Phytochemistry 151 (2018) 78–90

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Biochemical properties of a bacterially-expressed Bowman-Birk inhibitor from *Rhynchosia sublobata* (Schumach.) Meikle seeds and its activity against gut proteases of *Achaea janata*

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ARTICLE INFO

Article history:
Received 3 July 2017
Received in revised form
15 February 2018
Accepted 20 February 2018

Keywords:
Achaea janata
Bowman-Birk inhibitor
E. coli
Helicoverpa armigera
Trypsin-like gut proteases

ABSTRACT

Crude proteinase inhibitors (CPIs) extracted from the seeds of *Rhynchosia sublobata*, a wild relative of pigeon pea showed pronounced inhibitory activity on the larval gut trypsin-like proteases of lepidopteran insect pest – *Achaea janata*. Consequently, a full-length cDNA of Bowman-Birk inhibitor gene (*RsBBI1*) was cloned from the immature seeds of *R. sublobata*. It contained an ORF of 360 bp encoding a 119-amino acid polypeptide (13.3 kDa) chain with an N-terminus signal sequence comprising of 22 amino acids. The amino acid sequence and phylogenetic analysis together revealed that RsBBI1 exhibited a close relation with BBIs from soybean and *Phaseolus* spp. A cDNA sequence corresponding to *RsBBI1* mature protein (89 amino acid stretch) was expressed in *E. coli*. The recombinant rRsBBI1 protein with a molecular mass of 9.97 kDa was purified using trypsin affinity chromatography. The purified rRsBBI1 exhibited non-competitive mode of inhibition of both bovine trypsin (*Ki* of 358 ± 11 nM) and chymotrypsin (*Ki* of 446 ± 9 nM). Its inhibitory activity against these proteases was stable at high temperatures (>95 °C) and a wide pH range but sensitive to reduction with dithiothreitol (DTT), indicating the importance of disulphide bridges in exhibiting its activity. Also, rRsBBI1 showed significant inhibitory activity (IC_{50} = 70 ng) on *A. janata* larval gut trypsin-like proteases (AjGPs). Conversely, it showed <1% inhibitory activity (IC_{50} = 8 μg) on *H. armigera* larval gut trypsin-like proteases (HaGPs) than it has against AjGPs. Besides, *in vivo* feeding experiments clearly indicated the deleterious effects of rRsBBI1 on larval growth and development in *A. janata* which suggests it can be further exploited for such properties.

1. Introduction

Insect larvae feed on the vegetative and reproductive organs of plants and digest them with the aid of serine, cysteine, aspartic or metalloproteinases present in their gut environment (Terra and Ferreira, 1994). Upon infestation by larvae, plants display a myriad of defense responses, including the production of bioactive secondary metabolites and proteinaceous molecules such as α-amylase inhibitors, lectins, polyphenol oxidases and proteinase/protease inhibitors (PIs) (Furstenberg-Hagg et al., 2013). Serine PIs, which are active against many serine proteases found in the gastrointestinal tract of insects, are identified generally across the plant kingdom. They are further classified into eight different families viz. Kunitz inhibitors, Bowman-Birk inhibitors (BBIs), Potato inhibitor-I and Potato inhibitor-II, Mustard trypsin inhibitors, Squash inhibitors, Serpins and Cereal trypsin/α-amylase inhibitors (Mosolov and Valueva, 2005).

PIs are highly stable globular proteins constitutively expressed in storage organs such as seeds and tubers. They are also induced in

Abbreviations: AjGPs, *Achaea janata* larval gut trypsin-like proteases; AjGPIs, *A. janata* larval gut trypsin-like protease inhibitors; BBI, Bowman-Birk inhibitor; CI, Chymotrypsin inhibitor; CPIs, crude protease inhibitors; HaGPs, *Helicoverpa armigera* larval gut trypsin-like proteases; HaGPIs, *H. armigera* larval gut trypsin-like protease inhibitors; PIs, Protease inhibitors; RsBBI1, *Rhynchosia sublobata* BBI1 protein; rRsBBI1, Recombinant RsBBI1 protein; TI, Trypsin inhibitor.

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https://doi.org/10.1016/j.phytochem.2018.02.009
0031-9422/

vegetative organs of plants during biotic and abiotic stresses and participate in regulating endogenous proteolysis, seed development, and programmed cell death. In addition, they serve as seed reserves for sulfur-containing amino acids, cysteine (Jamal et al., 2013; Mosolov and Valueva, 2005). PIs act against insect pest by binding to their digestive proteases and block their proteolytic activity. This mechanism lowers the pool of essential amino acids in the insects' gut required for their growth and thereby causing mortality (Jongsma and Bolter, 1997). The PIs from wild relatives and non-host plants are more effective than the PIs from host plants in the management of the insect pests as the digestive enzymes present in their guts have not adapted to such PIs (Harsulkar et al., 1999; Jongsma et al., 1996). Several transgenic plants expressing PIs from the host or non-host plants are produced to counteract the insect pests (Duan et al., 1996; Hilder et al., 1987; Johnson et al., 1989; Macedo et al., 2015).

From an economic perspective, *Helicoverpa armigera* and *Achaea janata* are relatively important among the lepidopteran insect pests. *H. armigera,* being polyphagous has caused significant loss to many crops (Lammers and Macleod, 2007). Further, the management of *H. armigera* continued to be a major challenge as it has developed resistance to a variety of pesticides. Conversely, *A. janata* feeds on an oil-rich *Ricinus communis* and causes severe loss to this cash crop owing to its foliar feeding behavior (Sujatha et al., 2010).

The wild relatives of leguminous crops are known to harbor a valuable gene pool for biotic, abiotic and disease resistance traits (Mallikarjuna et al., 2011). Serine PIs such as BBIs are mostly identified in leguminous plants and they contain two reactive sites for trypsin and chymotrypsin inhibition. Besides, the larval gut environment of lepidopteran insects possessed chiefly trypsin-like and chymotrypsin-like proteases. Therefore, in the present study, a BBI (*RsBBI1*) gene was cloned and sequenced from the immature seeds of *R. sublobata*, a wild relative of pigeon pea. The recombinant RsBBI1 (rRsBBI1) expressed in *E. coli* was examined for its biochemical properties and inhibitory potential against AjGPs and HaGPs. Based on the *in vitro* studies, *in vivo* feeding bioassays were performed to reveal the importance of rRsBBI1 in inducing growth retardation and mortality of *A. janata* larvae.

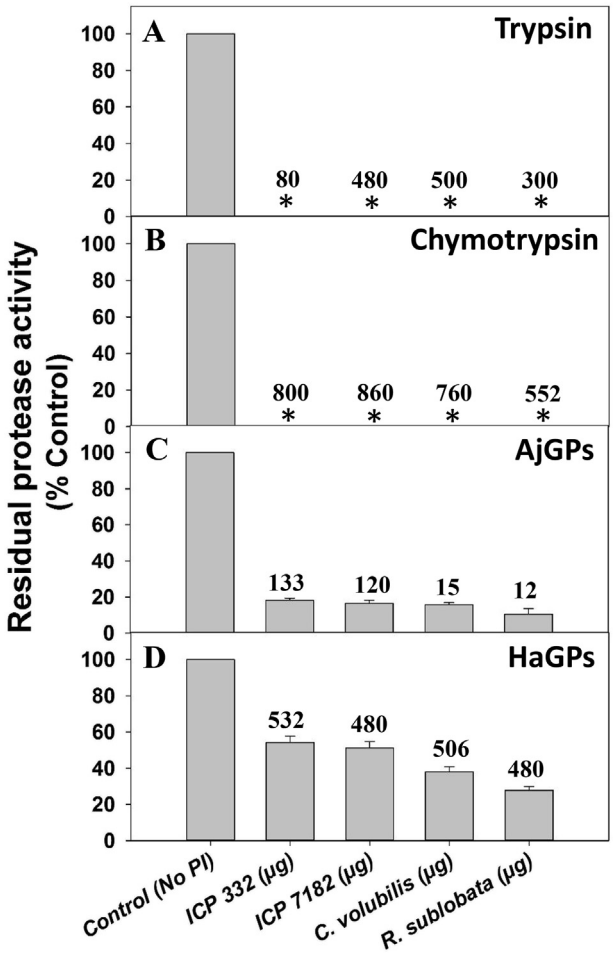

Fig. 1. Protease inhibition by seed CPI from cultivars and wild relatives of *C. cajan*. Residual protease activity of **(A)** bovine trypsin, **(B)** bovine chymotrypsin, **(C)** AjGPs and **(D)** HaGPs on incubation with CPI from seeds of *C. cajan* cultivars (ICP 332, ICP 7182) and wild relatives [*C. volubilis* (ICP 15774), *R. sublobata* (ICP 15868)]. Control bars represent the 100% activity of the different proteases in the absence of CPI. An asterisk (*) indicates the complete loss of protease activity in the presence of CPI. The values indicated above the bars are the corresponding CPI concentrations required to obtain maximum inhibition of respective proteases.

2. Results

2.1. Effect of seed crude PI on gut trypsin-like proteases

Seed crude PI (CPI) extracts of cultivars (ICP 332 and ICP 7182) and wild relatives (*C. volubilis* and *R. sublobata*) of pigeon pea were compared for their inhibitory potential against AjGPs and HaGPs in a wide range of concentration, using trypsin and chymotrypsin as reference controls (data not shown). The amount of CPI required from cultivars and wild relatives to cause maximum inhibition in activity of different proteases varied considerably (Fig. 1A–D). For example, among the bovine proteases tested, an amount of 80 µg of CPI from *C. cajan* cultivar ICP 332 was required to cause 100% inhibition in the activity of trypsin (Fig. 1A). Conversely, an amount of 552 µg of CPI was required from *C. cajan* wild relative *R. sublobata* to cause 100% inhibition in the activity of chymotrypsin (Fig. 1B). Among the larval gut trypsin-like proteases tested, the CPI from both cultivars and wild relatives could not inhibit the activity of AjGPs and HaGPs completely. However, the CPI from *R. sublobata* caused 85 ± 3% inhibition in the activity of AjGPs and 62 ± 5% inhibition in the activity of HaGPs at 12 µg and 480 µg, respectively (Fig. 1C and D). These results indicate *R. sublobata* CPI is 40-fold less active against HaGPs than AjGPs.

