Characterization of Bowman-Birk and Kunitz Protease Inhibitors from Selected Legumes with Special Reference to Development of a Rapid Protocol for Their Isolation

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BY

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DECLARATION

I, **Mariyamma Gujjarlapudi**, hereby declare that the work presented in this thesis entitled **"Characterization of Bowman-Birk and Kunitz Protease Inhibitors from Selected Legumes with Special Reference to Development of a Rapid Protocol for Their Isolation"** has been carried out by me under the supervision of Prof. K. Padmasree in the Department of Biotechnology & Bioinformatics, School of Life Sciences, University of Hyderabad. This work has not been submitted for any degree or diploma of any other University or Institute.

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CERTIFICATE

This is to certify that the thesis entitled **"Characterization of Bowman-Birk and Kunitz Protease Inhibitors from Selected Legumes with Special Reference to Development of a Rapid Protocol for Their Isolation"** submitted by **Mrs. Mariyamma Gujjarlapudi**, bearing registration number **14LTPH01** in partial fulfillment of the requirements for award of **Doctor of Philosophy** in the **Department of Biotechnology and Bioinformatics, School of Life Sciences** is a bonafied work carried out by her under my guidance and supervision.

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

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

A. Published in the following journals:

1. Acta Physiology Plantarum, DOI: 10.1007/s11738-015-1991-8
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Course Code	Name	Credits	Pass/Fail
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Dedicated to my Parents

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Samuel Moses - Rajya Lakshmi

Abbreviations

AjTPs	*Achaea janata* midgut trypsin-like proteases
AjTPIs	*A. janata* midgut trypsin-like protease inhibitors
BAPNA	N-α-benzoyl-DL-arginine-*p*-nitroanilide
BBI	Bowman-Birk inhibitor
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CpBBI	*Cajanus platycarpus* Bowman-birk inhibitor
CpKI	*Cajanus platycarpus* Kunitz inhibitor
CBB	Coomassie brilliant blue
CD	Circular dichroism
CI	Chymotrypsin inhibitor
DTT	Dithiothreitol
FPLC	Fast protein liquid chromatography
GLUPHEPA	N-glutaryl-L-phenylalanine-p-nitroanilide
HaTPs	*Helicoverpa armigera* midgut trypsin-like proteases
HaTPIs	*H. armigera* gut trypsin-like protease inhibitors
IC_{50}	Half-maximal inhibitory concentration
IDA	Iodoacetamide
KI	Kunitz Inhibitor
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MJ	Methyl jasmonate
PIs	Protease inhibitors
PVP	Polyvinylpyrrolidone
ROS	Reactive oxygen species
RsBBI	*Rhynchosia sublobata* Bowman-Birk inhibitor
RsKI	*R. sublobata* Kunitz inhibitor
TCA	Trichloroacetic acid
TI	Trypsin inhibitor
VrBBI	*Vigna radiata* Bowman-Birk inhibitor
VrKI	*V. radiata* Kunitz inhibitor
SBBI	Soybean Bowman-Birk inhibitor
SlTPs	*Spodoptera litura* midgut trypsin-like proteases
SlTPIs	*S. litura* midgut trypsin-like protease inhibitors

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Abstract of Thesis

Protease/proteinase inhibitors (PIs) are the defense molecules ubiquitously present in seeds and induce upon stress conditions in leaves. Legume seeds are the repositories of PIs, especially Bowman-Birk inhibitor (BBI) and Kunitz inhibitor (KI), and possess insecticidal as well as therapeutic properties. Due to narrow molecular mass differences, separation of these inhibitors from a single seed variety is tedious. In the current study, a rapid protocol (<24h) was developed to purify BBI and KI from mature, dry seeds of *Vigna radiata*, *Cajanus platycarpus* and *Rhynchosia sublobata*, by using mild trichloroacetic acid (TCA) extraction and affinity column chromatography followed by sodium acetate extraction. The purified BBI/KI was confirmed by western blot and MALD-TOF studies. The purified BBI(s) (VrBBI-8.9 kDa, CpBBI-7.8 kDa and RsBBI-9.2 kDa) showed inhibition against both trypsin and chymotrypsin, while KI(s) (VrKI-19.2 kDa, CpKI-19.4 kDa and RsKI-19.2 kDa) showed inhibition against trypsin only. The trypsin/chymotrypsin inhibitory (TI/CI) activity of both BBI(s) and KI(s) are stable at a wide range of pH (2-12). However, BBI/KI showed differential stability against temperature (20-90 ^{o}C) and DTT treatment as evidenced by *in vitro* assays, CD and fluorescence spectroscopic studies.

Lepidopteran insect pests such as *Achaea janata* and *Helicoverpa armigera* are the major insect pests causing severe damage to various economically important crops. Several previous studies reported that non-host PIs are more potent than host PIs in controlling insect pests. The seed varieties (*C. platycarpus* and *R. sublobata*) used in the present study are non-host plants to *A. janata* and wild relatives to *H. armigera*. Besides, *V. radiata* is a non-host plant to *A. janata*, but host plant to *H. armigera*. Hence, the inhibitory potential of purified BBI(s)/KI(s) was studied against these two insect pests by *in vitro* assays and *in vivo* feeding experiments. Low IC_{50}, reduced larval weight, formation of larval-pupal & pupa-adult intermediates, and decreased survival rate suggest that BBI(s) are potent in the management of *A. janata*, while KI(s) are potent in the management of *H. armigera*. Besides, among all the BBI(s)/KI(s), the RsKI inhibited breast cancer cell lines (MCF7) significantly (33%) than cervical cell lines (HeLa). Furthermore, both BBI(s) and KI(s) inhibited (≤60%) the growth of *Staphylococcus aureus*, a gram-positive pathogenic bacterium suggesting them as excellent candidates for developing novel anti-microbial agents.

As it is evidenced that PIs are induced in leaves upon stress, an attempt was made to address the following questions using black gram (*Vigna mungo*) as a model plant (i) whether PIs are induced in leaves of black gram; (ii) if induced, what is the nature of induced PIs, and (iii) does induced PIs differ with seed PIs in their biochemical and insecticidal properties. To address these questions, the induction of PIs was monitored at different time intervals in black gram leaves after wounding, methyl jasmonate treatment and *H. armigera* infestation. The activity of PIs was monitored as trypsin and chymotrypsin inhibitory units. As the induced PIs showed both TI and CI activity, a characteristic feature of Bowman-Birk inhibitor (BBI) family, real-time PCR was performed with BBI-specific primers. An increase in mRNA expression levels with BBI-specific primers indicates that PIs induced in black gram belong to BBI family. Further, an increase in H_2O_2 (ROS) content was observed under all the given treatment conditions indicating that ROS acts as a signaling molecule for PI induction.

The induction of PIs is high in leaves treated with methyl jasmonate as compared with other treatments. Thus, the PIs induced in leaves upon methyl jasmonate treatment and PIs present in seeds as storage proteins were purified to homogeneity using ammonium sulfate fractionation and different chromatography techniques to compare their biochemical and insecticidal properties. Leaf and seed PIs exhibited a molecular mass of 9.7 kDa & 8.2 kDa, respectively, and confirmed by performing dot-blot assay with anti-BBI antibody. Leaf and seed PIs varied in their stability against pH (2-12) i.e. leaf PI showed significant TI and CI activities at pH 7.0 and lost its activity at highly acidic or alkaline conditions, whereas the TI and CI activities of seed PI is stable from pH 2-12.Besides, both leaf and seed PIs showed stable TI and CI activities against a wide range of temperatures and lost their activity upon DTT treatment. Further, in-gel activity staining studies of leaf and seed PIs showed significant inhibitory activity against midgut trypsin-like proteases of Lepidoptera insects such as *A. janata, Spodoptera litura* and *H. armigera.* However, the *in vitro* insecticidal potential of seed PI is high when compared with leaf PI. Therefore, we restricted to perform *in vivo* feeding experiments with seed PI and evaluated its effect on the growth and development of *A. janata* and *S. litura.* The results obtained manifested the use of seed PIs in the management of *S. litura* and *A. janata*.

Chapter 1

Introduction and Review of Literature

Introduction and Review of Literature

The world population is anticipated to increase from 7.8 billion in 2021 to 8.6 billion in 2031, necessitating an increase in global food productivity to feed the growing population (OECD-FAO agricultural outlook 2022-2031). According to the global report on food crisis, around 193 million people in 2021 and about 258 million people in 2022 were in the worst food crisis (GRFC, 2023). The primary reason behind the food crisis is crop losses due to several abiotic factors such as water, temperature, and availability of nutrients and biotic factors such as weeds, pests, and pathogens. However, 20-25% of crop losses are caused due to pests and pathogens annually (Jamiołkowska and Kopacki, 2020). Farmers used various synthetic pesticides such as carbamine, pyrethrin and pyrethroid for several decades to control plant pests and pathogens (Fathipour and Sedaratian, 2013; Saha et al., 2020). These synthetic pesticides comprise organochlorine derivatives and chlorinated hydrocarbon, which are less effective against pests and degrade slowly (Bassil et al., 2007; Aktar et al., 2009; Jayaraj et al., 2016; Rani et al., 2021). The cost of pesticides used for pest control is more than US $10 billion per annum. Despite killing the pests, these pesticides also affect beneficial organisms and leave dangerous residues on soil and food (Sharma et al., 2019). Many pesticides have been banned as their chemical residues remain on fruits and vegetables, causing food and environmental pollution (Swarnam and Velmurugan, 2013; Curl et al., 2019).

The alternative approach to control insect pests and pathogens is the integrative pest management strategy, which combines biological, cultural, chemical and physical tools to reduce environmental, health, and economical risks. However, pests adapt to these control methods and acquire resistance (Abate et al., 2000). Therefore, it has become necessary to search for an alternative means of pest control to minimize insect

resistance. One such alternative strategy is the exploitation of plant's own defense molecules, such as lectins, ribosome-inactivating proteins, amylase inhibitors, and protease inhibitors, which offer several advantages in better pest management (Murdock and Shade, 2002; Agale et al., 2017; Marrone, 2019). However, the mechanism behind the entomotoxic activity of all these defense compounds may vary, for example: (i) lectins interact with the glycoproteins of insects and interrupt different physiological functions in insects (Vandenborre et al., 2011); (ii) ribosome-inactivating proteins (RIPs) depurinate rRNAs and arrest protein synthesis during translation (Bertholdo-Vargas et al., 2009; Zhu et al., 2018); (iii) amylases are the essential enzymes of insect midgut and provide nutrients to insects by digesting plant starch and proteins. Amylase inhibitors act on these amylases and affect insect's life cycle (Franco et al., 2002; Gupta et al., 2013; Yu et al., 2017), and (iv) protease inhibitors act on the midgut proteases of insect pests and retard their growth and development (Fan and Guo-Jiang, 2005; Bateman and James, 2011; Lokya et al., 2020).

Protease inhibitors (PIs) are one among the important defense molecules with highly proven inhibitory activity against insect pests and pathogens (Haq et al., 2004; Jain et al., 2022). PIs are small defense molecules present in storage organs such as seeds and tubers (1-10%) and induced in leaves and flowers upon exposure to various biotic and abiotic stress conditions (Fan and Guo-Jiang, 2005; Bateman and James, 2011; Islam et al., 2017).

Distribution, localization, and physiological functions of PIs

PIs have been widely distributed throughout the plant kingdom and are majorly characterized from Gramineae, Leguminosae and Solanaceae families. A few biochemical investigations on the intracellular localization of PIs indicated the presence of PIs in the

cytoplasm of cotyledons and parenchyma of leaf and stem (Baumgartner and Chrispeels, 1976; Chrispeels and Baumgartner, 1978; Weng et al., 2003). The localization studies carried out by Horisberger and Vonlanthen (1983) on thin sections of cotyledon and embryonic axis of *Glycine max* revealed the localization of BBI majorly in all protein bodies, nucleus, cytoplasm, and intercellular spaces to some extent. Further, KI is found to be localized in the cell wall, protein bodies, and cytoplasm present between lipid-containing spherosomes and the nucleus. Further, visualization of the nucleus showed the occurrence of PIs in chromatin deposits and nucleolus. Also, various biotic and abiotic stress conditions are known to induce PIs. For example, the wound inducible PIs from tomato and potato are reported to be accumulated in vacuolar protein bodies of the cell (Shumway et al., 1976; Hollaender-Czytko et al., 1985). A serine PI from *Solanum americanum* (SaPIN2) is expressed in the phloem of stems, leaves, and roots (Xu et al., 2004). The studies of Hernandez-Nistal et al. (2009) reported two cell wall localized KIs in chickpea during seed germination and seedling growth. Tagging of cysteine PIs with green fluorescent protein (GFP) revealed their presence in endoplasmic reticulum and golgi network of onion epidermal cells (Martinez et al., 2012).

PIs are mainly involved in (i) *regulation of endogenous proteases during seed germination and maturation*. For example, PIN2 purified from *S. americanum* inhibited endogenous proteases during seed development (Chye et al., 2006; Sin et al., 2006; Hartl et al., 2011); (ii) *protection against seed predators, pathogens, and pests*. For example, the PIs present in potato tubers and legume seeds showed insecticidal, antifungal and antibacterial activity (Kim et al., 2009; Marathe et al., 2019; Cotabarren et al., 2020); (iii) *act as seed storage proteins*. For example, the high content of PIs in seeds and their decrease during seed germination was observed in several studies indicating that PIs serve as a source of essential sulfur-containing amino acids for the germinating seed (Duranti,

2006; Grosse-Holz and van deer Hoorn, 2016) and (iv) *regulation of programmed cell death*. For example, an ectopic expression of cystatin (an endogenous cysteine PI gene) blocked PCD in soybean (Solomon et al., 1999). Similarly, the KI present in *Arabidopsis thaliana* regulated PCD during flower development (Boex-Fontvieille et al., 2015).

Classification of PIs

PLANT-PIs database (http://bighost.area.ba.cnr.it/PLANT-PIs) was developed by Leo et al. (2002) to avail the information related to plant PIs and their genes. This database contains almost 351 plant PIs isolated from 129 species of plants. Depending on the class of proteases they inhibit, PIs are mainly classified into the following four types such as serine, cysteine, aspartate and metallo PIs (**Fig. 1.1**; Haq et al., 2004; Bhattacharjee et al., 2012; Clemente et al., 2019; Cotabarren et al., 2020).

(i) Serine PIs

Serine PIs are the most abundant type of PIs reported from most plant varieties. Based on their molecular weight, cysteine content, the number of disulfide bridges, and their abundance in different plant species, serine PIs are subdivided into Bowman-Birk inhibitors, Kunitz inhibitors, mustard trypsin inhibitors, serpins, potato type-I and type II inhibitors, squash inhibitors and cereal trypsin/alpha-amylase inhibitors (Rachel and Sirisha, 2014; Marathe et al., 2019; Shakeel et al., 2019).

Bowman-Birk inhibitors (BBIs)

BBIs are reported to be more abundant in legume seeds than other seed varieties (Habib and Fazili, 2007; Rodriguez-Sifuentes et al., 2020; Herwade et al., 2021; Sultana et al., 2022). For the first time, BBI was isolated and characterized from soybean seeds by two scientists Bowman and Birk (Bowman, 1946; Birk et al., 1963). Soybean BBI is the most

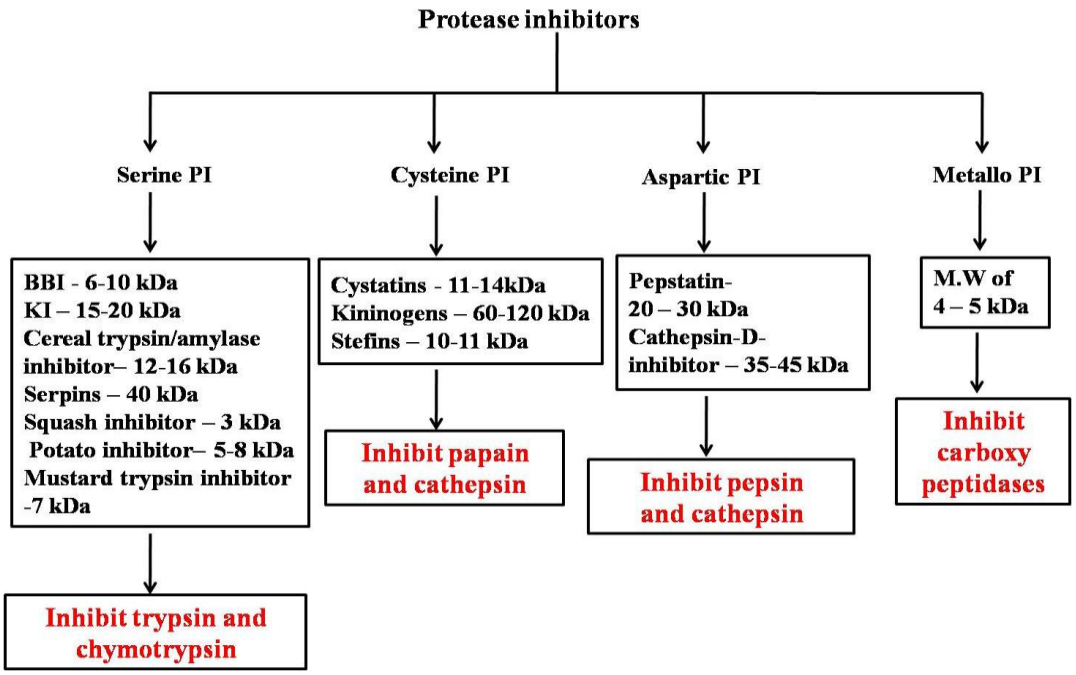

Fig. 1.1. Classification of protease inhibitors based on the type of proteases they inhibit. Adapted from Shamsi et al. (2016) and modified.

studied and well-characterized PI hence regarded as the classical marker for all BBI types of PIs. In general, dicot BBIs possess a low molecular weight of 8-10kDa with a single polypeptide chain and two reactive sites inhibiting proteases such as trypsin and chymotrypsin. The P_1 residue of the first reactive site is Lys/Arg, and the second reactive site is more variable: Arg/Phe/Tyr/Leu or Ala. Besides, the P1′ residue of both the first and second reactive site is a conserved Ser residue (Qi et al., 2005). The BBIs in monocots exist in two different classes: The first class possessed 16kDa molecular mass with two reactive sites and the second class possess 8kDa molecular mass with single reactive site (Prakash et al., 1996).

The structure of BBI is highly stabilized by its compactness due to involvement of fourteen cysteine residues to form seven disulfide bridges between Cys_1-Cys_{14}; Cys_2-Cys_6; Cys_3-Cys_{13}; Cys_4-Cys_5; Cys_7-Cys_9; Cys_8-Cys_{12}; and Cys_{10}-Cys_{11} (**Fig.1.2**; Birk, 1985; Odani et al., 1986; Tashiro et al., 1987).

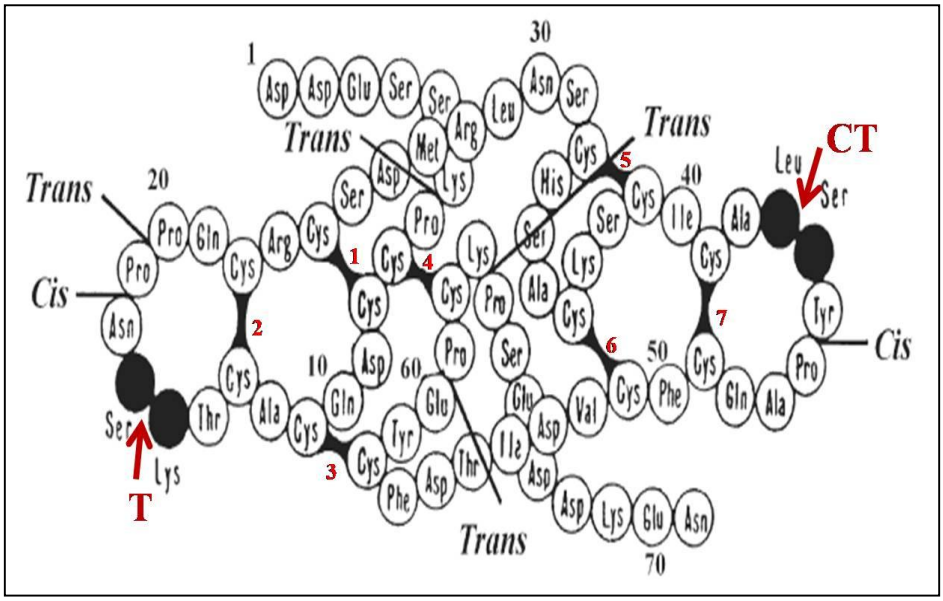

Fig. 1.2. Structure of Bowman-Birk inhibitor. Red color arrows indicate trypsin (T) and chymotrypsin (CT) inhibitor sites. The numbers indicated in red color font represent the disulfide bridges. The P1 residues in trypsin and chymotrypsin inhibitor sites include lysine and leucine, respectively (Adapted from Odani and Ikenaka, 1972).

Kunitz inhibitors (KIs)

KIs were first discovered in legume seeds and further reported from several plant species, such as cereals and some members of the Solanaceae family. Soybean KI was the first reported serine type of PI in 1945 by a scientist named 'Kunitz,' and it possessed 181 amino acids long single polypeptide chain, and two disulfide bridges (Koide and Ikenaka, 1973; Lehle et al., 1996; Oliva et al., 2010). Of the total reported 438 different eukaryotic KTI entries (UniProtKB database), 263 are reviewed, among which 51 are of plant origin (Bendre et al., 2018). Generally, KIs have a molecular mass of ~18-20kDa with two polypeptide chains and two disulfide bridges. These two polypeptide chains are initially synthesized as a single polypeptide that is further cleaved into two chains during post-translational modifications (Bonturi et al., 2022). However, the no. of polypeptide chains

and disulfide bonds varied between different plant KIs (Oliva et al., 2010, Luiza Vilela Oliva et al., 2011).

The KI structure consists of the beta-trefoil fold, in which twelve antiparallel beta-sheets are connected by the loops varying in conformation, amino acid composition, and length (Azarkan et al., 2011; Bendre et al., 2018). They contain a single reactive site inhibiting either trypsin or chymotrypsin (**Fig. 1.3**). But the KI from winged bean (*Psophocarpus tetragonolobus L.*) is double-headed and can bind to two chymotrypsin molecules simultaneously. Crystallographic studies of winged bean KI revealed that the first inhibitory loop is located at the same position as other plant KIs (Gln63-Phe64-Leu65-Ser66-Leu67-Phe68-Ile69), and the second inhibitory loop is located between Asn38-Glu39-Pro40-Cys41-pro42-Leu43 (Dattagupta et al., 1999; Bendre et al., 2018).

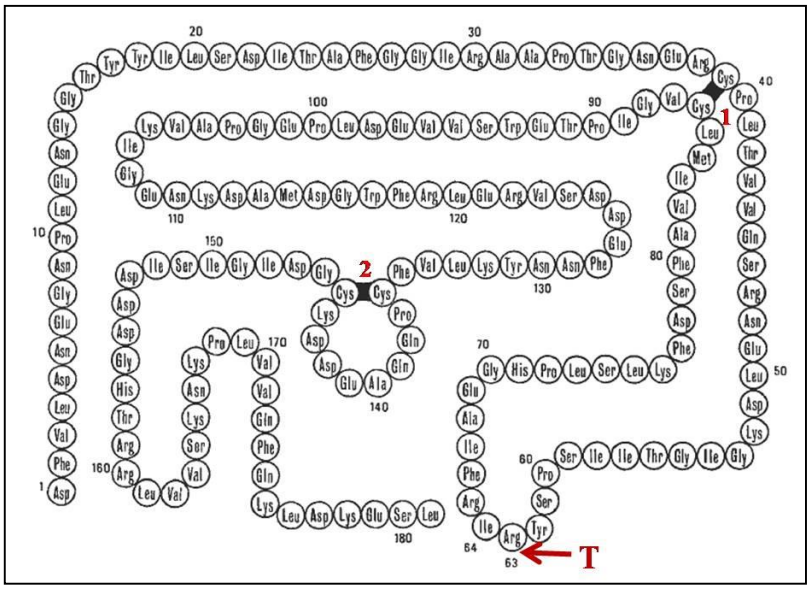

Fig. 1.3. Primary structure of Kunitz inhibitor purified from Soybean (SKTI). The red color arrow indicates the trypsin inhibitor site, and the numbers represented in red color font indicates the disulfide bonds (Adapted from Koide and Ikenaka, 1973).

In general, the PIs contain several isoforms of an inhibitor. For example, ten SKTI isoforms were reported to express in soybean (Krishnan, 2001). Further, the effect of individual isoforms also varies on their cognate proteases. *Erythrina latissima L.* seeds

contain two isoforms: one is effective against chymotrypsin but not trypsin, and the second isoform is specific to trypsin only. Apart from serine proteases, KIs are also known to inhibit other proteases, such as aspartic and cysteine proteases (Renko et al., 2012). The potential role of KIs in defense against pests and pathogens was studied from different plants, as evident from the up-regulation of gene expression on wounding and pest attack (Major and Constable, 2008; Philippe et al., 2009; Botelho et al., 2014; Arnaiz et al., 2018; Bonturi et al., 2022).

Cereal trypsin/alpha-amylase inhibitors

Cereal trypsin/alpha-amylase PIs can inhibit the serine-type proteases as well as the amylases (Gadge et al., 2015). Among them, most are active against amylase enzymes, and few of them have double activity. Barley alpha-amylase/subtilisin inhibitor (BASI) is a well-studied inhibitor with both subtilisin and alpha-amylase inhibitory activity (Franco et al., 2002). Barely PIs are 120-160 amino acids long with either four or five disulfide bonds. Moreover, Kunitz-like alpha-amylase inhibitors comprise around 180 amino acids with only two disulfide bonds (Leah and Mundy, 1989; Volpicella et al., 2011). Another example of this family includes Ragi amylase/trypsin inhibitor (RATI) that forms a stable complex with both amylase and trypsin enzymes simultaneously. RATI is around 122 amino acids long peptide chain, consisting of five disulfide bonds and two inhibitory sites. The alpha-amylase inhibitor region is located at the N-terminal end, and the trypsin inhibitory site is at the C-terminal end (Alam et al., 2001). PIs from maize and wheat are active against either trypsin or alpha-amylase (Mundy et al., 1983; Orengo et al., 1994; Alam et al., 2001; Svensson et al., 2004).

Serpins

Serpins are 40 kDa proteins with 300-500 amino acids long, consisting of 8-9 alpha helices, three beta-sheets, and a canonical reactive site loop in their structure (Huntington

et al., 1997). Serpins are reported from several plant varieties, such as *A. thaliana*, pumpkin, wheat, barley, and oats (Rosenkrands et al., 1994; Yoo et al., 2000; Ahn et al., 2009). Serpins are also known as suicide inhibitors due to their unique mode of inhibition, which is different from other PIs. Initially, serpins bind reversibly to their target protease through their exposed canonical reactive site loop. Upon binding, the exposed reactive site loop gets cleaved at the specific site (P1-P1' site), resulting in a typical change in their structure and stability. These conformational changes lead to its covalent binding with the active site of target protease, and hence an irreversible complex of serpins and protease formed (Cohen et al., 2019).

Squash PIs

Squash inhibitors, also known as Cucurbitaceae type-peptides, are the smallest inhibitors (3kDa) with 27-33 amino acids long polypeptide chain and a knot-like structure formed by three disulfide bonds. They also contain secondary structural elements like α-helix and β-sheets, and their N-terminus is protected by pyroglutamate. They majorly inhibit chymotrypsin, elastase and trypsin (Hellinger and Gruber, 2019). These are found in the seeds of zucchini, summer squash, cucumber, watermelon, red bryony, spaghetti squash, and fig leaf gourd. Proteases inhibited by squash PIs include plasmin, Xa and XII blood clotting factors, trypsin, cathepsin, and kallikrein (Otlewski et al., 1990; Otlewski and Krowarsch, 1996; Chiche et al., 2004).

Potato-type I (PIN 1) and potato-type II PIs (PIN 2)

PIN 1 and PIN 2 are accumulated mainly in the plant's reproductive and storage organs but are structurally different from each other. PIN 1 consists of four mixed parallel and antiparallel beta-sheets opposite to a single alpha helix. It does not consist of any disulfide bonds in its structure. However, the reactive site consists of several hydrogen bonds that provide stability. The molecular weight of PIN 1 is around 8kDa (Clore et al.,

1987; McPhalen and James, 1987; Turra and Lorito, 2011). These are found in organs of many plant species, such as *Amaranthus hypochondriacus* (stems and seeds), sweet potato (developing leaves and fruits), tomato (developing leaves and fruits), rice (shoot apical meristems and germinating seeds), and tobacco (etiolated leaves and floral buds) (Volpicella et al., 2011). Members of PIN 2 are primarily found in the *Solanaceae* family, such as *Nicotiana tabacum, S. americanum, S. lycopersicum* and *Capsicum annum* (Kong and Ranganathan, 2008; Turra et al., 2020). It consists of multiple inhibitory repeat domains joined by five to six amino acid linker regions. These inhibitory repeat domains are 50-55 amino acids long that release from their parent molecules upon cleavage at linker regions. Disulfide bonds stabilize the inhibitory domains (Barrette-Ng et al., 2003; Schirra and Craik, 2005; Mishra et al., 2010; Yadav et al., 2021). PIN I is effective against chymotrypsin, trypsin, and subtilisin, while PIN II inhibits trypsin and chymotrypsin (Clemente and Domoney, 2006).

Mustard trypsin inhibitors (MSI)

MSI is found in *Sinapis alba*, *A. thaliana,* wild relatives of *Brassicaceae* such as *Diplotaxis tenuifolia,* and *D. muralis* (Volpicella et al., 2001; Volpicella et al., 2009; Zhao et al., 2019). As its name suggests, it was first identified from the seed of mustard. The first isolated MSI is 63 amino acids long, cysteine, and glycine-rich single polypeptide chain. The molecular weight is around 7 kDa and was found to inhibit bovine trypsin. MSI isolated from *A. thaliana* contains two antiparallel beta-sheets and one alpha helix. It consists of four disulfide bonds. Three disulfide bonds connect the alpha-helix and inhibitory loop to the beta-sheet, whereas the fourth disulfide bridge connects the N and C termini (Zhao et al., 2002; Volpicella et al., 2011).

(ii) Cysteine PIs (CPIs)

After the serine-type proteases, the second most abundant protease is cysteine (Laskowsi Jr and Kato, 1980; Rustgi et al., 2018). CPIs consist of a central five-turn alpha-helix wrapped by the five-stranded antiparallel beta-sheet. Plant CPI is around 12-16 kDa without disulfide bonds and forms a reversible complex with target proteases (Dong et al., 2020; Balbinott and Margis, 2022). The N-terminal alpha-helix of Plant CPIs possess a conserved glycine residue responsible for its inhibitory activity (Machleidt et al., 1989; Margis et al., 1998). Oryzacystatins (OC-I and OC-II) are the best-studied plant CPIs, consisting of 102-107 amino acids in their structure. These are identified in rice caryopses and are active against papain (OC-I) and cathepsin (OC-II) (Kondo et al., 1990; Misaka et al., 1996). Cystatins play a significant role in protecting plants against biotic and abiotic stress conditions (Martinez et al., 2016; Mangena, 2020; Shibao et al., 2021).

(iii) Aspartic PIs (APIs)

APIs are rare and studied from few plant species such as tomatoes, potatoes and wheat. They are small proteins with a 25-27 kDa molecular weight and two disulfide bridges (Rachel and Sirisha, 2014). The inhibitor of Cathepsin-D was first identified API in potatoes. The molecular weight of the Cathepsin-D inhibitor is around 27 kDa and possesses sequence similarity with soybean Kunitz inhibitors (Mares et al., 1989; Guo et al., 2015).

(iv) Metallo PIs (MPIs)

Metallo proteases are the least inhibited proteases by plant PIs (Laskowsi Jr and Kato, 1980). MPI is found in the *Solanaceae* family, including tomato (fruit and wounded leaves) and potato (constitutively in tubers and leaves upon induction). The potato MPI is a 39 amino acids small globular protein with a molecular weight of around 4.2 kDa and

active against metallo carboxypeptidase. The structure of MPI consists of 3_{10} helix and two beta-sheets stabilized by three disulfide bridges (Gonzalez et al., 2003; Diez-Diaz et al., 2004; Volpicella et al., 2011). Potato metallo carboxypeptidase inhibitor binds to its cognate proteases like enzyme-substrate, even though its inhibitory site is located on the C-terminus tail rather than in a stabilized loop. The exposed C-terminus upon cleavage of the P1-P1' bond in the carboxypeptidase inhibitor coordinates with the Zn^{2+} of the carboxypeptidase active site and blocks the catalytic site of the enzyme (Gomes et al., 2011).

Elicitation of PIs in leaves and flowers

Plants emit various volatile substances from their leaves, flowers and fruits to communicate with other plants, attract pollinators, and these plants are less susceptible to pests (Pichersky and Gershenzon, 2002). Synthesis of these volatile compounds is induced by specific external challenges caused by pests, pathogens, herbivores, or adverse environmental conditions (Pare and Tumlinson, 1999). Furthermore, these volatile compounds act as signals in the air, communicating between the plants affected and their neighboring plants (Arimura et al., 2000). Jasmonic acid (JA) and its derivatives, like methyl jasmonate (MJ), are volatile in nature and play a significant role as a signaling molecule in plant cellular responses, plant-plant interaction, and plant-herbivores interaction (Cheong and Choi, 2003). The MJ is an essential cellular regulator in various developmental processes of plants, from seed germination to seed maturation (Creelman and Mulpuri, 2002; Wasternack and Hause, 2002; Mohamed and Latif, 2017). The JA levels in plants vary based on tissue, cell type and developmental stage. For example, a high level of jasmonate responsive gene expression was observed in mesophyll cells and bundle sheath cells of soybean leaves as compared to epidermal cells (Franceschi et al., 1983; Huang et al., 1991). The interactions of local or systemic signaling molecules such

as chitoson, abscisic acid, oligogalacturonic acid, systemin and some electrical and hydraulic signals initiate the JA-induced defense mechanism (Bhattacharjee et al., 2012). In the case of insect bite, volicitin, a component present in oral secretions of insects, acts like linolenic acid and induces the synthesis of PIs through the octadecanoid pathway (Alborn et al., 1997). The inducible synthesis of PIs upon exposure to biotic or abiotic stress conditions is shown in **figure. 1.4**.

The exogenous application of JA/MJ on plants helps to tolerate abiotic stress conditions. For example, the foliar application of MJ on two maize genotypes showed tolerance to salinity stress by decreasing the uptake of Na^+ in roots (Shahzad et al., 2015). Similarly, a rapid increase of JA was observed in *A. thaliana*, citrus, and soybean under drought stress, and plants tend to show normal growth (Balbi and Devoto, 2008;

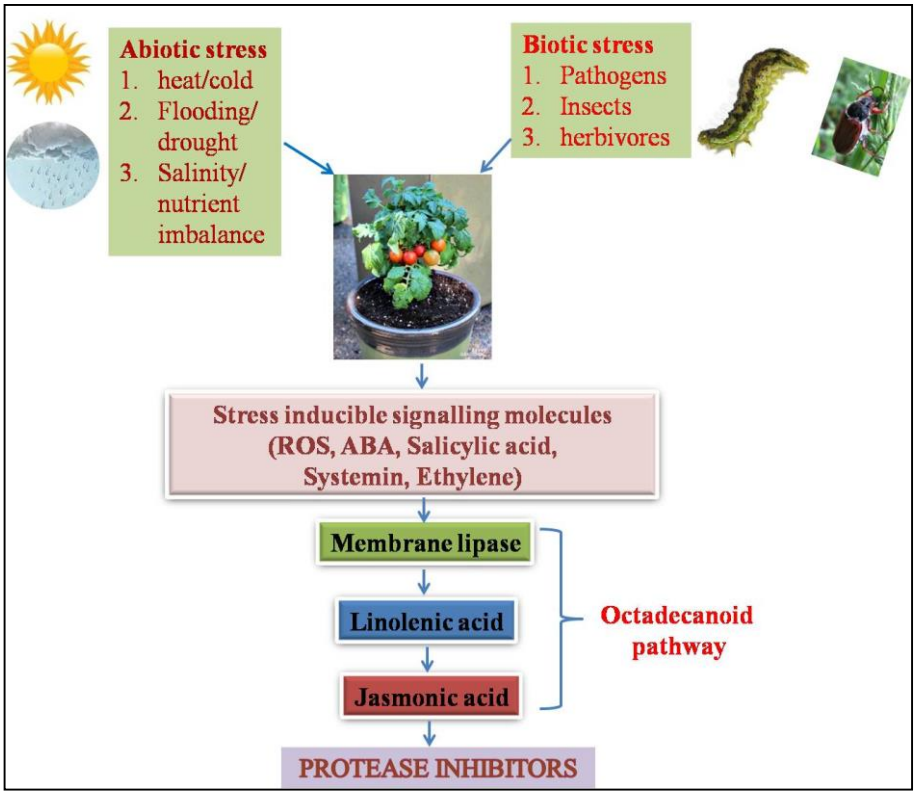

Fig. 1.4. Induction of PIs upon exposure to various biotic and abiotic stress conditions through jasmonic acid (Adapted from Swathi et al., 2021).

Mohamed and Latif, 2017). Likewise, the *A. thaliana* plants showed up-regulation of JA accumulation in response to drought and high light stress, whereas the JA mutants were damaged (Balfagon et al., 2019). Further, the studies of Ahmad et al. (2017) on the role of JA in heavy metal stress, like cadmium (Cd) stress in faba beans, revealed that JA enhanced plant development by increasing chlorophyll content and biomass yield. Similarly, the foliar application of MJ on plants under boron and lead stress enhanced photosynthesis, antioxidant enzyme synthesis, and decreased lipid peroxidation through ROS scavenging mechanism (Aftab et al., 2011; Bali et al., 2019). A transgenic plant was developed by introducing serine PIs isolated from sugar beet and subjected to salt stress. The plants expressing PI genes maintained high chlorophyll and deferred senescence, indicating that PIs might significantly inhibit chloroplast deterioration (Savic et al., 2019). Apart from serine PIs, cysteine PIs are also induced as defense molecules during abiotic stress conditions. *Nicotiana benthamiana* plants transformed with *Jatropha curcas* cysteine PIs showed potent salinity resistance compared to non-transformed plants (Li et al., 2015).

Several previous studies have reported the induction of PIs in leaves upon exposure to biotic and abiotic stresses. Green and Ryan (1972) observed the induction of PIs upon wounding the tomato and potato leaves by Colorado potato beetle. Further, induction of PIs (CanPI-4, -7 and -10) was also observed by subjecting the leaves of *C. annum* to wounding and treatment with oral secretions of pod borer *Helicoverpa armigera* (Mishra et al., 2012). The inducible expression of both serine and cysteine PI genes was observed in wheat leaves upon infestation with Colorado potato beetle larvae (*Oulema melanopus*) (Wielkopolan et al., 2018). Furthermore, the studies of Malefo et al. (2020) also showed the overexpression of BBI genes upon drought stress in *A. thaliana* plants. The recent studies by Eberl et al. (2021) also showed up-regulation of eight Kunitz

trypsin inhibitors in the leaves of black poplar (*Populus nigra*) upon invasion with three insect pests such as *Amata mogadorensis*, *Lymantria dispar* and *Phratora vulgatissima* .

Mode of action of PIs in the digestive system of insect pests

Insects feed on plants to fulfill their nutritional requirements for proteins and carbohydrates. Digestive proteases present in the insect midgut help in the proteolysis of these proteins into essential amino acids that aid in the growth and development of insets (Akbar et al., 2018). Generally, PIs block the insect midgut proteases in two ways, i.e., (i) reversible and (ii) irreversible mode of inhibition (Clemente et al., 2019). In reversible inhibition, PIs bind to proteases through the reactive site loop in a substrate-like manner and form a protease-PI complex which degrades the protease, and the active inhibitor dissociates to form a complex once again with other proteases (**Fig. 1.5**).

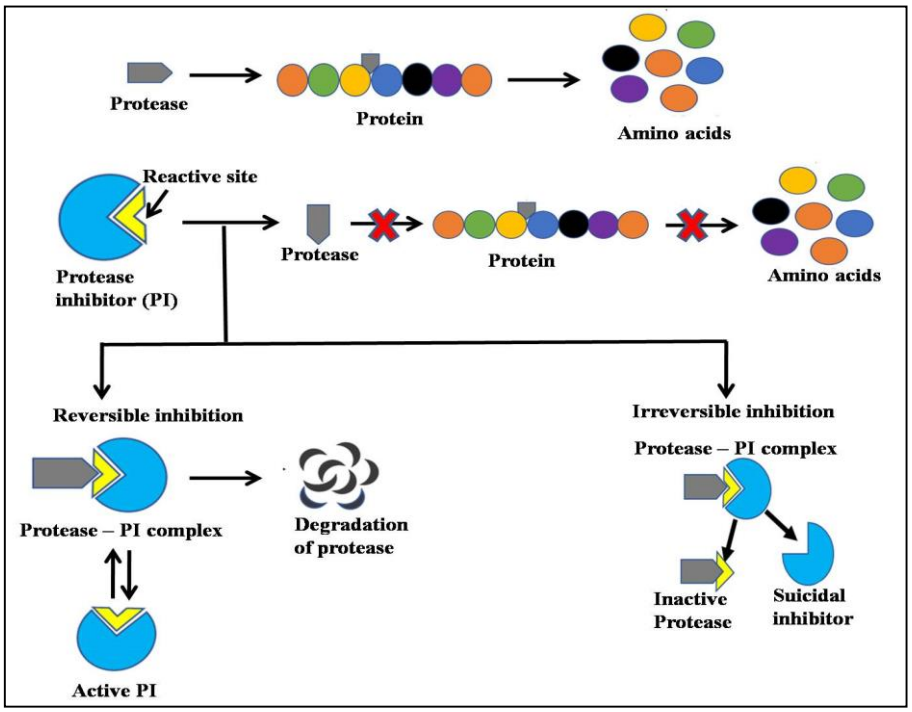

Fig. 1.5. Mode of action of PIs in inhibiting the target proteases. Adapted from Sultana et al. (2022) and modified.

In irreversible inhibition, PIs bind to the active site of proteases and form a protease-PI complex. This complex cleaves the peptide bonds of PIs resulting in the inactivation of both protease and PI (suicidal inhibitor) (**Fig. 1.5**; Sultana et al., 2022). The inhibition of proteases by PIs leads to amino acid deficiency and reduced nutritional absorption resulting in growth retardation, developmental abnormalities, low fertility, and fecundity (Napoleao et al., 2019).

General methods used for purification of PIs and biochemical characterization

The primary step in the purification of any PI from seed material is the preparation of crude extract, which involves grinding the seeds into a fine powder followed by depigmentation and defatation with several washes of acetone and hexane, respectively. The filtered dry powder is extracted into a suitable buffer containing 1% polyvinylpyrrolidone (PVP), stirred overnight at 4 ^{o}C, and subjected to centrifugation. The supernatant containing crude PI is then subjected to ammonium sulfate fractionation. The PI activity (usually against trypsin or chymotrypsin) is estimated after each fractionation, and the fractions with maximum inhibitory activity are dialyzed to remove salts. Further, the desalted protein is passed through the DEAE-Cellulose ion exchange column followed by CNBr-activated sepharose 4B and Sephadex G-50 columns to obtain PI in its pure form (Prasad et al., 2010a). Protein quantity is measured after each step of purification by using BCA kit method. The purification pattern of PIs is visualized by Tricine SDS-PAGE followed by silver staining. The exact molecular mass is determined by Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF) intact mass analysis, and in-gel activity is visualized by reverse zymography. Another important aspect is identifying the type of PI family, which is done by MALDI MS-MS analysis and N-terminal sequencing of purified PIs. Besides, the biochemical properties such as stability towards a wide range of temperatures, pH, and reducing agents such as DTT are

measured by pre-incubating the sample in the appropriate buffer (pH 2-12) or temperature (37 to 100 ^{o}C) or reducing agent followed by performing the protease inhibition assay. Further, the structural stability is evaluated by Circular dichroism (CD) spectroscopy (Prasad et al., 2010a; Dabhade et al., 2013; Swathi et al., 2021).

Applications of PIs

PIs and Lepidopteran insect pests

Lepidopteran insect pests such as castor semi-looper *Achaea janata* (Swathi et al., 2014), sorghum stem borer *Chilo partellus* (Panchal and Kachole, 2016), pod borer *H. armigera* (Swathi et al., 2015; Banerjee et al., 2017), tobacco hornworm *Manduca sexta* (Kessler et al., 2006), fall armyworm *Spodoptera frugiperda* (Ramalho et al., 2018), and cotton leafworm *S. litura* (Vasudev and Sohal, 2019) are the major defoliators causing yield losses to several economically important crops. These pests are generally controlled by using chemical pesticides and *Bacillus thuringiensis* (Bt)-derived products. However, long-term application of these compounds resulted in pest resistance and reduced efficacy. Hence, research on the application of plant-derived compounds with potential insecticidal activity has gained prominence in recent years (Pandey et al., 2022). Among them, PIs from non-host plants as well as wild varieties are prominent sources to control the growth of various insect pests as compared to host plant PIs since insect's gut digestive enzymes have not been adapted to non-host plants (Swathi et al., 2012, 2016). Serine PIs are extensively studied for their insecticidal activity, as serine-type proteases are the major proteases in the midgut of insects (Srinivasan et al., 2006; Furstenberg-Hagg et al., 2013).

PIs in the management of *H. armigera*

H. armigera, also known as cotton boll worm, feeds voraciously on different plant parts starting from seedling to the maturation stage (Younis et al., 2015). Several previous

studies on the usage of PIs in the management of *H. armigera* revealed that the insecticidal activity of PIs purified from host plants such as *Cicer arietinum, Gossypium herbaceum, Momordica charantia* and *Sorghum bicolor* is comparatively low when compared with the insecticidal potential of PIs purified from non-host plants such as *Arachis hypogaea*, *Rhynchosia sublobata* and *P. tetragonolobus* (Giri et al., 2003; Kuhar et al., 2013; Swathi et al., 2014; Mohanraj et al., 2019). Various studies have been carried out to evaluate the significance of wild relatives of pigeon pea. Initially, feeding of *H. armigera* larvae with crude protein extracts of *C. platycarpus* accessions showed growth retardation, and larval mortality, followed by pupal and adult deformities (Swathi et al., 2015). This was further strengthened by *in vivo* feeding experiments with purified PIs from *R. sublobata* and *C. platycarpus* seeds. Upon feeding, >95% of larvae could not continue their life cycle, indicating the significance of wild relative PIs in the management of *H. armigera* (Swathi et al., 2016; Mohanraj et al., 2018).

Further, the purified alpha-amylase/trypsin inhibitor from pigeon pea seeds inhibited the midgut proteases and reduced the growth of *H. armigera* in the *in vivo* feeding assay (Giri and Kachole, 1998; Gadge et al., 2015). Apart from pigeon pea, PINII PI from *C. annum* also inhibited *H. armigera* gut proteases in the *in vitro* assay and showed increased mortality and reduced weight when PINII was fed to larvae via an artificial diet (Tamhane et al., 2007). In comparison, PINII from tobacco inhibited only the midgut trypsin activity of *H. armigera* and could not inactivate the chymotrypsin activity (Stevens et al., 2013). Furthermore, the Kunitz PIs purified from *Butea monosperma* seed showed inhibition towards the midgut enzymes of *H. armigera* with IC_{50} of 2.0 µg/ml, and the *in vivo* feeding experiments showed a dose-dependent decrease in larval weight and mortality. Fertility and fecundity declined along with an extension of larval-pupal duration in *H. armigera* life cycle (Jamal et al., 2015).

The role of various biomolecules involved in the insecticidal potential of PIs was evident by transcriptomic and proteomic analysis. For example, proteomic analysis of *H. armigera* midgut upon feeding with multi-domain recombinant *C. annum* protease inhibitor (CanPI-7), revealed the downregulation of protease genes at early time points (0.5-6 h) and upregulation of trypsin, chymotrypsin, and aminopeptidase genes at later time points (12-48 h) (Lomate et al., 2018). In addition, several antioxidant genes such as cytochrome P450, Glutathione S-transferase, and thioredoxin were upregulated upon feeding with PIs, indicating that PIs are causing some oxidative stress in insect midgut which leads to the production of antioxidant enzymes (Kaur and Sohal, 2016).

PIs in the management of *A. janata*

A. janata, also called as castor semilooper, is a major pest of castor and causes severe yield losses in castor plants. However, it can infect other crops such as tomato, banana, cabbage, mango, mustard and pomegranate. The effect of PIs on the growth and development of *A. janata* was evaluated by *in vitro* and *in vivo* feeding experiments. The studies of Budatha et al. (2008) identified and characterized three trypsin-like and one elastase-like serine midgut proteases from *A. janata* as molecular targets which interact with Cry toxins and proteinase inhibitors. Similarly, the BBI purified from black gram (BgPI) inhibited the midgut trypsin-like proteinases of *A. janata* and also resulted in reduction in size and changes in the morphology of larvae, pupae, and adults might be due to the inhibition of proteases involved in the metamorphosis of insects and changes in the enzyme systems involved in the formation of pupal and adult tissues (Prasad et al., 2010b). Further, the *in vitro* screening studies of Prasad et al. (2009) on cultivars and wild relatives of pigeon pea revealed that 14 cultivars and 8 wild relatives are more (50-fold) potent in inhibiting the midgut proteases of *A. janata* than bovine pancreatic trypsin.

The *in vivo* feeding of *A. janata* on PIs purified from pigeon pea showed a significant reduction in larval growth and survival rates, followed by pupal and adult deformities. (Prasad et al., 2010b; Swathi et al., 2014). Apart from native BBI, the recombinant BBI cloned from *R. sublobata* seeds also showed insecticidal potential against *A. janata*, in terms of low IC_{50} and (70 ng) reduced growth and developmental delay in larvae and pupae (Mohanraj et al., 2018).

PIs in the management of *S. litura*

S. litura, also called cotton leafworm, infests more than 112 plant species belonging to 40 plant families. The host plants of *S. litura* include tobacco, potato, chickpea, jute, groundnut, maize, rice, cabbage, etc. It is responsible for severe yield losses in ground nut, potato, cowpea, tobacco and soybean (Sundar et al., 2018). Several chemical pesticides followed by integrated pest management strategies and Bt toxins are also applied to control these insect pests (Yinghua et al., 2017; Zhong et al., 2019). However, the larvae showed marginal mortality rates upon the above treatments, possibly due to increased resistance or decreased binding sites in epithelial gut tissue (Hallad et al., 2011). In this scenario, the exploitation of PIs became an alternative approach to control these insect pests. The PIs purified from host plants such as *C. cajan* and *V. mungo* inhibited the midgut trypsin-like and chymotrypsin-like proteases to the extent of 14% and 28%, respectively (Prasad et al., 2010b). In the *in vivo* feeding experiment also, the host PIs showed low to moderate effects on the growth and development of *S. litura*. Further, the PI purified from rice bean and maize showed significant inhibition effects in both *in vitro* and *in vivo* studies (Tamayo et al., 2000; Sharma et al., 2019). Feeding of PI purified from *Adenanthera pavonina* resulted in 42% reduction in *S. litura* larval weight, followed by 38% and 44% reduction in pupal and adult weight, respectively (Velmani et al., 2019).

PIs in the management of other insect pests

Apart from lepidopteran insect pests, several PIs were reported as potential candidates in inhibiting the insects belonging to Diptera, Coleoptera and Hemiptera. The *in vivo* studies on insects have demonstrated that feeding of an artificial diet containing different concentrations of soybean-based BBI has affected the growth of *Anticarsia gemmatalis*, *Bactrocera cucurbitae,* and *Apis mellifera* (Belzunces et al., 1994; Kaur et al., 2017; Mendonca et al., 2020). Another major pest *Callosobruchus maculatus*, which widely affects the *Vigna unguiculata*, showed a reduction in the weight of larvae when fed on an artificial diet containing Kunitz-type inhibitors isolated from the seeds of *Adenanthera pavonina* (Macedo et al., 2004). Further, KI isolated from seeds of *Poecilanthe parviflora* showed significant inhibition in the activity of midgut trypsin proteases of four larvae, including *Corcyra cephalonica, Diatraea saccharalis, S. frugiperda,* and *Anagasta kuehniella* (Garcia et al., 2004). The KI purified from seeds of *Acacia polyphylla* produced an anti-nutritional effect on *A. kuehniella* in the *in vivo* assay (Machado et al., 2013a).

The *in vitro* assay of wheat PIN I PIs against midgut proteases of *Tenebrio molitor*, *Plodia interpunctella* showed a significant effect in inhibiting the midgut proteases of these insect pests (Di Gennaro et al., 2005). The studies of Wang et al. (2007) also demonstrated the inhibitory activity of *S. americanum* PIN II PIs both in native (purified) form as well as recombinant form. Purified native PIN II strongly inhibited subtilisin protease and midgut protease of *Trichoplusia ni*, while recombinant PIs strongly inhibited chymotrypsin and showed poor inhibitory activity against subtilisin. Similarly, a trypsin inhibitor purified from *Phaseolus vulgaris* (220 µg) showed 89% reduction in larval growth of *Pieris brassicae* and two days delay in hatching as compared to control larvae (Negi et al., 2018).

Along with serine PIs, cysteine PIs also shows inhibitory activity against different insect pests. A cysteine PI purified from *Araucaria angustifolia* seeds showed inhibitory activity on proteases of *C. maculatus* digestive tract (Sallai et al., 2020). Cysteine PIs are found to have a negative impact on several insect pests, such as *Frankliniella occidentalis, C. maculatus, Leptinotarsa decemlineata,* and *Anagasta obtectus* (Murdock et al., 1988; Hines et al., 1991; Annadana et al., 2002). An *in vitro* study on the effect of oryzacystatin (purified from rice seeds) against midgut proteases of *Sitophilus oryzae,* and *Tribolium castaneum* showed significant inhibitory activity (Liang et al., 1991). A multidomain wheat cystatin showed insecticidal activity against the Colorado potato beetle (*Leptinotarsa decemlineata*) and inhibited its larval growth (Christova et al., 2018). Further, a novel CPI purified from *Enterobolium contortisiliquum* showed an inhibitory effect against *C. maculatus* in terms of larval weight reduction (92% at 1% w/w; Nunes et al., 2021).

The effect of different PIs on the growth and development of various insect pests is represented in **Table 1.1**.

Biomedical applications

Proteases play a crucial role in controlling several human physiological processes, such as cellular growth, homeostasis of intracellular and extracellular protein turnover, metabolism, differentiation, invasion, migration and apoptosis, which otherwise lead to a condition called pathogenesis (Bauvois, 2004; Moffitt et al., 2010; Petzold et al., 2012; Cid-Gallegos et al., 2022).

