Characterization of Bowman-Birk Inhibitor from Peanut and Evaluation of its Insecticidal and Anticoagulant Properties

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By

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DECLARATION

I hereby declare that the work presented in this thesis entitled **"Characterization of Bowman-Birk Inhibitor from Peanut and Evaluation of its Insecticidal and Anticoagulant Properties"** has been carried out by me under the supervision of Prof. K.P.M.S.V. 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 Inhibitor from Peanut and Evaluation of its Insecticidal and Anticoagulant Properties"** submitted by **Vadthya Lokya,** bearing registration number **12LTPH02** in partial fulfillment of the requirements for award of Doctor of Philosophy in the **Department of Biotechnology & Bioinformatics, School of Life Sciences** is a bonafide work carried out by him under my supervision and guidance.

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

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

A. Published in the following Peer-reviewed Journals.

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Lokya Vadthya

Abbreviations

PnCPI	Peanut crude proteinase inhibitor extract
PIs	Proteinase inhibitors
BBIs	Bowman-Birk inhibitors
PnBBI/rPnBBI	Native peanut BBI/Recombinant peanut BBI
SBBI	Soybean Bowman-Birk inhibitor
PMSF	Phenylmethylsulfonyl fluoride
TLCK	*N*α-Tosyl-L-lysine chloromethyl ketone hydrochloride
TPCK	*N*p-Tosyl-L-phenylalanine chloromethyl ketone
BSA	Bovine serum albumin
PVP	Polyvinylpyrrolidone
BAPNA	*N*-α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride
GLUPHEPA	*N*-glutaryl-L-phenylalanine-*p*-nitroanilide
H. armigera	*Helicoverpa armigera*
S. litura	*Spodoptera litura*
TI	Trypsin inhibitor
CI	Chymotrypsin inhibitor
HaGP/SlGP	*H. armigera/S. litura* midgut proteases
HaTP/SlTP	*H. armiger/S. litura* midgut trypsin-like proteases
HaCP/SlCP	*H. armigera/S. litura* midgut chymotrypsin-like proteases
HaTPI/SlTPI	*H. armigera/S. litura* midgut trypsin-like protease inhibitor
2-DE	Two-dimensional gel electrophoresis
IPG	Immobiline pH gradient
IEF	Isoelectric focusing
P^I	Isoelectric point
DTT	Dithiothreitol
IDA	Iodoacetamide
CD	Circular dichroism
IC_{50}	Half maximal inhibitory concentration
VTE	Venous thromboembolism
DVT	Deep vein thrombosis
PE	Pulmonary embolism
SPR	Surface plasmon resonance
RU	Response Unit
K_a	Association rate constant
K_d	Dissociation rate constant
K_D	Equilibrium dissociation constant

Contents

This thesis is dedicated to

My parents

Chapter 1

Introduction and Review of Literature

Introduction and Review of Literature

Insect pests have been a significant threat to agriculture by ravaging crops and food stocks. A great global challenge of the present century was to meet the food demand of expanding world-wide population besides maintaining its production and conserving biodiversity (Bradshaw et al., 2016). The crop losses due to herbivorous insect pests are estimated to be quite high in both developed and developing countries. The reported pre-harvest and post-harvest crop losses are equivalent to 10-16% of agricultural yield (Bebber et al., 2013; Bradshaw et al., 2016). Annually, three million tonnes of pesticides worth US$ 40 billion are applied throughout the world to control insect pests (Pimentel and Peshin, 2014). In this scenario, usage of biological control agents in the management of insect pests is a milestone to secure the food as well as to avoid the impact of synthetic pesticides on the environment. Parallel to the increased application of biocontrol agents in the management of insect pests, there is also an urgent need to explore for new biocontrol agents, since insect pests adapt and evolve dynamically to survive and sustain in nature.

In India, which is dependent on the agricultural-based economy, the estimated regular crop losses due to phytophagous insect pests were US$ 36 billion (Singh et al., 2014; Dhaliwal et al., 2015; Arora and Dhawan, 2017). For instance, in the year 2000, cotton is the most affected crop (50% loss) among the economically important crops, and the loss is much higher when compared with the pre-green revolution era. However, in the year 2002, the introduction of Bt cotton in India decreased the crop loss and made a significant improvement in the agricultural economy as it expanded from 50,000 ha in 2002 to 11.6 million ha in 2014 (James, 2014). Likewise, plant Proteinase Inhibitors (PIs) are identified as alternative biocontrol agents which effectively inhibit the proteolysis and render detrimental effects on larval growth and development (Ryan, 1990; De Leo et al., 2002). There are several reports on transgenic plants expressing PIs which conferred substantial resistance to lepidopteran insect pests such as *Helicoverpa armigera* and *Spodoptera litura*. These pests are known to feed on several economically important crops such as cotton,

pigeon pea, chickpea, corn, peanut, several vegetables and horticultural crops (CAB International, 2018; Hilder and Boulter, 1999; Haq et al., 2004; Macedo et al., 2015). Therefore, the present study is aimed towards isolation and characterization of insecticidal properties of a specific family of PIs "Bowman-Birk inhibitors (BBIs)" from an advanced interspecific variety (4368-1) of peanut which is developed in ICRISAT by crossing a synthetic amphidiploid generated from wild Arachis species with a tetraploid cultivar for the inheritance of resistant traits (Mallikarjuna et al., 2011; Fávero et al., 2015). Further, these BBIs are examined for their biochemical properties.

PIs also gained significant importance in the field of medicine due to their intrinsic ability to inhibit the action of specific proteases involved in the pathophysiology of several human diseases (Turk, 2006; Oliva and Sampaio, 2009; Scott and Taggart, 2010). Though PIs are considered as antinutritional factors, several recent studies reported their role in various therapeutic interventions (Losso, 2008; Srikanth and Chen, 2016). They are known to possess several therapeutic properties such as anti-inflammatory (Ware et al., 1999; Losso, 2008; Safavi and Rostami, 2012; Kobayashi, 2013; Routray, 2018; Shamsi et al., 2018; Rodrigues et al., 2019), anti-tumour (Kennedy, 1998; Ferreira et al., 2013; Srikanth and Chen, 2016; Laparra et al., 2019), anticoagulant (Hayashi et al., 1994; Oliva et al., 1996; Pinto et al., 2010; Patil et al., 2012; Brito et al., 2014; Salu et al., 2014; Hamad et al., 2017), anti-Alzheimer (Shamsi et al., 2016; Akbari et al., 2019) anti-leishmanial (Pramanik et al., 2019) and anti-HIV (Fang and Ng, 2015; Ma et al., 2018) activities. Therefore, in the present study, peanut BBI is examined for its anticoagulant properties parallel to its insecticidal potential.

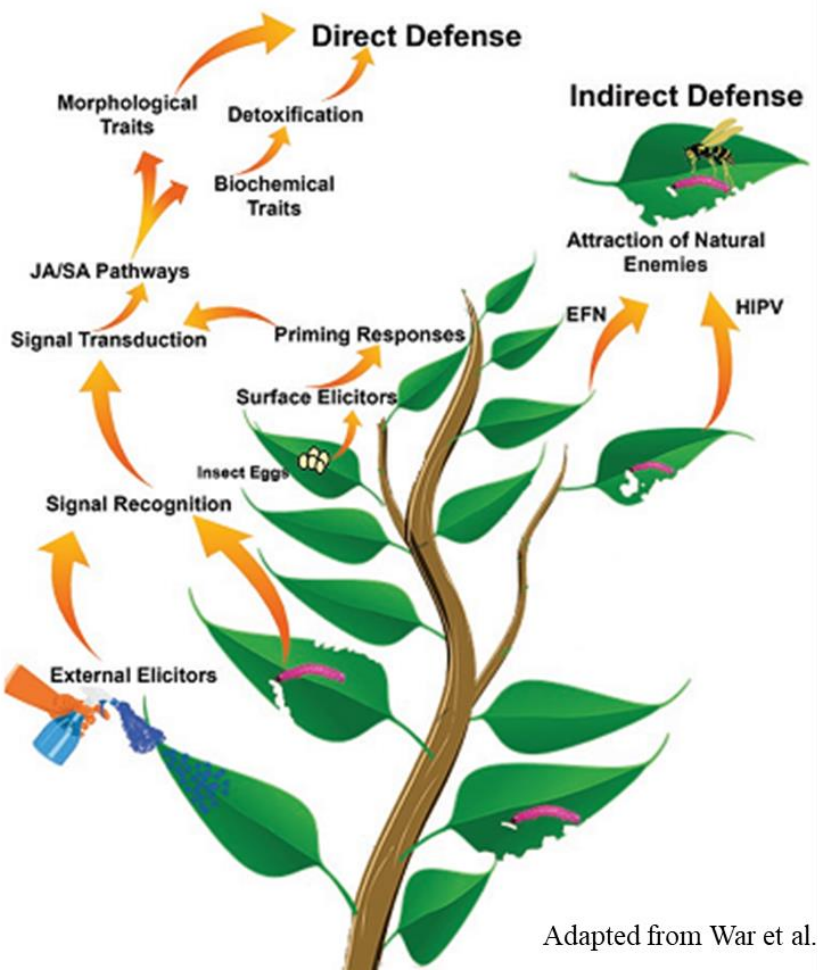
1.1. Plant Proteinase Inhibitors

Insect-Plant co-evolution is a continuous process which resulted in the diversification of their morphological, biochemical and behavioural traits in both organisms under the natural selection pressure (Zhao et al., 2009; Karban, 2011; War et al., 2012). In this scenario, plants respond to

insect herbivory by producing various biochemical and morphological defense responses which affect the insect growth and development directly or indirectly besides attracting natural enemies of insect pests by its volatiles (**Fig. 1.1**; Karban, 2011; Matthias, 2018; War et al., 2018). The biochemical defense response of plants includes an array of toxic secondary metabolites, enzymes and enzyme inhibitors such as ribosomal inactivating proteins, lectins, arcellins, chitinases, amylase inhibitors and PIs. Thus, PIs are known to play a significant role in plant defense response against insect pests (Jamal et al., 2013; Zhu-Salzman and Zeng, 2015).

Plant PIs are small natural antagonists of proteinases which binds to them by standard substrate like mechanism (Laskowski and Kato, 1980). They are ubiquitously distributed in plants (Richardson, 1977; Shamsi et al., 2016), animals (España et al., 2007; Ng et al., 2012) and microorganisms (Ishihara et al., 2006; Bijina et al., 2011; Harish and Uppuluri, 2018). In plants, PIs combat against herbivorous insects (Ryan, 1990; Swathi et al., 2014; Mohanraj et al., 2018), nematodes (Williamson and Hussey, 1996; Ali et al., 2017) and microbial pathogens (Paiva et al., 2013; Rustgi et al., 2018) by inhibiting their cognate proteases. The insecticidal potential of PIs has been attributed to inhibition of proteases involved in protein digestion which in turn results in deprivation of amino acids essential for their developmental growth and reproduction (De Leo et al., 2002; Prasad et al., 2010a). PIs are majorly found in storage and reproductive organs of plants as compared to vegetative parts (Ryan, 1990; Shewry, 2003; Padul et al., 2012). They are expressed constitutively and induced by mechanical wounding, pathogen and herbivore attack in various parts of the plant (Lawrence and Koundal, 2002; Srinivasan et al., 2009; War et al., 2012). Induction of PI gene expression occurs through the octadecanoid and phenylpropanoid pathways mediated through jasmonic acid and salicylic acid, respectively (Koiwa et al., 1997; Zhao et al., 2009; Scott et al., 2010). The defensive role of PIs in plant protection was established by the following observation where some of the insect larvae showed abnormal growth and development when reared upon the soybean products (Mickel and Standish, 1947). Subsequently, the toxic

effect of soybean trypsin inhibitor has been shown on the larvae of *Tribolium confusum* (Lipke et al., 1954). Besides these early studies, there have been many examples of plant PIs which are known to act against several phytophagous insect species by their unique action of protease inhibition (Chen, 2008; Haq et al., 2004; Stevens et al., 2012; Parmar et al., 2017; Hou et al., 2018; Jamal et al., 2019).



Adapted from War et al. (2018).

Fig. 1.1. Direct and indirect plant defense responses against phytophagous insect pests: Priming of plant defense response through Jasmonic acid (JA)/Salicylic acid (SA) pathway and induction of morphological and biochemical defensive traits in response to insect infestation, oviposition or external elicitors is a part of the direct defense. Production of different volatile compounds by plants to attract natural insect enemies is a part of the indirect defense. (EFN - extrafloral nectar; HIPV - herbivore-induced plant volatiles).

1.2. Physiological functions of plant PIs

In addition to a defensive role in plants, PIs are also involved in several critical endogenous functions such as regulation of endogenous proteinase activity in various organs during seed dormancy, germination and several other developmental stages (Sin and Chye, 2004; Chye et al., 2006). They serve as a storage protein in seeds and tubers, and found in abundance with high cysteine and basic amino acid content and stabilize other proteins during desiccation phase of seed maturation (Richardson, 1991; Mosolov and Valueva, 2005).

In seeds, the storage reserves of PIs are gradually hydrolyzed and mobilized during germination (Richardson, 1977; Birk, 2003; Shewry, 2003). However, accumulation of high content of PIs in seeds and their subsequent decrease during germination imply that they are the source of essential amino acids for structural protein synthesis. PIs are known to exist as several isoinhibitors. The isoinhibitors which appear in the early stage of seed development were electrophoretically distinct from those inhibitors present in mature seeds (Harsulkar et al., 1997; Sreerama and Gowda, 1998). Further, isoinhibitors found in cotyledons of germinated seeds differed in their N-terminal amino acid length possibly due to proteolytic cleavage of mature seed inhibitors (Kumar et al., 2002). Thus, the appearance of an array of isoinhibitors with unequal N-terminal amino acid length during different stages of plant life cycle suggest their specific defensive role throughout the plant growth against various pests and pathogens (Clemente and Domoney, 2006).

PIs are also known to play a significant role in the regulation of programmed cell death which occur in different organs of the plant during all developmental stages (Kosslak et al., 1997; Solomon et al., 1999; Boex-fontvieille et al., 2015). Besides their endogenous functions, PIs induced in response to herbivory attack defend them by blocking their gut proteases, which results in the deprivation of essential amino acids. This mechanism of action in turn leads to retarded

larval growth, decreased fertility and fecundity (Ryan, 1990; Zhu-Salzman and Zeng, 2015; Swathi et al., 2016). Similarly, PIs are also induced in response to various abiotic stresses (Srinivasan et al., 2009; Othman et al., 2014; Rehman et al., 2017; Subburaj et al., 2017). Thus, PIs support the plant growth by regulating protease activity and serve as an essential amino acid reserve during seed germination and development (Hartl et al., 2011).

1.3. Classification of PIs

Plant PIs are a diverse group of proteins which have been intensively investigated so far and classified based on several different aspects. They are small in size, possess compact structure harbouring one or more inhibitory domains specific to different classes of proteases. Initially, PIs are classified into four families based on their catalytic mechanism (Laskowski and Kato, 1980): (1) serine proteinase inhibitors, (2) aspartic proteinase inhibitors, (3) cysteine proteinase inhibitors and (4) metalloproteinase inhibitors. Based on their primary structure De Leo et al. (2002) categorised PIs into different protein families which can be linked to their sequence summary, structural and functional properties deduced from the literature for easy availability and access of information. The database of PLANT-PIs (http://plantpis.ba.itb.cnr.it/) contains information of about 495 inhibitors and their isoinhibitors identified from 195 plant species.

According to Birk (2003), plant PIs are classified based on their structure-function relationship such as Bowman-Birk serine protease inhibitors, Soybean trypsin (Kunitz) inhibitors, Cereal trypsin/α-amylase inhibitors, Mustard trypsin inhibitors, Cysteine protease inhibitors, Metallo carboxypeptidase inhibitors, Serpins, Squash inhibitors and Potato type I & II (Pin I & Pin II) inhibitors (Grosse-Holz and van der Hoorn, 2016; Rustgi et al., 2018). However, PIs have been evolved under selection pressure from inhibitors containing a single inhibitory domain to those of multidomain inhibitor to neutralize a variety of proteases from phytophagous insect pest and pathogens as a part of plant defense mechanism (Lopes et al., 2004).

Krowarsch et al. (2003) distinguished all plant PIs based on their mechanism of inhibition: (1) Canonical inhibitors, which have convex inhibitory loop complementary against concave active site of the enzyme and resembles that of an ideal substrate (2) Non-canonical inhibitors, interact through their N-terminal segment besides extensive secondary interactions which involves a segment other than its active site and (3) Serpins which are similar to canonical inhibitors. However, cleavage of one peptide bond in the inhibitory binding loop leads to structural changes. During the course of evolution, plants produced an array of PIs varying in their primary protein sequence to a tertiary structure to interact with numerous classes of proteases produced in response to herbivory. As a result, a new system of classification has been established which provided more comprehensive information rather than simple classification system which is based on protease specificity (Jongsma and Beekwilder, 2011; Rawlings et al., 2004).

The PIs which inhibit proteases/proteinases have gained significant importance in the field of medicine and biotechnology applications. Hence, there was a need for creating a more organised database to facilitate the storage and retrieval of information of these molecules to the research community. The group of Rawlings et al. (2004) created the MEROPS database (https://www.ebi.ac.uk/merops/inhibitors/) as an integrated source of different proteases, their substrates and inhibitors, which has a comprehensive classification system. They classified PIs into forty-eight families based on similarity in their primary structure. Further, based on three-dimensional structure, thirty-one families are grouped into twenty-six clans which facilitate the simple system of nomenclature. Further, Rawlings and co-workers (2014) organised PIs into seventy-six families, based on sequence homology of inhibitory domains. In this classification, PIs are categorised into simple inhibitors which contains the single inhibitory domain and complex inhibitors with multiple inhibitory domains. Subsequently, Rawlings et al. (2018) relocated the MEROPS database to EMBL-EBI (http://www.ebi.ac.uk/merops/) which has hierarchical classification such as protein species, families, clans and their identifier at each level.

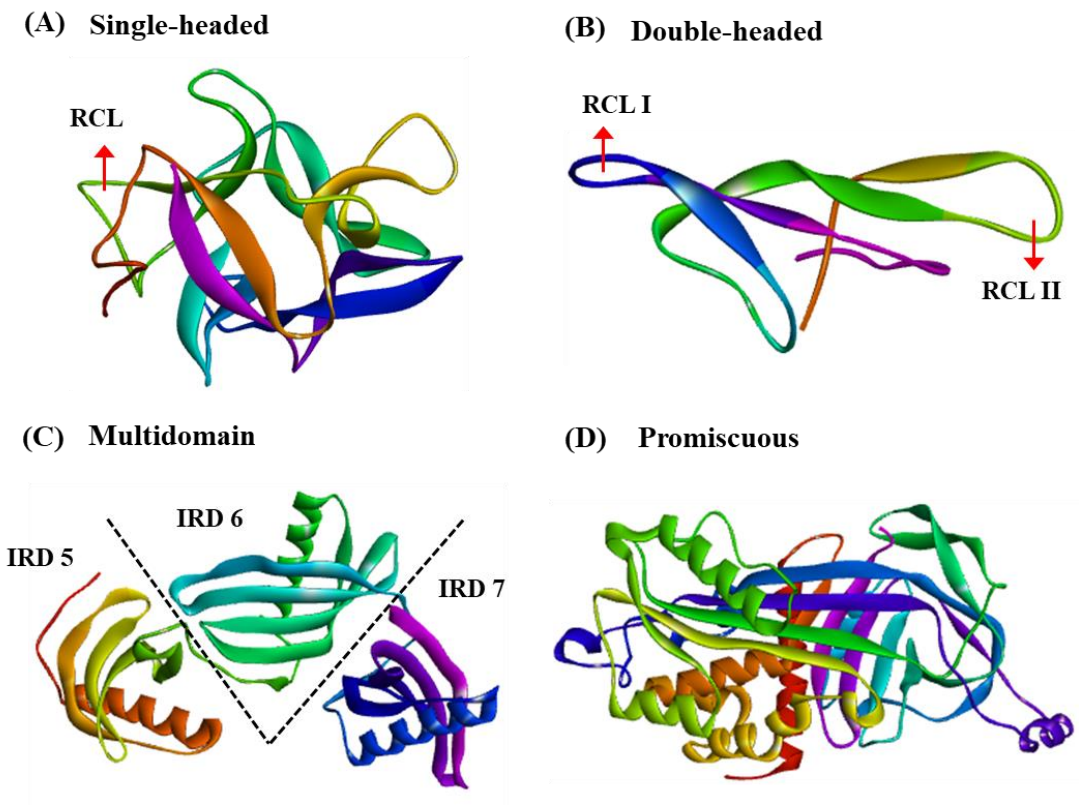


Fig. 1.2. Three-dimensional structures of different classes of plant PIs: (**A**) Single-headed *Bauhinia bauhinioides* Kunitz inhibitor (BbKI) with single reactive centre loop (RCL), PDB ID: 4ZOT; (**B**) Double-headed soybean Bowman-Birk inhibitor possess RCL1 and RCL2 at opposite ends and can bind to two molecules of proteases at any given time, PDB ID: 1K9B; (**C**) Potato multicystatin containing eight repeating units of cystatin (only three inhibitory repeat domains (IRDs) were shown). Each IRD bind to one cysteine protease and they are separated by trypsin-sensitive domain, PDB ID: 4LZI; (**D**) AtSerpin1 from *Arabidopsis thaliana* feature an RCL, which display a proteas target sequence as a bait. Seripins target various serine and cysteine proteases with remarkable trapping mechanism in which cleavage of RCL loop leads to the formation of serpin-protease irreversible complex similar to bait, PDB ID; 3LE2.

More recently, Grosse-Holz and van der Hoorn (2016) described the new type of classification of 'multifunctional' plant PIs such as their biological function, mechanism and evolution. They are (1) Single-headed inhibitors (e.g. Kunitz inhibitors and monocotyledonous

BBI), (2) Double-headed inhibitors [(e.g. BBIs, barley α-amylase/subtilisin inhibitors (BASI) and ragi bifunctional inhibitors (RBI)], (3) Multidomain inhibitors (Multicystatin & Pin II) and (4) Promiscuous inhibitors (Serpins) (**Fig. 1.2**).

1.3.1. Serine PIs (SPIs)

They are the extensively studied class of plant PIs which specifically bind to serine proteases of plant and animal origin such as trypsin, chymotrypsin and elastase. Among SPIs, the Bowman-Birk and Kunitz inhibitors are well characterized in terms of evolution, structural-functional relationship and their biological significance. In addition to their biotechnological applications in crop protection, they also found to have some pharmacological benefits due to their inhibitory activity towards serine proteases involved in human pathology (Clemente and Arques, 2014; Shamsi et al., 2016; Srikanth and Chen, 2016; Clemente et al., 2019).

1.3.1.1. Bowman-Birk inhibitors (BBIs)

The bifunctional PIs belong to the BBI family are classified into I12 families of MEROPS database (Rawlings et al., 2014). BBIs are most extensively studied plant PI besides Kunitz inhibitor and typically found in dicotyledonous Leguminosae family and monocotyledonous Poaceae family (Mello et al., 2001; Garcia et al., 2004; Bhattacharyya et al., 2006). In general, dicot BBIs possessed the molecular mass of 6-9 kDa with two inhibitory domains protruding in the opposite direction from a single molecule (Birk, 1985). These two inhibitory reactive sites can bind to two molecules of proteases simultaneously and independently, and form a stable complex by the standard mechanism of protease inhibition Laskowski and Kato (1980). The BBIs in monocots existed as two distinct classes: The first class possessed 16 kDa size with two reactive sites and the second class of BBI possessed 8-kDa size with one reactive site while the second reactive site has become non-functional due to the loss of four cysteine residues (C_3-C_{13}, C_{10}-C_{11}). The structure of BBIs from legumes has highly conserved cysteine residues framework and their disulfide bond network [C_1-C_{14}, C_2-C_6, C_3-C_{13}, C_4-C_5, C_7-C_9, C_8-C_{12}, C_{10}-C_{11}] (Chen et al., 1992;

Lin et al., 1993; Mello et al., 2003; Qi et al., 2005). The homology in the conserved sequence of BBIs suggests the evolution of double-headed inhibitors from ancestral single-headed BBI by the process of gene duplication and mutations in their reactive site loop residues (Prakash et al., 1996; Mello et al., 2003). So far, the three-dimensional structure of BBIs are resolved in *Arachis hypogaea* (Suzuki et al., 1987, 1993), *Glycine max* (Werner and Wemmer, 1992; Voss et al., 1996), *Hordeum vulgare* (Song et al., 1999), *Lens culinaris* (Ragg et al., 2006), *Medicago scutellata* (Catalano et al., 2003; Capaldi et al., 2007), *Vigna angularis* (Tsunogae et al., 1986) and *Vigna unguiculata* (Barbosa et al., 2007; Rao and Suresh, 2007). The broader specificity of dicot BBIs towards its cognate proteases is attributed to variation in reactive site P1-P1′ residues of the N-terminal inhibitory loop such as Arg-Ser/Lys-Ser/Ala-Ser and C-terminal inhibitory loop with Arg-Ser. Besides, P5′ variants of sunflower cyclic trypsin inhibitor (SFTI) with 14 residues showed improved inhibitory activity and specificity towards cognate proteases (Li et al., 2019).

The structural features of peanut BBI differed in several aspects with classical soybean BBI (**Fig. 1.3**, Suzuki et al., 1987; Qi et al., 2005). The N-terminal reactive site loop of peanut BBI has a unique structure with eleven residues instead of nine residues and contain Arg-Arg at P1-P1' positions. The two additional residues Phe-Glu which are present at P6'-P7' positions is uncommon among BBIs. Also, the N-terminal reactive loop of peanut BBI inhibits both trypsin as well as chymotrypsin whereas C-terminal reactive loop is specific towards trypsin in contrast to BBIs from other legumes (Norioka and Ikenaka, 1983; Suzuki et al., 1987). The phylogenetic analysis revealed that peanut BBIs are grouped into a distinct secondary branch which is far away from soybean BBI and also one of the inter-branch point was close to the monocotyledonous BBIs (Mello et al., 2003). These distinctive properties emphasize that peanut BBIs are a subgroup of dicotyledonous BBI and differ from all other known legumes BBI (Norioka and Ikenaka, 1983; Suzuki et al., 1987; Prakash et al., 1996; Mello et al., 2003). Thus, peanut BBI is considered as an intermediate of monocot and dicot plants (Mello et al., 2003). Together, BBIs are small, compact

and globular in structure and contain seven intramolecular disulfide bonds which preserve their functional properties (Joshi et al., 2013; Kumar and Gowda, 2013a; He et al., 2017; Mohanraj et al., 2019).

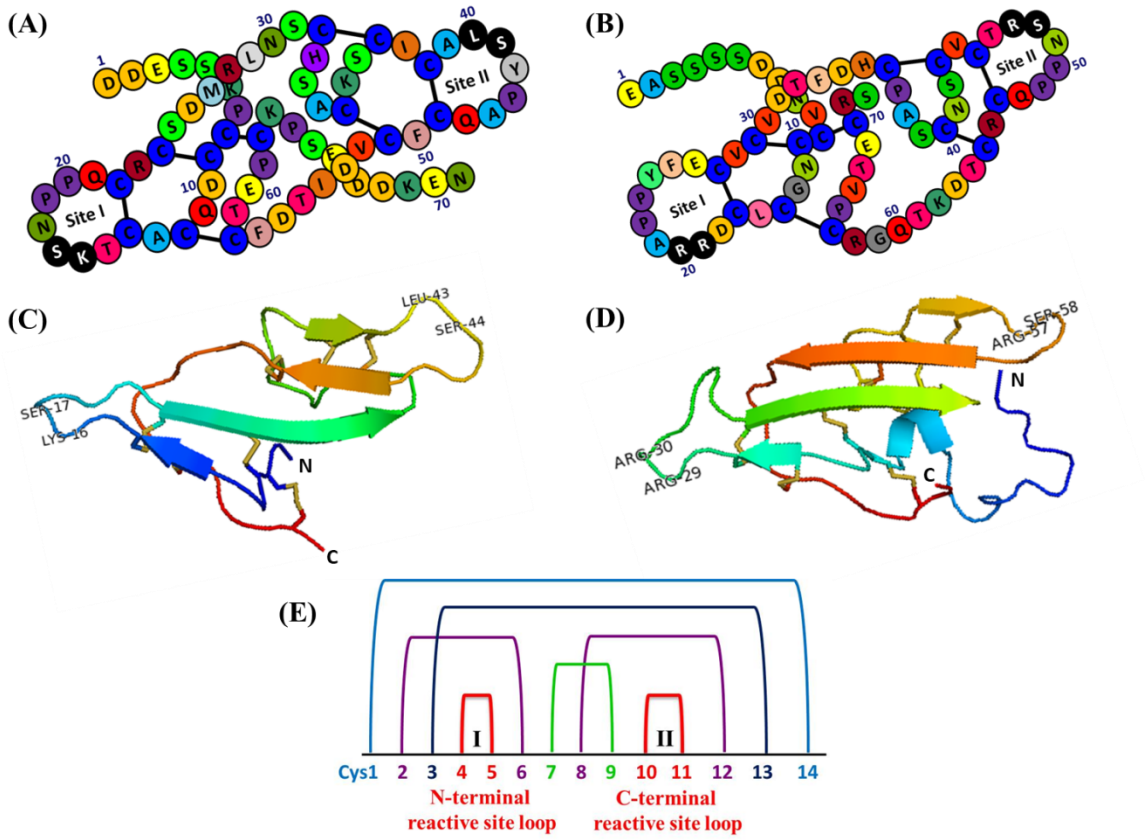


Fig. 1.3. The primary and tertiary structure of Bowman-Birk inhibitors: Amino acid sequence of BBIs from **(A)** soybean and **(B)** peanut, showing the N and C-terminal reactive site loops. Three-dimensional structure of BBIs from **(C)** soybean and **(D)** peanut, depicting their reactive site amino acids and an array of disulfide bonds. (**E**) Conserved disulfide bonding pattern occurs among the fourteen cysteine residues of BBIs which stabilize its structure. The block colour circles indicate P1 and P1′ amino acid residues of RCL. Adapted from Odani and Ikenaka (1972) and Suzuki et al. (1987) and modified.

The BBIs adopt a canonical disulfide-linked reactive site loop structure due to the presence of cis-Pro at P3' reversing an antiparallel β-strand (Mello et al., 2003; Barbosa et al., 2007). They

bind to their cognate proteases with exposed and convex reactive site loop which is complementary to the active site of the proteases (Krowarsch et al., 2003). BBIs exist as different isoforms, and they show self-association tendency depending on their concentration (Giri et al., 2003; Swathi et al., 2014; Mohanraj et al., 2019). The oligomeric structures of BBIs are known to be stabilized by hydrogen bonds among exposed hydrophobic patches of electrically charged clusters formed due to disulfide bonds (Silva et al., 2005; Rao and Suresh, 2007; Joshi et al., 2013). In case of horse gram BBI, dimers are known to be stabilized by the electrostatic interaction between Lys^{24} and $Asp^{75/76}$ residues of N, C-terminal ends of two different inhibitors, respectively (Kumar et al., 2004; Muricken and Gowda, 2010). The formation of an intramolecular C-terminal hook-like structure stabilized by salt-bridge between D76-K71, enable to form a dimer through intermolecular salt-bridge (D75-K24) between two different monomers (Kumar et al., 2015). However, the self-association phenomenon of BBI serves in molecular packing as seed storage protein (Barbosa et al., 2007; Kumar et al., 2015). Overall, BBIs from legume species share the following features: (i) low molecular mass, high cysteine content and occurrence of several isoinhibitors which exhibit self-association, (ii) double-headed structure with two identical reactive site loops and (iii) stability at various adverse conditions due to their structural makeup. In contrast, BBI with three repetitive inhibitory domains have been described in rice (Qu et al., 2003). Also, a cyclic trypsin inhibitor with 14 amino acids residues was reported in sunflower seeds, and it is homologous to an inhibitory domain of BBI (Luckett et al., 1999; Korsinczky et al., 2001; Li et al., 2019). Further, BBI with unique structural features was found in dicotyledonous *Maclura pomifera* (MpBBI) (Indarte et al., 2017). The MpBBI polypeptide possessed Arg41 at 'P1' with a molecular mass of 7.37 kDa, five disulfide bonds and found to be a potent inhibitor of trypsin. Also, the first reactive loop contains unique acidic amino acid residues 'CEEESRC' composition, whereas the second loop contained conserved 'CTRMNPPQC' region related to other BBIs from the plants.

1.3.1.2. Kunitz inhibitors (KIs)

KIs are extensively reported from Fabaceae, Solanaceae and Poaceae families (Mosolov and Valueva, 2005; Habib and Fazili, 2007). The KIs possessed the following typical characteristic features: (i) molecular mass of 14-24 kDa, (ii) single polypeptide chain containing one reactive site with β-trefoil structure which is stabilized by two disulfide bonds and (iii) capable of inhibiting serine, cysteine and aspartic proteases (Oliva et al., 2010; Oddepally et al., 2013; Jamal et al., 2015; Bendre et al., 2018). KIs containing two polypeptide chains were also found in *Archidendron ellipticum* (Bhattacharyya et al., 2006), *Leucaena leucocephala* (Oliva et al., 2000) and *Cassia grandis* (Brandão-Costa et al., 2018) and they are held by disulfide bonds. Besides, KIs with three disulfide bonds (Bronsoms et al., 2011), single disulfide bond (Macedo et al., 2007; Bezerra et al., 2016) and no disulfide bonds (Araújo et al., 2005; Hansen et al., 2007; Zhou et al., 2015). According to the MEROPS database, KIs are grouped in the I3B family and existed mostly in higher plants, and absent in green algae genome sequence (Rawlings et al., 2009). The KIs share typical β-trefoil structure with possible reactive site amino acids Arg-Ser/Arg-Lys/Ala which are responsible for their specificity towards different target proteases (Oliva et al., 2010; Bendre et al., 2018).

Several KIs have been isolated, characterised and exploited for their insecticidal as well as the therapeutic role (Major and Constabel, 2008; Srikanth and Chen, 2016; Bendre et al., 2018; Jamal et al., 2019). Besides, KIs are also known to exert antimicrobial (Da Silva Bezerra et al., 2016) and antifungal activities (Wang and Ng, 2006; de Oliveira et al., 2018). They are also induced by biotic (Müller et al., 2016) and abiotic (Ledoigt et al., 2006) stresses. KIs isolated from *Pithecellobium dumosum* (PdKI) and *Rhynchosia sublobata* (RsKI) remarkably inhibited the midgut serine protease activity of coleopteran (*Callosobruchus maculatus* and *Zabroted subfasciatus*) and several lepidopteran (*Alabama argillacea,Telchin licus, Achaea janata and H. armigera*) larvae (Rufino et al., 2013; Mohanraj et al., 2019). The AtKTI from *Arabidopsis*

thaliana conferred resistance against polyphagous spider mite *Tetranychus urticae* through bifunctional inhibitory activity against serine- and cysteine proteases (Arnaiz et al., 2018). Similarly, KI isolated from *Tamarindus indica* and *Butea monosperma* exhibited effective antibiosis against destructive polyphagous insect pests *H. armigera* (Pandey and Jamal, 2014; Jamal et al., 2015). A unique KI from *Adenanthera pavonina* with dual function such as trypsin inhibitory and chitin-binding activity impaired the digestion process of *Plodia interpunctella* which in turn lead to significant retardation in growth and mortality of the pest (de Oliveira et al., 2019). Despite their insecticidal properties, KI isolated from *Leucaena leucocephala* (LlTI) possessed two polypeptide chains and inhibited blood coagulation and fibrinolytic enzymes (Souza-Pinto et al., 1996; Oliva et al., 2000). The TKI from *Tamarindus indica* (Patil et al., 2012) and CrataBL from the bark of *Crataeva tapia* (Salu et al., 2014, 2018) inhibited the coagulation factors involved in thromboembolic diseases. Further, the *Bauhinia* seeds which are rich in KIs showed potent anticoagulant properties by inhibiting various coagulation factors (Sampaio et al., 1996; Nakahata et al., 2006; Oliva and Sampaio, 2009; Salu et al., 2014; Brito et al., 2014; Zhou et al., 2015; Srikanth and Chen, 2016).

1.4. Peanut PIs

Peanut (*Arachis hypogaea* L.) is a crucial legume crop and it is rich in protein (17-31%) and edible fat (Ofuya and Akhidue, 2005). It primarily originated as a seed crop in South America and now its cultivation has taken over worldwide. However, it is majorly produced in Asia (68%) and Africa (24%). According to the Food and Agriculture Organization of the United Nations (FAOSTAT-2018), India is the second leading producer of peanuts after China and the USA stands at third rank. This herbaceous legume is mostly grown for the production of vegetable oil. However, the by-products obtained after oil extraction contain several functional compounds such as polyphenols, antioxidants, vitamins, minerals, proteins and fibres that could be added as supplements in many processed foods (Arya et al., 2016). Besides, peanuts are also known to

contain resveratrol which has cardioprotective properties and it also improves blood flow to the brain, thus significantly reducing the risk of stroke (Mohan and Karthika, 2014). Apart, it has several bioactive health benefits such as anti-inflammatory, anti-oxidant, anti-cancer, anti-platelet aggregation, hypolipidemic, hypoglycemic and satietogenic (suppresses appetite for food) properties (Awad et al., 2000; Chang et al., 2006; Christman et al., 2018; Lee et al., 2018).

The study on peanut PIs has started as early in the 1970s by Hochstrasser et al. revealed the amino acid sequences of PIs which existed as a tetramer (17 kDa) and also suggested the possibility for the presence of several other small molecular weight PIs. Later, several BBI isoforms from peanut were isolated and their amino acid sequence was determined followed by elucidation of three-dimensional structures, and their molecular evolution (Norioka et al., 1982; Norioka and Ikenaka, 1983; Suzuki et al., 1987, 1993). The different isoinhibitors of BBI isolated from peanut has similar amino acid sequences but differed in their amino acid length at N-terminal end. Among the isoinhibitors, BBI A-II has a long chain of amino acids and generated A-I, B-I and B-III isoinhibitors upon proteolytic cleavage. However, isoinhibitor B-II has a slightly different amino acid sequence and showed 81% similarity with all other peanut BBI isoforms. Besides, peanut BBIs showed distinct structural and functional properties as compared to BBIs from other legumes. These include non-identical reactive site loops, N-terminal reactive loop containing twin Arg at P_1, P_1' and its specificity towards trypsin and chymotrypsin (Norioka and Ikenaka, 1983; Suzuki et al., 1987; Mello et al., 2003). So far, several trypsin inhibitors isolated from different peanut varieties displayed anti-carcinogenic (Ahmad et al., 2019) and satietogenic action (Serquiz et al., 2016). Besides, peanut BBI and Kunitz protease inhibitor genes are also known to be induced during both biotic (Müller et al., 2016) and abiotic stress tolerance (Drame et al., 2013).

1.5. Proteases

The enzymes hydrolysing peptide bonds are called proteases/peptidases and they are broadly classified as exopeptidases and endopeptidases. However, the term ‘proteinase’ is used when a protease acts as an ‘endopeptidase’ (Barrett et al., 2004). Proteolytic enzymes are ubiquitously distributed in all living organisms and regulate every aspect of their physiology and development by controlling the synthesis, turn over and protein function (Turk, 2006; Lopez-Otin and Bond, 2009). The specificity of proteases towards their substrates depends on their localisation, amino acid sequence, biochemical and structural properties of their active site. However, based on the mechanism of catalysis, proteases are categorised into five different classes, such as serine, cysteine, threonine, aspartic, and metalloproteases. In the first three classes, the active site amino acids (Ser, Cys and Thr) are utilized as a nucleophile to attack the peptide bond of the substrate molecule from which their class names are derived. In contrast, aspartic and metalloproteases use an activated water molecule as a nucleophile (Barrett et al., 2004).

1.5.1. Serine proteases

The name serine protease is derived from its amino acid ‘serine’ present in the catalytic triad and it is responsible for specific catalysis, and majorly includes trypsin, chymotrypsin, and elastase. They are abundantly found in the digestive system of lepidopteran larvae and are involved in the process of digestion (Srinivasan et al., 2006; Sarate et al., 2012), human blood coagulation (Walsh and Ahmad, 2002) and many other pathophysiological conditions (Turk, 2006; Heutinck et al., 2010). Serine proteases are categorized into different types based on their specificity towards P1 residues of the substrates. The trypsin-like proteases cleave the peptide bond after carboxy terminus of positively charged amino acids Lys/Arg, chymotrypsin cleaves next to the amino acid residue with large hydrophobic side chains such as Phe/Tyr/Leu and elastase cleaves at smaller hydrophobic residues like Ala/Val (Tyndall et al., 2005). The identification and biochemical

characterization of these proteases from any biological sample could be studied by using specific chemical inhibitors (PMSF/TLCK/TPCK) and their substrates. Each of these chemical inhibitors have a different mode of inhibition: PMSF sulfonylates hydroxyl groups in the active site serine residues, TLCK alkylate the histidine-46 of trypsin-like proteases and TPCK alkylate the histidine-57 of α-chymotrypsin. A conventional serine protease has a catalytic triad of Asp, His and Ser in their active site, where the serine residue act as a nucleophile and His serves as the proton donor. All serine proteases are similar in their structural configuration but differ in their catalytic triad and dyad, which include Ser-His-Glu, Ser-Lys/His, His-Ser-His (Hedstrom, 2002).

During insect feeding, the ingested food material mostly consists of macromolecules such as polysaccharides, proteins and fats. As plant tissue possesses sub-optimal protein content, often nitrogen becomes the limiting factor for many phytophagous insects. Therefore, complete hydrolysis of plant proteins is crucial to obtain essential amino acids for insect survival (Mattson, 1980; Bala et al., 2018). However, the proteins need to be broken down into individual components, which is mediated by different classes of proteases in the digestive system of insects before being absorbed and utilised (Sarate et al., 2012). The different orders of economically important insect pests predominantly use digestive proteases belonging to one particular class (Terra and Ferreira, 1994). The lepidopteran, dipteran and hymenopteran species mainly use serine proteases (Wolfson and Murdock, 1990; Purcell et al., 1992). In contrast, hemipteran and coleopteran species tend to use cysteine proteases (Murdock et al., 1987; Martinez et al., 2016). The lepidopteran larvae which feed on green plants majorly depend on serine proteases which accounts for 95% of total gut protease content. Among the serine proteases, trypsin-like proteases are abundant, which account for 90% while chymotrypsin-like proteases are 5% and elastases are 1%. All other proteases such as aminopeptidases, carboxypeptidases, cathepsin B-like, and metalloproteases account to rest 4% (Patankar et al., 2001; Srinivasan et al., 2006).

1.6. Insect response to plant PIs

PIs are one among the essential defensive traits in plants to combat against phytophagous insects and pathogens. They defend effectively against a broad spectrum of insect orders such as lepidoptera (Jadhav et al., 2016), hemiptera (Azzouz et al., 2005) and coleoptera (Zhu-Salzman et al., 2003). In general consumption of plant PIs inevitably alters the digestive efficiency of insect larvae which further result in stunted growth, delay in larval/pupal development, early mortality or decrease in fecundity and fertility. However, the outcome of the whole process is based on the PI type/origin and concentration in the diet (Gatehouse, 2011; Macedo et al., 2015). Besides, the phytophagous insects alter the digestive protease complement in response to consumption of PIs and trigger the counter defense response in different ways such as: (i) compensatory overproduction of existing digestive proteases, (ii) increased expression of PI-insensitive digestive proteases and (iii) production of PI hydrolysing proteases (**Fig. 1.4,** Girard et al., 1998; Giri et al., 1998; Zhu-Salzman et al., 2003).

Thus, the majority of insects use multiple defense strategies as described above to bypass the anti-nutritional effect of PIs in their diet. The intrinsic ability of insects to change their gut protease complement by switching on several gene copies of different proteases allows them to thrive on broad host range (Lopes et al., 2004; Kipgen and Aggarwal, 2014). Nevertheless, several studies have been conducted to generate transgenic plants expressing diverse kind of defensive genes to combat against insect adaptation strategies. These include transgenic plants with (a) potent PI gene alone, (b) combination of PI genes which synergistically inhibit sensitive and insensitive proteases, (c) PI genes in combination with cry toxin and (d) pool of different defensive genes together (Zhu-Salzman et al., 2003; Dunse et al., 2010b; Gatehouse, 2011; Tajne et al., 2013; Tanpure et al., 2017; Hamza et al., 2018; Boddupally et al., 2018). More recently, researchers have made numerous attempts to overcome the vulnerability of resistance development in phytophagous insect pests through gene stacking/pyramiding, transplastomic

engineering, protein engineering/3-D modelling, RNAi and CRISPR/Cas9-mediated approaches (Price and Gatehouse, 2008; Santamaria et al., 2012; Ni et al., 2017; Rauf et al., 2019; Cagliari et al., 2019).

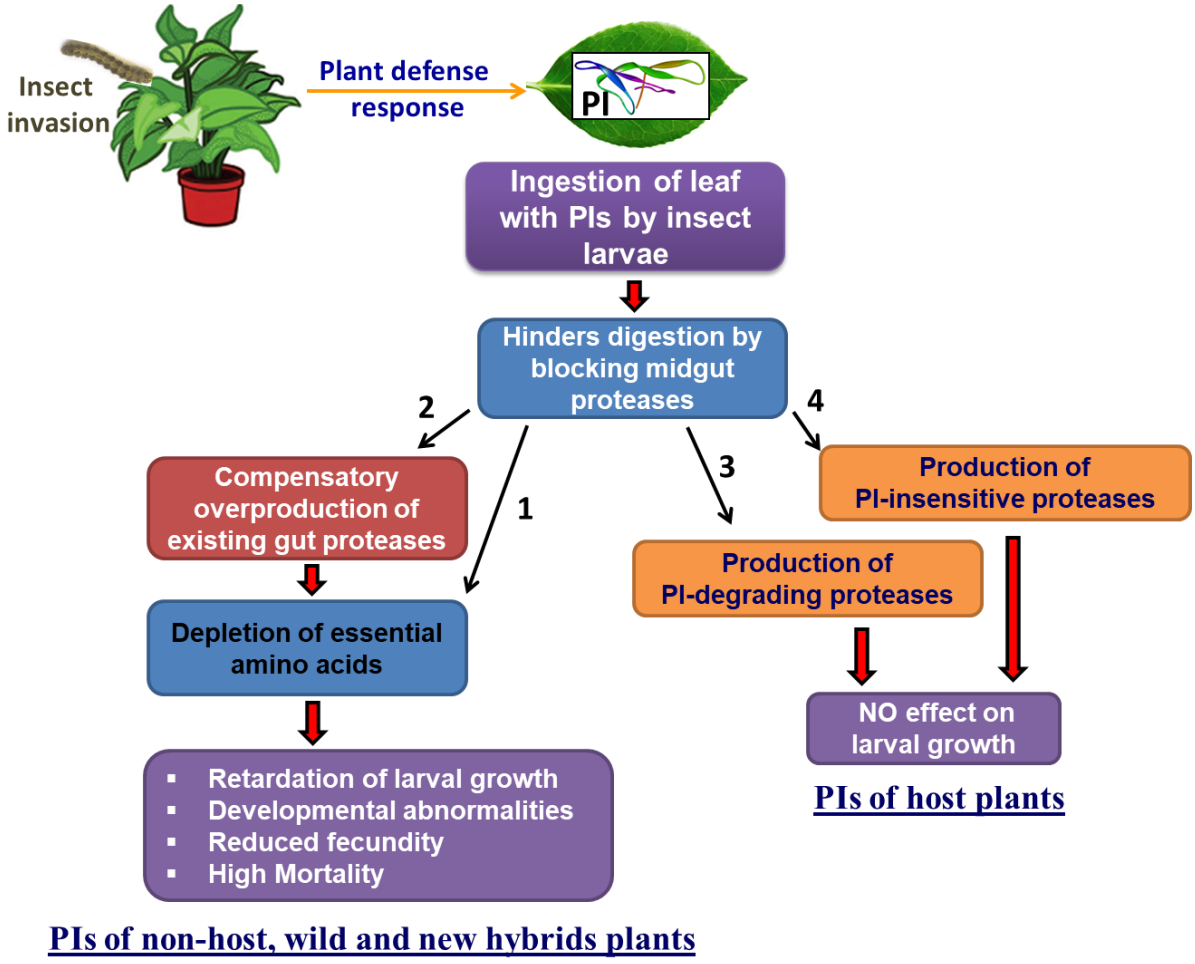


Fig. 1.4. Mechanism of action of dietary PIs and insect response: Antimetabolic effect of PIs is exerted by blocking the midgut digestive proteases. A decrease in digestive efficiency leads to deprivation of essential amino acids which in turn leads to retardation of larval growth, developmental abnormalities, reduced fecundity and high mortality, respectively. The counter-reaction of insect is mediated through (i) overproduction of existing proteases, (ii) production of PI-degrading proteases and (iii) synthesis of PI-insensitive proteases, to neutralize the effect of dietary PIs. Case 1 and 2 indicates PIs from non-host/wild/hybrid plants effectively control the pest while case 3 and 4 are the part of insect adaptation mechanism against PIs from host-plants.

1.7. Transgenic plants expressing plant PIs

As of now, several insecticidal molecules such as *B. thuringiensis* endotoxin, PIs, lectins and chitinases independently or synergistically conferred substantial resistance against several insect pests (Murdock and Shade, 2002; Abdeen et al., 2005; Tajne et al., 2013; Chen et al., 2014; Tabashnik and Carrière, 2017). Among the natural plant defense molecules, PIs attracted more attention in developing transgenic plant resistant to lepidopteran insect pests which rely on serine proteases for digestion (Jamal et al., 2013, 2019). Ingestion of PIs impaired insect digestion by forming a stable complex with specific proteases which in turn deplete essential amino acids required for normal growth and development of larvae (Prasad et al., 2010a; Swathi et al., 2014; War et al., 2018).

