solid ammonium sulphate was added slowly with constant stirring to obtain 30% relative saturation. After standing for 2 h at 4°C, the whole content was centrifuged at 10000 rpm for 45 min at 4°C. The resultant pellet was re-suspended with appropriate volume of the same extraction buffer. In similar fashion, 30-60% and 60-90% saturations were also achieved. Using 10 kDa cut off dialysis bags, the three fractions of *C. arietinum* extract were dialyzed with 1.5 L of 100 mM Sodium phosphate (pH 7.2) buffer at 4°C overnight.

Trypsin inhibition assay

Following dialysis, all three fractions 0-30, 30-60 and 60-90% along with the aqueous crude were tested

for their trypsin and chymotrypsin (SRL Chemicals) inhibition. Briefly, the steps involved as follows; 0.2 g of casein was mixed with 0.8 g of agar, adjusted to a pH of 7.2 and made up to 100 mL with deionised water. The casein agar stock was then autoclaved (15 lbs) for 30 min. On a sterile Petri plate, about 20 mL of casein agar was poured and allowed to solidify. To 5 µg (5 µL from 1 mg/mL) of bovine pancreatic trypsin 15 and 30 µL of respective samples were added in two different sets. The reaction volume was made to 40 µL with sample buffer and preincubated at 37°C for 40 min. For the positive control, 5 µg of bovine pancreatic trypsin was added with 35 µL of the sample buffer. Following the incubation, all samples were loaded onto the wells made in the solidified casein agar plate and kept at 37°C for overnight. Since 30-60% fraction exhibited maximum inhibition towards, this fraction was concentrated and subjected for further purification.

Ion exchange chromatography

Using AKTA FPLC purifier system, 30-60% fraction of *C. arietinum* was subjected for anionic exchange chromatography with 1 mL HiTrap Q HP column (GE Healthcare). The column was pre-equilibrated with 100 mM Sodium phosphate (pH 7.2) buffer. After injecting sample, the flow through was collected by washing the column with the starting buffer. Following this, the bound proteins were eluted at the flow rate of 1 mL/min with a linear saline gradient of NaCl from 0-0.8 M in a total of 30 column volume (30 mL). The peak fractions as observed by absorption at UV-280 nm were further analysed for their trypsin inhibition activity.

Determination of Km and V_{max}

The trypsin inhibition activity was assayed at various concentrations of the substrate using 1.0% casein as stock in a reaction volume of 100 µL. Briefly, to a clean sterile 1.5 mL of several Eppendorf tubes, 1 µg of the purified *C. arietinum* trypsin inhibitor (CATIN) was mixed with 5µg of bovine pancreatic trypsin. Following the incubation at 37°C for 30 min, a substrate with increasing concentrations (2 µg to 45 µg of 1.0% casein) was added to each tube and again incubated at 37°C for 30 min. At the end of the second incubation, the reaction was terminated by adding 10% ice-cold trichloroacetic acid (TCA) and incubated on ice for 30 min. All samples were then centrifuged at 10000 rpm for 40 min. Simultaneously, the proteolysis activity of bovine pancreatic trypsin was also carried out (in the absence of CATIN) using 1.0% casein. As mentioned earlier, to the several tubes 5 µg of bovine pancreatic trypsin was added with substrate of increasing concentrations (2 µg to 45 µg of 1.0% casein). After incubation at 37°C for 30 min, the reaction was terminated by using 10% ice-cold TCA. All the samples were then centrifuged at 10000 rpm for 40 min. The clear supernatants containing the liberated digestive fragments were analysed for their absorption at UV-280 nm. Km and V_{max} were determined from the michaelis-menten equation using graph pad prism (Version 6.0, La Jolla, California). The same sets of procedures were followed for the kinetic studies of CATIN towards bovine pancreatic chymotrypsin. As performed for bovine pancreatic trypsin, the caseinolytic activity of bovine pancreatic chymotrypsin was also carried out in the presence and absence of CATIN.

