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Purification and characterization of Bowman-Birk and Kunitz isoinhibitors from the seeds of *Rhynchosia sublobata* (Schumach.) Meikle, a wild relative of pigeonpea

S. Soundappan Mohanraj[a], Mariyamma Gujjarlapudi[b,1], Vadthya Lokya[b,1], Nalini Mallikarjuna[c], Aparna Dutta-Gupta[d], Kollipara Padmasree[b,*]

[a] Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad, 500 046, Telangana, India
[b] Department of Biotechnology & Bioinformatics, School of Life Sciences, University of Hyderabad, Hyderabad, 500 046, Telangana, India
[c] Legumes Cell Biology, Grain Legumes Program, International Crop Research Institute for Semi-Arid Tropics, Hyderabad, 502 324, Telangana, India
[d] Department of Animal Biology, School of Life Sciences, University of Hyderabad, Hyderabad, 500 046, Telangana, India

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ABSTRACT

Rhynchosia sublobata, a wild relative of pigeonpea, possesses defensive proteinase/protease inhibitors (PIs). Characterization of trypsin specific PIs (RsPI) separated from seeds by column chromatography using 2-D gel electrophoresis and Edman degradation method identified *R. sublobata* possessed both Bowman-Birk isoinhibitors (RsBBI) and Kunitz isoinhibitors (RsKI). A quick method was developed to separate RsBBI and RsKI from RsPI based on their differential solubility in TCA and acetate buffer. N-terminus sequencing of RsBBI and RsKI by MALDI-ISD ascertained the presence of Bowman Birk and Kunitz type isoinhibitors in *R. sublobata*. RsBBI (9216 Da) and RsKI (19,412 Da) exhibited self-association pattern as revealed by western blotting with anti-BBI antibody and MALDI-TOF peptide mass fingerprint analysis, respectively. RsBBI and RsKI varied significantly in their biochemical, biophysical and insecticidal properties. RsBBI inhibited the activity of trypsin (Ki = 128.5 ± 4.5 nM) and chymotrypsin (Ki = 807.8 ± 23.7 nM) while RsKI (Ki = 172.0 ± 9.2 nM) inhibited the activity of trypsin alone, by non-competitive mode. The trypsin inhibitor (TI) and chymotrypsin inhibitor (CI) activities of RsBBI were stable up to 100 °C. But, RsBBI completely lost its TI and CI activities on reduction with 3 mM DTT. Conversely, RsKI lost its TI activity on heating at 100 °C and retained > 60% of its TI activity in presence of 3 mM DTT. CD spectroscopic studies on RsBBI and RsKI showed their secondary structural elements in the following order: random coils > β-sheets/β-turns > α-helix. However, RsKI showed reversible denaturation midpoint (Tm) of 75 °C. Further, the significant inhibitory activity of RsBBI (IC_{50} = 24 ng) and RsKI (IC_{50} = 59 ng) against trypsin-like gut proteases of *Achaea janata* (AjGPs) and *Helicoverpa armigera* (HaGPs) suggest them as potential biomolecules in the management of *A. janata* and *H. armigera,* respectively.

1. Introduction

Seeds are the vehicles for continuity of next generation and contain various proteinaceous enzyme inhibitors such as amylase inhibitors and proteinase/protease inhibitors (PIs) (Furstenberg-Hagg et al., 2013). PIs are expressed constitutively in reproductive organs or induced in vegetative organs during biotic and abiotic stresses (Jamal et al., 2013; Yamchi et al., 2017). They also act as pseudosubstrates of proteases and stabilize them during desiccation. The PIs are rapidly degraded during seed germination to release essential amino acids and they reappear in cotyledons to protect them from invading pests and pathogens. They also take part in programmed cell death in plants. Bowman-Birk

Abbreviations: AjGPs, *A. janata* midgut trypsin-like proteases; AjGPIs, *A. janata* midgut trypsin-like protease inhibitors; BAPNA, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide; BBI, Bowman-Birk inhibitor; CI, Chymotrypsin inhibitor; CPIs, crude protease inhibitors; GLUPHEPA, *N*-glutaryl-L-phenylalanine-*p*-nitroanilide; HaGPs, *H. armigera* midgut trypsin-like proteases; HaGPIs, *H. armigera* midgut trypsin-like protease inhibitors; IEF, Isoelectric focusing; MALDI-ISD, Matrix-assisted laser desorption ionization insource decay; MALDI-TOF, Matrix-assisted laser desorption ionization time of flight; PIs, Proteinase/protease inhibitors; RsBBI, *R. sublobata* Bowman-Birk isoinhibitors; RsKI, *R. sublobata* Kunitz isoinhibitors; RsPI, *R. sublobata* trypsin specific protease inhibitors; TI, Trypsin inhibitor

* Corresponding author. Department of Biotechnology and Bioinformatics, School of Life Sciences, University of Hyderabad, Hyderabad, 500 046, Telangana, India.
E-mail addresses: kpssl@uohyd.ac.in, kpssl@uohyd.ernet.in (K. Padmasree).

[1] Contributed equally as second author.

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inhibitors (BBIs) and Kunitz inhibitors are widely studied among serine-type PIs across Leguminosae family members. The BBIs are 6–9 kDa proteins with seven intrachain disulfide bridges to stabilize its structure and contained two reactive sites for trypsin and chymotrypsin on opposite sides of the molecule. Conversely, the Kunitz inhibitors are ca. 20 kDa proteins with two disulfide bridges and contained a single reactive site for its cognate proteases such as trypsin or chymotrypsin (Mosolov and Valueva, 2005).

Insects induce huge yield loss in crop plants by feeding on them. In response to feeding, the PIs expressed as a part of the defense mechanism in crop plants inhibit the digestive proteases present in the insect gut and thereby reduce the bioavailability of free amino acids (Macedo et al., 2015). During the process of host-pest coevolution, insect gut proteases are adapted to the PIs of domesticated crop plants by overexpressing the existing proteases or synthesizing alternative proteases (Jongsma and Bolter, 1997; Santamaría et al., 2018). In this scenario, PIs from few non-host plants and wild relatives are suggested as potential candidates to inhibit the gut proteases of host insects since their guts are not exposed to such kind of PIs earlier. Many transgenic plants expressing PIs from non-host and wild relatives are reported to counteract the insect pests (Abdeen et al., 2005; Dunse et al., 2010; Hamza et al., 2018; Macedo et al., 2015; Rehman et al., 2017). Therefore, a continuous screening of non-hosts and wild relatives is essential to identify effective ecofriendly PIs to target economically important insect pests such as *Helicoverpa armigera* and *Achaea janata* (Jamal et al., 2013; Losvik et al., 2017). Controlling *H. armigera* (a polyphagous pest) and *A. janata* (a major pest on *Ricinus communis*) has been a major challenge since they are efficient in overcoming defense mechanisms of host plant and chemical pesticides due to their broad host range and voracious feeding behaviour.

Rhynchosia, a wild relative of *Cajanus cajan* is a non-host plant to both *H. armigera* and *A. janata* and known to harbor a rich pool of defensive proteins including PIs. The studies on different species of Rhynchosia indicated the presence of PIs active against trypsin-like gut proteases of *H. armigera* in their seeds (Chougule et al., 2003; Parde et al., 2012). Further studies with the crude protease inhibitors (CPIs) from *R. sublobata* seeds showed remarkable reduction in the activity of gut trypsin-like proteases from *H. armigera* and *A. janata* (Mohanraj et al., 2018). Therefore, it was hypothesized that the seeds of *R. sublobata* might possess different kinds of PIs to defend the gut trypsin-like proteases of both *H. armigera* and *A. janata*. Furthermore, as several classes of PIs are active against trypsin, it is important to identify and understand the properties of PIs from *R. sublobata*. Thus, in the present study, PIs which are active against gut trypsin-like proteases of *H. armigera* and *A. janata* are purified and characterized for their biochemical and biophysical properties.

2. Results and discussion

2.1. Purification of R. sublobata protease inhibitors "RsPI" by FPLC

The 20–60% $(NH_4)_2SO_4$ fractionated CPI was resolved into two peaks in trypsin-affinity column when eluted with 0.01 N HCl (Fig. 1A). The peak 2 with TI activity was further loaded on a Sephadex G-50 fine gel filtration chromatography column to remove any high MW contaminants retained in the trypsin affinity fractions. The pool of peak 2 fractions with significant TI activity was referred to as *R. sublobata* trypsin specific protease inhibitors "RsPI" (Fig. 1B) and it showed two major bands when separated in Tricine SDS-PAGE (Fig. 1C). The lower major band apparently possessed molecular mass of ca. 13 kDa or 8 kDa when compared with commercially available protein molecular weight marker (PMWM) and soybean Bowman-Birk Inhibitor (SBBI), respectively. This discrepancy is very common in TIs, particularly among those which are known to self-associate depending on the method of molecular weight determination such as ultra-centrifugation, size exclusion chromatography, SDS-PAGE, 2-D gel electrophoresis or MALDI-TOF mass spectrometry (Bergeron and Nielsen, 1993 and references therein; Swathi et al., 2014, 2016). However, molecular mass of lower band is assumed to be closer to 8 kDa considering its similar migration pattern with SBBI as compared to PMWM marker. In contrast, the upper band which showed molecular mass of ca. 20 kDa is well in range with both PMWM and SBBI. Further, this discrepancy in molecular mass was monitored by loading both SBBI and RsPI in all gels performed in this study.

2.2. IEF of RsPI and identification of RsBBI and RsKI by Edman degradation method

The RsPI is resolved into several isoinhibitors on IEF followed by second-dimension PAGE. The isoinhibitor spots in the 8 kDa region were distributed between pI 4.3 and pI 6.2, while the isoinhibitors spots in the 20 kDa region were distributed between pI 4.4 and pI 6.7 (Fig. 2A). Further, the 2DE combined with in-gel TI activity assay indicated isoinhibitor spots in 20 kDa region are active against trypsin even after reduction of RsPI with DTT/IDA whereas the isoinhibitor spots in 8 kDa region are inactive (Fig. 2B). These results suggest RsPI possess isoinhibitors from atleast two different PI families and the structural stability of isoinhibitors from 8 kDa region is influenced by redox-sensitive disulfide bridges.

