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Research article

Structural and functional characterization of proteinase inhibitors from seeds of *Cajanus cajan* (cv. ICP 7118)

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ABSTRACT

Proteinase inhibitors (C11PI) from mature dry seeds of *Cajanus cajan* (cv. ICP 7118) were purified by chromatography which resulted in 87-fold purification and 7.9% yield. SDS-PAGE, matrix assisted laser desorption ionization time-of-flight (MALDI-TOF/TOF) mass spectrum and two-dimensional (2-D) gel electrophoresis together resolved that C11PI possessed molecular mass of 8385.682 Da and existed as isoinhibitors. However, several of these isoinhibitors exhibited self association tendency to form small oligomers. All the isoinhibitors resolved in Native-PAGE and 2-D gel electrophoresis showed inhibitory activity against bovine pancreatic trypsin and chymotrypsin as well as *Achaea janata* midgut trypsin-like proteases (AjPs), a devastating pest of castor plant. Partial sequences of isoinhibitor (pI 6.0) obtained from MALDI-TOF/TOF analysis and N-terminal sequencing showed 100% homology to Bowman-Birk Inhibitors (BBIs) of leguminous plants. C11PI showed non-competitive inhibition against trypsin and chymotrypsin. A marginal loss (<15%) in C11PI activity against trypsin at 80 °C and basic pH (12.0) was associated with concurrent changes in its far-UV CD spectra. Further, *in vitro* assays demonstrated that C11PI possessed significant inhibitory potential (IC_{50} of 78 ng) against AjPs. On the other hand, *in vivo* leaf coating assays demonstrated that C11PI caused significant mortality rate with concomitant reduction in body weight of both larvae and pupae, prolonged the duration of transition from larva to pupa along with formation of abnormal larval-pupal and pupal-adult intermediates. Being smaller peptides, it is possible to express C11PI in castor to protect them against its devastating pest *A. janata*.

1. Introduction

The castor oil plant, *Ricinus communis*, is a plant species of the Euphorbiaceae family. Castor seed is the source of castor oil which has a wide variety of uses. The seeds contain between 40–60% of oil that is rich in triglycerides, mainly ricinolein. Castor seed oil has special chemical and physical properties. Its bio-degradable and eco-friendly nature makes it a vital industrial raw material for more than 700 industrial products, including high quality lubricants, paints, coatings, plastics, soaps, medications for skin infections and cosmetics (Ogunniyi, 2006). The recent application of castor oil is its use as biofuel for the production of biodiesel with reduced sulfur emission. Further, traditional ayurvedic medicine considered castor oil as the king of medicinals for curing arthritic diseases (Kalaiselvi et al., 2003). It has many therapeutical uses including anti-inflammatory and free radical scavenging activity (Ilavarasan et al., 2006; Saini et al., 2010), anti-diabetic effect (Rao et al., 2010) and hepato-protective activity (Visen et al., 1992).

Among the pests that damage the castor field, *Achaea janata* (castor semilooper) is a major feeder which causes about 30–70% loss in its production. Several recent studies indicated that among pest management methods used for crop protection, development of insect resistance by incorporating genes that express proteins with insecticidal activity is a novel approach (Dunse et al., 2010;

Abbreviations: AjPs, *Achaea janata* midgut trypsin-like proteases; AjPIs, *Achaea janata* midgut trypsin-like proteinase inhibitors; BBIs, Bowman-Birk inhibitors; IEF, isoelectric focusing; MALDI-TOF/TOF MS, matrix assisted laser desorption ionization time-of-flight mass spectrometry; PIs, proteinase inhibitors.

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Smigocki et al., 2013). It is therefore important to select appropriate candidate genes such as proteinase inhibitors (PIs), lectins and amylase inhibitors for expression in transgenic plants to strengthen the plant defense against the target pest in a sustainable manner (Foissac et al., 2000). Among them, PIs which are present in storage organs like seeds and tubers are promising candidates that confer resistance against insect pests (Benjakul et al., 2000). In plants, serine PIs are well documented class of inhibitors and are ubiquitous in nature (Haq et al., 2003). However, most lepidopteran pests like *A. janata*, *Helicoverpa armigera* and *Spodoptera litura* principally depend on serine proteases for digestion of proteins consumed through food (Chikate et al., 2013; Srinivasan et al., 2006; Telang et al., 2003; Budatha et al., 2008). When ingested by larvae, PIs inhibit digestive proteases leading to starvation of the insect for essential amino acids (Giri et al., 2004). The insects respond to this situation by overproducing gut proteases to compensate for the inhibited activity of digestive proteases. But, synthesis of additional proteases further deplete the pool of essential amino acids and result in developmental abnormalities and growth retardation (Oliva et al., 2010). Further, during the process of this co-evolution between plants and insects, adaptation of insects against plant PIs is the main limitation for the PI based defense strategy. Insect herbivores have developed multiple adaptive mechanisms viz. (i) over expression of PI-sensitive enzymes (De Leo et al., 1998); (ii) proteolytic cleavage of PIs by insect proteases (Yang et al., 2009); (iii) expression of proteases that are insensitive to inhibition by PIs (Brito et al., 2001). However, to combat this multifaceted adaptive mechanism by the insects, the identification of potential PIs which could target insect's digestive proteases from non-host plants is essential and would definitely make the insect pests adaptation more difficult (Lopes et al., 2004).

Thus, during this current scenario of host-pest co-evolution, pigeonpea (*Cajanus cajan*), a crop plant of the order Fabales, family Leguminosae has been reported as the potential source of (PIs) against wide varieties of lepidopteran larvae (Chougule et al., 2003; Lomate and Hivrale, 2011; Padul et al., 2012; Parde et al., 2012; Prasad et al., 2010a, 2010b, 2009). Earlier reports suggested that different varieties of *C. cajan* possessed PIs resistant to proteases of *A. janata* (Prasad et al., 2010a, 2009). The cultivars ICP 14770 and ICP 7118 of *C. cajan* were found to be the best non-host resource of potential PIs effective against gut proteases of *A. janata* (Prasad et al., 2010a, 2009; Swathi et al., 2012). The PIs from ICP 14770 were found to belong to BBI family (Prasad et al., 2010b). However, some of the earlier reports indicated the existence of Kunitz inhibitors in *C. cajan* (Haq and Khan, 2003). Therefore, in the present study, we purified the PIs from ICP 7118 and, characterized structurally and functionally to reconfirm that the PIs which were effective in controlling *A. janata* belonged to BBI family.

2. Methods

2.1. Seed material and chemicals

Seeds of *C. cajan* (cv. ICP 7118 or C11) were obtained from International Crops Research Institute for Semi-Arid Tropics (ICRISAT). *A. janata* insects were obtained from Directorate of Oil seeds Research (DOR), Hyderabad, India. Bovine serum albumin (BSA), bovine pancreatic trypsin and α-chymotrypsin were procured from Sisco Research Laboratory (Mumbai, India). DEAE-cellulose, cyanogen bromide-activated-Sepharose 4B, Sephadex G-50, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), *N*-glutaryl-L-phenylalanine-*p*-nitroanilide (GLUPHEPA), BBI, sorbitol, tricine, gelatin and Coomassie Brilliant Blue (CBB) R-250 were purchased from Sigma (St. Louis, MO). Immobiline dry strips (pH 4–7 linear, 11 cm), IPG buffer (4–7 linear), dithiothreitol (DTT) and iodoacetamide (IDA) were procured from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Bicinchoninic acid (BCA) protein estimation kit was purchased from Thermo Scientific (USA). Amicon filters (3 kDa cut-off) were purchased from Millipore Corporation, USA. All other chemicals and reagents used were of analytical grade.