CD spectra of CATIN

The far Ultraviolet (UV) Circular Dichroism (CD) measurements of CATIN were carried out in `Jasco J815 polarimeter. Quartz cuvettes of 0.1 cm path length were used in the far - UV region at a scanning speed of 50 nm/min. The CATIN at a concentration of 0.1 mg/mL in 100 mM Sodium phosphate buffer (pH 7.2) was used for the study at normal room temperature. Data were collected in triplicates and the average spectrum was taken for processing after the baseline correction with 100 mM Sodium phosphate buffer (pH 7.2). An online tool K2D2 was used to predict the secondary structure of CATIN[36].

Poly acrylamide gel electrophoresis

The molecular weight analysis of the purified CATIN was carried out using 12% SDS-PAGE vertical slab electrophoresis apparatus as described[37] and stained with Coomassie Brilliant blue G-250.

Results and Discussion

The dialyzed fractions 0-30, 30-60 and 60-90% of *C. arietinum* aqueous extract were tested for their trypsin inhibition activity by a radial diffusion method using casein as a substrate. From the results, it was observed that among all other fractions, 30-60% exhibited maximum inhibitory activity as shown in (Fig. 1) and hence was concentrated to 0.5 mL before proceeding to further purification. Using an anion exchange chromatography, the concentrated 30-60% fraction of *C. arietinum* was purified whose chromatogram has been represented in (Fig. 2).

Following the chromatographic run, all the peak fractions were then pooled and concentrated. Using

casein agar plate, these peaks were analysed for their trypsin inhibition activity (Fig. 3).

From the activity analysis, compared to the peak fractions maximum trypsin inhibition activity was observed in the flow through. Therefore, this fraction was further subjected to the kinetic and other characterization studies.

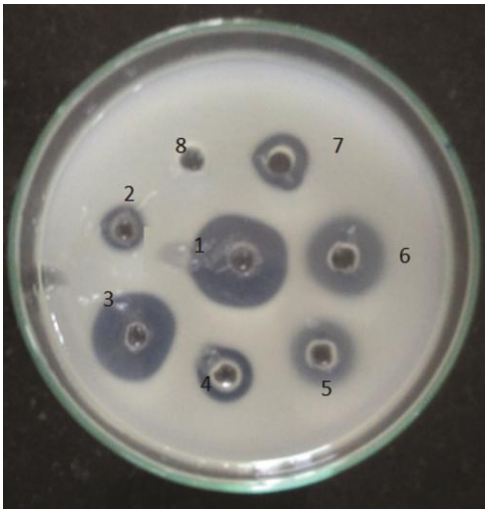

Fig 1 — Trypsin inhibition activity of the dialyzed fractions (1) 5 µg bovine pancreatic trypsin (positive control); (2 and 3) 15 and 30 µL of 0-30 % fraction; (4 and 5) 15 and 30 µL of 30-60 % fraction; (6 and 7) 15 and 30 µL of 60-90 % fraction; and (8) 100 mM sodium phosphate buffer (negative control)

Determination of V_{max} and Km

Kinetic parameters *e.g.* V_{max} & Km for the purified inhibitor from *C. arietinum* were determined. For trypsin inhibition, V_{max} was found to be 0.077 µM/min and Km was 26 µM whereas, for the chymotrypsin inhibition V_{max} was found to be 0.063 µM/min and Km was 25 µm.

CATIN-trypsin

UV based enzyme inhibition kinetics explained the action of *C. arietinum* Trypsin Inhibitor (CATIN) on trypsin. 1.0% casein was used as the substrate at various concentrations from 2 to 16 µM in the assay. From the plot of substrate velocity curves (Fig. 4A & 4B) V_{max} and Km values were calculated for trypsin inhibition activity with and without CATIN. Due to the addition of CATIN to trypsin, V_{max} is varied and Km remained the same. Hence, the inhibitor has been charecterized as noncompetitive inhibitor towards trypsin. The inhibitor may bind on the other side of the catalytic site of trypsin to stop the enzymatic action. The double reciprocal Linweiver B plot (1/S *vs* 1/V) calculates inhibitory constant Ki as 0.02 µM which was obtained from the interception of (V_{max}/Km) on X-axis. Similar kind of non-competitive mode of inhibition has been found from the earlier reports of trypsin inhibitors purified from *Coccinia grandis* L.[38], *Erythrina velutina*[39] and *Jatropha curcas* L.[40]. The Ki