2.2. Cloning and sequencing of RsBBI1

In an attempt to clone BBI from *R. sublobabta* (wild relative), a 250 bp RT-PCR product was amplified from the cDNA generated using oligo dT primer (Fig. 2A). Consequently, a full-length transcript sequence of *RsBBI1* was obtained after 5′ and 3′ RACE experiments (Fig. 2B). Primary 5′ and 3′ RACE products obtained were ~250 bp and ~450 bp and the secondary PCR with the nested primers yielded 207 bp and 361 bp, respectively. Development of smaller products by the expected number of bases than the primary RACE products preliminarily confirmed the desired gene amplification. Contig sequence, generated out of the nucleotide sequences of RACE products, yielded complete sequence (505 bp) information of *RsBBI1* transcript comprising of an open reading frame of 360 bp encoding 119 amino acids, 21 bp of 5′ UTR and 109 bp of 3′ UTR ending with a polyA tail (Fig. 2C). BLASTn of cDNA sequence obtained showed greater identity with several other proteinase inhibitors belonging to BBI family (Supplementary Fig. 1). The complete *RsBBI1* CDS sequence was submitted to NCBI GenBank (accession # KT119632.2).

In silico analysis of RsBBI1 protein sequence of 119 amino acids

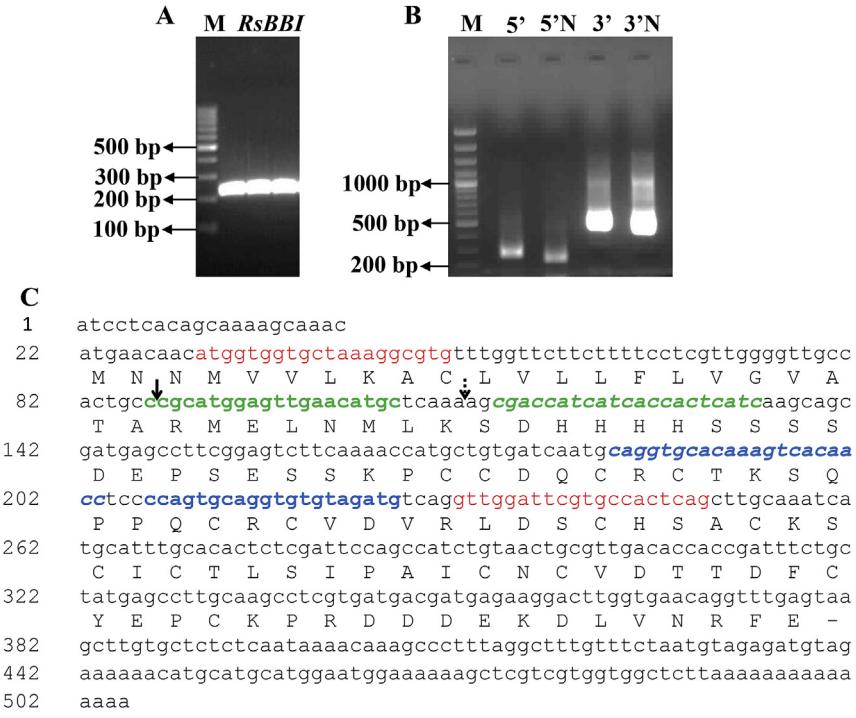

Fig. 2. Synthesis of full-length cDNA and cloning of *RsBBI1*. (A) PCR-amplified partial cDNA fragment (~250 bp) of *RsBBI1* from immature seeds of *R. sublobata*. Lane M is loaded with 100 bp DNA ladder and lanes 2-4 contained partially amplified *RsBBI1* gene product; **(B)** RACE amplified 5′ and 3′ fragments of *RsBBI1*. Lane M contained 100 bp DNA ladder, lanes 5′ and 5′N are loaded with the 5′ RACE primary and nested gene products and lanes 3′ and 3′N are loaded with 3′ RACE primary and nested gene products of *RsBBI1*; **(C)** Complete cDNA (505 bases) of *RsBBI1* and *in silico* translated amino acid sequence. The straight arrow indicates the end of the signal peptide and the dotted arrow indicates the starting point of the protein (89 amino acid stretch) overexpressed in *E. coli*. Primer sequences are shown in colour. Red colour indicates the primers used to get the partial sequence, green colour indicates the 3′ RACE forward primers and blue colour indicates the 5′ RACE reverse primers. The nested primers are italicised. Further details are indicated in Supplementary Table 1. The complete *RsBBI1* sequence is submitted to NCBI with accession number KT119632.2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

with a predicted mass of 13.303 kDa (Fig. 2C) using TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/) revealed the presence of a twenty-two-amino acid "MNNMVVLKACLVLLFLVGVATA" signal peptide. However, N-terminus sequencing of native BBI shows that it begins with serine followed by aspartic acid (Data not shown). Therefore, only 89 amino acids stretch mature protein with serine at N-terminus was cloned and expressed using pET23a (Fig. 2C).

Multiple sequence alignment with a pre-protein sequence of 119 amino acids revealed the alignment of RsBBI1 cysteine residues with the conserved cysteine residues (fourteen) backbone of BBIs from other leguminous plants (Fig. 3). The RsBBI1 possessed 'Lys-Ser' and 'Leu-Ser' at the P1-P1′ positions of trypsin (TKSQPPQ) and chymotrypsin (TLSIPAI) reactive site loops, respectively. However, the P1-P1′ residues in the trypsin reactive site are conserved, while those in the chymotrypsin reactive site of RsBBI1 matched with the BBIs from *Phaseolus*, *Glycine* and *Vigna* spp. (Fig. 3).

2.3. Phylogenetic analysis and 3D modeling of RsBBI1

The phylogenetic relationship of RsBBI1 from *R. sublobata* with BBIs from other legumes and cereals revealed, it is grouped between *Phaseolus* and *Glycine* spp. on one end, and between *C. cajan* and *Vigna* spp. at the other end of the phylogenetic tree (Fig. 4A). Further, *C. cajan* BBI outgrouped from RsBBI1 substantiating its evolutionary association with its wild gene pool. Furthermore, the BBIs from monocots were outgrouped from the dicots suggesting that though widely distributed among the Leguminosae members, they are highly conserved amongst dicots and monocots during evolution.

A three-dimensional structure was predicted for RsBBI1 from residues 44 to 113 using soybean BBI (PDB ID-1BBI) as a template (Fig. 4B). The modeled RsBBI1 represented a symmetrical protein with both trypsin and chymotrypsin reactive site loops on opposite sides. In 3D modeling, the amino acids identical between RsBBI1 and the template 1BBI are represented by the same color (Fig. 4C).

2.4. Overexpression and purification of rRsBBI1 from E. coli

The purification profile of rRsBBI1 was analyzed using 15% Tricine SDS-PAGE (Fig. 5D) and the trypsin inhibitory activity of rRsBBI1 was visualized in gelatin SDS-PAGE (Fig. 5E). The induced cell lysates of *E. coli* (*trxB*$^-$, *gor*$^-$) SHuffle T7 express cells transformed with pET23a-*RsBBI1* construct encoding a recombinant peptide of mature RsBBI1 of 89 amino acids (9970 Da) showed a dimeric band of ~20 kDa (Fig. 5D-lane 7; 5E-lane 6). However, this band does not appear in host cells transformed with vector alone i.e. pET23a without *RsBBI1* construct (negative control) (Fig. 5A-lane 7; 5B-lane 6). In-gel activity staining studies using gelatin SDS-PAGE clearly showed the trypsin inhibitory activity of rRsBBI1 protein expressed from the IPTG induced cell lysate (Fig. 5E). Conversely, such trypsin inhibitory bands do not appear at the corresponding position in the negative control (Fig. 5B). The elution profile of rRsBBI1 using trypsin affinity column is depicted in Fig. 5F and the chromatogram from negative control is devoid of any protein peak (Fig. 5C).

The purified rRsBBI1 with a yield of 2.5 ± 0.3 mg L^{-1} culture showed a single band in both SDS-PAGE as well as in-gel activity staining studies (Fig. 5D and E). But, intact mass MALDI-TOF analysis of rRsBBI1 denoted two peaks with molecular masses of 9970.87 Da and 9693.74 Da (Fig. 6). However, the theoretical mass (9971.02 Da) of 89 amino acid rRsBBI1 matched well with the peak