Several transgenic plants expressing plant PIs have been developed which successfully defended against several economically important insect pests (Sharma et al., 2000; Koundal and Rajendran, 2003; Haq et al., 2004; Stevens et al., 2012; Macedo et al., 2015). The first transgenic tobacco plant expressing cowpea trypsin inhibitor (CpTI) conferred resistance against *H. virescens* (Hilder et al., 1987) and *H. zea* (Hoffmann et al., 1992) and resulted in reduced insect growth and increased mortality. Consequently, transgenic tobacco plant expressing SPI-II retarded the growth of *Manduca sexta* larvae (Johnson et al., 1989). The transgenic sugarcane expressing SKTI and SBBI retarded the growth of *Diatraea saccharalis* (Falco and Silva-Filho, 2003). Also, *Nicotiana benthamiana* plant expressing *Beta vulgaris* serine proteinase inhibitor gene (BvSTI) significantly reduced the larval biomass of *S. frugiperda*, *S. exigua* and *M. sexta* and resulted in developmental abnormalities (Smigocki et al., 2013). Besides, *Nicotiana tabacum* trypsin inhibitor gene (NtPI) has conferred resistance against various abiotic stresses parallel to resistance against *S. litura*, and *H. armigera* (Srinivasan et al., 2009).

Transgenic tomato expressing multi-domain CanPI7 showed enhanced tolerance to *H. armigera* (Tanpure et al., 2017). Further, co-expression of barley serine (BTI-CMe) and cysteine (Hv-CPI2) protease inhibitors in tomato plant has recorded a notable reduction in *Tuta absoluta* larval biomass, deformities in adult wings and reduced fertility. This study also demonstrated that expression of cystatin promoted the plant defense by inducing co-expression of an endogenous wound inducible PI2 gene, increasing the number of glandular trichomes and altering the emission of volatile organic compounds (Hamza et al., 2018). Furthermore, several researchers developed a variety of transgenic plants expressing (a) more than one type of PIs against both sensitive and insensitive proteases and (b) combination of PIs and other defensive gene like lectins to overcome the insect adaptive mechanisms (Abdeen et al., 2005; Stevens et al., 2012; Chen et al., 2014; Macedo et al., 2015). In these lines, a fusion protein 'Bt-PI' reduced the development of insect resistance by targeting PIs towards insensitive proteases of adaptive insect (Gatehouse, 2011; Zhu et al., 2012; Tajne et al., 2013).

The mechanism by which PIs mediate their anti-metabolic effect on insects differs among species and the level of their adaptive capabilities. However, it is necessary to have a complete understanding of target insect midgut physiology and biochemistry while developing a strategy for insect control (Zhu-Salzman and Zeng, 2015; War et al., 2018). The next generation of transgenic technology has been developed to achieve the sustainable pest management which includes generation of transgenic plants through gene stacking (Dunse et al., 2010b; Santamaria et al., 2012) and RNAi mediated approach (Bhatia et al., 2012). The research group of Chen et al. (2014) have developed *N. benthamiana* transgenic plant containing an engineered plastid with three defensive genes "sporamin, cystatin and chitinase" which are specifically expressed in leaf and root plastids. Expression of this gene stack conferred broad spectrum of resistance against two Spodoptera insect species, two phytopathogenic fungi and tolerance to osmotic stress. Thus, production of transgenic plants expressing a combination of defence genes to confer resistance

against a single or multiple insects could be a successful strategy for efficient pest management (Cui et al., 2011; Macedo et al., 2015; Parmar et al., 2017).

1.8. Plant PIs and human health

The intrinsic ability of legume BBIs to inhibit serine proteases involved in several human pathophysiologies received significant interest in the current biomedical field (**Fig. 1.5;** Rakashanda et al., 2012; Safavi and Rostami, 2012; Srikanth and Chen, 2016). BBIs are water-soluble, functionally stable at physiological pH and a wide range of temperature, and resistant to proteolytic digestion (Qi et al., 2005; Muranova et al., 2019). They are small and compact in their structure and are known to inhibit several serine proteases (Mello et al., 2003; Qi et al., 2005). For instance, the classical BBI and KI from soybean inhibited the activity of several diverse proteases such as trypsin, chymotrypsin, elastase, cathepsin G and several kinases of human origin (Losso, 2008; Srikanth and Chen, 2016). Serine proteases are key players in various biological processes which include digestion, cell signalling, cell proliferation, immune responses and blood coagulation. They have gained considerable significance as target molecules of many human chronic diseases (Turk, 2006; Losso, 2008).

The laboratory of Correa (1981) has reported that the rate of occurrence of breast, colon and prostate cancer was low in a population consuming beans, rice and maize seeds. Similarly, higher consumption of nuts and legumes showed an inverse relation with the occurrence of cardiovascular diseases and various types of cancer (Clemente and Arques, 2014; Aune et al., 2016; Guasch-Ferré et al., 2017; Lee et al., 2018). Hence, legume BBIs are considered as functional food and the few explored applications of BBIs in biomedical field include: anti-carcinogenic (Kennedy, 1993; Losso, 2008; Clemente and Arques, 2014; Joanitti et al., 2018), anti-inflammatory (Safavi and Rostami, 2012; Srikanth and Chen, 2016; Bortolozzo et al., 2018), anti-HIV (Fang and Ng, 2015; Ma et al., 2018), promotion of gastrointestinal health

(Clemente et al., 2011; Vergnolle, 2016) and cardioprotective role (Hojima, 1980; Oliva et al., 2000; Nakahata et al., 2011; Borodin et al., 2013; Brito et al., 2014; Salu et al., 2018).

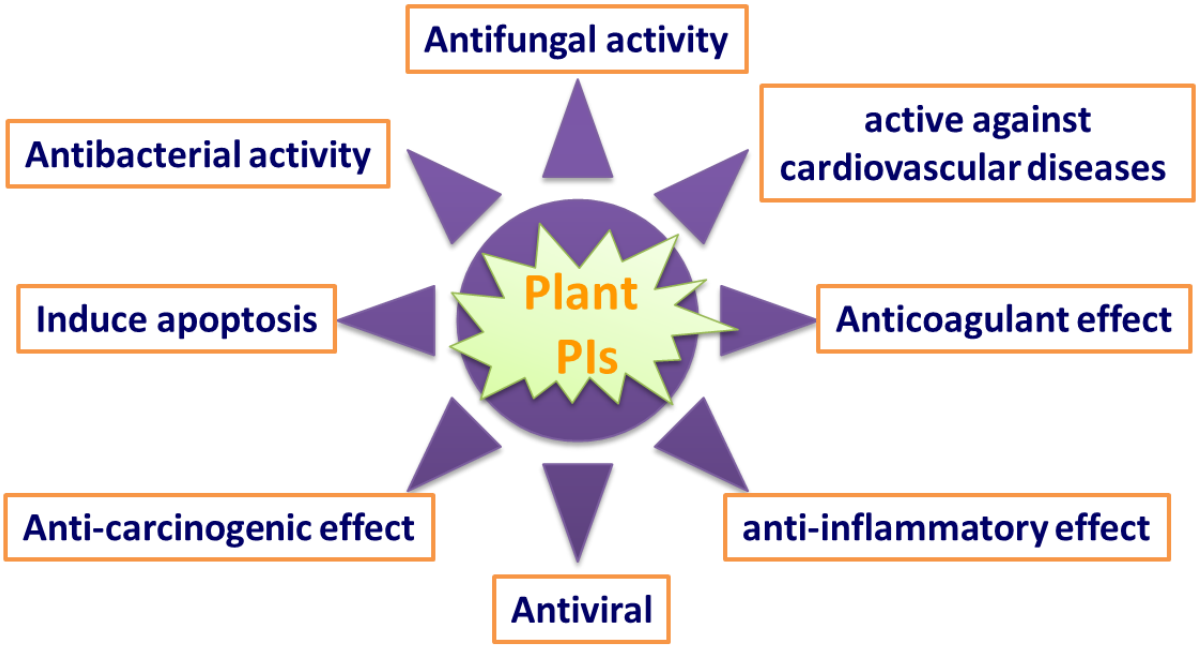


Fig. 1.5. Pharmacological benefits of plant PIs: The significance of PI-protease interaction associated with several biomedical applications. Proteases play an essential role in regulating biological processes. Any imbalance in proteolytic activity results in a diseased state. Several proteases have been identified as a target of many therapeutic drugs including PIs to control the disease.

Among the well-characterised plant PIs, Bowman-Birk family inhibitors are exploited more in cancer prevention and treatment. Furthermore, the most encouraging fact is that the FDA has given the "Investigational New Drug" status to Bowman-Birk inhibitor concentrate (BBIC) of soybean (Kennedy, 1998). Several human clinical trials are under investigation in patients with benign prostatic hyperplasia, oral leukoplakia and ulcerative colitis (Armstrong et al., 2000, 2013; Malkowicz et al., 2001; Lichtenstein et al., 2008). The basis for the chemopreventive property of BBIs originated from its inhibitory potential towards serine proteases involved in tumorigenesis (Losso, 2008; Rakashanda et al., 2012; Eatemadi et al., 2017). The soybean BBI has shown the suppressive effect on colon and anal gland inflammation in rodents upon exposure to a

carcinogenic agent (Billings et al., 1990). In the dimethylhydrazine (DMH) rat model, soybean BBI reduced the colorectal tumour growth without any adverse effect (Kennedy et al., 2002). Further, soybean BBI also showed the suppressive effect on human colon cancer cells (HT29) and disrupted the cell cycle by blocking the G0-G1 phase (Clemente et al., 2010). Also, BBIC stimulated the connexin 43 expression and prevented the growth of prostate tumours in transgenic rats (McCormick et al., 2007; Tang et al., 2009). The *in vitro* and *in vivo* studies revealed that soybean BBI acts as a potential inhibitor of the 26S proteasome in MCF7 breast cancer cells and indicated that BBI is able to arrest the G1/S phase of the cell cycle (Chen et al., 2005). It is also proven that soybean PIs inhibit human ovarian cancer cells by the various mechanism of action (Kobayashi, 2013).

BBIs from chickpea (*Cicer arietinum*) inhibited the viability of breast cancer (MDAMB231) and prostate cancer cell lines (PC-3 & LNCaP) (Magee et al., 2012). Similarly, black-eyed pea trypsin/chymotrypsin inhibitor named 'BTCI' induced apoptosis in human breast adenocarcinoma through multiple mechanisms of ROS production and mitochondrial impairment followed by 20S proteasome inhibition (Mehdad et al., 2016). The Kunitz-type trypsin inhibitor from *Enterolobium contortisiliquum* 'EcTI' inhibited the trypsin, chymotrypsin, plasma kallikrein and plasmin. Also, EcTI is also reported to suppress the breast (SkBr-7 & MCF-7), leukaemia (K562 & THP-1) and colorectal (HCT116 & HT29) cancer cell lines (Nakahata et al., 2011). Further, BBI and BBIC are reported to directly scavenge reactive oxygen species in their vicinity, thereby decreasing the prevalence of cancer (Arbogast et al., 2007). Besides, the *in silico* and *in vitro* studies of Borodin et al. (2013) demonstrated that soybean SBTI prolonged the blood clotting time and prevented the fibrinolysis. Also, BBIs derived from soybean inhibited HIV replication in macrophages and activated IFN-β mediated signalling pathway which is known to boosts the intracellular innate immunity (Ma et al., 2018).

Alzheimer's is a neurodegenerative disorder which affects memory and behaviour due to accumulation of β-Amyloid peptide, thereby leading to dysfunction of autophagy and apoptosis. Treatment of amyloid induced PC12 cells with soybean derived BBI decreased the expression of Bax/Bcl2 ratio and increased the expression of beclin1, Bnip3, Atg5, and several other genes involved in autophagy. These studies suggest that BBI prevent the accumulation of β-Amyloid peptide by inducing autophagy and also showed the neuroprotective property by decreasing apoptosis (Akbari et al., 2019).

1.9. Blood coagulation and Role of PI, as anticoagulants

The process of formation of a blood clot and its consequent dissolution followed by repair of injured tissue is characterized as 'hemostasis' (Butenas and Mann, 2002). In normal physiological conditions, development of a blood clot at the site of injury is initiated by the process of vascular constriction, the formation of a loose platelet plug followed by fibrin clot formation by activation of thrombin is called thrombus (Heemskerk et al., 2002; Furie and Furie, 2008). The process of hemostasis is categorized into primary and secondary events. However, primary events involve the formation of a loose platelet plug and secondary haemostasis form a thrombus accelerated by many coagulation factors (**Fig. 1.6**). This complex process of blood clotting is led by the intrinsic and extrinsic pathways, where numerous proteases termed as 'clotting factors' are involved along with the participation of various cofactors, Ca^{+2} and phospholipids. Biochemically, these proteases/clotting factors belong to serine proteases (Palta et al., 2014; Chaudhry and Babiker, 2018). However, any imbalance in normal haemostasis (eucoagubility) process leads to severe abnormal conditions such as bleeding and thrombosis, due to hypocoagubility and hypercoagubility states, respectively. Thus, the fine balance between blood clotting and fibrinolysis is crucial for health.

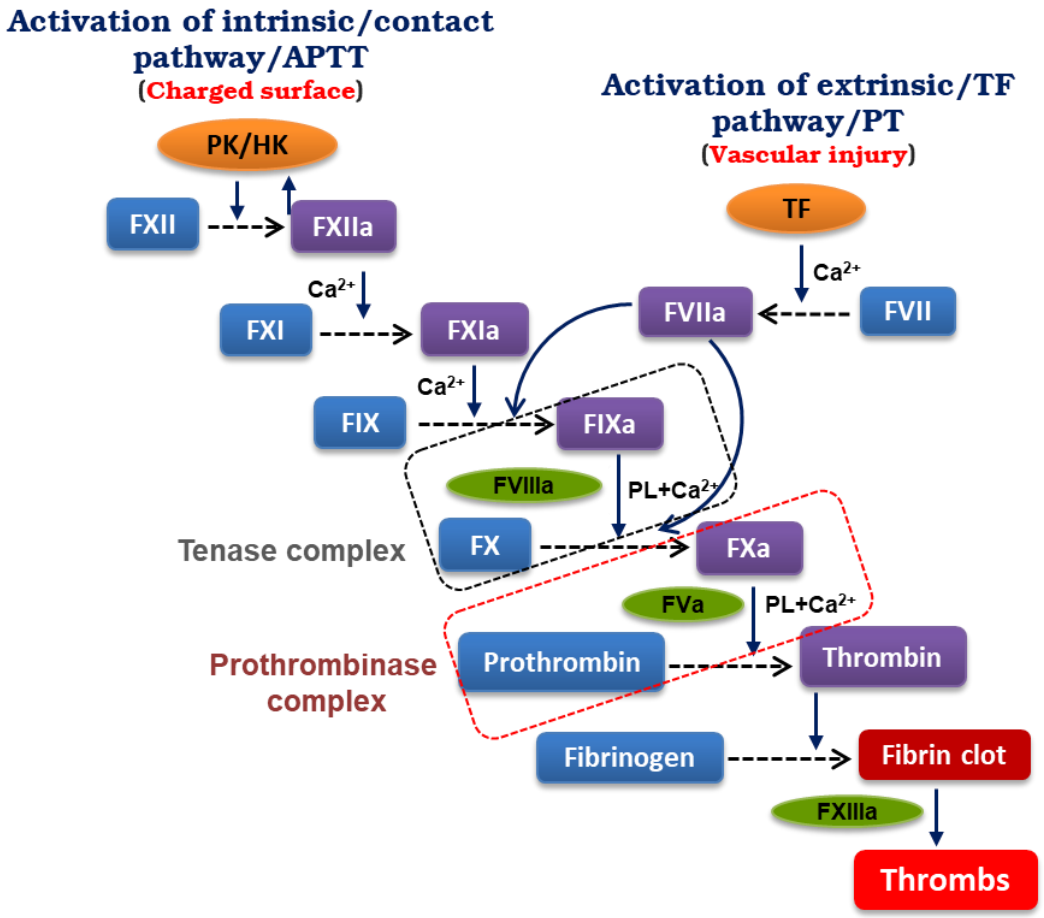


Fig. 1.7. Blood coagulation cascade: Schematic representation of the coagulation pathway where numerous coagulation factors (serine proteases) are involved in downstream activation of coagulation cascade leading to thrombus formation. The extrinsic pathway is initiated in response to the exposure of tissue factor (TF) of endothelial cells due to external damage of the vessel and clinically measured as prothrombin time (PT). The intrinsic pathway is initiated by exposure of endothelial collagen (negative charge surface) and is clinically measured as activated partial thromboplastin time (APTT). Both extrinsic and intrinsic pathways converge at a point of the common pathway which involves activation of FX and prothrombin ultimately leads to the formation of fibrin which further stabilizes the platelet plug. (PK-plasma kallikrein and HK-high molecular weight kininogen).

1.9.1. Extrinsic/tissue factor pathway

The tissue factor pathway is initiated upon vascular injury by exposure of membrane glycoprotein called tissue factor (TF) which is present on the surface of subendothelial tissue. TF binds to inactive 'FVII' and converts it into enzymatically active form 'FVIIa' by cleaving a single peptide bond. TF-FVIIa complex cleaves and activates FX to FXa of the common pathway and FIX to FIXa of the intrinsic pathway. The tissue factor has greater biological importance as it creates a link between both extrinsic and intrinsic pathways of coagulation. Therefore, the complex of TF-FVIIa is believed to be a key step of the clotting cascade (Lasne et al., 2006; Owens and Mackman, 2010).

1.9.2. Intrinsic/contact pathway

The intrinsic/contact pathway of blood coagulation is also called as a kallikrein-kinin system where FXIIa activates prekallikrein to kallikrein and then kallikrein cleaves high molecular weight kininogen (HK) to release bradykinin. When blood comes in to contact with negatively charged surfaces, FXII activates itself to FXIIa and is also reciprocally activated by kallikrein. The FXIIa is involved in downstream activation of FXI, FIX, FX and FII (thrombin), leading to thrombus formation in the presence of Ca^{2+} ions and phospholipids (PL). The intrinsic pathway of coagulation has a limited role in hemostasis and largely contribute to the formation of pathological intravascular thrombosis (Gailani and Renné, 2007). Besides, the intrinsic pathway is also known to have pathological significance during the hyperlipidemic state, bacterial infection, septicemia and typhoid fever (Schmaier and McCrae, 2007; Grover and Mackman, 2019).

1.9.3. Common pathway

This pathway is involved in activation of 'FX to FXa' followed by cleavage of prothrombin to thrombin, which in turn convert soluble fibrinogen to insoluble fibrin clot called 'thrombus'.

The assembly of tenase complex (Ca^{2+}, cofactor VIIIa, FIXa and FX) is involved in activation of FX to FXa and prothrombinase complex (Ca^{2+}, cofactor V, FXa and prothrombin) is involved in rapid generation of thrombin. Besides, thrombin activates FXIII to FXIIIa, which further stabilises the clot by covalent crosslinks between fibrin polymers (Palta et al., 2014).

1.9.4. Regulation of blood coagulation

A fine balance between blood clotting and bleeding is maintained in the body under normal physiological conditions. The coagulation system has different stages of regulation through the expression of several protease inhibitors. The endothelial expression of heparan sulfate activates antithrombin III which mainly decreases the production of thrombin as well as FIXa, FXa, FXIa and FXIIa (Opal et al., 2002; Ezihe-Ejiofor and Hutchinson, 2013). The endothelial release of annexin V prevents the binding of coagulation factors while tissue factor pathway inhibitor controls the FVIIa-TF complex (Dahm et al., 2008). Besides, thrombin regulates several steps of coagulation by activation of FXI and cofactors FV, FVIII, FXIII and feedback inhibition through activation of plasminogen to plasmin. However, protein C pathway majorly contributes to hampering the propagation of coagulation cascade by inhibiting specific cofactors FVa and FVIIIa in the presence of protein S and PL (Rigby and Grant, 2004).

1.9.5. Assessment of blood coagulation efficiency

The prothrombin time (PT) and activated partial thromboplastin time (APTT) assays are used to assess deficiencies in clotting factors and the presence of natural serine proteinase inhibitors/synthetic anticoagulant drugs like heparin and warfarin. A normal PT was 11 to 13 sec which is a clinical representative of TF and common pathways. APTT assay assesses the defects in the intrinsic and common pathway of blood coagulation and normal APTT range is less than 35 sec which usually expressed in International Normalised Ratio (INR) (Turgeon, 2005).

1.9.6. Blood coagulation disorders

The normal blood fluidity was maintained and regulated as a result of many proteolytic events of coagulation factors, cofactors, endothelium and plasmin-mediated fibrinolysis (Furie and Furie, 2008). However, imbalance in the regulatory system leads to bleeding or thrombotic implications associated with the deleterious consequences. Indeed, deficiency in FXIII, FIX leads to a severe bleeding disorder such as haemophilia A and B, respectively (Palta et al., 2014). On the other hand, thromboembolism is the formation of blood clots in the blood circulation system which in turn restricts blood flow and deprives of oxygen supply leading to the deadly medical condition of stroke or heart attack (ISTH steering committee, 2014). Venous thromboembolism (VTE) generally includes deep-vein thrombosis (DVT) and pulmonary embolism (PE), which affected 7-14 million people worldwide (Raskob et al., 2014). DVT is a state of thrombus formation in deep veins of legs or pelvis and its subsequent dissolution followed by migration of thrombi through the heart to pulmonary vasculature is called PE (Goldhaber, 2010; Di Nisio et al., 2016). Besides, various types of cancers and inflammatory diseases are associated with thrombosis, which accounts for the second most leading cause of death after cancer itself (Donnellan and Khorana, 2017).

1.9.7. Risk factors of venous thromboembolism (VTE)

Major acquired risk factors of VTE include hospitalisation, immobility, obesity, smoking, hypertension, diabetes, consumption of alcohol, birth control pills, nutrition, ageing, stress, pregnancy and cancer (Anderson and Spencer, 2003; Stein et al., 2005; Heit et al., 2016). Primarily hospitalised patients concerned for major surgeries related to cancer, congestive heart failure, chronic obstructive pulmonary disease and chronic kidney disease are more susceptible to VTE (Goldhaber, 2010). In addition, hereditary risk factors include deficiency in natural anticoagulants such as antithrombin III, protein C and protein S. However, most of the VTE

complications are preventable through the appropriate administration of cost-effective antithrombotic agents (Mahan et al., 2011; Raskob et al., 2014).

1.9.8. Preventive measures of VTE

The pharmacological approach for prevention and treatment of VTE using various traditional anticoagulants such as heparins and warfarin gained significant importance for several decades (Al-Horani and Desai, 2016). In recent years, new generation anticoagulants were developed using applications of recombinant DNA technology and structure-based drug designing with increased efficacy and convenience (Hirsh et al., 2005; McRae and Eikelboom, 2007; Joppa et al., 2018). All the current anticoagulants in the market fundamentally target the proteases involved in the coagulation cascade, thereby limiting the coagulation process. Inhibition of coagulation factors has emerged as a critical point of intervention for the development of antithrombotic agents.

Plant PIs, in particular, Kunitz type inhibitors are extensively investigated and reported for their anticoagulant activity by both *in vitro* and *in vivo* studies (Brito et al., 2014). Thrombosis is a common core pathology of major cardiovascular diseases such as myocardial infarction, stroke and venous thromboembolism (VTE) (ISTH steering committee for world thrombosis day, 2014). The "Virchow's triad" proposed by Virchow in 1856 postulated that stasis, changes in the vessel wall or changes in the blood could lead to thrombosis (**Fig. 1.7**). Also, any imbalance in the levels of coagulation enzymes or natural anticoagulants contributes to thrombotic risk which in turn leads to heart attack and stroke. The commercial anticoagulants available in the market are targeted towards the serine proteases in the coagulation pathway for prophylaxis and treatment of thromboembolic disorders (Al-Horani and Desai, 2016). Similarly, the natural plant PIs are also known to inhibit the action of these serine proteases (Turk, 2006; Shamsi et al., 2016). Thus, PIs could be exploited in the clinical field as an 'anticoagulant' molecules. The seeds of Bauhinia species are abundant in Kunitz type inhibitors and they inhibited bovine trypsin, chymotrypsin,

human plasmin and plasma kallikrein (Nakahata et al., 2006; Oliva and Sampaio, 2008; Brito et al., 2014). The primary structure of *Bauhinia bauhiniodes* kallikrein inhibitor (BbKI) is similar to other plant Kunitz inhibitors but lack disulfide bonds in their structure (Oliva et al., 1996).

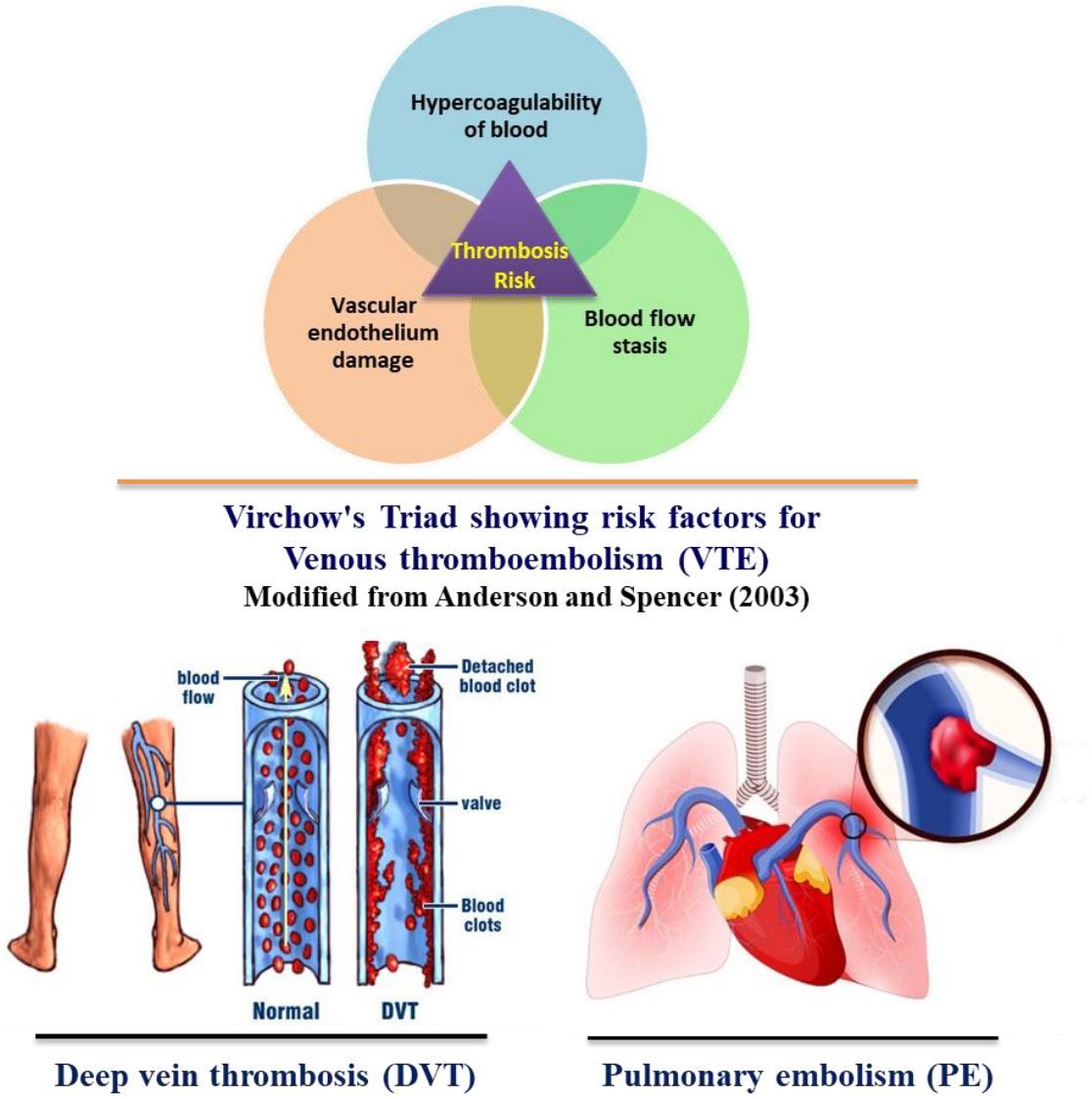


Fig. 1.6. Venous thrombosis and its risk factors: Virchow's triad showing the significant causes of venous thromboembolism (VTE) such as: 'stasis' of blood flow, vascular damage and hypercoagulability which are influenced by various factors. VTE includes deep vein thrombosis (DVT) which occurs in deep veins of legs and pelvis. Pulmonary embolism (PE) occurs due to dislodging of thrombi and dispersal through the blood circulatory system and obstruction of blood flow in the lungs.

Ferreira et al. (2013) generated a recombinant *B. bauhinioides* kallikrein inhibitor (rBbKIm) containing the 'RGD/RGE' motifs of the BrTI from *B. rufa* which is a potent inhibitor of trypsin, chymotrypsin and human plasma kallikrein. Human plasma kallikrein is also known to be a key player of the intrinsic coagulation cascade and it is reported that Bauhinia SPI inhibits PK along with FXa and XIIa and reduced the normal blood coagulation efficiency (Oliva et al., 1996). The plant PIs which inhibit the blood coagulation factors have been reported from several plant sources such as *Cucurbita maxima* (Krishnamoorthi et al., 1990), *Momordica charantia*, *Lagenaria leucantha* and *Luffa cylindrica* (Hayashi et al., 1994), *Leucaema leucocephala* (Oliva et al., 2000), *Caesalpinia echinata* (Cruz-Silva et al., 2004), *Tamarindus indica* (Patil et al., 2012), *Erythrina velutina* (Machado et al., 2013a) and *B. bauhiniodes* (Brito et al., 2014).

Chapter 2

Rationale of the Study and Objectives

Rationale of the Study and Objectives

Lepidopteran insect larvae are the most devastating pests responsible for substantial losses of many economically important crops and making crops susceptible to pathogens. The cotton bollworm '*H. armigera'* is the most severe pest worldwide causing substantial economic losses in cotton, pulses, grain legumes and vegetables (García, 2006; Rajapakse and Walter, 2007). Similarly, cotton leafworm '*S. litura'* is a voracious feeder and phytophagous insect pest majorly on cotton, chilli, soybean, groundnut and many other vegetables (Fand et al., 2015; Cheng et al., 2017). The setting of broad host range for these pests arises from key physiological characteristics such as polyphagy, high mobility and fecundity which facilitate them to survive in adverse conditions (Fitt, 1989). However, massive application of chemical pesticide to control insect pests on crop plants results in enormous economic burden, negative impact on beneficial insects and causes non-target insect resistance (Aktar et al., 2009; Gill and Garg, 2014). Additionally, it also causes a negative impact on ecology and food safety in relation to human health (Haq et al., 2004; Mahmood et al., 2016).

Though the usage of chemical pesticide in agriculture cannot be ignored, there is a need to develop target-specific compounds with low persistence leading to sustainable crop protection. The conventional approach which involves introgression of qualitative traits for host-plant resistance is slow and challenging to achieve (Sharma et al., 2000; Varshney et al., 2013). Besides, IPM practices have been followed to enhance plant resistance to pest and pathogens while minimising environmental risk (Pretty and Bharucha, 2015; Dara, 2019). On the other hand, plant PIs attracted much attention for developing transgenic plants resistant to insect pests (Parde et al., 2012; Jamal et al., 2013; Clemente et al., 2019).

Plant PIs are defensive proteins which act as a feeding deterrent for grazing animals and invasion of phytophagous insects by inhibiting their midgut proteases. PIs are often present in

stem, flowers, leaves, seeds and tubers of the plants and are also induced in response to wounding, insect and pathogen attack and during abiotic stress (Shewry, 2003; Drame et al., 2013; Rehman et al., 2017). The PIs accumulated in specific organs such as seeds and tubers contribute to < 20% of the total protein content in many plants (Ryan, 1990; Meulenbroek et al., 2012). Ingestion of PIs impairs insect digestion where PIs form a stable complex with larval midgut digestive proteases, which in turn affect larval growth and development (Macedo et al., 2011; War et al., 2018). This inhibitory action leads to a decrease in the availability of essential amino acid, which further causes retardation in larval growth and development, and mortality of larvae (**Chapter 1, Fig. 1.4**; De Leo et al., 2002; Zhu-Salzman and Zeng, 2015). Plant PIs could be an alternative potential substitute for *Bacillus thuringiensis* toxin for controlling phytophagous insect pests. Despite insect counter-defence/adaptation mechanism, several transgenic plants expressing plant PIs have been generated which confer resistance against various phytophagous insect pests (Haq et al., 2004; Macedo et al., 2015; Jamal et al., 2019; Clemente et al., 2019).

In addition to their insecticidal properties, PIs are also known to possess anticoagulant property similar to commercial anticoagulants such as heparin and warfarin (Shamsi et al., 2016; Srikanth and Chen, 2016). The state of excessive blood coagulation in the human body was associated with several deadly thromboembolic disorders. In that, venous thromboembolism (VTE) is a multifactorial and third most common vascular disease which occurs due to the formation of a blood clot in veins and obstructs the blood flow in the body (Anderson and Spencer, 2003; Moheimani and Jackson, 2011). The VTE includes deep vein thrombosis (DVT) which arises due to thrombus formation in deep veins of leg, groin or arm and pulmonary embolism (PE) which occurs when DVT clots dislodge to the lungs and block the blood supply, and often becomes fatal. VTE has become a leading cause of death worldwide. However, 10 million cases were reported annually across the globe (Heit et al., 2016; Phillippe, 2017). The VTE is more prevalent among US and Europe population, leading to ~3.0-5.4 lakhs death

every year, respectively (Mahan et al., 2011; ISTH steering committee for world thrombosis day, 2018). VTE is a life-threatening condition often preventable but occur without well-recognised symptoms. Hence it is unrecognised/undiagnosed by the patient or healthcare professionals due to lack of awareness unlike a heart attack, stroke, breast cancer, prostate cancer and AIDS etc. (Wendelboe et al., 2015; Almodaimegh et al., 2017). The significant risk factors of VTE include a person having hip/knee surgery, hospitalisation, long period bedrest, cancer, age, birth control pills, family history, smoking, alcohol and pregnancy (Goldhaber, 2010; Di Nisio et al., 2016). The symptoms of DVT include swelling, redness and pain or tenderness of calf, ankle or foot while PE includes chest pain, shortness of breath, rapid breathing and rapid heart rate (Phillippe, 2017). VTE prophylaxis and treatment has become a substantial economic burden on the healthcare system. The available preventive or treatment measures include the use of blood thinners/anticoagulants.

The currently available commercial anticoagulants used for treating VTE precisely target the serine proteases of coagulation cascade such as thrombin and FXa. The traditional broad-spectrum anticoagulant class such as heparins and coumarins have been ruling from the past 60 years in prophylaxis and treatment of thrombotic disorders (Weltermann et al., 2003; Moheimani and Jackson, 2011). Anticoagulant heparins such as unfractionated heparin, low molecular weight heparin composed of a heterogeneous mixture of glycosaminoglycans and polysulfated glycosaminoglycans, respectively, are activators of antithrombin (Hirsh and Raschke, 2004). In the coumarin class, warfarin is a vitamin K antagonist and it is most commonly used for the prophylaxis of VTE either independently or in combination with low molecular weight heparin. However, each of these molecules have their own disadvantages. The disadvantages associated with warfarin include drug-drug and drug-food interaction (Ageno et al., 2012). Similarly, heparin is associated with non-specific binding affinity, thrombocytopenia, osteoporosis and risk of contamination. It also requires routine dose-adjustment and monitoring

(McRae and Eikelboom, 2007; Garcia et al., 2012). Recently, a new class of target-specific peptidomimetic oral anticoagulants were developed with higher efficacy and safety over traditional anticoagulants (Wong et al., 2007; Al-Horani and Desai, 2016). They act as direct inhibitors of FXa [rivaroxaban, apixaban, edoxaban and betrixaban] and thrombin [hirudins, argatroban and dabigatran] (Pinto et al., 2010; Lee and Ansell, 2011; Al-Horani and Desai, 2016). The various types of treatment available for thromboembolic disorders includes the use of commercial anticoagulants [low molecular weight heparin, enoxaparin and oral anticoagulants such as apixaban, dabigatran, rivaroxaban and edoxaban], mechanical devices [compression stocking and special filters] and thrombolytic therapy using tissue plasminogen activator (Ay et al., 2017). Though these new generation anticoagulants have some advantage over heparin and coumarins, all clinically used anticoagulants are associated with severe life-threatening bleeding complications and problem in their reversibility (Joppa et al., 2018).

Therefore, search for a new class of alternative anticoagulant agents with enhanced efficacy and easy availability particularly from plant source will help to overcome the limitations of regularly used antithrombotic drugs. In this scenario, plant PIs such as Kunitz and Squash family inhibitors have been ascertained as novel anticoagulants by their specificity towards factors involved in the coagulation cascade (**Table, 2.1**; Oliva et al., 1996, 2000; Patil et al., 2012; Brito et al., 2014). In contrast, legume BBIs are less explored for their anticoagulant properties, despite their well-established role in the prevention of cancer, cardiovascular, and inflammatory diseases (Losso, 2008; Clemente and Arques, 2014; Srikanth and Chen, 2016; Joanitti et al., 2018).

Table 2.1. Inhibition constant (Ki) values of the different squash and Kunitz type plant protease inhibitors against blood coagulation factors/proteases.

PI source	Inhibitory constant (Ki)			Reference
	PK	FXIIa/FXIa	FXa	
Hordeum vulgare (BTI)	2.8×10^{-7} M	1.1×10^{-7} M	ND	Chong and Reeck, 1987
Cucurbita maxima (CMTI-V)	ND	4.1×10^{-8} M	ND	Krishnamoorthi et al., 1990
Momordica charantia MCTI-I MCTI-II MCTI-III	 1.1×10^{-4} M 1.0×10^{-4} M 1.4×10^{-4} M	 1.3×10^{-8} M 5.6×10^{-8} M 1.6×10^{-6} M	 1.0×10^{-4} M 1.4×10^{-6} M 5.9×10^{-5} M	Hayashi et al., 1994
Cucurbita maxima CMTI-III	 1.3×10^{-4} M	 7.0×10^{-8} M	 2.3×10^{-5} M	Hayashi et al., 1994
Lagenaria leucantha LLTI-II LLTI-III	 2.7×10^{-5} M 2.0×10^{-4} M	 1.4×10^{-6} M 4.2×10^{-6} M	 4.1×10^{-5} M 1.9×10^{-5} M	Hayashi et al., 1994
Luffa cylindrica LCTI-II LCTI-III	 2.0×10^{-5} M 3.8×10^{-5} M	 7.5×10^{-8} M 3.8×10^{-9} M	 7.8×10^{-4} M 1.0×10^{-4} M	Hayashi et al., 1994
Bauhinia variegate (SPI)	8.0×10^{-8} M	10.8×10^{-8} M	ND	Oliva et al., 1996
Bauhinia ungulate (SPI)	0.7×10^{-8} M	7.4×10^{-8} M	1.4×10^{-8} M	Oliva et al., 1996
Leucaena leucocephala (LlTI)	6.3×10^{-9} M	ND	ND	Oliva et al., 2000
Caesalpinia echinata (CeKI)	3.1×10^{-9} M	0.18×10^{-9} M	0.49×10^{-9} M	Cruz-Silva et al., 2004
Bauhinia rufa (BrTI)	14×10^{-9} M	ND	ND	Nakahata et al., 2006
Tamarindus indica (TKI)	ND	ND	2.2×10^{-7} M	Patil et al., 2012
Bauhinia baunoides (rBbKIm)	3.6×10^{-9} M	ND	ND	Ferreira et al., 2013
Crataeva tapia (CrataBL)	ND	ND	8.6×10^{-6} M	Ferreira et al., 2013 Salu et al., 2014
Zea mays (CHFI)	ND	3.2×10^{-9} M 5.4×10^{-6} M	ND	Korneeva et al., 2014
Bauhinia baunoides (BbKI)	2.4×10^{-9} M	ND	ND	Brito et al., 2014
Caesalpinia echinata (rCeEI)	1.0×10^{-9} M	ND	ND	Cruz-Silva et al., 2016
Delonix regia (DrTI)	5.25×10^{-9} M	3.1×10^{-7} M/ 1.3×10^{-6} M	ND	Salu et al., 2018
Acacia schweinfurthii (AsTI)	1.6×10^{-9} M	ND	ND	Salu et al., 2018

PK-human plasma kallikrein; ND-not determined

India is the second-largest producer of peanut besides china followed by Nigeria and the United States and it is also cultivated in tropical and subtropical regions of the world (Arya et al., 2016; Stalker, 2017). However, the cultivated peanut (*A. hypogaea* L.) is an allotetraploid susceptible to several foliar, soil born diseases and pest attack leading to a remarkable loss in crop yield (Wightman and Rao, 1994). Contrarily, the primary gene pool of several diploid wild Arachis species showed a high level of resistance to several diseases and insect pests (Holbrook and Stalker, 2003; Mallikarjuna et al., 2011; de Paula et al., 2017). Introgression of these resistant traits into the cultivar Arachis species has been difficult due to differences in ploidy level and genomic incompatibility which cause hybrid failure or sterility (Tallury et al., 2005). However, the approach of generating amphidiploids/synthetic hybrids from wild Arachis parental species by colchicine treatment paved the way for introgression of useful traits into the cultivated peanut species (Garcia et al., 2006; Fávero et al., 2015). So far, several interspecific hybrids of peanut have been developed with increased resistance to biotic and abiotic conditions (Mallikarjuna et al., 2004; Kumari et al., 2014; de Paula et al., 2017; Stalker, 2017).

A comparative study on the inhibitory activity of different peanut varieties ascertained that crude proteinase inhibitor extract of different interspecific hybrids has prominent trypsin inhibitor (TI) and *S. litura* midgut trypsin-like protease inhibitor (SlTPI) activity than commercial varieties (**Fig. 2.1;** Swathi, (PhD thesis, 2016). Therefore, peanut interspecific hybrid (4368-1) variety with high SlTPI activity was chosen in the present study. Also, the presence of various BBI isoforms and its crystal structure has been elucidated in some cultivars of peanut (Norioka et al., 1982; Suzuki et al., 1987). The identification and characterization of BBI in interspecific hybrid varieties of peanut which is known to possess biotic resistant traits against pests or pathogens is not explored so far (Suzuki et al., 1987; Mallikarjuna et al., 2011).

Therefore, the present study was undertaken to purify trypsin specific BBIs from an interspecific hybrid variety (4368-1) of peanut (PnBBI) and production of recombinant Bowman-

Birk isoinhibitor (rPnBBI) in the *E. coli* system. Further, it involved biochemical and biophysical characterization of both native PnBBI and rPnBBI followed by evaluation of their insecticidal as well as anticoagulant potential. These insights might provide a strong basis to develop insect-resistant transgenic crop plants with increased pest resistance and enlighten the functional food properties of peanut that is rich in BBIs and promotes humans health. Further, understanding the biological nature, structural and functional properties of inhibitors and their target proteases unveil the new approaches in preventive and therapeutic interventions.

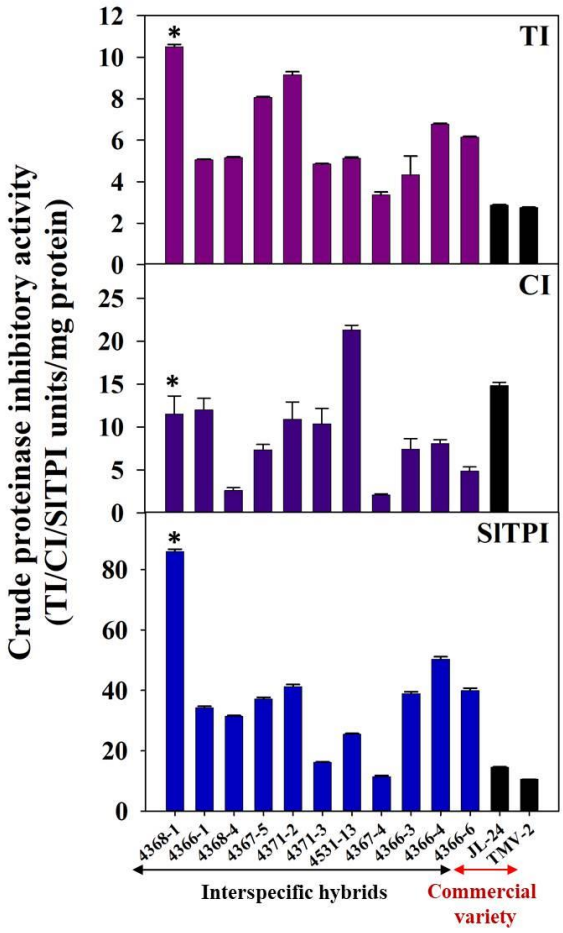


Fig. 2.1. Comparative inhibitory activity of different peanut varieties against bovine trypsin, chymotrypsin and *S. litura* midgut trypsin-like proteases (SlTPs): Crude proteinase inhibitor extract of different interspecific hybrids and commercial varieties of peanut are incubated with different proteases and residual protease activity was determined after addition of corresponding substrates. The accession of peanut which exhibited maximum trypsin inhibitor (TI) and *S. litura* midgut trypsin-like protease inhibitor (SlTPI) activity were indicated by an asterisk.

Based on the above rationale, the present study was aimed with the following objectives.

Objectives of the study

1. Purification and biochemical characterization of Bowman-Birk Inhibitor (PnBBI) from seeds of peanut interspecific hybrid variety (4368-1).

2. Evaluation of the insecticidal potential of PnBBI against agriculturally important insect pests *Helicoverpa armigera* and *Spodoptera litura.*

3. Cloning, expression and purification of a recombinant BBI isoform from peanut interspecific hybrid variety (rPnBBI) and evaluation of its biochemical properties.

4. A comparative study on the anticoagulant properties of native PnBBI and rPnBBI using *in vitro* assays and surface plasmon resonance studies.

Chapter 3

Materials and Methods

Materials and Methods

3.1. Seed material

Peanut seeds (Interspecific advanced hybrid variety 4368-1) were provided by the International Crop Research Institute for Semi-Arid Tropics (ICRISAT) located in Patancheru, Hyderabad, Telangana, India.

3.2. Chemicals and others

Bovine serum albumin, bovine pancreatic α-trypsin and α-chymotrypsin, Polyvinylpyrrolidone (PVP), casein, Ethylenediaminetetraacetic acid (EDTA), water-saturated phenol, sodium acetate and β-mercaptoethanol were procured from Sisco Research Laboratory (SRL), Mumbai, India. CNBr activated Sepharose-4B, Sephadex G-50 fine grade, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA), *N*-glutaryl-L-phenylalanine-*p*-nitroanilide (GLUPHEPA), soybean trypsin and chymotrypsin inhibitor (SBBI), phenylmethylsulfonyl fluoride (PMSF), *N*α-Tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), *N*p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), TRI reagent, tricine, gelatin, lithium dodecyl sulphate, guanidium thiocyanate, isoamyl alcohol, isopropyl thiogalactopyranoside (IPTG), CH_3OCO-D-CHA-Gly-Arg-*p*NA-AcOH substrate and Coomassie brilliant blue R-250 were purchased from Sigma Aldrich, Saint Louis, USA. Immobiline pH gradient dry strips (IPG strips), IPG buffer, Dithiothreitol (DTT), Iodoacetamide (IDA), urea, thiourea, 3-[(3-Cholamidopropyl) dimethylammonio]-1-Propanesulfonate hydrate (CHAPS), CM5 sensor chips, amine coupling kit and HEPES 10X buffer were procured from GE Healthcare Bio-Sciences, Uppsala, Sweden. Bicinchoninic acid (BCA) protein estimation kit, Verso cDNA synthesis kit, 50 bp as well as 1 kb DNA ladder, protein molecular mass standard and 3 kDa cut-off snakeskin dialysis membrane were purchased from Thermo Fisher Scientific, USA. Amicon Ultra centrifugal filter units were procured from Millipore Corporation, USA. The vector pTWIN1, Sap1, Pst1, BamH1, Nde1, *E. Coli* DH5α, BL21 (DE3) and all PCR components were purchased from New England Biolabs. SYBR Green PCR Master Mix purchased from Takara Bio, Shiga, Japan. Plasmid preparation and agarose gel extraction kits were procured from Qiagen, GmbH, Hilden, Germany. Luria-Bertani medium, agar-agar and ampicillin were purchased from Hi-Media Laboratory, Mumbai, India. FIXa, FXa, FXIIa and human plasma kallikrein were purchased from Enzyme Research Laboratories, USA. FXIa, substrates H-D-CHA-Gly-Arg-*p*NA.2AcOH and H-D-But-CHA-Arg-*p*NA.2AcOH were purchased from SEKISUI Diagnostics, American Diagnostica GmbH, Germany. Multimode plate reader (TECAN, Infinite), the flat bottom 96 well plates from TAESONS and heparin was

purchased from Samarth Life sciences (Mumbai, India) and all other chemicals and reagents used were of analytical grade.

3.3. Protein estimation

Protein content was determined by Bicinchoninic acid (Smith et al., 1985) and Bradford method (Bradford, 1976) using BSA as a standard.

3.4. Purification of peanut native Bowman-Birk Inhibitor (PnBBI)

3.4.1. Preparation of peanut crude proteinase inhibitor (PnCPI) extract

Mature, dry peanuts were ground into fine powder and depigmented by three washes of acetone followed defatted with hexane thrice. The filtered peanut powder was allowed to air-dried followed by PnCPI extraction with 1:6 (w/v) 50 mM Tris-HCl, pH 8.0 containing 1% PVP by mild stirring at 4 °C for overnight. The clear supernatant obtained from centrifugation at 10,000 rpm for 20 min was collected and used as PnCPI extract for further purification of PIs (**Fig. 3.1**; Prasad et al., 2010b).