Several PIs were reported to have therapeutic properties such as anti-inflammatory, anti-coagulant, anti-cancer, and anti-microbial properties and tested for their efficacy in controlling human diseases including kidney failures, high blood

Table 1.1. List of BBIs and KIs purified from various plant sources and their effect on target insect-pests

S.NO	Name of PI	Source	Type of Study	Concentration	Effect on the target pest	References
			H. armigera			
1.	BBI	*Psophocarpus tetragonolobus*	*In vitro*	----	94% inhibition in trypsin-like midgut protease activity	Giri et al. (2003)
2.	PI	*Cocculus hirsutus*	*In vitro* and *in vivo*	5000 TIU/ml	58% reduction in larval weight of third-instar larvae	Bhattacharjee et al. (2009)
3.	PI	*Albizia lebbeck*	*In vitro* and *in vivo*	1 mg/gm	70% mortality rate	Hivrale et al. (2013)
4.	PI	Soybean cultivars MAUS-61 and MAUS-158.	*In vitro*	IC_{50} - 66.28 µg and 69.06 µg, respectively	91% and 90% inhibition in total midgut protease activity	Ghodke et al. (2013)
5.	BBI	*Dolichos biflorus*	*In vitro* and *in vivo*	10,000 TIU and 3,140 CIU	68 % reduction in larval weight and 51% mortality rate	Kuhar et al. (2013)
6.	KI	*Butea monosperma*	*In vitro* and *in vivo*	IC_{50} - 2.0 µg/ml	50 % reduction in larval weight	Jamal et al. (2015)
7.	PI	*C. platycarpus*	*In vitro*	IC_{50} – 240 ng	3178 HGPI units/mg protein	Swathi et al. (2016)
8.	KI	*P. tetragonolobus*	*In vitro* and *in vivo*	1.5 µg/ml	48% reduction in larval weight and 72% mortality rate	Banerjee et al. (2017)
9.	BBI	*A. hypogaea*	*In vitro* and *in vivo*	IC_{50} - 40 ng	42% reduction in larval weight	Lokya et al. (2020)
10.	BBI	*S. surattense*	*In vivo*	300 µg/ml	46% reduction in larval weight	Herwade et al. (2022)

11.	TI	*Pigeonpea*	*In vivo*	5,10 and 15 µg/gm	68% reduction in larval weight and 25% mortality rate	Shaikh et al. (2022)
A. janata						
12.	PI	*C. cajan cultivars and wild relatives*	*In vitro*	-----	658-1351 *A. janata* trypsin-like inhibitory units/mg protein	Prasad et al. (2009)
13.	BBI	*C. cajan and V. mungo*	*In vitro* and *in vivo*	IC_{50} - 100 ng	92% reduction in larval weight and 100% mortality rate	Prasad et al. (2010b)
14.	BBI	*C. cajan*	*In vitro* and *in vivo*	IC_{50} - 78 ng	55-71% reduction in larval weight	Swathi et al. (2014)
15.	BBI	*R. sublobata*	*In vitro*	IC_{50} - 40 ng	80% inhibition in trypsin-like midgut protease activity	Mohanraj et al. (2019)
S. litura						
16.	KI	*Momocardia charantia*	*In vitro* and *in vivo*	12 trypsin inhibitory units/mg protein	70% reduction in larval weight	Telang et al. (2003)
17.	KI	*Archidendron ellipticum*	*In vitro* and *in vivo*	150 µM	61% reduction in larval weight	Bhattacharya et al. (2007)
18.	BBI	*C. cajan and V. mungo*	*In vitro* and *in vivo*	0.05% w/v	21% and 39% of reduction in larval weight, respectively	Prasad et al. (2010b)
19.	KI	*P. vulgaris*	*In vitro* and *in vivo*	6400 TIU/mg protein	29% inhibition in trypsin-like midgut protease activity, 22% reduction in larval weight, and 13% mortality rate	Mittal et al. (2014)
20.	BBI	*V. umbellata*	*In vitro*	1.35 ml/ reaction	97% and 81% inhibition in trypsin-like and chymotrypsin-like midgut protease activity,	Katoch et al. (2015)

					respectively.	
21.	KI	*Cassia occidentalis*	*In vivo*	133 µg/ml	50% larval mortality rate	Vasudev et al. (2015)
22.	PI	*Leucaena leucocephala*	*In vivo*	25 – 800 µg/ml	48% reduction in larval weight	Vasudev and Sohal (2015)
23.	KI	*C. glauca*	*In vitro* and *in vivo*	25 – 800 µg/ml	70% inhibition in trypsin-like midgut protease activity and 60% reduction in larval weight	Vasudev and Sohal (2019)
24.	KI	*V. umbellata*	*In vitro* and *in vivo*	75% - 100% composition of the diet	80% and 69% inhibition in trypsin-like and chymotrypsin-like midgut protease activity, respectively, and 80-100% mortality	Sharma (2019)

S.NO	Name of PI	Source	Target pest	Type of Study	Concentration	Effect on the target pest	References
25.	KI	*Adenanthera pavonina*	*C. maculatus*	*In vitro and in vivo*	0.5 % (w/w)	40% reduction in larval weight and 50% mortality rate	Macedo et al. (2004)
26.	TI	*T. indica*	*Ceratitis capitata*	*In vitro and in vivo*	4 % (w/w)	34% mortality rate	Araujo et al. (2005)
27.	TI	*Crotalaria pallida*	*C. capitata*	*In vitro* and *in vivo*	2-4% (w/w)	38% reduction in larval weight and 15% mortality rate	Gomes et al. (2005)
28.	TI	*G. max*	*C. capitata*	*In vivo*	0.25-0.5 %	20% mortality rate	Silva et al. (2006)
29.	TI	*G. max*	*Bactrocera cucurbitae*	*In vivo*	LC_{50} = 74.21 ppm	40% inhibition of pupation and 3.71 days delay in the developmental period	Kaur et al. (2009)
30.	PI	*Piptadenia moniliformis*	*C. capitata*	*In vitro and in vivo*	0.1 mg/ml	50% reduction in larval weight and 50% mortality rate	Cruz et al. (2013)
31.	TI	*G. max*	*Aegorhinus superciliosus*	*In vitro and in vivo*	10 mM	100% mortality rate	Medel et al. (2015)
32.	PI	*Pisum sativum*	*B. cucurbitae*	*In vivo*	400 µg/ml	27% reduction in larval weight	Kaur and Sohal (2016)
33.	TI	*Sapindus mukorossi*	*B. cucurbitae*	*In vitro*	625 µg/ml	90% reduction in larval weight	Samiksha et al. (2019)

pressure, obesity, cardiovascular diseases, and different types of cancers (breast, colon, cervical and prostate) (**Fig. 1.6**; Alvares et al., 2014; Regulski et al., 2015; Srikanth and Chen, 2016; Martins et al., 2018; Clemente et al., 2019; de Freital et al., 2020; Cid-Gallegos et al., 2022).

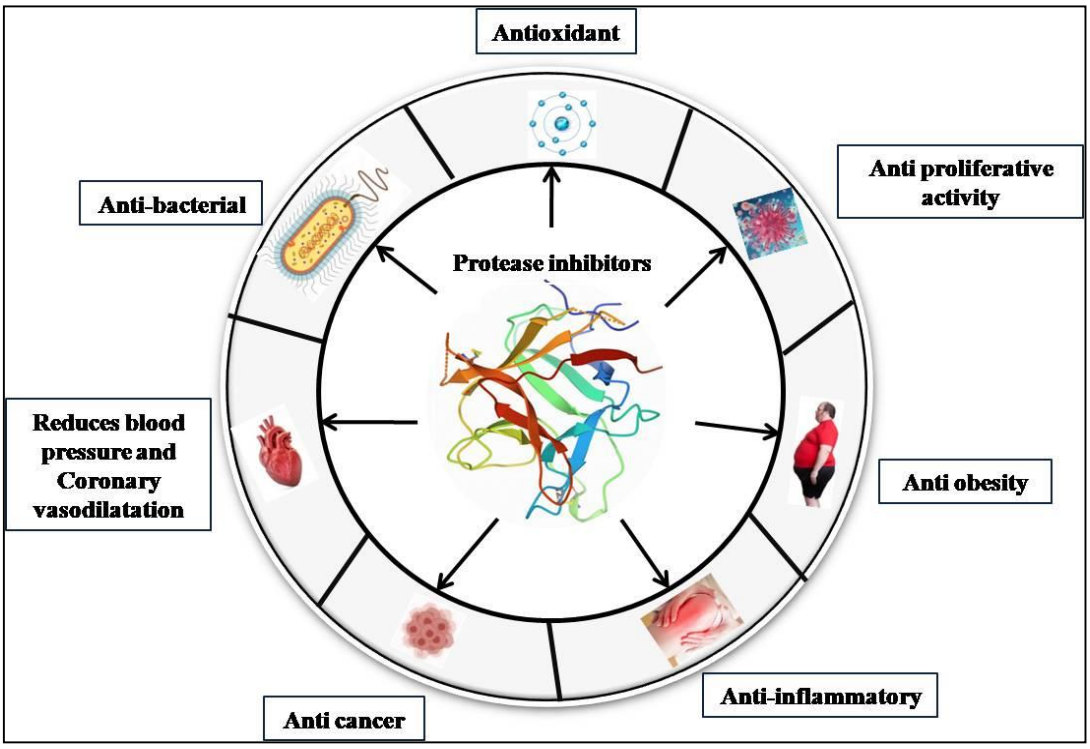

Fig. 1.6. Therapeutic properties of PIs. Adapted from Cid-Gallegos et al. (2020) and modified.

Several *in vitro* and *in vivo* studies have established the potential of plant PIs to hamper different stages of carcinogenesis. The studies of Kennedy et al. (2002) and De Paula et al. (2012) demonstrated the chemo-preventive role of BBIs purified from soybean seeds on rats infected with colon cancer in terms of reduced incidence and frequency of tumors. Similarly, BBI purified from *G. max* controlled cell growth in human cancer cell lines (HT29) by blocking serine proteases involved in cell cycle (G_0-G_1 phase), whereas BBIs purified from *V. unguiculata* seeds inhibited breast cancer cell viability by arresting S and G_2

phase (Clemente et al., 2010; Joanitti et al., 2010). Further, BBI purified from chickpeas also showed the ability to avoid the transformation of normal cells to malignant cells through chymotrypsin inhibition (Clemente and Arques, 2014). Besides, BBIs extracted from mung bean and pea seeds exhibited pharmacological activity against colorectal cancer through apoptosis induction, reduction in β-catenin protein levels, and tumor growth inhibition (Aviles-Gaxiola et al., 2020).

Several *in vivo* animal studies also reported the application of PIs as anticancer agents, which indicated that dietary BBI from legumes, including soybean (Clemente et al., 2010), peas (Clemente et al., 2006) and lentils (Ragg et al., 2006) can prevent or suppress gastrointestinal inflammatory processes and cancer (Srikanth and Chen, 2016). Evidence suggests that serine proteases might be the primary targets during the early stages of cancer progression (Gitlin-Domagalska et al., 2020). Hence a strong attentiveness has emerged in employing PIs as inhibitors of proteases. Another advantage of PIs, particularly BBIs can reach the colon in an active form due to their stability to extreme pH and thermal conditions within the gastrointestinal tract and retain their ability to inhibit serine proteases (Muricken and Gowda, 2010). PIs purified from *Erythrina velutina* seeds, having both trypsin and chymotrypsin inhibitory activity retarded the progression and invasiveness of gastric ulcers in rats. In addition, PIs, when administered together with peptides, such as Lunasin, enhanced the chemo-preventive function by controlling the activity of endogenous proteases and reached the target tissues in intact and active form and reduced the incidence of cancer in mice (de Lumen, 2005; Hsieh et al., 2010; Lima et al., 2017).

PIs when combined with antibiotics are helpful in improving the effect of antibiotics against antibiotic-resistant bacteria (Cristina Oliveira de Lima et al., 2019; Cid-Galligos et al., 2022). PIs are the potent alternative antibacterial agents to commonly existing overused antibiotics to which many pathogenic bacteria emerge with resistance (Supuran et al., 2002).

BBI type PI (14.3 kDa) isolated from leaves of *Coccinia grandis (L.)* act as a potent antibacterial agent against many pathogenic bacteria, including *Staphylococcus aureus* and *Bacillus subtilis* with MIC value of 1 mg/mL (Satheesh et al., 2011). Fitsulin (4 kDa) is a trypsin inhibitor derived from leaves of *Cassia fistula*, and it showed more efficient antibacterial activity in purified form than in partially purified form against *B. subtilis, S. aureus, E. coli and K. pneumonia* (Arulpandi et al., 2012). Similarly, a trypsin inhibitor (49 kDa) isolated from seeds of *Albizia amara* inhibited *B. subtilis* (MIC 16 µg/mL) and *Pseudomonas aeruginosa* (MIC 2 µg/mL) (Dabhade et al., 2016).

Trypsin inhibitor (JcTI-I) extracted from *J. curcas* inhibited proteases of *S. aureus* to 100% and proteases of *Salmonella enterica subsp. Enterica serovar cholerasuis* to 85% with MIC value of less than 5 µg/mL (Costa et al., 2014). RfIP1(22.5 kDa) is a serine PI derived from *Rhamnus frangula* exhibited strong antibacterial activity against *B. cereus*, *B. subtilis*, *E. faecalis*, *S. epidermidis*, *S. aureus*, *E. coli*, *K. pneumonia*, *P. aeruginosa* and *S. enterica* (Bacha et al., 2017). Further, the serine PI isolated from *Sophora japonica* seeds inhibited the growth of *Streptococcus pneumonia*, *S. aureus* and *B. subtilis* (Nabi et al., 2018). Besides, a study on BBI purified from *Luetzelburgia auriculata* seeds caused the death of *S. aureus* by disturbing the membrane integrity (Martins et al., 2018).

Viral proteases are essential for their replication and inhibit antiviral response. Hence, PIs are widely screened for their antiviral properties. One such broad-spectrum antiviral PI from the serpin family showed antiviral effect against viruses such as influenza viruses, Herpes simplex virus (HSV), Hepatitis C virus (HCV), and Human immunodeficiency virus (HIV). The chances of HIV acquisition have been reduced at increased expression levels of serpins (Whitney et al., 2011; Harish and Uppuluri, 2018). The studies of Fang et al. (2010) demonstrated that purified Kunitz-type PIs from Korean large black soybeans were effective in inhibiting the reverse transcriptase activity of HIV-1. Similarly, BBI purified from broad

beans showed around 100% inhibition of HIV-1 reverse transcriptase at the concentration of 196 µM (Ye et al., 2001). Further, a chymotrypsin inhibitor from *Acacia confusa* also inhibited the reverse transcriptase enzyme of HIV-1 (Lam and Ng, 2010).

It is also interesting to understand PIs can control obesity as well. BBIs reduced obesity by enhancing the levels of cholecystokinin, a hormone responsible for satiety (Chandra and Liddle, 2007; Serquiz et al., 2016). The studies of Christina Oliveira De Lima et al. (2019) suggested that trypsin inhibitors are potential candidates for controlling obesity and metabolic syndromes by stimulating cholecystokinin synthesis. Further, BBI also suppressed autoimmune inflammation and neuronal loss in mice affected with multiple sclerosis (Qi et al., 2005; Touil et al., 2008). Furthermore, treatment with BBI in mice delayed the onset of autoimmune encephalitis and reduced its severity by increasing interleukin 10 (IL-10) secretions (Dai et al., 2012). Besides, several *in vitro* and *in vivo* studies were performed to demonstrate the anticoagulant activity of plant PIs, particularly KIs. The KIs purified from seeds of *Bauhinia* species inhibited human kallikrein and plasma kallikrein along with bovine trypsin and chymotrypsin (Brito et al., 2014). Similarly, the KI purified from *Delonix regia* showed anticoagulation properties by inhibiting plasma kallikrein, factor XIa and factor XIIa (Salu et al., 2019). Further, the peptides (KI) derived from *D. regia* significantly extended the time of artery occlusion and modified the platelet adhesion and aggregation without any change in bleeding time (De Souza et al., 2023).

Chapter 2

Scope, Rationale, Objectives, and Approach of the Study

Scope, Rationale, Objectives, and Approach of the Study

2.1. Scope of the work

Plants produce a variety of natural bioactive compounds including carbohydrates, lipids, proteins, carotenoids, polyphenols and terpenes. Several proteins and peptides such as chitinases, glucanases, thaumatin-like proteins, peroxidases, ribonuclease-like proteins and protease inhibitors with potential targets in agronomy and medicine have been purified and well studied (Harvey et al., 2015; Shamsi et al., 2018; Zhu et al., 2018). Among them, PIs are an attractive class of protein molecules present in animals, plants and microorganisms which limit or inhibit the activity of their target proteases (Qi et al., 2005; Bendre et al., 2018).

PIs play significant functions in plants ranging from seed germination to protection against insect pests (Harish and Uppuluri, 2018; Hellinger and Gruber, 2019). In plants, PIs represent 10% of the whole protein and provide the sources of carbon, nitrogen and sulfur required during seed germination (Haq et al., 2004; Singh et al., 2020). Along with storage PIs, the expression of PIs in aerial parts upon stress conditions is also well documented. Further, the expression and abundance of these PIs vary based on the plant variety, tissue location, maturation stage, time of harvest, and coexistence with other PIs and their isoforms (Sin and Chye, 2004; Gitlin-Domagalska et al., 2020).

Apart from plant defense, the potential of PIs as therapeutic agents in treating several human diseases, such as inflammatory, neurodegenerative, cardiovascular and respiratory problems has been exemplified (Souza et al., 2014; Srikanth and Chen, 2016; Cid-Gallegos et al., 2022). Further, multiple roles of PIs have been reported such as antitumor, antihypertensive, anticoagulant, antioxidant and antimicrobial properties (Machado et al., 2013b; Popovic et al., 2013; Shamsi et al., 2016; Martins et al., 2018).

2.2. Rationale of the study

Lepidopteran insects are the primary pests causing severe damage to crop plants of economic importance. The major devastating Lepidopteran pests include *S. litura*, *A. janata* and *H. armigera* (**Fig. 2.1**). Further, serine proteases were identified as essential midgut proteases of the major insect order Lepidoptera (Macedo et al., 2004; Sharma, 2015; Anderson et al., 2016). PIs act as anti-nutritional compounds by hampering the proteolytic activity of proteases present in the midgut of insect pests and inhibit the synthesis of essential amino acids. Also, PIs form inactive complexes with digestive proteases and decrease their levels (Pusztai et al., 2004; Jongsma and Beekwilder, 2011; Popova and Mihaylova, 2019).

Legume seeds are regarded as the repositories of PIs as the concentration of PIs is higher in these seeds than in cereals and other seeds (Rodriguez-Sifuentes et al., 2020). Among plant PIs, serine PIs such as BBI and KI are majorly found in the leguminous family members and they showed concrete inhibitory properties against *H. armigera* and *A. janata*. For example, the recombinant BBI (rRsBBI) purified from *R. sublobata* seeds showed specific inhibition against midgut proteases of *A. janata* in the *in vitro* inhibition assays with an IC_{50} of 70 ng. It also affected the growth and development of castor semi-looper, *A. janata*, when fed on castor leaves coated with rRsBBI. But the same protein did not significantly affect midgut proteases of *H. armigera* (IC_{50} of 8µg) (Mohanraj et al., 2018). The KI isolated from the mature, dry seeds of *R. sublobata* showed significant inhibition (IC_{50} of 59 ng) against midgut proteases of *H. armigera* in the *in vitro* inhibition experiments. But the purified BBI did not show a significant effect (IC_{50} of 8.85 µg) on midgut proteases of *H. armigera*, indicating that BBI and KI are highly specific in their insecticidal properties (**Fig. 2.2**; Mohanraj et al., 2019). Therefore the application of BBI and KI in their pure form might be more effective in the management of these insect pests.

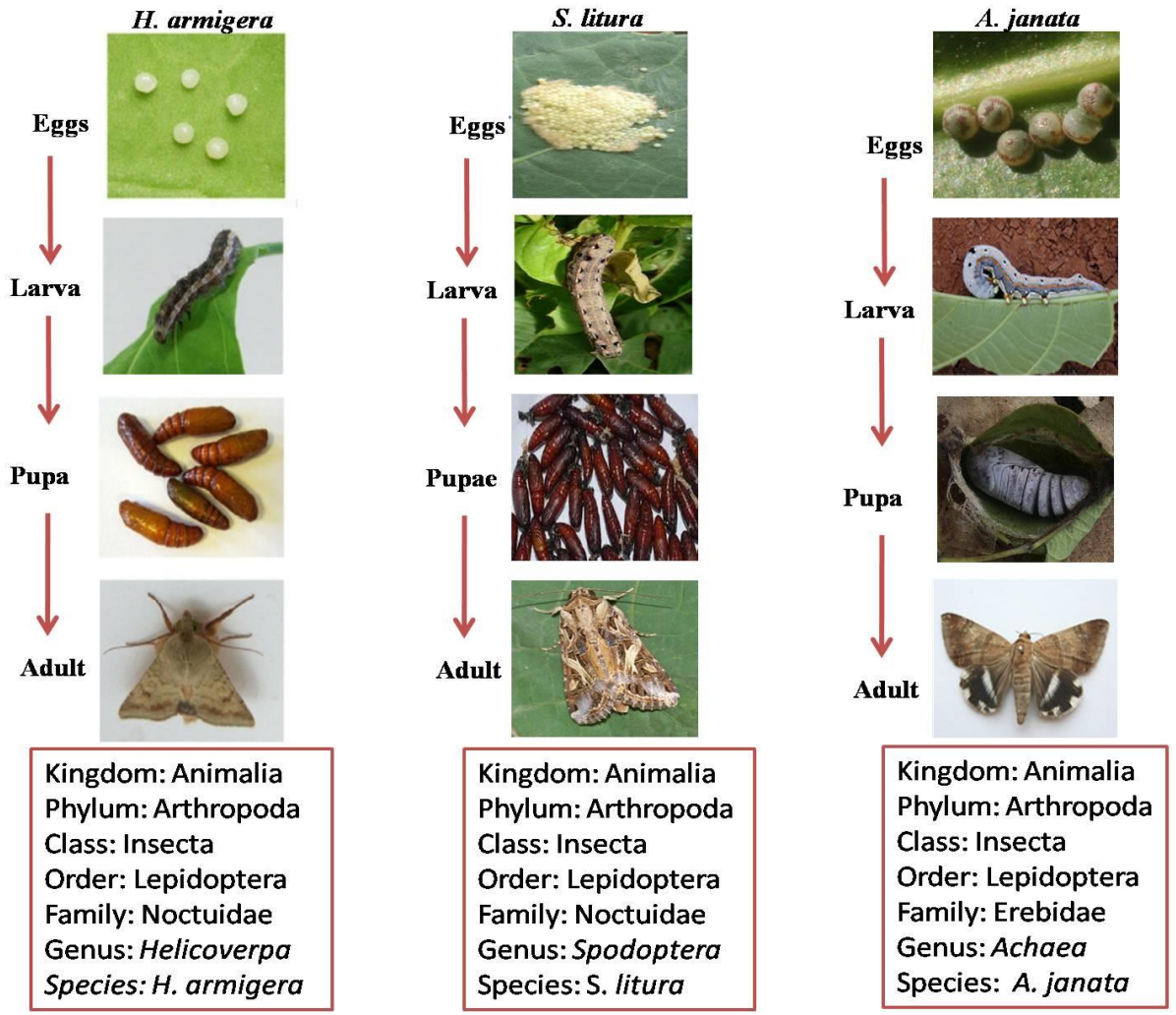

Fig: 2.1. Lepidopteran pests of economically important crop plants such as cotton, groundnut, soybean, sorghum, green gram, red gram, black gram, other legume plants (*H. armigera* and *S. litura*) and castor (*A. janata*).

Protein purification is the fundamental step for analyzing the biochemical/biophysical properties of individual proteins and to identify their interactions with other proteins, DNA/RNA or metabolites. Various protein purification strategies were used based on desired scale and downstream applications. However, the basic strategy of protein purification is the removal of all contaminants (nucleic acids, lipids, polysaccharides, and other proteins) while retaining the yield of the desired protein as much as possible. But most protein purification protocols result in high purification fold but lead to low yield recovery (Burgess and Deutscher, 2009; Lee, 2017). Also, the classical protein purification method involves several steps, such as crude extract preparation, ammonium sulfate fractionation, and passing through different columns (ion exchange, affinity, gel filtration, reverse phase chromatography), which is very laborious and time taking (Bhattacharyya and Babu, 2009; Prasad et al., 2010a; Dantzger et al., 2015; Dias et al., 2017; Lokya et al., 2020). Therefore, selection of a specific method for the purification of a desired protein is essential.

Several research groups purified BBI and KI from independent seed samples through the classical method (Bhattacharyya et al., 2006; Kuhar et al., 2013; Mohanraj et al., 2019; Lokya et al., 2020). But the information on the purification of both BBI and KI from the same starting seed material is very scanty and limited to a few seed varieties. BBI and KI are separated from soybean seeds by preparative gel filtration followed by isoelectric focusing in the narrow pH (3-6) range in the sucrose gradient (Catsimpolas, 1969). Later, Norioka et al. (1988) studied the distribution of BBI and KI in leguminous seeds by dissolving the 80% $(NH_4)_2SO_4$ saturated crude extract in 6M urea and concentrated using speed vac concentrator. Further, the sample was passed through a series of three gel filtration columns connected in series (precolumn – 4x100mm, TSK-Gel G3000SW, 7.5x600mm G3000SW & 7.5x600mm G2000SW) and tested for their

inhibitory potential against trypsin and chymotrypsin. Based on gel filtration patterns of inhibitors, it was assumed that *Mimosa invisa*, *Leucaena leuco*, *Pueraria lobata*, *P. tetragonolobus* and soybean have both BBI and KI in their seeds.

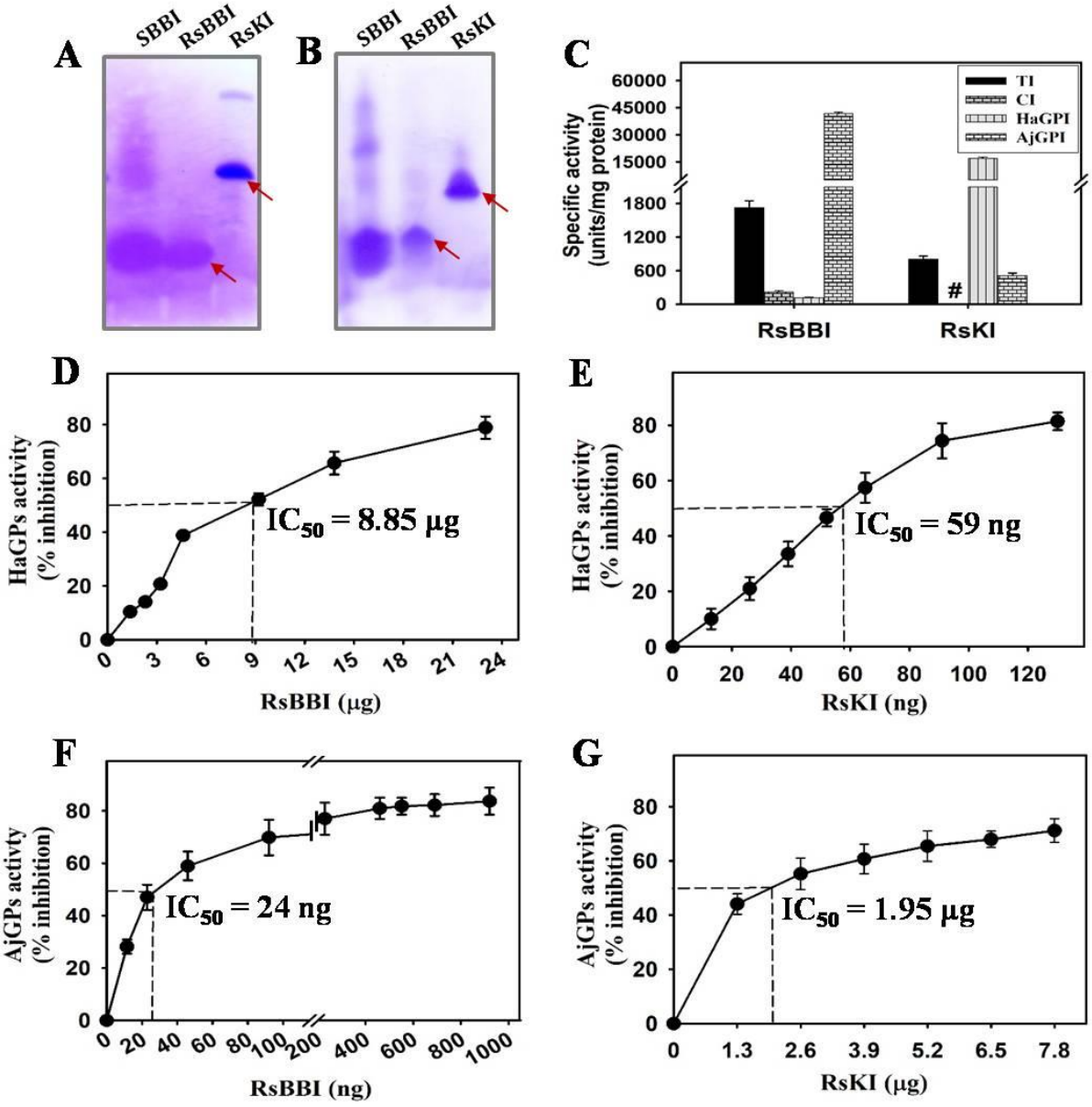

Fig. 2.2. Differential effects of BBI and KI purified from *R. sublobata* on midgut proteases of *A. janata* and *H. armigera*, respectively, using the classical method of purification involving ammonium sulfate fractionation, affinity and gel filtration chromatography (Mohanraj et al., 2019).

Both BBI and KI are also purified from *Clitoria ternatea* seeds by acetate extraction, ammonium sulfate fractionation followed by subjecting to Sephadex G-50 gel filtration column, CM-sepharose CL-4B ion exchange column and anhydrotrypsin affinity chromatography with purification fold of 22%, and 27% and yield recovery of 7%, and 3%, respectively (Macedo and Xavier-Filho, 1992). Also, Terrada et al. (1994) purified BBI and KI from *Canavalia lineata* seeds by heating the crude extract at 65 ^{o}C for 10 min and acidified in 2N HCl for 30 min and centrifuged. The obtained supernatant was subjected to ammonium sulfate fractionation (60%) and passed through the Sephadex G-50 column and DEAE Toyopearl 650 mm column (two times). The collected fractions were lyophilized, dialyzed, and passed through the hydroxyapatite column (2.5x15cm). Further, the inhibitors obtained were passed through anhydrotrypsin-sepharose column. The overall procedure resulted in the purification of two BBIs and one KI with yield recovery of 19%, 9% and 1%, respectively. However, the specific activity of these proteins is very low i.e. 3.35, 3.80 and 1.2 TIU/mg protein, respectively. The studies of Giri et al. (2003) purified BBI and KI from winged bean (*P. tetragonolobus*) seeds by subjecting the crude extract to heat treatment at 60 ^{o}C for 30 min and subjected to centrifugation. The clear supernatant obtained was passed through the Sephadex G-75 column. As the fractions obtained through this protocol contained both BBI and KI, they were separated by using 10% native polyacrylamide preparative gel tubes. The purified BBI and KI showed a specific activity of 26.1 and 16.5 TI units/mg protein, respectively.

The studies of Mohanraj et al. (2019) from the current laboratory developed a method to separate BBI and KI from the seeds of *R. sublobata* in two steps. Initially, the crude protein extract was subjected to ammonium sulfate fractionation followed by chromatographic techniques (affinity and gel filtrations) to obtain a PI pool which is enriched with BBI and KI. In the second step, the PI pool was subjected to TCA

extraction and sodium acetate extraction sequentially to separate BBI and KI in pure form. However, the overall purification procedure described by Mohanraj et al. (2019) to separate BBI and KI was more costly, laborious and time-consuming, but it resulted in high specific activity (1780, 837 TIU/mg protein, respectively) yield recovery (21.06%, 2.51%, respectively) and purification fold (50.16 and 23.59 - fold, respectively) of both BBI and KI. Separation of BBI and KI from single starting material is difficult because of the oligomeric nature of BBI and its close molecular weight with KI. Besides, BBI and KI varied in their functional properties such as insecticidal and therapeutic properties. Hence, it would be more beneficial if BBI and KI were separated within a short time and low cost.

Apart from the accumulation of PIs as storage proteins in seeds, the induction of PIs in aerial parts such as leaves and flowers upon exposure to various external stimuli was also reported in some plants (Leon et al., 2001; Gunjegaonkar and Shanmugarajan, 2019). The accumulation of PIs varies based on the plant variety, seed stage, and tissue type (Sels et al., 2008). The expression levels of PIs were found to be up-regulated upon insect herbivory, wounding, and pathogenic invasion by activating several signaling molecules, which act locally and systemically (Green and Ryan, 1972; Singh et al., 2008; Botelho et al., 2014). Jasmonic acid and its derivatives, such as MJ are critical regulators of plant defense and induce the synthesis of PIs (Singh et al., 2008; Jiang and Yan, 2018). The exogenous application of MJ on several plants resulted in the accumulation of PIs in both treated and neighboring plants (Lomate and Hivrale, 2012; Islam et al., 2017). Further, MJ plays a vital role in various stages of plant development, including seed germination, root growth, flowering, fruit ripening and seed development (Radhika et al., 2010; Sirhindi et al., 2020). But the information on the purification of inducible PIs is limited.

Thus in light of the lacunae discussed above, the following objectives were taken up in the present study:

2.3. Objectives of the study

1) Development of a rapid process for purification of Bowman-Birk inhibitor (BBI) and Kunitz inhibitor (KI) from the seeds of *Vigna radiata, Cajanus platycarpus* and *Rhyncosia sublobata* and their validation using biochemical and biophysical studies.
2) Evaluation of insecticidal, anticancer and antibacterial properties of BBI and KI purified from the seeds of *V. radiata, C. platycarpus* and *R. sublobata*.
3) Comparison of the biochemical properties and insecticidal potential of purified protease inhibitors induced in leaves and expressed in seeds of *Vigna mungo.*

2.4. Approach of the study

The major lepidopteran pests such as *H. armigera*, *S. litura* and *A. janata* are highly polyphagous, and application of PIs from non-host or wild relative would be beneficial to avoid the possibility of resistance. The seed varieties used in the present study *C. platycarpus* and *R. sublobata* are non-host (wild relatives) to both *A. janata* and *H. armigera*, while *V. radiata* is non-host plant to *A. janata* and host plant to *H. armigera*. Hence, the BBI and KI were purified from *V. radiata*, *C. platycarpus* and *R. sublobata* seeds by subjecting the crude PI extract to mild trichloroacetic acid (TCA) extraction and then applied to trypsin-affinity chromatography using AktaPrime plus. Among all the PIs purified, the abundance of BBI was more when compared with KI in all the seed varieties used. Further, western blot and MALDI-TOF studies confirmed the purified BBI and KI. The inhibitory activity of the PIs was studied by *in vitro* assays and in-gel activity staining studies by using gelatin, which acts as a substrate for proteases. The undigested PIs were visualized as blue bands on staining with CBB. Further, the PIs

were analyzed for their inhibition kinetics against trypsin and chymotrypsin. PIs are known to be structurally stabilized by disulfide bridges and non-covalent interactions. Hence, the structural stability of all the purified PIs was analyzed by performing CD and Fluorescence spectroscopy at different temperatures and DTT concentrations.

The *in vitro* inhibitory potential of BBIs and KIs against midgut trypsin-like proteases of *H. armigera* (HaTPs) and midgut trypsin-like proteases of *A. janata* (AjTPs) was evaluated by calculating specific activity and IC_{50}. Further, the insecticidal potential of these PIs was evaluated using *in vivo* feeding experiments. The first instar larvae of *H. armigera* and *A. janata* were used for *in vivo* feeding experiments. The reduction in larval growth, mortality rate, and formation of intermediates was recorded after each instar stage. Further, the anticancer properties of BBI and KI have been tested against breast cancer epithelial cells (MCF 7, MDA-MB-231 cell lines) and cervical cancer epithelial cells (HeLa cell lines) by performing cytotoxicity MTT assay. Furthermore, the antibacterial activity of purified PIs was tested against *S. aureus*, a gram-positive bacterium by the broth micro-dilution method.

PIs were reported to be induced upon exposure to several biotic and abiotic stress conditions (Green and Ryan, 1972; Islam et al., 2017). The presence of PIs in seeds of *V. mungo* was well reported. However, the induction of PIs in aerial parts was not reported so far. Besides, *V. mungo* is a non-host plant to *A. janata* and host plant to *H. armigera* and *S. litura*. Hence, the accumulation of PIs in *V. mungo* leaves was examined by MJ treatment, mechanical wounding, and infestation with *H. armigera*. The induction of PIs upon different treatments was quantified by *in vitro* inhibition assay with proteases like trypsin and chymotrypsin, using BAPNA and GLUPHEPA as substrates. BBI primers were designed, and real-time PCR was performed to confirm the type of PI induced under the given treatment. The amount of reactive oxygen species such as H_2O_2

released was measured *in vitro* by using potassium iodide. The PIs induced upon MJ treatment in leaves and PIs present in seeds of *V. mungo* were purified and confirmed by dot blot assay. The leaf and seed PI were purified using ammonium sulfate fractionation followed by passing through different chromatography columns and analyzed for their biochemical and insecticidal properties against *A. janata*, *H. armigera* and *S. litura*.

Chapter 3

Materials and Methods

Materials and Methods

3.1. Seed material

Wild relatives of pigeon pea: *C. platycarpus* (ICPW 68) and *R. sublobata* seeds were received from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India. The seeds were multiplied in greenhouse of the University of Hyderabad and used in the present study. Besides, the seeds of *V. radiata* (ML 267) and *V. mungo* (*Cv*. T9) were obtained from a local market in Hyderabad.

3.2. Chemicals

Acetone, ammonium persulfate (APS), bovine pancreatic trypsin and chymotrypsin, bovine serum albumin, casein, ethylenediaminetetraacetic acid (EDTA), hexane, lithium chloride, polyvinylpyrrolidone (PVP), sodium acetate, trichloroacetic acid, triton X-100, β-mercaptoethanol and tetramethylethylenediamine (TEMED) were procured from Sisco-Research laboratory, Mumbai, India. Coomassie brilliant blue R-250, cyanogen bromide (CNBr) activated sepharose 4B, gelatin, isoamyl alcohol, lithium dodecyl sulfate, methyl jasmonate (MJ), N-α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA), N-glutaryl-L-phenylalanine-*p*-nitroanilide (GLUPHEPA), Sephadex G-50 fine grade, soybean trypsin-chymotrypsin inhibitor (SBBI) and tricine were procured from Sigma-Aldrich, USA. All the components related to PCR were procured from New England Biolabs, USA. PCR master mix (SYBR Green) was purchased from Takara, Japan. DNA ladder, protein standard marker, bicinchoninic acid (BCA) protein estimation kit, 3-kDa cut-off snake skin dialysis membrane (3kDa), and Verso cDNA synthesis kit were obtained from Thermo Fischer Scientific, USA. Amicon ultra centrifugal filter units were procured from Millipore Corporation, USA. All other reagents and chemicals used in the present study were of analytical grade.

3.3. Purification of BBI and KI from leguminous seeds

Preparation of crude protein extract (CPE)

Mature, dry seeds (10 g) of *V. radiata, C. platycarpus* and *R. sublobata* were ground into a fine powder and subjected to three successive washes of acetone and hexane (1:3 w/v) to remove pigments and fats, respectively. The powder obtained was air dried and extracted with 1:6 (w/v) volumes of Tris-HCl (50 mM, pH 8.0) containing 1% polyvinylpyrrolidone for 3 h at 4 ^{o}C under constant stirring and centrifuged at 4 ^{o}C for 15 minutes at 12,000 rpm. The supernatant obtained was collected and labeled as crude protein extract (CPE).

Separation of BBI and KI using TCA extraction

The CPE was subjected to mild TCA extraction (2.5%) by heating at 70 ^{o}C for 10 min, followed by centrifugation at 12,000 rpm at room temperature (RT) for 5 min. The supernatant (S_{1a}) containing TCA soluble BBI was adjusted to pH 8.0 using Tris-HCl (400 mM) and subjected to acetone precipitation (1:4 v/v). The pellet (P_1) obtained after the first cycle of TCA extraction was subjected to the second cycle of TCA extraction by dissolving in Tris-HCl (50 mM, pH 8.0). The supernatant (S_{1b}) obtained was subjected to acetone precipitation as described above to collect any leftover BBI. The acetone-precipitated samples from both supernatants (S_{1a} and S_{1b}) were centrifuged at 12,000 rpm for 15 min (4 ^{o}C), and the corresponding protein pellets were dissolved in Tris-HCl (50 mM, pH 8.0). Besides, the pellet (P_2) obtained after the second cycle of TCA extraction, which is enriched with KI was dissolved in Tris-HCl (50 mM, pH 8.0).

Purification of BBI and KI using affinity chromatography

Both the BBI-enriched and KI-enriched samples were passed through CNBr-activated trypsin sepharose 4B column, which is pre-equilibrated with washing buffer (50 mM Tris-HCl containing 100 mM NaCl, pH 8.0). The unbound proteins are removed using washing buffer. The protein (BBI/KI) bound to the column was eluted using HCl (0.01N) and neutralized with Tris-HCl to pH 8.0, as described in Prasad et al. (2010a, c). The eluted fractions (1.0 ml) with significant trypsin inhibitory (TI) activity are concentrated using Amicon centrifugal filters (3 kDa).

The BBI fraction collected from the trypsin sepharose 4B column was pure and free from contaminants. However, the KI fraction obtained after passing through the trypsin sepharose 4B column showed some impurities. As the Kunitz inhibitors are found to be soluble in acetate buffers (Macedo and Xavier-Filho, 1992), the KI fraction containing impurities was extracted into sodium acetate buffer (100 mM, pH 4.0) by mixing at 1:2 ratios (v/v) and heating at 70 ^{o}C for 10 min. The supernatant (S_2) collected after centrifugation at 12,000 rpm for 5 min at RT was adjusted to pH 8.0 with Tris-HCl (100 mM) and subjected to acetone precipitation. The protein pellet containing KI was dissolved in 50 mM Tris-HCl (pH 8.0). Both the pure BBI and KI proteins were stored at -20 ^{o}C until further use. Thus, the schematic protocol used for the separation and purification of BBI and KI from mature seeds of *V. radiata*, *C. platycarpus* and *R. sublobata* is shown in **figure 3.1**.

3.4. Protein estimation

The protein content of the CPE and all other purified samples was estimated by using the BCA kit and BSA as a standard (Smith et al., 1985).

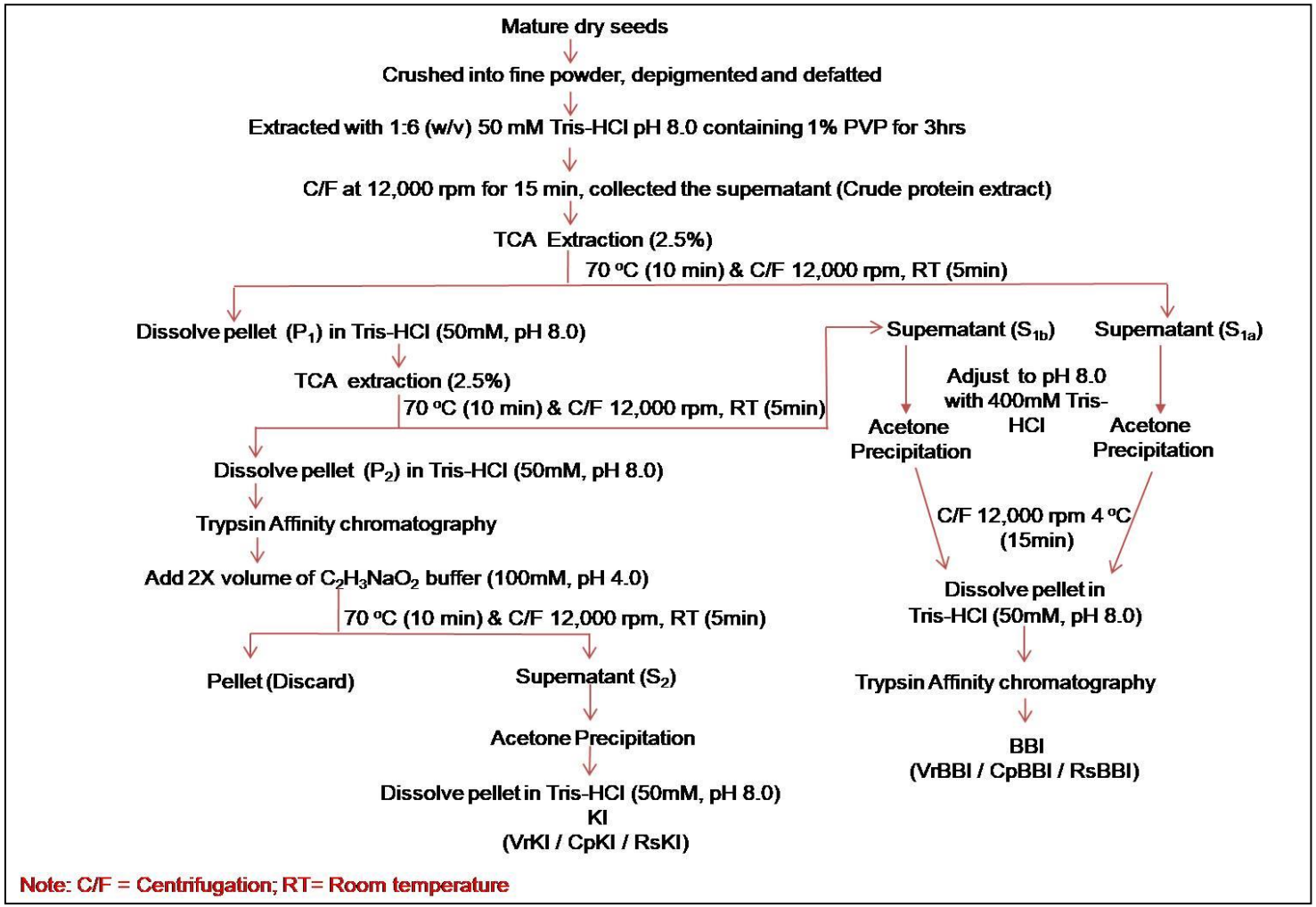

Fig. 3.1. Schematic diagram depicting the protocol for rapid separation and purification of BBI and KI from leguminous seeds of *V. radiata*, *C. platycarpus* and *R. sublobata*.

3.5. Protease inhibition assay

The inhibitory activity of purified PI (BBI/KI) against different serine proteases (bovine trypsin and chymotrypsin) and midgut trypsin-like proteases of *A. janata*, *H. armigera* and *S. litura* was determined by monitoring the reduction in the activity of these proteases (AjTPs, HaTPs and SlTPs, respectively - Please refer to section 3.8 for midgut protease extraction). For this, the assay mixture containing PIs in assay buffer [50 mM Tris-HCl and 20 mM $CaCl_2$ at pH 8.2 for trypsin/AjTPs (or) pH 7.8 for chymotrypsin and 50 mM Glycine-NaOH at pH 10.5 for HaTPs/SlTPs] was incubated with respective proteases (trypsin/chymotrypsin and AjTPs/HaTPs/SlTPs) for 15 min at 37 °C as described in Prasad et al. (2010a, b). Further, the samples were incubated with respective substrates (BAPNA or GLUPHEPA) at 37 ºC for 45 min, and the reaction was stopped by adding 30% acetic acid (Erlanger et al., 1961; Mueller and Weder, 1989). Thus the residual protease activity was evaluated by monitoring the decrease in absorbance at 410 nm. The amount of inhibitor required to block 50% activity of the cognate protease was referred to as one trypsin inhibitor (TI) unit or chymotrypsin inhibitor (CI) unit or *H. armigera* midgut trypsin-like protease inhibitor (HaTPI) unit or *A. janata* midgut trypsin-like protease inhibitor (AjTPI) unit or *S. litura* midgut trypsin-like protease inhibitor (SlTPI) unit (Mohanraj et al., 2019).

3.6. Electrophoresis

Tricine SDS-PAGE

Tricine SDS-PAGE (15%) was performed according to Schagger et al. (2006) using 4% stacking gel and 15% resolving gel under non-reducing conditions. All the samples obtained during various purification steps were examined electrophoretically along with a protein standard marker and commercial Soybean BBI (SBBI - 8.0 kDa), which is dissolved in Tris-HCl (50 mM, pH 8.0) since it is well known that the mobility of PIs (especially BBI) was

much slower when compared with standard protein marker (Prasad et al., 2010a). The protein bands were visualized by silver nitrate staining (Chevallet et al., 2006).

Gelatin SDS-PAGE

Tricine SDS-PAGE (15%) incorporated with 1% gelatin was performed to visualize the in-gel activity of purified PI (BBI/KI). After electrophoresis, the gels were washed with Triton X-100 for 15 min (three times) to remove SDS, followed by washing with water (three times) to remove any traces of Triton X-100. Later, the gels were incubated for 1 h at 37 ^{o}C with appropriate proteases dissolved in buffer solutions of 0.1 M Tris-HCl, pH 8.2 (trypsin/AjTPs), 0.1 M Tris-HCl, pH 7.8 (chymotrypsin) and 0.1 M Glycine-NaOH, pH 10.5 (SlTPs/HaTPs). The protein bands active against these proteases were visualized in blue color on a white background after staining with Coomassie brilliant blue R-250 (Lokya et al., 2020).

3.7. Characterization

Western blotting

For immunodetection, the PI (BBI/KI) are transferred from Tricine SDS-PAGE (15%) onto a nitrocellulose membrane activated in methanol using a transfer buffer containing Tris-HCl (25 mM, pH 8.0), glycine (192 mM), and methanol (20%) at 20 V for overnight at 4 °C. The membrane was blocked with skimmed milk powder in TBS (198 mM Tris-HCl, pH 7.6, and 150 mM NaCl) at RT for 2 h to avoid non-specific binding of the antibody. After blocking, the membrane was probed with anti-serum against BBI (1:500) for 2 h, followed by secondary antibody 'anti-rabbit IgG' (1:5000) conjugated with alkaline phosphatase for 2 h at RT. Finally, the blot was washed three times with TBS containing 0.2% Tween 20 and developed with 5-Bromo-4-chloro-3-indoyl phosphate/Nitro-blue-tetrazolium (BCIP/NBT) solution (Mohanraj et al., 2019).

MALDI intact mass and MS-MS analysis

The molecular mass of the purified proteins was determined by the mass spectroscopic [Electrospray-ionization Quadrupole Time of Flight (ESI-Q-TOF) or MALDI-TOF) method. The sample was diluted with 1% formic acid and was analyzed on the ESI-Q-TOF instrument for intact molecular weight by direct infusion. The raw data obtained were deconvoluted by MassLynx 4.1 WATERS software. In contrast, the molecular mass of protein in MALDI-TOF was determined by mixing the sample with α-cyano-4-hydroxy-cinnamic acid matrix, and the spectra obtained were analyzed using Flex analysis version 3.1 software. For MALDI MS-MS analysis, after destaining the gels, the BBI/KI bands were cut from CBB-stained Tricine SDS-PAGE gels and digested with trypsin. The ions generated were identified by using the mascot search engine.

Inhibition kinetics

The inhibition constant (K_i) was determined by Lineweaver-Burk plot where increased concentrations of PI (BBI/KI) were incubated at 37 °C for 15 min in fixed concentrations (1 M) of either trypsin or chymotrypsin. Further, various substrate concentrations (0.125, 0.25, 0.375, 0.5, 0.625, and 0.75 mM), i.e., BAPNA for trypsin and GLUPHEPA for chymotrypsin, were added and incubated at 37 °C for 45 min. The K_i of each sample was calculated by plotting the obtained absorbance values at 410 nm in a Lineweaver-Burk plot using Sigma Plot software 12.0, Enzyme Kinetics Module 1.3 (Prasad et al., 2010a).

Stability studies

To understand the temperature stability of the purified PI (BBI/KI), they were incubated at a wide range of temperatures from 37 - 100 ^{o}C for 30 min. Likewise, to understand the stability against a wide range of pH (2 to 12), the PIs were pre-incubated in the following buffers for

30 min: Glycine-HCl (pH 2-3), sodium acetate-acetic acid (pH 4-5), sodium phosphate buffer (pH 6), Tris-HCl (pH 7-9) and Glycine-NaOH (pH 10-12). The effect of DTT on the stability of PIs was determined by incubating them with various concentrations of DTT (e.g., 0 to 3 mM for BBI and 0 to 200 mM for KI) at 56 °C for 45 min, followed by incubation with iodoacetamide at twice the DTT concentration in the dark for 1 h. The residual TI and CI activities after each treatment were estimated using BAPNA or GLUPHEPA as substrates (Mohanraj et al., 2019; Lokya et al., 2020).

Circular Dichroism (CD) spectroscopy

CD spectrophotometer (JASCO J-810, Japan) was used to examine the changes in the secondary structure of purified PI (BBI/KI). The instrument was constantly flushed with nitrogen gas during the experiment. The CD scan was recorded at 190-260 nm (far UV region) with the following settings: scan speed of 50 nm/min; cuvette path length of 2 mm; data pitch and bandwidth of 1 nm; and protein solution (0.2 mg/ml). Before recording the protein sample scan(s), the buffer scan(s) were recorded under the same conditions and subtracted from the protein spectra. The structural stability of PIs from 20 to 90 °C was evaluated by taking the far-UV spectra with the Peltier thermostat at intervals of 10 °C (Lokya et al., 2020). To examine the impact of the reducing agent on BBI/KI, the samples were incubated with different concentrations of DTT ranging from 0.1 to 2 mM for 15 min before recording the spectra. The composition of secondary structural elements was analyzed using the Spectramanager 2.0 software. The unit of ellipticity was represented in millidegrees.

Fluorescence spectroscopy

The fluorescence emission spectra were recorded using a fluorescence spectrofluorometer (Jasco FP-8500, Japan). A quartz cell with a path length of 1.0 cm was used, and the spectra

were observed at 25 °C by using a circulatory water bath. Before taking the emission spectrum of protein samples, the buffers were scanned at the same conditions and extracted from protein spectra. The purified protein (0.2 mg/ml) sample(s) were excited at a wavelength of 280 nm, and the emission spectrum was recorded between 300-450 nm. The effect of various temperatures on PIs was assessed by recording the fluorescence emission from 20 to 90 °C, and the effect of DTT (2 mM) was examined by incubating the samples at different periods before taking the spectra (Haq and Khan, 2003).

3.8. Insecticidal potential of PIs

Insects

H. armigera eggs were obtained from the National Bureau of Agricultural Insect Resources (NBAIR: NBAII-MP-NOC-01), Bangalore and ICRISAT, Hyderabad. *A. janata* larvae were obtained from the Indian Institute of Oilseeds Research, Hyderabad, India, and *S. litura* eggs were obtained from NBAIR (NBAII-MP-NOC-02), Bangalore, India.

Rearing of *A. janata, H. armigera* and *S. litura* larvae

The egg mass of *A. janata*, *H. armigera* and *S. litura* was allowed to hatch on small castor leaves and maintained in an insect culture room under the following conditions: temperature 26 ±1 ^{o}C, relative humidity 65 ± 5%, and photoperiod of 14:10 h (L:D). After hatching, the *A. janata* larvae were maintained on castor leaves, while *H. armigera* and *S. litura* were maintained on a chickpea-based synthetic diet. The synthetic diet was prepared according to Gupta et al. (2000) with minor changes and include the following components: overnight soaked chickpea (Bengal gram - 55 g), cholesterol (55 mg), L-ascorbic acid (1.3 g), casein (5 g), yeast powder (10 g), methyl-p-hydroxybenzoate (1 g), sorbic acid (0.52 g), streptomycin

sulfate (100 mg), formaldehyde solution (0.5 ml), one multivitamin capsule, one vitamin E capsule, agar-agar (6.5 g) and 360 ml distilled water (Swathi et al., 2016).

In vivo feeding bioassays of *A. janata, H. armigera* and *S. litura*

For *in vivo* feeding experiments, the first instar larvae of *A. janata* were allowed to feed on castor leaves coated with different concentrations (1, 2, 4 and 8 µg/cm^2) of PIs (BBI/KI). Control larvae were grown on castor leaves coated with Tris-HCl (50 mM, pH 8.0). In contrast, the effect of PIs on the larval growth and development of *H. armigera* and *S. litura* was assessed by feeding first instar larvae on an artificial diet supplemented with PIs at different concentrations (0.01, 0.025, and 0.05%) until pupal emergence. Control larvae were fed on an artificial diet without PIs until pupal emergence. The weight of control and PI-supplemented larvae was recorded after each instar stage. Reduction in larval growth and deformities in pupal and adult stages was illustrated through photographs.

Preparation of insect midgut extract

The fifth instar larvae of control insects (*A. janata, H. armigera* and *S. litura*) were narcotized for 45 min on ice. The midguts were dissected by using iso-osmotic saline and stored at -80 °C until further use. The gut tissue was homogenized with a glass homogenizer by adding 0.1 M Tris-HCl (pH 8.2) for *A. janata* and 0.1 M Glycine-NaOH (pH 10.5) for *H. armigera, S. litura* and centrifuged at 12,000 rpm for 10 min at 4 ^{o}C. The clear supernatant containing *A. janata* midgut trypsin-like proteases (AjTPs), *H. armigera* midgut trypsin-like proteases (HaTPs) or *S. litura* midgut trypsin-like proteases (SlTPs) were used for *in vitro* inhibition assays as mentioned in section 3.5.

3.9. Anticancer properties of purified PIs

The anticancer properties of purified PI (BBI/KI) were evaluated on MCF7 and MDA-MB-

231 cancerous breast epithelial cells, HeLa cancerous cervical epithelial cells and MCF10A normal non-cancerous breast epithelial cells. All the cell lines were obtained from National Centre for Cell Sciences (NCCS) Pune, India. The commercially available soybean BBI (SBBI) was used as standard in all the experiments. The cytotoxicity MTT assay was performed as described in Arunasree (2010). After preliminary experiments, a final concentration of 10 µg of each purified PI (BBI/KI) was used in the MTT assay.

3.10. Antibacterial properties of purified PIs

The antibacterial properties of purified PI (BBI/KI) were evaluated using Methicilin-sensitive *S. aureus* (MSSA-29213), procured from the American Type Culture Collection (ATCC), USA. The antibacterial activity was evaluated using the broth micro-dilution method as per the Clinical and Laboratory Standards Institute (CLSI, 2012) guidelines. Briefly, 1% of the overnight grown MSSA culture in LB broth was inoculated into 10 ml of fresh LB medium and allowed to grow until the log phase. The bacterial cells were then diluted to 50 McFarlands as per the CLSI protocol, and 100 µl of bacterial cells were seeded into a 96-well plate. Different concentrations (2-10 µg) of the purified PI (BBI/KI) were added into the wells, and the total volume of the wells was made up to 200 µl with LB media and incubated for 12-16 h at 37 ^{o}C. After incubation, 20 µl of 5 mg/ml MTT was added and incubated for another 3 h at 37 ^{o}C. The formazan crystals formed were dissolved in 50 µl of DMSO, and the absorbance was read at 595 nm. The 1% growth was calculated as a fraction of control cells without the BBI or KI.

3.11. Induction of PIs in leaves

Plant treatments and preparation of leaf crude extract

Black gram (*V. mungo*) plants were maintained in a greenhouse chamber with 10 h light and

14 h dark cycles. After 45 days, wounds were inflicted across the mid vein of the leaf using a hole punching machine on one set of plants, and the other set of plants was sprayed with MJ (100 µM) solution. For insect infestation, 3rd instar *H. armigera* larvae were gently placed on 45-day-old back gram seedlings using a brush and allowed to feed on its leaves (Botelho et al., 2008; Chen et al., 2022). Control plants were kept aside in another chamber. After 6, 12, 24, 36, 48, 60, and 72 h of each treatment, both the control and treated leaves were collected and frozen in liquid nitrogen and preserved at -80 ºC until further use.

The leaves collected after different treatments were directly weighed and extracted with pre-chilled 0.1 M sodium phosphate buffer, pH 7.6 (1:10 w/v), in a pre-cooled mortar and pestle. The extract was centrifuged (7500 g) at 4 ºC for 30 min. The supernatant obtained was collected, labeled as crude extract, and maintained at -20 ºC until further use. Protein estimation, trypsin and chymotrypsin inhibition assays were performed as described in sections 3.4 and 3.5, respectively.