Further, the N-terminal sequencing of the isoinhibitor (spot 1) with pI 4.9 and molecular mass ca. 20 kDa by Edman degradation method revealed the following 20 amino acid sequence "DFVLDTDGDPLQNGGRYFIL" which showed 70% identity to Trypsin inhibitors A (ITRA_SOYBN) & B (ITRB_SOYBN) and 75% identity to Kunitz trypsin inhibitors 1 (KTI1_SOYBN) and 2 (KTI2_SOYBN) of soybean from UniProt data base (Fig. 2A and C). Conversely, N-terminal sequencing of the isoinhibitor (spot 2) with pI 5.0 and molecular mass ca. 8 kDa revealed the following amino acid sequence "SDHHHHHSSSSDEP" which showed 43–71% identity to the N-terminus of BBIs from *Macrotyloma axillare* (IBB4_MACAX), soybean (IBB1_SOYBN), *Phaseolus vulgaris* (IBB2_PHAVU) and *P. acutifolius* (IBB_PHAAT), respectively from the above database (Fig. 2A and D). Also, N-terminal sequencing performed for spot 3 of pI 6.0 and molecular mass ca. 20 kDa revealed the sequence of first five amino acids as "DFVLD" and they were similar to that obtained for spot 1 (Fig. 2A). Taken together, these results suggested the presence of Bowman-Birk isoinhibitors (RsBBI) and Kunitz isoinhibitors (RsKI) in the seeds of *R. sublobata*.

Though leguminous plants mostly possessed PIs from BBI family, a very few leguminous species such as *Glycine max*, *Peuraria lobata*, *Mucuna pruriens*, *Psophocarpus tetragonolobous*, *Clitoria ternatea* and *Canavalia lineata* including *C. cajan* were reported to have both BBI and Kunitz inhibitors in their seeds (Giri et al., 2003; Macedo and Xavier-Filho, 1992; Norioka et al., 1988; Terada et al., 1994a, 1994b). Perhaps, the presence of BBI and Kunitz inhibitors among these different plants including *R. sublobata* might provide biochemical resistance to withstand the damage caused by more than one insect species.

2.3. A rapid method for the separation of RsBBI and RsKI

A variety of protocols are applied by various research groups to separate BBIs and Kunitz inhibitors present in a single seed variety which included (i) multiple chromatographic steps; (ii) preparative native gels; and (iii) application of different processing steps after extraction into a suitable organic solvent (Deshimaru et al., 2002; Giri et al., 2003; Macedo and Xavier-Filho, 1992). All these methods were highly time-consuming and involved a tedious process. Also, in the present study, separation of RsBBI and RsKI from RsPI using the principles of chromatography was found to be very difficult for the following reasons: (a) both RsBBI and RsKI are TIs (Fig. 2C and D); (b) close proximity in the molecular mass of RsBBI and RsKI (Figs. 1C and 2A); and (c) most of the isoinhibitors of RsBBI and RsKI showed overlapping in their pI values (Fig. 2A). Besides, usage of the columns such

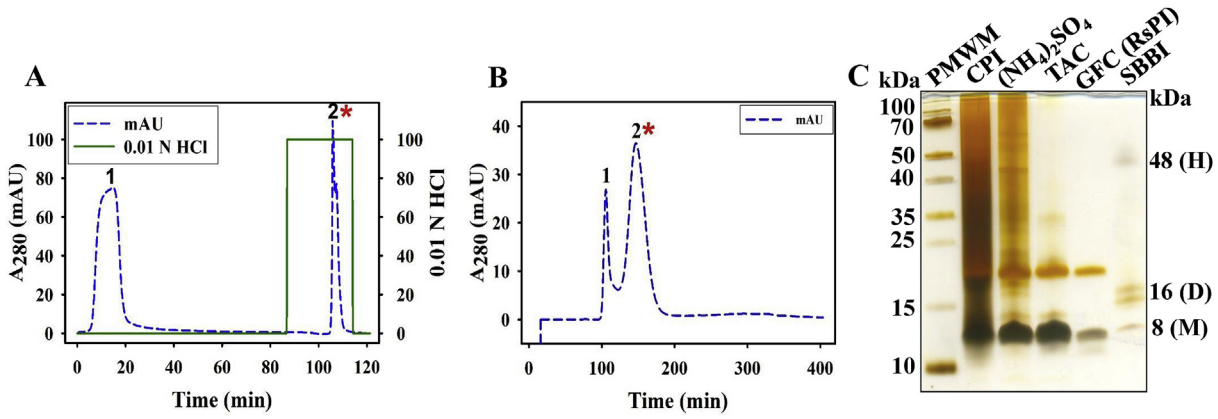


Fig. 1. FPLC purification of 'RsPI' from *R. sublobata* seeds. Elution profiles of **(A)** trypsin-affinity and **(B)** gel filtration columns. The protein peaks active against trypsin were marked with an asterisk (*); **(C)** Tricine SDS-PAGE (15%) showing different fractions of purification protocol under non-reducing conditions. Lane 1 is loaded with Protein molecular weight marker (PMWM) and lanes 2–6 are loaded with 5 or 10 µg of corresponding protein sample. The gel was stained with silver nitrate. CPI – crude protease inhibitor, TAC – Trypsin affinity chromatography, GFC – Gel filtration chromatography and SBBI – Soybean BBI.

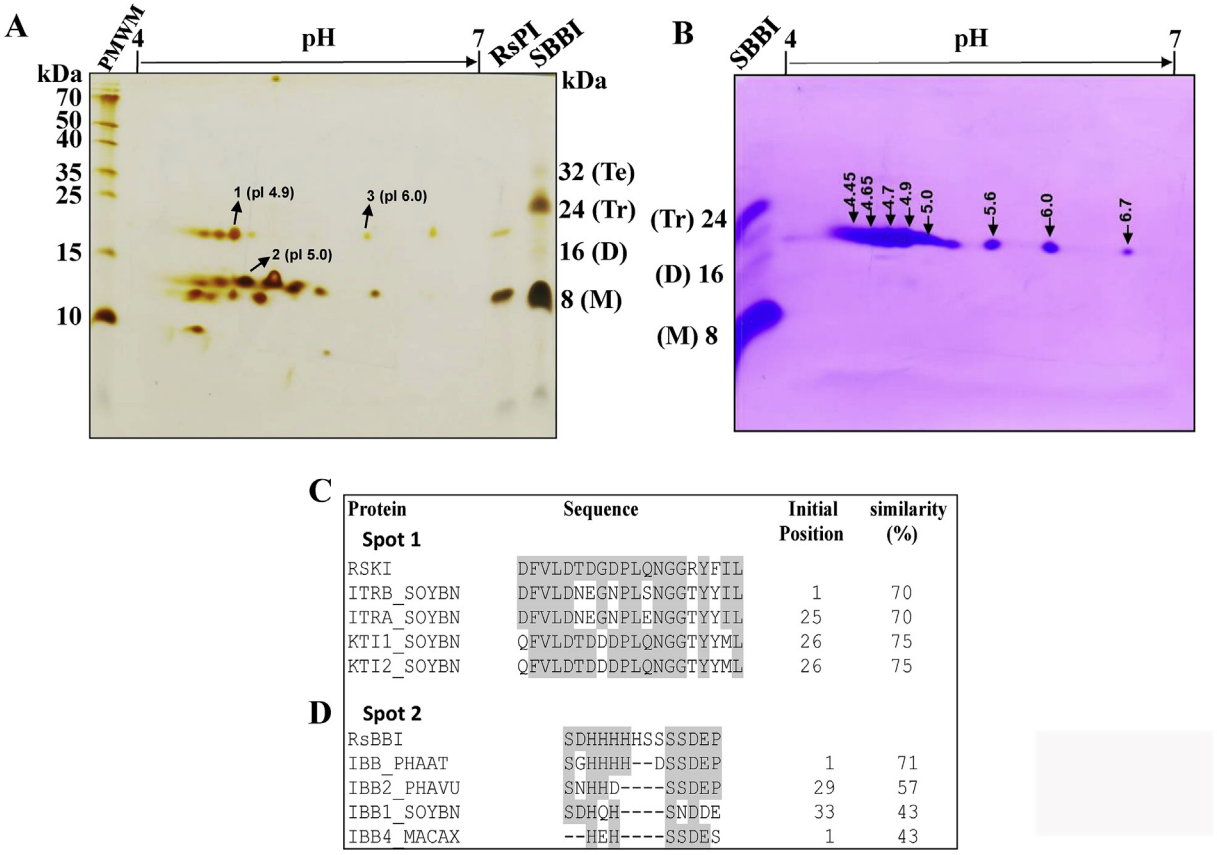


Fig. 2. Two-dimensional gel electrophoresis (2DE) of RsPI and N-terminal sequencing of isoinhibitor spots from RsPI. (A) 2DE of RsPI after reduction with DTT and IDA treatment and **(B)** in-gel trypsin like inhibitory activity of RsPI after 2DE. RsPI (30 µg) was subjected to IEF using pH 4–7, 11 cm strip and the spots were resolved on 15% Tricine SDS-PAGE. 2 µg of RsPI and SBBI were loaded on to the neutral end of 2DE gel as controls. The gel was stained with silver nitrate. Gelatin is incorporated into Tricine SDS-PAGE (15%) for visualization of active spots. 50 µg of RsPI was resolved. SBBI (5 µg) was loaded as positive control for in-gel activity visualization and the gel was stained with CBB R-250. Clustal alignment of the N-terminal sequences obtained for **(C)** spot 1 (RsKI) and **(D)** spot 2 (RsBBI) with known inhibitor sequences from UniProt database. Identical amino acids are shaded in grey colour. Other details were as described in materials and methods. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

as Superdex G-75 and MonoQ Sepharose, and procedures like 10 kDa cut-off spin columns or simple SDS-PAGE gel elution techniques could not separate RsBBI and RsKI from RsPI. The hydrophobic interaction chromatography using Phenyl Sepharose 6 fast flow method separated the RsBBI and RsKI from RsPI on decreasing ammonium sulfate gradient (Data not shown). But, this procedure involved extensive time-consuming post processing steps such as buffer exchange and sample concentration to isolate them in pure forms. Therefore, during this study, a short and quick method was introduced to separate RsBBI and RsKI from RsPI based on their differential solubility properties in TCA and sodium acetate, respectively (Fig. 3A).