2.2. Crude protein preparation and purification of C11PI

The seed powder prepared from mature dry seeds was extracted in to 50 mM Tris–HCl, pH 8.0 containing 1% polyvinylpyrrolidone in 1:6 (w/v) ratio under mild continuous stirring for overnight at 4 °C as described in Prasad et al. (2009). The clear supernatant obtained after centrifuging twice at 10,000 rpm for 20 min (4 °C) was subjected to 0–25%, 25–75% and 75–100% $(NH_4)_2SO_4$ fractionation for 1 h (4 °C). The corresponding precipitates were dialyzed (3.0 kDa cut-off) against 50 mM Tris–HCl pH 8.0 and estimated for protein (Smith et al., 1985) as well as trypsin inhibitor (TI) activity. The 25–75% $(NH_4)_2SO_4$ fraction with maximum TI activity was purified by passing sequentially through DEAE-cellulose column, cyanogen bromide-activated-trypsin Sepharose 4B column and Sephadex G-50 column using AKTA prime plus (GE healthcare) fast protein liquid chromatography (FPLC) system. The eluted fractions (1.0 ml) from different chromatography columns were analyzed for total protein (A_{280}) and TI activity. The protein fractions showing significant TI activity were pooled, dialyzed, concentrated using Freeze dryer (Labconco)/Amicon filters (3.0 kDa cut-off) and stored as "C11PI" at −20 °C for further use.

2.3. Proteinase inhibitor assay and determination of inhibitor constant (K_i)

The inhibitory activity of C11PI was evaluated against pancreatic trypsin/chymotrypsin/*A. janata* midgut trypsin-like proteases (AjPs), respectively, as described in Prasad et al. (2010b). After addition of respective proteases, the assay mixture was incubated for 15 min at 37 °C. The residual protease activity was determined after 45 min incubation at 37 °C with 1 mM BAPNA (Erlanger et al., 1961) or GLUPHEPA (Mueller and Weder, 1989) and the reaction was terminated with 30% acetic acid (v/v). One TI/chymotrypsin inhibitor (CI)/*A. janata* midgut trypsin-like proteinase inhibitor (AjPI) unit was defined as the amount of C11PI required to inhibit 50% of the BAPNA or GLUPHEPA hydrolysis by trypsin/AjPs and chymotrypsin, respectively. The K_i values of C11PI against trypsin and chymotrypsin were determined by pre-incubating the respective enzymes with increasing concentrations of C11PI [20, 50 and 100 nM for trypsin (or) 500, 2000 and 5000 nM for chymotrypsin] for 15 min followed by 45 min incubation at 37 °C with different concentrations of BAPNA or GLUPHEPA (0.125, 0.165, 0.250, 0.375, 0.500, 0.625 and 0.750 mM), respectively. The K_i values were estimated from the Lineweaver–Burk plots using Sigma Plot 11.0, Enzyme Kinetics Module 1.3 (Systat Software Inc., San Jose, California, USA).

2.4. Electrophoresis

Tricine-SDS-PAGE was performed using 4% stacking gel and 15% separating gel as described by Schagger and Jagow (1987) under reducing and non-reducing conditions. C11PI was reduced with 50 mM DTT at 56 °C for 1 h followed by alkylation with 2-fold molar excess of IDA (100 mM) for 45 min in dark at room temperature (25 °C). The protein molecular mass standards (Puregene, Genetix, India) ranging from 4.6 to 180 kDa were used. Also, commercially available purified soybean trypsin chymotrypsin inhibitor (Bowman-Birk Inhibitor, BBI) with molecular mass 8.0 kDa

prepared in 50 mM Tris–HCl pH 8.0 was loaded (5 μg/μl) as a reference protein. Native-PAGE was carried out in 4% stacking and 12.5% separating gels as described by Laemmli (1970). Proteins were detected by staining with either CBB R-250 (0.1%) or silver nitrate.

The TI/CI/AjPI bands were visualized in activity staining gels by performing gelatin-Native-PAGE or gelatin-SDS-PAGE (Felicioli et al., 1997). However, the gels performed under denaturing conditions were washed thoroughly with 2.5% Triton-X-100 to remove SDS. After hydrolysis of gelatin by corresponding proteases, i.e., trypin/chymotrypsin/AjPs, the gel was washed with distilled water to remove the excess enzymes and stained with CBB R-250. The presence of TI/CI/AjPI bands were identified by the appearance of dark blue bands in a clear background due to complex formation of the unhydrolyzed gelatin with stain.

In two-dimensional (2-D) gel electrophoresis, Isoelectric focusing (IEF) was performed with Immobilized pH gradient (IPG) strips pH 4–7 (linear) using Ettan IPGPhor 3 IEF system (GE Healthcare) following the manufacturer's instructions. IPG strips were rehydrated overnight with C11PI contained in either IEF rehydration buffer (7.0 M urea, 2.0 M thiourea, 4% CHAPS and 40 mM DTT) for reducing conditions or 10% sorbitol for non-reducing conditions along with 1.0% IPG buffer. IEF was performed at maximum current setting of 75 μA per strip by following the manufacturer's instructions. After IEF, the second dimension was performed by tricine-SDS-PAGE or Gelatin-SDS-PAGE as described above. However, under reducing conditions after IEF, each strip was equilibrated separately with DTT (50 mM) and IDA (100 mM) for 20 min each in equilibration buffer followed by 1x running buffer for 10 min.

2.5. Mass spectrometry and N-terminal sequencing

The peptide sequence (in-gel digestion) and the molecular mass (intact mass analysis) of C11PI either in non-reducing or reducing conditions was determined by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/TOF), by using a Bruker Daltonics Autoflex III smart beam instrument (Bruker Daltonics, Bremen, Germany) equipped with Nd:YAG-laser (355 nm) and operated in linear mode for intact mass analysis, and reflectron mode for peptide mass sequencing with α-cyano-4-hydroxy-cinnamic acid (CHCA) matrix according to the method described by Shevchenko et al. (1996) with minor modifications. The gel plug of C11PI isoinhibitor (pI 6.0) was reduced and alkylated with 10 mM DTT and 55 mM IDA before subjecting to digestion with either trypsin (12.5 μg/μl) or Lys-C (100 ng/μl). The supernatant containing the peptide mixture was desalted using C-18 ZipTip™ (Millipore Corporation, USA), concentrated using speed vac and analyzed by MALDI-TOF-TOF in CHCA matrix. The spectra from MALDI-MS and MALDI-MS-MS ionization were searched using Mascot search engine or Biotools (Bruker Daltonics, version 3.1). Further, the isoinhibitor (pI 6.0) of C11PI was electroeluted into 10 mM CAPS buffer from PVDF membrane as described in Prasad et al. (2010b) and N-terminus was sequenced by Edman's degradation method using Applied Biosystems Procise Sequencer (Model No. 492).