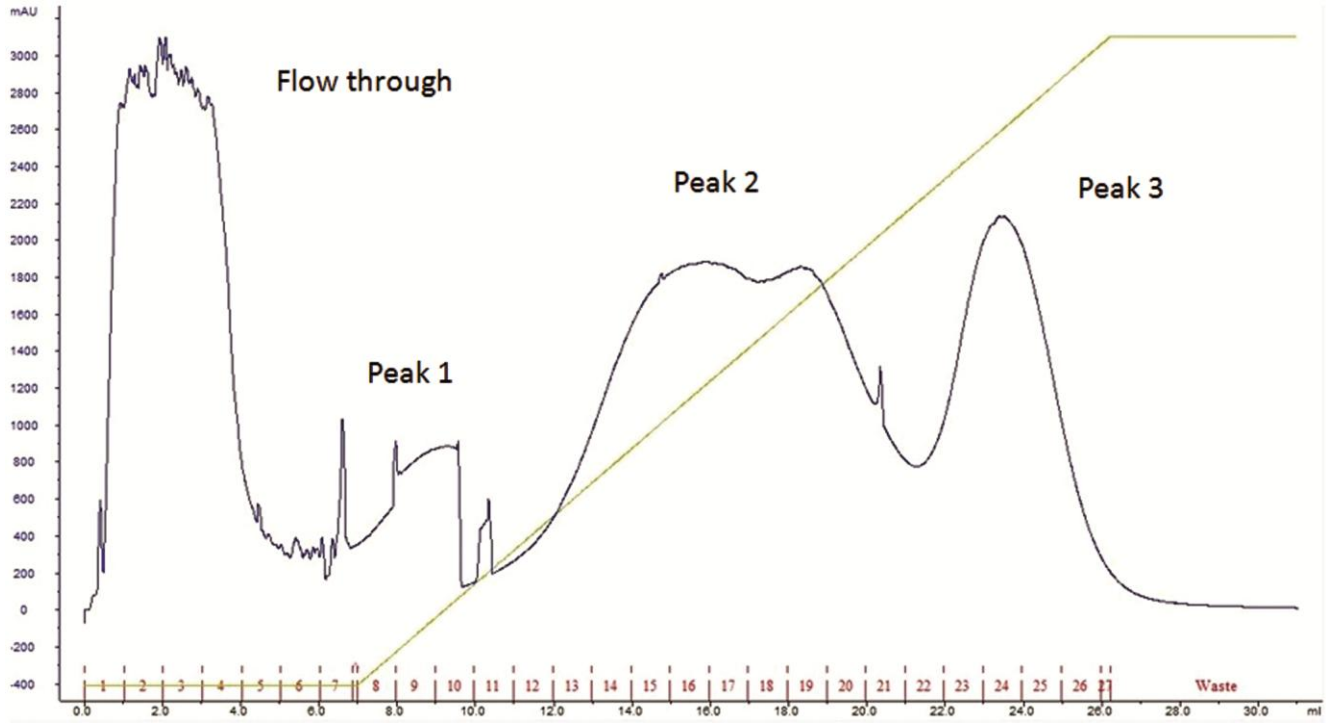

Fig 2 — Chromatogram of 30-60% fraction of *Cicer arietinum* L. by anion exchange chromatography

value of CATIN reported for *C. arietinum* correlates well with the Ki value reported in these inhibitors.