In the first phase, RsBBI was separated from RsPI by extracting into 2.5% TCA at 70 °C. Subsequently, the supernatant 'S1' enriched with RsBBI is subjected to acetone precipitation and the pellet was

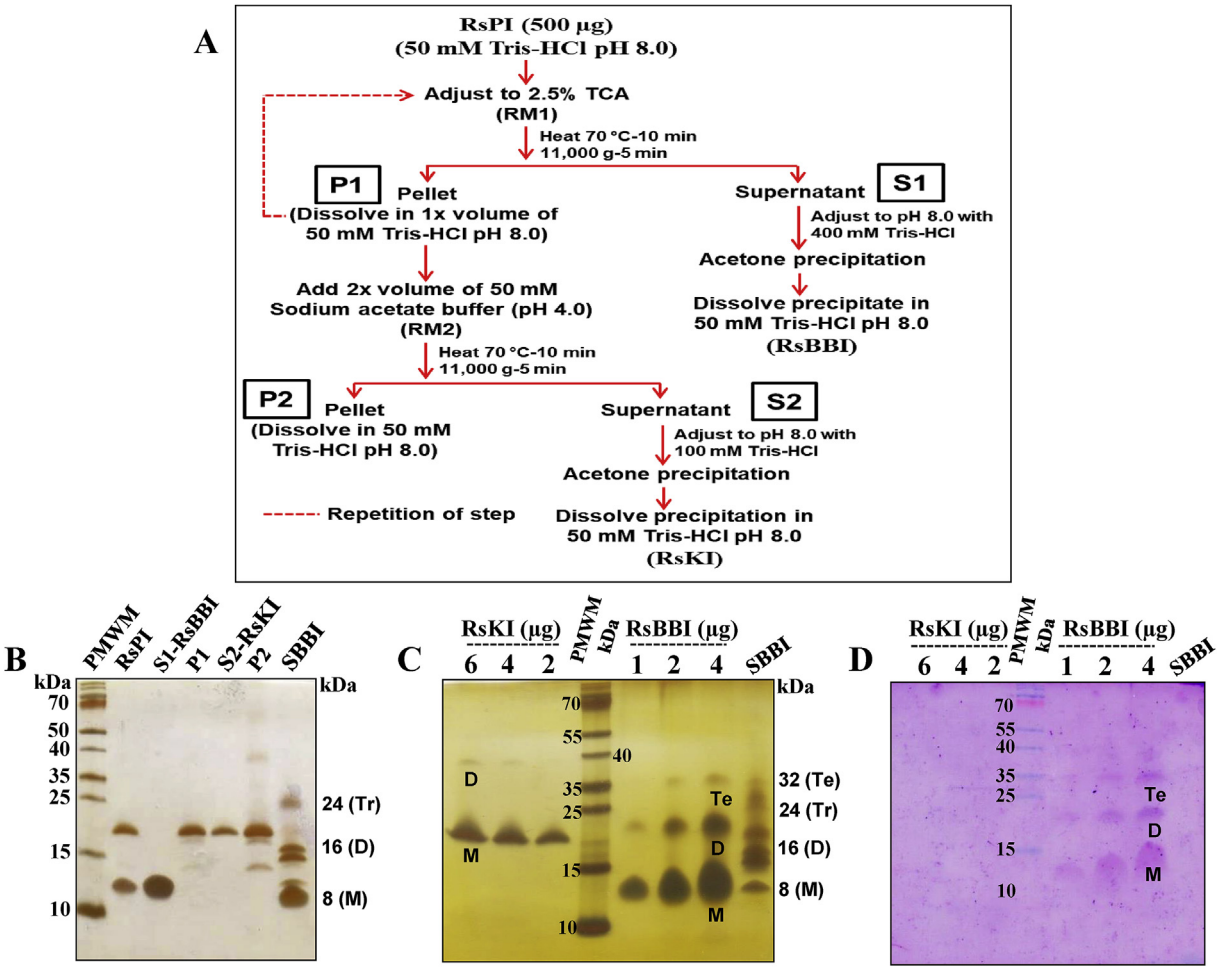


Fig. 3. Separation of RsBBI and RsKI from RsPI & immunoblot analysis. (A) Flow diagram for separation of RsBBI and RsKI from RsPI. The dotted line (—) indicate the repetition of the step; **(B)** Visualization of separation of RsBBI and RsKI on Tricine SDS-PAGE (15%) under non-reducing condition. 2 µg of protein was loaded in each lane and the gel was stained with silver nitrate; **(C)** Concentration dependent oligomerisation of RsBBI and RsKI on Tricine SDS-PAGE (15%); **(D)** Western blot of RsBBI and RsKI on 15% Tricine SDS-PAGE probed with anti-BBI antibody from *C. cajan*. SBBI and RsKI did not react with anti-BBI antibody. M, D, Tr, Te refers to monomer, dimer trimer and tetramer bands. 1, 2 and 4 µg of RsBBI; 2, 4 and 6 µg of RsKI and 5 µg of SBBI were loaded. Further details were as described in materials and methods sections 3.5. and 3.6.

solubilized in 50 mM Tris-HCl, pH 8.0. This step was repeated to extract the BBI totally from RsPI. In the second phase, RsKI is separated from RsPI by extracting the P1 pellet after TCA extraction into 50 mM Sodium acetate buffer, pH 4.0. The supernatant 'S2' enriched with RsKI is subsequently subjected to acetone precipitation and the pellet was solubilized in 50 mM Tris-HCl, pH 8.0 (Fig. 3A). TCA is known to increase the hydrophobicity of the proteins by exposing their hydrophobic core and thereby induce bulk protein precipitation. Hence, exposure of RsPI to 2.5% TCA at 70 °C resulted in precipitation of RsKI with hydrophobic core as pellet "P1" (Fig. 3A and B). Conversely, BBIs are low molecular mass, globular, water soluble proteins with hydrophilic core and surface hydrophobic patch (Silva et al., 2005). These properties of RsBBI allowed it to be retained in the soluble fraction (S1) on mild TCA precipitation and it exhibited self-association pattern (homo-dimer and -trimer) in solution. Since, Kunitz inhibitors are also reported to be soluble in acetate buffers (Macedo and Xavier-Filho, 1992), the extraction of RsKI enriched pellet 'P1'with Sodium acetate (pH 4.0) retained RsKI in the supernatant 'S2'. Thus, a simple and cost-effective method is developed to separate RsBBI and RsKI from *R. sublobata* within a short period of 2 h. This protocol yielded 4.26 mg of RsBBI and 1.08 mg of RsKI from 10 g of seeds accounting to 50- and 23-fold of purification, respectively. RsBBI is highly active against bovine trypsin enzyme (1780.28 TI units/mg protein) than RsKI (837.03 TI units/mg protein; Table 1).

2.4. Confirmation of RsBBI and RsKI by immunodetection, mass spectrometry and 2DE

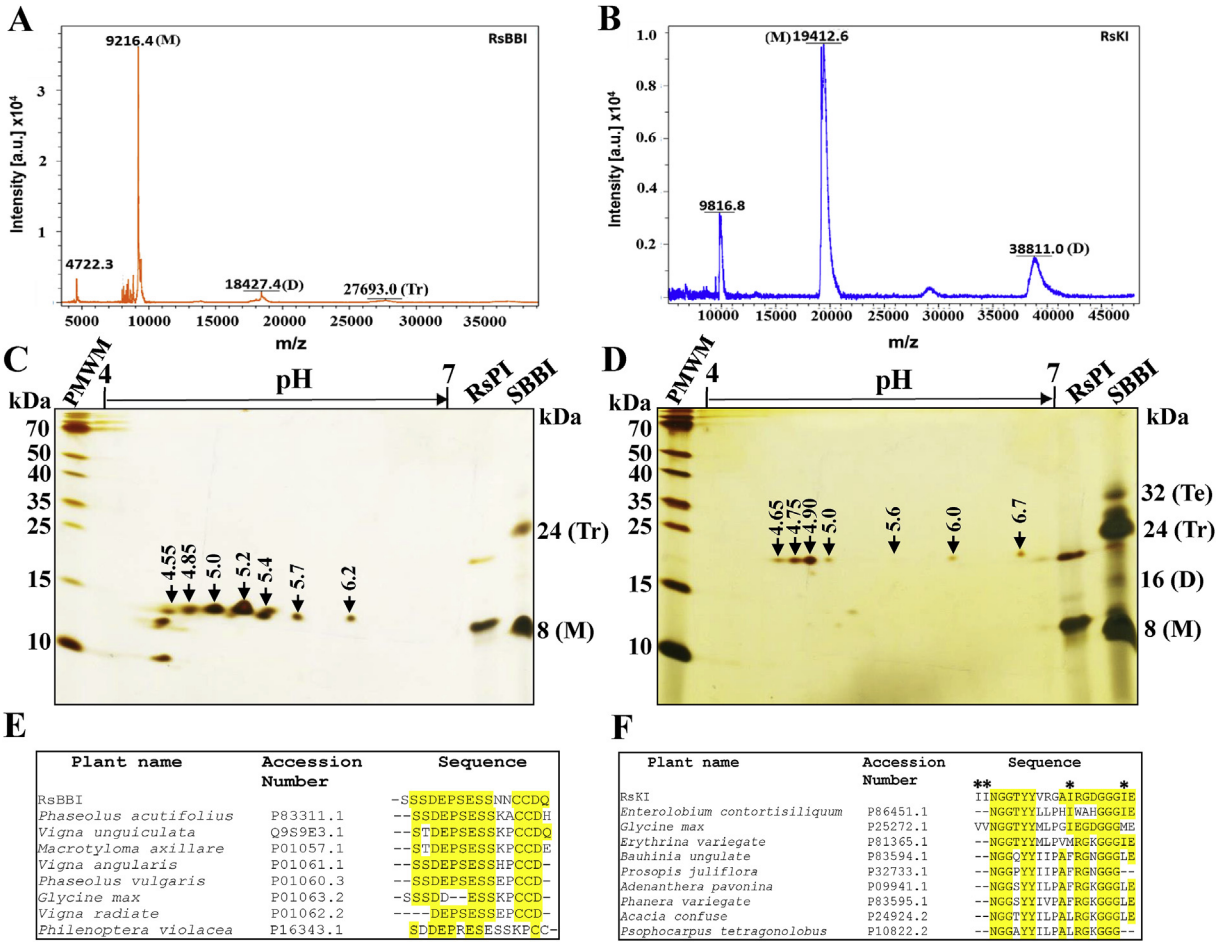
The homogeneity of RsBBI and RsKI separated from RsPI was visualized in 15% Tricine SDS-PAGE (Fig. 3B). Loading of RsBBI and RsKI at increasing concentrations demonstrated the presence of these PIs in higher order conformations (Fig. 3C). Further, probing with anti-BBI antibody revealed the existence of RsBBI as monomer, dimer and tetramer (Fig. 3D). Furthermore, RsKI which existed as monomer and dimer did not show any reaction with anti-BBI antibody. This in turn confirmed the absence of any traces of RsBBI in S2 (Fig. 3A–D). Besides, the peptide mass fingerprint (PMF) spectra of the higher order bands of RsBBI (dimer and tetramer) and RsKI (dimer) with corresponding monomers demonstrated their self-association property (Supplementary Figs. 1 and 2). Furthermore, when an identical peak from all the PMF spectra of RsBBI (ca. 1708 Da) and RsKI (ca. 1201 Da) were ionized (MS/MS) and de novo sequenced (Supplementary Fig. 3A,B,C; Fig. 4A and B) the obtained sequence matched with the related BBIs and Kunitz inhibitors (Supplementary Fig. 5A and B).

