Purification and characterization of proteinase inhibitors from red gram (*Cajanus cajan*) and black gram (*Vigna mungo*) and evaluation of their insecticidal potential

Thesis submitted to the University of Hyderabad

for the Degree of

DOCTOR OF PHILOSOPHY

By

E. Ramanjaneya Prasad
Enrolment No. 03LPPH10

Supervisor**: Dr. K.P.M.S.V. Padmasree**

Department of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad 500 046, INDIA

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DEPARTMENT OF PLANT SCIENCES
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD-500 046
INDIA

DECLARATION

I hereby declare that the work presented in this thesis entitled **"Purification and characterization of proteinase inhibitors from red gram (*Cajanus cajan*) and black gram (*Vigna mungo*) and evaluation of their insecticidal potential"**, has been carried out by me under the supervision of Dr. K.P.M.S.V. Padmasree in the Dept. of Plant Sciences, School of Life Sciences, University of Hyderabad and this work has not been submitted for any degree or diploma of any other University or Institute.

E. Ramanjaneya Prasad
(Candidate)
Enrol. No. 03LPPH10

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

DEPARTMENT OF PLANT SCIENCES
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD-500 046
INDIA

CERTIFICATE

This is to certify that **Mr. E. Ramanjaneya Prasad** has carried out the research work embodied in the present thesis entitled "**Purification and characterization of proteinase inhibitors from red gram (*Cajanus cajan*) and black gram (*Vigna mungo*) and evaluation of their insecticidal potential"**, for the degree of **Doctor of Philosophy** under my supervision in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad.

Dr. K.P.M.S.V. Padmasree
Supervisor
Department of Plant Sciences

Head
Department of Plant Sciences

Dean
School of Life Sciences

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E. Ramanjaneya Prasad

Dedicated to my parents

Abbreviations

BAPNA	*N*-α-benzoyl-DL arginine-*p*-nitroanilide
BBI	Bowman-Birk inhibitor
BgPI	Black gram proteinase inhibitor
BPB	Bromophenol blue
CBB	Coomassie Brilliant Blue
CD	Circular Dichroism
CHD	1,2-Cyclohexanedione
CI	Chymotrypsin inhibitor
CV	Column volume(s)
DEPC	Diethyl pyrocarbonate
DTT	Dithiothreitol
GLUPHEPA	*n*-glutaryl-L-phenylalanine-*p*-nitroanilide
MALDI-TOF	Matrix-assisted laser desorption/ionization – time of flight
NAI	*N*-Acetylimidizole
NBS	*N*-Bromosuccinimide
PIs	Proteinase inhibitor(s)
pI	isoelectric point
PMSF	Phenyl methyl sulfonyl fluoride
RgPI	Red gram proteinase inhibitor
SKTI	Soybean Kunitz trypsin inhibitor
TEMED	N,N,N',N'-Tetramethylethylenediamine
TI	Trypsin inhibitor
TLCK	Nα-p-Tosyl-L-Lysine Chloromethyl Ketone
TNBS	2,4,6-Trinitrobenzenesulfonic acid
TPCK	N-p-Tosyl-L-Phenylalanine Chloromethyl Ketone
Tricine	N-tris(hydroxymethyl) methyl glycine

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General Introduction

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General Introduction

The current scenario:

The world population by the end of 2000 crossed 6.0 billions, and was expected to cross 10 billions by 2050 (U.S. Census Bureau, 2006). As on today 230 million tons of protein per year is required for the maintenance of this population. Since a substantial amount of protein comes from animals, the feed needed for livestock is even greater (Carlini and Grossi-de-Sa, 2002). Hence there is a need to increase the agricultural production to meet the demand of ever expanding population. This can be possible through improved harvest yields of major crops from existing cultivated land. One practical means of increasing yield is possible by way of minimizing losses due to insect pests (Oerke and Dehne, 2004). Insects are not only responsible for massive direct losses of productivity as a result of their herbivory, but also cause massive indirect losses due to their role as vectors for various plant pathogens. These losses occur despite the extensive use of pesticides and fungicides. In the absence of such crop protection measures losses would be much more serious (Hilder and Boulter, 1999). World wide crop losses without the use of pesticides and other non-chemical control strategies is estimated to be about 70% of crop production, amounting to US $ 400 billion. The world wide pre-harvest losses due to insect pests, despite the use of insecticides are 15% of total production representing over US $ 100 billion (Krattiger, 1997).