CATIN-chymotrypsin

The substrate-velocity curve of chymotrypsin caseinolysis in presence and absence of CATIN (Fig. 4C & 4D) were also carried out. The result revealed that both the V_{max} and Km values for the presence and absence of CATIN were varied. Hence, it can be assumed that CATIN exhibits a competitive mode of inhibition towards chymotrypsin. In this case, the protein may bind on the active site pocket of chymotrypsin. Based on the relationship between V_{max} and Km, inhibtory constant Ki was calculated as 33 µM. The LB-Plot also confirmed the Ki value from the intersection of V_{max}/Km. Generally, plant protease inhibitors are reported to exhibit more preference towards trypsin than chymotrypsin. These kinetic values proved that CATIN as a non-competitive inhibitor towards trypsin (Fig. 4A & 4B) and as competitive inhibitor towards chymotrypsin (Fig. 4C & 4D).

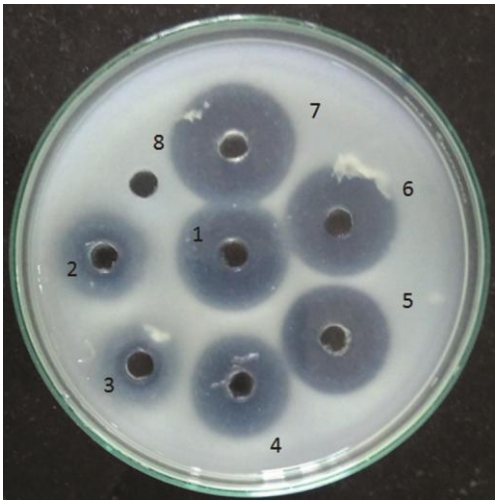

Fig 3 — Trypsin inhibition activity of the peak fractions obtained from anion exchange chromatography: (1) 5 µg bovine pancreatic trypsin (positive control); (2 and 3) flow through [15 and 30 µL]; (4 and 5) Peak 2 [15 and 30 µL]; (6 and 7) Peak 3 [15 and 30 µL]; and (8) 100 mM Sodium phosphate buffer (negative control)

In 1976, Smirnoff *et al.* have reported a low molecular weight trypsin and chymotrypsin inhibitor purified from *C. arietinum*[21]. Though the polyacrylamide-gel electrophoresis (in presence of sodium dodecyl sulphate) has showed a pure protein with a relative molecular weight of ~ 10 kDa, analysis of amino acid composition has revealed the molecular weight of the purified inhibitor as 8.3 kDa. Apart from possessing similar amino acid composition for the trypsin and chymotrypsin inhibition, the reported inhibitor has also been found similar to the trypsin inhibitors purified from the lima-bean, soya beans and groundnut[21]. While on chemical treatment with maleylation has reduced the trypsin inhibition activity significantly (up to 85%), inhibition towards chymotrypsin remained unaffected. The inhibitor