Intact mass analysis of purified 'RsBBI' and 'RsKI' using MALDI-TOF mass spectroscopy studies confirmed that they existed as small oligomers in solution (Fig. 4A and B). The appearance of (i) three peaks for RsBBI with molecular masses 9216.4 Da (monomer), 18,427.4 Da (dimer) and 27,693.0 Da (trimer), and (ii) two peaks for RsKI with

Table 1
Purification of trypsin-specific protease inhibitors (RsPI) from mature seeds of *R. sublobata*. RsBBI and RsKI were separated from RsPI as described in Fig. 3A and their specific activity and purification fold were determined.

Purification step	Total protein (mg)	Total activity (TI units)	Yield recovery (%)	Specific activity (TI units/mg protein)	Purification fold
Crude protease inhibitors	1014.43	36000	100	35.48	1.00
Ammonium sulfate fractionation (20–60%)	477.4	29684	82.45	62.17	1.75
Affinity chromatography	51.36	15250	42.36	296.92	8.36
Gel filtration chromatography (RsPI)	9.0	9964	27.67	1107.11	31.20
RsBBI	4.26	7584	21.06	1780.28	50.16
RsKI	1.08	904	2.51	837.03	23.59

molecular masses 19,412.6 Da (monomer) and 38,811.0 Da (dimer) corroborated with the higher ordered conformation observed in SDS-PAGE (Fig. 3C). However, the appearance of RsBBI in trimeric state during intact MALDI-TOF analysis and tetrameric state in Tricine SDS-PAGE could be due to variation in the technique applied and the concentration of RsBBI used (Fig. 3C and 4A). Sismilarly, the oligomeric nature of SBBI which is used as a control varied significantly in different gels (Figs. 1C,2A and 3B,C). Thus, the results from the present study revealed that RsBBI self-associate to form dimeric, trimeric and tetrameric states (Figs. 3C,D and 4A). Oligomerisation of BBIs is well-known and occurred as a very common phenomenon when compared with Kunitz inhibitors (Birk, 1985; Brand et al., 2017; Losso, 2008). The purified BBIs from cultivars of *C. cajan* were reported to possess self-association tendency and existed from monomeric to pentameric states (Prasad et al., 2010c; Swathi et al., 2014). Thermodynamically, the existence of BBIs in solution as monomer is suggested as an unfavorable reaction (Campos et al., 2004; Paiva et al., 2006). Perhaps, oligomerisation of BBIs might increase their thermal stability and resistance to enzyme digestion (Kumar et al., 2004). The BBI from *Vigna ungiculata* is reported to form a very tight dimeric structures (Rao and Suresh, 2007). The studies of Gennis and Cantor (1976) hypothesized existence of dimeric and tetrameric structures, and their transitions at equilibria for



E

Plant name	Accession Number	Sequence
RsBBI		-SSSDEPSESSNNCCDQ
Phaseolus acutifolius	P83311.1	--SSDEPSESSKACCDH
Vigna unguiculata	Q9S9E3.1	--STDEPSESSKPCCDQ
Macrotyloma axillare	P01057.1	--STDEPSESSKPCCDE
Vigna angularis	P01061.1	--SSDEPSESSHPCCD-
Phaseolus vulgaris	P01060.3	--SSDEPSESSEPCCD-
Glycine max	P01063.2	-SSSDD--ESSKPCCD-
Vigna radiate	P01062.2	----DEPSESSEPCCD-
Philenoptera violacea	P16343.1	SDDEPRESESSKPCC-

F

Plant name	Accession Number	Sequence
RsKI		IINGGTYYVRGAIRGDGGGIE
Enterolobium contortisiliquum	P86451.1	--NGGTYYLLPHIWAHGGGIE
Glycine max	P25272.1	VVNGGTYYMLPGIEGDGGGME
Erythrina variegate	P81365.1	--NGGTYYMLPVMRGKGGGIE
Bauhinia ungulate	P83594.1	--NGGQYYIIPAFRGNGGGLE
Prosopis juliflora	P32733.1	--NGGPYYIIPAFRGNGGG--
Adenanthera pavonina	P09941.1	--NGGSYYILPAFRGKGGGLE
Phanera variegate	P83595.1	--NGGSYYIVPAFRGKGGGLE
Acacia confuse	P24924.2	--NGGTYYILPALRGKGGGLE
Psophocarpus tetragonolobus	P10822.2	--NGGAYYILPALRGKGGG--

Fig. 4. MALDI-TOF mass spectrum, 2-D gel electrophoresis and MALDI-ISD of RsBBI and RsKI. Intact mass of RsBBI **(A)** and RsKI **(B)** at 4000–40000 m/z and 5000–50000 m/z, respectively. M, D and Tr represent the monomer, dimer and Trimer peaks while m/z 4722.3 and 9816.8 represent the halfmers of RsBBI and RsKI; **(C)** & **(D)** IEF of RsBBI (20 µg) and RsKI (20 µg) followed by Tricine SDS-PAGE (15%) under reducing conditions. The gels were stained with silver nitrate and the relative pI values of individual isoinhibitor spots are indicated with arrows. M, D, Tr, Te refers to monomer, dimer, trimer and tetramer bands; ClustalW alignment of the MALDI-ISD obtained N-terminal sequence of **(E)** RsBBI and **(F)** RsKI with known BBIs and Kunitz inhibitors from related plants; Asterisk '*' indicate the possibility of Leucine in place of Isoleucine as they possess identical mass and can't be distinguished in MALDI analysis (Supplementary Fig. 6B). The search criterion in NCBI is limited to Isoleucine, Non-redundant protein sequences and Plants (Taxid: 3193). Identical amino acids are shaded in yellow colour. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

black-eyed pea trypsin-chymotrypsin-inhibitor and TI. The opposite electrostatic potential between the TI domain of one molecule of BBI and the CI domain of second molecule of BBI render them together during formation of higher order conformations (Catalano et al., 2003). Precisely, Lys^{24} at the TI reactive site of first monomer is shown to interact with the Asp^{76} of the CI reactive site of second monomer in BBI of *Dolichos biflorus* (Kumar et al., 2004). But, the dimerization of Kunitz inhibitors as observed in RsKI (Figs. 3B and 4B) is rarely found and reported from species such as *C. platycarpus, Canavalia lineata* and *Schizolobium parahyba* (Swathi et al., 2016; Teles et al., 2007; Terada et al., 1994a).

Further, IEF of RsBBI and RsKI followed by SDS-PAGE on second dimension visualized the different isoinhibitors of Bowman-Birk and Kunitz family in the acidic range (Fig. 4C and D). Since, both plants and insects are evolving independently and together, presence of multiple isoinhibitors in plants is suggested to play a strategic role in protecting themselves against invading predators (Domoney et al., 1995; Harsulkar et al., 1999; Zhu-Salzman and Zeng, 2015). The polymorphic nature of TIs is also evident in several wild relatives of *C. cajan* such as *C. volubilis*, *C. crassicaulis* and *C. scarabaeoides* along with other crop plants like *Cicer arietinum*, *G. max* and *V. ceratotropis* (Chougule et al., 2003; Kollipara et al., 1994; Konarev et al., 2002a,b; Krishnamurthy et al., 2013; Patankar et al., 1999; Prasad et al., 2009). Further, multiple isoinhibitors have been arisen in plants, possibly due to events such as gene duplication/multi-gene products and/or post translational modifications both at N- and C-terminal ends (Giri et al., 2003).

Furthermore, the following N-terminal sequences, 'SSSDEPSESSNNCCDQ' and 'I/LI/LNGGTYYVRGAI/LRGDGGGI/LE' are identified by MALDI-ISD spectrum in RsBBI and RsKI (Supplementary Fig. 6A and B). The BLAST analysis of these sequences showed a significant identity to BBIs from Phaseolus, Vigna, Horse gram and soybean and Kunitz inhibitors from soybean and tree legumes (Fig. 4E and F). Besides, the overlap in N-terminal sequences obtained through MALDI-ISD with those sequences obtained through Edman degradation (Figs. 2C,D and 4E,F), further strengthened the evidences for existance of both RsBBI and RsKI in *R. sublobata* seeds.

The studies of Giri et al. (2003) attempted to separate individual TIs from the mixture of partially purified winged bean TIs using preparative gel electrophoresis. However, the method applied did not result in separation of individual TIs. Further, the studies of Prasad et al. (2010a,b) and Swathi et al. (2014, 2016) also used pool of isoinhibitors to examine the kinetics or biochemical properties of BBI or Kunitz/miraculin like PIs purified from cultivars/wild-relatives of *C. cajan* and *V. mungo*. Thus, due to limitation in the techniques available to separate individual isoinhibitors, we used RsBBI and RsKI to evaluate their biochemical, biophysical and insecticidal properties.

2.5. Visualization of activity, titration and inhibition kinetics of RsBBI and RsKI

In-gel activity staining studies revealed both RsBBI and RsKI are active against bovine pancreatic trypsin (Fig. 5A). However, RsBBI but not RsKI is active against chymotrypsin (Fig. 5B). RsBBI is bound to its cognate proteases trypsin and chymotrypsin at 1:2 and 3:4 M ratio (Fig. 5C) and RsKI is bound to trypsin at 1:2 M ratio (Fig. 5D). In general, the PIs from BBI family were double headed while PIs from Kunitz inhibitor family were single headed (Mosolov and Valueva, 2005). RsBBI corroborated well with the reported BBIs from leguminous plants in possessing inhibitory domains for trypsin and chymotrypsin. *In silico* three-dimensional modelling of RsBBI1 also predicted it as a symmetrical protein with both trypsin and chymotrypsin reactive site loops on opposite sides (Mohanraj et al., 2018). But, RsBBI neutralized chymotrypsin activity in a unique pattern (3:4 ratio) although 80% of enzyme activity is inhibited at 1:2 M ratio. Also, RsKI differed from most earlier known Kunitz inhibitors in possessing two trypsin inhibitory domains (Fig. 5A and D). Occurrence of such double headed Kunitz inhibitors is found to be rare in nature and reported from the following plants earlier: *Leucaena leucocephala* (Sattar et al., 2004), black soybean (Lin and Ng, 2008), *Adenanthera pavonina* (Migliolo et al., 2010), tamarind (Patil et al., 2012) and winged bean (Bhattacharjee et al., 2014). However, the exact nature of the number of TI/CI reactive sites on RsBBI and RsKI has to be studied in detail as it could also be due to oligomerisation. But, the existence of dual TI sites on BBI and Kunitz inhibitors of plants is not uncommon.