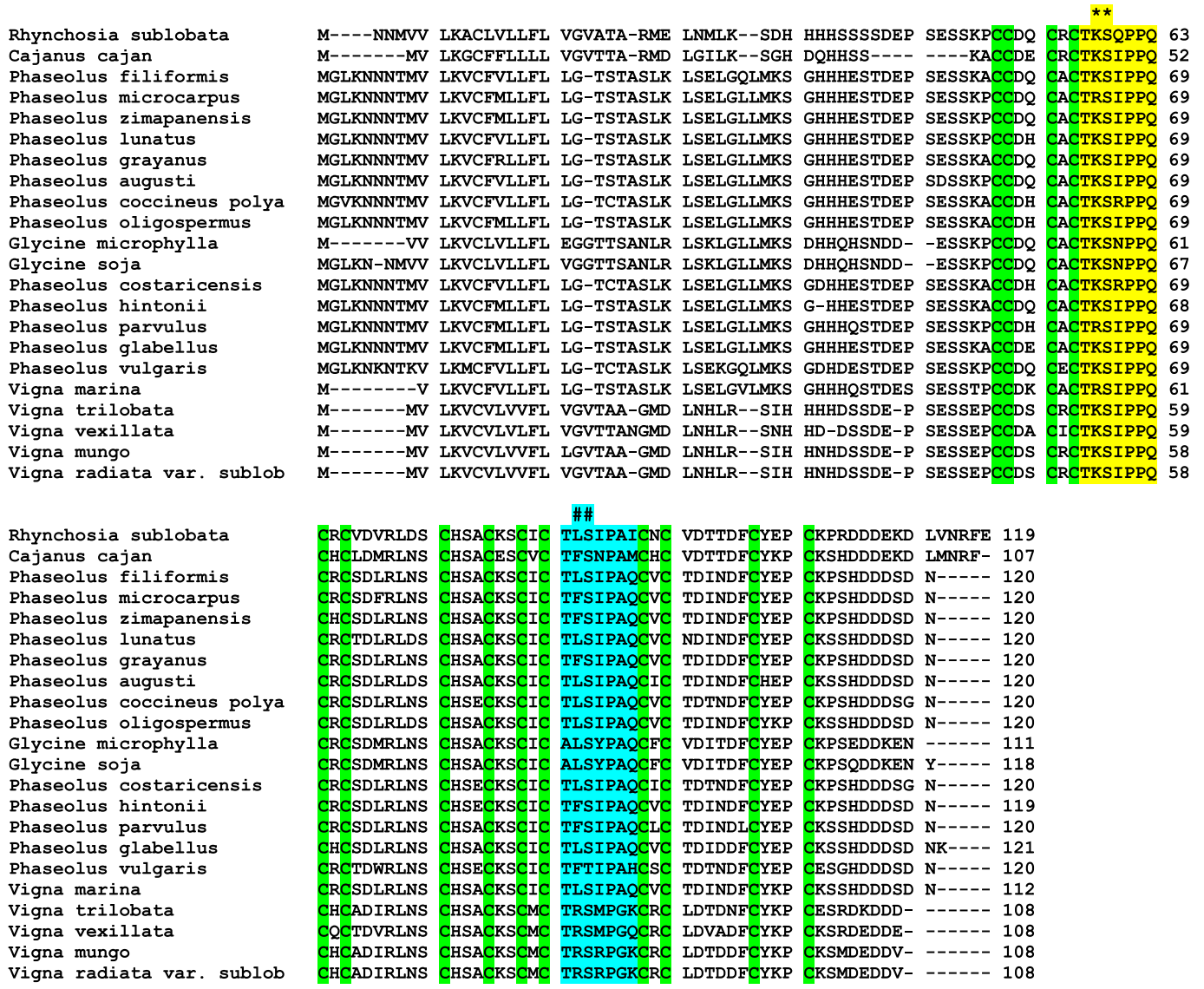

Fig. 3. Multiple sequence alignment of RsBBI1 with the reported BBIs. The trypsin and chymotrypsin reactive site loop residues are indicated in yellow and cyan colour and the corresponding reactive site amino acids are labeled with '**' and '##,' respectively. The fourteen-cysteine residue backbone of RsBBI1 showed matching with the reported BBIs as indicated by green colour. All the BBI sequences were downloaded from NCBI database. Accession numbers of the different Clustal W aligned BBIs are as follows: *Cajanus cajan* - KYP42282.1; *P. filiformis* - CAL69281.1; *P. microcarpus* - CAL64060.1; *P. zimapanensis* - CAQ52360.1; *P. lunatus* - CAL51268.1; *P. grayanus* - CAQ52359.1; *P. augusti* - CAL51269.1; *P. coccineus* - CAQ58092.1; *P. oligospermus* - CAL51270.1; *G. microphylla* - AAO89510.1; *G. soja* - BAB86783.1; *P. costaricensis* - CAL69279.1; *P. hintonii* - CAQ52357.1; *P. parvulus* - CAL69237.1; *P. glabellus* - CAL69238.1; *P. vulgaris* - CAQ34829.1; *V. marina* - ABD97867.1; *V. trilobata* - ABD91574.1; *V. vexillata* - ABD97866.1; *V. mungo* - AKC45532.1; *V. radiata* - ABD91575.1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of 9970.87 Da. Besides, the appearance of an additional peak with a molecular mass of 9693.74 Da in MALDI-TOF analysis could be due to cleavage of an arginine residue present at 87th position by trypsin bound to Sepharose column during affinity chromatography of rRsBBI1. As cleavages occur at the far end of C-terminus and the 14 cysteine residues that stabilize the rRsBBI1 are present within the 87 amino acids stretch, apparently, peptide with mass 9693.74 Da might exhibit inhibitory activity against both trypsin and chymotrypsin along with 9970.87 Da polypeptide.

2.5. Biochemical characterization of rRsBBI1

The purified rRsBBI1 inhibited the activity of both trypsin and chymotrypsin enzymes. However, the inhibitory activity of rRsBBI1 was more pronounced against trypsin as compared to chymotrypsin. Trypsin lost ~75% of its activity when the molar ratio of rRsBBI1 to trypsin was 0.5 and lost its activity completely as the molar ratio increased to 1.0 (Fig. 7A). Conversely, chymotrypsin lost 85% of its activity when the molar ratio of rRsBBI1 to chymotrypsin was 1.0 (Fig. 7A). Furthermore, chymotrypsin lost its activity completely as the molar ratio increased to 2.0. However, a linear extrapolation to obtain 100% inhibition indicated that rRsBBI1 binds to trypsin and chymotrypsin apparently at 1:0.75 and 1:1 molar ratio, respectively. Practically it is not possible for one molecule of rRsBBI1 to bind with 0.75 molecules of trypsin; it is assumed trypsin binds to rRsBBI1 at 1:1 molar ratio *a la* chymotrypsin (Fig. 7A). Enzyme kinetics analysis by Lineweaver-Burk plot indicated rRsBBI1 inhibited both trypsin ($Ki = 358 \pm 11$ nM) and chymotrypsin ($Ki = 446 \pm 9$ nM) in a non-competitive mode (Fig. 7B and C).