3.12. Quantitative RT-PCR analysis (qRT-PCR)

Treated (wounded/MJ treated/*H. armigera* infested) and control leaf material (1 g) was ground into a fine powder using liquid nitrogen in a pre-cooled mortar and pestle. To the powdered tissue, a cocktail of pre-warmed extraction buffer (1 ml) containing 2% cetyl trimethyl ammonium bromide (CTAB), PVP (2%), EDTA (25 mM), spermidine (0.5g/L), Tris-HCl (100 mM, pH 8.0), β-mercaptoethanol was added and subjected to centrifugation at 10,000 rpm for 10 min at 4 ºC. The supernatant obtained was extracted twice with isoamyl alcohol: chloroform (1:1 ratio) by centrifuging (10,000 rpm) at 4 ºC for 10 min. LiCl (10 M) was added to the supernatant and centrifuged at 10,000 rpm for 20 min. The obtained pellet was dissolved in 500 µl of SSTE (1 M sodium chloride, 0.5% SDS, 100 mM Tris, 1 mM EDTA). To this, an equal volume of chloroform: isoamyl alcohol (24:1) was added and

centrifuged (10,000 rpm) for 10 min at 4 ºC. Two volumes of ethanol was added to the supernatant and precipitated at -70 ºC for 1 h followed by centrifugation (10,000 rpm) for 10 min at 4 ºC. The pellet containing pure RNA was washed thrice with ethanol (75%), air-dried, and dissolved in RNase-free water.

First-strand cDNA was synthesized from total RNA (1 µg) in a 20 µl reaction mixture containing oligo (dT) primers and reverse transcriptase according to the manufacturer's instructions (VERSO cDNA synthesis kit). Reverse transcription was performed at 42 ºC for 1 h, followed by heating at 70 ºC for 5 min. The cDNA was PCR amplified using BBI gene (NCBI accession No. KP966296.1) specific primers (forward 5'-GACTCAAGCGATGAGC CTTC-3' and reverse 5'-TCCATGGATTTGCAAGGTTT-3'). The qRT-PCR (10 µl) reaction mixture contains 2.5 µl cDNA, 5 µl SYBR Green PCR master mix, 0.5 µl of each forward and reverse primer, and 1.5 µl of sterilized water. ROX was used as a reference dye. The qRT-PCR was performed according to the manufacturer's instructions (StepOnePlus Real-Time PCR System, Applied Biosystems, USA) by using the following PCR program: initial denaturation at 95 ºC for 5 min, followed by 40 cycles : 10 sec at 95 ºC (denaturation), 35 sec at 55 ºC (annealing), and 15 sec at 72 ºC (extension). Actin is used as a reference gene, and relative quantification analysis was determined by the ΔΔCT method (Lokya et al., 2020).

3.13. Determination of hydrogen peroxide (H_2O_2) content during induction of PIs

Leaves collected after each treatment are homogenized with TCA (0.1% w/v) at 4 ºC. The homogenate was centrifuged at 12,000 g for 15 min at 4 ºC. The supernatant was collected and used for the quantitative measurement of H_2O_2. The reaction mixture (1 ml) contains an aliquot (250 µl) of supernatant, 250 µl of potassium phosphate buffer (10 mM, pH 7.0), and 500 µl of potassium iodide (1 M). The absorbance was read at 390 nm, and the concentration of H_2O_2 was calculated using the relevant standard as shown in **figure. 3.2.** The final

concentration of H_2O_2 was expressed as µmol/g fresh weight of leaf sample (Velikova et al., 2000).

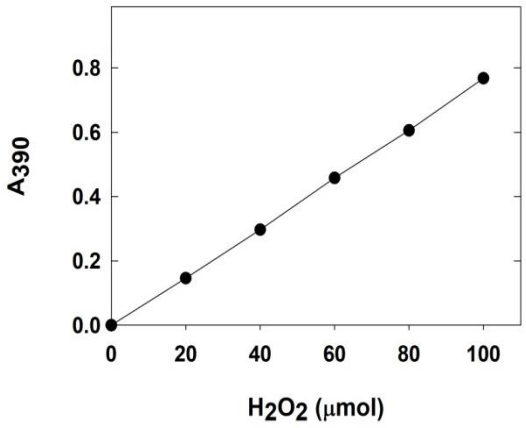

Fig. 3.2. Standard curve for H_2O_2 determining the concentration of H_2O_2 (0-100 µmol) versus absorbance at 390 nm.

3.14. Purification of PIs from leaves and seeds of *V. mungo* by using ammonium sulfate fractionation

To purify PI from MJ treated leaves, the crude extract obtained from MJ treated leaves (72 h) was subjected to 0-20% followed by 20-60% ammonium sulfate fractionation. The final pellet obtained after centrifuging (12,000 rpm, 20 min at 4 ^oC) the 20-60% ammonium sulfate fraction was dissolved in 50 mM Tris-HCl (pH 8.0) and subjected to dialysis against the same buffer. The dialyzed fraction containing significant TI activity was applied on to the trypsin sepharose 4B column pre-equilibrated with washing buffer (50 mM Tris-HCl containing 100 mM NaCl, pH 8.0). The protein bound to the column was eluted with 0.01N HCl and neutralized to pH 8.0 with Tris-HCl. The fractions with significant TI activity were pooled and concentrated using amicon filters (3 kDa cut-off). The pure protein obtained was labeled as leaf PI and stored at -20 ^oC until further use. The schematic protocol used to purify inducible PI from leaves of *V. mungo* is shown in **figure 3.3.**

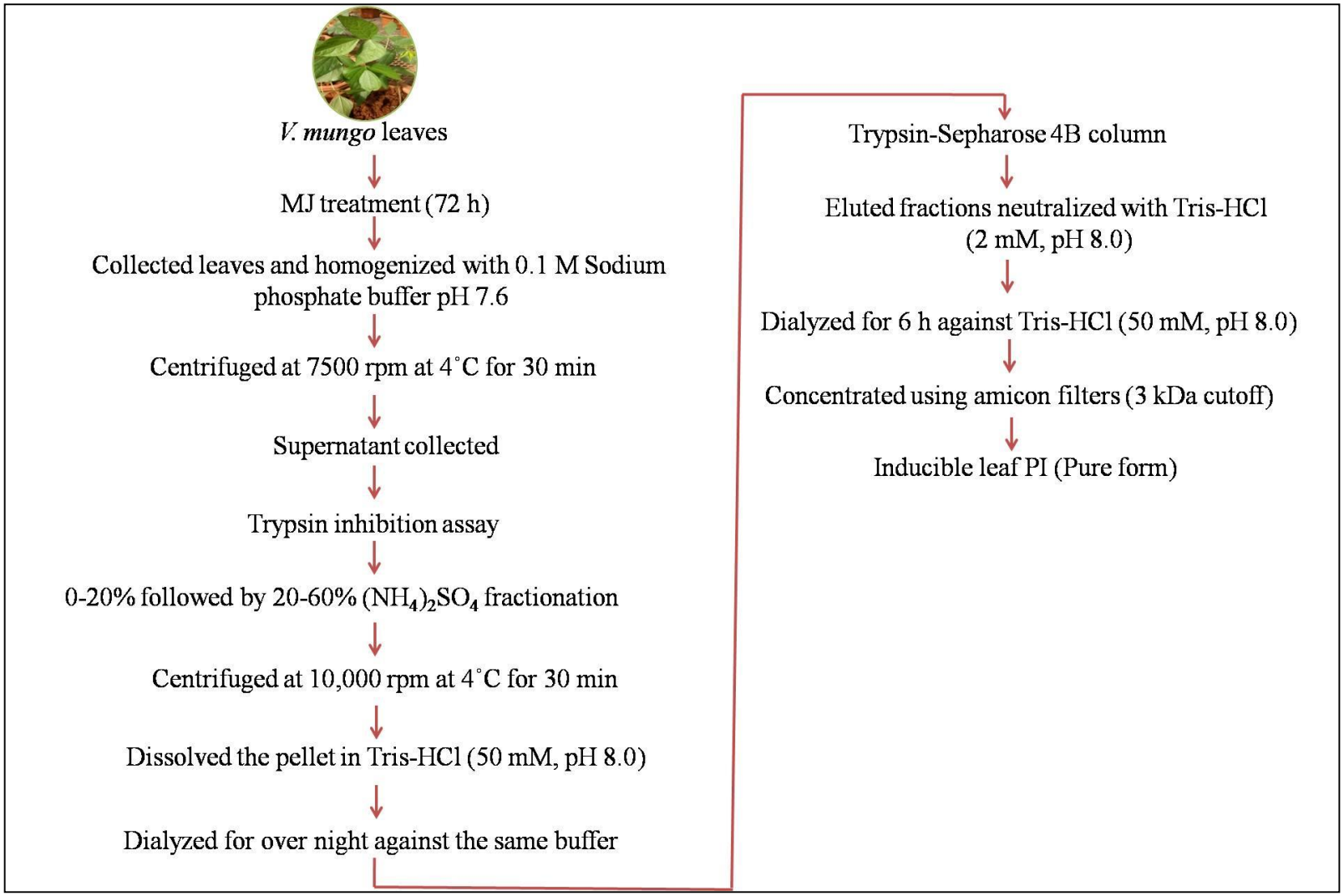

Fig. 3.3. Schematic diagram depicting the protocol for purification of inducible PI from leaves of *V. mungo*

To purify seed PI, the CPE was prepared from mature, dry seeds (50 g) of *V. mungo* by grinding them into a fine powder and subjecting it to three successive washes of acetone and hexane (1:3 w/v) to remove pigments and fats, respectively. The powder obtained was air dried and extracted with 1:6 (w/v) volumes of Tris-HCl (50 mM, pH 8.0) containing 1% polyvinylpyrrolidone for overnight at 4 ^{o}C under constant stirring and centrifuged at 4 ^{o}C for 15 minutes at 12,000 rpm. The supernatant obtained was labeled CPE and subjected to 0-20% followed by 20-80% ammonium sulfate fractionation. The final pellet obtained was dissolved in Tris-HCl (50 mM, pH 8.0) and dialyzed overnight against the same buffer. The dialyzed protein with significant TI activity was applied on to the trypsin sepharose 4B column. The eluted protein with prominent TI activity was concentrated, dialyzed, and subjected to gel filtration chromatography (Sephadex G-50 column prepared by suspending the dry resin in 50 mM Tris-HCl, pH 8.0 for overnight). The protein fractions were collected at a flow rate of 60 ml/h and examined for TI activity (absorbance at 410 nm) and protein content (absorbance at 280 nm). The pure protein obtained was labeled as seed PI. The schematic protocol used to purify PI from seeds of *V. mungo* is shown in **figure 3.4.**

The protein concentration of all the samples was estimated by BCA kit method. Tricine SDS-PAGE, in-gel activity, intact mass analysis, functional stability, and *in vivo* feeding bioassays were performed as mentioned in sections 3.6, 3.7 and 3.8, respectively.

3.15. Statistical analysis

The data shown is mean ± SE of three biological replicates, and the statistical differences were determined by one-way ANOVA analysis and Tukey's test ($P \leq 0.05$) using sigma plot software. All the FPLC chromatograms shown in the present study were the best representative of four to five purifications performed on different days, each time with

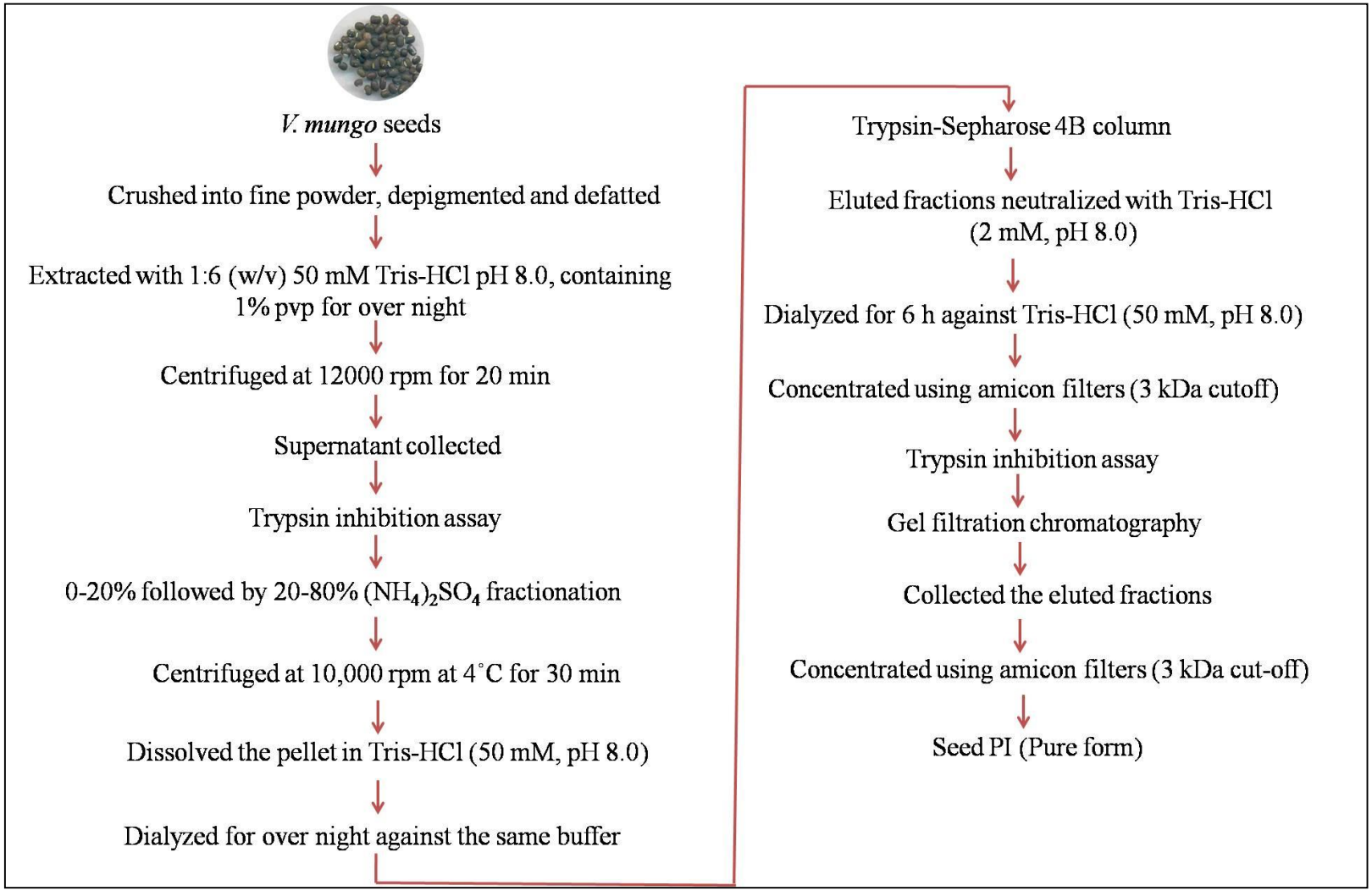

Fig. 3.4. Schematic diagram depicting the protocol for purification of PI from seeds of *V. mungo*

three to four cycles. All the tricine-SDS and in-gel activity staining gels shown in the present study are the best representative of PIs purified from three independent experiments with independent batch of seeds. The molecular weights of PIs shown in the present study are representative of two replicates of PIs purified from two independent experiments performed on different days. CD and fluorescence emission spectra represented in the present study are the average of three scans. All the stability experiments were repeated three to four times, each with protein purified on different days. The specific activity of PIs represented in the present study against serine proteases and midgut trypsin-like proteases of *A. janata*, *H. armigera* and *S. litura* is the representative of three independent experiments each time with three replicates. The *in vivo* effect of PIs represented in the present study against *A. janata*, *H. armigera* and *S. litura* is representative of three independent experiments, each time with 20-25 larvae fed with PIs purified from the independent batch of seeds.

Chapter 4

Development of a rapid process for purification of Bowman-Birk inhibitor and Kunitz inhibitor from the seeds of *V. radiata*, *C. platycarpus* and *R. sublobata*, and their validation using biochemical and biophysical studies

Development of a rapid process for purification of Bowman-Birk inhibitor and Kunitz inhibitor from the seeds of *V. radiata*, *C. platycarpus* and *R. sublobata*, and their validation using biochemical and biophysical studies

4.1. Introduction

Legume seeds are the natural repositories of PIs, *viz*. BBI and KI, the primary defense molecules involved in protecting plants against various biotic (pests and pathogens) and abiotic (salinity, drought, and heavy metal) stresses (Ryan, 1990; Sin and Chye, 2004; Gomes et al., 2011; Islam et al., 2017; Marathe et al., 2019; Swathi et al., 2021). Apart from stress tolerance, BBI and KI possess several applications in the biomedical field, such as anti-coagulant, anti-inflammatory, anti-tumor, anti-oxidant, and anti-microbial properties (Fang et al., 2010; Machado et al., 2013; Costa et al., 2014; Souza et al., 2014; Dias et al., 2017; Shamsi et al., 2018; Gitlin-Domagalska et al., 2020). BBIs are highly stable small proteins (Mr of ~ 8-10 kDa) and possess two inhibitory loops active against two identical or non-identical proteases and fourteen cysteine residues which form seven disulfide bridges. KIs are proteins of Mr ~18-24 kDa and contain a single reactive site and four cysteine residues that form two disulfide bridges. However, both families are reported to show either competitive or non-competitive mode of inhibition against trypsin and chymotrypsin (Singh and Rao, 2002; Macedo et al., 2004; Qi et al., 2005; Lingaraju and Gowda, 2008; Dantzger et al., 2015; Clemente et al., 2019).

In general, most of the studies focused on purifying either BBI or KI from the seeds of various leguminous plants by subjecting crude protein extract (CPE) to ammonium sulfate fractionation (20-80%) and usage of at least two or more chromatography columns such as ion exchange, affinity, gel filtration, and HPLC, etc. (Haq and Khan, 2003; Wang et al., 2006; Kansal et al., 2008; Prasad et al., 2010a, c; Swathi et al., 2016; Lokya et al., 2020). All these protocols are associated with more financial investment, tedious, and time-consuming. Also, very few studies focused on

separating both BBI and KI from a single or specific leguminous seed variety, possibly due to the limitations such as their close molecular mass and specificity against trypsin (Terada et al., 1994; Giri et al., 2003; Mohanraj et al., 2019). Further, the separation of BBI and KI during the purification process is even more complex due to the oligomeric nature of BBI (Devaraj and Manjunatha, 1999; Deshimaru et al., 2002; Calderon et al., 2005; Bhattacharya and Babu, 2009). Despite these challenges, the studies of Mohanraj et al. (2019) developed a two-step method to purify BBI and KI from the seeds of *R. sublobata* as follows (i) ammonium sulfate fractionation of CPE followed by dialysis and chromatographic (affinity and gel filtration) techniques to obtain a trypsin specific PI pool which contained both BBI as well as KI and (ii) TCA extraction of the PI pool to separate BBI and KI in their pure form. Though the second step of the purification procedure is rapid enough (2 h), the first step is not cost-effective and similar to that of the above-mentioned methods and involved more labor and time.

Considering the applications of BBI and KI in both agro and pharma industries, the present study is intended to further shorten the duration for separation and purification of these PIs from mature seeds while simplifying the process developed by Mohanraj et al. (2019) to make it more affordable (Sin and Chye, 2004; Fang et al., 2010; Machado et al., 2013; Clemente et al., 2019; Marathe et al., 2019; Gitlin-Domagalska et al., 2020). In this method, the CPE is directly subjected to mild TCA extraction while heating at 70 ^{o}C since it is well-known from past studies that the trypsin and/or chymotrypsin inhibitory activities of both BBI and KI are stable at this temperature (Macedo et al., 2007; Oddeypally et al., 2013; Mohanraj et al., 2019; Lokya et al., 2020). Also, a combination of TCA and heat treatment eliminates both acid-sensitive and heat-labile proteins from the CPE. Besides, TCA is known to induce the precipitation of proteins containing a hydrophobic core. Therefore, upon heating (70 ^{o}C) and centrifugation of TCA treated

protein sample, the BBI, which contains a hydrophilic core, remains in supernatant while KI which contains a hydrophobic core precipitate (Schmidt et al., 2005; Barbosa et al., 2007; Gitlin-Domagalska et al., 2020). Subsequently, both the supernatant and pellet fractions are subjected to trypsin-affinity chromatography to purify BBI and KI, respectively. Thus, an improved, ultrafast, efficient method of TCA separation and affinity capture scheme was applied in the present study for purifying BBI and KI present in single seed variety. The mature seeds of *V. radiata*, *C. platycarpus*, and *R. sublobata* are used in this study. Further, the BBI and KI obtained from these seeds are validated by analyzing their biochemical and biophysical properties.

4.2. Results

Rapid purification and confirmation of BBI and KI from CPE of *V. radiata*, *C. platycarpus*, and *R. sublobata* seeds

The depigmented and defatted CPE from the mature seeds of *V. radiata*, *C. platycarpus* and *R. sublobata* containing both BBI and KI were subjected to TCA extraction at 70 ^{o}C. Both the supernatant (S_{1a} and S_{1b}) and pellet (P_2), which are enriched with BBI and KI, respectively, are subjected to trypsin-affinity chromatography soon after treatment with TCA (Refer to **Fig. 3.1, Chapter 3**). These inhibitor proteins which are bound to the trypsin column, are identified in peak II fractions during elution with 0.01N HCl (**Fig. 4.1A-F**). The pure proteins with significant TI activity obtained from the affinity chromatography of supernatant are labeled as 'VrBBI' (*V. radiata*), 'CpBBI' (*C. platycarpus*), and 'RsBBI' (*R. sublobata*) (**Fig. 4.1A-C**). Further, the KI-enriched trypsin-affinity fractions obtained from the pellet are subjected to sodium acetate extraction to remove any contaminants that persisted during affinity chromatography (**Fig. 4.1D-F**). Thus, the pure proteins obtained after sodium acetate extraction are labeled as 'VrKI' (*V. radiata*), 'CpKI' (*C. platycarpus*) and 'RsKI' (*R. sublobata*). This protocol

resulted in the separation of ≤4.5 mg of BBI (VrBBI, CpBBI, and RsBBI) and ≤1.34 mg of KI (VrKI, CpKI, and RsKI), respectively, from 10 g seed material with ≤ 55-fold and ≤ 26-fold purity (**Table 4.1**).

The purity of BBI and KI obtained from *V. radiata*, *C. platycarpus*, and *R. sublobata* seeds was visualized in Tricine SDS-PAGE (15%) run under non-reducing conditions (**Fig. 4.2A-C**). The VrBBI and CpBBI existed as both monomer and dimer while RsBBI existed as mono, di and trimeric form as evidenced by increasing the concentration of the protein in Tricine SDS-PAGE (**Fig. 4.3A-C**). However, ESI/MALDI studies revealed that VrBBI, CpBBI, and RsBBI showed a predominant monomeric peak at 8.92, 7.92, and 9.21 kDa and a minor dimeric peak at 17.81, 15.77, and 18.42 kDa, respectively, between m/z 5,000-20,000 (**Fig. 4.4A-C**). Conversely, VrKI, CpKI, and RsKI showed only a monomeric peak at 19.25, 19.48, and 19.28 kDa, respectively (**Fig. 4.4D-F**).

Immunodetection studies after probing with anti-BBI antibodies confirmed the low molecular mass proteins as VrBBI (8.92 kDa), CpBBI (7.92 kDa), and RsBBI (9.21 kDa) in the western blot. The BBI from *V. radiata* and *R. sublobata* appeared as a single band, whereas in *C. platycarpus*, it appeared as two bands which indicated the presence of BBI as two isoforms in *C. platycarpus*. On the contrary, the wells loaded with VrKI, CpKI and RsKI did not show any bands when probed with anti-BBI antibodies indicating that these samples are pure and free from contamination of any BBI traces (**Fig. 4.5**). Further, the MALDI MS-MS analysis of the purified BBI(s) and KI(s) showed sequence similarity with other BBI and KI sequences available in the NCBI database. For this, the pure BBI (VrBBI/CpBBI/RsBBI)

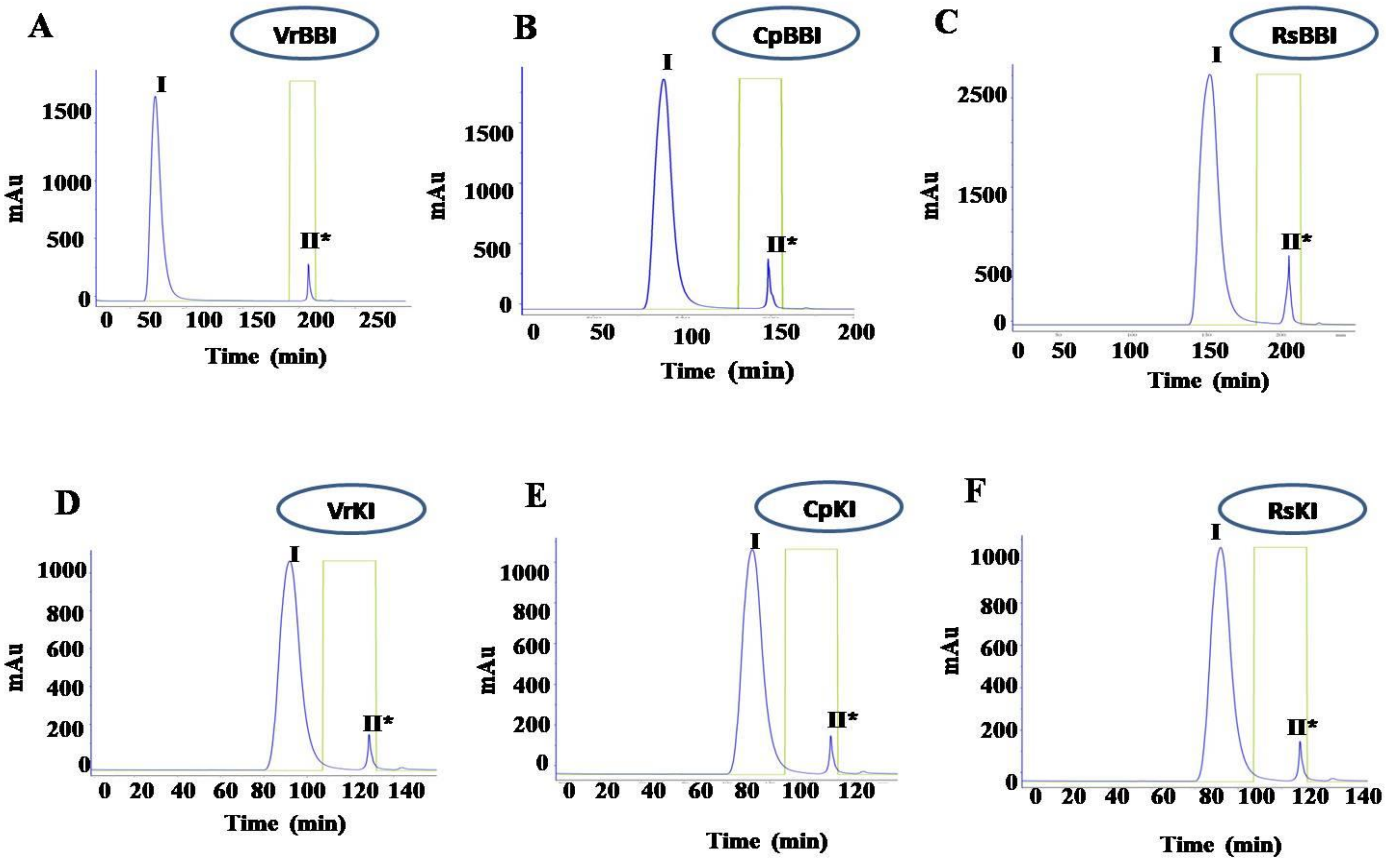

Fig. 4.1. Purification of BBI and KI from mature seeds of *V. radiata*, *C. platycarpus*, and *R. sublobata*. Elution profile of trypsin sepharose 4B column loaded with supernatants (S_{1a} + S_{1b}) enriched with (A) VrBBI; (B) CpBBI; (C) RsBBI, and pellets enriched with (D) VrKI; (E) CpKI and (F) RsKI, respectively, after TCA extraction. Peak I indicates the flow through, and peak II with an asterisk (*) indicates the active protein fraction.

Table 4.1. Purification of VrBBI/VrKI, CpBBI/CpKI, and RsBBI/RsKI from mature, dry seeds (10 g) of *V. radiata*, *C. platycarpus* and *R. sublobata*, respectively. One TI unit is defined as the amount of BBI/KI required to inhibit 50% of trypsin activity. Specific activity is defined as the number of TI units per mg of protein. The data shown here is the representative of four to five independent experiments performed on different days.

Purification step(s)	Total protein (mg)	Total activity (TI units)	Yield Recovery (%)	Specific Activity (TI units/mg protein)	Purification (fold increase)
***V. radiata* seeds**					
Crude protein extract	858	25588	100	30	1
TCA extraction	235	12525	49	53.2	1.8
Pure BBI	**3.5**	**4584**	**18**	**1309**	**44**
Pure KI	**1.34**	**1042**	**4**	**778**	**26**
***C. platycarpus* seeds**					
Crude protein extract	900	34587	100	38.4	1
TCA extraction	340	16321	27	48	1.25
Pure BBI	**3.5**	**4946**	**14.3**	**1413**	**37**
Pure KI	**1.0**	**980**	**2.83**	**980**	**25.5**
***R. sublobata* seeds**					
Crude protein extract	954	34496	100	36.2	1
TCA extraction	304	22858	66.3	75.2	2.1
Pure BBI	**4.5**	**8912**	**26**	**1980**	**55**
Pure KI	**1.2**	**1046**	**3.03**	**886**	**24.5**

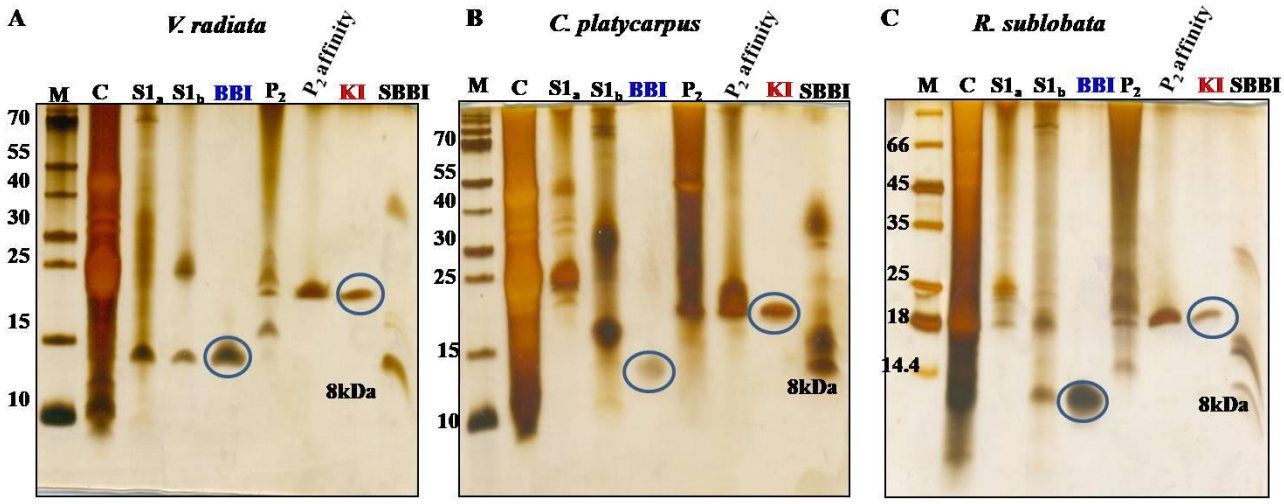

Fig. 4.2. Non-denaturing Tricine SDS-PAGE (15%) showing the purification pattern of (A) VrBBI & VrKI; (B) CpBBI & CpKI and (C) RsBBI & RsKI. The gels are loaded with the following; lane 1: protein standard marker, lane 2: CPE (10 µg), lane 3: S_{1a} (5 µg), lane 4: S_{1b} (5 µg), lane 5: active fraction from S_{1a} and S_{1b} after trypsin affinity chromatography (pure BBI - 1.0 µg), lane 6: Pellet (P_2) obtained after the second cycle of TCA extraction (5 µg), lane 7: active fraction from P_2 after trypsin affinity chromatography (2.5 µg), lane 8: supernatant obtained after sodium acetate extraction (pure KI - 2.5 µg) and lane 9: SBBI (2.5 µg). Blue color circles indicate pure BBI and KI fractions.

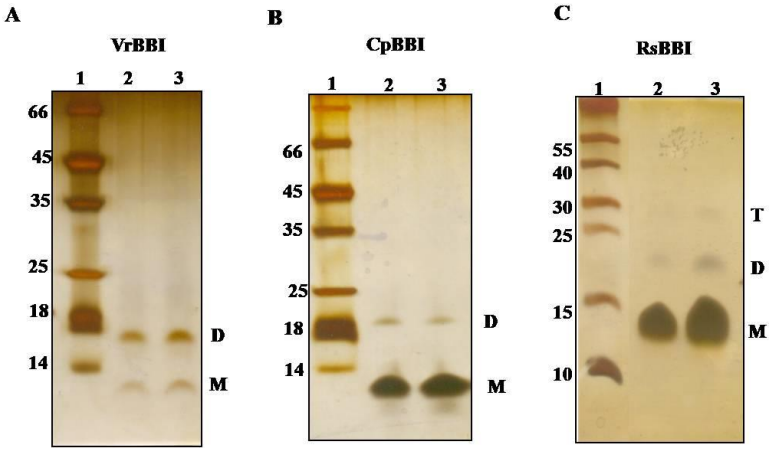

Fig. 4.3. Self-association pattern of BBI(s). Tricine SDS-PAGE (15%) showing concentration-dependent oligomerization of (A) VrBBI; (B) CpBBI and (C) RsBBI. The gels are loaded with the following: Lane 1: Protein standard marker, lane 2: 2 µg of purified BBI(s) and lane 3: 4 µg of purified BBI(s). M, D, and T indicate monomer, dimer and trimer, respectively.

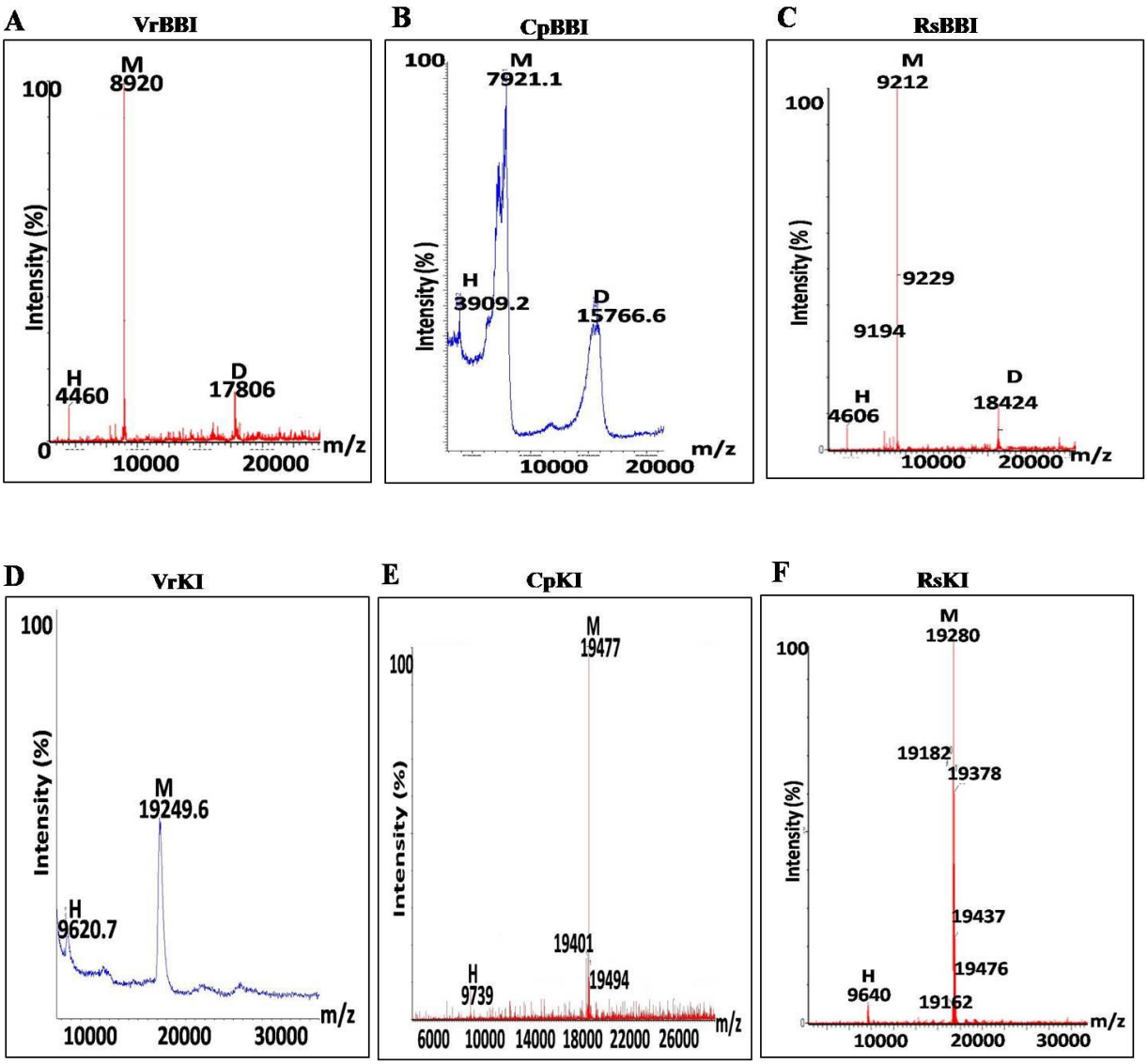

Fig. 4.4. Intact mass analysis of purified BBI(s)/KI(s) under non-reducing conditions. (A) MALDI-ESI-Q-TOF analysis of VrBBI; (B) MALDI-TOF analysis of CpBBI; (C) MALDI-ESI-Q-TOF analysis of RsBBI; (D) MALDI-TOF analysis of VrKI; (E) MALDI-ESI-Q-TOF analysis of CpKI and (F) MALDI-ESI-Q-TOF analysis of RsKI. H, M, and D indicate halfmer, monomer, and dimer, respectively.

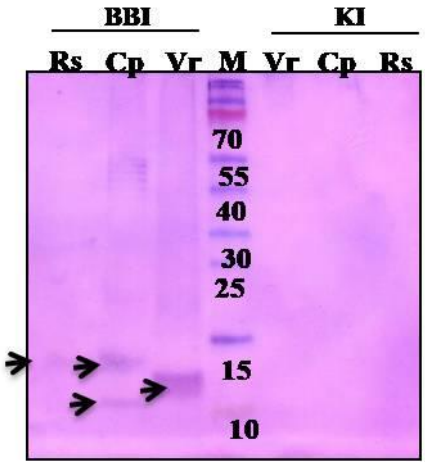

Fig. 4.5. Confirmation of purified BBI(s). Immunodetection of VrBBI, CpBBI, and RsBBI on nitrocellulose membrane probed with anti-BBI antibody. Lane 1: RsBBI (5µg), lane 2: CpBBI (5µg), lane 3: VrBBI (5µg), lane 4: protein standard marker, lane 5: VrKI (5µg), lane 6: CpKI (5µg), and lane 7: RsKI (5µg). VrKI/CpKI/RsKI did not show any cross-reactivity with anti-BBI antibody. Arrow indicates the pure BBI bands formed after probing with an anti-BBI antibody.

and KI (VrKI/CpKI/RsKI) bands isolated from CBB-stained Tricine SDS-PAGE gels were digested with trypsin, and the peptide mass fingerprint (PMF) spectrum of the corresponding proteins was represented in **figures. 4.6A, 4.7A, 4.8A, 4.9A, 4.10A, and 4.11A**. Further, the mascot score histogram of all the BBI(s) and KI(s) purified in the present study showed significant scores: VrBBI (43); CpBBI (60); RsBBI (94); VrKI (62); CpKI (129) and RsKI (170) with *Pisum sativum* or *Glycine max* BBI/KI (**Figs. 4.6B, 4.7B, 4.8B, 4.9B, 4.10B and 4.11B**). Furthermore, various ions obtained after MALDI MS-MS showed the following sequence coverage: VrBBI (14%); CpBBI (43%); RsBBI (50%); VrKI (30%); CpKI (44%), and RsKI (75%) with *P. sativum* or *G. max* BBI and KI (**Figs. 4.6C, 4.7C, 4.8C, 4.9C, 4.10C and 4.11C**). Finally, the Blast P analysis of the partial amino acid sequence obtained from MALDI MS-MS analysis of the purified VrBBI (MELMNKKVMMK) showed 100% similarity with BBI from *P. sativum*, whereas CpBBI (SCICALSYPAQCFCVDITDFCYEP CKP SEDDKEN) and RsBBI (CLDTTDFCYKPCK) showed 100% matching with BBI from *G. max* accessions available in NCBI database (**Figs. 4.6D, 4.7D and 4.8D**). Besides, the partial amino acid sequence obtained from MALDI MS-MS analysis of the purified VrKI (GKGGGIEVDSTGK), CpKI (ESLAKKNHGLSR), and RsKI (GKGGGIEVDSTGK) showed 100% matching with Kunitz inhibitor of *G. max* and *G. soja* accessions available in NCBI database (**Figs. 4.9D, 4.10D and 4.11D**).

Biochemical and biophysical characterization of BBI and KI from *V. radiata, C. platycarpus*, and *R. sublobata*

The VrBBI, CpBBI, and RsBBI showed 1309 ± 62, 1413 ± 78, and 1980 ± 125 TI units/mg protein and 240 ± 17, 450 ± 28, and 342 ± 20 CI units/mg protein, while VrKI, CpKI, and RsKI showed 778 ± 55, 980 ± 69 and 886 ± 63 TI units/mg protein and 10 ± 1.2, 12.5 ± 1.8 and 4.5 ± 0.5 CI units/mg protein, respectively (**Table 4.1; Fig. 4.12A and B**). Further, the

MALDI MS-MS analysis of VrBBI

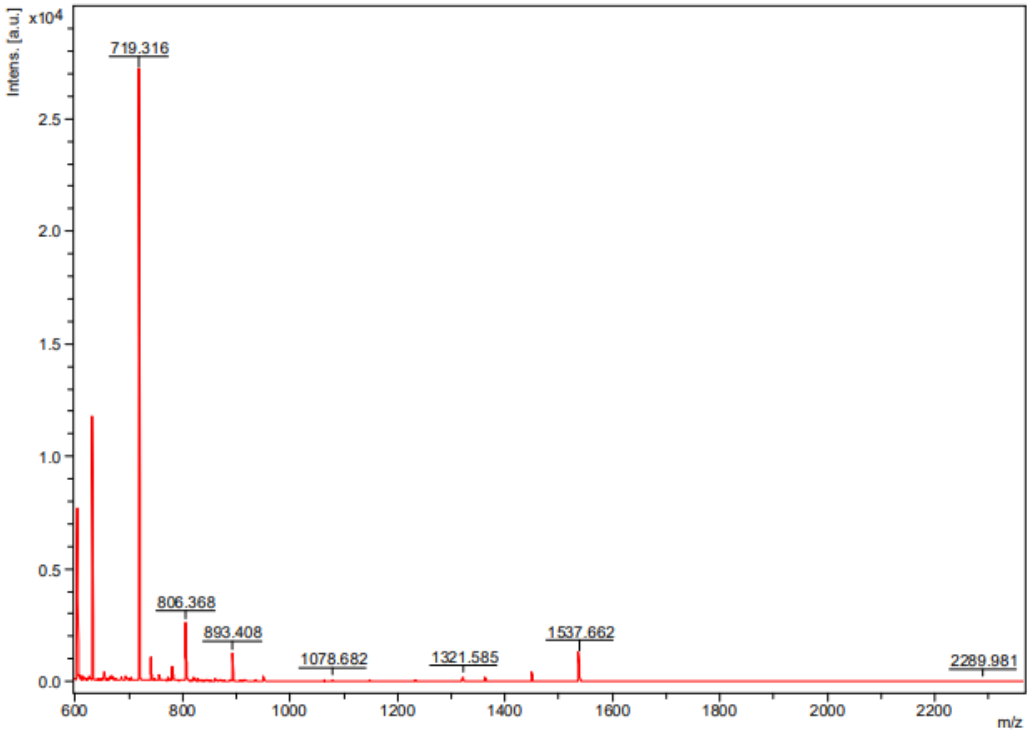

Fig. 4.6A. Peptide mass fingerprint (PMF) spectrum obtained after tryptic digestion of VrBBI protein band (Please refer to Fig. 4.2A, lane 5).

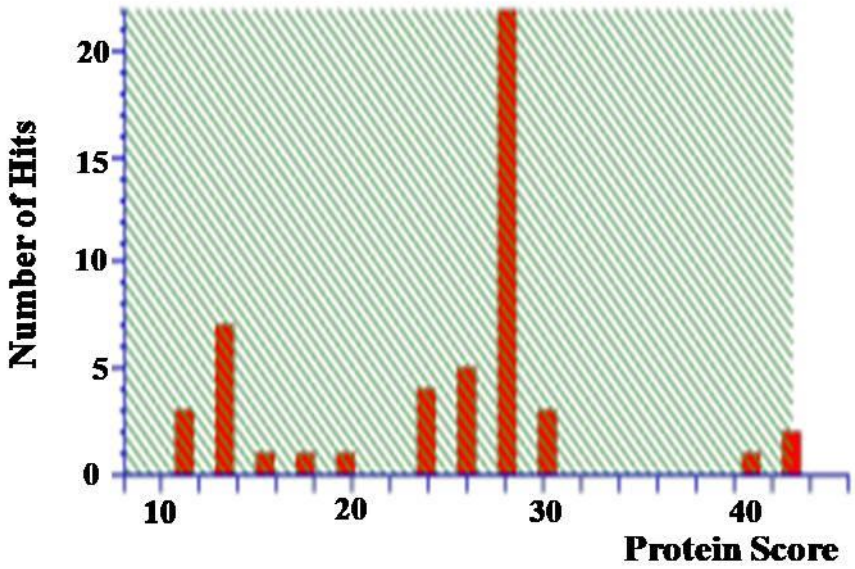

Fig. 4.6B. Mascot score histogram of VrBBI showing a protein score of 43 with Trypsin/chymotrypsin inhibitor (Fragment) OS=*Pisum sativum* OX=3888 GN=ti2B PE=3 SV=1.

Protein Summary Report

Format As: Protein Summary | Help

Significance threshold p< 0.05 | Max. number of hits 20

Preferred taxonomy: All entries

Re-Search All | Search Unmatched

Index

	Accession	Mass	Score	Description
1.	Q8VX21	12245	43	Trypsin/chymotrypsin inhibitor (Fragment) OS=Pisum sativum OX=3888 GN=ti2B PE=3 SV=1
2.	Q8VX24	12210	43	Trypsin/chymotrypsin inhibitor (Fragment) OS=Pisum sativum OX=3888 GN=ti4F PE=3 SV=1
3.	Q41066	13387	42	Seed trypsin/chymotrypsin inhibitor TI5-72 OS=Pisum sativum OX=3888 GN=TI572 PE=2 SV=1
4.	Q8VX19	12206	30	Trypsin/chymotrypsin inhibitor (Fragment) OS=Pisum sativum OX=3888 GN=ti5F PE=3 SV=1
5.	Q8VX18	12125	30	Trypsin/chymotrypsin inhibitor (Fragment) OS=Pisum sativum OX=3888 GN=ti6V PE=3 SV=1
6.	Q8VX22	12097	30	Trypsin/chymotrypsin inhibitor (Fragment) OS=Pisum sativum OX=3888 GN=ti8V PE=3 SV=1
7.	Q8VX10	13314	29	Trypsin/chymotrypsin inhibitor OS=Pisum sativum OX=3888 GN=ti7V PE=2 SV=1
8.	L7N9M1	13345	29	BBI inhibitor OS=Lathyrus sativus OX=3860 PE=3 SV=1
9.	L7N9M8	13301	29	BBI inhibitor OS=Lathyrus sativus OX=3860 PE=2 SV=1
10.	L7N9M9	13397	29	BBI inhibitor OS=Lathyrus sativus OX=3860 PE=3 SV=1
11.	L7N9N3	13328	29	BBI inhibitor OS=Lathyrus sativus OX=3860 PE=3 SV=1
12.	L7N9P1	13373	29	BBI inhibitor OS=Lathyrus sativus OX=3860 PE=3 SV=1
13.	L7N9P3	13383	29	BBI inhibitor OS=Lathyrus sativus OX=3860 PE=2 SV=1
14.	L7N9S6	13342	29	BBI inhibitor OS=Lathyrus sativus OX=3860 PE=3 SV=1
15.	L7N9T1	13343	29	BBI inhibitor OS=Lathyrus sativus OX=3860 PE=3 SV=1
16.	L7N9X3	13319	29	BBI inhibitor OS=Lathyrus sativus OX=3860 PE=3 SV=1
17.	L7N9Y6	13365	29	BBI inhibitor OS=Lathyrus sativus OX=3860 PE=2 SV=1
18.	L7N9Y8	13365	29	BBI inhibitor OS=Lathyrus sativus OX=3860 PE=2 SV=1
19.	Q8VX09	13362	29	Trypsin/chymotrypsin inhibitor OS=Pisum sativum OX=3888 PE=2 SV=1
20.	Q8VX23	12316	28	Trypsin/chymotrypsin inhibitor (Fragment) OS=Pisum sativum OX=3888 GN=ti3F PE=3 SV=1

MATRIX SCIENCE MASCOT Search Results

Protein View: Q8VX21

Trypsin/chymotrypsin inhibitor (Fragment) OS=Pisum sativum OX=3888 GN=ti2B PE=3 SV=1

Database:	Bowman_birk_VignaSP
Score:	43
Expect:	0.055
Monoisotopic mass (M_r):	12245
Calculated pI:	6.23
Taxonomy:	**Pisum sativum**

Sequence similarity is available as **an NCBI BLAST search of Q8VX21 against nr**.

Search parameters

MS data file:	E:\Maldi\DATA 70\PMF MSMS\GGBBI(Green gram)\0_P5\1\1SRef\pdata\1\peaklist.xml
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	**Carbamidomethyl (C)**
Variable modifications:	**Oxidation (M)**
Mass values searched:	33
Mass values matched:	4

Protein sequence coverage: 14%

Matched peptides shown in ***bold red***.

```
  1 MELMNKKVMM KLALMVFLLS FAANVVNARF DSTSFITQVL SNGDDVKSAC
 51 CDTCLCTKSD PPTCRCVDVG ETCHSACDSC ICALSYPPQC QCFDTHKFCY
101 KACH
```

Unformatted sequence string: **104 residues** (for pasting into other applications).

Sort by ◉ residue number ○ increasing mass ○ decreasing mass
Show ◉ matched peptides only ○ predicted peptides also

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
1 - 6	781.3545	780.3472	780.3510	-4.77	0	-.MELMNK.K + Oxidation (M)
1 - 7	893.4075	892.4003	892.4510	-56.9	1	-.MELMNKK.V
7 - 11	668.3279	667.3206	667.3397	-28.6	1	K.KVMMK.L + 2 Oxidation (M)
98 - 101	617.3166	616.3093	616.2679	67.1	0	K.FCYK.A

Fig. 4.6C. Mascot search results showing protein hits and sequence coverage of the corresponding peptides obtained from VrBBI with Trypsin/chymotrypsin inhibitor (Fragment) OS=*Pisum sativum* OX=3888 GN=ti2B PE=3 SV=1**.**

Plant source	Accession no.	Sequence	% Similarity
VrBBI		MELMNKKVMMK	
Pisum sativum	Q41066.1	MELMNKKVMMK	100
Lathyrus sativus	ADV40031.1	MELMNKKAMMK	90
Lens orientalis	CAH04446.1	MVLMNKKTMMK	81
Medicago sativa	CAA56254.1	MELMMNKKAMM	81

Fig. 4.6D. BLAST-P analysis of the peptide sequence obtained from MALDI MS-MS analysis of VrBBI with the homologous BBI sequences available in the NCBI database. The obtained sequence has shown 100% identity with *Pisum sativum* BBI. Identical amino acids are represented in yellow color.

MALDI MS-MS analysis of CpBBI

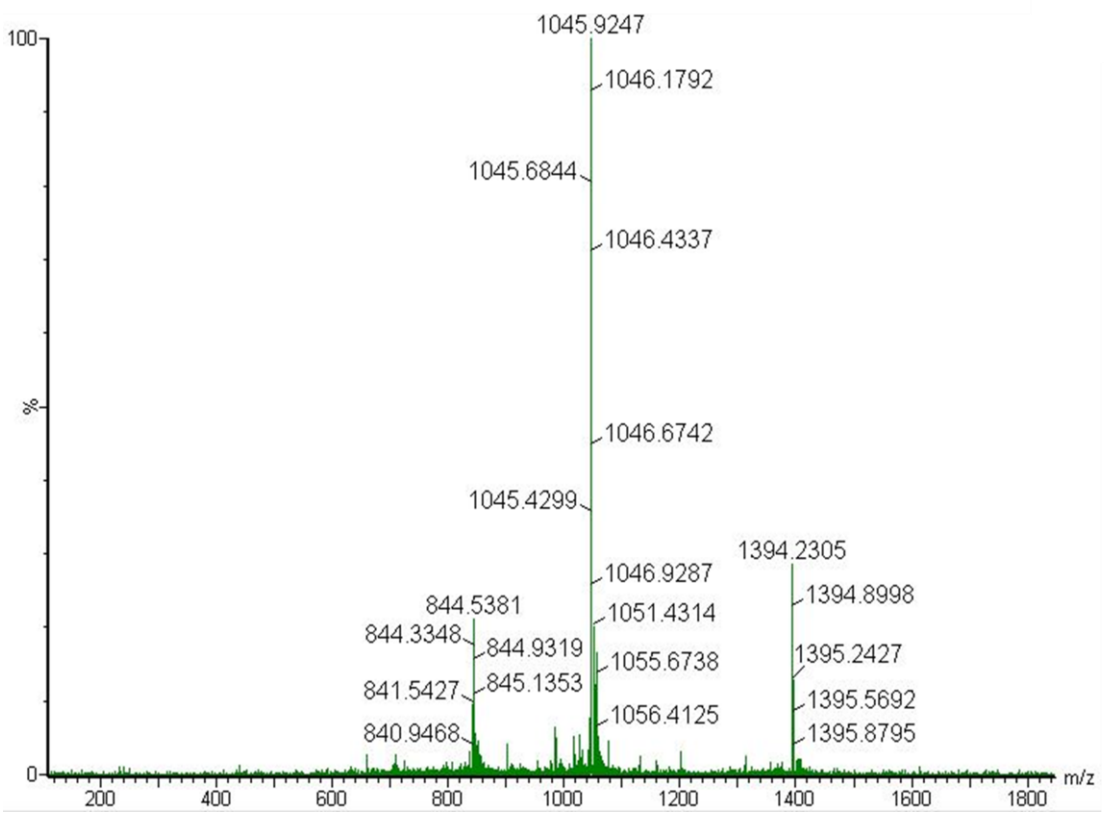

Fig. 4.7A. Peptide mass fingerprint (PMF) spectrum obtained after tryptic digestion of CpBBI protein band (Please refer to Fig. 4.2B, lane 5).

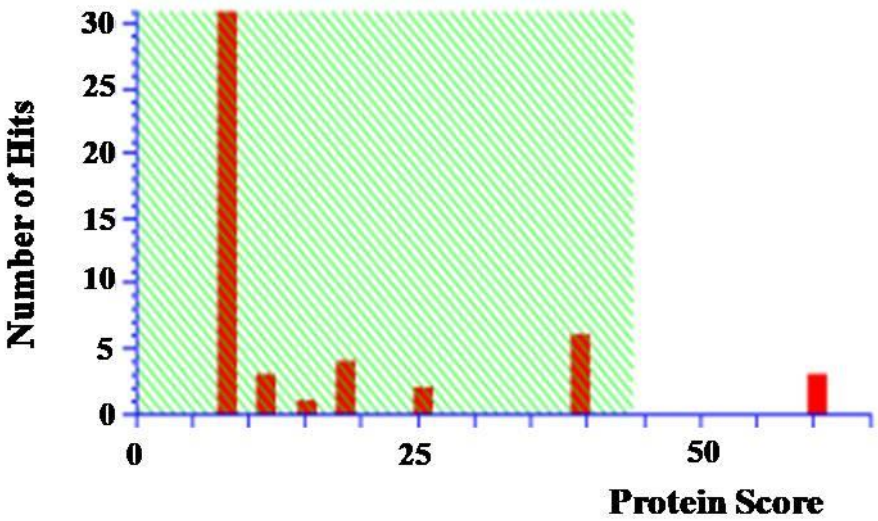

Fig. 4.7B. Mascot score histogram of CpBBI showing a protein score of 60 with Bowman-Birk type proteinase inhibitor OS=*Glycine max* OX=3847 PE=1 SV=2.

Protein Summary Report

Format As: Protein Summary — Help

Significance threshold p< 0.05 — Max. number of hits 10

Preferred taxonomy: All entries

Re-Search All | Search Unmatched

Index

	Accession	Mass	Score	Description
1.	P01055	12939	60	Bowman-Birk type proteinase inhibitor OS=Glycine max OX=3847 PE=1 SV=2
2.	Q53ZY0	12939	60	Bowman-Birk protease inhibitor OS=Glycine max OX=3847 PE=3 SV=1
3.	Q84LF5	13106	60	Bowman-Birk protease inhibitor OS=Glycine microphylla OX=45693 PE=3 SV=1
4.	A0A0R0IF61	13239	39	BOWMAN_BIRK domain-containing protein OS=Glycine max OX=3847 GN=GLYMA_09G158900 PE=3 SV=1
5.	C6SVL8	13920	38	BOWMAN_BIRK domain-containing protein OS=Glycine max OX=3847 PE=2 SV=1
6.	I1L3Q3	13896	38	BOWMAN_BIRK domain-containing protein OS=Glycine max OX=3847 GN=GLYMA_09G158500 PE=3 SV=1
7.	I1MAC4	13856	38	BOWMAN_BIRK domain-containing protein OS=Glycine max OX=3847 GN=GLYMA_14G117700 PE=3 SV=1
8.	Q8RU23	13856	38	Bowman-Birk type proteinase isoinhibitor A2 OS=Glycine soja OX=3848 GN=WSTI-A2 PE=2 SV=1
9.	A0A0B2NX54	13870	38	Bowman-Birk type proteinase inhibitor OS=Glycine soja OX=3848 GN=D0Y65_024320 PE=3 SV=1
10.	Q9SBA9	5097	27	Bowman-Birk proteinase inhibitor (Fragment) OS=Glycine max OX=3847 PE=2 SV=1

MASCOT Search Results

Protein View: P01055

Bowman-Birk type proteinase inhibitor OS=Glycine max OX=3847 PE=1 SV=2

Database:	Bowman_birk_VignaSP
Score:	60
Expect:	0.0015
Monoisotopic mass (M_r):	12939
Calculated pI:	5.82
Taxonomy:	Glycine max

Sequence similarity is available as **an NCBI BLAST search of P01055 against nr**.