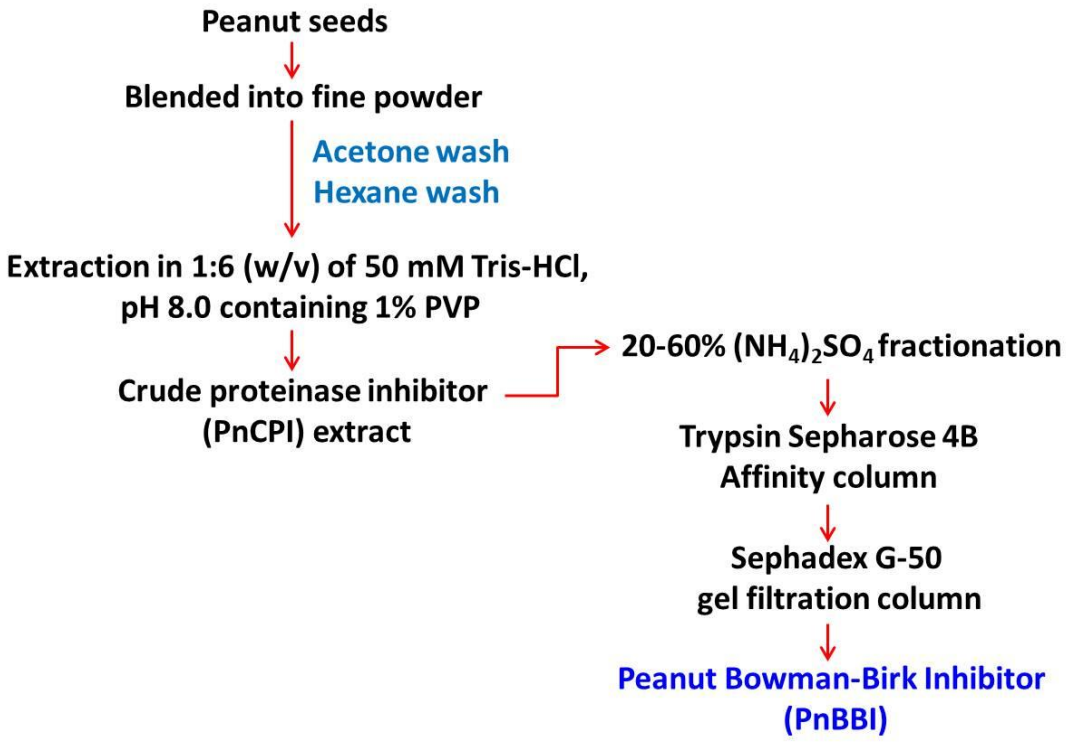


Fig. 3.1. Schematic diagram of PnBBI purification: Representation of various steps involved in the purification of PnBBI from peanut interspecific hybrid variety (4368-1) by using trypsin affinity and gel filtration columns. Further details are as described in section 3.4.

3.4.2. $(NH_4)_2SO_4$ fractionation

The clear PnCPI extract was subjected to 0-20%, 20-60%, 60-80% $(NH_4)_2SO_4$ fractionation by mild stirring for ~45 min followed by centrifugation at 10,000 rpm for 20 min. The precipitated protein in each fractionation step was dissolved and dialyzed (3 kDa cut-off snakeskin dialysis membrane) against 50 mM Tris-HCl, pH 8.0. The fraction showing prominent TI activity (Refer section 3.5) was used for further purification using AKTA prime plus (GE Healthcare Life sciences) fast protein liquid chromatography (FPLC) system by affinity and size exclusion chromatography columns.

3.4.3. CNBr Sepharose trypsin affinity chromatography

The dialyzed protein fraction (20-60%) was loaded onto trypsin bound CNBr-activated Sepharose 4B column (XK 16/20, 3cm), pre-equilibrated with 50 mM Tris-HCl, pH 8.0 containing 100 mM NaCl. The bound trypsin specific proteins were eluted by 0.01 N HCl with a flow rate of 60 ml/h and subsequently neutralized with 20 µl of 2.0 M Tris-HCl, pH 8.0. The 1.0 ml fractions possessed significant TI activity were assembled and subjected to buffer exchange (3 kDa c/o dialysis membrane) followed by freeze-drying (Labconco, USA).

3.4.4. Size exclusion chromatography

The concentrated affinity fraction pool was loaded onto Sephadex G-50 fine column (XK 16/100, 85 cm), pre-equilibrated with 50 mM Tris-HCl, pH 8.0 at a flow rate of 30 ml/h and subsequently collected in 1.0 ml tubes. The fractions showing TI activity were pooled, concentrated using 3-kDa cut-off Amicon ultra centrifugal filters and named as peanut native BBI (PnBBI) than stored at -20 ºC for further use.

3.5. Proteinase inhibitor assay

Inhibitory activity of PnCPI/PnBBI against bovine trypsin or chymotrypsin and midgut trypsin-like (HaTP/SlTP) or chymotrypsin-like (HaCP/SlCP) proteases of *H. armigera* and *S. litura* was determined by the addition of an increasing amount of inhibitor to the corresponding proteinase and monitoring its residual activity at 410 nm (**Table 3.1**; Refer section 3.11.4). Assay mixture (1.0 ml) consists of PI in assay buffer composed of 50 mM Tris-HCl containing 20 mM $CaCl_2$ either at pH 8.2 for analysing trypsin inhibitory activity (TI) or pH 7.8 for chymotrypsin inhibitory activity (CI) or 50 mM Glycine-NaOH at pH 10.5 for midgut trypsin and chymotrypsin-like protease inhibitory activities of *H. armigera* (HaTPI/HaCPI) and *S. litura* (SlTPI/SlCPI). In the assay mixture, trypsin (10 µg) or chymotrypsin (80 µg) or midgut protease extract of *H. armigera* (HaGP) or *S. litura* (SlGP) equivalent to one protease unit was added and incubated

at 37 °C for 15 min (Refer section 3.11.5). Residual trypsin/trypsin-like or chymotrypsin /chymotrypsin-like protease activity was determined in the presence of 1 mM BAPNA (Erlanger et al., 1961) or GLUPHEPA (Mueller and Weder, 1989), respectively. The reaction was terminated after incubating for 45 min at 37 °C by adding 0.2 ml of 30% acetic acid. The activity of PI(s) was expressed as TI / CI / HaTPI / HaCPI / SlTPI / SlCPI units/mg protein. One protease inhibitor unit (TI / CI / HaTPI / HaCPI / SlTPI / SlCPI) was defined as the amount of inhibitor protein required to inhibit 50% hydrolysis of BAPNA and GLUPHEPA by the corresponding proteases.

Table 3.1. Method of proteinase inhibitor assay: Suitable assay buffer containing protease and PI was incubated for 15 min at 37 °C. This was followed by quantification of residual protease activity using the respective substrates as described in section 3.5. The relative inhibitory activity was calculated with respect to control OD. One proteinase inhibitor unit is defined as the amount of PI required to inhibit the corresponding protease activity by 50%.

S. No	Assay buffer (ml)	Proteinase inhibitor (µl)	Protease (ml)	Incubation for 15 min@37 °C	Substrate (ml)	Incubation for 45 min@37 °C	30% Acetic acid	OD @ 410 nm
Blank	0.5	-	-					-
Control	0.5	-	0.5					
Test 1	0.490	10	0.5					
Test 2	0.480	20	0.5		1.0		0.2 ml	
Test 3	0.470	30	0.5					
Test 4	0.460	40	0.5					
Test 5	0.450	50	0.5					

3.6. Electrophoresis

3.6.1. Tricine SDS-PAGE

Tricine SDS-PAGE was performed as described by Schägger (2006) using 4% stacking gel and 15% separating gel under reducing and non-reducing conditions. The samples were reduced with 50 mM DTT at 56 °C for 1 h followed by alkylation with a 2-fold molar excess of iodoacetamide for 45 min in the dark at ambient temperature. The pre-stained protein molecular mass standard was used along with commercial soybean BBI (8 kDa) to minimize the differences in molecular mass of PIs. The gel was stained with silver nitrate method (Chevallet et al., 2006).

3.6.2. Native PAGE

Native-PAGE was carried out in 4% stacking and 12.5% separating gels as described by Laemmli (1970). The gel was stained with CBB R-250.

3.6.3. Gelatin SDS-PAGE

In-gel activity staining was performed by casting resolving gel with 1% gelatin as a protease substrate as described by Felicioli et al. (1997). After electrophoresis, gels were washed twice for 20 min each with 2.5% (v/v) Triton X-100 followed by distilled water. The gels were then incubated in 0.1 M Tris-HCl containing 20 mM $CaCl_2$, pH 8.2 for trypsin or pH 7.8 for chymotrypsin and 0.1 M Glycine-NaOH, pH 10.5 for SlGP extracts (Refer section 3.5). After hydrolysis of gelatin by corresponding proteinases at 37 °C, the gels were washed with distilled water and stained with CBB R-250. The presence of inhibitory band(s) active against trypsin/chymotrypsin/SlGPs was identified by the dark blue band(s) against a clear background due to complex formation between un-hydrolysed gelatin and CBB stain. Commercially available soybean BBI was used as a marker protein.

3.6.4. Two-dimensional gel electrophoresis (2-DE)

Immobiline pH gradient (IPG) dry strip (pH 4-7, 11 cm, L) was rehydrated overnight with 200µl of rehydration buffer containing 100 µg protein under reducing conditions (7.0 M urea, 2.0 M thiourea, 4% CHAPS, 50 mM DTT and 1% IPG buffer) or native conditions (10% sorbitol containing 1% IPG buffer). Isoelectric focusing (IEF) was performed using Ettan IPGPhor3 Isoelectric focusing system (GE Healthcare) at current setting of 50 µA per strip as per the manufactures instructions. Voltage is applied as follows: 500 V - 500 Vh; linear gradient to 1000 V - 800 Vh; ramping gradient to 6000 V - 8800 Vh and final focusing at 6000 V - 4500 Vh. After IEF, each strip was equilibrated for 20 min with 50 mM DTT followed by 100 mM IDA in equilibration buffer (6 M urea, 30% glycerol and 2% SDS in 0.1 M Tris-HCl, pH 8.8) for reduced 2-DE or in equilibration buffer without DTT and IDA for non-reduced 2-DE. The second-dimension was performed in 15% Tricine SDS-PAGE or gelatin SDS-PAGE as described in Section 3.6.3. The pre-stained protein molecular mass standards and soybean BBI were used as markers and gels were stained with CBB R-250.

3.7. Mass spectrometry

The molecular mass of native PnBBI and rPnBBI was determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF), facilitated by proteomics facility of the School of Life Sciences, UoH. The saturated solution of a matrix, α-cyano-4-

hydroxycinnamic acid (CHCA, prepared in 50% ACN and 0.1% TFA in water) was mixed with analyte protein in 1:1 ratio and spotted on MALDI metal target plate as described by Prasad et al. (2010c). The resulting air-dried solid material was irradiated with laser pulses (Nd: YAG laser) which generate a short burst of ions accelerated to a fixed amount of kinetic energy under vacuum. These ions travel in a flight tube according to their size, detected in linear mode and produce time-of-flight (TOF) spectrum (Bruker Daltonics, Autoflex III smartbeam instrument, Bremen, Germany). For MALDI MS-MS analysis, the isoinhibitor spot (pI 5.9) from the reduced 2-DE gel was excised, reduced with DTT (10 mM) and alkylated with IDA (55 mM) before tryptic digestion (12.5 µg/µl). The purified peptide mixture was spotted on a MALDI target plate and peptide mass fingerprint (PMF) spectrum was generated (MALDI-TOF). This was followed by second ionization of high-intensity PMF peaks (MALDI TOF-TOF) using Biotools (Bruker Daltonics, Version 3.1). Further, protein was identified using Mascot MS/MS ion search based on raw data of different peptides. The following peptide sequence obtained was subjected to 'BLASTp' search analysis against the SwissProt database and closely resembled sequences were aligned using the Clustal Omega tool. The mass spectrometry protein identification data has been deposited to the ProteomeXchange Consortium (Deutsch et al., 2017) via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD016933.

3.8. Stability to temperature, pH, and reducing agent dithiothreitol

The effect of temperature on the inhibitory activity of native PnBBI or rPnBBI was assessed by incubating them at a range of temperatures (20-90 °C) for 30 min. The effect of pH was also determined by incubating them for 30 min in the following buffers at a final concentration of 50 mM: Gly-HCl (pH 2-3), sodium acetate-acetic acid (pH 4-5), sodium phosphate buffer (pH 6-7), Tris-HCl (pH 8-9) and Gly-NaOH (pH 10-12). Effect of DTT on their inhibitory activity was assessed by incubating them with increasing concentration of DTT (0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 mM) at 56 °C for 1 h followed by alkylation with IDA for 45 min in the dark. The residual TI and CI activities of native and recombinant PnBBI were determined under various conditions as described above by using BAPNA and GLUPHEPA as substrates in assay buffer indicated in section 3.5.

3.9. Circular Dichroism spectroscopy

CD spectroscopy was used to determine the secondary structural elements of native and recombinant PnBBI at far-UV (190-260 nm) range using JASCO J-810 spectropolarimeter. The spectrum of various PnBBI concentrations prepared in 10 mM PBS (0.2-0.6 mg/ml) was

recorded at 25 °C using the following parameters: scan speed '50 nm/min', data pitch and bandwidth of '1 nm', and cuvette path length '2 mm' in which '0.5 ml' of protein solution (PnBBI/rPnBBI) was added. Effect of different buffers from acidic to basic pH range on the secondary structure was determined by incubating the inhibitor protein (0.3 mg/ml) in respective pH buffer as described in the section (3.7) for 1 h. The structural stability of inhibitors at various temperatures was assessed by recording the far-UV CD spectra for every 10 °C interval from 20-90 °C using the Peltier thermostat. Similarly, the effect of DTT on inhibitor structure was determined by reducing with 2 mM DTT followed by incubation with 4 mM IDA as described in the section (3.7) before recording the far-UV CD spectra. The final spectrum is an average of five scans and the nitrogen gas was constantly supplied during the operation. The obtained CD spectra were analysed using DichroWeb online server (Whitmore and Wallace, 2004, 2008). The unit of ellipticity was represented as millidegrees.

3.10. Surface plasmon resonance (SPR)

Biacore T200 instrument of GE Healthcare was used to determine biomolecular interactions of ligand PnBBI/rPnBBI with analytes such as trypsin, chymotrypsin, human blood coagulation factors such as plasma kallikrein (PK), FXIIa, FXIa, FIXa and FXa (Refer section 3.13.2). The principle involved in this analysis is the SPR phenomenon that occurs in thin conducting films at an interspace between the medium of different refractive index (**Fig. 3.2A**; Douzi, 2017). Binding kinetics of different analytes with immobilized inhibitors (PnBBI/rPnBBI) was determined by the characteristics such as association (Ka) and dissociation (Kd) rate constants. A typical SPR sensorgram represents four phases such as association, steady/equilibrium, dissociation and regeneration phase (**Fig. 3.2B**). Immobilization of ligand PnBBI/rPnBBI on the surface of versatile carboxymethyl dextran-5 (CM5) sensor chip was achieved by principle of amine coupling chemistry according to the manufacturer's instructions. The dextran matrix on the sensor chip surface, flow cell - 2 (Fc-2) was first activated with a mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N hydroxysuccinimide (NHS) to generate reactive succinimide esters. Initially, pH scouting experiments were performed to determine the optimum pH value required for maximum immobilization of ligand molecule (**Fig. 3.3A**). Then, PnBBI/rPnBBI ligand was diluted to the concentration of 100µg/ml in 10mM such sodium acetate buffer pH 4.5, passed over the sensor chip surface, where the esters react spontaneously with uncharged amino groups to link the ligand covalently to the dextran. This amine coupling procedure resulted in immobilization of approximately ~455.3 RU of PnBBI/rPnBBI to the chip (**Fig. 3.3B**). The residual active NHS esters were blocked by passing ethanolamine-HCl after

sufficient immobilization and the reference flow cell (Fc-1) was treated in the same way without ligand. Then, initial binding experiments were performed to evaluate the immobilization of the ligand molecule before the kinetic study (**Fig. 3.3C**).

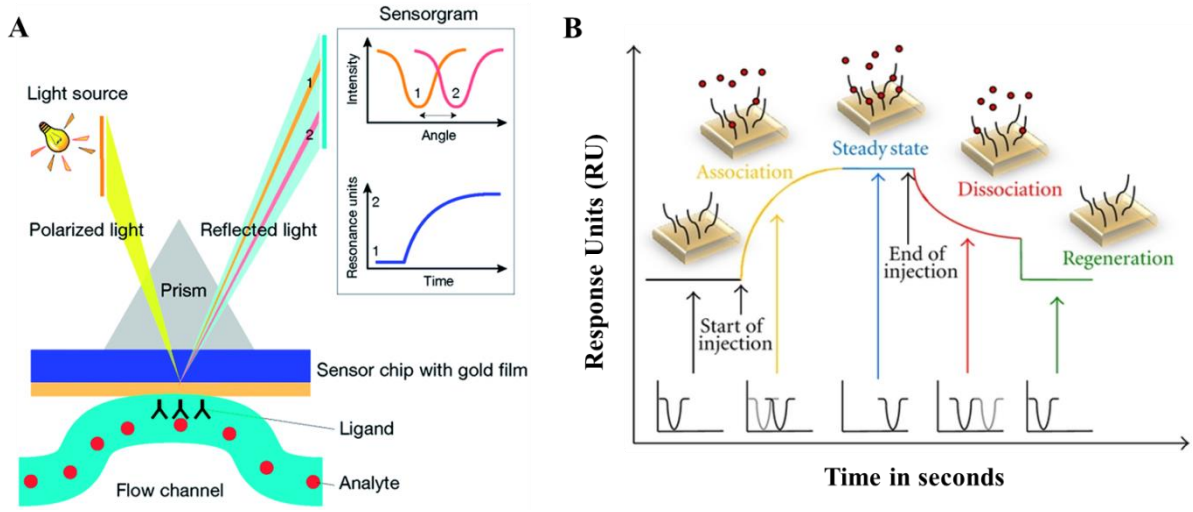


Fig. 3.2. Schematic view of SPR detection principle and typical sensorgram of kinetic study: (A) The incident polarized light is coupled to a glass prism on the biosensor chip which is coated with a thin layer of gold and integrated with a flow channel for continuous flow of a buffer. At a defined incidence angle, the SPR phenomenon is seen as a dip in the intensity of the reflected light, characteristic of the specific angle of reflection. The shift of the angle of reflection from position 1 to position 2 reveals a change in the composition of the medium near the gold film as a result of the binding between the ligand and analyte. The angular variations are recorded in resonance units (RU) and plotted against time in a sensorgram (Adapted from Brogioni and Berti, 2014) and **(B)** Interactions of analytes (red dots) passing through the solution over the surface of immobilized ligand, while SPR sensors detect a refractive index changes during the analyte binding to ligand within the detection area as a change of resonance angle. The angular variations are recorded in resonance units and plotted against time in a sensorgram showing different phases of the kinetic cycle such as association, steady-state, dissociation and regeneration.

The kinetic experiments were performed by passing over different concentrations of analytes (thrice) on the sensor chip at a flow rate of 30µl/min, contact time of 180 sec and dissociation time of 600 sec using 0.1 M HEPES buffer containing 1.5 M NaCl, 30 mM EDTA and 0.5% surfactant P20 at pH 7.4 (or) 0.1 M PBS containing 27 mM KCl and 1.37 M NaCl at pH 7.4 under RT (25 ºC). Regeneration involves removal of bound analyte and this was achieved by passing the Glycine-HCl at pH 2.0 or 5 M NaCl with a contact time of 30 sec after each cycle and stabilization period of 60 sec. Biomolecular interactions on the sensor chip are represented

as a change in Resonance/Response Units (RU) in a sensorgram which was plotted as RU against time in sec. One RU corresponds to a change in surface concentration of approximately 1 pg/mm^2. Obtained data was analysed using BIAevaluation software (version 4.0, GE Healthcare Life Sciences) with Langmuir fit model of 1:1 binding.

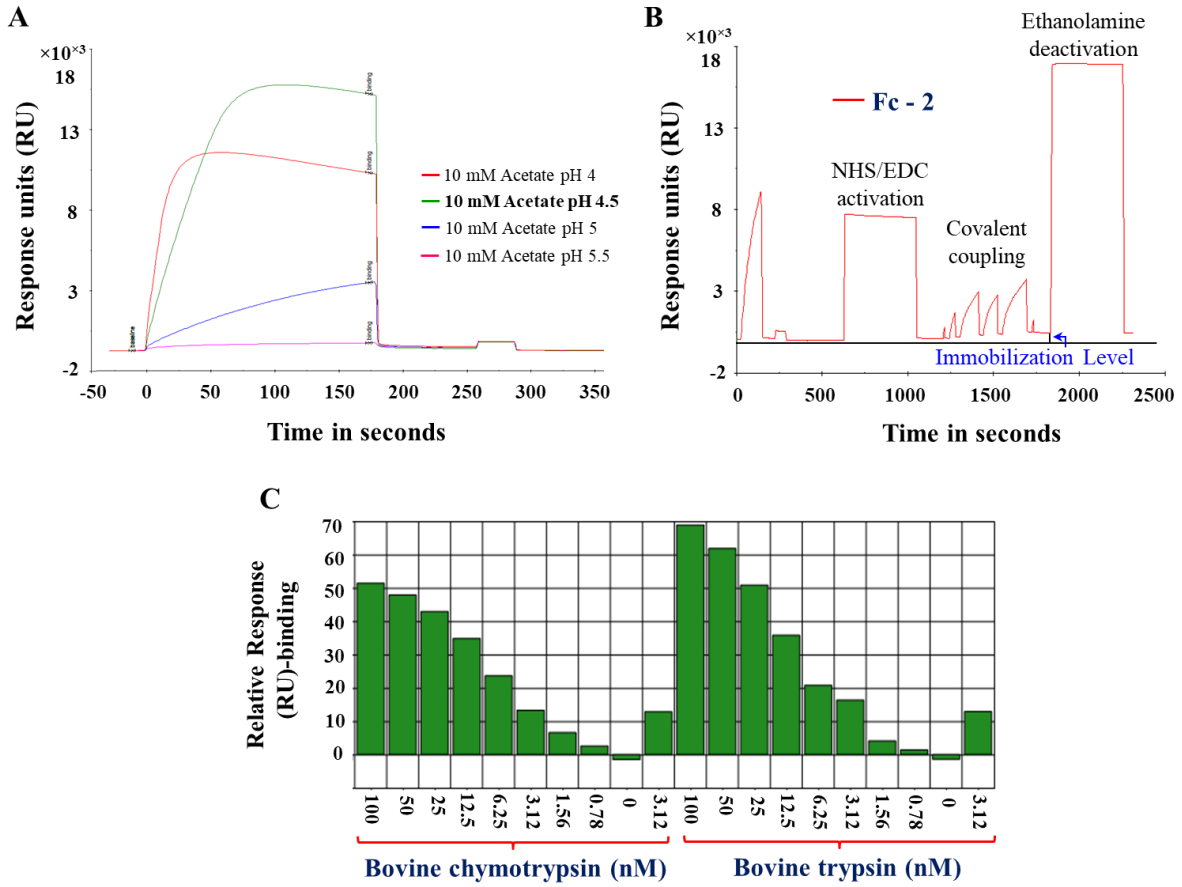


Fig. 3.3. Immobilization of PnBBI/rPnBBI on CM5 sensor chip (Fc-2): (A) pH scouting experiment in which maximum binding of ligand was observed at pH 4.5; **(B)** Immobilization of ligand includes series of steps such as: surface activation by a mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N hydroxysuccinimide (NHS) to generate reactive succinimide esters, covalent coupling and inactivation of active NHS esters by ethanolamine after sufficient coupling of ligand molecules and (**C**) Binding evaluation of analytes bovine trypsin and chymotrypsin on the surface of immobilized ligand PnBBI/rPnBBI before performing SPR kinetic studies. Different concentrations of analytes were prepared in HEPES buffer, pH 7.4 and sequentially passed over the surface of an immobilized ligand. Each binding cycle involves association, dissociation and regeneration phases.

3.10.1. SPR competitive binding assay by capture method

The competitive binding experiment was performed to determine the specificity of two reactive sites of double-headed ligand (PnBBI) towards trypsin and chymotrypsin by following the Biacore T200 control software (capture method module). The flow cell (Fc2-1) of CM5 sensor chip immobilized with PnBBI was confined with saturated (500 nM) levels of first analyte (chymotrypsin/trypsin) followed by passing over (30 µl/min) different concentrations (3.12, 6.25, 12.5, 25, 50 and 100 nM) of second analyte (trypsin/chymotrypsin). The capture process was regenerated by removing both the analytes from PnBBI surface. The disproportion in the number of bound molecules of trypsin or chymotrypsin was interpreted by the difference in RU.

3.11. Evaluation of the insecticidal potential of PnBBI

3.11.1. Insects

The egg mass of *H. armigera* and *S. litura* was procured from the National Bureau of Agricultural Insect Resources (NBAII-MP-NOC-01: *H. armigera* and NBAII-MP-NOC-02: *S. litura*), Bengaluru, India. The classification, global distribution of species and its life-cycle, and the most damaged crop plants by these insect pests were depicted in **Fig. 3.4**.

3.11.2. Rearing of *Helicoverpa armigera* and *Spodoptera litura* larvae

The egg mass was allowed to hatch on fresh castor leaves in insect culture facility which is maintained at 27±1°C, 60±5 RH and 16:8 h photoperiod. Newly hatched larvae were reared and maintained on a chickpea-based artificial diet, provided in insect culture plates containing individual cages. The artificial diet was prepared as described by Gupta (2000) with minor modifications. It contain overnight soaked chickpea (Bengal gram-55 g), yeast powder (10 g), casein (5 g), L-ascorbic acid (1.3 g), cholesterol (55 mg), methyl-p-hydroxybenzoate (1 g), sorbic acid (0.52 g), streptomycin sulphate (100 mg), formaldehyde solution (0.5 ml), one multivitamin capsules, one vitamin E capsule, agar-agar (6.5 g) and 360 ml distilled water (Swathi et al., 2016).

3.11.3. *In vivo* feeding bioassay of *H. armigera* and *S. litura*

The effect of PnBBI on the growth of *H. armigera* and *S. litura* was examined by performing the *in vivo* feeding assay. The newly hatched larvae on fresh castor leaves were transferred onto the chickpea-based artificial diet (Gupta et al., 2000; Swathi et al., 2016). The second instar *H. armigera* larvae were transferred on to a test diet supplemented with low (0.001%), moderate (0.0025%) and high (0.005%) doses of PnBBI which is equivalent to 250, 625 and 1250 HaTPI units/gram-diet.

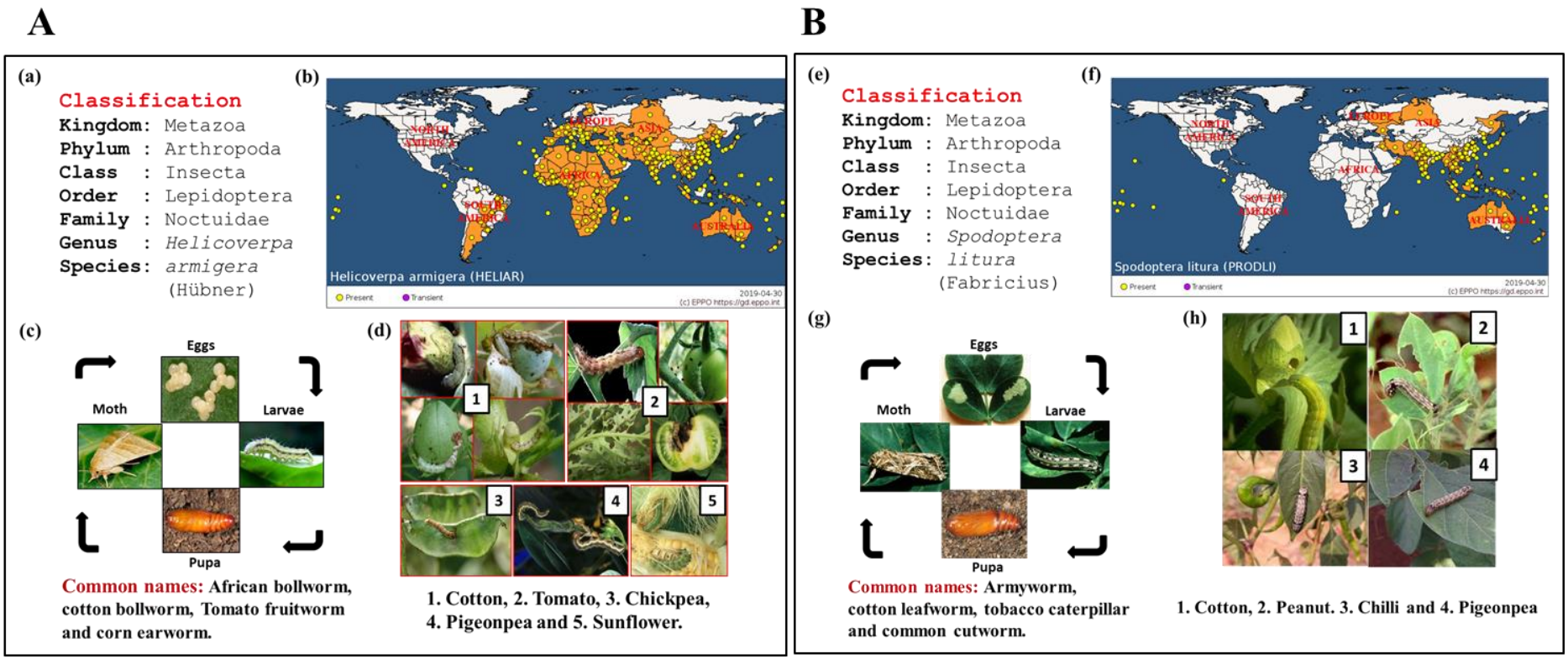


Fig. 3.4. A general information of devastating insect pest (A) *Helicoverpa armigera* and (B) *Spodoptera litura*: (**a&e**) Classification; (**b&f**) Global distribution of species; (**c&g**) Life cycle - metamorphosis and (**d&h**) Crops affected by both phytophagous insect pests, respectively.

Similarly, second instar larvae of *S. litura* were transferred on to diet supplemented with 0.025% PnBBI. For each concentration, 30 numbers of larvae (equal size and weight) were chosen for the study and kept in individual cages of insect culture plates. The control larvae were maintained on diet without PnBBI. Observations record was maintained throughout the experiment whereas larval body weights were recorded on day 0, 3, 6 and 9 and larvae were dissected for the collection of gut tissue in 200µl of Trizol reagent for RNA isolation. Midgut was collected in 0.1 M Glycine-NaOH, pH 10.5 on day 9/5th instar larvae for protease activity determination.

3.11.4. Preparation of larval midgut protease extract

The 5th instar larvae of *H. armigera* and *S. litura* actively feeding on an artificial diet with and without PnBBI were dissected dorsally after narcotising them on ice for 30 min. The midguts were collected in iso-osmotic saline and stored at -80 °C until use. The midgut of *H. armigera*/*S. litura* was homogenised in 0.1 M Glycine-NaOH, pH 10.5 using glass homogeniser in ice-cold condition. The supernatant obtained after centrifugation (twice) at 12,000 rpm for 20 min at 4 °C was collected and named as HaGP/SlGP extract. These extracts were further used for assessment of trypsin/chymotrypsin-like protease activity.

3.11.5. Midgut protease activity

The protease activity (HaTP / HaCP / SlTP / SlCP - Refer section 3.5) was analysed by monitoring the formation rate of *p*-nitroanilides from BAPNA or GLUPHEPA (1mM) at 410 nm using UV-visible spectrophotometer (UV-1700, Shimadzu, Japan) as described by Mohanraj et al. (2019). The molar extinction coefficient (M^{-1} cm^{-1}) of *p*-nitroanilide is 8,800 at 410 nm. One protease unit is described as an aliquot of HaGP/SlGP extract which gives ~1 OD at 410 nm after incubating with their respective substrates for 45 min at 37 °C. Further, the activity of these gut proteases was expressed as moles of *p*-nitroanilide released/min/mg protein as described in Swathi et al. (2014). BAPNA and GLUPHEPA were dissolved in DMSO.

3.11.6. Zymography

One dimensional electrophoresis was performed using 4% stacking and 7.5% separating gel under native conditions. The total HaGP/SlGP profile of larvae fed on a diet supplemented with PnBBI was visualized by casein zymography (Prasad et al., 2010a). Different protease types were determined by incubating various synthetic inhibitors such as PMSF (20 mM), TLCK (10 mM) and TPCK (10 mM) with HaGPs/SlGPs at 30 °C for 30 min before loading into the gel. For 2-DE, HaGP extracts were subjected to acetone precipitation and the obtained pellet was washed thrice with 80% chilled acetone following centrifugation at 12,000 rpm for 5 min at 4 °C. The final

pellet was dissolved in 2-DE native rehydration buffer containing 10% NP40, 5% glycerol and 0.5% IPG buffer. The IPG strips (pH 4-7, 11cm) were rehydrated overnight with 200µl of a solution containing 8µg of midgut protein followed by IEF. After IEF, IPG strip was equilibrated in 50 mM Tris-HCl, pH 8.8 containing 2% SDS and 30% glycerol for 20 min. The separation in second-dimension separation was carried out in 10% SDS-PAGE. After electrophoresis, the gel was washed twice with 2.5% (v/v) Triton X-100. The excess detergent was removed by washing with distilled water. The gels were incubated in a pre-chilled buffer (0.1 M Glycine-NaOH, pH 10.5) containing 1% casein for 30 min under mild shaking. The gels were incubated in a water bath at 37 °C for 1 h during casein hydrolysis. The solution was drained and washed with distilled water. The protease activity is visualised as clear zones (bands/spots) in the gel after staining with CBB R-250.

3.11.7. Quantitative Real-time PCR analysis

3.11.7.1. RNA preparation

Midgut tissue (pool of six guts free from food material) was collected in a microcentrifuge tube containing TRI reagent at time intervals of day3, 6 and 9 from *H. armigera* larvae fed on a diet in presence and absence of PnBBI as described in Section 3.11.5. The midgut tissue was homogenized on ice with a plastic pestle by further addition of 0.5 ml TRI reagent (Sigma-Aldrich, St Louis, Mo, USA). The tissue was homogenized using micro pestle at ice-cold conditions and centrifuged at 13,000 rpm. The supernatant was mixed with 200 µl of chilled chloroform, vortexed and centrifuged at 13,000 rpm. To the upper layer collected, an equal volume of isopropanol was added to precipitate RNA. Finally, the RNA pellet was washed thrice with 75% ethanol, air-dried and suspended in RNase free water. The quantity and integrity of RNA was determined using Nanodrop (Thermo Scientific, USA) and agarose gel electrophoresis, respectively.

3.11.7.2. Primer

The nucleotide sequences of *H. armigera* midgut trypsin- and chymotrypsin-like (*HaTry* 1, 4, 6, 7, 8; *HaChy* 1, 2, 4) proteases are adapted from Chougule et al. (2005), Chikate et al. (2013) and Mahajan et al. (2013). These set of genes were selected based on phylogenetic divergence and examined their relative expression levels in larvae fed on control and diet supplemented with different concentrations of inhibitor. The list of primers and their accession numbers were provided in **Table 3.2**.

3.11.7.3. cDNA preparation and gene expression analysis by using qRT-PCR

The first strand of DNA was synthesised by adding 1µg of total RNA using Verso cDNA synthesis kit as per manufacturer's instructions. The obtained cDNA was subjected to 1:40 dilution and selected midgut trypsin- and chymotrypsin-like genes (**Table 3.2**) were amplified using their respective primers and analysed by agarose gel (1%) electrophoresis. The 40S ribosomal protein gene (RpS18) was used as a reference for normalization.

Table 3.2. List of primers used for quantitative real-time PCR analysis. Gene-specific primers for midgut trypsin-like (*HaTry*) and chymotrypsin-like (*HaChy*) proteases of *H. armigera*, along with housekeeping gene of ribosomal protein S18.

Name	GenBank ID	Forward/ Reverse	Sequence	No. of bases
HaTry1	EU982841	F	GAGGACACAGATGTGGAGGGG	21
		R	GAACACACGGAATTCAGCCACG	22
HaTry4	EF600059	F	GTGCTACCCCTTCTGATTC	19
		R	AACTTGTCGATGGAGGTGAC	20
HaTry6	Y12276	F	CCATCGCCGGTGCCAACTA	19
		R	CTGAACGTGACGCAACTGCTC	21
HaTry7	Y12271	F	CAGAGGATTGTGGGTGGTTCG	21
		R	GCGGTGAGGATAGCCCTGTT	20
HaTry8	Y12286	F	GGGCTACTGGTGCCTTCAACG	21
		R	CAGAGTCATACACGTCACCGACG	23
HaChy1	Y12287	F	GCA CCA GAC TGA ACA CCG CTA G	22
		R	GCG ATG TTG CCA GAA GTA GCA ACG	24
HaChy2	EU325550	F	GACTTGTCAGGTGGCCAGGCTG	22
		R	GCGATTCTGGTACCGCCGGAGAAC	24
HaChy4	Y12273	F	CACCATCTTCATCTTCCAATCCGTGTGC	28
		R	GTGTTGATACGAGTACCACCGAAGAAC	27
RpS18	XM_021328334.1	F	GCG TGC TGG AGA ATG TAC TG	20
		R	GCC TGT TGA GGA ACC AGT CT	20

The relative transcript abundance of selected genes was determined by qRT-PCR using the StepOnePlus Real-Time PCR system (Applied Biosystems, USA) and ROX as reference fluorescent dye. The qRT-PCR (10µl) reaction mixture contains 1.0µl of cDNA, 5.0µl of 2X SYBR Green PCR Master Mix, 0.5µl of each forward and reverse primers and 3.0µl of sterilized water. PCR conditions were maintained at 94 °C for 10 min (initial denaturation), 40 cycles of 94 °C for 15 s followed by 60 °C for 1 min for annealing and extension. The respective changes of gene expression were assessed by the $2^{-\Delta\Delta CT}$ method using the cycle threshold (C_T) value of qRT-PCR analysis (Livak and Schmittgen, 2001). The C_T value of genes of interest (GOI) was normalized against the C_T value of a housekeeping gene (RpS18) and ΔC_T value (ΔC_T = $GOIC_T$ – $RpS18C_T$) was generated. Later, $\Delta\Delta C_T$ ($\Delta\Delta C_T$ = Test ΔC_T – Control ΔC_T) value generated is finally represented in log2 fold change of $2^{-\Delta\Delta CT}$.

3.12. Cloning, expression and purification of recombinant PnBBI

3.12.1. Extraction buffer

The extraction buffer (0.1 M Tris-HCl, pH 9.5) contained 10 mM EDTA, 2% (w/v) lithium dodecyl sulfate, 0.6 M NaCl and 0.4 M trisodium citrate. β-Mercaptoethanol (final concentration 5%) was added before use.

3.12.2. Phenol mixture

Water saturated phenol containing 35% (w/v) of Guanidium thiocyanate and $1/10^{th}$ volume of 2 M sodium acetate (pH 4). It was stored at 4 °C.

3.12.3. RNA extraction procedure

Total RNA was extracted according to the procedure of Suzuki et al. (2004). The extraction buffer, mortar, pestle, microcentrifuge tubes and pipette tips were sterilized by autoclaving. Mature peanut seeds (200 mg) were blended into powder using motor and pestle with liquid nitrogen as a grinding medium. The obtained fine powder was transferred into a clean microcentrifuge tube containing 20 volumes of the extraction buffer and vortexed for 60 sec. The supernatant obtained after centrifugation at 12000×g for five min at RT were transferred into a new microcentrifuge tube and an equal volume of chilled chloroform: isoamyl alcohol mixture (24:1) was added, and mixed by inversion for ten times. Then, the clear upper phase was transferred into a clean microcentrifuge tube after centrifugation at 12000×g for 5 min at 4 °C. An equal volume of phenol mixture was added, mixed by inversion and incubated for 3 min at RT followed by addition of chloroform: isoamyl alcohol mixture at half the volume of phenol mix and vigorously shaken. The clear upper phase obtained after centrifugation at 12000×g for 5 min

at 4 °C was transferred into a clean microcentrifuge tube and 0.6 volume of isopropanol was added followed by vortexing and incubation for 10 min at RT. The pellet obtained after centrifugation was washed thrice with 75% ethanol, allowed for air dry for 5 min and dissolved in 40 µl of RNase free water.

3.12.4. Assessment of RNA quality and cDNA synthesis

Isolated total RNA was quantified and validated for its quality preliminarily by using Nanodrop (Thermo Scientific, USA) spectrophotometer readings at 260/280 and 260/230 nm, respectively. Further, the RNA quality was determined by separating on 1% agarose gel. cDNA was synthesized by using Verso cDNA synthesis kit as per the manufacture instructions.

3.12.5. *In Silico* translation

BBTI gene sequence was subjected to ExPASy translate (https://web.expasy.org/translate/) tool. The 240 bp CDS encode 80 amino acids peptide which starts with methionine and end with serine (**Fig. 3.5A**).

3.12.6. Primers

The *Arachis hypogaea* Bowman-Birk trypsin inhibitor (BBTI) gene (GenBank: AY330200.1) sequence containing 430 bp was retrieved from NCBI database. The oligonucleotide primers were designed by using the IDT OligoAnalyzer tool (**Fig. 3.5B**). The first set of primers complementary to N and C-terminal of the gene of interest lacking restriction sites were used for initial amplification of cDNA. The second set of primers with cloning sites were used for directional cloning of the target gene into the pTWIN1 vector.

3.12.7. Construction of recombinant pTWIN1-PnBBI vector

The recombinant construct of pTWIN1-PnBBI was generated as described in Kumar and Gowda (2013b). pTWIN1 expression vector has 7.375 kb in size, N-terminal chitin-binding domain-DnaB (CBD-DnaB) intein and C-terminal chitin-binding domain-Mxe (CBD-Mxe) intein as fusion partners which has pH-induced self-cleavage property. This CBD-intein mediated system helps in ease purification of recombinant protein using Chitin beads affinity chromatography as per the supplier's instruction. In the present study, we have selected the N-terminal fusion partner CBD-DnaB intein which is encoded by 675 bp (225 amino acids) for PnBBI protein.

(A)

>gb|AY330200.1|:82-324 Arachis hypogaea Bowman-Birk trypsin inhibitor mRNA, complete cds

```
1|
ggc cgc gcc aag gta gca ctg ttg ctt ttc ctt gtg gga ctt tca gcc acc gtt gaa gct
 G   R   A   K   V   A   L   L   L   F   L   V   G   L   S   A   T   V   E   A
gtc cgc ctt gac cca agc ttg atg ctc tca cag gtg ata aac aat att ggc gaa gca tca
 V   R   L   D   P   S   L   M   L   S   Q   V   I   N   N   I   G   E   A   S
tca tct tca gac gac aat gtt tgc tgc aat ggc tgt cta tgc gac cgt agg gcc cca cca
 S   S   S   D   D   N   V   C   C   N   G   C   L   C   D   R   R   A   P   P
tat ttc gag tgt gtt tgt gtt gac acg ttc gat cat tgc cct gcg tct tgc aac tcc tgc
 Y   F   E   C   V   C   V   D   T   F   D   H   C   P   A   S   C   N   S   C
gtt tgc aca agg tct aat cct cca cag tgc cgt tgc acc gac aaa act caa ggc cgt tgc
 V   C   T   R   S   N   P   P   Q   C   R   C   T   D   K   T   Q   G   R   C
cct gta aca gaa tgt cgt tct tga agc aat taa gtt ccc tta ata aat aat aaa ttg cat
 P   V   T   E   C   R   S   -   S   N   -   V   P   L   I   N   N   K   L   H
atg cag tgt aac ttg cat att gca atg tat gtg atg atg agg caa agt cac ctt aga ctt
 M   Q   C   N   L   H   I   A   M   Y   V   M   M   R   Q   S   H   L   R   L
tgt ttt cat
 C   F   H  |430
```

(B)

Primer ID	Sequence	No. bases	GC (%)	Tm (°C)
BBTI-F	5'- ATG CTC TCA CAG GTG ATA AAC -3'	21	42.9	52.4
BBTI-R	5'- AGA ACG ACA TTC TGT TAC AG -3'	20	40	49.7
BBTI-Sap1-F	5'- GGT GGT TGC TCT TCC AAC ATG CTC TCA CAG -3'	30	53.3	64
BBTI-Pst1-R	5'- GGT GGT CTG CAG TTA AGA ACG ACA TTC TG -3'	29	48.3	60.8

Fig. 3.5. Peanut Bowman-Birk trypsin inhibitor (BBTI) mRNA sequence analysis: (A) The complete mRNA sequence of BBTI gene (430 bp) contained 243 bp CDS (82-324 bp) retrieved from NCBI database (AY330200.1). *In silico* translation of BBTI sequence by using ExPasy translation tool in which 243 bp CDS encode 80 amino acids peptide starts with methionine and **(B)** First set of primers (BBTI-F & R) complementary to N and C-terminal end of the CDS, and highlighted in both figure A & B with yellow colour. Second set of primers (BBTI-Sap1-F and BBTI-Pst1-R) additionally contains restriction sites for Sap1 and Pst1 and extra supporting bases helps in directional cloning. Both restriction sites were underlined in red colour.

PnBBI cDNA was amplified by Set-I primers using Pfu polymerase. (Thermocycler steps as follows, 1. T = 98 °C - 5 min; 2. T = 98 °C - 10 sec; 3. Tm = 52 °C - 30 sec; 4. T = 72 °C - 30 sec; 5. GO TO 2 REP 34; 6. T = 72 °C - 10 min; 7. HOLD @ 4 °C) The amplified gene product was analysed by separating on 1% agarose gel at 100V for 30 min. Restriction sites were incorporated into the amplified gene of interest using Set-II primers (Tm = 60 °C) which helps in directional cloning into the pTWIN1 vector. The amplified gene product was separated on an agarose gel and extracted using a Qiagen gel extraction kit as per the manufacturer protocol. Purified DNA fragment and pTWIN1 vector were subjected to sequential digestion with restriction enzymes SapI (Cutsmart buffer) and PstI (Buffer 3.1) and the purified digested products were subjected to ligation (3:1 molar ratio of vector: insert) at 4 ºC for overnight.

3.12.8. Transformation

Chemically competent *E. coli* DH5α cells were used for the transformation of the pTWIN1-PnBBI vector. Positive clones obtained on ampicillin LB agar plates were confirmed by colony PCR and restriction digestion with NdeI and BamHI while the SapI site was lost during the ligation. Further, the clone was validated by dideoxynucleotide sequencing method using gene-specific and vector-specific primers.

3.12.9. Heterologous expression of rPnBBI

Recombinant plasmid pTWIN1-PnBBI was transformed into *E. coli* BL21 (DE3) expression strain. Transformed *E. coli* cells were grown (0.8 OD at 600 nm) in Luria-Bertani (LB) medium containing 100µg/ml ampicillin at 37 ºC. The culture was induced with 0.6 mM IPTG at 16 ºC for 17 hours. The cell pellet obtained from one-litre culture after centrifugation at 5000 rpm for 10 min was suspended in 100 ml of chilled lysis buffer (50 mM Tris-HCl, pH 8.0 containing 300 mM NaCl and 10 mM EDTA). The cell pellet was subjected to sonication and centrifuged twice at 10,000 rpm for 20 min. The supernatant was named as clarified cell lysate and which was further used for purification of rPnBBI.

3.12.10. Purification of rPnBBI

Initially, the clarified cell lysate containing fusion protein CBD-DnaB-PnBBI was subjected to purification by using chitin beads. The following process resulted in low yield and half of the rPnBBI was in the unbound protein fraction (flow-through), because of the premature autocleavage of fusion partner (CBD-DnaB) during in vivo expression or while preparing the clarified cell extract. These observations directed us to use trypsin affinity column alternatively which could bind to both fusion and free rPnBBI of clarified cell lysate. This was followed by

passing through size exclusion chromatography column to remove the fusion partner which was cleaved during pH shift of affinity column elution.

3.12.11. Molecular modelling

Peanut Bowman-Birk trypsin inhibitor primary protein sequence was obtained from the NCBI (Protein ID: AAP93913.1) database. After BLASTp analysis against Swiss PDB, the obtained structures similar to PnBBI with maximum sequence coverage and identity was selected and used as a template for homology modelling. Modeller 9.19 version was used to model the protein as per its python scripts described in the tutorial. The generated rPnBBI model was validated by using PDBsum generate (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html) and visualized by PyMOL (https://pymol.org/2/). Further, multiple sequence alignment of rPnBBI with similar sequences obtained after BLASTp search were done using Clustal Omega online tool.

3.13. Anti-coagulation properties

3.13.1. PT and APTT assay

The inhibitory effect of native and recombinant PnBBI on the prothrombin time (PT) and activated partial thromboplastin time (APTT) periods of human blood coagulation system was evaluated by using commercial PT (Neoplastine-Stago) and APTT (Cephascreen-Stago) reagents from DIAGNOSTICA STAGO, France. Samples were outsourced for analysis at Apollo Hospital, Department of Hematology, Hyderabad. The various concentrations of inhibitors (PnBBI-4, 16µg; rPnBBI-8, 16 and 32µg) were added to 400µl of fresh citrated normal human plasma and incubated for 15 min at 25 °C. PT and APTT reagents were added (50µl) to the incubation mixture and clotting time was measured by using the instrument-Stago (STA Compact max 2) coagulation analyzer. Heparin (20 International Units) was used as positive drug control. The clotting time in the absence of inhibitors was determined in a similar manner by adding buffer.