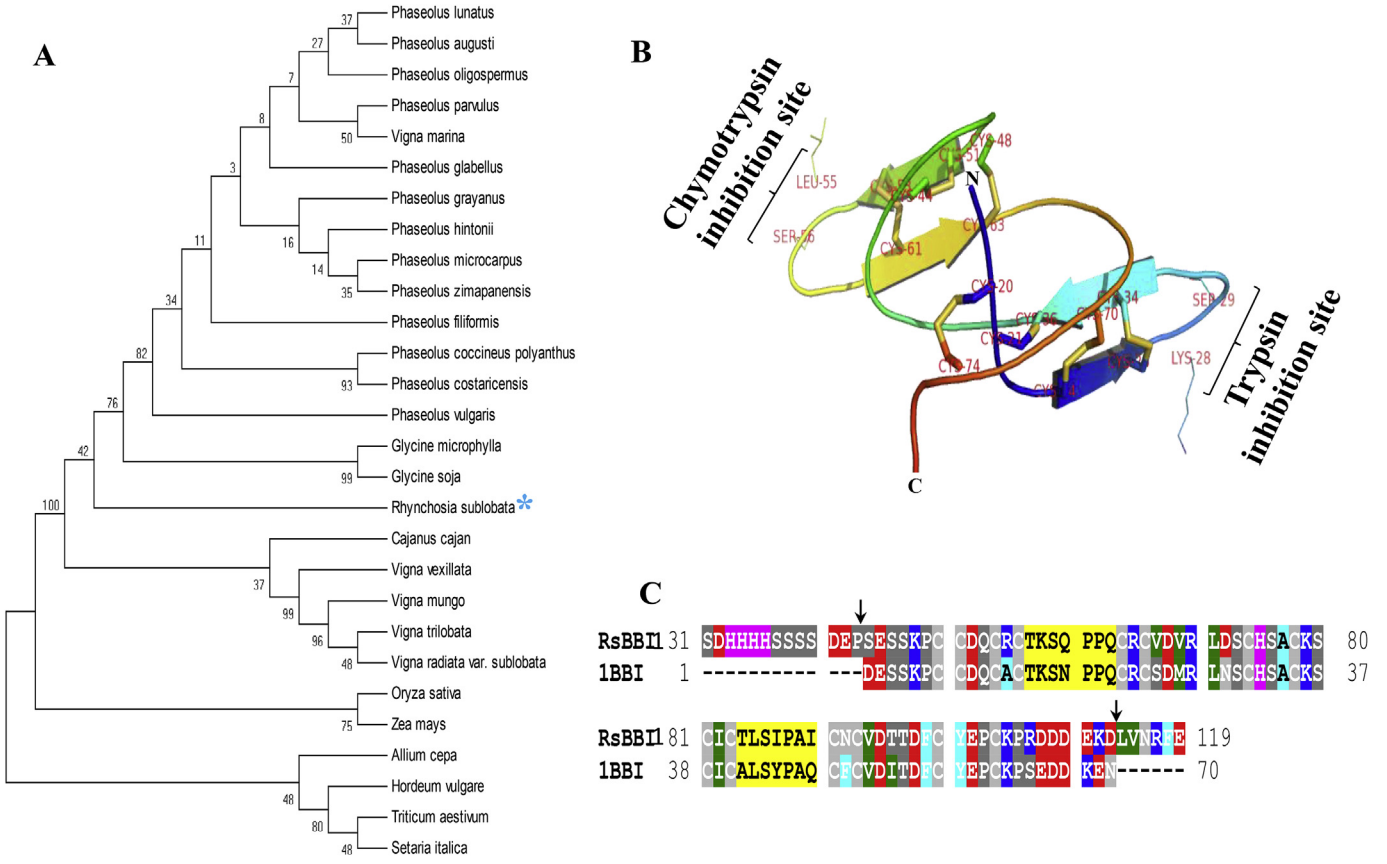

Fig. 4. Phylogenetic analysis and molecular modeling of RsBBI1. (A) Phylogenetic analysis of deduced RsBBI1 with reported BBIs was performed in MEGA 6.0 software using the Neighbor-Joining algorithm with a bootstrap of 1000. The BBIs from individual plants are indicated in the phylogenetic tree. The bootstrapping values were mentioned at the branches to depict the grouping robustness. All the sequences were downloaded from NCBI. Accession Numbers for BBIs from *P. filiformis*; *P. microcarpus*; *P. zimapanensis*; *P. lunatus*; *C. cajan*; *P. grayanus*; *P. augusti*; *P. coccineus*; *P. oligospermus*; *G. microphylla*; *G. soja*; *P. costaricensis*; *P. hintonii*; *P. parvulus*; *P. glabellus*; *P. vulgaris*; *V. marina*; *V. trilobata*; *V. vexillata*; *V. mungo*; *V. radiata* were as indicated in Fig. 3; the accession numbers of other BBIs were as follows: *Hordeum vulgare* - BAK01561.1; *Oryza sativa* - CAB88209.1; *Triticum aestivum* - ABX84379.1; *Zea mays* - NP_001150715.1; *Setaria italica* - XP_004986446.1; *Allium cepa* - BAB88746.1. The position of *R. sublobata* in the predicted evolutionary tree is marked with asterisks '*'. **(B)** The predicted three-dimensional SWISS-MODEL of RsBBI1 protein from amino acids 44 to 113 was obtained by using 1BBI from soybean as the template. The disulfide bonds, trypsin, and chymotrypsin reactive sites were visualized using PyMol software and represented as sticks. The N- and C-terminus ends were marked as N and C, respectively. **(C)** Pairwise alignment of RsBBI1 and 1BBI sequences: Identical amino acids are shaded in the same color. The inhibitory domains of trypsin (TKSQPPQ) and chymotrypsin (TLSIPAI) are shaded in yellow color. Arrows indicate the stretch of RsBBI1 protein sequence modeled according to the template BBI. Note: Few amino acids present in RsBBI1 at both N and C-terminal were absent in 1BBI. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The inhibitory activity of rRsBBI1 was stable during heat treatment from 37 to 100 °C. The loss in TI and CI activities of rRsBBI1 was <10% even upon heating at 100 °C for 30 min (Fig. 8A). Similarly, the inhibitory activity of rRsBBI1 against both trypsin and chymotrypsin was stable at a wide range of pH from 2.0 to 12.0 (Fig. 8B). However, rRsBBI1 lost its TI and CI activities completely on reduction with DTT at 1.0 and 10 mM concentrations, respectively (Fig. 8C). Secondary structural analysis of rRsBBI1 at far-UV region revealed it to be consisting of 29.3% β-sheets, 11.8% β-turns, 56.8% random coils and 2.0% α-helices (Fig. 8D).

2.6. Inhibitory activity of rRsBBI1 on larval gut trypsin-like proteases

The rRsBBI1 showed significant increase in specific activity against trypsin (7.6-fold), chymotrypsin (25-fold) and AjGPs (17-fold) except for HaGPs as compared to CPI (Fig. 9A). The specific activities of rRsBBI1 against trypsin, chymotrypsin, AjGPs and HaGPs were 656 ± 16 TIU/mg protein, 523 ± 20 CIU/mg protein, 14,285 ± 500 AjGPI units/mg protein and 125 ± 10 HaGPI units/mg protein, respectively. The specific activity of rRsBBI1 against HaGPs was <1% when compared with the specific activity against AjGPs (Fig. 9A). These results corroborated well with the IC_{50} values obtained for rRsBBI1 against AjGPs (IC_{50} of 70 ± 2.0 ng) and HaGPs (IC_{50} of 8.0 ± 0.5 μg), suggesting that rRsBBI1 has significant inhibitory potential against AjGPs but not against HaGPs (Fig. 9B and C).

2.7. Effect of rRsBBI1 in larval growth retardation and mortality

Feeding of rRsBBI1 coated castor leaves to second instar *A. janata* larvae caused significant retardation in their growth as compared to larvae fed on control leaves (Fig. 9D). At the end of 11th day of feeding on leaves coated with rRsBBI1 at 2, 4 or 8 μg cm^{-2} leaf area, the body weight of the larvae decreased significantly up to 84% of its control weight (Fig. 9D). Along with the reduction in body weight, some of the larvae showed mortality. Thus, the mortality rate of *A. janata* larvae fed on rRsBBI1 increased from 33 ± 5% (2 μg/cm^2) to 60 ± 7% (8 μg/cm^2) of control level in a dose-dependent manner (Fig. 9D inset).

3. Discussion

R. sublobata, a wild relative of pigeon pea exhibited several

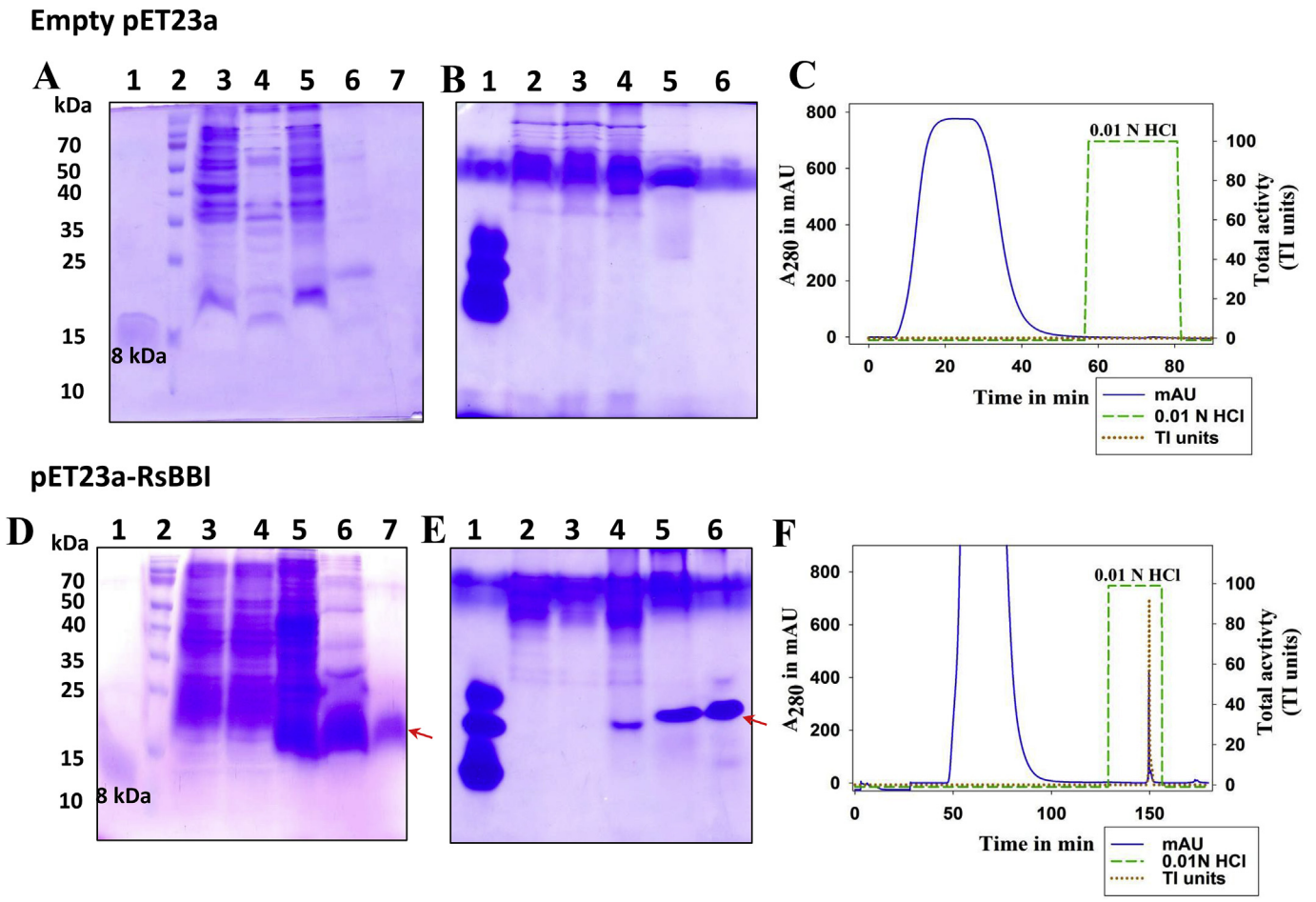

Fig. 5. Heterologous expression of RsBBI1. (A) Expression profile of empty pET23a vector (control) on 15% SDS-PAGE; **(B)** in-gel trypsin inhibitory activity of heat treated *E. coli* lysate with empty vector in gelatin SDS-PAGE; **(C)** trypsin specific affinity chromatogram from heat treated *E. coli* lysate with empty vector showing no peak on elution with 0.01 N HCl; **(D)** Expression profile of pET23a-*RsBBI1* construct on 15% SDS-PAGE; **(E)** in-gel trypsin inhibitory activity of heat treated *E. coli* lysate with pET23a-*RsBBI1* construct in gelatin SDS-PAGE and **(F)** trypsin specific affinity chromatogram from heat treated *E. coli* lysate with pET23a-*RsBBI1* construct showing the elution of rRsBBI1 with 0.01 N HCl. Lanes of figure A and D are loaded with: (1) soybean BBI; (2) PMW marker; (3-7) cell lysates of untransformed SHuffle cells, plasmid transformed but un-induced, IPTG induced, 80 °C heat treated and the affinity chromatogram fractions. Lanes 1-6 of gelatin SDS-PAGE of figure B and E are loaded with the fractions in the same order as stated above without PMW marker. Arrows indicate the pure rRsBBI1 and its in-gel trypsin inhibitor band. Soybean BBI is loaded as the positive marker. The gels were stained with CBB R-250.