Insect pests are capable of evolving to biotypes that can adapt to new situations. For instance, they overcome the effect of toxic materials or bypass natural or artificial plant resistance, which further confounds the problem (Roush and McKenzie, 1987). This problem is more acute in the tropics and subtropics, where the climate provides a highly condusive environment for a wide range of insects and necessitates massive efforts to suppress the population densities of different pests in order to achieve an adequate supply

of food (Lawrence and Koundal, 2002). In order to feed the ever expanding population, crop protection plays a vital and integral role in the modern day agricultural production to minimize the yield losses. Currently, the crop protection practice in such agricultural systems relies exclusively on the use of agrochemicals, although a few specific cases do exist where inherent varietal resistance and biological control have been successfully employed. The exclusive use of chemical pesticides not only results in rapid build-up of resistance by insects to such compounds, but their non-selectivity affects the balance between pests and natural predators, and is generally in favour of pests (Metcalf, 1986).

Therefore, an integrated pest management (IPM) programme, comprising a combination of practices including the judicious use of pesticides, crop rotation, field sanitation and exploitation of inherently resistant plant varieties would provide the best option (Meiners and Elden, 1978). The last option includes the use of transgenic crops, expressing foreign insecticidal genes which could make a significant contribution to sustainable agriculture and thus could be an important component of IPM (Boulter, 1993). A milestone in this field was established about 22 years ago, with the introduction of the genetic material coding for an entomotoxic protein into a tobacco plant from the bacterium *Bacillus thuringiensis* (Bt) (Vaeck et al., 1987). Nowadays a large number of Bt containing products are in the market in the United States as well as in some other countries.

However, in spite of the tremendous impact brought about by this new technology, there is some concern regarding the biosafety of Bt proteins for mammals (Vazquez-Padron et al., 2000), as well as some unsolved questions related to the ecological impacts of this new class of bioinsecticides. Bt toxins used at present is not adequate to target all the pests, such as sap-sucking pests and pests of stored products (Gatehouse, 2008). Hence, there has been a need to discover new effective plant genes which would offer greater resistance against these pests. An alternative strategy could be

to take advantage of the plant's own defense mechanisms. For example, by manipulating the expression of their endogenous defense proteins, or by introduction of an insect control gene derived from another plant. In this context, several different genes encoding toxic proteins have been introduced into crop genomes in order to confer insect and pathogen resistance. Many of these plants are now being tested in field conditions or awaiting commercialization (Hilder and Boulter, 1999; Mosolov and Valueva, 2008).

Plant defense proteins:

Plants have evolved certain defense mechanisms most of which are concentrated in the seeds since these are the vehicles for propagation and survival of the species. Seed tissues may accumulate a wide array of defensive compounds constitutively or after induction that confer resistance against phytophagous predators and infection by viruses, bacteria, fungi, nematodes, etc. (Carlini and Grossi-de-sa, 2002). The plant proteins that are involved in defense mechanisms are lectins, ribosome-inactivating proteins, inhibitors of proteolytic enzymes and glycohydrolases (Koiwa et al., 1997; Domoney, 1999). The class of inhibitors of proteolytic enzymes comprises various protein types which include amylase and proteinase inhibitors (Garcia-Olmedo et al., 1987). Other plant proteins involved in the complex mechanisms of defense are the arcelins (Osborn et al., 1988), chitinases (Cohen, 1993), canatoxin (Carlini et al., 1997) and modified forms of storage proteins (Sales et al., 2000).

However, the components of defense system of some plants might have been lost during the artificial selection imposed by domestication. One example of this process are the arcelins, a family of proteins displaying insecticidal properties are now found only in wild accessions of the *Phaseolus vulgaris* (Cardona et al., 1990), and coding genes have somehow been silenced in the domesticated plant (Mirkov et al., 1994). This silence can be reversed if the expression of phaseolin (the main storage protein of the domesticated bean) is partially blocked. Then, the arcelins are accumulated at high levels in *P. vulgaris*

seeds increasing their resistance against bruchid attacks (Hartweck et al., 1997). Similarly, the susceptibility of the *Vigna unguiculata* to predation by *Callosobruchus maculatus* is supposed to be related to an increased digestibility of vicilins (a major class of storage proteins) by the insect's enzymes, as compared to the protein found in resistant seeds from Nigerian cowpea (Sales et al., 2000).

