Purification, Identification and Characterization of insecticidal protease inhibitors from *Rhynchosia sublobata* (Schumach.) Meikle seeds against *Helicoverpa armigera* and *Achaea janata*: Molecular cloning of novel *RsBBI* and *RsKI*

Thesis submitted to the University of Hyderabad

for the Degree of

DOCTOR OF PHILOSOPHY

By

S. S. MOHANRAJ
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Department of Plant Sciences
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University of Hyderabad
Hyderabad 500 046, INDIA

September 2017

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DEPARTMENT OF PLANT SCIENCES
SCHOOL OF LIFE SCIENCES
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INDIA

DECLARATION

I hereby declare that the work presented in this thesis entitled **"Purification, Identification and Characterization of insecticidal protease inhibitors from *Rhynchosia sublobata* (Schumach.) Meikle seeds against *Helicoverpa armigera* and *Achaea janata*: Molecular cloning of novel *RsBBI* and *RsKI*"**, has been carried out by me under the supervision of Dr. K.P.M.S.V. Padmasree in the Dept. of Plant Sciences, School of Life Sciences, University of Hyderabad and this work has not been submitted for any degree or diploma of any other University or Institute.

S. S. Mohanraj
(Candidate)
Enrol. No. 10LPPH19

Dr. K.P.M.S.V. Padmasree
(Supervisor)

CERTIFICATE

This is to certify that the thesis entitled **"Purification, Identification and Characterization of Insecticidal Protease Inhibitors from *Rhynchosia sublobata* (Schumach.) Meikle Seeds against *Helicoverpa armigera* and *Achaea janata*: Molecular Cloning of Novel *RsBBI* and *RsKI*"** submitted by **S. S. Mohanraj** bearing registration number **10LPPH19** in partial fulfilment of the requirements for the award of Doctor of philosophy in the **Department of Plant Sciences**, **School of Life Sciences** is a bonafide work carried out by him under my supervision and guidance.

This thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for award of any degree or diploma.

Part of the work performed in relation to this thesis have been:

A. Published in the following publication:

1. Acta Physiologiae Plantarum (2015) 37:1-11.
2. Functional Plant Sciences and Biotechnology (2012) 6:82-85.

B. Presented in the following conferences

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2. ISSP South Zonal Seminar on Physiological and Molecular Interventions for Improving Crop Productivity, ANGRAU, Bapatla on 23rd Jan 2013 (National).
3. A.P. Science congress-2013, UoH, Hyderabad on 14-16th Nov 2013 (National).

Further, the student has passed the following courses towards fulfillment of coursework requirement for Ph.D.

Sl. No	Course Code	Name	Credits	Pass/Fail
1.	PL 801	Research methodology	4	PASS
2.	PL 802	Research Ethics & Management	2	PASS
3.	PL 803	Lab Work	4	PASS
4.	PL 804	Biostatistics	2	PASS

Supervisor **Head of Department** **Dean of School**

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S. S. Mohanraj

Abbreviations

AjGPIs	*A. janata* gut trypsin-like protease inhibitors
AjGPs	*Achaea janata* gut trypsin-like proteases
BAPNA	N-α-benzoyl-DL arginine-*p*-nitroanilide
BBI	Bowman-Birk inhibitor
BCA	Bicinchoninic acid
C. cajan	*Cajanus cajan*
CBB	Coomassie Brilliant Blue
CD	Circular Dichroism
CI	Chymotrypsin inhibitor
CV	Column volume(s)
DTT	Dithiothreitol
FPLC	Fast protein liquid chromatography
GLUPHEPA	n-glutaryl-L-phenylalanine-*p*-nitroanilide
HaGPIs	*H. armigera* gut trypsin-like protease inhibitors
HaGPs	*Helicoverpa armigera* gut trypsin-like proteases
IDA	Iodoacetamide
IEF	Isoelectric focussing
Ki	Inhibition constant
MALDI-ISD	MALDI – In source decay
MALDI-TOF	Matrix-assisted laser desorption/ionization – time of flight
pI	isoelectric point
PIs	Protease inhibitors
rRsBBI	Recombinant RsBBI protein
RsBBI	*Rhynchosia sublobata* BBI protein
RsKI	*Rhynchosia sublobata* kunitz inhibitor protein
RsPI	*Rhynchosia sublobata* protease inhibitor
TI	Trypsin inhibitor
Tricine	N-tris(hydroxymethyl) methyl glycine
2-DE	Two dimensional gel electrophoresis

Contents

Dedicated to

My Parents &

To my Beloved Teacher

Mr. P. Sivashanmugam

Chapter 1

General Introduction

INTRODUCTION

1.1. Agriculture and human population

The total population of the world was estimated as 7.3 billion by mid-2015. India is the second most populous country with approximately 1.3 billion people accounting for 18% of total population. The population is predicted to grow continuously up to 9.7 billion by the year 2050 where in India it is predicted to reach 1.7 billion (Figs. 1.1A,B; Nations, 2015a, b). As the population continues to grow, there will be a major demand for the food. But, the area of land converted for cultivation in the past 50 years was mostly stable (Fig. 1.2A). In this scenario, high yielding and disease resistant crop varieties have to be generated regularly to meet the increasing demand for food production. However, when we look back into the history, in the last 50 years, there is a parallel rise in both human population and the global food production (Fig. 1.2B; Land, 2009). This balance was achieved with the advent of green revolution followed by the availability of food at an affordable price along with medical and technological advancements (Fig. 1.3A; Evenson and Gollin, 2003).

The green revolution was formulated in the early 1960s by developing high yielding and disease resistant varieties with regular irrigation, fertilizer, and pesticide application to the crop land (Fig. 1.3A). Green revolution improved the agricultural yields of wheat and rice by several fold in India (Fig. 1.3B). Also, the green revolution had put an intense selection pressure on one of the original shareholders of the plants, i.e., the insect pests. Further, large-scale monocultures of high yielding varieties and injudicious use of chemical pesticides added to increased pressure on the insects leading to development of resistance in majority of the pests and this has become a serious threat to agriculture (Shiva, 1993). The estimated production loss was about 70% accounting to US$ 400 billion without the use of pesticides. However, after pesticide use, the insecticide resistant insect caused only 15% loss in agricultural yield globally

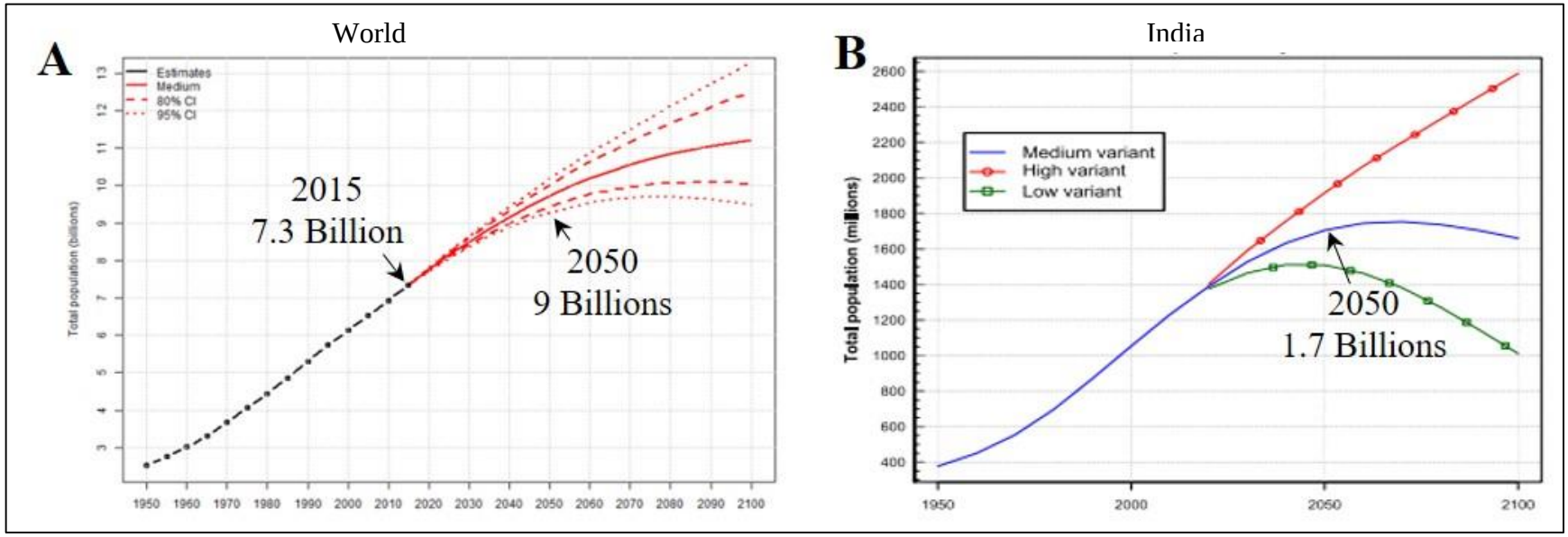


Fig. 1.1. The population estimate. (A) Population estimate of the world from 1950-2015 and the population prediction for the year 2015-2100 at medium-variant, 80 and 95 percent confidence interval projection; (B) Population estimate of India in 2015 and the estimates from 2015-2100. Source – World Population Prospects: The 2015 Revision, Volume I and II.

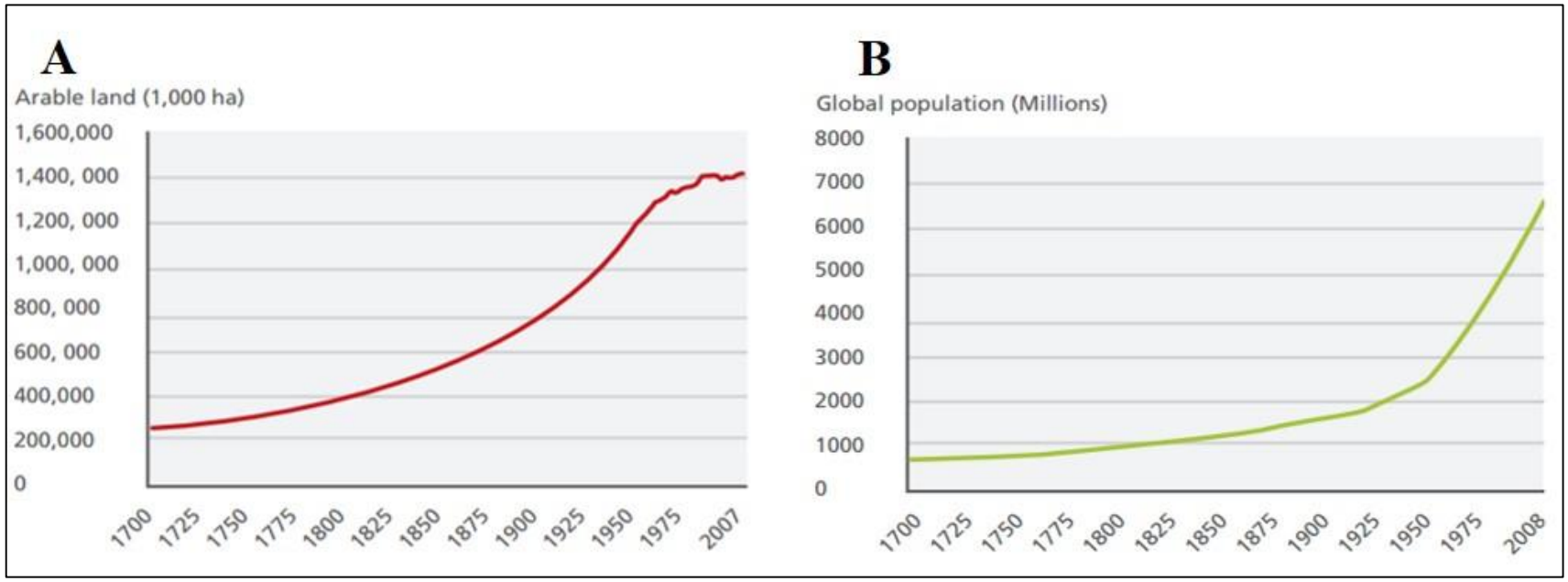


Fig. 1.2. Arable land and world population dynamics. (A) Increase in global farmland from 1700 to 2007 in 1,000 ha; (B) Increase in global human population from 1700 to 2008 in millions. Source – The Land Commodities Global Agriculture and Farmland Investment Report (2009).

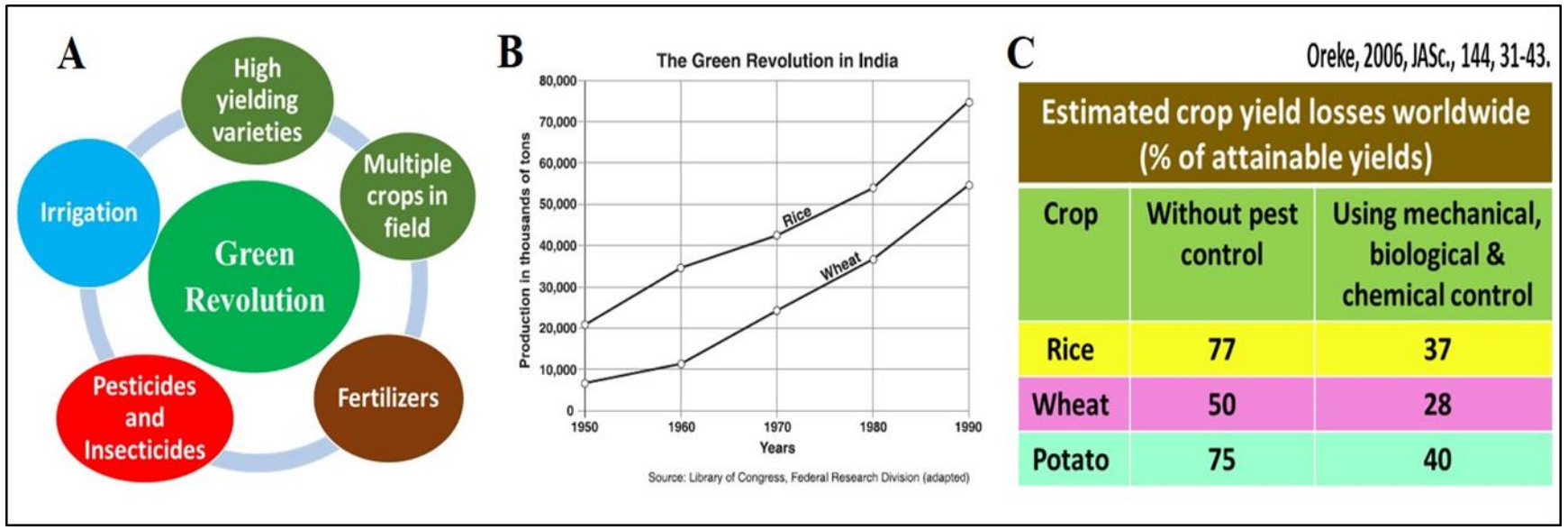


Fig. 1.3. Green revolution and its impact on crop yield and insect pests. (A) Various agricultural strategies formulated by Norman Borlaug (1961) to achieve higher agricultural yields per unit area (Green Revolution); (B) The impact of the green revolution in India towards wheat and rice production; (C) Estimated loss of crop yields (Rice, Wheat and Potato) globally in the presence and absence of pest control strategies. Data adapted from Oerke (2006).

accounting to US$ 100 billion (Fig. 1.3C; Krattiger, 1997; Oerke et al., 1994). Thus, there is an immediate need to eradicate resistant insects as well as to control further development of resistance in them to increase agriculture production to satiate the ever increasing human population.

1.2. Development of resistance in insect and its control measures

Insects are one of the major stakeholders of the plant and plant products. They are controlled with the use of pesticides, which block the nervous and digestive system and thereby leading to mortality of the larvae. Apart, insect growth regulators such as juvenile hormone and ecdysone which impart a long term developmental defects on the insects are used in the pest control strategy. Recently, blocking the excretory system of insects with ion-channel inhibitors was also suggested as an effective pest control strategy (Fig. 1.4A; Ruiz-Sanchez and O'Donnell, 2015). In spite of the usage of different insect control strategies, the rapid development of resistance to pesticides is due to less generation time, larger clutch size and sperm mixing during mating. (Fordyce, 2005; Karaağaç, 2012). The insects also exhibit metabolic resistance towards insecticides using the enzymes such as esterases, glutathione-S-transferase and cytochrome P450 monooxygenases. Glutathione-S-transferases function by conjugating insecticides to reduced glutathione and reductive dechlorination of chlorinated pesticides to produce easily excretable water soluble metabolites. Cytochrome P450 monooxygenases function by hydroxylation of O- or N-alkyl groups; aliphatic or aromatic groups and oxidation of ester, nitrogen and thioether groups present in the insecticides to detoxify them. On the other hand, esterases hydrolyze the ester bonds in the insecticides. Apart from metabolic resistance, dominant, recessive and point mutations in genes also contribute to the development of insecticide resistance. For example, resistance towards organophosphates which inhibit acetylcholine esterase by blocking the nerve signal transduction is observed in

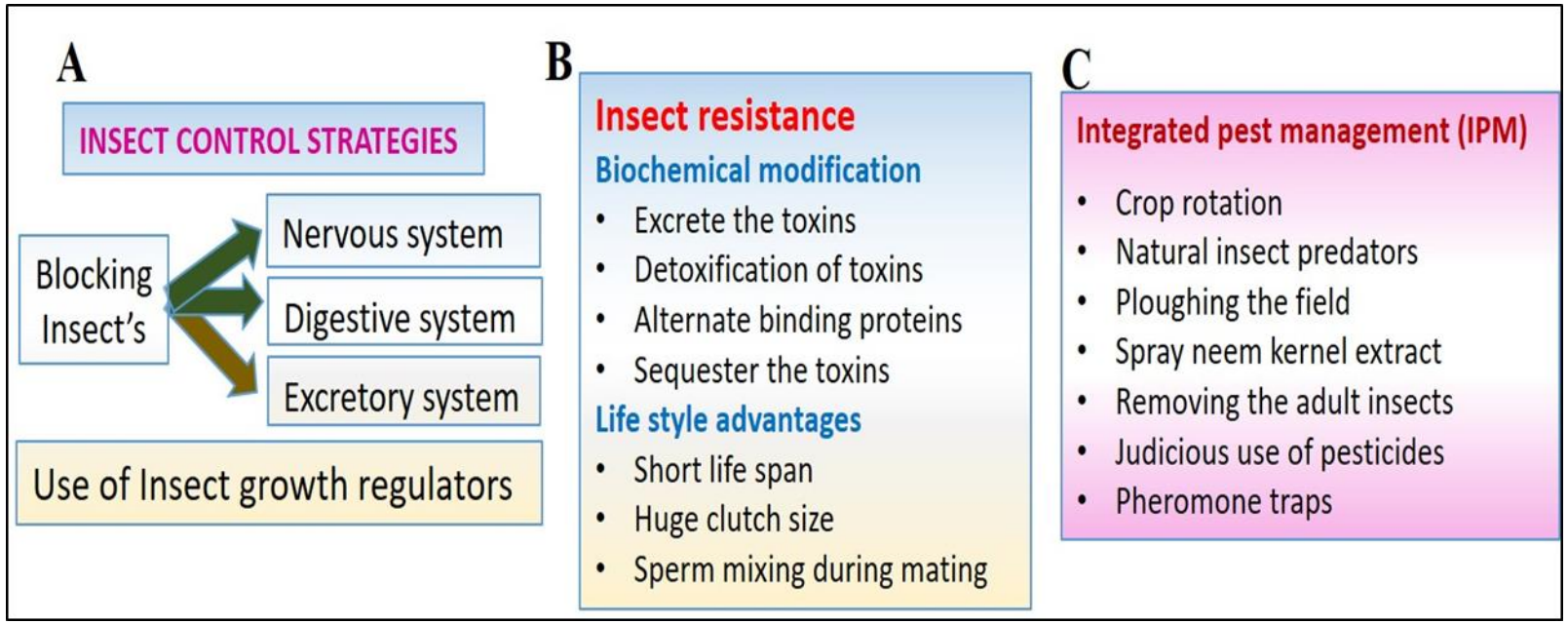


Fig. 1.4. Combat against insects. (A) List of insect control strategies; (B) Insect resistance by biochemical and lifestyle mechanisms; (C) Integrated pest management strategies to kill insects and control resistance development which in turn increased the yield and decreased the application of chemical pesticides, hence more eco-friendly.

Helicoverpa armigera. The resistant *H. armigera* insects express mutated acetylcholine esterase generated through point mutation. Hence, it cannot bind to organophosphates (Ahmad, 2007; Karaağaç, 2012). Also, *H. armigera* express multiple sensitive and insensitive trypsin as well as chymotrypsin proteases which are alternatively expressed upon feeding on host and non-host plant protease inhibitors (Chougule et al., 2005). Moreover, insect resistance is more pronounced in the hot spots of diversity, the tropics and subtropics where climate plays a pivotal role. Further, insects also tend to develop alternate methods to sequester or expel the toxins. For example, pyrrolizidine alkaloids are sequestered after reduction by cytochrome P450 into lipophilic tertiary alkaloids in *Creatonotos transiens* which diffuse passively through gut epithelium. But in *Longitarsus jacobeae*, the hydrophilic pyrrolizidine N-oxides are not reduced and cross the gut epithelium through carriers and sequestered (Duffey and Stout, 1996; Opitz and Müller, 2009; Petschenka and Agrawal, 2016). In few cases a varietal resistance has also been developed to achieve a significant control of a variety of insect population. Thus it has become a threat to the farmers in obtaining potential crop yields in terms of quantity and economy (Fig. 1.4B). However, a combination of different practices such as judicious use of pesticides, crop rotation, field sanitation and exploitation of inherent resistant plant varieties together called as "Integrated Pest Management (IPM)" was developed to increase agricultural production (Fig. 1.4C; Metcalf and Luckmann, 1994; Noor-ul-Ane et al., 2015). Further, integrating foreign genes into the crop plants to improve its resistance is considered as a final option. This approach is also cost effective to achieve a greater control of insects and hence, a significant contributor for IPM practices (Fitt, 2000; Haq et al., 2004). For example, a foreign gene from the bacteria *Bacillus thuringiensis* (Bt) encoding a toxic crystal protein (Cry) was first transferred in to tobacco plant and the transformed plant showed potent toxic effects on tobacco hornworm (Vaeck et al., 1987). But, the Bt toxins cannot control the sap-sucking pests (Chougule and Bonning, 2012). However, several recent reports indicated that lepidopteran

insects had developed tendency for resistance against Bt toxins (Jin et al., 2013; Kumari and Kumar, 2015; Tabashnik, 2015). Further, to control the development of Bt toxin resistance in insects, it was suggested to augment two different Bt toxins into the same plant. For example, on co-expressing Cry1Ac and Cry2Ab in cotton while Cry1Ac and Cry1C in broccoli exhibited a delayed resistance development in cotton bollworm and diamondback moth, respectively (Wei et al., 2015; Zhao et al., 2003). Now, there are a large number of Bt-incorporated products available in the U.S. market (Carrière et al., 2015). Apart from insects developing resistance, there is a massive societal resistance regarding the usage of Bt toxin expressing edible crops which lead the Indian government to stop the field trials of Bt crops (Jayaraman, 2010). However, owing to the merit of transgenic technology, the new Indian government has approved the field trials of 21 different transgenic crops including rice, cotton, maize (corn), mustard, brinjal and chickpea (Kumar, 2015).

Another strategy to counter the insects is to tune the expression of plant's defense proteins or incorporation of potent insecticidal genes from insect resistant wild varieties in to cultivated susceptible crop plants (Carlini and Grossi-de-Sá, 2002; Chen, 2008; Peumans and Van Damme, 1995). However, to obtain a holistic control of insects of multiple orders, gene pyramiding, where expression of a combination of plant's defense genes from the same or different plants together with or without Bt toxin was suggested (Chan et al., 2015; Joshi and Nayak, 2010; Senthilkumar et al., 2010; Tajne et al., 2014). As this strategy decreased the attack of targeted insects in many plants, it could serve as an eco-friendly technology to control insect pests and the development of resistance in them (Carrière et al., 2010). However, this technology warrants the prior identification of the potent plant genes involved in pyramid construction from either cultivar or wild gene pools. Since the plant pest interaction is co-evolutionary, there is a need for understanding the mechanism of resistance development in the insect so as to apply suitable strategies to control them.

1.3. Plant defense proteins

Plants developed a variety of defense compounds which are expressed inducibly and constitutively. The direct defensive responses exhibited by plants through the bioactive compounds / secondary metabolites target the nervous, endocrine and digestive organs (Rosenthal and Berenbaum, 2012). For example, the plants limit the nutrition to pests at both pre- and post-ingestion and induce toxicity by physical damage as well as chemical disruption in the pests (Fig. 1.5A). These bioactive compounds include alkaloids, benzoxazinoids, cyanogenic glucosides, glucosinolates, non-protein amino acids, phenolics and terpenoids (Furstenberg-Hagg et al., 2013). The defense proteins are also a form of plant's primary defense response and comprised of lectins, chitinases, ribosome inactivating proteins (RIPs), arcelins, canatoxins, modified storage proteins and protease inhibitors (Fig. 1.5B). These defense molecules mainly target insect digestion. Chitin, a major component of the exoskeleton and peritrophic matrix of the insect gut along with glycoproteins form a non-cell layer covering the insect intestinal lumen (Zhu et al., 2016). The chitinases produced by plants are a type of plant pathogenesis-related proteins and they act by digesting the intestinal lining of the insects. Similarly, lectins are sugar binding proteins present in the storage organs of Leguminosae family members (Peumans and Van Damme, 1995). When ingested by insects, the lectins bind to the intestinal wall glycoprotein and reduce the nutrient absorption (Chrispeels and Raikhel, 1991). Many crop plants like tomato, potato, maize, groundnut, mustard, finger millet, cotton, lychee, banana, grape, wheat and rice have been transformed with the chitinase gene to develop resistance against insect and fungal attack (Cletus et al., 2013).

The RIPs are N-glycosidases which depurinate the A4324 base in the 28S rRNA loop and thereby block the protein synthesis in the ingested animal/insect (Walsh et al., 2013). The RIPs are initially identified from the plants and are divided into two groups:0 A single domain 30 kDa toxicprotein (Activity domain) and a dual domain toxin where the toxic-A domain is

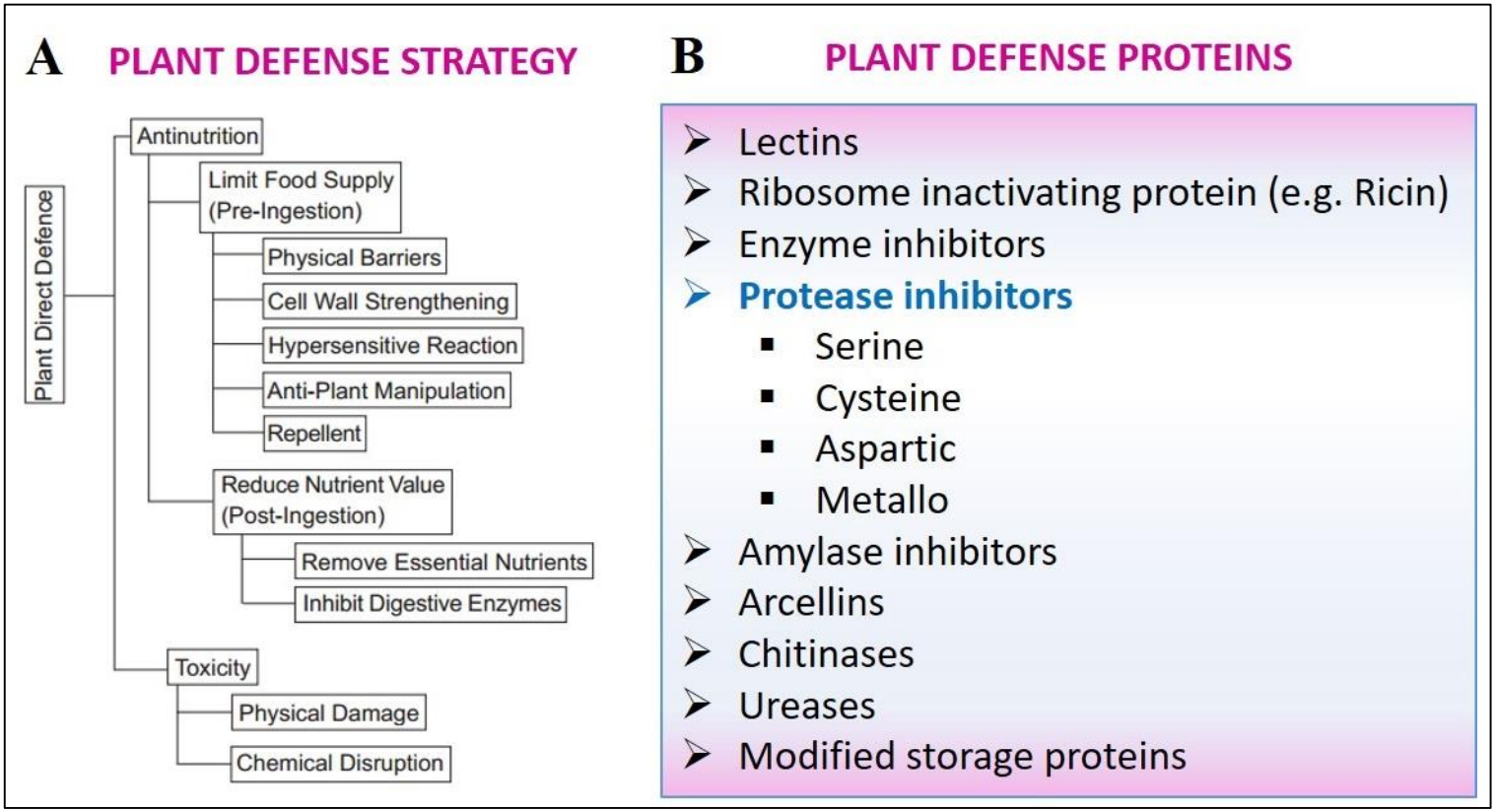


Fig. 1.5. Plant's primary defense. (A) List of primary defense strategies observed in plants. Figure adapted from Chen (2008); (B) List of primary defense proteins synthesized by plants against insect pests.

linked to a lectin (Binding or B-domain) through a disulfide linkage. The latter type of toxin which include ricin and abrin are prevalent in *Ricinus communis* and *Abrus precatorius* seeds, respectively (Carlini and Grossi-de-Sá, 2002). The lectin domain of the RIP toxin bind to D-galactose or *N*-acetylgalactosamine present in the cell membrane and is engulfed into the acidic endocytic vescicle by receptor mediated internalization. In the endocytic vesicle, the two domains will be separated and the toxic A domain insert itself into the vesicle and enters the cytoplasm, and inhibit the protein synthesis (Montecucco, 1998). In plants, the toxins are stored in the modified vesicles called protein bodies. During wounding or viral infection these RIPs are released and inhibit the protein synthesis inducing cell death whereby mitigating the infection (Virgilio et al., 2010). Considering their toxic nature, RIPs are designated as category B biological warfare and used in the past by the army and terrorist alike to kill people (Walsh et al., 2013). Since RIPs are toxic to humans, they are not used in crop resistance improvement.

Domestication of the plants lead to the loss of defense proteins and thereby plant resistance. For example, arcelins are found in the wild beans *Phaseolus vulgaris* (Cardona et al., 1990; Osborni et al., 1988) but not in its cultivar species (Mirkov et al., 1994). However, arcelins which exhibit resistance against bruchids are found to be expressed in the cultivar seeds when the expression of phaseolin, a major storage protein is partially decreased (Hartweck and Osborn, 1997). Likewise, vicilin is a major storage protein of cowpea. The bruchid (*Callosobruchus maculatus*) digests this protein comparatively higher than the Nigerian variety of cowpea which showed resistance against the bruchid (Sales et al., 2000).

1.4. Proteases

Proteases are omnipresent in all the life forms from Archae to bacteria to plants and humans (Neurath, 1984). They play a role in catalyzing the hydrolysis of a peptide bond in the protein and thereby influence many biological processes such as protein catabolism, apoptosis,

senescence, blood coagulation, signal transduction, inflammation and enzyme activation (Chang and Yang, 2000; Neurath and Walsh, 1976; Pham, 2006; Riewald and Ruf, 2002). Any impairment in the regulation of proteases leads to pathological conditions such as lung emphysema, cardiovascular cancer, acquired immune deficiency syndrome (AIDS), Alzheimer's disease and rheumatoid arthritis (Bergers and Coussens, 2000; Davis et al., 2012; Hamada et al., 2015; Lomas et al., 2002; Stubbs and Bode, 1994). Though proteases perform a myriad of functions, only 2% of the total genome code for proteases (Barrett et al., 2012). The proteases are classified based on their active site amino acids into Serine proteases (Ser and His), cysteine proteases (Cys), aspartic proteases (Asp) and metalloproteases containing metallic ions such as Zn^{2+}, Ca^{2+}, Mn^{2+} in their active sites (Barrett et al., 2012; Neurath, 1984). Apart from many functions in the homeostasis of other living organisms, the proteases play a significant role in the growth and metamorphosis of insects. For example, the digestive enzymes such as trypsin and chymotrypsin which belonged to serine proteases are present in significant amount in the guts of Lepidopteran insects (Table 1.1; Berenbaum, 1980; Terra and Ferreira, 1994). Plants are a rich source of all the four classes of proteases (Losso, 2008; Rawlings et al., 2004). Plant proteases such as papain, ficin and bromelain are used in brewing, milk clotting, meat softening, digestion, treatment of viral disorders and cancer (González-Rábade et al., 2011). Conversely, the protease inhibitors (PIs) which play a vital role in regulating the action of proteases are also abundant in the plant kingdom. PIs perform a myriad of functions by controlling these proteolytic enzymes.

1.5. Plant protease inhibitors (PIs)

PIs are a form of plant's primary ubiquitously found defense compound. They are low molecular weight proteins and are natural antagonists of the proteases. These PIs bind to the active site of the proteases reversibly with a very low dissociation constant. This mechanism

Table 1.1. Different classes of insect pests and their major type of digestive gut proteases.

Type of insect	Major gut enzyme	Type of protease
Lepidopteran	Serine protease	Trypsin and chymotrypsin
Coleopteran	Cysteine proteases,	Cathepsin-A like protease
	Aspartic proteases	Cathepsin-D like protease
Dipteran	Serine protease	Trypsin

Table 1.2. List of plant PIs, the types of proteases inhibited, mass and distribution in the plant kingdom. Adapted from Laing and McManus 2002.

Protease inhibited	Name of the inhibitor family	Mass (kDa)	Known distribution
Serine	Kunitz	19-24	Legumes, potato, cereal and Arabidopsis
	Bowman-Birk	6-9.5	Legumes, maize, rice, wheat and barley
	Cereal trypsin/amylase	11.5-14	Cereals
	Mustard seed inhibitor	6.6-7.1	Brassicaceae, members of Arabidopsis
	Proteinase inhibitor 1	7.2-9.1	Solanaceous species, cereals and legumes
	Proteinase inhibitor 2	5-6	Solanaceous species
	Serpin	42-44	Cereals
	Squash	3-3.5	Cucurbit seeds
Cysteine	Cystatin	10-16	Cereals, potato, soybean and Arabidopsis
	Multicystatin	6-87	Potato and sunflower
	Kunitz	20	Potato
	Pineapple Bromelain	5.8-5.9	Pineapple
Aspartic	Kunitz	20-21	Potato
	SQAPI	10.5	Squash
	Wheat inhibitor	58	Wheat
Metallo	Carboxy-peptidase	4.1-4.3	Solanum

potentially neutralizes the enzyme activity of proteases and thereby block their function. PIs are available in the range of 1 to 10% of the total protein in the mature storage organs like seeds and tubers (Lawrence and Koundal, 2002; Ryan, 1973). The PIs control a variety of physiological functions by regulating diverse proteases (Richardson, 1977). PIs are studied mostly from Leguminosae, Solanaceae and Gramineae family members of the plants (Richardson, 1991).

1.6. Classification of PIs

Plant PIs are classified into serine, cysteine, aspartic and metallo PIs based on their ability to inhibit the specific type of proteases (Table 1.2; Ryan, 1990). The PIs are further grouped into many families based on their primary and tertiary structures, the amino acid present in the reactive site and the number and position of disulfide bonds present in them (Laskowski Jr and Kato, 1980). The majority of the PIs identified from the plants inhibit the serine-type proteases followed by cysteine, aspartate and metalloproteases. Apart from the single headed inhibitors which inhibit a single type of proteases, there are many inhibitors which inhibit two proteases simultaneously (Bode and Huber, 1993). On the same context, there are also many cross-class inhibitors which inhibit a different type of proteases through multiple reactive sites or contain single reactive site which inhibits more than one type of proteases (Heibges et al., 2003; Rawlings et al., 2004; Svensson et al., 2004). A database for plant PIs depicting the plant source links to protein, gene sequence, and tertiary structure is available. The database contained 351 plant PIs apart from isoinhibitors from 129 species of plants and grouped into ten families including the suicidal serpin type inhibitor based on the primary protein structure (De Leo et al., 2002).

1.7.Serine PIs

1.7.1. Serpins

Serpins are serine protease inhibitors, widespread throughout the evolution from Archaea to bacteria to eukaryotes including plants and humans to viruses (Irving et al., 2000; Rawlings et al., 2004). Serpins are proteins with molecular mass above 40 kDa and represent a unique, irreversible mode of inhibition of proteases. Serpins consist of three β- sheets, 8 to 9 α-helices and an exposed canonical reactive site loop which upon binding to the proteases is cleaved at the P1-P1' site which in turn lead to a typical change in its structure followed by covalent binding of the PI to the protease. Hence, the Serpins are also named suicide inhibitors (Elliott et al., 2000; Patston and Gettins, 1996; Whisstock et al., 1998). Serpins are isolated and purified from the seeds of wheat (Rosenkrands et al., 1994), barley (Lundgard and Svensson, 1989) and pumpkin phloem sap (Yoo et al., 2000). The genome of *A. thaliana* contains 21 Serpin family genes (Ahn et al., 2009). Cross class inhibitors of Serpin family which inhibit cysteine proteinase 'Metacaspase 9' are also observed in *A. thaliana* (Vercammen et al., 2006). Serpin1 from *Arabidopsis thaliana* showed effective growth inhibition on Spodoptera litura and the nymphs of pea aphid *Acyrthosiphon pisum* (Alvarez-Alfageme et al., 2011).

Apart from the irreversible Serpins, plants contain a variety of reversible serine PIs. These reversible serine PIs are classified based into seven types on their primary, tertiary structure, number and position of disulfide bonds as follows.

1.7.2. Kunitz inhibitors

Kunitz inhibitor was the first PI to be isolated and purified in crystal form from soybean by Kunitz in 1945. It is also the first plant PI to be characterized and studied in detail (Kunitz, 1945, 1946, 1947). Kunitz inhibitor isolated from soybean contained single polypeptide chain

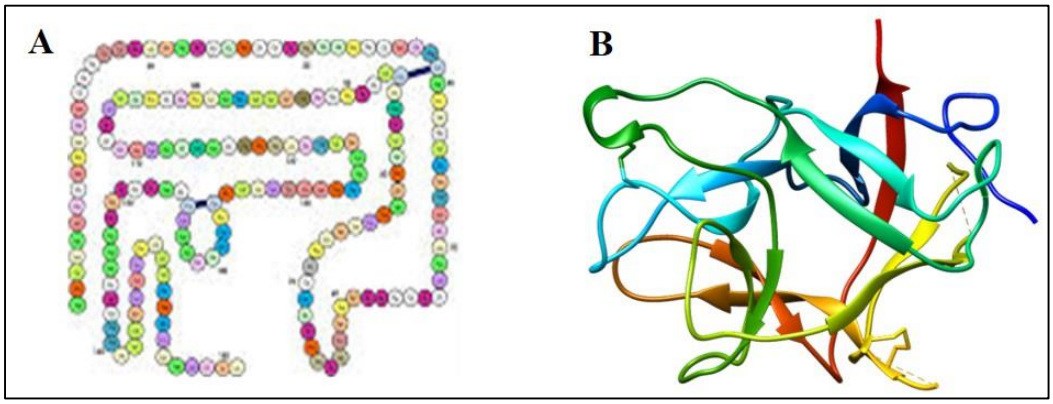


Fig. 1.6. Soybean Kunitz inhibitor. (A) The two-dimensional schematic representation of Kunitz inhibitor showing two disulfide bonds. Figure adapted from Meester et al. (1998); (B) Three-dimensional structure of soybean Kunitz inhibitor containing β-sheets and random coils (PDB id: 1AVU).

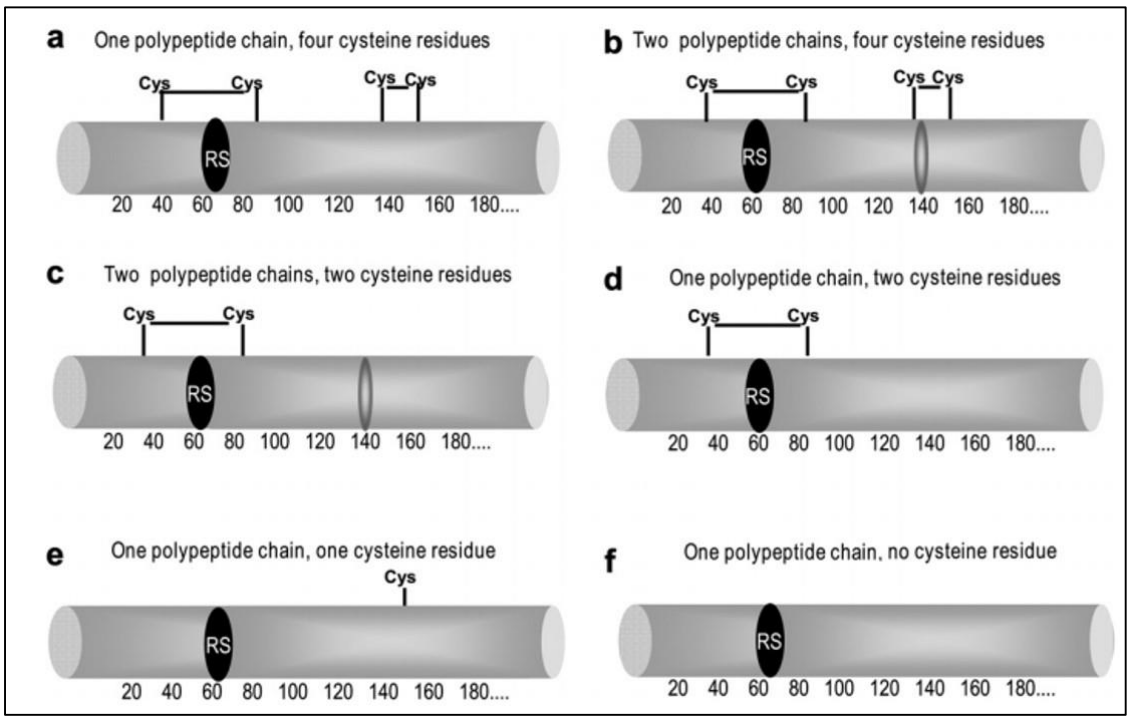


Fig. 1.7. Sub-classification of Kunitz inhibitors identified from many plants based on the number of polypeptides, cysteine residues and disulfide bonds around the reactive site (RS). Figure adapted from Oliva et al. (2010).

with 181 amino acids and two disulfide bridges (Figs. 1.6A,B; Oliva et al., 2010). It is a potent inhibitor of trypsin protease and interact by reactive site amino acids Arg63 and Ile64 (P1-P1') located within the disulfide bond stabilized (Cys39-Cys86) reactive site loop (Song and Suh, 1998). However, Kunitz inhibitors with two polypeptide chains are also identified from the ancient subfamilies of Mimosaceae and Caesalpinieae belonging to Leguminosae. In general, Kunitz inhibitors existed as ~20 kDa molecular mass protein with two polypeptide chain containing two disulfide bridges (Koide and Ikenaka, 1973; Lehle et al., 1996).

In Mimosaceae, Kunitz inhibitors with two polypeptide chains of different lengths are identified. The α-chain is constituted by about 140-150 amino acids of mass 15 kDa and the β-chain is constituted by about 40-50 residues of mass 5 kDa (Macedo et al., 2000; Mello et al., 2001). Both the chains are synthesized as a single polypeptide. During post-translational modification it is cleaved into two chains but linked through inter-chain disulfide bridges. The reactive site is located within the α-chain (Ryan, 1973; Ryan, 1990). The number and position of the disulfide bridges in the Kunitz inhibitors of plants is variable. Thus, several combinations are possible such as: one or two polypeptide chains with two disulfide bonds; one or two polypeptide chain with one disulfide bond and one polypeptide chain with single cysteine residue or no cysteine residue (Fig. 1.7; Luiza Vilela Oliva et al., 2011; Oliva et al., 2010).

Kunitz inhibitors are proteins with a single reactive site and inhibit serine proteases. However, plants contain several types of combinations. Kunitz inhibitors from *Psophocarpus tetragonolobus* possess two reactive sites for chymotrypsin (Shibata et al., 1986). Apart from a few dual headed inhibitors, Kunitz inhibitors which inhibit cross-class proteases are also reported. For instance, Kunitz inhibitors from potato inhibit aspartic and cysteine proteases along with serine proteases (Ishikawa et al., 1994; Pouvreau et al., 2001). Kunitz inhibitors which inhibit subtilisin, leucocyte elastase and cathepsin D were also reported from potato and tomato. Further, Kunitz inhibitors from Bauhinia are known to inhibit human cathepsin L,

neutrophil elastase, plasma kallikrein, factor XIIa, factor Xa and plasma (Vilela Oliva and Sampaio, 2008). Further, plants contain many isoforms of the protein which are expressed in different tissues at different developmental stages (Hernández-Nistal et al., 2009; Huang et al., 2007; Jiménez et al., 2008). Over the years, many isoinhibitors are catalogued from many different plant species while soybean genome alone contained ten different Kunitz inhibitors (Jofuku and Goldberg, 1989). Kunitz inhibitors purified from the seeds of *Poincianella pyramidalis* and *Entada acaciifolia* exhibited potent insecticidal activity against flour moth, *Anagasta kuehniella* (de Oliveira et al., 2014; Guimarães et al., 2015) while Kunitz inhibitors from *Pithecellobium dulce* and *Tamarindus indica* exhibited potent insecticidal activity against *H. armigera* (Pandey and Jamal, 2014; Pandey et al., 2015). Moreover, Kunitz inhibitors from the seeds of *Piptadenia moniliformis* and *Talisia esculenta* exhibited insecticidal activity against *Anthonomus grandis* and *Diatraea saccharalis* (Cruz et al., 2013; Maria das Graças et al., 2012).

1.7.3. Bowman-Birk inhibitors (BBIs)

BBIs are first identified by D. E. Bowman and characterized by Y. Birk from soybean (Birk et al., 1963; Bowman, 1946). BBIs are well-studied group of inhibitors parallel to Kunitz inhibitors. BBIs are widely identified from Legumes and cereals. They are abundant in seeds apart from many other plant tissues. Over the years many crystal as well as solution structures of independent BBI or BBI bound to specific protease are solved (Catalano et al., 2003; de la Sierra et al., 1999; Park et al., 2004; Ragg et al., 2006; Rao and Suresh, 2007; Song et al., 1999; Voss et al., 1996; Werner and Wemmer, 1992). BBI from soybean is considered as the classical model for solving BBIs from other plant species. BBIs are low molecular weight proteins of mass 6 to 9 kDa and consists of around 80 amino acids in length. BBIs are double headed PIs which can inhibit trypsin and chymotrypsin independently or simultaneously. The reactive sites

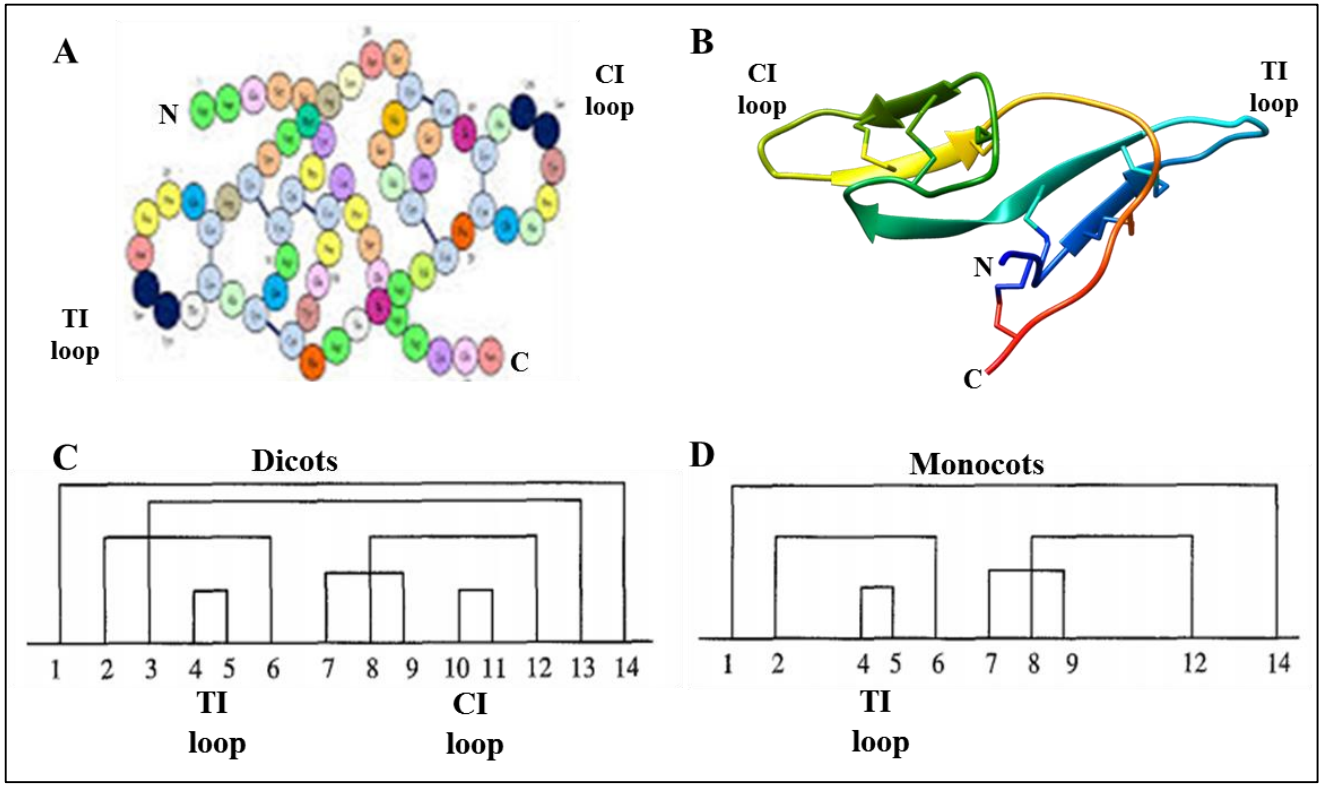


Fig. 1.8. The structure of BBIs. (A) Primary structure of BBI indicating the array of disulfide bonds and inhibitor loops. Figure adapted from Odani and Ikenaka, 1973; (B) Three-dimensional structure of soybean BBI showing trypsin inhibitor (TI) and chymotrypsin inhibitor (CI) loops. The seven disulfide bonds are represented as sticks (PDB id: 1K9B); The disulfide bond map of (C) BBIs in dicots containing seven disulfide bridges; and (D) the monomeric BBIs of monocots with five disulfide bridges were presented along with the trypsin reactive site loop. Figure modified from Prakash et al. (1996).

are present at opposite ends of the molecule allowing it to inhibit different proteases exclusive of each other interactions (Figs. 1.8A,B). Thus, BBIs are observed to host two domains of 30 amino acids each with similar secondary structures containing independent reactive site loop. In general, trypsin reactive site is located at the N-terminal with Lys or Arg at the P1 position, and chymotrypsin reactive site is located at the C-terminal side with hydrophobic amino acid (Figs. 1.8A,B) at the P1 position. Each domain contains two antiparallel β-sheets connected by the reactive site loop. BBIs are structurally stable proteins with seven disulfide bridges. Among them, four are present in the N-terminal domain and three are present in the C-terminal domain (Fig. 1.8C). BBI is a highly soluble globular protein and lacks the hydrophobic core. Instead hydrophobic patches are present on the surface which allow the protein to form higher ordered self-associated forms to increase the stability of the protein. BBIs also exist as multiple isoinhibitors and are expressed in different parts of the plants at different time points and performs various functions (De Paola et al., 2012; Rackis and Gumbmann, 1981).