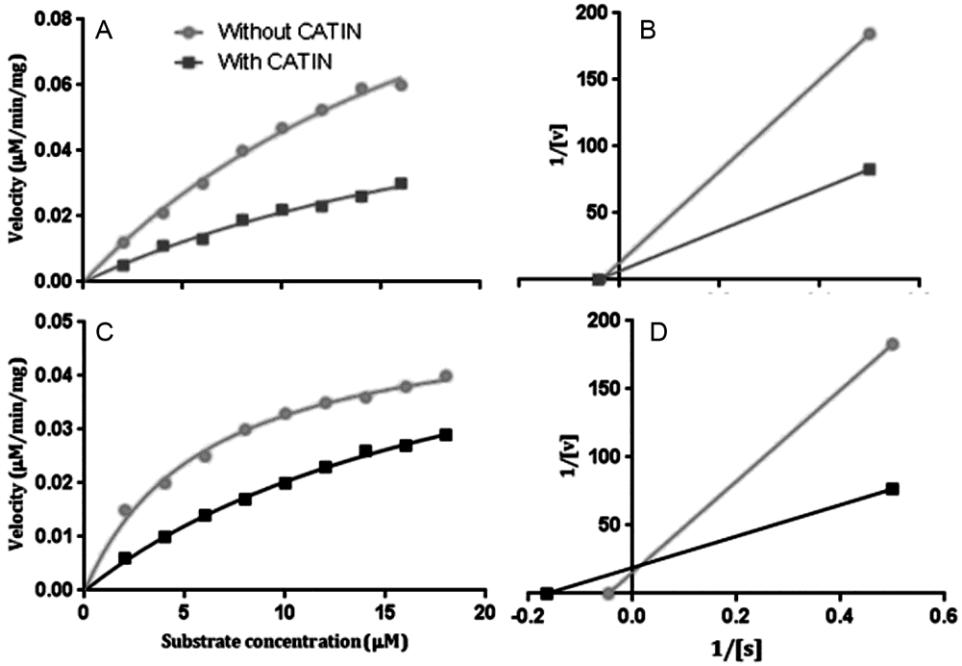

Fig 4 — Determination of kinetic parameters such as V_{max} & Km of the purified CATIN from *Cicer arietinum* L. towards (A & B) Trypsin; and (C & D) Chymotrypsin

formed complexes with trypsin and chymotrypsin at molar ratios of 1:1.5[21]. Similarly, Belew *et al.* have reported a set of iso inhibitors of trypsin and chymotrypsin purified from the crude extract of *C. arietinum*[26]. The iso inhibitors had a molecular weight of about 10 kDa as determined by molecular-sieve chromatography on Sephadex G-75. Their activity remained stable in various pH ranges and temperatures up to 75°C. As for the inhibition towards trypsin and chymotrypsin, they exhibited similar kind of inhibition towards chymotrypsin but differed in their inhibitory activities towards trypsin, indicating the presence of separate sites for the two enzymes[26]. The Bowman-Birk soybean inhibitor and the lima bean inhibitor also have independent inhibitory sites for trypsin and chymotrypsin, indicating the structural similarities that exist between these inhibitors[31].

Apart from those low molecular weight trypsin inhibitors, in 2005, a trypsin inhibitor from *C. arietinum* with an apparent molecular mass of 20 kDa has also been reported[41]. Representing the Kunitz-type family of inhibitors it showed inhibitory activity exclusively for trypsin and Helicoverpa armigera gut proteases (HGP) but possessed no inhibition towards chymotrypsin. Another 18 kDa protein from *C. arietinum* (CLAP) that resembles cyclophilin-like proteins and possesses antifungal and anti-HIV-1 reverse transcriptase activities have been reported[42]. The insecticidal potential against HGP by a 30 kDa trypsin inhibitor from *C. arietinum* has also been reported[43].

Purification and biochemical studies of trypsin inhibitors from legume seeds especially from *C. arietinum* have been well documented. While few inhibitors tend to show activity exclusively against trypsin and HGP[41-43] having a molecular weight ranging from 18-30 kDa, the earlier reports of inhibitors purified from *C. arietinum* were found to inhibit trypsin and chymotrypsin through different binding mechanisms[21,26,31]. As observed in those earlier reports, *C. arietinum* trypsin inhibitor reported in this study can also be assumed to possess different sites for trypsin and chymotrypsin inhibition as evidenced by the enzyme kinetic results.

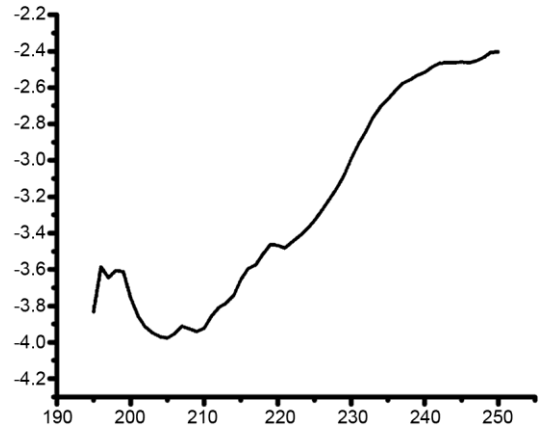

Fig 5 — CD Spectra profile for the purified CATIN from *Cicer arietinum* L. indicating the presence of random coils

CD spectra analysis of CATIN

The standard far UV-CD spectrum predicts the secondary structure content at 25°C with of 1 mg/mL (pH 7.2) CATIN. The negative peak at 195 nm and 200 nm reveals that the protein highly composed of random coils (Fig. 5).