Search Parameters

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Type of search         : MS/MS Ion Search
Enzyme                 : Trypsin
Fixed modifications    : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values            : Monoisotopic
Protein Mass           : Unrestricted
Peptide Mass Tolerance : ± 20 ppm
Fragment Mass Tolerance: ± 0.6 Da
Max Missed Cleavages   : 1
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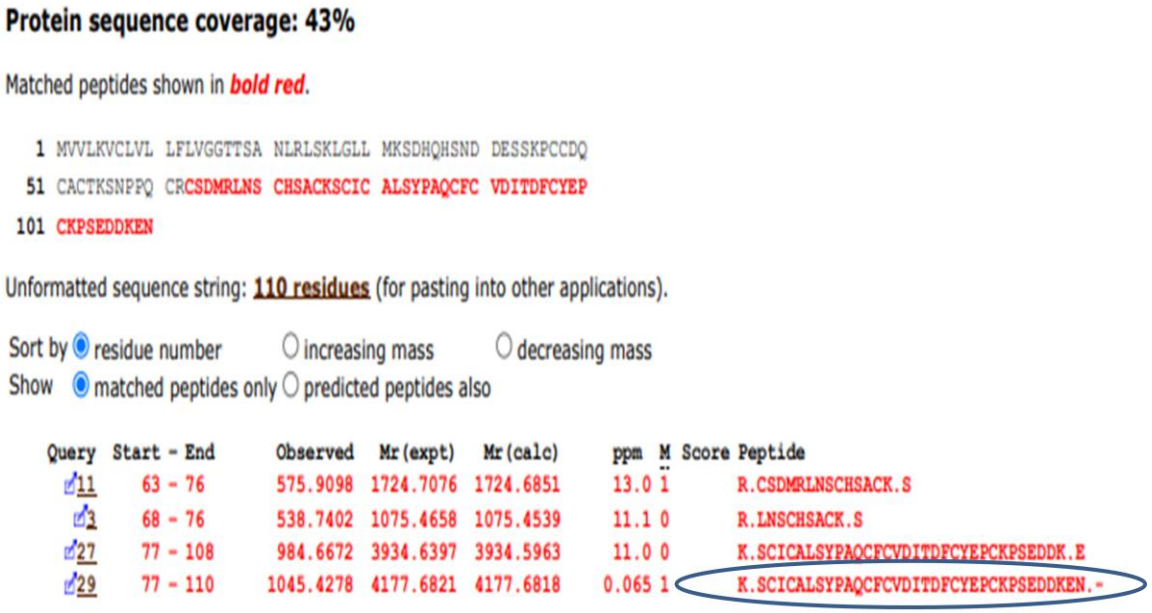

Protein sequence coverage: 43%

Matched peptides shown in ***bold red***.

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  1 MVVLKVCLVL LFLVGGTTSA NLRLSKLGLL MKSDHQHSND DESSKPCCDQ
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101 CKPSEDDKEN
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Unformatted sequence string: **110 residues** (for pasting into other applications).

Sort by ◉ residue number ○ increasing mass ○ decreasing mass
Show ◉ matched peptides only ○ predicted peptides also

Query	Start - End	Observed	Mr(expt)	Mr(calc)	ppm	M	Score	Peptide
11	63 - 76	575.9098	1724.7076	1724.6851	13.0	1		R.CSDMRLNSCHSACK.S
3	68 - 76	538.7402	1075.4658	1075.4539	11.1	0		R.LNSCHSACK.S
27	77 - 108	984.6672	3934.6397	3934.5963	11.0	0		K.SCICALSYPAQCFCVDITDFCYEPCKPSEDDK.E
29	77 - 110	1045.4278	4177.6821	4177.6818	0.065	1		K.SCICALSYPAQCFCVDITDFCYEPCKPSEDDKEN.-

Fig. 4.7C. Mascot search results showing protein hits and sequence coverage of the corresponding peptides obtained from CpBBI with Bowman-Birk type proteinase inhibitor OS=*Glycine max* OX=3847 PE=1 SV=2.

Plant source	Accession no.	Sequence	% Similarity
CpBBI		SCICALSYPAQCFCVDITDFCYEPCKPSEDDKEN	
Glycine max	ACU13291.1	SCICALSYPAQCFCVDITDFCYEPCKPSEDDKEN	100
G. microphylla	AAO89510.1	SCICALSYPAQCFCVDITDFCYEPCKPSEDDKEN	100
Abrus precatorius	XP_027357745.1	TCICALSYPPQCRCVDTTDFCYEPCK-SSDDD--	87
Phaseolus coccineus	CAQ58092.1	SCICTLSIPAQCVCTDTNDFCYEPCKPSHDD	77

Fig.4.7D. BLAST-P analysis of the peptide sequence obtained from MALDI MS-MS analysis of CpBBI with the homologous BBI sequences available in the NCBI database. The obtained sequence has 100% identity with *G. max* BBI. Identical amino acids are represented in yellow color.

MALDI MS-MS analysis of RsBBI

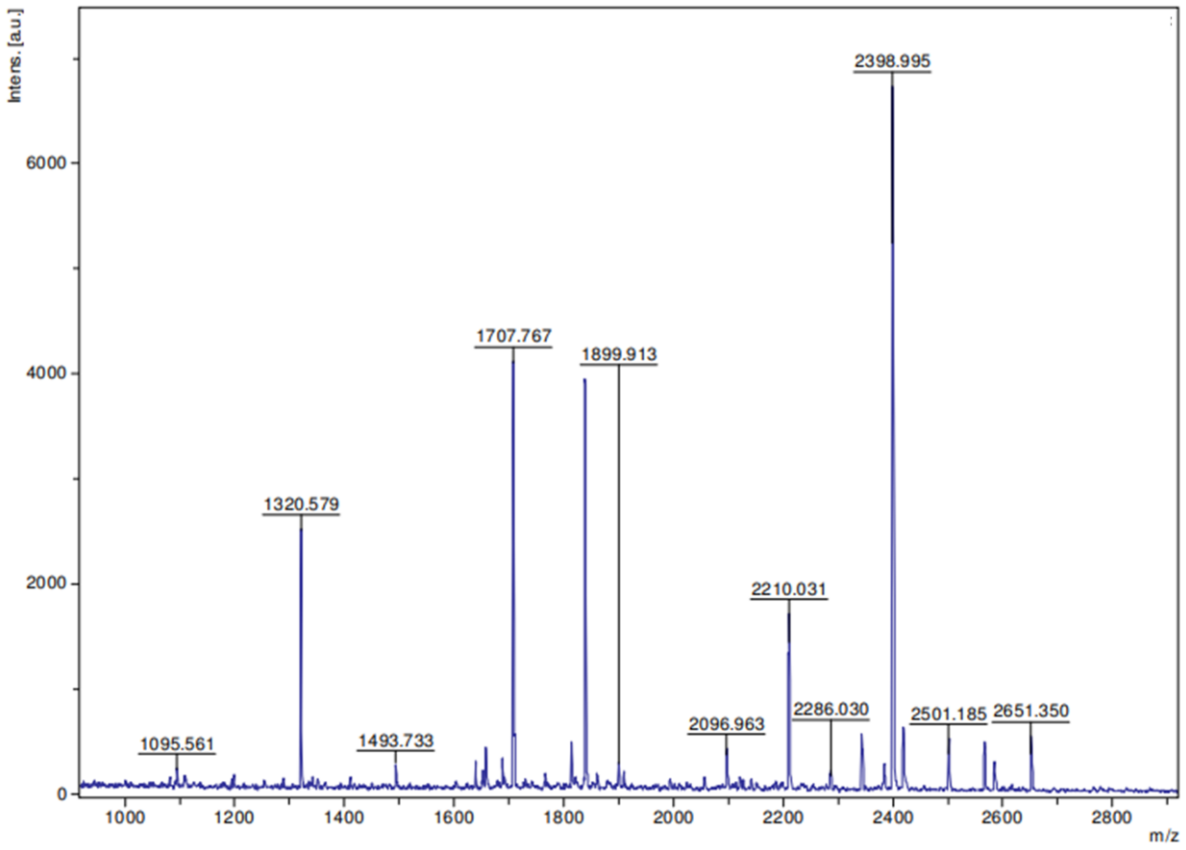


Fig. 4.8A. Peptide mass fingerprint (PMF) spectrum obtained after tryptic digestion of RsBBI protein band (Please refer to Fig 4.2C, lane 5).

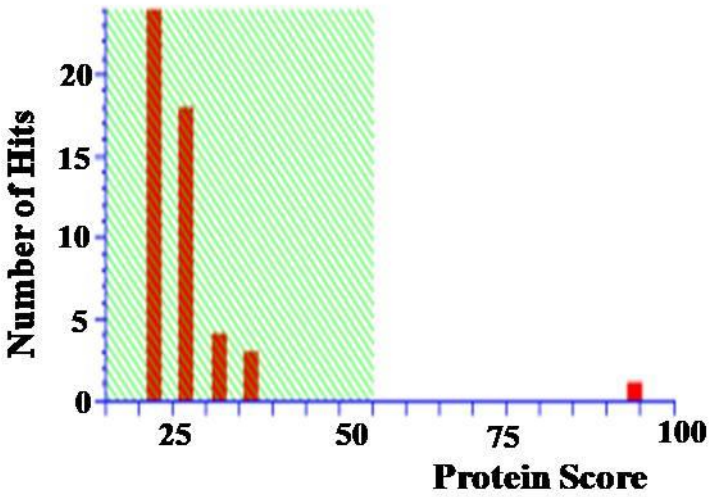


Fig. 4.8B. Mascot score histogram of RsBBI showing a protein score of 94 with IBB1_SOYBN, Bowman-Birk type proteinase inhibitor OS=*Glycine max* OX=3847 PE=1 SV=2.

Protein Summary Report

Format As | Protein Summary (deprecated) | Help

Significance threshold p< 0.05 Max. number of hits 20

Preferred taxonomy All entries

Re-Search All | Search Unmatched

Index

	Accession	Mass	Score	Description
1.	IBB1_SOYBN	12939	94	Bowman-Birk type proteinase inhibitor OS=Glycine max OX=3847 PE=1 SV=2
2.	FLOT2_MEDTR	53204	36	Flotillin-like protein 2 OS=Medicago truncatula OX=3880 GN=FLOT2 PE=2 SV=1
3.	SR542_SOLLC	55433	35	Signal recognition particle 54 kDa protein 2 OS=Solanum lycopersicum OX=4081 PE=2 SV=1
4.	HSP70_LUPPO	28617	35	Heat shock 70 kDa protein (Fragment) OS=Lupinus polyphyllus OX=3874 PE=2 SV=2
5.	CADH1_ARACO	39674	32	Probable cinnamyl alcohol dehydrogenase 1 OS=Aralia cordata OX=29746 GN=CAD1 PE=2 SV=1
6.	CCSA_ANTAG	38564	32	Cytochrome c biogenesis protein CcsA OS=Anthoceros angustus OX=48387 GN=ccsA PE=2 SV=1
7.	H1_PEA	28006	31	Histone H1 OS=Pisum sativum OX=3888 PE=2 SV=1
8.	GLTB_MAIZE	176545	31	Ferredoxin-dependent glutamate synthase, chloroplastic OS=Zea mays OX=4577 GN=GLSF PE=1 SV=1
9.	ADH2_SOLLC	41755	29	Alcohol dehydrogenase 2 OS=Solanum lycopersicum OX=4081 GN=ADH2 PE=2 SV=2
10.	KADC_MAIZE	24909	28	Adenylate kinase, chloroplastic OS=Zea mays OX=4577 GN=ADK1 PE=1 SV=1
11.	IBB1_ARAHY	8426	28	Bowman-Birk type proteinase inhibitor A-II OS=Arachis hypogaea OX=3818 PE=1 SV=1
12.	CCR1_MAIZE	70704	28	Probable serine/threonine-protein kinase CCRP1 OS=Zea mays OX=4577 GN=CCRP1 PE=1 SV=1
13.	IDI2_CLAXA	33257	28	Isopentenyl-diphosphate Delta-isomerase II OS=Clarkia xantiana OX=3938 GN=IPI2 PE=3 SV=1
14.	FTSIH_CHLAT	76400	28	Peptidoglycan D,D-transpeptidase FtsI homolog OS=Chlorokybus atmophyticus OX=3144 GN=ftsI PE=3 SV=1
15.	FER2_MAIZE	15362	27	Ferredoxin-2, chloroplastic OS=Zea mays OX=4577 GN=FDX2 PE=1 SV=1
16.	CALR_BERST	48125	27	Calreticulin OS=Berberis stolonifera OX=33814 PE=2 SV=1
17.	RPOC2_HORVU	170412	27	DNA-directed RNA polymerase subunit beta'' OS=Hordeum vulgare OX=4513 GN=rpoC2 PE=3 SV=1
18.	BIP_SPIOL	73775	27	Luminal-binding protein OS=Spinacia oleracea OX=3562 GN=HSC70 PE=2 SV=1
19.	FLOT6_MEDTR	52412	27	Flotillin-like protein 6 OS=Medicago truncatula OX=3880 GN=FLOT6 PE=2 SV=1
20.	YMF31_MARPO	6966	26	Uncharacterized mitochondrial protein ymf31 OS=Marchantia polymorpha OX=3197 GN=YMF31 PE=4 SV=1

MATRIX SCIENCE MASCOT Search Results

Protein View: IBBC2_SOYBN

Bowman-Birk type proteinase inhibitor C-II OS=Glycine max OX=3847 PE=1 SV=2

Database:	SwissProt
Score:	61
Expect:	0.035
Monoisotopic mass (M_r):	9993
Calculated pI:	4.63
Taxonomy:	Glycine max

Sequence similarity is available as **an NCBI BLAST search of IBBC2_SOYBN against nr**.

Search parameters

MS data file:	peaklist.xml
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Oxidation (M)
Mass values searched:	51
Mass values matched:	6

Protein sequence coverage: 50%

Matched peptides shown in ***bold red***.

```
 1 MELNLFKSDH SSSDDESSKP CCDLCMCTAS MPPQCHCADI RLNSCHSACD
51 RCACTRSMPG QCRCLDTTDF CYKPCKSSDE DDD
```

Unformatted sequence string: **83 residues** (for pasting into other applications).

Sort by ◉ residue number ○ increasing mass ○ decreasing mass
Show ◉ matched peptides only ○ predicted peptides also

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
42 - 51	1219.4943	1218.4870	1218.4870	-0.0033	0	R.LNSCHSACDR.C
42 - 56	1867.7415	1866.7342	1866.7342	0.0086	1	R.LNSCHSACDRCACTR.S
52 - 63	1483.6022	1482.5949	1482.5948	0.026	1	R.CACTRSMPGQCR.C
57 - 63	835.3549	834.3477	834.3476	0.038	0	R.SMPGQCR.C
64 - 76	1707.7175	1706.7103	1706.7103	-0.0082	0	R.CLDTTDFCYKPCK.S
64 - 83	2470.9319	2469.9246	2469.9247	-0.030	1	R.CLDTTDFCYKPCKSSDEDDD.-

Fig. 4.8C. Mascot search results showing protein hits and sequence coverage of the corresponding peptides obtained from RsBBI with Bowman-Birk type proteinase inhibitor OS=*Glycine max* OX=3847 PE=1 SV=2.

Plant source	Accession No.	Sequence	% similarity
RsBBI		CLDTTDFCYKPCK	
G. max	AAA33952.1	CLDTTDFCYKPCK	100
C. cajan	KYP42282.1	CVDTTDFCYKPCK	92
C. lineata	AAB30146.1	CVDTTDFCYKPCK	92
V. unguiculata	pir|S09415	CLDIADFCYKPCK	85
M. truncatula	AES80153.2	CYDITDFCYKPC-	83

Fig.4.8D. BLAST-P analysis of the peptide sequence obtained from MALDI MS-MS analysis of RsBBI with the homologous BBI sequences available in the NCBI database. The obtained sequence has shown 100% identity with *G. max* BBI. Identical amino acids are represented in yellow color.

MALDI MS-MS analysis of VrKI

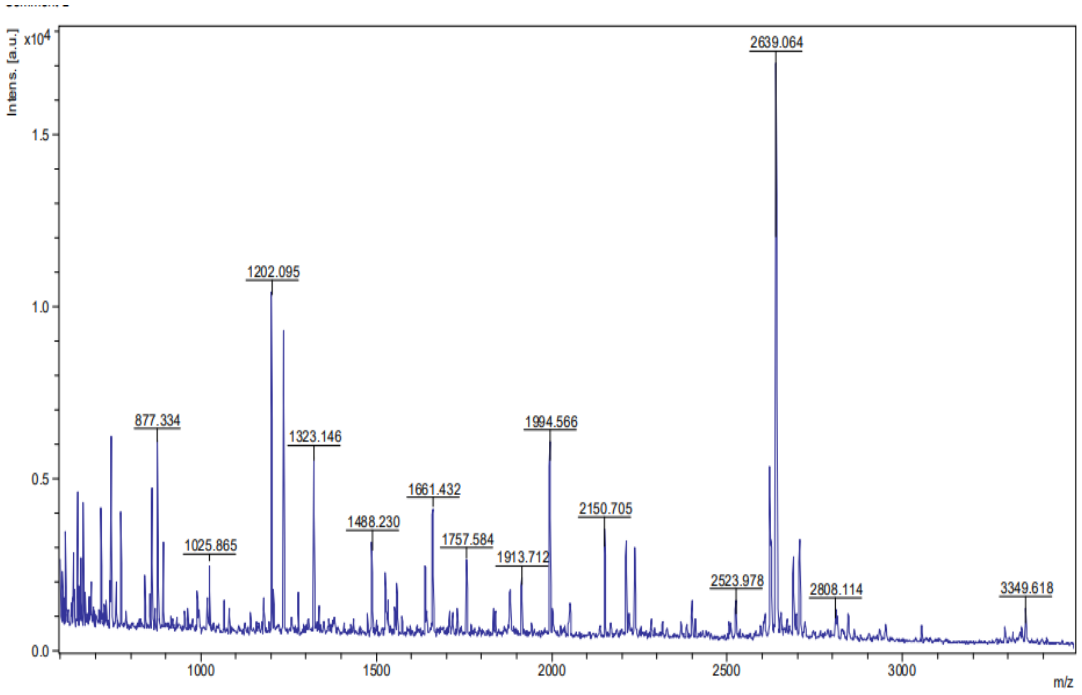


Fig. 4.9A. Peptide mass fingerprint (PMF) spectrum obtained after tryptic digestion of VrKI protein band (Please refer to Fig 4.2A, lane 8).

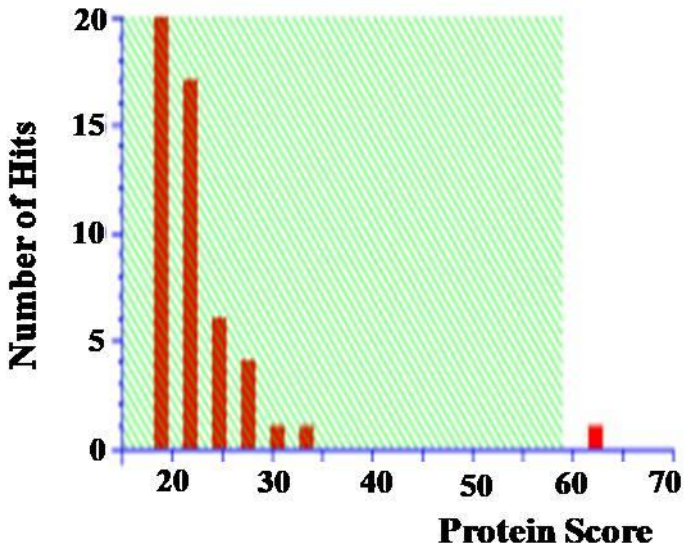


Fig. 4.9B. Mascot score histogram of VrKI showing a protein score of 62 with Kunitz type-trypsin inhibitor KTI1 OS=*Glycine max* OX=3847GN=KTI1 PE=2 SV=1.

Protein Summary Report

Format As: Protein Summary Help

Significance threshold p< 0.05 Max. number of hits 20

Preferred taxonomy: All entries

Re-Search All Search Unmatched

Index

	Accession	Mass	Score	Description
1.	KTI1_SOYBN	22817	62	Kunitz-type trypsin inhibitor KTI1 OS=Glycine max OX=3847 GN=KTI1 PE=2 SV=1
2.	ATPF_CYCTA	20960	35	ATP synthase subunit b, chloroplastic OS=Cycas taitungensis OX=54799 GN=atpF PE=3 SV=1
3.	PSY_ONCHC	47683	30	Phytoene synthase, chloroplastic OS=Oncidium hybrid cultivar OX=141207 GN=PSY PE=2 SV=1
4.	Y1215_ARATH	62839	29	WEB family protein At1g12150 OS=Arabidopsis thaliana OX=3702 GN=At1g12150 PE=2 SV=1
5.	IBB1_WHEAT	6732	28	Bowman-Birk type proteinase inhibitor I-2B (Fragment) OS=Triticum aestivum OX=4565 PE=1 SV=1
6.	PBL18_ARATH	42954	27	Probable serine/threonine-protein kinase PBL18 OS=Arabidopsis thaliana OX=3702 GN=PBL18 PE=2 SV=1
7.	C71BD_ARATH	56753	27	Cytochrome P450 71B13 OS=Arabidopsis thaliana OX=3702 GN=CYP71B13 PE=2 SV=1
8.	CTU1_ORYSJ	41702	26	Cytoplasmic tRNA 2-thiolation protein 1 OS=Oryza sativa subsp. japonica OX=39947 GN=NCS6 PE=2 SV=1
9.	TYDC1_PETCR	49099	26	Tyrosine decarboxylase 1 (Fragment) OS=Petroselinum crispum OX=4043 GN=TYRDC-1 PE=2 SV=1
10.	ATPF_WELMI	21001	26	ATP synthase subunit b, chloroplastic OS=Welwitschia mirabilis OX=3377 GN=atpF PE=3 SV=1
11.	LSM7_ARATH	10824	25	Sm-like protein LSM7 OS=Arabidopsis thaliana OX=3702 GN=LSM7 PE=1 SV=1
12.	Y1112_ARATH	92590	25	G-type lectin S-receptor-like serine/threonine-protein kinase At1g11280 OS=Arabidopsis thaliana OX=3702 GN=At1g11280
13.	SCI1B_TOBAC	18708	25	Style cell-cycle inhibitor 1-B OS=Nicotiana tabacum OX=4097 GN=SCI1B PE=2 SV=2
14.	KN12C_ARATH	235843	23	Kinesin-like protein KIN-12C OS=Arabidopsis thaliana OX=3702 GN=KIN12C PE=1 SV=1
15.	TYDC4_PETCR	57362	23	Tyrosine decarboxylase 4 OS=Petroselinum crispum OX=4043 GN=TYRDC-4 PE=2 SV=1
16.	TRXH5_ORYSJ	15469	23	Thioredoxin H5 OS=Oryza sativa subsp. japonica OX=39947 GN=Os05g0480200 PE=2 SV=1
17.	TYDC3_PETCR	58224	23	Tyrosine decarboxylase 3 OS=Petroselinum crispum OX=4043 GN=TYRDC-3 PE=2 SV=1
18.	MORF5_ARATH	26236	23	Multiple organellar RNA editing factor 5, chloroplastic/mitochondrial OS=Arabidopsis thaliana OX=3702 GN=MORF5 PE=1 S
19.	TBL7_ARATH	51098	23	Protein trichome berefringence-like 7 OS=Arabidopsis thaliana OX=3702 GN=TBL7 PE=3 SV=1
20.	FB290_ARATH	54197	22	F-box protein At5g51380 OS=Arabidopsis thaliana OX=3702 GN=At5g51380 PE=2 SV=1

Parameters used:

Protein View: KTI1_SOYBN

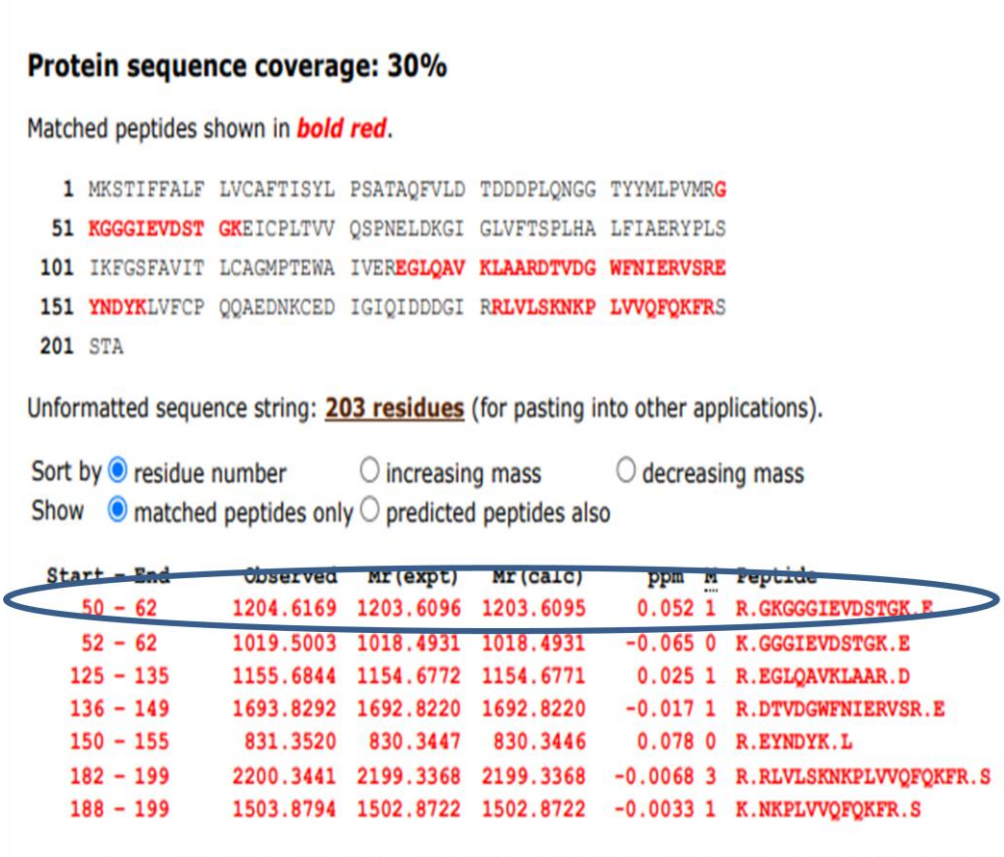
Kunitz-type trypsin inhibitor KTI1 OS=Glycine max OX=3847 GN=KTI1 PE=2 SV=1

Database:	SwissProt
Score:	62
Expect:	0.024
Monoisotopic mass (M_r):	22817
Calculated pI:	4.97
Taxonomy:	Glycine max

Sequence similarity is available as **an NCBI BLAST search of KTI1_SOYBN against nr**.

Search parameters

MS data file:	peaklist.xml
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Oxidation (M)
Mass values searched:	31
Mass values matched:	7



Protein sequence coverage: 30%

Matched peptides shown in ***bold red***.

```
  1 MKSTIFFALF LVCAFTISYL PSATAQFVLD TDDDPLQNGG TYYMLPVMRG
 51 KGGGIEVDST GKEICPLTVV QSPNELDKGI GLVFTSPLHA LFIAERYPLS
101 IKFGSFAVIT LCAGMPTEWA IVEREGLQAV KLAARDTVDG WFNIERVSRE
151 YNDYKLVFCP QQAEDNKCED IGIQIDDDGI RRLVLSKNKP LVVQFQKFRS
201 STA
```

Unformatted sequence string: **203 residues** (for pasting into other applications).

Sort by ◉ residue number ○ increasing mass ○ decreasing mass

Show ◉ matched peptides only ○ predicted peptides also

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
50 - 62	1204.6169	1203.6096	1203.6095	0.052	1	R.GKGGGIEVDSTGK.E
52 - 62	1019.5003	1018.4931	1018.4931	-0.065	0	K.GGGIEVDSTGK.E
125 - 135	1155.6844	1154.6772	1154.6771	0.025	1	R.EGLQAVKLAAR.D
136 - 149	1693.8292	1692.8220	1692.8220	-0.017	1	R.DTVDGWFNIERVSR.E
150 - 155	831.3520	830.3447	830.3446	0.078	0	R.EYNDYK.L
182 - 199	2200.3441	2199.3368	2199.3368	-0.0068	3	R.RLVLSKNKPLVVQFQKFR.S
188 - 199	1503.8794	1502.8722	1502.8722	-0.0033	1	K.NKPLVVQFQKFR.S

Fig. 4.9C. Mascot search results showing protein hits and sequence coverage of the corresponding peptides obtained from VrKI with Kunitz trypsin inhibitor KTI1 OS=*Glycine max* OX=3847GN=KTI1 PE=2 SV=1.

Plant source	**Accession no.**	**Sequence**	**% similarity**
VrKI		GKGGGIEVDSTGK	
G. max	KAH1265567.1	GKGGGIEVDSTGK	100
G. soja	KHN34551.1	GKGGGIEVDSTGK	100
C. cajan	XP_020224397.1	GKGGGIERVRTGK	77
L. leucocephalia	P83036.2	GKGGGLELARTG-	66

Fig. 4.9D. BLAST-P analysis of the peptide sequence obtained from MALDI MS-MS analysis of VrKI with the homologous KI sequences available in the NCBI database. The obtained sequence has shown 100% identity with *G. max* KI. Identical amino acids are represented in yellow color.

MALDI MS-MS analysis of CpKI

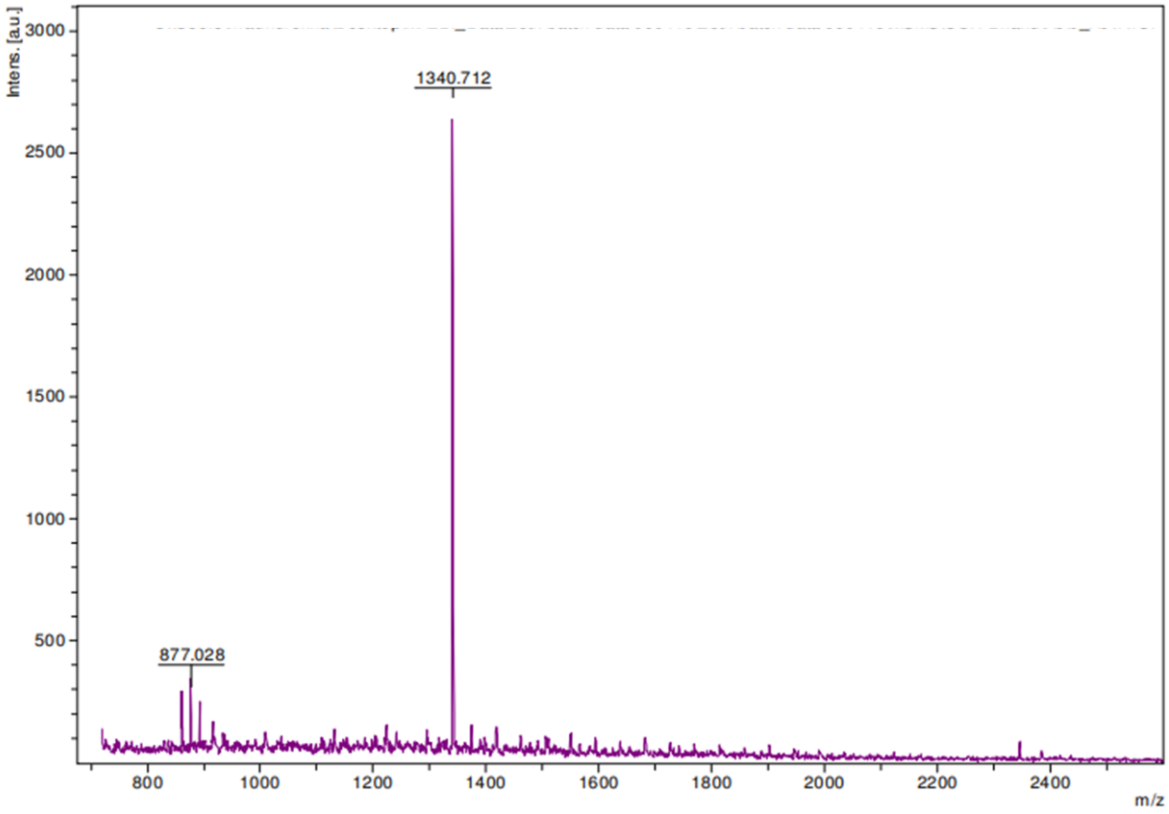


Fig. 4.10A. Peptide mass fingerprint (PMF) spectrum obtained after tryptic digestion of CpKI protein band (Please refer to Fig 4.2B, lane 8).

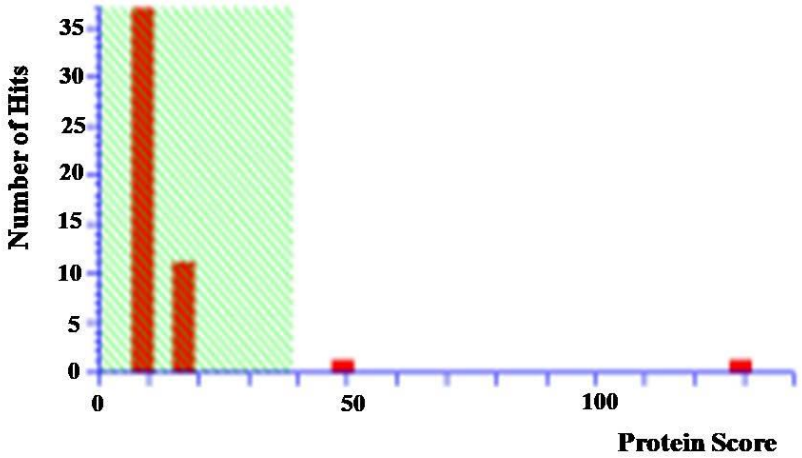


Fig. 4.10B. Mascot score histogram of CpKI showing a protein score of 129 with trypsin inhibitor OS=*Glycine max* OX=3847GN=KTI3 PE=1 SV=2.

Protein Summary Report

Format As: Protein Summary — Help

Significance threshold p<: 0.05 — Max. number of hits: 30

Preferred taxonomy: All entries

Re-Search All | Search Unmatched

Index

	Accession	Mass	Score	Description
1.	ITRA_SOYBN	24275	129	Trypsin inhibitor A OS=Glycine max OX=3847 GN=KTI3 PE=1 SV=2
2.	ITRB_SOYBN	20256	46	Trypsin inhibitor B OS=Glycine max OX=3847 PE=1 SV=1
3.	RR3_SOYBN	24918	19	30S ribosomal protein S3, chloroplastic OS=Glycine max OX=3847 GN=rps3 PE=3 SV=1
4.	CYT_SOYBN	5272	18	Cystatin (Fragment) OS=Glycine max OX=3847 PE=1 SV=1
5.	RPOA_SOYBN	38980	18	DNA-directed RNA polymerase subunit alpha OS=Glycine max OX=3847 GN=rpoA PE=3 SV=1
6.	IBBD2_SOYBN	10316	17	Bowman-Birk type proteinase inhibitor D-II OS=Glycine max OX=3847 PE=1 SV=2
7.	RR11_SOYBN	15106	14	30S ribosomal protein S11, chloroplastic OS=Glycine max OX=3847 GN=rps11 PE=3 SV=1
8.	HSOP1_SOYBN	64480	14	Hsp70-Hsp90 organizing protein 1 OS=Glycine max OX=3847 GN=HOP1 PE=1 SV=2
9.	HS22M_SOYBN	23969	14	Heat shock 22 kDa protein, mitochondrial OS=Glycine max OX=3847 GN=HSP23.9 PE=2 SV=1
10.	DMC1_SOYBN	37679	13	Meiotic recombination protein DMC1 homolog OS=Glycine max OX=3847 PE=2 SV=1
11.	AOX2_SOYBN	38275	13	Ubiquinol oxidase 2, mitochondrial OS=Glycine max OX=3847 GN=AOX2 PE=1 SV=2
12.	RR12_SOYBN	13860	13	30S ribosomal protein S12, chloroplastic OS=Glycine max OX=3847 GN=rps12-A PE=3 SV=2
13.	GSTX3_SOYBN	26112	13	Glutathione S-transferase 3 OS=Glycine max OX=3847 GN=GST3 PE=1 SV=1
14.	SDL5A_SOYBN	68628	13	Dynamin-related protein 5A OS=Glycine max OX=3847 PE=2 SV=1
15.	RR8_SOYBN	15732	12	30S ribosomal protein S8, chloroplastic OS=Glycine max OX=3847 GN=rps8 PE=3 SV=1
16.	C93A1_SOYBN	58232	12	3,9-dihydroxypterocarpan 6A-monooxygenase OS=Glycine max OX=3847 GN=CYP93A1 PE=1 SV=1
17.	GLNA2_SOYBN	39355	12	Glutamine synthetase cytosolic isozyme 2 OS=Glycine max OX=3847 PE=1 SV=1
18.	EFGC1_SOYBN	87187	12	Elongation factor G-1, chloroplastic OS=Glycine max OX=3847 GN=fusA1 PE=3 SV=2
19.	1433B_SOYBN	28018	12	14-3-3-like protein B (Fragment) OS=Glycine max OX=3847 GN=GF14B PE=2 SV=1
20.	C71D8_SOYBN	57713	12	Cytochrome P450 71D8 OS=Glycine max OX=3847 GN=CYP71D8 PE=2 SV=1
21.	SDLCA_SOYBN	68712	12	Dynamin-related protein 12A OS=Glycine max OX=3847 PE=1 SV=1
22.	HSP70_SOYBN	71291	12	Heat shock 70 kDa protein OS=Glycine max OX=3847 GN=HSP70 PE=3 SV=1
23.	C93A3_SOYBN	58445	12	Cytochrome P450 93A3 OS=Glycine max OX=3847 GN=CYP93A3 PE=2 SV=1
24.	RT13_SOYBN	16824	12	Small ribosomal subunit protein S13, mitochondrial OS=Glycine max OX=3847 GN=RSP13 PE=3 SV=1

Protein View: ITRA_SOYBN

Trypsin inhibitor A OS=Glycine max OX=3847 GN=KTI3 PE=1 SV=2

Database: SwissProt
Score: 129
Expect: 5.3e-011
Nominal mass (M_r): 24275
Calculated pI: 4.99
Taxonomy: Glycine max

Sequence similarity is available as an NCBI BLAST search of ITRA_SOYBN against nr.

Search parameters

MS data file: `peaklist.xml`

Enzyme: Trypsin: cuts C-term side of KR unless next residue is P.

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Mass values searched: 32
Mass values matched: 16

Protein sequence coverage: 44%

Matched peptides shown in ***bold red***.

```
  1 MKSTIFFLFL FCAFTTSYLP SAIADFVLDN EGNPLENGGT YYILSDITAF
 51 GGIRAAPTGN ERCPLTVVQS RNELDKGIGT IISSPYRIRF IAEGHPLSLK
101 FDSFAVIMLC VGIPTEWSVV EDLPEGPAVK IGENKDAMDG WFRLERVSDD
151 EFNNYKLVFC PQQAEDDKCG DIGISIDHDD GTRRLVVSKN KPLVVQFQKL
201 DKESLAKKNH GLSRSE
```

Unformatted sequence string: 216 residues (for pasting into other applications).

Sort peptides by ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Show predicted peptides also

Start	End	Observed	Mr(expt)	Mr(calc)	Delta	M	Peptide
55	62	815.1473	814.1401	814.3933	-0.2532	0	R.AAPTGNER.C
72	76	618.1293	617.1221	617.3020	-0.1800	0	R.NELDK.G
77	89	1432.6653	1431.6580	1431.8198	-0.1618	1	K.GIGTIISSPYRIR.F
88	100	1479.9785	1478.9712	1479.8562	-0.8850	1	R.IRFIAEGHPLSLK.F
131	135	560.0130	559.0057	559.2966	-0.2908	0	K.IGENK.D
131	143	1537.9090	1536.9017	1537.6983	-0.7966	1	K.IGENKDAMDGWFR.L
144	156	1628.7018	1627.6945	1627.7478	-0.0533	1	R.LERVSDDEFNNYK.L
147	156	1230.5717	1229.5644	1229.5200	0.0443	0	R.VSDDEFNNYK.L
184	189	701.0958	700.0885	700.4595	-0.3711	1	R.RLVVSK.N
185	189	545.0359	544.0287	544.3584	-0.3298	0	R.LVVSK.N
190	202	1556.7102	1555.7029	1555.9086	-0.2057	1	K.NKPLVVQFQKLDK.E
200	207	903.1419	902.1346	902.5073	-0.3726	1	K.LDKESLAK.K
203	207	547.5023	546.4950	546.3013	0.1937	0	K.ESLAK.K
203	208	675.8857	674.8785	674.3963	0.4822	1	K.ESLAKK.N
203	214	1339.5977	1338.5904	1338.7368	-0.1464	2	K.ESLAKKNHGLSR.S
209	216	899.1665	898.1592	898.4237	-0.2644	1	K.NHGLSRSE.-

Fig. 4.10C. Mascot search results showing protein hits and sequence coverage of the corresponding peptides obtained from CpKI with trypsin inhibitor OS=*Glycine max* OX=3847GN=KTI3 PE=1 SV=2.

Plant source	**Accession no.**	**Sequence**	**% similarity**
CpKI		ESLAKKNHGLSR	
G. max	BAF95192.1	ESLAKKNHGLSR	100
G. soja	AGC92015.1	ESLAKKNHGLSR	100
G. tomentella	BAG68489.1	DSLVKKNHGLSR	91

Fig. 4.10D. BLAST-P analysis of the peptide sequence obtained from MALDI MS-MS analysis of CpKI with the homologous KI sequences available in the NCBI database. The obtained sequence has shown 100% identity with *G. max* KI. Identical amino acids are represented in yellow color.

MALDI MS-MS analysis of RsKI

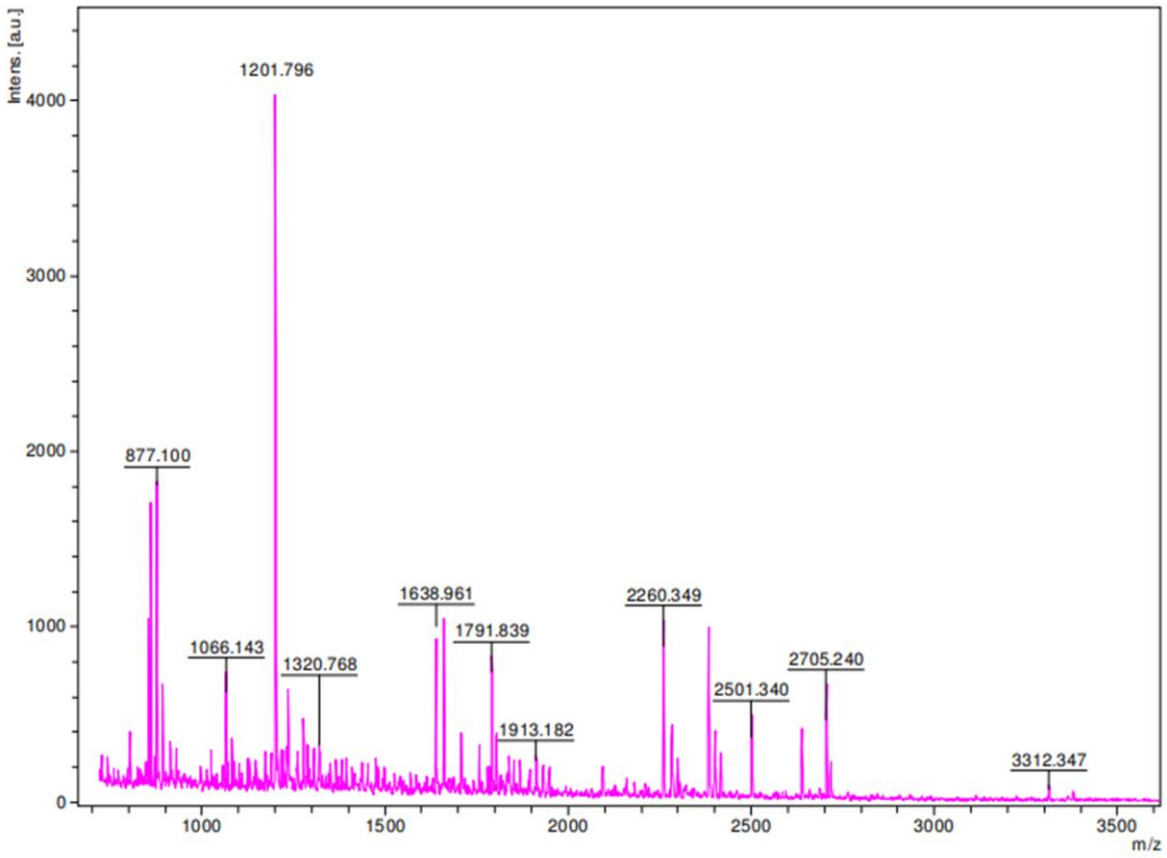


Fig. 4.11A. Peptide mass fingerprint (PMF) spectrum obtained after tryptic digestion of RsKI protein band (Please refer to Fig 4.2C, lane 8).

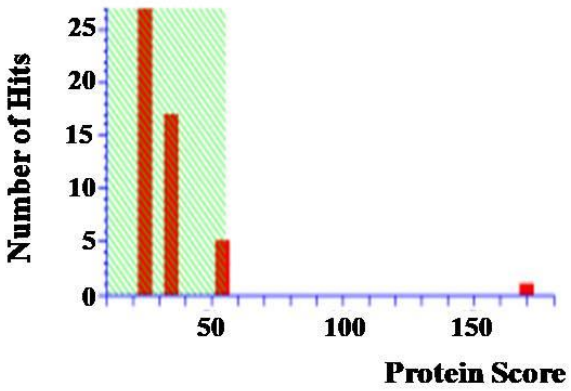


Fig. 4.11B. Mascot score histogram of RsKI showing a protein score of 170 with KTI1_SOYBN, Kunitz-type trypsin inhibitor KTI1 OS=*Glycine max* OX=3847 GN=KTI1 PE=2 SV=1.

Protein Summary Report

Format As: Protein Summary (deprecated) — Help

Significance threshold p< 0.05 Max. number of hits 20

Preferred taxonomy: All entries

Re-Search All | Search Unmatched

Index

	Accession	Mass	Score	Description
1.	KTI1_SOYBN	22817	170	Kunitz-type trypsin inhibitor KTI1 OS=Glycine max OX=3847 GN=KTI1 PE=2 SV=1
2.	GALE1_PEA	39251	58	UDP-glucose 4-epimerase OS=Pisum sativum OX=3888 GN=GALE PE=2 SV=1
3.	UGE1_PEA	39221	58	Bifunctional UDP-glucose 4-epimerase and UDP-xylose 4-epimerase 1 OS=Pisum sativum OX=3888 GN=UGE1 PE=1 SV=2
4.	MTB2_SOLLC	64686	55	Transcription factor MTB2 OS=Solanum lycopersicum OX=4081 GN=MTB2 PE=2 SV=1
5.	MTB1_SOLLC	68668	51	Transcription factor MTB1 OS=Solanum lycopersicum OX=4081 GN=MTB1 PE=1 SV=1
6.	MTB3_SOLLC	58094	51	Transcription factor MTB3 OS=Solanum lycopersicum OX=4081 GN=MTB3 PE=2 SV=1
7.	TI214_NUPAD	222881	39	Protein TIC 214 OS=Nuphar advena OX=77108 GN=TIC214 PE=3 SV=1
8.	CEMA_STAPU	68189	38	Chloroplast envelope membrane protein OS=Staurastrum punctulatum OX=102822 GN=cemA PE=3 SV=1
9.	RPOC2_CHLRE	358877	38	DNA-directed RNA polymerase subunit beta'' OS=Chlamydomonas reinhardtii OX=3055 GN=rpoC2 PE=2 SV=1
10.	IF1A_ONOVI	16545	37	Eukaryotic translation initiation factor 1A OS=Onobrychis viciifolia OX=3882 PE=2 SV=2
11.	MFP1_TOBAC	82453	37	MAR-binding filament-like protein 1-1 OS=Nicotiana tabacum OX=4097 GN=MFP1-1 PE=2 SV=1
12.	PRS6A_SOLLC	47704	36	26S proteasome regulatory subunit 6A homolog OS=Solanum lycopersicum OX=4081 GN=TBP1 PE=2 SV=1
13.	RPOC2_LIRTU	157839	35	DNA-directed RNA polymerase subunit beta'' OS=Liriodendron tulipifera OX=3415 GN=rpoC2 PE=3 SV=1
14.	BIP8_TOBAC	32436	35	Luminal-binding protein 8 (Fragment) OS=Nicotiana tabacum OX=4097 GN=BIP8 PE=2 SV=1
15.	GLGL3_SOLTU	53854	34	Glucose-1-phosphate adenylyltransferase large subunit 3, chloroplastic/amyloplastic OS=Solanum tuberosum OX=4113 GN=AGPS3
16.	ACCD_CAPBU	55681	33	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta, chloroplastic OS=Capsella bursa-pastoris OX=3719 GN=accD
17.	PRS6A_BRACM	47747	32	26S proteasome regulatory subunit 6A homolog OS=Brassica campestris OX=3711 GN=TBP1 PE=2 SV=1
18.	RR3_MORIN	24897	32	30S ribosomal protein S3, chloroplastic OS=Morus indica OX=248361 GN=rps3 PE=3 SV=1
19.	NNJA3_MAIZE	28245	32	Ninja-family protein 3 OS=Zea mays OX=4577 PE=2 SV=1
20.	NNJA4_MAIZE	28096	30	Ninja-family protein 4 OS=Zea mays OX=4577 PE=2 SV=1

MATRIX SCIENCE MASCOT Search Results

Protein View: KTI1_SOYBN

Kunitz-type trypsin inhibitor KTI1 OS=Glycine max OX=3847 GN=KTI1 PE=2 SV=1

Database:	SwissProt
Score:	170
Expect:	1.9e-13
Monoisotopic mass (M_r):	22817
Calculated pI:	4.97
Taxonomy:	Glycine max

Sequence similarity is available as **an NCBI BLAST search of KTI1_SOYBN against nr**.

Search parameters

MS data file:	RS KI.mgf
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Oxidation (M)

Protein sequence coverage: 75%

Matched peptides shown in ***bold red***.

```
  1 MKSTIFFALF LVCAFTISYL PSATAQFVLD TDDDPLQNGG TYYMLPVMRG
 51 KGGGIEVDST GKEICPLTVV QSPNELDKGI GLVFTSPLHA LFIAERYPLS
101 IKFGSFAVIT LCAGMPTEWA IVEREGLQAV KLAARDTVDG WFNIERVSRE
151 YNDYKLVFCP QQAEDNKCED IGIQIDDDGI RRLVLSKNKP LVVQFQKFRS
201 STA
```

Sort by ◉ residue number ○ increasing mass ○ decreasing mass
Show ◉ matched peptides only ○ predicted peptides also

Query	Start – End	Observed	Mr(expt)	Mr(calc)	ppm	M	Score	Peptide
21	50 – 62	1204.7676	1203.7603	1203.6095	125	1		R.GKGGGIEVDSTGK.E
11	52 – 62	1019.5142	1018.5070	1018.4931	13.6	0		K.GGGIEVDSTGK.E
48	63 – 78	1841.9301	1840.9228	1840.9241	-0.66	0		K.EICPLTVVQSPNELDK.G
53	79 – 96	1941.1064	1940.0991	1940.0884	5.53	0		K.GIGLVFTSPLHALFIAER.Y
68	79 – 102	2642.4871	2641.4798	2641.4996	-7.48	1		K.GIGLVFTSPLHALFIAERYPLSIK.F
1	97 – 102	720.3388	719.3315	719.4218	-125	0		R.YPLSIK.F
65	103 – 124	2455.2234	2454.2161	2454.2076	3.47	0		K.FGSFAVITLCAGMPTEWAIVER.E
2	125 – 131	744.4486	743.4413	743.4177	31.7	0		R.EGLQAVK.L
18	125 – 135	1155.7428	1154.7355	1154.6771	50.6	1		R.EGLQAVKLAAR.D
40	132 – 146	1762.8249	1761.8177	1761.8798	-35.3	1		K.LAARDTVDGWFNIER.V
28	136 – 146	1351.6046	1350.5974	1350.6204	-17.1	0		R.DTVDGWFNIER.V
66	136 – 155	2506.2402	2505.2330	2505.1561	30.7	2		R.DTVDGWFNIERVSREYNDYK.L
19	147 – 155	1173.5385	1172.5312	1172.5462	-12.7	1		R.VSREYNDYK.L
4	150 – 155	831.3637	830.3564	830.3446	14.2	0		R.EYNDYK.L
56	150 – 167	2260.3486	2259.3413	2260.0106	-296	1		R.EYNDYKLVFCPQQAEDNK.C
31	156 – 167	1448.6949	1447.6876	1447.6766	7.65	0		K.LVFCPQQAEDNK.C
35	168 – 181	1618.7753	1617.7680	1617.7305	23.2	0		K.CEDIGIQIDDDGIR.R
41	168 – 182	1774.8733	1773.8660	1773.8316	19.4	1		K.CEDIGIQIDDDGIRR.L
60	168 – 187	2315.2893	2314.2820	2314.1951	37.6	2		K.CEDIGIQIDDDGIRRLVLSK.N
52	182 – 197	1897.1488	1896.1415	1896.1673	-13.6	2		R.RLVLSKNKPLVVQFQK.F
49	188 – 203	1850.0544	1849.0471	1849.0210	14.1	2		K.NKPLVVQFQKFRSSTA.-

Fig. 4.11C. Mascot search results showing protein hits and sequence coverage of the corresponding peptides obtained from RsKI with Kunitz inhibitor OS=*Glycine max* OX=3847GN=KTI1 PE=2 SV=1.

Plant source	**Accession no.**	**Sequence**	**% similarity**
RsKI		GKGGGIEVDSTGK	
G. max	KAH1265567.1	GKGGGIEVDSTGK	100
G. soja	KHN34551.1	GKGGGIEVDSTGK	100
C. cajan	XP_020224397.1	GKGGGIERVRTGK	77
P. tetragonolobus	P10821.1	- -GGGIEAAATG -	70
L. leucocephalia	P83036.2	GKGGGLELARTG-	66

Fig. 4.11D. BLAST-P analysis of the peptide sequence obtained from MALDI MS-MS analysis of RsKI with the homologous KI sequences available in the NCBI database. The obtained sequence has shown 100% identity with *G. max* KI. Identical amino acids are represented in yellow color.

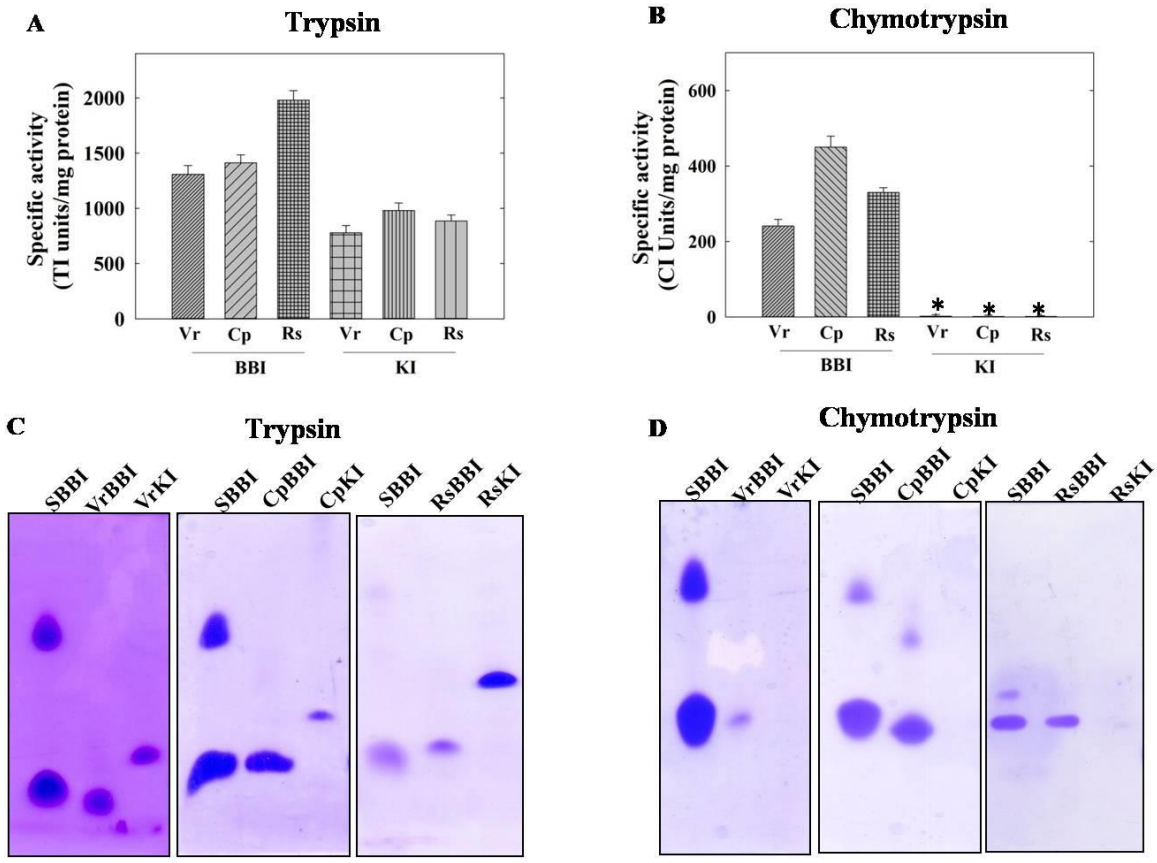


Fig. 4.12. *In vitro* inhibitory activity of purified PIs against serine proteases. The specific activity of VrBBI, CpBBI, RsBBI, VrKI, CpKI, and RsKI against (A) bovine pancreatic trypsin and (B) chymotrypsin. * As CI activity of KI(s) is very low, it is not visible in the graph; (C, D) Visualization of in-gel activity of VrBBI, CpBBI & RsBBI (2 µg) and VrKI, CpKI & RsKI (2 µg) against trypsin and chymotrypsin in SDS-PAGE (15%) incorporated with gelatin. SBBI (2 µg) was used as a reference marker. Bands were visualized after staining with Coomassie Brilliant blue R-250.

in-gel activity staining studies against trypsin and chymotrypsin correlated well with these *in vitro* studies (**Fig. 4.12C and D**). However, no bands are identified against VrKI, CpKI, and RsKI when incubated with chymotrypsin. Besides, these PIs showed a non-competitive mode of inhibition against trypsin and/or chymotrypsin. The inhibitory constant (K_i) of VrBBI, CpBBI, and RsBBI against trypsin was 116.3 ± 2.5, 125.4 ± 3.2, and 127.8 ± 3.5 nM, and chymotrypsin was 936.6 ± 15.8, 704.4 ± 10.6 and 807.5 ± 13.8 nM, respectively (**Figs. 4.13A-C and 4.14A-C**). Also, the VrKI, CpKI, and RsKI inhibited trypsin with a K_i of 150.4 ± 4.5, 133.6 ± 3.9, and 182.1 ± 5.8 nM, respectively (**Fig. 4.15A-C**).