Enzyme inhibition kinetics analysis by Lineweaver-Burk plot revealed that RsBBI and RsKI inhibited the trypsin/chymotrypsin enzymes by non-competitive mode. The *Ki* of RsBBI and RsKI against trypsin were 128.5 ± 4.5 nM and 172.0 ± 9.2 nM (Fig. 5E and G) while the *Ki* of RsBBI against chymotrypsin was 807.8 ± 23.7 nM (Fig. 5F). The BBIs purified from seeds of different leguminous plants exhibited *Ki* values at a wide range from < 1 nM to 52 μM towards trypsin (Ragg et al., 2006; Ramasarma et al., 1994). However, the *Ki* of RsBBI against trypsin (2-fold) and chymotrypsin (4.6-fold) was less as compared to BBIs from cultivars of *C. cajan* (Prasad et al., 2010a,b,c; Swathi et al., 2014). In contrast, the *Ki* of RsKI against trypsin was several fold higher as compared to the reported non-competitive Kunitz inhibitors from *Erythrina velutina* (*Ki* of 10 nM) and *Prosopis juliflora* (0.59 nM) (Machado et al., 2013; Oliveira et al., 2002). The *Ki* values of the BBIs and Kunitz inhibitors purified form seeds of different leguminous plants against trypsin and chymotrypsin are listed in Supplementary Tables S1 and S2, respectively.

2.6. Biochemical stability of RsBBI and RsKI

There is no remarkable loss in the TI and CI activities of RsBBI on heating up to 100 °C (Fig. 6A). Similarly, the TI and CI activities of RsBBI were stable at alkaline pH. However, at less acidic (pH 5.0) or neutral pH (pH 7.0), a decrease (> 40%) in TI activity but not CI activity was observed (Fig. 6B). Such a decrease in TI activity was correlated to the precipitation of PIs at their isoelectric pH (Figs. 2A and 4C; Prasad et al., 2010c). Conversely, RsBBI lost 100% of its TI and CI activities after reduction with 1.0 mM and 3 mM DTT, respectively (Fig. 6C). The loss in TI and CI activities of RsBBI on reduction with DTT confirmed the importance of redox-sensitive disulphide bridge network in maintenance of RsBBI structure (He et al., 2017). The effect of temperature, pH and DTT treatment on the TI activity of RsKI was quite different from RsBBI. The TI activity of RsKI is stable up to 70 °C. But, it lost > 80% of its TI activity when incubated at 100 °C (Fig. 6D). There was a marginal loss (< 10%) in TI activity of RsKI at acidic/neutral/alkaline pH (Fig. 6E). In contrast to RsBBI, the TI activity of RsKI was quite stable to the action of DTT. RsKI lost complete (100%) TI activity when incubated with DTT at a concentration as high as 200 mM (Fig. 6F). Such stability in the TI activity of Kunitz inhibitors after reduction with DTT was also reported from *Putranjiva roxburgii* (Chaudhary et al., 2008), *Inga laurina* (Macedo et al., 2007) and *Plathymenia foliolosa* (da Silveira Ramos et al., 2008).

2.7. Secondary structural analysis of RsBBI and RsKI

At far-UV (190–260 nm) region, the secondary structural elements existed in the following order in RsBBI: random coils (56%) > β-sheets (21%) > β-turns (18%) > α-helices (5%) while in RsKI, they existed as: random coils (62.9%) > β-turns (23.8%) > β-sheets (7%) > α-helices (6%). Thermal treatment of RsBBI showed only a marginal decrease in its ellipticity when the temperature was raised from 25 to 90 °C. But, this effect was reversed on cooling down from 90 to 25 °C suggesting RsBBI is stable to heat treatment (Fig. 7A and B). Also, the changes observed in mean residue ellipticity of RsBBI at 203 nm on increasing the temperature to as high as 90 °C indicate RsBBI is highly stable and resistant to thermal denaturation (Supplementary Fig. 7A and B). BBIs are structurally stable due to its rich network of seven disulphide bridges and β-sheets which prevent the RsBBI from

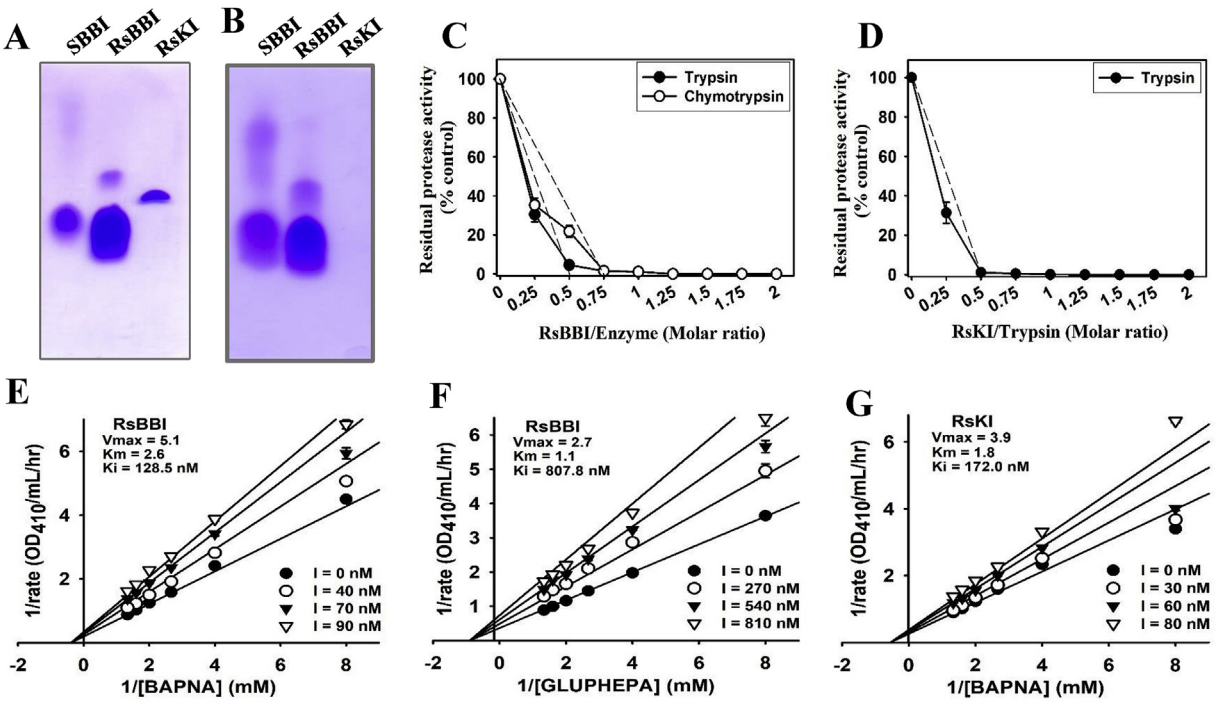


Fig. 5. Visualization of inhibitory activity, titration curves and enzyme inhibition kinetics of RsBBI and RsKI against trypsin and chymotrypsin. Visualization of in-gel activity of RsBBI (5 μg) and RsKI (5 μg) against **(A)** bovine trypsin and **(B)** bovine chymotrypsin in gelatin SDS-PAGE (15%) under non-reducing conditions. SBBI (2 μg) was loaded as positive control. The inhibitor bands were visualized after staining with CBB R-250. Molar ratios of **(C)** RsBBI and **(D)** RsKI against trypsin/chymotrypsin are determined as the intercept of y-coordinate reaching the zero activity (dotted lines); Lineweaver-Burk plot depicting non-competitive mode of inhibition after incubating with increasing concentrations of **(E)** RsBBI (40, 70 and 90 nM) against trypsin; **(F)** RsBBI (270, 540, 810 nM) against chymotrypsin, and **(G)** RsKI (30, 60 and 80 nM) against trypsin at different concentrations of BAPNA/GLUPHEPA (0.125, 0.250, 0.375, 0.500, 0.625 and 0.750 mM); The values are the mean ± SE of at least three independent assays each with three replicates. The purity of the proteases trypsin and chymotrypsin was visualized on 15% Tricine SDS-PAGE by staining with silver nitrate (Supplementary Fig. 8).

undergoing any changes in their secondary structures even after heating to 90 °C and thereby render the protein to retain both its TI and CI activities (Figs. 6A and 7A,B). Such structural stability was reported in BBIs from horse gram (Kumar and Gowda, 2013) and black gram (Prasad et al., 2010b). Conversely, RsKI is sensitive to thermal treatment and showed a remarkable decrease in its ellipticity when heated at 80 °C (Fig. 7D). However, the ellipticity was reversed on cooling down from 90 to 25 °C (Fig. 7E). Moreover, the mean residue ellipticity of RsKI at 199 nm revealed a sigmoidal curve on increasing temperature which started at 70 °C and ended at 90 °C with a *Tm* of 75 °C and reversed upon cooling from 90 to 25 °C (Supplementary Fig. 7C and D). Such structural plasticity of Kunitz inhibitors in temperature induced unfolding and refolding was reported from *C. cajan* (*Tm* = 63 °C; Haq and Khan, 2005) and *C. selloi* (*Tm* = 68 °C; Yoshizaki et al., 2007).

Further, any change in pH from 8.0 to 2.0 or 12.0 resulted in a marginal decrease in the ellipticity of RsBBI (Fig. 7C). The ellipticity exhibited by RsKI did not vary at pH 4.0 and 6.0 as compared to pH 8.0. But, a marginal increase in the ellipticity of RsKI was observed when incubated at pH 2.0 or 12.0 (Fig. 7F). These results suggest that RsBBI is structurally more stable than RsKI at different temperature and pH treatments.

2.8. Inhibitory activity of RsBBI and RsKI against HaGPs and AjGPs

R. sublobata is a wild relative of *C. cajan*. Given the potential of PIs from wild relatives in inhibiting the gut proteases of insect pests, the inhibitory activity of RsBBI and RsKI was examined against HaGPs and AjGPs (Fig. 8A–G). In-gel activity staining studies suggest RsBBI and RsKI were active against both AjGPs and HaGPs (Fig. 8A and B). Despite the marginal differences showed during in-gel activity staining studies, quantification of the specific activity of both RsBBI and RsKI showed wide difference against AjGPs and HaGPs (Fig. 8A–C). The specific activity of RsBBI against trypsin, chymotrypsin, AjGPs and HaGPs are found to be in the following order: AjGPIs (41,666 ± 950 units/mg protein) > TI (1730 ± 120 units/mg protein) > CI (217 ± 25 units/mg protein) > HaGPIs (113 ± 10 units/mg protein). Conversely, the specific activity of RsKI against the respective enzymes were in the following order: HaGPIs (16,950 ± 750 units/mg protein) > TI (807 ± 55 units/mg protein) > AjGPIs (513 ± 45 units/mg protein). However, no activity was detected for RsKI against chymotrypsin (Fig. 8C).