3.13.2. Coagulation factors inhibition assay

The inhibitory effect of PnBBI/rPnBBI on human blood clotting factors were determined by adding different concentrations of inhibitors to the different proteases: FIXa (2.8µg), FXa (30ng), FXIa (50ng) in 50 mM Tris-HCl, pH 8.3 containing 5 mM calcium chloride, 0.2 mM sodium chloride; FXIIa (300ng) and human plasma kallikrein (300ng) in 50 mM Tris-imidazole pH 7.9 containing 150 mM NaCl. After 10 min pre-incubation, the proteolytic activity of the various factors was measured by the addition of respective substrates at 37 ºC. The synthetic chromogenic substrates used were: CH_3OCO-D-CHA-Gly-Arg-*p*NA-AcOH (0.8 mM) for FIXa, FXa and FXIa; H-D-CHA-Gly-Arg-pNA-2AcOH (0.25 mM) for FXIIa and H-D-But-CHA-Arg-pNA.2AcOH

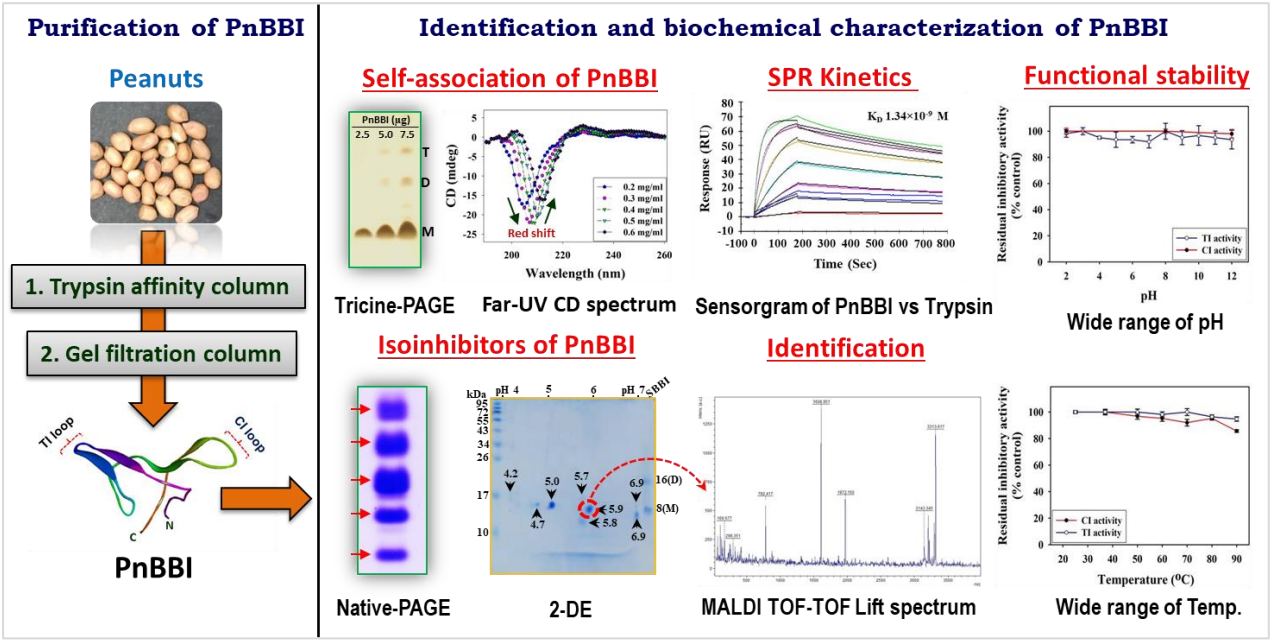
(0.5 mM) for plasma kallikrein. Finally the amidolytic activity was recorded at every one min interval immediately soon after adding respective substrates spectrophotometrically at 405 nm for 20 min. The total assay volume was made up to 200µl with respective buffers in flat bottom 96 well plate and control was made without inhibitor. Relative per cent inhibition in the activity of various factors was calculated with reference to control OD at linear phase. The supplied proteases and their substrates were carefully reconstituted with respective buffers as per the manufactures instructions and stored at -80 °C until use.

3.14. Statistical analysis

The data shown is Mean ± SE/SD of three biological replicates. Statistical differences were determined by one-way ANOVA followed by Tukey test at a significance level of $P \leq 0.05$ using Sigma plot, version 12.0, Systat Software Inc., San Jose, CA.

Chapter 4

Purification and biochemical characterization of Bowman-Birk inhibitor (PnBBI) from interspecific hybrid variety (4368-1) of peanut



Purification and biochemical characterization of Bowman-Birk inhibitor (PnBBI) from interspecific hybrid variety (4368-1) of peanut

4.1. Introduction

Proteolysis is a key physiological process catalysed by specific proteases in plants, animals and microorganisms (Lopez-Otin and Bond, 2009). The exogenous and endogenous proteolytic activity of four mechanistic classes (serine, cysteine, aspartate and metallo-) of proteases are highly regulated by corresponding proteinase/protease inhibitors (PIs). In higher plants, PIs are constitutively expressed in storage organs and induced in vegetative organs in response to wounding/herbivory during the invasion of microbial pathogens or infestation by insect pests (Ryan, 1990; Shewry, 2003; Rehman et al., 2017). PIs are known to play a role in the regulation of programmed cell death and tolerance against abiotic stress (Drame et al., 2013; Boex-fontvieille et al., 2015). Several studies established the use of PIs in the management of agriculturally important insect pests (Shamsi et al., 2016; War et al., 2018; Clemente et al., 2019). In this regard, serine PIs such as Kunitz and Bowman-Birk inhibitors (BBIs) are receiving considerable attention as a part of Integrated Pest Management (IPM) since lepidopteran insect pests depend predominantly on trypsin- and chymotrypsin-like digestive proteases for attaining energy (Sharma et al., 2000; Srinivasan et al., 2006; Swathi et al., 2016). The Kunitz PIs possess typical characteristics such as a molecular mass of 14-24 kDa, single polypeptide chain containing one reactive site with β-trefoil structure stabilized by two disulfide bonds and inhibit serine, cysteine and aspartic proteases (Bendre et al., 2018). On the other hand, BBIs are ~8 kDa proteins with two reactive sites specific for trypsin and chymotrypsin and are stabilized by seven intramolecular disulphide bonds (Macedo et al., 2015). These canonical serine PIs follows the standard mechanism of inhibition by interacting with the active site of proteases by tight binding reaction (Laskowski and Qasim, 2000; Bateman and James, 2011).

Peanut (*Arachis hypogaea* L.) is one of the important oilseed legume crops of tropical and semi-arid tropical countries of Asia, Africa and America (Arya et al., 2016). The cultivated varieties of peanut are tetraploid and known to have inadequate levels of resistance to several biotic and abiotic constraints. Contrarily, the closely related wild relatives of peanut are diploid and possessed several useful disease/pest resistant traits (Holbrook and Stalker, 2003; de Paula et al., 2017). The genetic barrier of 'diploid vs tetraploid' prohibited the gene flow from wild-relatives to cultivars and thereby resulted in a narrow genetic base of peanut (Stalker, 2017). In this scenario, allotetraploid and amphidiploid synthetic peanut varieties have been generated which possessed several desirable traits with wide genetic variability of wild germplasm (Mallikarjuna et al., 2011). This provided an opportunity for introgression of resistance traits from eminent synthetic varieties into cultivated peanut gene pool by developing interspecific hybrid varieties (Mallikarjuna et al., 2011; Kumari et al., 2014).

So far, several isoinhibitors of BBI have been isolated from different varieties of peanut and characterized their biochemical properties (Norioka et al., 1982; Norioka and Ikenaka, 1983; Boateng et al., 2006). However, Peanut BBI has distinct structural properties as compared to the BBI from other legumes such as non-identical reactive site loops, unique N-terminal reactive site residues and their specificity towards cognate proteases (Suzuki et al., 1987; Qi et al., 2005). In addition, peanut trypsin inhibitors are known to possess satietogenic and cancer-preventive properties (Serquiz et al., 2016; Ahmad et al., 2019), involved in biotic (Harsulkar et al., 1999; Telang et al., 2003; Muller et al., 2017), and abiotic stress tolerance (Drame et al., 2013). Though, the presence of various PIs in cultivars of peanut was reported, its identification and characterization in interspecific hybrid peanut varieties were not yet explored. Therefore, the present study is focused on purification and detailed biochemical characterization of PIs from interspecific hybrid peanut variety (4368-1) which are known to possess beneficial traits against biotic and abiotic stresses.

4.2. Results

4.2.1. Purification of peanut proteinase inhibitor (PnBBI)

The 20-60% $(NH_4)_2SO_4$ fraction of peanut crude proteinase inhibitor (PnCPI) extract showing prominent TI activity was subjected to Sepharose 4B trypsin affinity chromatography. The trypsin bound proteins (peak II) were eluted using 0.01 N HCl and neutralized with Tris base (**Fig. 4.1A**). The eluted fractions with TI activity were pooled and loaded onto the Sephadex gel permeation column. Among the obtained peaks, protein fractions (peak II) which showed prominent TI activity were pooled and concentrated (**Fig. 4.1B**). The progressive increase in the purity of protein during the sequential steps of purification was clearly evident in SDS-PAGE. Also, an increase in the number of protein bands (monomer, dimer and tetramer) was observed with an increase in loading concentration of purified protein from 2.5-10 µg (**Fig. 4.1C, lanes 5-7**). The present protocol yielded pure protein with 167-fold purification and 37.5% yield recovery (**Table 4.1**). Further, non-reducing gelatin SDS-PAGE in-gel activity staining studies demonstrated the presence of TI and CI activities similar to commercial soybean BBI (**Fig. 4.1D,E**). Hence, it is named as "PnBBI".

4.2.2. Determination of molecular mass and self-association pattern

Electrophoretic separation of PnBBI resolved it as a single protein with a molecular mass of ~10 kDa on non-reducing Tricine SDS-PAGE (**Fig. 4.1C, lane 5**). However, analysis of intact PnBBI molecular mass by MALDI-TOF revealed a predominant peak of 6733.394 Da, as a monomer and a minor peak of 13741.216 Da, as a dimer (**Fig. 4.2A**). PnBBI existed as several isoinhibitors apparently with similar molecular mass and each isoinhibitor showed variability in their abundance (inset of **Fig. 4.2A**) on Native-PAGE and 2-D electrophoresis (**Fig. 4.2B,E**). Nevertheless, all the electrophoretically separated isoinhibitors exhibited resistance against bovine trypsin and chymotrypsin digestion (**Fig. 4.2C,D,F,G**). The observed self-association tendency

and presence of TI and CI activities of PnBBI are in agreement with the characteristic features of PIs belonging to BBI family.

On the other hand, appearance of several new monomeric bands (~6-10 kDa) was observed in Tricine SDS-PAGE under reducing conditions, parallel to the disappearance of dimeric and tetrameric bands which further confirms the existence of PnBBI in several isoforms as well as higher-ordered oligomeric forms (**Fig. 4.1C, lanes 8-10**). Further, soybean BBI (~8 kDa) was used as a reference to eliminate the discrepancy in molecular mass and electrophoretic migration of self-associated BBIs (**Fig. 4.1C, lane 11;** Swathi et al., 2014). However, the mass of PnBBI was considered as "6.73 kDa" as evident in MALDI-TOF study.

Table 4.1. Purification of PnBBI from mature seeds (10 g) of peanut interspecific advanced hybrid variety (4368-1). The data shown here is a representative of the purification protocol for PnBBI from three independent biological replicates.

Purification step	Total protein (mg)	Total activity (TIU)[a]	Yield recovery (%)	Specific activity (TIU/mg)[b]	Purification fold change
Crude extract	713	6000	100	8.4	1
$(NH_4)_2SO_4$ Fractionation (20-60%)	490	5164	86	10.5	1.3
Trypsin-Sepharose 4B	8.4	2500	41.6	298	35.5
Sephadex G-50	1.6	2250	37.5	1406	167

[a] One TI unit is equal to 40-60% inhibition of trypsin enzyme activity by PnBBI.

[b]Specific inhibitory activity is defined as the number of TIU per milligram of PnBBI.

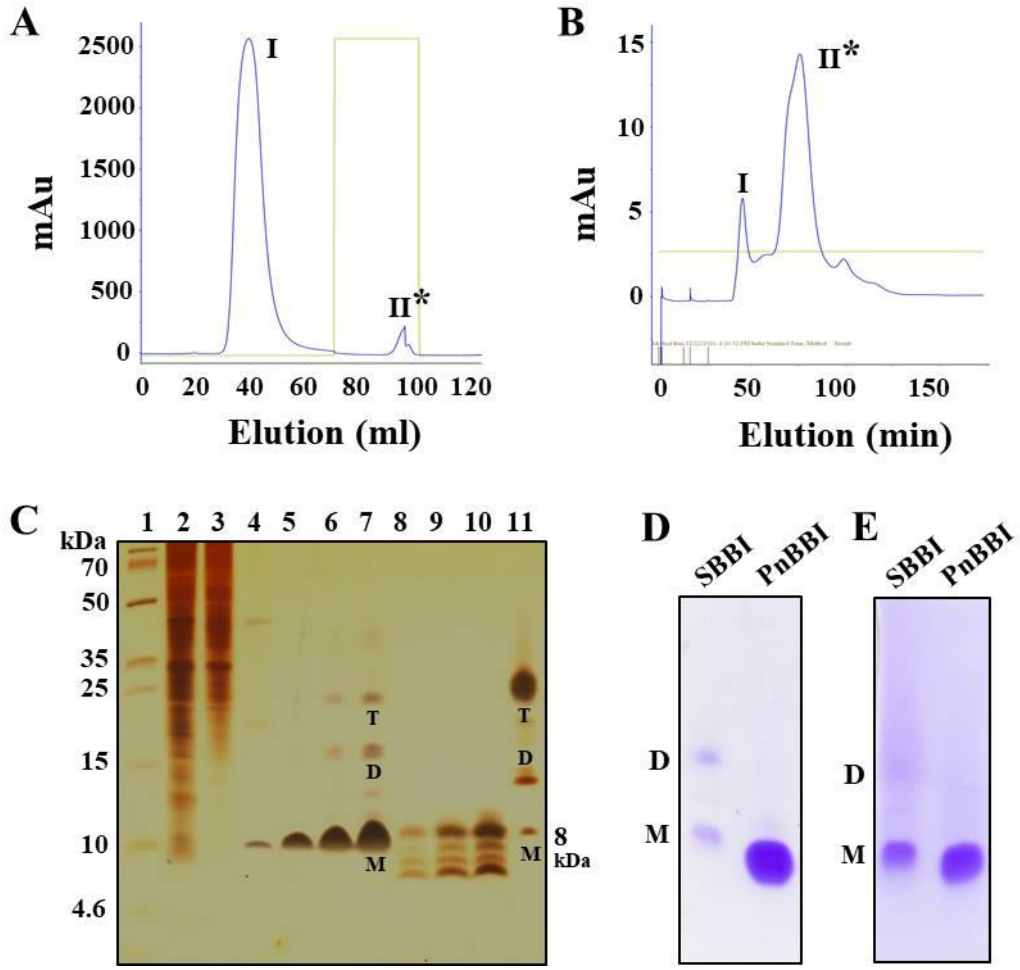


Fig. 4.1. Purification profile of PnBBI and its in-gel activity. Elution profile of **(A)** trypsin-Sepharose 4B column loaded with 20-60% $(NH_4)_2SO_4$ active fraction; **(B)** Sephadex G-50 fine column loaded with active peak II fraction pool of trypsin affinity column; **(C)** Tricine SDS-PAGE (15%) showing purification profile and self-association pattern of PnBBI: lane 1, molecular weight marker designated in kDa; lane 2, peanut crude protein extract (20 µg); lane 3, 20-60% $(NH_4)_2SO_4$ protein fraction (15 µg); lane 4, active fraction pool (Peak II) of trypsin affinity column (10 µg); lanes 5-7 and 8-10 active fraction pool (peak II) of gel filtration column under non-reducing and reducing conditions with increased protein concentration (2.5, 5 and 10 µg), respectively; lane 11, soybean BBI (5 µg) was used as a reference. Non-reducing gelatin SDS-PAGE (15%): Lane 1, soybean BBI (5 µg); lane 2, PnBBI (5 µg) active against **(D)** bovine pancreatic trypsin and **(E)** chymotrypsin, respectively. Asterisks indicate active peak with inhibitory activity against trypsin. M-monomer, D-dimer and T-tetramer. The data shown here is the representative of three biological replicates.

4.2.3. Two-dimensional gel electrophoresis and MALDI-TOF/TOF analysis

Two-dimensional electrophoretic separation of PnBBI under non-reducing conditions resolved as several isoinhibitor and its higher-order forms in pH range between 4-7 (**Fig. 4.2E**). Further, gelatin in-gel activity staining studies demonstrated the resistance of these isoinhibitor spots to hydrolysis against both trypsin and chymotrypsin (**Fig. 4.2F,G**). The high-intensity isoinhibitor spot at pI 5.9 resolved under reducing conditions in 2-D electrophoresis was excised and subjected to MALDI MS-MS analysis (**Fig. 4.3A**). The MS-MS ionization of PMF peak 3313.817 m/z obtained the following amino acid sequence "APPYFECVCVDTFDHCPASCNSCVCTR" which matched to *Arachis hypogaea* BBI A-II in Mascot MS-MS ions search with a score of 20 and 38% sequence coverage (**Fig. 4.4A-D**). Further, Blastp search of the obtained sequence showed 100% identity with BBIs from *A. hypogaea* (A-I, B-III, BBTI and B-II) and 48-60% similarity with other legume BBIs such as *Lathyrus sativus*, *Vicia faba*, *Rhynchosia sublobata*, *Vigna angularis*, *Lupinus albus* and *Vigna radiata*. Multiple sequence alignment of the obtained sequence is in accordance with the pattern of conserved cysteine residue framework of BBIs (**Fig. 4.3B**).

4.2.4. Kinetic analysis using SPR

SPR analysis has been widely used to study the biomolecular interactions in the real-time and label-free environment. The ligand PnBBI showed stable interactions with both trypsin (Ka 4.18×10^{5} $M^{-1}s^{-1}$; Kd 0.00056 s^{-1}) and chymotrypsin (Ka 7.76×10^{5} $M^{-1}s^{-1}$; Kd 0.00172 s^{-1}) during SPR kinetic analysis. The obtained dissociation equilibrium constant emphasize the relative stability of PnBBI-trypsin (K_D 1.34×10^{-9} M) complex over PnBBI-chymotrypsin (K_D 2.22×10^{-9} M) complex with slower dissociation rates (**Fig. 4.5A,B**). On the other hand, quantitative binding analysis of trypsin and chymotrypsin to double-headed PnBBI was determined by SPR competitive binding assay (**Fig. 4.5C,D**). These results suggest the chymotrypsin saturated PnBBI surface showed greater capacity to bind with trypsin molecules

which is evident by maximum RU of 12.6. In contrast, trypsin saturated PnBBI surface showed a lower capacity to bind with chymotrypsin molecules which is evident by maximum RU of 2.46.

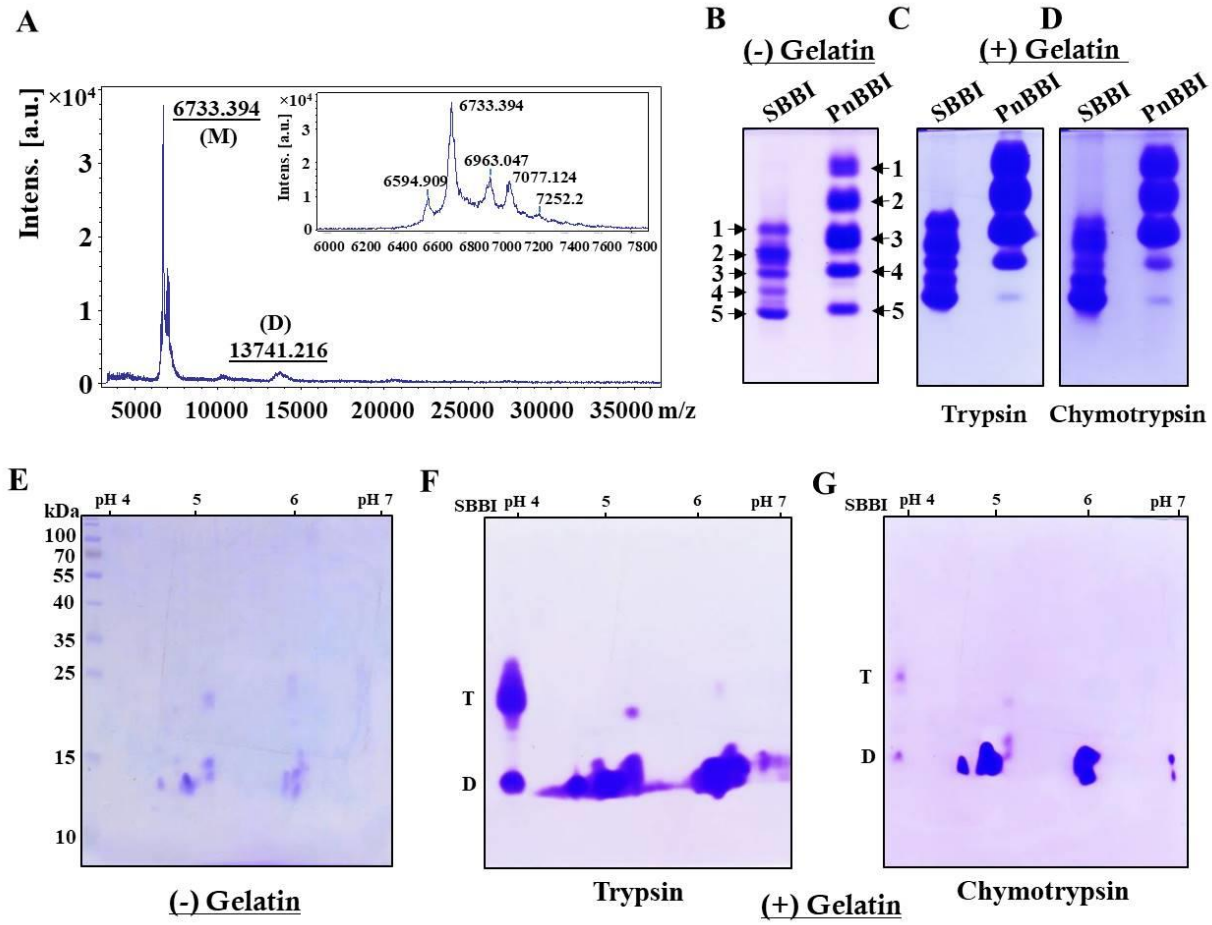
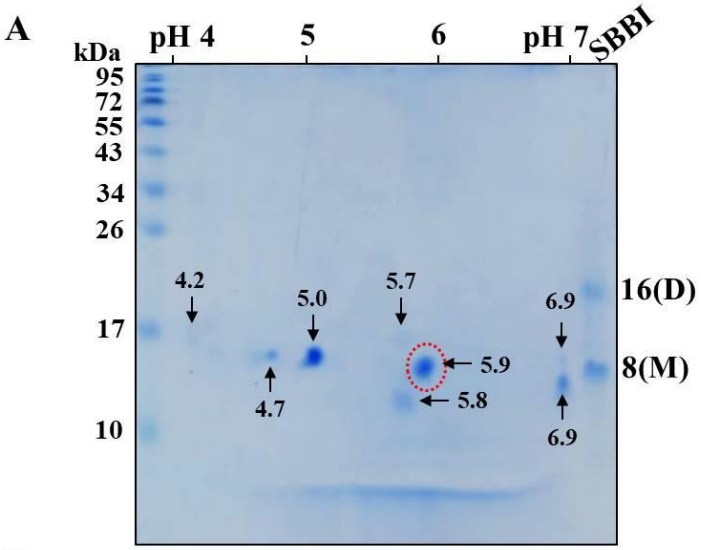


Fig. 4.2. Molecular mass determination and electrophoretic visualization of PnBBI isoinhibitors, and in-gel activity staining. (A) MALDI-TOF intact mass spectrum of PnBBI from 3000-37000 *m/z* representing a high-intensity peak (monomer) with a molecular mass of 6733.394 Da, and its self-associated dimer (13741.216 Da). Inset: zoomed spectrum at 6000-7800 *m/z* represents the peanut isoinhibitors; **(B)** Visualization of PnBBI isoinhibitors (20 µg) resolved on native gel electrophoresis (12.5%). In-gel activity staining of PnBBI resolved on gelatin Native-PAGE active against bovine **(C)** trypsin and **(D)** chymotrypsin; **(E)** 2-D electrophoretic separation of PnBBI (70 µg) isoinhibitors (IEF, pH 4-7, 11cm, L) under non-reducing conditions and respective in-gel activity staining against bovine **(F)** trypsin and **(G)** chymotrypsin. The second dimension was performed on SDS-PAGE (15%) under non-reducing conditions. Gels were stained with CBB R-250. Soybean BBI (SBBI) is loaded as a positive control in Native-PAGE as well as activity staining studies. D-dimer and T-tetramer.



B

Plant source	Accession No.	Sequence	No.of Res.	(%) Sim.
PnBBI		APPYFECVCVDT-FDH-CPASCNSCVCTR	27	100
A.hypogaea(A-II)	0908247B	APPYFECVCVDT-FDH-CPASCNSCVCTR	27	100
A.hypogaea(A-I)	0908247A	APPYFECVCVDT-FDH-CPASCNSCVCTR	27	100
A.hypogaea(B-I)	0908247C	APPYFECVCVDT-FDH-CPASCNSCVCTR	27	100
A.hypogaea(B-III)	0908247E	APPYFECVCVDT-FDH-CPASCNSCVCTR	27	100
A.hypogaea(BBTI)	AAP93913.1	APPYFECVCVDT-FDH-CPASCNSCVCTR	27	100
A.hypogaea(B-II)	0908247D	APPYFECTCGDT-FDH-CPAACNKCVCTR	27	85
L.sativus	ADV40042.1	------CRCVDIR-ET-CHSACNSCVCT-	20	60
V.faba	P24661.1	------CRCVDVG-ER-CHSACNSCVC--	19	58
R.sublobata	ALA09300.2	-PP--QCRCVDVRLDS-CHSACKSCICT-	24	54
V.angularis	P01061.1	-PP--QCQCADIRLDS-CHSACKSCMCTR	25	52
L.albus	P85172.1	-PP--QCRCTDIG-ET-CHSACKSCICTR	24	50
V.radiata	P01062.2	-PP--ECHCANIRLNS-CHSACKSCICTR	25	48

Fig. 4.3. Two-dimensional gel electrophoresis and MALDI TOF-TOF analysis of PnBBI. (A) Isoelectric focusing (pH 4-7, 11 cm, L) of PnBBI (70 µg) was performed under standard reducing conditions as described in methods (Chapter 3, section 3.6.4). The second dimension was performed on SDS-PAGE (15%) under reducing conditions and the gel was stained with CBB R-250. Molecular mass standards and soybean BBI were used as a reference and positive control, respectively. The 2-DE gel picture shown is the representative of three biological replicates; **(B)** The isoinhibitor spot with pI 5.9 (indicated by a dotted circle of **Fig. 3A**) was subjected to trypsin digestion followed by MALDI TOF-TOF analysis. The resulting peptide sequence from Mascot MS/MS ions search was aligned with the homologous BBI sequences of NCBI database using Clustal Omega tool. The obtained sequence has shown 100% identity with peanut BBI.

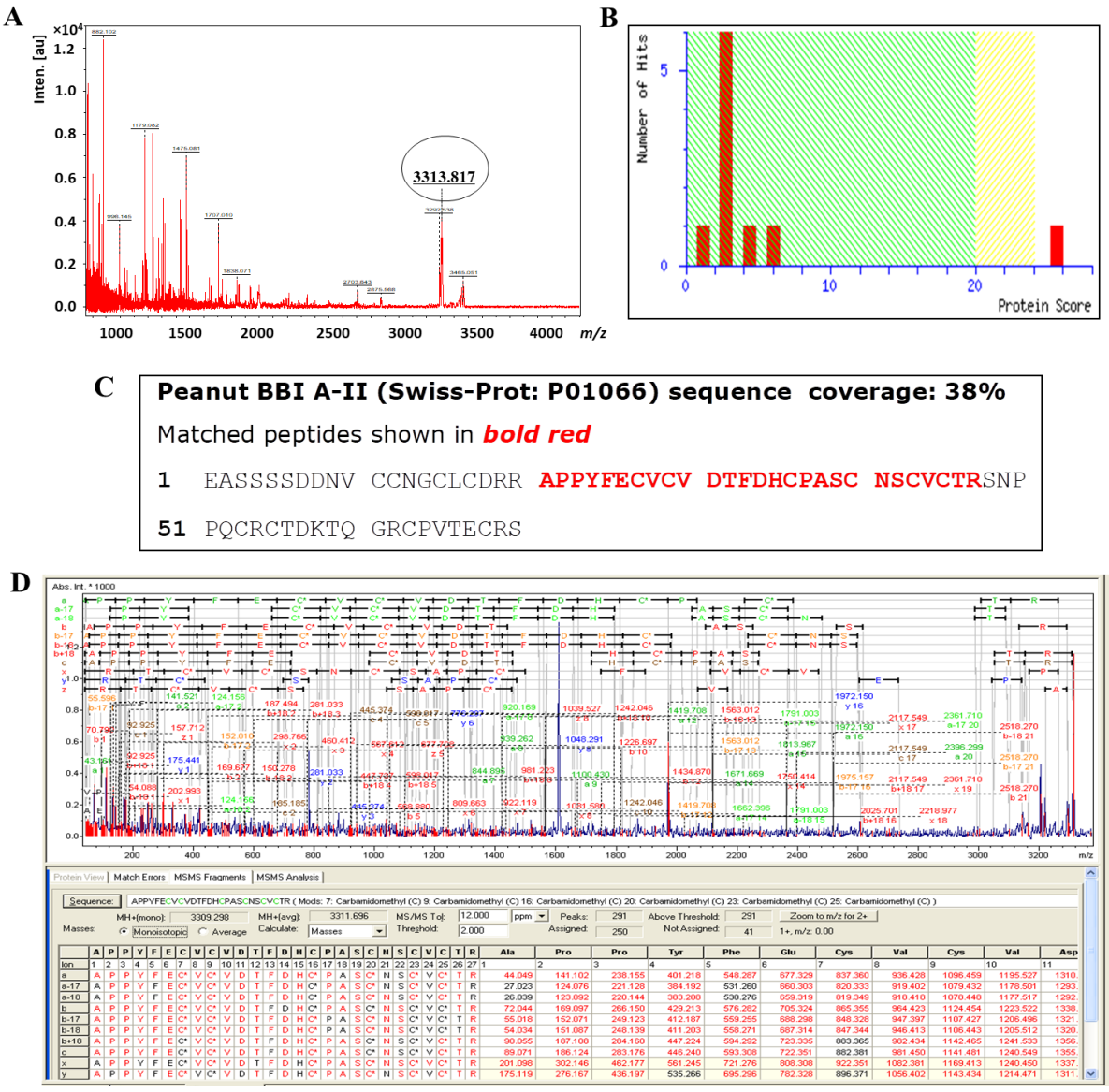


Fig. 4.4. MALDI-TOF-TOF analysis of PnBBI isoinhibitor spot (pI 5.9). (**A**) Peptide mass fingerprint (PMF) spectra; (**B**) Mascot MS-MS ions search result and (**C**) Peptide sequence obtained after MS-MS ion search showed 38% coverage with protein sequence of peanut BBI A-II and (**D**) Lift spectrum from PMF peak with m/z 3313.817 generated by Biotool software after digestion of PnBBI isoinhibitor spot pI 5.9 with trypsin. (refer **method section 3.7** and **Fig. 4.3A**).

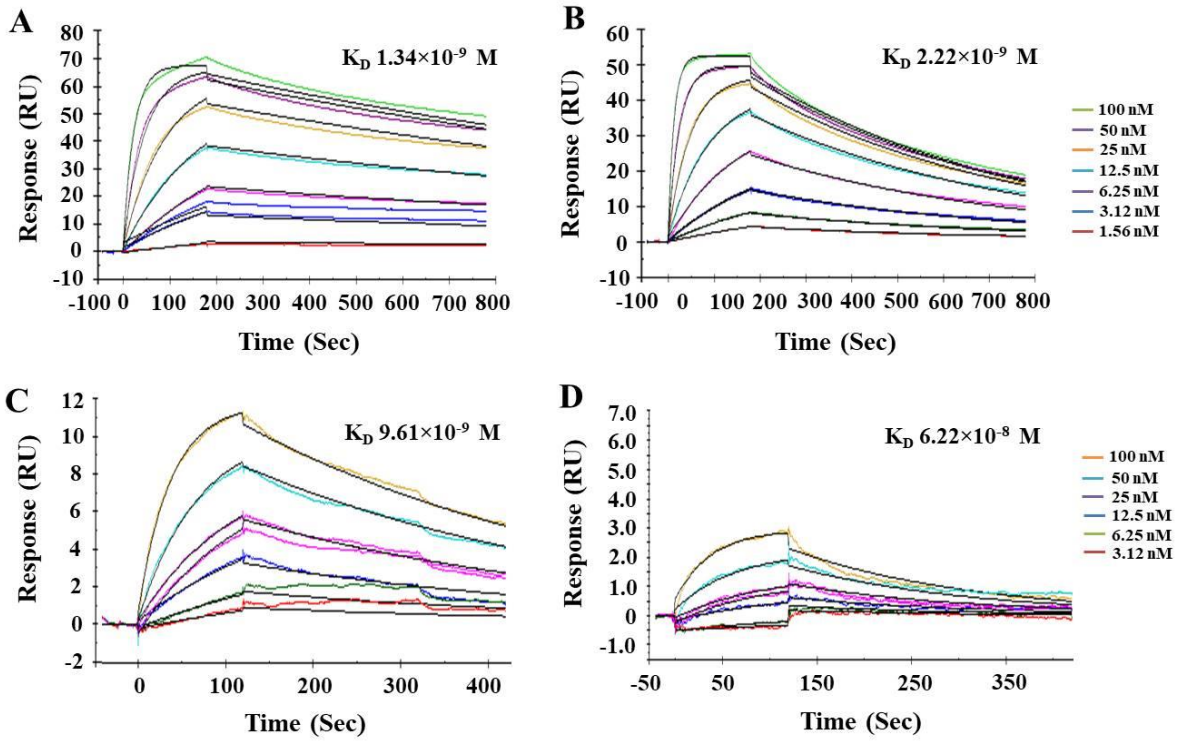


Fig. 4.5. Biacore surface plasmon resonance (SPR) analysis of PnBBI. Sensogram plots generated by SPR kinetic analysis demonstrate the association and dissociation characteristics between immobilized ligand (PnBBI) and analytes, bovine **(A)** trypsin and **(B)** chymotrypsin; Competitive binding studies between ligand and analytes where **(C)** chymotrypsin (500 nM) was captured on PnBBI ligand and trypsin was passed through as an analyte and **(D)** trypsin (500 nM) was captured on PnBBI ligand and chymotrypsin was passed over as an analyte. A range of trypsin/chymotrypsin concentrations (1.56, 3.12, 6.25, 12.5, 25, 50 and 100 nM) prepared in 10 mM HEPES, pH 7.4 are passed over the surface of PnBBI sequentially and the curves obtained were further analysed by Langmuir fit model of 1:1 binding. One response unit (RU) corresponds to a change of 1 pg/mm^2 in surface protein concentration i.e., analytes.

4.2.5. Functional stability

The TI and CI activities of PnBBI was stable even after incubating at a wide range of pH (2-12) and temperature (20-90°C). PnBBI lost <5% of TI activity and <14% of CI activity under the above extreme conditions (**Fig. 4.6A,B**). In contrast, the TI, CI activities of PnBBI were completely (100%) lost upon incubation with an increasing concentration of reducing agent (DTT) followed by alkylation (IDA). The TI activity of PnBBI was found to be much sensitive to DTT reduction compared to CI activity in which 14% of TI and 80% of CI activity was retained at 0.2 mM DTT (**Fig. 4.6C**). This result emphasizes the vital role of disulfide bonds in stabilizing the structure of trypsin reactive loop of PnBBI. The superior affinity of PnBBI towards trypsin was evident by molar inhibition studies, in which PnBBI binds with trypsin at 1:2 stoichiometry but not with chymotrypsin (**Fig. 4.6D**).

4.2.6. Structural stability

Circular dichroism spectroscopy is the most useful technique for studying protein secondary structure in solution. The DichroWeb analysis of far-UV CD spectrum revealed the preponderance of β-sheets (47%) and random coils (44%) over α-helix (9%) in PnBBI. The secondary structure of PnBBI has not shown much variation when incubated under different pH or temperatures (**Fig. 4.7A,B**). However, a significant distortion in the secondary structure of PnBBI was observed upon reduction and alkylation with DTT and IDA, respectively (**Fig. 4.7C**). In contrast, there was a shift in the negative ellipticity maxima of spectrum towards higher wavelength i.e., from 205 - 212 nm with an increase in PnBBI concentration from 0.2 to 0.6 mg/ml (**Fig. 4.7D**).

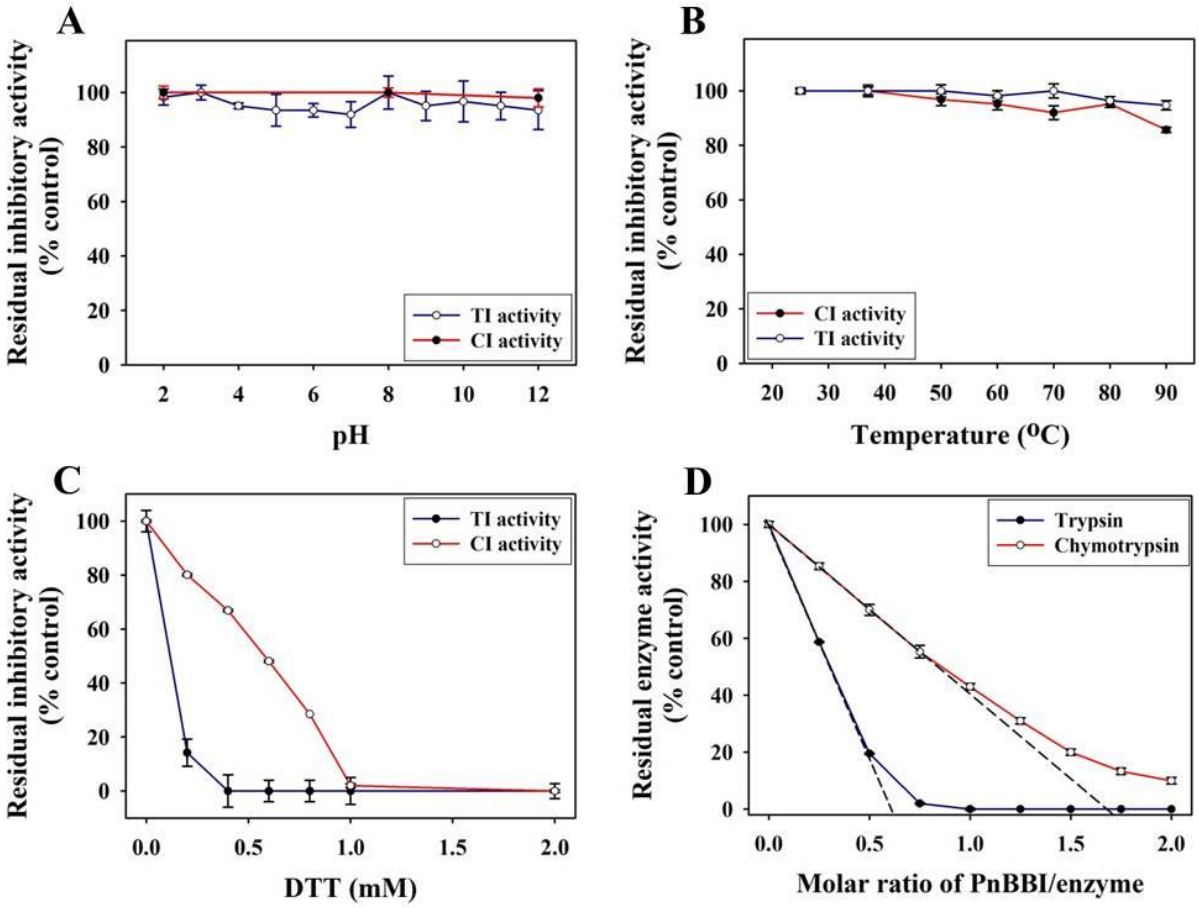


Fig. 4.6. Functional stability of PnBBI. The TI and CI activities of PnBBI were monitored at a broad range of **(A)** pH; **(B)** temperature and **(C)** reducing agent Dithiothreitol (DTT). The amount of PnBBI equivalent to one TI or CI units was incubated at different pH, temperatures or DTT concentration and residual inhibitory activities against trypsin and chymotrypsin was monitored using respective substrates as described in methods (Section 3.8); **(D)** Titration curve of trypsin and chymotrypsin inhibition by PnBBI. A fixed amount of enzyme (1 µM trypsin and/or 2 µM chymotrypsin) was mixed with different concentrations of PnBBI and the residual protease activity was determined. The x-intercept where the corresponding proteases are completely inhibited by the inhibitor was the molar binding ratio. The data represented is mean ± SE of three biological replicates.

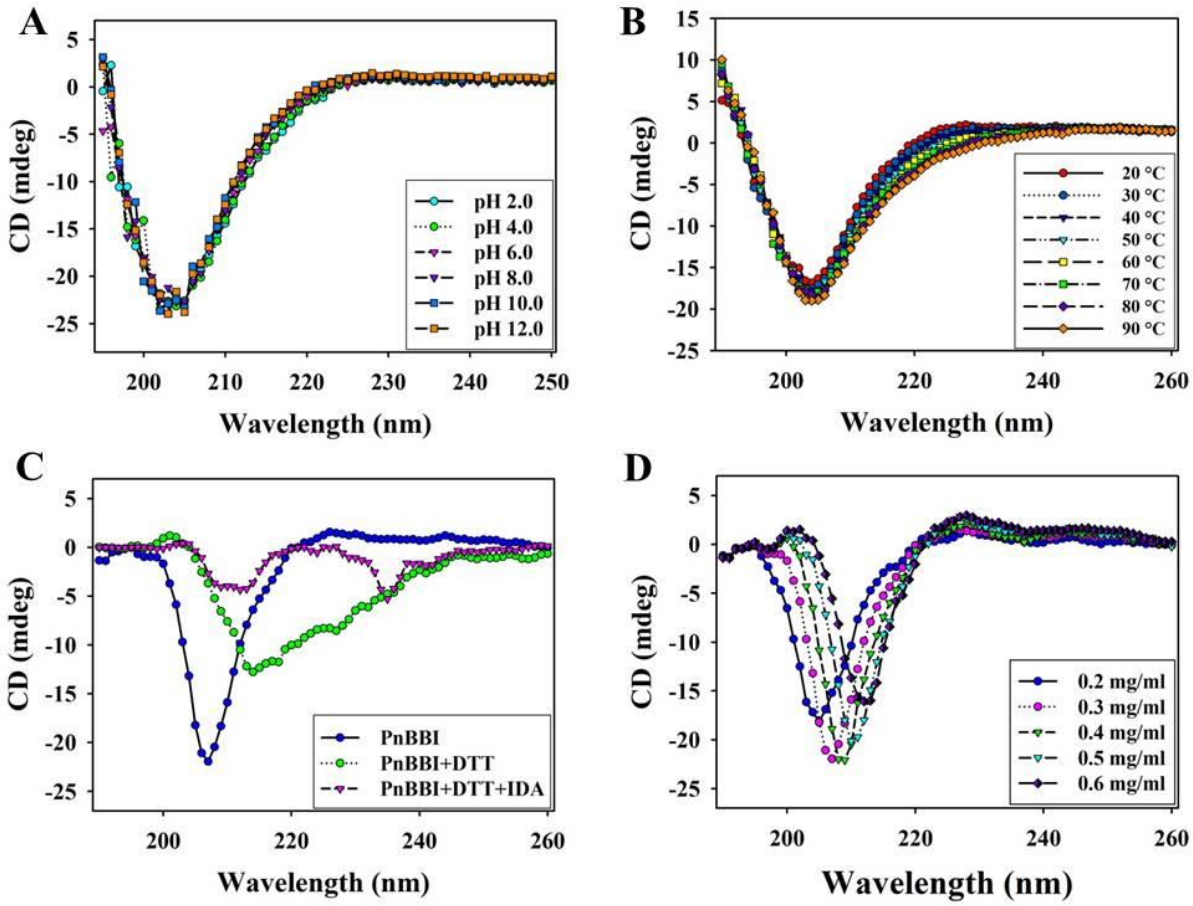


Fig. 4.7. Structural stability of PnBBI. Far-UV (190-260 nm) secondary structure of PnBBI was determined by CD spectroscopy at different **(A)** pH from 2.0 to 12.0; **(B)** temperatures from 20 to 90 °C; **(C)** DTT (2 mM) reduction followed by IDA (4 mM) alkylation and **(D)** increasing concentration (0.2-0.6 mg/ml) of PnBBI prepared in 10 mM PBS, pH 7.4 as described in method section 3.9.

4. 3. Discussion

Peanut seeds are rich in protein, edible fats and various bioactive molecules such as PIs (Norioka et al., 1982; Arya et al., 2016). PIs are proteinaceous enzyme inhibitors isolated from several legumes and explored for many applications including therapeutic as well as insecticidal agents (Srikanth and Chen, 2016; Clemente et al., 2019). Though the presence of various BBI isoforms and its crystal structure has been elucidated in some cultivars of peanut, its identification or characterization in interspecific hybrid peanut varieties which are known to possess biotic

resistant traits against pests or pathogens was not yet explored (Suzuki et al., 1987; Mallikarjuna et al., 2011). This is the first report to showing the biochemical and biophysical properties of a BBI isolated from an interspecific hybrid variety (4368-1) of peanut.

The PnBBI was isolated using salting-out precipitation and purified to homogeneity using trypsin affinity and size exclusion chromatography techniques (**Fig. 4.1A,B**). The purity and yield recovery of PnBBI obtained in the present study is much superior when compared with PIs isolated from other plant sources such as mung bean (Klomklao et al., 2011), fenugreek (Oddepally et al., 2013), sombreiro (Dantzger et al., 2015) and pigeon pea cultivars (Swathi et al., 2014) and wild relatives (Swathi et al., 2016; Mohanraj et al., 2019), (**Table 4.1**). The discrepancy observed in molecular mass of PnBBI determined by Tricine SDS-PAGE (~10 kDa) as well as MALDI-TOF (6.73 kDa) analysis is not uncommon among the BBIs (**Figs. 4.1C, lane 5; 4.2A**; Swathi et al., 2014; Mohanraj et al., 2019). However, the observed molecular mass of PnBBI in SDS-PAGE is comparable to commercial soybean BBI. The retarded electrophoretic migration of PIs could be due to their compact structural makeup (Gennis and Cantor, 1976; Bergeron and Nielsen, 1993; He et al., 2017). Though the molecular mass of PnBBI (6.73 kDa) varied marginally, its biochemical properties such as structural and functional stability corroborated well with soybean BBI (Mello et al., 2003; Gu et al., 2014). Further, the amino acid sequence identity of isoinhibitor spot (pI 5.9) with known plant BBIs during MALDI MS-MS analysis confirmed the purified protein belongs to BBI family (**Fig. 4.3A,B**). Also, peanut BBI resembled with other known plant PIs in its crystal structure and biochemical properties except for non-identical reactive site loops and their specificity towards proteases being inhibited (**Fig 4.3B**; Suzuki et al., 1987; Qi et al., 2005). Further, the characteristic features such as low molecular mass and binding specificity of PnBBI towards serine proteases (trypsin or chymotrypsin) is in line with several isoinhibitors of *V. angularis* (Ishikawa et al., 1985), *V. faba* (Gupta et al., 2000) and *L. sativus*

(Rocco et al., 2011). Such BBIs identified earlier in pigeonpea and black gram through MALDI-TOF-TOF studies (Prasad et al., 2010b,c; Swathi et al., 2014).

In the present study, concentration-dependent self-association of PnBBI was visible in Tricine SDS-PAGE (**Fig. 4.1C, lanes 5-7**). However, the higher-ordered structures were dissociated after reduction with DTT as monomers (6-10 kDa), suggesting the key role of disulfide bonds in self-association (**Fig. 4.1C, lanes 8-10**). Previous studies indicated that the oligomeric structures of BBIs are known to be stabilized by hydrogen bonds among exposed hydrophobic patches of electrically charged clusters formed due to disulphide bonds (Silva et al., 2005; Rao and Suresh, 2007; Joshi et al., 2013). In case of horse gram, dimers shown to be stabilized by the electrostatic interaction between Lys^{24} and $Asp^{75/76}$ residues of N, C-terminal ends (Kumar et al., 2004; Muricken and Gowda, 2010). However, the mechanism of self-association may vary among various BBI molecules. The distinctive self-association characteristic feature of PnBBI was further validated by concentration-dependent far-VU CD spectra (**Fig. 4.7D**). The observed "red shift" from 205 to 212 nm in negative CD band together with decreased intensity was attributed to "absorption flattening" phenomena (Wallace and Teeters, 1987; Manzo et al., 2015). The systematic arrangement of chromophores (oligomer) and their random distribution in the sample perhaps might give differential absorption by a stepwise increase in the concentration of PnBBI. Similar changes in CD spectra associated with protein aggregation were reported earlier even with other proteins such as seed globulins (Mäkinen et al., 2016), antimicrobial peptides (Gopal et al., 2012) and monoclonal antibodies (Joshi et al., 2014). However, the formation of dimer or further oligomers does not induce any steric hindrance while reacting with trypsin which is also evident in 2-DE in-gel activity staining study (**Fig. 4.2F,G**). Besides, the self-association of BBIs not only provides stability, but also important in molecular packing as a seed storage protein (Hogg, 2003; Kumar et al., 2015). All the isoinhibitors visualized on one and two-dimensional gel electrophoresis conferred resistance against trypsin

and chymotrypsin digestion (**Fig. 4.2C,D,F,G;** Prasad et al., 2010a; Swathi et al., 2016; Mohanraj et al., 2019). The prominent TI activity was evidenced through more extensive protective zones against trypsin as compared to chymotrypsin during gelatin in-gel activity staining (**Fig. 4.2F**). However, the existence of BBI as several isoinhibitors is a part of plant defensive trait to combat against pests and their counter defense (Giri et al., 2003; Lopes et al., 2004; War et al., 2018).

The greater inhibitory potential of PnBBI towards trypsin over chymotrypsin is also evident through stoichiometric assays as well as SPR binding analysis (**Figs. 4.5C,D; 4.6D**). However, the differential inhibitory potential of double-headed PnBBI was attributed to its reactive site amino acids and their specificity towards cognate proteases. Further, the stoichiometric molar ratio of PnBBI and trypsin was found to be 1:2 which suggests both reactive sites can interact with trypsin in the absence of chymotrypsin. In contrast, chymotrypsin has not shown such distinctive stoichiometry, though it was inhibited by PnBBI (**Fig. 4.6D**; Prasad et al., 2010c; Satheesh and Murugan, 2012). These results taken together suggest that one of the reactive sites of PnBBI is flexible to bind either trypsin or chymotrypsin. Similar binding ratio (1:2) was reported earlier in case of trypsin inhibitors isolated from *L. albus* (Scarafoni et al., 2008), *Vigna mungo* (Prasad et al., 2010b), *Artocarpus heterophyllus* (Lyu et al., 2015) and *Psophocarpus tetragonolobus* (Banerjee et al., 2017).