The TI and CI activities of VrBBI, CpBBI, and RsBBI are stable with a marginal loss (≤15%) when heated at 100 ^{o}C (**Fig. 4.16A and B**). Conversely, the TI activity of VrKI, CpKI, and RsKI was stable up to 70 ^{o}C with ≤10% loss in activity. However, they lost ≥90% of activity when heated up to 100 ^{o}C (**Fig. 4.16C**). Besides, the TI and CI activities of all the purified PIs were stable at different pH conditions (pH 2.0 - 12.0) with ≤15% loss in activity (**Fig. 4.17A-C**). Also, the reduction of VrBBI, CpBBI, and RsBBI with DTT (1 mM) resulted in ≥90% loss in both TI and CI activities. However, the activities are completely lost when treated with 3 mM DTT (**Fig. 4.18A and B**). In contrast, the TI activity of VrKI, CpKI, and RsKI was stable even after treatment with 1 mM DTT. But, a 50% reduction in TI activity at 25 mM, and a complete loss in TI activity at 200 mM DTT concentration were observed in VrKI, CpKI, and RsKI, respectively (**Fig. 4.18C**).

Further, the CD spectroscopy studies revealed that random coils are more predominant than β-turns/β-sheets followed by α-helices in both BBI and KI from *V. radiata*, *C. platycarpus*, and *R. sublobata* (**Table 4.2**). A marginal change in the ellipticity of VrBBI, CpBBI, and RsBBI at ~202 nm when heated from 20 to 90 °C suggests that they are structurally stable at high temperatures (**Fig. 4.19A-C**). But VrKI, CpKI, and RsKI showed a

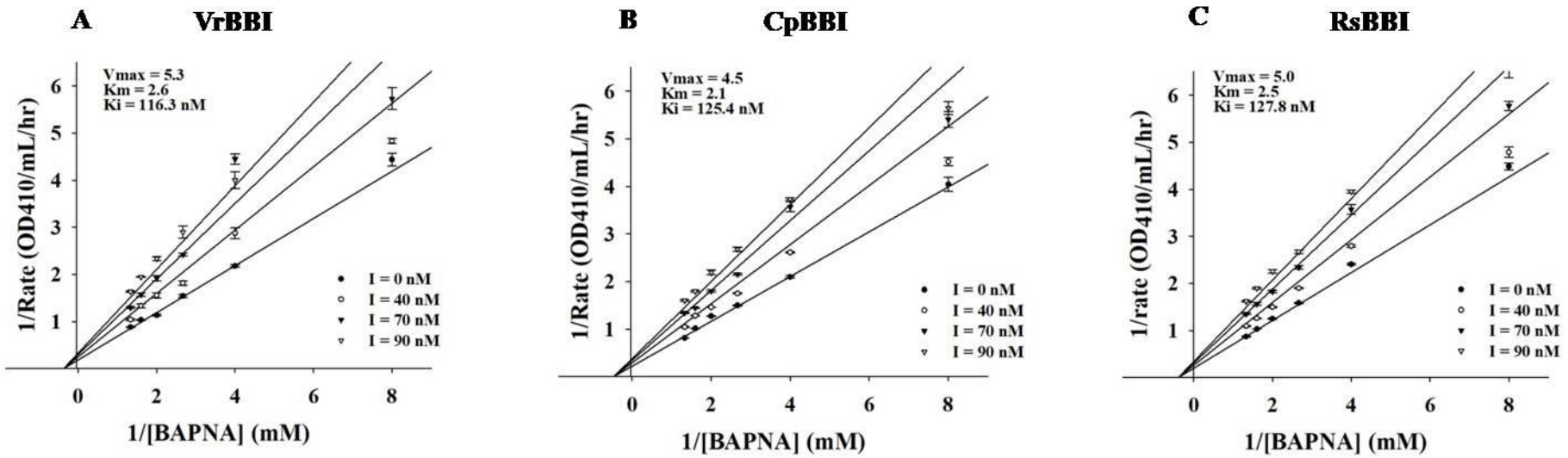


Fig. 4.13. Enzyme inhibition kinetics of BBI(s) against trypsin. Lineweaver-Burk plot showing the non-competitive mode of inhibition after incubating with increasing (40, 70 and 90 nM) concentrations of (A) VrBBI; (B) CpBBI and (C) RsBBI against trypsin (1 µM) at a wide range of concentrations (0.125, 0.25, 0.375, 0.5, 0.625 and 0.75 mM) of BAPNA. The values are the mean ± SE of at least three independent assays, each with three replicates.

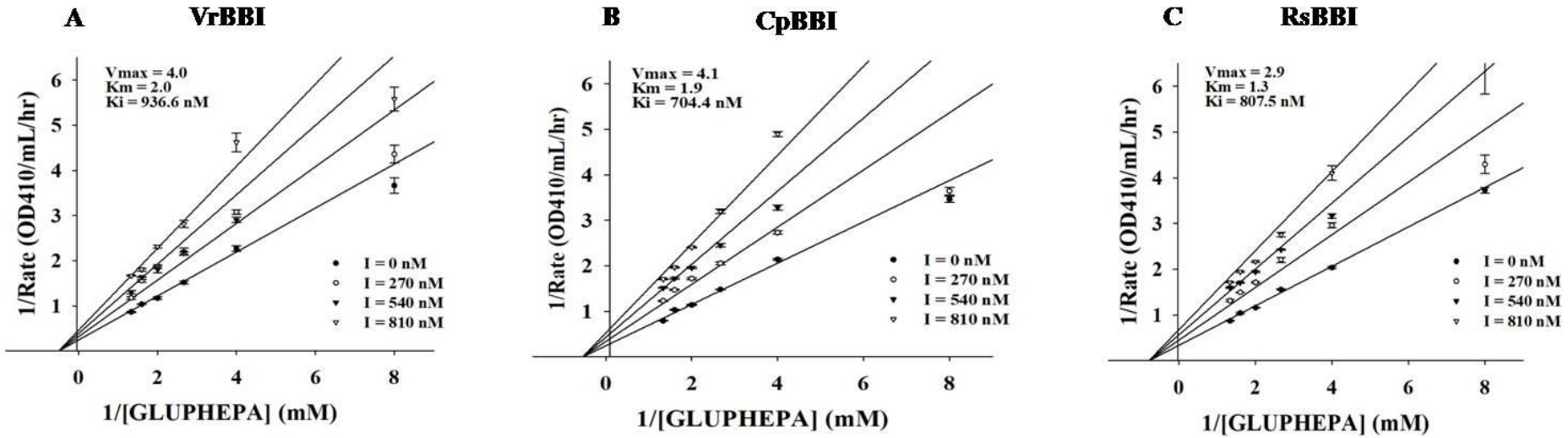


Fig. 4.14. Enzyme inhibition kinetics of BBI(s) against chymotrypsin. Lineweaver-Burk plot showing the non-competitive mode of inhibition after incubating with increasing (270, 540, and 810 nM) concentrations of (A) VrBBI; (B) CpBBI and (C) RsBBI against chymotrypsin (1 µM) at a wide range of concentrations (0.125, 0.25, 0.375, 0.5, 0.625 and 0.75 mM) of GLUPHEPA. The values are the mean ± SE of at least three independent assays, each with three replicates.

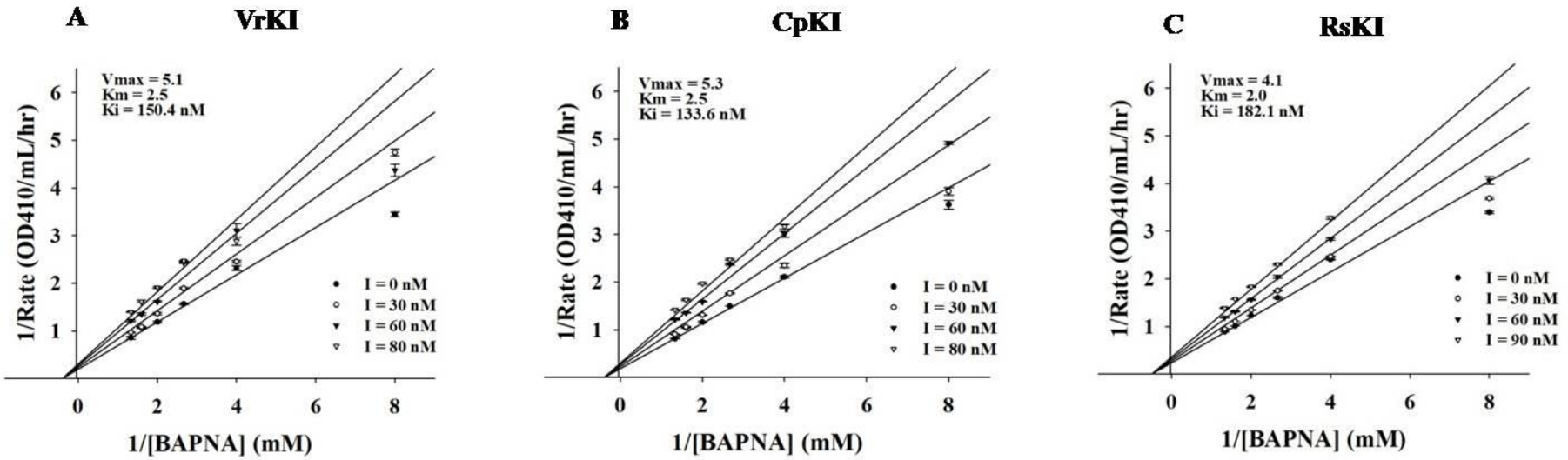


Fig. 4.15. Enzyme inhibition kinetics of KI(s) against trypsin. Lineweaver-Burk plot showing the non-competitive mode of inhibition after incubating with increasing (30, 60, and 80 nM) concentrations of (A) VrKI; (B) CpKI and (C) RsKI against trypsin (1 µM) at a wide range of concentrations (0.125, 0.25, 0.375, 0.5, 0.625 and 0.75 mM) of BAPNA. The values are the mean ± SE of at least three independent assays, each with three replicates.

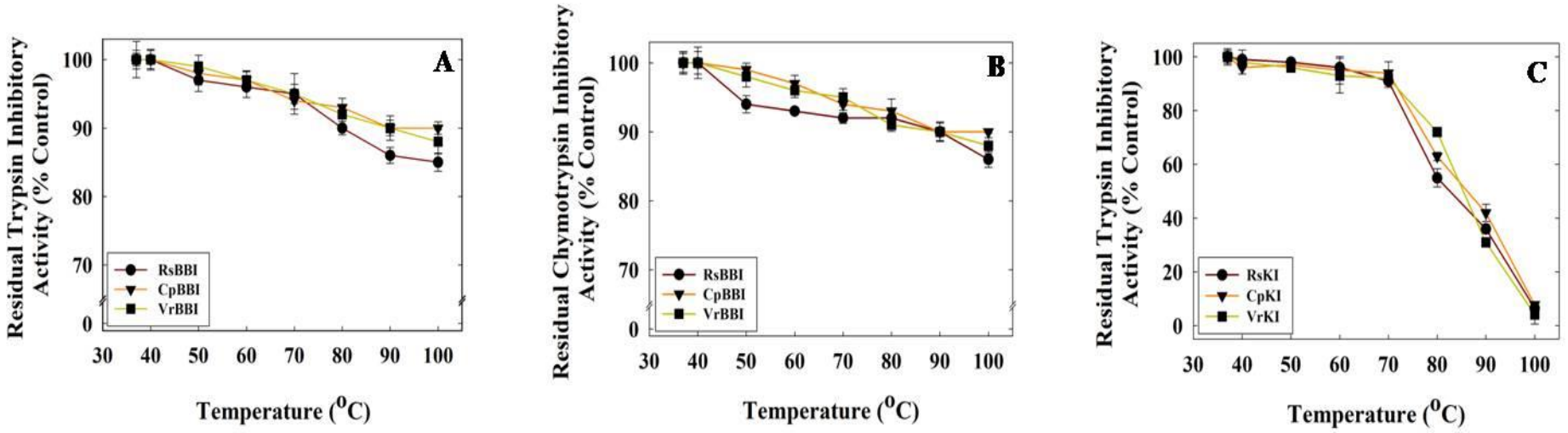


Fig. 4.16. Functional stability of BBI(s) and KI(s) against a wide range of temperatures (20-100 ^{o}C). The effect of temperature on (A) TI; (B) CI activity of VrBBI/CpBBI/RsBBI and (C) TI activity of VrKI/CpKI/RsKI was determined after incubating the PI sample for 30 min at indicated temperature. The residual protease inhibitor activity was measured as described in the materials and methods. The data represented is the mean ± SE of three biological replicates.

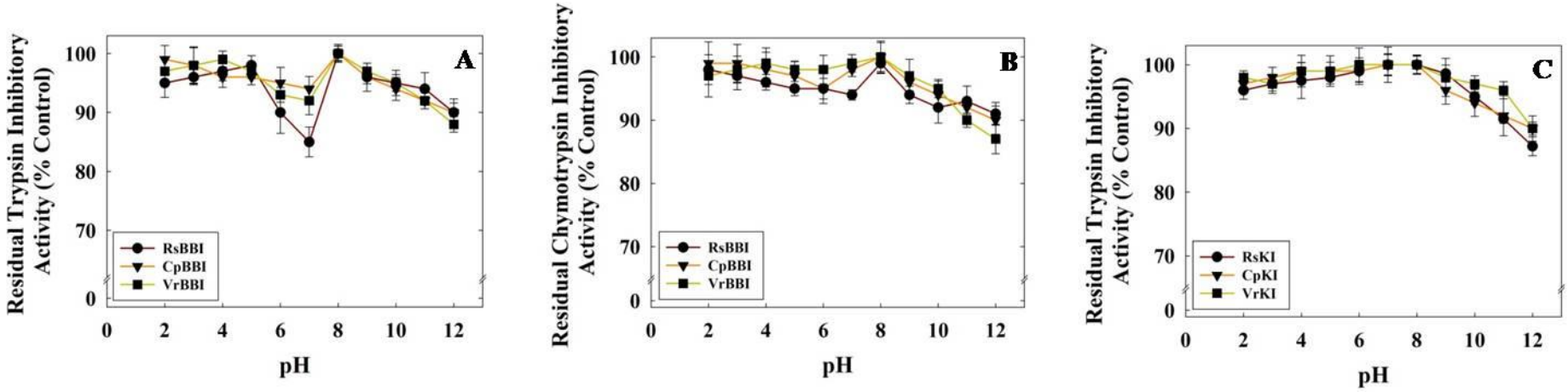


Fig. 4.17. Functional stability of BBI(s) and KI(s) against a wide range of pH (2.0-12.0) conditions. The effect of pH on (A) TI; (B) CI activity of VrBBI/CpBBI/RsBBI and (C) TI activity of VrKI/CpKI/RsKI was determined by incubating the PI sample at described pH for 30 min. The details of the buffer(s) used and the determination of the residual protease inhibitor activity were described in the materials and methods. The data represented is the mean ± SE of three biological replicates.

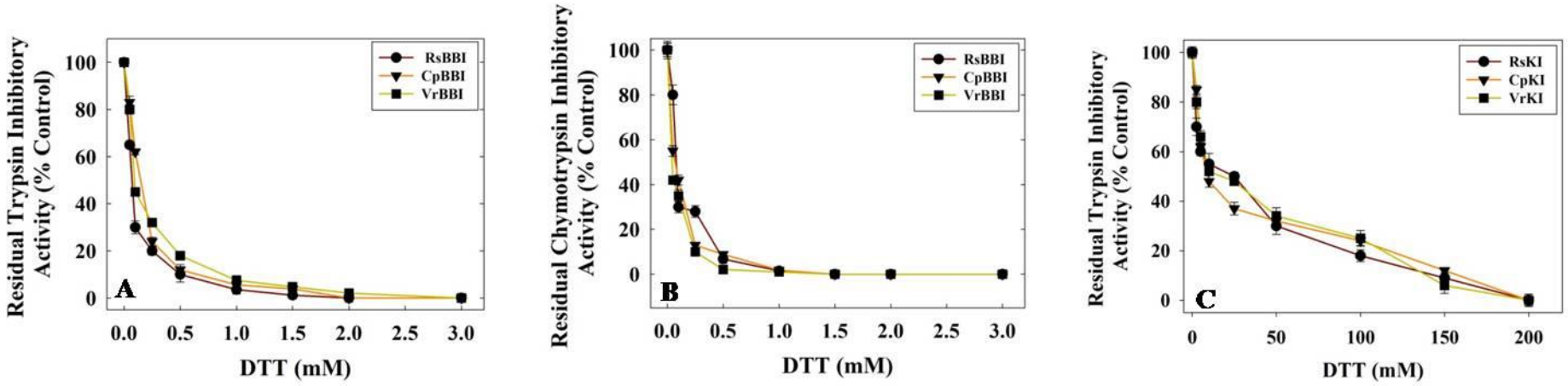


Fig. 4.18. Functional stability of BBI(s) and KI(s) upon treatment with different concentrations of DTT. The effect of DTT on (A) TI; (B) CI activity of VrBBI/CpBBI/RsBBI and (C) TI activity of VrKI/CpKI/RsKI was determined after incubating the PI sample at indicated concentration of DTT for 1 h. The residual protease inhibitor activity was measured as described in the materials and methods. The data represented is the mean ± SE of three biological replicates.

Table. 4.2. The composition of secondary structural elements (α-helix, β-sheets, β-turns, and random coils) in BBI(s) and KI(s). The obtained values are the average of three scans, and the data is analyzed using Spectramanager 2.0 software.

Protein	**α-helix (%)**	**β-sheets (%)**	**β-turns (%)**	**Random coils (%)**
VrBBI	5.9	15.3	19.4	59.4
CpBBI	6.3	19	18.6	56
RsBBI	5.4	20.7	17.8	56.2
VrKI	7.1	7.1	19.2	66.6
CpKI	7.9	11.2	20.3	61.2
RsKI	5.6	11.6	28.3	54.6

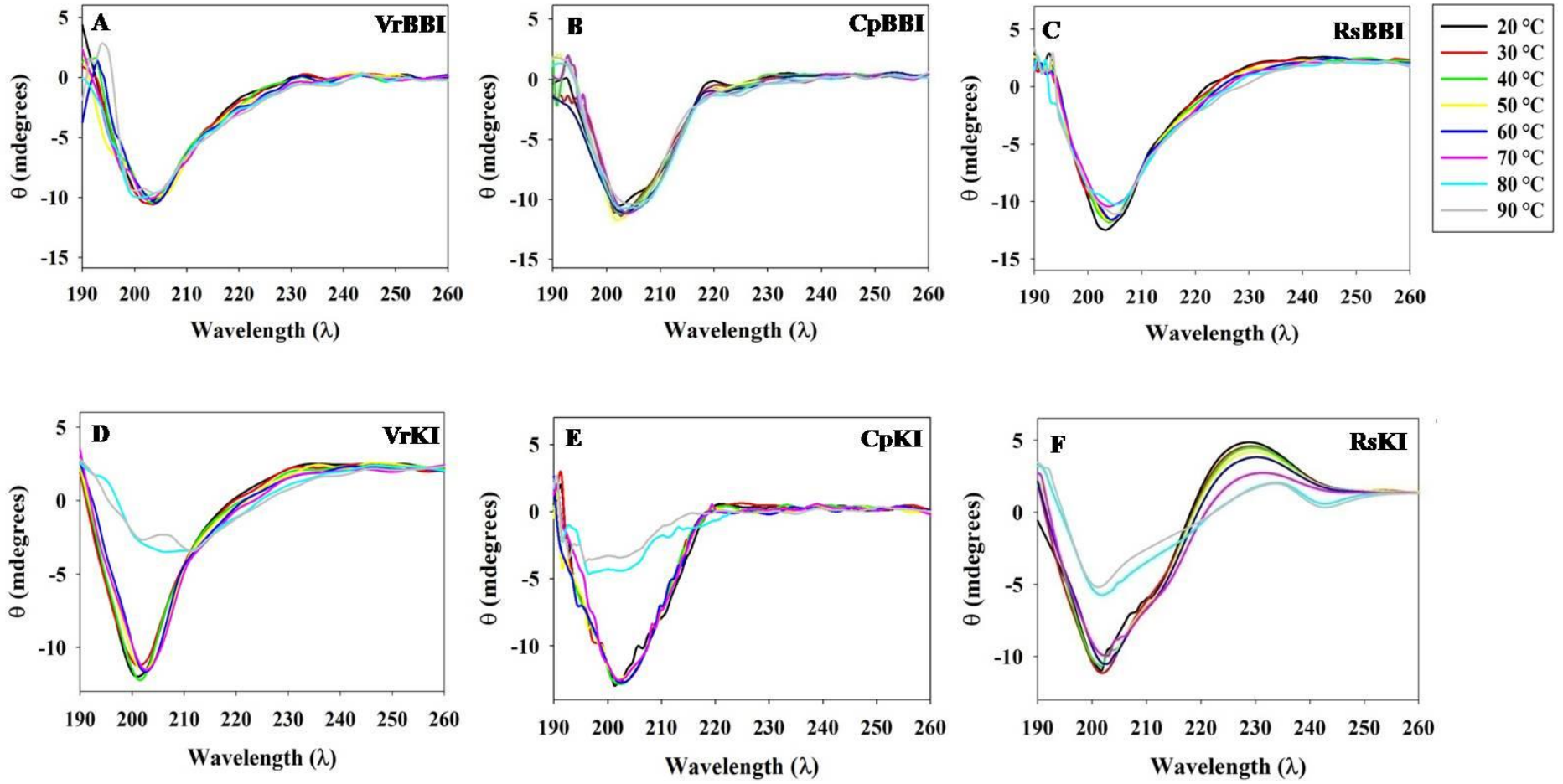


Fig. 4.19. Structural stability of BBI(s) and KI(s) upon temperature treatment. Far-UV (190-260 nm) CD spectra depicting the change in the ellipticity of (A-C) VrBBI/CpBBI/RsKI and (D-F) VrKI/CpKI/RsKI on increasing the temperature from 20 to 90 °C. The final spectrum is an average of three scans, and other details are as described in materials and methods.

drastic decrease in their ellipticity at ~199 nm when heated at (or) above 80 °C (**Fig. 4.19D-F**). Conversely, upon treatment with different concentrations of DTT, VrBBI, CpBBI, and RsBBI showed a significant decrease in their ellipticity, whereas VrKI, CpKI, and RsKI didn't show such variation in their ellipticity (**Fig. 4.20A-F**). These results further confirm the greater stability of VrKI/CpKI/RsKI against DTT as compared to VrBBI/CpBBI/RsBBI.

The protein folding dynamics of BBI and KI was analyzed by fluorescence emission spectra. VrBBI exhibited maximum emission at 350 nm along with a minor shoulder peak at 304 nm, while CpBBI and RsBBI showed maximum emission at 342 nm and did not show any shoulder peak. However, upon heating from 20 to 90 °C, a progressive decrease in fluorescence intensity was observed in VrBBI, CpBBI, and RsBBI (**Fig. 4.21A-C**). In the case of VrKI, CpKI, and RsKI, the maximum emission was recorded at 338, 344, and 342 nm, respectively and the fluorescence intensity decreased gradually when heated from 20 to 90 °C (**Fig. 4.21D-F**). Upon treatment with DTT, VrBBI showed a significant drop in fluorescence intensity after 1 h incubation, CpBBI retained 20-30% of fluorescence intensity even after 12 h incubation, and RsBBI showed a significant drop in intensity after 12 h incubation with DTT. These results indicate that the reduction of disulfide bonds totally quenched fluorescence emission in the case of VrBBI but not CpBBI and RsBBI (**Fig. 4.22A-C**). Conversely, VrKI, CpKI, and RsKI are more stable than VrBBI/CpBBI/RsBBI upon treatment with DTT as evidenced by incubation at different time periods (**Fig. 4.22D-F**).

4.3. Discussion

Development of a novel purification protocol to separate BBI and KI from CPE of leguminous seeds

Protein purification is the fundamental step for analyzing the structure and function of individual proteins. Purification of BBI and KI would be of interest as they possess a wide

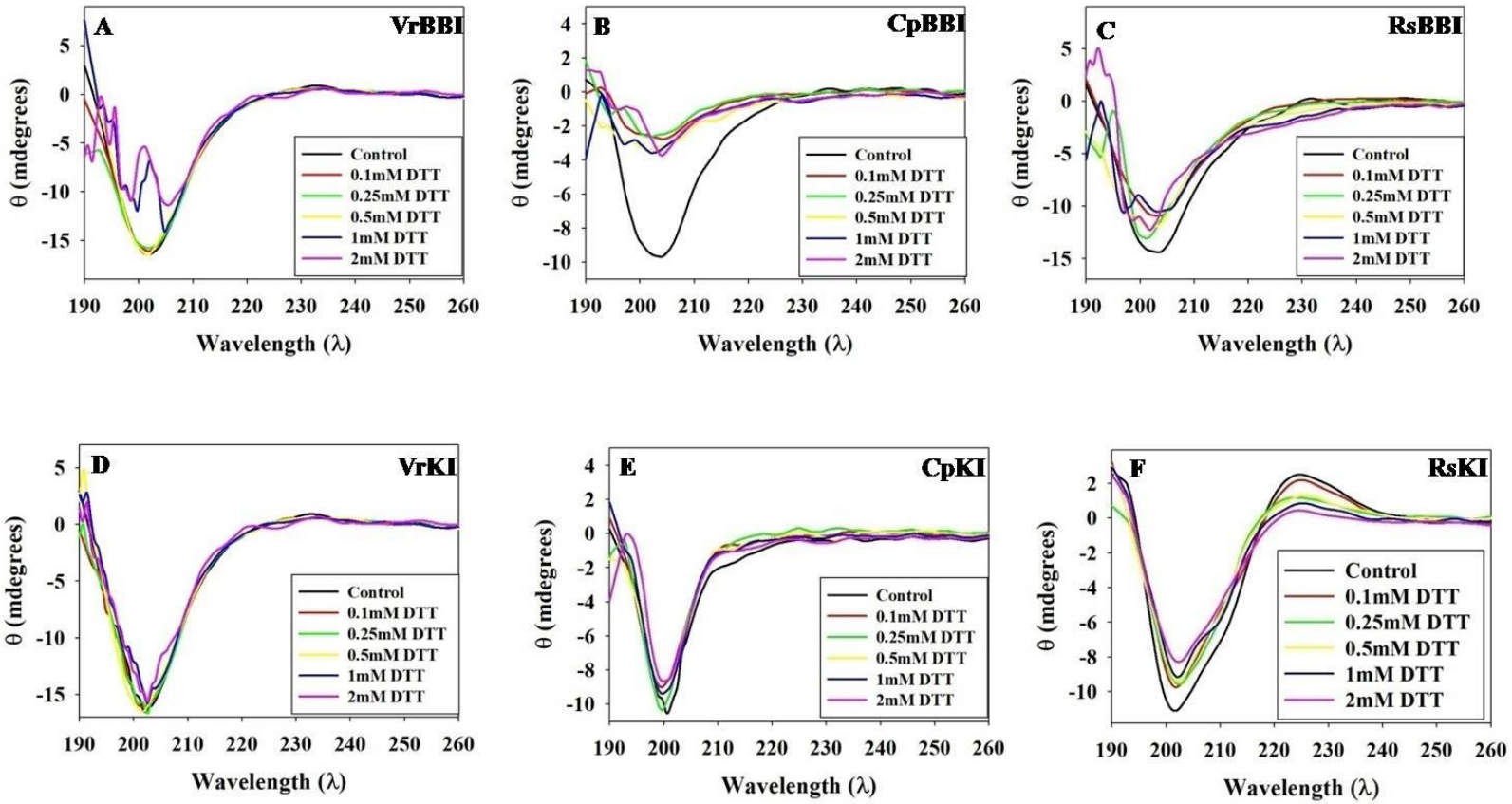


Fig. 4.20. Structural stability of BBI(s) and KI(s) upon DTT treatment. Far-UV CD (190-260 nm) spectra depicting the change in the ellipticity of (A-C) VrBBI/CpBBI/RsKI and (D-F) VrKI/CpKI/RsKI upon treatment with different concentrations of DTT (0.1 to 2 mM). The final spectrum is an average of three scans, and other details are as described in materials and methods.

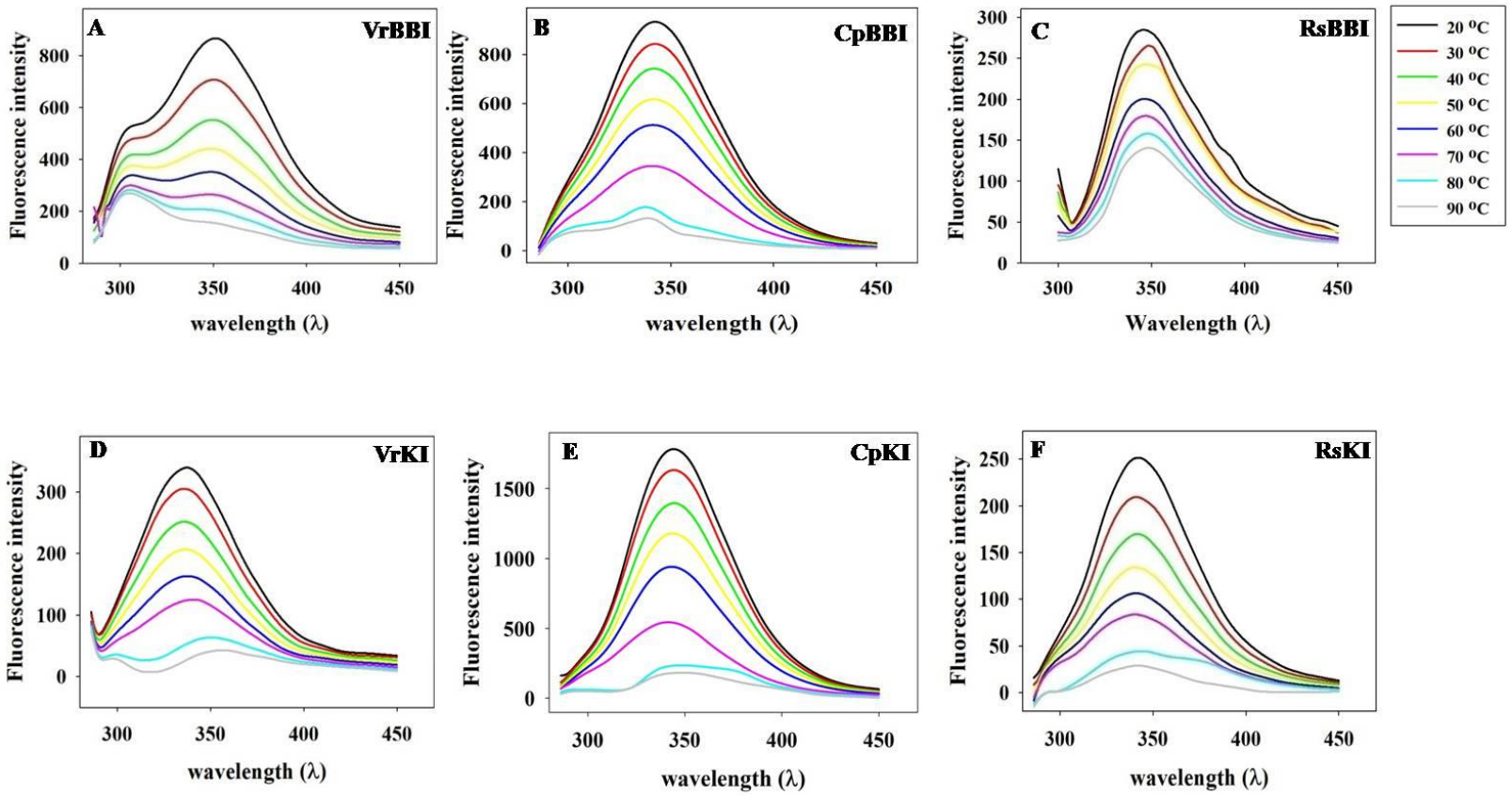


Fig. 4.21. Fluorescence emission spectra of BBI(s) and KI(s). Change in fluorescence intensity of (A-C) VrBBI/CpBBI/RsBBI and (D-F) VrKI/CpKI/RsKI upon increasing the temperature from 25 to 90 ^{o}C. The final spectrum is an average of three scans, and other details are as described in materials and methods.

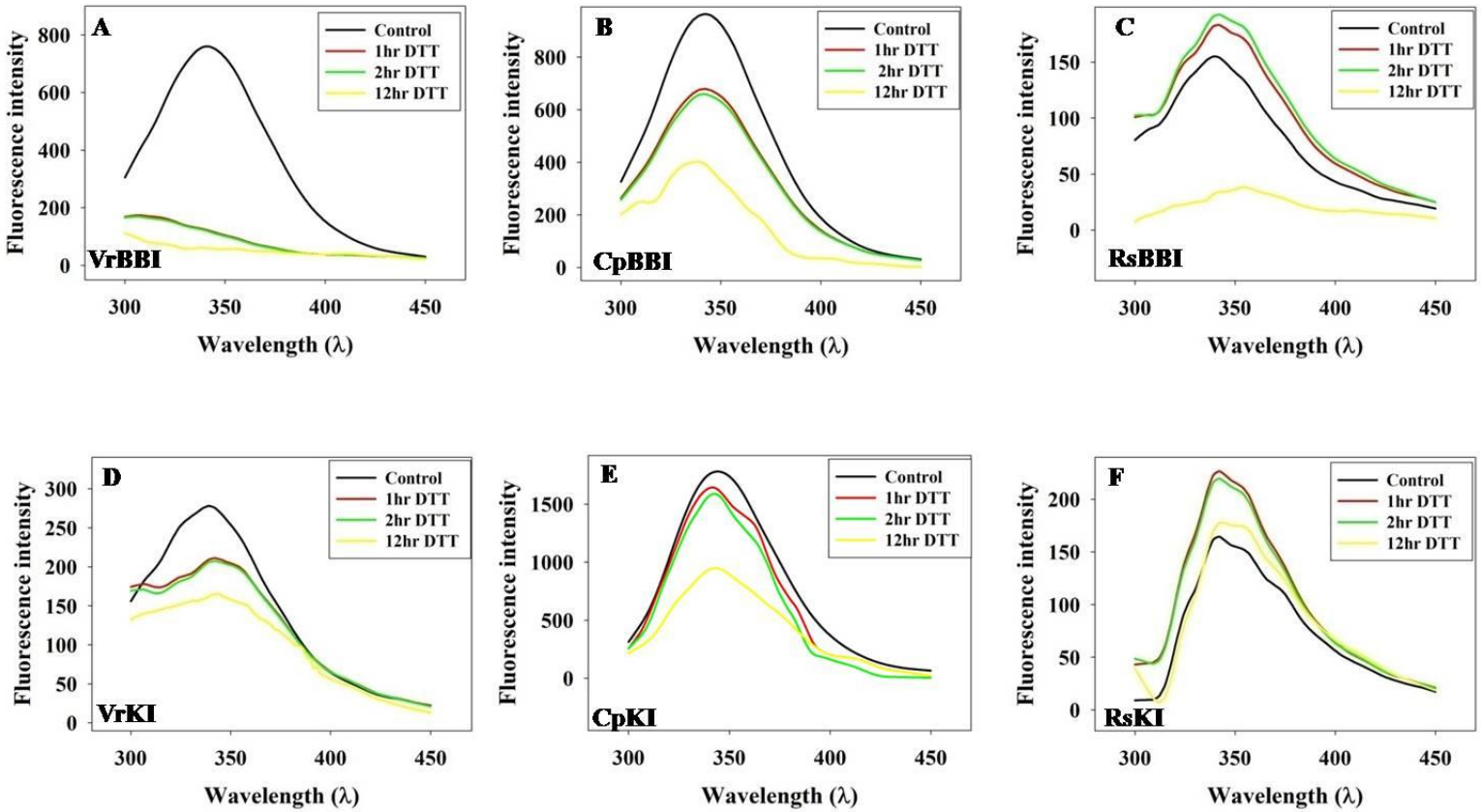


Fig. 4.22. Fluorescence emission spectra of BBI(s) and KI(s). Change in fluorescence intensity of (A-C) VrBBI/CpBBI/RsBBI and (D-F) VrKI/CpKI/RsKI upon treatment with 2 mM DTT for different time periods. The final spectrum is an average of three scans, and other details are as described in materials and methods.

range of properties starting from insecticidal to therapeutic applications and are present in almost all leguminous seeds (Prasad et al., 2010b; Kuhar et al., 2013; Jamal et al., 2015; Clemente et al., 2019; Lokya et al., 2020). Further, the BBI and KI differ (i) minutely in their molecular mass; (ii) in oligomeric pattern; (iii) in the number of reactive sites, and (iv) in the number of disulfide bridges. Hence, different strategies are required to separate and purify them, and it would be beneficial to develop a protocol to recover both BBI and KI from the same starting seed material to save resources. Thus, the present study is focused on the separation and purification of both BBI and KI simultaneously from the CPE prepared from leguminous seeds of *V. radiata*, *C. platycarpus*, and *R. sublobata* using a less laborious and simplified protocol in < 24h as follows: (i) CPE prepared from mature seeds was directly subjected to mild TCA extraction at 70 °C; (ii) the supernatant obtained after TCA extraction was subjected to acetone precipitation and passed through trypsin sepharose 4B column to obtain pure BBI and (iii) the pellet obtained after TCA extraction was passed through trypsin Sepharose 4B column and subsequently subjected to sodium acetate extraction to obtain pure KI (**Figs. 3.1, 4.1, and 4.2**). In the present study, two cycles of TCA extraction were applied to separate BBI and KI (**Fig. 3.1**). However, the number of TCA extraction cycles may be increased based on the amount and ratio of BBI to KI present in a particular seed variety.

The recovery of both BBI and KI from CPE of *V. radiata* and *C. platycarpus* was not yet reported. However, a few studies showed the purification of PIs with varying molecular mass from these seed varieties by using heat treatment, acid precipitation, ammonium sulfate fractionation, ion exchange, affinity, gel filtration chromatography, and reverse phase HPLC techniques (**Table 4.3**). Nevertheless, when compared with the techniques applied in these studies to purify PI, TI, BBI, or KI, the protocol developed in the present study eliminated several purification steps. Further, the specific activity, purity, and yield recovery of BBI or

Table 4.3. A comparative study on the yield recovery, specific activity and purity of PIs isolated from *V. radiata* and *C. platycarpus* seeds using various purification steps.

Seed variety	Purification steps used	Name of the PI purified	Molecular Mass	Yield recovery (%)	Specific activity (TIU/mg protein)	Purification fold	Reference
V. radiata	Crude extract, TCA extraction, affinity chromatography and sodium acetate extraction	BBI	8.2kDa	18	1309	44	Present study Gujjarlapudi et al. (2023)
		KI	19.2kDa	4	778	26	
	Crude extract, acid precipitation, ammonium sulfate fractionation, ion exchange and affinity chromatography	PI	13.6kDa	1.07	2500	12.5	Haq et al. (2005)
	Crude extract, ammonium sulfate precipitation, gel filtration and ion exchange chromatography	PI	10kDa	ND	ND	ND	Wang et al. (2006)
	Crude extract, heat denaturation, ammonium sulfate fractionation, ion exchange and gel filtration chromatography	TI	47kDa	22.8	166.10	58.1	Kansal et al. (2008)
	Crude extract, heat treatment (90^{o} C), ammonium sulfate fractionation and gel filtration chromatography	TI	14kDa	30.25	405.63	13.51	Klomklao et al. (2011)
C. platycarpus	Crude extract, TCA extraction, affinity chromatography sodium acetate extraction	BBI	7.9kDa	14.3	1413	37	Present study Gujjarlapudi et al. (2023)
		KI	19.4kDa	2.83	980	25.5	
	Crude, ammonium sulfate fractionation, ion exchange, affinity and gel filtration chromatography	PI	9.6kDa	6.9	358	21.7	Swathi et al. (2016) (ICPW 63)

ND – Not determined

KI obtained with *V. radiata* and *C. platycarpus* in the present study using the rapid TCA extraction method is much higher when compared with other methods (**Table 4.3**).

Further, purification of both BBI and KI from the same starting seed material has been earlier reported in *R. sublobata* and a few other seed varieties, such as *Clitoria ternatea*, *Canavalia lineata* and *P. tetragonolobus* by using the above-mentioned classical methods of purification (ammonium sulfate fractionation, affinity and gel filtration chromatography) combined with TCA/sodium acetate extraction, preparative native PAGE and isoelectric focusing (**Table 4.4**). As the above-depicted protocol is very time-consuming, the present study took the advantage of using TCA extraction and affinity chromatography to recover both BBI and KI present in single seed variety without compromising their yield recovery, purification fold, and specific activity within short time (**Table 4.4**).

Confirmation of BBI and KI isolated from *V. radiata*, *C. platycarpus*, and *R. sublobata* using biochemical, biophysical, and MALDI-TOF studies

In general, the BBI and KI isolated from various leguminous seeds showed characteristic differences in the following biochemical and biophysical properties: (i) molecular mass; (ii) number of inhibitor sites; (iii) stability towards temperature/pH, and (iv) stability against DTT.

The molecular mass of BBI (8.9 kDa, 7.9 kDa and 9.2 kDa) and KI (19.2 kDa, 19.4 kDa and 19.2 kDa) obtained from *V. radiata*, *C. platycarpus* and *R. sublobata* in the present study (**Fig. 4.4A-F**) matched well with BBIs and KIs purified in previous studies from other leguminous seeds such as *A. hypogaea* (Lokya et al., 2020), *Archidendron ellipticum* (Bhattacharyya et al., 2006), *C. cajan* (Prasad et al., 2010c; Swathi et al., 2014), *Clitoria fairchildiana* (Dantzger et al., 2015), *C. lineata* (Terada et al., 1994), *Lens culinaris* (Ragg et al., 2006), *Piptadenia moniliformis* (Cruz et al., 2013), *Pithecellobium dulce* (Pandey et al., 2015), *P. tetragonolobus* (Giri et al., 2003), *S. surattense* (Herwade et al., 2021), Soybean

Table 4.4. A comparative study on yield recovery, specific activity and purity of both BBI and KI and their isoforms (if any) isolated from different seed varieties using various purification steps.

Seed Variety	Purification steps used	Name of PI purified	Molecular Mass	Yield recovery (%)	Specific activity (TIU/mg protein)	Purification fold	Reference
C. ternatea	Crude extract, ammonium sulfate fractionation, gel filtration, ion exchange and affinity chromatography	BBI-I BBI-II	12kDa 7kDa	ND	ND	ND	Macedo and Xavier (1992)
		KI	20kDa	ND	ND	ND	
C. lineata	Crude extract, heat treatment, acidification, ammonium sulfate, gel filtration, ion exchange, hydroxyapatite and affinity chromatography	BBI-I BBI-II	8kDa 8kDa	19 9	3.35 3.89	ND	Terada et al. (1994)
		KI	20.5kDa	1	1.20	ND	
P. tetragonolobus	Crude extract, heat treatment, gel filtration chromatography and electro-elution from preparative native gels	BBI-I BBI-II BBI-III	9kDa 8kDa 7kDa	ND	16.5	ND	Giri et al. (2003)
		KI-I KI-II KI-III KI-IV	28kDa 24kDa 24kDa 20kDa	ND	26.1	ND	
R. sublobata	Crude extract, ammonium sulfate fractionation, affinity and gel filtration chromatography, followed by TCA and Sodium acetate extraction	BBI	9.2kDa	21.06	1780	50.16	Mohanraj et al. (2019)
		KI	19.4kDa	2.51	837.03	23.59	
	Crude extract, TCA extraction, affinity chromatography and sodium acetate extraction	BBI	9.2kDa	26.0	1980	55	Present study
		KI	19.4kDa	3.03	886	24.5	
V. radiata	Crude extract, TCA extraction, affinity chromatography and sodium acetate extraction	BBI	8.2kDa	18	1309	44	Present study Gujjarlapudi et al. (2023)
		KI	19.2kDa	4	778	26	
C. platycarpus	Crude extract, TCA extraction, affinity chromatography and sodium acetate extraction	BBI	7.9kDa	14.3	1413	37	Present study Gujjarlapudi et al. (2023)
		KI	19.4kDa	2.83	980	25.5	

ND – Not determined

(Zhou et al., 2017), and *V. mungo* (Prasad et al., 2010a). In most cases, the molecular mass of PI was determined either by SDS-PAGE, gel-filtration chromatography, or MALDI-intact mass analysis. The molecular mass of BBI from the same seed species varied marginally between different techniques applied because of their compact structure, ambiguous oligomeric pattern in solution, and differential mobility in SDS-PAGE due to the presence of disulfide bridges (**Figs. 4.2 - 4.4**; Dantzger et al., 2015; Swathi et al., 2016; He et al., 2017).

BBIs are well-known to form highly ordered oligomers which is evident from several seed varieties such as *A. hypogaea* (Lokya et al., 2020), *C. cajan* (Swathi et al., 2014), *Dolichos biflorus* (Kumar et al., 2004; Honda et al., 2018), *Medicago scutellata* (Catalano et al., 2003), *P. sativum* (de la Sierra et al., 1999), and *V. unguiculata* (Rao and Suresh, 2007). In *V. radiata*, *C. platycarpus*, and *R. sublobata*, BBI existed as dimeric forms (**Figs. 4.3 and 4.4**). The hydrophobic contacts and ion pair interactions might contribute to the observed oligomeric pattern of BBI (Koepke et al., 2000; Barbosa et al., 2007). Also, this self-association tendency of BBI facilitates its tight packing in seeds and thereby maintenance of its physiological function as a plant storage protein (Gitlin-Domagalska et al., 2020). Further, the BBI and KI purified in the present study from *V. radiata*, *C. platycarpus*, and *R. sublobata* are confirmed by probing with an anti-BBI antibody and MALDI-TOF studies (**Figs. 4.5 - 4.11**). The partial sequences obtained after tryptic digestion of VrBBI, CpBBI & RsBBI as well as VrKI, CpKI & RsKI during MALDI-TOF studies showed significant matching with the BBI and KI of legume seeds in the NCBI database.

BBIs from dicot plants comprise a binary arrangement of two inhibitory reactive sites, hence, can interact independently with two proteases (Birk et al., 1985; Qi et al., 2005). Whereas KIs contain a single reactive site and can interact with only one protease (Oliva et al., 2010; Bendre et al., 2018). The *in vitro* inhibition assays and in-gel activity staining studies from the present study revealed that VrBBI, CpBBI, and RsBBI possessed two reactive sites

as evidenced by significant inhibitory potential against trypsin and chymotrypsin, whereas VrKI, CpKI, and RsKI possessed inhibitory potential against trypsin but not chymotrypsin confirming the presence of a single reactive site (**Fig. 4.12A-D**). Also, the inhibition kinetics studies revealed that VrBBI/VrKI, CpBBI/CpKI, and RsBBI/RsKI bind strongly to trypsin as compared to chymotrypsin (**Figs. 4.13 - 4.15**). This property was reported for BBIs from several seed varieties such as *C. cajan* (Prasad et al., 2010c), *G. soja* (Deshimaru et al., 2002), *Lens culinaris* (Ragg et al., 2006) and *V. mungo* (Prasad et al., 2010a). Further, the crystal structure (PDB ID: 3myw) of *V. radiata* BBI with porcine trypsin showed hydrogen bond interactions that were similar to those identified between trypsin-BTCI chymotrypsin complex (PDB ID: 3RU4) and *S. surattense*-bovine trypsin (Honda et al., 2018; Herwade et al., 2022). Since bovine trypsin and porcine trypsin share 82% identity in their coding sequences, VrBBI and bovine trypsin may possess similar E-EI interactions as described in Honda et al. (2018) and Herwade et al. (2022).

BBIs are familiar for possessing high cysteine content and thereby disulfide bridges which offer them greater structural and functional stability against a wide range of pH and temperatures (Bateman and James, 2011). The TI and CI activities of BBIs are stable towards a wide range (20-90 °C) of temperatures (**Fig. 4.16A and B**). The TI and CI activities of VrBBI, CpBBI & RsBBI as well as TI activity of VrKI, CpKI & RsKI showed significant stability from pH 2.0 to 12.0 (**Fig. 4.17A-C**). In contrast, the TI activity of KIs, which was stable up to 70 °C was lost significantly when heated at 80 °C (**Fig. 4.16C**). This loss in TI activity of KI at temperatures above 80 ^{o}C is aligned with loss in its ellipticity at 202 nm in the corresponding CD spectra when heated above 80 ^{o}C (**Fig. 4.19D-F**). Further, the fluorescence emission spectra indicated that VrKI, CpKI or RsKI undergo marginal changes in their hydrophobic core surrounding tryptophan residues at high temperatures, i.e., 80 ^{o}C (**Fig. 4.21D-F**).

Proteins generally consist of intrinsic fluorophores, including aromatic amino acids such as tyrosine, tryptophan, and phenylalanine. BBIs in some species are known to be devoid of tryptophan residues. The observed changes in the fluorescence emission spectra of VrBBI/CpBBI/RsBBI after treatment with temperature or DTT could be due to the contribution of tyrosine residues. Further, the manifestation of λ_{max} at a wavelength >340 nm in VrBBI/CpBBI/RsBBI indicates the ionization of tyrosine to tyrosinate (**Fig. 4.21A-C**). Such tyrosinate emission is reported in several BBIs and other proteins lacking tryptophan residue in their structure (Szabo et al., 1978; Jordano et al., 1983; Kumar and Gowda, 2013).

The presence of disulfide bridges plays a significant role in maintaining the structure of a protein, which in turn has a substantial implication on its function. In the present study, the effect of the reduction of cysteine residues present in BBI and KI from *V. radiata*, *C. platycarpus* and *R. sublobata* with DTT correlated to their structural and functional stability (**Figs. 4.18A-C, 4.20A-F and 4.22A-F**). The changes in the fluorescence emission spectra suggest that VrBBI has undergone significant conformational changes as compared to CpBBI and RsBBI in the hydrophobic region closer to tyrosine residues, irrespective of treatment with DTT for 1 h or 12 h (**Fig. 4.22A-C**). Further, the conformational changes observed after DTT treatment in VrBBI/CpBBI/RsBBI are also reflected in their corresponding CD spectra and TI/CI activity (**Figs. 4.18A & B, and 4.20A-C**; Krishnan and Murugan, 2015). A total loss in TI and CI activities of VrBBI/CpBBI/RsBBI was observed after treatment with 1 mM DTT (**Fig. 4.18A and B**). However, the loss in the ellipticity of CpBBI was much more than that of VrBBI and RsBBI at 202 nm (**Fig. 4.20A-C**).

In contrast to BBIs, the KIs are much more stable to DTT treatment. The VrKI/CpKI/RsKI did not show any loss in their TI activity at 1 mM DTT. But, they lost ≥50% of their activity at 50 mM DTT (**Fig. 4.18C**). Corroborating with these *in vitro* studies, VrKI, CpKI, and RsKI did not show any significant changes in the ellipticity of their CD spectra

even at 2 mM DTT concentration (**Fig. 4.20D-F**). However, the fluorescence emission spectra varied between VrKI, CpKI, and RsKI, possibly due to differences in protein folding in their hydrophobic core closer to tryptophan residues (**Fig. 4.22D-F**).

Thus, a decrease in fluorescence intensity observed during (i) denaturation of KIs (VrKI, CpKI & RsKI) at high temperatures and (ii) reduction of disulfide bonds in BBIs (VrBBI, CpBBI & RsBBI) upon DTT treatment is attributed to changes in the conformation of these proteins. These results are in agreement with fluorescence studies of KI from *Albizzia kalkora* (Zhou et al., 2008), *C. cajan* (Haq and Khan, 2003) and *Trigonella foenum-graecum* (Pallavi and Rajender, 2021), and BBI from *D. biflorus* (Ramasarma et al., 1995).

(P.T.O)

Highlights of the study

- ✓ A rapid (<24h) protocol was developed to separate and purify both BBI & KI from crude protein extracts of *V. radiata*, *C. platycarpus* and *R. sublobata* seeds by mild TCA extraction and trypsin affinity chromatography.
- ✓ The purified BBI (VrBBI/CpBBI/RsBBI) and KI (VrKI/CpKI/RsKI) are confirmed by immunodetection and MALDI-TOF studies.
- ✓ The purified BBI and KI are validated by their differential stability towards temperature and DTT as evidenced by both biochemical and biophysical (CD and fluorescence spectroscopy) studies as follows:

Property	BBI	KI
Molecular weight	VrBBI - 8.92 kDa CpBBI - 7.92 kDa RsBBI - 9.21 kDa	VrKI - 19.25 kDa CpKI - 19.48 kDa RsKI - 19.28 kDa
Inhibitory activity	Both trypsin and chymotrypsin inhibition	Only trypsin inhibition
Confirmation	Western blotting and MALDI MS-MS analysis	MALDI MS-MS analysis
Functional stability ➢ **Temperature** ➢ **DTT**	• Stable from 20-100 ^{o}C • Unstable - 100% loss at 0.1 mM	• Unstable from 80 ^{o}C • Stable up to 25 mM
Structural stability ➢ **Temperature** ➢ **DTT**	• Marginal change in ellipticity at 202 nm • Major change in ellipticity	• Major change in ellipticity at 199 nm above 70 ^{o}C • No change in ellipticity
Protein dynamics ➢ **Temperature** ➢ **DTT**	• Drop in fluorescence intensity • Confirmational changes occured	• Partial unfolding at 80 ^{o}C • Drop in fluorescence intensity

Chapter 5

Evaluation of insecticidal, anticancer and antibacterial properties of BBI and KI purified from the seeds of *V. radiata*, *C. platycarpus* and *R. sublobata*

Evaluation of insecticidal, anticancer and antibacterial properties of BBI and KI purified from the seeds of *V. radiata, C. platycarpus,* and *R. sublobata*

5.1. Introduction

PIs gained significant importance in both agriculture and pharma industry as they possess several biological applications such as insecticidal, anticancer, anticoagulant, antiobesity, antimicrobial, and anti-inflammatory properties (Shamsi et al., 2016; Srikanth and Chen, 2016; Araujo et al., 2019; Singh et al., 2020; Cotabarren et al., 2020). The preliminary action of PIs in controlling insect pests or mammalian diseases is by inhibiting the proteases (trypsin, chymotrypsin, pepsin, cathepsin, kallikrein, papain, carboxypeptidase A and B), which play a crucial role in the survival of insect pests and progression of many human diseases such as cancer, HIV, Alzheimer's, cardiovascular and rheumatoid arthritis (Turk et al., 2008; Rakashanda et al., 2013; Bacha et al., 2017; Clemente et al., 2019).

PIs are reported to possess insecticidal properties against several lepidopteran pests, such as castor semi-looper *A. janata* (Swathi et al., 2014), sorghum stem borer *C. partellus* (Panchal and Kachole, 2016), pod borer *H. armigera* (Swathi et al., 2015, Banerjee et al., 2017), tobacco hornworm *M. sexta* (Kessler et al., 2006), fall armyworm *S. frugiperda* (Ramalho et al., 2018), and cotton leafworm *S. litura* (Vasudev and Sohal, 2019). They are known to block the digestive proteases of insects imparting anti-nutritional effects and thereby hinder several vital processes, including growth and development. As the major proteases of these insect midguts are reported to be of serine-type, the effect of serine protease inhibitors such as BBI and KI has been tested on the growth and development of these insect pests by antifeedant assays and transgenic studies (Falco and Silva-Filho, 2003; Abdeen et al., 2005; Quilis et al., 2007; Luo et al., 2009; Srinivasan et al., 2009; Prasad et al., 2010b; Quilis et al., 2014; Cingel et al., 2015).

During the course of evolution, both plant PIs and insect proteases co-evolved. However, insects frequently tend to escape the effects of PIs by either over-expressing existing proteases or expressing new alternative proteases for their survival (Gruden et al., 1998; Dunse et al., 2010a, b; Zhu-Salzman and Zeng, 2015; Mitchell et al., 2016). To overcome this scenario, it is essential to exploit PIs from non-host plants and wild varieties since the insect midgut environment has not been adapted to synthesize digestive enzymes against the PIs from these plant resources (Chougule et al., 2003; Parde et al., 2010). The studies by Swathi et al. (2012) have reported excellent insecticidal effects of PIs from non-host plants (both cultivars and wild accessions of pigeon pea) against *A. janata*. Similarly, the PIs reported from non-host plants of *H. armigera*, such as *P. tetragonolobus*, *S. tuberosum* and *A. hypogaea*, showed significant inhibitory effect on its midgut proteases when compared with PIs from host plants such as *Cicer arietinum, C. cajan* and *Gossypium arboreum* (Harsulkar et al., 1999). Thus, continuous screening of non-host plants or wild relatives helps to identify PIs suitable for the management of larval forms of insect pests that voraciously feed on aerial parts (leaves, pods, and seeds) of plants.