2.6. Stability of C11PI and circular dichroism (CD) spectroscopy

The effect of temperature on TI activity of C11PI was evaluated by incubating at a wide range of temperatures (20 °C, 37 °C, 60 °C and 80 °C) for 30 min. After cooling the samples to room temperature (25 °C), the residual TI activity was assayed at 37 °C in presence of BAPNA as described above (Section 2.3). The effect of pH on TI activity of C11PI was evaluated by incubating in the following buffers at a final concentration of 50 mM: glycine-HCl (pH 2.0), Tris–HCl (pH 8.0) and glycine-NaOH (pH 12.0), respectively. After incubation at 37 °C for 1 h in the respective buffers, the residual TI activity was assayed at pH 8.2 as indicated above.

CD spectroscopy was used to determine the secondary structure of C11PI at far-UV (190–250 nm) using JASCO J-810 spectropolarimeter at a scan speed of 50 nm/min. The entire instrument including the sample chamber was constantly flushed with nitrogen gas during the operation. Buffer scans were recorded under the same conditions and subtracted from the spectra of C11PI before further analysis. The data analysis of CD spectra was performed using Spectra Manager – II™ software.

2.7. Rearing of A. janata insects and assay of larval gut enzymes

The egg masses of *A. janata* were allowed to hatch on a moist filter paper and reared in clean plastic tubs. The larvae were fed with fresh castor leaves every day. The culture was maintained in insect culture room at 25 ± 1 °C temperature, 60 ± 5% relative humidity and 14:10 h light–dark photoperiod.

The midguts from $4^{th}/5^{th}$ instar larvae were dissected into iso-osmotic saline (0.15 M NaCl) solution as described in Girard et al. (1998). The gut tissue was homogenized in 50 mM Tris–HCl containing 20 mM $CaCl_2$ (pH 8.2) and centrifuged twice at 12,000 rpm for 10 min at 4 °C. The supernatant containing midgut proteases was collected and stored at −20 °C for AjPs and AjPI assays. The assay for AjPs (pH 8.2) and chymotrypsin-like (pH 7.8) proteases was carried out as described in Budatha et al. (2008) using BAPNA and GLUPHEPA as chromogenic substrates. One protease unit (trypsin-like/chymotrypsin-like) is defined as the amount of enzyme that increases the absorbance by 1.0 optical density after 45 min incubation. The molar extinction coefficient (M^{-1} cm^{-1}) for *p*-nitroanilide at 410 nm is equivalent to 8800.

2.8. In vivo effect of C11PI on growth and development of A. janata

The effect of C11PI on *A. janata* growth and development was examined by performing the leaf coating assays. Fresh castor leaves were coated with different concentrations (2, 4 and 8 μg/cm^2 leaf area) of C11PI and the control leaf was coated with 50 mM Tris–HCl, pH 8.0. The second instar larvae of *A. janata* ($n = 20$) were allowed to grow either on the control leaf or the treated leaf. The mortality rate along with morphological changes between control and treated were recorded through photographs as well as by monitoring the larval/pupal weights at regular time intervals.

2.9. Statistical analysis

All experiments were carried out at least three times each with three replications, and the mean ± SE was represented. Statistical differences were determined by one-way ANOVA followed by Tukey test at $P < 0.05$ using Sigma-Plot, version 11.0, software (San Jose, CA, USA).

3. Results and discussion

3.1. Purification of proteinase inhibitors

The proteins from 25–75% $(NH_4)_2SO_4$ precipitated fraction with maximum TI activity were resolved into two peaks with a linear gradient (5 ml/min) of 0.1–1.0 M NaCl in 50 mM Tris–HCl pH 8.0 on DEAE-cellulose column (Fig. 1A). Protein fractions (peak I) with TI activity were further resolved in to two peaks when applied on to a trypsin-Sepharose 4B affinity column. The bound proteins were