Apart from leguminous plants, BBIs are also identified from the monocots such as rice (Qu et al., 2003), wheat (Odani et al., 1986), barley (Park et al., 2004) and millets (Tashiro et al., 1990). Monocotyledonous plants contain two types of BBIs. Group 1 BBIs are double headed dimers with mass of 16 kDa, while group 2 BBIs are single reactive site monomers with mass of 8 kDa. Higher mass group 1 BBIs were suggested to be derived from group 2 BBIs during evolution by gene duplication. Group 2 monocot BBIs contains five disulfide bonds while seven disulphide bonds were present in dicot BBIs (Fig. 1.8D; Odani et al., 1986; Tashiro et al., 1990).

Recently, BBIs from the Compositae family gained prominence as peptide inhibitor for their novel ultra-low molecular mass. BBI isolated from seeds of sunflower, *Helianthus annuus*, is a 14 residue cyclic peptide containing the canonical six residues reactive site loop stabilized by the disulfide bridge. Sunflower BBI peptide is a potent inhibitor of bovine trypsin

with *Ki* of 100 pM as compared to the soybean BBI with *Ki* of 610 pM (Luckett et al., 1999; Voss et al., 1996). This ultra-simplified natural BBI portrays the crux of inhibition with well-placed P3-P3' residue containing inhibitor loop. This BBI structure revolutionized the protein engineering field by allowing researchers to study the function of individual residues in the reactive site loop. This approach led to synthesis of many different BBI peptides (Hilpert et al., 2005; Rafał et al., 2011) and greater understanding of the reactive site and its application in drug discovery towards many human complications including cancer, inflammation and liver failure (Bautista et al., 2000; Lesner et al., 2011). Further, BBIs from many plant seeds exhibited insecticidal activtiy against a variety of insect pests. For exampls, BBIs purified from *Clitoria fairchildiana*, *Albizia lebbeck* and *Cajanus cajan* exibted potent insecticidal activty against *Anagasta kuehniella*, *Pieris brassicae* and *Manduca sexta*, respectively (Dantzger et al., 2015; Prasad et al., 2010c; Sharma et al., 2012).

1.7.4. Cereal α-amylase/trypsin inhibitors

Amylase/trypsin inhibitor is a unique family of PIs which inhibit the serine-type proteases along with the amylases which digest α-1,4-glucosidic bonds present in the glycogen and starch. This class of PI is present exclusively in plants. Although, a few proteins characterized to date possess both the activity, a majority of the inhibitors are active against amylase class of enzymes. The first amylase/trypsin inhibitor was identified and characterized from ragi, *Eleusine coracana* Gaertn. The ragi amylase/trypsin inhibitor (RATI) has mass of 13 kDa and possessed 122 amino acids (Campos and Richardson, 1983; Shivaraj and Pattabiraman, 1981). The three-dimensional structure revealed four alpha helices arranged as two antiparallel helices with a short antiparallel β-sheet (Fig. 1.9A). The structure is stabilized by five disulfide bonds with two independent reactive site domains, one each for amylase and trypsin, respectively, at the N-terminal and C-terminal ends of the inhibitor. RATI exhibit high

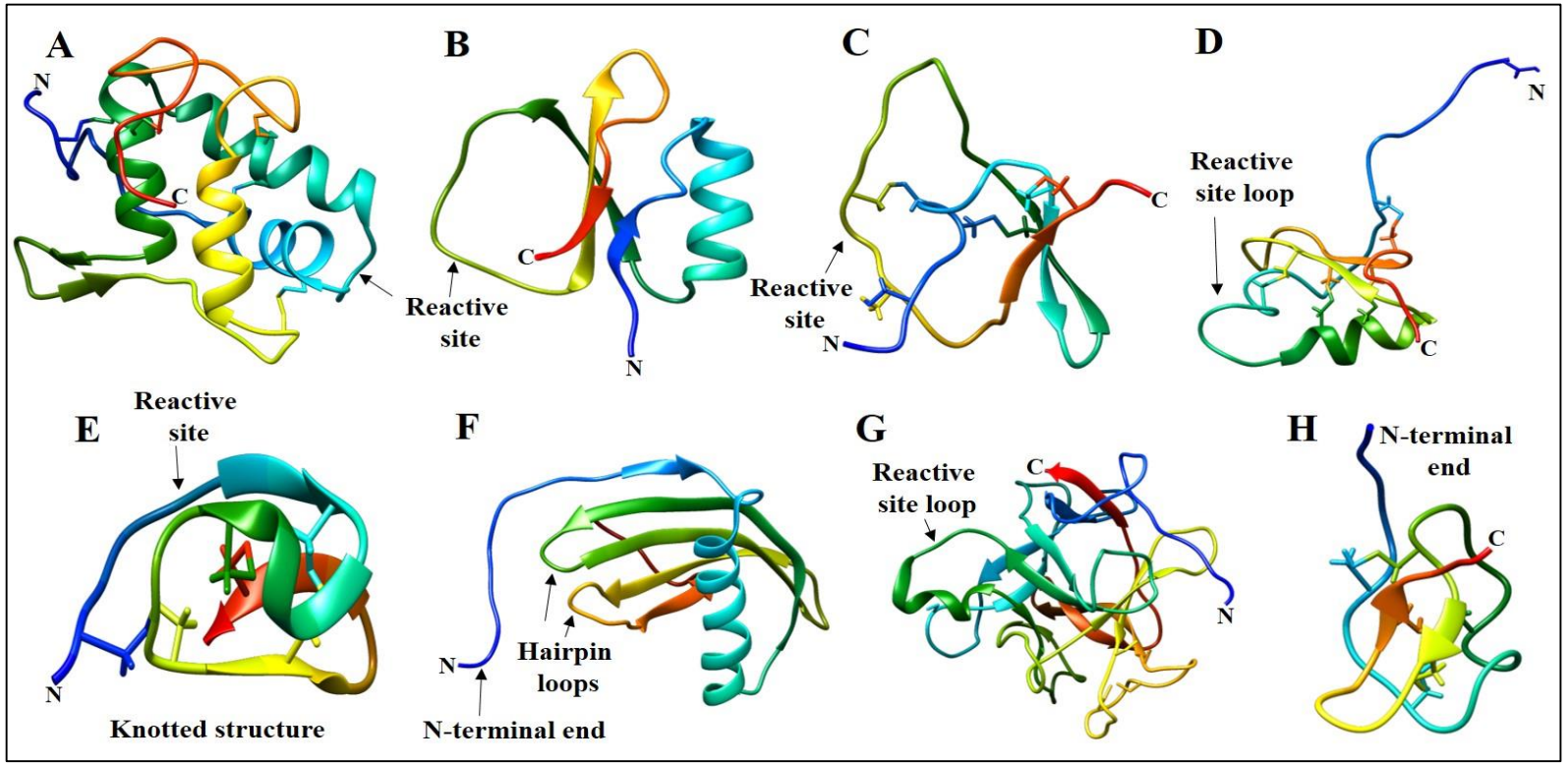


Fig. 1.9. Three-dimensional structure of different plant PIs. (A) Cereal bifunctional α-amylase/trypsin inhibitor crystallized from ragi (finger millet, *Eleusine coracana*), PDB id: 1B1U; (B) PIN 1 type inhibitor, PDB id: 2CI2; (C) PIN2 type inhibitor, PDB id: 2JZM; (D) Mustard type TI isolated from *Arabidopsis thaliana*, PDB id: 1JXC; (E) Squash TI, PDB id: 2IT7; (F) Cysteine PI isolated from potato, PDB id: 1EQK; (G) Kunitz-type aspartate PI glycoprotein can inhibit trypsin and aspartate protease cathepsin-D, isolated from potato, PDB id: 5DZU; (H) Metallo-carboxy PI of potato, PDB id: 1H20. The reactive site loops are marked, and the disulfide bonds are represented as sticks.

competitive inhibition towards trypsin with *Ki* of 1.2 nM (Maskos et al., 1996; Strobl et al., 1995). RATI is a canonical type inhibitor and form stable ternary complex simultaneously with both amylase and trypsin enzymes (Alam et al., 2001). This dual class protease inhibitor is also identified from barley (Mundy et al., 1983), maize (Svensson et al., 2004) and wheat (Orengo et al., 1994). Recently this cross-class inhibitor has been identified and characterized from red gram, *Cajanus cajan* with significant growth reducing activity against *H. armigera* (Gadge et al., 2015; Giri and Kachole, 1998). This class of inhibitor has also been postulated to play a crucial role in designing the transgenic plants against insect herbivory (Kaur and Gupta, 2015). However, amylase/trypsin inhibitors identified from wheat are shown to induce inflammation in human intestine through activation of Toll-like receptor 4 (Junker et al., 2012). Therefore, considering the hazardous effect of amylase/trypsin inhibitors on human health, proper cooking might be a useful strategy before consuming to utilize insect resistant transgenic plants and avoiding untoward complications in man.

1.7.5. Potato Type 1 inhibitors (PIN1)

PIN1 PIs are broadly present in the plant kingdom and are expressed at different developmental, spatial and stress conditions. PIN1 inhibitors are identified from the etiolatedleaves and flower buds of tobacco (Heitz et al., 1993; Kuo et al., 1984), germinating rice seeds and apical meristem (Wang et al., 2008b), stems and seeds of amaranthus (Valdés-Rodríguez et al., 1999), barley (Svendsen et al., 1982) and young leaves as well as fruits of sweet potato and tomato (Lincoln et al., 1987; Wang et al., 2003). On wounding, a spike in the accumulation of PIN1 was observed in tomato leaves (Lee et al., 1986). PIN1 inhibitors are 8 kDa inhibitors consisting of four mixed parallel and antiparallel β-sheets opposite to a single α-helix with no disulfide bonds but many hydrogen bonds stabilizing the reactive site (Fig. 1.9B; Clore et al., 1987a; McPhalen and James, 1987). The reactive site is positioned between the second and third β-sheet forming a wedge-shaped structure (McPhalen and James, 1988).

PIN1 from Cucurbit though possessed a single disulfide bridge it is not involved in stabilizing the reactive site conformation. Also, neither reduction of disulphide bridge to sulphydryl groups nor heat treatment inactivated the PIN1 inhibitors (Cai et al., 1995). Further, the PIN1 inhibitors from maize and wheat inhibited the gut proteases of *S. littoralis*, and *H. armigera*, *Plodia interpunctella* and *Tenebrio molitor*, respectively (Di Gennaro et al., 2005; Tamayo et al., 2000).

1.7.6. Potato Type 2 inhibitors (PIN2)

PIN2 inhibitors are another form of serine-type PIs identified from Solanaceae family from potato tubers (Christeller and Laing, 2005). They are identified from different parts of plants like leaves, flowers, fruits and phloem of many Solanaceae members, Jasmine tobacco flowers, tobacco leaves (Atkinson et al., 1993; Pearce et al., 1993), and *Capsicum annum* (Tamhane et al., 2007). PIN2 inhibitors are low molecular mass multi-gene family proteins of 2-8 inhibitory repeat domains. These inhibitors are produced as a single pro-protein and forms 6-7 individual inhibitors targeting multiple proteases such as chymotrypsin, trypsin, elastase, Pronase E and subtilisin after post-translational modifications (Fischer et al., 2015; Lee et al., 1999a; Tamhane et al., 2007). PIN2 inhibitors are structurally dissimilar to PIN1 inhibitors in having triple stranded antiparallel β-sheet, single turn 3_{10}-helix, long loops, and four disulphide bonds with unique circularization of the N and C-terminal protein sequence together forming another inhibitory domain (Barrette-Ng et al., 2003; Schirra and Craik, 2005; Tamhane et al., 2007). Crystal structure of a single domain PIN2 inhibitor is shown in Figure 1.9C. Further, PIN2 inhibitors from nightshade and pepper inhibited the gut proteases of *S. litura* and *Trichoplusia ni* and *H. armigera*, respectively (Tamhane et al., 2007; Wang et al., 2007).

1.7.7. Mustard trypsin inhibitors

Mustard trypsin inhibitor was first isolated and sequenced from the mustard seeds, *Sinapis alba*. It is a 63 amino acid single polypeptide of mass 7 kDa with four disulfide bonds (Menegatti et al., 1992). It is highly active against trypsin with *Ki* of 0.001 nM and less active against chymotrypsin with *Ki* of 1000 nM (Volpicella et al., 2001). The inhibitor is expressed in the latter stages of seed maturation and also found to be wound inducible (Ceci et al., 1995). Further, mustard trypsin inhibitors were also identified from the wild relatives of Brassicaceae, *Diplotaxis tenuifolia* and *D. muralis* (Volpicella et al., 2009). Similar trypsin inhibitor was identified in *Arabidopsis thaliana* and its solution structure was identified by nuclear magnetic resonance (NMR). The three-dimensional structure revealed a unique fold with a single α-helix and two antiparallel β-strands with β-turn connecting the two strands (Fig. 1.9D). While, three disulfide bonds bridge the α-helix and the β-sheet, the fourth disulphide bond connect the N and C-terminal ends of the protein (Zhao et al., 2002). Mustard trypsin inhibitor undergoes a standard mechanism of inhibition and is observed to be effective in controlling the insect pests. The transgenic tobacco overexpressing mustard trypsin inhibitor was found to impair the fertility of Lepidopteran larvae such as *S. littoralis*, *Mamestra brassicae* and *Plutella xylostella* (De Leo et al., 2001a; De Leo and Gallerani, 2002).

1.7.8. Squash trypsin inhibitors

Squash trypsin inhibitors are first identified from the seeds of pumpkin and *Cucurbita maxima*, and later from many other members of the squash family (Polanowski et al., 1979; Wieczorek et al., 1985). The squash inhibitors are perhaps the PIs with least molecular mass next to the sunflower trypsin inhibitor. It consisted of 27-34 amino acids residues and molecular mass of ~3 kDa. The NMR structure revealed that it contains six cysteine residues forming three disulfide bonds and it is folded into a knottin-like structure (Fig. 1.9E; Felizmenio-

Quimio et al., 2001; Heitz et al., 2001). Their small, circular and rigid structure paved the way for protein discovery and engineering including a database (Wang et al., 2008a). Squash inhibitors are found to be effective in inhibiting proteases of blood plasma such as activated Hagemann factor, kallikrein, plasmin, thrombin, along with elastase (Grzesiak et al., 2000; McWherter et al., 1989). A novel independent but interconvertible cis-trans isomeric squash inhibitors were identified from wax gourd, *Benincasa hispida* (Atiwetin et al., 2006). More recently squash inhibitors isolated from bitter gourd seeds are found to be highly effective against Lepidopteran insect larvae such as *H. armigera* and *S. litura* (Telang et al., 2003).

1.8. Cysteine PI

Cysteine PIs are also ubiquitous in nature and found in plants, animals and invertebrates (Rawlings and Barrett, 1990). In plants, the cysteine PIs are first identified from rice and later reported from many crops plants such as wheat, maize, soybean, sugarcane, cowpea, potato, cabbage, carrot, papaya, apple, avocado, chestnut and Job's tears (Haq et al., 2004). Cysteine PIs are broadly classified into three families viz. stefins, cystatins and kininogens based on disulfide bridges and their localization (Barrett et al., 1986). Cystatins are low molecular weight (12-16 kDa) PIs lacking any disulfide bridges. They contain five antiparallel β-sheets encompassing a central α-helix (Fig. 1.9F; Nagata et al., 2000). Cystatins are potent inhibitors of papain and the various forms of C-terminal chain extended cystatins also inhibit the legumains (Martinez et al., 2007). Cystatins inhibit cysteine proteases by inserting two hairpin loops and the N-terminal end into the active site of the proteases (Bode et al., 1988). Cystatins are involved in endogenous regulation of cysteine proteases released during the rupture of protein bodies and apoptosis. They are also found to regulate the protein turnover and proteolysis during seed maturation as well as seed germination (Corre-Menguy et al., 2002; Hong et al., 2007). Moreover, cystatins are expressed during various abiotic stresses such as water deficit, salinity, heat and cold stress (Valdés-Rodríguez et al., 2007), and in biotic stress

against various insect pests, nematodes and fungi to inhibit their digestive proteases (Kuroda et al., 1996; Samac and Smigocki, 2003; Wang et al., 2008c). Further, cystatins are implicated as an anti-cancer agent through inhibition of cathepsin-B which in turn control the cell invasion rate of human breast cancer cells (Gianotti et al., 2008). Further, the barley cystatin exhibited potent insecticidal activity, increased mortality and decreased fecundity towards *C. maculatus* (El-latif, 2015). Moreover, transgenic sugarcane expressing cane cysteine protease inhibitor 1 gene decreased damage caused by *Sphenophorus levis* by exhibiting 50% reduction in weight gain of this insect larvae (Schneider et al., 2017).

1.9. Aspartic PIs

Aspartic PIs are rarely found in plants and hence they are relatively less studied. Inhibitors of Cathepsin-D, a lysosomal aspartic protease, was first identified from potato (Mareš et al., 1989). The cathepsin-D inhibitor is of 27 kDa with sequence similar to soybean Kunitz inhibitors and unusual in their function by inhibiting trypsin, chymotrypsin along with cathepsin-D but not the other aspartic proteases such as pepsin, renin or cathepsin-E (Ryan, 1990). The structure of cathepsin-D inhibitor from potato is recently crystallized (Fig. 1.9G; Guo et al., 2015). Aspartic PIs are also reported from tomato, sunflower and cardon flowers (Frazão et al., 1999; Park et al., 2000; Werner et al., 1993). They are present in the seeds, the phloem exudates of plants and involve in seed germination (Christeller et al., 2006; Kulkarni and Rao, 2009). Aspartic PIs are also reported to exhibit insecticidal activity against the coffee berry borer, *Hypothenemus hampei* which rely on aspartic proteases for digestion (Molina et al., 2014).

1.10. Metallo-PIs

The metallo-PIs are a small family of PIs identified from potato and tomato which inhibit the metallocarboxypeptidases (Graham and Ryan, 1981; Rancour and Ryan, 1968).

Metallo-carboxy PIs possess low molecular mass (~4.2 kDa) and reported to contain 39 residues. They are globular and knotted where the structure is stabilized by three disulfide bridges containing a 3_{10} helix and two small β-sheets devoid of any α-helix (Fig. 1.9H; González et al., 2003). The C-terminal Gly39-Gly35 tail on binding to the proteases cleaves the Gly39 allowing the Val38 to co-ordinate with the Zn^{2+} ions present in the protease thereby inhibiting the enzyme (Molina et al., 1994). These PIs are expressed constitutively in tubers and induced in leaves upon wounding (Volpicella et al., 2011). The metallocarboxy PIs function in biotic stress by inhibiting the carboxypeptidases present in the gut of *H. zea* (Bayés et al., 2006). Further, these metallocarboxy PIs inhibits pancreatic carboxypeptidase A and B of many animals (Hass and Ryan, 1981). Potato metallocarboxy PIs are structural analogs of human epidermal growth factors (EGF). Hence, they act as anticancer agents by binding to the EGF receptors on the cancer cells thereby preventing the receptor dimerization and further downstream signaling (Blanco-Aparicio et al., 1998).

1.11. Mode of action

PIs of plant origin bind to proteases in both canonical and non-canonical manner (Laskowski and Qasim, 2000). The canonical inhibitors such as the serine PIs bind to the proteases by projecting the reactive site loop in a substrate-like manner (Bode and Huber, 1992; Read and James, 1986). The reactive sites contain a cleavable substrate like peptide bond (P1-P1') stabilized by a disulfide bridge. The PIs have low K_m and K_{cat} which allows the inhibitor to bind tightly with the proteases and ensure slow hydrolysis (Laskowski Jr and Kato, 1980; Laskowski Jr et al., 1987). Upon binding, the peptide bond in the PIs may be cleaved by the enzyme, but the inhibitor remains bound to the enzyme because of the interaction of other amino acids in the PI reactive site and the active site amino acids of the protease (Walker et al., 1998).

In non-canonical inhibitors the reactive site is not rigid due to the absence of disulfide bonds as shown in serine-type PIs. However, they show similarity to the canonical inhibitors in containing a substrate like reactive site with low K_m to block the function of proteases. Non-canonical inhibitory mechanisms in the plant are exhibited by the potato metallocarboxy PIs and the cystatin type inhibitors. In metallocarboxy inhibitors, the C-terminal glycine (G39) residue of the inhibitor is cleaved by the protease thereby allowing the valine (V38) to coordinate with the Zn^{2+} atom and tryptophan (W28) to undergo hydrophobic interaction in the reactive site (Clore et al., 1987b; González et al., 2003; Rees and Lipscomb, 1982). In cystatin type PIs, the collective action of three segments of the inhibitor, the N-terminal trunk, the QxVxG loop and a second loop containing an aromatic amino acid block the active site of proteases and thereby inhibit its activity (Stubbs et al., 1990).

1.12. Physiological functions

PIs take part in many physiological processes of plant development. PIs were best known for their ability to bind to the endogenous proteases. The first studied PIs were the barley seed cystatins and are known to participate in the process of seed germination. The cystatins were found to be abundant in the seeds, but on germination, they were found to decrease with concomitant increase in the cysteine proteases (Mikola and Enari, 1970). Similar patterns of PI abundance w.r.t. vicilin peptide hydrolase inhibitor was observed in mung bean during seed germination (Baumgartner and Chrispeels, 1976). Likewise, oryzacystatin I and II inhibiting cysteine proteases oryzains in rice seed (Abe et al., 1987); phytocystatins in wheat (Simińska and Bielawski, 2015), corn (Abe et al., 1992) and amaranthus grains (Valdés-Rodríguez et al., 2007) was observed. Apart from cystatins, other class of PIs such as aspartic PIs from *Vigna radaiata* (Kulkarni and Rao, 2009); trypsin inhibitors from lettuce (Shain and Mayer, 1965) and job's tears (Ohtsubo et al., 1989); metallo-PIs from buckwheat (Belozerskii et al., 1982); BBIs from horse gram (Kumar et al., 2002), pigeon pea (Godbole et al., 1994a)

and soybean (Dia et al., 2012) were also found to play a significant role in seed germination. The accumulation of PIs in seeds at the early seed developmental stage and their decrease during early germination indicate their importance in preventing premature degradation of storage proteins in seeds. PIs reappear in the cotyledons to protect against pests (Mosolov and Valueva, 2005).

PIs are present at high concentrations in the storage organs such as seeds and tubers and they contain a high content of sulphur containing amino acids and lysine residues which are utilized during germination. The high content of PIs in seeds and their subsequent decrease during germination imply that the essential amino acids from PIs could be diverted towards the synthesis of other structural proteins. This mechanism substantiates that the PIs act as storage proteins (Duranti, 2006; Richardson, 1991). Further, the PIs are thought to have originated from the ancestors of 30 residues seed storage protein, 2S globulin. The barley α-amylase inhibitor sequences are homologous to 2S globulin and other PIs from cereals and legumes such as BBI, Kunitz inhibitors and maize amylase/trypsin inhibitors (Kreis et al., 1985; Ryan, 1990). Apart from them, the storage proteins such as sporamin from potato and taro tubers (Shewry, 2003), discortin from *Dioscorea batata* (Hou et al., 2000), and globulins of *Colocasia esculenta* also inhibits trypsin (Shewry, 2003).

PIs also play a significant role in programmed cell death of plants. The programmed cell death happens in different organs of the plants such as trichomes, root cap, stem parenchyma, in flower development and reproduction, leaf senescence, and cells around the wounded and diseased leaves (Greenberg, 1996; Pennell and Lamb, 1997). Similar to animals, the programmed cell death in plants is mediated by cysteine proteases and are regulated by the cystatins present in them (Solomon et al., 1999).

Apart from the above, PIs are induced in the plants to defend against grazing insect larvae. The PIs mimic the substrate binding site loop conformation of their cognate protease and is stabilized by disulfide bonds many times. As a mimic of the regular substrate, it is possible that PI could be cleaved by the proteases. But, the low dissociation constant (10^{-7} to 10^{-14} M) exhibited by the PIs, render them to bind to the proteases for a very long time and make the proteases unusable. PIs expressed in the plants during insect wounding bind to the digestive proteases present in the larval gut and inhibit their function. This in turn deprives the larvae for essential amino acids from the ingested food. Prolonged inhibition of digestive proteases by PIs leads to stunted growth, decreased fertility and fecundity in insects. For example, PIs from *Capsicum annum* negatively regulated the growth and decreased the fertility of *Chilo partellus* (Jadhav et al., 2016). Similar effects were observed on *H. armigera* by *Cassia fistula* seed PIs, bitter gourd seed trypsin inhibitor and PIN2 PIs from *C. annum* (Pandey et al., 2016; Tamhane et al., 2007; Telang et al., 2003). Moreover, BBIs from *C. cajan* exhibited growth retardation in *M. sexta* and *Achaea janata* larvae (Prasad et al., 2010a; Prasad et al., 2010c; Swathi et al., 2014). This mechanism of action of PIs on gut proteases allowed many research groups to develop transgenic crop plants expressing PIs to control the economically important insects.

1.13. Application of PIs

1.13.1. Transgenic plants

Many economically important insects hampering the crop yield belong to the orders Lepidoptera, Diptera, and Coleoptera and rely on their gut serine and cysteine proteases for digestion. Hence, most of the transgenic plants expressing PIs active against these different class of proteases were developed (Gatehouse, 2011; Losvik et al., 2017). Many PI genes isolated from different plants were expressed in host plants under the control of either

constitutive or wound-inducible promoters. The first transgenic plant expressing PI was the tobacco and it was transformed with cowpea seed trypsin inhibitor which exhibited resistance to *Heliothis virescens*. Since then, many transgenic plants expressing PIs resistant to a variety of insect pests were developed (Table 1.3). However, during the course of host-pest coevolution, the insects developed resistance by either over producing the sensitive proteases or expressing insensitive proteases or simply excreting the PIs (Zhu-Salzman and Zeng, 2015). Second level of transgenic plants expressing more than one PI in a single plant were developed to overcome insensitive proteases (Chen et al., 2014; Senthilkumar et al., 2010). Apart, transgenic plants expressing PIs along with lectins were developed which deprived the insects to excrete the ingested PIs as lectins bind to its gut walls (Li et al., 2005; Zhu-Salzman et al., 2003). Further, Bt toxin-PI fusion proteins reduced the development of insect resistance while the PI targeted the insensitive proteases (Gatehouse, 2011; Tajne et al., 2014; Zhu et al., 2012). Also, the transgenic cotton expressing both PIN1 and PIN2 inhibitors which act independently against both sensitive and insensitive gut proteases of the *H. armigera* was developed (Dunse et al., 2010a; Dunse et al., 2010b). More recently, plastid engineered transgenic plants expressing lethal doses of multiple PIs and chitinases which showed resistance against biotic and abiotic stress was developed (Chen et al., 2014). Thus, the concept of "designer transgenic plants" where multiple PIs active against single or multiple insects are present in a single plant was put forth to sustain both plants and mankind.

1.13.2. Functional food additive

Food plays a significant role in the health of an individual and culture of a society. Different cultures has different food styles and hence their role in prevalence or controlling diseases. For instance, the prevalence of cancer is more in Americans than the Asians. But comparatively less in Japanese since they consume more soybean in their diet (Kennedy, 1998). Soybean seeds host a rich percentage of BBIs. Food containing a compound or a molecule

Table 1.3. Transgenic plants expressing different PIs active against insect pests.

Proteinase inhibitor	Plant transformed	Targeted pest	References
Kunitz inhibitor	Tobacco	*Spodoptera litura*	McManus et al. (1999)
	Sugarcane	*Diatraea saccharalis*	Falco and Silva-Filho (2003)
	Rice	*Nilaparvata lugens*	Lee et al. (1999b)
	Sweet potato	*Cyclas spp.*	Cipriani et al. (1999)
	Potato/tobacco	*S. littoralis*	Marchetti et al. (2000)
	Tomato	*Manduca sexta*	Meyer et al. (2016)
BBI	Tobacco	*S. litura*	Sane et al. (1997)
		Heliothis virescens	Hilder et al. (1987)
		S. frugiperda	Pujol et al. (2005)
	Rice	*Chilo suppressalis, Sesamia inferens*	Xu et al. (1996)
	Cabbage	*H. armigera*	Hao and Ao (1997)
		Pieris rapae	Fang et al. (1996)
	Pigeonpea	*H. armigera*	Lawrence and Koundal (2001)
	Strawberry	*Otiorynchus sulcatus*	Graham et al. (1997)
PIN2	Tobacco	*M. sexta*	Johnson et al. (1989)
		Chrysodeixis eriosoma	McManus et al. (1994)
		H. armigera, S. litura	Luo et al. (2009)
	Sugarcane	*Antitrogus consanguineous*	Nutt et al. (1999)
	Rice	*Sesamia inferens*	Duan et al. (1996)
		Scirpophaga incertulas	Rao et al. (2009)
	Poplar	*Chilo suppressalis*	Vila et al. (2005)
		Plagiodera versicolora	Klopfenstein et al. (1997)
Mustard trypsin inhibitor	Tobacco	*Plutella xylostella*	De Leo et al. (2001b)
		S. littoralis	De Leo et al. (1998)
	Oilseed rape	*S. littoralis*	De Leo and Gallerani (2002)
	Arabidopsis	*Mamestra brassicae*	De Leo et al. (2001a)
Cystatin	Potato	*Spodoptera littoralis*	
		Myzus persicae	Gatehouse et al. (1996)
		Leptinotarsa decemlineata	Cingel et al. (2015)
			Cingel et al. (2016)
	Poplar	*L. decemlineata*	Lecardonnel et al. (1999)
		Chrysomela tremulae	Leplé et al. (1995)
	Oilseed rape	*Myzus persicae*	Rahbé et al. (2003)
		Ceutorhynchus assimilis	Girard et al. (1998)
	Rice	*Sitophilus zeamais*	Irie et al. (1996)

	Lissorhoptrus brevirostris	Armas et al. (2009)
Sugarcane	*Sphenophorus levis*	Schneider et al. (2017)
Soybean	*Riptortus clavatus*	Ishimoto et al. (2012)
Maize	*Tetranychus urticae*	
	Brevipalpus chilensis	Carrillo et al. (2011)

which help to decrease the incidence of a disease is called as a functional food (Arai, 1996). BBIs are considered as functional food because of their ability to inhibit the proteases expressed in many disease states (Losso, 2008). BBIs were identified as a chemopreventive agent and was given an investigative new drug status by Federal Drug Administration in 1992. BBI and BBI concentrate (BBIC) were reported to directly scavenge reactive oxygen species in their vicinity thereby decreasing the prevalence of cancer (Arbogast et al., 2007). PIs are also suggested to be a satiating agent for their ability to inhibit the digestive proteases in animals which in turn induce the release of cholecystokinin, a satiating hormone in the stomach (Losso, 2008).

1.13.3. Chemopreventive

The BBIs are known to possess anti-cancer activities. It was shown in the breast cancer cell line that they function by inhibiting the proteolytic enzymes of proteasomes thereby hindering the protein catabolism (Souza Lda et al., 2014). Proteasomes are an array of trypsin, chymotrypsin, and caspase-like proteases which play a central role in intracellular protein degradation (Sterz et al., 2008). BBI containing dual inhibitory sites inhibit proteasome's chymotrypsin primarily than trypsin, thereby arresting cancer cells at G1/S, G2/M phase of cell cycle and induces apoptosis (Chen et al., 2005; Joanitti et al., 2010). Many BBIs and BBICs purified from different plants were reported for their anti-cancer activity on a variety of cancer cell lines. The potential of BBIs as therapeutic molecule against cancer in multiple cell lines along with their IC_{50} values was represented in Table 1.4.

1.13.4. BBI as a drug in clinical trials

Following the *in vitro* activity of BBIs on cancer cell lines, six clinical trials were carried out using human models (Table 1.5.). One such human trial was to control oral leukoplakia by soybean BBIC. Initially, the dose range of BBIC from 25 to 800 chymotrypsin

Table 1.4. Potential inhibition of multiple cancer cell lines by different plant PIs *in vitro* and their IC_{50} values.

Sl. No.	Source	Cell line	IC_{50}	References
1.	*Lavatera cashmeriana* PI (LC-PI)	A549 Cells LC-pi I LC-pi II	 54 µg/mL 38 µg/mL	Rakashanda et al. (2015)
2.	Lentil BBI	HT29 CCD-18Co	32±2 µM (not affected)	Caccialupi et al. (2010)
3.	Brown kidney bean BBI	MCF7 HepG2 WRL68	71.52 µM No effect No effect	Chan et al. (2013)
4.	Soy BBI	MCF 7 Proteasome	88 µM 20 µM	Chen et al. (2005)
5.	Pea BBI	HT29	rTI1B (46 µM)	Clemente et al. (2005)
6.	Soy BBI	HT29 CCD-18Co	IBB1-39.9 µM IBBD2-48 µM	Clemente et al. (2010)
7.	Chickpea BBI Kidney bean Soybean Mung bean	MDAMB-231 PC-3 and LNCaP LNCaP LNCaP LNCaP	25–400 µg/ml 200, 400µg/ml 50, 100 µg/ml 100, 200 µg/ml	Magee et al. (2012)
8.	Soy BBI	U2OS cell	200, 400µg/ml	Saito et al. (2007)
9.	Soy BBI & Soy BBI concentrate (BBIC)	A2780 C30 &C200	100 µg/ml	Wan et al. (1998)
10.	Soy BBI & BBIC	MCF7 SCC61 & SQ20B HeLa, HeLa-R1, & HeLa-R3, MCF10, HTori3, C3H10T1/2	100 µg/ml	Zhang et al. (1999)
11.	Black-eyed pea Trypsin/Chymotrypsin Inhibitor (BTCI)	MCF7	200mM (This is the conc used not IC_{50})	Souza Lda et al. (2014)
12.	*Vigna mungo* PI (16 kDa)	Hep G2 MCF7	No inhibition	Cheung et al. (2009)

Table 1.5. List of clinical trials completed using BBI as a drug.

Sl. No.	Protease inhibitor	Disorder examined	Clinical trial phase	Dosage/day	References
1	BBIC	Oral leukoplakia	Phase I	800 CIU	Armstrong et al. (2000a)
			Phase IIa	200-1066 CIU	Armstrong et al. (2000b)
			Phase IIb	300 CIU twice/day	Armstrong et al. (2013)
2	BBIC	Prostatic hyperplasia	Phase I	100-800 CIU	Malkowicz et al. (2001)
3	BBIC	Ulcerative colitis	Phase I	800 CIU	Lichtenstein et al. (2008)
4	BBIC	Prostate cancer	---	800 CIU	Kennedy (2005)

inhibitor unit (CIU) and dose toxicity of BBIC on human subjects was analyzed (Armstrong et al., 2000a). This study found that BBI was rapidly taken up and the metabolic products were excreted in 24-48 hrs in urine. Following this study, phase IIa clinical trial was carried out with BBIC dose range of 200-1066 CIU per day (Armstrong et al., 2000b). This phase IIa clinical study which was carried out for one month exhibited 31% clinical response from patients. The total lesion area decreased from 615 mm^2 to 438 mm^2 in oral leukoplakia patients. However, the phase IIb clinical trials performed for six months with 300 CIU twice per day did not show significant effects on oral leukoplakia patients, as the decrease in mean lesion area of placebo and BBIC treatment was 17.1% and 20.6%, respectively (Armstrong et al., 2013). However, the authors suggested that this could be due to the decrease in the bioavailability of the new formulation due to the freezing of the BBIC by the suppliers. Hence, though the initial results of BBI as an anti-cancer agent is promising, further concrete evidences are needed to establish BBI as a green chemopreventive drug. Apart from oral leukoplakia, BBIC also exhibited potent activity against prostatic hyperplasia and ulcerative colitis (Hawkins et al., 1997; Wan et al., 1999). The phase I clinical trials carried out on prostatic hyperplasia and ulcerative colitis patients exhibited encouraging results when compared to placebo which warrants further studies at a larger samples and at multiple centers (Lichtenstein et al., 2008; Malkowicz et al., 2001).

1.13.5. Other applications of PIs

Serine proteases such as elastase, cathepsin G, and mast cell chymase play a major role in regulating inflammation. These enzymes induce hypersensitive response and degrade the extracellular matrix of the infected tissues (Annahazi et al., 2009; Shimoda et al., 2007). Abnormal release of elastase, a serine protease induce tissue inflammation mediated damage in many diseases such as lung emphysema, chronic bronchitis, cystic fibrosis, septic shock, adult respiratory distress

syndrome and rheumatoid arthritis (Adkison et al., 2002; Antunes and Rocco, 2011; Kim and Criner, 2013; Le Gars et al., 2013; Schouten et al., 2008). Many plant PIs were reported to control the proteases involved in the above mentioned diseases and thereby mitigate it. For example, serine-cysteine inhibitor from *Bauhinia bauhinioides* decreases lung emphysema and reduce inflammation in mice (Almeida-Reis et al., 2017); soybean BBI and tamarind serine PI inhibited the human neutrophil elastase (Fook et al., 2005; Larionova et al., 1997) while Kunitz inhibitor from *Erythrina velutina* seeds mitigated the sepsis in Swiss mice (Machado et al., 2013). Moreover, soybean BBI was reported to be effective in experimental autoimmune encephalomyelitis and thereby control autoimmune multiple sclerosis (Safavi and Rostami, 2013). Due to their intrinsic anti-inflammatory activity and stability at extreme acidic and alkaline conditions, BBIs were implicated as colorectal chemo-preventive agent (Clemente and Arques, 2014). Further, Kunitz inhibitors were reported as effective anti-coagulating agents (Machado et al., 2013). Moreover, plant PIs exhibit anti-bacterial, anti-fungal and anti-viral properties (Kondo et al., 1992; Paiva et al., 2013; Srikanth and Chen, 2016). Also, Kunitz type PIs from *Leucaena leucocephala* were reported to be effective in controlling dipteran insect larvae, *Aedes aegypti* (Almeida Filho et al., 2017).

Chapter 2

Scope, Rationale, Objectives and Approach of the study

Scope, Rationale, Objectives and Approach of the study

2.1. Scope of the work

Protease inhibitors (PIs) are globular proteins expressed constitutively in the storage organs of plants such as seeds and tubers (Grosse-Holz and Hoorn, 2016) or induced in vegetative parts such as leaves during biotic and abiotic stresses (Drame et al., 2013; Parmar et al., 2017; Tiwari et al., 2015). They function in regulating endogenous proteolysis, seed development and programmed cell death (Chye et al., 2006; Ryan, 1973; Xu et al., 2001) and act as the reserve store of essential amino acids during abiotic stress (Mandal et al., 2002) or as defense molecules during biotic stresses such as animal grazing, insect feeding and microorganism infection (Jamal et al., 2013; Tamayo et al., 2000).

The insects digest the ingested plant material using the digestive proteases present in their gut. The digestive proteases produced in the larval guts are classified into four types based on their catalytic mechanism: serine, cysteine, aspartic and metalloproteases (Losso, 2008). However, serine proteases are of considerable interest since most of the lepidopteran insects depend on them for digestion of ingested food (Wolfson and Murdock, 1990). Therefore, PIs active against serine proteases gained importance among the defense proteins produced by plants to counteract the insect attack (Furstenberg-Hagg et al., 2013). After ingestion, PIs bind to the digestive proteases present in the insect guts, block their function and thereby decrease their feeding rate. To counteract plant's defensive PIs, the insects respond by either over producing inhibitor-sensitive proteases or synthesizing new inhibitor-insensitive proteases. This mechanism lowers the amount of essential amino acids present in the gut which in turn retard the growth and development, ultimately leading to mortality of the insect (Banerjee et al., 2017; Jongsma and Bolter, 1997). This mechanism of action of PIs is being exploited to control the pest attack on crop plants (Macedo et al., 2015; Machado et al., 2017).

Kunitz inhibitors and Bowman-Birk inhibitors (BBI) from leguminous plants are well known for their potent insecticidal properties. They function in crop protection by blocking the insect gut proteases (Shamsi et al., 2016). The BBIs are proteins with molecular mass in the range of 7-9 kDa and five to seven disulphide bridges. The Kunitz inhibitors are proteins with molecular mass of ~20 kDa and in general contain single polypeptide chain with two disulphide bridges (Mosolov and Valueva, 2005; Oliva et al., 2010). The PIs from non-host/wild plants are suggested to be efficient candidates in controlling the insect pests which sustain on their host plants. This response is possible because the gut digestive enzymes of insects grown on their host plants have not been exposed to such type of non-host plant PIs earlier (Harsulkar et al., 1999; Sandhya et al., 2017). But, a major intriguing question is how far that all the non-host/wild-relative plant PIs would be effective in controlling the insects which grow on their host plants. Nevertheless, many transgenic plants expressing PIs from non-host/wild relatives are known to counteract the insect pests successfully (Abdeen et al., 2005; Dunse et al., 2010b; Macedo et al., 2015; Rehman et al., 2017).

2.2. Rationale of the study

India is the world leader in the production of red gram and castor oil. *H. armigera* and *A. janata* are two economically important insect pests which feed on these crop plants. *H. armigera* is a polyphagous insect pest which feeds on more than 200 varieties of plant species including major crop plants like cotton, chickpea, pigeon pea, groundnut, soybean, maize, sorghum, tomato and sunflower (Fitt, 1989). This feeding habitat has spread across many economically important crop plants leading to an increase in the monetary loss to a bigger margin (Lammers and Macleod, 2007). Controlling *H. armigera* is a major challenge since they are very successful in overcoming both the host plant's defense and chemical pesticides (Ahmad, 2007; Armes et al., 1994). Successful control of this insect can lead to a massive

increase in crop yield. Hierarchical classification of *H. armigera* and its feeding on multiple crop plants were shown in Figure 2.1.

In India, the castor seeds are produced mainly in Gujarat followed by a distantly placed Rajasthan state (Pavaskar and Kshirsagar, 2013). Castor is an oil-rich cash crop cultivated for its oil containing seeds which have a myriad of industrial benefits. Castor oil is used mainly in the production of automobile oils, paints, varnishes, cosmetics and skin creams (Ogunniyi, 2006). Apart from industrial applications, castor oil is also used in medicine as a laxative, labor inducer during pregnancy (Tunaru et al., 2012), and it has excellent anti-inflammatory properties apart from many other therapeutic properties (Scarpa and Guerci, 1982). Castor plants are attacked by a variety of lepidopteran insect pests like *Spodoptera litura*, *Amascata albistriga,* and *Achaea janata*. Among them, *A. janata* is specific to castor and its larvae feeds on the leaves voraciously. It can defoliate the entire plant leaving only the main stem. Fully grown castor can tolerate up to 25% of leaf damage. However, uncontrolled damage caused by *A. janata* larvae can lead to the death of young plants or decrease yield in the older plants. Though Bt toxins showed prominence in the control of *A. janata* in the castor (Malathi et al., 2006), there is a lacuna in the biotechnological application of eco-friendly agents to manage this insect where PIs and other plant's defense related proteins could fill in. Hierarchical classification of *A. janata*, pictures of castor plant, its inflorescence, *A. janata* larva, worldwide and nationwide production of castor was shown in Figure 2.2.

Successful control of these insect pests can lead to a huge increase in crop yields. A variety of control measures were applied by scientists starting from plant's own defense molecules like proteinase inhibitors (PIs) (Johnston et al., 1993), crude leaf extracts of plants (Ahmad et al., 2015), toxic proteins from the bacterium *Bacillus thuringiensis* (Bravo et al., 2011), viral-based control in the form of nucleopolyhedrovirus (NPV) (Moscardi, 1999) and

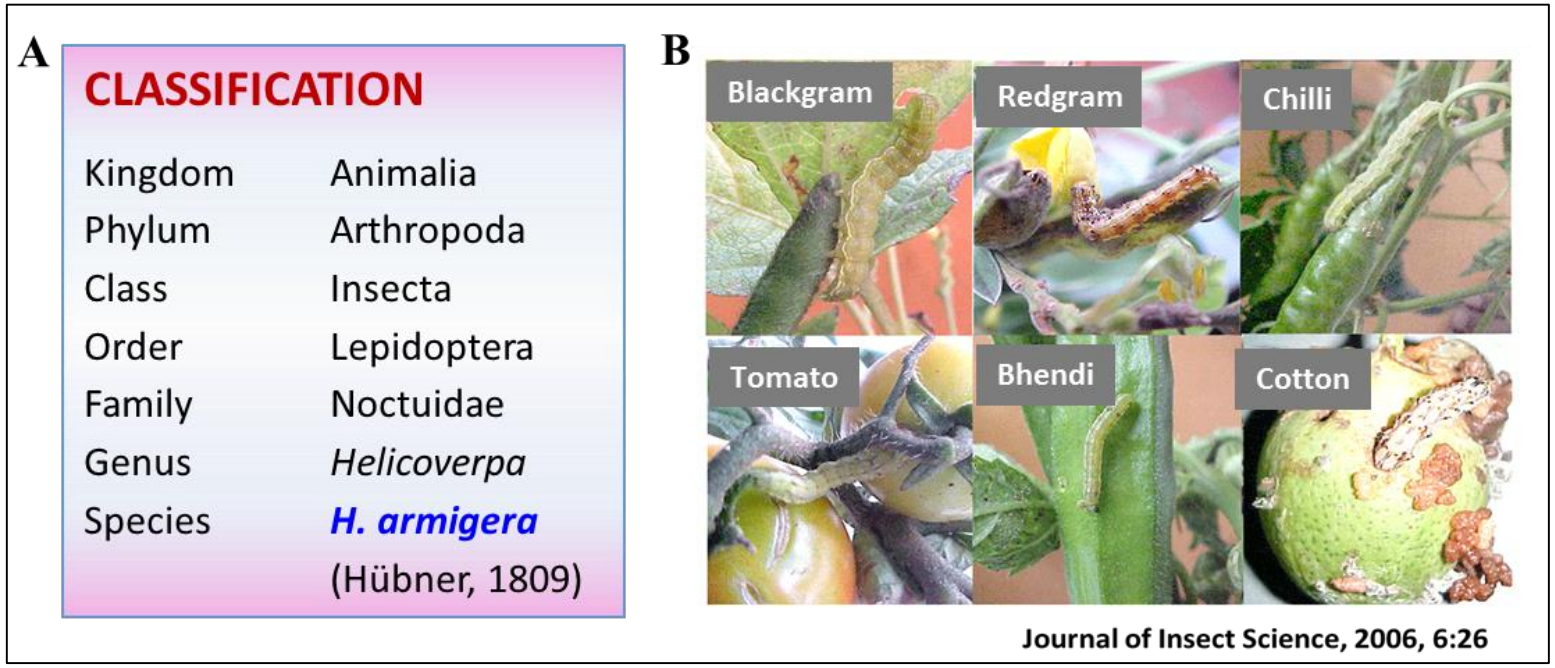


Fig. 2.1. (A) Taxonomical classification of Lepidopteran insect pest, *H. armigera*; (B) Pictures of polyphagous *H. armigera* feeding on multiple crops such as black gram, red gram, chili, tomato, bhendi and cotton. Figure modified from Subramanian and Mohankumar (2006).