RsBBI inhibited AjGPs to the maximum extent of 84% of control with an IC_{50} of 24 ng as compared to HaGPs (79% of control) which showed an IC_{50} of 8.85 μg (Fig. 8D and F). India is the world leader in castor oil production. The farmers control the castor pests such as *A. janata* and *Spodoptera litura* using chemical pesticides such as Quinalphos, Chloropyriphos, carbaryl, monocrotophos, and endosulfan. These pesticides are harmful to both mankind and environment (Gahukar, 2015). In this scenario, probing for natural pest control agents such as the crude leaf extracts of *Solanum melanogena* containing BBI and secondary metabolites was reported to inhibit the activity of *A. janata* gut proteases (Devanand and Rani, 2011). The BBIs from cultivar varieties of *C. cajan* (ICP 7118 and ICP 14770) and recombinant RsBBI from *R. sublobata* retarded the growth and development of *A. janata* during *in vivo* feeding assay (Mohanraj et al., 2018; Prasad et al., 2010a; Swathi et al., 2014). However, the IC_{50} of wild RsBBI (24 ng) against AjGPs observed in the present study is much lower (3.25-fold) as compared to BBIs (78 ng) from *C. cajan* cultivar (Fig. 8F; Prasad et al., 2010a; Swathi et al., 2014).

Conversely, RsKI inhibited HaGPs to the maximum extent of 81% of control with an IC_{50} of 59 ng as compared to AjGPs (71% of control) which showed an IC_{50} of 1.95 μg (Fig. 8E and G). Several TIs from

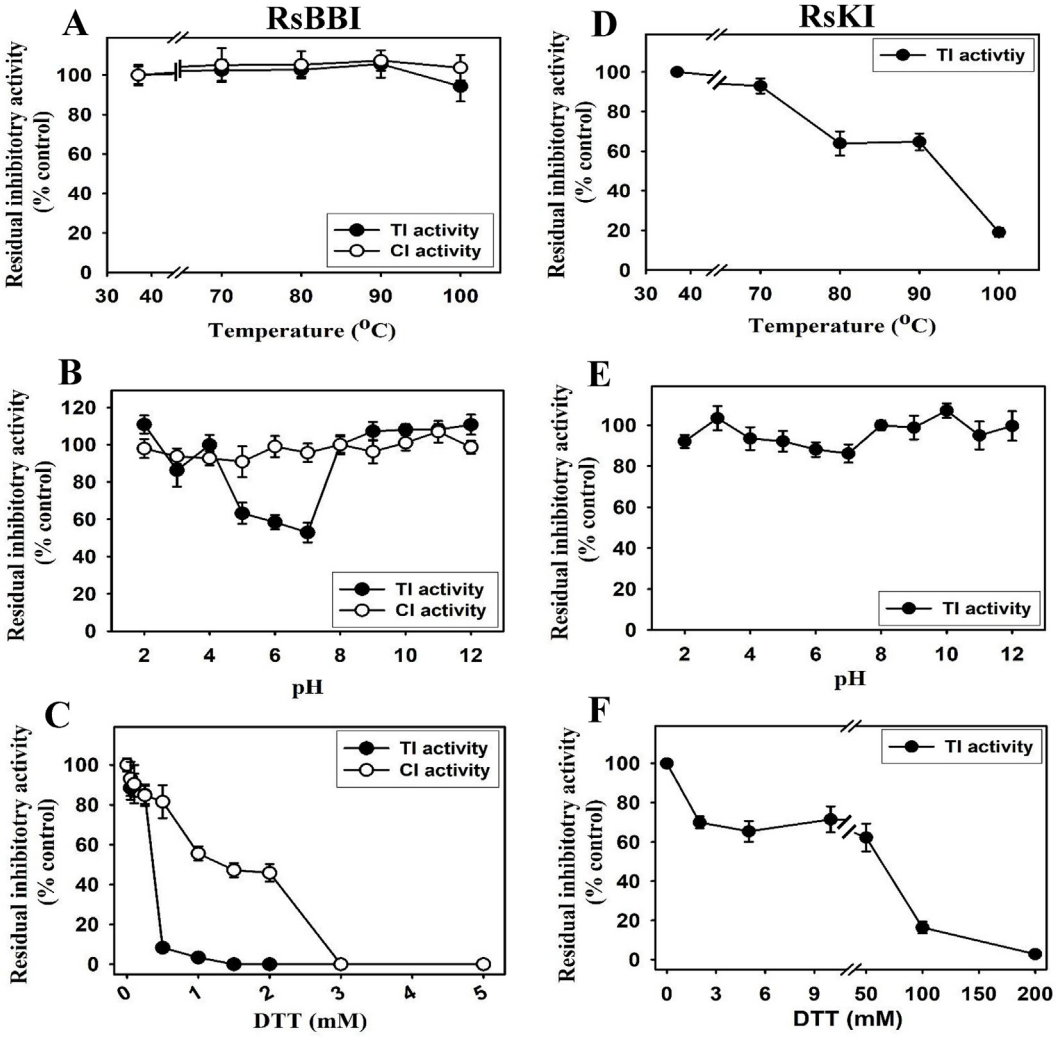


Fig. 6. Effect of temperature, pH, and DTT on the inhibitory activities of RsBBI and RsKI. The TI and CI activities of RsBBI and RsKI after incubating for 30 min at indicated **(A,D)** temperature, **(B,E)** pH and **(C,F)** DTT concentration, respectively. The residual protease activity was measured as described in materials and methods section 3.10. The values are the mean ± SE of three independent assays each with three replicates.

different PI families such as squash inhibitor from *Momordica charantia* (Telang et al., 2009b); PIN1 inhibitor from Capsicum (Tamhane et al., 2005); Kunitz inhibitors from *P. tetragonolobus* (Telang et al., 2009a); *Cicer arietinum* (Srinivasan et al., 2005); *Acacia senegal* (Babu and Subrahmanyam, 2010); and miraculin/kunitz inhibitors from *C. platycarpus* (Swathi et al., 2016) showed effective inhibition against HaGPs. However, the inhibition of gut trypsin-like protease activity at the nano gram range indicate the importance of RsBBI and RsKI as potential biopesticides in the management of *A. janata* and *H. armigera*.

3. Materials and methods

3.1. Experimental

R. sublobata (Schumach.) Meikle (ICP 15868) seeds were obtained from International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, India. *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), *N*-glutaryl-L-phenylalanine-*p*-nitroanilide (GLUPHEPA) and soybean BBI were supplied by Sigma-Aldrich, USA. CNBr activated Sepharose, Sephadex G-50 fine and DTT were procured from GE Healthcare Biosciences Corp., USA. 3X crystals of bovine pancreatic α-trypsin and α-chymotrypsin, and all other chemicals were procured from Sisco Research Laboratories, Mumbai, India. *H. armigera* eggs were procured from National Bureau of Agricultural Insect Resources (NBAIR), Bengaluru, India. *A. janata* larvae were collected from the surrounding fields of the University of Hyderabad. The insects were reared in insect culture room maintained at temperature of 26 ± 1 °C, RH of 65 ± 5% and 14:10 h light-dark photoperiod.

3.2. Purification of trypsin specific PIs (RsPI) from R. sublobata seeds

The CPIs were extracted from seed powder in 50 mM Tris-HCl pH 8.0 with 1% PVP (1:6 w/v) by mild stirring overnight at 4 °C as described in Prasad et al. (2010c). The clear supernatant obtained after centrifugation (twice) at 10,000 g for 20 min at 4 °C was subjected to 20–60% $(NH_4)_2SO_4$ fractionation. The pellet obtained was dissolved in 50 mM Tris-HCl pH 8.0, dialyzed and applied onto trypsin-Sepharose 4B column (XK 16/20, 3 cm; FPLC AkTAprime plus, GE Healthcare Life Sciences, USA). The unbound protein was washed with five column volumes of 50 mM Tris-HCl, pH 8.0 containing 100 mM NaCl and the bound protein was eluted with 0.01 N HCl. The eluted peak fractions (1 mL) neutralized with 50 mM Tris were pooled up and concentrated in a freeze dryer (FreeZone −105 °C 4.5 L Benchtop, Labconco, USA). The concentrated sample was subjected to size exclusion chromatography by loading on to a Sephadex G-50 fine column (XK 16/100, 85 cm). After equilibration of column, the sample was eluted with 50 mM Tris-HCl, pH 8.0. The eluted peak fractions (1 mL) containing TI activity was concentrated using 3.0 kDa cut-off filter. Thus, the purified pool of