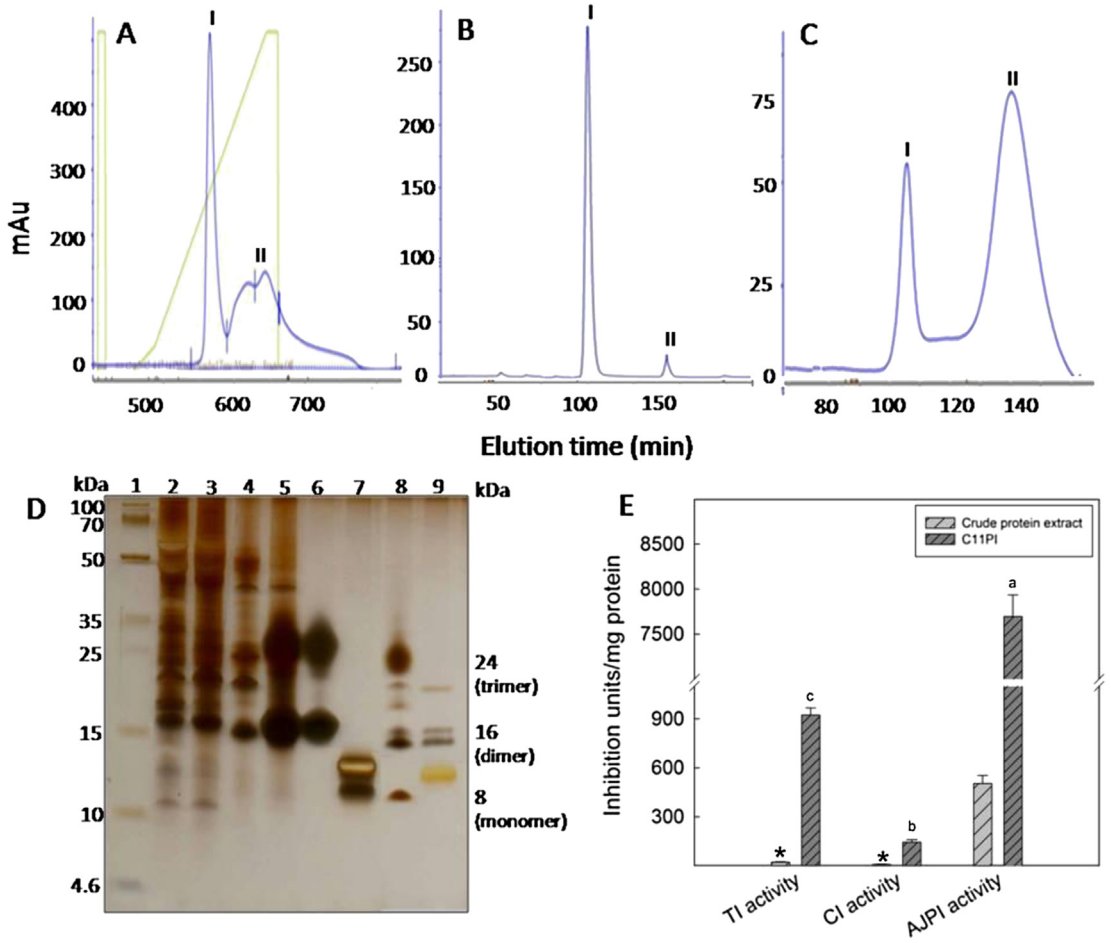


Fig. 1. Purification pattern and inhibitory activity of C11PI. Elution profiles of (A) DEAE-cellulose column loaded with 25–75% $(NH_4)_2SO_4$ fraction; (B) trypsin-Sepharose 4B column loaded with peak I fraction of ion-exchange column; (C) Sephadex G-50 column loaded with peak II fraction of affinity column. The eluted peak II fractions with TI activity were pooled and named as 'C11PI'; (D) Tricine SDS-PAGE (15%) showing different fractions of purification: lane 1, molecular mass standards; lane 2, crude protein (20 μg); lane 3, 25–75% $(NH_4)_2SO_4$ fraction (20 μg); lane 4, peak I fractions of DEAE-cellulose column (20 μg); lane 5, peak II fractions of trypsin-Sepharose 4B column (10 μg); lane 6, peak II fractions (C11PI) of Sephadex G-50 column (2.5 μg); lane 7, C11PI (2.5 μg) after reduction (50 mM DTT) and alkylation (100 mM IDA); lane 8, Commercially available purified soybean BBI (5 μg); lane 9, Soybean BBI (5 μg) after reduction (50 mM DTT) and alkylation (100 mM IDA). The molecular mass and self association pattern of soybean BBI is shown on right side of the gel; (E) Inhibitory activities of C11 crude protein and C11PI against bovine pancreatic trypsin, chymotrypsin and AjPs, respectively. The represented values are mean ± SE of three independent experiments each with three replications. Different lowercase alphabetical letters indicate statistically significant difference ($P < 0.05$). *The TI and CI activities of C11 crude protein are not clearly visible.

eluted (1 ml/min) with 0.01 N HCl and subsequently neutralized with 2.0 M Tris-base (Fig. 1B). Affinity chromatography was proven to be a very convenient way of purifying PIs (Macedo et al., 2000; Prasad et al., 2010b). Minor contaminants present in active fractions (peak II) from affinity column were eliminated by performing size exclusion chromatography using Sephadex G-50 column (Fig. 1C and D). The peak II fractions collected in 50 mM Tris–HCl pH 8.0 (0.5 ml/min) with prominent TI activity were pooled and here by referred as 'C11PI', and used in subsequent characterization studies. The present protocol resulted in higher purification fold of C11PI (87-fold) when compared to RgPI (66.5-fold) from ICP 14770, though there was reduction in the yield of C11PI (7.9%) when compared to RgPI (55%). The wide differences in purification fold and yield recovery between C11PI and RgPI could have possibly arisen due to variation in variety and $(NH_4)_2SO_4$ fraction used for purification along with handling of purification protocol through FPLC and manual columns, respectively (Table 1; Prasad et al., 2010b).

Table 1
Purification of proteinase inhibitors from *Cajanus cajan* (cv. ICP 7118 or C11) seeds.

Purification step	Total protein (mg)	Total activity (TI units)[a]	Yield recovery (%)	Specific activity[b] (TI units/mg protein)	Purification (fold)
Crude extract	5760	60,768	100.0	10.6	1.0
$(NH_4)_2SO_4$ fraction (25–75%)	1235	37,963	62.5	30.7	2.9
DEAE-cellulose column	116	11,223	18.5	96.7	9.1
Trypsin-Sepharose 4B column	20.4	5,192	8.5	254.5	24.0
Sephadex G-50 column	5.2	4,800	7.9	923.0	87.0

[a] One TI unit is defined as the amount of C11PI required to inhibit 50% of BAPNA hydrolysis by trypsin.
[b] Specific activity is defined as the number of TI units/mg of protein.

3.2. Inhibitory activity against serine proteases

C11PI exhibited significant inhibitory activity against trypsin (923 ± 43 TI units/mg protein) and chymotrypsin (144 ± 15 CI units/mg protein) when compared to crude protein which showed 22 ± 2.5 TI units/mg protein and 8.0 ± 1.2 CI units/mg protein, respectively (Fig. 1E). This coupled activity of C11PI against trypsin and chymotrypsin is possibly due to the existence of two tandem homologous inhibitory domains on the same polypeptide chain, which is a distinctive feature of BBI type PIs (Catalano et al., 2003). The Lineweaver–Burk plots indicated that C11PI interacts with these proteases in a non-competitive manner (Prasad et al., 2010b; Yan et al., 2009; Satheesh and Murugan, 2012; Prasad et al., 2010c). However, the affinity of C11PI towards trypsin (K_m value 0.62 ± 0.04) was >4.0-fold higher when compared to chymotrypsin (K_m value 2.71 ± 0.56). Further, the observed K_i values of C11PI against trypsin (K_i value 272 ± 18.7 nM) and chymotrypsin (K_i value 3725 ± 204 nM) are well within the range (0.1–52000 nM) observed earlier with several other leguminous BBIs (Zhang et al., 2008; Scarafoni et al., 2008). The stoichiometry ratio of C11PI with trypsin was found to be 0.5 which indicate that one molecule of C11PI can bind to two molecules of trypsin. But, such precise stoichiometric ratio was not shown by C11PI with chymotrypsin (data not shown). Furthermore, C11PI also showed significant AjPI activity (7696 ± 240 AjPI units/mg protein), which was 8.0-fold higher when compared to TI activity. These results indicate that C11PI is a potent inhibitor of AjPs analogous to bovine pancreatic trypsin and chymotrypsin.

3.3. Intact mass analysis

Electrophoretic separation of C11PI in SDS-PAGE showed two bands with molecular masses of ~16 kDa and ~25 kDa under non-reducing conditions and two bands with molecular masses of >9 kDa under reducing conditions, which correlated well with commercially available purified soybean BBI (Fig. 1D). Further, MALDI-TOF/TOF analysis revealed four peaks with 8385.682, 16771.148, 25157.323 and 33542.540 Da, respectively under non-reducing and one peak with 9195.702 Da under reducing conditions (Fig. 2A–D). These results suggest that C11P1 existed as small oligomers/isoinhibitors under native conditions which is essential for its molecular packing as a storage protein in seeds (Prasad et al., 2010b; Barbosa et al., 2007). The self association tendency of BBIs