Further, SPR kinetics suggests PnBBI forms more stable complex with trypsin K_D 1.34×10^{-9} M compared to chymotrypsin K_D 2.22×10^{-9} M (**Fig. 4.5A,B**). In this study, the PnBBI-chymotrypsin complex is able to bind with 5-fold excess (12.6 RU) of trypsin but not reciprocated by PnBBI-trypsin complex (2.46 RU), (**Fig. 4.5C,D**). These results corroborate well with the reports of Norioka et al. (1982), in which peanut BBI-chymotrypsin complex prone to bind with trypsin by slow release of its partner chymotrypsin but not seen in case of BBI-trypsin complex. PnBBI also possessed excessive anti-parallel β-sheets and random coils which is a core

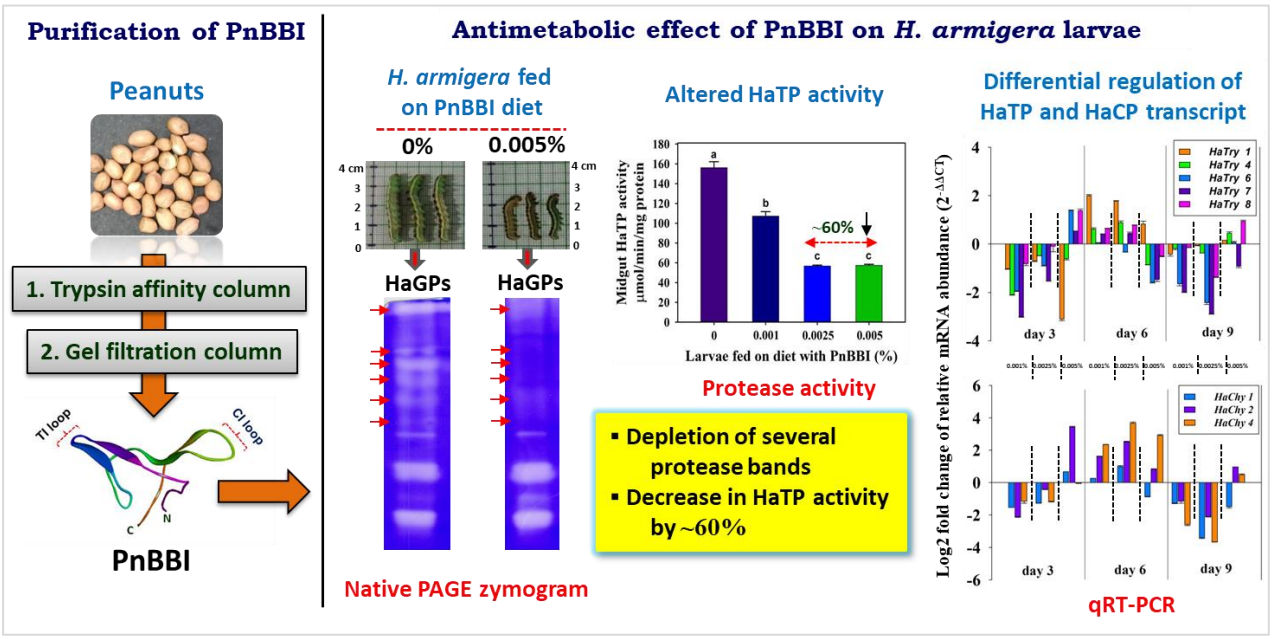
structural feature of BBIs (**Fig. 6**; Voss et al., 1996; Qi et al., 2005; Banerjee et al., 2017). Also, the function of PnBBI in terms of its TI and CI activity was stable at a broad spectrum of pH and temperature, and corroborated well with its unaffected Far-UV CD spectra (**Figs. 4.6A,B; 4.7A,B**). The structural features of PnBBI such as compactness, seven disulfide bonds and hydrogen-bonding network possibly aided stability to PnBBI under different adverse conditions (Tamura et al., 1994; Zavodszky et al., 2001; Zhou et al., 2005; Joshi et al., 2013). In contrast, the structural and functional stability of PnBBI was greatly affected by reducing agent dithiothreitol, particularly in its TI activity as compared to CI activity (**Figs. 4.6C; 4.7C**). Also, the comparative resistance of its CI activity towards DTT reduction was found to be higher than BBIs from *Cajanus cajan* (Prasad et al., 2010c) and lower than rRsBBI from *R. sublobata* (Mohanraj et al., 2018). In fact, the disulfide bonds stabilizing the CI loop might have been buried and inaccessible to DTT as compared to trypsin reactive site loop structure of PnBBI (He et al., 2017). Further, the observed characteristics such as low molecular mass, the existence of several isoinhibitors in a self-association pattern, presence of TI and CI activity, typical secondary structural components and MALDI-MS/MS analysis identified the purified protein as BBI.

Highlights

- ✓ PnBBI is purified to homogeneity with 167-fold purification and 37.5% yield recovery by sequentially passing through two chromatography columns.
- ✓ The PnBBI possessed a molecular mass of ~6.73 kDa and existed as several isoinhibitors which are resistant to trypsin and chymotrypsin digestion.
- ✓ The bifunctional PnBBI showed more affinity towards trypsin as compared to chymotrypsin.
- ✓ Its functional and structural integrity was stable over a broad range of pH and temperature.
- ✓ Disulfide bonds play a significant role in the maintenance of PnBBI structure, function and self-association pattern.

Chapter 5

Evaluation of the insecticidal potential of PnBBI against lepidopteran insect pest *Helicoverpa armigera*



Evaluation of the insecticidal potential of PnBBI against lepidopteran insect pest *Helicoverpa armigera*

5.1. Introduction

H. armigera (Lepidoptera: Noctuidae) is one among the devastating insect pests of agriculture, globally accounting to an estimated loss of US$ 2 billion annually (EPPO, 2019; Tay et al., 2013). It infests more than 200 plant species including economically important agricultural crops such as cotton, tomato, pigeon pea, chickpea, sorghum, cowpea, maize, tobacco and peanut (Pratissoli et al., 2015). The direct feeding habit of *H. armigera* larvae on flowering and fruiting bodies of the plant is cited as an important rationale for a significant loss in crop yield (McGahan et al., 1991). Though various pesticides from both synthetic and biochemical origin are employed, control of *H. armigera* invasion remained challenging due to its survival and adaptability to unstable habitats and seasonable changes (Fitt, 1989; Tabashnik and Carrière, 2017). The enormous flexibility in midgut protease expression of *H. armigera* upon feeding on different protein-rich parts of the plant allowed it to adapt and augment polyphagous nature (Patankar et al., 2001; Chikate et al., 2013; Zhu-Salzman and Zeng, 2015).

The expression of larval midgut proteases switches among several gene copies of serine, cysteine, aminopeptidases depending on their developmental stage and diet composition (Sarate et al., 2012; Kipgen and Aggarwal, 2014). In addition, insects use different defensive strategies to minimize the anti-nutritional effect of ingested plant PIs such as (i) compensatory overproduction of existing digestive proteases, (ii) expression of inhibitor insensitive digestive proteases and (iii) production of PI hydrolysing proteases (Bown et al., 1997; Girard et al., 1998; Jongsma and Bolter, 1997; Zhu-Salzman et al., 2003; Macedo et al., 2015). Further, the extensive usage of pesticides causes deleterious effects on natural ecosystem such as loss of biodiversity, human health hazards, pest resistance and a surge of secondary pests, ultimately leading to outbursts of pests (Aktar et al., 2009; Nicolopoulou-Stamati et al., 2016). Therefore, it is

necessary to develop novel eco-friendly strategies such as application of PIs to manage the damage caused by *H. armigera* and reduce the hazardous pesticide use including organophosphates, synthetic pyrethroids, carbamates and endosulfan (Sharma et al., 2000; Downes et al., 2017). In general, the PIs bind firmly and irreversibly to the active site of digestive enzymes and attenuate their activity, which in turn impair the protein turnover in various metabolic processes, eventually limiting their growth and development (Zhu-Salzman and Zeng, 2015). Also, the resistance mediated by PIs from wild-relatives or non-host or hybrid plants is more advantageous than host plant resistance due to inexposure of pest midgut proteases to PIs from such plants (Parde et al., 2010; Pandey et al., 2014; Swathi et al.,2016; de Paula et al., 2017).

In fact, most of the cultivar peanut varieties were tetraploid and possessed inadequate levels of resistance to several biotic and abiotic constraints. In contrast, the closely related wild relative peanut diploids possessed several useful disease/pest resistant traits (Garcia et al., 2006; de Paula et al., 2017; Stalker, 2017). This provided an opportunity for introgression of resistant traits from wild germplasm via generating amphidiploids/synthetic varieties into cultivated peanut gene pool (Mallikarjuna et al., 2011; Kumari et al., 2014). Likewise, foliar disease resistance in peanut was achieved by introgression of traits from synthetic amphidiploids namely ISATGR 278-18 (*A. duranesis* x *A. batizocoi*) and ISATGR 5B (*A. magna* x *A. batizocoi*) into five (ICGV 91114, ICGS 76, ICGV 91278, JL 24 and DH 86) cultivars (Kumari et al., 2014). Also, pest (*Spodoptera litura*) resistant peanut hybrids were developed by ingression of traits from amphidiploid An13 (*A. magna* V 13751 x *A. kempff-mercadoi* V 13250) into *A. hypogaea* cv. IAC OL4 (Mallikarjuna et al., 2004; Fávero et al., 2015). Therefore, the present study was aimed to evaluate insecticidal properties of PnBBI isolated from interspecific hybrid variety of peanut (4368-1) against most economically important pest *H. armigera*.

5.2. Results

5.2.1. Effect on *H. armigera* larval growth

The inhibitory potential of PnBBI against *H. armigera* larval growth was examined under both *in vitro* and *in vivo* conditions. Under *in vitro* conditions, PnBBI exhibited strong inhibitory activity against trypsin-like HaGPs (25,000 HaTPI U/mg) as compared to bovine trypsin (1406 TI U/mg) and chymotrypsin (57 CI units/mg protein) (**Fig. 5.1**). Also, PnBBI is a potential inhibitor of HaTP (IC_{50} = 40 ng) as compared to soybean BBI (IC_{50} = 16.5 µg). In contrast, PnBBI has not shown much variation with soybean BBI in its inhibitory potential against chymotrypsin (**Table 5.1**). Further, *in vivo* feeding experiments demonstrated dose-dependent retardation in growth of *H. armigera* larvae upon feeding with increasing concentrations (0.001%, 0.0025% and 0.005%) of PnBBI. Among the given treatments, maximum reduction (~42% of control) in body mass of larvae fed on high dose (0.005%) of PnBBI was observed on day 6 (**Fig. 5.2A,B**). The antifeedant potential of PnBBI was further evaluated by comparing the activity of serine proteases in midgut of larvae fed on various doses of PnBBI using *in vitro* studies. A gradual decrease (~63%) in the activity of HaTPs was observed in larvae fed on the moderate and high dose of PnBBI as compared to larvae fed on control diet (**Fig. 5.3**). However, the chymotrypsin-like activity of HaGPs are not in detectable range in control as well as treated larval gut extracts (data not shown).

5.2.2. In-gel HaGP activity profile and their inhibition

The activity of HaGP(s) in larvae fed on control diet was detected as 10-12 distinct protease bands on casein Native-PAGE. The disappearance of this protease band pattern upon incubation with PMSF demonstrated the predominance of serine proteases in midguts of *H. armigera* larvae. However, the protease activity bands 1-5 (group-I) disappeared when incubated with trypsin-like protease inhibitor TLCK, but not by chymotrypsin-like protease inhibitor TPCK. Also, protease

band 9 disappeared in the presence of TPCK but not in presence of TLCK. These results suggest that group-I bands belong to trypsin-like midgut proteases and band 9 belong to chymotrypsin-like protease in HaGP extract (**Fig. 5.4A**).

Table 5.1. Comparison of inhibitory potential of PnBBI and soybean BBI towards Bovine trypsin, chymotrypsin and trypsin-like midgut proteases of *H. armigera* (HaTPs). The data represented is mean ± SE of three biological replicates.

Protease	**Half-maximal inhibitory concentration (IC_{50})**	
	PnBBI (µg)	Soybean BBI (µg)
Bovine trypsin	0.730 ± 0.01	3.4 ± 0.09
Chymotrypsin	17.54 ± 0.92	16.1 ± 0.85
HaTPs	0.040 ± 0.003	16.5 ± 0.65

Note: IC_{50} is defined as the amount of inhibitor required to inhibit 50% of the corresponding protease activity.

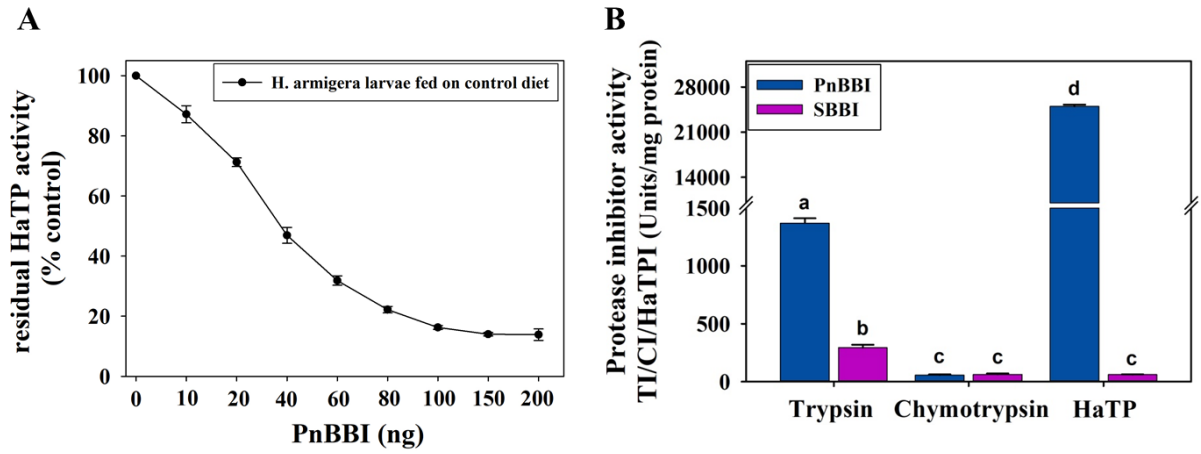


Fig. 5.1. Inhibitory potential of PnBBI by *in vitro* studies: (**A**) Inhibition of *H. armigera* midgut trypsin-like protease (HaTP) activity at wide range of PnBBI concentrations and (**B**) A comparative inhibitory profile of PnBBI with soybean BBI towards bovine trypsin, chymotrypsin and HaTP. The specific activity is defined as number of protease inhibitor units per milligram of PnBBI. The data represented is mean ± SE of three biological replicates. Different lower case letters 'a, b, c and d' indicate statistically different at a significant level ($P < 0.05$) as compared to control.

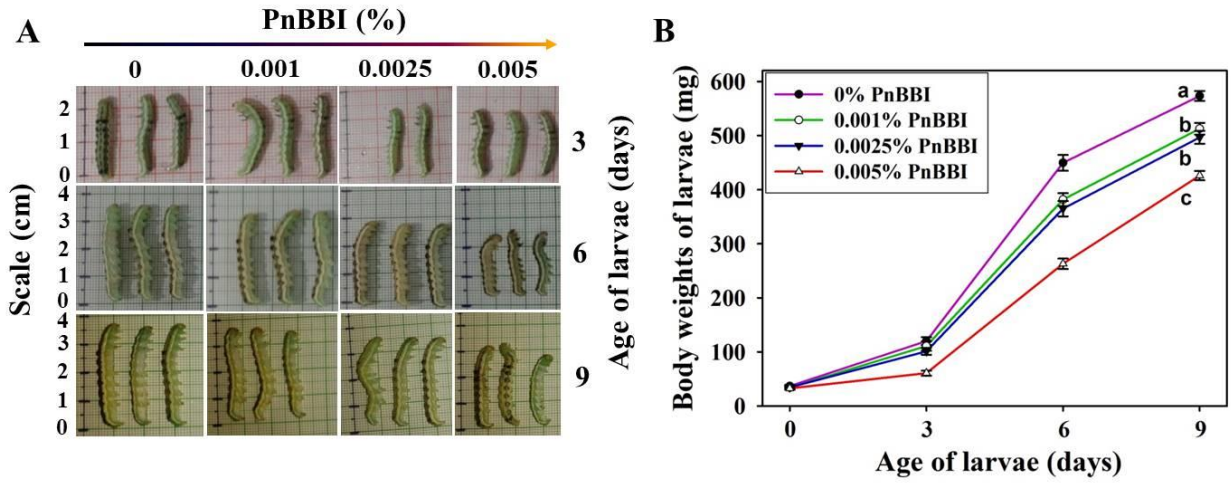


Fig. 5.2. Insecticidal potential of PnBBI: (**A**) Photographs depicting the reduction in size of *H. armigera* larvae fed on increasing doses (0.001%, 0.0025% and 0.005%) of PnBBI as compared to larvae fed on control diet at the end of day 3, 6 and 9 of feeding and (**B**) Depletion in mean larval body weights after exposure to PnBBI. The data represented is mean ± SE of three biological replicates. Different lower case letters 'a, b and c' indicate statistically different at a significant level ($P < 0.05$) as compared to control.

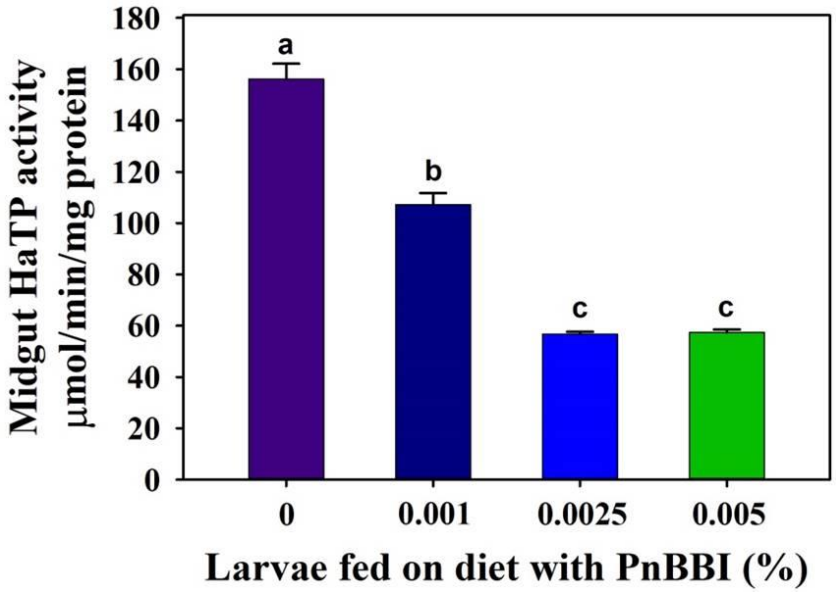


Fig. 5.3. PnBBI induced changes in the midgut HaTP activity: The biochemical changes with respect to decreased HaTP activity in larvae reared on diet containing PnBBI as compared to control. The data represented is mean ± SE of three biological replicates. Different lower case letters 'a, b and c' indicate statistically different at a significant level ($P < 0.05$) as compared to control.

Mimicking the treatment with TLCK, the activity of HaTPs (bands 1-5 of HaGPs) was decreased gradually with increasing concentration of PnBBI in the diet supplemented to larvae. Apart, at high dose (0.005%) of PnBBI, the activity of protease band 9 (HaCP) increased when compared with the control sample (**Fig. 5.4B-D**). A significant divergence was observed in protease profiles of larvae fed on a high dose of PnBBI when compared with the control. It was further resolved by using 2-DE coupled zymography technique. HaGPs of larvae fed on the control diet showed a greater number of active protease spots ranging between pH 3 to 10. In contrast, several of these protease spots disappeared in zymogram of HaGPs extracted from larvae fed on a high dose (0.005%) of PnBBI as indicated in box 1 and 2 (**Fig. 5.5A,B**).

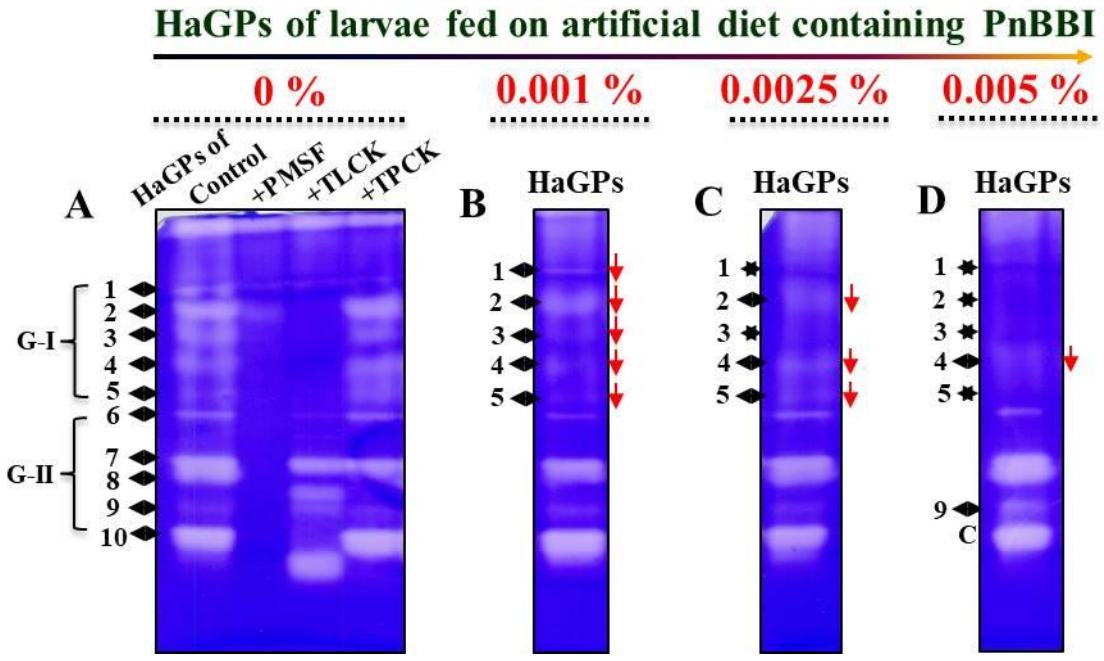


Fig. 5.4. Comparative HaGPs profile of larvae fed on PnBBI. Casein Native-PAGE zymogram of HaGPs extracted from larvae (day9) fed on diet with **(A)** No PnBBI (Lane1: HaGPs, lane2: HaGPs+PMSF, lane3: HaGPs+TLCK and lane4: HaGPs+TPCK); **(B)** 0.001% PnBBI; **(C)** 0.0025% PnBBI and **(D)** 0.005% PnBBI (down headed arrow are an indicative of decreased protease expression, *indicate diminished protease expression and letter 'C' indicate chymotrypsin-like protease).

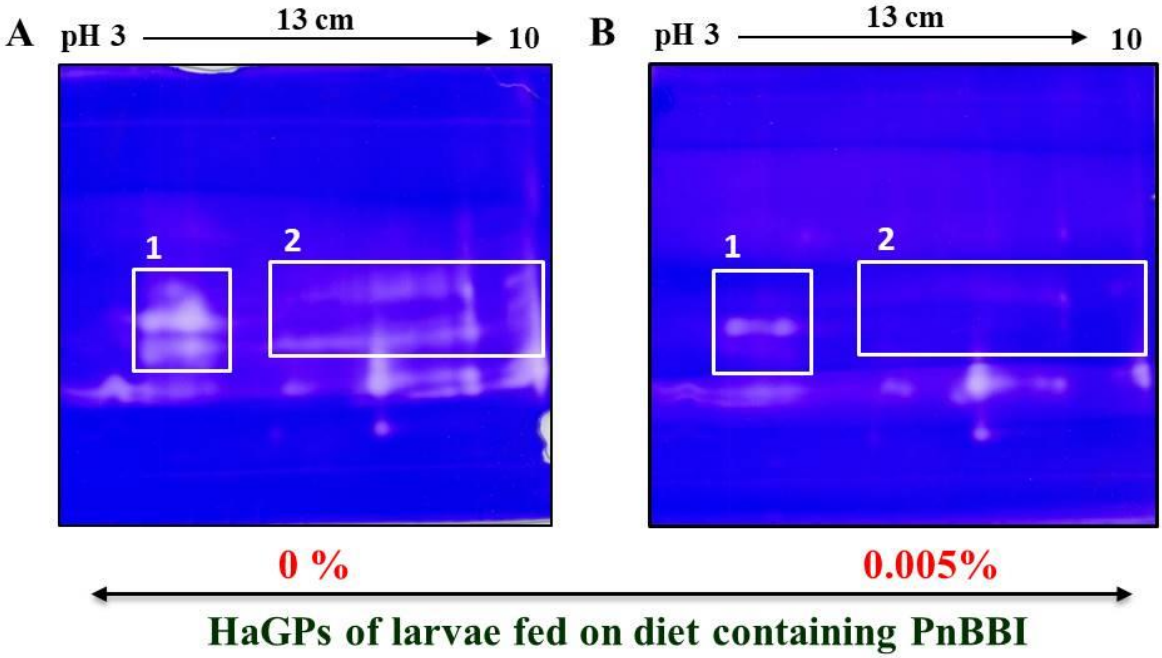


Fig. 5.5. Differential 2-DE zymography of HaGPs from larvae fed on **(A)** control diet and **(B)** 0.005% PnBBI supplemented diet. An equal amount of protein (8 µg) from HaGP extract was subjected to IEF followed by SDS-PAGE (10%) and zymography as described in methods section 3.11.6. The respective clear zones against the dark blue background are the result of casein digestion due to the presence of proteases. The areas highlighted with boxes on 2-DE zymograms represent the disappearance of several protease isoforms upon PnBBI ingestion. The gel pictures are the selective representatives of two biological replicates.

5.2.3. qRT-PCR analysis of HaGPs

Total RNA was extracted by using TRI reagent and cDNA was amplified by using primers of housekeeping gene RpS18 (**Fig. 5.6A,B**). The quantitative real-time PCR analysis revealed the relative transcript abundance of HaTPs (*HaTry*1, *HaTry*4, *HaTry*6, *HaTry*7, *HaTry*8) and HaCPs (*HaChy*1, *HaChy*2, *HaChy*4) in larvae fed on low (0.001%), moderate (0.0025%) and high (0.005%) dose of PnBBI at the end of day3, day6 and day9 (**Fig. 5.7**). The five HaTP-like (*HaTry* 1, 4, 6, 7, 8) and two HaCP-like (*HaCry* 2, 4) genes were selected from the study of Chikate et al. (2013) and Mahajan et al. (2013), and the remaining *HaChy* 1 was adopted from Chougule et al. (2005). At the end of day3 and day9, relative transcript abundance of HaTPs was down-regulated

(< 3-fold) at low as well as at a moderate dose of PnBBI, while most of the HaTPs (except *HaTry*1/*HaTry*4/ *HaTry*7) were up-regulated (< 2-fold) at a high dose of PnBBI. In contrary, at the end of day6, the relative transcript abundance of HaTPs was increased (< 2-fold) at a low or moderate dose of PnBBI, while most of the HaTPs (except *HaTry*1) were down-regulated (< 2-fold) at a high dose of PnBBI (**Fig. 5.7A**). The relative transcript abundance of HaCPs were down-regulated (< 3.7-fold) similar to HaTPs at the end of day3 and day9 in the presence of low or moderate dose of PnBBI. However, the transcript levels *HaChy*2 was up-regulated significantly at the end of day3 (< 3.7-fold) in the presence of a high dose of PnBBI. In contrast, at the end of day6, the transcript levels of all HaCPs (except *HaChy*1 at high dose) were up-regulated (< 4-fold) under low, moderate and high dose of PnBBI (**Fig. 5.7B**).

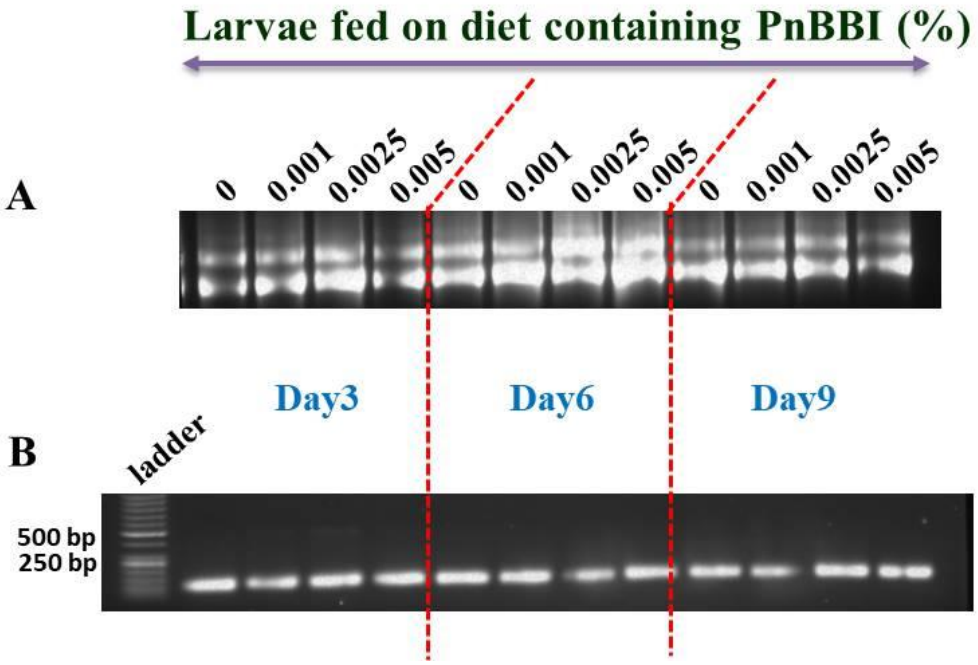


Fig. 5.6. (**A**) Total RNA isolation from midgut epithelial tissue of *H. armigera* larvae fed on artificial diet containing PnBBI on different days of feeding and (**B**) cDNA amplification was performed using specific primers of housing keeping gene (ribosomal protein S18, RpS18) for normalization. The midgut tissue from six individual larvae was pooled in a vial containing TRI reagent and further subjected to RNA extraction.

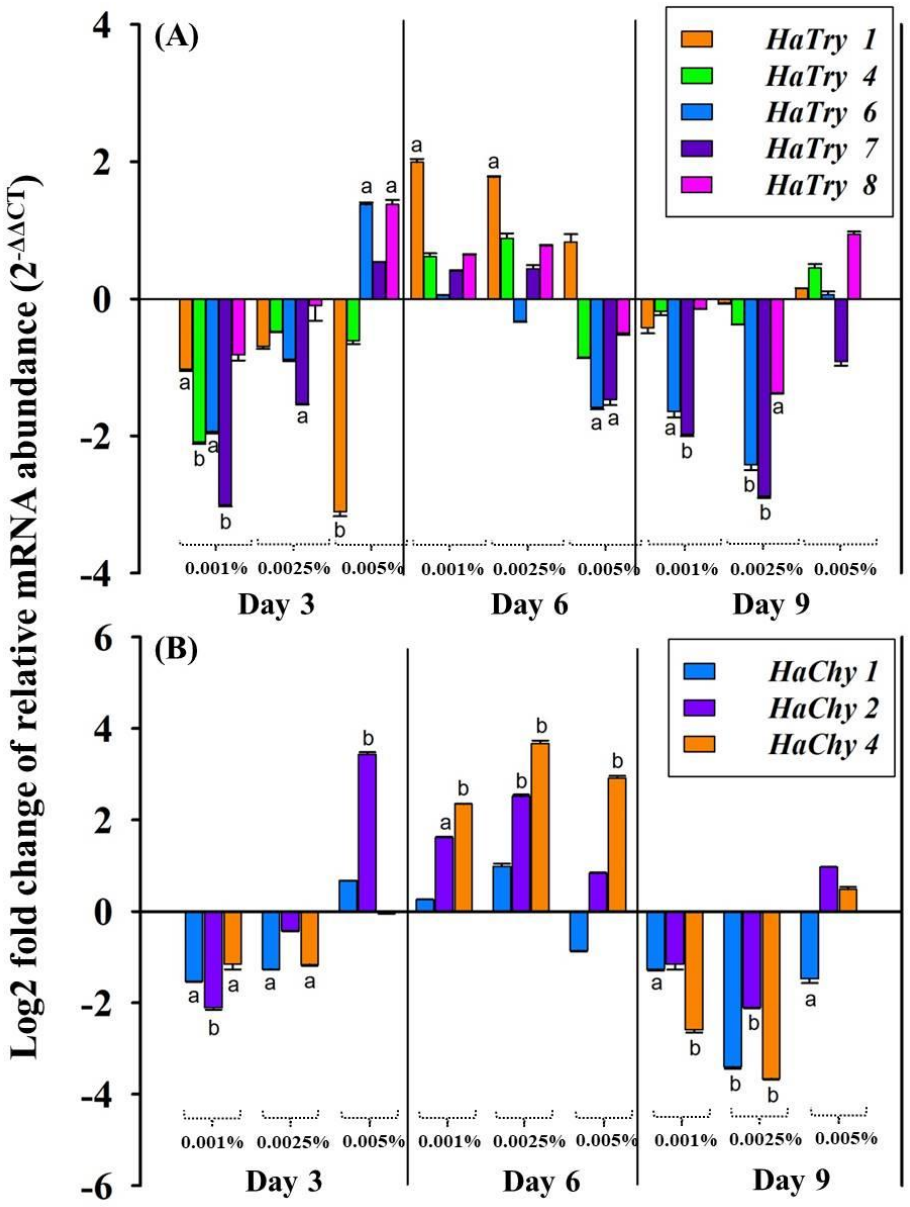


Fig. 5.7. qRT-PCR analysis of midgut serine proteases of *H. armigera* larvae fed on PnBBI. Changes in transcript abundance of **(A)** midgut trypsin-like (*HaTry* 1, 4, 6, 7 and 8) and **(B)** chymotrypsin-like (*HaChy* 1, 2 and 4) proteases from midgut tissue of *H. armigera* larvae after rearing on artificial diet containing different concentration of PnBBIs (0%, 0.001%, 0.0025% and 0.005%) for 3, 6 and 9 days. The fold difference in transcript abundance of these candidate genes was calculated using Livak ($2^{-\Delta\Delta CT}$) method and final values are expressed in log (base2) fold changes. Further details were as described in the methods section 3.11.7. The data shown is mean ± SD of triplicates. The transcript abundance of trypsin- and chymotrypsin-like genes of the larvae fed on PnBBI incorporated diet was compared with control diet-fed larvae. The lower case letters 'a & b' assigned for transcripts which showed >1 and 2-fold difference in their abundance, respectively.

5.3. Discussion

H. armigera uses multiple defense strategies while feeding upon various host plant material. The digestive proteases and their flexibility in expression play a prime role in contributing major support for its survival, adaptability and polyphagous nature (Padul et al., 2012; Jamal et al., 2013; Zhu-Salzman & Zeng, 2015; War et al., 2018). At any given time, alkaline trypsin-like serine proteases are the major constituents of the midgut despite changes in digestive protease profile with developmental stage and type of diet it consumes (Srinivasan et al., 2006). In this scenario, targeting these proteases with PnBBI which possessed significant inhibitory activity against HaTPs would be a potential approach to control the growth of larvae (**Fig. 5.1**; Harsulkar et al., 1999; Chougule et al., 2005; Parde et al., 2010). Hence, the present study was undertaken to evaluate the *ex vivo* and indigenous PnBBI effects on modulation in expression profiles of trypsin- and chymotrypsin-like gut proteases, their activity and associated changes in larval growth.

The strong insecticidal potential of PnBBI was primarily represented by low IC_{50} values (40 ng/ml) and high maximal inhibition of HaTP (86%) as compared to the other reported PIs (**Table 1; Fig. 5.1A**; Babu and Subrahmanyam, 2010; Jamal et al., 2014; Pandey et al., 2014; Swathi et al., 2016; Banerjee et al., 2017; Mohanraj et al., 2019). Hence, the antifeedant effect of PnBBI against *H. armigera* at morphological as well as protease expression level was further examined by biochemical and molecular approaches. In the present study, a significant reduction (40-50%) in larval body weight was observed with the intake of high dose PnBBI (0.005%). These observations are in accordance with the *in vitro* studies emphasizing the efficacy of PnBBI in inhibiting midgut proteases which in turn led to impaired protein digestion and hindered larval growth (**Fig. 5.2A,B; 5.3**).

The modulation in the expression of various digestive proteases suggests that diet make a significant impact on the survival and adaptation of the insect (Chougule et al., 2005). The

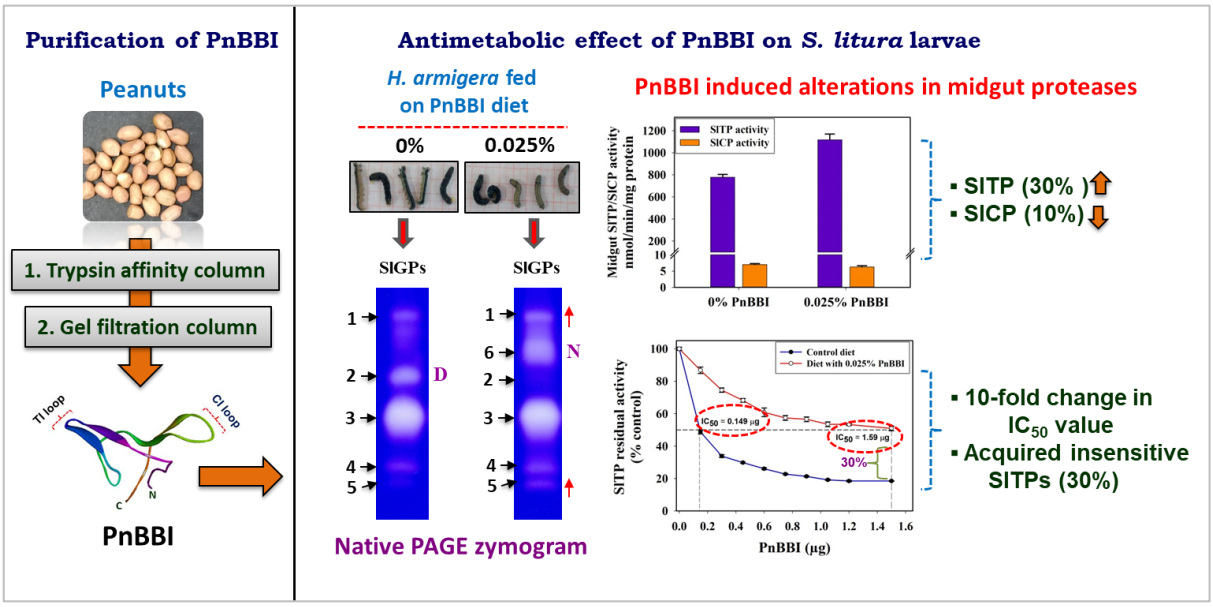
observations of the present study are in agreement with the earlier reports (Harsulkar et al., 1999; Patankar et al., 2001; Telang et al., 2003; Chougule et al., 2005). Also, the HaTPI potential of PnBBI in limiting larval growth is further evident by the loss of HaTP activity (~63%) in larvae fed on high dose PnBBI diet (**Figs. 5.1; 5.2; 5.3**). The one and two-dimensional zymography further confirmed these observations. The comparative HaGP profiles on native PAGE zymogram revealed the depletion of several trypsin-like proteases in larvae fed on PnBBI diet (**Figs. 5.4; 5.5**). However, the proteases represented in group-II (6-10) has not shown any changes in their expression in response to PnBBI ingestion except a slight increase in the intensity of protease band 9 (**Fig. 5.4D**). The inhibition in the activity of protease isoform 9 suggests that it belong to chymotrypsin-like protease and its increased expression might be compensatory response to minimise the effect of the PnBBI. Interestingly, the disappeared proteases which belong to trypsin-type were inhibited by the PnBBI. However, remaining protease bands (6-10) were neither inhibited nor changed in their expression levels in response to PnBBI. The PnBBI mediated modulations in gut protease activity of *H. armigera* larvae were further supported by serine protease transcripts analysis using qRT-PCR. PnBBI prominently influenced the expression of HaTP and HaCP transcripts in a dose and exposure time-dependent manner. At any observed time of interval, the larvae exposed to low and moderate dose of PnBBI exhibited a similar pattern in expression of HaTP and HaCP transcripts when compared with those fed on a high dose of PnBBI (**Fig. 5.7**). Overall, the low and moderate dose of PnBBI ingestion regulated HaTP/HaCP transcripts by a signature pattern of down-up-down expression after feeding on 3^{rd}, 6^{th} and 9^{th} day, respectively. Conversely, a high dose of PnBBI fed larvae exhibited a pattern of up-down-up expression on 3^{rd}, 6^{th} and 9^{th} day, respectively, with few exceptions. Thus, the expression pattern of trypsin- and chymotrypsin-like proteases in response to PnBBI ingestion was similar to the fashion of trypsin expression after ingesting rCanPI-7 (Mahajan et al., 2013).

Highlights

- ✓ PnBBI is a potent inhibitor of HaTPs with IC_{50} of 40 ng (25000 HaTPI units/mg protein).
- ✓ Ingestion of PnBBI retarded the *H. armigera* larval growth by >40%.
- ✓ A decrease in larval body mass was associated with ~63% decline in the HaTP activity.
- ✓ PnBBI had a strong influence on trypsin-like midgut proteases of larvae as evident by the disappearance of large number of protease isoforms on 1-DE & 2-DE zymogram.
- ✓ PnBBI modulated the expression and activity of trypsin-like & chymotrypsin-like midgut proteases.

Chapter 6

Evaluation of the insecticidal potential of PnBBI against lepidopteran insect pest *Spodoptera litura*



Evaluation of the insecticidal potential of PnBBI against lepidopteran insect pest *Spodoptera litura*

6.1. Introduction

The tobacco cutworm *Spodoptera litura* (Lepidoptera: Noctuidae) is a polyphagous insect pest of many economically important crops and covers at least 120 host plant species (CAB International, 2018). In recent years, it became the most destructive insect pest leading to massive crop losses in the Asian-pacific region due to its high reproductive rate and dispersal capability (Babu et al., 2015; Fand et al., 2015). This tobacco caterpillar damage major economically important crops such as tobacco, cotton, castor, groundnut, chilli, cauliflower, pulses, many fruits and vegetables throughout tropical Asia, Australia and Pacific Islands (Xue et al., 2010; Yooboon et al., 2019; Vasudev and Sohal, 2019). Continuous usages of chemical pesticides to protect the crops from armyworm cause a negative impact on the environment and human health. Moreover, *S. litura* is resistant to commonly used insecticides such as pyrethroids, organophosphates, carbamates, indoxacarb and fipronil etc., (Kranthi et al., 2002; Ahmad et al., 2007; Maqsood et al., 2017). These issues together encouraged the scientific community to seek for biomolecules with insecticidal activity such as proteinase inhibitors (PIs), chitinases and lectins as a part of sustainable, non-hazardous and effective pest control measures (Parde et al., 2012; Jamal et al., 2013; Chen et al., 2014; War et al., 2018). Thus, usage of PIs have become a part of integrated pest management (IPM) similar to other biological pesticides (Sharma et al., 2000; Haq et al., 2004).

Plant PIs block the proteases of insect midgut which play a crucial role in the digestion of proteins consumed through natural food and thus creates nutrient deficiency (Gatehouse et al., 2000; Macedo et al., 2015). Though, the process of co-evolution enabling the insect to overcome the action of PIs by swapping over the synthesis of new gut proteases. The new proteases are either sensitive or insensitive to such PIs or modified to a class of different substrate specificity.

For instance, a chymotrypsin-like activity might be adapted alternative to trypsin-like activity (Bown et al., 1997; Jongsma and Bolter, 1997; Bayes et al., 2005; Lopes et al., 2006; Dunse et al., 2010a; de Oliveira et al., 2013; Zhu-Salzman and Zeng, 2015; Spit et al., 2016). Despite the adaptive strateges exhibited by the insect pests, PIs isolated from wild plant species, hybrid plant varieties and non-host plants, which are distinct and not exposed to such pests might provide substantial and durable resistance towards insect pests (Harsulkar et al., 1999; Parde et al., 2010; Prasad et al., 2010a; Pandey et al., 2014; Swathi et al., 2014; Mohanraj et al., 2018). So far, several plant PIs have been proved to provide resistance against destructive insect pest *S. litura* at various levels such as *in vivo* bioassays (Telang et al., 2003; Katoch et al., 2015; Vasudev and Sohal, 2019) and development of transgenic plants (McManus et al., 1999; Chen et al., 2014; Saikhedkar et al., 2019; Zhu et al., 2019).

Therefore, in the present study, the antimetabolic effect of PnBBI purified from interspecific hybrid peanut variety (4368-1) was evaluated against the larvae of *S. litura*.

6.2. Results

6.2.1. Inhibitory properties of PnBBI

Inhibitory activity of PnBBI towards bovine trypsin and chymotrypsin as well as trypsin-like midgut proteases of *S. litura* (SlTP) was evaluated by *in vitro* assays. In which PnBBI exerted ~4.75-fold higher inhibitory activity against SlTP as compared to bovine trypsin with IC_{50} of 0.149 µg and 0.73 µg, respectively (**Fig. 6.1A; Table 6.1**). Further, isoinhibitors of PnBBI separated on non-reducing 2-DE gel displayed resistance to digestion by *S. litura* midgut proteases (SlGPs) similar to the resistance shown by soybean BBI (**Fig. 6.1B**). These results ascertained 'PnBBI' as a potential inhibitor of SlGPs and the insecticidal efficacy of PnBBI was further evaluated by *in vivo* feeding bioassay.

Table 6.1. Comparative inhibitory potential of PnBBI and soybean BBI towards bovine trypsin, bovine chymotrypsin and trypsin-like midgut proteases of *S. litura* (SlTPs) larvae. Data represent Mean ± SE of three biological replicates.

Protease	Half-maximal inhibitory concentration (IC_{50})	
	PnBBI (µg)	Soybean BBI (µg)
Bovine trypsin	0.730 ± 0.01	3.4 ± 0.09
Chymotrypsin	17.54 ± 0.92	16.1 ± 0.85
SlTP	0.149 ± 0.012	18.9 ± 0.003

Note: IC_{50} is defined as the amount of inhibitor required to inhibit 50% of the corresponding protease activity.

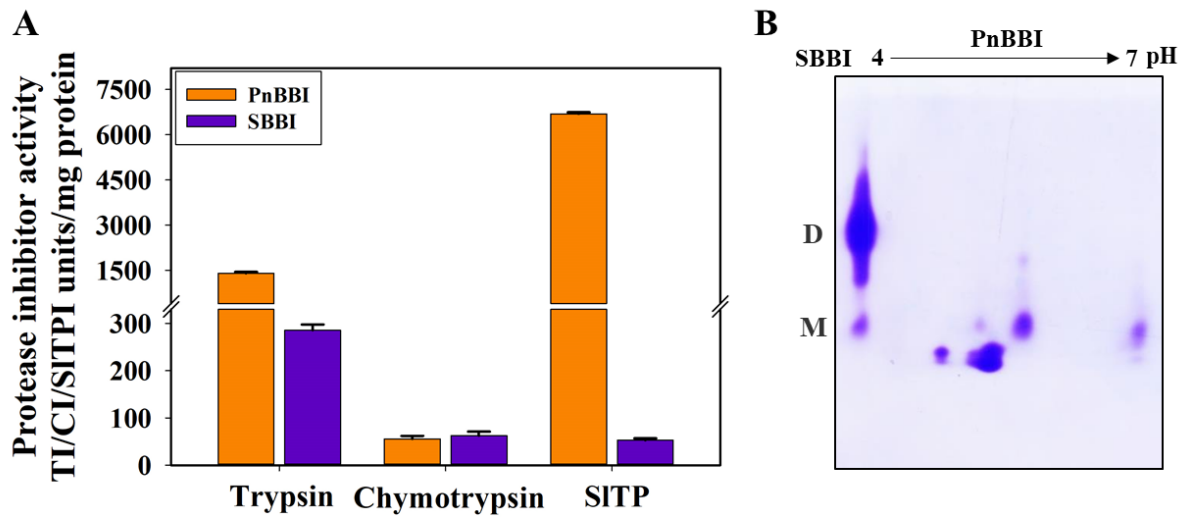


Fig. 6.1. Inhibitory potential of PnBBI: (A) Specific inhibitory activity of PnBBI towards bovine trypsin, bovine chymotrypsin and SlTP was determined by *in vitro* assay using chromogenic substrates and **(B)** Two-dimensional gelatin SDS-PAGE showing PnBBI isoinhibitors resistant to SlGPs digestion for 2 h at 37 °C. Rehydrated IPG strip (11 cm) with PnBBI (70 µg) was subjected to IEF under non-reducing condition followed by gelatin SDS-PAGE. Commercial soybean BBI was used as a standard for in-gel activity staining. M-monomer and D-dimer. The data represent Mean ± SE of three biological replicates.

6.2.2. *In vivo* feeding bioassay

Insecticidal efficacy of PnBBI towards polyphagous insect pest *S. litura* was assessed by allowing the larvae to feed on a chickpea-based artificial diet supplemented with 0.025% PnBBI. The morphological changes in *S. litura* larvae with respect to their size upon feeding with PnBBI were depicted in the photographs (**Fig. 6.2A**). Further, PnBBI retarded the growth of *S. litura* larvae as revealed by their decreased body weight by 17.25% and 13.0% at the end of 4^{th} instar and 5^{th} instar stage, respectively (**Fig. 6.2B**).

6.2.3. Response of *S. litura* midgut proteases to PnBBI ingestion

The possible changes in the midgut proteases of *S. litura* larvae after PnBBI ingestion was determined by estimating SlTP and chymotrypsin-like (SlCP) activity and their inhibition by PnBBI. The SlTP activity was drastically increased by ~30% in larvae fed on PnBBI diet as compared to control diet. In contrast, SlCP activity was decreased by ~10% in response to PnBBI ingestion (**Fig. 6.3A**). Further, total midgut protease profile of larvae fed on control and PnBBI supplemented diet was visualized by native PAGE zymography. Interestingly, the comparative protease profile revealed that among the five prominent protease bands identified in larvae fed on the control diet, the band 2 disappeared with concomitant emergence of a new protease band 6 after exposure to PnBBI diet. Likewise, two low-intensity proteases (bands 1 and 5) showed increased expression in response to PnBBI ingestion. Thus, the increased SlTP and decreased SlCP activity corroborated with the newly emerged and diminished proteases, respectively (**Fig. 6.3B**). Such alterations in midgut protease expression pattern are observed as a part of the compensatory defensive response of insects to escape from the antinutritional effect of PIs consumed through diet.