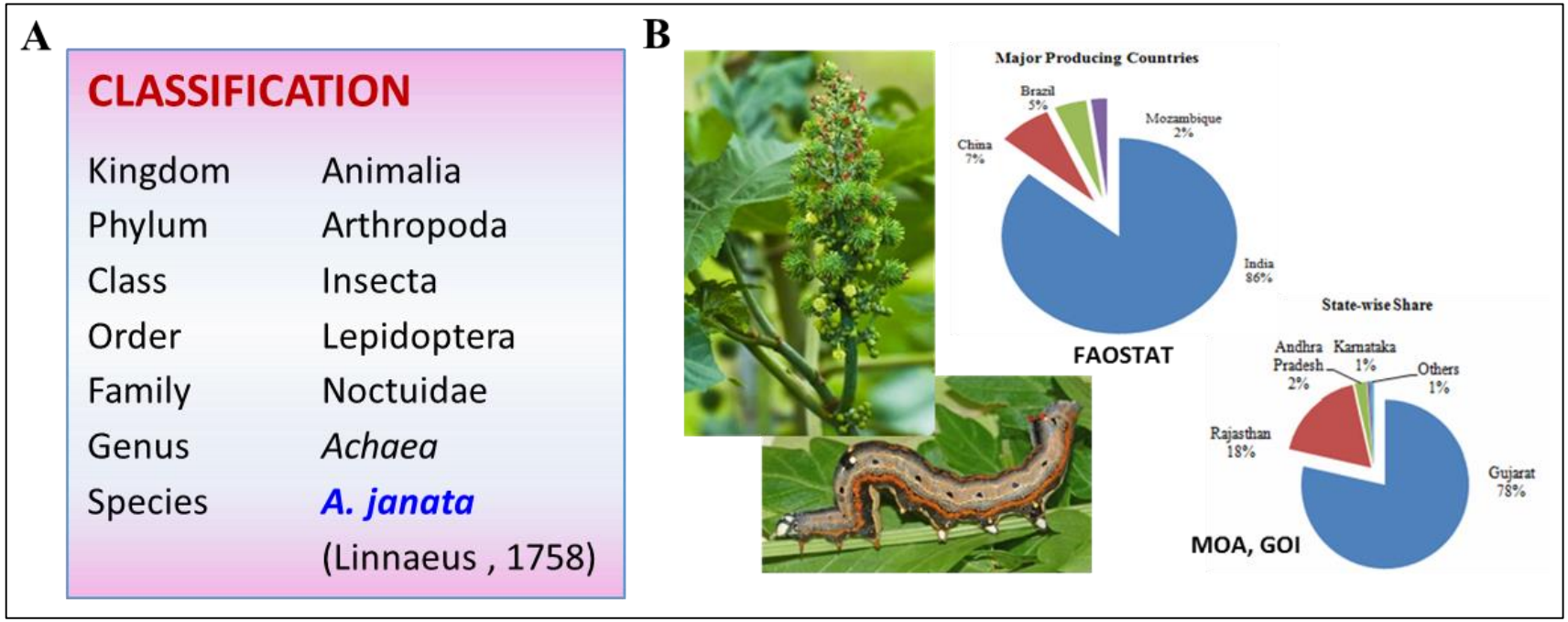


Fig. 2.2. (A) Taxonomical classification of a Lepidopteran insect pest, *A. janata*; (B) Pictures of the inflorescence of the castor plant, *A. janata* insect pest, production of castor in the world (FAO, USA) and state wise production of castor in India (MOA, GoI).

more recently RNA interference (RNAi) based strategies (Kola et al., 2015). Bt-based crystal (Cry) 1Ac protein expressed in transgenic plants appeared promising initially to control *H. armigera* (Bravo et al., 2011). However, recent reports stated that the insect developed resistance. But, the pyramiding of transgenic plants with Cry1Ac and Cry2Ab was suggested to slow down the process of resistance development (Jin et al., 2015; Wei et al., 2015). Further, recent biotechnological developments in dsRNA-based RNAi studies showed promising results, though it involved a lot of whole genome sequencing and transcriptome analysis of other related predator insects and all other consumers of the specific transformed plant is required in order to eliminate cross activity (Kola et al., 2015). Also, the effect of this dsRNA on human health was not studied systematically and the safe food status is yet to arrive. Hence, the above Bt toxin and RNAi based strategies have both scientific and societal problems which are yet to be addressed. In this scenario, PIs which belong to plants own defensive mechanism is much more promising in a long run when applied together with IPM strategy. As the host and pest, both are known to evolve together during evolution, a continuous screening of non-host/wild-relatives would be beneficial to identify potent ecofriendly PIs against these economically important insect pests (Jamal et al., 2013; Losvik et al., 2017).

Rhynchosia, a wild relative of *C. cajan* is considered as an efficient candidates for controlling a variety of insect pests as it is known to harbor an important gene pool for biotic, abiotic and disease resistance traits. These traits could be introgressed into the cultivars by rDNA technology because of its placement in the quaternary gene pool of Cajanus (Fig. 2.3A; Mallikarjuna et al.,2007). Almost all the major organs of *Rhynchosia* spp. are reported to be economically important starting with edible seeds (Kalidass and Mohan, 2012), leaves enriched with therapeutically important bioactive compounds (Bakshu and Venkata Raju, 2001) and roots containing a polysaccharide with anti-cancer properties (Jia et al., 2015). On the crop protection perspective, different species of Rhynchosia seeds are reported to contain defensive

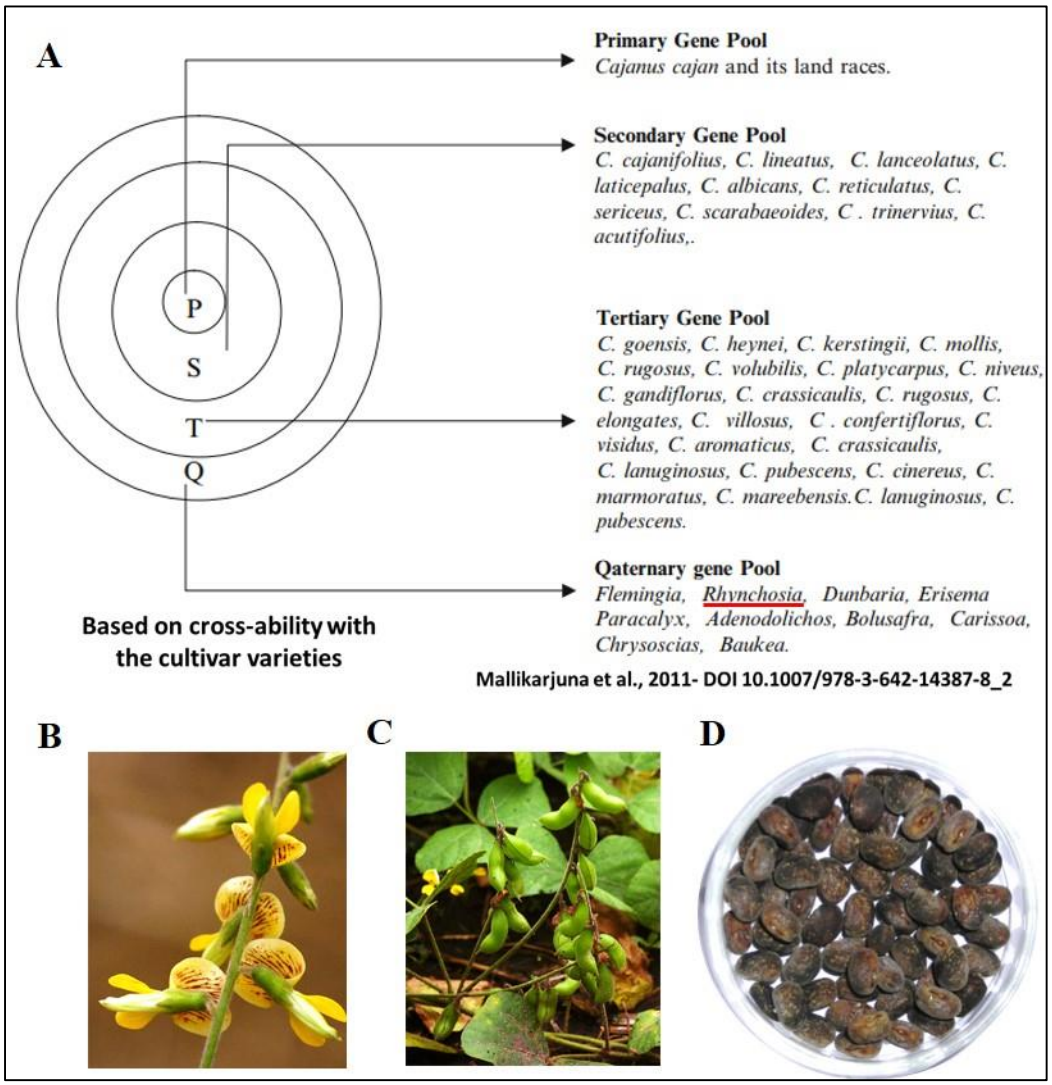


Fig. 2.3. *C. cajan* gene pool. (A) Classification of *C. cajan* and its wild gene pool based on its crossability with each other. Rhynchosia is grouped under quaternary gene pool of *C. cajan* is underlined in red colour. Figure adapted from Mallikarjuna et al. (2011); (B) Flower; (C) pod; and (D) dry seeds of *R. sublobata*. Figures B and C are adapted from Bart Wursten©, Flora of Mozambique.

inhibitor proteins active against gut proteases of insect pest *H. armigera* (Chougule et al., 2003). These features suggest Rhynchosia as an interesting plant of study to purify PIs from its seeds and characterize their insecticidal properties. *R. sublobata* is one of the species under the genus of Rhynchosia. The flower, pod and seed pictures of *R. sublobata* are presented in Figure 2.3B-D. Further, preliminary studies with *R. sublobata* seed crude PI showed that it significantly inhibited the gut proteases of *H. armigera* and *A. janata* while the effect was marginal among the other insects tested such as *Spodoptera litura*, *Papilio demoleus*, *Amsacta albistriga* and *Daphnis nerii* (Fig. 2.4). In this context, it is important and interesting to design the following objectives in the present study. All these studies pave way to develop transgenic crop plants resistant to *H. armigera* and *A. janata* in future.

2.3. Objectives of the present study

1. Identification and separation of Bowman-Birk and Kunitz inhibitors from trypsin specific PIs (RsPI) purified from *R. sublobata* seeds: Evaluation of their biochemical properties and activity against gut trypsin-like proteases of *A. janata* and *H. armigera*.
2. A comparative study on the growth retardation potential and biocidal activity of protease inhibitors from *C. cajan* wild relative (*R. sublobata*) and cultivar (BDN1) on castor semilooper *A. janata*.
3. Molecular cloning of *RsBBI* and *RsKI* genes: overexpression in *Escherichia coli* and functional characterization of recombinant RsBBI1 for insecticidal activity against *A. janata*.

2.4. Approach of the study

PIs are produced by all life forms and are ubiquitous in plant kingdom. Many of the plant PIs function as regulators of both abiotic and biotic stress in plants. Though both these aspects of protection rendered by plant PIs were well utilized by many research groups (Mosolov and Valueva, 2011; Rehman et al., 2017; Srinivasan et al., 2009; Tiwari et al., 2015), the biotic

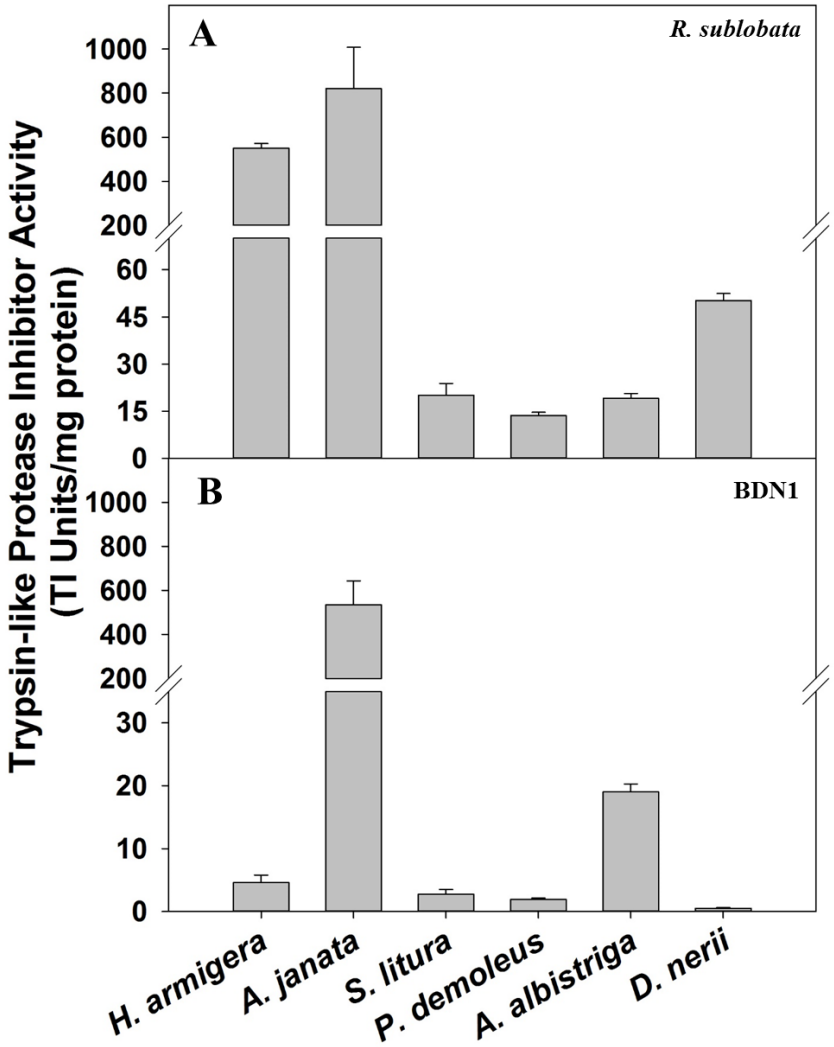


Fig. 2.4. *In vitro* inhibition of gut trypsin-like protease of *A. janata*, *S. litura*, *P. demoleus*, *A. albistriga* and *D. nerii* by the crude PI extracts from the seeds of (A) *R. sublobata* and (B) red gram cultivar variety BDN1.

stress resistance has a greater significance (Chen et al., 2014; Ussuf et al., 2001). PIs act as pseudo-substrates with low dissociation constant and function by binding to the active site of different class of proteases present in the insect gut and thereby inhibit the process of digestion.

Serine proteases such as trypsin and chymotrypsin cleave the protein at the C-terminal end of Lys/Arg and Phe/Tyr/Trp, respectively. Therefore, the protease activity is quantified using the artificial substrates such as BAPNA (Erlanger et al., 1961) for trypsin and GLUPHEPA (Mueller and Weder, 1989) for chymotrypsin. Both the substrates yield *p*-nitroanilide which has absorbance at 410 nm when cleaved by respective proteases. Further, the activity of PIs present in the sample is quantified by following the decrease in absorbance at 410 nm. The insect gut proteases were obtained by homogenizing the midgut from fifth instar larvae of different insects. The PIs were purified from the seed crude PI extract by successive anion exchange, trypsin-affinity, and gel filtration chromatography by AktaPrime plus. The mass of purified protein was obtained using matrix assisted laser desorption ionization time of flight (MALDI-TOF). The identity of protein present in *R. sublobata* was determined by Edman degradation after two dimensional electrophoresis (2-DE). The inhibitor activity of the PIs was visualized on gelatin incorporated SDS-PAGE. Since gelatin is a general substrate for most of the proteases, the undigested PIs were visualized as blue bands on staining with CBB.

Both BBIs and Kunitz inhibitors identified in the present study were separated based on their intrinsic differential solubility in Trichloroacetic acid (TCA) and sodium acetate buffer (pH 4.0). PIs are generally present in the plant seeds as multiple isoinhibitors and each isoinhibitor is known to be expressed in different physiological conditions (Godbole et al., 1994a; Kumar et al., 2002) or abiotic stress conditions (Harsulkar et al., 1999). Therefore, the isoinhibitors of both BBIs and Kunitz inhibitors were visualized on 2-DE. The molecular mass of individual PIs was analyzed by MALDI-TOF and the N-terminal sequence of each of PIs was identified by MALDI in source decay (MALDI-ISD). Being an enzyme inhibitor, both PIs

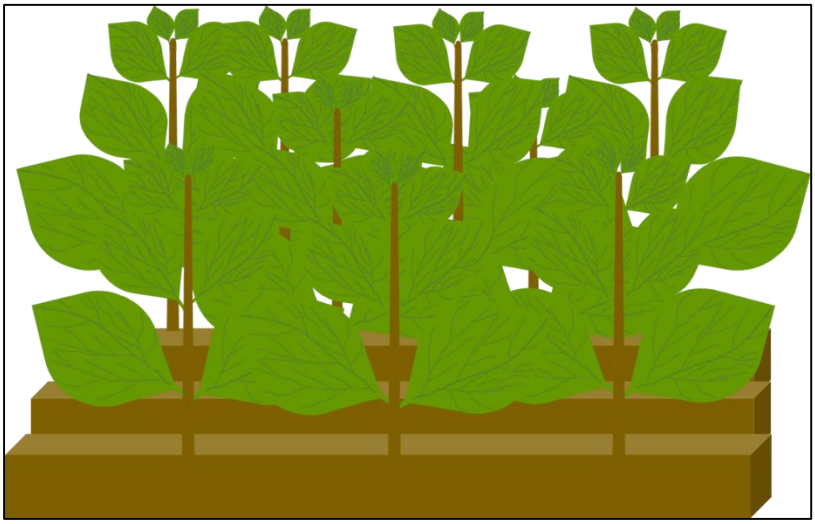
were analyzed for their inhibition kinetics against trypsin and chymotrypsin. In general, the PIs are known to be structurally stabilized by intra- and inter-molecular disulphide bridges, and non-covalent interactions. Hence, both the PIs were examined for their secondary structural stability at far UV-region under different temperature and pH conditions using CD spectroscopy. Further, the two purified PIs were examined for their *in vitro* insecticidal activity against the gut proteases of *H. armigera* and *A. janata*.

Among the two PIs separated, the concentration of BBI is more (~80%) in the overall PI pool and it inhibited the activity of gut protease of *A. janata* with an IC_{50} at nano gram level which is 83 times more than that of the Kunitz inhibitor. Hence, to further evaluate the insecticidal potential of *R. sublobata* BBIs, insect bioassays was performed by no choice feeding method using neonatal first instar and actively feeding third instar stage larvae of *A. janata.* BDN1PI purified from the red gram cultivar seeds of BDN1 was used to make a comparative study.

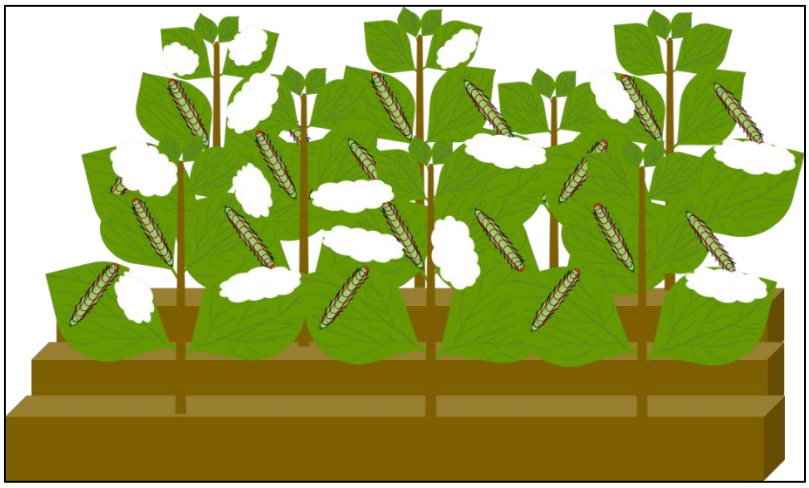
To utilize the defense potential of the wild species of *R. sublobata*, the complete gene sequence of both *RsBBI* and *RsKI* was determined by rapid amplification of cDNA ends (RACE). To further substantiate the insecticidal potential of the sequenced *RsBBI*, the RsBBI was expressed in *E. coli* Shuffle T7 express cells using pET23a plasmid. The expressed recombinant protein (rRsBBI1) was purified by trypsin affinity chromatography. The pure rRsBBI1 was evaluated for its biochemical properties and growth retardation potential of *A. janata*.

Chapter 3

Identification and separation of Bowman-Birk and Kunitz inhibitors from trypsin specific PIs (RsPI) purified from *R. sublobata* seeds: Evaluation of their biochemical properties and activity against gut trypsin-like proteases of *A. janata* and *H. armigera*.



Normal plant field



Insect infested plant field

Identification and separation of Bowman-Birk and Kunitz inhibitors from trypsin specific PIs (RsPI) purified from *R. sublobata* seeds: Evaluation of their biochemical properties and activity against gut trypsin-like proteases of *A. janata* and *H. armigera*.

3.1. Introduction

Cajanus cajan is an important staple food of India and many developing countries. It is grown widely in the semi-arid tropics and subtropics. It is preferred by marginalized farmers for its ability to grow in harsh abiotic and biotic stress conditions. *C. cajan* grains are rich source of protein with cheaper price in developing countries and *H. armigera* is the major pest on this crop plant. Since *H. armigera* is resistant to insecticides, IPM practices (Fig. 1.4C) are being followed to control them (Sharma et al., 2015). However, *C. cajan* is also known to contain many insecticidal defence proteins and PIs are major among them (Sandhya et al., 2017). Both Kunitz inhibitors and BBIs which belong to serine PIs are identified in the seeds of *C. cajan* (Godbole et al., 1994b; Norioka et al., 1988). Further, Norioka et al. (1988) suggested that the BBIs would be retained while Kunitz inhibitors might be lost during the course of evolution. However, the recently obtained draft genome sequencing results of *C. cajan* (variety-Asha) revealed the presence of many Kunitz inhibitors along with BBIs in their genome (Varshney et al., 2012).

The BBIs which are purified from the dry seeds of *C. cajan* cultivar variety (ICP 14770) exhibited significant inhibitory effect on the gut proteases of *A. janata* as compared to *H. armigera* (Prasad et al., 2010a). On the other hand, the seeds from many wild accessions of *C. cajan* were screened for their *in vitro* PI activity against *H. armigera* gut proteases. Among the different accessions tested, *C. crassus*, *C. lanceolatus*, *C. acutifolius*, *C. platycarpus*, *C. albicans*, *Flemingia bracteata*, *Rhynchosia rothii*, *R. sublobata*, and *R. bracteata* exhibited more than or equal to 80% inhibition of *H. armigera* gut trypsin-like protease activity (Chougule et al., 2003; Parde et al., 2012). In *C. platycarpus*, a wild relative from tertiary gene

pool of *C. cajan* which exhibited potent inhibitory activity (IC_{50} = 480 ng) against *H. armigera* gut proteases contained Kunitz/miraculin like PIs in its seeds (Swathi et al., 2016). However, the preliminary experiments performed with *R. sublobata*, a wild accession of *C. cajan* exhibited potent inhibition against the gut trypsin-like proteases of both *H. armigera* and *A. janata* (Fig. 2.3). Taken together these studies, it was hypothesized that *R. sublobata* might possess one or more PIs which may act independently or synergistically to inhibit the gut trypsin-like proteases from *H. armigera* and *A. janata*. So far, BBIs are purified mainly from the dry seeds of *C. cajan* (ICP 14770; ICP 7118; Prasad et al., 2010b; Swathi et al., 2014) whereas Kunitz inhibitors are purified from the overnight soaked seeds (TAT-10; PUSA 33; Godbole et al., 1994; Haq and Khan, 2003). Soaking may trigger the germination process which in turn induce the expression of new PIs. Further, the wild relatives of *C. cajan* exhibited more number of isoinhibitor bands in native PAGE than their cultivars (Chougule et al., 2003; Parde et al., 2012; Prasad et al., 2009).

Considering these aspects, the first part of this study focused on purification of trypsin specific PIs (RsPI) from *R. sublobata* seeds. In the second part, the Bowman-Birk inhibitors (RsBBI) and Kunitz inhibitors (RsKI) identified in RsPI were separated and characterized their biochemical properties and insecticidal potential against trypsin-like gut proteases of *A. janata* and *H. armigera*.

3.2. Materials and methods

3.2.1. Materials

R. sublobata (Schumach.) Meikle (ICP 15868) seeds were obtained from International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, India. *H. armigera* eggs were procured from National Bureau of Agricultural Insect Resources (NBAIR), Bengaluru, India. *A. janata* larvae were collected from the surrounding castor field maintained

in University of Hyderabad. The insects were maintained in insect culture room at 26 ± 1 ^{o}C, RH of 65 ± 5% and 14:10 h light-dark photoperiod.

3.2.2. Purification of seed PIs (RsPI) from *R. sublobata*

The crude PIs were extracted from mature dry seed powder in 50 mM Tris-HCl, pH 8.0 with 1% PVP (1:6 w/v) by mild stirring for overnight at 4 ^{o}C as described in Prasad et al. (2010c). The clear supernatant obtained after centrifugation (twice) at 10,000 g for 20 min at 4 ^{o}C was subjected to 20-60% $(NH_4)_2SO_4$ fractionation. The obtained pellet was dissolved in 50 mM Tris-HCl pH 8.0 and dialyzed against the same buffer. The fractionated protein was applied onto DEAE-Sepharose (XK 26/20, 14 cm) column pre-equilibrated with 50 mM Tris-HCl pH 8.0. The bound protein was eluted with a linear gradient of 0.5-1.5 M NaCl contained in the same buffer. The eluted peak fractions (1 mL) were examined for trypsin inhibitor (TI) activity. The collected active protein peak was dialyzed against 50 mM Tris-HCl, pH 8.0 and further applied onto a trypsin-Sepharose 4B affinity column (XK 16/20, 3cm). 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) were neutralized with 50 mM Tris-HCl, pH 8.0, pooled up and concentrated using a freeze dryer. The concentrated affinity fractions was subjected to size exclusion chromatography by loading on to a Sephadex G-50 fine column (XK 16/100, 85 cm). The column was equilibrated and eluted with 50 mM Tris-HCl, pH 8.0. The eluted peak fractions (1mL) containing TI activity was concentrated using 3.0 kDa cut-off membrane filter. Thus, the purified pool of trypsin specific PIs was labelled as "RsPI" and stored at -20 ^{o}C for further use. Protein estimation was performed using the Bicinchoninic acid method.

3.2.3. Tricine SDS-PAGE, two-dimensional gel electrophoresis (2-DE) and N-terminal sequencing of RsPI

The molecular mass of trypsin-specific PIs present in RsPI was determined by Tricine SDS-PAGE as described by Schagger (2006) followed by silver nitrate staining. The 2-DE of RsPI was performed as described in the manufacturer's instructions. 100 µg of RsPI was dissolved in 200 µL of sample solubilization buffer (7M urea, 2M thiourea, 4% CHAPS, 1% IPG buffer and 40 mM DTT) and applied onto an IPG strip (11 cm, pH 4-7) by passive rehydration overnight. IEF was performed at 20 ºC 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 14-18% Tricine SDS-PAGE gel. After 2-DE, the gel was stained with colloidal Coomassie and spots were visualized.

For N-terminal sequencing, the isoinhibitor spots separated in 2-DE were transferred onto a sequencing grade PVDF (0.22 µm) membrane using 10 mM CAPS buffer, pH 11.0 at 30 V and 4 ºC 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 19.2 kDa (Spot 1); pI 5.0 and mass 9.2 kDa (Spot 2) and pI 6.0 and mass 19.2 kDa (Spot 3) were cut from PVDF membrane, destained in 50 % methanol and submitted for N-terminal sequencing. The N-terminal sequences were aligned using ClustalW2 with the related protein sequences from UniProt database to identify percent similarity.

3.2.4. Separation of RsBBI and RsKI

RsPI was purified as indicated in section 3.2.2. However, anion exchanger column was excluded from purification protocol of RsPI. The BBI and Kunitz inhibitors were separated

from RsPI based on their differential solubility in TCA and aqueous acetate buffer (Fig. 3.4A) by modifying the protocols of Bowman (1946); Macedo and Xavier-Filho (1992), 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 so as to attain its concentration at 2.5% in the reaction mixture (RM1). The RM1 was heated at 70 ^{o}C for 10 min and centrifuged at 11,000 g for 5 min. The pH of the supernatant (S1) from RM1 was adjusted to pH 8.0 with 400 mM Tris-HCl. To this, 4x volumes of cold acetone was added and kept at -20 ^{o}C for 1 hour. The protein obtained in acetone precipitate was air dried and dissolved in 50 mM Tris-HCl, pH 8.0 and labeled as RsBBI, and stored at -20 ^{o}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 ^{o}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 100 mM Tris-HCl, pH 8.0. The S2 was acetone precipitated as described above. The acetone precipitate was air dried and dissolved in 50 mM Tris-HCl, pH 8.0 and labeled as RsKI, and stored at -20 ^{o}C.

3.2.5. 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 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 ^{o}C, followed by incubation for 2 hrs at 37 ^{o}C in the respective proteases dissolved in 0.1 M Tris-HCl, pH 8.0 (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 staining.

3.2.6. MALDI-TOF & MALDI-ISD

For MALDI-TOF, the RsBBI and RsKI were mixed independently with equal volumes of α-cyano-4-hydroxy-cinnamic acid (CHCA) matrix. 2 µl of the reaction mixture 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. For MALDI-ISD, the sample 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 3.0, and the annotated sequences were searched in pBLAST to confirm the identity of the PIs.

3.2.7. Insect midgut extraction

Midguts were excised from 5th instar larvae of *H. armigera* and *A. janata*, and the gut digestive enzymes were extracted as described in Swathi et al. (2014). The larvae narcotized on the ice were dissected dorsally in saline (0.15 M NaCl). The guts 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 ^{o}C. The supernatant was collected from respective homogenates, and labeled as *H. armigera* midgut trypsin-like proteases (HaGPs) and *A. janata* midgut trypsin-like proteases (AjGPs) and stored at -20 ^{o}C for further use.

3.2.8. Assay of proteases and protease inhibitors (RsPI, RsBBI & RsKI)

The assay of proteases and PIs was performed as described in Prasad et al. (2010c). The protease activity was determined by monitoring the increase in absorbance at 410 nm due to the formation of *p*-nitroanilides on hydrolysis of BAPNA, catalyzed by trypsin (10 µg) or an appropriate aliquot of HaGPs/AjGPs which yield 1.0 OD or GLUPHEPA, catalyzed by chymotrypsin (80 µg). The assay buffer 50 mM Tris-HCl containing 20 mM $CaCl_2$ at pH 8.2

or pH 7.8 was used for the analysis of trypsin/AjGPs and chymotrypsin, respectively. In contrast, 50 mM Glycine-NaOH, pH 10.5 was used for the analysis of HaGPs activtiy. To determine the inhibitory activity of RsPI, RsBBI or RsKI, the assay mixture was incubated with an appropriate quantity of the respective PIs at 37 ^{o}C for 15 min before the residual protease activity was determined. Subsequently after incubation with 1 mM BAPNA/GLUPHEPA for 45 min at 37 ^{o}C, the reaction was terminated by adding 30% acetic acid to bring its final concentration to 3%. One trypsin inhibitory (TI), *H. armigera* midgut trypsin-like protease inhibitory (HaGPI), *A. janata* midgut trypsin-like protease inhibitory (AjGPI) or chymotrypsin inhibitory (CI) unit is defined as the amount of RsPI, RsBBI or RsKI required to inhibit 50% hydrolysis of BAPNA or GLUPHEPA, respectively by trypsin/HaGPs/AjGPs or chymotrypsin enzymes.

3.2.9. Inhibition kinetics and titration studies

Inhibition constant (*Ki*) of RsBBI against both trypsin and chymotrypsin was determined after incubating it at different concentrations (40, 70 and 90 nM; 270, 540, 810 nM) against a fixed concentration (1 µM) of trypsin or chymotrypsin for 15 min at 37 ^{o}C. This was followed by addition of different concentrations (0.125, 0.25, 0.375, 0.5, 0.625, 0.75 mM) of substrates, BAPNA for trypsin and GLUPHEPA for chymotrypsin and incubated at 37 ^{o}C for 45 min. Similarly, the inhibition constant (*Ki*) for RsKI was determined by incubating it at different concentrations (30, 60 and 80 nM) against 1 µM trypsin. The *Ki* values were determined using Sigma Plot 12.5 (Systat Software Inc. San Jose, California).

Further, the residual trypsin and chymotrypsin activities at different molar ratios (0.0-2.0) of RsBBI or 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).

3.2.10. 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 ^{o}C). The residual TI and CI activity was measured after cooling the samples to room temperature as described in section 3.2.8. The sample incubated at 37 ^{o}C is used as a control. Similarly, the stability of RsBBI and RsKI against pH was determined after incubating them at different pH from 2.0 to 12.0 at 37 ^{o}C for 1 hour 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 of RsBBI and RsKI against DTT was determined by incubating it at different concentrations (0.00 to 5.0 mM) with RsBBI or (0.0 to 200 mM) with RsKI at 56 ^{o}C for 45 min followed by incubation with iodoacetamide at double the concentration of DTT in the dark for 1 hour. The residual TI and CI activities of RsBBI and RsKI was measured as described in section 3.2.8.

3.2.11. 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 for RsKI and RsBBI were recorded at a final concentration of 0.2 µg/µl dissolved individually in 10 mM Tris-HCl (pH 8.0). The secondary structural elements were estimated using Spectra Manager 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 ^{o}C using Peltier thermostat.

The temperature-induced unfolding and refolding of RsBBI and RsKI were measured by increasing the temperature from 25 to 90 ^{o}C and cooling from 90 to 25 ^{o}C at the far-UV

region. The thermal transient unfolding midpoint (Tm) 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 ^{o}C per minute was maintained. The effect of pH on the secondary structural elements was determined by incubating both RsKI and RsBBI at 37 ^{o}C for 1 hour in respective buffers as described in Section 3.2.10 at 10 mM concentrations and cooled down to room temperature and the CD spectra were recorded.

3.2.12. 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).

3.3. Results

3.3.1. Purification of Trypsin-specific PIs "RsPI" by FPLC

The 20-60% $(NH_4)_2SO_4$ fractionated crude PI extract is resolved into two peaks upon loading on a weak anion exchanger DEAE-Sepharose column and eluting with a linear gradient of NaCl (Fig. 3.1A). The peak 1 fractions with TI activity was passed through a Trypsin-affinity column and the bound protein with TI activity (peak 2) was eluted with 0.01 N HCl (Fig. 3.1B). High MW contaminants retained in the trypsin affinity fractions were removed by further loading onto a Sephadex G-50 fine gel filtration chromatography column. The peak 2 fractions which showed significant TI activity was referred to as "RsPI" (Fig. 3.1C) and it showed two major bands with molecular mass of approximately 10 and 20 kDa in Tricine SDS-PAGE (Fig. 3.1D). RsPI was purified by 10.8 folds with a yield recovery of 29% (Table. 3.1).

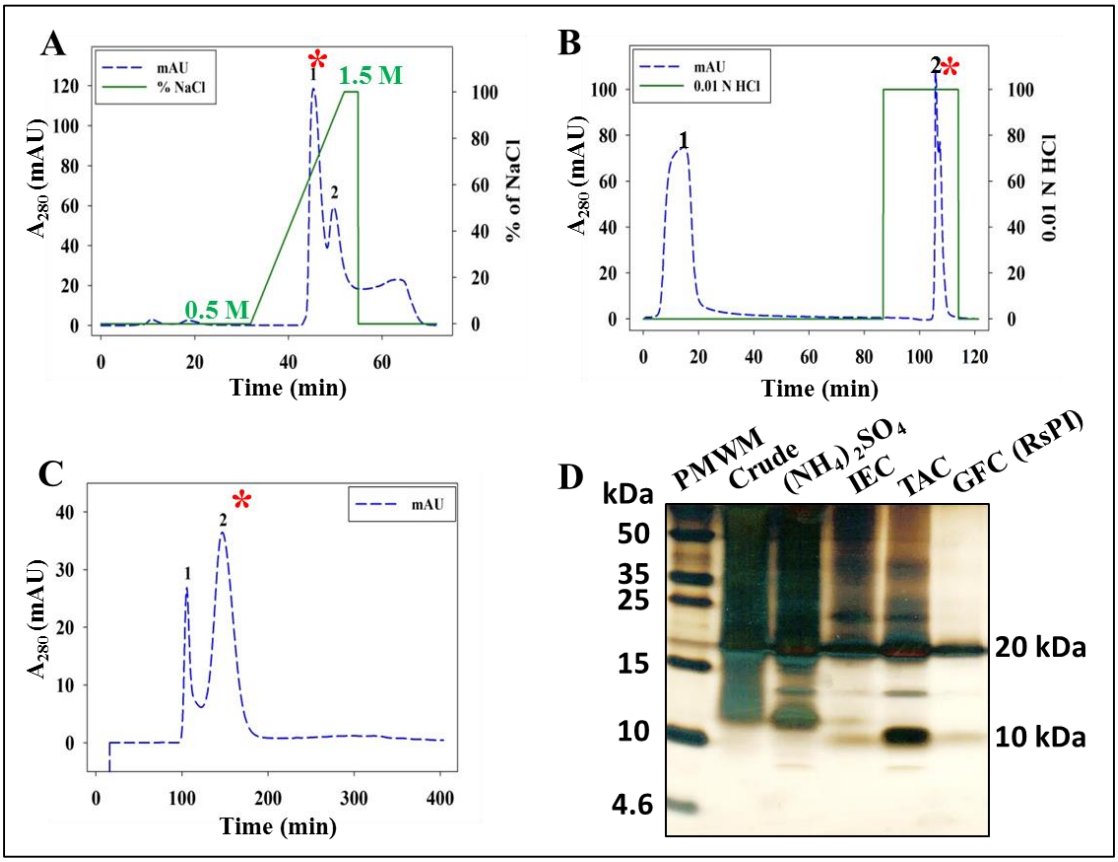


Fig. 3.1. FPLC purification of trypsin-specific PIs "RsPI" from *R. sublobata* seeds. Elution profiles of (A) ion-exchange (DEAE-Sepharose) column loaded with 20-60% $(NH_4)_2SO_4$ fraction of crude PI extract. The bound proteins were eluted with a linear gradient (0-100%) of 0.5-1.5 M NaCl; (B) trypsin-affinity column loaded with active fraction pool from the ion-exchange column; (C) gel filtration column loaded with active fraction pool from the affinity column. The protein peaks active against trypsin were marked with an asterisk (*); (D) Tricine SDS-PAGE (18%) showing different fractions of purification protocol under non-reducing conditions. Lanes 1 – 6 are loaded with PMW marker, crude PI extract (10 µg), $(NH_4)_2SO_4$ fraction (10 µg), active fraction pool from ion exchange column (IEC-5 µg), trypsin affinity column (TAC-10 µg), RsPI from gel filtration column (GFC-5 µg). The gel was stained with silver nitrate.

Table 3.1. Purification of trypsin-specific PIs "RsPI" from *R. sublobata* mature seeds using FPLC.

Purification step	Total protein (mg)	Total activity (TI units) [1]	Yield recovery (%)	Specific activity (TI units/mg protein) [2]	Purification fold
Crude PI extract	148.32	8425.80	100	56.80	1.00
Ammonium sulfate fractionation (20-60%)	49.38	6924.95	82.19	140.22	2.47
Ion exchange chromatography	31.04	5352.97	63.52	172.45	3.04
Affinity chromatography	6.095	3476.83	41.26	570.44	10.04
Gel filtration chromatography	3.99	2453.83	29.12	614.99	10.82

[1] One TI unit is defined as the amount of PI required to inhibit 50% of BAPNA hydrolysis.

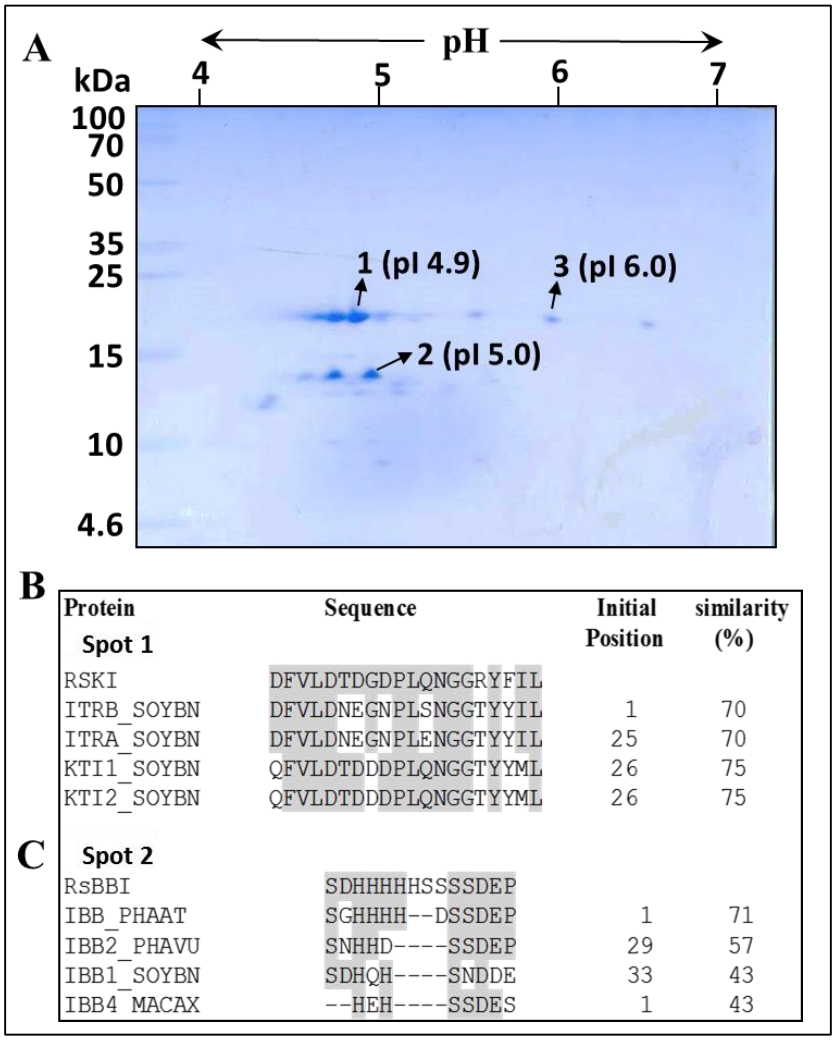
[2] Specific activity is the number of TI units per mg protein.

3.3.2. IEF of RsPI and identification of RsBBI and RsKI

The RsPI is resolved into several isoinhibitors on IEF followed by 2-DE. The isoinhibitor spots in the 10 kDa region were distributed between pI 4.3 to pI 5.2, while the isoinhibitors spots in the 20 kDa region were distributed between pI 4.4 to pI 6.6 (Fig. 3.2A). The N-terminal sequencing of the isoinhibitor with pI 4.9 and molecular mass 19.2 kDa (spot 1, Figs. 3.2A,B) revealed the following 20 amino acid sequence "DFVLDTDGDPLQNGGRY FIL". It showed 70% similarity to Trypsin inhibitors A (ITRA_SOYBN) & B (ITRB_SOYBN) and 75% similarity to Kunitz trypsin inhibitors 1 (KTI1_SOYBN) & 2 (KTI2_SOYBN) of soybean (Fig. 3.2B). In contrast, N-terminal sequencing of the isoinhibitor with pI 5.0 and molecular mass 9.2 kDa (spot 2, Figs. 3.2A,C) revealed the following amino acid sequence "SDHHHHHSSSSDEP" and it showed 43 to 71% similarity to the N-terminus of BBIs from *Macrotyloma axillare*, (IBB4_MACAX), soybean (IBB1_SOYBN), *Phaseolus vulgaris* (IBB2_PHAVU) and *P. acutifolius* (IBB_PHAAT), respectively (Fig. 3.2C). Also, N-terminal sequencing performed for spot 3 of pI 6.0 and MW 19.2 kDa (Fig. 3.2A) revealed that the sequence of first five amino acids "DFVLD" were similar to that obtained for spot 1. Together, IEF and N-terminal sequencing of RsPI demonstrated that both BBI and Kunitz family PIs were present in the seeds of *R. sublobata*, and possibly existed as several isoinhibitors in the 10 kDa and 20 kDa region of Tricine SDS-PAGE and collectively referred to as 'RsBBI' and 'RsKI', respectively (Figs. 3.2A-C). Further, the disappearance of all isoinhibitor spots in the 10 kDa region after tryptic digestion indicated that they all might belong to BBI superfamily (Fig. 3.3).

3.3.3. Separation of RsBBI and RsKI

RsBBI and RsKI were separated from RsPI based on their differential solubility properties in TCA and sodium acetate buffer (Fig. 3.4A). RsBBI was separated from RsPI by



B

Protein	Sequence	Initial Position	similarity (%)
Spot 1			
RSKI	DFVLDTDGDPLQNGGRYFIL		
ITRB_SOYBN	DFVLDNEGNPLSNGGTYYIL	1	70
ITRA_SOYBN	DFVLDNEGNPLENGGTYYIL	25	70
KTI1_SOYBN	QFVLDTDDDPLQNGGTYYML	26	75
KTI2_SOYBN	QFVLDTDDDPLQNGGTYYML	26	75

C

Spot 2			
RsBBI	SDHHHHHSSSSDEP		
IBB_PHAAT	SGHHHH--DSSDEP	1	71
IBB2_PHAVU	SNHHD----SSDEP	29	57
IBB1_SOYBN	SDHQH----SNDDE	33	43
IBB4_MACAX	--HEH----SSDES	1	43

Fig. 3.2. Identity of PIs present in RsPI. (A) 2-DE of RsPI after reduction with DTT and IDA treatment: 50 µg of protein was focused on pH 4-7, 11 cm IPG strip, and resolved on 18% Tricine SDS-PAGE under reducing conditions and the gel was stained with CBB; Clustal alignment of the N-terminal sequence obtained for (B) spot 1 and (C) spot 2 with the known inhibitor sequences from Uniprot database. The aligned sequences related to spot 1 (RsKI) included - ITRB-Trypsin inhibitor B, ITRA-trypsin inhibitor A, KTI1-Kunitz trypsin inhibitor 1 and KTI2-Kunitz trypsin inhibitor 2, all from soybean; and spot 2 (RsBBI) included - IBB_ PHAAT-BBI from *P. acutifolius*, IBB2_PHAVU-BBI from *P. vulgaris*; IBB1_SOYBN-BBI from soybean, and IBB4_MACAX- BBI from *M. acutifolius*. Identical amino acids are shaded in grey colour.

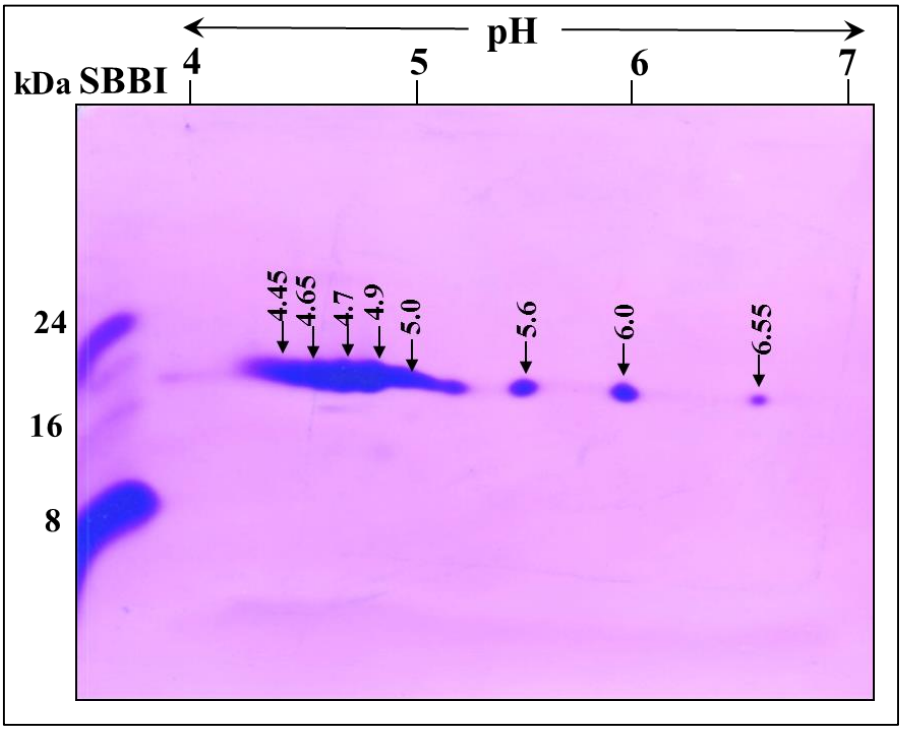


Fig. 3.3. Isoinhibitor spots of RsPI active against trypsin under reducing conditions. RsPI (50 µg) was subjected to IEF using pH 4-7, 11 cm strip. Electrophoresis in second dimension was performed in 18% gelatin SDS-PAGE. After electrophoresis, the gel was washed in Triton X-100 followed by incubation with trypsin enzyme as described in materials and methods section 3.2.5. The isoinhibitor spots active against trypsin were visualized after staining with CBB R-250. The gel shown here is the selective representation from three replicates of RsPI purified from three independent experiments. Soybean BBI (SBBI, 5 µg) was loaded as positive control.