The studies of Mohanraj et al. (2019) demonstrated that the BBI purified from *R. sublobata*, a wild relative of pigeon pea showed significant *in vitro* inhibitory activity against midgut trypsin-like proteases of *A. janata* (AjTPs), while KI purified from the same variety showed potent *in vitro* inhibitory activity against midgut trypsin-like proteases of *H. armigera* (HaTPs). This variation in the activity of BBI and KI isolated from the same seed variety, i.e., *R. sublobata* against gut proteases of specific lepidopteran larvae, indicates the significance of each of these PIs in pest management.

H. armigera does not infest the wild relatives of pigeon pea: *C. platycarpus* and *R. sublobata*. Hence, they are considered as non-host plants to *H. armigera*. In contrast, *H. armigera* infests *V. radiata*. Hence it is regarded as a host plant to *H. armigera*. However,

A. janata can not infest all these three plants. Hence, they are considered as non-host plants to *A. janata* (**Fig. 5.1**). Thus, the BBI(s) and KI(s) purified from these seed varieties were examined for their insecticidal activity against *A. janata* and *H. armigera* by *in vitro* inhibition assays and *in vivo* feeding experiments.

Globally, the burden of cancer incidence and mortality is steadily increasing. It is anticipated that there will be 28.4 million cancer cases worldwide by 2040, which reflects a 47% increase as compared to 19.3 million cases that existed in 2020 (Sung et al., 2021). More than 60% of the cancer drugs (for example, vinblastine, vincristine, etoposide, paclitaxel, topotecan and docetaxel) are derived from natural sources like plants (Cragg et al., 2012; Newman and Cragg, 2012; Cragg and Newman, 2018). Apart from the above-mentioned natural anticancer drugs, plants also possess PIs with anticancer activities. PIs are known to inhibit serine and metallo proteases, which are regarded as the most important proteins involved in cell migration, invasion, angiogenesis, metastasis, and growth of tumors (Murphy and Gavvrilovic, 1999; Rudek et al., 2002). Hence, PIs are considered as potential therapeutic compounds for treating different stages of several human cancers (Ferreira et al., 2013; Souza et al., 2014; Mehbad et al., 2016).

Besides, PIs are also reported to have antimicrobial properties. Serine PIs such as BBI and KI isolated from *Acacia nilotica*, *C. grandis*, *J. curcas* and *Luetzelburgia auriculata* effectively controlled the growth of several gram-positive and gram-negative bacteria such as *Salmonella typhimurium*, *E. coli* and *S. aureus* (Kim et al., 2005; Costa et al., 2014; Martins et al., 2018; Karray et al., 2020). The antimicrobial and anticancer properties of the PIs isolated from the seed materials used in the current study were not revealed so far. Therefore, an attempt was made to evaluate the anticancer and antimicrobial properties along with the insecticidal properties of the BBI and KI purified from *V. radiata*, *C. platycarpus*, and *R. sublobata* seeds.

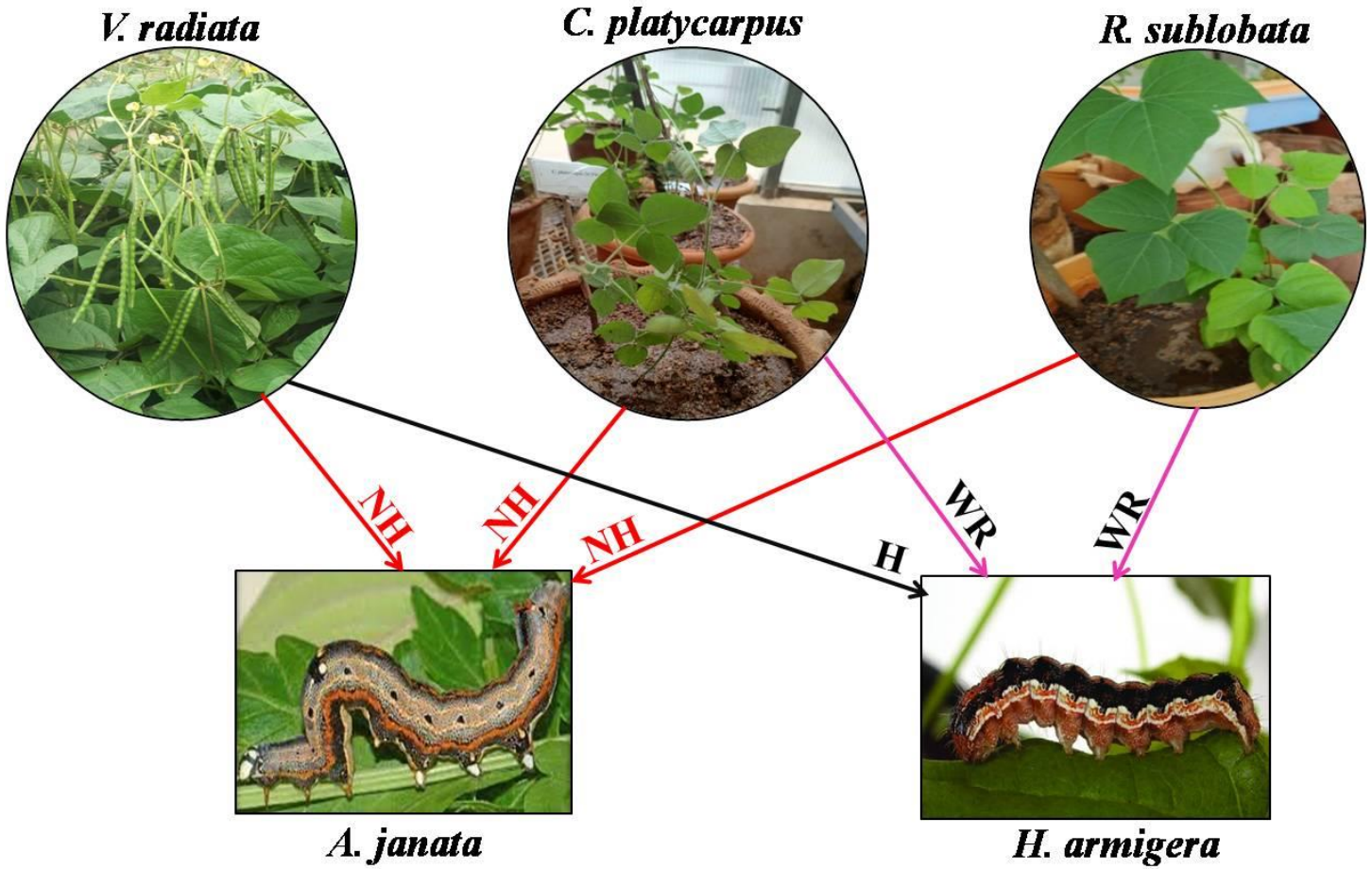


Fig. 5.1. Different plants (seed material) used in the present study to purify BBI and KI and their interaction with *A. janata* and *H. armigera*. The red color arrows indicate non-host (NH) plants of *A. janata*. The black color arrow indicates the host-plant (H) of *H. armigera*. The pink color arrows indicate wild-relatives (WR; non-host) of *H. armigera*.

5.2. Results

Inhibitory activity of BBI and KI from *V. radiata, C. platycarpus* and *R. sublobata* against AjTPs and HaTPs

The inhibitory potential of the purified BBI(s) (VrBBI/CpBBI/RsBBI) and KI(s) (VrKI/CpKI/RsKI) varied significantly against AjTPs and HaTPs. The VrBBI, CpBBI, RsBBI, VrKI, CpKI and RsKI showed a specific activity of 21,200 ± 521, 25,560 ± 652, 33,940 ± 985, 1548 ± 118, 683 ± 57 and 478 ± 45 AjTPI units/mg protein against AjTPs and 458 ± 41, 922 ± 73, 215.5 ± 15, 18,520 ± 345, 26,240 ± 665 and 21,360 ± 758 HaTPI units/mg protein against HaTPs, respectively (**Fig. 5.2A and B**).

Further, BBIs exhibited potent inhibitory activity against AjTPs up to an extent of 86% with very low IC_{50} values (VrBBI - 96ng, CpBBI - 84ng and RsBBI - 22ng) as compared to IC_{50} values of KIs (VrKI - 4.2µg, CpKI - 2.6µg and RsKI - 3.5µg) which inhibited the activity of AjTPs upto an extent of 85% (**Fig. 5.3A-F**). Conversely, KIs inhibited HaTPs up to an extent of 80% with low IC_{50} values (VrKI - 150ng, CpKI - 80ng and RsKI - 42ng) as compared to IC_{50} values of BBIs (VrBBI - 6.0µg, CpBBI - 4.2µg and RsBBI - 7.5µg) which inhibited the activity of HaTPs upto an extent of 84% (**Fig. 5.4A-F**). These results demonstrated that BBIs purified from *V. radiata*, *C. platycarpus* and *R. sublobata* seeds are specific and possess potential inhibitory activity toward AjTPs. In contrast, KIs are specific and possess potential inhibitory activity towards HaTPs.

Insecticidal properties of BBI and KI from *V. radiata, C. platycarpus,* and *R. sublobata* against *A. janata* and *H. armigera*

As VrBBI/CpBBI/RsBBI and VrKI/CpKI/RsKI showed variation in their specific activity and IC_{50} values against the AjTPs and HaTPs, the effect of BBI(s)/KI(s) on different developmental (instar) stages of *A. janata* and *H. armigera* was evaluated by performing *in*

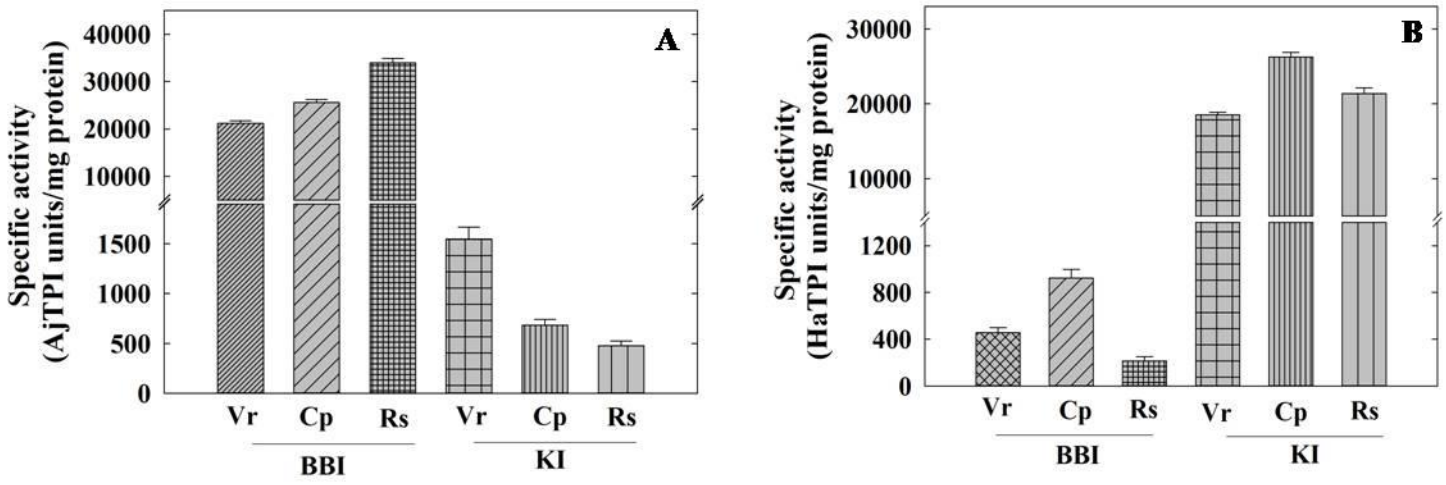


Fig. 5.2. *In vitro* inhibitory potential of BBI(s) and KI(s) against midgut trypsin-like proteases of *A. janata* and *H. armigera*. The specific activity of VrBBI/CpBBI/RsBBI and VrKI/CpKI/RsKI against (A) AjTPs and (B) HaTPs. The data represented is the mean ± SE of three biological replicates.

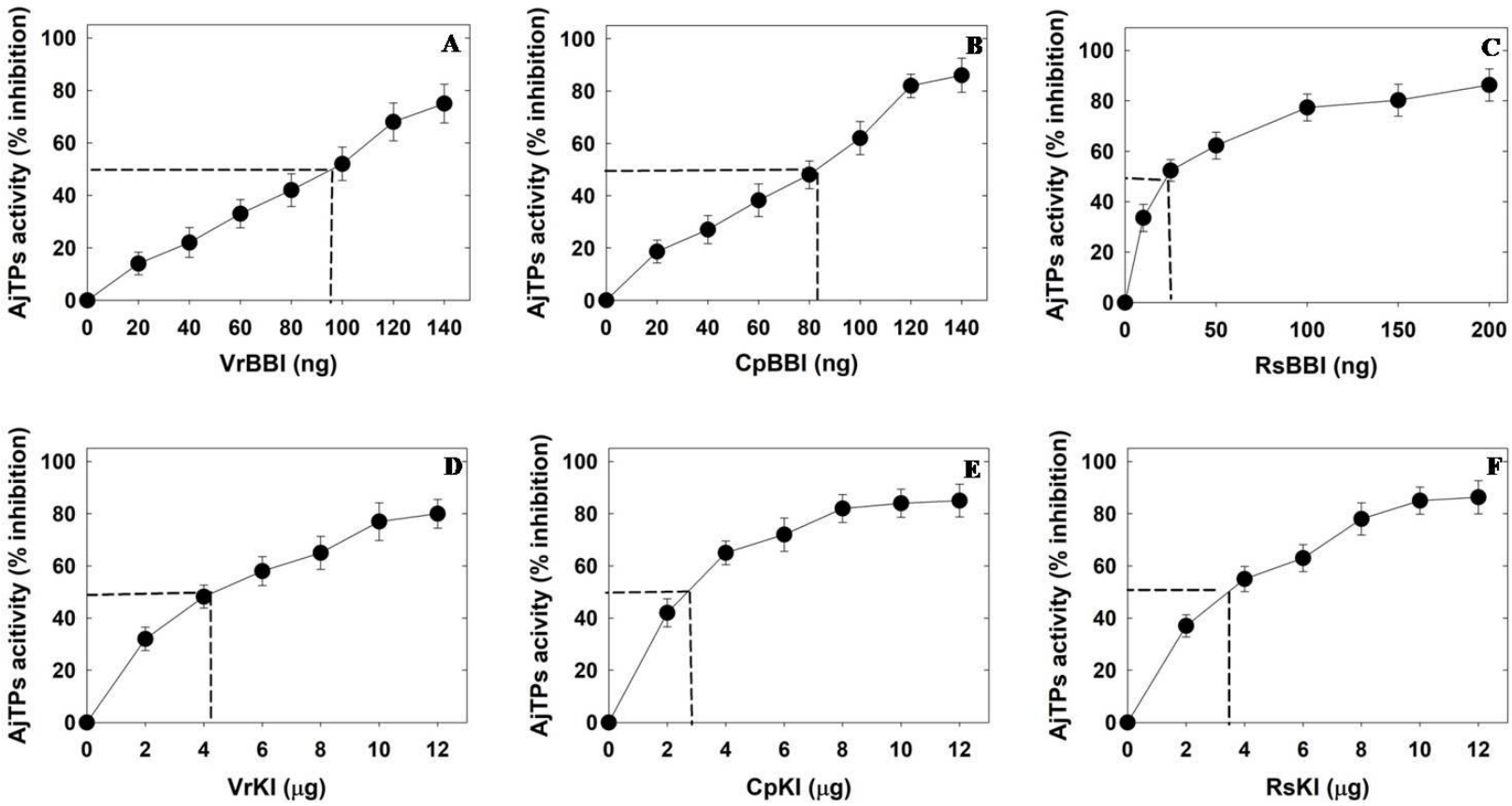


Fig. 5.3. *In vitro* Inhibitory potential of BBI(s) and KI(s) against the midgut trypsin-like proteases of *A. janata* larvae. Half-maximal inhibitory concentration (IC_{50}) of VrBBI/CpBBI/RsBBI (A-C) and VrKI/CpKI/RsKI (D-F) against AjTPs. The data represented is the mean ± SE of three biological replicates.

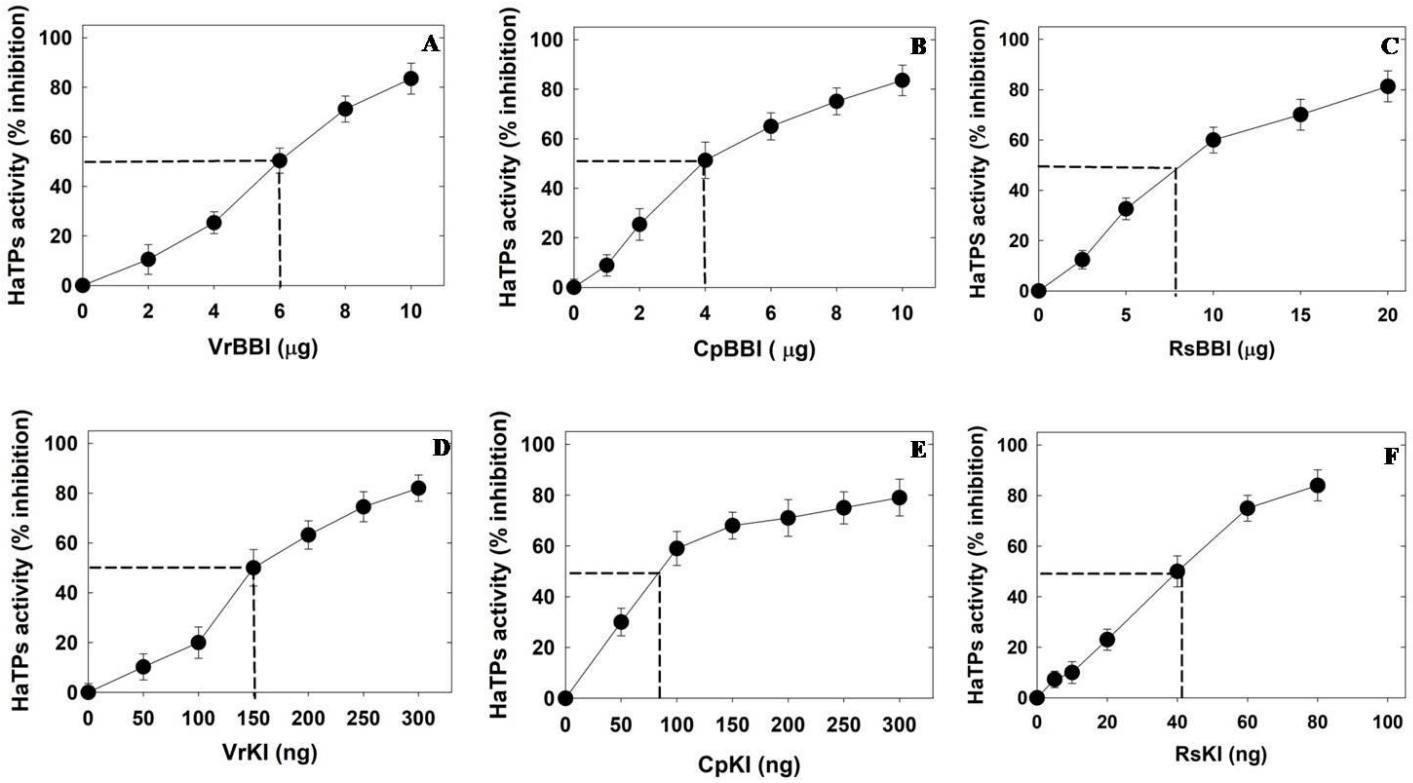


Fig. 5.4. *In vitro* inhibitory potential of BBI(s) and KI(s) on midgut trypsin-like proteases of *H. armigera* larvae. Half-maximal inhibitory concentration (IC_{50}) of VrBBI/CpBBI/RsBBI (A-C) and VrKI/CpKI/RsKI (D-F) against HaTPs. The data represented is the mean ± SE of three biological replicates.

vivo feeding experiments. Weights of control and BBI(s)/KI(s) supplemented larvae were taken after each instar stage (second, third, fourth and fifth) and analyzed for their weight reduction.

The *A. janata* larvae fed on leaves coated with BBI(s)/KI(s) showed variation in their weight reduction at each instar stage in a concentration (1, 2, 4 and 8 µg/cm^2) dependent manner as compared to larvae fed on control diet, i.e. leaves coated with buffer (**Fig. 5.5A-F**). Upon feeding with VrBBI/CpBBI/RsBBI, for example, at 8 µg/cm^2, the second instar larvae showed ≤82% reduction in their weight, whereas the VrKI/CpKI/RsKI (8 µg/cm^2) supplemented larvae showed ≤29% weight reduction as compared to control larvae. Further, in the third instar stage, the VrBBI/CpBBI/RsBBI supplemented *A. janata* larvae showed ≤76% reduction in their weight, and VrKI/CpKI/RsKI fed larvae showed ≤28% reduction in their weight as compared to control larvae. With reference to the fourth instar stage, the VrBBI/CpBBI/RsBBI fed larvae lost ≤82% of their weight, whereas VrKI/CpKI/RsKI fed larvae showed ≤37% reduction in their larval weight with reference to control larvae. Also, the fifth instar *A. janata* larvae showed ≤80% reduction in their larval weight upon feeding with VrBBI/CpBBI/RsBBI when compared with control larvae. Besides, the fifth instar *A. janata* larvae fed upon castor leaves coated with VrKI/CpKI/RsKI exhibited ≤33% reduction in their body weight as compared to control larvae. However, the overall effect of BBI(s)/KI(s) on weight reduction of *A. janata* larvae did not vary much on different instar stages upon treatment with 8 µg/cm^2 concentration (**Fig. 5.5A-F**). The pictorial representation of reduction in body weight of different instar stages of *A. janata* larvae after treatment with 8 µg/cm^2 concentration of BBI (VrBBI/CpBBI/RsBBI) or KI (VrKI/CpKI/RsKI) is shown in **figures. 5.6A, B and 5.7A, B.**

Besides, feeding of *A. janata* larvae with VrBBI/CpBBI/RsBBI (8 µg/cm^2) also resulted in ≤64% reduction in their corresponding pupal weights as compared to control pupae (**Fig. 5.8A**).

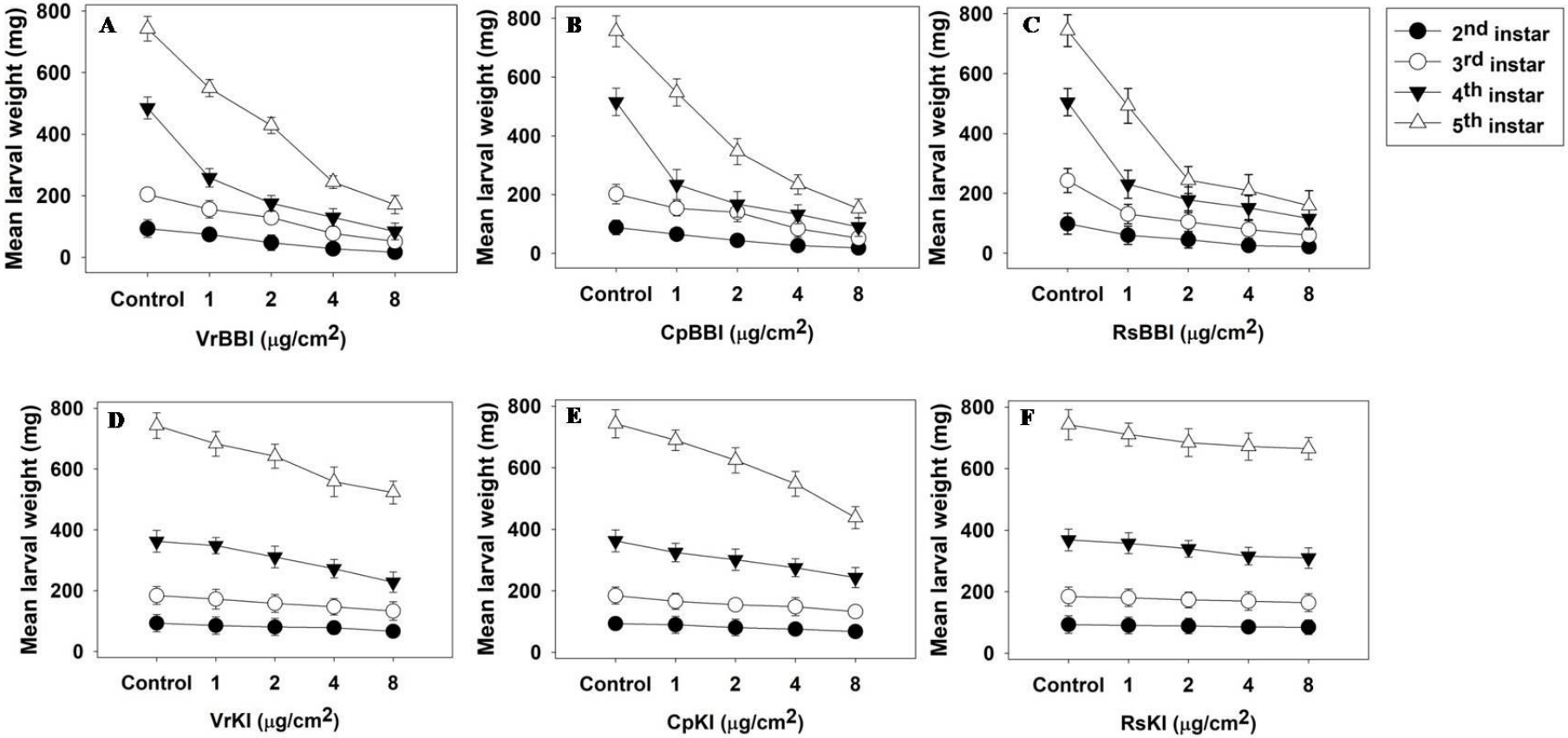


Fig. 5.5. The effect of purified BBI(s)/KI(s) from *V. radiata*, *C. platycarpus* and *R. sublobata* on the larval weight at second, third, fourth, and fifth instar stages of *A. janata* upon feeding with different concentrations (1, 2, 4 and 8 µg/cm^2) of (A) VrBBI; (B) CpBBI; (C) RsBBI; (D) VrKI; (E) CpKI and (F) RsKI, respectively. The data represented is the mean ± SE of three biological replicates. Other details are as described in materials and methods.

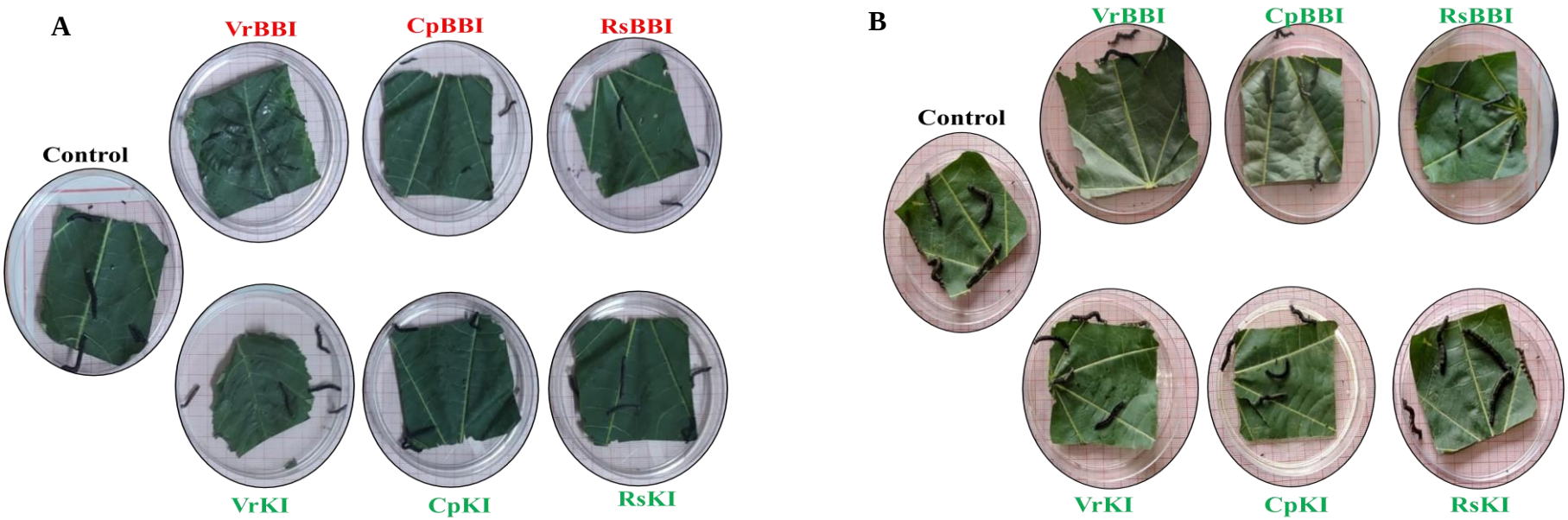


Fig. 5.6. Effect of VrBBI/CpBBI/RsBBI and VrKI/CpKI/RsKI on the larval growth of *A. janata*. Pictorial representation of the effect of feeding the castor leaves coated with BBIs/KIs (8 µg/cm^2) on (A) second and (B) third instar larvae of *A. janata*. Control leaves were coated with Tris-HCl (50 mM, pH 8.0). Other details are as described in materials and methods.

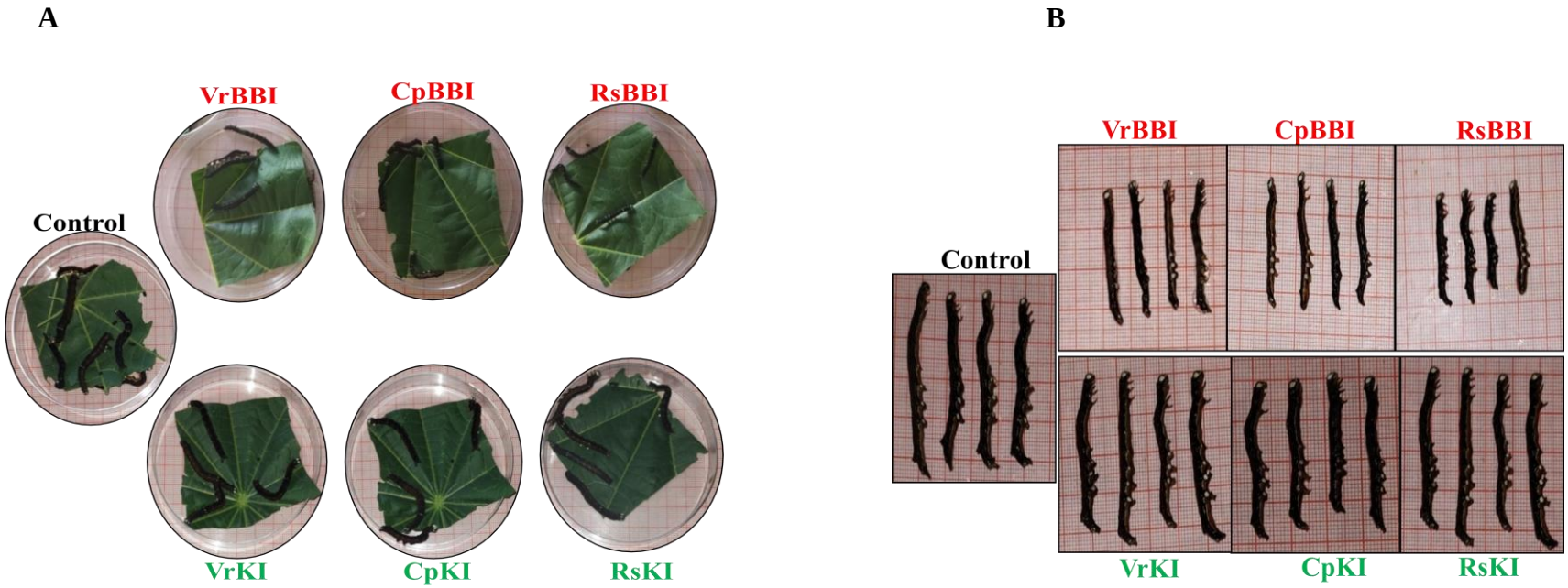


Fig. 5.7. Effect of VrBBI/CpBBI/RsBBI and VrKI/CpKI/RsKI on the larval growth of *A. janata*. Pictorial representation of the effect of feeding the castor leaves coated with BBIs/KIs (8 µg/cm^2) on (A) fourth and (B) fifth instar larvae of *A. janata*. Control leaves were coated with Tris-HCl (50 mM, pH 8.0). Other details are as described in materials and methods.

The pictorial representation in the reduction of pupal weights was shown in **figure. 5.8B**. Further, a delay in pupal emergence (10-12 days), formation of larval-pupal (≤50%), as well as pupal-adult intermediates (≤25%) was observed (**Fig. 5.8B and C; Table 5.1**). Apart, ≤35% mortality rate was observed in *A. janata* larvae upon feeding with VrBBI, CpBBI or RsBBI coated leaves (**Table 5.1**). In the larvae fed upon VrKI, CpKI or RsKI neither pupal weight reduction nor mortality was observed, but it resulted in growth deformities like the formation of larval-pupal intermediates but not pupal-adult intermediate formation (**Fig. 5.8B**). The defective pupae and adults formed after feeding with VrBBI, CpBBI, RsBBI or CpKI are indicated by dotted circles in the **figure. 5.8B and C**.

Similarly, the effect of BBI(s)/KI(s) on different developmental (instar) stages of *H. armigera* was examined by supplementing the synthetic diet with them at different concentrations (0.01, 0.025 and 0.05%). The *H. armigera* larvae fed on a diet supplemented with BBI(s)/KI(s) showed variation in their larval weight reduction at each instar stage in a concentration (0.01, 0.025 and 0.05%) dependent manner as compared to larvae fed on control diet i.e., without BBI(s)/KI(s) (**Fig. 5.9A-F**). Upon feeding with VrBBI/CpBBI/RsBBI, for example, at 0.05% concentration, the second instar larvae showed ≤42% reduction in their weight, whereas VrKI/CpKI/RsKI (0.05%) supplemented larvae showed ≤82% weight reduction, as compared to control larvae. Further, in the third instar stage, the VrBBI/CpBBI/RsBBI supplemented *H. armigera* larvae showed ≤40% reduction in their weight, and VrKI/CpKI/RsKI fed larvae showed ≤70% reduction in their body weight when compared with control larvae. With reference to the fourth instar stage, the VrBBI/CpBBI/RsBBI fed larvae lost ≤36% of their weight, whereas VrKI/CpKI/RsKI fed larvae showed ≤68% reduction in their weight as compared with the control larvae. Also, the fifth instar *H. armigera* larvae showed ≤40% reduction in their body weight upon feeding with VrBBI/CpBBI/RsBBI supplemented artificial diet.

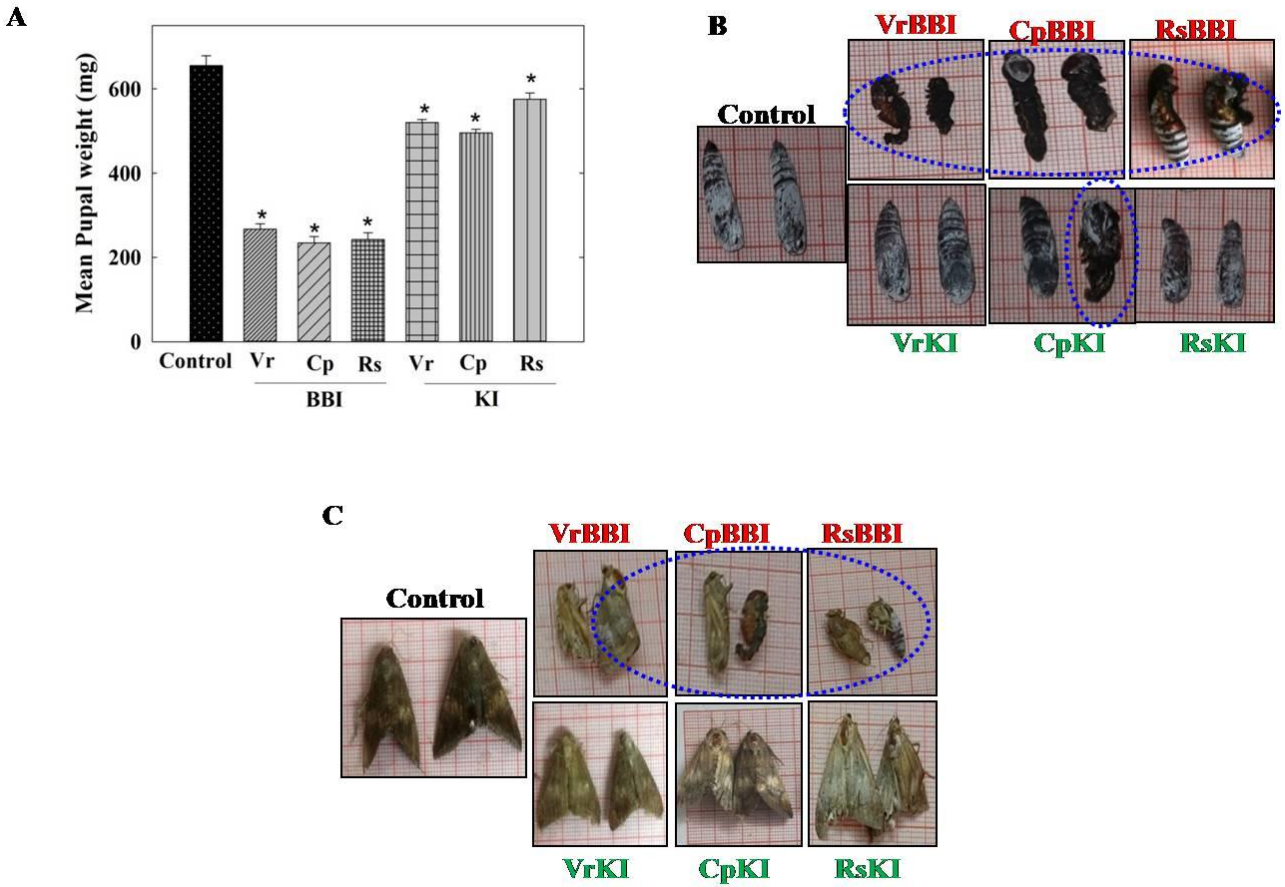


Fig. 5.8. Effect of VrBBI/CpBBI/RsBBI and VrKI/CpKI/RsKI on pupal and adult formation of *A. janata*. The effect of feeding the *A. janata* larvae on castor leaves coated with 8 µg/cm^2 concentration of BBI(s)/KI(s) on (A) mean larval weight of pupae; (B) formation of pupae and (C) emergence of adults. Blue color dotted circles indicate the malformed pupae and adults. * indicates the statistical significance $P<0.05$. Other details are as described in the materials and methods.

Table 5.1. Effect of BBI(s)/KI(s) on overall growth and development of *A. janata* (n=20). The larvae were allowed to feed on leaves coated with BBI(s)/KI(s) from the first to fifth instar stage at a concentration of 8 µg/cm^2. Other details are as described in materials and methods.

Protein (8 µg/cm^2)	**Larval-pupal Intermediates (% Total larvae)**	**Pupal - adult intermediates (% Total larvae)**	**Mortality rate (% Total larvae)**
Control	0	0	0
VrBBI	40 ± 4	15 ± 5	20 ± 2
CpBBI	50 ± 6	10 ± 3	35 ± 4
RsBBI	30 ± 3	25 ± 2	25 ± 1
VrKI	0	0	0
CpKI	10 ± 1	0	0
RsKI	0	0	0

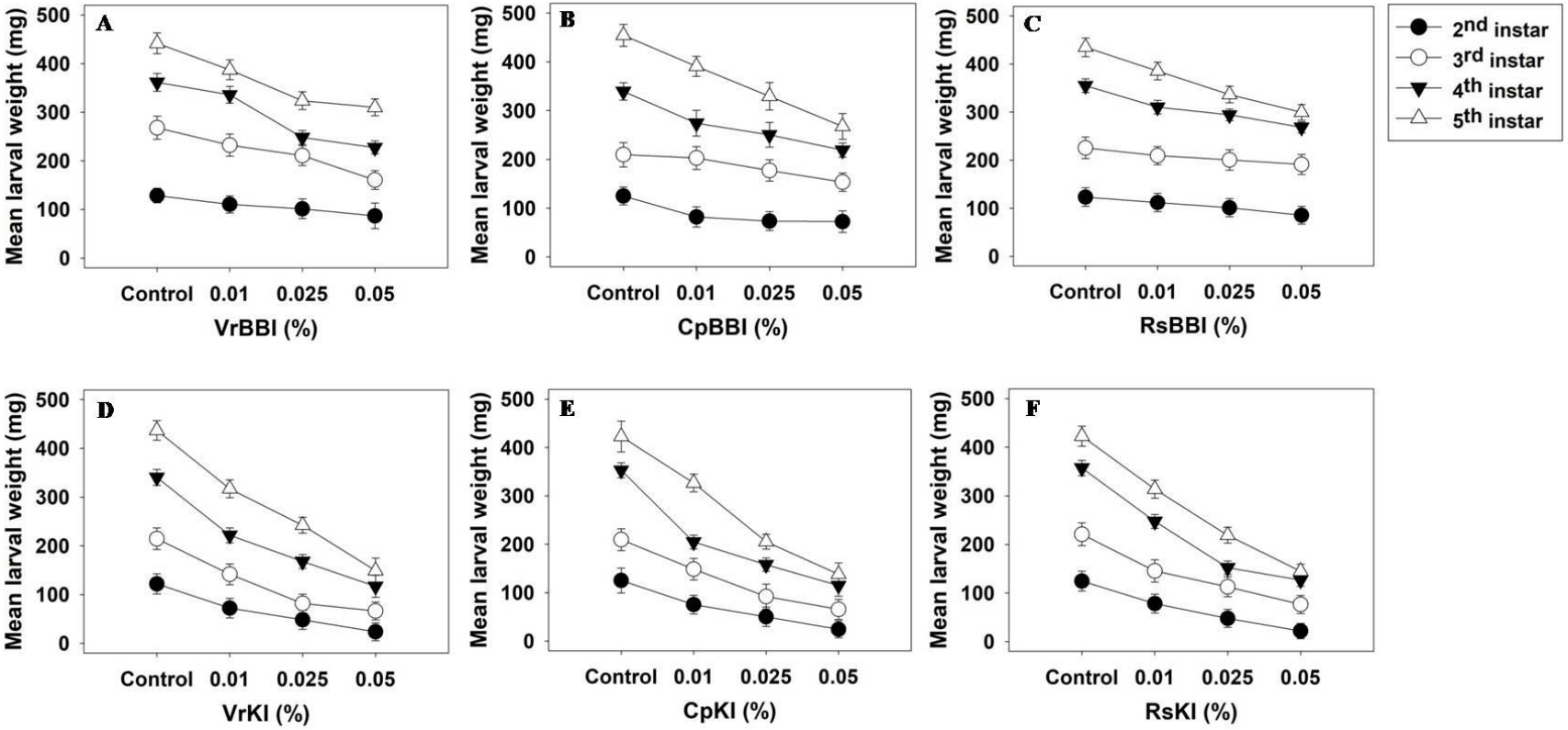


Fig. 5.9. The effect of purified BBI(s)/KI(s) from *V. radiata*, *C. platycarpus* and *R. sublobata* on the larval weight at second, third, fourth and fifth instar stages of *H. armigera* upon feeding with different concentrations (0.01, 0.025 and 0.05%) of (A)VrBBI; (B) CpBBI; (C) RsBBI; (D) VrKI; (E) CpKI and (E) RsKI, respectively. Other details are as described in materials and methods. The data represented is the mean ± SE of three biological replicates.

Besides, the fifth instar *H. armigera* larvae fed upon VrKI/CpKI/RsKI supplemented diet exhibited ≤68% reduction in their body weight as compared to control larvae. The overall effect of BBI(s) on weight reduction of *H. armigera* larvae did not vary much on different instar stages upon treatment with 0.05% concentration. However, treatment with 0.05% KI concentration marginally decreased its effect on the weight reduction of *H. armigera* larvae from the second (≤82%) to fifth instar (≤68%; **Fig. 5.9A-F**). The pictorial representation of reduction in weight of different instar stages of *H. armigera* larvae after treatment with 0.05% concentration of BBI (VrBBI/CpBBI/RsBBI) or KI (VrKI/CpKI/RsKI) is shown in **figures. 5.10A, B and 5.11A, B.**

Upon feeding of *H. armigera* larvae with VrBBI/CpBBI/RsBBI, there was no delay in pupal formation, and the formation of larval-pupal intermediates was not significant (≤12%) while the mortality rate was zero (**Table 5.2**). In contrast, a delay in pupal emergence (8-12 days), pupal weight reduction (≤57%) along with larval-pupal deformities (≤52%) and formation of pupal-adult intermediates (≤30%) was observed upon feeding with 0.05% of KIs (**Fig. 5.12A-C; Table 5.2**). Furthermore, larvae fed with VrKI/CpKI/RsKI showed ≤36% mortality rate, while such mortality was not observed in control as well as VrBBI/CpBBI/RsBBI supplemented larvae. The larval-pupal and pupal-adult intermediates formed after feeding with VrKI/CpKI/RsKI/CpBBI are pictorially shown in the **figure. 5.12B and C**. The defective pupae and adults formed after feeding with VrKI/CpKI/RsKI/CpBBI are indicated by dotted circles in the **figure. 5.12B and C**.

The reduction in growth and mortality caused to *A. janata* and *H. armigera* larvae might be due to the inhibition of gut proteases by BBI/KI.

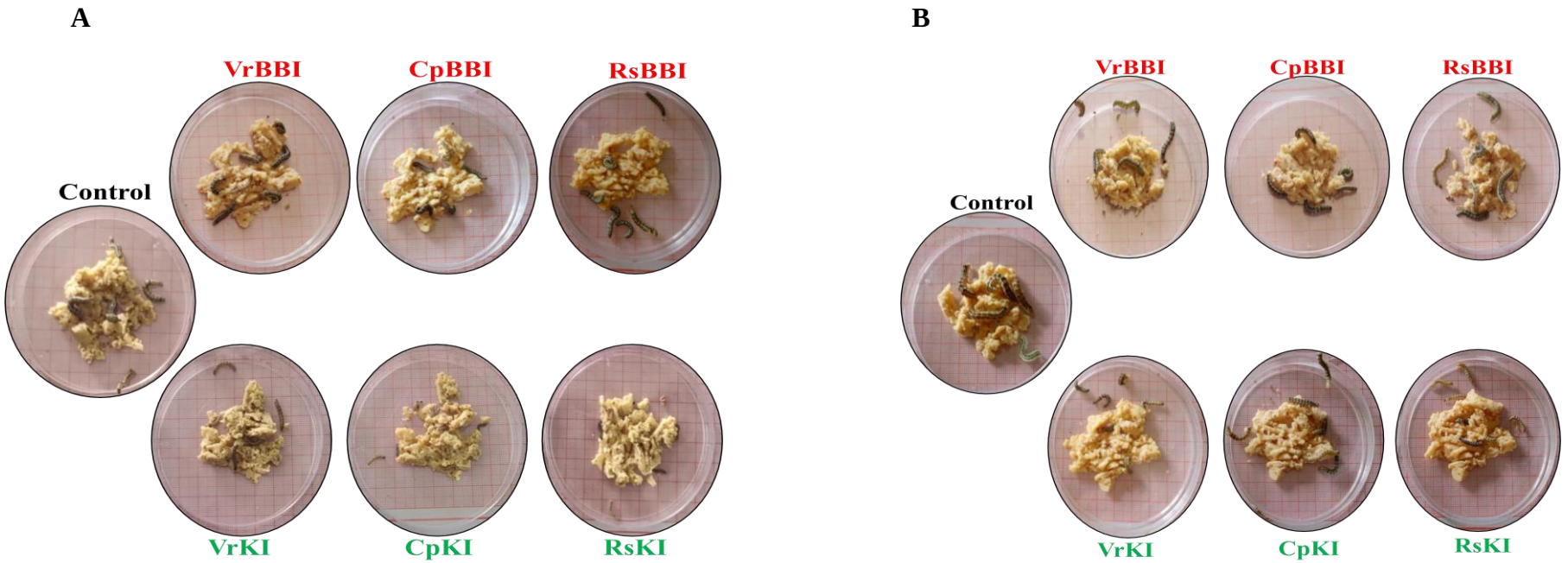


Fig. 5.10. Effect of VrBBI/CpBBI/RsBBI and VrKI/CpKI/RsKI on larval growth of *H. armigera*. Pictorial representation of the effect of feeding the synthetic diet supplemented with BBI(s)/KI(s) at a concentration of 0.05% on (A) second and (B) third instar larvae of *H. armigera*. Other details are mentioned in materials and methods.

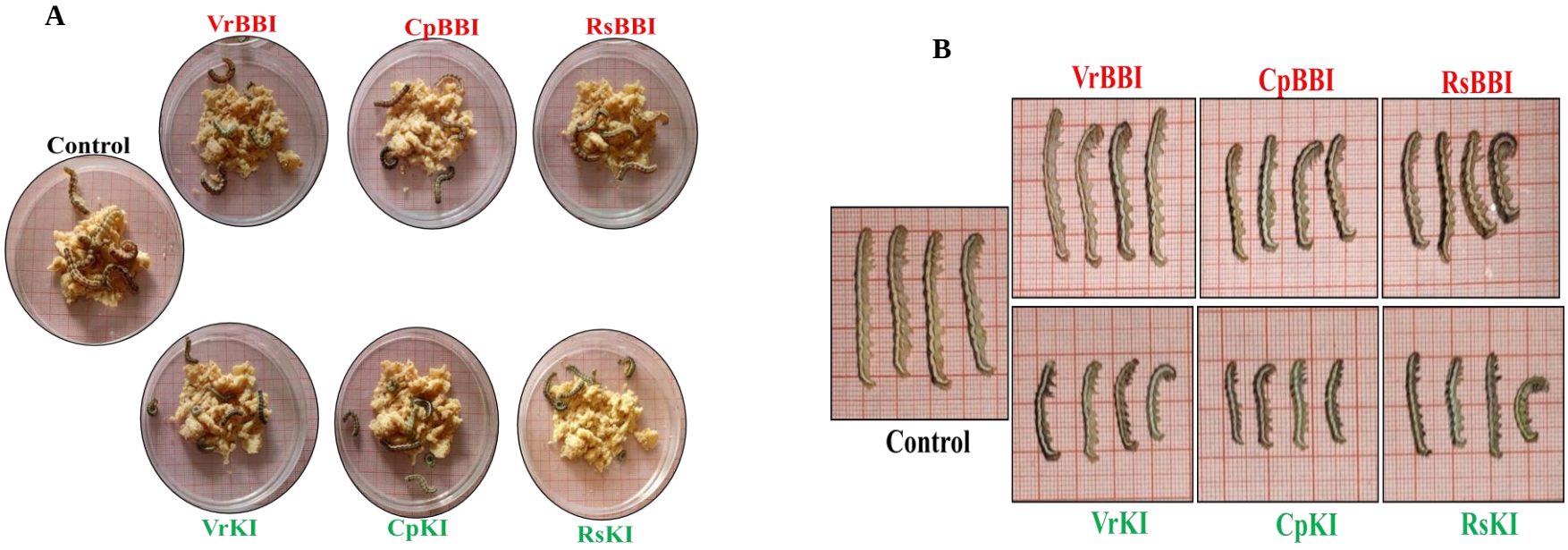


Fig. 5.11. Effect of VrBBI/CpBBI/RsBBI and VrKI/CpKI/RsKI on larval growth of *H. armigera*. Pictorial representation of the effect of feeding the synthetic diet supplemented with BBI(s)/KI(s) at a concentration of 0.05% on (A) fourth and (B) fifth instar larvae of *H. armigera*. Other details are mentioned in materials and methods.

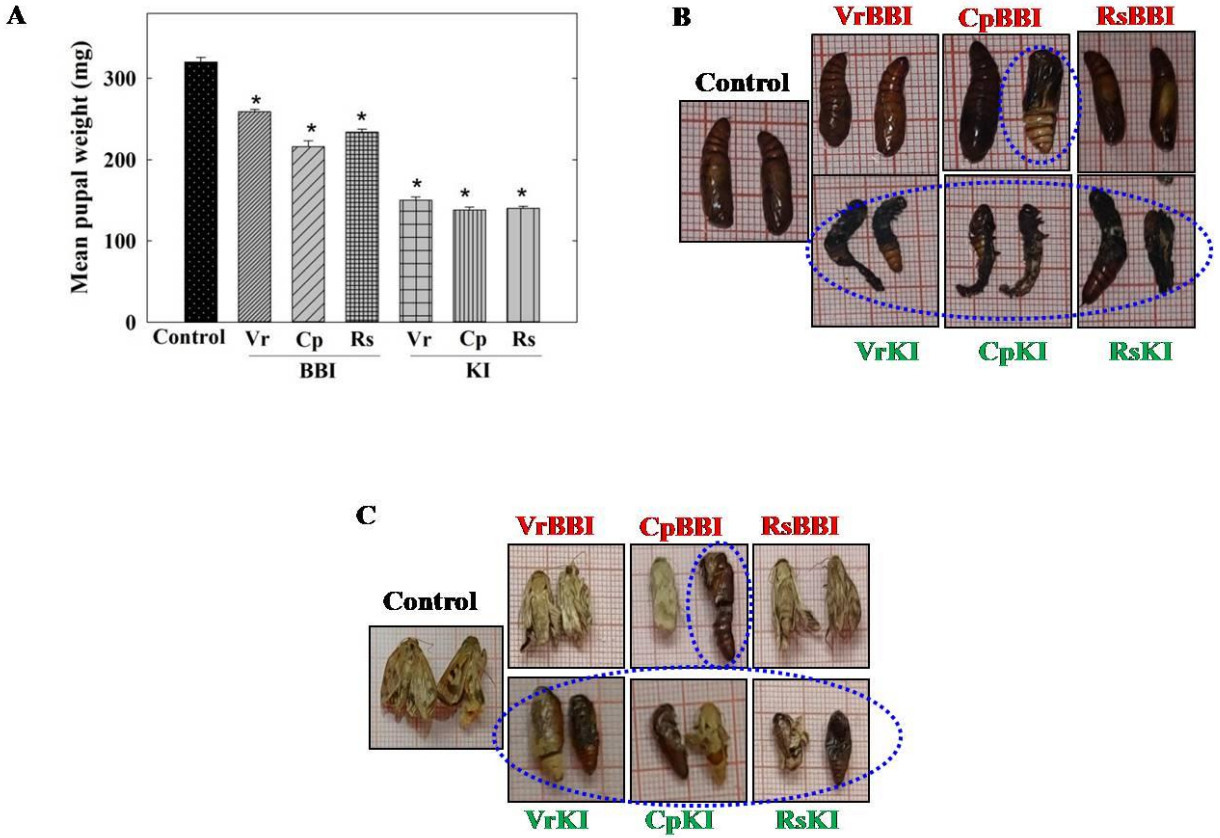


Fig. 5.12. Effect of BBI(s) and KI(s) on the pupal and adult formation of *H. armigera*. The effect of feeding the *H. armigera* larvae on a synthetic diet supplemented with 0.05% concentration of BBI(s) and KI(s) on (A) mean larval weight of pupae; (B) formation of pupae and (C) emergence of adults. Blue color dotted circles indicate the larval-pupal and pupal-adult intermediates**.** * indicates the statistical significance $P<0.05$. Other details are mentioned in the materials and methods.

Table 5.2. Effect of BBI(s)/KI(s) on overall growth and development of *H. armigera* (n=25). The larvae were allowed to feed on a synthetic diet supplemented with BBI(s)/KI(s) from the first to fifth instar stage at a concentration of 0.05%. Other details are as described in materials and methods.

Protein (0.05%)	**Larval-pupal intermediates (% Total larvae)**	**Pupal-adult intermediates (% Total larvae)**	**Mortality rate (%Total larvae)**
Control	0	0	0
VrBBI	8 ± 1	0	0
CpBBI	12 ± 1	5 ± 1	0
RsBBI	0	0	0
VrKI	40 ± 3	12 ± 1	20 ± 1
CpKI	48 ± 4	20 ± 2	28 ± 2
RsKI	52 ± 6	30 ± 4	36 ± 3

Anticancer activity of purified BBI/KI from *V. radiata, C. platycarpus* and *R. sublobata*

The anticancer effects of purified BBI/KI from *V. radiata, C platycarpus,* and *R. sublobata* seeds were evaluated on two breast cancer cell lines (MCF7, MDA-MB-231) and one cervical cancer cell line (HeLa). The MTT assay conducted with various cancerous cell lines revealed that among all the purified BBI(s) (VrBBI/CpBBI/RsBBI) and KI(s) (VrKI/CpKI/RsKI), RsKI showed remarkable inhibition in the growth of MCF7 (33%) than in MDA-MB-231 (14%) and HeLa (7.5%) cell lines as compared to non-cancerous cell line (MCF10A) (**Fig. 5.13**). Soybean BBI (SBBI) used as a positive control in the present study, showed inhibition in the growth of different cell lines in the following order: MCF7 (37%) > HeLa (11.43%) > MDA-MB-231 (9.8%) as compared to non-cancerous cell line (MCF10A) (**Fig. 5.13**).

In contrast, other BBI(s) and KI(s) showed differential effects on various cell lines. The CpBBI showed a marginal reduction in the growth of all cancerous cell lines. While VrBBI, RsBBI, VrKI and CpKI showed proliferation in the growth of different cancer cell lines, including MCF10A (control) (**Fig. 5.13**). Taken together, the results suggest that the effect of RsKI on different cancer cell lines is similar to that of SBBI.