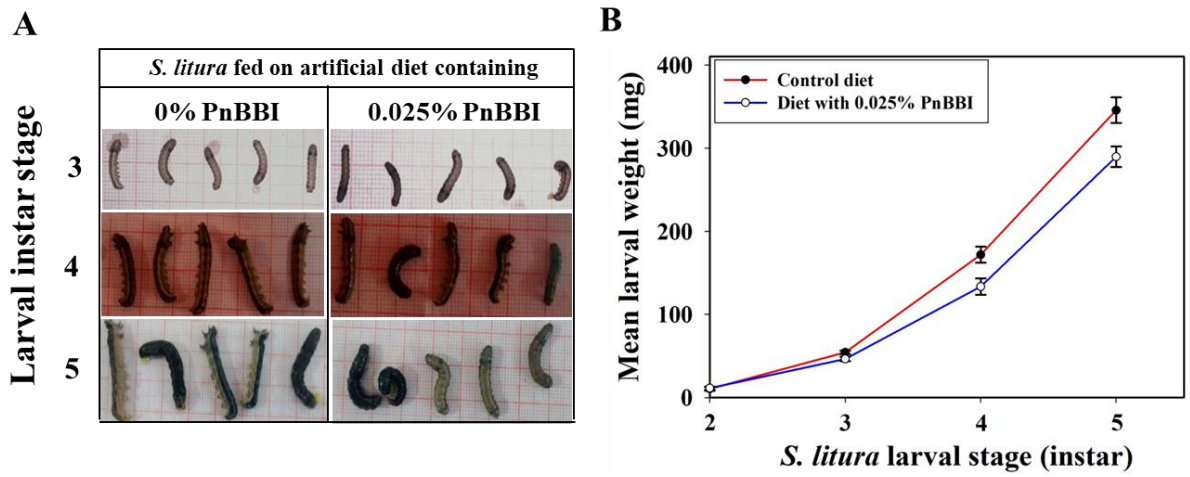


Fig. 6.2. Antimetabolic effect of PnBBI on *S. litura* larval growth: (**A**) Photograph depicting morphological changes with reference to their size after feeding of larvae on diet supplemented with 0.025% PnBBI as compared to control diet (no PnBBI) and (**B**) Larval body weight of corresponding *S. litura* fed on diet with and without PnBBI. During *in vivo* feeding bioassay, 30 numbers of equal size second instar larvae were taken in each group and maintained till 5^{th} instar stage. The data represent Mean ± SE.

Furthermore, the distinctive changes in the activity of SlTP extracted from larvae fed on control and PnBBI incorporated diet was determined by *in vitro* inhibition assay after incubating at a wide range of concentrations of PnBBI (**Fig. 6.3C,D**). The obtained results revealed that SlTPs altered their specificity towards PnBBI to some extent during post-PnBBI exposure. The SlTPs extracted from larvae fed on control diet displayed maximum inhibition of 80% at 1.5 µg of PnBBI. In contrast, SlTPs from larvae exposed to PnBBI showed maximum inhibition of 50% even after incubation at 2 µg of PnBBI. As a result, the IC_{50} value of PnBBI towards SlTP increased from 0.149 µg to 1.50 µg, which account to ~10-fold change after exposure of larvae to PnBBI. Besides, the results suggest *S. litura* larvae acquired ~30% insensitivity which is evident by its *in vitro* SlTP activity (**Fig. 6.3C,D**).

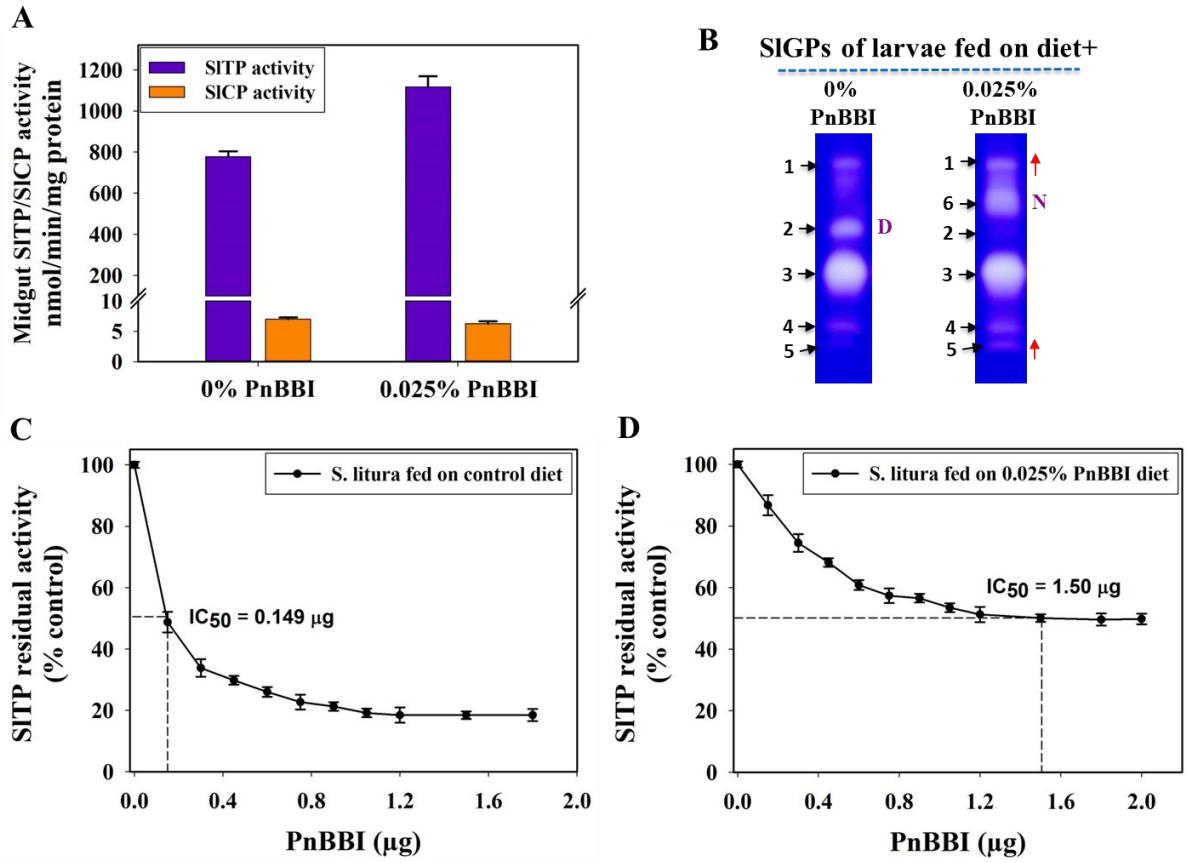


Fig. 6.3. PnBBI induced alterations in midgut trypsin- and chymotrypsin-like activity of 5th instar *S. litura* larvae: (A) *In vitro* quantification of SlTP and SlCP activity of larvae fed on control and PnBBI supplemented diet; **(B)** Casein zymogram showing SlGP profile of larvae fed on a diet with and without PnBBI. *In vitro* inhibition of SlTP activity of larvae fed on (**C**) control and (**D**) PnBBI supplemented diet at a wide range of PnBBI concentrations in the assay mixture. The data represent Mean ± SE of three biological replicates. The letter 'D' and 'N' are an indicative of diminished and newly emerged protease bands, respectively.

6.2.4. Differential two-dimensional gel electrophoresis

The differences in the midgut proteome profile of *S. litura* larvae fed on control and PnBBI supplemented diet was analysed by 2-DE. Precisely, 299 proteins were identified by 2-DE platinum software in which 14 proteins were up-regulated (Spot IDs: 12, 15, 25, 56, 57, 58, 78, 81, 116, 165, 181, 182, 185 and 192) and 19 proteins were down-regulated (Spot IDs: 6, 24, 30, 35, 38, 41, 55, 75, 91, 93, 119, 141, 151, 157, 162, 163, 175, 276, 289 and 299) by >1.5-fold in larvae fed on PnBBI diet as compared to larvae fed on control diet (**Figs. 6.4A,B; 6.5; 6.6**).

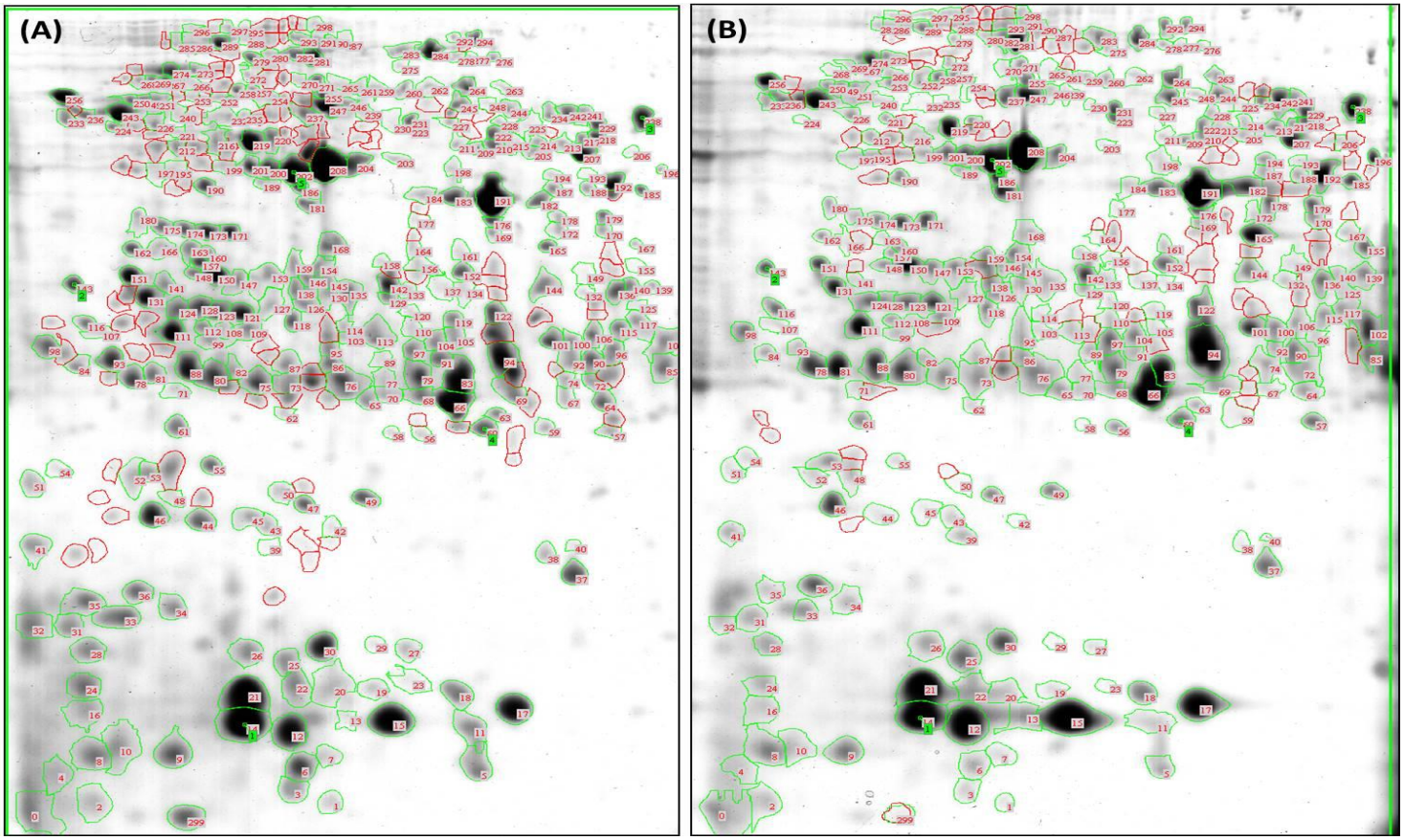


Fig. 6.4. Two-dimensional gel electrophoresis: Differential midgut proteome profile of *S. litura* larvae fed on (**A**) control diet and (**B**) PnBBI (0.025%) supplemented diet. IPG strip (11 cm) was rehydrated with buffer containing 300 µg of *S. litura* midgut proteome under reducing conditions and subjected to IEF followed by 12.5% SDS-PAGE. The differentially expressed proteins were analysed by using Image master 2-DE platinum software version 7.0, GE Healthcare, Life Sciences. All the protein spots were identified by their match IDs in both the gels.

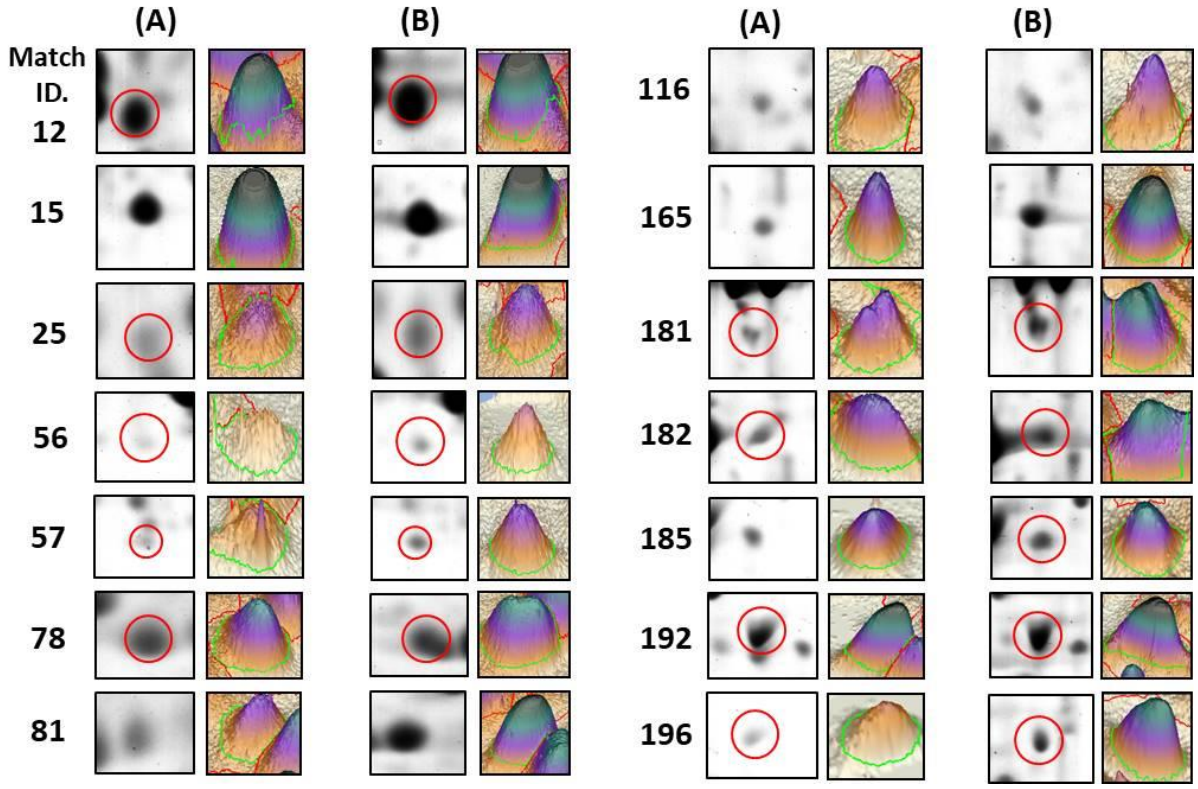


Fig. 6.5. Visualization of up-regulated midgut proteins in response to PnBBI ingestion: *S. litura* larvae fed on diet **(A)** without PnBBI and **(B)** supplemented with PnBBI (0.025%). The proteins which are up-regulated by >1.5-fold in the gel (**Fig. 6.4)** were shown in their corresponding 3D view. Match IDs represent for both panel A and panel B.

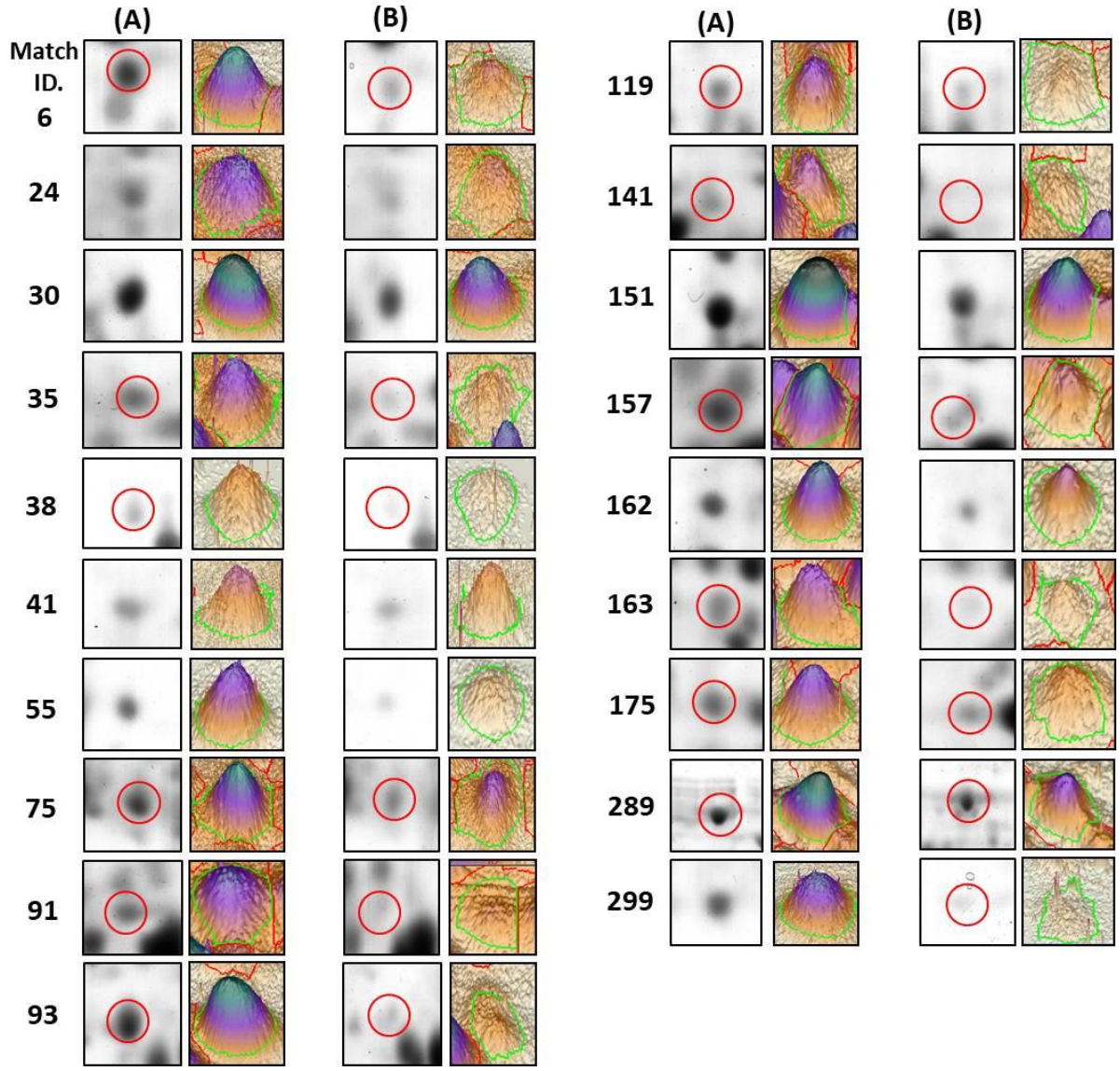


Fig. 6.6. Visualization of down-regulated midgut proteins in response to PnBBI ingestion: *S. litura* larvae fed on diet **(A)** without PnBBI and **(B)** supplemented with PnBBI (0.025%). The proteins which are down-regulated by >1.5-fold in the gel (**Fig. 6.4)** were shown in their corresponding 3D view. Match IDs represent for both panel A and panel B.

6.3. Discussion

The polyphagous insect pest *S. litura* has huge economic importance due to their enormous host range, high reproductive capacity, high mobility and adaptability (Jongsma et al., 1995; de Oliveira et al., 2013; Fand et al., 2015). Therefore, it is essential to explore new insecticidal molecules, particularly from botanical sources paving the way for sustainable pest management. The interspecific hybrid varieties of peanut are identified to possess resistant traits against microbial pathogens and insect pests (Mallikarjuna et al., 2011). However, the biochemical basis for such resistance in these hybrid varieties has not been identified so far. Since, several PIs including BBI and KI are known to possess insecticidal potential, the PnBBI purified in the present study (Chapter 4) is examined for its potential to control the growth of *S. litura* larvae in the current chapter (McManus et al., 1999; Bhattacharyya et al., 2007; Chen et al., 2014; Katoch et al., 2015; Vasudev and Sohal, 2019).

6.3.1. Inhibitory potential of PnBBI towards SlTP and SlGP

The *in vitro* studies ascertained 'PnBBI' as a potential bioinsecticide against *S. litura* based on the following characteristics: (i) IC_{50} of 149 ng; (ii) Specific activity of 6,711 SlTPI units/mg proteins (*S. litura* midgut trypsin-like protease inhibitor) and (iii) Resistance to hydrolysis by SlGPs. The maximum inhibition (80%) in SlTP activity shown by PnBBI is comparable with rice bean (*Vigna umbellata*) trypsin inhibitor (Katoch et al., 2015). Also, the SlTPI activity of PnBBI was determined to be high as compared to other TIs isolated from *Vigna mungo* (Prasad et al., 2010a), *Phaseolus vulgaris* (Mittal et al., 2014) and *Archidendron ellipticum* (Bhattacharyya et al., 2006). However, PnBBI was found to have less (3.72-fold) SlTPI activity as compared to its HaTPI activity (25,000 HaTPI units/mg protein), (**Chapter 5; Fig. 6.1**).

6.3.2. Alteration in SlTPs sensitivity towards ingested PnBBI

In contrast to the significant inhibitory potential of PnBBI towards SlTP during *in vitro* assays, the *in vivo* antifeedant effect of PnBBI on *S. litura* larval growth was marginal (<17%) (**Fig. 6.2B**). However, the larvae reared on 0.025% PnBBI which is equivalent to 1,666 SlTPI units/g diet substantially altered the midgut serine protease complement and the activities of SlTP as well as SlCP as compared to larvae fed on a normal diet (**Fig. 6.3**). The biochemical changes in larval midgut were associated with >30% elevation in SlTP activity with a subsequent decline in SlCP activity by ~10% (**Fig. 6.3A**). Such insect counter-responses were also observed in cowpea bruchids when exposed to soybean cystatin N (Zhu-Salzman et al., 2003), *S. litura* reared on AeTI from *A. ellipticum* (Bhattacharyya et al., 2007), *Anagasta kuehniella* fed on AcKI from *Acacia polyphylla* (Machado et al., 2013b) and *S. frugiperda* fed on EATI/SPI from *Entada acaciifolia/Glycine max* (Brioschi et al., 2007; de Oliveira et al., 2013). This kind of feedback response was suggested to be a part of insect adaptation mechanism to compensate the declined midgut protease activity by dietary PI and their defense (Jongsma et al., 1995; Macedo et al., 2015). Despite the plasticity in midgut protease expression, insects are also known to produce new class of PI degrading proteases and PI-insensitive proteases to escape the deleterious consequences of dietary PI (Girard et al., 1998; Zhu-Salzman et al., 2003; Ahn et al., 2007; Dunse et al., 2010a).

Further, comparative SlGP profile showed remarkable changes correlating with altered SlTP and SlCP activities (**Fig. 6.3A,B**). However, the newly emerged protease band 6 could be responsible for the increased SlTP activity and this protease band apparently seems to be insensitive to the ingested PnBBI. Also, the concomitant diminution in protease band 2 substantiates for decreased SlCP activity (**Fig. 6.3A,B**). This was also further evident by *in vitro* inhibition studies at a wide range of PnBBI concentrations. The maximum inhibition in SlTP

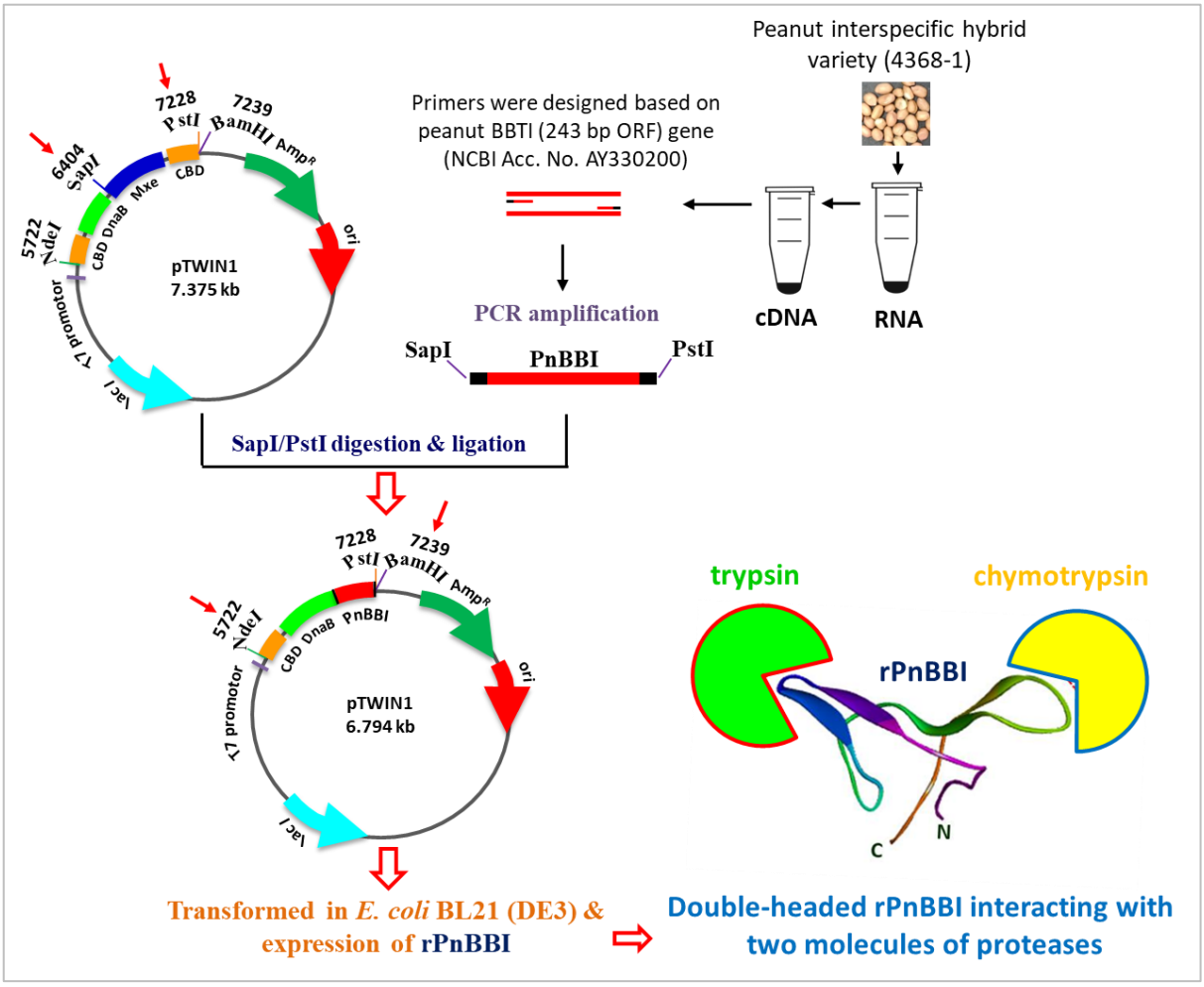
activity was found to be 80% in larvae fed on PnBBI-free diet, while it was reduced to 50% after exposure to PnBBI (**Fig. 6.3C,D**). Consequently, a ~10-fold increase in IC_{50} value of PnBBI towards SlTPs associated with acquired (~30%) insensitive SlTP activity suggests that *S. litura* has developed compensatory adaptive mechanism upon feeding with PnBBI (**Fig. 6.3**). In fact, synthesis of alternative PI-insensitive proteases was found to be derived from multiple gene families which help them to survive on a wide range of host plants (Bown et al., 1997; Broadway, 1997). Nevertheless, few amino acid substitutions in existing protease structures could alter them into insensitive to dietary PIs (Lopes et al., 2004; Dunse et al., 2010a). Therefore, identification of these differentially expressed midgut proteins might unveil the mechanism of molecular resistance to PnBBI (**Figs. 6.4; 6.5; 6.6;** Huang et al., 2004; Meng et al., 20110).

Highlights

- ✓ PnBBI act as a potent inhibitor of SlTPs with IC_{50} of 149 ng.
- ✓ Ingestion of PnBBI delayed the *S. litura* larval growth but not to a significant extent at chosen concentration.
- ✓ The significant biochemical changes are ~30% increase in SlTP activity and ~10% decrease in SlCP activity.
- ✓ The larval counter defense was mediated through induction of ~30% insensitive SlTP activity.
- ✓ As a result, the IC_{50} of PnBBI towards SlTPs was increased by 10-fold and showed resistance to PnBBI.
- ✓ PnBBI altered midgut proteome of *S. litura* significantly as evidented by 2-DE.

Chapter 7

Cloning and expression of recombinant PnBBI isoinhibitor and evaluation of its biochemical properties



Cloning and expression of recombinant PnBBI isoinhibitor and evaluation of its biochemical properties

7.1. Introduction

The dicotyledonous Bowman-Birk inhibitors (BBIs) are double-headed proteinaceous molecules abundant in legumes and cereal grains. They are compact, small (6-9 kDa) and possess two inhibitory domains protruding in opposite directions from a single molecule (Birk, 1985). They can interact with two molecules of proteases independently and form a stable complex by Laskowski's standard mechanism of protease inhibition (Laskowski and Qasim, 2000). These globular protein structures contain seven disulfide bonds which stabilizes the overall structure of inhibitor even at wide range of pH and temperature (Mello et al., 2003; Prasad et al., 2010b; Joshi et al., 2013; Swathi et al., 2014; He et al., 2017; Mohanraj et al., 2019). BBIs adopt the canonical disulfide-linked reactive site loop structure due to presence of cis-Pro at P3' position reversing an antiparallel β-strand which existed in all known BBI structures (Bode and Huber, 1992; Qi et al., 2005).

The BBI molecules are known to play a significant role in maintaining human health through its ability to inhibit serine proteases involved in various pathophysiological conditions (Turk, 2006). The biomedical applications of BBIs include anticancer (Kennedy, 1993; Losso, 2008; Clemente and Arques, 2014; Joanitti et al., 2018), anti-inflammatory (Safavi and Rostami, 2012; Srikanth and Chen, 2016; Bortolozzo et al., 2018), anti-HIV (Fang and Ng, 2015; Ma et al., 2018), immunomodulatory functions (Pramanik et al., 2019), promote gastrointestinal health (Clemente et al., 2011; Vergnolle, 2016) and cardioprotective role (Hojima, 1980; Oliva et al., 2000; Nakahata et al., 2011; Borodin et al., 2013; Brito et al., 2014; Salu et al., 2018). Nevertheless, soybean BBI concentrate has attained investigational new drug status by USFDA (Armstrong et al., 2000; Malkowicz et al., 2003) and clinical trials are underway at phase IIb trial for oral leukoplakia (Armstrong et al., 2013; Clemente and Arques, 2014). BBI/BBIC from soybean is also under investigation against multiple sclerosis encephalomyelitis (Gran et al., 2006;

Touil et al., 2008), prostatic hyperplasia (Malkowicz et al., 2001; Kennedy et al., 2002) and ulcerative colitis (Lichtenstein et al., 2008). Despite the pharmacological benefits, BBIs also have insecticidal potential against various economically important insect pests (**Chapter 5**; Haq et al., 2004; Jamal et al., 2013; Macedo et al., 2015; Clemente et al., 2019).

The natural plant sources contain mixed varieties of PIs and their isoinhibitors (Giri et al., 2003). However, the separation of these isoforms by chromatography techniques was found to be challenging since they are similar in their size and structure, and slightly vary in few amino acid residues (Norioka et al., 1982; Mohanraj et al., 2019). In most of the studies, PIs have been characterized in the form of isoinhibitors pool and/or mixed type of inhibitors. Despite the various applications of BBIs, production of a single molecular species by rDNA technology and its application in agriculture/pharmacology is scanty. Moreover, many of the studies reported that production of recombinant BBI requires an additional step of refolding to attain its function (Flecker, 1987; Li et al., 2004; Vogtentanz et al., 2007). Nevertheless, a very few studies successfully produced fully functional recombinant BBIs (Bijola et al., 1994; Muricken and Gowda, 2010; Kumar and Gowda, 2013b; Katoch et al., 2015; Indarte et al., 2017; Mohanraj et al., 2018). Hence, the present study is aimed towards the production of recombinant BBI from peanut using pTWIN1 expression system in *E. coli* BL21 (DE3) strain.

A cDNA clone (AY330200.1) of Bowman-Birk trypsin inhibitor (BBTI) isolated from peanut seed cDNA library contained 243 bp ORF which encoded for 80 amino acids mature protein with Met at its N-terminal end (Boateng et al., 2005). The deduced amino acid sequence of BBTI is identical to isoinhibitors of *A. hypogaea* BBI: A-II, A-I, B-I and B-III (Norioka et al., 1982; Norioka and Ikenaka, 1983). The trypsin inhibitors isolated from different peanut varieties displayed anti-carcinogenic (Ahmad et al., 2019) and satietogenic action (Serquiz et al., 2016). Further, peanut BBI and KI genes are known to be involved in biotic (Müller et al., 2016) and

abiotic stress tolerance (Drame et al., 2013). Therefore, structural and functional characterization of recombinant peanut BBI is essential for its agricultural and therapeutic applications.

7.2. Results

7.2.1. Primary structure analysis of peanut BBTI

The protein sequence of peanut BBTI (AAP93913.1) showed similarity with other known legume BBI in their conserved cysteine residues framework **(Fig. 7.1)**. However, among two reactive sites, the N-terminal reactive site loop contained eleven residues "CDRRAPPYFEC" unlike to other legumes BBI which have nine amino acid residues. In this reactive site loop, Phe and Glu which are present at 29^{th} and 30^{th} position of 80 amino acid sequence are additional residues as compared to other legumes BBI. Further, peanut BBTI also possessed unique Arg-Arg residues at P_1,-P_1' position while most of the legume BBIs contain Arg-Ser/Lys-Ser. However, the C-terminal reactive site loop is composed of nine residues "CTRSNPPQC" which are similar in BBIs from other plant sources. These interesting characteristic features of peanut BBTI prompted us for cloning and production of recombinant BBI protein (PnBBI) from peanut interspecific hybrid variety (4368-1) for further biochemical characterisation.

7.2.2. RNA isolation and cloning of peanut BBI isoinhibitor gene (*PnBBI*) from 4368-1

The complete gene sequence of BBTI (AY330200.1) composed of 430 bp was subjected to in-silico translation by using ExPASy-translate tool where a CDS of 240 bp encoding for 80 amino acids mature peptide was revealed (**Fig. 7.2A**). RNA extracted from mature peanut (4368-1) seeds was intact and pure with a 260/280 ratio of 2.13 and 260/280 ratio 1.93 (**Fig. 7.2B**). The ORF containing 243 bases was amplified using gene-specific primers and the amplicon showed expected molecular size on an agarose gel electrophoresis (**Fig. 7.2C**). The purified gene product and vector pTWIN1 were subjected to sequential digestion with Sap1 and Pst1 restriction enzymes followed by ligation in the presence of T4 DNA ligase. The transformed positive

colonies on the Amp-LB agar plate were identified by colony PCR using gene-specific primers followed by restriction enzyme digestion (**Fig. 7.2D**).

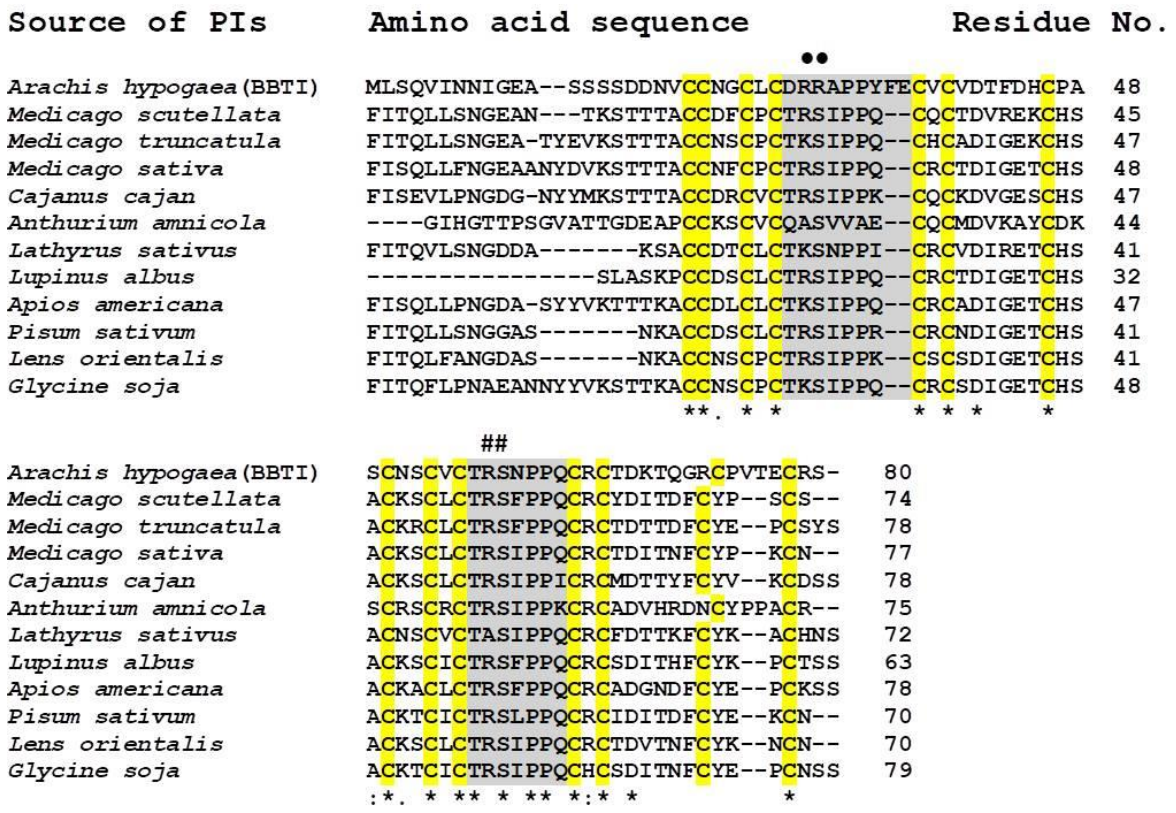


Fig. 7.1. Multiple sequence alignment of *A. hypogaea* Bowman-Birk inhibitor (BBTI) (GenBank protein ID: AAP93913.1) with other legume BBIs from NCBI-BLASTp search. Conserved cysteine residue framework was highlighted in yellow colour, N and C-terminal reactive site loop residues were highlighted in light grey colour. The dots and hash symbols indicate P1, P1′ amino acid residues of corresponding N and C-terminal reactive site loop. Peanut BBTI showed similarity with other BBIs from *M. scutellata* (AAQ10729.1), *M. truncatula* (XP_003623947.1), *M. sativa* (CAA56253.1), *C. cajan* (KYP64429.1), *A. amnicola* (JAT63041.1), *L. sativus* (ADV40040.1), *L. albus* (P85172.1), *A. Americana* (BAF50740.1), *P. sativum* (CAC24564.1), *L. orientalis* (CAH04448.1) and *G. soja* (KHM99387.1). Identical residues are indicated by asterisks '*' and conserved residues are indicated by a colon '**:**'.

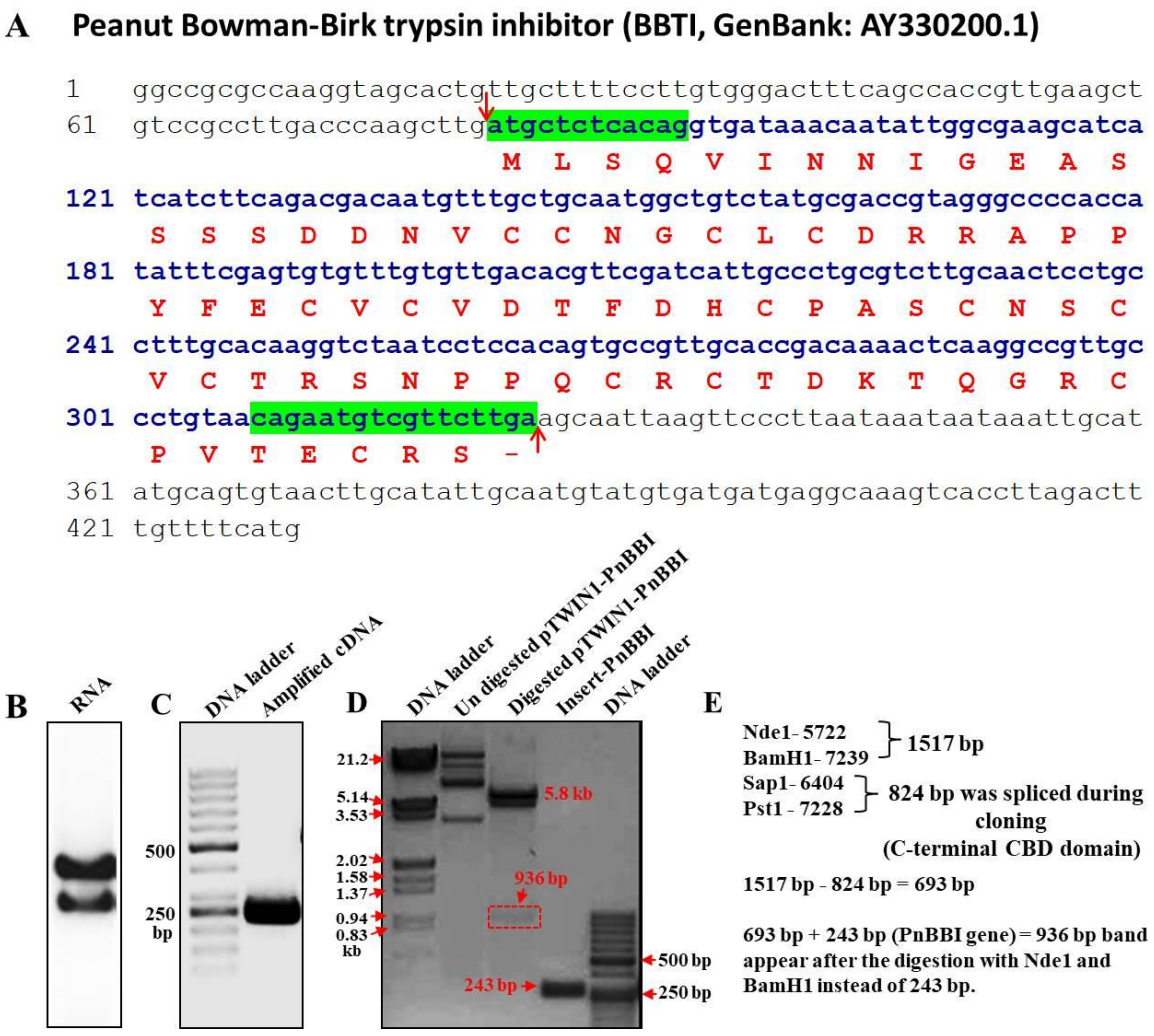


Fig. 7.2. Cloning of peanut BBI (PnBBI) gene from interspecific hybrid variety (4368-1): (**A**) *In silico* translation of BBTI gene (GenBank Accession no. AY330200.1) representing 240 bp ORF encoded 80 amino acid polypeptide (indicated by red color arrows); (**B**) total RNA isolated from interspecific hybrid variety (4368-1) of peanut was resolved on agarose gel; (**C**) cDNA amplification of 4368-1 with peanut BBTI gene-specific primers including SapI and PstI cloning sites; (**D**) restriction digestion of recombinant construct with alternative NdeI and BamHI and (**E**) technical clarification for liberation of bigger size fragment (936 bp) which include gene of interest. Both forward and reverse primer sequences are marked with green color.

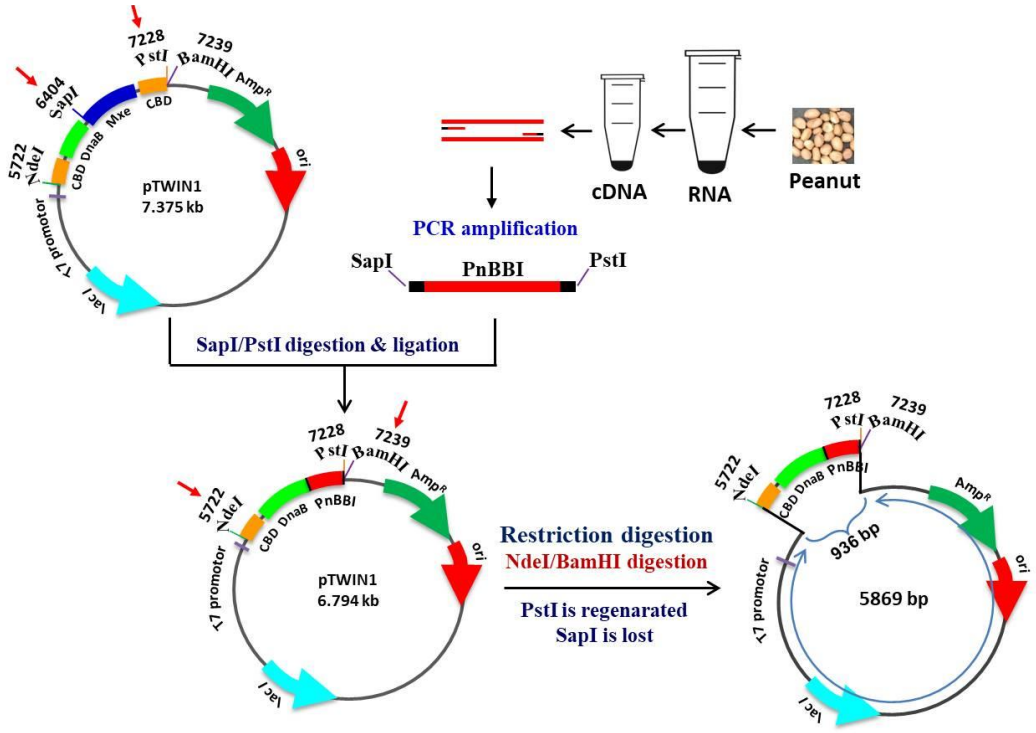


Fig. 7.3. Pictorial representation of PnBBI gene cloning and its confirmation: The vector pTWIN1 and amplified peanut BBI gene were digested with SapI and PstI enzymes followed by ligation. The clone was confirmed by restriction digestion using NdeI/BamHI. PnBBI gene was cloned beside C-terminal end of the CBD-DnaB intein fusion protein (225 amino acids encoded by 675 bases) which has a self-cleavage property in response to change in pH.

However, the SapI site was lost after cloning and the resulting Nde1 and BamH1 digestion liberates bigger size DNA fragment (936 bp) from recombinant construct instead of the original size of the insert (240 bp) (**Figs. 7.2D**). The schematic representation of PnBBI gene cloning and its confirmation was illustrated (**Fig. 7.3**). Further, the dideoxynucleotide sequencing of the recombinant plasmid with Ssp DnaB-intein I vector-specific forward primer and gene-specific reverse primers resulted in 100% homology with *A. hypogaea* Bowman-Birk trypsin inhibitor gene sequence except for single base variation in the stop codon (**Fig. 7.4**).

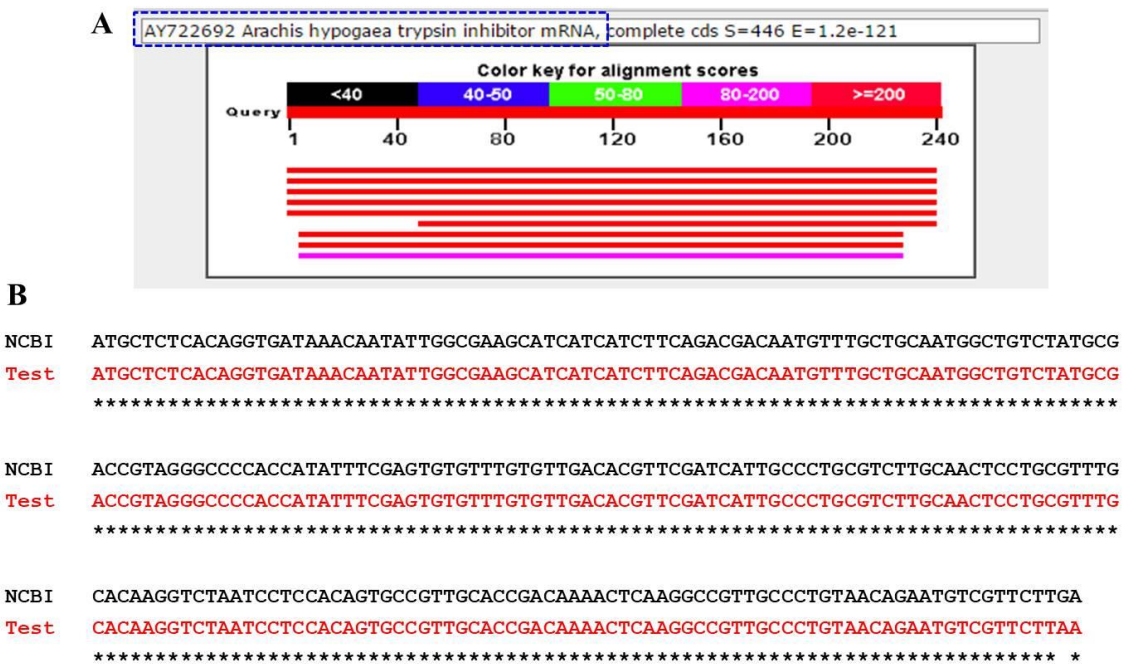


Fig. 7.4. Sanger's sequencing result of recombinant construct CBD-DnaB-PnBBI: (**A**) Nucleotide Blast search result identified as peanut trypsin inhibitor gene and (**B**) multiple sequence alignment of test result and existing BBTI gene sequence from NCBI database was found to be identical except single nucleotide base change in the stop codon.