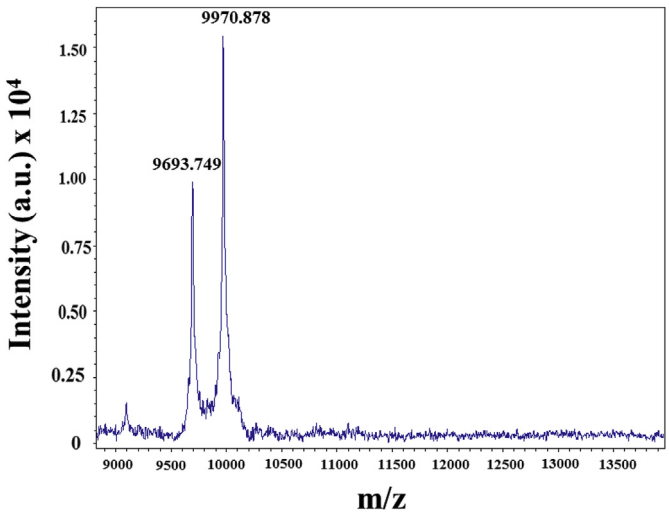

Fig. 6. Intact mass analysis of rRsBBI1 by MALDI-TOF. The rRsBBI1 purified from trypsin affinity column is resolved into two peaks with *m/z* 9693.749 Da and 9970.878 Da, respectively.

defense traits including PIs that are active against gut proteases of *H. armigera* (Chougule et al., 2003; Mallikarjuna et al., 2011). The CPI from *R. sublobata* inhibited the activity of AjGPs (85%) and HaGPs (62%) to the maximum extent at concentrations (12 and 480 μg) that are much lower than any other cultivar (ICP 332 and ICP 7182) or wild relative (*C. volubilis*) chosen in the present study (Fig. 1C and D). The CPI from *R. sublobata* also showed high activity against AjGPs, but low against HaGPs (Fig. 9A). Therefore, in the present study, an attempt was made to clone, overexpress and characterize the recombinant BBI from the immature seeds of *R. sublobata.*

3.1. Molecular characterization of RsBBI1

The complete cDNA sequence of '*RsBBI1*' obtained by using RACE procedure encoded a polypeptide of 119 residues with a molecular mass of 9971.02 Da, fourteen cysteine residues and two inhibitory sites, the typical characteristic features possessed by BBI molecules (Figs. 2C and 3, Figs. 4C and 6; Qi et al., 2005). The predicted three-dimensional structure of RsBBI1 also indicated it exists as a double-headed inhibitor with two of its reactive site loops at opposite ends to facilitate binding with its cognate proteases trypsin and

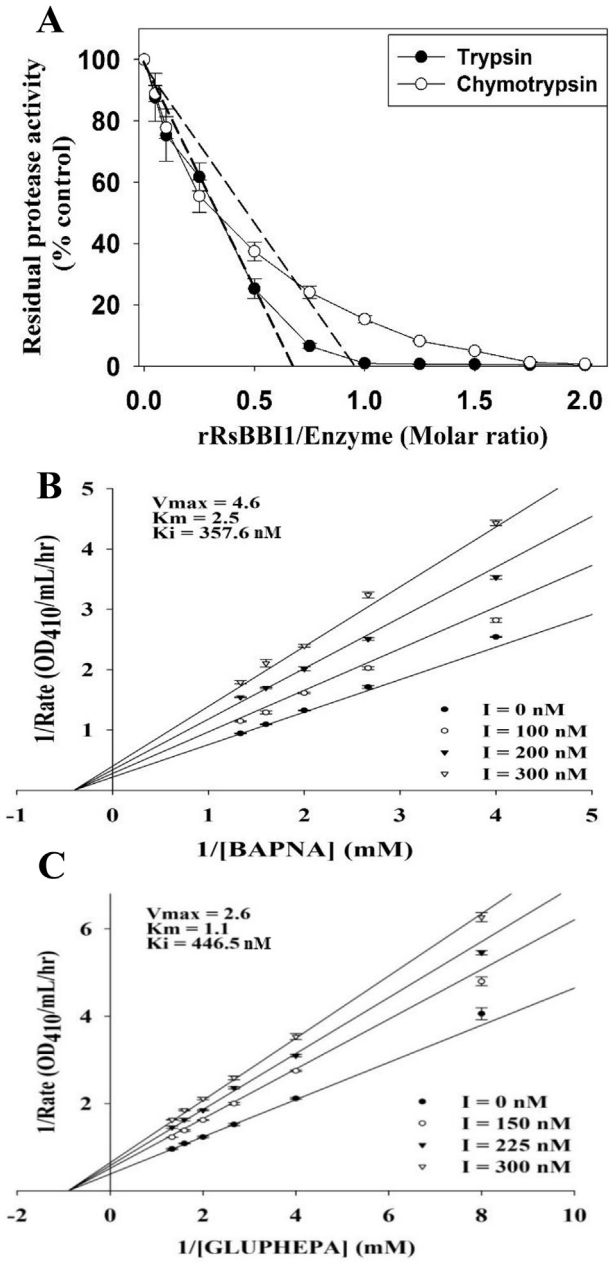

Fig. 7. Enzyme inhibition kinetics of rRsBBI1. (A) Titration curves of trypsin and chymotrypsin inhibition by rRsBBI1. The rRsBBI1 at increasing concentrations was added to a fixed 1 μM concentration of trypsin and chymotrypsin enzymes, respectively. The residual activity of trypsin or chymotrypsin was determined as described in section 4.10. The molar ratio of the rRsBBI1 to the trypsin or chymotrypsin was the intercept of x-coordinate when the tangent was extrapolated to the zero activity (Knights and Light, 1976). Enzyme inhibition kinetics of rRsBBI1 with bovine pancreatic **(B)** trypsin and **(C)** chymotrypsin by Lineweaver-Burk plot showing non-competitive nature of inhibition along with Vmax, Km, and *Ki* values. The data shown are mean ± SE of at least three different independent experiments each with three to four replicates.

chymotrypsin both independently and simultaneously (Fig. 4B; Voss et al., 1996). The reactive site loops for trypsin and chymotrypsin inhibition possessed 'TKSQPPQ' and 'TLSIPAI' at P2, P1, P1′, P2′, P3′, P4′ and P5′ residues, respectively (Fig. 3). The existence of 'Lys' and 'Leu' in the trypsin and chymotrypsin inhibitory sites ascertained this gene belonged to BBI family (Fig. 3; Laskowski Jr and Kato, 1980). The occurrence of 'KS' and 'LS' residues at the P1-P1′ positions of trypsin and chymotrypsin inhibitory sites along with 'Thr' conserved at P2 position of both reactive sites is necessitated to exhibit inhibitory activity and to facilitate efficient binding of BBIs to its cognate enzymes (Fig. 3; Gariani et al., 1999 and Brauer et al., 2003). Further, the existence of 'Gln' at the P2′ position of the trypsin reactive site loop occurring rarely is observed in rRsBBI1 in concurrence with two other BBIs from IBB_VICAN of *Vicia sativa* subsp. *nigra* and IBB3_SOYBN of *Glycine max* (Fig. 3). Conversely, the BBIs from *C. cajan, Phaseolus, Glycine or Vigna* spp. possessed Ile, Arg or Asn at P2′ position (Fig. 3). Similarly, the amino acid residues at P2′ and P5′ positions in the chymotrypsin reactive site loop of RsBBI1 varied in the BBIs of the above-mentioned species. These variations may justify the placement of *R. sublobata* in the quaternary gene pool of wild relatives of *C. cajan* and phylogenetic tree between *G. soja* and *C. cajan* (Fig. 4A; Mallikarjuna et al., 2011).

3.2. *Biochemical characterization of* rRsBBI1

The BBIs are known to be stable at temperatures as high as 90 °C (Osman et al., 2002; Prasad et al., 2010b). Therefore, heat denaturation step (80 °C, 30 min) was adapted during purification of BBIs as reported for intrinsically disordered proteins to eliminate the heat-sensitive digestive proteases from the cell lysate (Fig. 5D–F; Livernois et al., 2009). Low molecular mass detected in intact mass MALDI-TOF analysis also corroborated well with the ExPASy translated sequence of rRsBBI1 (Figs. 2–6). The following observations were made in the midst of trypsin and chymotrypsin suggesting rRsBBI1 was folded appropriately into its native conformation during purification process: (i) interaction with proteases at 1:1 molar ratio; (ii) exhibition of non-competitive mode of enzyme kinetics; (iii) appearance of trypsin or chymotrypsin inhibitory bands in gelatin SDS-PAGE; (iv) retention of TI and CI activities at a wide range of temperature and pH and (v) loss of TI and CI activity on reduction with DTT (Figs. 5E, 7-8). The present study is also suggesting rRsBBI1 is functionally very stable in terms of its TI and CI activities when heated up to 100 °C or exposure to acidic/alkaline pH (Fig. 8A and B). These results were consistent with earlier reports of BBIs purified from the seeds of *Dioclea glabra* (Bueno et al., 1999), *G. soja* (Deshimaru et al., 2002), *L. albus* (Scarafoni et al., 2008), *Cratylia mollis* (Paiva et al., 2006), *P. coccineus* (Pereira et al., 2007) and *D. biflorus* (Singh and Rao, 2002).