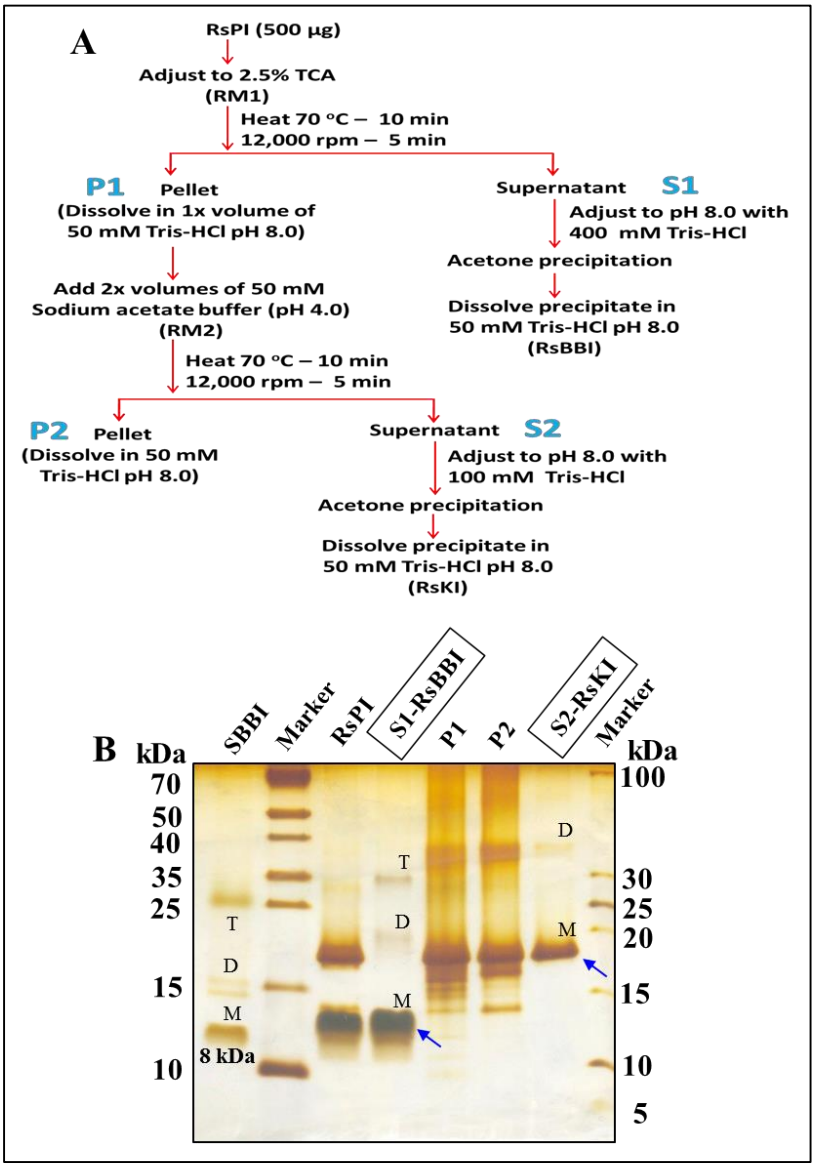


Fig. 3.4. Separation of RsBBI and RsKI from RsPI. (A) Flow diagram for separation of RsKI and RsBBI from RsPI. Further details are as described in section 3.2.4; (B) Purity of RsBBI and RsKI on Tricine SDS-PAGE (18%) under non-reducing condition. Lane 1 – soybean BBI (SBBI), 2 – PMW marker, 3 – RsPI, 4 – S1-RsBBI, 5 – pellet 'P1', 6 – pellet 'P2', 7 – S2-RsKI, 8 – PMW marker. 5 µg of protein was loaded in each lane. RsBBI existed as monomer (M), dimer (D) and trimer (T) while RsKI existed as monomer (M) and dimer (D). The gel is stained with silver nitrate.

extracting into 2.5% TCA at 70 ^{o}C (Fig. 3.4B). The supernatant 'S1' obtained after TCA extraction is enriched with RsBBI and subsequently subjected to acetone precipitation. The acetone pellet containing 'RsBBI' is solubilized in 50 mM Tris-HCl, pH 8.0. In solution, the RsBBI existed as monomer, dimer and trimer (Figs. 3.4B, 3.6A). RsKI is separated from RsPI by extracting the P1 pellet from TCA extraction into 50 mM Sodium acetate buffer, pH 4.0. The supernatant (S2) obtained after acetate extraction is enriched with RsKI and subsequently subjected to acetone precipitation. The RsKI recovered in acetone pellet is solubilized in 50 mM Tris-HCl, pH 8.0. In solution, RsKI existed as monomer and dimer (Figs. 3.4B, 3.6B). The purity of RsBBI and RsKI was visualized in 18% Tricine SDS-PAGE (Fig. 3.4B). This protocol resulted in a total yield of 4.19 mg of RsBBI and 1.11 mg of RsKI accounting to approximately 50- and 23-folds of purification, respectively, taken from 10 g of seeds. RsBBI is highly active against bovine trypsin enzyme (1730 TIU/mg protein) when compared with RsKI (807 TIU/mg protein) (Fig. 3.5A). Thus, a highly time-saving, simple and cost-effective scheme was formulated to separate both RsBBI and RsKI from RsPI within a time frame of 2 hrs. Further, in-gel activity visualization studies indicated that both RsBBI and RsKI were active against bovine pancreatic trypsin, AjGPs and HaGPs (Figs. 3.5B,D&E). However, RsBBI but not RsKI is active against chymotrypsin (Fig. 3.5C).

3.3.4. Confirmation of RsBBI and RsKI using mass spectrometry and 2-DE

MALDI-TOF MS analysis revealed that both RsBBI and RsKI existed as small oligomers (Figs. 3.6A,B). The appearance of (i) three peaks for RsBBI with molecular masses 9,216.4, 18,427.4 and 27,693 Da and (ii) two peaks for RsKI with molecular masses 19,412.6 and 38,811 Da corroborated well with the trimeric and dimeric nature of RsBBI and RsKI observed in SDS-PAGE (Fig. 3.4B). IEF of both RsBBI and RsKI followed by 2-DE analysis confirmed that they existed as isoinhibitors in the acidic range. A total of five isoinhibitors of RsBBI and eight isoinhibitors of RsKI were observed (Figs. 3.7A,B). The N-terminal

A

Purification step	Total protein (mg)	Total activity (TI units)	Yield recovery (%)	Specific activity (TI units/mg protein)	Purification fold
Crude PI extract	950.76	33140	100	34.86	1
Ammonium sulfate fractionation (20-60%)	614.8	32248	97.3	52.45	1.5
Affinity chromatography	46.49	13076	39.5	281.67	8.07
Gel filtration chromatography	8.94	9753	29.43	1056.67	30.31
RsBBI	4.19	7249	21.87	1730.07	49.63
RsKI	1.11	896	2.7	807.2	23.15

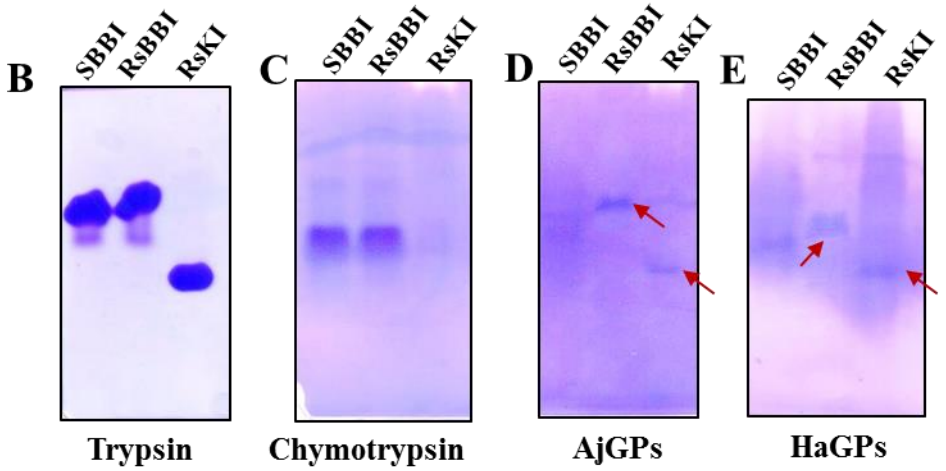


Fig. 3.5. Analysis of the purity and yield of RsBBI & RsKI and their in-gel activity. (A) Purification table indicating the yield, purification fold and specific activity of RsBBI and RsKI obtained from *R. sublobata* seeds; Visualization of in-gel activity of RsBBI and RsKI against (B) bovine trypsin; (C) bovine chymotrypsin; (C) AjGPs; and (D) HaGPs. Lane 1 – soybean BBI (SBBI - 5 µg), 2 – RsBBI (10 µg) and 3 – RsKI (10 µg) in gelatin SDS-PAGE (18%) under non-reducing conditions. After electrophoresis of RsBBI and RsKI, the gels were washed with Triton X-100 and incubated in their cognate protease solutions as described in materials and methods section 3.2.5. The inhibitor bands were visualized after staining with CBB R-250.

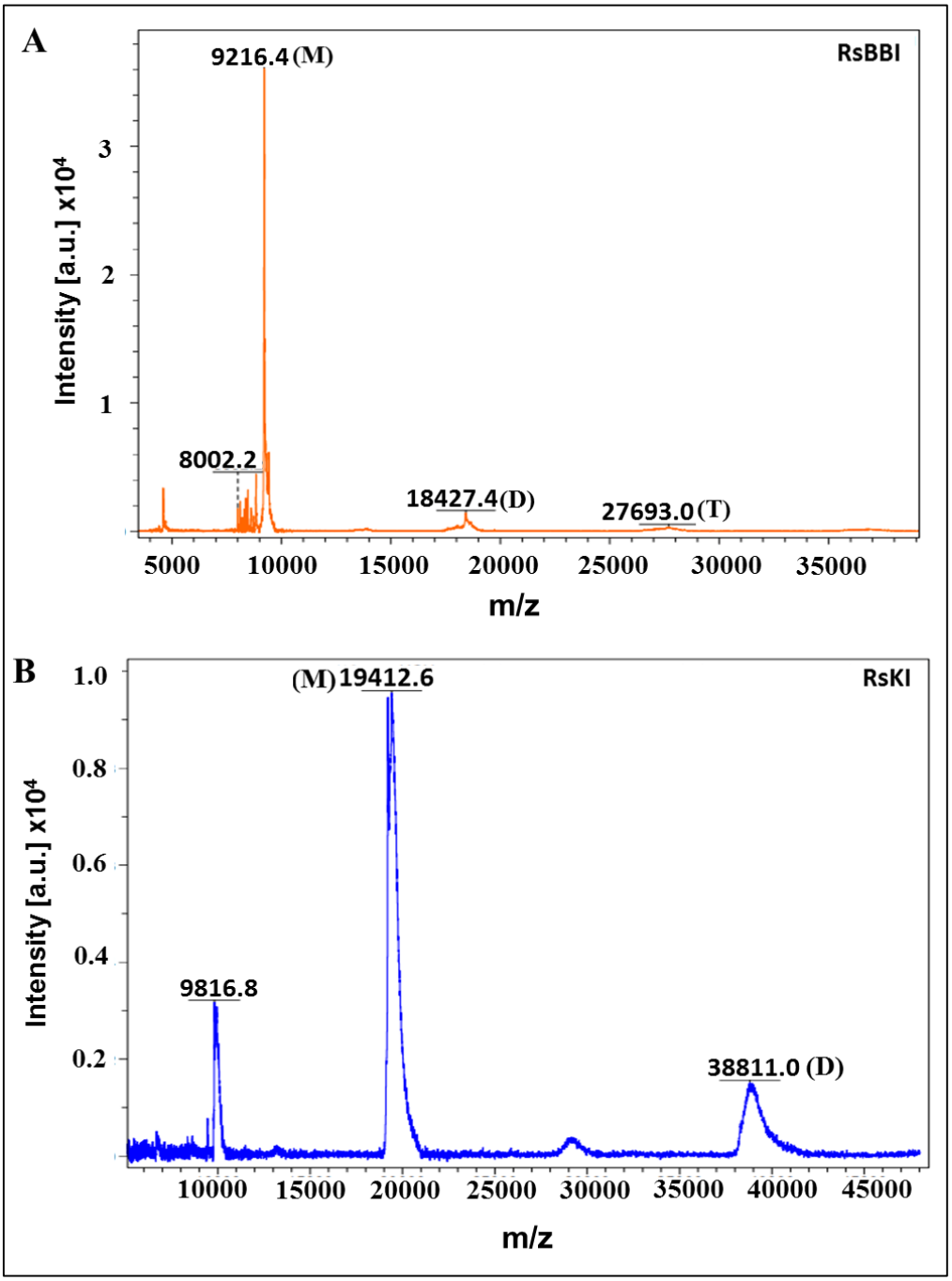


Fig. 3.6. MALDI-TOF mass spectrum of RsBBI and RsKI under non-reducing conditions. (A) 4000-40000 m/z representing the monomer (M), dimer (D) and trimer (T) of RsBBI and (B) 5000-50000 m/z representing the monomer (M) and dimer (D) of RsKI.

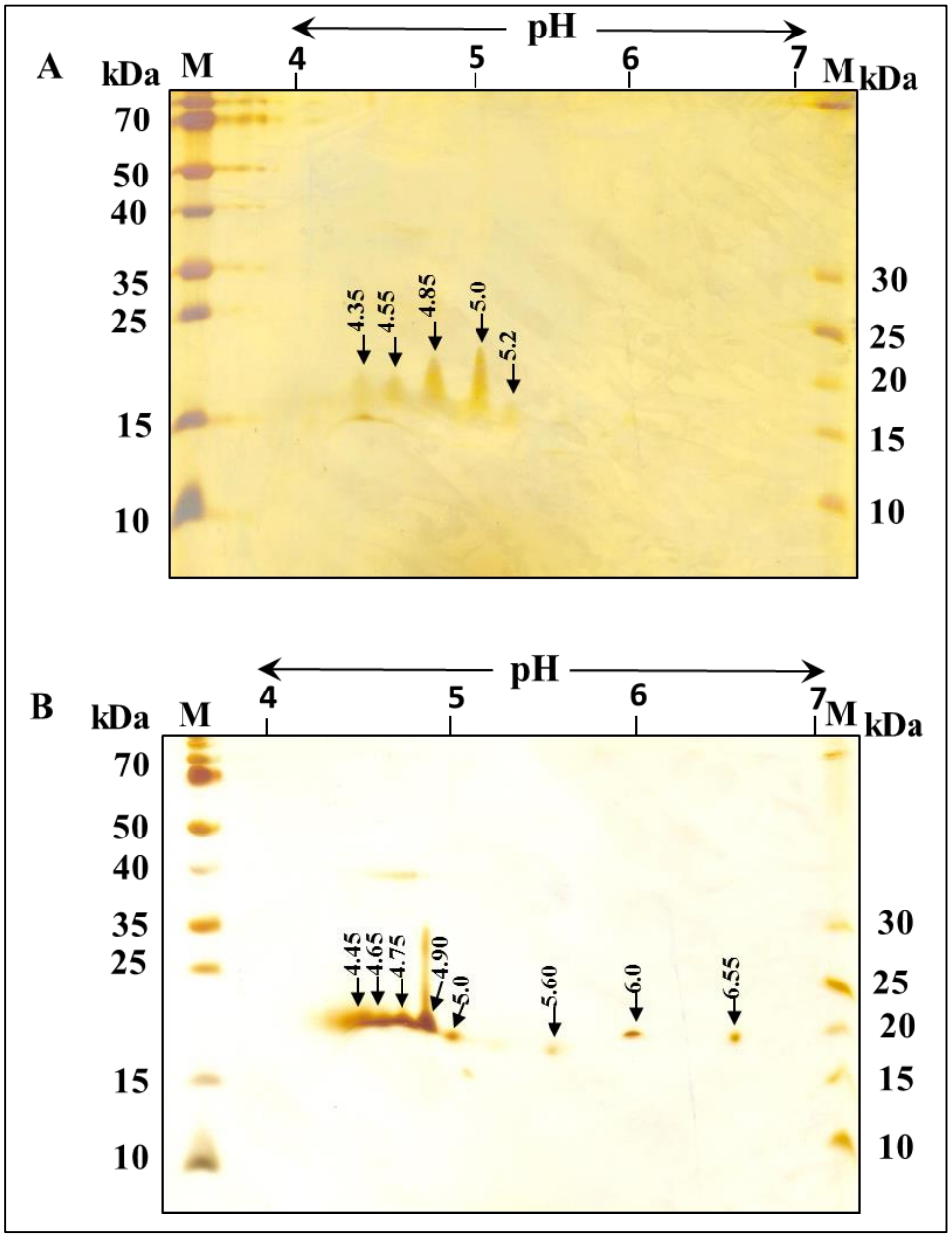


Fig. 3.7. Separation of isoinhibitors of RsBBI and RsKI in 2-DE. (A) RsBBI (25 µg) and (B) RsKI (25 µg) were separated on pH 4-7, 11 cm IPG strips and the second dimension was performed in 18% Tricine SDS-PAGE under reducing conditions. The molecular mass markers (5 µg) are loaded on either side of the strip in second dimension. The gels were stained with silver nitrate to visualize the corresponding isoinhibitor spots. The isoinhibitors spots with their relative pI values are indicated with arrows.

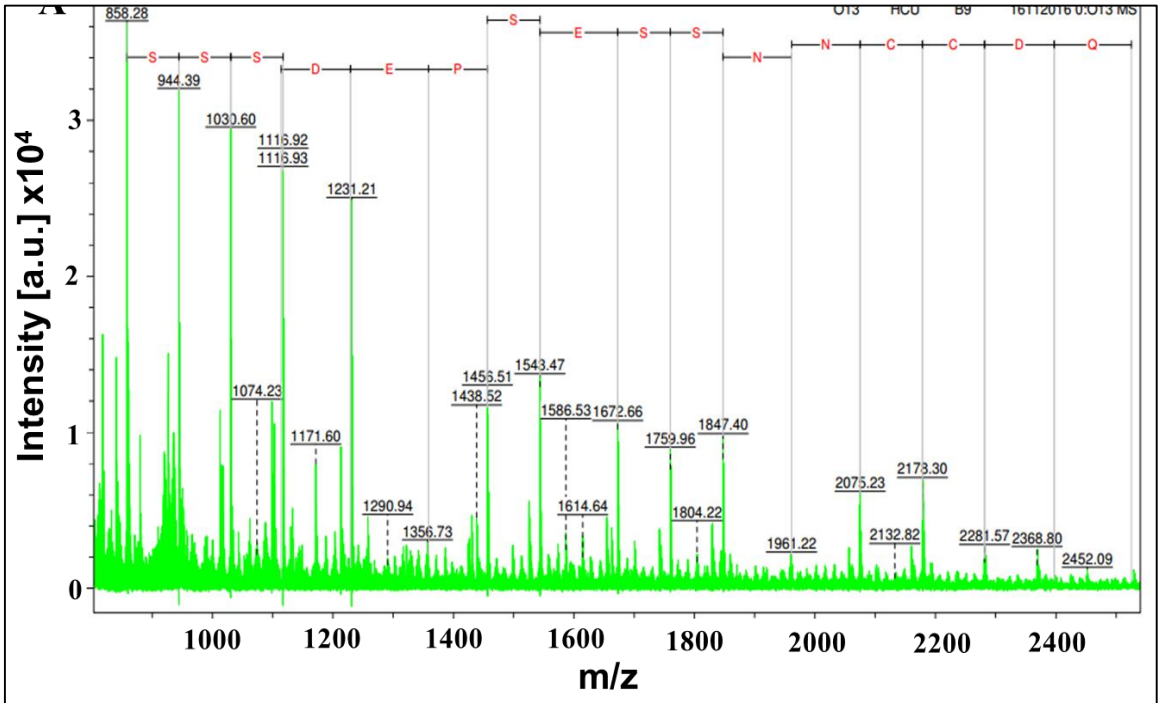
sequences of RsBBI and RsKI as identified by MALDI-ISD spectrum showed the following sequences: 'SSSDEPSESSNNCCDQ' (Fig. 3.8A) and 'I/LI/LNGGTYYVRGAI/LRGDGGG I/LE' (Fig. 3.9A), respectively. Further, BLAST analysis of N-terminal sequence of RsBBI showed a significant matching to BBIs from Phaseolus, Vigna, Horse gram and soybean (Fig. 3.8B) while N-terminus of RsKI showed significant matching to Kunitz inhibitors from soybean and tree legumes (Fig. 3.9B). Further, the sequences obtained through MALDI-ISD showed overlapping with the sequences obtained through Edman degradation (Figs. 3.2B,C).

3.3.5. *Ki* determination of RsBBI and RsKI

Ki determination by Lineweaver-Burk plot revealed that RsBBI inhibited the trypsin and chymotrypsin enzymes by non-competitive mode. The *Ki* of RsBBI against trypsin was more significant (128.5 ± 4.5 nM) than chymotrypsin (807.8 ± 23.7 nM) (Figs. 3.10A,B). Further, RsBBI showed binding with its cognate proteases trypsin and chymotrypsin in 1:2 and 1:1 molar ratios, respectively (Fig. 3.11A). However, RsKI inhibited trypsin alone (*Ki* = 172.0 ± 9.2 nM) in non-competitive mode at 1:2 molar ratio (Figs. 3.10C, 3.11B).

3.3.6. Stability of RsBBI and RsKI

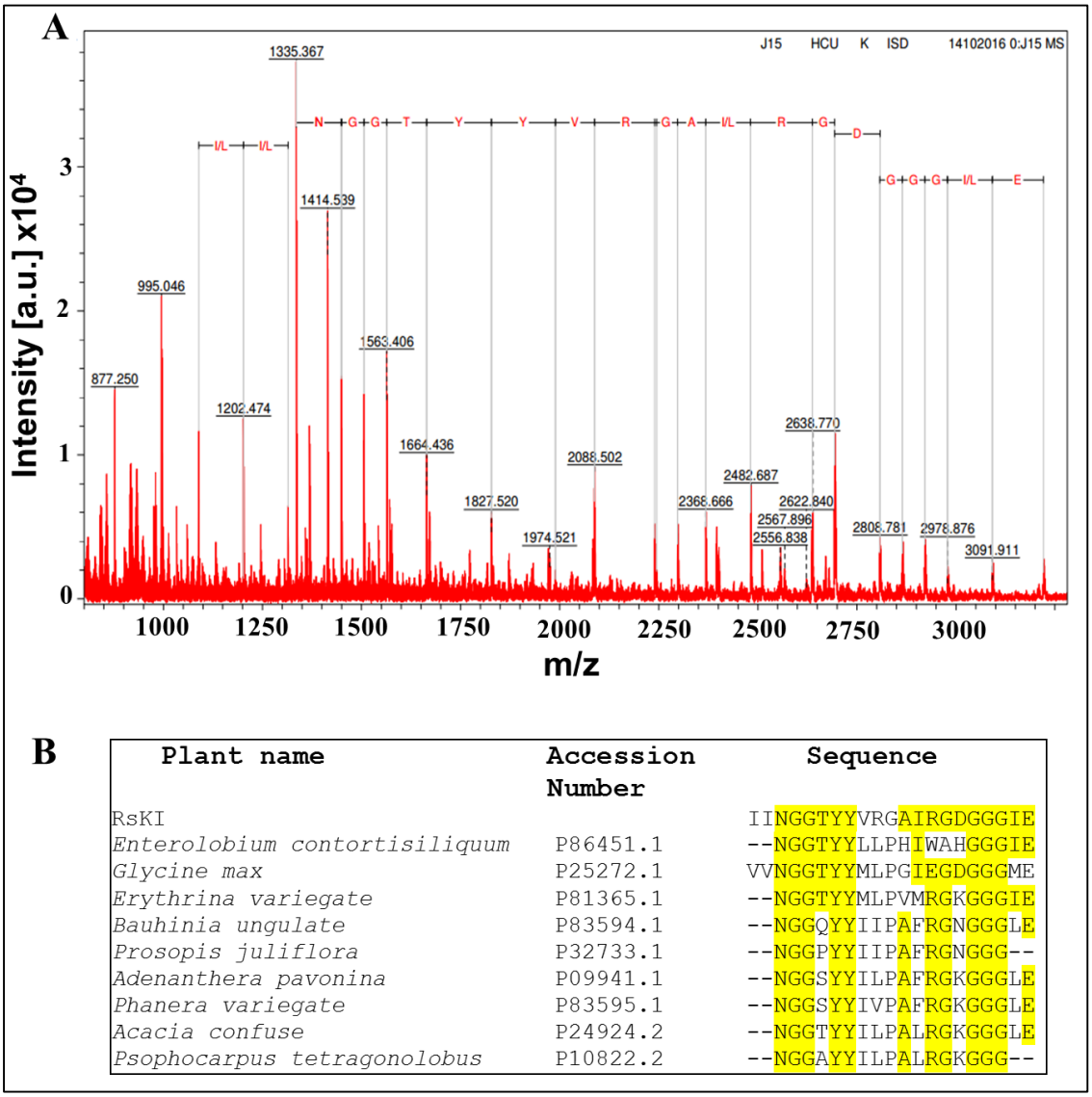
The inhibitory activity of RsBBI against trypsin and chymotrypsin was stable even when heated up to 100 ^{o}C (Fig. 3.12A). Similarly, the trypsin inhibitory activity (TI) and chymotrypsin inhibitory activity (CI) of RsBBI was stable at alkaline pH. However, at less acidic (pH 5.0) or neutral (pH 7.0), a 40% decrease in TI activity but not CI activity was observed (Fig. 3.12B). In contrast, RsBBI lost 100% of its TI and CI activities on reduction with 1.5 mM and 3 mM DTT, respectively (Fig. 3.12C). The stability of RsKI activity against temperature, pH and DTT was quite different from that of RsBBI. The TI activity of RsKI decreased when heated above 70 ^{o}C. For example, it lost >80% of its TI activity when incubated at 100 ^{o}C (Fig. 3.12D). But, there was a marginal loss of <10% in its TI activity at acidic/



B

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-

Fig. 3.8. Mass based N-terminal sequencing of RsBBI. (A) N-terminal MALDI-ISD spectrum of RsBBI and (B) ClustalW alignment of the MALDI-ISD obtained RsBBI sequence with other known BBIs from related plants. All the relevant protein sequences indicated with their accession numbers were downloaded from NCBI using the search criterion limited to "Non-redundant protein sequences" and Plants (Taxid: 3193). Identical amino acids are shaded in yellow colour.



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. 3.9. Mass based N-terminal sequencing of RsKI. (A) N-terminal MALDI-ISD spectrum of RsKI and (B) ClustalW alignment of the MALDI-ISD obtained RsKI sequence with the known Kunitz inhibitors from related plants. All the relevant protein sequences indicated with their accession numbers were downloaded from NCBI using the search criterion limited to "Non-redundant protein sequences" and Plants (Taxid: 3193). Identical amino acids are shaded in yellow colour.

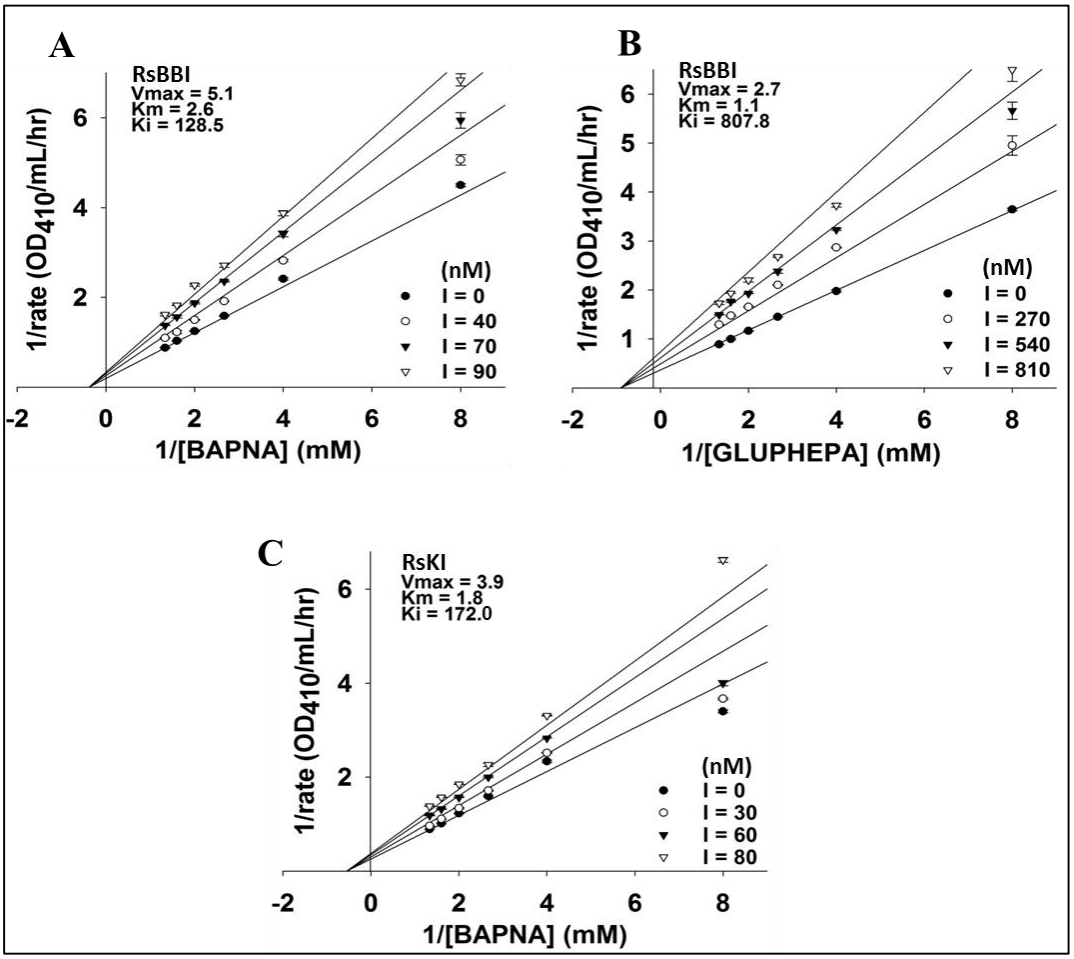


Fig. 3.10. Enzyme inhibition kinetics of RsBBI and RsKI. Lineweaver-Burk plot depicting non-competitive mode of inhibition of RsBBI against (A) trypsin and (B) chymotrypsin and RsKI against (C) trypsin enzyme. Inhibition kinetics was measured at different concentrations of the substrate BAPNA/GLUPHEPA (0.125, 0.250, 0.375, 0.500, 0.625 and 0.750 mM) against constant (1 µM) concentration of trypsin/chymotrypsin enzyme. The values are the mean ± SE of at least three independent experiments each with three replicates.

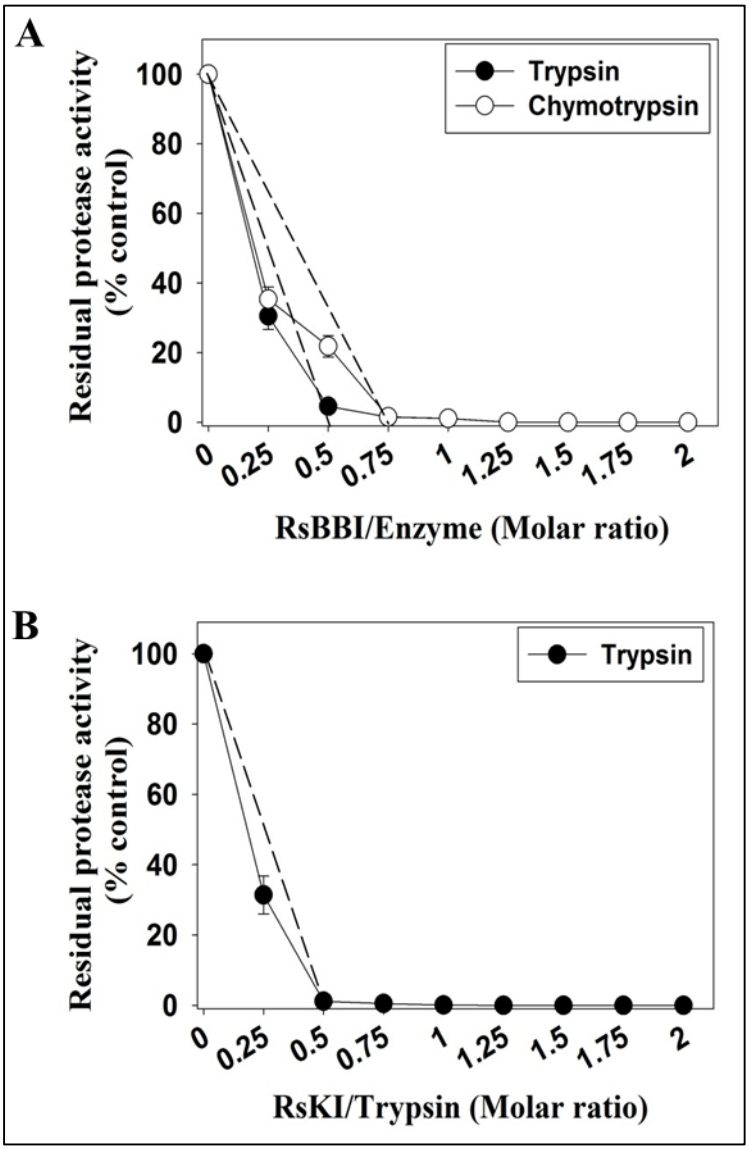


Fig. 3.11. Titration curves of trypsin and chymotrypsin inhibition by RsBBI and RsKI. Increasing concentrations of (A) RsBBI and (B) RsKI was added to a fixed 1 µM concentration of their cognate proteases (trypsin, chymotrypsin). The residual protease activity was determined as described in section 3.2.8. The molar ratio of the inhibitor (RsBBI or RsKI) to the trypsin or chymotrypsin was the intercept of x-coordinate, when the tangent was extrapolated to the zero activity. The values are the mean ± SE of at least three independent assays each with three replicates.

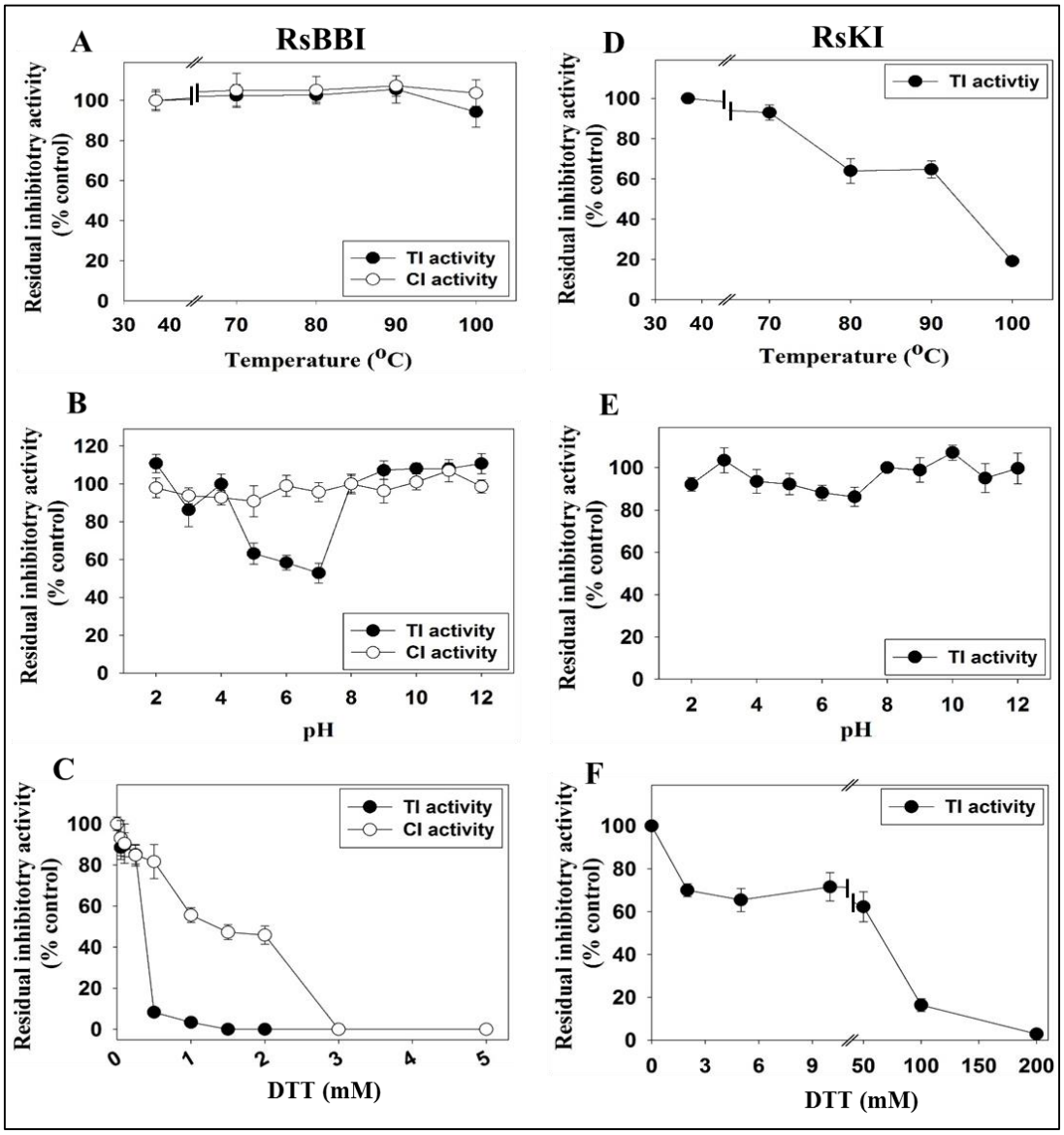


Fig. 3.12. Effect of temperature, pH and DTT on the inhibitory activity of RsBBI and RsKI. The TI and CI activity of RsBBI after incubating for 30 min at indicated (A) temperature, (B) pH and (C) DTT concentration; The TI activity of RsKI after incubating for 30 min at indicated (D) temperatures, (E) pH and (F) DTT concentration. The residual protease activity was measured as described in materials and methods section 3.2.8. The values are the mean ± SE of three independent assays each with three replicates.

neutral/alkaline pH (Fig. 3.12E). In contrast to RsBBI, the TI activity of RsKI was quite stable to the action of DTT. For e.g., it lost >40% of its TI activity when incubated with 2 mM DTT and lost 100% of its TI activity after incubating at a concentration as high as 200 mM DTT (Fig. 3.12F).

3.3.7. Secondary structural analysis of RsBBI and RsKI

At far-UV (190-260 nm) region, the secondary structural elements existed as following 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 ^{o}C. However, this effect was reversed on cooling down from 90 to 25 ^{o}C which indicate that RsBBI is stable to heat treatment (Figs. 3.13A,B). Also, the mean residue ellipticity at 203 nm revealed that the RsBBI is highly stable and resistant to denaturation even at temperature as high as 90 ^{o}C (Figs. 3.14A,B). In contrast, RsKI was sensitive to thermal treatment and showed a remarkable decrease in its ellipticity when heated above 80 ^{o}C (Fig. 3.13D). But, the ellipticity was reversed on cooling down from 90 to 25 ^{o}C (Fig. 3.13E). Further, the mean residue ellipticity observed for RsKI at 199 nm revealed a sigmoidal curve which started at 70^{o}C and ended at 90^{o}C with a *Tm* of 75 ^{o}C and it was reversed upon cooling from 90 to 25 ^{o}C (Figs. 3.14C,D). Similar to temperature, any change in pH (2.0 to 12.0) caused only a marginal decrease in the ellipticity of RsBBI as compared with ellipticity at pH 8.0 (Fig. 3.13C). In contrast, ellipticity exhibited by RsKI did not vary at pH 4.0 and 6.0 as compared to pH 8.0. However, a marginal increase in its ellipticity was observed when incubated at pH 2.0 or 12.0 (Fig. 3.13F). These results suggest that RsBBI is structurally more stable than RsKI at different temperature and pH.

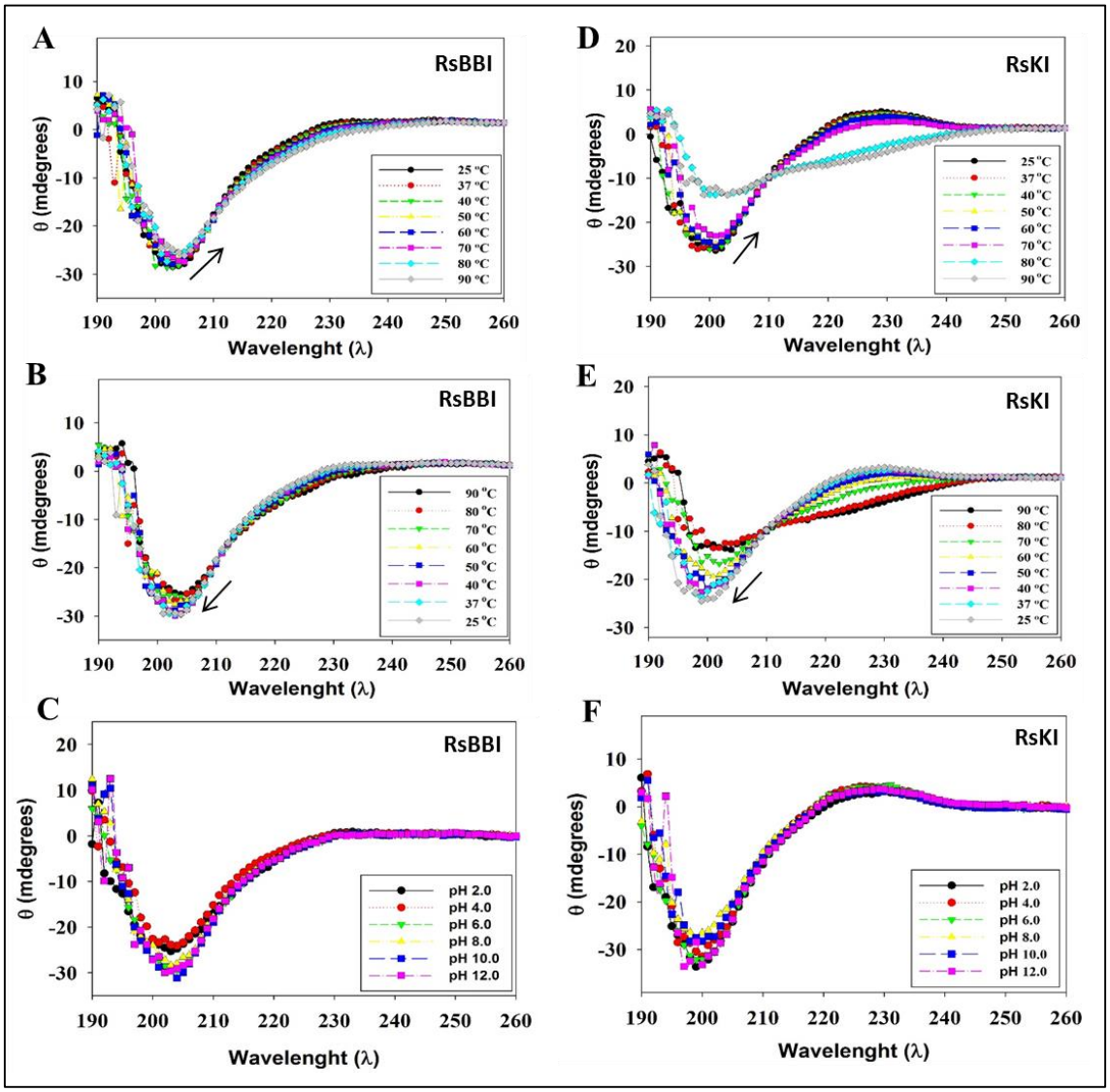


Fig. 3.13. Effect of temperature and pH on the conformation of RsBBI and RsKI at far-UV (190-260 nm). CD spectra indicating the change in ellipticity of (A) RsBBI and (D) RsKI on increasing the temperature from 25 to 90 ^{o}C; CD spectra indicating the recovery of ellipticity in (B) RsBBI and (E) RsKI by gradually decreasing the temperature from 90 to 25 ^{o}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). Concentration of the purified RsBBI or RsKI used to obtain the CD spectrum was 0.2 mg/mL. Further details are described in materials and methods section 3.2.11.

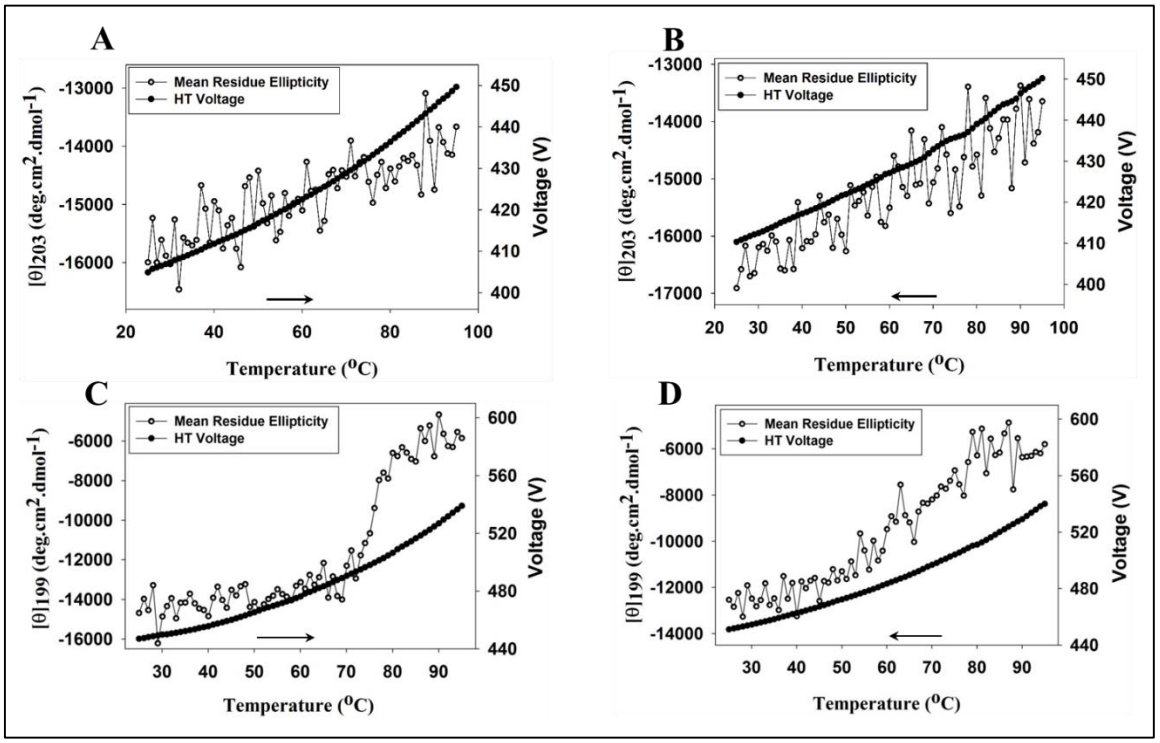


Fig. 3.14. Thermal transition of RsBBI and RsKI. Changes in mean residue ellipticity of (A) RsBBI at 203 nm and (C) RsKI at 199 nm on increasing the temperature from 25 to 90 °C; recovery in mean residue ellipticity of (B) RsBBI and (D) RsKI on decreasing the temperature from 90 to 25 °C. Concentration of the purified RsBBI or RsKI used to obtain the CD spectrum was 0.2 mg/mL. Further details are as described in materials and methods section 3.2.11.

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

The specific activity of RsBBI against trypsin and AjGPs are significantly higher than RsKI by 2.14-fold and 81-fold, respectively (Fig. 3.15A) while the specific activity of RsKI against HaGPs was 150 fold higher than RsBBI (Fig. 3.15A). However, the specific activity of RsKI against chymotrypsin was not detected (Fig. 3.15A). Further, the activity of HaGPs and AjGPs decreased by 79% and 84% of control with an IC_{50} of 8.85 µg and 24 ng, respectively when incubated with RsBBI at a wide range of concentrations (Figs. 3.15B,C). In contrast, the activity of HaGPs and AjGPs was inhibited by 81% and 71% of control with an IC_{50} of 59 ng and 1.95 µg, respectively when incubated with RsKI at a wide range of concentrations (Figs. 3.15D,E). Taken together, the results suggest that RsBBI possessed significant potential to inhibit AjGPs while RsKI possessed potential to inhibit HaGPs.

3.4. Discussion

3.4.1. Identity of trypsin specific PIs present in *R. sublobata* seeds

PIs are one among the major defense strategies used by plants against grazing insects (Mosolov and Valueva, 2005). The studies with PIs from wild relatives of pigeonpea including *R. sublobata* recognized their importance in inhibiting the trypsin-like gut proteases of *H. armigera* (Chougule et al., 2003). In the present study, the identity of trypsin-specific PIs (RsPI) present in the mature seeds of *R. sublobata* was revealed.

The separation of RsPI (trypsin specific PI pool) into two discrete bands of low molecular mass (10 kDa and 20 kDa) in Tricine SDS-PAGE and the disappearance of 10 kDa but not of 20 kDa isoinhibitor spots which are active against trypsin under reducing condition indicated that RsPI possessed PIs from at least two different families (Figs. 3.1D, 3.3). Further, N-terminal sequencing and pBLAST search of the isoinhibitor spots (pI 4.9 and pI 5.0) together

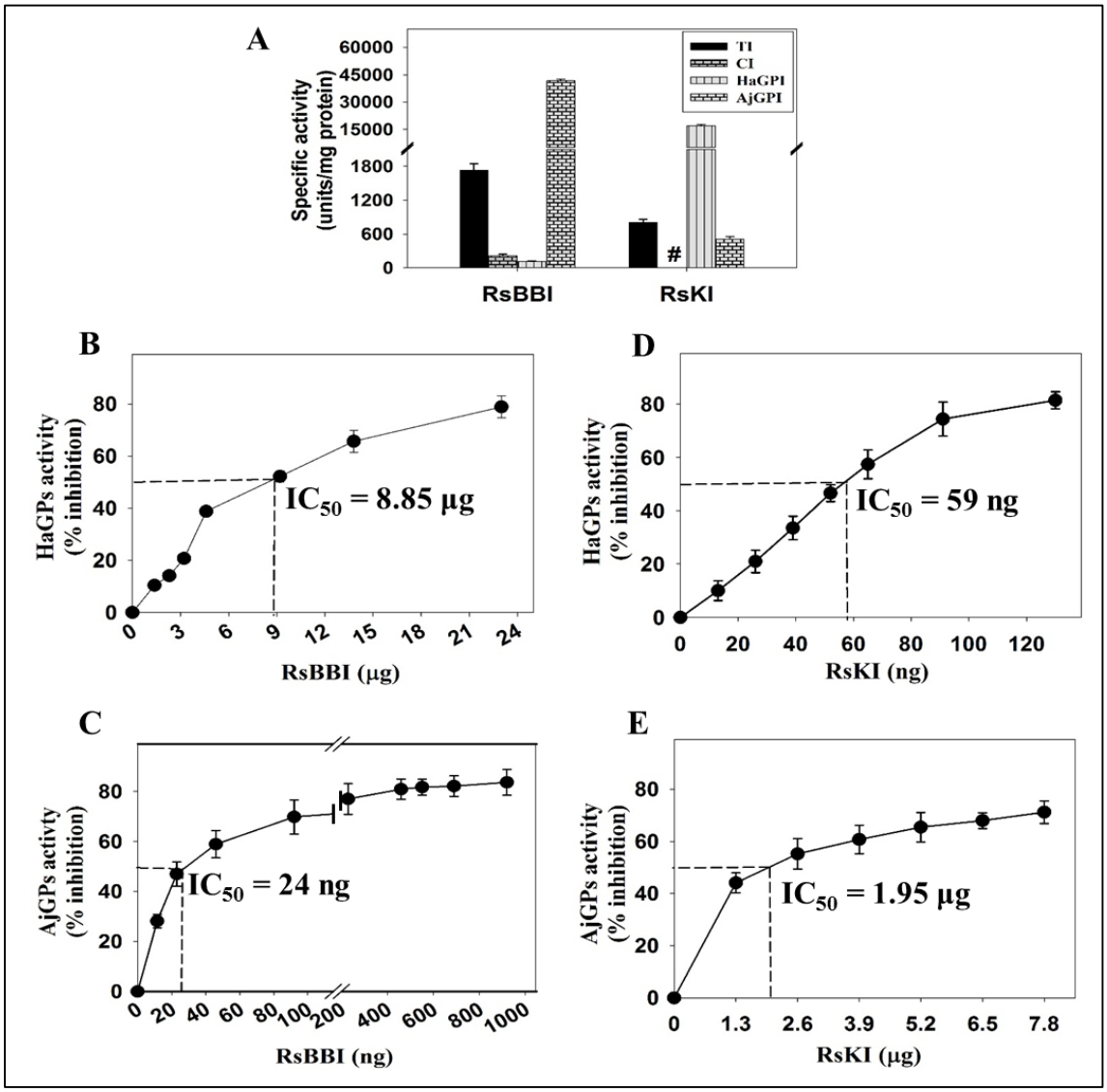


Fig. 3.15. Evaluation of inhibitory activity of RsBBI and RsKI against trypsin, chymotrypsin, HaGPs and AjGPs. (A) The specific activity of RsBBI and RsKI against the trypsin, chymotrypsin, HaGPs and AjGPs. The symbol '#' represent CI activity is not detected for RsKI; Half-maximal inhibitory concentrations (IC_{50}) of RsBBI and RsKI against (B,D) HaGPs and (C,E) AjGPs, respectively. The results are the mean ± S.E. of at least three independent assays each with three replicates.

confirmed the presence of both BBI and Kunitz inhibitors in the seeds of *R. sublobata* (Figs. 3.2B,C). Though leguminous plants mostly possessed PIs from BBI family, a very few leguminous species such as *G. 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; Terada et al., 1994b).