The co-evolution of plants and their predators has led to an adaptation of phytophagous insects to the deterrent compounds present in the plants they feed on. There are several reports in the literature describing the lack of effect of plant proteinase and α-amylase inhibitors upon the digestive enzymes of the insects adapted to a particular plant species (Broadway, 1996; Chrispeels, 1996; Jongsma and Bolter, 1997). However the same inhibitors on the other hand are very efficient in blocking enzymes from mammalian source or from other insects that do not feed on that plant. Thus, the genetic engineering of plants aiming to increase resistance to insect predation or to phytopathogens may rely exclusively on the repertoire of genes found in plants (Chrispeels, 1996).

Proteinases:

Proteinases or proteolytic enzymes catalyze the cleavage of peptide bonds in proteins. The term "protease" includes both "endopeptidases" and "exopeptidases" whereas, the term "proteinase" is used to describe only "endopeptidases" (Ryan, 1990). Since most of the plant inhibitors target mainly endopeptidases, the term proteinase inhibitor is used. Proteinases are classified according to their mechanism of catalysis and the amino acid present in the active center as serine proteinases, with a serine and histidine; cysteine proteinases, with a cysteine; aspartic proteinases, with an aspartate group and metalloproteinases, with a metallic ion (Zn^{2+}, Ca^{2+} or Mn^{2+}) (Neurath, 1984). Proteolysis is a key process in all living organisms, but must be highly regulated which otherwise could be very hazardous to their natural environment. Therefore it is not

surprising that a large number of naturally occurring PIs have been described in animals, plants and microorganisms and have been extensively studied in order to elucidate their structural and functional properties (Hibbetts et al., 1999; Haq et al., 2005; Fan and Wu, 2005).

Proteinase inhibitors (PIs):

PIs are the natural antagonists of proteinases. They are small proteins which are quite common in nature and also present in all life forms (Fritz, 2000). Most PIs interact with their target proteinases by contact with the active (catalytic) site of the proteinase, resulting in the formation of a stable proteinase-inhibitor complex and loss of enzymatic activity (Norton, 1991). PIs have an enormous diversity of function by regulating the proteolytic activity of their target proteinases (Leung et al., 2000).

Plant PIs:

In plants PIs are present as one of the most abundant class of proteins. Most storage organs such as seeds and tubers contain PIs, which inhibits different types of enzymes (Lawrence and Koundal, 2002). Seeds usually accumulate PIs during maturation and the concentrations of these inhibitors vary between 1-10% of the total seed proteins (Ryan, 1981). All these proteins share a common trait, the ability to form complexes with proteinases, within which the enzymes lose their activity (Laskowski and Kato, 1980; Bode and Huber, 2000). Because of their ability to inhibit the enzymes involved in the digestive processes of humans and animals, plants PIs have been referred to as ‘‘antinutritional compounds’’ so far. Some of them have now been favorably reconsidered in view of the potential exploitation of their biological properties in pharmacological and medical applications (Scarafoni et al., 2007). Majority of PIs studied in plant kingdom originates from three main families namely Leguminosae, Solanaceae and Gramineae (Richardson, 1991).

Classification of plant PIs:

Proteinases are classified based on the active site residues involved in hydrolysis, as serine, cysteine, aspartic and metallo proteinases (Barrett, 1999). Hence, the classification of PIs generally follows the proteinase catalytic type (Laskowski and Kato, 1980). Thus there are many families of the inhibitors that inhibit serine proteinases, several families that inhibit cysteine proteinases and others that inhibit aspartate and metallo-proteinases (**Table 1.1**). In addition, there are multi-headed inhibitors which have more than one active site on one PI peptide chain (Bode and Huber, 2000) and PIs that consists of multiple peptide chains with different specificities, and as well as multifunctional PIs that have only one active site but inhibit more than one PI class (Bode and Huber, 2000).