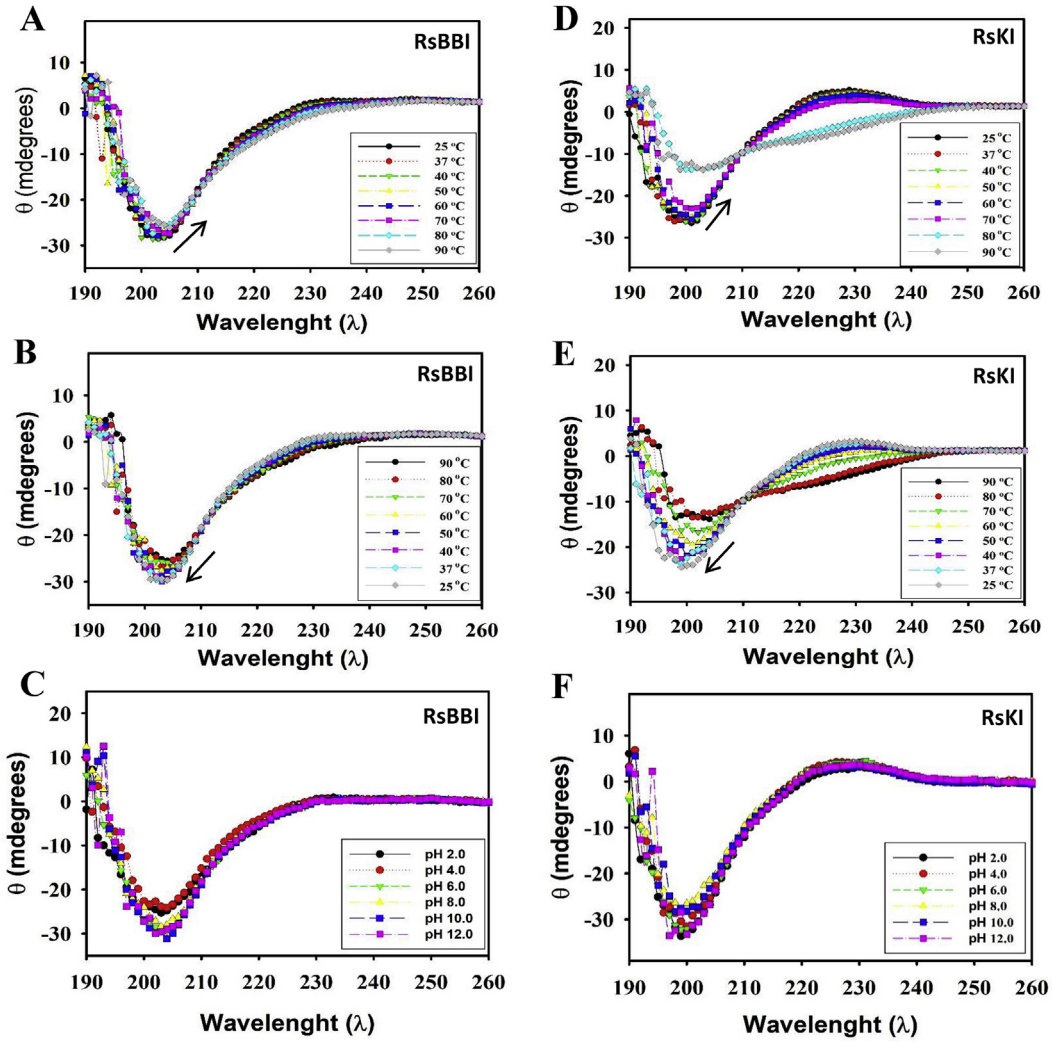


Fig. 7. Effect of temperature, pH, and DTT on the structural stability of RsBBI and RsKI at far-UV (190–260 nm). CD spectra indicating the change in ellipticity of **(A)** RsBBI and **(D)** RsKI on increasing the temperature from 25 to 90 °C; CD spectra indicating the recovery of ellipticity in **(B)** RsBBI and **(E)** RsKI when the temperature is decreased from 90 to 25 °C; CD spectra indicating the changes in ellipticity of **(C)** RsBBI and **(F)** RsKI at different pH (2.0, 4.0, 6.0, 8.0, 10.0, 12.0). The concentration of RsBBI or RsKI used to obtain the CD spectrum was 0.2 mg/mL. Further details were as described in materials and methods section 3.13.

trypsin specific PIs was labeled as "RsPI" and stored at −20 °C until further use. Protein estimation was performed using the Bicinchoninic acid method.

3.3. Tricine SDS-PAGE and two-dimensional gel electrophoresis (2DE) of RsPI

The molecular mass of trypsin-specific PIs present in RsPI was determined using Tricine SDS-PAGE as described by Schagger (2006) followed by silver staining. The 2DE of RsPI was performed as described in the manufacturer's instructions (GE Healthcare Life Sciences, USA). 30 µg of RsPI was dissolved in 200 µL of sample preparation solution (7M urea, 2M thiourea, 4% CHAPS, 1% IPG buffer and 40 mM DTT) and applied on to an 11 cm IPG strip (pH 4–7) by passive rehydration overnight. IEF was performed at 20 °C and 70 µA current as follows: 500 V for 2 h; linear gradient to 1000 V for 800 Vh; linear ramping gradient to 6000 V for 8800 Vh and final focusing at 6000 V for 4500 Vh. After IEF, the strip was equilibrated with 5 mL of SDS equilibration buffer (6M urea, 50 mM Tris-HCl pH 8.8, 29.3% Glycerol, 2% SDS and 0.002% bromophenol blue) containing 10 mg/mL DTT followed by 25 mg/mL IDA and 5 mL of Tricine SDS-PAGE upper tank buffer, each for 30 min. The second dimension was performed on 15% Tricine SDS-PAGE gel. After 2DE, the gel was stained with silver nitrate and the spots were visualized.

3.4. N-terminal sequencing of isoinhibitor spots from RsPI

The N-terminal sequencing of the isoinhibitor spots from RsPI separated in 2DE was performed by transferring them on to a sequencing grade PVDF (0.22 µm) membrane using 10 mM CAPS buffer, pH 11.0 at 30 V and 4 °C overnight. The transfer of isoinhibitors was visualized by staining for 5 min in 0.3% CBB R-250 (methanol and water, 40:60 v/v). Three isoinhibitor spots of pI 4.925 and mass ca. 20 kDa (Spot 1); pI 5.0 and mass ca. 8 kDa (Spot 2) and pI 6.0 and mass ca. 20 kDa (Spot 3) were cut from PVDF membrane, destained in 50% methanol and their N-terminus was sequenced (Procise Sequencer - model 492 coupled with HPLC system - model 785 A; Applied Biosystems, USA). The N-terminal sequences were ClustalW2 aligned with the related protein sequences from UniProt database to identify percent similarity.

3.5. Separation of Bowman-Birk isoinhibitors (RsBBI) and Kunitz isoinhibitors (RsKI) from RsPI

RsBBI and RsKI were separated from RsPI based on their differential solubility in TCA and aqueous acetate buffer (Fig. 3A) by modifying the protocols of Bowman (1946) and, Macedo and Xavier-Filho (1992),

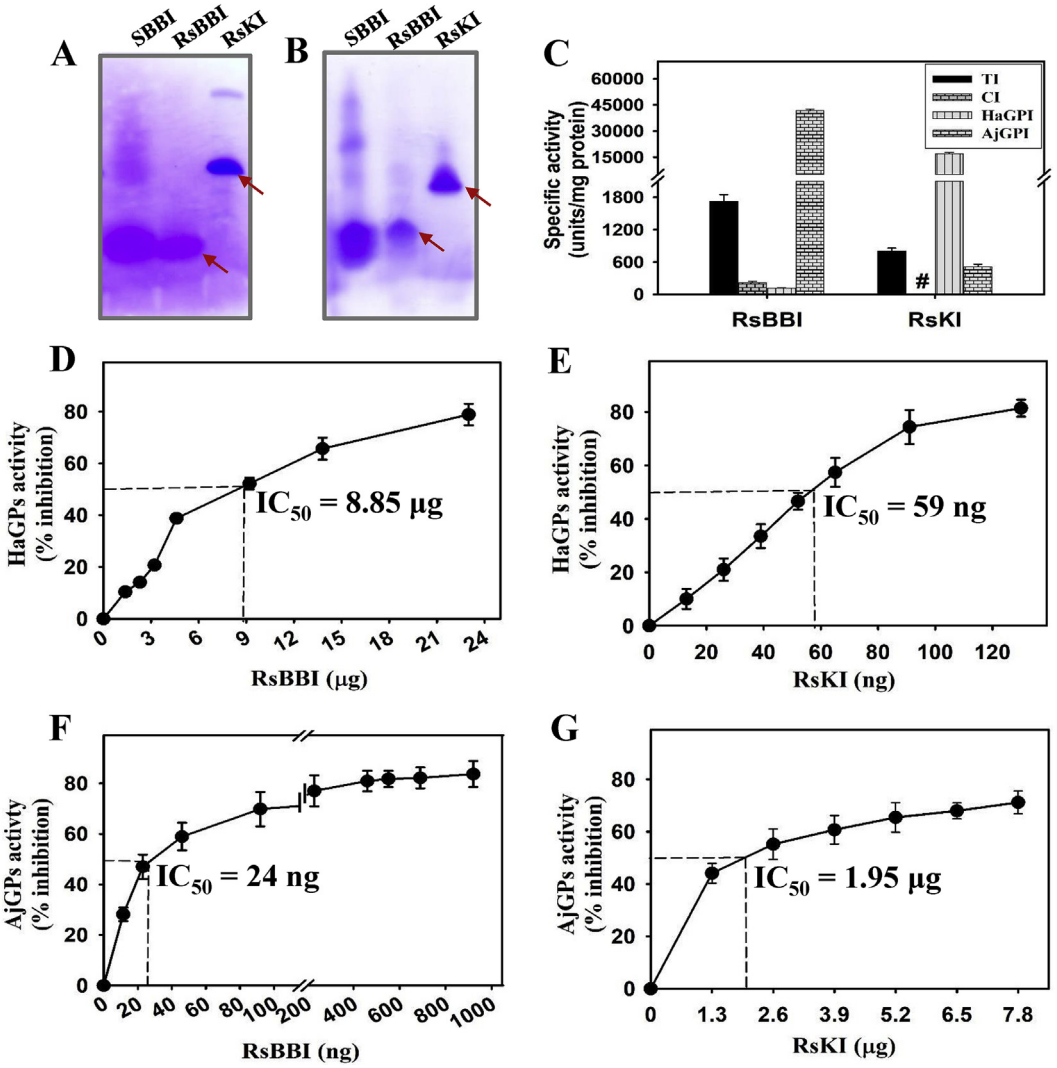


Fig. 8. Evaluation of the inhibitory activity of RsBBI and RsKI against AjGPs and HaGPs. Visualization of in-gel activity of RsBBI (5 µg) and RsKI (5 µg) against **(A)** AjGPs and **(B)** HaGPs in gelatin SDS-PAGE (15%) under non-reducing conditions. SBBI (5 µg) was loaded as positive control. The inhibitor bands were visualized after staining with CBB R-250. Arrows '→' indicate the undigested PI band active against respective proteases; **(C)** The specific activity of RsBBI and RsKI against HaGPs and AjGPs enzymes. The symbol '#' indicate that CI activity was not detected in RsKI; Half-maximal inhibitory concentrations (IC_{50}) of RsBBI and RsKI against **(D,E)** HaGPs and **(F,G)** AjGPs, respectively. The results are the mean ± S.E. of at least three independent assays each with three replicates. Further details were as described in materials and methods section 3.10.

respectively. An aliquot of RsPI containing 500 µg of protein was taken in 1 mL of 50 mM Tris-HCl, pH 8.0 and 20% TCA was added to attain a final concentration of 2.5% in the reaction mixture (RM1). The RM1 was heated to 70 °C for 10 min and centrifuged at 11,000 g for 5 min. The supernatant (S1) was adjusted to pH 8.0 with 400 mM Tris-HCl and acetone precipitated. The precipitate was air dried, and dissolved in 50 mM Tris-HCl (pH 8.0) and labeled as RsBBI. It is stored at −20 °C until further use. The precipitate obtained with TCA in RM1 was dissolved in 300 µL of 50 mM Tris-HCl, pH 8.0 and the above step was repeated to remove any traces of BBI left in the pellet. The final pellet (P1) thus obtained from RM1 was dissolved in 200 µL of 50 mM Tris-HCl, pH 8.0 and extracted in 50 mM Sodium acetate buffer, pH 4.0 (1:2, v/v). This reaction mixture (RM2) was heated at 70 °C for 10 min and centrifuged at 11,000 g for 5 min. The pellet (P2) from RM2 was discarded while the supernatant (S2) was adjusted to pH 8.0 with 100 mM Tris-HCl. The S2 fraction obtained was acetone precipitated, air dried, dissolved in 50 mM Tris-HCl (pH 8.0), labeled as RsKI and stored at −20 °C.