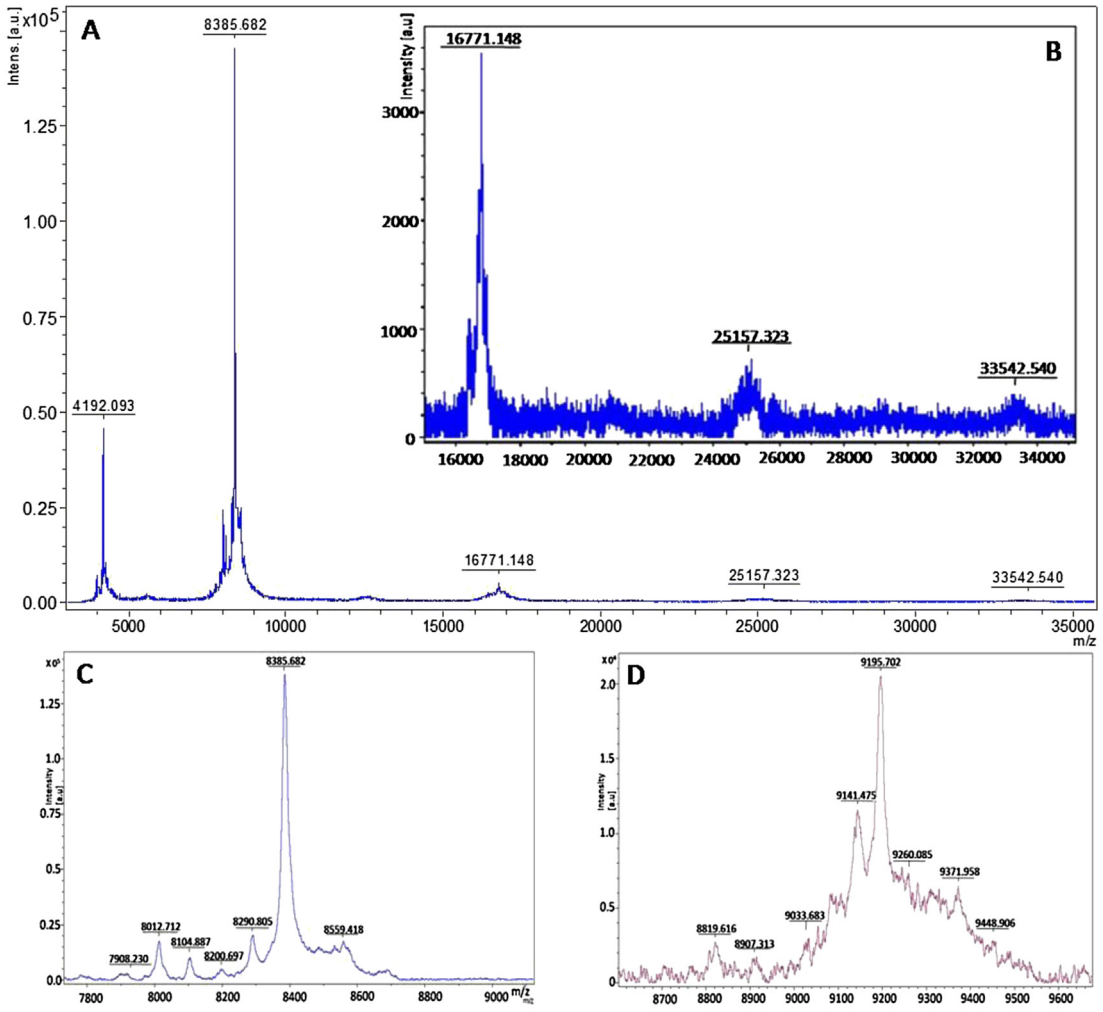


Fig. 2. MALDI-TOF mass spectrum of C11PI under non-reducing conditions between: (A) 4000–35,000 *m*/*z* and (B) 16000–35,000 *m*/*z* representing dimer (16771.148 Da), trimer (25157.323 Da), tetramer (33542.540 Da) of C11 monomer (8385.682 Da). Further, C11PI existed as several isoinhibitors under both (C) non-reducing and (D) reducing conditions. The shift in the mass of monomers by 810 Da after reduction and alkylation indicate that C11PI possessed 14 cysteine residues. The mass spectra shown here are representative of C11PI purified from at least three to four different batches of seeds.

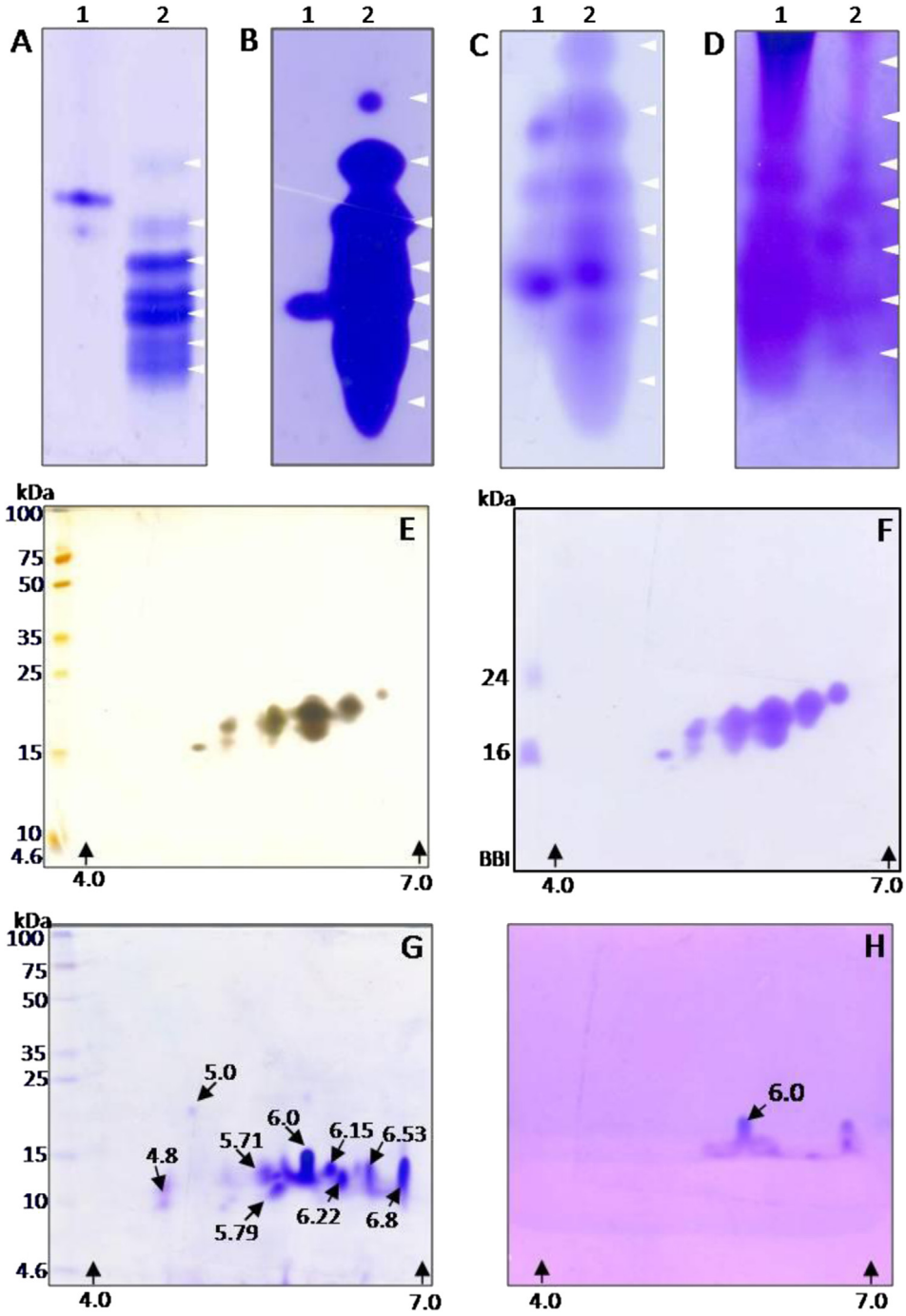


Fig. 3. (A) Isoinhibitors of C11PI (50 μg) separated in one-dimensional native electrophoresis and gelatin activity staining of corresponding isoinhibitors against: (B) bovine pancreatic trypsin, (C) bovine pancreatic chymotrypsin and (D) AjPs; (E) Isoinhibitors of C11PI (30 μg) separated in 2-D gel electrophoresis at IEF (pH 4–7 L) under native conditions and (F) gelatin activity staining of corresponding isoinhibitors against bovine pancreatic trypsin; (G) Isoinhibitors of C11PI (100 μg) separated under reducing conditions at IEF (pH 4–7 L) and (H) gelatin activity staining of corresponding isoinhibitors against AjPs. Second dimension was performed using SDS-PAGE (15%). Gels were stained with CBB R-250 or silver nitrate. Soybean BBI was used as a standard. Further details are described in materials and methods. Each gel shown here is a selective representative from three to four replicates of C11PI purified from at least three to four independent batches of seeds.