3.4.2. Development of rapid method for separation of RsBBI and RsKI

The separation of BBIs and Kunitz inhibitors present in a single seed variety using multiple chromatographic steps, preparative native gels or processing differently after extracting into a suitable organic solvents is well known (Deshimaru et al., 2002; Giri et al., 2003; Macedo and Xavier-Filho, 1992). However, these methods are highly time-consuming and involved a tedious process. Also, in the present study, apparantly, the separation of RsBBI and RsKI from RsPI using the principles of chromatography was found to be very difficult for the following reasons: (i) both RsBBI and RsKI are trypsin inhibitors (Figs. 3.1B,C,3.5B,3.11A,B); (ii) the difference in molecular mass of RsBBI (~10 kDa) and RsKI (~20 kDa) is very narrow (Fig. 3.2A,3.6A,B); (iii) RsBBI has the tendency to self associate, hence, the molecular mass of RsBBI dimer (~20 kDa) and the RsKI monomer (~20 kDa) are very close (Figs. 3.4B,3.6A); and (iv) most of the isoinhibitors of both RsBBI and RsKI showed overlapping pI values (Fig. 3.2A). Further, in spite of usage of the following columns or procedures such as Superdex G-75, MonoQ Sepharose, 10 kDa cut-off spin columns or simple SDS-PAGE gel elution, apparently RsPI could not be separated into RsBBI and RsKI. Although the hydrophobic interaction chromatography (HIC) using Phenyl Sepharose 6 fast flow separated the RsBBI and RsKI based on decreasing ammonium sulfate gradient (Data not shown), the procedure involved time-consuming extensive post processing protocols such as buffer exchange and concentration steps to separate them into their pure forms. Therefore in

this study, a short and quick method was developed to separate RsBBI and RsKI based on their differential solubility properties in TCA (Fig. 3.4A).

In principle, TCA increases the hydrophobicity of the protein by exposing its hydrophobic core and thereby induce bulk protein precipitation. RsKI being more hydrophobic as compared to BBI is obtained in the pellet fraction 'P1' as compared to BBI when RsPI was subjected to 2.5% TCA precipitation at 70 ^{o}C (Fig. 3.4B). The BBIs are known to have low molecular mass, globular, completely water soluble proteins with hydrophilic core, and surface hydrophobic patch which renders the monomers to self-associate as dimers and trimers in solution (Silva et al., 2005). This behavior perhaps allowed RsBBI to be retained in the soluble fraction (S1) on mild TCA precipitation. Further, Kunitz inhibitors were reported to be soluble in acetate buffers (Macedo and Xavier-Filho, 1992). Thus, extraction of RsKI enriched pellet 'P1'with Sodium acetate (pH 4.0) retained RsKI in the supernatant 'S2'. Thus, this protocol demonstrated the efficient separation of both RsBBI and RsKI from RsPI in less than two hours' time period (Fig. 3.4). Further, oligomerisation of BBIs is very common (Figs. 3.4B,3.6A). But, dimerization of Kunitz inhibitors as observed by RsKI (Figs. 3.4B, 3.6B) is limited and reported from *Schizolobium parahyba*, and *Canavalia lineata* (Teles et al., 2007; Terada et al., 1994a). Further, the MALD-ISD data confirmed the presence of both RsBBI and RsKI in *R. sublobata* seeds which in turn validated the effectiveness of the purification scheme adapted to separate RsBBI and RsKI from RsPI (Figs. 3.8,3.9). Furthermore, both RsBBI and RsKI were resolved into multiple isoinhibitors spots in the 2-DE gel which in turn might have a strategic role in plant defense (Figs. 3.7A,B; Harsulkar et al., 1999).

3.4.3. RsBBI and RsKI possessed differential biochemical properties

The PIs from BBI family were double headed while PIs from Kunitz inhibitor family were single headed (Mosolov and Valueva, 2005). In the present study, though the RsBBI

corroborated well with other BBIs from leguminous plants in possessing two inhibitory domains for trypsin and chymotrypsin, RsKI differed from other known Kunitz inhibitors in possessing two trypsin inhibitory domains (Fig. 3.11A,B). Such double headed Kunitz inhibitors occurred rarely in nature and so far reported from tamarind (Patil et al., 2012), winged bean (Bhattacharjee et al., 2014) and *Adenanthera pavonina* (Migliolo et al., 2010). Though the BBIs purified from leguminous plants exhibited *Ki* values in the range of 0.1 nM to 52 μM against trypsin (Ragg et al., 2006; Ramasarma et al., 1994), the *Ki* (128.5±4.5 nM) of RsBBI against trypsin was less than the *Ki* (≤292 nM) observed for BBIs from *C. cajan* cultivars. Similarly, the *Ki* (807.8±23.7 nM) of RsBBI against chymotrypsin was 3 to 4.5-fold less than the *Ki* (≤3725 nM) for BBIs from *C. cajan* cultivars (Fig. 3.10A,B; Prasad et al., 2010c; Swathi et al., 2014). Further, the *Ki* (172±9.2 nM) of RsKI against trypsin was higher as compared to the other known non-competitive Kunitz inhibitors which possessed *Ki* of 0.1 nM in *Erythrina velutina* (Fig. 3.10 C; Machado et al., 2013) and 0.59 nM in *Prosopis juliflora* (Oliveira et al., 2002).

The TI and CI activities of BBIs including RsBBI are stable to changes in temperature and pH treatments but sensitive to DTT/IDA reduction (Figs. 3.12A-C; Prasad et al., 2010b; Swathi et al., 2014). In contrast, the TI activity of RsKI is stable to changes in pH (2.0-12.0) and DTT but sensitive to temperature (Figs. 3.12D-F). When heated above 70 ^{o}C it lost >90% of its TI activity (Fig 3.12A). Kunitz inhibitors which were stable to DTT reduction were reported from *Putranjiva roxburgii* (Chaudhary et al., 2008), *Inga laurina* (Macedo et al., 2007) and *Plathymenia foliolosa* (da Silveira Ramos et al., 2008). Further, RsKI which is related to a small group of Kunitz inhibitors from *C. cajan* (*Tm*=63^{o}C) (Haq and Khan, 2005) and *C. selloi* (*Tm*=68^{o}C) (Yoshizaki et al., 2007), showed structural denaturation at a higher temperature (*Tm*=75^{o}C) and renatured on cooling down to room temperature (Figs. 3.14C,D). Similar to other known BBIs from horse gram (Kumar and Gowda, 2013a) and black gram

(Prasad et al., 2010b) RsBBI is structurally stable due to its rich network of seven disulphide bridges and β-sheets which prevent the proteins from undergoing any changes in their secondary structures even after heating to 90 ^{o}C thus rendering the protein retain both its TI and CI activities (Figs. 3.13A,3.14A,B).

3.4.4. Insecticidal potential of RsBBI and RsKI against *H. armigera* and *A. janata*

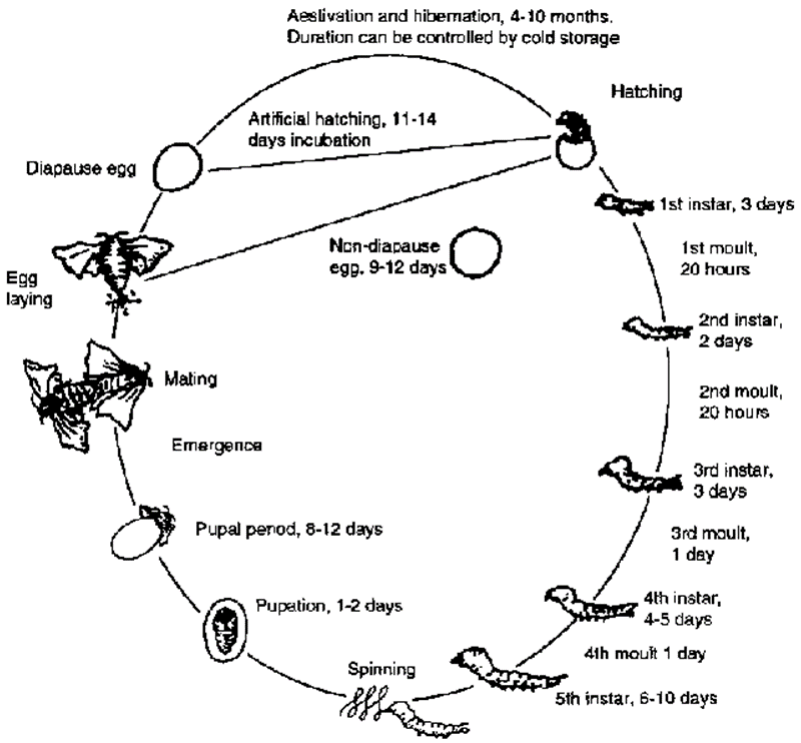
R. sublobata is a wild relative of *C. cajan*. Given the potential of PIs from wild-relatives in inhibiting the gut proteases of insect pests, the inhibitory activity of RsBBI and RsKI was examined on HaGPs and AjGPs (Figs. 3.15A-E). The RsBBI and RsKI showed differential inhibitory potential against HaGPs and AjGPs. The significant loss in the activity of HaGPs in presence of RsKI, but not RsBBI unravels that the earlier reports on effective inhibition of HaGPs by the crude PIs of red gram wild relatives including *R. sublobata* is due to the Kunitz inhibitors present in them (Figs. 3.15C,D; Chougule et al., 2003; Parde et al., 2012). Several trypsin inhibitors from different PI families were reported to show effective inhibition against the HaGPs. For e.g., squash inhibitor from *Momordica charantia* (Telang et al., 2009b), PIN1 inhibitor from Capsicum (Tamhane et al., 2005), and Kunitz inhibitors from *P. tetragonolobus* (Telang et al., 2009a), *Cicer arietinum* (Srinivasan et al., 2005) and *Acacia senegal* (Babu and Subrahmanyam, 2010). However, the IC_{50} of RsKI at concentration observed as low as 59 ng in the present study suggest its potential in controlling the growth and development of *H. armigera* (Fig. 3.15D).

Further, India is the world leader in castor oil production, and the farmers control the castor pests using chemical pesticides such as Quinalphos, Chloropyriphos, carbaryl, monocrotophos, and endosulfan which are harmful to humans and environment (Gahukar, 2015). The crude leaf extracts of eggplant, *Solanum melanogena* which contained high concentrations of BBI along with secondary metabolites inhibited the activity of AjGPs

(Devanand and Rani, 2011). Further, the BBIs from cultivar varieties (ICP 7118, and ICP 14770) of *C. cajan* induced high mortality rates in *A. janata* when fed up on leaves coated with BBI at 2 µg cm^{-2} (Fig. 3.15C; Prasad et al., 2010a; Swathi et al., 2014). However, the observed IC_{50} of RsBBI (24 ng) as compared to cultivar varieties (78 ng) against AjGPs indicate the significant potential of RsBBI in controlling the larval growth and inducing mortality in *A. janata*.

Chapter 4

A comparative study on the growth retardation potential and biocidal activity of protease inhibitors from *C. cajan* wild relative (*R. sublobata*) and cultivar (BDN1) on castor semilooper *A. janata*.



Representative life cycle of insect.

(Adapted from https://goo.gl/images/W4Iq0z)

A comparative study on the growth retardation potential and biocidal activity of protease inhibitors from *C. cajan* wild relative (*R. sublobata*) and cultivar (BDN1) on castor semilooper *A. janata*

4.1. Introduction

Castor is one of the cash crops which has earned a lot of interest among farmers for its ability to withstand low rainfall and its potential in export market (Pavaskar and Kshirsagar, 2013). *A. janata* is one of the devastating insect pests of castor plants. It defoliates the entire crop in a short time by feeding voraciously. Though mature plants can withstand up to 25% of foliage loss, extensive loss of leaves decrease the yield in older plants and cause death in younger plants. IPM strategies are suggested for the control of *A. janata* using natural enemies like parasitoids, birds, pheromone traps, spraying neem kernel extract or chemical pesticide malathion and Bt toxins at or above 25% loss of foliage by the insects (Sujatha et al., 2010). But on the field, farmers are frequently resorting to hazardous chemical pesticides like endosulfan, dichlorvos, dimethoate, metasystox, triazophos and acephate to control the insects (Gahukar, 2015). However, such injudicious use of chemical pesticides and continued Bt toxin stress, induced resistance in lepidopteran insects such as *Helicoverpa armigera* (ffrench-Constant, 2013; Jin et al., 2013b; Wei et al., 2015) and *Plutella xylostella* (Baxter et al., 2011; Furlong et al., 2013; Sayyed et al., 2008). In this scenario, PIs effective against the gut proteases of insect pests could improve the defense potential of the plants or improve the efficiency of Bt toxin expressing transgenic plants (Lawrence and Koundal, 2002; Sharma, 2015).

C. cajan is a non-host plant to *A. janata*. Hence it can't feed on it. Earlier reports from our laboratory indicated that Bowman-Birk inhibitors (BBIs) purified from seeds of *C. cajan* cultivars (ICP 14770 and ICP 7118) were highly effective in inducing mortality and controlling the growth and development of larvae when fed to them at first and second instar stages, respectively (Prasad et al., 2010a; Swathi et al., 2014). But, most of the insects actively feed

from their third instar stage. Also, the insecticidal potential of the wild relatives of *C. cajan* was not examined so far for their *in vivo* insecticidal potential against *A. janata*. In this context, an attempt was made in the present study to compare the insecticidal potential of the PIs purified from the seeds of *R. sublobata* and BDN1 on *A. janata* by supplementing them along with the feed at the neonatal first instar stage and actively feeding third instar stage. The studies from 3rd chapter indicated that the RsPI pool contains 80% of RsBBI and 20% of RsKI (Fig. 3.3C). Also, RsBBI exhibited high inhibition potential against *A. janata* at nano gram level (IC_{50} = 24 ng; Fig. 3.10C) which is 81-fold higher than that exhibited by RsKI (IC_{50} = 1.95 µg; Fig. 3.10E). Hence, to examine the growth retardation potential and mortality effects of the RsBBI on *A. janata*, RsPI pool was fed directly to first and third instar larvae of *A. janata.* Furthermore, considering the possibility that cultivars of *C. cajan* are enriched in BBIs (Prasad et al., 2010c; Swathi et al., 2014), we choose a new cultivar BDN1 in this study to make a comparison and identify BBI among wild relatives and cultivars of *C. cajan* wich is best suitable for the management of *A. janata*.

4.2. Materials and methods

4.2.1. Materials

The seed materials of *R. sublobata* (ICP 15868) and red gram cultivar variety BDN1 were obtained from International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, India. Absorption measurements were performed in UV-1700 Spectrophotometer (Shimadzu, Japan). The temperature was maintained in water bath Model F12 (Julabo, Germany). Benzylarginylpara nitroanilide (BApNA) was procured from Sigma (Bangalore, India). The bicinchoninic acid reagent was procured from Thermo Scientific, India. DEAE Sepharose, CNBr activated Sepharose 4B, and Sephadex G-50 were procured from GE

Healthcare Biosciences Pvt. Ltd., India. Tris base, trypsin enzyme, and sodium chloride were procured from Sisco Research Laboratory, Mumbai, India.

4.2.2. Purification of PIs

RsPI was purified as described in Chapter 3, Figure 3.1 whereas BDN1PI from red gram seeds was purified according to Prasad et al. (2010c). In brief, crude PI extracts from seeds were obtained after de-pigmenting and de-fatting the seed powder using acetone and hexane, respectively. To the air-dried powder 50 mM Tris-HCl, pH 8.0 containing 1% PVP (1:6 W/V) was added and stirred overnight at 4ºC. The crude PI obtained after centrifugation at 11,000 *g* was subjected to ammonium sulphate fractionation. The PIs were purified from 20-60% ammonium sulfate fraction by passing through different chromatography columns: anion exchange, trypsin affinity, and gel filtration columns. The protein obtained was concentrated and stored at -20ºC as BDN1PI. The protein concentration was determined by the Bicinchoninic acid method.

4.2.3. SDS-PAGE, Gelatin Native PAGE and MALDI-TOF mass spectrometry

The purity of BDN1PI was visualized in 18% Tricine SDS-PAGE as per Schagger (2006). Inhibitory activity of BDN1PI against trypsin enzyme was visualized in gelatin incorporated native PAGE as described in Felicioli et al. (1997). After electrophoresis, the gel was washed and incubated successively with 0.1 mg/mL trypsin solution in 0.1 M Tris-HCl, pH 8.2 for 15 min at 4 ºC and 1 hour at 37 ºC. The bands active against trypsin were visualized by CBB R-250 staining. Intact mass of pure BDN1PI was analyzed by MALDI-TOF mass spectrometry (Autoflex II Bruker Daltonics, Bremen, Germany). 1 µL of BDN1PI was mixed with 1 µL of a saturated solution of CHCA matrix in 50% ACN and 0.1% TFA in water and was spotted on MALDI target plate. The intact mass was analysed in linear mode in the range of 1-30 kDa.

4.2.4. Insect rearing and larval gut protease preparation

A. janata larvae were cultured on fresh castor leaves, and the pupae were kept in moist sand for generation of adults. The adults were fed through a cotton swab immersed in 20% honey solution containing 200 mg vitamin-E, and allowed to lay eggs on fresh castor leaves. The next generation larvae were used for *in vivo* feeding experiments. *A. janata* insects were reared in insect culture room at 26 ± 1 ^{o}C and 65 ± 5% relative humidity with 14:10 hour light:dark photoperiod. The larvae of *H. armigera*, *S. litura*, *P. demoleus*, *A. albistriga* and *D. nerii* were obtained from the fields surrounding the University of Hyderabad. For the preparation of gut trypsin-like proteases, the 5th instar larvae were chosen and narcotized on ice for 15 min and dissected dorsally in 0.15 M saline solution. The obtained guts were homogenized in the same solution, centrifuged at 11,000 *g* for 20 min at 4 ^{o}C and the supernatant was labelled as respective gut proteases, and stored at -20 ^{o}C .

4.2.5. Protease inhibitor (PI) assay

PI assay was performed according to Prasad et al. (2010c) The protease assay was performed using the substrate BAPNA or GLUPHEPA which yields yellow colored *p*-nitroanilide with maximum absorption at 410 nm on cleavage by trypsin or chymotrypsin, respectively. The assays were performed in 50 mM Tris-HCl, pH 8.2 or pH 7.8 containing 20 mM $CaCl_2$ for trypsin and chymotrypsin, respectively. 10 µg of trypsin or 80 µg of chymotrypsin which yield an optical density of 1.0 was titrated with PIs and used as a source of protease. For the assay of inhibitory activity against gut trypsin-like proteases, larval gut proteases which yield 1.0 OD were taken in respective assay buffers (*A. janata* – 50 mM Tris-HCl, pH 8.2; *H. armigera*, *S. litura*, *P. demoleus*, *A. albistriga* and *D. nerii* – 50 mM Glycine-NaOH, pH 10.5). PIs from crude extracts or purified PIs were mixed with trypsin or chymotrypsin and incubated for 15 min at 37 ^{o}C, followed by addition of 1 mM BAPNA or 1

mM GLUPHEPA and incubated at 37 ºC for 45 min. The reaction was stopped by adding 30% acetic acid. The reduction in OD was monitored at 410 nm. One inhibitor unit is defined as the amount of protein which induces a 50% decrease in the OD. All the assays were performed thrice, and the experiments were repeated three times.

4.2.6. Stability studies of BDN1PI

The stability of BDN1PI at a wide range of temperature and pH conditions, and after reduction with DTT was determined as described in Swathi et al. (2014). In brief, BDN1PI was incubated at different temperature (37, 40, 50, 60, 70, 80, 90 & 100 ºC) for 30 min, or different pH (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0) at 37 ºC for 30 min, or reduced with different concentrations of DTT (0.0 mM to 1.0 mM) and alkylated with twice the concentration of iodoacetamide. The residual TI and CI activities were determined as percent control after each treatment. The molar ratio of BDN1PI/protease (0.0 to 2.0) was determined by adding increasing concentrations of BDN1PI to a fixed 1 µM concentration of either trypsin or chymotrypsin proteases. The residual PI activity was measured as described in section 4.2.5.

4.2.7. Feeding experiment

The effect of RsPI and BDN1PI on growth kinetics of *A. janata* was performed i*n vivo* by no choice feeding experiment where castor leaf is coated with 2, 4 or 8 µg of PIs per square cm of leaf area. The leaves coated with respective PI concentration was air dried at room temperature and fed to both first and third instar larvae of *A. janata* for 14 and 7 days, respectively. After the PI treatment, the insects were allowed to complete their life cycle on normal castor leaves. Castor leaves coated with 50 mM Tris-HCl, pH 8.0 were fed to the control group of larvae. The reduction in the growth of treated insect groups was calculated by considering the average weight of control group as 100%. The leaves were changed twice a

day, and the weights were recorded on alternate days. A total of 15 insects were taken in each of the test insect groups.

4.2.8. Statistics

All the experiments were repeated thrice, and the mean ± SE was represented. Statistical differences were determined by one-way ANOVA followed by Post hoc Tukey test at $P<0.05$ using Sigma-Plot, version 12.5, software (San Jose, CA, USA).

4.3. Results

4.3.1. *In vitro* assay of seed crude PIs from *R. sublobata* and BDN1 against gut trypsin-like proteases of different lepidopteran larvae

The crude PI extracts of both *R. sublobata* and BDN1 seeds were examined for their potential to inhibit the larval gut trypsin-like protease activity in lepidopteran insect pests such as *A. janata, S. litura, P. demoleus, A. albistriga* and *D. neeri*. The PIs from *R. sublobata* exhibited significant inhibitory activity against larval gut enzymes of *A. janata* with 820±187 TIU. In contrast, the effect of PIs from *R. sublobata* on larval gut enzymes of *S. litura, P. demoleus, A. albistriga* and *D. neeri* was marginal with 20±3, 13±1, 19±1.5 and 50±2 TIU, respectively (Chapter 2; Fig. 2.4A). Similarly, the PIs from BDN1 showed potent inhibitory activity against larval gut enzymes of *A. janata* with 534±108 TIU, while they showed marginal effect on larval gut enzymes of *S. litura, P. demoleus, A. albistriga* and *D. neeri* with 2.7±0.7, 1.9±0.2, 19±1.2 and 0.5±0.1 TIU, respectively (Chapter 2; Fig. 2.4B).

4.3.2. Purification of RsPI

The trypsin specific PIs (RsPI) was purified from *R. sublobata* seeds as described in Chapter 3 – Figure 3.1.

4.3.3. Purification of BDN1PI

BDN1PI was purified from the seed crude PI extract using ammonium sulphate fractionation followed by anion exchange, trypsin affinity, and gel filtration chromatography. Different chromatograms of BDN1PI were shown in Figures 4.1A-C. The purity of BDN1PI was observed on 18% Tricine SDS-PAGE and MALDI-TOF analysis. The purified BDN1PI existed as multiple bands of MW ~16 kDa, 24 kDa and 32 kDa in SDS-PAGE (Fig. 4.1D). Intact mass analysis revealed that BDN1PI existed as multiple isoinhibitors in the low molecular mass range of 6800-8100 Da (Fig. 4.2A). Taken together, the results suggest that BDN1PI exhibited self-association pattern and existed as small oligomers up to tetramers (Fig. 4.1D). Further, the Gelatin Native-PAGE showed that the isoinhibitors of BDN1PI were active against bovine pancreatic trypsin proteases (Fig. 4.2B). The purification protocol resulted in 51.9 fold purification of BDN1PI with yield recovery of 18.6% (Table 4.1).

4.3.4. Biochemical characterization of BDN1PI

BDN1PI purified from BDN1 seeds were also active against both trypsin and chymotrypsin proteases (Fig. 4.3). BDN1PI retained the TI and CI activities even after heating for 30 min at a wide range of temperature from 37 to 100 ^{o}C (Fig. 4.3A). Further, on incubating BDN1PI at different pH (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 & 12.0), it was revealed that the TI activity of BDN1PI was stable at alkaline pH whereas <15% decrease in TI activity was observed at pH 5.0–7.0. The CI activity of BDN1PI was stable across the pH range tested. However, a 30% spike in activity was observed at pH 6.0 (Fig. 4.3B). In contrast, the BDN1PI was sensitive to reduction with DTT. BDN1PI lost 100% of both TI and CI activities on reduction with 1.0 mM DTT (Fig. 4.3C). Further, BDN1PI inhibited trypsin proteases in 1:2 molar ratio but no stoichiometry was observed for chymotrypsin inhibition (Fig. 4.3D).

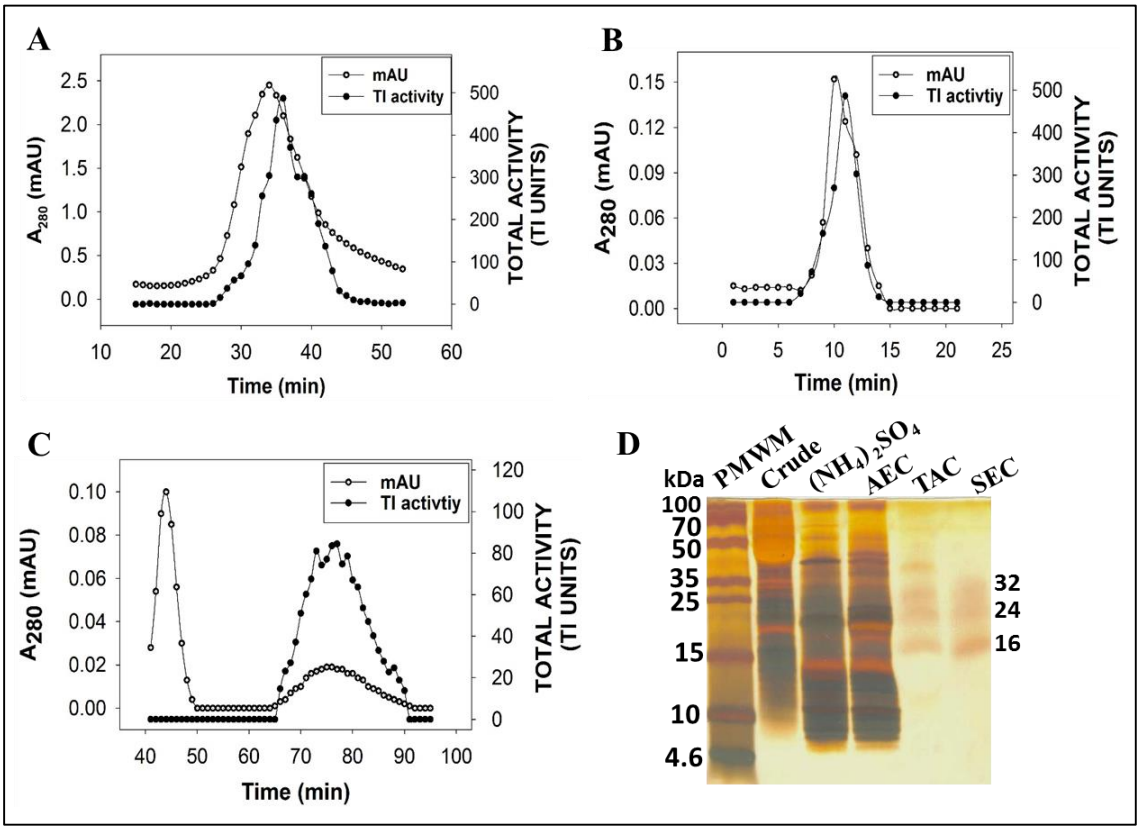


Fig. 4.1. Purification of BDN1PI from the seeds of red gram cultivar variety BDN1. Elution profiles of (A) anion exchange (DEAE Sepharose) column loaded with 20-60% $(NH_4)_2SO_4$ fraction of crude PI extract, (B) trypsin affinity chromatogram loaded with 28^{th}-44^{th} fractions of DEAE column; (C) gel filtration chromatogram loaded with fractions 6-14 from affinity column. The active fraction pool from each chromatogram was collected, pooled and concentrated, and proceeded to successive step of chromatography. The active fractions after gel filtration chromatography were collected, concentrated and stored at -20 ^{o}C as purified BDN1PI. (D) Tricine SDS-PAGE (18%) showing different fractions of purification protocol under non-reducing conditions. Lanes 1 – 6 are loaded with PMW marker, crude PI extract (10 µg), $(NH_4)_2SO_4$ fraction (10 µg), active fraction pool from anion exchange column (AEC-5 µg), trypsin affinity column (TAC-10 µg) and BDN1PI from size exclusion column (SEC-5 µg). The gel was stained with silver nitrate.

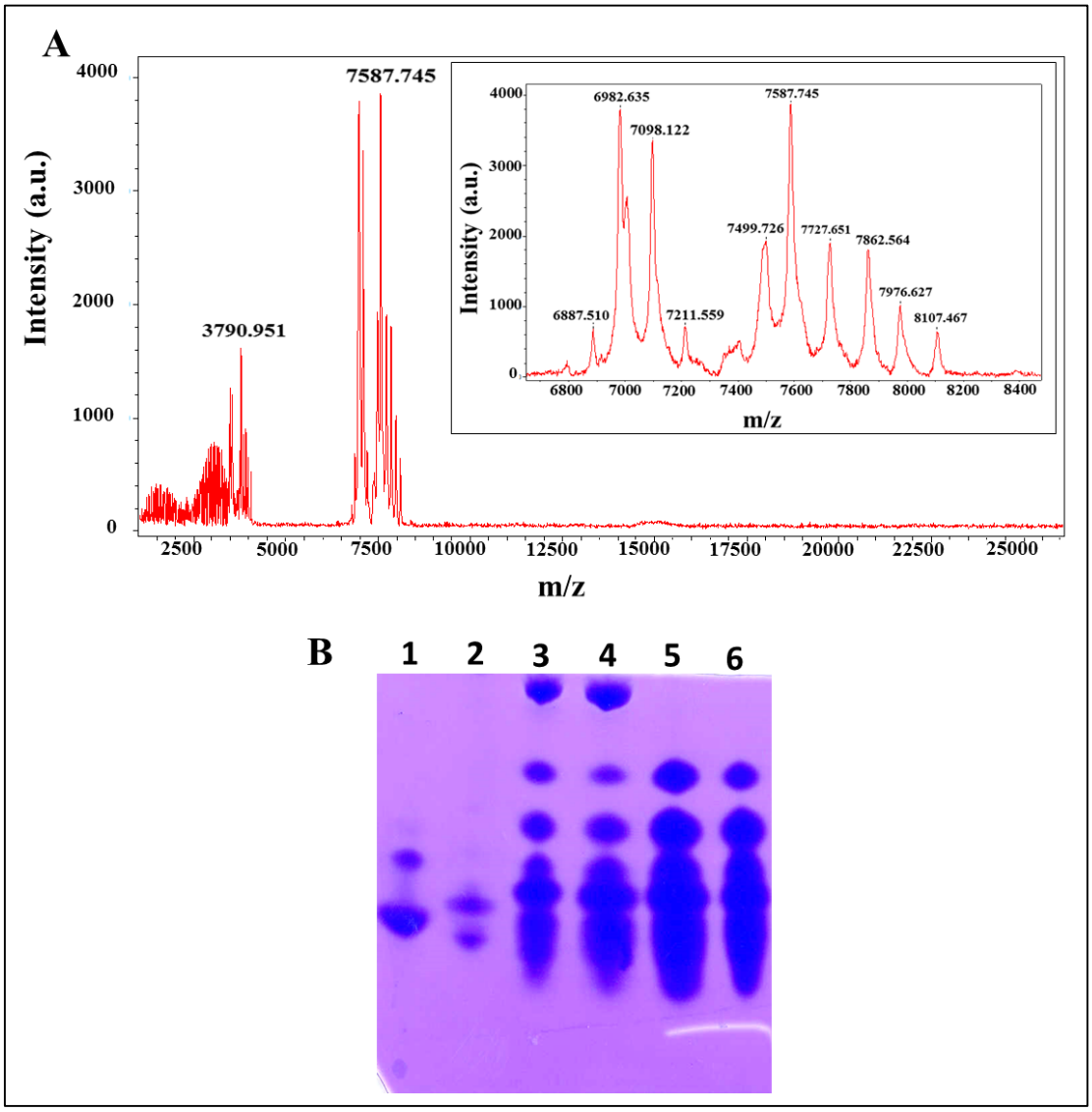


Fig. 4.2. Intact mass analysis and visualisation of trypsin inhibitor activity of BDN1PI on Gelatin Native-PAGE (15%). (A) MALDI-TOF spectrum of BDN1PI at 0-25000 Da under non-reducing condition, inset is the spectrum zoomed between 6800-8400 m/z representing the isoinhibitors; (B) In-gel trypsin inhibitor activity in different purification fractions of BDN1PI was visualized in Gelatin Native-PAGE. Lanes 1-7 are loaded with soybean BBI (5 µg), crude PI extract (50 µg), $(NH_4)_2SO_4$ fraction (50 µg), active fraction pool from anion exchange column (50 µg), trypsin affinity column (25 µg) and BDN1PI from size exclusion column (15 µg). The gel was stained with CBB R-250.

Table 4.1. Purification of BBIs from red gram cultivar variety BDN1 seeds.

Purification step	Total protein (mg)	Total activity (TI units) [1]	Yield recovery (%)	Specific activity (TI units/mg protein) [2]	Purification fold
Crude PI extract	617.1	10894.00	100	17.7	1.00
Ammonium sulfate fractionation (20-60%)	70.93	6130.00	56.26	86.42	4.88
Ion exchange chromatography	43.36	5920.00	54.34	136.53	7.71
Affinity chromatography	5.23	5042.00	46.28	964.05	54.57
Gel filtration chromatography	2.204	2025.00	18.59	918.78	51.91

1 One TI unit is equal to 50% reduction in the trypsin enzyme activity.

2 Specific activity is equal to the number of trypsin inhibitor units per mg protein.

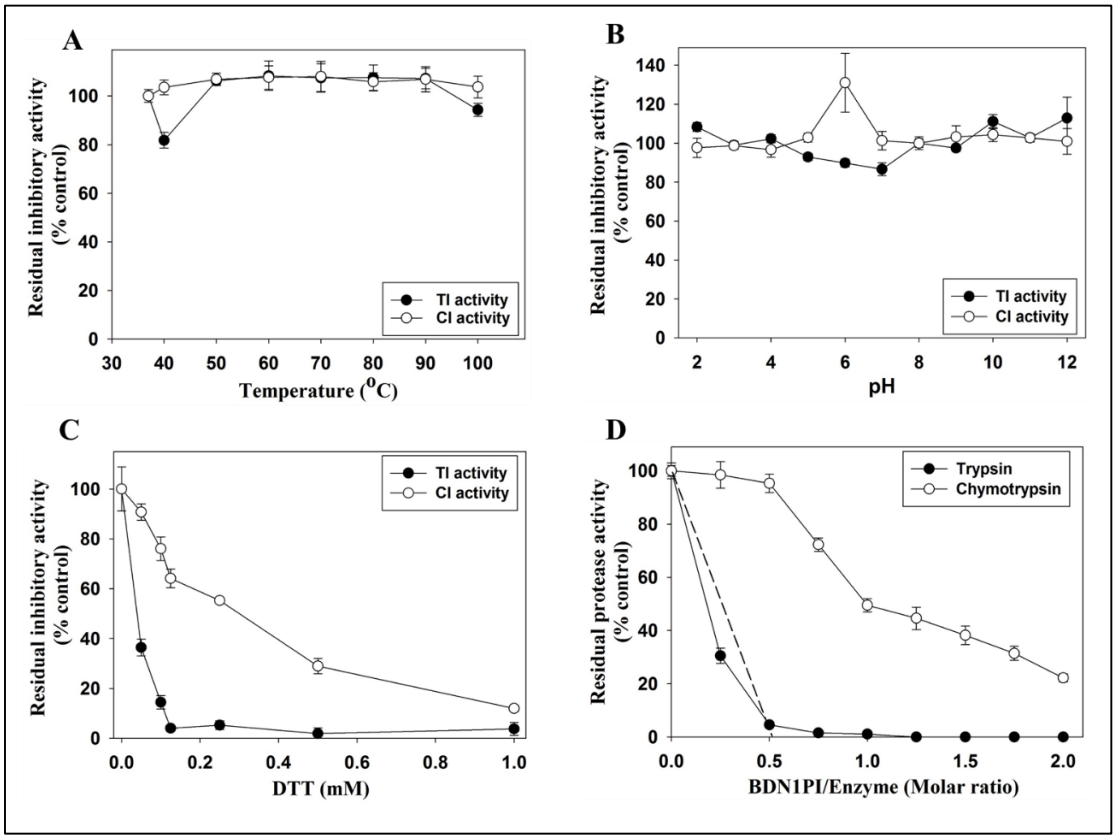


Fig. 4.3. Biochemical characterization of BDN1PI. The TI and CI activities of BDN1PI after incubation for 30 min at indicated (A) temperature, (B) pH and (C) DTT concentration. The quantity of BDN1PI yielding one TI or CI unit was taken in control or test sample. The test samples were incubated at various temperatures, pH or DTT concentration as described in materials and methods section 4.2.6, and analyzed for their residual PI activity. The obtained PI activity was compared with control samples to obtain the percent residual protease activity. For temperature treatments, sample incubated at 37 ºC was taken as a control, for pH treatment, sample incubated at pH 8.0 was taken as control. For DTT treatments, sample without DTT was taken as control. (D) Titration curves of trypsin and chymotrypsin inhibition by BDN1PI. Increasing concentration of BDN1PI was added to the fixed 1 µM concentration of either trypsin or chymotrypsin proteases. The molar ratio of BDN1PI to the protease was the intercept of x-coordinate when the tangent was extrapolated to zero activity. The residual protease activity was measured as described in methods section 4.2.5. The values are mean ± SE of three independent assays each with three replicates.

4.3.5. Effect of *in vivo* feeding of RsPI and BDN1PI on first instar larvae of *A. janata*

The purified RsPI and BDN1PI were examined for their effects on the growth retardation kinetics of newly hatched first instar larvae of *A. janata*. The larvae were allowed to feed on castor leaves coated with 2, 4 and 8µg of PI per square cm for 14 days. Larvae which were fed with RsPI at concentrations mentioned above induced 73±10, 77±8 and 80±12% reduction in weight gain as compared to controls (Fig. 4.4A1). Likewise, BDN1PI induced 80±7, 88±1, 89±3% reduction in larval weight gain as compared to controls when fed at respective concentrations (Fig. 4.4B1). Further, RsPI induced 53±6, 52±4 and 67±2% mortality rate whereas BDN1PI induced 70±3, 80±2 and 81±1% mortality rate in the first instar larvae fed up on PIs at the respective concentrations for 14 days (Table 4.2). The larvae which survived were allowed to grow on the normal castor leaves to complete their life cycle. The pictures of pupae and adult developed from survived larvae after feeding on RsPI and BDN1PI were depicted in Figures 4.4A2-A4 and Figures 4.4B2-B4, respectively.

4.3.6. Effect of *in vivo* feeding of RsPI and BDN1PI on third instar larvae of *A. janata*

The average weight of the control third instar larvae after feeding for seven days was 888±61 mg. The growth retardation potential of RsPI and BDN1PI on the third instar larvae of *A. janata* was examined after feeding upon them for seven days. RsPI coated at 2, 4 and 8 µg per square cm on castor leaves induced 63±2, 65±5 and 64±4% reduction in weight gain as compared to controls in third instar larvae (Fig. 4.5A). Likewise, BDN1PI induced 45±21, 62±3 and 66±3% reduction in weight gain as compared to controls of third instar larvae (Fig. 4.5B). Further, RsPI induced 27±7, 48±2 and 50±1% mortality rate whereas BDN1PI induced 13±2, 27±2 and 40±7% mortality rate in *A. janata* after feeding at the above mentioned concentrations of PIs (Table 4.2). This was followed by feeding of survived larvae on normal castor leaves to complete their life cycle. The pictures of pupae and adults of *A. janata* developed from survived

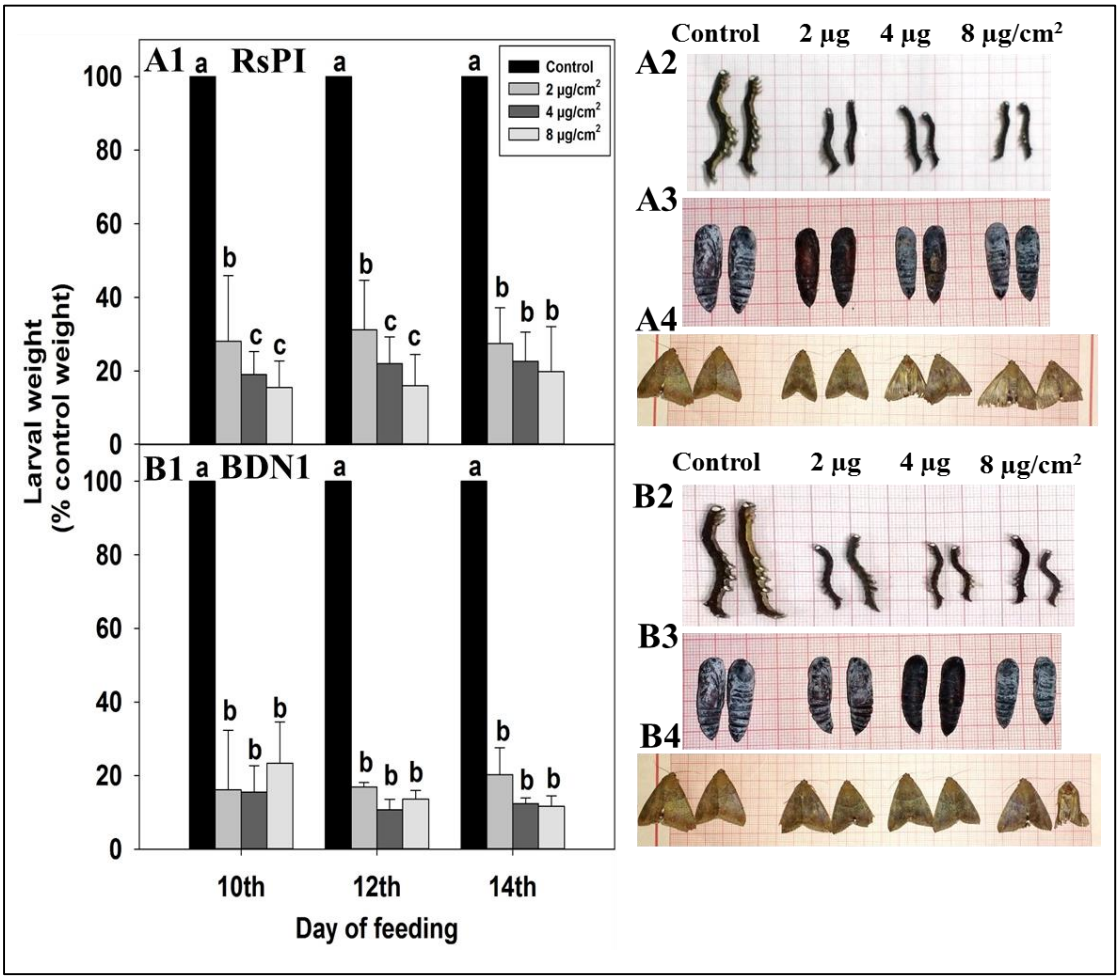


Fig. 4.4. Growth retardation studies of RsPI and BDN1PI on first instar larvae of *A. janata*. (A1) Average weight of first instar larvae of *A. janata* fed up on castor leaves coated with RsPI at a concentration of 2, 4 and 8 µg per square cm for fourteen days. At the end of 14 days, the average weight of the control larvae was found to be 505±6 mg. The pictures of (A2) larvae after 14th day of feeding on RsPI; (A3) pupal and (A4) adult pictures of *A. janata* developed from survived larvae after 14 days of feeding on RsPI at their first instar stage. Likewise, (B1) average larval weight and (B2) larval pictures of *A. janata* at 14th day up on feeding on BDN1PI; (B3) pupal and (B4) adult pictures of *A. janata* developed from survived larvae after 14 days of feeding on BDN1PI at their first instar stage. All the experiments were performed thrice, and the results are mean ± SE of three different experiments. Statistics were performed as described in materials and methods section 4.2.8. Different lowercase alphabetical letters indicate statistically significant difference ($P<0.05$).

Table 4.2. Effect of RsPI and BDN1PI on first and third instar larvae of *A. janata* after *in vivo* feeding.

Stage	PI concentration (µg per square cm leaf area)		Average weight of each larvae (mg)	Reduction in larval growth (% control)	Survival rate (% control) [c]	Mortality rate (% control) [c]
Control		0	505 ± 6	0	100	0
First instar [a]	RsPI	2	139 ± 51	73 ± 10	47 ± 7.10	53 ± 6.45
		4	115 ± 51	77 ± 8	48 ± 2.85	52 ± 4.29
		8	101 ± 63	80 ± 12	33 ± 3.42	67 ± 2.57
	BDN1	2	100 ± 5	80 ± 7	30 ± 2.85	70 ± 3.15
		4	62 ± 7	88 ± 1	20 ± 3.42	80 ± 2.50
		8	58 ± 13	89 ± 3	19 ± 2.15	81 ± 1.19
Control		0	888 ± 61	0	100	0
Third instar [b]	RsPI	2	329 ± 3	63 ± 2	73 ± 6.65	27 ± 7.52
		4	312 ± 23	65 ± 3	52 ± 6.50	48 ± 2.23
		8	350 ± 12	61 ± 4	50 ± 2.52	50 ± 1.05
	BDN1	2	474 ± 153	45 ± 21	87 ± 1.35	13 ± 2.00
		4	337 ± 17	62 ± 3	73 ± 1.65	27 ± 2.01
		8	302 ± 27	66 ± 3	60 ± 6.65	40 ± 7.34

[a] reduction in larval growth after 14 days of feeding from the first instar

[b] reduction in larval growth after 7 days of feeding from the third instar

[c] rate after respective days of feeding with respective PIs

larvae after feeding on RsPI or BDN1PI for seven days were depicted in Figures 4.5A2-A4 and Figures 4.5B2-B4, respectively.

4.3.7. Effect on pupal weights of larvae fed upon RsPI and BDN1PI

The pupae developed from the survived first instar larvae grown on 2, 4 or 8 µg of RsPI showed weights at 70±9, 61±16 and 87±3% of control pupal weight. Likewise, the pupae developed from survived larvae fed on respective concentrations of BDN1PI showed weights at 99±1, 90±1 and 93±1% of control pupal weight (Fig. 4.6).

Also, the pupae developed from the survived third instar larvae grown on 2, 4 or 8 µg of RsPI showed weights at 83±8, 89±16 and 70±2% of control pupal weight. Likewise, the pupae developed from survived larvae fed on respective concentrations of BDN1PI showed 80±8, 74±3 and 80±1% of control pupal weight (Fig. 4.6). The abnormal larval-pupal intermediate and malformed pupa of *A. janata* from the first instar larvae derived upon feeding with 2 and 4 µg per square cm of RsPI was shown in Figure 4.6B.