(i) Serine PIs:

Serine PIs are universal throughout the plant kingdom and have been described in many plant species and the number of known and partially characterized serine PIs are enormous (Haq et al., 2004). They reported from a variety of plant sources and are most studied class of PIs. All the serine PI families shown in **Table 1.1** are competitive inhibitors and inhibit the proteinases with a similar standard mechanism (Laskowski and Kato, 1980; Bode and Huber, 2000). However, some non-competitive type of inhibitors are also reported (Macedo et al., 2003; Araujo et al., 2005; Oliveira et al., 2007).

In general, serine PIs behave as pseudo-substrates, with the amino acid at position P_1 of the inhibitor determining the specificity for the enzyme, either trypsin or chymotrypsin (Bode and Huber, 1992). In spite of differences in primary structure and topology, the reaction center structure and mechanism of action are well preserved among serine PIs (Qi et al., 2005). Some of the plant serine PIs are bifunctional molecules being able to inhibit trypsin as well as α-amylase (Strobl et al., 1995; Haq et al., 2005).

Kunitz-inhibitor family: The Kunitz inhibitor family is a widespread family first described in legumes (Laskowski and Kato, 1980), but found in wide range of species including *Arabidopsis thaliana,* cereals, and solanaceous species (Ishikawa et al., 1994). A protein exhibiting the activity of a trypsin inhibitor was isolated by Kunitz from soybean (*Glycine max*) and crystallized in 1945 (Kunitz, 1945). This protein was the first PI to be purified from a plant source and characterized in detail. The inhibitor, known as soybean Kunitz trypsin inhibitor (SKTI), contains 181 amino acid residues and has two disulfide bonds (**Fig. 1.1**). The active site of the inhibitor, localized inside a peptide loop (limited by the disulfide bond between Cys39 and Cys86 residues) contains amino acid residues Arg63-Ile64 in the position P1-P1'.

Further large number of Kunitz type inhibitors were detected in leguminous plants, mostly belonging to subfamilies Mimosaceae and Caesalpinieae, which comprise more ancient representatives of the family (Norioka et al., 1988). The majority of the inhibitors appear as proteins with a molecular weight of about 20 kDa, containing 160–200 amino acid residues joined together into a single polypeptide chain and two disulfide bonds (Cavalcanti et al., 2002; Macedo et al., 2003; Liao et al., 2007; Lingaraju and Gowda, 2008). The inhibitors are also composed of two polypeptide chains of unequal size. The larger subunit (α-chain) contains 140-150 amino acid residues with its molecular weight 15 kDa, and the smaller subunit (β-chain) contains 40-50 residues with its molecular weight around 5 kDa. The chains are linked via a disulfide bond (Delgado-Vargas et al., 2004; Bhattacharyya et al., 2006, 2007b; Zhou et al., 2008). The active site is located within the α-chain (Odani et al., 1980). The two chains are formed as a result of proteolytic cleavage of the original single chain molecule and it is not clear why the presence of two-chain inhibitors is limited to species of the subfamily Mimosaceae in leguminous plants.