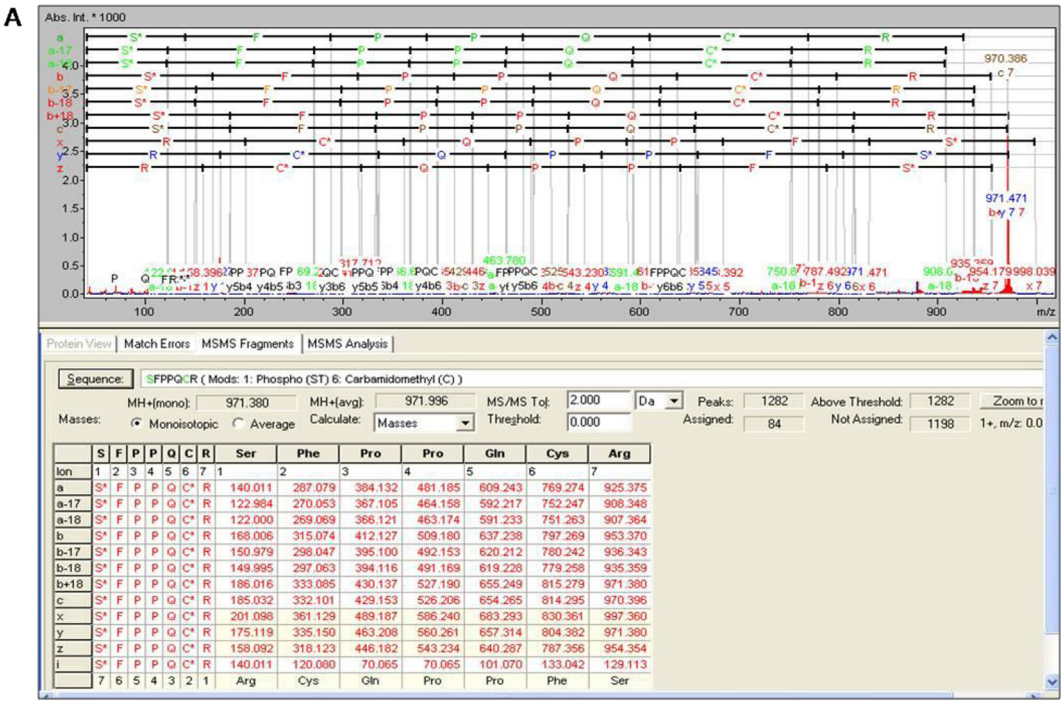
was suggested through formation of strong network of hydrogen bonds possibly due to (i) exposed hydrophobic surface patches with buried charged residues and (ii) rigid, constrained and electrically charged monomeric structure stabilized through disulphide bonds (Barbosa et al., 2007). The hydrogen bond formed between N-terminal K^{24} (P_1) of one monomer and C-terminal D^{76} of the other monomer was responsible for dimer formation or self association behavior of BBIs in horse gram. Further, the superimposition of monomers also did not induce any steric hindrances to interact with trypsin molecules in dimers (Kumar et al., 2004). In the present study, the increase in molecular mass of monomers by 810 Da (57 Da for each cysteine residue) under reducing conditions indicate the existence of seven disulfide bonds in C11PI to maintain the 3-D structure of monomers and thereby hydrogen bonds between individual monomers which stabilize their self association behavior (Fig. 2A and D).

3.4. Detection of isoinhibitors

BBIs are the products of multi-gene families and the derivation of multiple isoforms has been associated with protein processing at both the amino and carboxylic ends (Domoney et al., 1995). It has been also reported that in a co-evolving system of plants and insects, both of them evolve with new forms of PIs and proteases as a defense strategy to counteract each other. Therefore it is not surprising that C11PI existed as several isoinhibitors (Figs. 1D and 2C and D), which was also evident through native PAGE and 2-D gel electrophoresis (Fig. 3A, E and G). All the isoinhibitors resolved in native PAGE exhibited inhibitory activities against trypsin, chymotrypsin and AjPs (Fig. 3B–D). The isoelectric point (pI) of these various isoinhibitors ranged from 4.8 to 6.8 (Fig. 3E and H) and this broad pI range in BBIs could have arisen due to the anomalous distribution of polar and non-polar amino acid residues in their primary structure (Losso, 2008). All the isoforms (dimeric/trimeric) resolved in 2-D gel electrophoresis under native conditions or reducing conditions showed significant inhibitory activity against trypsin/AjPs (Fig. 3F and H). BBI was suggested as thermodynamically unfavorable for the protein to exist as monomer in solution. Therefore, all the resolved isoinhibitors were restricted to exist as dimer/trimer which is evident by SDS-PAGE and MALDI-TOF/TOF (Figs. 1D, 2A and 2B and 3E) (Prasad et al., 2010b; Campos et al., 2004; Paiva et al., 2006). However, the electrophoretic migration of isoinhibitors under reducing conditions was comparatively slow possibly due to incomplete reduction of disulphide bonds resulting in different three dimensional shapes of the C11PIs, which was found to be very common among BBIs (Fig. 3H); (Bergeron and Nielsen, 1993).

3.5. Determination of partial amino acid sequences

The isoinhibitor (pI 6.0) which showed relatively high inhibitory potential against AjPs was subjected to MALDI-TOF/TOF analysis and N-terminal sequencing (Fig. 3H). MALDI-MS-MS ionization of



PI plant source	Accession No. (NCBI)	Sequence	(%) Similarity with C11PI
***Cajanus cajan* (C11)**		**SFPPQCR**	
Lupinus albus	P85172.1	SFPPQCR	100
Medicago scutellata	P80321.2	SFPPQCR	100
Medicago truncatula	XP_003623935.1	SFPPQCR	100
Aegilops tauschii	EMT15035.1	SIPPQCR	86
Arachis hypogaea	AAP93913.1	SNPPQCR	86
Glycine max	NP_001237767.1	SIPPQCR	86
Lathyrus sativus	ADV40045.1	SYPPQCR	86
Medicago sativa	CAA56254.1	SIPPQCR	86
Triticum urartu	EMS65202.1	SFPPMCR	86

Fig. 4. (A) Lift spectrum and Biotools display of peak *m/z* 970.386 from tryptic digested C11PI isoinhibitor (pI 6.0). ClustalW2 alignment of internal peptide sequences: (B) 'SFPPQCR' obtained from tryptic digested C11PI for *m/z* 970.386 and (C) 'MELMNKKVMMK' obtained from PMF after Lys-C digestion of C11PI, which showed similarity with several BBIs from NCBI database. (D) N-terminal sequence of C11PI obtained after Edman's degradation exhibiting similarity with several BBIs: RgPI, *Cajanus cajan* (ICP 14770) (Prasad et al., 2010b); DE-3, DE-4, *Macrotyloma axillare* BBI (Joubert et al., 1979); PcBBI1, *Phaseolus coccineus* BBI (Pereira et al., 2007); TBPI, *Phaseolus acutifolius* BBI (Campos et al., 2004); CLTI-I, *Canavalia lineata* BBI (Terada et al., 1994); HGI-3, *Dolichos biflorus* BBI (Sreerama et al., 1997); MBTI-F, *Vigna radiata* BBI (Wilson and Chen, 1983); AB-I, *Vigna angularis* BBI (Ishikawa et al., 1985); CMTI2, *Cratylia mollis* BBI (Paiva et al., 2006). The lift spectrum and PMF shown here is a representative of C11PI purified from at least three to four different batches of seeds.

the peak *m*/*z* 970.386 from trypsin digestion revealed the sequence 'SFPPQCR' in Biotools, which showed 86–100% identity to the conserved region of chymotrypsin reactive site of various BBIs in NCBI database (Fig. 4A and B; Supplementary Fig. 1A and B). The peptide mass fingerprint (PMF) obtained after Lys-C digestion of C11PI using Mascot PMF search revealed the sequence 'MELMNKKVMMK' which showed 45–100% similarity to the signal sequence of several BBIs (Fig. 4C; Supplementary Fig. 2). Though the existence of several BBI analogs across the plant kingdom brings about several differences in terms of amino acid sequence, but in most cases the reactive site loop residues are conserved. Further, the N-terminal sequence 'DQHHSSKACCDECRCTKKIP' also exhibited 60–100% similarity to BBIs (Fig. 4D). Thus, the partial amino acid sequences obtained in the present study further confirm that C11PI belong to BBI-type inhibitors of leguminous plants.