3.6. Western blotting and immunodetection of RsBBI

Tricine SDS-PAGE containing RsBBI and RsKI was equilibrated in transfer buffer (25 mM Tris-HCl, 192 mM Glycine and 20% Methanol) for 15 min and the PIs were transferred on to nitrocellulose membrane at 20 V for overnight at 4 °C. The membrane was blocked with 5% non-fat dry milk powder in TBS (198 mM Tris-HCl pH 7.6 and 150 mM NaCl) at RT for 2 h. Later, it was incubated in 1:500 dilution of anti-BBI antibody for 2 h. This was followed by washing with TBS containing 0.2% Tween 20 (TBST) and incubation with 1:5000 dilution of goat anti-Rabbit IgG antibody conjugated with alkaline phosphatase for 2 h at RT. After three washes with TBST, the blot was developed using BCIP/NBT (5-bromo-4-chloro-3-indoyl phosphate/Nitro-blue-tetrazolium) solution (Vishwakarma et al., 2016).

3.7. Visualization of in-gel activity of RsPI, RsBBI and RsKI

The in-gel activity of RsPI, RsBBI and RsKI against their cognate proteases was visualized on gelatin-incorporated Tricine SDS-PAGE as described by Felicioli et al. (1997). After electrophoresis, the gels were

washed thrice with 2.5% Triton X-100 for 15 min each at 25 °C, followed by incubation for 2 h at 37 °C with the respective proteases dissolved in 0.1 M Tris-HCl, pH 8.2 (trypsin/AjGPs), 0.1 M Tris-HCl, pH 7.8 (chymotrypsin) or 0.1 M Glycine-HCl, pH 10.5 (HaGPs) buffer solutions, respectively. The inhibitory protein bands/isoinhibitor spots were visualized by CBB R-250 staining.

3.8. MALDI-TOF, MALDI-ISD, MALDI-TOF PMF and MALDI-MS-MS analysis of RsBBI and RsKI

For MALDI-TOF, RsBBI and RsKI were mixed independently with equal volumes of α-cyano-4-hydroxy-cinnamic acid (CHCA) matrix. The reaction mixture (2 µl) was spotted on MALDI target plate and allowed to crystallize at room temperature. The ions generated from sample spot after laser shot were collected in linear mode and analyzed for the intact mass (Autoflex III smart beam instrument equipped with Nd:YAG laser; Bruker Daltonics, Bremen, Germany). For MALDI-ISD, RsBBI or RsKI was mixed with 1,5-diaminonaphthalene (DAN) matrix in 2:1 ratio to spot on MALDI plate. ISD spectrum of BSA is used to calibrate the instrument. The ISD spectra obtained from RsBBI and RsKI were annotated using FlexAnalysis 3.4, and the annotated sequences were searched in pBLAST to confirm the identity of PIs. For MALDI-TOF PMF analysis, the individual RsBBI (mono-, di- or tetramer) or RsKI (mono- or dimer) bands were cut from 15% Tricine SDS-PAGE and the protein was digested with trypsin enzyme overnight at 4 °C. The eluted peptides were observed for PMF peaks as mentioned above. The obtained PMF spectra were stacked using FlexAnalysis 3.4. The individual peaks were further ionized and denovo sequenced using Biotools 2.2 software.

3.9. Insect midgut extraction

The 5th instar larvae of *H. armigera* and *A. janata* were narcotized on ice and dissected dorsally in saline (0.15 M NaCl). The digestive enzymes from midguts were extracted as described in Swathi et al. (2014). The excised midguts were collected and homogenized in 50 mM Glycine-HCl, pH 10.5 for *H. armigera* or 50 mM Tris-HCl, pH 8.2 for *A. janata* and centrifuged at 10,000 g for 15 min at 4 °C. The supernatant was collected from respective homogenates, labeled as HaGPs and AjGPs, and stored at −20 °C for further use.

3.10. Assay of proteases and PIs

The assay of proteases (trypsin, chymotrypsin, AjGPs or HaGPs) was performed as described in Prasad et al. (2010c) and Swathi et al. (2014). The protease activity was determined by monitoring the rate of formation of *p*-nitroanilide from a chromogenic substrate BAPNA/GLUPHEPA (1 mM) at 37 °C after incubating for 45 min. BAPNA was used as a substrate for trypsin, HaGPs and AjGPs while GLUPHEPA was used as a substrate for chymotrypsin. The assay buffers contained 50 mM Tris-HCl and 20 mM $CaCl_2$ at either pH 8.2 for trypsin and AjGPs or pH 7.8 for chymotrypsin. Conversely, the assay buffer for HaGPs contained 50 mM Glycine-NaOH at pH 10.5. The reaction was terminated with 30% acetic acid (v/v) and the absorbance at 410 nm was recorded in UV–visible spectrophotometer (UV-1700, Shimadzu, Japan). The molar extinction coefficient (M^{-1} cm^{-1}) for *p*-nitroanilide at 410 nm is equivalent to 8800. One unit of trypsin, chymotrypsin, AjGP or HaGP is defined as the amount of enzyme or gut extract which increases the absorbance of reaction medium by 1.0 O.D.

The assay of PIs (RsPI, RsBBI or RsKI) was assessed indirectly by monitoring the inhibition in the activity of proteases. After incubating PIs with respective proteases (trypsin, chymotrypsin, AjGPs and HaGPs) for 15 min at 37 °C, the decrease in the activity of proteases was determined (Swathi et al., 2014). One unit of TI, CI, *A. janata* gut trypsin-like protease inhibitor (AjGPI) or *H. armigera* gut trypsin-like protease inhibitor (HaGPI) was defined as the amount of RsPI, RsBBI or RsKI required to inhibit 50% hydrolysis of BAPNA or GLUPHEPA by their respective proteases under the optimal assay conditions. The IC_{50} of RsBBI and RsKI against HaGPs and AjGPs was determined by monitoring the activity of respective proteases after incubating with RsBBI and RsKI at a wide range of concentrations.

3.11. Inhibition kinetics and titration studies

Inhibition constant (*Ki*) was determined by incubating RsBBI or RsKI at a concentration in the increasing order against a fixed concentration (1 µM) of either trypsin or chymotrypsin at 37 °C for 15 min. This was followed by addition of different concentrations (0.125, 0.25, 0.375, 0.5, 0.625, 0.75 mM) of enzyme substrate, i.e., BAPNA for trypsin and GLUPHEPA for chymotrypsin and incubated at 37 °C for 45 min. The *Ki* values were determined using Sigma Plot 12.5 (Systat Software Inc. San 1 Jose, California).

Further, the residual trypsin and chymotrypsin activities at different molar ratios (0.25–2.0) of RsBBI and RsKI to trypsin/chymotrypsin were determined by titrating different concentrations of RsBBI or RsKI with a fixed concentration (1 µM) of its cognate protease as described in Prasad et al. (2010c).

3.12. Stability studies

The effect of temperature, pH, and DTT on the TI and CI activities of RsBBI and RsKI was determined as described below. The temperature stability studies were performed by incubating RsBBI or RsKI for 30 min at a high temperature (70, 80, 90 and 100 °C). The residual TI and CI activities were measured as described in section 3.10 after cooling the samples to room temperature. The sample incubated at 37 °C is used as a control. Similarly, the stability in the TI/CI activities of RsBBI and RsKI against pH was determined after incubating them at different pH from 2.0 to 12.0 at 37 °C for 1 h using the following buffers at 50 mM concentration: Glycine-HCl (pH 2–3), sodium acetate-acetic acid (pH 4–5), sodium phosphate (pH 6.0), Tris-HCl (pH 7–9) and Glycine-NaOH (pH 10–12). Also, the stability in the TI/CI activities of RsBBI and RsKI against DTT was determined by incubating it at different concentrations, i.e., up to 5 mM with RsBBI or 200 mM with RsKI at 56 °C for 45 min followed by incubation with iodoacetamide at twice the concentration of DTT in the dark for 1 h. The residual TI and CI activities of RsBBI and RsKI was measured at specific pH and DTT concentration as described in section 3.10.

3.13. Circular dichroism spectroscopy

CD spectra were recorded at a scan speed of 50 nm/min with three accumulations using 1 mm path length cuvette. Secondary structural elements were probed at far-UV region (190–260 nm). CD spectra of RsKI and RsBBI were recorded at a final protein concentration of 0.2 µg/µl dissolved individually in 10 mM Tris-HCl (pH 8.0). The secondary structural elements were estimated using SpectraManager 2.0. The effect of temperature on secondary structural elements of both RsBBI and RsKI was monitored at 25, 37, 40, 50, 60, 70, 80 and 90 °C using Peltier thermostat.

The temperature-induced unfolding and refolding of RsBBI or RsKI was measured by increasing the temperature from 25 to 90 °C and decreasing back from 90 to 25 °C at the far-UV region. The thermal transient midpoint (*Tm*) during unfolding was determined at a fixed wavelength of 199 nm for RsKI and 203 nm for RsBBI, where maximum ellipticity was observed. The temperature slope of 1 °C per minute was maintained. The effect of pH on the secondary structural elements was determined by incubating both RsBBI and RsKI at 37 °C for 1 h in 10 mM concentrations of respective buffers as described in Section 3.12. The samples were cooled down to room temperature and the CD spectra were recorded.

3.14. Statistical analysis

The data shown were mean ± standard error of at least three independent experiments each with three replicates. Statistics were performed using SigmaStat 12.5 (Systat Software Inc., San Jose, CA).

4. Conclusion

In the present study trypsin-specific PIs (RsPI) were purified from the seeds of *R. sublobata*, a wild relative of pigeonpea using chromatographic techniques. Bowman-Birk isoinhibitors (RsBBI) and Kunitz isoinhibitors (RsKI) present in RsPI are purified to homogeneity by exploiting their differential solubility in TCA and sodium acetate buffer, sequentially. The identity of RsBBI and RsKI was confirmed through N-terminal sequencing by Edman degradation method and mass spectrometry studies. The self-association pattern of RsBBI and RsKI was demonstrated by SDS-PAGE, immunoblotting with anti-BBI antibody, MALDI- intact mass and MALDI-TOF-TOF analysis, respectively. The TI and CI activities of RsBBI and RsKI correlated with their structural stability at a wide range of temperature, pH and DTT concentration. Further, *in vitro* studies against AjGPs and HaGPs suggested the potential of RsBBI and RsKI in the management of *A. janata* and *H. armigera*, respectively.

Author contributions

Conceived and designed the experiments: KP; Experimental material: NM; Performance of the experiments: SSM, VL and GM; Analysis of the data: SSM and KP; Manuscript writing: SSM; Manuscript editing: KP; Critical suggestions during experimental design and manuscript editing: ADG.

Conflict of interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2018.12.018.

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