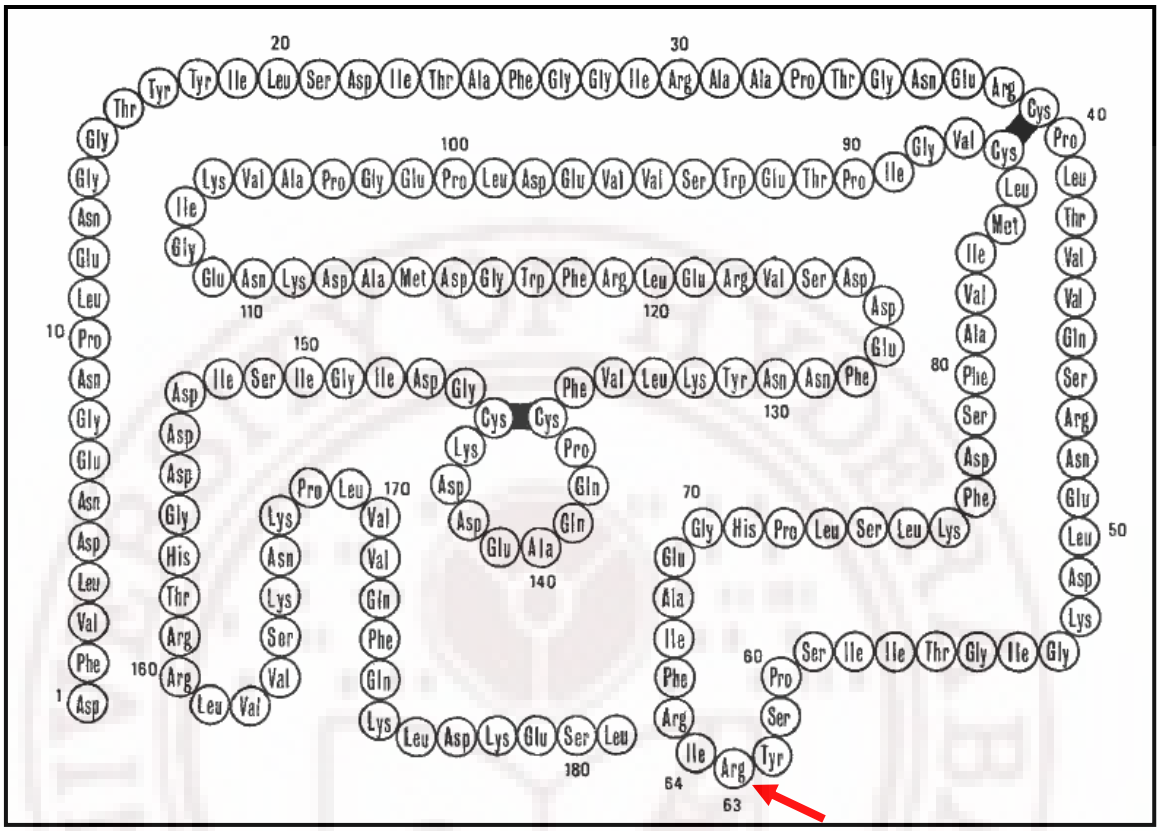

Fig. 1.1. Amino acid sequence of soybean Kunitz trypsin inhibitors (SKTI). Figure adapted from Koide et al. (1973). Arg at 63 position is the P_1 residue indicated with red arrow.

The majority of inhibitors of the Kunitz family have one active site. The inhibitor from asparagus pea (*Psophocarpus tetragonolobus* L.) is an exception, since it is capable of binding two chymotrypsin molecules at the same time. The first active site is located in the same position as the active sites of other members of the family (Shibata et al., 1988). The location of the second site has been tentatively determined, based on crystal X-ray structure data (Dattagupta et al., 1999). In general, the PIs contains several isoforms of an inhibitor. Thus, at least ten SKTI genes are found in the soybean genome, which are expressed in distinct parts of the plant or at different stages of its development (Krishnan,

2001). Individual isoforms may differ considerably in their effects on the enzymes. For example, the seed of *Erythrina latissima* L. contain two inhibitors, one of which (DE-1) acts on chymotrypsin, but not trypsin, whereas the other (DE-3) is a specific trypsin inhibitor. Certain inhibitors also inactivate other serine proteinases, such as tissue plasminogen activator (Joubert, 1987) and blood plasma proteinases (Batista et al., 1996; Nakagaki et al., 1996).

Bowman-Birk Inhibitors (BBIs) family: The BBI family is one of the most widespread groups of serine PIs, with members found in many botanical families. The family is named after Bowman (1946) and Birk (Birk et al., 1963), who were the first to identify and characterize a member of this family from soybean. The soybean inhibitor is now the most-well-studied member of this family and is often referred as the classic BBI. These are particularly abundant in legume seeds compared with their concentrations in other legume organs or in other plant families. The inhibitors have been mainly found in legumes and cereals (Laing and McManus, 2002; Norioka and Ikenaka, 1983).