4.4. Discussion

4.4.1. Crude PIs from *R. sublobata* and BDN1 *Vs* inhibitory activity on gut trypsin-like proteases of *A. janata*

The crude PI extracts of *R. sublobata* and BDN1 seeds were screened for their potential inhibitory activity against gut trypsin-like proteases of different lepidopteran insects (Chapter 2–Fig. 2.4). Of the six insects screened, *P. demoleus* and *D. nerii* feeds on cultivated citrus trees and toxic oleander leaves, respectively. *A. janata*, *S. litura*, and *A. albistriga* are the insect pests of castor. *S. litura* and *A. albistriga* are polyphagous pests which feed on castor upon choice whereas *A. janata* feeds mainly on castor apart from its polyphagous activity reported towards pomegranate, guava, rose, ber, cocoa, coconut, etc. (Basappa, 2013). *A. janata* has an

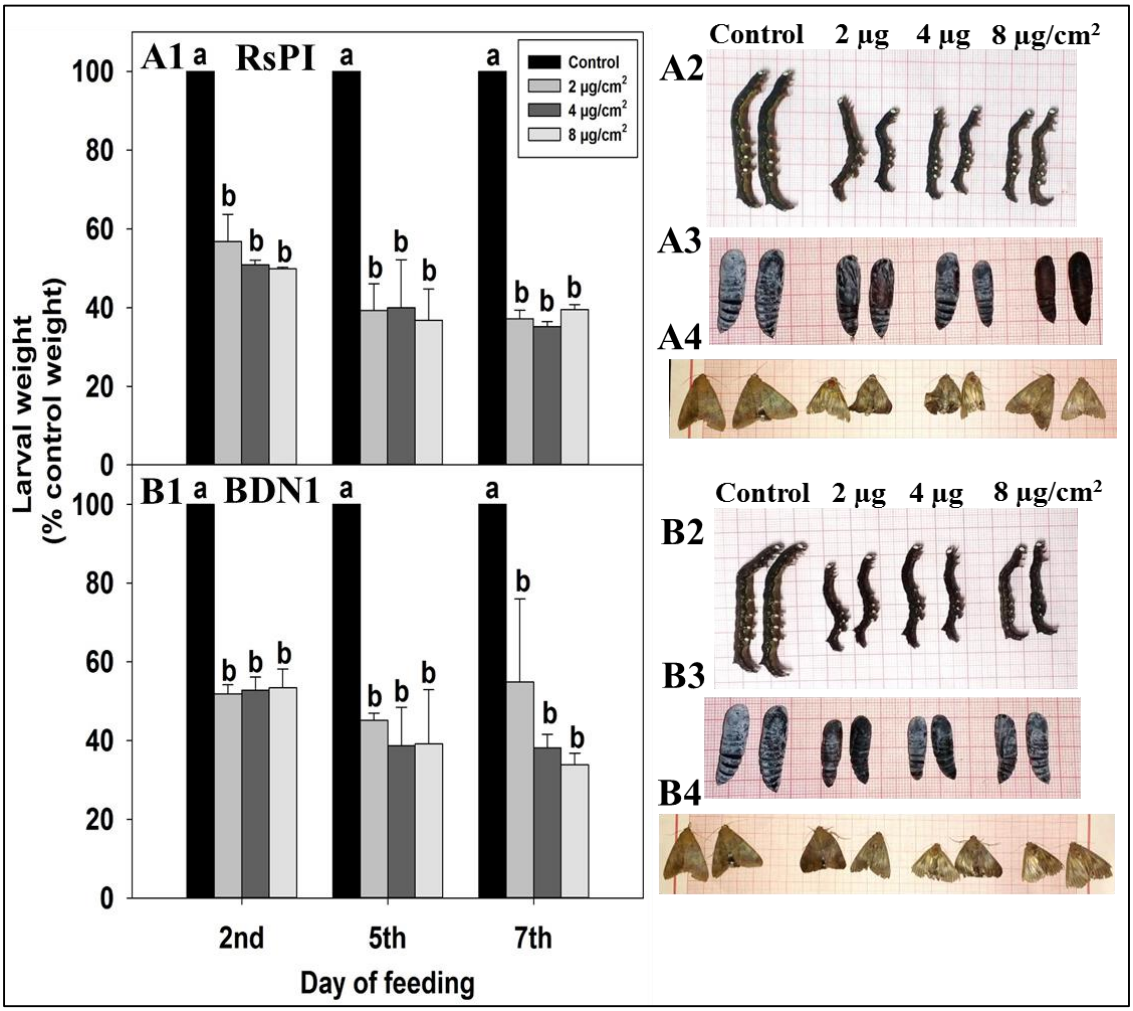


Fig. 4.5. Growth retardation studies of RsPI and BDN1PI on third instar larvae of *A. janata*. (A1) Average weight of third instar larvae of *A. janata* fed up on castor leaves coated with RsPI at a concentration of 2, 4 or 8 µg per square cm for seven days. At the end of seven days, the average weight of the control larvae was found to be 888±6 mg. The pictures of (A2) larvae after 7th day of feeding on RsPI; (A3) pupal and (A4) adult pictures of *A. janata* developed from survived larvae after 7 days of feeding on RsPI at their third instar stage. Likewise, (B1) larval weights and (B2) larval pictures of *A. janata* at 7th day up on feeding on BDN1PI; (B3) pupal and (B4) adult pictures of *A. janata* developed from survived larvae after seven days of feeding on BDN1PI at their third instar stage. All the experiments were performed thrice, and the results are mean ± SE of three different experiments. Statistics were performed as described in materials and methods section 4.2.8. Different lowercase alphabetical letters indicate statistically significant difference ($P<0.05$).

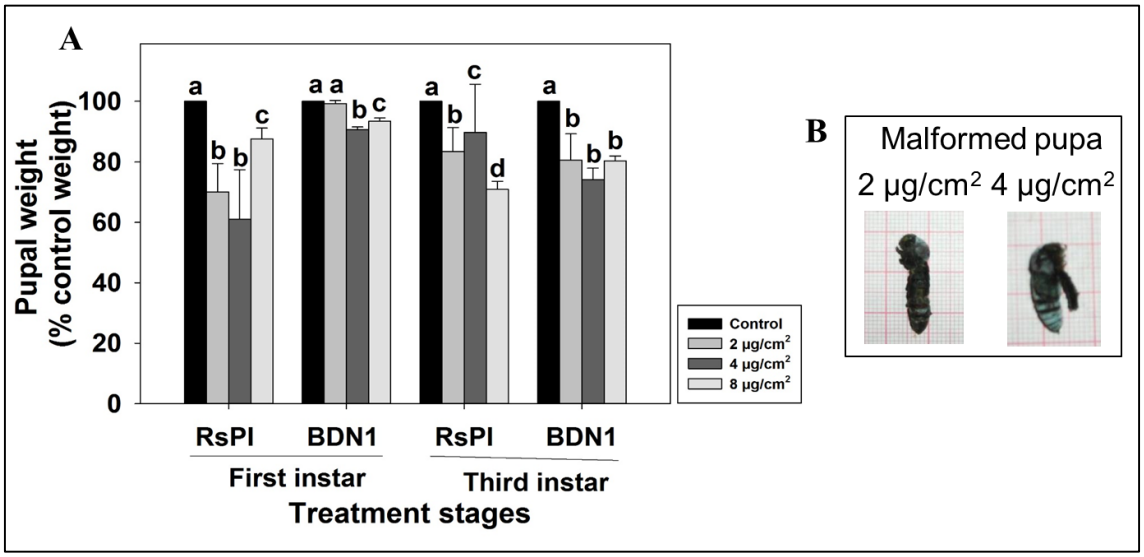


Fig. 4.6. (A) Average weight of pupa derived from first and third instar larvae of *A. janata* upon feeding with RsPI or BDN1PI at different concentrations. The average weight of control pupae obtained from both first and third instar group was 678±122 mg **(B)** A representative of larval-pupal intermediate and malformed pupa formed upon feeding of first instar larvae of *A. janata* with RsPI at 2 and 4 µg per square cm. The results are the mean±SE of three different replicates. Statistics was performed as described in materials and methods section 4.2.8. Different lowercase alphabetical letters indicate statistically significant difference ($P<0.05$).

alkaline pH in its gut environment where the majority of the enzymes belonged to trypsin-like serine proteases and contained a minor content of elastase and chymotrypsin-like proteases (Budatha et al., 2007; Prasad et al., 2009; Swathi et al., 2014). Initial screening analysis confirmed that the crude PI extracts of both *R. sublobata* and BDN1 effectively inhibited the activity of larval gut trypsin-like proteases of *A. janata* than other insects tested in this study. However, the activity of crude PIs from *R. sublobata* was more pronounced than BDN1 against larval gut trypsin-like proteases of *A. janata* (Fig. 2.4A,B). Therefore, an attempt was made to compare the growth retardation potential and mortality inducing capacity of trypsin specific PIs purified from *R. sublobata* and BDN1 seeds.

4.4.2. PIs from *R. sublobata* and BDN1 seeds, and their biochemical characteristic features

RsPI on SDS-PAGE obtained two bands of mass 10 kDa and 20 kDa (Chapter 3 – Fig. 3.1D). Further, the N-terminal sequencing of few spots from these two bands revealed the presence of BBI and Kunitz inhibitors in their seeds (Chapter 3-Figs. 3.2A-C). BBI and Kunitz inhibitors are the two major types of PIs reported from the Leguminosae family. BBIs are small globular proteins with MW in the range of 7-9 kDa whereas Kunitz inhibitors are single or two polypeptide globular proteins with MW of ~20 kDa (Mosolov and Valueva, 2005). BBI is a Janus-faced dual inhibitor which inhibits both trypsin and chymotrypsin proteases simultaneously or independently whereas Kunitz inhibitors bind only to trypsin enzyme (Grosse-Holz and Hoorn, 2016). BBIs are known to undergo self-association in solution to form higher order structures such as dimers, trimers, etc. whereas Kunitz inhibitors rarely undergo dimerization when there is a free cysteine residue (Losso, 2008). BBI isolated from ICPL 332 reported to undergo self-association and both monomer and dimer were observed in gel, while MALDI intact mass analysis revealed up to pentamer (Prasad et al., 2010c). This feature was further strengthened with the finding from BDN1PI which also exhibited

oligomeric nature as evident from Tricine SDS-PAGE (Fig. 4.1D). These double headed BBIs from leguminous plants are stabilized by seven disulphide bridges which renders them to remain structurally stable at high temperatures (Voss et al., 1996). Corroborating with the above general characteristics, BDN1PI existed as i) several low molecular mass isoinhibitors in the range of 7000-8000 Da (Fig. 4.2A,B); ii) small oligomers in SDS-PAGE (Fig. 4.1D); iii) inhibited both trypsin and chymotrypsin proteases (Fig. 4.3D); iv) stable at wide range of temperature and pH conditions (Figs. 4.3A,B) and v) lost both TI and CI activity on reduction with DTT (Figs. 4.3C) which together confirms that the PI purified from BDN1 seeds are BBIs.

4.4.3. Growth retarding potential and larvicidal activity of RsPI and BDN1PI on the neonatal first instar and actively feeding third instar larvae of *A. janata*

Earlier, the PI purified from red gram cultivar variety ICPL 332, when fed to first instar larvae for 6 days, showed a potent decrease in larval weight gain. Further feeding on normal castor leaves for next 20 days resulted in mortality of larvae (Prasad et al., 2010a). Also, the PI purified from a different red gram cultivar variety ICP 7118 when fed to second instar larvae throughout its life cycle induced a dose-dependent decrease in weight gain and resulted in formation of larval-pupal intermediates in *A. janata* (Swathi et al., 2014). However, PIs purified from BDN1 seeds when fed to first instar larvae for 14 days showed that 81% of the insects were dead with 89% reduction in weight gain (Table 4.2). The effect of PIs on insect digestion and growth reduction was reported from Capsicum, winged bean, bitter gourd and soybean Kunitz and BBI inhibitors against *Helicoverpa armigera* (Johnston et al., 1993; Tamhane et al., 2005; Telang et al., 2003; Telang et al., 2009a); bitter gourd, *Archidendron ellipticum* and soybean Kunitz inhibitor against *S. litura* (Bhattacharyya et al., 2007; McManus and Burgess, 1995; Telang et al., 2003); *Adenathera pavonia* PI against *Anagasta kuehniella* (Macedo et al., 2010). Further, as shown in this paper, RsPI, when fed to first instar larvae showed significant effects on growth retardation though the mortality was less as compared to

BDN1PI. When fed to third instar larvae, both BDN1PI and RsPI induced a significant reduction in the larval weight as well as induction of mortality comparable to each other. However, the mortality rate was slightly higher in RsPI than in BDN1PI.

4.4.4. BBIs act as a potent insecticidal agent of *A. janata* larvae

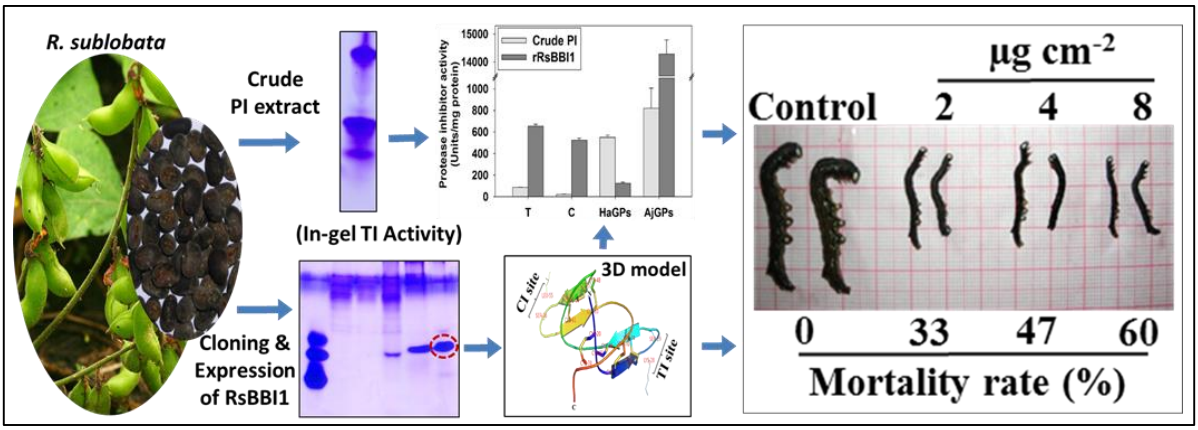
From the literature, transgenic plants expressing non-host PIs from plants such as cowpea and winged bean were observed to be resistant against rice stem borer (Mochizuki et al., 1999; Xu et al., 1996). However, the insects are also able to overcome the effects of PIs by overproducing PI sensitive proteases or producing different proteases to counter the expressed PIs (Bown et al., 1997; Macedo et al., 2015; Wu et al., 1997; Zhu-Salzman and Zeng, 2015). Hence, to counter the insect resistance, expression of multiple inhibitor genes, gene pyramiding (Abdeen et al.; Dunse et al., 2010b; Senthilkumar et al., 2010) or gene fusion strategies (Pardo-López et al., 2009; Zhu-Salzman et al., 2003) were carried out in plants. Also, a lethal level of defense gene expression was obtained in plant leaves through plastid gene expression (Chen et al., 2014). Further, when applied in the field, plants expressing PI genes in combination with IPM practices would yield an excellent strategy to destroy few initial resistant larvae thereby delaying the development of resistance which ultimately reduce the use of chemical pesticides. In terms of *A. janata*, other research groups also examined for the larvicidal effects using plant leaf extracts (Devanand and Rani, 2011), and identified the secondary metabolite and their growth inhibition activity towards this insect (Rani and Devanand, 2013; Sreelatha et al., 2010; Yadav et al., 2014). However, azadirachtin from the neem plant was found to be superior in growth inhibition of *A. janata* larvae, and it also function by decreasing the digestion in the larval gut by inhibiting the production of trypsin enzymes by midgut cells (Timmins and Reynolds, 1992). Thus, neem kernel extract was adopted as one of the ingredients of IPM strategy (Satyagopal et al., 2014). Further, the leaf extract of *Solanum melongena* which

contained BBI is shown to inhibit the growth of third instar larvae of *A. janata* along with other secondary metabolites in the leaf (Devanand and Rani, 2011).

To enhance the crop plant's resistance to insect pests and obtain a good yield, there is a clear need for the identification of resistance genes and their incorporation into the crop plants. Thus, this chapter provides the potential effect of BBIs on *A. janata* from cultivars of red gram at the first instar stage and wild relative *R. sublobata* at the third instar stage. These different BBIs when expressed together may effectively control this larva on the field since each of them compensate for the other at different stages of the larval growth. However, it is also imperative to blend transgenic technology with IPM practices to delay the development of insect resistance.

Chapter 5

Molecular cloning of *RsBBI* and *RsKI* genes: Overexpression in *E. coli* and functional characterization of recombinant RsBBI1 for insecticidal activity against *A. janata*



Isolation of RsBBI from R. sublobata seeds

Molecular cloning of *RsBBI1* and *RsKI* genes: Overexpression in *E. coli* and functional characterization of recombinant RsBBI1 for insecticidal activity against *A. janata*

5.1. Introduction

Many different PIs are produced in plants both constitutively and on induction under various stress conditions. The function of plant PIs as an effective regulator in controlling several abiotic and biotic stresses is well established (Drame et al., 2013; Joshi and Nayak, 2010; Mosolov and Valueva, 2005, 2011). Constitutive overexpression PIs from both monocots and dicots help the plants to overcome the different forms of abiotic stress (Guo et al., 2015b; Srinivasan et al., 2009; Tiwari et al., 2015). Also, several plant PIs are expressed in many transgenic crops to control agricultural insect pests (Table 1.3). However, before generating transgenic plants, prior information such as the identification of PIs from a specific plant, its *in vivo* activity against a specific insect and determination of the nucleotide sequence of the PI are very essential. The results from the present study (chapter 3) indicated that *R. sublobata* possessed both BBI and Kunitz inhibitors in their seeds. Moreover, the separated PIs exhibited effective *in vitro* inhibition against gut trypsin-like proteases of *H. armigera* and *A. janata* at nano gram level (Fig. 3.10). Further, the combined RsPI pool of PIs containing majorly of RsBBI1 (~80%) exhibited potent insecticidal activity against *A. janata* at both first and third instar larval stages (Chapter4; Figs. 4.4, 4.5). Furthermore, the studies of Mallikarjuna et al. (2011) reported that it is difficult to introgress the accessions of Rhynchosia with the cultivars of *C. cajan* by conventional breeding methods. Hence, to utilize the potential of *R. sublobata* PIs in improving the defence system of *C. cajan* and castor, it is imperative to elucidate the nucleotide sequence of both the proteins. Also, it is equally important to establish the potential of these PI gene sequences against the specific insects by bioassays before attempting to integrate these genes in to either *C. cajan* or castor. Therefore, in this chapter, the gene sequences of both the RsBBI1 and RsKI were elucidated. Further, the *RsBBI1* gene was

expressed in *E. coli* and purified. The recombinant RsBBI1 was validated for its biochemical and structural properties, insecticidal potential against *H. armigera* (*in vitro*) and *A. janata (in vitro* and *in vivo*).

5.2. Experimental

R. sublobata seeds (Accession No. 15868) were obtained from the International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, India and grown in a greenhouse at University of Hyderabad, Hyderabad, India. Glycine, MOPS, EDTA, NaCl, SDS, bentonite, formaldehyde, phenol solution saturated with 0.1 M citrate buffer (pH 4.5), lithium chloride, bovine pancreatic trypsin, and chymotrypsin were procured from Sisco Research Laboratories, Mumbai, India. Agarose, isopropyl-β-D-thiogalactoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (x-gal), dimethyl sulfonyl fluoride (DMSF), 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 supplied by Sigma-Aldrich, USA. CNBr activated Sepharose and dithiothreitol (DTT) were procured from GE Healthcare Biosciences Corp., USA. Pfu polymerase, Taq polymerase, and RACE kit were obtained from Thermo Fischer Scientific, India. Gel elution kit and plasmid isolation kit were procured from Qiagen, India.

5.2.1. 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 100mM Glycine-NaOH at pH 9.0, 40 mM EDTA, 100 mM NaCl, 2% SDS and 0.05% Bentonite was added to a finely ground seed powder (100 mg) under liquid N_2 and shaken vigorously before incubating at 42ºC for 20 min. Subsequently, the sample was centrifuged at 11,000 *g* for 20 min at 4ºC and, the supernatant obtained was extracted sequentially with phenol (pH 4.5),

phenol/chloroform (1/1, v/v) and chloroform. The upper aqueous phase was mixed with 12 M LiCl at one-third of its volume and incubated at 80^{o}C for 3h. The sample was centrifuged at 11,000 *g* for 10 min at 4^{o}C. The pellet obtained was dissolved in RNA lysis buffer (500 µL) containing an equal volume of phenol/chloroform/isoamyl alcohol (25/2/1, v/v/v). The sample was vortexed, incubated at 55^{o}C for 5 min and centrifuged at 11,000 *g* for 10 min at 4^{o}C. Further, this step was repeated by adding an equal volume of chloroform/isoamyl alcohol (24/1, v/v) to the upper aqueous phase. Finally, RNA was precipitated by adding 2.2 volumes of 100% ethanol to the upper aqueous phase. The RNA pellet obtained after centrifugation at 11,000 *g* for 10 min at 4^{o}C was air dried and dissolved in RNase/DNase-free water to quantify using NanoDrop. The integrity of RNA was visualized in the formaldehyde-agarose gel using MOPS buffer.

5.2.2. Partial gene amplification of *RsBBI* and *RsKI*

From the isolated RNA, complementary strand DNA (cDNA) was synthesized by following manufacturer's instruction (Verso, cDNA synthesis kit) using an oligo-dT primer. The forward and reverse primers 'RsBBI-F' 'RsBBI-R' for amplifying the partial BBI gene sequence from *R. sublobata* were designed based on the soybean BBI isoinhibitors D-II sequence (NCBI: NM_001249286.1). For amplifying the partial gene sequence of RsKI, degenerate primers RsPI-F and RsPI-R were designed based on the conserved bases between the 2-DE obtained N-terminal sequences and the matched protein sequences in the BLAST analysis (Chapter – 3, Figs. 3.2A & B; Table 5.1). The primers were designed using the OligoAnalyzer 3.1 and verified for the hairpin, self-dimer, and heterodimer (http://eu.idtdna.com/calc/analyzer). A partial *RsBBI* and *RsKI* gene sequences were amplified in a thermal cycler by the following program: 90 ^{o}C-2 min for denaturation of DNA followed by 35 cycles of amplification consisting of denaturation at 90 ^{o}C for 30 s, annealing at 55 ^{o}C for 30 s, extension at 72 ^{o}C for 70 s. The reaction mix was loaded onto 1.5% agarose gel and

Table 5.1. List of primers used to obtain the complete sequence of *RsKI* and *RsBBI* from the immature seeds of *R. sublobata*.

Primer name	Sequence (5'-3') of the primer designed	Nucleotide position*
RsBBI-F	ATGGTGGTGTTAAAGGTGTG	31-50
RsBB1-R	CTGAGTGGCATGAATTCAATC	231-250
Race-RsBBI 3'R	CATCTACACACCTGCACTGG	207-226
Race-RsBBI 3'NR	GGTTGTGACTTTGTGCACCTG	183-203
Race-RsBBI 5'F	CCGCATGGAGTTGAACATGC	87-106
Race-RsBBI 5'NF	CGACCATCATCACCACTCATC	114-134
RsPI-F	GAYTTYGTNYTNGAYACNG	Not applicable
RsPI-R	NACYTTYTGRAAYTGNACNAC	Not applicable
RsKI-F	TTCGACCTGTTCGACACG	121-154
RsKI-R	CTCAGATGATGATCTAGCTCTCTG	616-639
Race-RsKI 3'R	TTGGAACTGGACGACCAAAG	599-618
Race-RsKI 3'NR	GTCGTCACCAGAACAGAACAC	508-528
Race-RsKI 5'F	GGGAAACCAGTGACGCTTTA	280-299
Race-RsKI 5'NF	AAGGACCCGCTGTTAAACTC	413-432
GeneRacer™ 5′Primer	CGACTGGAGCACGAGGACACTGA	Not applicable
GeneRacer™ 5′Nested Primer	GGACACTGACATGGACTGAAGGAGTA	Not applicable
GeneRacer™ 3′Primer	GCTGTCAACGATACGCTACGTAACG	Not applicable
GeneRacer™ 3′Nested Primer	CGCTACGTAACGGCATGACAGTG	Not applicable
RsBBI1-NdeI-F	TAGCCATATGAGCGACCATCATC	112-124
RsBBI1-XhoI-R	ATGCCTCGAGTCACTCAAACCTGTTCAC	364-378

Note: * Nucleotide position concerning the *RsKI* and *RsBBI* sequence as indicated in Fig. 5.1C and Fig. 5.8C, respectively.

visualized for the amplified gene product. The observed gene specific band was excised and gel extracted (Qiagen, gel extraction kit). The eluted partial gene of *RsBBI* and *RsKI* was quantified and directly sequenced using RsBBI-F and RgPI-F primer, respectively. The obtained sequence was analyzed using BLASTn. The DNA sequencing of all samples was performed in Applied Biosystems 3130 xl Genetic Analyzer facilitated by Sandor proteomics, Hyderabad, India.

5.2.3. 5' and 3' RACE for amplifying full-length transcript of *RsBBI* and *RsKI*

The complete sequence of *RsBBI* and *RsKI* were obtained by following the RACE protocol as described in manufacturer's instruction (GeneRacer kit, Invitrogen). For 5' RACE and 3' RACE amplification, the corresponding partial *RsBBI* and *RsKI* gene sequences based RACE primers were designed along with the nested primers (Race-RsBBI 3'R, Race-RsBBI 3'NR, Race-RsBBI 5'F, Race-RsBBI 5'NF for *RsBBI* and Race-RsKI 3'R, Race-RsKI 3'NR, Race-RsKI 5'F, Race-RsKI 5'NF for *RsKI*, Table 5.1) using IDT Oligo server. The primers were examined for the hairpin, self-dimer and heterodimer with the kit supplied primer sequences.

In brief, the following three steps were followed in 5' RACE protocol: 1) Dephosphorylation of the mRNA, 2) Removal of mRNA 5' cap and 3) GeneRacer RNA oligo ligation at the 5' end of the decapped mRNA followed by cDNA synthesis. For 3' RACE, the mRNA was directly used. The cDNA was synthesized for both 5' and 3' RACE using GeneRacer oligo dT RNA. The mRNA was initially incubated at 70 ^{o}C for 5 min to remove RNA secondary structures and later at 42 ^{o}C for 50 min to synthesize both 5' and 3' RACE cDNAs.

The 5' and 3' ends of *RsBBI* and *RsKI* were amplified from 5' and 3' RACE cDNA in a thermal cycler 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 (Invitrogen, USA) by the following program: activation of the enzyme and denaturation of DNA at 90ºC for 5 min followed by 35 cycles of amplification consisting of denaturation at 90ºC for 30 s, annealing at 55ºC for 30 s and extension at 72ºC for 70 s. Finally, 3'ATP overhangs were added using Taq polymerase at 72ºC for 10 min. The amplified products were visualized on 1.5% agarose gel, and the gene-specific amplification was recognized by differences in the sizes of primary and nested PCR products. The amplified products are gel eluted and ligated into pTZ57R/T vector (Thermo Fischer Scientific, Mumbai, India) and transformed into *E. coli* DH5α. The positive colonies were selected by a blue/white colony screening followed by colony PCR using 5' and 3' RACE nested primers. Plasmids isolated from the positive colonies were sequenced using an M13 reverse primer. The full-length cDNA sequence of *RsBBI1-3* and *RsKI1&2* transcripts were deduced from the contig sequence constructed by the amplified 5' and 3' RACE sequences from different colonies.

5.2.4. Three-dimensional (3D) structure prediction

The complete gene sequence of *RsBBI1* and *RsKI1* were submitted to ExPASy translate tool (http://web.expasy.org/translate/) to obtain the ORF. The obtained protein sequences were submitted to TargetP 1.1 Server online (http://www.cbs.dtu.dk/services/TargetP/) for signal peptide prediction. The predicted mature RsBBI1 and RsKI1 protein sequences were 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 the secondary structures, disulfide bridges and inhibitory sites.

5.2.5. Phylogenetic analysis

BBI and Kunitz inhibitor protein sequences from different plants were downloaded from National Center for Biotechnology Information – NCBI (www.ncbi.nlm.nih.gov). The downloaded BBI and Kunitz protein sequences were ClustalW aligned with the translated RsBBI1 & RsKI1 and a phylogenetic tree was constructed by the neighbour-joining method with a bootstrap of 1000 using Mega 6.0 software.

5.2.6. pET23a-*RsBBI1* construct preparation

The N-terminal sequence analysis of a 2-D separated trypsin-specific BBI purified from *R. sublobata* seeds revealed the presence of Ser-Asp-His residues at its N-terminus (Chapter-3, Fig. 3.2C). Therefore, in the present study, an 89 amino acid stretch (31-119) *RsBBI1* gene which started with similar amino acid (Ser-Asp-His) sequence at its N-terminus was cloned while leaving the first 30 amino acid residues. The *RsBBI1* gene was amplified using forward and reverse primers (RsBBI1-NdeI-F and RsBBI1-XhoI-R; Table 5.1), and Pfu polymerase using the PCR program: activation of the enzyme and denaturation of cDNA at 90^{o}C for 5 min followed by 35 cycles of amplification consisting of denaturation at 90^{o}C for 30 s, annealing at 54^{o}C for 30 s and extension at 72^{o}C for 70 s. The amplified product was gel purified, and the yielded *RsBBI1* fragment, and pET23a plasmids were digested individually with XhoI at 37^{o}C overnight followed by NdeI at 37^{o}C for 2 h. The reaction was terminated on incubation at 65^{o}C for 20 min. The digested *RsBBI1* and pET23a were gel eluted and ligated using T4 DNA ligase (New England Bio Labs Incorporation, UK) by incubating at 37^{o}C for 2 h. The pET23a-*RsBBI1* construct 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 DNA sequencing confirmed the *RsBBI1* sequence.

5.2.7. Heterologous expression and purification of rRsBBI1

The pET23a-*RsBBI1* plasmid was transformed into *E. coli* Shuffle T7 express competent cells (NEB, UK) by heat shock at 42ºC for 90 s and plated on LB-Amp plates. A single colony was picked and inoculated into LB-Amp broth and incubated at 37ºC. The overnight grown culture was inoculated into 1 L of LB-Amp broth on the following day and incubated at 37ºC until the culture reaches ~1.1 OD_{600} units. Later, the culture was induced to express the rRsBBI1 with 0.4 mM IPTG at 30ºC for 8 h. 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 80ºC for 30 min and chilled on ice. The supernatant was collected after centrifugation at 11,000 *g*. The rRsBBI1 was purified from the supernatant using trypsin coupled CNBr-Sepharose column in FPLC AKTAprime plus (1 mL Flow rate at 25ºC and 1 Bar pressure) and the bound rRsBBI1 was eluted with 0.01 N HCl. The eluted rRsBBI1 was neutralized with 50 mM Tris-HCl (pH 8.0), concentrated and stored at -20ºC until further use. The purification profile was represented in 15% SDS-PAGE as per Laemmli (1970) and in-gel trypsin inhibitor activity of BBI was visualized as described by Felicioli et al. (1997).

5.2.8. Rearing of larvae and extraction of gut enzymes

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 University of Hyderabad, Hyderabad, India. Both the larvae were reared and maintained at 26 ± 1ºC 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 whereas *H. armigera* larvae were fed on an artificial diet as described in Gupta et al. (2000). The fifth instar larvae were narcotized on ice for 15 min, and the midguts were dissected and collected in iso-osmotic saline (0.15M NaCl). The gut tissues were homogenized in two volumes of 50 mM Glycine-NaOH

(pH 10.5) for *H. armigera* or 50 mM Tris-HCl containing 20 mM $CaCl_2$ (pH 8.2) for *A. janata* and centrifuged at 11,000 *g* for 15 min at 4°C. The supernatant collected was used as the gut enzyme extract to analyze the trypsin-like protease activity and stored at -20°C.

5.2.9. *In vitro* gut trypsin-like protease activity and *in vivo* feeding assays

The assay for gut trypsin-like protease(s) of *A. janata* (AjGPs) or *H. armigera* (HaGPs) was performed as described in Swathi et al. (2014). The AjGPs or HaGPs activity was determined by monitoring the rate of formation of *p*-nitroanilide from a chromogenic substrate BAPNA (1 mM) at 37°C in 50 mM Tris-HCl containing 20 mM $CaCl_2$ (pH 8.2) for *A. janata* or 50 mM Glycine-NaOH (pH 10.5) for *H. armigera*. One AjGP or HaGP unit is defined as the amount of enzyme extract that increases the absorbance of reaction medium by 1.0 O.D. after incubation with BAPNA for 45 min. The molar extinction coefficient (M^{-1} cm^{-1}) of *p*-nitroanilide is equivalent to 8,800 at 410 nm. AjGP or HaGP activity was analyzed from at least three to four independent larval gut enzyme extractions prepared from two/three different biological sets of *A. janata* and *H. armigera*.

The *in vivo* feeding of *A. janata* larvae was performed as described in Prasad et al. (2010a). The second instar larvae were allowed to grow on castor leaf coated with rRsBBI1 at 2, 4 and 8 µg per cm^2 by no choice feeding method. 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 the larvae was monitored on alternative days. Approximately 15 larvae were used in each treatment, and at least three independent experiments were performed to evaluate the insecticidal activity of rRsBBI1 on *A. janata*.

5.2.10. Protease inhibitor assay

The inhibitory activity of rRsBBI1 was assessed against an aliquot of pancreatic trypsin 10 µg), chymotrypsin (80 µg) or appropriate volumes of HaGPs/AjGPs which give 1.0 optical

density due to the formation of *p*-nitroanilide when incubated with their respective substrates BAPNA/GLUPHEPA at 1 mM concentration for 45 min at 37°C. 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. But, the assay buffer for HaGPs contained 50 mM Glycine-NaOH at pH 10.5 (Prasad et al., 2010c; Swathi et al., 2014). However, the activity of different proteases was measured after incubating with rRsBBI1 for 15 min at 37°C. The reaction was terminated with 30% acetic acid (v/v), and the absorbance at 410 nm was recorded in a UV-visible spectrophotometer (UV-1700, Shimadzu, Japan). The molar extinction coefficient (M^{-1} cm^{-1}) for *p*-nitroanilide at 410 nm is equivalent to 8,800. One trypsin inhibitor (TI), chymotrypsin inhibitor (CI), *A. janata* gut trypsin-like protease inhibitor (AjGPI) or *H. armigera* gut trypsin-like protease inhibitor (HaGPI) unit was defined as the amount of rRsBBI1 required to inhibit 50% hydrolysis of BAPNA or GLUPHEPA by relevant proteases under the optimal assay conditions.

5.2.11. 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 different concentrations (100, 200 and 300 nM) for 15 min at 37°C. This was followed by incubation with corresponding substrates at various 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 (Systat Software Inc. San Jose, California). Further, the residual trypsin/chymotrypsin activities at different molar ratios (0.05 to 2.0) of rRsBBI1 to trypsin/chymotrypsin were determined by titrating different concentrations of rRsBBI1 with a fixed concentration (1µM) of trypsin or chymotrypsin, respectively (Prasad et al., 2010c).

5.2.12. Stability studies

The effect of temperature, pH, and DTT on the TI and CI activities of rRsBBI1 was examined as described in Prasad et al. (2010c). Temperature stability studies were performed by incubating rRsBBI1 for 30 min at a wide range of temperatures (37, 40, 50, 60, 70, 80, 90, 100 ^{o}C) using a thermostated water bath (Julabo F10). The residual TI and CI activities of rRsBBI1 was determined by the standard method as described in section 5.2.10 after cooling the samples to room temperature. Similarly, the stability of TI and CI activities of rRsBBI1 at different pH from 2.0 to 12.0 was determined by incubating rRsBBI1 at 37^{o}C for 1 h using the following buffers at 50 mM concentration: Glycine-HCl (pH 2-3), sodium acetate-acetic acid (pH4-5), sodium phosphate (pH 6.0), Tris-HCl (pH 7-9) and Glycine-NaOH (pH 10-12). The residual TI or CI activity of rRsBBI1 in each sample was measured as described above. The effect of reducing agent on TI and CI activities of rRsBBI1 was determined after incubating rRsBBI1 with different concentrations of DTT (0.05 - 10.0 mM) in 25 mM NH_4HCO_3 at 56^{o}C for 45 min. Subsequently, the samples were incubated in the dark for 1 h with iodoacetamide at twice the amount of corresponding DTT concentration to terminate the reaction before analyzing the residual TI and CI activities.

5.2.13. Circular dichroism (CD)

Secondary structural elements were determined in a J-1500 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a peltier type temperature controller and a thermostated cell holder. The nitrogen gas was continuously flushed on to the instrument including sample chamber while recording the spectra. The ellipticity was measured at far-UV region (190-260 nm) in a 1 mm path length cuvette at a scan speed of 50 nm/min. A minimum of three scans were acquired at 25^{o}C using 0.05 mg/mL of rRsBBI1 in 5 mM Tris-HCl (pH 8.0) containing 5 mM NaCl. The secondary structural elements were estimated using SpectraManager 2.0 software after subtracting the buffer spectra from rRsBBI1 spectra.

5.2.14. Statistical Analysis

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

5.3. Results

5.3.1. Cloning and sequencing of *RsKI*

A partial *RsKI* of approximately 500 bp length was amplified from the synthesized cDNA (Fig. 5.1A). Further, 5' and 3' ends of the transcript were amplified by 5'and 3'RACE, respectively (Fig. 5.1B). RACE experiments yielded smaller nested products by the expected number of bases than the primary RACE products, confirming the desired gene amplification. DNA sequencing of the nested products obtained from 5' and 3'RACE showed 527 bp and 346 bp, respectively. Sequence alignment of 5' and 3'RACE products yielded complete *RsKI1* mRNA transcript of 758 bases, and it contained 597 bp open reading frame (ORF), 45 bp 5' UTR and 116 bp 3' UTR (Fig. 5.1C). The complete *RsKI1* CDS sequence was submitted to NCBI GenBank with accession number KT119633.1. Further, the generated cDNA library contained another gene *RsKI2* which is a 770 bp gene containing 45 bp of 5' UTR, 597 bp of ORF and 128 bp of 3' UTR. Further, RsKI1 vary with RsKI2 in the ORF only at the 115th nucleotide position (A→T) and it codes for Phenylalanine at the P1 position of reactive site at 90th amino acid (L→F; Fig. 5.2&5.3). Further, analysis of *RsKI1* by BLASTn confirmed that the sequence obtained belonged to the Kunitz inhibitor family (Fig. 5.4).

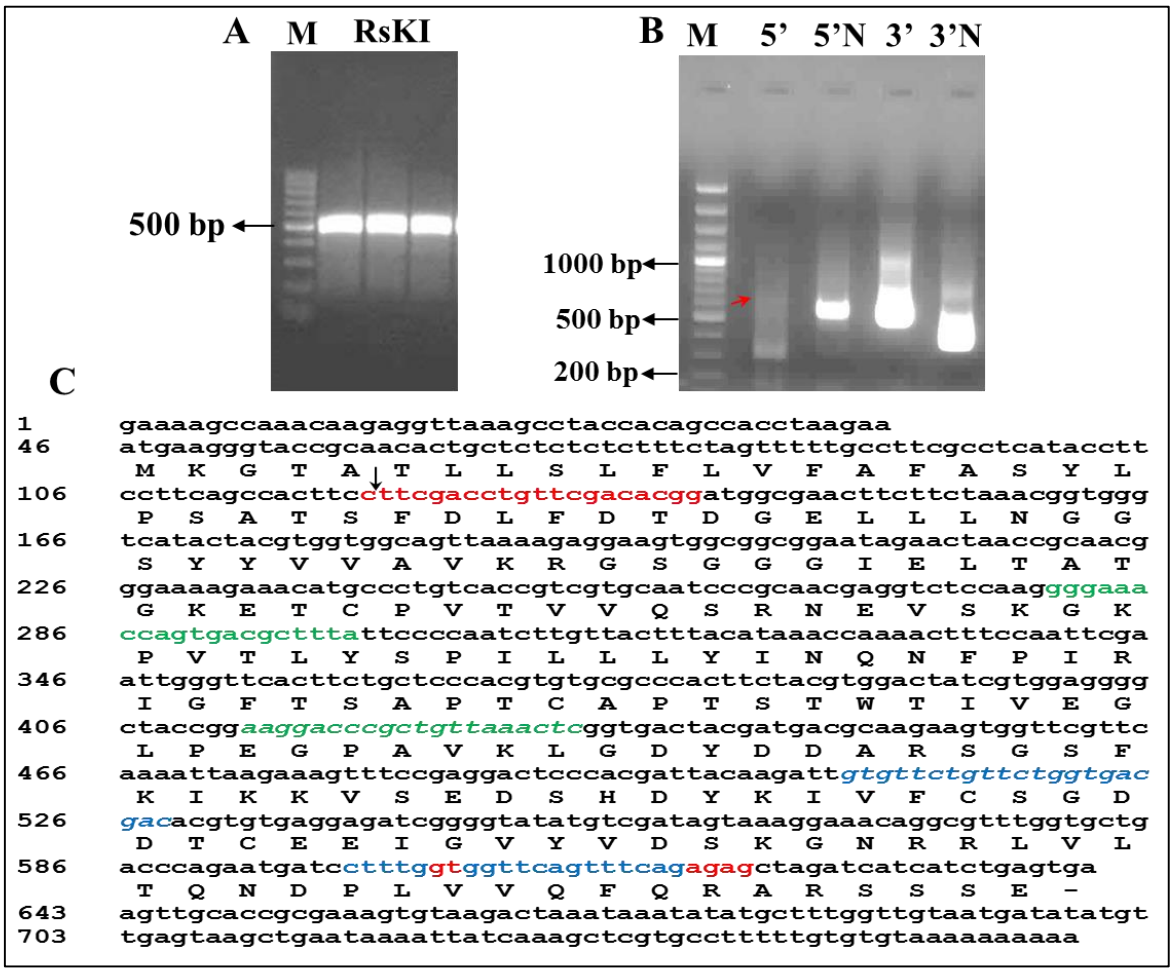


Fig. 5.1. Synthesis of full length cDNA and cloning of *RsKI*. (A) PCR-amplified partial cDNA fragment (~500 bp) of *RsKI* from immature seeds of *R. sublobata*. Lane 1 (M) is loaded with 100 bp DNA ladder and lanes 2-4 contained partially amplified *RsKI* gene product; **(B)** RACE amplified 5' and 3' fragments of *RsKI*. Lane M contained 100 bp DNA ladder and Lanes 5' and 5'N are loaded with the 5' RACE primary and nested gene products, Lanes 3' and 3'N are loaded with 3' RACE primary and nested gene products of *RsKI*; **(C)** Complete cDNA (758 bases) of *RsKI1* and *in silico* translated amino acid sequence. The arrow indicates the end of the signal peptide and the starting point of the mature protein (173 amino acid stretch). Primer sequences are shown in color. Red color indicate the primers used to get the partial sequence, green color indicate the 3'RACE forward primers, and blue color indicate the 5'RACE reverse primers. Nested primers are italicized. The complete *RsKI1* sequence is submitted to NCBI with accession number KT119633.1.

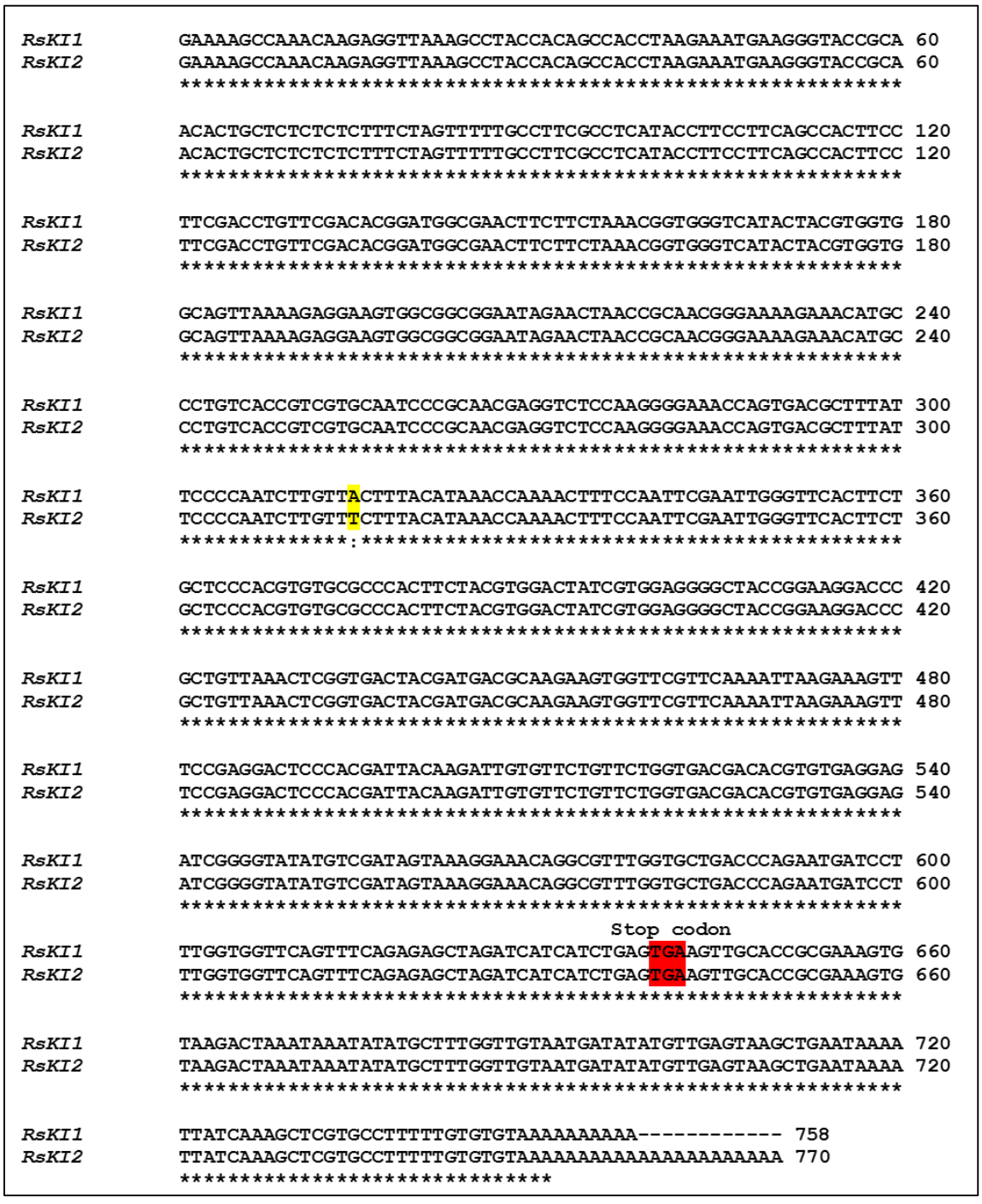


Fig. 5.2. Pairwise alignment of the *RsKI* gene sequences obtained from the immature seeds of *R. sublobata* by RACE procedure. The nucleotide differing in both the genes are colored in yellow and the stop codon is marked in red colour. Identical nucleotides are marked with asterisks '*' and conserved mutation is marked with colon ':'.

```
                  Signal peptide
RsKI1        MKGTATLLSLFLVFAFASYLPSATSFDLFDTDGELLLNGGSYYVVAVKRGSGGGIELTAT 60
RsKI2        MKGTATLLSLFLVFAFASYLPSATSFDLFDTDGELLLNGGSYYVVAVKRGSGGGIELTAT 60
             ************************************************************

RsKI1        GKETCPVTVVQSRNEVSKGKPVTLYSPILLLYINQNFPIRIGFTSAPTCAPTSTWTIVEG 120
RsKI2        GKETCPVTVVQSRNEVSKGKPVTLYSPILFLYINQNFPIRIGFTSAPTCAPTSTWTIVEG 120
             ****************************:*******************************

RsKI1        LPEGPAVKLGDYDDARSGSFKIKKVSEDSHDYKIVFCSGDDTCEEIGVYVDSKGNRRLVL 180
RsKI2        LPEGPAVKLGDYDDARSGSFKIKKVSEDSHDYKIVFCSGDDTCEEIGVYVDSKGNRRLVL 180
             ************************************************************

RsKI1        TQNDPLVVQFQRARSSSE 198
RsKI2        TQNDPLVVQFQRARSSSE 198
             ******************
```

Fig. 5.3. Pairwise alignment of the protein sequences of RsKI1 and RsKI2 deduced from the respective gene sequences. The cysteine residues are marked in green and the reactive site P1-P1' amino acids are marked in yellow. Note that both RsKI1 and RsKI2 are differing only at the reactive site P1 position. *RsKI1* contain 'Leu' whereas *RsKI2* contain 'Phe' at the P1 position. The signal peptide is marked in cyan colour. Identical amino acids are marked with asterisks '*' and conserved mutation is marked with colon ':'.

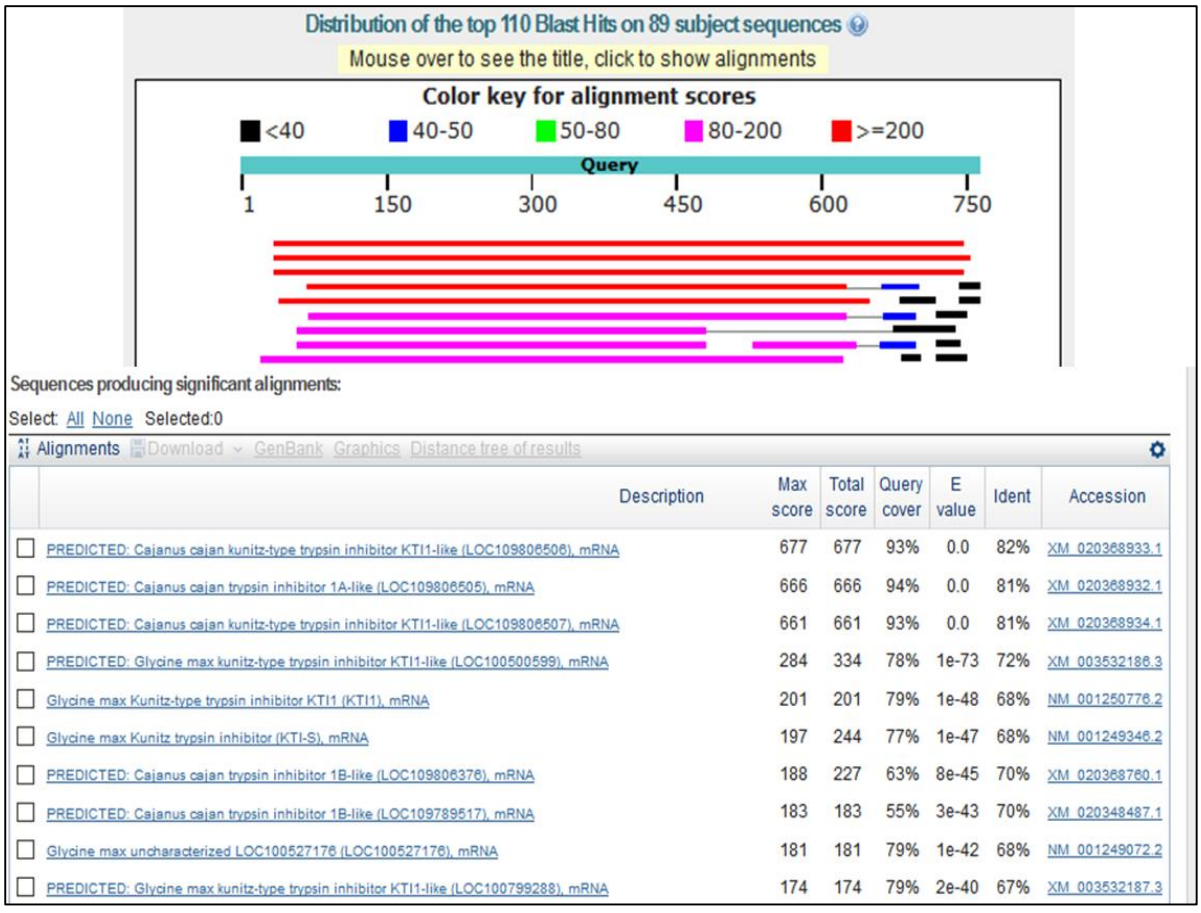


Fig. 5.4. BLASTn analysis of the *RsKI1* obtained through RACE procedure indicate that the isolated sequence is a member of *C. cajan* and soybean Kunitz trypsin inhibitor family.