Antimicrobial activity of BBI and KI from *V. radiata, C. platycarpus,* and *R. sublobata*

The antibacterial activity of purified BBIs/KIs was evaluated on Methicillin-sensitive *S. aureus* (MSSA), a gram-positive bacterium. In general, VrBBI, CpBBI, and RsBBI have shown antibacterial effect compared to SBBI, which did not show any inhibition. However, both BBI(s) and KI (s) did not show any dose-dependent inhibition. Besides, the optimal concentration for inhibiting the growth of bacteria varied between different BBIs and KIs. VrBBI & VrKI showed maximum inhibition (60%) in the bacterial growth at a concentration of 8 µg (**Fig. 5.14A and D**), and CpBBI/CpKI showed 40% inhibition in the growth at a

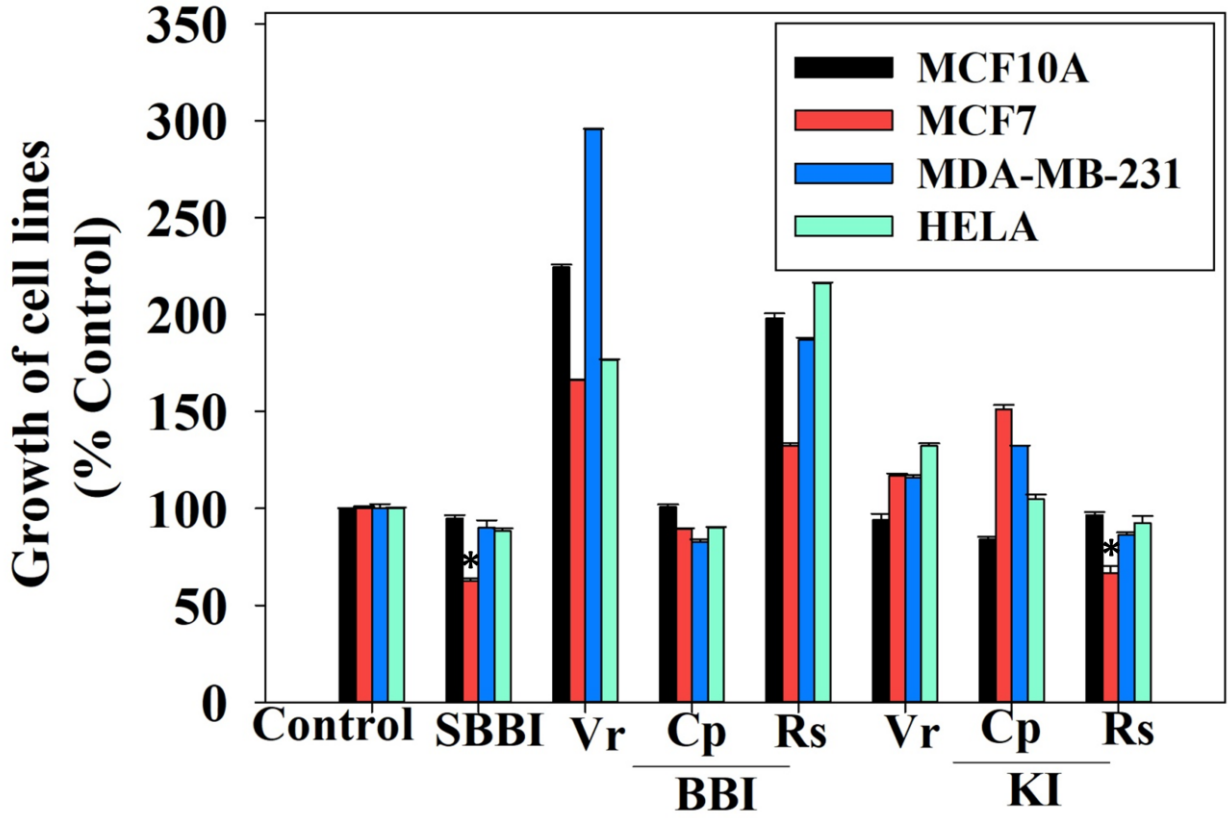


Fig. 5.13. Anticancer activity of BBIs/KIs against breast cancer (MCF-7 and MDA-MB-231) and cervical cancer (HeLa) epithelial cell lines. MCF-10A is used as non-cancerous cell line, and commercially available Soybean BBI was used as standard. * indicates the statistical significance $P<0.05$. The data represented is the mean ± SE of three biological replicates, and other details are as mentioned in materials and methods.

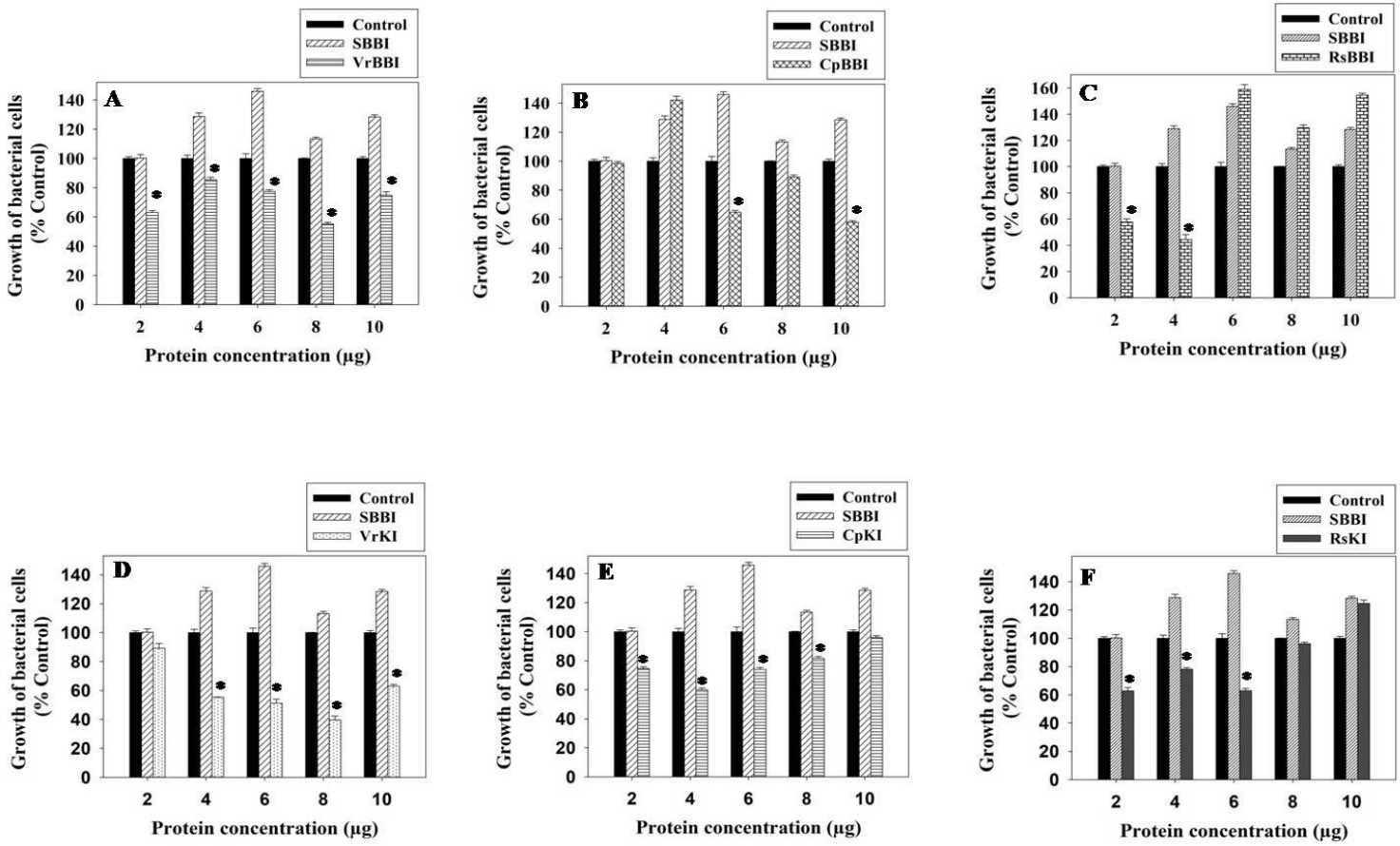


Fig. 5.14. Anti-bacterial activity of BBI(s) and KI(s). Effect of (A) VrBBI; (B) CpBBI; (C) RsBBI; (D) VrKI; (E) CpKI and (F) RsKI on the growth of *Methicillin-sensitive S. aureus,* a gram-positive bacterium. Commercially available Soybean BBI (SBBI) was used as standard. * indicates the statistical significance $P<0.05$. The data represented is the mean ± SE of three biological replicates, and other details are as mentioned in materials and methods.

concentration of 6 and 4 µg, respectively (**Fig. 5.14B and E**). Besides, RsBBI (60% inhibition) and RsKI (40% inhibition) were effective at low concentrations of 4 and 6 µg, respectively, and upon increasing the concentrations, the inhibition decreased (**Fig. 5.14C and F**).

5.3. Discussion

Differential responses of *A. janata* and *H. armigera* larvae to BBI(s) and KI(s)

Proteases are the essential enzymes in the larval gut of insects as they hydrolyze the proteins and release amino acids essential for their survival and metamorphosis. Most of the proteases in the larval gut of lepidopteran insects belong to the serine family (e.g., trypsin and chymotrypsin), and a few others belong to aspartic, metallo, and cysteine protease family (Patankar et al., 2001; Srinivasan et al., 2006; Tabatabaei et al., 2011). The *in vitro* inhibition assays, along with IC_{50} values obtained with purified BBI(s)/KI(s), revealed that VrBBI, CpBBI, and RsBBI are effective in inhibiting the gut proteases of *A. janata*. In contrast, VrKI, CpKI, and RsKI are effective in inhibiting the gut proteases of *H. armigera* (**Figs. 5.2 - 5.4**). Subsequently, the feeding experiments further confirmed the specificity of VrBBI/CpBBI/RsBBI in inhibiting the growth of larvae at various developmental stages of *A. janata* (**Figs. 5.5 - 5.7**). Besides, VrBBI, CpBBI, and RsBBI caused remarkable mortality rates (20-35%) and formed inactive larval-pupal (40-50%) and pupal-adult (10-25%) intermediates when supplemented in the diet to *A. janata* larvae (**Fig. 5.8**; **Table 5.1**). Thus, the life cycle of ≤ 95% of the *A. janata* larvae treated with VrBBI/CpBBI/RsBBI was terminated. These effects of VrBBI, CpBBI, and RsBBI on *A. janata* larvae are almost similar to the effects caused by BBIs purified from *C. cajan* (both cultivar and wild-type) using conventional methods, including those of ammonium sulfate fractionation and chromatography columns (Prasad et al., 2010c; Swathi et al., 2014; Mohanraj et al., 2018).

Further, the IC_{50} of VrKI/CpKI/RsKI against trypsin-like gut proteases of *H. armigera* (<150 ng - **Fig. 5.4**) is much lower than the IC_{50} of PIs pool purified from ICPW 63 variety (IC_{50} of 240 ng) of *C. platycarpus* (Swathi et al., 2016). These results indicate that separating BBI and KI is more effective in controlling *H. armigera*. Further, the IC_{50} (42 ng) of RsKI obtained in the present study is similar to the IC_{50} (59 ng) of RsKI purified from *R. sublobata* using the classical method of ammonium sulfate fractionation and chromatography columns followed by TCA extraction (Mohanraj et al., 2019)

Besides, purified VrKI, CpKI, and RsKI caused a significant reduction in larval weights of *H. armigera* at each instar level and ≤36% mortality rate (**Figs. 5.9 - 5.11; Table 5.2**), perhaps due to starvation of larvae as the KIs bind to midgut trypsin-like proteases (Johnston, 1993). Further feeding of larvae on KI supplemented diet showed abnormality in molting, which in turn resulted in the formation of larval-pupal as well as pupal-adult intermediates. Also, the formation of adults from larvae is very low, while most of the adults formed are abnormal (**Fig. 5.12; Table 5.2**). Thus, ≤98% of *H. armigera*, fed upon a diet containing KIs, could not continue their life cycle. Thus, the inhibition potential of VrKI/CpKI/RsKI against *H. armigera* is several times higher than that shown in previous studies by Srinivasan et al. (2005), Jamal et al. (2015) and Shaikh et al. (2022).

The results of the current study revealed that BBI and KI purified from *C. platycarpus* and *R. sublobata* were more effective in controlling both *A. janata* and *H. armigera*, when compared with BBI and KI purified from *V. radiata*. Perhaps, this could be due to the possibility that *C. platycarpus* and *R. sublobata*, both are non-host plants to *A. janata* and host plants (wild relative) to *H. armigera*, and midgut proteases of these larvae were not exposed to the BBI/KI present in wild relatives of *C. cajan*.

Effect of BBI and KI on the growth of cancerous cell lines

PIs are the major components of several seeds (soybean, rice, potato, corn, and cereals) which are consumed by humans regularly. As proteases play a crucial role in the progression of many human diseases, pharmaceutical companies are showing interest in using PIs as medicine for disease control as they possess inhibitory activity against different proteases (Shamsi et al., 2016; Cotabarren et al., 2020). Among them, several *in vitro* and *in vivo* studies have established the significance of plant PIs in controlling the proteases involved in different stages of carcinogenesis (Kennedy and Wan, 2002; de Paula-Carli et al., 2012; Cid-Gallegos et al., 2022). As PIs are stable to extreme pH (acidic or basic) conditions, they can reach the target site in active form and inhibit serine/metallo proteases involved in cancer progression (Saito et al., 2007; Oliveira et al., 2017). However, very few PIs are taken up to clinical trials like soybean BBI, while the anticancer properties of the PIs from other legume seed varieties are still unknown. In the present study, among all the purified PIs, RsKI inhibited the growth of MCF7 breast cancer cell lines (**Fig. 5.13**) when compared with other cancerous cell lines (MDA-MB 231 and HeLa) which is in correlation with several earlier studies reported with PIs from *C. arietinum* (12-15% inhibition in the growth of MDA-MB 231 cell line at 25 µg/ml concentration of PI - Magee et al., 2012), *Lavatera cashmeriana* (36% inhibition in the growth of MCF 7 cell line at 50 µg/ml concentration of PI - Rakashanda et al., 2013), *M. scutellata* (5% reduction in the growth of MCF 7 cell line at 50 µg/ml concentration of TI - Lanza et al., 2004) and *V. unguiculata* (40% inhibition in the growth of MCF 7 and MDA-MB 231 cell line at 300 µM concentration of BBI - Mehbad et al., 2016).

Effect of BBI and KI on the growth of *S. aureus*

One of the biggest threats to public health in the twenty-first century is the emergence of antibacterial resistance, which ensues when changes in bacteria lead to a decrease in the

effectiveness of the medications used to treat infections. In the 88 pathogen-drug combinations examined by Murray et al. (2022), about 12.7 million deaths that occurred in 2019 were directly attributed to antimicrobial resistance. Six major bacteria, which include *E. coli*, *S. aureus*, *Klebsiella pneumoniae*, *S. pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* are responsible for more than 2,50,000 antibacterial resistance-related deaths. Further, the two pathogens, *S. aureus* and *E. coli*, accounted for roughly half of the antibacterial resistance-related deaths. The only pathogen-drug combination that had more than 1,00,000 deaths is attributed to methicillin-resistant *S. aureus*, and it is the second leading pathogen causing antimicrobial resistance-related deaths (Murray et al., 2022). The VrBBI/CpBBI/RsBBI and VrKI/CpKI/RsKI purified in the present study inhibited the growth of *S. aureus* with varied (2-10 µg) optimal concentrations (**Fig. 5.14A-F**). The results obtained in the present study is in correlation with growth inhibition studies shown by PIs purified from seeds of *J. curcas* (5 µg/ml - Costa et al., 2014) and *A. nilotica* (20 µg/disc - Mehmood et al., 2020). Thus, the preliminary results obtained in the present study depict that both BBI(s) and KI(s) purified from *V. radiata*, *C. platycarpus* and *R. sublobata* can be explored further as antibacterial and anti-infective agents.

Highlights of the study

- ✓ *In vitro* inhibition assays conducted with BBI(s) and KI(s) purified from *V. radiata*, *C. platycarpus* and *R. sublobata* revealed that VrBBI/CpBBI/RsBBI have the potential to inhibit the midgut trypsin-like proteases of *A. janata* (IC_{50}<100 ng), whereas VrKI/CpKI/RsKI have the potential to inhibit the midgut trypsin-like proteases of *H. armigera* (IC_{50}<150 ng).

- ✓ VrBBI/CpBBI/RsBBI significantly inhibited the larval growth of *A. janata*, whereas VrKI/CpKI/RsKI retarded the growth of *H. armigera* larval growth.

- ✓ Feeding of VrBBI/CpBBI/RsBBI to *A. janata* and VrKI/CpKI/RsKI to *H. armigera* larvae resulted in a significant mortality rate, larval-pupal, and pupal-adult intermediate formation.

- ✓ Among all the PIs examined, RsKI was found to be effective in inhibiting the growth of MCF 7 compared to MCF10A.

- ✓ All the BBIs and KIs showed potency to inhibit the growth of bacterial cells. However, the optimal concentration for inhibition of growth varied between different PIs.

Chapter 6

Comparison of the biochemical properties and insecticidal potential of purified protease inhibitors induced in leaves and expressed in seeds of *V. mungo*

Comparison of the biochemical properties and insecticidal potential of purified protease inhibitors induced in leaves and expressed in seeds of *V. mungo*

6.1. Introduction

Pests and pathogens, including more than 200 insects, fungi, viruses, bacteria, and nematodes, are responsible for crop yield loss (Bebber and Gurr, 2015; Savary et al., 2019). Plants develop several defense molecules to cope with pest and pathogen attacks. One of the well-known defense molecules that significantly respond to pest or pathogen infestation is protease inhibitors (PIs), and known to accumulate in different plant tissues and sub-cellular organelles such as cell wall, protein bodies, cytosol, nucleus, and phloem (Habib and Fazili, 2007; Cohen et al., 2019). These PIs can be classified into two main categories: constitutive and inducible PIs. Constitutive PIs are synthesized prior to pest or pathogen attack and is considered as an energy-consuming process as plants need to invest some amount of ATP to maintain the constitutive PIs. Further, insects may develop resistance or adapt to these constitutive PIs (host PIs). Besides, induced PIs are synthesized in a time period ranging between a few minutes to several hours after insect attack and establish an enhanced defensive capacity in plant parts far away from the site of injury and protect the plant from succeeding invasion. As inducible PIs accumulate only after pest attack, the chances for adaptation of insects to inducible PIs are minimal, and the plant's energy investment is less (Leon et al., 2001; Padul et al., 2012).

The accumulation of PIs in different plant parts in response to various stress conditions such as wounding, pest or pathogen attack, and environmental stimuli is elicited by several signaling molecules. Among them, jasmonic acid (JA) and its derivatives - methyl jasmonate (MJ) and jasmonyl isoleucine play a major role in triggering plant defense responses (Feussner and Wasternack, 2002; Cheong and Choi, 2003; Anjum et al., 2011). The extensive studies of Ryan and co-workers established the involvement of JA in response

to wounding or insect attack in tomato and potato plants (Green and Ryan, 1972; Moura and Ryan, 2001). Further, the foliar application of JA and its methyl ester (MJ) in various plants, such as *C. annum*, *C. cajan*, and *Vitis vinifera*, enhanced the plant's resistance to various pests indicating that JA is a crucial component that regulates plant's defense against pests and pathogens via the synthesis of PIs (Farmer et al., 2003; Belhadj et al., 2006; Tamhane et al., 2009).

Black gram (*V. mungo*) is one of the vital pulse crops grown throughout India, and the avoidable quantitative losses (7-35%) to black gram crops are caused by insect pests such as pod borers, sucking pests, and flower feeders (Singh et al., 2017; Radhika et al., 2018; Yadav et al., 2019). Black gram contributes to about 10% of the national pulse production, and India is one of the largest producers and consumers of black gram. Several earlier studies reported the purification of PIs from the seeds of *V. mungo* and evaluated them for their biochemical properties and biological applications (Benjakul, 2001; Cheung et al., 2009; Prasad et al., 2010a). But, the synthesis of inducible PIs in *V. mungo* leaves upon exposure to stress conditions, their purification, and characterization was not reported yet. Considering these facts, in the present study, an attempt was made to address the following questions (i) whether PIs are induced in leaves of *V. mungo* up on exposure to biotic and abiotic stress; (ii) if induced, what is the nature of induced PIs, and (iii) does induced PIs differ from seed PIs in their biochemical and insecticidal properties. To address these questions, the induction of PIs was monitored at different time intervals in *V. mungo* leaves after wounding, exposure to MJ, and *H. armigera* infestation. The activity of inducible PIs was monitored as trypsin and chymotrypsin inhibitory units. Further, the PIs induced in leaves (MJ treated) and PIs present in seeds were purified using different chromatography techniques and evaluated for any difference in their biochemical and insecticidal properties.

6.2. Results

Induction of PIs in *V. mungo* leaves upon wounding, MJ treatment, and *H. armigera* infestation

In *V. mungo*, mechanical wounding, MJ application, or infestation with *H. armigera* resulted in the induction and significant accumulation of PIs in leaves as compared to leaves collected from control plants without any treatment. The *in vitro* inhibition assay of the crude extracts prepared from the wounded, MJ treated, and *H. armigera* infested leaves showed both trypsin inhibitory (TI) (**Fig. 6.1A-C**) and chymotrypsin inhibitory (CI) activities in response to all stimuli (**Fig. 6.2A-C**). Induction of PIs was observed from 6 h of treatment under all the given treatment conditions and was maximum at 72 h upon wounding (4.2 ± 0.4 TIU/mg protein and 3.1 ± 0.3 CIU/mg protein) and MJ treatment (5.6 ± 0.7 TIU/mg protein and 3.3 ± 0.2 CIU/mg protein) (**Figs. 6.1A, B and 6.2A, B**). However, during the *H. armigera* infestation, the TI and CI activities reached a maximum at 24 - 36 h (4.5 ± 0.5 TIU/mg protein and 3.0 ± 0.2 CIU/mg protein) of treatment and stable up to 72 h (**Figs. 6.1C and 6.2C**). Further, induction of PIs was higher in MJ treated leaves than in other treatments.

Since the induced PIs showed both TI and CI activities, a characteristic feature of the Bowman-Birk inhibitor (BBI) family of PIs, real-time PCR analysis was performed using BBI-specific gene primers to confirm the nature of induced PIs. Correlating with the TI and CI activities, relative expression of BBI-specific genes was high at 72 h in samples subjected to wounding and MJ treatment (**Fig. 6.3A and B**), while samples subjected to *H. armigera* infestation showed maximum expression levels between 36 to 72 h (**Fig. 6.3C**). A significant increase (> 2 fold) in BBI gene expression was observed under all given stress treatments as compared to control leaves (**Fig. 6.3A-C**).

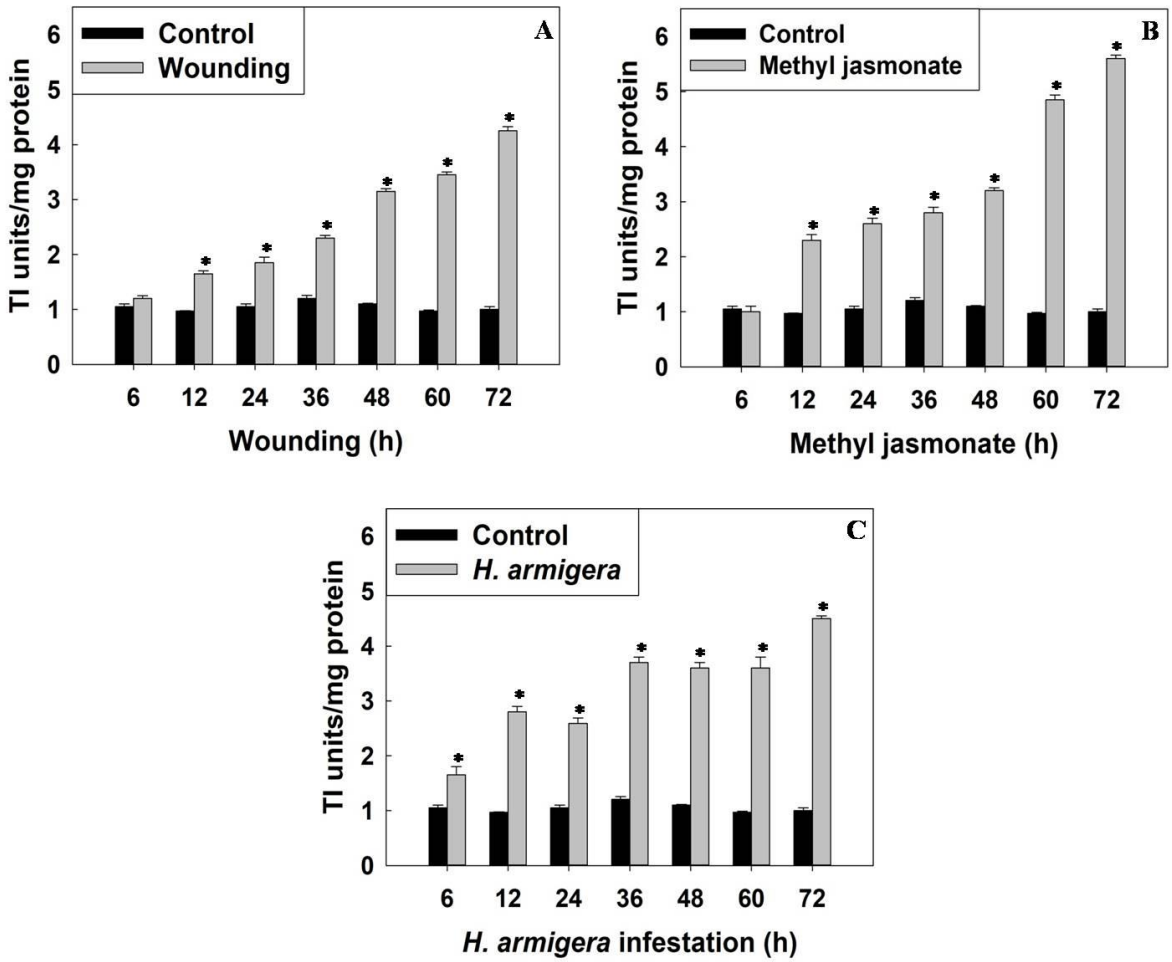


Fig. 6.1. Effect of (A) wounding; (B) methyl jasmonate treatment and (C) *H. armigera* infestation on the induction of PIs. The TI activity of induced PI was monitored at different time intervals in *V. mungo* leaves. The control and treated leaves are represented in black and grey color bars, respectively. * indicates the statistical significance $P<0.05$.

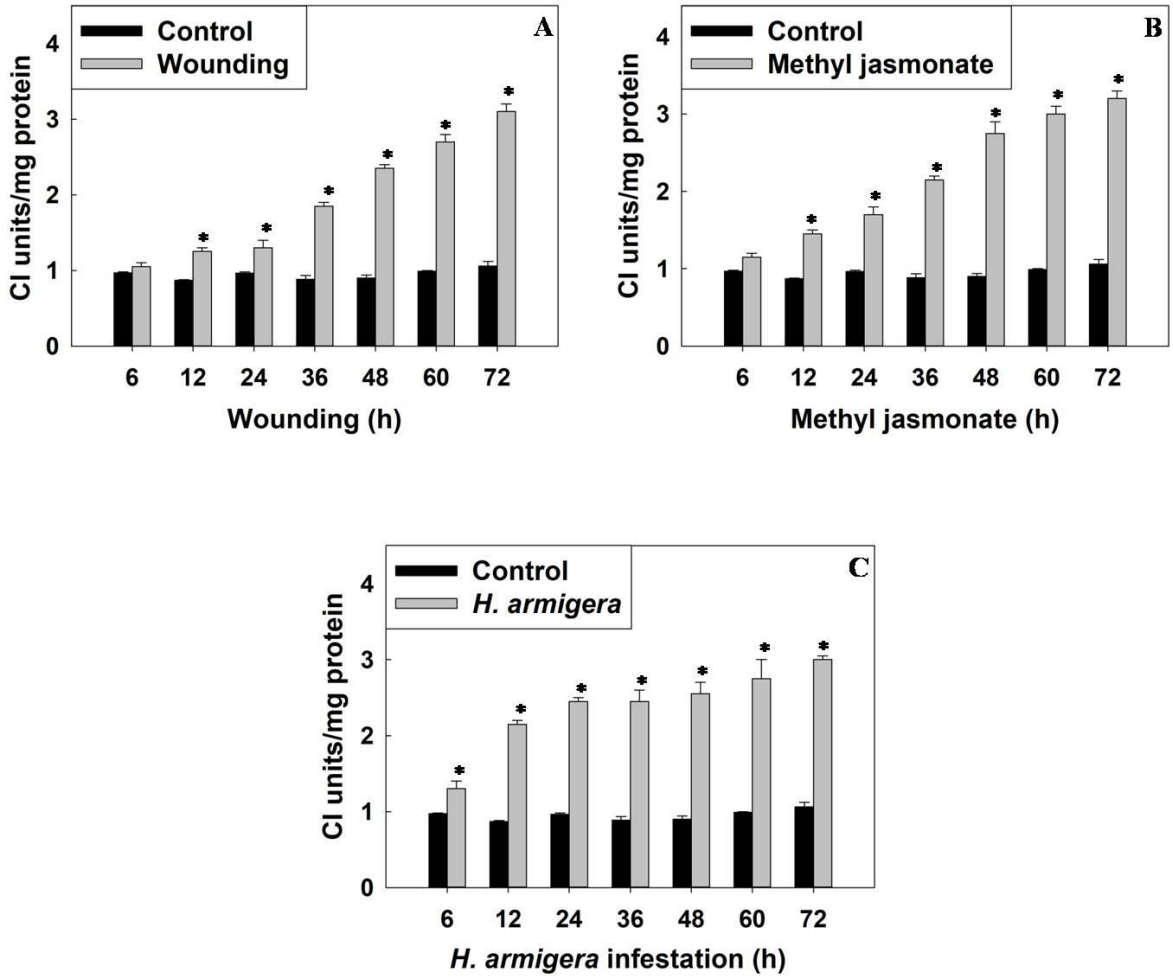


Fig. 6.2. Effect of (A) wounding; (B) methyl jasmonate treatment and (C) *H. armigera* infestation on the induction of PIs. The CI activity of induced PI was monitored at different time intervals in *V. mungo* leaves. The control and treated leaves are represented in black and grey color bars, respectively. * indicates the statistical significance $P<0.05$.

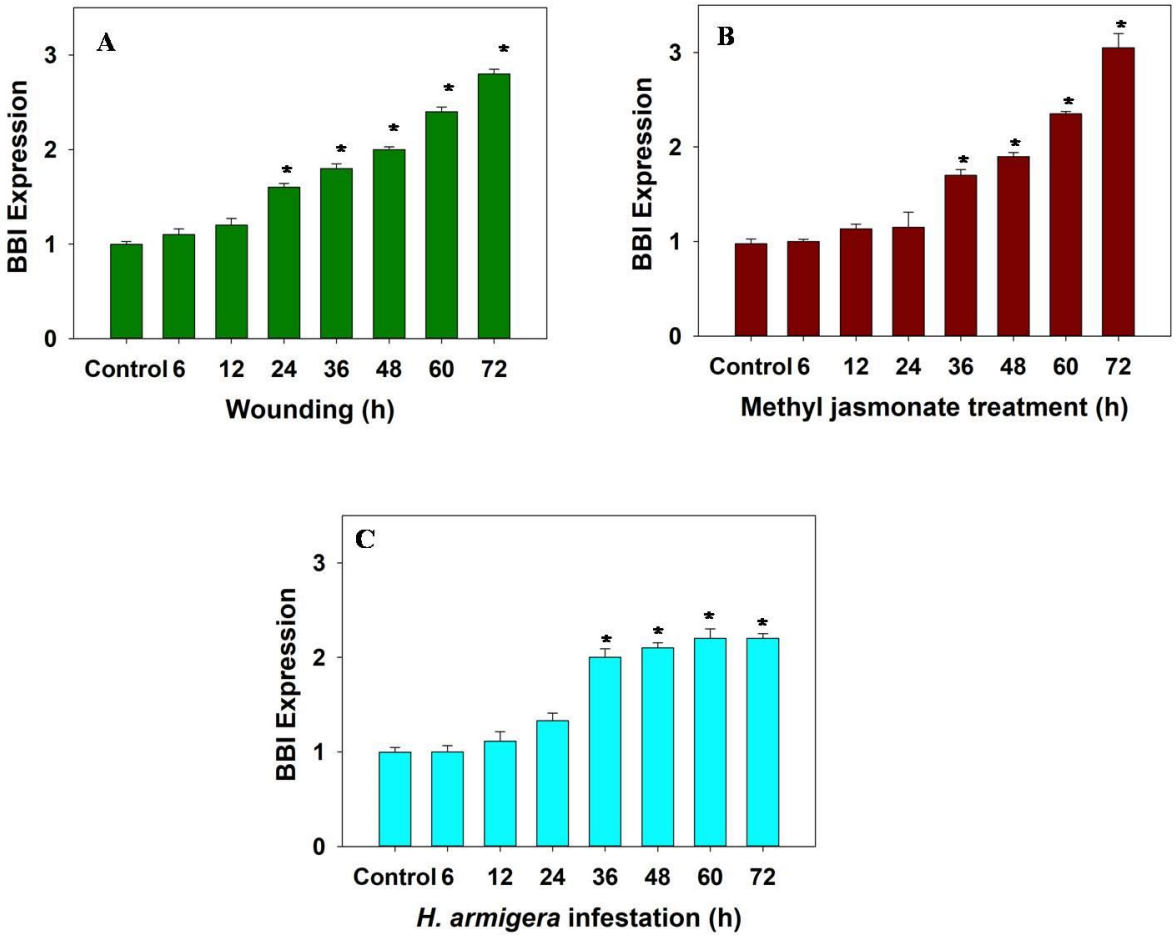


Fig. 6.3. BBI expression profile in *V. mungo* leaves upon (A) wounding; (B) methyl jasmonate treatment and (C) *H. armigera* infestation. The transcript abundance was quantified by the ΔΔCT method by using actin as a reference gene. * indicates the statistical significance P<0.05. Other details are as described in materials and methods.

In living cells, ROS are the primary components released in response to any biotic or abiotic stress. Thus, in light of the crucial role played by ROS in signal-mediated defense mechanisms, the amount of H_2O_2 (a major reactive oxygen species) accumulated in leaves upon wounding, MJ treatment, and *H. armigera* infestation was measured (**Fig. 6.4A-C**). In the case of wounding and MJ treatment, high levels of H_2O_2 were detected at 6 h ($\leq$ 520 ± 9.5 µmol g^{-1} fresh weight) as compared to control, and it reduced gradually at 72h (**Fig. 6.4A and B**). Whereas in the case of *H. armigera* infestation, an increase in H_2O_2 was observed between 12 h and 24 h (1400 ± 23 µmol g^{-1} fresh weight) (**Fig. 6.4.C**). The increase in H_2O_2 was (>2 fold) high in leaves infested with *H. armigera* when compared with the H_2O_2 content of wounded and MJ treated leaves.

Purification of leaf PI and seed PI

In the present study, the PIs induced in leaves and PIs present in seeds as storage proteins were purified to differentiate and evaluate their biochemical and insecticidal properties. Since the TI and CI activities of PIs are slightly higher in MJ-treated leaves than in other treatments, the crude extract of MJ treated leaves (72 h) was used to purify proteins induced in leaves. Thus, the CPE was subjected to ammonium sulfate fractionation (0-20% followed by 20-60%) and passed through CNBr-activated sepharose 4B column after dialysis in Tris-HCl (50 mM, pH 8.0). The unbound protein (peak I) was washed out, and the protein bound to column (peak II) was collected. The peak II fractions showing significant TI activity were concentrated using 3 kDa cut-off amicon filters and used for characterization studies (**Fig. 6.5**). From 100 g of MJ treated leaf tissue, 3.5 mg of protein was obtained with a specific activity of 215 ± 10.7 TIU/mg protein. Besides, the yield recovery and purification fold of leaf PI were found to be 15% and 37-fold, respectively (**Table 6.1**).

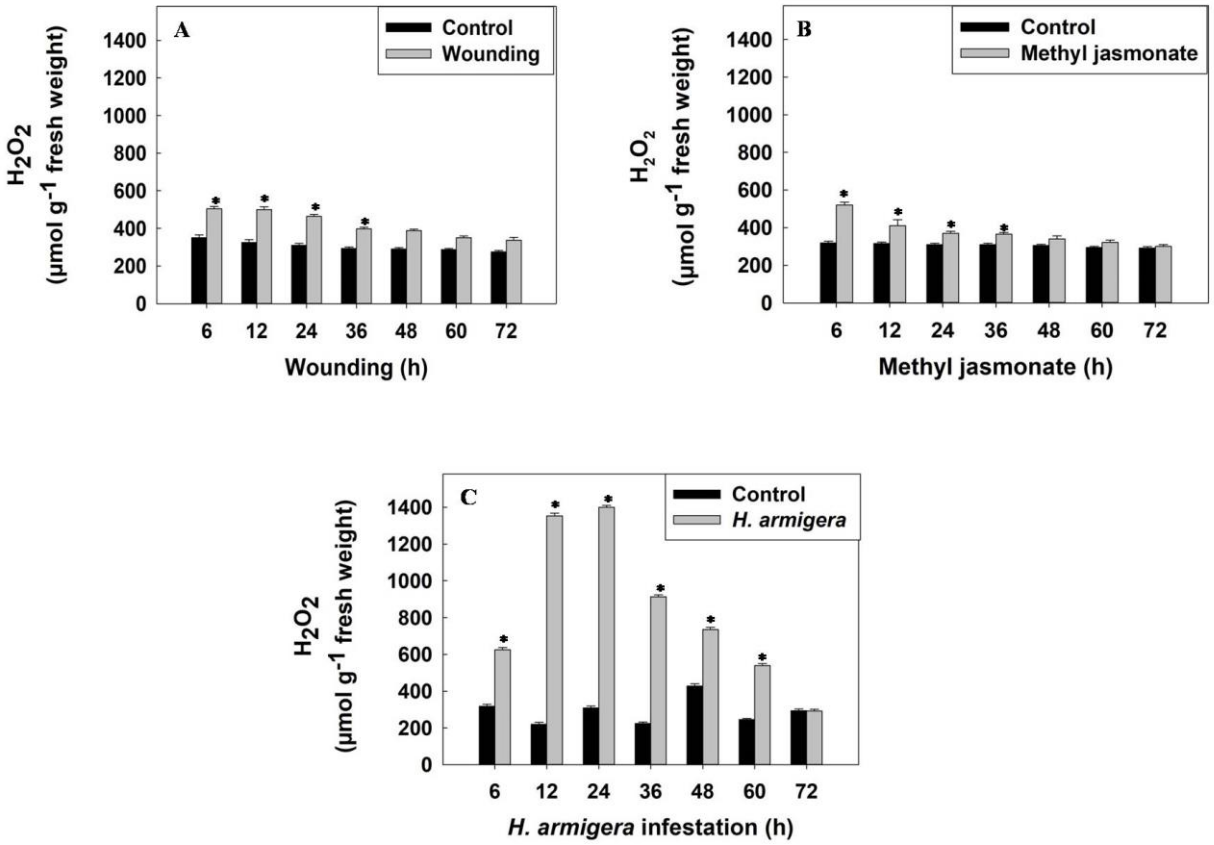


Fig. 6.4. Estimation of H_2O_2 content (µmolg^{-1}FW) in *V. mungo* leaves after (A) wounding; (B) methyl jasmonate treatment and (C) *H. armigera* infestation at different time intervals. The control and treated leaves are represented in black and grey color bars, respectively. * indicates the statistical significance $P<0.05$.

For purification of PIs from seeds, the CPE was subjected to 0-20% followed by 20-80% ammonium sulfate fractionation. The protein pellet obtained was dissolved in 50 mM Tris-HCl and dialyzed against the same buffer for 24 h. The salt-free protein obtained after dialysis was subjected to trypsin affinity chromatography. The unbound protein was washed with wash buffer, and the protein-bound to column (peak II) was eluted with 0.01N HCl (**Fig. 6.6A**). The eluted protein was neutralized and tested for TI activity and dialyzed overnight at 4 ^{o}C. The affinity fraction(s) enriched in TI activity was passed through the Sephadex G-50 column to remove high molecular weight minor contaminants, and the pure protein (PI) was collected using 50 mM Tris-HCl (**Fig. 6.6B**). The specific activity of purified PI was found to be 983 ± 36.1 TIU/mg protein, and the protein was purified to 116-fold with yield recovery of 33%, respectively (**Table 6.1**). The obtained pure protein was used for its characterization.

Biochemical characterization of leaf PI and seed PI

The electrophoretic profile of PI purified from both leaves and seeds showed a single band in Tricine-SDS-PAGE between 10-15 kDa with reference to standard molecular weight markers and ~ 8 kDa with reference to soybean BBI (**Fig. 6.7A and B**). Further, intact mass analysis of the leaf PI exhibited an oligomeric pattern with three intense peaks possessing a molecular mass of 9.763 kDa (monomer), 19.526 kDa (dimer), and 39.05 kDa (tetramer), respectively (**Fig. 6.8A**). Besides, the intact mass analysis of seed PI showed a single peak with a molecular mass of 8.209 kDa (**Fig. 6.8B**). Further, the formation of blue color spots in the dot blot assay against PI(s) from leaves and seeds upon probing with anti-BBI antibodies revealed that the PI purified from both leaves and seeds of *V. mungo* belonged to the BBI family (**Fig. 6.9**).

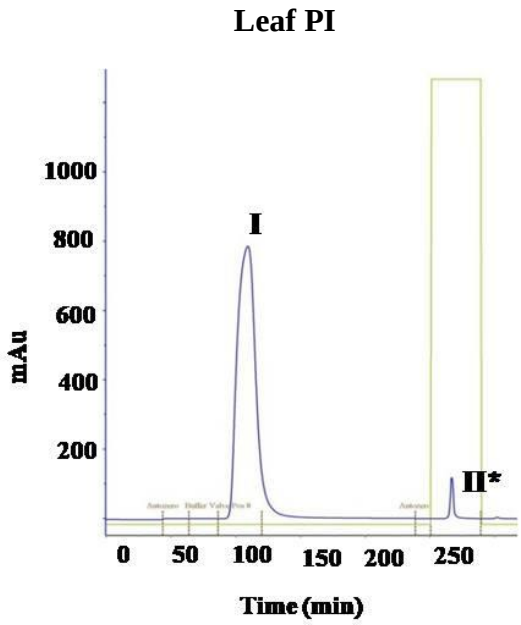


Fig. 6.5. Elution profile of CNBr activated trypsin sepharose 4B column loaded with ammonium sulfate fractionation (20-60%) pool of MJ treated leaves. PI with significant TI activity was recovered in peak II*. Other details are as described in materials and methods.

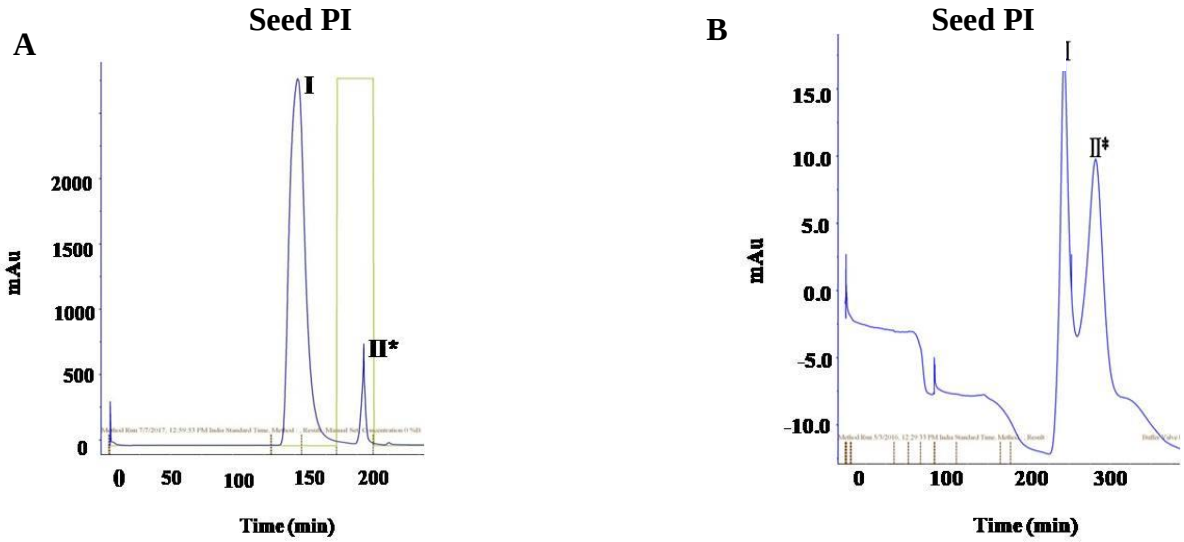


Fig. 6.6. Elution profile of (A) CNBr activated trypsin sepharose 4B column loaded with ammonium sulfate fractionation (20-80%) pool of seeds; (B) Sephadex G-50 column loaded with trypsin affinity active fraction pool. PI with significant TI activity was recovered in peak II*. Other details are as described in materials and methods.

Table 6.1. Purification of PIs induced in methyl jasmonate-treated leaves (100 g) and expressed in mature seeds (50 g) of black gram (*V. mungo*). One trypsin inhibitor (TI) unit is defined as the amount of leaf/seed PI required to inhibit 50% of trypsin activity and specific activity is the number of TI units per mg of protein. Other details are as mentioned in materials and methods.

The data shown here is representative of three independent experiments performed on different days.

Purification Step	Total protein (mg)	Total activity (TI units)	Yield Recovery (%)	Specific Activity (TI units/mg protein)	Purification (fold)
Leaf PI					
Crude protein extract	836	5000	100	5.8	1
$(NH_4)_2SO_4$ (20-60%)	220	3045	61	14	2.4
Affinity chromatography	3.5	750	15	215	37
Seed PI					
Crude protein extract	3556	30,400	100	8.5	1
$(NH_4)_2SO_4$ (20-80%)	1222	26,666	88	21.8	2.6
Affinity chromatography	35.1	11,000	36	314	37
Gel filtration chromatography	10.2	10,000	33	983	116

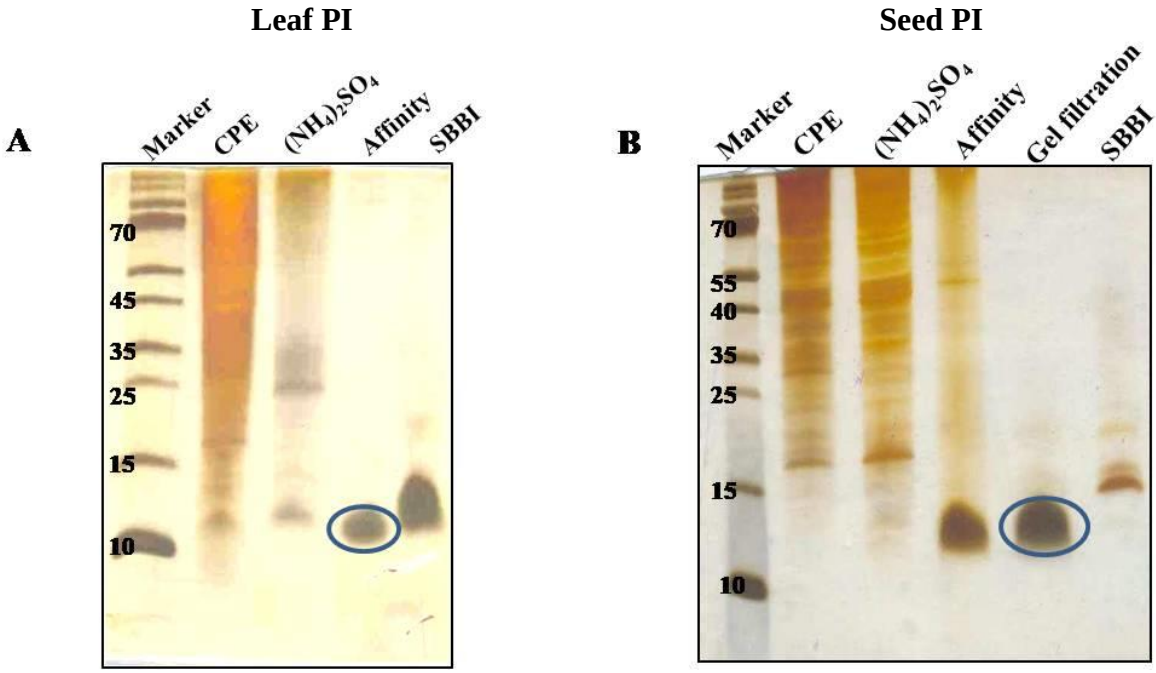


Fig. 6.7. Tricine SDS-PAGE (15%) showing the purification pattern of (A) leaf PI loaded with molecular marker, crude protein extract (CPE-10 µg), 20-60% ammonium sulfate fraction (10 µg), and affinity fraction (5 µg), and commercially procured Soybean Bowman-Birk inhibitor (SBBI) (2 µg); (B) seed PI loaded with molecular marker, crude protein extract (25 µg), 20-80% ammonium sulfate fraction (10 µg), affinity fraction (10 µg), gel filtration fraction (10 µg), and SBBI (1 µg). The blue color circle indicates the pure leaf PI and seed PI proteins. The other details are as mentioned in materials and methods.

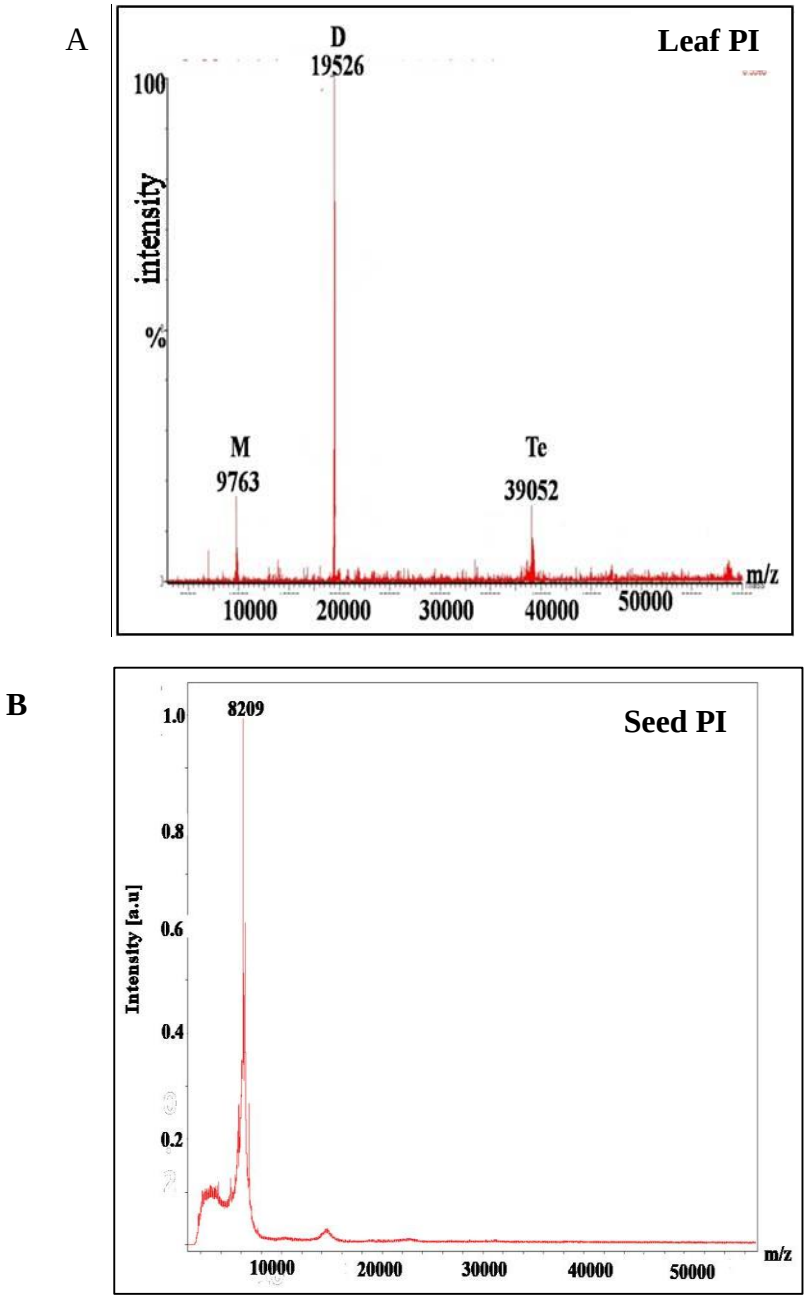


Fig. 6.8. Intact mass analysis of leaf and seed PI. (A) MALDI-ESI-Q-TOF analysis of leaf PI and (B) MALDI-TOF analysis of seed PI between 10000-50000 m/z. M, D, and Te indicate monomer, dimer, and tetramer, respectively. Other details are as described in materials and methods.

As BBIs contain two inhibitory loops, one for trypsin and the other for chymotrypsin, the inhibitory potential of leaf PI and seed PI was tested against serine proteases such as bovine pancreatic trypsin and chymotrypsin, respectively, by *in vitro* inhibition assays. The specific activity of leaf PI against trypsin and chymotrypsin was found to be 215 ± 10.7 TIU/mg protein and 43.6 ± 3.2 CIU/mg protein, respectively, while the specific activity of seed PI against trypsin and chymotrypsin was found to be 983 ± 36.1 TIU/mg protein and 195 ± 8.6 CIU/mg protein, respectively (**Fig. 10A and B**). Further, in-gel activity staining studies revealed two active bands with leaf PI and a single active band with seed PI upon incubating the gels with trypsin and chymotrypsin, respectively (**Fig. 6.10C and D**).

The stability in the TI/CI activity of leaf PI and seed PI was analyzed by incubating them at a wide range of temperatures (37-100 ^{o}C), pH (2.0-12.0), and different concentrations of a denaturing agent, DTT (0.01 to 3 mM). The TI/CI activity of leaf PI was stable from pH 3.0 to pH 9.0 with a minimal loss of ≤15% than the TI/CI activity observed at optimal pH (8.0). Also, ≥50% loss in TI/CI activity of leaf PI was observed at highly acidic (pH 2.0) and alkaline conditions (pH 10.0-12.0) (**Fig. 6.11A**). In contrast, seed PI showed stable TI/CI activity at both acidic (pH 2.0-5.0) and basic (pH 8.0-12.0) pH conditions but lost activity around neutral pH (pH 6.0-7.0) by ≤40% (**Fig. 6.11B**). A concentration-dependent decrease in TI/CI activity was observed with both leaf and seed PI upon incubating them with DTT. However, both leaf PI and seed PI lost their TI/CI activity completely upon incubating in the presence of high concentrations (≤3 mM) of DTT (**Fig. 6.11C and D**). Further, both leaf PI and seed PI showed maximum TI and CI activities at assay temperature (37 ^{o}C) (**Fig. 6.11E and F**). However, there was only a minimal loss (≤10%) in TI and CI activities of both leaf and seed PIs, even after heating them at temperatures as high as 90 ^{o}C.

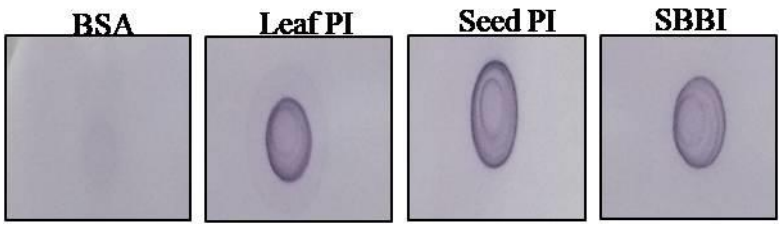


Fig. 6.9. Dot-blot analysis of leaf and seed PI after probing with anti-BBI antibody (1: 500 dilution). 2 µg of each protein was added. BSA was used as a negative control, and SBBI was used as a positive control. Other details are as described in materials and methods.