BBI from legumes such as *G. max* and pea (*Pisum sativum*), are proteins consist of a single polypeptide chain with molecular masses in the range of 6-9 kDa (Clemente and Domoney, 2006). They usually comprise two distinct binding loops (**Fig. 1.2**), responsible for the inhibition of two enzyme molecules, that may be same or different enzymes (Singh and Appu Rao, 2002). Such inhibitors can interact simultaneously and independently with two target proteinases without any substantial conformational change. BBI-like proteins interact with the enzymes they inhibit *via* their binding loops that adopt a characteristic canonical proteinase inhibitory conformation, similar to that of a bound substrate (Bode and Huber, 2000). The resulting non-covalent complex renders the proteinase inactive. The first reactive site in these inhibitors is usually specific for trypsin, chymotrypsin and elastase (Qi et al., 2005). The active site configuration in these inhibitors is stabilized by the presence of seven conserved disulfide bonds (Qi et al.,

2005). A particular feature of the BBI family is the high content of cysteine residues involved in a highly conserved array of disulphide bridges (Birk, 1985). Circular dichroism and fluorescence studies have demonstrated that the 14 half-cysteine residues, involved in seven disulphide bridges, play a major role in maintaining the structural stability of BBI-like proteins (Ramasarma et al., 1995).

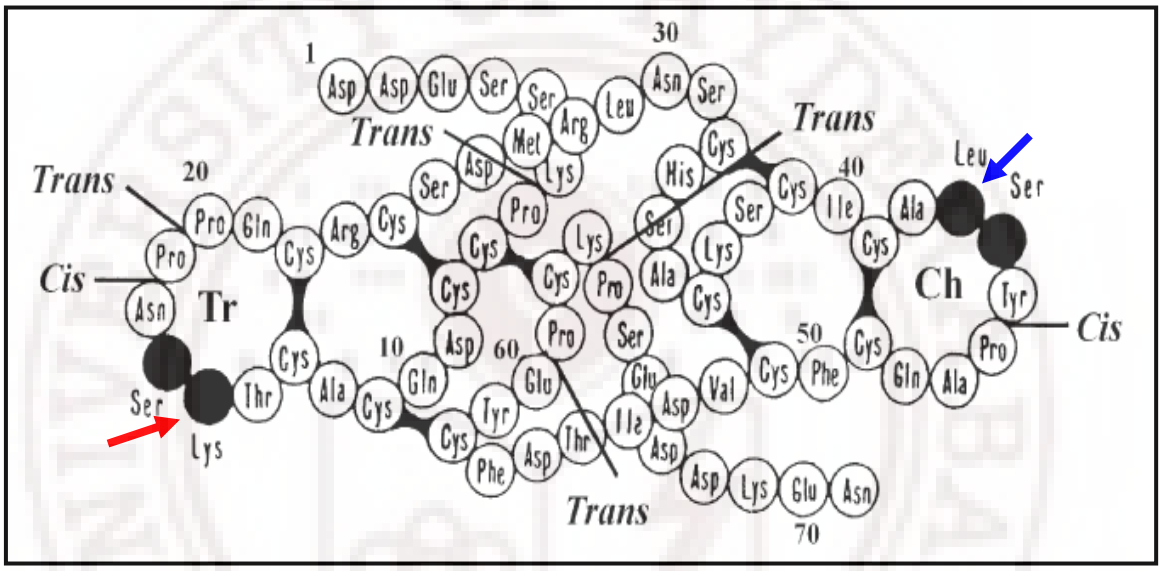

Fig. 1.2. Primary structure of soybean Bowman-Birk inhibitor. Figure adapted from Odani and Ikenaka (1972). Lys and Leu are the P_1 residues (indicated with red and blue) in the inhibitory site for trypsin and chymotrypsin, respectively.

BBIs from monocotyledonous plants are of two types. One group consists of a single polypeptide chain with a molecular mass of about 8 kDa. They have a single reactive site. Another group has a molecular mass of 16 kDa with two reactive sites (Prakash et al., 1996). It has been suggested that larger inhibitors have arisen from smaller ones by gene duplication (Odani et al., 1986). In the case of double-headed BBIs, it has been found that the relative affinity of binding of proteases is altered when one site is already occupied. Peanut (*Arachis hypogea*) inhibitor has been found to exhibit no activity against chymotrypsin when preoccupied with trypsin and vice versa (Tur et al.,

1972). In the same way, the activity of soybean BBIs decreases by 100-fold when trypsin is bound at the other site (Gladysheva et al., 1999). The BBI family of PIs contains a unique disulfide-linked nine-residue loop that adopts a characteristic canonical conformation. The loop is called protease-binding loop and binds the protease in a substrate-like manner (Clemente and Domoney, 2006).

