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Structural–functional insights of single and multi-domain *Capsicum annuum* protease inhibitors

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ABSTRACT

Pin-II protease inhibitors (PIs) are the focus of research interest because of their large structural–functional diversity and relevance in plant defense. Two representative *Capsicum annuum* PI genes (*CanPI-15* and *-7*) comprising one and four inhibitory repeat domains, respectively, were expressed and recombinant proteins were characterized. β-Sheet and unordered structure was found predominant in CanPI-15 while -7 also displayed the signatures of polyproline fold, as revealed by circular dichroism studies. Inhibition kinetics against bovine trypsin indicated three times higher potency of CanPI-7 ($K_i \sim 57$ μM) than -15 (~184 μM). Activity and structural stability of these CanPIs were revealed under various conditions of pH, temperature and denaturing agent. Structure prediction, docking studies with proteases and mass spectroscopy revealed the organization of multiple reactive site loops of multi domain PIs in space as well as the steric hindrances imposed while binding to proteases due to their close proximity.

1. Introduction

Proteinase inhibitors (PIs) are widely spread in nature and form complexes with proteases thereby resulting into loss of their proteolytic activity. Serine PIs are well known as plant defense proteins due to their high efficiency in inhibiting digestive proteases of feeding insects and thus imparting anti-nutritional effects on them [1–3]. The Potato inhibitor-II (Pin-II) or Pot-II family of serine PIs, predominantly found in Solanaceae is extensively studied at gene as well as protein level and its functional co-relation to insect defense has been established [4]. The striking feature of Pin-II PIs is the presence of variable number of inhibitory repeat domains (IRDs) (1–8) forming multi-domain precursor proteins. A conserved Pin-II PI protein consists of an endoplasmic reticulum signal peptide of 25 amino acids (aa) followed by variable number of IRDs of ~50 aa which are separated by 5 aa long linkers. The sequence of IRDs is highly variable; however, the presence of eight cysteines, a single proline residue and an active site either for trypsin or chymotrypsin inhibition is conserved throughout IRDs. The cysteines are involved in formation of four disulfide bonds, which stabilize the repeat structure [5,6].

The three-dimensional structures of several Pin-II PIs, single- as well as two-domain, have been determined either by X-ray crystallography or NMR and these give a good outline of the structure and dynamics of this class [5,7–10]. However, there have been few studies on multi-domain inhibitors giving insights into their domain orientations, binding to proteases and stoichiometry [5,11]. Possibly because the precursors PIs are processed at linker region(s) by plant endogenous proteases to release IRD(s) [11], it has been difficult to characterize the multi-domain inhibitors from natural plant sources.

Capsicum annuum Pin-II PIs (CanPIs), displaying high isoform diversity with PIs of 1- to 4-IRDs, have been isolated and characterized to assess their defense potential against Lepidopteran proteases [2,12]. In the present study, single domain CanPI-15 (1-IRD) and four-domain CanPI-7 (4-IRD) were characterized for their protease inhibitory specificity and inhibition kinetics was studied to evaluate binding. Transitions in the structure of rCanPIs under varying conditions were monitored using biophysical techniques. CD spectroscopy, structure prediction and docking studies rendered insight into the conformational stability and/or flexibility, structure and binding mechanisms of multi-domain Pin-II PIs, respectively. We report biochemical and structural reasons for better efficiency of multi-domain inhibitors against target proteases and their high stability imparted by disulfide bonds assigning them key role in insect control strategies.

2. Materials and methods

2.1. Cloning, recombinant expression and purification of CanPIs

The cDNAs encoding the mature peptide region of *CanPIs* (*CanPI-15* and *-7*) were cloned in ligation-independent cloning

Abbreviations: CanPIs, *C. annuum* proteinase inhibitors; CD, circular dichroism; CI, chymotrypsin inhibition; IRDs, inhibitory repeat domains; MRE, mean residual ellipticity; PI, proteinase inhibitor; TI, trypsin inhibition.

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(LIC) compatible expression vector pMCSG7 [13] for recombinant expression in *Escherichia coli*. *E. coli* Origami B (DE3) cells were transformed with pMCSG7-CanPI-15 and pMCSG7-CanPI-7. Single recombinant *E. coli* colonies were initially grown overnight at 37 °C in 10 ml LB medium supplemented with antibiotics (Ampicillin, Kanamycin, and Tetracycline). The pre-culture was used to inoculate 1 L 'Terrific Broth' (TB) medium with appropriate antibiotics and allowed to grow until the OD (600 nm) reached 0.6–0.8. Cells were induced with IPTG (0.5 mM) overnight at 21 °C and harvested by centrifugation.

The cell pellet was solubilized in ice cold cell lysis buffer A (50 mM Tris–HCl, pH 8.0; 300 mM NaCl; 2% glycerol) and disrupted by sonication (0.5 s pulse with 0.5 s intervals for 10 min) using an Ultrasonic Disruptor UD-201 (Tomy, Tokyo). The supernatant and pellets were separately collected by centrifugation for 45 min at 10,000×*g*, 4 °C (RS-4S rotor, Kubota). The supernatant was loaded on Ni–NTA resin (Qiagen, Valencia, CA, USA) and purified using standard affinity chromatography. The fusion protein was eluted with buffer B (50 mM Tris–HCl, pH 8.0; 300 mM NaCl; 2% glycerol; 250 mM imidazole). The His-tag was cleaved using *Tobacco etch virus* (TEV) protease at a protease to target protein ratio of 1:100 (w/w) at RT for 12 h. Additional Ni–NTA purification was performed to remove the cleaved tag and collect the protein in flow through. This was applied on Sephadex S-75 for further purification.