BBIs are known to possess seven disulfide bridges providing high stability against temperature and pH (Prasad et al., 2010a; b; Swathi et al., 2014). The X-ray structure of BBI-A from soybean indicated it possesses five disulfide bridges on the surface and two disulfide bridges buried in the core (He et al., 2017). BBIs also show self-aggregation in solution. However, such aggregates affecting their interaction with cognate proteases are not observed in the present study may be due to the usage of dilute rRsBBI1 solutions (Brand et al., 2017). The incubation of rRsBBI1 with DTT resulted in a remarkable loss in its TI and CI activities. This could be due to destabilization of the reactive site scaffold of rRsBBI1 on reduction with DTT, a characteristic feature of BBIs (Qi et al., 2005). Conversely, the CI activity of rRsBBI1 is more resistant to DTT as compared to BBIs from cultivars of *C. cajan* (Fig. 8C; Prasad et al., 2010b). For example, the BBIs from *C. cajan* lost 50% of its CI activity at ~0.1 mM DTT concentration while RsBBI1 lost identical activity after incubation with 1.0 mM DTT concentration. Thus, rRsBBI1 from *R. sublobata* is structurally more stable over BBIs from *C. cajan* cultivar. Secondary structural elements of rRsBBI1 observed in far-UV CD spectra showed similarity with the reported BBIs in possessing a high percentage of β sheets and random coils (Voss et al., 1996). The horse gram BBI followed a 'two-state' mode of unfolding in presence of DTT indicating the hyper-reactive nature

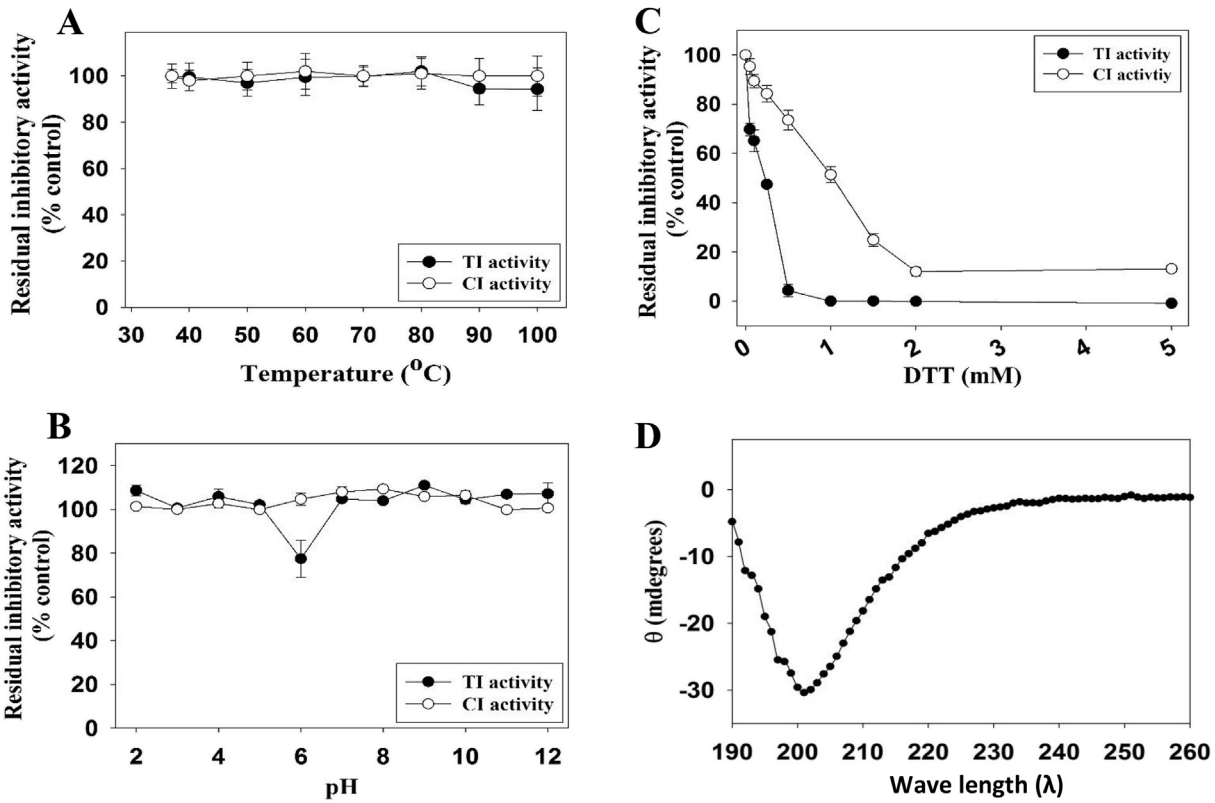

Fig. 8. Biochemical stability and secondary structure of rRsBBI1. Stability of rRsBBI1 at various **(A)** temperatures, **(B)** pH and **(C)** DTT/IDA concentrations. Residual trypsin and chymotrypsin inhibitory activities were plotted against the appropriate condition; **(D)** CD spectra of rRsBBI1 at the far-UV region. The data shown are mean ± SE of at least three different independent experiments each with three to four replicates.

of disulfide bonds (Singh and Rao, 2002). Existence of high ellipticity at 201 nm also confirms the presence of disulfide bridges in rRsBBI1 (Kumar and Gowda, 2013). CD estimated secondary structural elements of rRsBBI1 also showed correlation with the modeled 3D structure of RsBBI1 (Figs. 4B and 8D).

Summarising the results from the present study: (i) specificity towards trypsin/chymotrypsin enzymes; (ii) stability against pH and temperature and (iii) presence of a high percentage of disulphide bridges, suggest that rRsBBI1 could be exploited for insecticidal, clinical and therapeutic applications (Clemente and Arques, 2014; Farinaz and Abdolmohamad, 2013; Souza Lda et al., 2014). However, in the present study, experiments were limited to testing the insecticidal potential of rRsBBI1 using *in vitro* and *in vivo* studies.

3.3. *Insecticidal potential of* rRsBBI1

rRsBBI1 exhibited a significant variation (114-fold) in its IC_{50} against AjGPs and HaGPs. This could be due to the susceptibility of AjGPs towards the non-host rRsBBI1 and the presence of relatively less rRsBBI1 susceptible HaGPs in the larval guts of *A. janata* and *H. armigera*, respectively. Moreover, it is well known that *H. armigera* possess multiple trypsin enzymes in its gut environment along with other proteases such as chymotrypsin and elastase. This may be the cause for the lower affinity of rRsBBI1 towards HaGPs and thereby lower protease inhibitory activity of rRsBBI1 against them (Bown et al., 1997; Chougule et al., 2005; Kuwar et al., 2015; Wu et al., 1997). Studies of Swathi et al. (2016) indicated Kunitz/Miraculin like PIs identified in *C. platycarpus*, another wild relative of pigeon pea effectively inhibited HaGPs. Similarly, several other serine PIs such as squash type inhibitor from bitter gourd (Telang et al., 2009), PIN-II type inhibitors from *Capsicum annum* and many Kunitz inhibitors (Jamal et al., 2013) effectively inhibit the gut proteases of *H. armigera*. Despite the existence of report on *G.* max BBI to induce mortality in *H. armigera*, although at a higher concentration range, the biocidal effect of rRsBBI1 on *H. armigera* could not be examined in the present study due to its limited inhibitory effect on the activity of HaGPs as compared to AjGPs (Fig. 9B and C; Johnston et al., 1993). Higher specific activity of *R. sublobata* CPI on HaGPs as compared to rRsBBI1 suggested the existence of PIs other than BBIs in the seed proteome of *R. sublobata*. The following results from the present study: (i) differential residual protease activity of AjGPs and HaGPs observed in presence of CPI from *R. sublobata* (Fig. 1C and D); (ii) higher specific activity of CPI as compared to rRsBBI1 against HaGPs (Fig. 9A), and (iii) higher IC_{50} values of rRsBBI against HaGPs than AjGPs (Fig. 9B and C) together with earlier literature warrants to further explore the genome of *R. sublobata* so as to identify new novel seed PIs active against *H. armigera*.

The specific activity of the non-host rRsBBI1 against AjGPs (14,285 AjGPI units/mg protein) was significantly higher than BBIs from *C. cajan* by approximately 2- fold (Swathi et al., 2014). The higher activity of rRsBBI1 over *C. cajan* BBIs against AjGPs could be attributed to the presence of 'Gln' at P2′ position of trypsin inhibitory loop in rRsBBI1 (Fig. 3) and the presence of susceptible trypsin-like proteases in the midguts of *A. janata*. But, the observed IC_{50} (70 ± 2 ng) of rRsBBI1 against AjGPs did not vary significantly from the IC_{50} (78–100 ng) of BBIs from cultivar varieties of *C. cajan* (Fig. 9C; Prasad et al., 2010b; Swathi et al., 2014). However, we suggest that rRsBBI1 is more efficient than *C. cajan* BBI in management of *A. janata* since the BBI from C. *cajan* is known to exist as several isoinhibitors, and they might all contribute collectively to