The presence of multiple variants of BBI-like proteins within and among species is well-documented. This polymorphism can be attributed both to the expression of distinct genes and to post-translational modifications of the primary gene products (Domoney, 1999; Domoney et al., 2002). In recent years, cyclic peptides based on inhibitory loops have been used as molecular tools with which the functional and biological properties of BBI and its variants can be investigated (McBride and Leatherbarrow, 2001). These peptides have allowed the effects of single amino acid changes on the functional properties of the proteinase-binding sites to be determined (McBride et al., 2002). Interestingly, a naturally occurring cyclic peptide (SFTI-1) has been isolated from sunflower (*Helianthus annuus*) that inhibits trypsin strongly (K_i = 100 pM) and shows homology to sequence within the inhibitory domain of BBI-like proteins, although derived apparently from a precursor that is unrelated to BBI (Mulvena et al., 2005).

Many aspects of the biology of BBI proteins have been investigated, ranging from structural features to functional and biological properties (Losso, 2008). The importance of these inhibitory sites differ from each other with respect to biological significance (Clemente and Domoney, 2006). The trypsin inhibitory site has been associated with protection of plants against insects and microorganisms, and also showed negative effect on bioavailability of dietary proteins. Conversely, the chymotrypsin inhibitory site has been implicated in cancer chemopreventive effects (Kennedy, 1998). BBI-like proteins have been considered to be major anti-nutritional compounds in legume seeds. Their

involvement in crop protection has been demonstrated clearly, although their physiological role as significant stores of sulphur amino acids in dormant seeds and/or as regulators of endogenous enzymes in plants is less clear (Richardson, 1991; Domoney, 1999). Recent work demonstrating that soybean BBI has cancer chemopreventive properties has led to a resurgence of interest in these proteins from the scientific community (Kennedy 1998). The soybean derived BBI with a well characterized ability to inhibit trypsin and chymotrypsin is particularly effective in suppressing carcinogenesis in a variety of *in vivo* and *in vitro* systems (Clemente et al., 2005; Kennedy et al., 2003).

Cereal trypsin/α-amylase inhibitor family: The members of this family have serine proteinase inhibitory activity and/or α-amylase inhibitory activity (Gourinath et al., 2000). A large number of inhibitors in this family have only α-amylase-inhibitory activity, however inhibitors from barley (*Hordeum vulgare*), rye (*Secale cereale*) and tall fescue (*Festuca arundinacea*) are active against trypsin (Odani et al., 1983). Maize (*Zea mays*) and ragi (*Elusine coracana*) inhibitors show dual activities and can inhibit serine proteinases as well as α- amylases (Mahoney et al., 1984). The cereal trypsin/α-amylase inhibitors consist of a single polypeptide chain containing five disulfide bonds with a molecular mass of about 13 kDa (Christeller and Laing, 2005). The structure of the ragi inhibitor resolved by NMR spectroscopy and its complex with α-amylase from yellow mealworm (*Tenebrio molitor*) by x-ray crystallography has shown that the proteinase-binding loop adopts a canonical conformation (Strobl et al., 1998).

Potato inhibitor I (PI-1) family: The inhibitors of this family are widespread in plants and have been described in many species, including potato tubers (Ryan and Balls, 1962), tomato fruit (Wingate et al., 1989), squash phloem exudates and in tomato leaves in response to wounding (Lee et al., 1986). Recently from the seeds of cucurbitaceous plant, *Momordica cochinchinensis* chymotrypsin inhibitor belonging to potato inhibitor-I family was reported. These inhibitors have the molecular mass of 8 kDa and are generally

monomeric. However isoinhibitors from potato tubers form an oligomeric structure consistent with a pentamer on molecular sieving chromatography. PI-1 family inhibitors are unusual in they generally lack disulphide bridges, which are typical of most inhibitor families, acting to assist in stabilizing the inhibitor structure. However, Cucurbit (Cai et al., 1995) and potato tuber inhibitors possess a single disulphide, but this is not involved in stabilizing the reactive site loop. The inhibitory mechanism in this family is considered to fit the standard model (Christeller and Laing, 2005).