Generally, most of the reported trypsin inhibitors were a beta sheet in nature. Though the CD spectra profile of CATIN is different from that of the existing trypsin inhibitors, a 7 kDa trypsin inhibitor from *Lupinus albus* L. and 17 kDa trypsin/subtilisin inhibitor from *Brassica nigra* L. were found to be composed of a majority of random coil[44-45]. Hence, CATIN can be correlated with these trypsin inhibitors which were further proved from the results of K2D2, online software confirming that CATIN contains only 2.1% of helical nature. The purified CATIN was analysed on a 12% SDS PAGE where a clear band corresponding to approximately 12-13 kDa has been observed (Fig. 6).

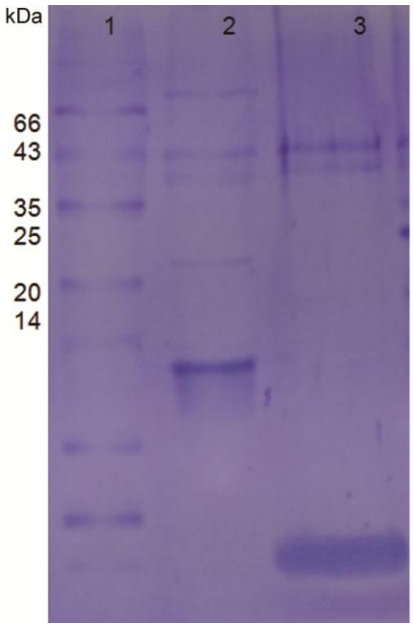

Fig 6 —SDS PAGE 12 % profile of the purified CATIN from *Cicer arietinum* L. Lane 1-Standard molecular marker (Molecular weight in kDa), 2-Bovine pancreatic trypsin and 3-Purified CATIN with a molecular weight of ~ 12-13 kDa

Conclusion

The aqueous crude extract of *Cicer arietinum* L. was tested for its trypsin inhibition activity by the radial diffusion method. After employing the conventional methods of purification steps such as ammonium sulphate precipitation and ion exchange chromatography, the purified protein from *C. arietinum* was tested for its protease inhibition activity. Throughout the purification process trypsin and chymotrypsin inhibition of CATIN was carried out using 1.0% case in as substrate. While the radial diffusion method has demonstrated the significant inhibition of CATIN against trypsin, the enzyme kinetic studies have shown that CATIN exhibits a non competitive and competitive mode of inhibition towards trypsin and chymotrypsin, respectively. In the case of trypsin inhibition, the inhibitor may bind on the other side of the catalytic site of trypsin as V_{max} are varied and Km remained the same. Trypsin inhibitors purified from *Coccinia grandis* L., *Erythrina velutina* and *Jatropha curcas* L. have been found to possess similar kind of non-competitive mode of inhibition[38-40]. As for the chymotrypsin inhibition, both the V_{max} and Km values for the presence and absence of CATIN were varied. Hence, it can be assumed that CATIN exhibits a competitive mode of inhibition towards chymotrypsin. For the trypsin inhibition, V_{max} and Km values were found to be 0.077 µM/min and 26 µM respectively, whereas, for the chymotrypsin inhibition values of V_{max} and Km were found to be 0.063 µM/min and 25 µM, respectively.The double reciprocal Linweiver-B plot (1/S *vs* 1/V) calculated the inhibitory constant Ki as 0.02 µM for trypsin inhibition and 33 µM for chymotrypsin inhibition. These values were obtained from the interception of (V_{max}/Km) on the X axis. The kinetic studies proved that the purified CATIN from *C. arietinum* not only inhibited trypsin but also chymotrypsin. The CD spectrum of the purified inhibitor has revealed that the secondary structure content has been highly composed of random coils which were supported by the reports of other low molecular weight trypsin inhibitors[44-45]. The apparent molecular mass was found to be approximately 12-13 kDa as observed from the electrophoresis result. To conclude, a low molecular weight (~12-13 kDa) serine protease inhibitor possessing both trypsin and chymotrypsin inhibition has been purified and characterized from the seeds of *C. arietinum.*

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