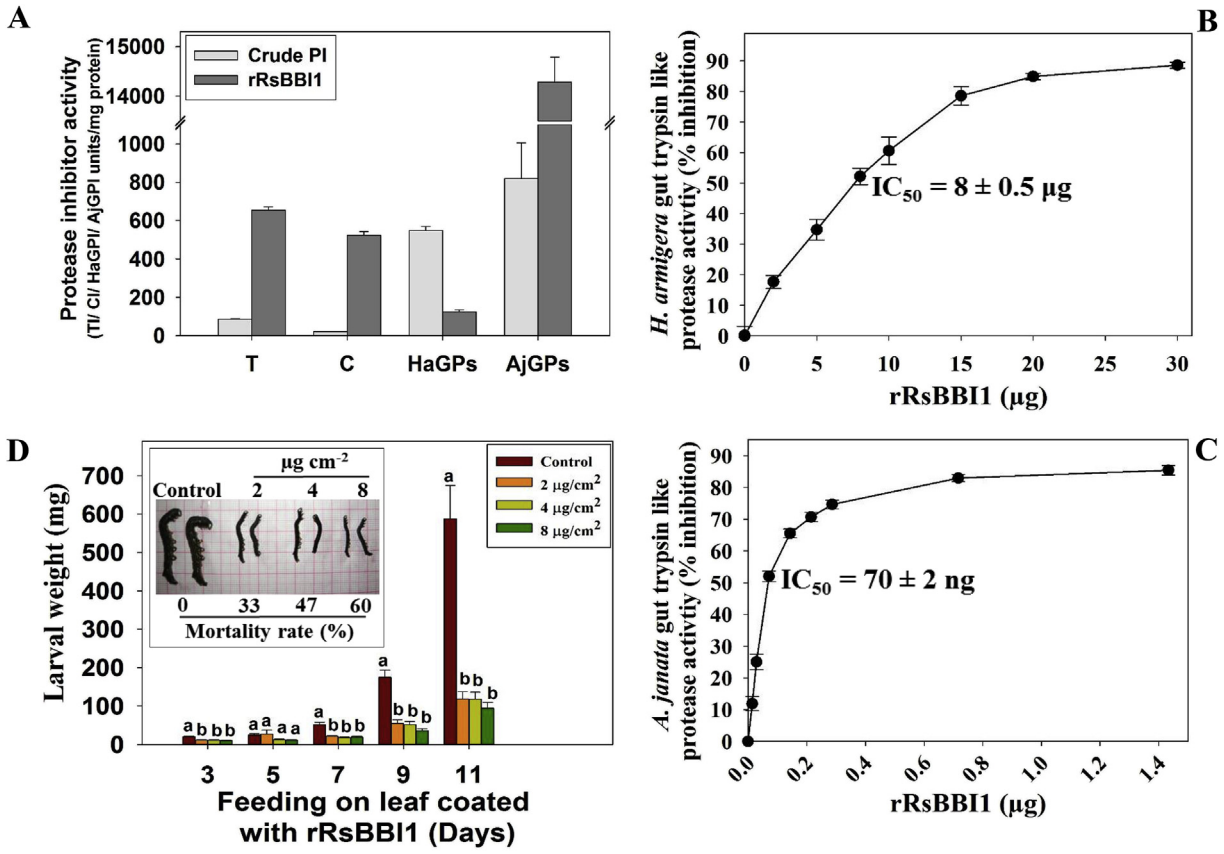

Fig. 9. (A) *In vitro* activity of *R. sublobata* mature seed CPI (light grey) and rRsBBI1 (dark grey) against trypsin (T), chymotrypsin (C), HaGPs, and AjGPs; **(B)** and **(C)** rRsBBI1 concentration-dependent inhibition in the activity of HaGPs and AjGPs. The IC_{50} values of rRsBBI1 required for the inhibition of gut proteases were indicated. **(D)** Larval weights of *A. janata* on different days after *in vivo* feeding of castor leaves coated with rRsBBI1 at 2, 4, and 8 µg cm^{-2} of leaf area. The inset shows the larval pictures and mortality rates of *A. janata* larvae after 11 days of feeding with rRsBBI at respective concentrations. Further details on statistical analysis of data were as described in materials and methods. Statistically significant differences ($P < 0.05$) were indicated in various lowercase alphabetical letters.

the observed insecticidal activity in *A. janata* (Fig. 9C and D; Prasad et al., 2010b; Swathi et al., 2014). Conversely, the studies on *in vivo* feeding of rRsBBI1 to the second instar larvae of *A. janata* indicated rRsBBI1 'as an independent BBI isoform' is able to act as a potent growth retarding agent (Fig. 9D). In line with this study, transgenic tobacco plants expressing cowpea trypsin inhibitor and rice plants expressing potato type 2 (PIN2) inhibitor exhibited resistance against *H. virensis* and *Sesamia inferens* (Duan et al., 1996; Hilder et al., 1987). But, *Manduca sexta* gut proteases were effectively inhibited by PIN2 rather than by PIN1 expressing tobacco plants (Johnson et al., 1989). In contrast, on feeding tobacco plants expressing giant taro PI to *H. armigera*, the insect survived by overexpressing alternate proteases such as chymotrypsin and elastase (Wu et al., 1997). Moreover, insects such as *Heliothis zea*, *H. armigera,* and *Spodoptera exigua* were able to overcome the effect of PIs by producing alternate proteases or overproducing sensitive proteases (Jongsma and Bolter, 1997; Wu et al., 1997). Altogether, these studies indicated insects alter their gut protease profile according to the type of inhibitors fed to them. In this context, targeting of multiple gut proteases and PI insensitive proteases related to a particular insect were suggested as a potential strategy to control the pests individually (Jongsma and Bolter, 1997). Pyramiding of PIs active against both sensitive and insensitive proteases induced high growth reduction and mortality in transgenic cotton expressing potato type I and II PI against *H. armgiera* (Dunse et al., 2010), and transgenic tobacco expressing sporamin, cystatin and chitinases against *S. litura* as well as *S. exigua* (Chen et al., 2014). Therefore, identifying PIs from non-host plants effective against a particular insect might be an added feature for such type of insect control strategy. Thus, RsBBI1 which effectively inhibited the activity of gut proteases and induced growth retardation and mortality in *A. janata* would be a potential candidate gene for the development of transgenic plants resistant to *A. janata* using pyrimading technology.

4. Experimental

R. sublobata seeds (Accession No. 15868) were obtained from the International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, India and cultured in a greenhouse for seed collection at University of Hyderabad, Hyderabad, India. *H. armigera* larvae were procured from National Bureau of Agricultural Insect Resources (NBAIR), Bengaluru, India. The larvae of *A. janata* were collected from the fields of the University of Hyderabad, Hyderabad, India. Agarose, chloroform, isoamyl alcohol, isopropanol, ethidium bromide, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), *N*-glutaryl-L-phenylalanine-*p*-nitroanilide (GLUPHEPA) and absolute ethanol were procured from Sigma-Aldrich, USA. CNBr activated Sepharose and dithiothreitol (DTT) were purchased from GE Healthcare Biosciences Corp., USA. Pfu polymerase, Taq polymerase and RACE kits were obtained from Thermo Fischer Scientific, India. Gel elution and plasmid isolation kits were procured from Qiagen, India. All other chemicals were procured from Sisco Research Laboratories, Mumbai, India.

4.1. Preparation of crude PI extract

The CPI extract from the mature seeds of two different cultivars (ICP 332 and ICP 7182) and wild relatives (*C. volubilis* – ICP 15774 and *R. sublobata* – ICP 15868) was prepared as described in Swathi et al. (2014). The seeds were ground to a fine powder, depigmented and defatted by washing thrice with acetone and hexane respectively. The filtrate was air dried and extracted with 50 mM Tris-HCl, pH 8.0 containing 1% PVP under a mild stirring condition at 4 °C overnight. The solution was centrifuged twice at 10,000 g for 20 min at 4 °C and the supernatant obtained was used as a CPI extract.

4.2. RNA isolation

RNA was isolated from the immature seeds of *R. sublobata* by using the modified protocol of Matilla et al. (1980). The RNA lysis buffer (1 mL) containing 100 mM Glycine-NaOH at pH 9.0, 40 mM EDTA, 100 mM NaCl, 2% SDS and 0.05% Bentonite was added to finely ground seed powder (100 mg) under liquid N_2 and shaken vigorously before incubating at 42 °C for 20 min. Subsequently, RNA was isolated from the sample by treating with phenol/chloroform and precipitation by 12 M LiCl. The RNA obtained was air dried, dissolved in RNase/DNase-free water and quantified using NanoDrop. The integrity of RNA was visualized in the formaldehyde-agarose gel using MOPS buffer.

4.3. Partial gene amplification of RsBBI1

The complementary DNA (cDNA) was synthesised from the total RNA using an oligo-dT primer by following the manufacturer's instructions (Verso, cDNA synthesis kit). The forward 'RsBBI1-F' and reverse 'RsBBI1-R' primers were designed using Oligo Analyzer 3.1 based on the soybean BBI isoinhibitor D-II sequence (NCBI:NM_001249286.1) so as to amplify the BBI gene sequence from *R. sublobata* (Supplementary Table 1). The partial *RsBBI1* cDNA fragment was amplified using the following program: 90 °C - 2 min of initial denaturation time, followed by 35 cycles of amplification (denaturation at 90 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 70 s). The PCR product of ~250 bp separated on an agarose gel was extracted (Qiagen, gel extraction kit) and subjected to DNA sequencing using 'RsBBI1-F' primer (Sandor proteomics, Hyderabad). The obtained sequence was analyzed using BLASTn.

4.4. 5′ and 3′ RACE for amplifying full-length transcript of RsBBI1

The complete transcript sequence of *RsBBI1* was obtained by 5′- and 3′-RACE experiments. The primary and nested primers for 3′ RACE (Race-RsBBI1 3′R; Race-RsBBI1 3′NR) and 5′ RACE (Race-RsBBI1 5′F and Race-RsBBI1 5′NF) were designed (Supplementary Table 1) based on the internal sequence of *RsBBI1* obtained by RT-PCR and the experiment was conducted as per manufacturer's instructions (GeneRacer kit, Invitrogen). The 5′ and 3′ ends of *RsBBI1* were PCR amplified from 5′ and 3′ RACE cDNA using gene-specific primers in combination with the kit-supplied primers. The nested PCR was performed with 1:100 dilution of the primary PCR product. The above products were amplified using proofreading Pfu polymerase (Phusion High-Fidelity DNA polymerase, Invitrogen, USA). The PCR program was same as mentioned above, with an additional initial denaturation step (5 min at 90 °C) required to activate the Pfu enzyme. At the end of the reaction, 3′ ATP overhangs were added using Taq polymerase after incubation at 72 °C for 10 min. The amplified products were visualized on 1.5% agarose gel, and the gene-specific amplification was preliminarily verified by monitoring the difference in the sizes of primary and nested PCR products. The amplified products were gel eluted and ligated into pTZ57R/T vector (Thermo Fischer Scientific, Mumbai, India) and transformed into *E. coli* DH5α. The positive colonies were distinguished by blue/white colony screening followed by colony PCR. Plasmids isolated from the positive colonies were subsequently subjected to DNA sequencing using vector primers. The full-length cDNA sequence of the *RsBBI1* transcript was deduced from the contig sequence constructed by the amplified 5′ and 3′ RACE product sequences.