In silico translation by ExPASy Translate tool indicated that the *RsKI1* (bp 46-636) codes for 198 amino acids (Fig. 5.1C). Further, signal peptide analysis of the translated protein showed that RsKI1 possessed a 25 amino acids "MKGTATLLSLFLVFAFASYLPSATS" signal sequence and 173 amino acids stretch mature protein sequence of mass 18,792 Da (Fig. 5.1C). Multiple sequence alignment of RsKI with the available Kunitz inhibitors from other leguminous plants are depicted in Figure. 5.5. The obtained RsKI is a chymotrypsin inhibitor containing 'Leu-Leu' or 'Phe-Leu' amino acids at the P1-P1' positions of reactive site (Fig. 5.3). RsKI contained four cysteine residues and their alignment with other Kunitz inhibitors were marked in green colour (Fig. 5.5).

5.3.2. Phylogenetic analysis and 3D modeling of RsKI

Proteins performing the same function in different plants are related to each other by their conserved amino acid sequence and structural motif, and these features are used to analyze the evolutionary relationship among them through sequence homology. The phylogenetic relationship of RsKI from *R. sublobata* with Kunitz inhibitors from other legumes and cereals revealed that RsKI is phylogenetically close to soybean Kunitz inhibitors (Fig. 5.6). RsKI out grouped from soybean Kunitz inhibitor though both of them belong to a single clade. Further, three-dimensional structure of RsKI was predicted from residues 27 to 196 using winged bean chymotrypsin inhibitor (PDB ID-1EYL) as a template (Fig. 5.7A). The modeled RsKI exhibited chymotrypsin reactive site. The amino acids which are identical between RsKI and the template 1EYL used for 3D modeling are represented by the same color in pair-wise alignment (Fig. 5.7 B).

```
RsKI          MKGTATLLSL FLVFAFAS-Y LPSATSFDLF DTDGELL-LN GGSYYVVAVK R-GSGGGIEL TATGKETCPV 67
ITRA_SOYBN    MK-STIFF-L FLFCAFTTSY LPSAIADFVL DNEGNPL-EN GGTYYILSDI T-AFGG-IRA APTGNERCPL 65
ICW3_PSOTE    -MKSTTFLAL FLLSAIISHL PSSTADDDLV DAEGNLV-EN GGTYYLLPHI W-AHGGGIET AKTGNEPCPL 67
ITRY1_BAUR    ---------- ---------- ------SVVL DTKGQPVRNA ADAYYLEPVA R-GDGG-LAL AKVGNEAEPK 42
ITRY_BAUVA    ---------- ---------- ------DTLL DTDGEVVRNN GGPYYIIPAF R-GNGGGLTL TRVGSETCPR 43
BBKI_BAUBA    ---------- ---------- ------SVVV DTNGQPVSNG ADAYYLVPVS H-GHAG-LAL AKIGNEAEPR 42
IFXA_BAUUN    ---------- ---------- ------DIVL DTDGKPV-NN GGQYYIIPAF R-GNGGGLEL TRVGRETCPH 42
DRTI_DELRE    ---------- ---------- ---SDAEKVY DIEGYPV-FL GSEYYIVSAI IGAGGGGVRP GRTRGSMCPM 46
ITRY_LEULE    ---------- ---------- ------QVLV DLDGDPLYNG M-SYYILPVA R-GKGGGLEL ARTGSESCPR 42
ICI2_CANLI    ---------- ---------- ---NDVDVVM DASSKPI-FP GGEYYIMPAI WGPPGGGVRL AKTRNSDCPV 46
ITRY_ENTCO    ---------- ---------- ------KELL DSDGDILRNG G-TYYILPAL R-GKGGGLEL AKTGDETCPL 42
ITRY_ACACO    ---------- ---------- ------KELL DADGDILRNG G-AYYILPAL R-GKGGGLTL AKTGDESCPL 42
IDE3_ERYCA    ---------- ---------- -------VLL DGNGEVV-QN GGTYYLLPQV W-AQGGGVQL AKTGEETCPL 41
IDE3_ERYLA    ---------- ---------- -------VLL DGNGEVV-QN GGTYYLLPQV W-AQGGGVQL AKTGEETCPL 41
IDE3_ERYVA    ---------- ---------- -------VLL DGNGEVV-QN GGTYYLLPQV W-AQGGGVQL AKTGEETCPL 41

RsKI          TVVQSRN--- EVSKGKPVTL YSPILLL-YI NQNFPIRIGF TS---APTCA PT-STWTIVE GLPE-GPAVK 128
ITRA_SOYBN    TVVQSRN--- ELDKGIGTII SSPYRIR-FI AEGHPLSLKF DSFAVIMLCV GIPTEWSVVE DLPE-GPAVK 130
ICW3_PSOTE    TVVRSPN--- EVSKGEPIRI SSQFLSL-FI PRGSLVALGF AN---PPSCA AS-PWWTVVD -SPQ-GPAVK 127
ITRY1_BAUR    AVVLDP---- NHRPGLTVRF ETPLRIN-II KESFFLNIKF VPS-------- ---SSESEVW EVRQQYPEGL 97
ITRY_BAUVA    TVVQASS--- EHSDGLPVVI SALPRSL-FI STSWRVTIQF VEA----TCI PKPSFWHIPQ DSELEG-AVK 104
BBKI_BAUBA    AVVLDP---- HHRPGLPVRF ESPLRIN-II KESYFLNIKF GPS------- ---SSDSGVW DVIQQDPIGL 97
IFXA_BAUUN    TVVQASS--- EISNGLPVMI AALPRTM-FI STAWRVSIQF LKVP---TCT PKPSYWHIPQ DSDMEG-SVE 103
DRTI_DELRE    SIIQEQ---S DLQMGLPVRF SSPEESQGKI YTDTELEIEF VEK---PDCA ES-SKWVIVK DSGEAR--VA 107
ITRY_LEULE    TVVQTRS--- ETSRGLPARL ASPYRIL-IG S-NIPLTIEF QPQK--PYSC HGHSSRSLQW KVEKTQ-MVK 104
ICI2_CANLI    TVLQDYG--- EVIFGQPVKF TLPGRGSGLI ITNTPVE-EF IKK---PECA SS-SKWSVFV DDEIEKACVG 108
ITRY_ENTCO    NVVQARG--- ETKRGRPAII WTPPRIA-IL TPAFYLNIEF QTKD--LPAC LREYSR-LPR EEEQHS-EVK 104
ITRY_ACACO    TVVQAQS--- ETKRGLPAVI WTPPKIA-IL TPGFYLNFEF QPRD--LPAC LQKYST-LPW KVEGESQEVK 105
IDE3_ERYCA    TVVQSPN--- ELSDGKPIRI ESRLRSA-FI PDDDKVRIGF AY---APKCA PS-PWWTVVE DEQE-GLSVK 102
IDE3_ERYLA    TVVQSPN--- ELSDGKPIRI ESRLRST-FI PDDDEVRIGF AY---APKCA PS-PWWTVVE DEQE-GLSVK 102
IDE3_ERYVA    TVVQSPN--- ELSNGKPIRI ESRLRSA-FI PDDDKVRIGF AY---APKCA PS-PWWTVLE DEQE-GLSVK 102

RsKI          LGDYDDARSG --SFKIKKVS -E-DSHD-YK IVFC---SGD DT--CEEIGV YVD-SKGNRR LVLTQNDPLV 187
ITRA_SOYBN    IGENKDAMDG --WFRLERVS -DDEFNN-YK LVFCPQQAED DK--CGDIGI SIDHDDGTRR LVVSKNKPLV 194
ICW3_PSOTE    LSQQKLPEKD ILVFKFEKVS --HSNIHVYK LLYCQHDEE- DVKCDQYIGI HRD-RNGNRR LVVTEENPLE 193
ITRY1_BAUR    AVKVTDTKSL VGPFRVEKEG ------EGYK IVYYPDRG-- --ETGLDIGL VHR-NE-KYY LAVKDGEPFV 155
ITRY_BAUVA    VGASDERFPL --EFRIERVS -----EDTYK LMHCSS---- TSDSCRDLGI SID-EEGNRR LVVRDENPLL 162
BBKI_BAUBA    AVKVTDTKSL LGPFKVEKEG ------EGYK IVYYPERG-- --QTGLDIGL VHR-ND-KYY LAVKDGEPCV 155
IFXA_BAUUN    VRV-DERFPL --EFRIEKVS -----EDAYK LMHCPS---- SSDSCRDLGI AID-EENNRR LVVRDGKPLL 160
DRTI_DELRE    IGGSEDHPQG ELVRGFFKIE KLGS--LAYK LVFCPK---S SSGSCSDIGI NYE-GRRSLV LKSSDDSPFR 171
ITRY_LEULE    IASSDEEQRL FGPFQIQPYR ------NHYK LVYCESESRN HHDDCRDLGI SID-DQQNRL LVVKNGDPLV 177
ICI2_CANLI    IGGHEDHPGE QVFSGTFTIQ KSRTPYNSYK LVFCE----S DSSTCSDIGR YDN-NEGGRR LILTHHNPFQ 173
ITRY_ENTCO    LAPKE-EAAA FGXEKLKPYR ------DDYK IVYCEGGS-- DDDSCKDLGI SID-DENNRR LVVKDGDPLA 164
ITRY_ACACO    IAPKEKEQFL VGSFKIKPYR ------DDYK LVYCEGNS-- DDESCKDLGI SID-DENNRR LVVKDGHPLA 166
IDE3_ERYCA    LSEDESTQFD -YPFKFEQV- --SDQLHSYK LLYCEG---- KHEKCASIGI NRD-QKGYRR LVVTEDYPLT 163
IDE3_ERYLA    LSEDESTQFD -YPFKFEQV- --SDKLHSYK LLYCEG---- KHEKCASIGI NRD-QKGYRR LVVTEDNPLT 163
IDE3_ERYVA    LSEDESTQFD -YPFKFEQV- --SDKLHSYK LLYCEG---- KHEKCASIGI NRD-QKGYRR LVVTEDNPLT 163
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RsKI          VQFQRARSSS E--------- -- 198
ITRA_SOYBN    VQFQKLDKES LAKKNHGLSR SE 221
ICW3_PSOTE    LVLLKAKSET ASSH------ -- 207
ITRY1_BAUR    FKIRKATDE- ---------- -- 164
ITRY_BAUVA    VRFKKANQDS EK-------- -- 174
BBKI_BAUBA    FKIRKATDE- ---------- -- 164
IFXA_BAUUN    VRFKEANQDS E--------- -- 171
DRTI_DELRE    VVFVKPRSGS ETES------ -- 185
ITRY_LEULE    VQFAKANRGG DD-------- -- 189
ICI2_CANLI    VVFMDASTFD GTIRSDG--- -- 190
ITRY_ENTCO    VRFVKAHRRG ---------- -- 174
ITRY_ACACO    VRFEKAHRSG ---------- -- 176
IDE3_ERYCA    VVLKKDESS- ---------- -- 172
IDE3_ERYLA    VVLKKDESS- ---------- -- 172
IDE3_ERYVA    VVLKKDESS- ---------- -- 172
```

Fig. 5.5. **Multiple sequence alignment of RsKI1 with the reported Kunitz inhibitors.** The chymotrypsin reactive site residues are indicated in yellow color and the cysteine residues were marked in green colour. All the Kunitz inhibitor sequences were downloaded from Uniprot data base. Accession numbers of the Clustal W aligned Kunitz inhibitors are from the following families: ITRA_SOYBN – *G. max*; ICW3_PSOTE – *Psophocarpus tetragonolobus*; ITRY1_BAUR – *Bauhinia rufa*; ITRY_BAUVA – *B. variegata*; BBKI_BAUBA – *B. bauhinioides*; IFXA_BAUUN – *B. ungulata*; DRTI_DELRE – *Delonix regia*; ITRY_LEULE – *Leucaena leucocephala*; ITRY_CANLI – *Canavalia lineata*; ITRY_ENTCO – *Enterolobium contortisiliquum*; ITRY_ACACO – *Acacia confusa*; IDE3_ERYCA – *Erythrina caffra*; IDE3_ERYLA – *E. latissima*; IDE3_ERYVA – *E. variegata*.

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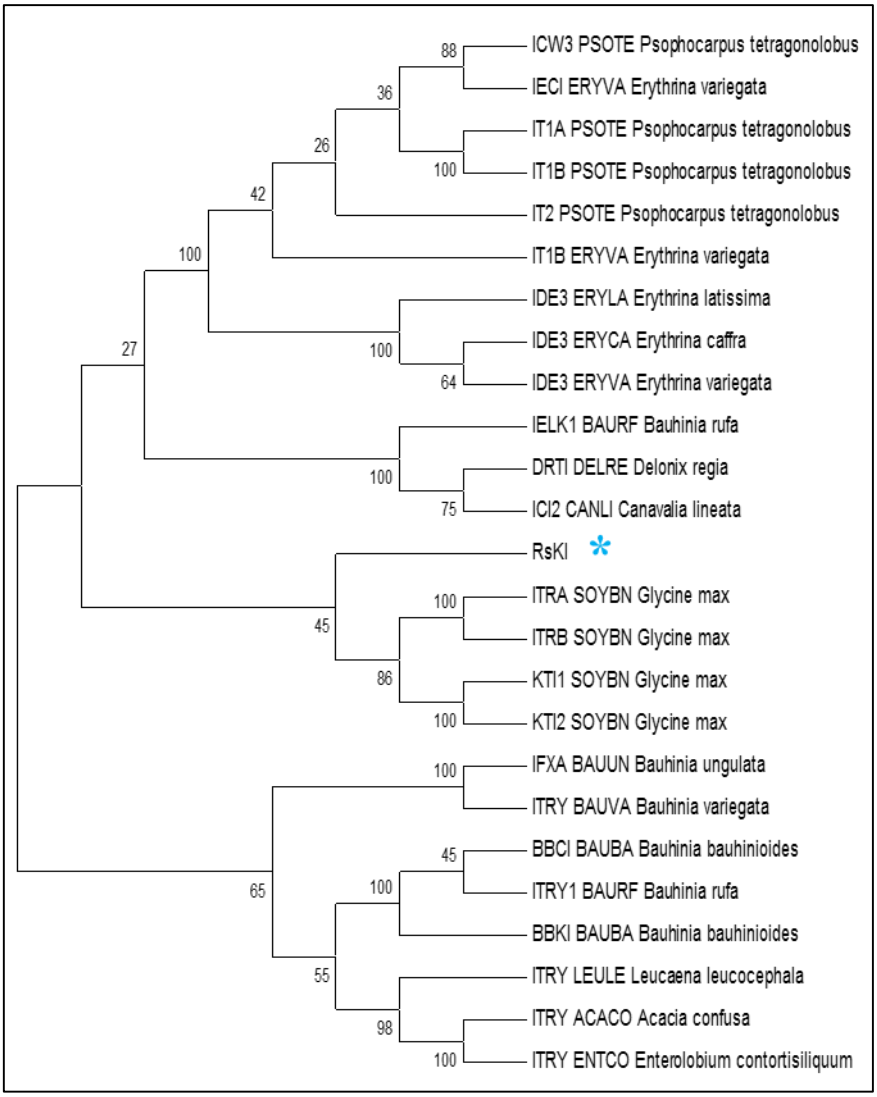

Fig. 5.6. Phylogenetic analysis of RsKI1. (A) Phylogenetic analysis of deduced RsKI with reported Kunitz inhibitors was performed in MEGA 6.0 software using the Neighbour-Joining algorithm with a bootstrap of 1000. The Kunitz inhibitors from individual plants are indicated in the phylogenetic tree. The bootstrapping values were mentioned at the branches to depict the grouping robustness. All the sequences were downloaded from Uniprot. The accession numbers and the plant names are indicated in the phylogenetic tree. The position of *R. sublobata* Kunitz inhibitor (RsKI1) in the tree is marked with asterisk '*'.

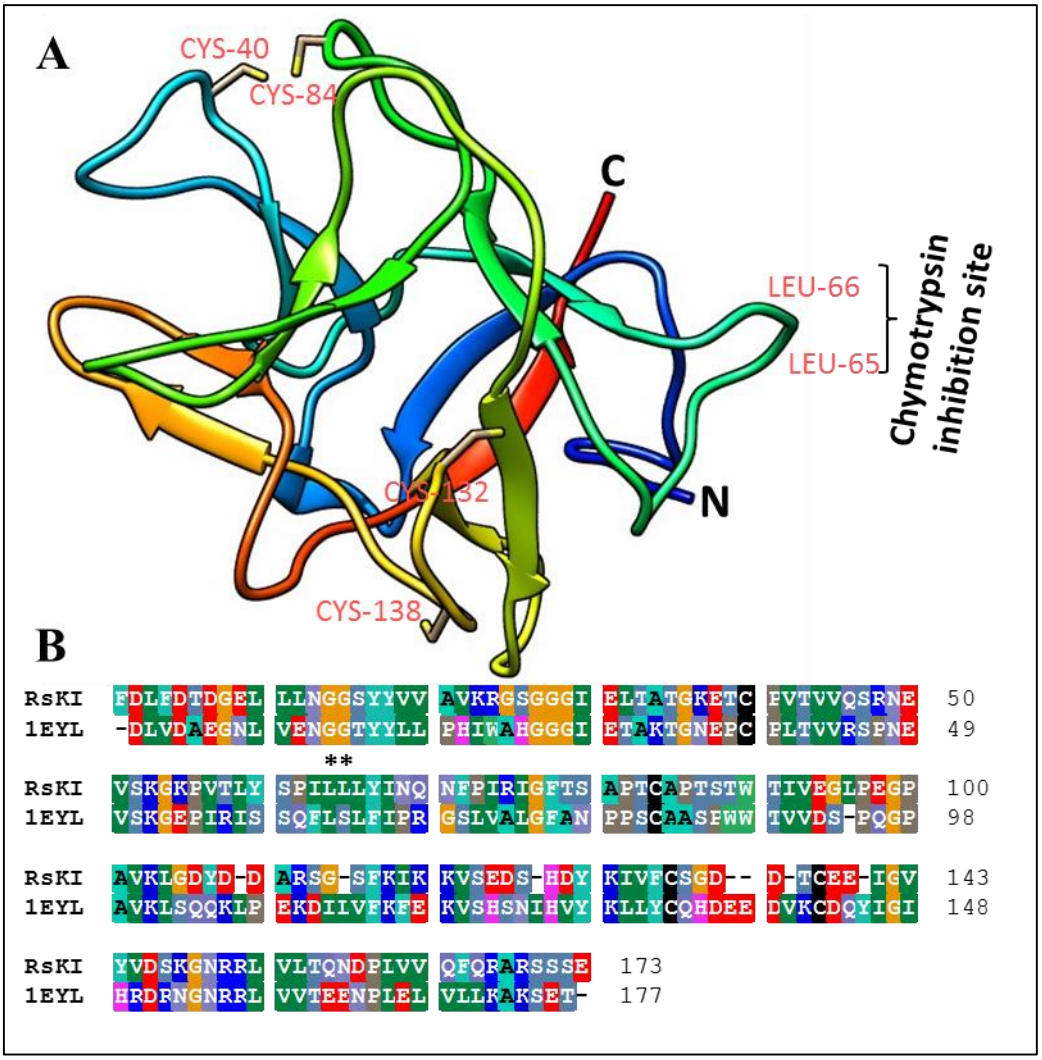

Fig. 5.7. Molecular modeling of RsKI1. (A) The predicted three-dimensional SWISS-MODEL of RsKI protein was obtained using 1EYL from winged bean as the template. The disulfide bonds, trypsin, and chymotrypsin reactive sites were visualized using PyMol software and represented as sticks. The N- and C-terminus ends were marked as N and C, respectively; **(B)** Pairwise alignment of RsBBI1 and 1BBI sequences: The reactive site P1-P1' amino acids are marked with asterisks '*'.

5.3.3. Cloning and sequencing of *RsBBI*

A partial *RsBBI* of approximately 250 bp length was amplified from the synthesized cDNA (Fig. 5.8A). Further, 5' and 3' ends of the transcript were amplified by 5'and 3'RACE, respectively (Fig. 5.8B). RACE experiments yielded smaller nested products by the expected number of bases than the primary RACE products, confirming the desired gene amplification. DNA sequencing of the nested products obtained from 5' and 3'RACE showed 207 bp and 361 bp, respectively. Sequence alignment of 5' and 3'RACE products yielded complete *RsBBI1* mRNA transcript of 505 bases, and it contained 360 bp open reading frame, 21 bp 5' UTR and 124 bp 3' UTR (Fig. 5.8C). The complete *RsBBI1* CDS sequence was submitted to NCBI GenBank with accession number KT119632.2.

Further, the cDNA library of the *R. sublobata* seeds showed two more BBI genes – *RsBBI2* and *RsBBI3* (Fig. 5.9). *RsBBI2* and *RsBBI3* contained 523 and 538 bp with 357 bp open reading frame which encodes a 118 residue protein. Further, *RsBBI2* contains 21 bp of 5' UTR and 145 bp of 3' UTR and *RsBBI3* contains 21 bp of 5' UTR and 160 bp of 3' UTR. RsBBI2 differs from both RsBBI1at the 45th amino acid (D→E) and in lacking the 119th Glutamate residue at the C-terminal end (Fig. 5.10). *RsBBI3* encodes the 45th Glu residue similar to *RsBBI2* but differs from *RsBBI1*&*2* in encoding 12 Cysteine residues which may form only 5 disulphide bridges (Fig. 5.10). The cysteine at the 6th and 14th position are replace by Gly (C→G) and Ser (C→S). Further, as per the general disulphide bond frame work of BBIs (Chapter1–Fig. 1.8C) Cys at the 6th position bonds with Cys at the 2nd position and Cys at 14th position bonds with the Cys at the 1st position. Moreover, Cys 6-Cys 2 appears as an accessory bond to the disulphide bridge (Cys4-Cys5) which holds the TI loop. Since Gly is present at the 6th position, it can't form disulphide bridge with the Cys 2 and hence may lead to a slightly distorted TI loop or in other words, a more flexible TI loop. On the other hand, Ser at the 14th Cys position can't form any disulphide bond with the Cys at the 1st position. But, it may form

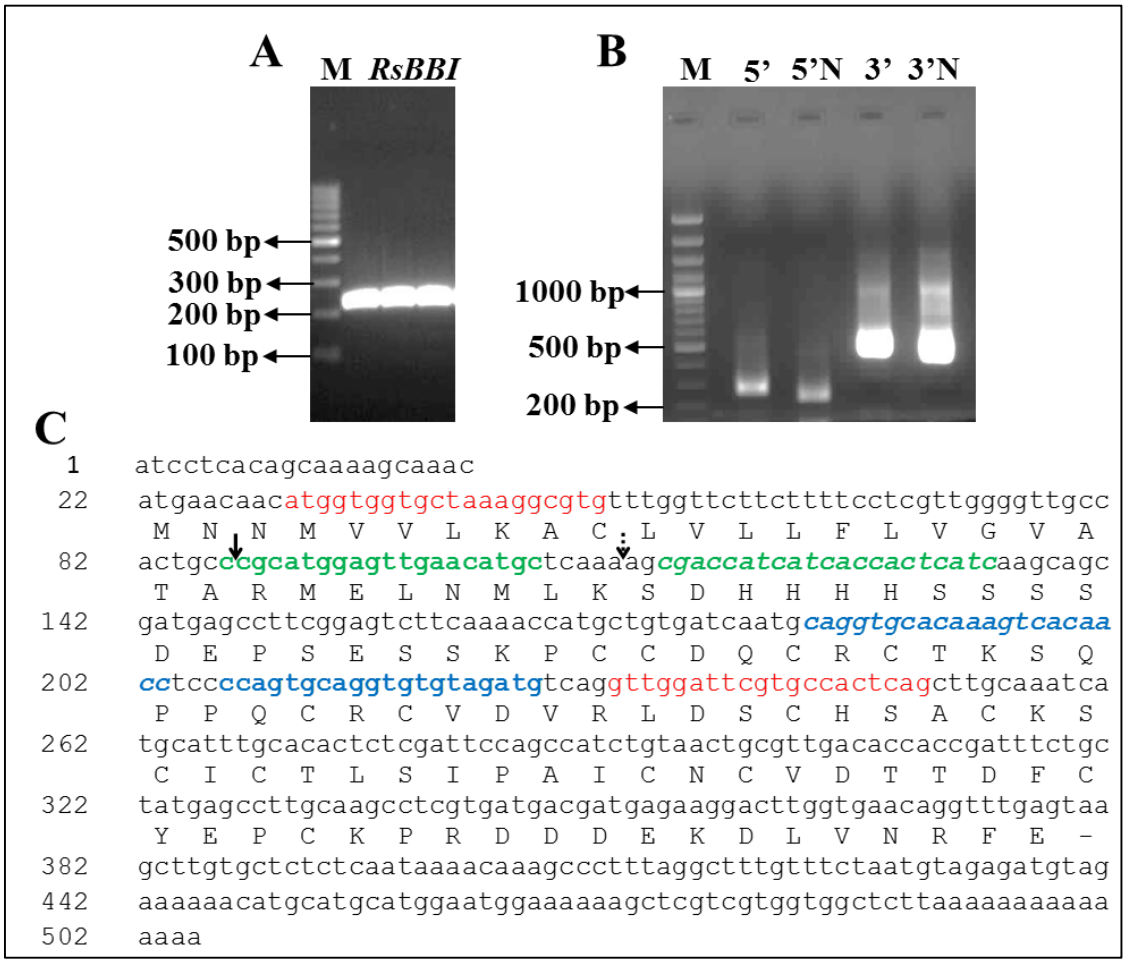

Fig. 5.8. Synthesis of full length cDNA and cloning of *RsBBI1*. (A) PCR-amplified partial cDNA fragment (~250 bp) of *RsBBI1* from immature seeds of *R. sublobata*. Lane 1 (M) is loaded with 100 bp DNA ladder and lanes 2-4 contained partially amplified *RsBBI1* gene product; **(B)** RACE amplified 5' and 3' fragments of *RsBBI1*. Lane M contained 100 bp DNA ladder and Lane 5' and 5'N are loaded with the 5' RACE primary and nested gene products, 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) heterologously expressed in *E. coli*. Primer sequences are shown in color. Red color indicate the primers used to get the partial sequence, green color indicate the 3'RACE forward primers, and blue color indicate the 5'RACE reverse primers. Nested primers are italicized. The complete *RsBBI1* sequence is submitted to NCBI with accession number KT119632.2.

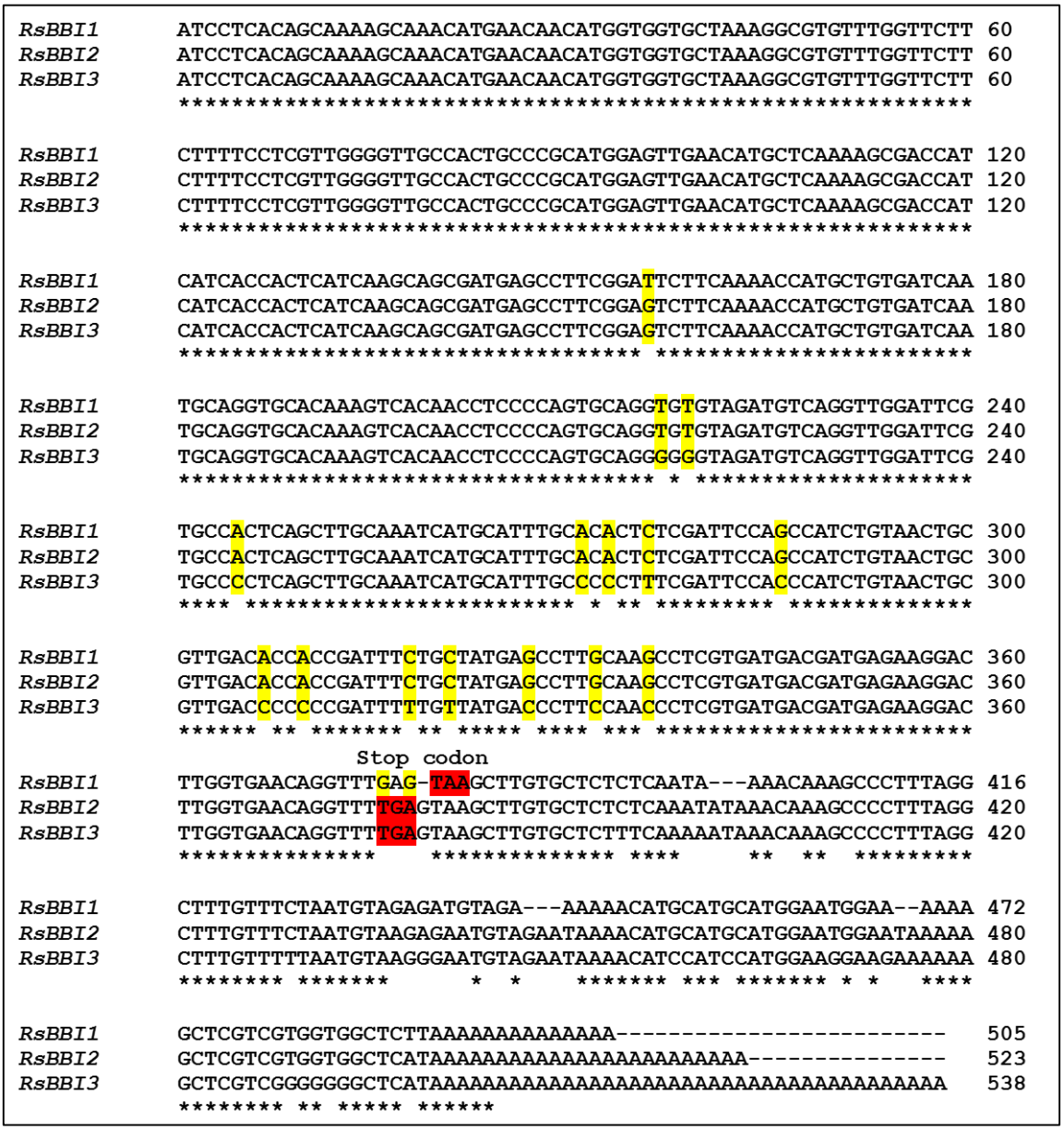

Fig. 5.9. ClustalW alignment of the *RsBBI* gene sequences obtained from the immature seeds of *R. sublobata* by RACE procedure. The nucleotide differing among themselves at the ORF are colored in yellow and the stop codons are marked in red colour. Identical nucleotides are marked with asterisks '*'.

```
                    Signal peptide                |<        Mature protein
RsBBI1      MNNMVVLKACLVLLFLVGVATARMELNMLKSDHHHHSSSSDEPSDSSKPCCDQCRCTKSQ 60
RsBBI2      MNNMVVLKACLVLLFLVGVATARMELNMLKSDHHHHSSSSDEPSESSKPCCDQCRCTKSQ 60
RsBBI3      MNNMVVLKACLVLLFLVGVATARMELNMLKSDHHHHSSSSDEPSESSKPCCDQCRCTKSQ 60
            ************************************************:***************

RsBBI1      PPQCRCVDVRLDSCHSACKSCICTLSIPAICNCVDTTDFCYEPCKPRDDDEKDLVNRFE 119
RsBBI2      PPQCRCVDVRLDSCHSACKSCICTLSIPAICNCVDTTDFCYEPCKPRDDDEKDLVNRF- 118
RsBBI3      PPQCRGVDVRLDSCPSACKSCICPLSIPPICNCVDPPDFCYDPSNPRDDDEKDLVNRF- 118
            ***** ******** ******** **** ******  ****:*.:*************
```

Fig. 5.10. ClustalW alignment of the protein sequences of RsBBI1, 2 and 3 deduced from the respective gene sequences. The cysteine residues and signal peptide are marked in green colour and yellow colour, respectively, and the identical amino acids are marked with asterisks '*'.

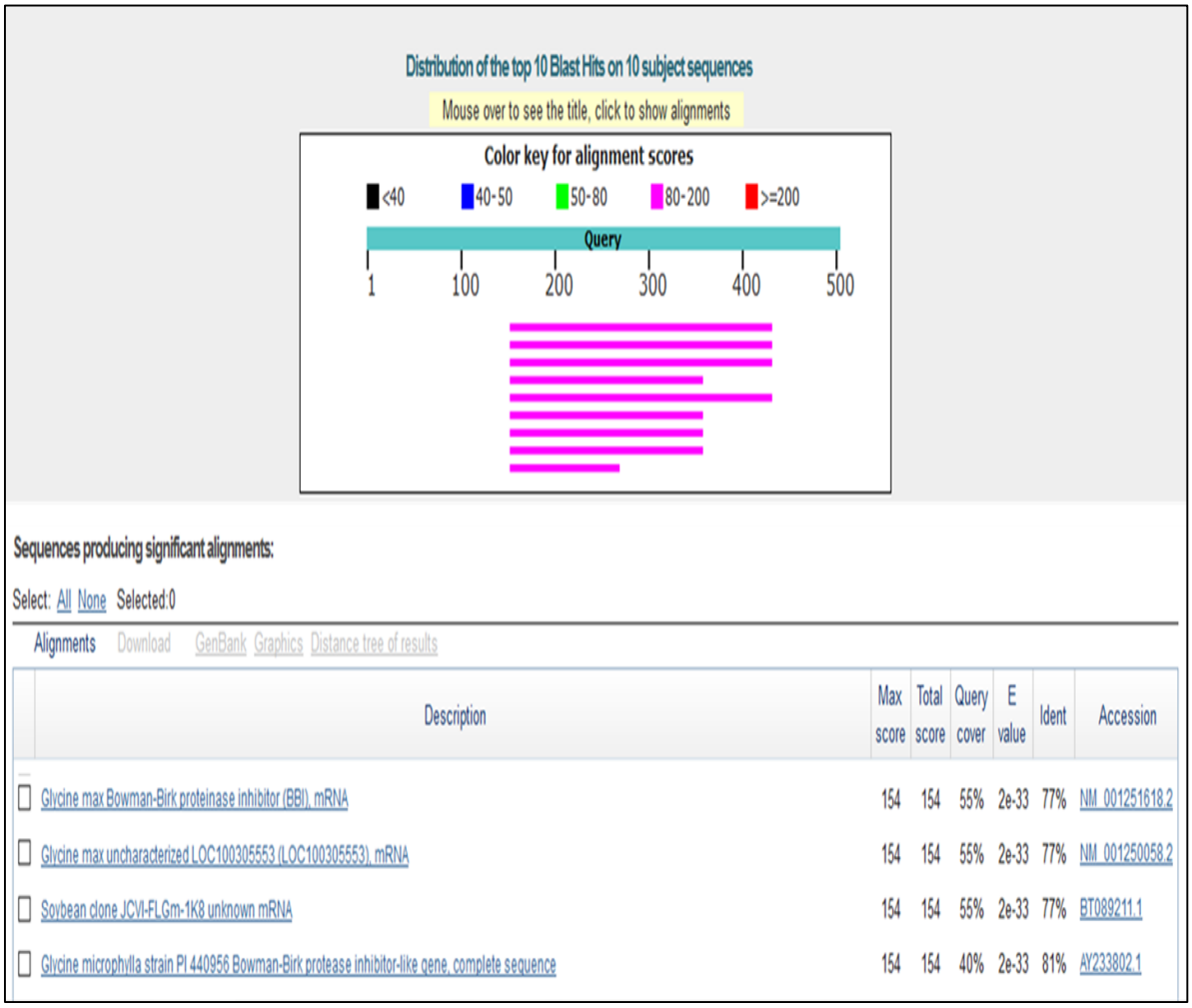

Fig. 5.11. BLASTn analysis of the *RsBBI1* obtained through RACE procedure. The isolated sequence showed homology with Bowman-Birk inhibitors.

hydrogen bond due to the presence of electronegative Oxygen atom in hydroxyl group which may retain the BBI fold by the non-covalent interaction rather than covalent disulphide bond. Apart from the cysteines, the following residues in RsBBI3 at position 75 (H→P), 84 (T→P), 89 (A→P), 96 (T→P), 97 (T→P), 102 (E→D), and 105 (K→N) are also different from *RsBBI1* (Fig. 5.10). Further, the analysis of *RsBBI1* by BLASTn confirmed that the sequence obtained belonged to the BBI superfamily (Fig. 5.11).

In silico translation by ExPASy Translate tool indicated that the *RsBBI1* (bp 22-381) codes for 119 amino acids of mass 13,303 Da (Fig. 5.6C). Further, signal peptide analysis of the translated protein showed that RsBBI1 possessed a 22 amino acid "MNNMVVLKACLVL LFLVGVATA" signal sequence. Furthermore, a short stretch consisting of 8 amino acids "RMELNMLK" of unknown nature is placed between the signal sequence and 89 amino acids stretch mature sequence which is expressed using pET23a as shown below (Fig. 5.8C). Multiple sequence alignment revealed that the cysteine residues of RsBBI1 aligned with the conserved 14 cysteine residues backbone of BBIs from other leguminous plants (Fig. 5.8). 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 showed matching with the BBIs from *Phaseolus*, *Glycine* and *Vigna* spp. (Fig. 5.12).

5.3.4. Phylogenetic analysis and 3D modeling of RsBBI1

The phylogenetic relationship of RsBBI1 from *R. sublobata* with BBIs from other legumes and cereals revealed that RsBBI1 is grouped between *Phaseolus* spp., and soybean at one end while placed between Vigna and pigeon pea at the other end of the phylogenetic tree (Fig. 5.13). Further, *C. cajan* BBI out grouped from RsBBI1 substantiating its evolutionary association with its wild gene pool. Furthermore, the BBIs from monocots are grouped out

```
                                                                                                **
Rhynchosia sublobata        M----NNMVV LKACLVLLFL VGVATA-RME LNMLK--SDH HHHSSSSDEP SESSKPCCDQ CRCTKSQPPQ 63
Cajanus cajan               M-------MV LKGCFFLLLL VGVTTA-RMD LGILK--SGH DQHHSS---- ----KACCDE CRCTKSIPPQ 52
Phaseolus filiformis        MGLKNNNTMV LKVCFVLLFL LG-TSTASLK LSELGQLMKS GHHHESTDEP SESSKACCDQ CACTKSIPPQ 69
Phaseolus microcarpus       MGLKNNNTMV LKVCFMLLFL LG-TSTASLK LSELGLLMKS GHHHESTDEP SESSKPCCDQ CACTRSIPPQ 69
Phaseolus zimapanensis      MGLKNNNTMV LKVCFMLLFL LG-TSTASLK LSELGLLMKS GHHHESTDEP SESSKPCCDQ CACTKSIPPQ 69
Phaseolus lunatus           MGLKNNNTMV LKVCFVLLFL LG-TSTASLK LSELGLLMKS GHHHESTDEP SESSKPCCDH CACTKSIPPQ 69
Phaseolus grayanus          MGLKNNNTMV LKVCFRLLFL LG-TSTASLK LSELGLLMKS GHHHESTDEP SESSKACCDQ CACTKSIPPQ 69
Phaseolus augusti           MGLKNNNTMV LKVCFVLLFL LG-TSTASLK LSELGLLMKS GHHHESTDEP SDSSKPCCDQ CACTKSIPPQ 69
Phaseolus coccineus polya   MGVKNNNTMV LKVCFVLLFL LG-TCTASLK LSELGLLMKS GHHHESTDEP SESSKACCDH CACTKSRPPQ 69
Phaseolus oligospermus      MGLKNNNTMV LKVCFMLLFL LG-TSTASLK LSELGLLMKS GHHHESTDEP SESSKACCDH CACTKSIPPQ 69
Glycine microphylla         M-------VV LKVCLVLLFL EGGTTSANLR LSKLGLLMKS DHHQHSNDD- -ESSKPCCDQ CACTKSNPPQ 61
Glycine soja                MGLKN-NMVV LKVCLVLLFL VGGTTSANLR LSKLGLLMKS DHHQHSNDD- -ESSKPCCDQ CACTKSNPPQ 67
Phaseolus costaricensis     MGLKNNNTMV LKVCFVLLFL LG-TCTASLK LSELGLLMKS GDHHESTDEP SESSKACCDH CACTKSRPPQ 69
Phaseolus hintonii          MGLKNNNTMV LKVCFMLLFL LG-TSTASLK LSELGLLMKS G-HHESTDEP SESSKACCDQ CACTKSIPPQ 68
Phaseolus parvulus          MGLKNNNTMV LKVCFMLLFL LG-TSTASLK LSELGLLMKS GHHHQSTDEP SESSKPCCDH CACTRSIPPQ 69
Phaseolus glabellus         MGLKNNNTMV LKVCFMLLFL LG-TSTASLK LSELGLLMKS GHHHESTDEP SESSKACCDE CACTKSIPPQ 69
Phaseolus vulgaris          MGLKNKNTKV LKMCFVLLFL LG-TCTASLK LSEKGQLMKS GDHDESTDEP SESSKPCCDQ CECTKSIPPQ 69
Vigna marina                M--------V LKVCFVLLFL LG-TSTASLK LSELGVLMKS GHHHQSTDES SESSTPCCDK CACTRSIPPQ 61
Vigna trilobata             M-------MV LKVCVLVVFL VGVTAA-GMD LNHLR--SIH HHHDSSDE-P SESSEPCCDS CRCTKSIPPQ 59
Vigna vexillata             M-------MV LKVCVLVLFL VGVTTANGMD LNHLR--SNH HD-DSSDE-P SESSEPCCDA CICTKSIPPQ 59
Vigna mungo                 M-------MV LKVCVLVVFL LGVTAA-GMD LNHLR--SIH HNHDSSDE-P SESSEPCCDS CRCTKSIPPQ 58
Vigna radiata var. sublob   M-------MV LKVCVLVVFL VGVTAA-GMD LNHLR--SIH HNHDSSDE-P SESSEPCCDS CRCTKSIPPQ 58
```

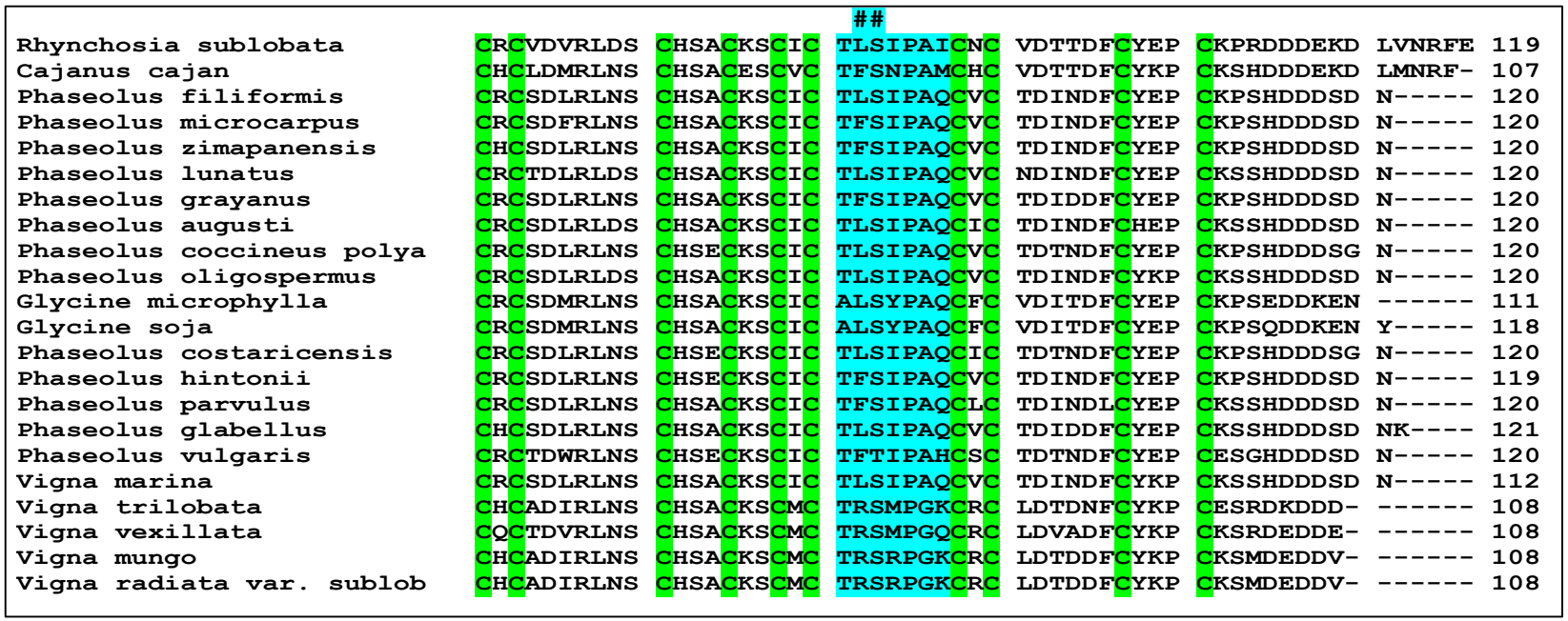

Fig. 5.12. **Multiple sequence alignment of RsBBI1 with the reported BBIs.** The trypsin and chymotrypsin reactive site loop residues are indicated in yellow and cyan color, and their corresponding reactive site amino acids are labeled with '**' and '##', respectively. The fourteen cysteine residue backbone of RsBBI1 which showed matching with the reported BBIs is indicated in green color. All the BBI sequences were downloaded from NCBI data base. Accession numbers of the different Clustal W aligned BBIs are as follows: *Cajanus cajan* - KYP42282.1; *Phaseolus filiformis* - CAL69281.1; *Phaseolus microcarpus* - CAL64060.1; *Phaseolus zimapanensis* - CAQ52360.1; *Phaseolus lunatus* - CAL51268.1; *Phaseolus grayanus* - CAQ52359.1; *Phaseolus augusti* - CAL51269.1; *Phaseolus coccineus* - CAQ58092.1; *Phaseolus oligospermus* - CAL51270.1; *Glycine microphylla* - AAO89510.1; *Glycine soja* - BAB86783.1; *Phaseolus costaricensis* - CAL69279.1; *Phaseolus hintonii* - CAQ52357.1; *Phaseolus parvulus* - CAL69237.1; *Phaseolus glabellus* - CAL69238.1; *Phaseolus vulgaris* - CAQ34829.1; *Vigna marina* - ABD97867.1; *Vigna trilobata* - ABD91574.1; *Vigna vexillata* - ABD97866.1; *Vigna mungo* - AKC45532.1; *Vigna radiata* - ABD91575.1.

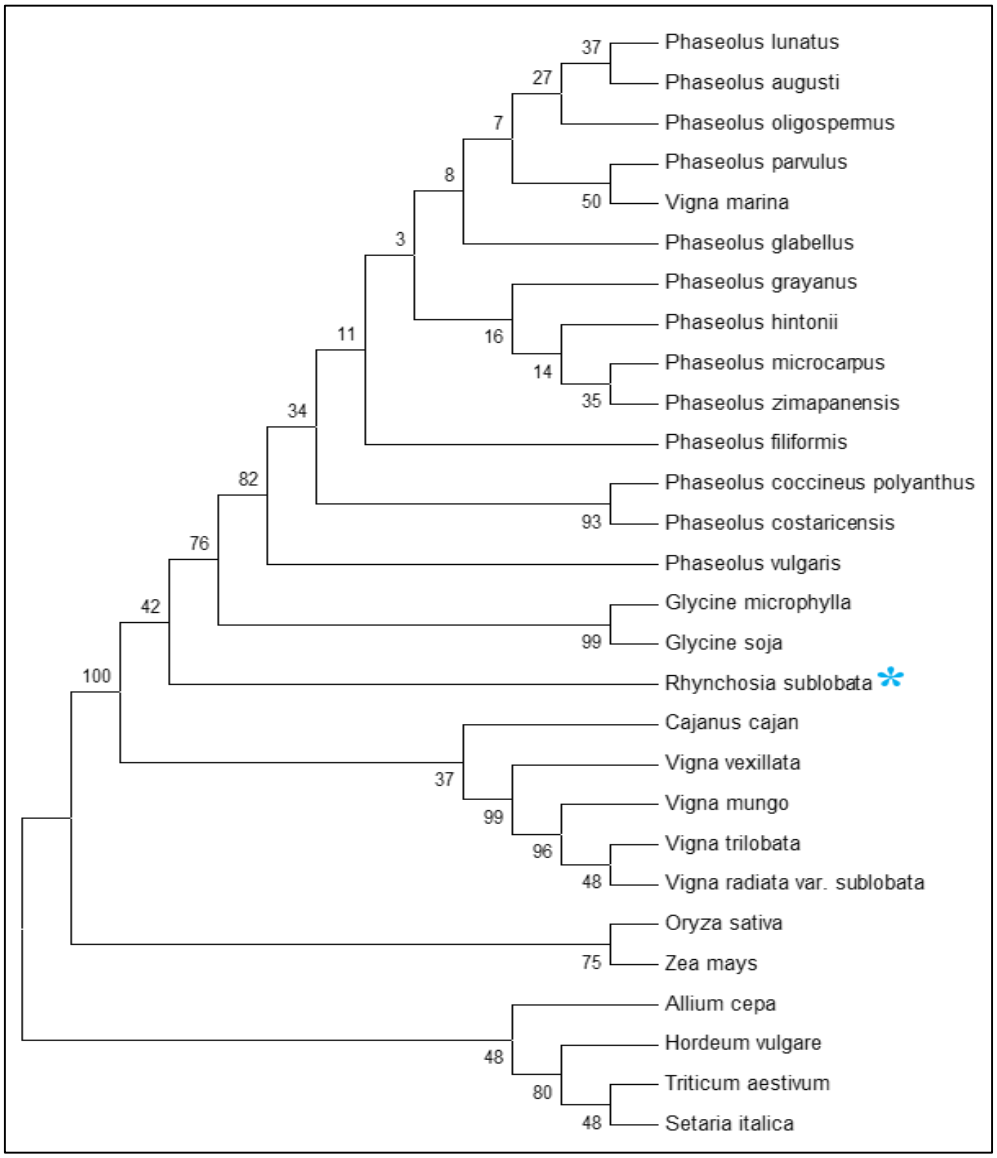

Fig. 5.13. **Phylogenetic analysis of RsBBI1.** Phylogenetic analysis of deduced RsBBI1 with reported BBIs as described in materials and methods section 5.2.5. The bootstrapping values were mentioned at the branches to depict the grouping robustness. Accession Numbers of NCBI for BBIs from *P. filiformis*; *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. 5.12; the accession numbers of other BBIs were as follows: *H. vulgare* - BAK01561.1; *O. sativa* - CAB88209.1; *T. aestivum* - ABX84379.1; *Z. mays* - NP_001150715.1; *Setaria italica* - XP_004986446.1; *Allium cepa* - BAB88746.1. The position of *R. sublobata* BBI in the tree is marked with asterisks '*'.

from the dicots, which clearly indicate that BBIs though widely distributed among the Leguminosae members, they are highly conserved among dicots and monocots during evolution.