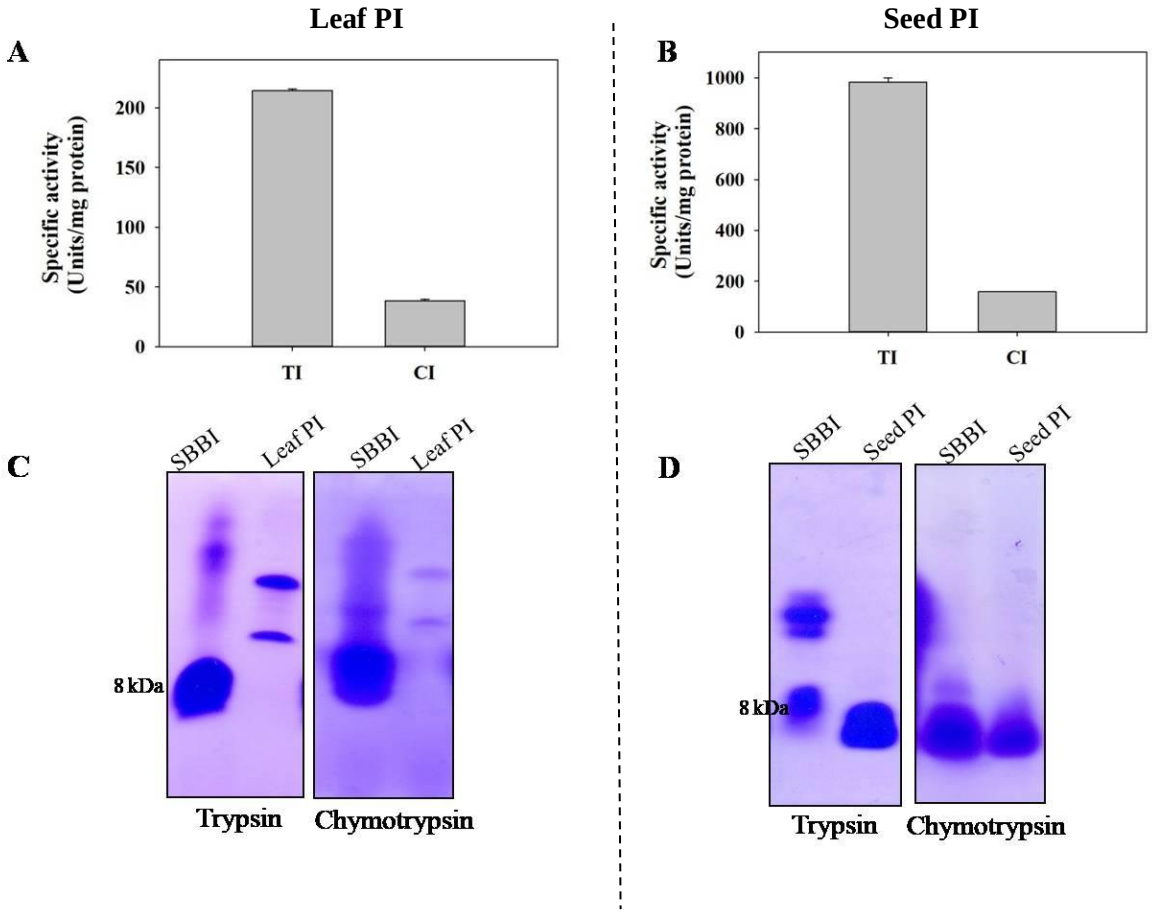


Fig. 6.10. *In vitro* inhibitory activity of purified PIs against serine proteases. The specific activity of (A) leaf PI and (B) seed PI against bovine pancreatic trypsin and chymotrypsin. Visualization of in-gel activity of (C) leaf PI and (D) seed PI against trypsin and chymotrypsin in gelatin SDS-PAGE (15%). 2 µg of each protein was loaded into each well. SBBI was used as a reference marker. Bands were visualized after staining with Coomassie Brilliant blue R-250. Other details are as described in materials and methods.

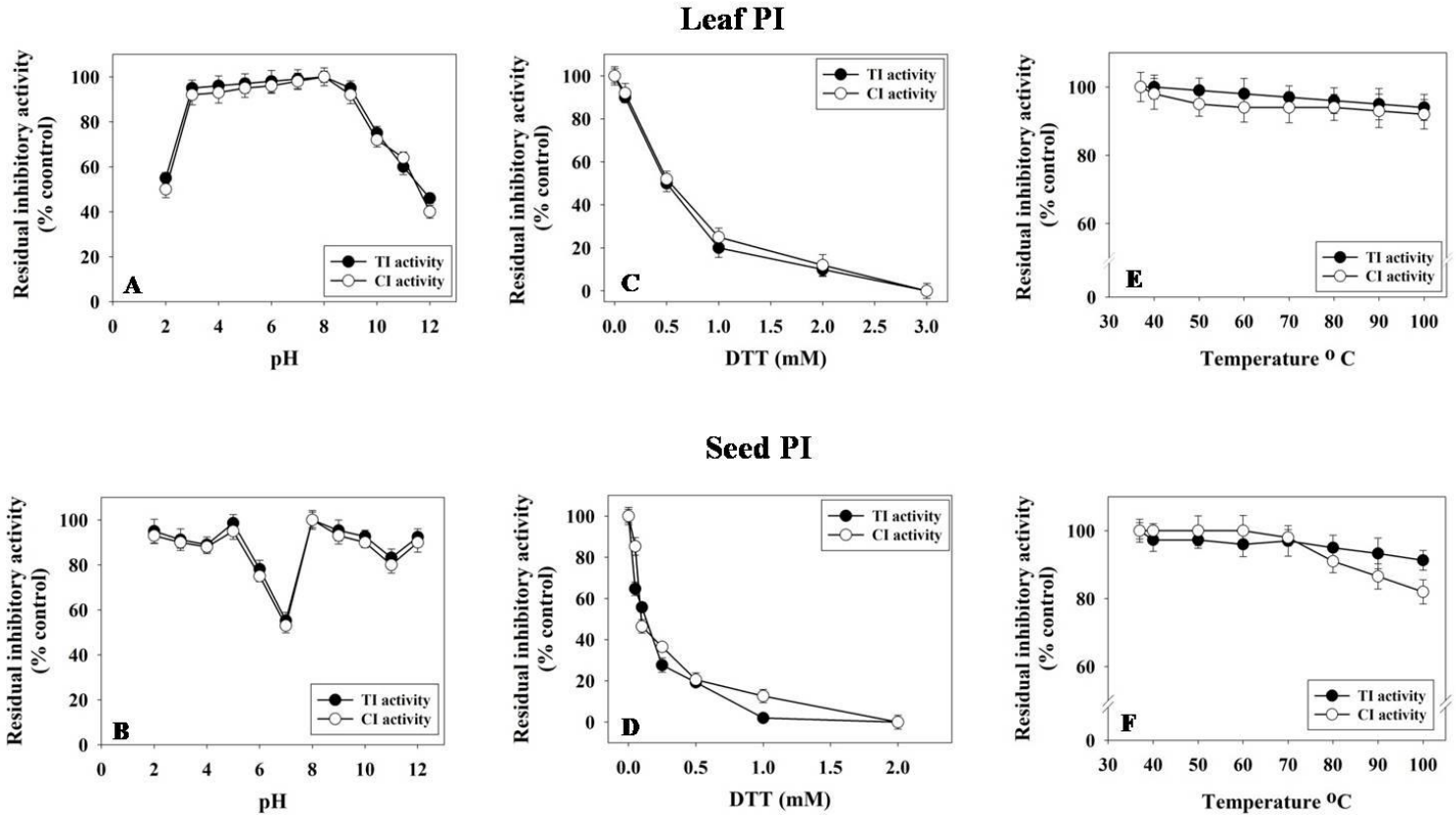


Fig. 6.11. Functional stability of leaf PI and seed PI against a wide range of pH, temperature, and DTT treatment. (A, B) The TI and CI activities against pH were assessed by incubating them at described pH for 30 min, (C, D) Effect of DTT on TI/CI activity was monitored by incubating them in different concentrations for 1 h and (E, F) Effect of temperature on TI/CI activity was assessed by incubating them for 30 min at indicated temperature, respectively. Residual TI/CI activity was measured using BAPNA and GLUPHEPA as substrates. Other details are as described in materials and methods.

Insecticidal potential of leaf PI and seed PI

As trypsin-like serine proteases are the major midgut proteases of lepidopteran insect pests, the potential of leaf/seed PI to inhibit the midgut proteases of *S. litura*, *A. janata*, and *H. armigera* was evaluated by *in vitro* assays and in-gel activity staining studies. *V. mungo* is a non-host plant to *A. janata*, a major castor pest, and host plant to *H. armigera* and *S. litura*. The leaf PI showed significant inhibitory potential against midgut trypsin-like proteases of *S. litura* (958 ± 53.6 SlTPI units/mg protein) than *A. janata* (569 ± 37.1 AjTPI units/mg protein) and *H. armigera* (450 ± 23.8 HaTPI units/mg protein), respectively (**Fig. 6.12A**). Contrarily, the seed PI showed several-fold higher specific activity against midgut trypsin-like proteases of insect pests than leaf PI. However, the inhibitory potential of seed PI was higher against midgut trypsin-like proteases of *A. janata* (7022 ± 462 AjTPI units/mg protein) when compared with *S. litura* (2065 ± 235 SlTPI units/mg protein) or *H. armigera* (935 ± 52 HaTPI units/mg protein) (**Fig. 6.12B**).

Further, the activity staining studies of leaf PI against midgut proteases of these insect pests revealed that both the monomer and dimer are effective in inhibiting the midgut proteases of *S. litura* and *A. janata* as compared to *H. armigera* (**Fig. 6.12C**). Besides, the activity staining studies with seed PI revealed that the single band observed in gelatin SDS-PAGE showed stronger inhibitory activity against midgut proteases of *A. janata* than midgut proteases of *S. litura* or *H. armigera* (**Fig. 6.12D**).

As the yield recovery and inhibitory potential of seed PI were higher than leaf PI, the *in vivo* feeding experiments were carried out with seed PI alone in the present study. Also, we restricted to evaluate the effect of seed PI on the growth and development of *A. janata* and *S. litura* based on the *in vitro* results shown above.

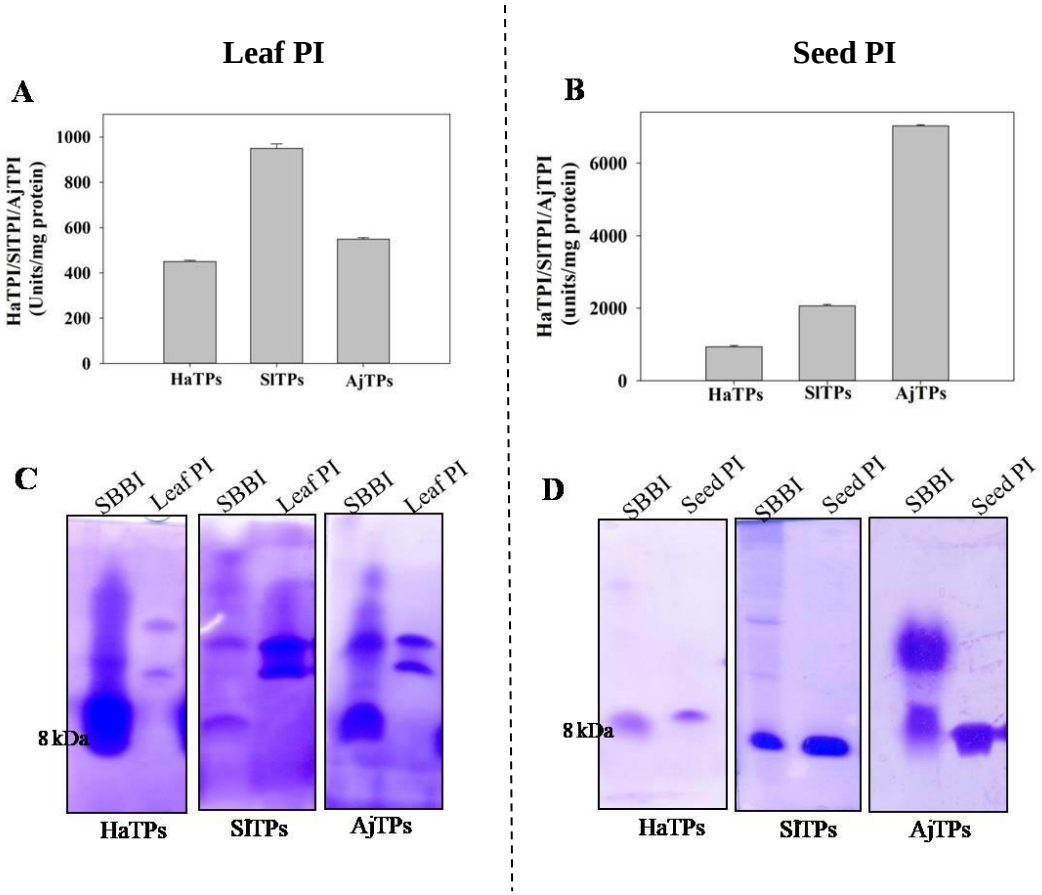


Fig. 6.12. *In vitro* inhibitory activity of leaf and seed PI. The specific activity of (A) leaf PI and (B) seed PI against midgut trypsin-like proteases of *H. armigera* (HaTPs), *S. litura* (SlTPs), and *A. janata* (AjTPs), respectively. Visualization of in-gel inhibitory activity of (C) leaf PI and (D) seed PI against HaTPs, SlTPs, and AjTPs, respectively, in gelatin SDS-PAGE. 2 µg of protein was loaded in each well, and SBBI was used as a reference marker. Bands were visualized after staining with Coomassie Brilliant blue R-250. Other details are as described in materials and methods.

The leaf coating experiments of *A. janata* revealed that a concentration-dependent reduction (2, 4, 8 µg/cm^2) in larval weight was observed when fed upon leaves coated with seed PI as compared to larvae fed on control leaves without PI treatment (**Fig. 6.13A**). The highest reduction in larval weight (≤86%) was observed at 8 µg/cm^2 of seed PI, and the mortality rate of larvae was found to be ≤40% at this treatment. Further, the larval-pupal (≤35%) and pupal-adult (≤20%) intermediate formation was also observed in larvae fed upon leaves coated with 8 µg/cm^2 of seed PI (**Fig. 13B and C; Table. 6.2**).

Likewise, *S. litura* larvae also showed a significant reduction in larval weight (≤70%) and mortality rate (≤30%) in a concentration-dependent manner (0.025, 0.05, and 0.1%) when allowed to grow on an artificial diet containing seed PI (**Fig. 6.14A**). Also, the formation of larval-pupal (≤40%) and pupal-adult (≤30%) intermediates were observed in larvae fed upon 0.1% seed PI (**Fig. 6.14B and C; Table 6.3**). The formation of malformed pupae and adults was found to be high in 0.1% seed PI treatment compared to low concentrations (0.025 and 0.05%) of seed PI treatment (**Table 6.3**).

6.3. Discussion

Correlation between the activity and expression of PIs induced in *V. mungo* leaves

PIs are the major defense proteins induced in leaves upon exposure to biotic or abiotic stress conditions (Bateman and James, 2011). Several previous studies reported that trypsin and chymotrypsin inhibitors are synthesized in all parts of the plants upon insect infestation, hormone treatment or mechanical wounding caused to even a single leaf by releasing and transporting proteinase inhibitor-inducing factor (Green and Ryan, 1972; Yu et al., 2018). Similarly, in the present study, trypsin and chymotrypsin inhibitors were induced in *V. mungo* leaves upon wounding, MJ treatment, and *H. armigera* infestation (**Figs. 6.1 and 6.2**). It was

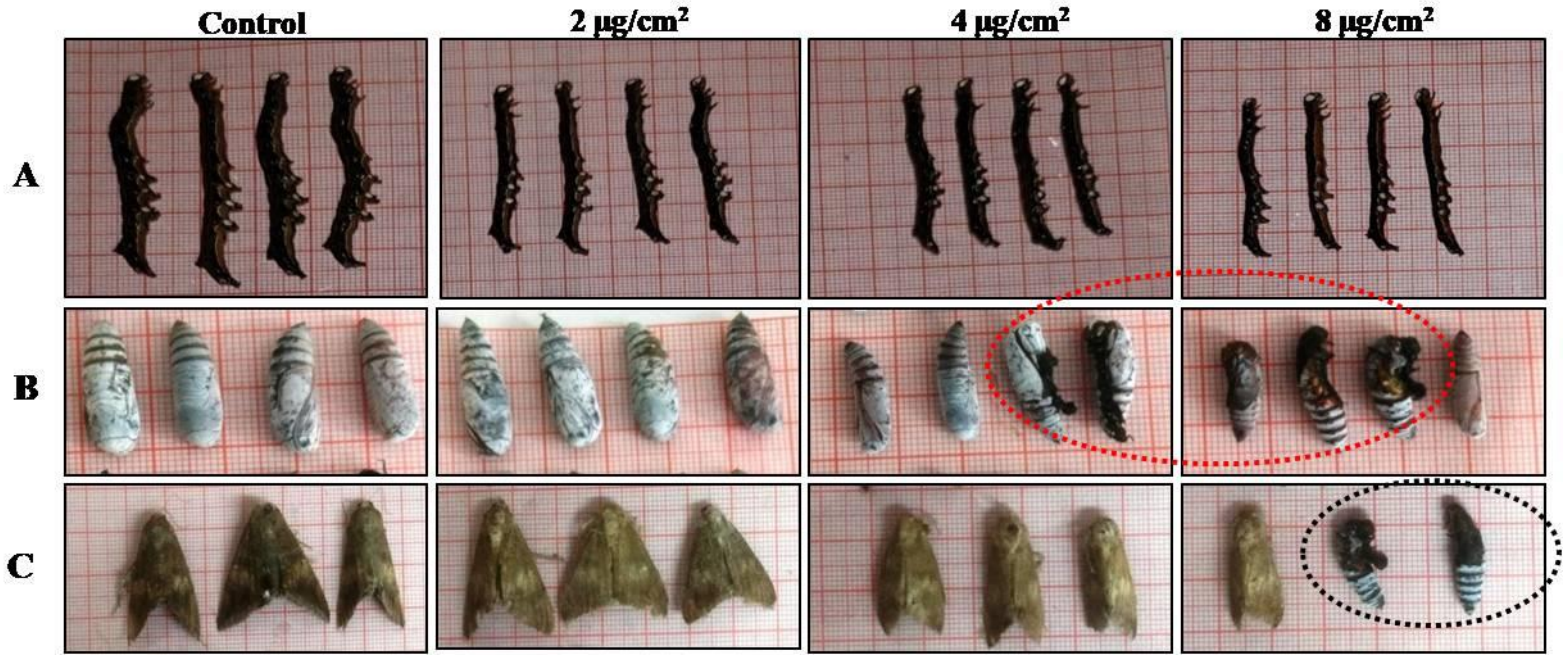


Fig. 6.13. *In vivo* inhibitory effect of seed PI on the growth and development of *A. janata* larvae (n=25). Photographs depicting the (A) reduction in the size of larvae; formation of abnormal (B) pupae and (C) adults upon feeding the castor leaves coated with seed PI (2, 4, and 8 µg/cm^2 leaf area) than larvae fed on leaves coated with buffer (control). The larval-pupal and pupal-adult intermediates formed upon treatment with seed PI were indicated in red and black color dotted circles, respectively. Other details are as described in materials and methods.

Table 6.2. Effect of seed PI on overall growth and development of *A. janata* (n=25). The larvae were allowed to feed on leaves coated with various concentrations (0, 2, 4, and 8 μg/cm^2) of seed PI from first to fifth instar stage. Other details are as described in materials and methods.

Concentration of seed PI (μg/cm^2 leaf area)	**Larval weight (% Reduction)**	**Larval mortality rate (%Total larvae)**	**Larval-pupal intermediates (% Total larvae)**	**Pupal -adult intermediates (% Total larvae)**
0	753.6 ± 26 (0)	0	0	0
2	437.08 ±17 (42.5)	10±1	0	0
4	275 ± 12 (63.47)	25±2	20±1	0
8	103.2 ± 9 (86.31)	40±3	35±3	20±2

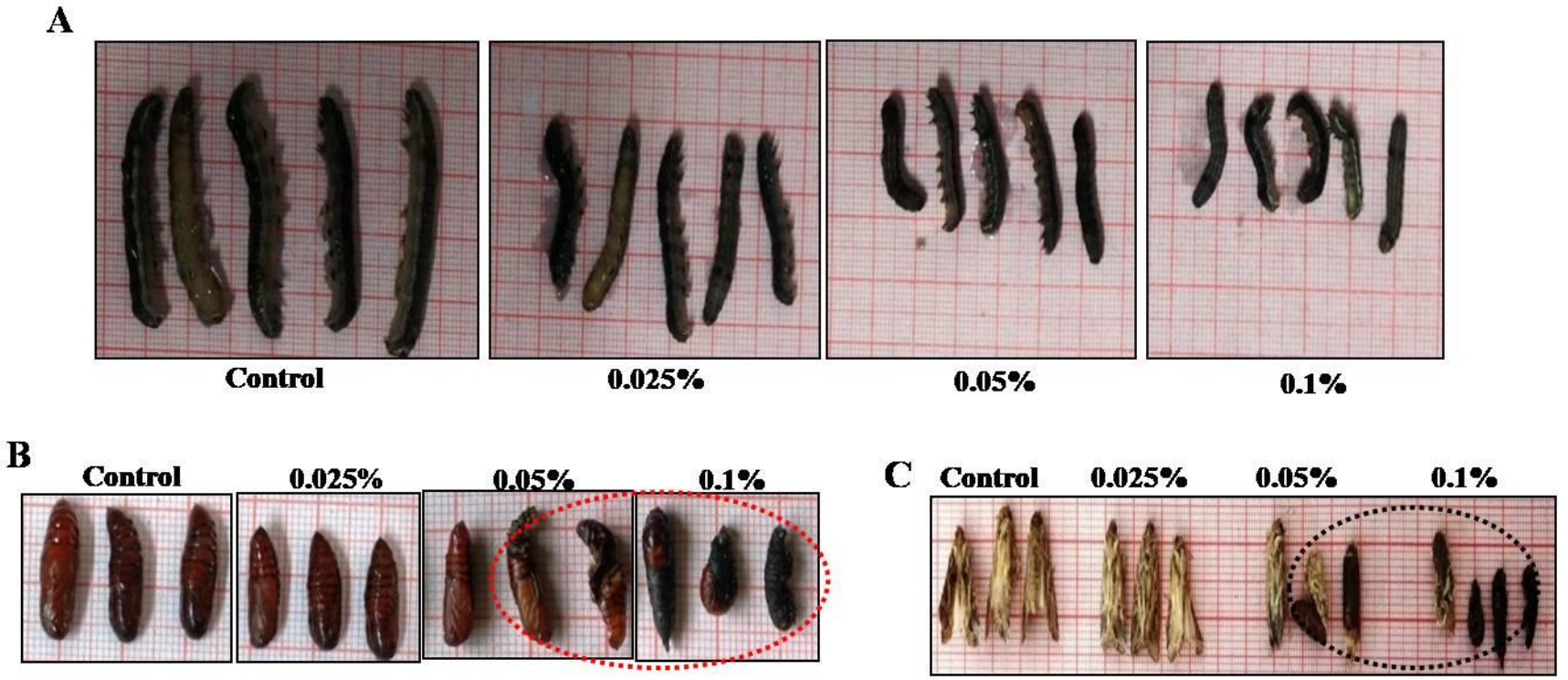


Fig. 6.14. *In vivo* inhibitory effect of seed PI on the growth and development of *S. litura* (n=25). Photographs depicting the (A) reduction in the size of larvae; formation of abnormal (B) pupae and (C) adults upon feeding with an artificial diet supplemented with seed PI than larvae fed on control diet without PI. The larval-pupal and pupal-adult intermediates formed upon feeding with seed PI were represented in red and black color dotted circles, respectively. Other details are as described in materials and methods.

Table. 6.3. Effect of seed PI on overall growth and development of *S. litura* (n=25). The larvae were allowed to feed on a diet supplemented with various concentrations (0.01, 0.025, and 0.05%) of seed PI from first to fifth instar stage. Other details are as mentioned in materials and methods.

Concentration of seed PI (%)	**Larval weight (% Reduction)**	**Larval mortality rate (% Total larvae)**	**Larval-pupal intermediates (% Total larvae)**	**Pupal-adult intermediates (% Total larvae)**
0	495±28 (0)	0	0	0
0.025	324±19(34)	5±1	0	0
0.05	283±11(42)	20±2	35±2	15±1
0.1	146±7.5 (70.5)	30±4	40±3	30±2

hypothesized that the increased rate of protease inhibitor synthesis might be due to an increased accumulation rate of protease inhibitor-inducing factor released from the wound site and its transport to the rest of the plant (Green and Ryan, 1972). Further, real-time PCR studies with BBI-specific gene primers showed that expression levels of BBI are increased upon any of the given treatments (**Fig. 6.3A-C**). Such induced expression of protease inhibitors genes (BBI, KI, potato inhibitor I, II, and aspartic and cysteine PI genes) was observed in response to wounding, MJ treatment, insect, aphid infestation, and several abiotic stress conditions (Wu and Haard, 2000; Chan et al., 2017; Malefo et al., 2020; Osmani et al., 2022). In the present study, the identification of both TI and CI activities in the crude extract and the increase in expression of BBI genes in *V. mungo* leaves under all given stress conditions suggest that the PIs induced in *V. mungo* leaves belong to the BBI family.

During evolution, plants tend to develop several regulatory components to overcome biotic and abiotic stress conditions. Among them, reactive oxygen species, such as H_2O_2, act as signaling molecules in various plant physiological processes and resistance against biotic or abiotic stress conditions (Quan et al., 2008). In the present study, an increase in H_2O_2 was observed in *V. mungo* leaves upon wounding, MJ treatment, or *H. armigera* infestation within 6 h of treatment (**Fig. 6.4A-C**), and this result is in correlation with the following studies: (i) H_2O_2 accumulation was observed in the cell wall and apoplast of the lima bean leaves near the damaged area within 3 h upon mechanical damage or infestation with *S. littoralis* (Maffei et al., 2006) and (ii) an intense burst of H_2O_2 within 3 h of aphid infestation (*Diuraphis noxia*) in wheat leaves (Moloi and Van der Westhuizen, 2006). All these observations demonstrated that wounding, MJ treatment, or infestation with *H. armigera* led to the synthesis of PIs using ROS as a signaling molecule in *V. mungo* leaves. Such induction of PIs upon wounding, herbivory infestation, and jasmonate treatment by using ROS as a signaling molecule was

reported earlier in plant species such as hybrid poplar and trembling aspen (Haruta et al., 2001; Torres et al., 2006).

Differential biochemical properties of PI purified from leaves and seeds of *V. mungo*

In the present study, chromatography techniques such as affinity and gel filtration were used to purify PI induced in leaves and those PI that are constitutively expressed in seeds (**Figs. 6.5 and 6.6**). The PI purified from mature seeds showed four-fold higher specific activity and 2.2-fold more yield than the PI purified from leaves after induction with MJ (**Table 6.1**). Besides, the qualities such as the purity, yield recovery, and specific activity of the PI purified from *V. mungo* seeds were higher when compared with PIs purified from seed varieties such as *D. biflorus, Vicia faba,* and *P. sativum* (**Table 6.1**; Ye et al., 2001; Kuhar et al., 2013; Kaur and Sohal, 2016). Also, such qualities for PI obtained from *V. mungo* leaves exposed to MJ were higher when compared with PIs purified from alfalfa leaves subjected to mechanical wounding (Brown and Ryan, 1984). Further, the molecular mass of PI purified from the seeds (8.2 kDa) and leaves (9.7 kDa) matched well with PIs reported earlier from leaves/seeds of *C. cajan* (Prasad et al., 2010c), *Phaseolus aureus* (Haq et al., 2005), and *Zea mays* (Tamayo et al., 2000) (**Fig. 6.8**). Also, the variation in molecular mass of each of the purified PI observed between Tricine SDS-PAGE and MALDI-TOF intact mass analysis could be due to slow-movement of PIs in Tricine SDS-PAGE as compared to standard protein molecular weight markers (**Figs. 6.7 and 6.8**). However, their movement and molecular mass (8 kDa) matched with soybean BBI (**Fig. 6.8**).

The PI purified from leaves as well as seeds of *V. mungo* belonged to the BBI family as revealed by dot blot assay using anti-BBI antibodies raised against *C. cajan* BBI (**Fig. 6.9**). One of the characteristic features of BBI is its existence in oligomeric forms or self-association pattern (Catalano et al., 2003; Rao and Suresh, 2007; Prasad et al., 2010a; Lokya et al., 2020). In the present study, such a self-association pattern was observed with PI purified

from leaves but not with BBI purified from seeds. The PI purified from *V. mungo* leaves existed predominantly in the dimeric form, and the existence of BBI in such dimeric form was reported earlier in plant varieties, such as *D. biflorus* and *V. unguiculata* (Kumar et al., 2004; Rao and Suresh, 2007). Besides, the existence of PI purified from the mature seeds of the T9 variety of *V. mungo* in the monomeric form in the present study matched well with the PI purified from the TAU-1 variety of *V. mungo* earlier (Prasad et al., 2010a).

Further, the *in vitro* inhibition assays and in-gel activity staining studies revealed that both leaf PI and seed PI showed inhibitory activity against bovine pancreatic trypsin and chymotrypsin (**Fig. 6.10**). These results are in agreement with PIs purified from the leaves of pigeon pea (Lomate and Hivrale, 2012) and seeds of red gram and peanut (Swathi et al., 2016; Lokya et al., 2020). However, the TI and CI activities of PIs purified from both leaves and seeds showed (i) similarity in their stability towards temperature & DTT (**Fig. 6.11C-F**) and (ii) variation in stability towards pH (**Fig. 6.11A and B**). The results obtained in the present study suggest that both leaf PI and seed PI showed significant stability towards temperatures up to 90 ^{o}C, which is evident by the retention of their TI and CI activities (**Fig. 6.11E and F**). The TI/CI activity of PI from leaves was stable from pH 3.0 to pH 9.0, while TI/CI activity of PI from seeds was stable at both acidic and basic pH but not at neutral pH (**Fig. 6.11A and B**). In the presence of DTT, both leaf and seed PI lost TI/CI activity at ≤3 mM concentration (**Fig. 6.11C and D**), possibly due to the reduction of disulfide bridges which play a significant role in the structural and functional stability of BBIs (He et al., 2017; Honda et al., 2018).

Taken together the biochemical properties examined in the present study, the following characteristic features suggest that the PIs purified from both leaves and seeds belong to BBI family: (i) low molecular mass, (ii) presence of both TI and CI activities, (iii) oligomeric nature (iv) immuno-detection with anti-BBI antibodies (v) stability in TI/CI activity against temperature, and (vi) loss of TI/CI activity upon DTT treatment.

Differential insecticidal potential of PI purified from leaves and seeds

PIs like BBI inhibit the serine proteases of insect midgut and disturb the coordination among digestive proteases leading to growth, development, and reproduction abnormalities in insects (Lawrence and Koundal, 2002). Hence the effect of leaf PI and seed PIs on midgut proteases of *A. janata*, *H. armigera*, and *S. litura* was evaluated by *in vitro* inhibition assay and activity staining studies. Both leaf PI and seed PI showed inhibition against the midgut trypsin-like proteases of *A. janata*, *H. armigera*, and *S. litura* (**Fig. 6.12**). The results obtained in the present study with leaf PI are in agreement with the PI purified from MJ-treated *C. cajan* leaves against *H. armigera*, and trypsin-inhibitors purified from MJ treated *Passiflora f. edulis flavicarpa* leaves against *Diatraea saccharalis* (Botelho et al., 2008; Lomate and Hivrale, 2012). Similarly, the results obtained with seed PI against *A. janata*, *H. armigera* and *S. litura* are in correlation with the PI purified from seeds of *C. cajan*, *C. arietinum* and *A. nilotica* (Srinivasan et al., 2005; Prasad et al., 2010b; Babu et al., 2012; Swathi et al., 2014; Mohanraj et al., 2019). However, the specific activity of seed PI against midgut proteases of these insect pests was higher when compared with leaf PI. Further, the seed PI showed potent inhibition against midgut trypsin-like proteases of *A. janata*, and *S. litura* than *H. armigera* (**Fig. 6.12**).

Effect of *in vivo* feeding of PI purified from mature seeds on *A. janata* and *S. litura*

As the *in vitro* inhibitory potential of seed PI was significant against midgut proteases of *A. janata* and *S. litura*, they were allowed to feed on seed PI-supplemented diet to evaluate its insecticidal activity. Upon feeding with increasing concentrations of seed PI, larvae of *A. janata* showed a significant reduction in their body weight (≤86%) and increased mortality rate (≤40%) than control larvae. Besides, the treated *A. janata* larvae also showed larval-pupal and pupal-adult intermediates formation (**Fig. 6.13; Table 6.2**). This effect of seed PI on the growth and development of *A. janata* larvae showed correlation with the effect of PIs purified from cultivars and wild relatives of *C. cajan*, and TAU-1 variety of *V. mungo* (Prasad et al.,

2010b; Swathi et al., 2014; Mohanraj et al., 2018). However, the insecticidal effect of seed PI, which was purified from the T9 variety of black gram in the present study, was comparatively higher when compared with the effect of PI purified from TAU-1 variety of *V. mungo*. This difference in weight reduction and mortality rate observed with PI from T9 and TAU-1 variety could be due to the difference in the feeding pattern of PIs to *A. janata* larvae (Prasad et al., 2010b).

The feeding experiments with *S. litura* larvae on seed PI-supplemented diet also showed a concentration-dependent reduction in their growth and body weight as compared to larvae fed on a control diet (**Fig. 6.14; Table 6.3**). Further, the larval mortality and larval-pupal and pupal-adult intermediates formation were also observed in treated larvae. These results are in corroboration with the previous reports of Prasad et al. (2010b) and Vasudev and Sohal (2016), where the purified PIs from TAU-1 variety of *V. mungo* and *G. max* showed low to moderate effect on the growth and development of *S. litura.* The present study reveals that the application of PIs purified from *V. mungo* seeds may provide a better approach to overcome the yield losses to crop plants caused by lepidopteran insect pests such as *A. janata* and *S. litura*.

Highlights of the study

- ✓ Protease inhibitors were induced in leaves of *V. mungo* upon wounding, MJ treatment, and *H. armigera* infestation using H_2O_2 as a signaling molecule.
- ✓ The CPE from leaves exposed to MJ or subjected to wounding or *H. armigera* infestation showed significant (i) TI/CI activity and (ii) increased expression in BBI genes in a time-dependent manner.
- ✓ The purification fold, yield recovery, and specific activity of the purified seed PI were comparatively higher than leaf PI.

- ✓ Immuno-detection studies, along with the existence of molecular mass below 10 kDa, self-association pattern, and stability in TI/CI activity towards temperature and loss in the corresponding activity after DTT treatment confirm that both the PIs purified from leaves and seeds belong to BBI family.

- ✓ The *in vitro* assays showed seed PI possessed more potential than leaf PI in inhibiting the midgut proteases of *S. litura, A. janata,* and *H. armigera.*

- ✓ *In vivo* feeding experiments with seed PI revealed its potency in the management of *A. janata* and *S. litura*.

Chapter 7

Summary and Conclusions

Summary and Conclusions

Protease inhibitors (PIs) are small defense proteins that protect plants against pest attacks by inhibiting their midgut proteases (Haq et al., 2004; Ramalho et al., 2018; Clemente et al., 2019; Marathe et al., 2019). Though extensive research has been carried out on elucidating the insecticidal potential of PIs, a continuous screening of PIs from non-host and wild-relatives is essential to overcome the possibility of insect resistance (Macedo et al., 2004; Sharma and Suresh, 2015; Mohanraj et al., 2019; Lokya et al., 2020).

The two serine PIs, such as BBI and KI are well characterized for their differential biochemical and functional properties. Generally, most of the seed varieties contain both BBI and KI. However, several earlier studies mainly focused on purifying either BBI or KI, while very few research groups have reported the recovery and purification of both BBI and KI from the same seed material by using a series of time-consuming and laborious steps. Hence, in the present study, **Chapter 4** describes a new protocol to purify BBI and KI from three model seed varieties such as *V. radiata*, *C. platycarpus*, and *R. sublobata* in a short time (<24h). In this method, the CPE is directly subjected to mild TCA (2.5%) extraction while heating at 70 ^{o}C. Further, the supernatant and pellet fractions are subjected to trypsin affinity chromatography to purify BBI and KI, respectively (**Fig. 4.1A-F**). The eluted BBI fractions are pure, while the KI enriched fractions showing some impurities are subjected to sodium acetate extraction (100 mM, pH 4.0) to obtain pure KI. About 0.35 to 0.45 mg pure BBI (VrBBI/CpBBI/RsBBI) and 0.1 to 0.134 mg of pure KI (VrKI/CpKI/RsKI) per gram seed were purified by using this method. The differences in the yield of BBIs and KIs might be due to the abundance of BBI in the seed itself when compared with KI. Further, the purification fold of the BBIs and KIs ranged between 37 to 55 fold and 24.5 to 26 fold, respectively (**Table 4.1**).

The electrophoretic profile of the purified BBI(s) and KI(s) showed a single monomeric band in Tricine-SDS-PAGE (**Fig. 4.2A-C**). Also, loading with increasing concentrations of protein, BBI(s) showed a self-association pattern (**Fig. 4.3A-C**). Further, the ESI/MALDI-TOF studies revealed that VrBBI, CpBBI and RsBBI showed a predominant monomeric peak at 8.92, 7.92 and 9.21 kDa, respectively, and a minor dimeric peak at 17.81, 15.77 and 18.42 kDa, respectively, between m/z 5,000-20,000 (**Fig. 4.4A-C**). Conversely, VrKI, CpKI and RsKI showed only a monomeric peak at 19.25, 19.48 and 19.28 kDa, respectively (**Fig. 4.4D-F**). The appearance of bands in the western blot after probing with an anti-BBI antibody confirmed the purified low molecular mass (<10kDa) proteins as BBIs. Further, the absence of bands in KI-loaded wells of western blot clearly indicated that the KI samples are pure and free from contamination of BBI (**Fig. 4.5**). MALDI MS-MS analysis of the purified BBIs and KIs showed 100% similarity with BBI/KI of *G. max*, and *P. sativum*, respectively (**Figs. 4.6-4.11**).

BBI(s) showed inhibition against both trypsin and chymotrypsin, whereas KI(s) inhibited trypsin alone in both *in vitro* assay and in-gel activity staining studies (**Fig. 4.12A-D**). Further, both BBI(s) and KI(s) showed a non-competitive mode of inhibition against their respective proteases (**Figs. 4.13-4.15**). The BBI(s) and KI(s) are stable at a wide range of pH conditions (2.0 and 12.0), with ≤15% loss in their TI and CI activities. Further, the BBI(s) are stable from 20 to 90 ^{o}C, whereas KI(s) lost their TI activity at or above 80 ^{o}C. Upon treatment with DTT, BBI(s) showed ≥ 90% loss in both TI and CI activities at 1 mM DTT concentration, while the TI activity of KI(s) was stable at 1 mM DTT (**Figs. 4.16-4.18**).

The CD spectroscopy studies revealed that both BBI and KI contain random coils predominantly than β-turns/β-sheets and α-helices (**Table 4.2**). Further, upon increasing the temperature from 20-90 ^{o}C, BBI(s) did not show any major changes in their ellipticity, while KI(s) showed a drastic change in their ellipticity at ~199 nm above 70 ^{o}C, indicating that

BBI(s) are more stable at high temperatures than KI(s) (**Fig. 4.19A-F**). Furthermore, upon DTT treatment, BBI(s) showed a significant decrease in their ellipticity, while KI(s) did not show any significant change in their ellipticity, indicating KI(s) are stable to DTT treatment when compared with BBI(s) (**Fig. 4.20A-F**). The protein dynamics studies using fluorescence spectroscopy revealed that both BBI(s) and KI(s) showed a decrease in fluorescence emission intensity upon increasing the temperature from 20 to 90 ^{o}C. However, KI(s) showed a red shift towards higher wavelength upon heating at or above 80 ^{o}C, indicating that KI(s) are partially unfolded at high temperatures (**Fig. 4.21A-F**). Among BBI(s), VrBBI is more sensitive to DTT treatment and showed conformational change when compared with CpBBI and RsBBI, while among KI(s), CpKI is more sensitive to DTT and showed conformational change than VrKI and RsKI (**Fig. 4.22A-F**).

As it is evidenced that BBI/KI possess several biological applications such as insecticidal and therapeutic properties, the BBI and KI purified from *V. radiata*, *C. platycarpus* and *R. sublobata* were evaluated for their potential in pest management and therapeutics. The two lepidopteran pests chosen in the present study are the voracious feeders of several economically important crop plants causing severe damage to crop yields. The wild relatives of pigeon pea: C. *platycarpus* and *R. sublobata* are non-host plants to *A. janata* and host plants to *H. armigera*. Similarly, *V. radiata* is a non-host plant to *A. janata* and host plant to *H. armigera* (**Fig. 5.1**). As the biological significance of the purified BBIs/KIs from these plant varieties are not reported, **Chapter 5** summarizes the insecticidal (*in vitro* inhibition assays, *in vivo* feeding experiments), anticancer and antibacterial properties (MTT assays) of BBIs and KIs purified from *V. radiata*, *C. platycarpus* and *R. sublobata*.

The *in vitro* studies conducted with the BBIs/KIs purified in the present study against midgut proteases of *A. janata* and *H. armigera* revealed that VrBBI/CpBBI/RsBBI are specific against trypsin-like midgut proteases of *A. janata* ($IC_{50} \leq 96$ng) and

VrKI/CpKI/RsKI are potent in inhibiting the trypsin-like midgut proteases of *H. armigera* ($IC_{50} \leq 150$ng**; Figs. 5.2-5.4**). Further, feeding of BBI(s)/KI(s) to *A. janata* and *H. armigera* larvae resulted in larval weight reduction at each instar stage (second, third, fourth and fifth) in a concentration-dependent manner as compared to larvae fed on control diet/leaf i.e., without BBI(s)/KI(s) (**Figs. 5.5A-F and 5.9A-F**). However, the overall effect of BBI(s) is more significant than KI(s) in reducing the *A. janata* larval weight (≤80%) and mortality (≤35%). Besides, feeding of *A. janata* larvae with BBI(s) also reduced their corresponding pupal weights (≤64%), followed by a delay in pupal emergence (10-12 days), formation of larval-pupal (≤50%) and pupal-adult intermediates (≤25%) (**Figs. 5.6-5.8; Table 5.1**). Further, the effect of KI(s) is more significant than BBI(s) in reducing the *H. armigera* larval weight with deleterious effects on its life cycle in terms of larval weight reduction (≤68%), mortality (≤36%), delay in pupal emergence (8-12 days), pupal weight reduction (≤57%), along with formation of larval-pupal (≤52%) and pupal-adult intermediates (≤30%) (**Figs. 5.10-5.12; Table 5.2**). Besides, the BBI and KI purified from *C. platycarpus* and *R. sublobata* were more significantly effective in inhibiting the growth and development of *H. armigera* and *A. janata* at all instar stages of the life cycle compared with BBI and KI purified from *V. radiata* (**Figs. 5.5-5.12**).

Among all the BBIs/KIs purified from *V. radiata, C. platycarpus* and *R. sublobata*, RsKI showed inhibition in the growth of breast cancer cell lines (MCF7 - 33%, MDA-MB-231 - 14%), than cervical cancer cell lines (HeLa - 7.5%; **Fig. 5.13**). Besides, all the BBIs and KIs purified in the present study inhibited the growth of *S. aureus*, a gram-positive pathogenic bacterium with varied optimal concentrations (**Fig. 5.14A-F**).

The importance of PIs present in seeds was elucidated well. However, the characterization and significance of PIs induced in leaves are poorly reported. Hence **chapter 6** summarizes the induction of PIs in leaves of *V. mungo* upon wounding, MJ

treatment, and *H. armigera* infestation at different time points (6, 12, 24, 36, 48, 60 and 72 h). The *in vitro* inhibition assay of the crude extracts prepared from the wounded, MJ treated, and

H. armigera infested leaves showed both TI and CI activities in response to all stimuli (**Figs. 6.1A-C and 6.2A-C**). However, the induction of trypsin and chymotrypsin inhibitors is high in MJ treated leaves when compared with wounded and *H. armigera* infested leaves. Since the induced PIs showed both TI and CI activities, a characteristic feature of Bowman-Birk inhibitor (BBI) family of PIs, real-time PCR analysis was performed using BBI-specific gene primers to confirm the nature of induced PIs. Correlating with the TI and CI activities, the relative expression of BBI-specific genes was high at 72 h in all the samples (**Fig. 6.3A-C**). In light of the crucial role played by ROS in signal-mediated defense mechanisms, the amount of H_2O_2 accumulated in leaves upon wounding, MJ treatment, and *H. armigera* infestation was measured. The increase in H_2O_2 was high in leaves subjected to wounding, MJ treatment or *H. armigera* infestation when compared with control, indicating that ROS might be acting as a signal during the induction of trypsin and chymotrypsin inhibitors (**Fig. 6.4A-C**).

To evaluate the differences in PIs induced in leaves and PIs constitutively present in seeds, both leaf PIs and seed PIs were purified by using ammonium sulfate fractionation and chromatography techniques (**Figs. 6.5 and 6.6**). The purification fold, yield recovery, and specific activity of the purified seed PIs (116 fold, 33% and 983 TIU/mg protein, respectively) were comparatively higher than purified leaf PI (37 fold, 15% and 215 TIU/mg protein, respectively; **Table 6.1**). Further, the leaf and seed PIs showed molecular mass between 10-15 kDa in Tricine SDS-PAGE and 9.7 & 8.2 kDa in intact mass analysis (**Figs. 6.7 and 6.8**). The existence of molecular weight below 10 kDa, dot blot with anti-BBI antibody, stability in TI and CI activities against temperature, and loss in activity after DTT

treatment confirmed that both the purified leaf and seed PIs belong to BBI family (**Figs. 6.6-6.11**). As *V. mungo* is a non-host plant to *A. janata* and a host-plant to *H. armigera* and *S. litura*, *in vitro* inhibition assay was performed to evaluate the efficacy of leaf and seed PIs in inhibiting the trypsin-like midgut protease activity of these three insect pests. The *in vitro* assays revealed that seed PI possessed more potential than leaf PI in inhibiting the midgut proteases of *S. litura, A. janata* and *H. armigera* (**Fig. 6.12A-D**). Further, feeding of seed PI to *S. litura* and *A. janata* showed a negative effect on the growth and development of *S. litura* and *A. janata* larvae in terms of reduction in larval weight, formation of pupal, adult intermediates and mortality, indicating the significance of seed PIs in the pest management (**Figs. 6.13A-C and 6.14A-C; Table 6.2 and 6.3**).

Major Conclusions

- ✓ A simple and rapid method (<24 h) was optimized for the separation and purification of both BBI and KI from the seeds of *V. radiata*, *C. platycarpus* and *R. sublobata*.
- ✓ Both purified BBI(s) [VrBBI, CpBBI and RsBBI] and KI(s) [VrKI, CpKI and RsKI] were confirmed by Western blot and MALDI-MS-MS studies.
- ✓ The purified BBI(s)/KI(s) varied significantly in their structural and functional stability against temperature and DTT.
- ✓ The *in vitro* assays and *in vivo* feeding experiments together revealed that BBIs are effective in the management of *A. janata* while KIs are effective in the management of *H. armigera*.
- ✓ The BBI/KI purified from wild relatives (*C. platycarpus* & *R. sublobata*) is more efficient in controlling the insect pests than that of BBI/KI purified from the cultivar variety (*V. radiata*).
- ✓ RsKI was effective in inhibiting the growth of MCF 7 breast cancer cell lines.
- ✓ VrKI is more potent in inhibiting the growth of *S. aureus*, than VrBBI, CpBBI/CpKI and RsBBI/RsKI.

- ✓ Leaf PI and seed PI purified from *V. mungo* showed all the characteristic features of the BBI family and inhibited the midgut proteases of *S. litura*, *A. janata* and *H. armigera*. However, the yield recovery and inhibitory activity of seed PI are high when compared with leaf PI.
- ✓ The *V. mungo* seed PI showed a significant insecticidal effect on the growth and development of *A. janata* and *S. litura*.

Future Prospectives

Protease inhibitors such as BBI and KI are reported as potential candidates in pest management. However, the role of various biological factors providing resistance to insects against PIs, such as microbes, upregulation or downregulation of proteins/metabolites/enzymes, is unclear. Hence, identifying these factors and combining PIs with other natural compounds that can inhibit these factors would help for better pest management and reduce yield losses. Further, PIs also possess therapeutic properties in treating several human diseases because PIs can reach the target site easily due to their stability at a wide range of pH conditions. However, as PIs are regarded as anti-nutritional agents, research must be done on human consumption of plant PIs and their efficacy after digestion.

Chapter 8

Literature Cited

Literature Cited

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1. **Mariyamma Gujjaralapudi**, Bharti Kotarya, Soundappan Mohanraj, Deepali Gupta, Elaprolu R Prasad, Arunasree M Kalle, Jagdish Jaba, Duraimurugan Ponnusamy, Kollipara Padmasree (2023) Development of a rapid process for purification of Bowman-Birk and Kunitz inhibitors from legume seeds, and evaluation of their biophysical, insecticidal, and anti-microbial properties. *International Journal of Biological Macromolecules*, **238**: 124050.

2. Marri Swathi, Vadthya Lokya, **Mariyamma Gujjarlapudi**, Shreya Verma, Pallabi Kisku, Nukapangu Sravan Kumar and Kollipara Padmasree (2021) Proteinase Inhibitors. In: Omkar (eds) Molecular Approaches for Sustainable Insect Pest Management. Springer, Singapore.

3. S. Soundappan Mohanraj, **Mariyamma Gujjarlapudi**, Vadthya Lokya, Nalini Mallikarjuna, Aparna Dutta-Gupta, Kollipara Padmasree (2019) Purification and characterization of Bowman-Birk and Kunitz inhibitors from seeds of *Rhynchosia sublobata* (Schumach.) Meikle, a wild relative of pigeonpea". *Phytochemistry*, **159**:159-171.

4. Marri Swathi, Soundappan S. Mohanraj, Vanka Swaroop, **Mariyamma Gujjarlapudi**, Nalini Mallikarjuna, Aparna Dutta-Gupta and Kollipara Padmasree (2015) Proteinase inhibitors from *Cajanus platycarpus* accessions active against pod borer *Helicoverpa armigera*. *Acta Physiology Plantarum* **37**:242.

5. **Mariyamma Gujjarlapudi,** Bharti Kotarya, Deepali Gupta, Kollipara Padmasree. Significance of Kunitz inhibitors over Bowman-Birk inhibitors of *Rhynchosia sublobata* in inhibiting the growth of *H. armigera* (**Under preparation**).

6. **Mariyamma Gujjarlapudi,** Bharti Kotarya, Deepali Gupta, Kollipara Padmasree. Potential use of methyl jasmonate inducible protease inhibitors from black gram leaves in the management of lepidopteran pests (**Under preparation**).

Patent Filed

7. Kollipara Padmasree, M. K. Aruna sree, **Mariyamma Gujjarlapudi**, Bharti Kotarya: Method for separation and purification of multifunctional proteins Bowman-birk and Kunitz inhibitors (2021) Intellectual property India, Application No. 202141017396 Application status: In order to grant under section 43, awaiting NBA approval.

Conferences and workshops attended

1. **Mariyamma Gujjarlapudi and Kollipara Padmasree** (2022) A comparative study on the biochemical and insecticidal properties of inducible and constitutive protease inhibitors purified from black gram (*Vigna mungo*). Symposium on Frontiers in Biotechnology and Bioinformatics, was held on 7th – 8th November 2022, at the School of Life Sciences, University of Hyderabad, Hyderabad, India, 500 046 (Oral presentation).

2. **Mariyamma Gujjarlapudi and Kollipara Padmasree** (2019) Purification and Biochemical characterization of inducible proteinase inhibitors from leaves of black gram and monitoring their insecticidal potential. National conference on "Future India Science and Technology: Research innovations in Biology" held on 14th and 15th February at Jain University, Bangalore, India, 560 011 (Poster presentation).

3. **Mariyamma Gujjarlapudi, Vanka Swaroop, Vadthya Lokya and Kollipara Padmasree** (2015) Biochemical characterization of host and non-host herbivore induced protease inhibitors in leaves of black gram (*Vigna mungo*). "Third international plant physiology congress on challenges and strategies in plant biology research" was held from 11th – 14th December at JNU, New Delhi, India, 110 067 (Poster presentation).

4. **Mariyamma Gujjarlapudi (2023)** Basic to Advanced Bioinformatics training course organized by centre of Bioinformatics training and research, Lucknow from 20th Feb to 2nd March 2023 (Participation).

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Development of a rapid process for purification of Bowman-Birk and Kunitz inhibitors from legume seeds, and evaluation of their biophysical, insecticidal, and antimicrobial properties

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ABSTRACT

Bowman-Birk inhibitor (BBI ~10 kDa) and Kunitz inhibitor (KI ~20 kDa) are serine protease/proteinase inhibitor(s) [PI(s)] ubiquitously found in several Leguminous plant species with insecticidal and therapeutic properties. Due to narrow molecular mass differences, the separation of these inhibitors from a single seed variety is tedious. The present study is aimed to develop a rapid protocol (<24 h) for purifying BBI and KI from legume seeds using mild trichloroacetic acid (TCA) extraction followed by trypsin-affinity chromatography. The mature seeds of *Vigna radiata* and *Cajanus platycarpus* are used as a model to purify BBI and KI using this protocol. The BBI and KI purified from the seeds of *V. radiata* are labeled as VrBBI & VrKI, and *C. platycarpus* are labeled as CpBBI & CpKI, respectively. These PIs are confirmed by immunodetection and MALDI-TOF studies and further characterized for their structural (CD & fluorescence spectroscopy) and functional properties (temperature & DTT stability). BBI(s) purified using the above process are effective in the management of castor semi-looper '*Achaea janata*', while KI(s) are effective in the management of pod borer '*Helicoverpa armigera*'. Besides, both BBI(s) and KI(s) have significant potential in controlling the growth of methicillin-sensitive '*Staphylococcus aureus*', a gram-positive pathogenic bacterium.

1. Introduction

Legume seeds are the repositories of protease/proteinase inhibitor(s) [PI(s)], *viz.* Bowman-Birk inhibitor (BBI) and Kunitz inhibitor (KI), the primary defense molecules involved in protecting plants against various biotic (pests and pathogens) and abiotic (salinity, drought, and heavy metal) stresses [1–6]. Apart from stress tolerance, BBI and KI possessed several applications in the biomedical field such as anti-coagulant, anti-inflammatory, anti-tumor, anti-oxidant, and anti-microbial properties [7–13]. BBIs are highly stable small proteins (Mr of ~8–10 kDa) and possess two inhibitory loops active against two identical or non-identical proteases and fourteen cysteine residues which form seven disulfide bridges. KIs are proteins of Mr ~18–24 kDa and contained a single reactive site and four cysteine residues that form two disulfide bridges. However, both families are reported to show either competitive or non-competitive mode of inhibition against trypsin and chymotrypsin

Abbreviations: AjTPs, *Achaea janata* midgut trypsin-like proteases; BAPNA, N-α-benzoyl-DL-arginine-*p*-nitroanilide; BBI, Bowman-Birk inhibitor; CI, chymotrypsin inhibitor; CpBBI, *Cajanus platycarpus* Bowman-Birk inhibitor; CpKI, *Cajanus platycarpus* Kunitz inhibitor; CPE, crude protein extract; GLUPHEPA, N-glutaryl-*L*-phenylalanine-*p*-nitroanilide; HaTPs, *Helicoverpa armigera* midgut trypsin-like proteases; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; PI(s), protease inhibitor(s); TCA, trichloroacetic acid; TI, trypsin inhibitor; VrBBI, *Vigna radiata* Bowman-Birk inhibitor; VrKI, *Vigna radiata* Kunitz inhibitor.

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Proteinase Inhibitors

7

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

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Sodium acetate

ABSTRACT

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

1. Introduction

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

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

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ORIGINAL ARTICLE

Proteinase inhibitors from *Cajanus platycarpus* accessions active against pod borer *Helicoverpa armigera*

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Abstract *Cajanus platycarpus*, a wild relative of *Cajanus cajan*, is an important source for various agronomically desirable traits, including resistance towards pod borer, *Helicoverpa armigera*. In the present study, the inhibitory activity of proteinase inhibitors (PIs) present in crude protein extracted from different accessions of *C. platycarpus* and cultivars of *C. cajan* was evaluated against *H. armigera* under in vitro and in vivo conditions. The PIs active against *H. armigera* gut trypsin-like proteinases (HGPs), referred to as 'HGPIs', were more pronounced in mature dry seeds of *C. platycarpus* accessions when compared with cultivars, which is also evident through gelatin activity staining studies. Therefore, the inhibitory activity of HGPIs was further evaluated in various plant organs of *C. platycarpus* accessions, such as leaves, flowers, pods, developing seeds at 8–10 days (DAP-I), 18–20 days (DAP-II), and 28–32 days after pollination (DAP-III). However, the HGPI activity was more pronounced in mature dry seeds > DAP-III > DAP-II > DAP-I > flowers > pods > leaves. The observed quantitative allocation of HGPIs closely resembled "Optimal Defense Theory". Further, bioassays demonstrated that there was a significant reduction in the body weight of the larvae fed upon crude PI extracts of *C. platycarpus* accessions with concomitant increase in mortality rate and the formation of larval–pupal intermediates. Nevertheless, such changes were not observed when the larvae were fed on crude PI extracts of C. *cajan* cultivars. These results suggest that the PI gene(s) from *C. platycarpus* accessions could be exploited in the management of *H. armigera* by introgression into *C. cajan* cultivars.

Keywords Gelatin activity staining · Optimal defense · Pigeonpea · Proteinase inhibitor · Proteinases

Abbreviations

CI	Chymotrypsin inhibitor
DAP	Days after pollination
HGPs	*Helicoverpa armigera* gut (trypsin-like) proteinases
HGPIs	*Helicoverpa armigera* gut (trypsin-like) proteinase inhibitors
PIs	Proteinase inhibitors
TI	Trypsin inhibitor

Introduction

Pigeonpea (*Cajanus cajan* L. Millsp) is one of the most important crops cultivated in southern Asia and eastern Africa (Sujana et al. 2008). Although more than 200 species of insects are known to infest this crop, its production is most affected by pod borer *Helicoverpa armigera* (Reed and Lateef 1990). *H. armigera* is polyphagous and well

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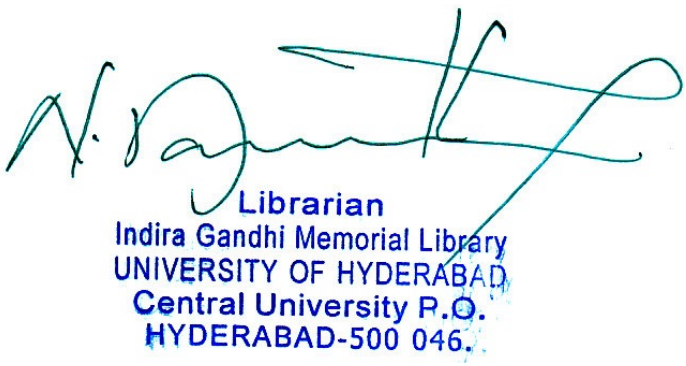
Characterization of Bowman-Birk and Kunitz Protease Inhibitors from Selected Legumes with Special Reference to Development of a Rapid Protocol for Their Isolation

by Mariyamma Gujjarlapudi



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