3.6. Effect of temperature and pH on trypsin inhibitory activity and secondary structure

The TI activity of C11PI was stable over a wide range of temperatures (20 °C–60 °C) and pH (2.0–8.0) possibly due to the (i) structural stability provided by disulfide bonds (Qi et al., 2005) and (ii) shielding of exposed hydrophobic residues of the monomers from the solvent by other subunits in the oligomer (Kumar et al., 2004). The secondary spectrum taken at far-UV (195–240 nm) revealed the predominance of β-sheets (62 ± 2%) and random coils (27 ± 2%) over β-turns (10 ± 0.5%). However, a marginal loss (15%) in TI activity of C11PI was observed when incubated at 80 °C or at basic pH (12.0), which could have arisen due to minor changes in native structural conformations caused by slow unfolding of 3-D structure and/or electrostatic repulsions, respectively (Catalano et al., 2003; Barbosa et al., 2007). Further, the ellipticity at 203 nm increased marginally at 80 °C and basic pH as evident from the far-UV secondary spectrum (Supplementary Fig. 3A and B). These conformational changes induced by temperature and pH are known to be reversible (Prasad et al., 2010b, 2010c), perhaps due to the structural flexibility provided by random coils. The absence of α-helices, which is also indicated in the present study is the characteristic feature of BBIs and their analogs (Losso, 2008).

3.7. Evaluation of insecticidal activity against A. janata

The role of PIs in combating against insect pests is well documented (Tripathi et al., 2014; Stevens et al., 2013; Rufino et al., 2013). The use of PIs in insect control strategies primarily depended on inhibition of digestive gut proteases of the target insects (Bhattacharyya et al., 2007; Chougule et al., 2008). The trypsin and chymotrypsin are the major proteases that contribute to more than 95% of the total digestive process in larval gut (Srinivasan et al., 2006). In view of the 100-fold difference between trypsin-like (80 nM min^{-1} mg^{-1} protein) and chymotrypsin-like (0.8 nM min^{-1} mg^{-1} protein) enzyme activities in $4^{th}/5^{th}$ instar larvae of *A. janata*, the evaluation of inhibitory potential of C11PI against these proteases was restricted to AjPs alone (Fig. 5A); (Prasad et al., 2009; Swathi et al., 2012). C11PI

C

PI plant source	Accession No. (NCBI)	Sequence	(%) Similarity with C11PI
Cajanus cajan (c11)		**MELMNKKVMMK**	
Pisum sativum	CAC93854.1	MELMNKKVMMK	100
Lathyrus sativus	ADV40041.1	MELMNKKAMMK	91
Lens culinaris	4450.1	MVLMNKKTMMK	82
Lens ervoides	CAH04453.1	MVLMNKKTMMK	82
Lens nigricans	CAH04454.1	MVLMNKKTMMK	82
Lens orientalis	CAH04448.1	MVLMNKKTMMK	82
Apios Americana	BAF50740.1	ME-MKKKVVMK	73
Medicago sativa	CAA56254.1	MELMMNKKAMM	54
Medicago truncatula	XP003623935.1	MVLMMNKKAMM	45

D

Protein	Initial Position	Sequence	(%) Similarity with C11PI
C11PI	**1**	**DQ-HHSSKACCDECRCTKKIP**	
RgPI	1	DQ-HHSSKACC----------	100
DE-3	7	DEPSESSKPCCDECACTKSIP	70
PCBBI	1	DEPSESSKACCDHCACTKSIP	70
TBPI	10	DEPSESSKACCDHCACTKSIP	70
CLTI-1	4	DDESESSKPCCDECKCTKSEP	65
DE-4	6	DGSSESSKPCCDLCTCTKSIP	65
HGI-3	7	DEPSESSKPCCDQCTCTKSIP	65
MBTI-F	9	DEPSESSEPCCDSCRCTKSIP	65
AB-I	9	DEPSESSEPCCDLCLCTKSIP	60
CMTI2	6	DE-SESSKPCCDKCQCTKSEP	60

Fig. 4. (*continued*).

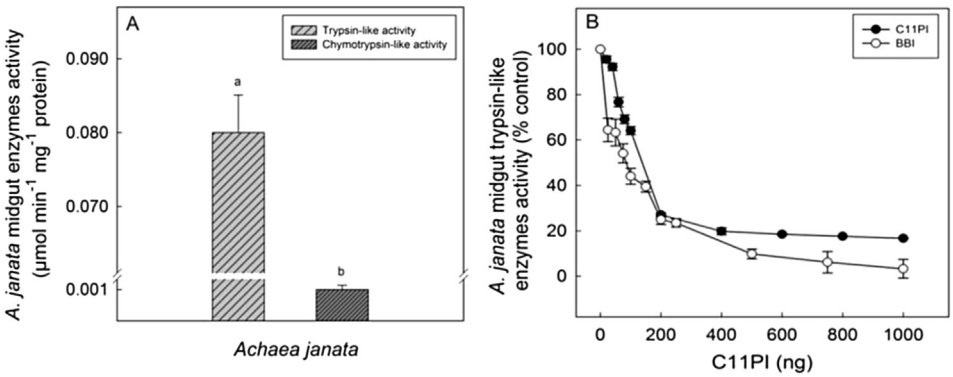


Fig. 5. *In vitro* inhibitory potential of C11PI against AjPs. (A) Trypsin-like and chymotrypsin-like protease activities in the midgut extracts of *A. janata*. Protease activities were measured as described in materials and methods; (B) Concentration dependent (5–1000 ng) inhibitory potential of C11PI against AjPs. Soybean BBI was used as a standard. The values shown here are mean ± SE of three independent experiments each with three replications. Different lowercase alphabetical letters indicate statistically significant difference ($P < 0.05$).

inhibited 50% of AjPs activity (IC_{50}) at 78 ng, corroborating with activity (IC_{50}) shown by soybean BBI at 70 ng (Fig. 5B). Further, the antibiosis of C11PI on larval growth and development was tested in a dose and time dependant manner using second instar larvae of *A. janata* ($n = 20$). After 10 days of growth, the weights of the larvae fed on leaves coated with C11PI (2–8 µg/cm^2) decreased drastically up to 55–71% of their controls (Fig. 6A and B). Similarly, the pupal weights of the respective larvae fed on C11PI (2–8 µg/cm^2) also decreased by 33–55% when compared to their controls which is statistically significant with $P < 0.05$ (Fig. 6C and D). The pronounced decrease in the larval/pupal weights might be due to tight binding of PIs to the active site of gut proteases, thus preventing protease-PI complex formation essentially irreversible. Thus, the inability to utilize ingested protein and to recycle