2.2. Inhibitory activity assays and kinetic analysis

Inhibitory assays using rCanPIs against bovine trypsin (50 mM) were performed using chromogenic substrate Benzoyl-L-arginyl-*p*-nitroanilide (BA*p*NA) as detailed in Tamhane et al. [14]. Michaelis–Menton constant (K_m) for trypsin was calculated by using various concentrations of BA*p*NA substrate (1–5 mM). Kinetic properties of rCanPIs were analyzed over a range of concentration of inhibitors (100–1000 μM). IC_{50} and K_i values for each inhibitor were calculated from the sigmoid curve and Cheng–Prusoff's classical equation, respectively [15]:

$$K_i = IC_{50}/(1 + [S]/K_m)$$

2.3. Fluorescence measurements and ANS binding studies

The rCanPI protein samples (100 μg/ml) were incubated in an appropriate buffer over the pH range of 2–10 for 4 h at 28 °C. The following buffers (25 mM) were used for these studies: Glycine–HCl for pH 1–3, acetate for pH 4–5, phosphate for pH 6–7, Tris–HCl for pH 8–9 and Glycine–NaOH for pH 10–12. Appropriate aliquots from samples were used to check for TI activity and fluorescence measurements for binding with 1-anilino-8-naphthalenesulfonate (ANS) were performed on Perkin Elmer LS 50B luminescence spectrometer at 28 °C. ANS is a hydrophobic dye which binds to solvent-exposed hydrophobic regions in a protein and shows increased fluorescence intensity and blue shift in the λ_{max} of emission [16]. The final ANS concentration used was 50 μM, excitation wavelength was 375 nm and total fluorescence emission was monitored between 400 and 550 nm. Slit widths of 7 nm each were set for excitation and emission monochromators and the spectra were recorded at 100 nm/min. Reference spectrum of ANS in the buffer was subtracted from the spectrum of the sample.

2.4. Circular dichroism (CD) measurements

The far UV CD spectra (in wavelength range of 195–300 nm) of rCanPI proteins (125 μg/ml) were recorded on a J-815 spectropolarimeter (Jasco, Tokyo, Japan) at 28 °C in a quartz cuvette. Each

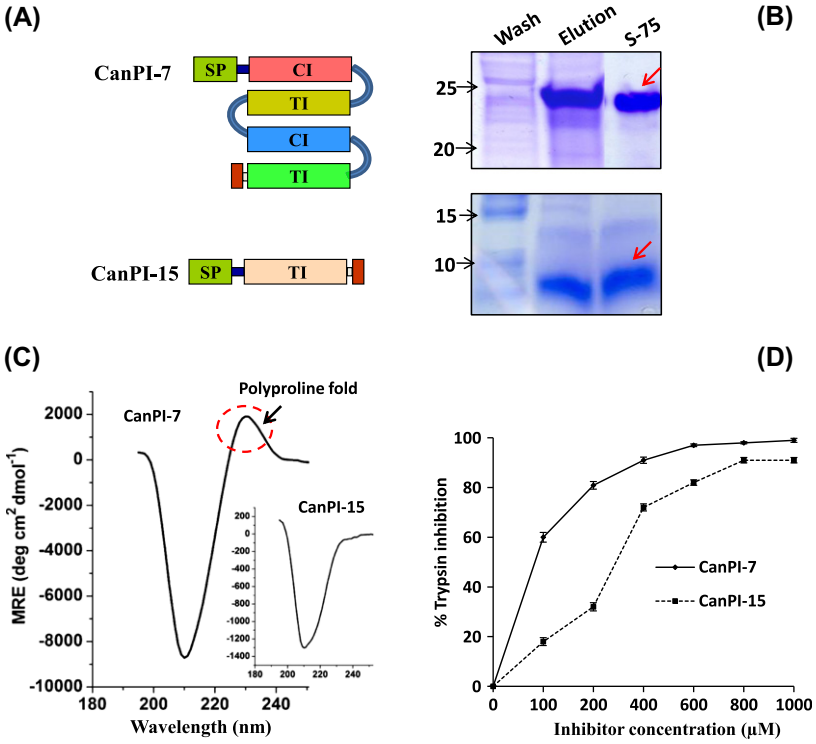

Fig. 1. (A) Diagrammatic representation of the gene structure of two *CanPIs* from *C. annuum*, with their signal peptide (SP), various IRD(s), linker region(s) and the stop codon. The IRDs varying in the aa sequence are shown in different colors. Trypsin inhibitory (TI) domains and chymotrypsin inhibitory (CI) domains are marked. (B) Purified recombinant CanPI-15 (6 kDa) and CanPI-7 (25 kDa) separated on 15% SDS–PAGE and stained with Coomassie Blue R250. Lane 1, wash with 20 mM of imidazole. Lane 2, fusion protein eluted with 250 mM of imidazole. Lane 3, purified through Sephadex-75 column. (C) Far UV CD spectra of rCanPIs. Signatures of polyproline fold observed in rCanPI-7. (D) Inhibition kinetics of CanPI-15 and -7 against bovine trypsin. The inhibition of trypsin follows a sigmoidal pattern with increasing concentration of the inhibitors.