7.2.3. Expression and purification of recombinant PnBBI

The *E. Coli* BL21 (DE3) strain containing recombinant plasmid (CBD-DnaB-PnBBI), expressed rPnBBI in the soluble form under the following optimal conditions: 0.6 mM IPTG, induction at > 0.8 OD and 16 °C. Initially, the clarified cell lysate obtained after sonication and centrifugation was subjected to purification using chitin beads. The following process resulted in reduced yield due to premature cleavage of fusion partner (CDB-DnaB intein) and the cleaved rPnBBI was found in unbound protein fraction (flow-through). These observations directed towards an alternative trypsin affinity column which have the ability to capture both fusion protein and free rPnBBI which is cleaved from its fusion partner (**Fig. 7.5A**). The elution fractions of trypsin affinity column possessing trypsin inhibitor (TI) activity were pooled, dialysed against 50 mM Tris-HCl, pH 8.0 and concentrated using a freeze-dryer (**Fig. 7.5B**). The process of trypsin affinity

elution (0.01 N HCl, pH 2.5) resulted in pH-induced self-cleavage of CBD-DnaB intein which is co-eluted with rPnBBI. Further, CBD-DnaB intein was eliminated from rPnBBI by passing through size exclusion chromatography column (**Fig. 7.5C**).

7.2.4. Determination of molecular mass of rPnBBI and its in-gel activity

The various fractions of recombinant protein expression and purification separated on SDS-PAGE indicated that rPnBBI is in the soluble fraction. However, fractions of vector control and un-induced cell lysate did not show its expression. The induced cell lysate fraction possessed two forms of a recombinant inhibitor such as rPnBBI with fusion partner CBD-DnaB intein and cleaved rPnBBI. CBD-rPnBBI fusion protein showed a molecular mass of 33 kDa whereas, cleaved rPnBBI migrated at ~10 kDa (**Fig. 7.6A**). However, the purified rPnBBI showed a molecular mass close to soybean BBI (7.9 kDa). Nevertheless, MALDI-TOF intact mass analysis of rPnBBI revealed its precise molecular mass as 7.53 kDa, which is considered for further study (**Fig. 7.6B**). The gelatin SDS-PAGE in-gel inhibitory activity studies of various fractions of recombinant protein expression and purification revealed that rPnBBI is resistant to bovine pancreatic trypsin and chymotrypsin digestion, respectively (**Fig. 7.7A,B**). During the process of protease digestion, the place where PI is located in the gel was protected and stained along with undigested gelatin against a transparent background. The fraction of induced cell lysate showed two active inhibitory bands. The upper band represent the fusion protein (CBD-DnaB-rPnBBI) and lower band represent the rPnBBI which is formed possibly due to pre-mature cleavage of a fusion protein (**Fig. 7.7A,B**).

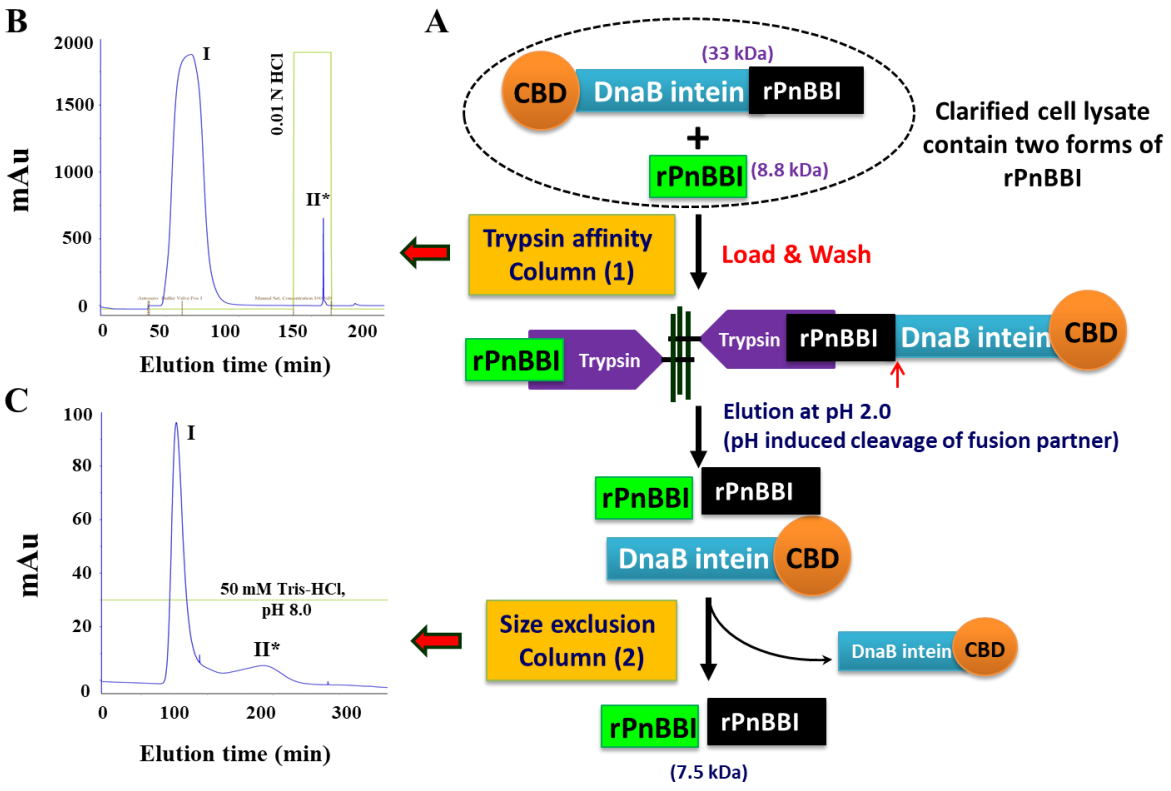


Fig. 7.5. Purification of recombinant PnBBI: (**A**) chromatogram of CNBr-Sepharose trypsin affinity column loaded with clarified cell lysate; (**B**) chromatogram of gel filtration column and (**C**) illustration of rPnBBI purification scheme and removal of its fusion partner CBD-DnaB intein which has pH-induced self-cleavage activity (indicated by arrow). In both chromatograms peak II exhibited trypsin inhibitor (TI) activity (indicated by an asterisk).

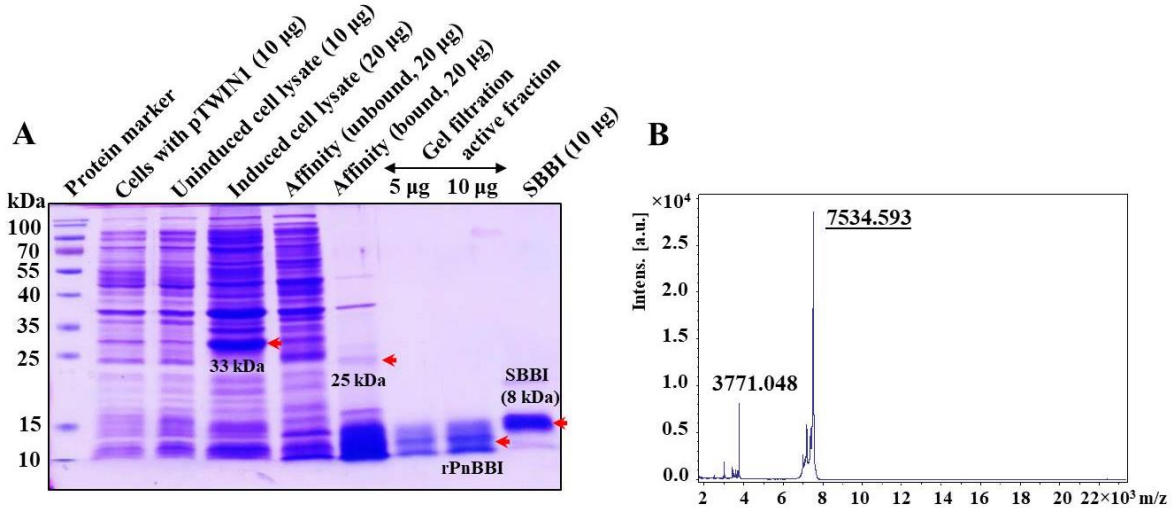


Fig. 7.6. Purification profile of rPnBBI and MALDI-TOF analysis: **(A)** SDS-PAGE (15%) showing the induced cell lysate containing 33 kDa fusion protein (CBD-DnaB-rPnBBI). The bound fraction of trypsin affinity column contained rPnBBI, CBD-DnaB fusion partner (25 kDa) and high molecular weight contaminants. Fraction from gel filtration column showed pure rPnBBI. Soybean BBI (7.9 kDa) is used as a reference to eliminate the discrepancy, since PIs are known to be migrated slowly as compared to standard molecular weight marker proteins and **(B)** MALDI-TOF intact mass analysis of purified rPnBBI showed a peak at m/z 7534.593.

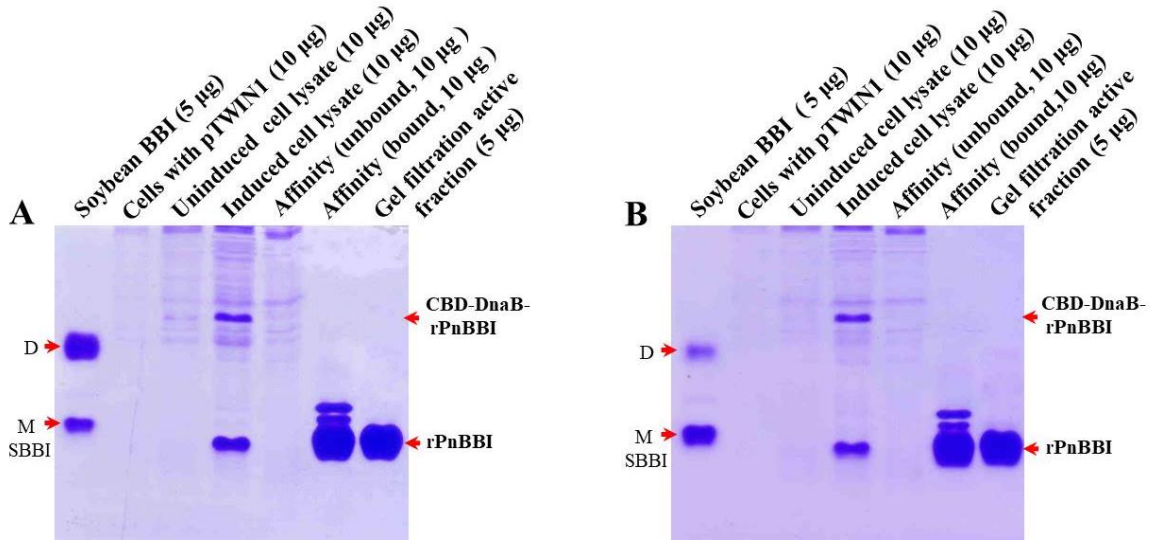


Fig. 7.7. Gelatin SDS-PAGE in-gel activity assay: The rPnBBI present in different fractions of protein expression and purification is active against bovine (**A**) trypsin and (**B**) chymotrypsin digestion. The clarified cell lysate contains two forms of rPnBBI: (i) higher inhibitory band represent fusion protein (CBD-DnaB-rPnBBI) and (ii) lower band represent rPnBBI which is liberated by pre-mature auto cleavage of a fusion protein. SBBI is used as a standard for in-gel activity staining.

7.2.5. Inhibitory potential of rPnBBI

rPnBBI showed differential inhibitory activity towards bovine trypsin and chymotrypsin with specific inhibitory activity of 520 TI and 26 CI units/mg protein. However, the inhibitory potential of rPnBBI was found to be half as compared to native Bowman-Birk isoinhibitors pool (**Fig. 7.8A**). Further, SPR kinetic analysis revealed the association and dissociation characteristics of bovine trypsin and chymotrypsin with immobilized rPnBBI. The analyte trypsin showed stable biomolecular interactions with an association rate constant of Ka 3.29×10^{6} $M^{-1}s^{-1}$ and a dissociation rate constant of Kd 2.93×10^{-4} s^{-1}. Similarly, chymotrypsin exhibited strong interaction with Ka 4.65×10^{6} $M^{-1}s^{-1}$ and Kd 3.56×10^{-4} s^{-1}. These results infer that complex formation with both trypsin and chymotrypsin involved fast association and slow dissociation rate. Also, the present study emphasize that PnBBI form a highly stable complex with both trypsin and chymotrypsin as indicated by their K_D 8.92×10^{-11} M and K_D 7.65×10^{-11} M, respectively (**Fig. 7.8B,C**).

7.2.6. Structural and functional stability of rPnBBI

Far-UV CD spectra of rPnBBI revealed its secondary structure components in the following proportion: 47% β-sheets, 44% random coil and 9% α-helix. Also, the overlapping spectra of native PnBBI and rPnBBI did not show any significant deviation (**Fig. 7.9A**). Further, incubation of rPnBBI at a wide range of pH and temperature did not show substantial changes in the ellipticity (**Fig. 7.9B,C**). Similarly, the TI activity of rPnBBI was stable throughout the acidic to basic pH range (**Fig. 7.9D)**. But, in case of temperature treatment <6% TI activity was reduced at 50 ºC and it is further extended to a loss of 25% at 90 ºC (**Fig. 7.9E**).

7.2.7. Molecular modelling and phylogenetic analysis

Three-dimensional structure of rPnBBI was built by Modeller 9.19 using *Medicago scutellata* BBI (PDB ID: 1MVZ) as a model template and visualised by Pymol (**Fig. 7.10**). The 3-D structure of

rPnBBI revealed the presence of two non-identical reactive site loops in opposite directions. The N-terminal reactive site loop consists of eleven amino acid residues "CDRRAPPYFEC", whereas the C-terminal loop contains nine amino acids "CTRSNPPQC". Both reactive site loops were stabilized by disulfide bonds in which P_1, P_1' amino acids in the N- and C-terminal inhibitory loops are Arg-Arg and Arg-Ser, respectively.

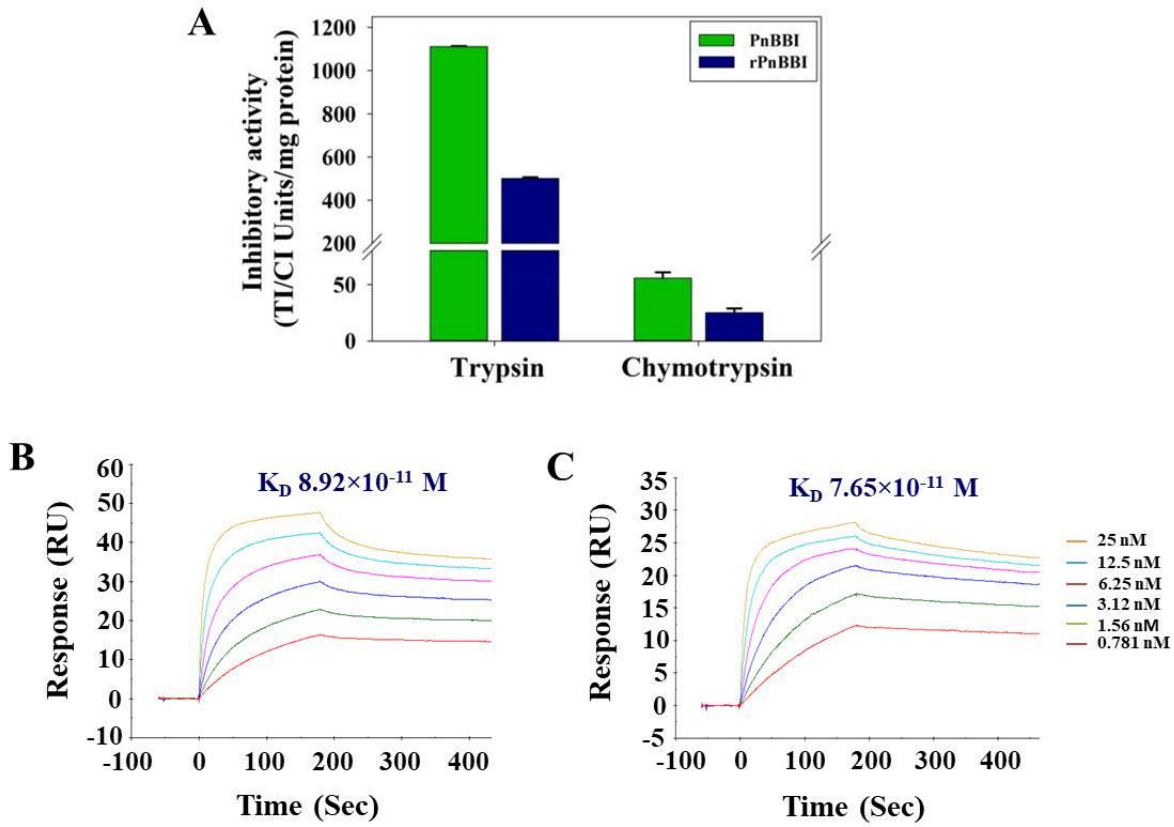


Fig. 7.8. Inhibitory potential of rPnBBI and SPR kinetic analysis: (**A**) Comparative inhibitory activity of PnBBI and rPnBBI against bovine trypsin and chymotrypsin. SPR sensorgrams showing biomolecular interactions of immobilized rPnBBI with analytes bovine (**B**) trypsin and (**C**) chymotrypsin. The different concentrations (0.78, 1.56, 3.12, 6.25, 12.5 and 25 nM) of analyte (trypsin/chymotrypsin) were prepared in 100 mM HEPES buffer and passed over the surface of rPnBBI thrice following regeneration. The obtained data was analysed by Langmuir fit model of 1:1 binding by using BIAevaluation 4.0 software (GE Healthcare, Life Sciences).

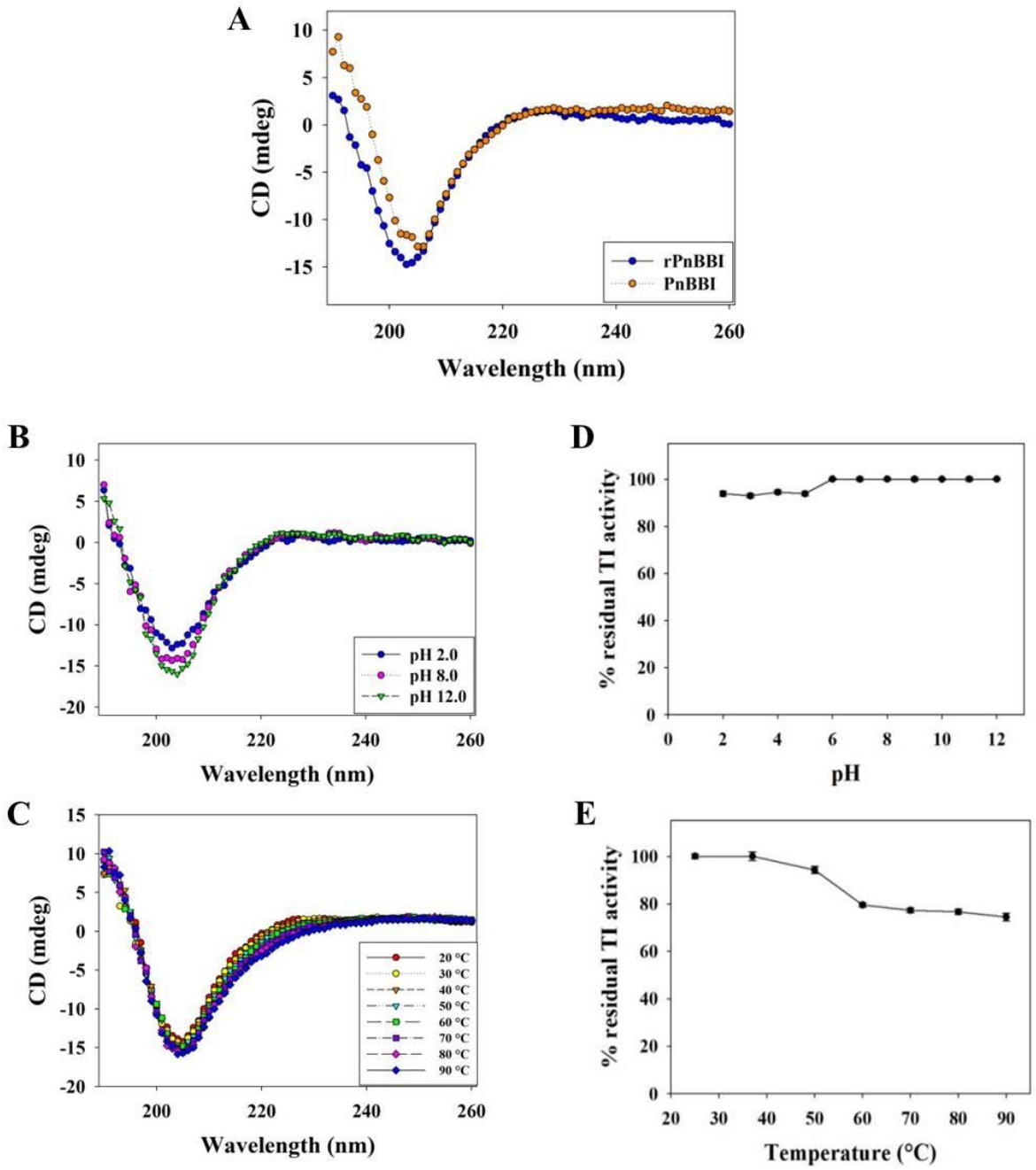


Fig. 7.9. Structural and functional stability of rPnBBI: (A) A comparative Far-UV (190-260 nm) CD spectra of PnBBI and rPnBBI. Effect of **(B)** pH and **(C)** temperature on far-UV CD spectra of rPnBBI. The TI activity of rPnBBI at a wide range of **(D)** pH and **(E)** temperature. The solution containing rPnBBI was incubated at respective conditions before recording CD spectra or performing *in vitro* TI assay. Further details are furnished in materials and methods (**Chapter 3**).

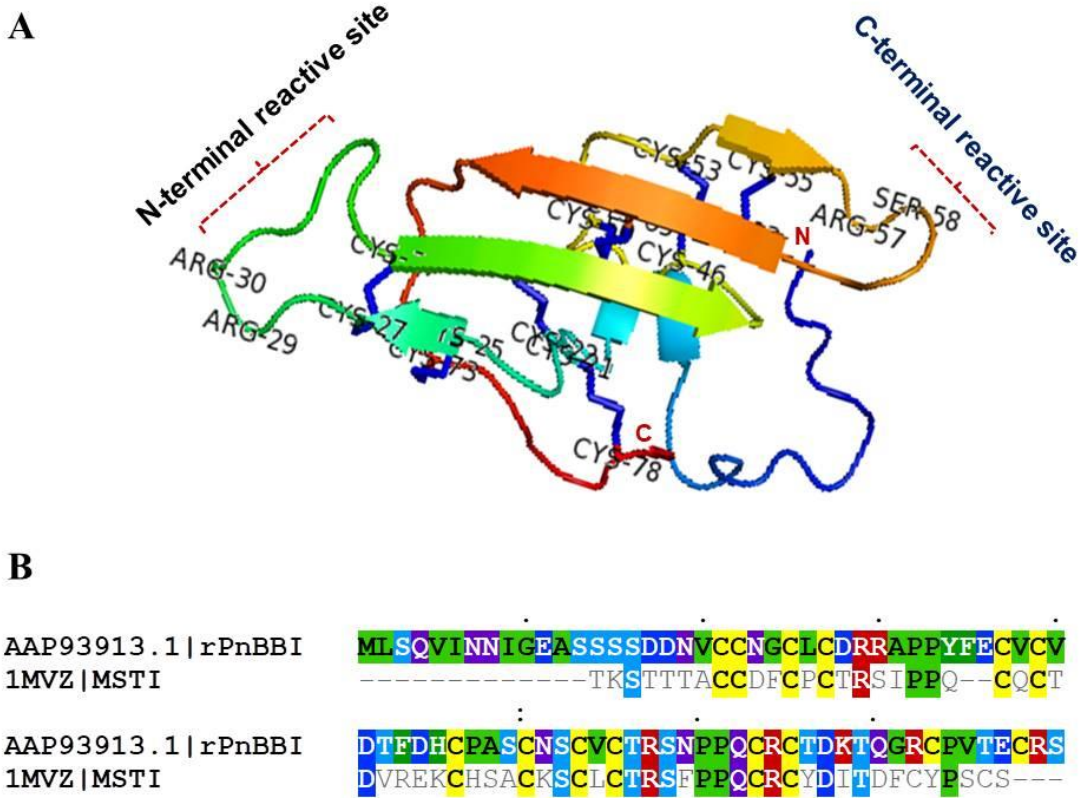


Fig. 7.10. Molecular modelling of rPnBBI: (A) Predicted three-dimensional structure of rPnBBI based on the structure of *Medicago scutellata* trypsin inhibitor (MSTI) as a template using Modeller 9.19 python scripts. The model was validated by generating a Ramachandran plot using PDBsumgenerate (http://www.ebi.ac.uk/thorntonsrv/databases/pdbsum/Generate.html). Three-dimensional structure of rPnBBI displayed seven disulfide bonds (highlighted in blue colour) with exposed N-terminal and C-terminal inhibitory loop structures and **(B)** Amino acid sequence alignment of rPnBBI with MSTI and their consensus shown in different colours.

The phylogenetic tree revealed the evolutionary relationship of peanut BBI with other legume and cereal BBIs (**Fig. 7.11**). PnBBI was placed along with dicotyledonous *Medicago* species and monocotyledonous *Coix lacryma* at one end while grouped with dicotyledonous *Lathyrus sativus* and *Pisum sativus* at other end of the phylogenetic tree. However, PnBBI is an ancestral species of many other legumes and classical soybean BBI which is generated from ancestral internal nodes of *Arachis* species. The root of the tree and its ancestral branch emerged

into monocots and dicots in which *Medicago* species have a close evolutionary relationship with monocots followed by *Arachis* species. Further, dicot and monocot BBIs are highly conserved during evolution, though they are broadly distributed among the leguminosae family.

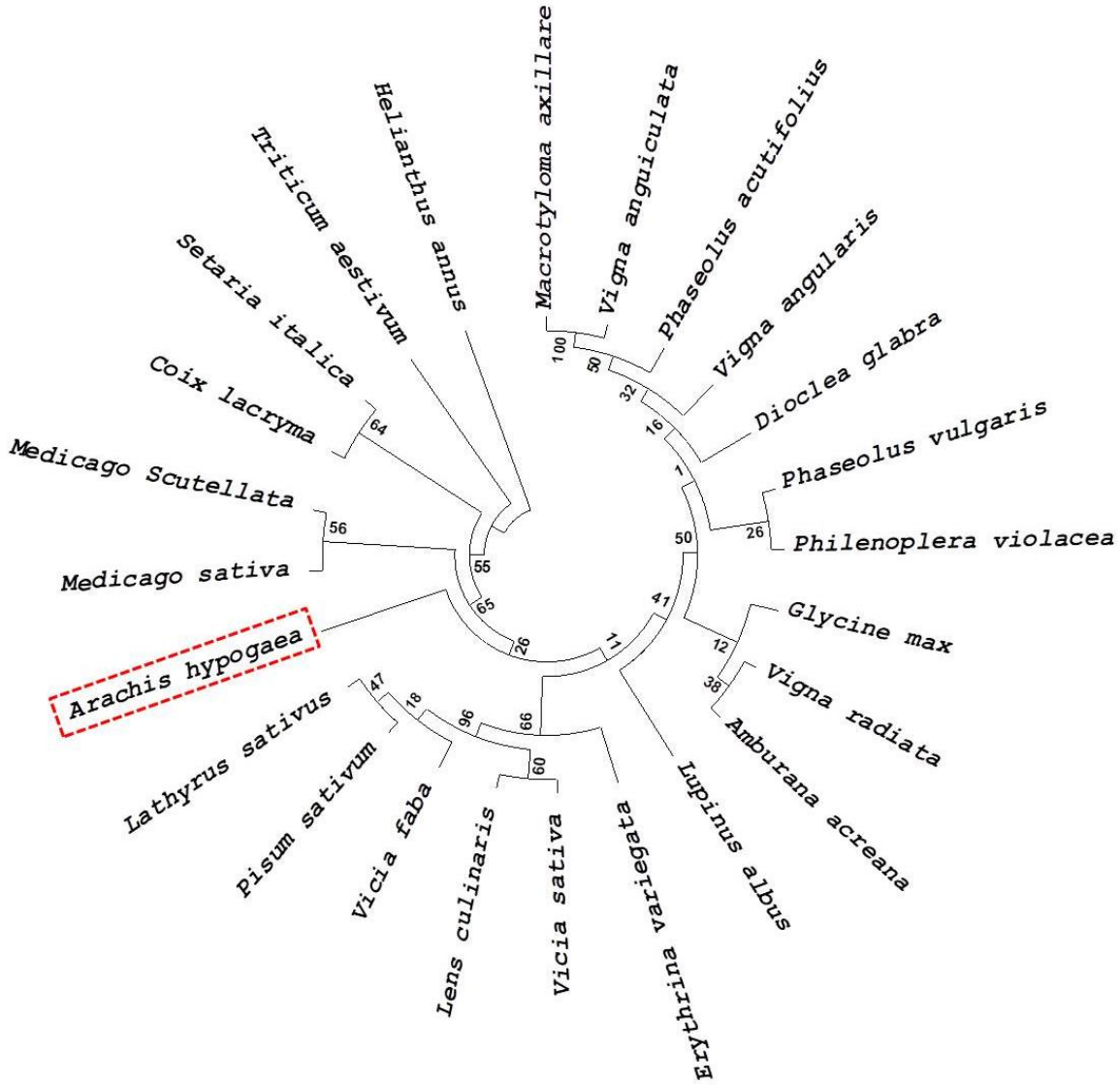


Fig. 7.11. Phylogenetic relationship of rPnBBI with other reported plant BBIs: Phylogenetic analysis was performed using MEGA 6.0 software using the Neighbour-Joining algorithm with a bootstrap of 500. The rPnBBI protein sequence was subjected to BLASTp search and obtained homologous sequences were aligned, and further used for the construction of the phylogenetic tree. The obtained bootstrap percentage values are indicated at branch points which reveal the robustness of characteristics that support the branch node split. The location of *Arachis hypogaea* was highlighted with a dotted line box.

7.3. Discussion

The PIs purified from the seeds using trypsin affinity column (**Chapter 4**) might possess trypsin inhibitors from different families which can exaggerate the significance of the study by their synergistic biological activity. The BBI purified from interspecific hybrid variety (4368-1) of peanut contained a pool of isoinhibitors and it is challenging to separate each of the isoinhibitor from the pool to study their ideal biochemical properties (Giri et al., 2003; Mohanraj et al., 2019). Hence, the present study aimed at the production of single isoinhibitor species using rDNA technology.

7.3.1. Cloning, expression and purification of rPnBBI

The method adopted from Suzuki et al. (2004) facilitated to obtain a good quality of RNA from dry peanut which is essential for gene cloning and protein expression studies (**Fig. 7.2A**). Besides, pTWIN1 vector system enhanced the solubility of fusion protein during induction at a lower temperature of 16 °C (Diao et al., 2007; Che et al., 2009; Kumar and Gowda, 2013b). The vector 'pTWIN1' of the IMPACT^{TM} protein purification system utilizes the inducible self-cleavage property of 'intein' for easy purification (Diao et al., 2007; Esipov et al., 2008). This system allows purification of recombinant protein in its native form and avoids the risk of using proteases, and chemicals to remove the affinity purification tag (Chong et al., 1997). Though, the N-terminal coupled purification tag of CBD was specific to chitin, affinity purification of rPnBBI using chitin beads resulted in massive yield loss due to premature cleavage of fusion partner (CBD-DnaB) from rPnBBI (**Fig**. **7.7**; Kumar and Gowda, 2013b). However, the usage of trypsin Sepharose affinity column enhanced the purification yield (**Fig. 7.7**). Also, a difference in mass of about 1,320 Da was observed between theoretical (8,800 Da) and predicted mass through MALDI-TOF (7,534 Da) analysis. This could be due to cleavage of the peptide at Lys (Residue 69) while passing through affinity column during purification process.

7.3.2. Characterization of biochemical properties

The discrepancy in molecular mass of purified rPnBBI on SDS-PAGE (~10 kDa) and MALDI-TOF (7.53 kDa) analysis has arisen due to unique slow migration property of BBI family protein on PAGE which is well documented in several reports (**Fig. 7.6**, Terada et al., 1994; Swathi et al., 2014; Mohanraj et al., 2019). However, the different fractions collected during expression and purification of rPnBBI showed prominent TI and CI bands on gelatin SDS-PAGE similar to soybean BBI (**Fig. 7.7**). The rPnBBI showed differential inhibition towards bovine trypsin and chymotrypsin where it possessed ~20-fold higher TI activity as compared to its CI activity. However, the inhibitory potential of rPnBBI was found to be decreased by 50% in terms of its TI and CI activity as compared to native PnBBI (**Fig. 7.8A**). Thus, the observed discrepancy in the inhibitory potential between native PnBBI and rPnBBI could have arisen possibly due to the presence of a heterogeneous pool of Bowman-Birk isoinhibitors in native PnBBI which might differ in their inhibition capacity. The SPR kinetic studies revealed that both trypsin and chymotrypsin formed a highly stable complex with rPnBBI and native PnBBI. However, rPnBBI showed strong affinity towards these proteases as compared to native PnBBI (**Fig. 7.8B,C; Chapter 4, Fig. 4.5A,B**).

The TI activity of rPnBBI was found to be unaffected at a wide range of pH (2-12) and temperature up to 50 °C. The loss in TI activity (~25%) of rPnBBI as compared to PnBBI at 60 °C could be possibly due to cleavage of short peptide (as described above, section 7.3.1) possessing Cys13 and Cys14 residues which are involved in disulfide bond formation with Cys1 and Cys3 (**Figs. 7.2A; 7.9E; Chapter 1, Fig. 1.3**). While, the secondary structure of rPnBBI as revealed by far-UV CD spectra was found to be as stable as native PnBBI isoinhibitor pool under different pH and temperature conditions (**Fig. 7.9**; Muricken and Gowda, 2010; Mohanraj et al., 2018).

Despite the vast therapeutic application of BBIs, efficient production systems are currently limited and it is essential to produce recombinant BBI and characterize their biological activity.

Several reports on recombinant BBI production have been facing challenges in terms of solubility, protein refolding, activation, loss of function, additional residues of fusion partner etc., (Flecker, 1987; Li et al., 1999; Clemente et al., 2004; Vogtentanz et al., 2007; Muricken and Gowda, 2010). However, effective production of several recombinant BBI has been reported from *Dolichos biflorus* (Kumar and Gowda, 2013b), *Psophocarpus tetragonolobus* (Bhattacharjee et al., 2014), *Vigna umbellata* (Katoch et al., 2014), *Maclura pomifera* (Indarte et al., 2017) and *Rhynchosia sublobata* (Mohanraj et al., 2018).

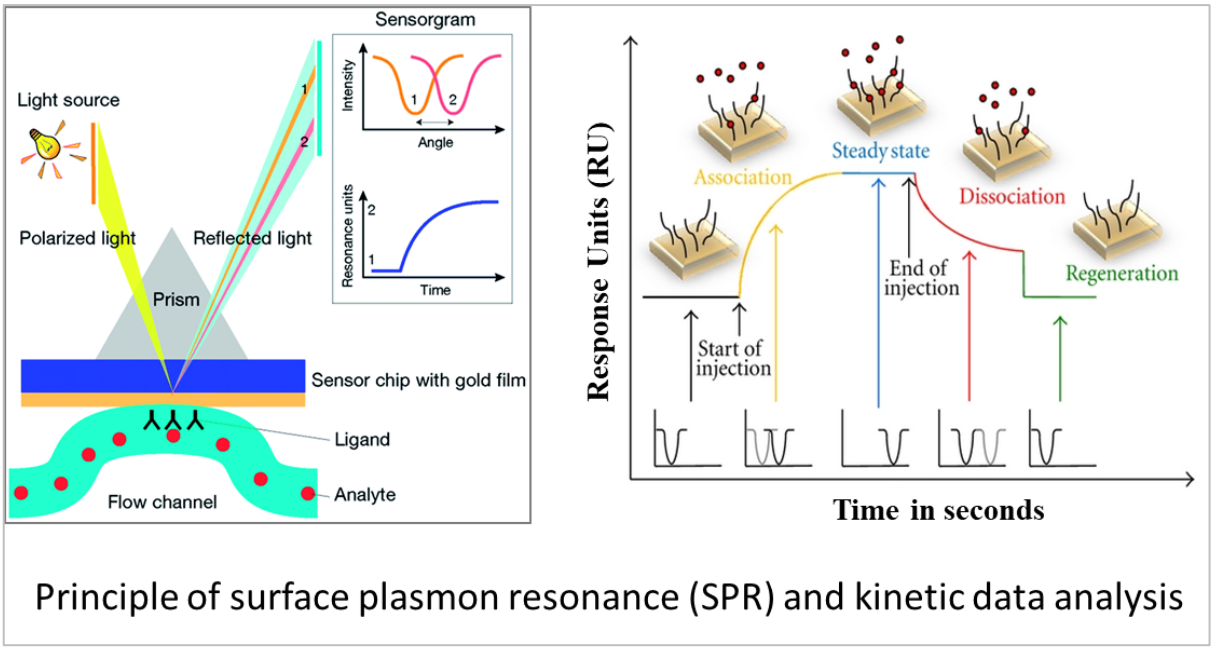
The 3-D structural analysis of rPnBBI demonstrated the presence of unique N-terminal reactive site loop with a eleven residues which is uncommon in other legumes BBIs (**Fig. 7.10**; Suzuki et al., 1987; Prakash et al., 1996; Qi et al., 2005). The eleven residues loop 'CDRRAPPYFEC' contains additional Phe-Glu residues at P5′- P6′ along with Arg-Arg at P1-P1′ position. However, the Arg29 and Arg30 at the first reactive site, and Arg57 at the second reactive site of rPnBBI attributed to its potent TI activity (Norioka and Ikenaka, 1983; Suzuki et al., 1987; Prakash et al., 1996; Mello et al., 2003). Similarly, a unique reactive loop with seven residues 'CEEESRC' was identified in MpBBI which is isolated from *Maclura pomifera* dicotyledonous plant (Indarte et al., 2017). The MpBBI polypeptide possessed Arg41 at P1 with five disulfide bonds and it is found to be a potent inhibitor of trypsin. In contrast, the second loop of conserved 'CTRMNPPQC' region was similar to other legume BBIs. The rPnBBI showed evolutionary divergence with other legume BBIs. Thus, the present study resulted in the successful production of a functional Bowman-Birk isoinhibitor 'rPnBBI' which can be further exploited in the field of agriculture and medicine.

Highlights

- ✓ Peanut Bowman-Birk inhibitor gene was successfully cloned into pTWIN1 vector.
- ✓ Maximum induction in soluble form was achieved at 0.6 mM IPTG and at 16 °C.
- ✓ Recombinant PnBBI was purified by using trypsin affinity columns instead of its fusion partner-specific chitin column.
- ✓ rPnBBI is a bifunctional inhibitor with a molecular mass of 7.53 kDa which exhibit TI and CI activity.
- ✓ A Far-UV CD spectrum of rPnBBI was found to be similar to PnBBI.
- ✓ SPR kinetics suggests that rPnBBI form a highly stable complex with both trypsin and chymotrypsin respectively.
- ✓ The structural and functional properties of rPnBBI were stable at diverse pH and temperatures (up to 50 °C).

Chapter 8

Examination of the anticoagulant properties of native PnBBI & rPnBBI: A comparative study using *in vitro* assays and surface plasmon resonance



Principle of surface plasmon resonance (SPR) and kinetic data analysis

Examination of the anticoagulant properties of native PnBBI & rPnBBI : A comparative study using *in vitro* assays and surface plasmon resonance

8.1. Introduction

The cotyledons of a peanut are rich source of proteins, edible fats and various proteinaceous (e.g. amylase inhibitors and proteinase inhibitors) and non-proteinaceous bioactive molecules such as resveratrol, phenolic acid and flavonoids to protect themselves from various diseases and pests (Stephens et al., 2010; Arya et al., 2016; Lee et al., 2018). However, bioactive molecules such as PIs from different varieties of peanut displayed both anti-carcinogenic (Ahmad et al., 2019) and satietogenic action (Serquiz et al., 2016). In general, PIs are also known to possess several therapeutic properties and promote human health (Losso, 2008; Srikanth and Chen, 2016). PIs from *Bauhinia* species are characterized well for their anticoagulant properties (Sampaio et al., 1996; Oliva et al., 1996; Nakahata et al., 2006; Oliva and Sampaio, 2009; Ferreira et al., 2013; Brito et al., 2014). Further, Kunitz inhibitors isolated from different plant sources are also known to selectively inhibit various coagulation factors (Grzesiak et al., 2000; Patil et al., 2012; Borodin et al., 2013; Machado et al., 2013a; Korneeva et al., 2014; Hamad et al., 2017; Salu et al., 2018). However, BBIs are less explored for their anticoagulant properties.

Thrombosis is a common underlying pathological phenomenon of major cardiovascular diseases including myocardial infarction, stroke and venous thromboembolism (VTE, ISTH steering committee for world thrombosis day, 2014). VTE is a multifactorial, third most common vascular disease which occurs due to the formation of a blood clot in veins and obstructs the blood flow in the body (Di Nisio et al., 2016). VTE includes deep vein thrombosis (DVT) and pulmonary embolism (PE). VTE has become a leading cause of death worldwide, while 7-14 million people are affected annually (Raskob et al., 2014) and it is more prevalent in the US and Europe population (Mahan et al., 2011; ISTH steering committee for world thrombosis day, 2018). Moreover, thrombosis is well known to be associated with inflammation, cancer, diabetes

and atherosclerosis (de Laforcade, 2012; Salu et al., 2014; Wu, 2015; Ay et al., 2017). VTE is a life-threatening condition often preventable but not as popularised as heart attack, stroke, breast cancer, prostate cancer and AIDS due to lack of awareness (Wendelboe et al., 2015; Almodaimegh et al., 2017).

Currently available VTE prophylaxis measures include use of anticoagulants [heparin, enoxaparin, warfarin and FDA approved direct oral anticoagulants (DOACs) such as apixaban, dabigatran, rivaroxaban, edoxaban] (Henry and Desai, 2010; Al-Horani and Desai, 2016; Ay et al., 2017; Joppa et al., 2018). Moreover, commercial antithrombotic drugs such as heparin and warfarin which are associated with non-specific drug-drug and drug-food interactions and bleeding complications need regular dose-adjustment, and monitoring (Hirsh and Raschke, 2004; McRae and Eikelboom, 2007; Ageno et al., 2012; Garcia et al., 2012). During recent years, the use of DOACs increased rapidly which has a better safety profile, but found to have issues of reversibility (Al-Horani and Desai, 2016; Joppa et al., 2018; Vranckx et al., 2018).

Therefore, a search for new alternative anticoagulant molecules with improved efficacy and easy availability is a major challenge in the current clinical field. Nevertheless, the *in vitro* anticoagulant effect of PIs isolated from interspecific hybrid variety of peanut has been reported earlier in the PhD thesis of Dr. Swathi Marri (2016) from our laboratory (**Fig. 8.3**). In the present study, we evaluated the (i) binding affinities of coagulation factors with the PnBBI and (ii) compared the anticoagulant potential of bacterially expressed rPnBBI with native PnBBI pool.

8.2. Results

8.2.1. Anticoagulant activity of PnBBI and rPnBBI

The inhibitory effect of PIs on blood coagulation process was assessed by prothrombin time (PT) and activated partial thromboplastin time (APTT) assay. The APTT assay revealed that both PnBBI and rPnBBI at 16 µg concentration prolonged the clotting time by 2.76 and 2.08-fold, respectively as compared to the standard blood clotting time (13 sec). However, the commercial anticoagulant 'heparin (20 IU)' prolonged the clotting time of APTT by 2.39-fold (**Fig. 8.1A,B**). In contrast, PT was not affected by these inhibitors which has standard clotting time of 32 sec (**Fig. 8.1C,D**).

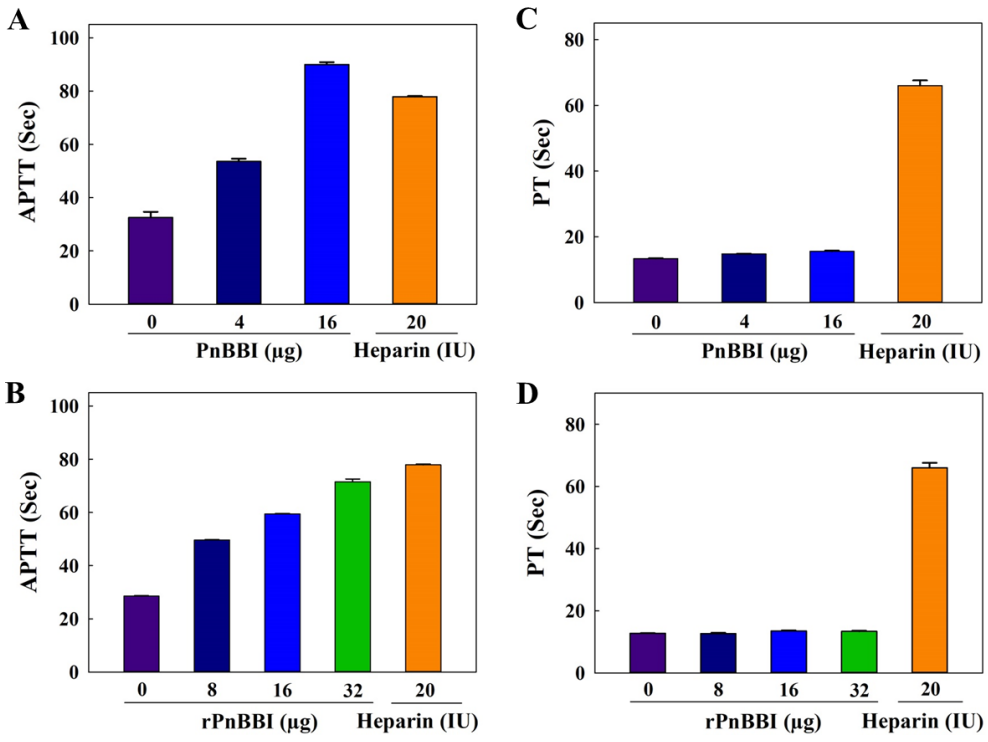


Fig. 8.1. Anticoagulant effect of PnBBI and recombinant PnBBI. Effect of PnBBI and rPnBBI on (**A and B**) APTT which clinically represents intrinsic/contact pathway of blood coagulation and (**C and D**) PT which clinically represents extrinsic/tissue factor (TF) pathway. Assays were performed by the addition of different concentrations of inhibitors to the citrated plasma and blood clotting time was determined by using coagulometer. The commercial anticoagulant heparin was used as a positive drug control. The coagulation time of controls (normal plasma) in APTT and PT pathways were 13 sec and 32 sec, respectively.

8.2.2. Inhibition of human plasma kallikrein (PK)

PK and high molecular weight kininogen (HK, non-enzymatic cofactor) plays an important role in the activation of inactive zymogen FXII to active FXIIa and initiate the intrinsic pathway of blood coagulation. Both PnBBI and rPnBBI inhibited the PK activity in a dose-dependent manner. PnBBI showed IC_{50} of ~2 µg and subsequent increase in the inhibitor concentration (up to 32 µg) resulted in the loss of PK activity by 87% (**Fig. 8.2A**). In contrast, rPnBBI exhibited IC_{50} of ~8 µg and inhibited the PK activity by 77% at 32 µg (**Fig. 8.2B**). SPR kinetics suggested that rPnBBI has higher affinity (K_D 2.49×10^{-9} M) toward PK as compared to the PnBBI (K_D 1.15×10^{-7} M) (**Fig. 8.2C,D**). In general, the strength of the biomolecular interactions typically measured by the equilibrium dissociation constant (K_D). For instance, smaller the K_D value greater the affinity between interacting partners.

8.2.3. Inhibition of FXIIa, FXIa and FIXa

Both PnBBI and rPnBBI exhibited an equivalent inhibitory potential towards FXIIa, FXIa and FIXa with IC_{50} of <0.55, <6.0 and 32 µg, respectively (**Figs. 8.3A-C; 8.5A-C**). A dose-dependent decrease in the activity of FXIIa was observed, while it was found to be declined by 89% and 82.55% at 2 µg of PnBBI and rPnBBI, respectively (**Figs. 8.3A; 8.5A**). Also, both PnBBI and rPnBBI showed a similar effect on FXIa by reducing its activity to 68% at 8 µg of protein (**Figs. 8.3B; 8.5B**). On the other hand, in the case of FIXa, approximately 50% activity was lost at 32 µg of PnBBI (or) rPnBBI (**Figs. 8.3C; 8.5C**). However, both inhibitors PnBBI and rPnBBI showed similar inhibitory tendency in the following order FXIIa > FXIa > FIXa. Further, SPR kinetic study revealed that FXIIa and FIXa formed stable complexes with PnBBI as reflected by their lower kinetic K_D 8.33×10^{-8} M and K_D 1.4×10^{-9} M in contrast to rPnBBI which showed K_D 1.99×10^{-7} M and K_D 5.22×10^{-7} M, respectively (**Figs. 8.4A,C; 8.6A,C**). Contrarily, the FXIa

showed more stable interactions with rPnBBI (K_D 7.44×10^{-9} M) as compared to PnBBI (K_D 1.82×10^{-7} M) in concomitance with their inhibitory potential (**Figs. 8.4B; 8.6B; Table 8.1**).