The three-dimensional structure was predicted from residues 44 to 113 of RsBBI1 using soybean BBI (PDB ID-1BBI) as a template (Fig. 5.14A). The modeled RsBBI1 represented a symmetrical protein with both trypsin and chymotrypsin reactive site loops on the opposite sides, a characteristic feature of BBI proteins. The amino acids which are identical between RsBBI1 and the template 1BBI used for 3D modeling are represented by the same color in pair-wise alignment (Fig. 5.14B).

5.3.5. Overexpression and purification of rRsBBI1

The pET23a-*RsBBI1* plasmid was transformed into *E. coli* Shuffle T7 express cells, which contain *trxB*$^{-}$, *gor*$^{-}$ mutation and a chromosomal copy of disulfide bond isomerase (DsbC). DsbC facilitates both formation of disulfide bonds in the cytoplasmic proteins and proper folding of the proteins by acting as a chaperone (de Marco, 2009).The mature rRsBBI1 (89 amino acids stretch) which contained fourteen cysteine residues was expressed using pET23a, as indicated in materials and methods section 5.2.7. However, pET23a without *RsBBI1* is used as a negative control (Figs. 5.15A-C). The purification profile of rRsBBI1 was shown in 15% Tricine SDS-PAGE (Figs. 5.15A,D) while the activity of rRsBBI1 against trypsin was visualized in gelatin SDS-PAGE (Figs. 5.15B,E). Further, purification of the rRsBBI1 through trypsin bound Sepharose column was depicted in Figures 5.15C and 5.15F.

The purified rRsBBI1 is observed as a single band in both SDS-PAGE & activity staining studies (Figs. 5.15D,E), while two peaks with molecular masses of 9970.87 Da and 9693.74 Da appeared in intact mass MALDI-TOF analysis (Fig. 5.16). The theoretical mass (9971.02 Da) of 89 amino acid rRsBBI1 coincided well with the peak of 9970.87 Da observed

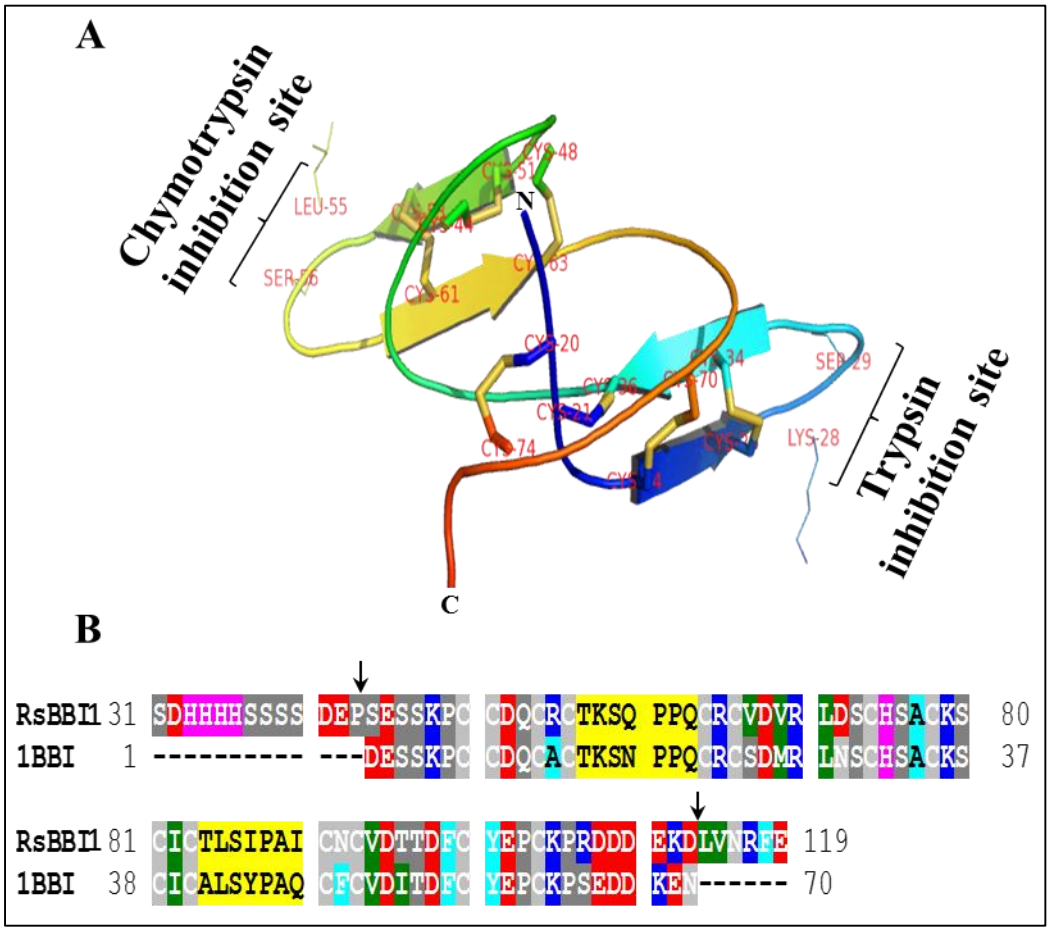

Fig. 5.14. Molecular modeling of RsBBI1. (A) The predicted three-dimensional SWISS-MODEL of RsBBI1 protein was obtained using 1BBI from soybean as the template. The disulfide bonds, trypsin, and chymotrypsin reactive sites were visualized using PyMol software and represented as sticks. The N- and C-terminus ends were marked as N and C, respectively; **(B)** 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.

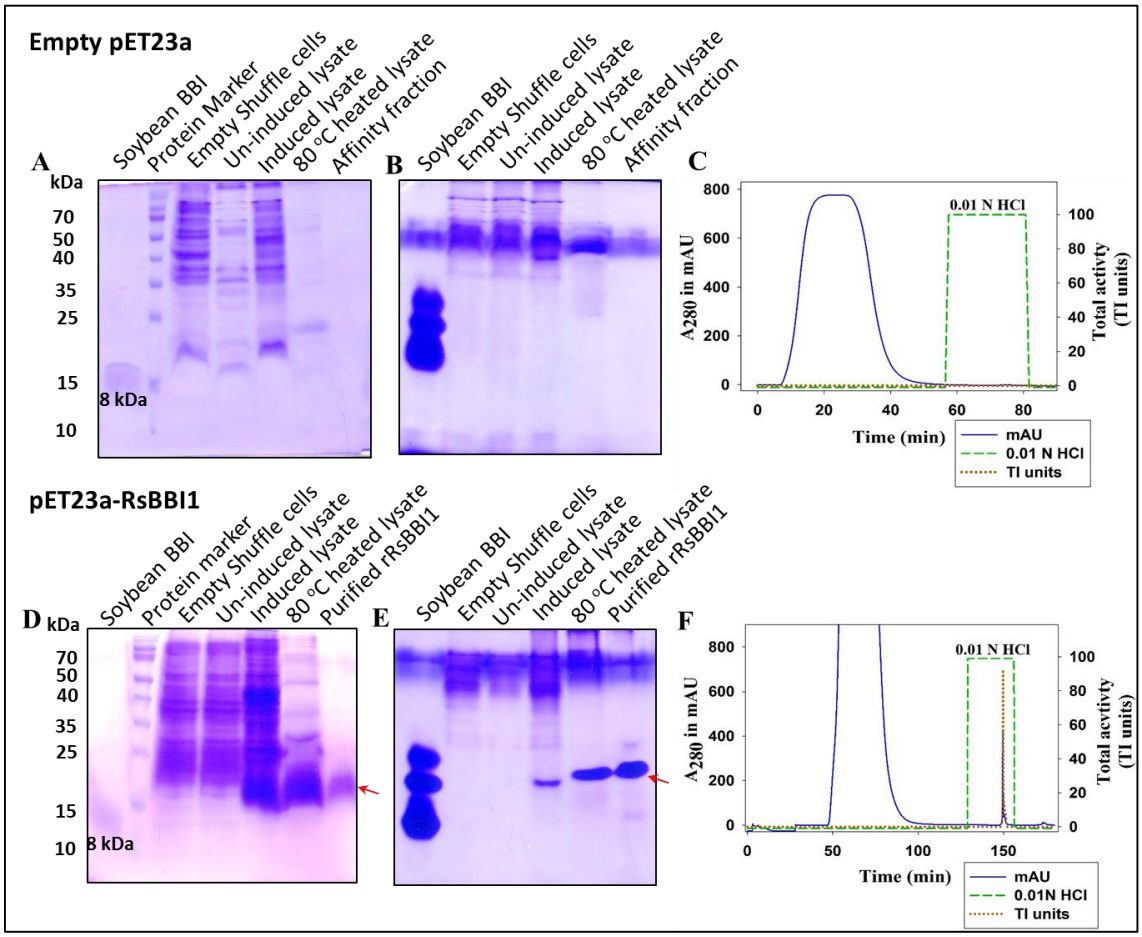

FIGURE 5.15. Heterlogous expression of RsBBI1. (A) Expression profile of empty pET23a vector (control) on 15% SDS-PAGE; **(B)** the corresponding in-gel trypsin activity staining of heat treated *E. coli* lysate with empty vector in gelatin SDS-PAGE; **(C)** the trypsin affinity chromatogram from heat 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)** the corresponding in-gel trypsin activity staining of heat treated *E. coli* lysate with pET23a-*RsBBI1* construct in gelatin SDS-PAGE and **(F)** the trypsin affinity chromatogram from heat treated *E. coli* lysate with pET23a-*RsBBI1* construct showing the elution of bound peak (rRsBBI1) with 0.01 N HCl. 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.

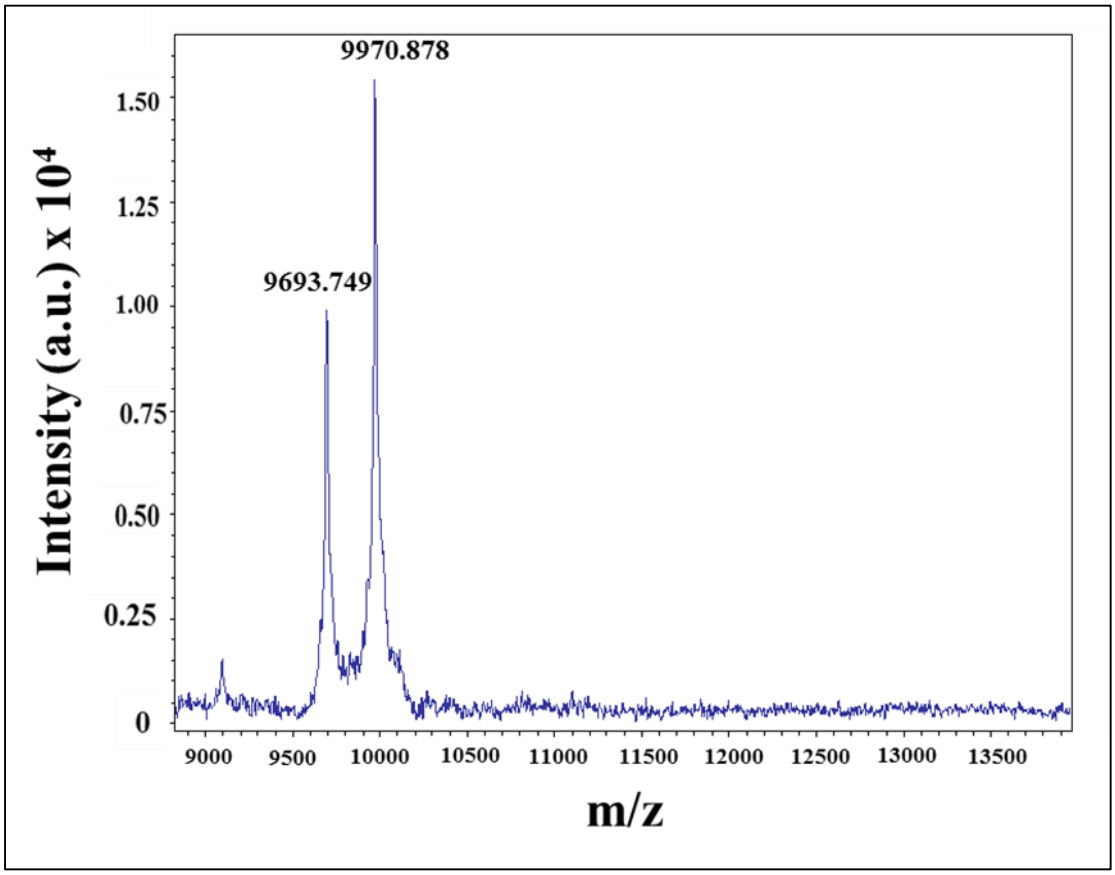

Fig. 5.16. **Intact mass analysis of rRsBBI1 by MALDI-TOF**. The rRsBBI1 purified from trypsin affinity column is resolved into two peaks with m/z 9693.749 Da and 9970.878 Da, respectively.

in MALDI-TOF analysis. However, the appearance of a new peak with a molecular mass of 9693.74 Da in MALDI-TOF analysis could be due to cleavage of an arginine residue present at the 87^{th} position by trypsin enzyme bound to Sepharose column during affinity chromatography of rRsBBI1. As cleavage happen to occur at the far end of C-terminus and the 14 cysteine residues which stabilize the protein are present within the 87 amino acids stretch, it is possible that the protein with mass 9693.74 Da might exhibit inhibitory activity against both trypsin and chymotrypsin enzymes along with 9970.87 Da polypeptide.

5.3.6. Biochemical characterization of rRsBBI1

The rRsBBI1 inhibited the activity of both trypsin and chymotrypsin enzymes. However, the inhibitory activity of rRsBBI1 against trypsin was higher as compared to chymotrypsin. The trypsin lost 94% of its activity when the molar ratio of rRsBBI1 to trypsin was 0.75 and further lost 100% activity as the molar ratio increased to 1.0 (Fig. 5.17A). In contrast, chymotrypsin lost 85% of its activity at 1.0 molar ratio and further lost 100% of its activity as the molar ratio of rRsBBI1 increased to 2.0 (Fig. 5.17A). 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. Since, practically it is not possible that one molecule of rRsBBI1 binding to 0.75 molecule of trypsin, we assume that similar to chymotrypsin, the trypsin enzyme also binds to rRsBBI1 at 1:1 molar ratio (Fig. 5.17A). Following these studies, enzyme kinetics analysis by Lineweaver-Burk plot indicated that rRsBBI1 inhibits both trypsin (*Ki* = 358 ± 11 nM) and chymotrypsin (*Ki* = 446 ± 9 nM) by non-competitive mode (Figs. 5.17B,C). Further, the observed kinetic studies also suggest that rRsBBI1 was folded properly into its native conformation, hence exhibited interaction with both trypsin and chymotrypsin.

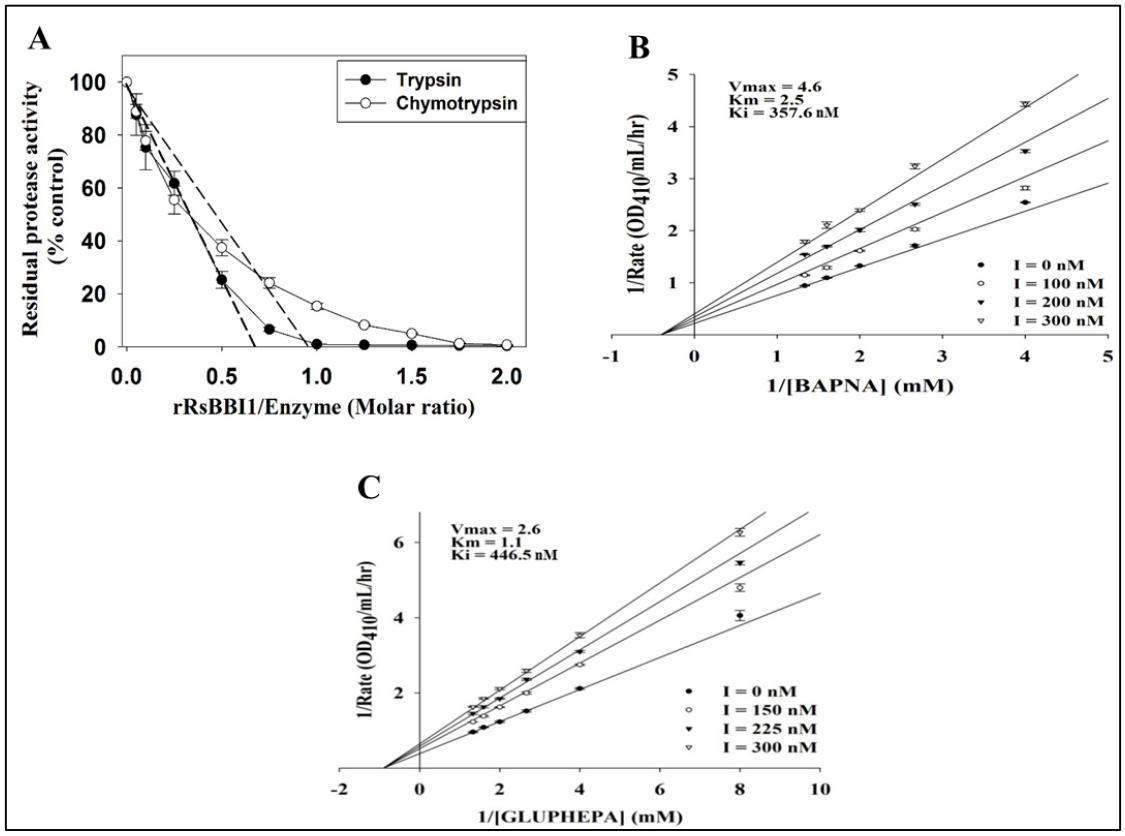

Fig. 5.17. **Enzyme inhibition kinetics of rRsBBI1. (A)** Titration curves of trypsin and chymotrypsin inhibition by rRsBBI1. The rRsBBI1 at increasing concentrations was added to a fixed 1 µM concentration of trypsin and chymotrypsin enzymes, respectively. The residual inhibitory activity of trypsin or chymotrypsin was determined as described in materials and methods section 5.2.10. The molar ratio of the inhibitor 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 against 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.

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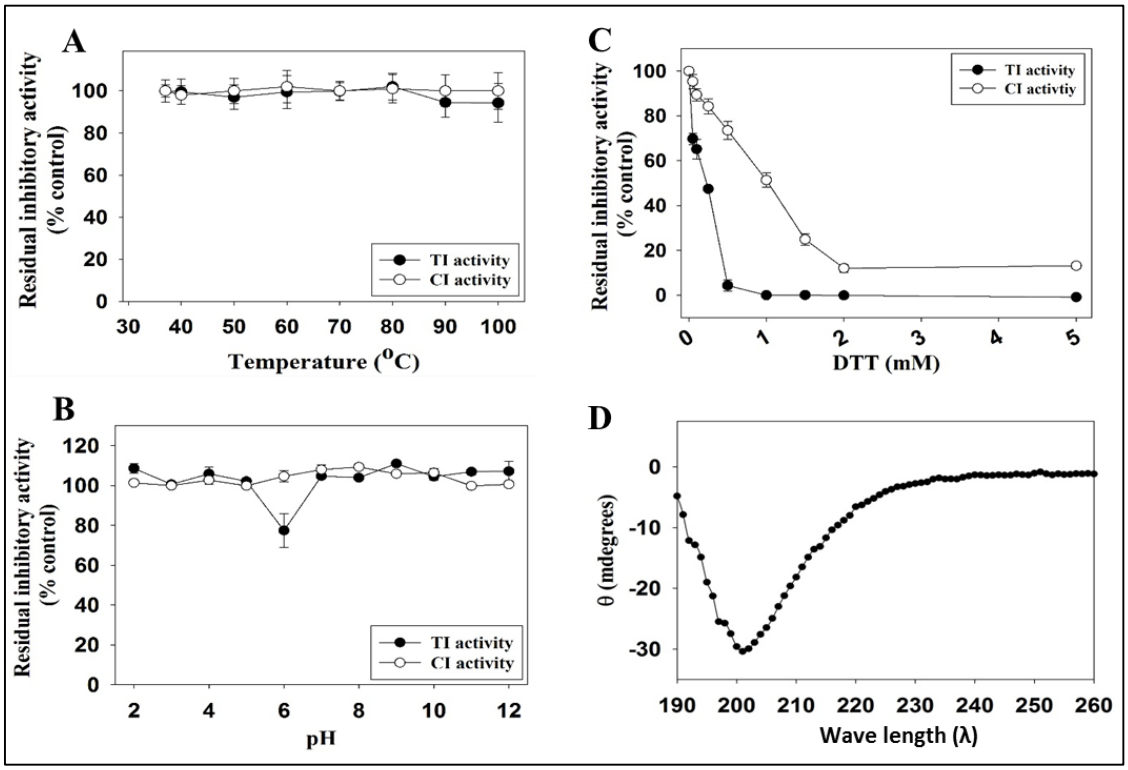

Fig. 5.18. **Biochemical stability and secondary structure of rRsBBI1.** Stability of rRsBBI1 at various **(A)** temperatures; **(B)** pH; and **(C)** on reduction with DTT/IDA; residual trypsin and chymotrypsin inhibitory activity were plotted against the appropriate condition; **(D)** CD spectra of 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.

The inhibitory activity of rRsBBI1 was stable to thermal treatments from 37 to 100^{o}C. The loss in TI and CI activities was negligible (<10%) even upon heating at 100^{o}C for 30 min (Fig. 5.18A). 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. 5.18B). But, rRsBBI1 lost 100% of its TI and CI activities on reduction with DTT at 1 mM and 10 mM concentrations, respectively (Fig. 5.18C). Secondary structural analysis of rRsBBI1 at far-UV region revealed that it consists of 29.3% β-sheets, 11.8% β-turns, 56.8% random coils and 2.0% α-helices (Fig. 5.18D). Further, the existence of high ellipticity at 201 nm confirms the presence of disulfide bridges in rRsBBI1 (Kumar and Gowda, 2013b). Thus, the CD estimated secondary structural elements of rRsBBI1 showed correlation with the modeled 3D structure of RsBBI1 (Fig. 5.14A).

5.3.7. Insecticidal activities of rRsBBI1

The rRsBBI1 was obtained at 2.5 ± 0.3 mg L^{-1} culture, and it showed a specific activity of 656 ± 16 TIU/mg protein and 523 ± 20 CIU/mg protein towards trypsin and chymotrypsin, respectively. Furthermore, the specific activity of rRsBBI1 against AjGPs (14,285 ± 500 AjGPI units/mg protein) was >20 fold higher as compared to trypsin or chymotrypsin, respectively. But, the specific activity of rRsBBI1 against HaGPs (125 ± 10 HaGPI units/mg protein) was < 25% of the specific activity against trypsin or chymotrypsin (Fig. 5.19A). These results corroborated well with the IC_{50} values of rRsBBI1 against AjGPs and HaGPs (Figs. 5.19B,C). The rRsBBI1 inhibited the trypsin-like proteases present in the larval gut of both *A. janata* and *H. armigera*. However, the inhibitory activity of rRsBBI1 against the gut trypsin-like proteases of *A. janata* (IC_{50} of 70 ± 2 ng) was more pronounced as compared to *H. armigera* (IC_{50} of 8 ± 0.5 μg) (Figs. 5.19B,C).

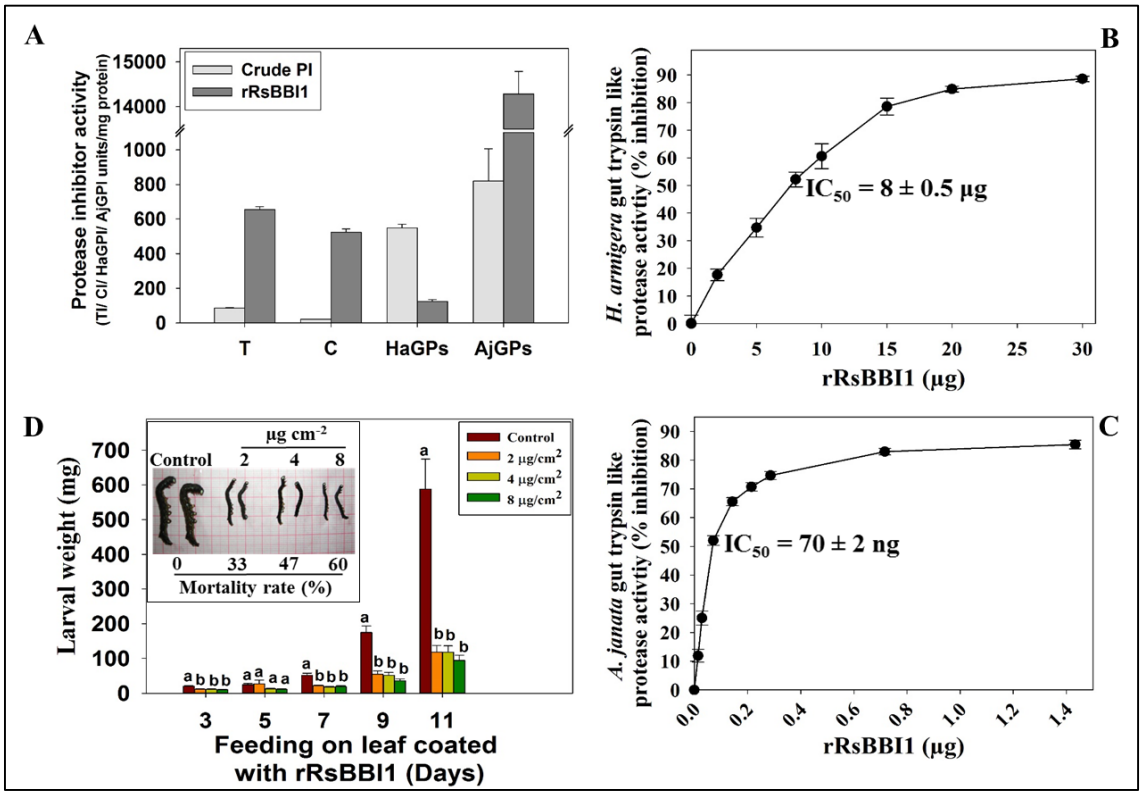

Fig. 5.19. (A) The *in vitro* activity of *R. sublobata* mature seed crude PI protein (light grey) and *E. coli* expressed rRsBBI1 (dark grey) against trypsin (T), chymotrypsin (C), HaGPs, and AjGPs; **(B)** and **(C)** rRsBBI1 concentration dependent inhibition of the activity of gut trypsin-like proteases of *H. armigera* and *A. janata*. 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 2, 4, and 8µg cm^{-2} of rRsBBI1. The inset shows the larval pictures and mortality rates of *A. janata* after 11 days of feeding with rRsBBI1 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.

In vivo feeding of rRsBBI1 coated castor leaves to *A. janata* larvae caused a significant reduction in their growth as compared to larvae fed on control leaves (Fig. 5.19D). At the end of the 11th Day of feeding, the weight of the larvae were reduced significantly by $\geq$84% of control larvae at 2, 4 or 8 μg/cm^2 of rRsBBI1 coated on the leaf (Fig. 5.19D). Further, the mortality rate of larvae fed on rRsBBI1 coated leaves increased in a dose dependent manner up to 60 ± 7% (Fig. 5.19D inset).

5.4. Discussion

5.4.1 Molecular Characterization of *RsKI*

The complete cDNA of *RsKI* sequence obtained using RACE procedure encoded a polypeptide of 198 amino acids including signal peptide. The mature protein contained four cysteine residues. Kunitz inhibitors generally contain two disulphide bridges (Oliva et al., 2010) and in agreement with this, RsKI also contained two disulphide bridges. Most of the Kunitz inhibitors reported generally contain single reactive site and inhibit either trypsin or chymotrypsin proteases. However, a few bifunctional Kunitz inhibitors can inhibit two different proteases simultaneously. For example, barley α-amylase/subtilase inhibitor (Leah and Mundy, 1989), and Kunitz inhibitors from *Adenanthera pavonina* and potato serine protease inhibitor inhibit simultaneously both trypsin and chymotrypsin (Migliolo et al., 2010; Valueva et al., 2000). However, RsKI1 and RsKI2 obtained in the present study contain only a single reactive site against chymotrypsin protease with either 'Leu-Leu' or 'Phe-Leu' at P1-P1' position of the reactive site.

5.4.2. Molecular characterization of *RsBBI1*

The complete cDNA sequence of *RsBBI1* obtained using RACE procedure encoded a polypeptide of 119 residues with molecular mass of 9971.02 Da and contained 14 cysteine residues as well as two inhibitory sites, the typical features of BBI molecules (Figs. 5.8C, 5.12,

5.13, 5.14 & 5.16; Qi et al., 2005). The predicted three-dimensional structure suggested that RsBBI1 existed as a double headed enzyme inhibitor with two reactive site loops one each for trypsin and chymotrypsin on opposite ends to facilitate its binding with its cognate proteases both independently and simultaneously (Fig. 5.14A; Voss et al., 1996). Further, the P2, P1, P1', P2', P3', P4' and P5'residues in the trypsin and chymotrypsin reactive site loop were identified as 'TKSQPPQ' and 'TLSIPAI,' respectively (Fig. 5.12). The existence of 'Lys' and 'Leu' in the trypsin and chymotrypsin inhibitory sites of RsBBI1 ascertained that the gene cloned belonged to BBI family (Fig. 5.12; Laskowski Jr and Kato, 1980). Both 'KS' and 'LS' residues at the P1-P1' positions and 'Thr' which is conserved at P2 position of both reactive sites are essential to exhibit inhibitory activity and to facilitate the efficient binding of BBIs to its cognate enzymes (Fig. 5.12; Gariani et al., 1999 and Brauer et al., 2003). Further, the occurrence of 'Gln' at the P2' position of the trypsin reactive site loop is observed rarely and it occurred in rRsBBI1 along with two other BBIs from IBB_VICAN of *Vicia sativa subsp. nigra and* IBB3_SOYBN of *Glycine max* (Fig. 5.12). In contrast, the BBIs from *C. cajan, Phaseolus, Glycine or Vigna spp.*possessed Ile, Arg or Asn in the corresponding position (Fig. 5.12). Similarly, the amino acid residues of chymotrypsin reactive site loop at P2' and P5' positions of RsBBI1 varied from the BBIs of these species. These variations perhaps justify the placement of *R. sublobata* in the quaternary gene pool of wild-relatives of *C. cajan* and in the phylogenetic tree of the RsBBI1 where *R. sublobata* out grouped from *G. max* and *C. cajan* (Fig. 5.13; Mallikarjuna et al., 2011).

In the present study, two genes of *RsKI* (*1&2*) and three genes of *RsBBI* (*1-3*) were obtained from the immature seeds of *R. sublobata* (Fig. 5.2, 5.3, 5.8 & 5.9). Of the two different group of genes, *RsKI1* and *RsBBI1* were ligated to the expression vector *pET23a* and transformed in to the expression host, *E. coli* Shuffle cells for overexpression. RsKI1 protein was not obtained in the soluble fraction from the *pET23a-RsKI1* transformed, overexpressed

cells. However, recombinant RsBBI1 protein was obtained in the soluble form from the *pET23a-RsBBI1* transformed, overexpressed cells and were purified by trypsin affinity chromatography.

5.4.3. Overexpression and biochemical characterisation of rRsBBI1

The BBIs are known to be stable at temperatures >90^{o}C (Osman et al., 2002; Prasad et al., 2010c). Therefore, heat denaturation step (80^{o}C, 30 min) was adapted during purification of BBIs as reported for intrinsically disordered proteins to eliminate the heat sensitive digestive proteases present in the cell lysate (Figs. 5.15D-F; Livernois et al., 2009). The low molecular mass of rRsBBI1 in MALDI-TOF correlated with the ExPASy translated sequence (Figs 5.8, 5.12–5.16). The following observations of rRsBBI1 against its cognate proteases trypsin and chymotrypsin strengthen that the rRsBBI1 was folded properly into its native conformation during purification: (i) a 1:1 molar ratio; (ii) non-competitive mode of enzymes kinetics; (iii) the in-gel activity staining studies; (iv) stability against temperature and pH and (v) loss of activity on reduction with DTT (Figs.5.15E,5.17,5.18).

The present study also revealed that the rRsBBI1s is functionally very stable in terms of its TI and CI activities at high temperatures up to 100^{o}C and at both acidic as well as alkaline pH conditions (Figs. 5.18A,B). Similar types of activity were identified for 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 possess seven disulfide briges and they were suggested to be responsible for their high stability against temperature and pH (Prasad et al., 2010a,b; Swathi et al., 2014). The X-ray stucture of BBI-A from soybean showed that it has five exposed disulfide and two buried disulfide bridges (He et al., 2017). In solution, BBIs are known to self-aggregate and such

aggregates which effect their interaction with cognate proteases are not observed in the present study, perhaps due to usage of diltute rRsBBI1 solutions (Brand et al., 2017). Together with the stability against pH and temperature, the presence of a high percentage of disulphide bridges and specificity towards trypsin/chymotrypsin enzymes suggested that the BBIs could be exploited for clinical and therapeutic applications (Clemente and Arques, 2014; Farinaz and Abdolmohamad, 2013; Souza Lda et al., 2014).

The disulphide bridges which stabilise the reactive site scaffold of rRsBBI1 were destabilised on reduction with DTT, a characteristic feature of BBIs (Qi et al., 2005). The incubation of rRsBBI1 with DTT resulted in a remarkable loss in its TI and CI activities. However, the CI activity of rRsBBI1 is more resistant to the action of DTT as compared to BBIs from the cultivars of *C. cajan* (Fig. 5.18C; 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 at > 1.0 mM concentration. Thus, the present study suggests that the rRsBBI1 from *R. sublobata* is structurally more stable over BBIs from *C. cajan* cultivar. The secondary structural elements of rRsBBI1 observed in far-UV CD spectra coincided well with the reported BBIs in possessing a high percentage of β sheets and random coils (Voss et al., 1996). The horse gram BBI was shown to follow the 'two-state' mode of unfolding in presence of DTT which indicate the hyper reactive nature of disulfide bonds (Rajesh Singh and Appu Rao, 2002). Also, the existence of high ellipticity at 201 nm confirms the presence of disulphide bridges in rRsBBI1 (Kumar and Gowda, 2013b). Further, the CD estimated secondary structural elements of rRsBBI1 showed correlation with the modelled 3D structure of RsBBI1 (Figs. 5.14A,5.18D).

5.4.4. Differential responses of *A. janata* and *H. armigera* towards rRsBBI1

rRsBBI1 exhibited 114-fold difference in their IC_{50} against AjGPs than HaGPs (Figs. 5.19C,D). This might be due to the susceptible *A. janata* gut trypsin proteases towards the non-

host rRsBBI1 and the presence of relatively less rRsBBI1 susceptible trypsin enzyme in HaGPs. Moreover, it is widely known that *H. armigera* possess multiple trypsin enzymes in their gut environment along with other proteases such as chymotrypsin and elastase which also may be a cause for the lower activity of rRsBBI1 towards HaGPs (Bown et al., 1997; Chougule et al., 2005; Kuwar et al., 2015; Wu et al., 1997). This study also revealed that the high activity of *R. sublobata* crude seed PI on HaGPs as reported by Chougule et al., (2003) could be due to the presence of several other PIs along with BBIs in the *R. sublobata* crude PI extract. But, the studies of Swathi et al. (2016) on *C. platycarpus*, one of the wild relative of *C. cajan* effectively inhibited HaGPs due to the Kunitz/Miraculin like PIs present in them. Apart from BBIs, other types of PIs such as squash type inhibitor from bitter gourd (Telang et al., 2009a), PIN-II type inhibitors from *Capsicum annum* and many Kunitz inhibitors (Jamal et al., 2013) were reported to effectively inhibit the gut proteases of *H. armigera*. Further, Johnston et al. (1993) indicated that a very high concentration of *G. max* BBI is required to induce mortality in *H. armigera*. The present report warrants to further explore the genome of *R. sublobata* to identify PIs active against *H. armigera*.

Further, 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 inhibitor loop in rRsBBI1 (Fig. 5.12) and the susceptible trypsin-like proteases in the *A. janata* gut. The observed IC_{50} (70 ± 2 ng) of rRsBBI1 against AjGPs did not vary significantly from the IC_{50} (78 to 100 ng) of BBIs from cultivar varieties of *C. cajan* (Fig. 5.19C; 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 BBIs from *C. cajan* is known to exist as several isoinhibitors, and they might all contribute together to the observed insecticidal activity against *A. janata* (Figs.

5.19C,D; Prasad et al., 2010b; Swathi et al., 2014). Further, preliminary *in vivo* feeding of rRsBBI1 to the second instar *A. janata* larvae indicated that rRsBBI1 is a potent growth retarding agent (Fig. 5.19D). Further, transgenic tobacco expressing cowpea trypsin inhibitor and rice plants expressing potato type 2 (PIN2) inhibitor exhibit resistance against *H. virensis* and *Sesamia inferens* (Duan et al., 1996; Hilder et al., 1987). However, of the tobacco plants expressing PIN1 (chymotrypsin inhibitor) and PIN2 (trypsin and chymotrypsin inhibitor) inhibitors, *Manduca sexta* was effectively inhibited by PIN2 expressing plants rather than PIN1 (Johnson et al., 1989). Further, on feeding tobacco plants expressing giant taro PI to *H. armigera*, the insect survived by over expressing alternate proteases such as chymotrypsin and elastase (Wu et al., 1997). Also, insects such as *Heliothis zea*, *H. armigera*, and *Spodoptera exigua* were able to overcome the effect of PIs by producing alternate proteases or over producing sensitive proteases (Jongsma and Bolter, 1997; Wu et al., 1997). Thus, from these studies, the insects alter their gut protease profile according to the type of inhibitors fed to them. Hence, targeting multiple gut proteases and PI insensitive proteases of a particular insect was suggested as a potential strategy (Jongsma and Bolter, 1997). In this line, recent studies of pyramiding PIs active against both sensitive and insensitive proteases induced high growth reduction and mortality as observed in transgenic cotton expressing potato type I and II PI against *H. armgiera* (Dunse et al., 2010b) and transgenic tobacco expressing sporamin, cystatin and chitinases against *S. litura* and *S. exigua* (Chen et al., 2014). Hence, identifying PIs from non-host plants effective against a particular insect is the first step towards insect control. In this aspect, RsBBI1 which effectively inhibited the activity of gut proteases of *A. janata* could be a potential candidate gene for the development of transgenic plants. However, further studies are warranted to counter this castor pest.

Chapter 6

Summary and Conclusions

Summary and Conclusions

PIs are a form of defense molecules produced by plants against insect pests. In this ever evolving world of plants and pests, identifying potent PIs from host/non-host plants or wild-relatives is imperative to effectively control the insect pests. *Rhynchosia* is a wild relative of *C. cajan* which is infested by *H. armigera*. However, it is a non-host to castor pest *A. janata*. Previous reports by Chougule et al. (2003) and Parde et al. (2012) stated that the crude PI extracts of *Rhynchosia* spp. effectively inhibited the gut trypsin-like proteases of *H. armigera.* Preliminary analysis of the crude PIs from *R. sublobata* indicated that it is active against the gut trypsin-like gut proteases of *H. armigera* along with *A. janata* (**Chapter 2**; Fig. 2.4). Identifying potential insecticidal PIs from *R. sublobata* could (i) broaden the narrow gene pool of closely related *C. cajan* and (ii) enhance the defense capability of unrelated castor plants (Figs. 2.2 & 2.4).

Chapter 3 describes the purification of trypsin-specific PIs present in the seeds of *R. sublobata* (RsPI) by ammonium sulphate fractionation (20-60%) followed by anion exchange, trypsin-affinity and gel filtration chromatography (Fig. 3.1). Two different PIs, Bowman-Birk inhibitor (RsBBI) and Kunitz inhibitor (RsKI) were identified from RsPI (Figs. 3.2, 3.8 & 3.9). Further, a short method was formulated to separate both RsBBI (MW = 9,216 Da) and RsKI (MW = 19,412 Da) from RsPI pool within two hours, by successive precipitation with 2.5% Trichloroacetic acid and 0.1M Sodium acetate buffer (Figs. 3.4 & 3.6). The protocol resulted in the purification of RsBBI and RsKI by 49.63-fold and 23.15-fold, with 21.87% and 2.7% yield recovery, respectively (Fig. 3.5A). In solution, both RsBBI and RsKI exhibited self-association property up to trimer and dimers, respectively as observed in SDS-PAGE and MALDI-TOF mass spectrum (Figs. 3.4B & 3.6). Further, 2-DE revealed that RsBBI and RsKI contained five isoinhibitors which range between pI 4.35 to pI 5.2 and eight isoinhibitors

between pI 4.45 to 6.55, respectively (Figs. 3.7A & B). ClustalW analysis of the N-terminal sequences of RsBBI and RsKI obtained through MALD-ISD confirmed that the separated PIs were indeed BBI and Kunitz inhibitors (Figs. 3.8 & 3.9). RsBBI is a dual inhibitor active against both bovine pancreatic trypsin and chymotrypsin whereas RsKI is active only against trypsin protease (Figs. 3.5B, C & 3.11). RsBBI is a non-competitive inhibitor which inhibit trypsin with *Ki* of 128.5 ± 4.5 nM and chymotrypsin with *Ki* of 807.8 ± 23.7 nM (Figs. 3.10A & B). RsKI is also a non-competitive inhibitor with *Ki* of 172.0 ± 9.2 nM (Fig. 3.10C). Biochemical analysis of RsBBI and RsKI revealed that RsBBI is stable at temperatures up to 100 ^{o}C. In contrast, RsKI is stable only up to 70 ^{o}C and lost its activity at 100 ^{o}C (Figs. 3.12A & D). Further, on incubating RsBBI from pH 2.0 to 12.0, the chymotrypsin inhibitory activity of RsBBI remained stable in the alkaline pH whereas a 40-50% reduction in the trypsin inhibitory activity of RsBBI was observed at pH 4.0-7.0 (Fig. 3.12B). In contrast, the trypsin inhibitory activity of RsKI was stable from pH 2.0 to 12.0 (Fig. 3.12E). However, RsBBI is sensitive to reduction with DTT where 100% loss of trypsin and chymotrypsin inhibitory activity was observed on reduction with 1.0 mM and 3.0 mM DTT, respectively (Fig. 3.12C). On the other hand, RsKI is relatively highly stable to DTT reduction and lost 100% activity only at 200 mM DTT (Fig. 3.12F). Further, CD spectrum indicated that RsBBI was stable to different temperature (25 to 95 ^{o}C) and pH (pH 2.0 to 12.0) whereas RsKI exhibited a decrease in ellipticity on heating at 80 and 90 ^{o}C. But, the ellipticity was regained on cooling down to room temperature (Figs. 3.12A-E). Further, mean residue ellipticity analysis of RsKI at 199 nm revealed that RsKI undergo two state sigmoidal reversible denaturation with *Tm* of 75 ^{o}C on heating from 25 to 95 ^{o}C and cooling down to 25 ^{o}C (Figs. 3.14C & D). Furthermore, RsBBI and RsKI exhibited potent differential inhibitory activity towards AjGPs and HaGPs. RsBBI inhibited AjGPs with IC_{50} of 24 ng and RsKI inhibited HaGPs with IC_{50} of 59 ng (Figs. 3.15C & D). RsBBI exhibited 81-fold higher activity against AjGPs than RsKI (Figs. 3.15C & E)

whereas RsKI exhibited 150-fold higher activity against HaGPs than RsBBI (Figs. 3.15B & D).

RsPI contained 80% of RsBBI and 20% of RsKI (Fig. 3.5A). **Chapter 4** describes the *in vivo* growth retardation and mortality properties of RsBBI against *A. janata* at first and third instar stages by feeding RsPI coated castor leaf. Further, BBIs from the seeds of *C. cajan* cultivar variety BDN1 (BDN1PI) was purified to 51-fold with 18% yield recovery and characterised (Figs. 4.1-4.3; Table 4.1). BDN1PI was used as a positive control for *in vivo* feeding experiments against *A. janata*. RsPI induced 80% growth retardation and 67% mortality in first instar larvae of *A. janata* when compared to 89% growth retardation and 81% mortality as induced by BDN1PI (Fig. 4.4; Table 4.2). On feeding to third instar *A. janata* larvae, RsPI induced 61% growth retardation and 50% mortality as compared to 66% growth retardation and 40% mortality as induced by BDN1PI (Fig. 4.5; Table 4.2).

In order to utilize the potential of PIs of *R. sublobata* i.e., RsBBI and RsKI to its maximum, the complete gene sequences of *RsBBI* and *RsKI* were elucidated in **Chapter 5** using RACE procedure (Figs. 5.1 & 5.8).Two chymotrypsin inhibitor *RsKI* gene sequences (*RsKI1 & RsKI2*) varying at the P_1 position of the reactive site were obtained (Figs. 5.2 & 5.3). The phylogenetic analysis indicated that the RsKI is closely related to soybean Kunitz inhibitor (Fig. 5.6). Further, three *RsBBI* gene sequences (*RsBBI1, RsBBI2* & *RsBBI3*) containing trypsin and chymotrypsin reactive site loops were elucidated (Figs. 5.8-5.10). *RsBBI1* encodes 119 amino acids with 14 cysteine residues. *RsBBI2* encodes 118 amino acids lacking the 119th Glutamate. Further, Glu is present at 45th position in *RsBBI2* instead of Asp as observed in RsBBI1. However, *RsBBI3* encodes 118 amino acids similar to *RsBBI2* but it encodes only 12 cysteine residues which may form only 5 disulphide bonds (Figs. 5.9 & 5.10). The phylogenetic analysis indicated that the RsBBI1 is closely related to BBIs from *Phaseolus* spp., and soybean

(Fig. 5.13). rRsBBI1 is overexpressed in *E. coli* and the purified 89 amino acid polypeptide obtained a molecular mass of 9970 Da by MALDI-TOF analysis (Figs. 5.15 & 5.16). rRsBBI1 inhibited both trypsin and chymotrypsin proteases by 1:1 molar ratio and exhibited non-competitive mode of inhibition against both trypsin (*Ki* = 358 ± 11 nM) and chymotrypsin (*Ki* = 446 ± 9 nM) proteases (Fig. 5.17). Further, rRsBBI1 is stable on heating up to 100 ^{o}C and at wide range of pH from 2.0 to 12.0, but sensitive to DTT reduction (Fig. 5.18). rRsBBI1 exhibited a very potent inhibitory activity against AjGPs with IC_{50} of 70 ± 2 ng whereas it exhibited an IC_{50} of 8 ± 0.5 µg against HaGPs (Figs. 5.19B & C). *In vivo* feeding of rRsBBI1 coated castor leaves to the second instar larvae of *A. janata* for 11 days exhibited 84% growth retardation and 60% mortality of the larvae (Fig. 5.19D).

Major conclusions

1. The trypsin-specific PIs (RsPI) from the seeds of *C. cajan* wild relative *R. sublobata* contained both RsBBI and RsKI inhibitors. They were, purified efficiently in short time using TCA/Sodium acetate buffer, and were characterised.
2. The non-competitive RsBBI and RsKI inhibitors exhibited differential biochemical properties.
3. RsBBI is highly active against the gut proteases of *A. janata* while RsKI is highly active against the gut proteases of *H. armigera*.
4. RsPI contained 80% of RsBBI which exhibited growth retardation properties and mortality on the first and third instar larvae of *A. janata* similar to BBIs from the seeds of *C. cajan* cultivar variety BDN1.
5. Complete CDS of *RsKI1* and *RsBBI1* genes isolated from immature seeds of *R. sublobata* was determined and they were phylogenetically related to soybean and *Phaseolus* spp.
6. Recombinant RsBBI1 expressed in *E. coli* is a non-competitive inhibitor of both trypsin and chymotrypsin, and it exhibited potent growth retardation and mortality against second instar larvae of *A. janata*.

Table 6.1. A comparative table indicating the biochemical properties of RsKI, RsBBI and rRsBBI1

Property	**RsKI**	**RsBBI**	**rRsBBI1**
Stability			
• Temp	Unstable at 80 °C	Stable at 100 °C	Stable at 100 °C
• pH	Stable (2.0-12.0)	≤40% TI loss (pH 5-7)	20% TI loss (pH 6.0)
• DTT	Not affected	100% loss TI-1 mM; CI-3 mM	100% loss TI-1 mM; CI-10 mM
Enzymes inhibited	Trypsin	Trypsin and Chymotrypsin	Trypsin and Chymotrypsin
Enzyme Kinetics	*Ki* = 172.0±9.2 nM	TI-*Ki* = 128.5±4.5 nM CI-*Ki*= 807.8±23.7 nM	TI-*Ki* = 357.6±11.4 nM CI-*Ki* =357.6±9.3 nM
Inhibition of gut proteases (IC_{50})	*H. armigera* = 59 ng *A. janata* = 1.95 µg	*H. armigera* = 8.85 µg *A. janata* = 24 ng	*H. armigera* = 8 µg *A. janata* = 70 ng

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Chapter 7

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1. Mohanraj S.S., Dutta-Gupta A., Padmasree K. 2017 Purification, identification and biochemical characterization of protease inhibitors from *Rhynchosia sublobata* seeds and their insecticidal activity against *Helicoverpa armigera* and *Achaea janata*. (**Under communication**).

2. Mohanraj S.S., Dutta-Gupta A., Padmasree K. 2017 *Rhynchosia sublobata* seed proteinase inhibitors and their growth inhibition potential at first and third instar larval stage of *Achaea janata.* (**Under preparation**).

3. Mohanraj S.S., Sarada D Tetali., Dutta-Gupta A., Padmasree K. 2017 Cloning, Heterologous Expression, Biochemical Characterization and Insecticidal activity of a novel Bowman-Birk Inhibitor from the young seed of Pigeonpea Wild-relative *Rhynchosia sublobata* (**under communication**).

4. Swathi M., **Mohanraj S.S**., Swaroop V., Gujjarlapudi M., Mallikarjuna N., Dutta-Gupta A., Padmasree K. (2015). Proteinase inhibitors from *Cajanus platycarpus* accessions active against pod borer *Helicoverpa armigera*. ***Acta Physiologiae Plantarum***, *37*(11), 1-11.

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