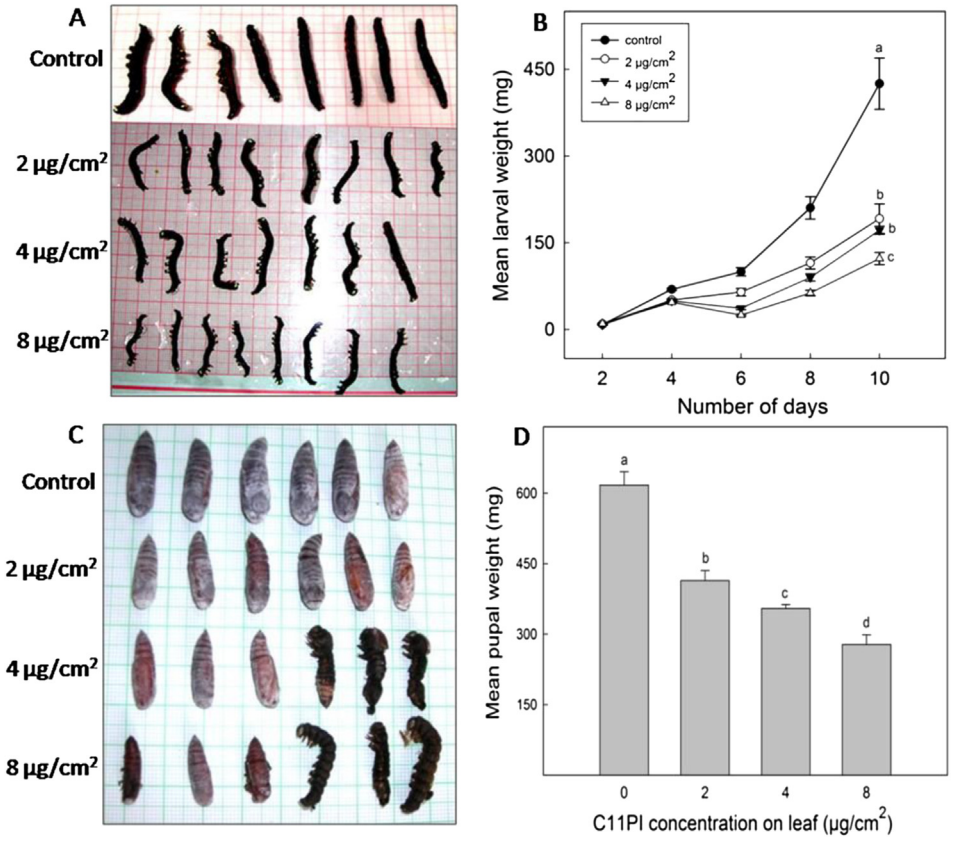


Fig. 6. *In vivo* effect of C11PI on growth and development of *A. janata*. (A) Development of *A. janata* larvae reared on castor leaf coated with or without C11PI at different concentrations (2, 4 and 8 µg/cm^2). The photographs of larvae were taken after 8 days of feeding on castor leaves coated with C11PI at indicated concentrations. Control leaves were treated with 50 mM Tris–HCl pH 8.0; (B) Mean weight of larvae after feeding on castor leaves coated with C11PI (2, 4 and 8) at different time intervals; (C) Effect of C11PI on development of pupae as observed in photograph taken after 16 days of rearing on C11PI coated castor leaves at respective concentrations. Dose dependent reduction in pupal size and larval-pupal as well as pupal-adult intermediates were observed in photograph. (D) Mean weight of pupae developed from respective larvae fed on different concentrations of C11PI (2, 4 and 8 µg/cm^2) on 16th day. The values shown here are mean ± SE of three independent experiments each with three replications ($n = 20$). Different lowercase alphabetical letters indicate statistically significant difference ($P < 0.05$).

Table 2
C11PI was coated on fresh castor leaf as described in materials and methods and provided to feed the larvae of *A. janata*. Reduction in larval growth was represented after 8 days of feeding on C11PI. Survival/mortality rate of larvae/pupae/adult was recorded after 25 days of feeding on C11PI. Percent intermediate formation was represented by considering emergence of both larval-pupal and pupal-adult intermediates. Data shown here is the average (±SE) values of triplicates from three independent experiments ($n = 20$ larvae). Different lowercase alphabetical letters indicate statistically significant difference ($P < 0.05$).

Concentration of C11PI (μg/cm^2 leaf area)	Reduction in larval growth (% control)	Survival rate (% control)	Mortality rate (% control)	Pupal formation time (days)	Intermediates formation (%)
0	0^a	100^a	0^a	10–16	0^a
2	45 ± 2.97^b	87 ± 6.30^b	13 ± 2.70^b	12–17	19 ± 2.56^b
4	57 ± 1.32^c	25 ± 1.91^c	75 ± 1.89^c	14–18	75 ± 5.21^c
8	70 ± 1.97^d	12 ± 2.39^d	88 ± 2.40^d	14–22	87 ± 7.94^d

digestive enzymes lead to a critical amino acid deficiency which ultimately affect the growth and development of the insect pest (Telang et al., 2009). At the end of 25th day, the mortality rate of larvae/pupae increased significantly up to 88% upon feeding on C11PI in a dose dependent manner. The feeding of C11PI (4–8 μg/cm^2) also lead to emergence of larval-pupal/pupal-adult intermediates (75–87% of control) together with a significant delay (~4–6 days) in their development (Fig. 6 and Table 2). These results further confirm the efficacy of PIs present in cultivars of *C. cajan* in combating against *A. janata* possibly by inhibiting the proteases related to development and metamorphosis (Prasad et al., 2010b; Chapman, 1982). Nevertheless, the present study is an example which demonstrated that PIs from non-host plant (*C. cajan*) to which *A. janata* has minimal or no prior exposure might be much useful for enhancing insect resistance of host plants (castor) through genetic engineering studies.

4. Conclusion

C11PI was purified to homogeneity using AKTA prime plus FPLC purification system. Several aspects of its biochemical properties such as their molecular mass, self-association pattern, presence of TI and CI activities, stability towards temperature and pH, analysis of MALDI MS–MS ions and N-terminal sequence suggest that C11PI belong to BBI-type serine PIs. Further, *in vitro* and *in vivo* assays using C11PI resulted in adverse effects on growth and development of *A. janata* which include significant reduction in larval and pupal weights, delay in pupal and adult transition time and formation of abnormal larval-pupal and pupal-adult intermediates. Thus, the results from the present study and earlier findings suggest that C11PI is a potential candidate to enhance the resistance of not only castor plant but also other crop plants which hosts *A. janata* (or) related noctuid lepidopteran pests through recombinant DNA technology.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2014.07.009.

Contributions

MS, VL and VS contributed to experiments; MS, manuscript preparation; NM, provided seeds for experimental purpose and participated in relevant discussions and manuscript editing; MK, performed MALDI-TOF/TOF studies; ADG, guided insect feeding experiments and critical reading of the manuscript; KPS, genesis of objectives, PI of the project, guidance in execution of experiments and manuscript editing.

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