4.5. Three-dimensional (3D) structure prediction

The complete gene sequence of *RsBBI1* was submitted to ExPASy Translate tool (http://web.expasy.org/translate/) and obtained ORF. The derived protein sequence was submitted to TargetP 1.1 Server online (http://www.cbs.dtu.dk/services/TargetP/) for prediction of the signal peptide. The predicted RsBBI1 protein sequence was submitted to SWISS-MODEL (http://swissmodel.expasy.org/interactive) for automatic modeling of the three-dimensional protein structure. The PDB file generated by SWISS-MODELL was visualized in PyMOL software for secondary structures, disulphide bridges and inhibitory sites.

4.6. Phylogenetic analysis

BBI protein sequences related to different plant species were downloaded from NCBI (www.ncbi.nlm.nih.gov). The downloaded BBI protein sequences were ClustalW aligned with the deduced protein sequence of RsBBI1, and a phylogenetic tree was constructed by the Neighbor-Joining method with a bootstrap of 1000 using Mega 6.0 software.

4.7. pET23a-RsBBI1 construct preparation

A cDNA construct encoding 89 amino acids stretch (Fig. 2C, 31-119 amino acids) was cloned into an expression vector, pET23a, as described below. The cDNA fragment was amplified using forward and reverse primers, RsBBI1-NdeI-F and RsBBI1-*Xho*I-R (Supplementary Table 1), and Pfu polymerase and subjected to PCR amplification as mentioned in section 4.4. The amplified product was gel purified, digested with *Xho*I followed by NdeI enzymes and directionally cloned into pET23a plasmid. The recombinant plasmid pET23a-*RsBBI1* was transformed into *E. coli* DH5α and selected against ampicillin by plating on Luria broth containing 100 μg/mL ampicillin (LB-Amp) plates. The positive colonies were selected based on colony PCR and their plasmids were subjected to DNA sequencing.

4.8. Overexpression and purification of rRsBBI1

The pET23a-*RsBBI1* plasmid was transformed into *E. coli* SHuffle T7 express competent cells (NEB, UK) by heat shock method. These host cells have a chromosomal copy of constitutively-expressed disulphide bond isomerase (DsbC), a chaperone assisting in proper folding of the cytoplasmic proteins (de Marco, 2009). Single colony transformants were cultured in LB-Amp broth and incubated at 37 °C. The overnight grown culture was inoculated into 1 L of LB-Amp broth on the following day and incubated at 37 °C until the culture reaches ~1.0 OD_{600} units. Later, the culture was induced using 0.4 mM isopropyl-β-D-thiogalactoside (IPTG) at 30 °C for 8 h to express the *rRsBBI1*. The cell pellet obtained was suspended in 50 mM Tris-HCl (pH 8.0) containing 500 mM NaCl and sonicated. The lysate was heated at 80 °C for 30 min and chilled on ice. The rRsBBI1 was purified from the supernatant collected after

centrifugation at 10,000 g using trypsin coupled CNBr-Sepharose column in fast protein liquid chromatography (FPLC) AKTAprime plus (1 mL Flow rate at 25 °C and 1 Bar pressure). The rRsBBI1 eluted with 0.01 N HCl was neutralized with 50 mM Tris- HCl (pH 8.0), concentrated and stored at −20 °C until further use. The purification profile was represented in 15% SDS-PAGE as per Laemmli (1970) and in-gel trypsin inhibitor activity of rRsBBI1 was visualized as described by Felicioli et al. (1997).

4.9. Rearing of larvae and extraction of gut enzymes

The larvae of *H. armigera* and *A. janata* were reared and maintained at 26 ± 1 °C with a light-dark photoperiod of 14:10 h and relative humidity of 65 ± 5% in insect culture room. The *A. janata* larvae were fed on castor leaves. Conversely, *H. armigera* larvae were fed on an artificial diet as described in Gupta et al. (2000). The fifth instar larvae were narcotised on ice for 15 min and the midgut content was extracted into two volumes of 50 mM Glycine-NaOH (pH 10.5) in case of *H. armigera* or 50 mM Tris-HCl containing 20 mM $CaCl_2$ (pH 8.2) for *A. janata.* The suspension of midgut content was centrifuged at 10,000 g for 15 min at 4 °C. The resulting supernatant enriched with AjGPs or HaGPs was stored as small aliquots at −20 °C until use.

4.10. Protease and protease inhibitor assays

The assay for trypsin, chymotrypsin, AjGPs or HaGPs was performed as described in Prasad et al. (2010b) 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 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 inhibitory activity of rRsBBI1 was assessed after incubating with respective proteases for 15 min at 37 °C. One unit of trypsin inhibitor (TI), chymotrypsin inhibitor (CI), *A. janata* gut trypsin-like protease inhibitor (AjGPI) or *H. armigera* gut trypsin-like protease inhibitor (HaGPI) was defined as the amount of rRsBBI1 required to inhibit 50% hydrolysis of BAPNA or GLUPHEPA by relevant proteases under the optimal assay conditions.

4.11. Leaf coating assay

It was performed using 2nd instar larvae of *A. janata.* The larvae were allowed to feed on castor leaves coated with rRsBBI1 at 2, 4 and 8 μg per cm^2 as described in Prasad et al. (2010a). Control leaves were coated with 50 mM Tris-HCl (pH 8.0). The feed was changed in the morning and evening, and the weight of each larva was monitored on alternate days. Approximately 15 larvae were used for each treatment and the data shown is mean ± S.E. of three biological sets.

4.12. Inhibition constant (Ki) determination and titration studies

The inhibition constant (*Ki*) of rRsBBI1 against both trypsin and chymotrypsin was determined after pre-incubation with respective enzymes (1 μM) at increasing concentrations for 15 min at 37 °C. This was followed by incubation with corresponding substrates at different concentrations for 45 min at 37 °C. BAPNA is used at 0.25, 0.375, 0.5, 0.625 and 0.75 mM while GLUPHEPA is used at 0.125, 0.25, 0.375, 0.5, 0.625 and 0.75 mM, respectively. The *Ki* values were determined using Sigma Plot 12.5 software (SystatSoftware Inc. San Jose, California). The residual trypsin/chymotrypsin activities at different molar ratios (0.05–2.0) of rRsBBI1 to trypsin/chymotrypsin were determined by titrating different concentrations of rRsBBI1 with a fixed concentration (1 μM) of trypsin or chymotrypsin, respectively (Prasad et al., 2010b).

4.13. Stability studies

The stability of rRsBBI1 to changes in temperature, pH or disulfide bridges was assessed as residual trypsin inhibitory (TI) or chymotrypsin inhibitory (CI) activity (Prasad et al. (2010b). To study the effect of temperature, rRsBBI1 was incubated for 30 min at different temperatures (37, 40, 50, 60, 70, 80, 90, 100 °C) using a thermostat controlled water bath (Julabo F10). The effect of pH from 2.0 to 12.0 was determined by incubating rRsBBI1 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). Furthermore, rRsBBI1 was incubated for 45 min with different concentrations of DTT (0.05–10.0 mM) in 25 mM NH_4HCO_3 at 56 °C. This was followed by incubation for 1 h in the dark with iodoacetamide at twice the amount of corresponding DTT concentration to terminate the reaction.

4.14. Circular dichroism (CD)

The changes in ellipticity were measured at far-UV region (190–260 nm) using a 1 mm path length cuvette at a scan speed of 50 nm/min in a J-1500 spectropolarimeter (Jasco, Tokyo, Japan). A minimum of three scans were acquired at 25 °C using 0.05 mg/mL of rRsBBI1 in 5 mM Tris-HCl (pH 8.0) containing 5 mM NaCl. Secondary structural elements were estimated using SpectraManager 2.0 software after subtracting the buffer spectra from rRsBBI1 spectra.

4.15. Statistical analysis

All the *in vitro* experiments were carried out at least three times, each with three replications and the mean ± SE was reported. Minimum three *in vivo* leaf coating assays were performed and the statistical differences were determined by one-way ANOVA followed by Tukey test at a significance level of $P \leq 0.05$ using SigmaPlot, version 12.5, software (San Jose, CA, USA).

5. Conclusions and future prospective

The present study revealed full-length cDNA sequence of a novel *BBI* gene from the immature seeds of a wild legume *R. sublobata*. The rRsBBI1 from *R. sublobata* differed from its cultivar *C. cajan* BBI at both trypsin and chymotrypsin reactive site loops. The mature sequence (from 31 to 119 amino acids) of *RsBBI1* is overexpressed in *E. coli* and purified by passing the protein lysate heated at 80 °C through trypsin affinity column. The TI and CI activities of rRsBBI1 are stable against a wide range of temperatures and pH. But these activities were lost on the reduction of rRsBBI1 with DTT. Also, the rRsBBI1 showed a significant *in vitro* inhibition potential against the gut proteases of *A. janata* but not *H. armigera*. Our results also contributed towards an understanding of the biochemical

properties of a Bowman-Birk isoinhibitor from *R. sublobata,* a wild relative of pigeon pea and its effect on larval growth retardation and mortality. Further exploration of related BBI isoinhibitors as well as other PI genes from the genome of *R. sublobata* would pave the path to examine and expand the insecticidal potential of this wild legume on other economically important lepidopteran insect pests, including *H. armigera* and their application in transgenic technology.

Author contributions

Conceived the concept: KP; designed the experiments: KP and SDT; Production of Experimental seed material: NM; Performance of the experiments: SSM; Analysis of the data: SSM, KP, SDT and ADG; Manuscript writing: SSM; Manuscript editing: KP, SDT and ADG.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgements

This work was supported by grants from the Department of Biotechnology (DBT), New Delhi, India (Ref. BT/PR 13261/AGR/05/489/2009/(letter I) dt. 21/12/2010) to KPS (PI) and ADG (Co-PI). SSM is thankful to UoH and DBT for JRF and SRF. We also thank Ms. Monika Kannan and Proteomics facility, SLS, UoH for help in MALDI-TOF experiments. The authors declare no conflict of interests.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.phytochem.2018.02.009.

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