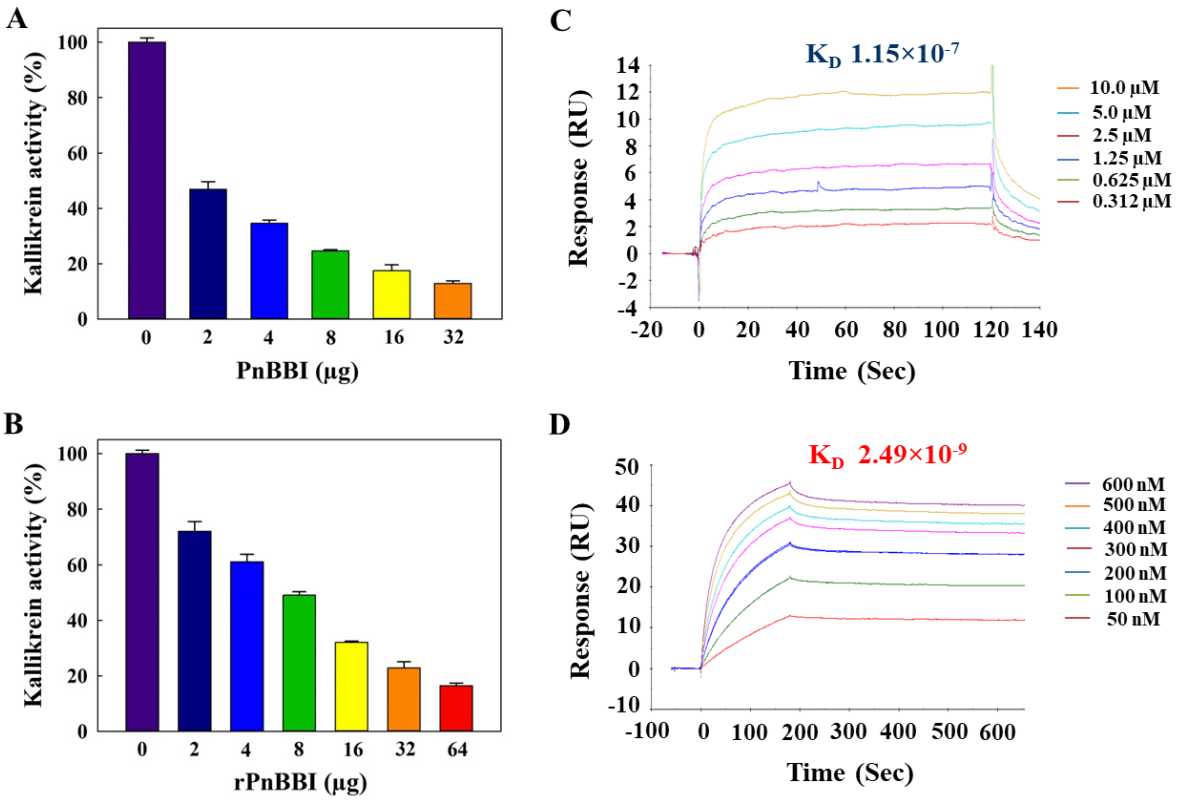


Fig. 8.2. Inhibitory action of PnBBI and rPnBBI towards plasma kallikrein (PK) and their SPR interaction kinetics: Increasing concentration of (**A**) PnBBI and (**B**) rPnBBIs were added to a fixed concentration of PK. After 10 min of incubation, the remaining PK amidolytic activity was determined by the addition of chromogenic substrate at 410 nm; In SPR kinetic study the various concentrations of PK were passed over the CM5 sensor chip surface, immobilized with (**C**) PnBBI and (**D**) rPnBBI. The sensorgram showing the association and dissociation pattern of PK is represented in the form of Resonance Units (RU). The obtained kinetic data was processed by BIAevaluation software using Langmuir 1:1 binding model.

8.2.4. Inhibition of FXa

FXa plays a significant role in the process of blood coagulation and considered as a convergence point of both intrinsic and extrinsic pathways. The rPnBBI exhibited IC_{50} of ~4 µg towards FXa while in case of PnBBI, it was found to be IC_{50} of ~16 µg (**Figs. 8.3D; 8.5D**). However, rPnBBI exerted 4-fold greater inhibitory potential towards FXa as compared to PnBBI. In contrast, both

PnBBI and rPnBBI displayed strong affinity towards FXa such as K_D 1.20×10^{-10} M and K_D 4.29×10^{-9} M, respectively (**Figs. 8.4D; 8.6D**).

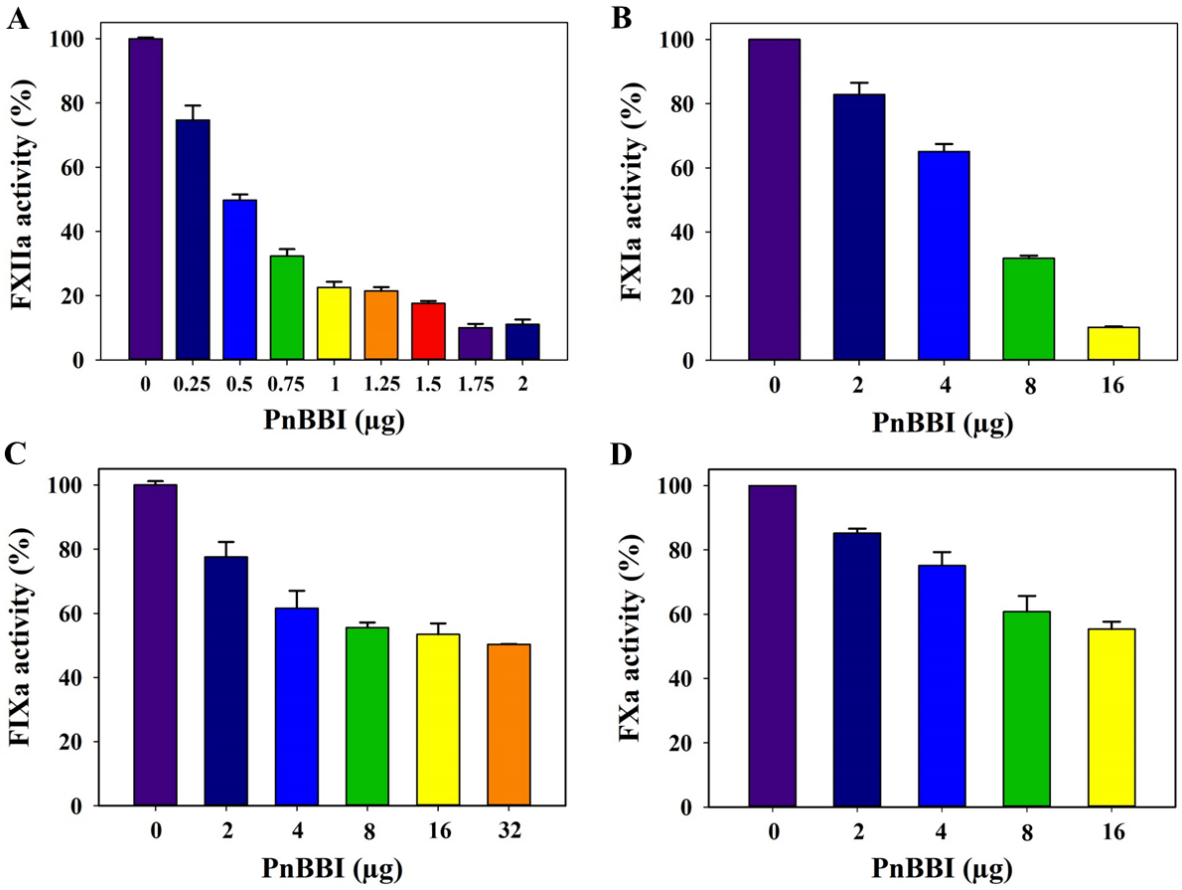


Fig. 8.3. Anticoagulant effect of PnBBI: Inhibitory action of PnBBI towards intrinsic pathway coagulation proteases (**A**) FXIIa; (**B**) FXIa; (**C**) FIXa and (**D**) FXa. Different concentrations of PnBBI were added to a fixed concentration of coagulation factors. After incubation for 10 min the residual amidolytic activity was determined by the addition of chromogenic substrate at 410 nm. Figure adapted from Dr. Swathi Marri (Thesis, 2016).

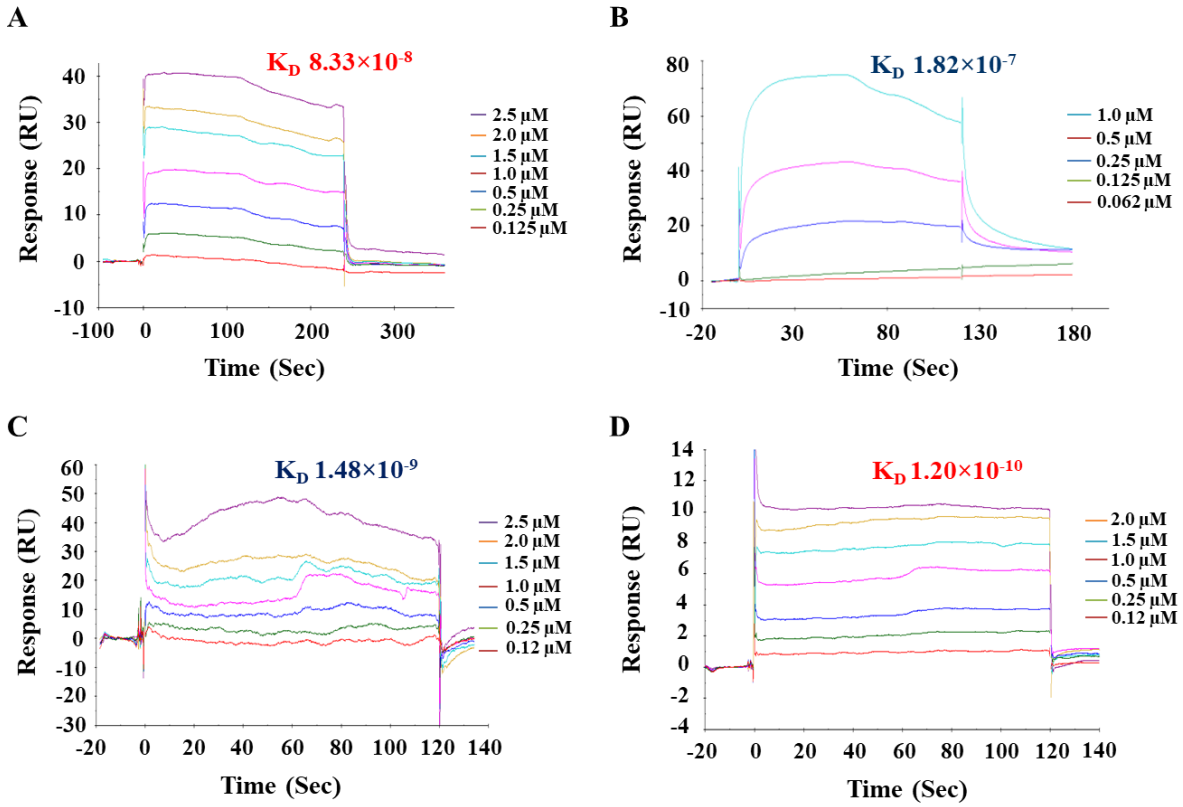


Fig. 8.4. SPR kinetics: The biomolecular interaction of immobilized PnBBI and proteases involved in the intrinsic pathway of blood coagulation (**A**) FXIIa; (**B**) FXIa; (**C**) FIXa and (**D**) FXa. The different concentrations of various coagulation factors were passed over the surface of immobilized PnBBI to determine the association and dissociation characteristics, represented in the form of change in Response Units (RU). The obtained kinetic data was processed by BIAevaluation software using Langmuir 1:1 binding model.

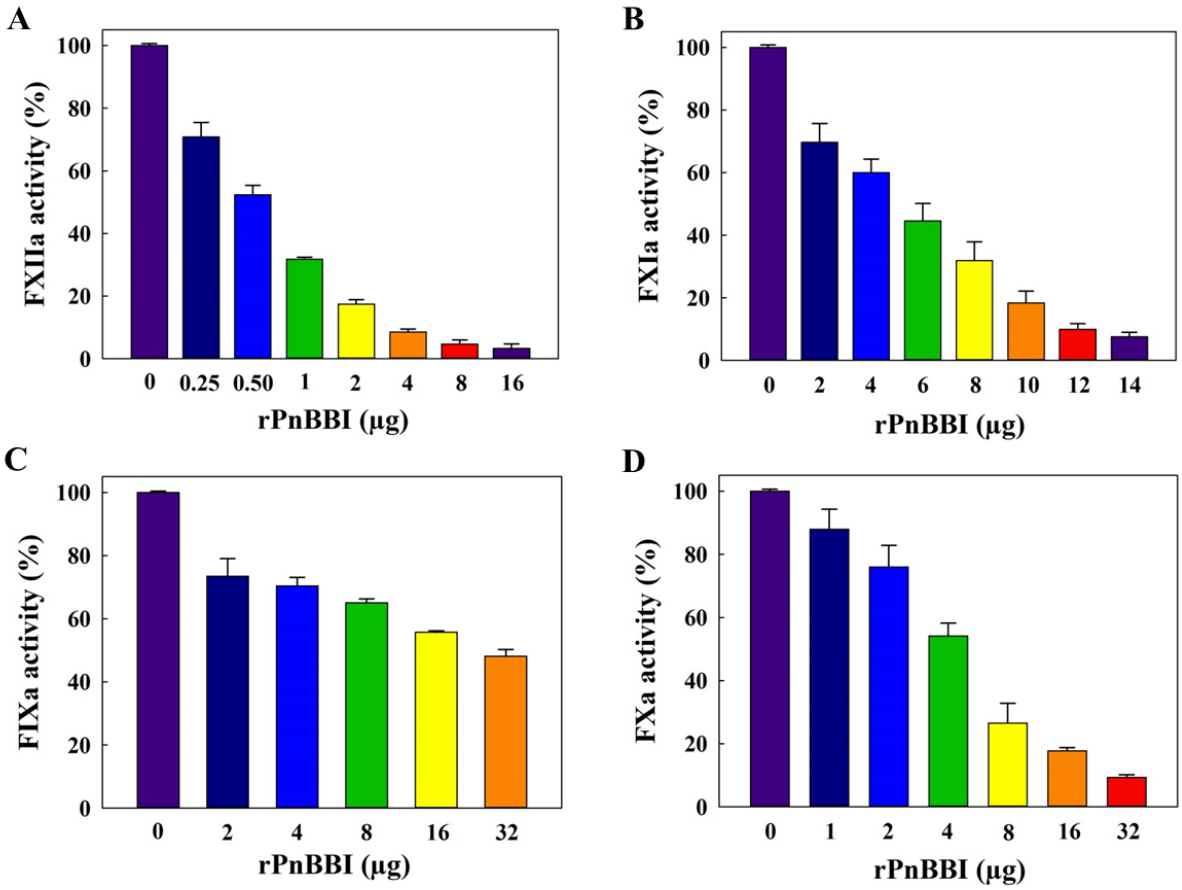


Fig. 8.5. Anticoagulant effect of recombinant PnBBI: Inhibitory action of rPnBBI towards intrinsic pathway coagulation proteases (**A**) FXIIa; (**B**) FXIa; (**C**) FIXa and (**D**) FXa. Different concentrations of rPnBBI were added to a fixed concentration. After incubation for 10 min the residual amidolytic activity was determined by the addition of chromogenic substrate at 410 nm.

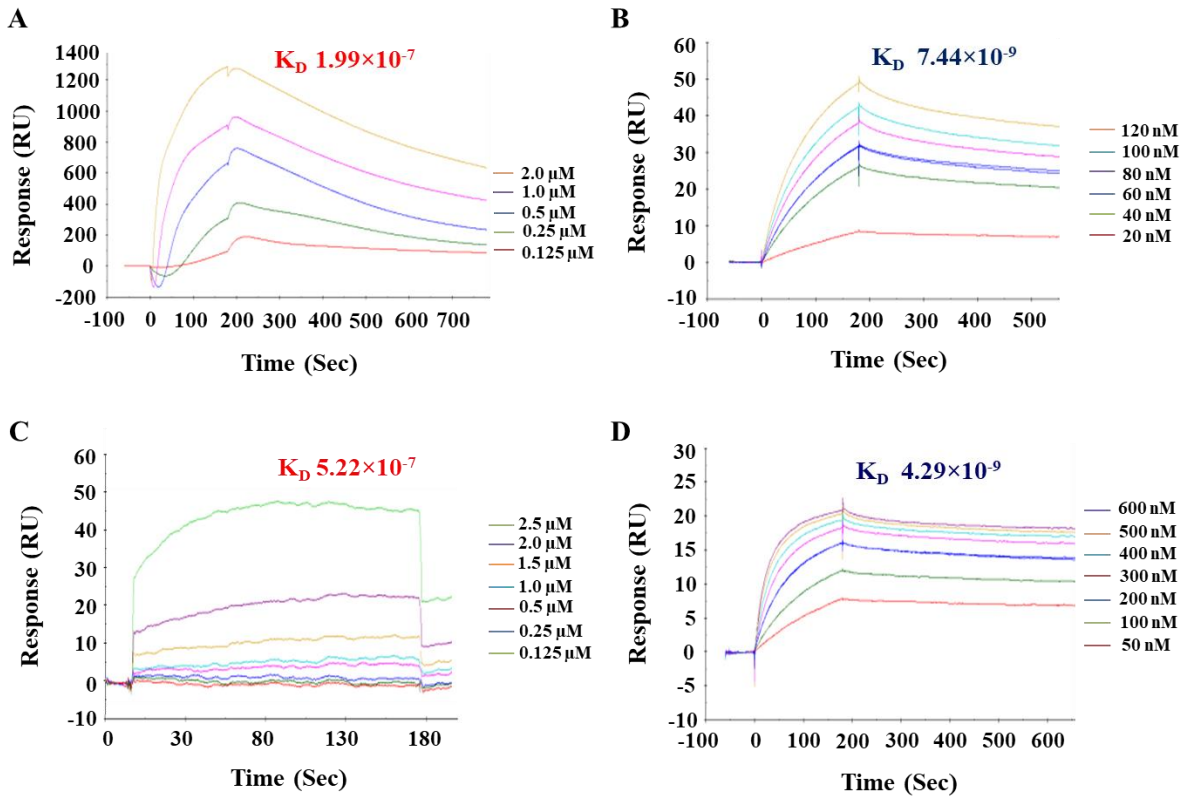


Fig. 8.6. SPR kinetics: The biomolecular interaction of immobilized rPnBBI and proteases involved in the intrinsic pathway of blood coagulation (**A**) FXIIa; (**B**) FXIa; (**C**) FIXa and (**D**) FXa. The different concentrations of various coagulation factors were passed over the surface of immobilized rPnBBI to determine the association and dissociation characteristics, represented in the form of change in Response Units (RU). The obtained kinetic data was processed by BIAevaluation software using Langmuir 1:1 binding model.

8.3. Discussion

Thrombosis plays a crucial role in the pathogenesis of several cardiovascular disorders and became a leading cause of illness and mortality throughout the world (de Laforcade, 2012; Goto and Tomita, 2013). Besides, the association of thrombosis with cancer is very well known (Lip et al., 2002; Blann and Dunmore, 2011; Ay et al., 2017). Therefore, an intensive search of antithrombotic molecules from natural food sources is beneficial and helpful to overcome the deleterious complications of current anticoagulant drugs.

The present study evaluated and compared the antithrombotic properties of rPnBBI with PnBBI by monitoring the activities of PK, FXIIa, FXIa, FIXa and Xa (**Table 8.1**). Indeed, both PnBBI and rPnBBI extended the average blood clotting time (APTT) by >2-fold which is significantly higher as compared to trypsin inhibitor from *Erythrina velutina* (EvTI), *Tamarindus indica* (TKI) , *Delonix regia* (DrTI) and *Acacia schweinfurthii* (AsTI) (Machado et al., 2013a; Patil et al., 2012; Salu et al., 2018). In contrast, PT which is clinically represented by the extrinsic pathway of blood coagulation was not affected by both PnBBI and rPnBBI (**Fig. 8.1C,D**). Also, similar results were reported in case of trypsin inhibitor from *Leucaena leucocephala* (LlTI), *E. velutina* (EvTI), *Bauhinia bauhinioides* (BbKI) and *Crataeva tapia* (CrataBL) where they did not affect PT but prolonged the APTT significantly (Oliva et al., 2000; Machado et al., 2013a; Brito et al., 2014; Salu et al., 2014).

On the other hand, APTT assay is a clinical indicator of the intrinsic pathway of coagulation. Activation of the intrinsic pathway has a limited role in hemostasis but extensively contribute to the pathogenesis of venous and arterial thrombosis (Wheeler and Gailani, 2016). However, the factors which initiate and amplify the intrinsic signals are the promising drug targets for thromboembolic disorders with minimal effect on protective hemostasis process (Gailani and Renné, 2007; Al-Horani and Desai, 2016; Grover and Mackman, 2019). Precisely, the PnBBI and rPnBBI inhibited the PK activity which is known to be involved in activation of FXII and platelet aggregation (**Fig. 8.2A,B**; Turk, 2006; Botos and Wlodawer, 2007). Similarly, inhibitors of PK isolated from Bauhinia species (BbKI) showed promising antithrombotic activity in an *in-vivo* animal model of arterial and venous thrombosis (Brito et al., 2014; Zhou et al., 2015). Further, Kunitz trypsin inhibitors from *D. regia* (DrTI) and *A. schweinfurthii* (AsTI) prevented arterial thrombus formation by interfering with PK and FXIa activity (Salu et al., 2018). Besides, both PnBBI and rPnBBI showed a similar inhibitory tendency towards other proteases of the coagulation pathway: FXIIa > FXIa > FIXa (**Figs. 8.3A-C; 8.5A-C**). Likewise, selective

inhibitors found in corn seeds (CTI) interacted with FXIIa in a canonical fashion (Hojima, 1980; Hamad et al., 2017).

Table 8.1. Inhibitory activity of PnBBI/rPnBBI towards factors involved in the intrinsic pathway of blood coagulation and their equilibrium dissociation constant K_D determined by SPR kinetics.

Proteases	**Half maximum inhibitory concentration (IC_{50})**		**Equilibrium dissociation constant (K_D) in M**	
	PnBBI (µg)	**rPnBBI (µg)**	**PnBBI**	**rPnBBI**
PK	2.0	8.0	1.15×10^{-7}	2.49×10^{-9}
FXIIa	0.50	0.55	8.33×10^{-8}	1.99×10^{-7}
FXIa	6.0	5.62	1.82×10^{-7}	7.44×10^{-9}
FIXa	32.0	32.0	1.48×10^{-9}	5.22×10^{-7}
FXa	16.0	4.0	1.20×10^{-10}	4.29×10^{-9}

FXa and thrombin which are known to play a central role in thrombus formation were targeted by many commercial anticoagulants such as heparin and DOACs (Al-Horani and Desai, 2016; Di Nisio et al., 2016). In the present study, though both PnBBI and rPnBBI inhibited the FXa activity, recombinant form showed 4-fold higher inhibitory potential as compared to the PnBBI (**Figs. 8.3D; 8.5D**). Also, several plant KIs such as EvTI, TKI and CrataBL are reported to inhibit the activity of FXa (Patil et al., 2012; Machado et al., 2013a; Ferreira et al., 2013). CrataBL is a combination of lectin and Kunitz type protease inhibitor isolated from the bark of *C. tapia* and it is also proven to be effective in preventing arterial thrombus formation and induce apoptosis through activation of caspase-3 in DU145, and PC3 cell lines (Ferreira et al., 2013; Salu et al., 2014).

Besides, the *in vitro* inhibition studies, the molecular interactions between PnBBI/rPnBBI and analytes PK, FXIIa, FXIa, FIXa and FXa was determined by using SPR spectrometer. SPR kinetics determine the association and dissociation characteristics of the binding partners in a

real-time label-free environment (Nguyen et al., 2015). However, this principle has been used in the field of drug discovery and development to screen and identify the lead compounds of a particular target molecule (Patching, 2014). In the present study, both PnBBI and rPnBBI recorded strong affinities towards PK, FXIIa, FXIa, FIXa and FXa which is evident by their K_D values obtained in nano-molar range (**Table 8.1**). However, PK showed higher affinity towards the single molecular species rPnBBI than native PnBBI pool. Formerly, several studies had determined the inhibition constant (Ki) or equilibrium dissociation constant (K_D). However, lower the Ki (or) K_D value the higher is its affinity. The K_D value 2.49×10^{-9} M of PK towards rPnBBI was equivalent to Ki of LlTI (Oliva et al., 2000), DrTI and AsTI (Salu et al., 2018). It is also reported to be lower than Ki of many squash and Kunitz type inhibitors. Further, the observed K_D for FXIIa towards PnBBI (K_D 8.33×10^{-8} M) and rPnBBI (K_D 1.99×10^{-7} M) were similar to the Ki of many reported anticoagulant PIs (**Chapter 2, Table 2.1**) such as MCTI-I, II, CMTI-III and LCTI-II (Hayashi et al., 1994), CMTI-V (Krishnamoorthi et al., 1990), BvTI and BuTI (Oliva et al., 1996), CeKI (Cruz-Silva et al., 2004), CHFI (Korneeva et al., 2014) and DrTI (Salu et al., 2018). However, the critical factor of coagulation cascade FXa has recorded the lowest K_D towards both PnBBI (1.20×10^{-10} M) and rPnBBI (4.29×10^{-9} M) as compared to reported anticoagulant PIs further added significance to this study (**Chapter 2, Table 2.1**). In contrast to the reported K_D, PnBBI was found to be more effective inhibitor of PK activity as compared to rPnBBI. However, both PnBBI and rPnBBI have approximately equal inhibitory potential in the following order FXIIa>FXIa>FIXa with the K_D range of 10^{-7} M to 10^{-9} M. On the other hand, rPnBBI is a potent inhibitor of FXa as compared to PnBBI pool in concomitance with their K_D (**Table 8.1**). Altogether, natural peanut BBIs inhibited the coagulation factors are involved in intrinsic pathway of blood coagulation and they could be exploited in the antithrombotic therapy (**Fig. 8.7**).

Highlights

- ✓ Inhibitory potential of both PnBBI and rPnBBI against proteases of intrinsic coagulation pathway followed similar pattern: FXIIa > FXIa > FIXa.
- ✓ Native PnBBI is more effective towards PK as compared to rPnBBI.
- ✓ rPnBBI is a potent inhibitor of key common pathway protease FXa than native PnBBI.
- ✓ SPR kinetic studies ascertained that both PnBBI & rPnBBI showed stable molecular interactions with coagulation factors PK, FXIIa, FXIa, FIXa and FXa.

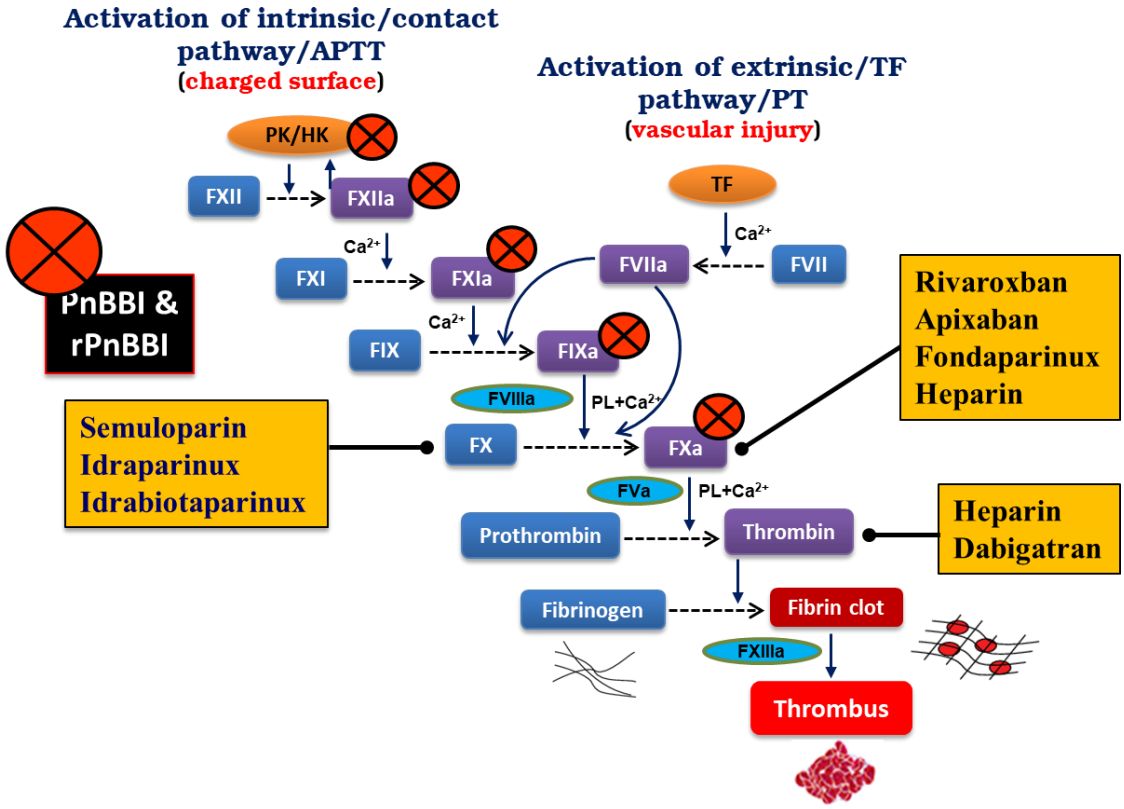


Fig. 8.7. Blood coagulation cascade and targets of various anticoagulants: Blood coagulation comprised of series of biochemical reactions which involves subsequent activation of inactive zymogens into active coagulation proteases. The process of blood coagulation initiated by intrinsic and extrinsic pathways converges into common pathway mediated through activation of FX to FXa which ultimately leads to thrombus formation. Most of the commercial antithrombotic drugs targets key enzymes of coagulation cascade such as FXa and FIIa (thrombin). In the particular study both PnBBI and rPnBBI showed significant anticoagulant potential by inhibiting the activity of multiple proteases of intrinsic pathway such as PK, FXIIa, FXIa, FIXa and FXa.

Chapter 9

Summary and conclusions

Summary and Conclusions

Plant defense proteinase inhibitors (PIs) act as feeding deterrents for grazing animals and insects by inhibiting their gut proteases. They are often found in seeds and tubers and induced in other vegetative parts of the plant in response to wounding, biotic and abiotic stresses (Shewry, 2003; Srinivasan et al., 2009; War et al., 2018). PIs accumulate in storage organs and contribute to < 20% of the total protein content in leguminous plants (Ryan, 1990; Meulenbroek et al., 2012). They combat pests by inhibiting their midgut digestive proteases, hinders digestion and creates an essential amino acids deficiency conditions which in turn leads to retarded larval growth, deformities during metamorphosis, reduced fecundity and mortality (Dunse et al., 2010a; Zhu-Salzman and Zeng, 2015). So far, numerous plant PIs has been isolated, characterized for their biochemical and insecticidal efficacy, and generated transgenic plants which express candidate PI genes (Fan and Wu, 2005; Macedo et al., 2015; Clemente et al., 2019). However, there is a need for continuous exploration of new proteinase inhibitor molecules from non-host/wild/hybrid plant species to overcome the insect adaptation mechanisms against PIs (Harsulkar et al., 1999; Parde et al., 2012; Jamal et al., 2013; de Paula et al., 2017; Mohanraj et al., 2019). Despite the biotechnological applications in agricultural crop protection, PIs are also known to have several therapeutic properties which promote human health (Losso, 2008; Shamsi et al., 2016; Srikanth and Chen, 2016). The biomedical applications of BBIs are geared up since the past decade (Shamsi et al., 2016; Srikanth and Chen, 2016). However, they are less explored as anticoagulants.

In the present study, **Chapter 4** describes the isolation of trypsin specific PIs from interspecific hybrid variety of peanut (*A. hypogaea*) using affinity and size exclusion chromatography columns. This isolation procedure facilitated 167-fold purification and 37.5% yield recovery (**Table 4.1**). The purified PnBBI is active against both trypsin and chymotrypsin. However, it is a potent inhibitor of trypsin as compared to chymotrypsin (**Table 4.2**). Similarly,

SPR kinetic study revealed that PnBBI-trypsin complex (K_D 1.34×10^{-9} M) is more stable than PnBBI-chymotrypsin complex (K_D 2.22×10^{-9} M; **Fig. 4.5A,B**). PnBBI existed as several isoinhibitors with close molecular mass range while predominant isoinhibitor peak showed a molecular mass of 6.73 kDa upon MALDI-TOF intact mass analysis (**Fig. 4.2**). These isoinhibitors separated on one and two-dimensional electrophoresis under native conditions showed resistance to bovine trypsin and chymotrypsin digestion (**Fig. 4.2E-G**). They showed self-association characteristic on Tricine SDS-PAGE with the occurrence of monomeric, dimeric, and tetrameric forms similar to the pattern shown by commercial soybean BBI. However, dithiothreitol reduction resulted in dissociation of higher-order forms into their monomers (**Fig. 4.1A**). The phenomenon of concentration-dependent self-association was further evident by characteristic red-shift (205-212 nm) in the far-UV CD spectrum (**Fig. 4.7D**). Further, isoinhibitor spot (pI 5.9) from the reduced 2-DE gel is identified as a Bowman-Birk inhibitor (BBI) by MALDI TOF-TOF analysis (**Figs. 4.3, 4.4**). Since PnBBI is bifunctional trypsin and chymotrypsin inhibitor, the variable specificity of PnBBI towards trypsin and chymotrypsin is validated by SPR competitive binding assay. The PnBBI-chymotrypsin complex tends to associate with trypsin while binding of chymotrypsin was not observed with PnBBI-trypsin complex (**Fig. 4.5C,D**). Similarly, PnBBI showed 1:2 stoichiometric binding ratio with trypsin, whereas chymotrypsin did not follow distinctive stoichiometry due to its poor inhibition (**Fig. 4.6D**). These observations emphasize that both inhibitory sites of PnBBI are specific to trypsin. PnBBI possessed secondary structural components in the following order β-sheets (47%) > random coil (44%) > α-helix (9%). Further, the structural and functional integrity of PnBBI was found to be stable over diverse pH and temperatures but sensitive to a reducing agent which infers the importance of disulfide bonds in the stabilization of its structure (**Figs. 4.6, 4.7**). Altogether, the following properties such as low molecular mass, the existence of isoinhibitors and their self-association, dual inhibitory activity against trypsin and chymotrypsin, structural and functional stability in diverse adverse conditions

and sensitive to reducing agent suggest that the PI isolated from interspecific hybrid variety (4368-1) of peanut belong to BBI family.

The PnBBI isolated in the present study inhibited trypsin-like midgut proteases of both *H. armigera* and *S. litura* with IC_{50} of 0.040 µg and 0.149 µg, respectively, which accounts to 3.72-fold higher HaTPI activity as compared to its SlTPI activity (**Figs. 5.1, 6.1**). Further, the antimetabolic efficacy of PnBBI against *H. armigera* was evaluated by feeding on an artificial diet supplemented with PnBBI (**Chapter 5**). Ingestion of high dose of (0.005%) PnBBI not only retarded the larval growth by >40% on day6 but also influenced the midgut protease complement significantly. The HaTP activity of larvae fed on moderate (0.0025%) and high dose PnBBI diet was declined by 63% (**Figs. 5.2, 5.3**). Further, native PAGE zymography of HaGP extracts from larvae fed on PnBBI diet showed dose-dependent depletion in serine protease bands (Group-I) as compared to larvae fed on control diet (**Fig. 5.4**). In contrast, the chymotrypsin-like protease (band 9 of Group-II) showed enhanced expression in larvae exposed to high dose PnBBI diet (**Fig. 5.4D**). Similarly, differential native 2-DE coupled zymography revealed the disappearance of several protease isoforms in larvae fed on a high dose of PnBBI as compared to larvae fed on control diet (**Fig. 5.5**). Further, ingestion of PnBBI modulated the expression of HaTP and HaCP transcripts in a dose-dependent manner. The larvae exposed to low and moderate dose of PnBBI showed a similar pattern of expression contrary to high dose PnBBI fed larvae (**Fig. 5.7**). Though in general the mechanisms of dynamic regulation and plasticity in proteases expression indicate the adaptation of insect pests to biopesticides. The results from present study emphasize that PnBBI retarded the growth of *H. armigera* larvae by effective inhibition of their midgut trypsin-like proteases and modulating their expression.

Chapter 6 described the effect of PnBBI (0.025%) ingestion on *S. litura* larval growth and midgut protease expression (**Fig. 6.2**). The PnBBI retarded the growth of *S. litura* marginally

(<17%) which is associated with >30% elevation in SlTP activity and a subsequent decline in SlCP activity by ~10% (**Fig. 6.3A**). Further, a comparative SlGP profile showed remarkable changes correlating with altered SlTP and SlCP activities. However, the newly emerged protease band 6 could be responsible for the increased SlTP activity and this protease band apparently seems to be insensitive to the ingested PnBBI. Also, the concomitant diminution in protease band 2 substantiates with the decreased SlCP activity (**Fig. 6.3A,B**). These observations were further validated by *in vitro* inhibitory studies with PnBBI at a wide range of concentrations. The maximum inhibition in SlTP activity was found to be 80% in larvae fed on PnBBI-free diet while it was reduced to 50% upon exposure to PnBBI (**Fig. 6.3C,D**). Consequently, a ~10-fold increase in IC_{50} value of PnBBI towards SlTPs suggests *S. litura* acquired ~30% of insensitive SlTPs during PnBBI exposure through diet as a part of their adaptation mechanism. Further, differential 2-DE of midgut proteome of 5^{th} instar larvae fed on control and 0.025% PnBBI diet revealed up-regulation of 14 proteins and down-regulation of 19 proteins which might provide further insight for the key mechanism of *S. litura* adaptation (**Figs. 6.4, 6.5, 6.7**). In this particular study, we suggest that *S. litura* effectively employed defensive compensatory response to minimize the antimetabolic effect of PnBBI.

In order to exploit the benefits of BBI, **Chapter 7** is involved in heterologous expression of full-length PnBBI isoinhibitor from peanut interspecific hybrid (4368-1) using pTWIN1 vector and *E. Coli* BL21 (DE3) expression strain. The purification of a fusion protein (CBD-DnaB-rPnBBI) by using chitin beads resulted in huge yield loss due to premature autocleavage of fusion partner CBD-Intein from the rPnBBI (**Figs. 7.6A, 7.7**). Alternatively, the trypsin affinity column facilitated the efficient purification of rPnBBI with a molecular mass of 7.53 kDa (**Fig. 7.6B**). Similar to the native PnBBI, the purified rPnBBI exhibited (i) differential inhibitory activity towards bovine trypsin and chymotrypsin and (ii) higher specificity towards trypsin than chymotrypsin. In contrast, the inhibitory potential of rPnBBI was found to be 50% lower as

compared to PnBBI (**Figs. 7.8A**). This discrepancy in inhibitory capacity could have arisen due to the presence of several isoinhibitors species in PnBBI which might differ in their inhibitory potential. Further, SPR kinetics revealed rPnBBI formed highly stable complexes with both trypsin and chymotrypsin (**Fig. 7.8B,C**). On the other hand, though secondary structure of rPnBBI was similar to PnBBI its TI activity varied with increase in temperature (**Figs. 4.6B; 7.9E**). The 3-D structure of rPnBBI illustrated a unique N-terminal reactive site loop with eleven residues which is uncommon in other legumes BBI (**Fig. 7.10**). The eleven residue loop 'CDRRAPPYFEC' contained Phe-Glu as additional residues at P5′- P6′ along with Arg-Arg at P1-P1′ position. However, the Arg29 and Arg30 at the first reactive site, and Arg57 at the second reactive site of rPnBBI attributed to its potent TI activity. Further, rPnBBI has evolutionary divergence with other legumes BBI and also produced in the form of single isoinhibitor species by rDNA technology (**Figs. 7.7; 7.11**). Therefore rPnBBI could be exploited in the therapeutic application.

Subsequently, the final part of the study **Chapter 8** assesses the anticoagulant properties of both PnBBI and rPnBBI. The preliminary APTT assay revealed PnBBI and rPnBBI prolonged blood coagulation time by >2.0-fold which is comparable with commercial anticoagulant heparin (**Fig. 8.1A,B**). APTT assay clinically represents intrinsic/contact pathway of blood coagulation which has a limited role in hemostasis and extensively contribute to the pathogenesis of venous and arterial thrombosis. Further, *in vitro* inhibition assay and SPR kinetics revealed that both PnBBI and rPnBBI inhibited the amydolytic activity of proteases involved in intrinsic pathway such as PK, FXIIa, FXIa, FIXa and FXa and formed stable complexes within the range of nanomolar K_D (**Table 8.1**). Comparatively, PnBBI is a more effective inhibitor of PK activity than rPnBBI as revealed by their IC_{50} values. However, both PnBBI and rPnBBI have approximately equal inhibitory potential against the factors of the intrinsic pathway in the following order: FXIIa>FXIa>FIXa. However, their K_D values are in the range of 10^{-7} M to 10^{-9} M. On the other

hand, rPnBBI is a potent inhibitor of FXa as compared to PnBBI (**Table 8.1**) Altogether, the bioactive molecules 'PnBBI or rPnBBI' from the natural food source peanut showed significant anticoagulant property which could be exploited in the antithrombotic therapy.

Major Conclusions

- PnBBI is a bifunctional trypsin/chymotrypsin inhibitor and its structural and functional integrity remained stable under diverse adverse conditions.
- PnBBI act as potent inhibitor of *H. armigera* larval growth by modulating the midgut protease expression and activity.
- The metabolic response of *S. litura* larvae was associated with induction of substantial trypsin-like insensitive proteases as a part of compensatory adaptive mechanism against PnBBI.
- Recombinant PnBBI isoinhibitor was successfully produced by using pTWIN1and *E. coli* BL21 (DE3) expression system.
- Both PnBBI and rPnBBIs showed significant anticoagulant potential which could be exploited in the prophylaxis of thromboembolic diseases.

Table 9.1. A comparative table indicating the biochemical properties of PnBBI and rPnBBI.

S. No.	Property	PnBBI	rPnBBI
1	Molecular mass (Da)	6594, 6733, 6963, 7077, 7252.	7534
2	Trypsin inhibitory activity	Yes K_D 2.22×10^{-9} M	Yes K_D 8.92×10^{-11} M
3	Chymotrypsin inhibitory activity	Yes K_D 1.34×10^{-9} M	Yes K_D 7.65×10^{-11} M
4	Temperature stability (90 °C)	Yes	Loss in TI activity by ~25%
5	pH stability (2.0-12)	Yes	Yes
6	Stability against DTT	No	No
7	Secodary structure	β-sheets>Random coils>α-helix	β-sheets>Random coils>α-helix

Chapter 10

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Publications

1. **Lokya V**, Swathi M, Mallikarjuna N and Padmasree K (2020) Response of midgut trypsin and chymotrypsin-like proteases of *Helicoverpa armigera* larvae upon feeding with peanut BBI: Biochemical and Biophysical characterization of PnBBI. Front. Plant Sci. 11:266. doi: 10.3389/fpls.2020.00266.
2. **Vadthya Lokya**, Marri Swathi, Sarada Devi Tetali, Nalini Mallikarjuna, Kollipara Padmasree. Anti-coagulant Bowman-Birk inhibitors form peanut (*Arachis hypogaea)*, (**Under preparation**).
3. Mohanraj, S. S., Gujjarlapudi, M[#]., **Lokya, V**[#]., Mallikarjuna, N., Dutta-Gupta, A., and Padmasree, K. (2019). Purification and characterization of Bowman-Birk and Kunitz isoinhibitors from the seeds of *Rhynchosia sublobata* (Schumach.) Meikle, a wild relative of pigeonpea. Phytochemistry, 159, 159-171. ([#]**equal contribution**).
4. Swathi, M., Mishra, P. K., **Lokya, V**., Swaroop, V., Mallikarjuna, N., Dutta-Gupta, A., and Padmasree, K. (2016). Purification and partial characterization of trypsin-specific proteinase inhibitors from pigeonpea wild relative *Cajanus platycarpus* L. (Fabaceae) active against gut proteases of lepidopteran pest *Helicoverpa armigera*. Frontiers in physiology, 7, 388.
5. Swathi, M., **Lokya, V**., Swaroop, V., Mallikarjuna, N., Kannan, M., Dutta-Gupta, A., and Padmasree, K. (2014). Structural and functional characterization of proteinase inhibitors from seeds of *Cajanus cajan* (cv. ICP 7118). Plant physiology and biochemistry, 83, 77-87.
6. Vinod K. Chauhan, Narender K. Dhania, **Vadthya Lokya**, Bhoopal Bhuvanachandra, Kollipara Padmasree, Aparna Dutta-Gupta (2019). Altered expression of aminopeptidase N isoform associated with Cry toxin tolerance in larvae of castor semilooper, *Achaea janata*. (**Under communication**).

Conference attended

1. **Lokya V** and Padmasree K. (2017) "Potential Bowman-Birk Inhibitors of human neutrophil cathepsin G and evaluation of its biochemical properties" International conference on Innovations in Pharma and Biopharma Industry: Challenges and Opportunities for Academy and Industry (**ICIPBI-2017**) held at School of Life Sciences, University of Hyderabad, Hyderabad on 20-22nd Dec 2017.
2. **Lokya V**, Swathi M, Tetali S D, Mallikarjuna N, Dutta-Gupta A, and Padmasree K. (2016) "Purification and characterization of Bowman-Birk Inhibitors from peanut: Evaluation of their insecticidal and pharmacological activities" 85th Annual Meeting of Society of Biological Chemists (**SBC-2016**), held at CSIR-Central Food Technological Research Institute, Mysuru on 21-24th Nov 2016.
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4. **Lokya V**, Padmasree K. (2016) "Purification and partial characterization of peanut proteinase inhibitors active against midgut proteases of *Achaea janata*" 2nd Andhra Pradesh Science Congress (**APSC-2016**) held at P. B. Siddhartha College of Arts & Sciences, Vijayawada, Andhra Pradesh on 7-9th Nov 2016.
5. **Lokya V**, Swathi M, Swaroop V, Mallikarjuna N, Dutta-Gupta A, and Padmasree K. (2015) "Purification and biochemical characterization of proteinase inhibitors from *Cajanus cajan* (ev. ICP7118): Evaluation of their insecticidal potential against *Achaea janata*" 2nd International Conference on "Frontiers in Biological Sciences (**InCoFIBS-2015**)" held at National Institute of Technology, Rourkela on 22-24th Jan 2015.

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Response of Midgut Trypsin- and Chymotrypsin-Like Proteases of *Helicoverpa armigera* Larvae Upon Feeding With Peanut BBI: Biochemical and Biophysical Characterization of PnBBI

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Proteinase/Protease inhibitors (PIs) from higher plants play an important role in defense and confer resistance against various insect pests as well as pathogens. In the present study, Bowman-Birk Inhibitor (BBI) was purified from mature seeds of an interspecific advanced hybrid peanut variety (4368-1) using chromatographic techniques. The biochemical and biophysical characteristics such as low molecular mass, presence of several isoinhibitors and higher-ordered dimer/tetramer, predominance of antiparallel β-sheets and random coils in secondary structure, reactive sites against trypsin and chymotrypsin, broad spectrum of stability toward extreme pH and temperature along with MALDI TOF-TOF analysis (ProteomeXchange identifier PXD016933) ascertained the purified biomolecule from peanut as BBI (PnBBI). Surface plasmon resonance competitive binding analysis revealed the bifunctional PnBBI is a trypsin specific inhibitor with 1:2 stoichiometry compared to chymotrypsin. A concentration-dependent self-association tendency of PnBBI was further confirmed by red shift in far-UV CD spectra. Furthermore, the insecticidal potential of PnBBI against *Helicoverpa armigera* was assessed by *in vitro* assays and *in vivo* feeding experiments. A significant reduction in larval body weight was observed with concomitant attenuation in the activity of midgut trypsin-like proteases of *H. armigera* (HaTPs) fed on PnBBI supplemented diet. The one and two-dimensional zymography studies revealed the disappearance of several isoforms of HaTP upon feeding with PnBBI. qRT-PCR analysis further suggests the role of PnBBI in not only inhibiting the activity of midgut trypsin and chymotrypsin-like proteases but also in modulating their expression. Taken together, the results provide a biochemical and molecular basis for introgressed resistance in peanut interspecific advanced hybrid variety against *H. armigera*.

Keywords: *Arachis hypogaea* (Fabaceae), PnBBI, two-dimensional zymography, circular dichroism, surface plasmon resonance, *Helicoverpa armigera* (Noctuidae), trypsin-like midgut proteases

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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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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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Purification and Partial Characterization of Trypsin-Specific Proteinase Inhibitors from Pigeonpea Wild Relative *Cajanus platycarpus* L. (*Fabaceae*) Active against Gut Proteases of Lepidopteran Pest *Helicoverpa armigera*

Marri Swathi[1], Prashant K. Mishra[2], Vadthya Lokya[2†], Vanka Swaroop[2†], Nalini Mallikarjuna[3], Aparna Dutta-Gupta[4] and Kollipara Padmasree[2]*

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Proteinase inhibitors (PIs) are natural defense proteins of plants found to be active against gut proteases of various insects. A pigeonpea wild relative *Cajanus platycarpus* was identified as a source of resistance against *Helicoverpa armigera*, a most devastating pest of several crops including pigeonpea. In the light of earlier studies, trypsin-specific PIs (CpPI 63) were purified from mature dry seeds of *C. platycarpus* (ICPW-63) and characterized their biochemical properties in contributing to *H. armigera* resistance. CpPI 63 possessed significant *H. armigera* gut trypsin-like proteinase inhibitor (HGPI) activity than trypsin inhibitor (TI) activity. Analysis of CpPI 63 using two-dimensional (2-D) electrophoresis and matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry revealed that it contained several isoinhibitors and small oligomers with masses ranging between 6 and 58 kDa. The gelatin activity staining studies suggest that these isoinhibitors and oligomers possessed strong inhibitory activity against *H. armigera* gut trypsin-like proteases (HGPs). The N-terminal sequence of the isoinhibitors (pI 6.6 and pI 5.6) of CpPI 63 exhibited 80% homology with several Kunitz trypsin inhibitors (KTIs) as well as miraculin-like proteins (MLPs). Further, modification of lysine residue(s) lead to 80% loss in both TI and HGPI activities of CpPI 63. In contrast, the TI and HGPI activities of CpPI 63 were stable over a wide range of temperature and pH conditions. The reported results provide a biochemical basis for pod borer resistance in *C. platycarpus*.

Keywords: gelatin activity staining, Kunitz trypsin inhibitor, mass spectrometry, miraculin-like proteins, two-dimensional electrophoresis

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Research article

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

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ABSTRACT

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

1. Introduction

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

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

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

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