Potato inhibitor II (PI-2) family: The members of this group have been reported only from the members of Solanaceae family. Initially characterized from potato tubers (Christeller and Laing, 2005), these inhibitors have been found in leaves, flowers, fruit and phloem of other solanaceaous species (Pearce et al., 1993). A low molecular-mass inhibitor of this family has been found to be constitutively present in Jasmme tobacco (*Nicotiana alata*) flowers (Atkinson et al., 1993). Six small wound-inducible PIs of this family have been reported from tobacco leaves (Pearce et al., 1993). An analysis of isolated inhibitor II proteins and genes has shown that they are composed of multiple repeat units, varying from one to eight (Antcheva et al., 2001; Miller et al., 2000; Choi et al., 2000). Inhibitors in this family have been reported to inhibit chymotrypsin, trypsin, elastase, oryzin, Pronase E and subtilisin.

Mustard trypsin inhibitor (MSI) family: These are small single polypeptide chain inhibitors with the molecular mass of about 7 kDa, found in the family Cruciferae, which includes *A. thaliana* (Laing and McManus, 2002). These inhibitors have been isolated and characterized from a number of species including white mustard (*Sinapis alba*) and rape (*Brassica napus*) (Volpicella et al., 2000). These inhibitors are expressed in seeds during their development and are also wound-inducible (De Leo et al., 2001). There is a possibility that these inhibitors evolved from BBIs (Menengatti et al., 1992) as the two inhibitors show about a 27% similarity of amino acids over 63 residues that make up the

mature mustard seed inhibitor. However, the alignment, while including only a one-residue gap, does not align the two reactive sites. The inhibitors followed the standard model in forming a tight binding complex with trypsin, and being cleaved by trypsin (Ceciliani et al., 1994).

Squash inhibitors family: The squash inhibitor family is a plant unique inhibitor family, have been described only in plants, and particularly form the seeds of Cucurbitaceae. It is the smallest inhibitor family known, consist of a small single peptide chain containing between 28 and 30 amino acids with molecular mass of 3.0 - 3.5 kDa, and P_1 at ~5^{th} amino acid from the N-terminal. The squash inhibitors also have three disulphide bridges and fold in a novel knottin structure (Heitz et al., 2001) with similarity to potato carboxypeptidase inhibitor. The members of this family have been described from many cucurbit families (Felizmenio et al., 2001). Seven serine PIs belonging to this family have been isolated and characterized from the seeds of wild cucumber (*Cyclanthera pedata*) (Kuroda et al., 2001). Recently two different but inter-convertible (cis-trans isomers) inhibitors have been isolated and characterized from seeds of wax gourd (*Benincasa hispida*) (Atiwetin et al., 2006). The small size of these inhibitors, combined with potential activity against important biological molecules such as Hageman factor, human leucocyte elastase and cathepsin G (McWherter et al., 1989), has made them particularly attractive for studying proteinase and inhibitor interactions.

(ii) Cysteine PIs:

Plant cystatins or phytocystatins are the second most studied class of inhibitors and have been identified and characterized from several plants like sunflower, rice, wheat, maize, soybean, sugarcane, cowpea, potato, cabbage, ragweed, carrot, papaya, apple fruit, avocado, chestnut, Job's tears, etc (Haq et al., 2004). The transgenic plants expressing cysteine PIs have shown the most promising results, probably because most phytophagous insects employ these proteinases in their digestive mechanism.

The rice cysteine PIs are well studied of all the cysteine PIs and are highly heat stable (Abe et al. 1987). Three dimensional structure analysis of oryzacystatin (OC-I), using NMR has showed a well defined main body consisting of amino acids from Glu^{13}-Asp^{97} and an α-helix with five stranded anti parallel β-sheet, while the N terminus (Ser^{2}-Val^{12}) and C terminus (Ala^{98}-Ala^{102}) are less defined (Nagata et al. 2000). Further, analysis has demonstrated OC-I to be similar to chicken cystatin which belongs to type-2 animal cystatin.

Aspartic PIs:

Aspartic PIs are a relatively less studied class partly due to their rarity of occurrence. Potato tubers possess an aspartic proteinase (cathepsin D) inhibitor that shares considerable amino acid sequence identity with SKTI (Marres et al., 1989). Cathepsin D inhibitor is the only well-characterized aspartic PI (Keilova and Tomasek, 1976). The cathepsin D inhibitor (27 kDa) is unusual as it inhibits trypsin and chymotrypsin as well as cathepsin D, but does not inhibit aspartyl proteases such as pepsin, rennin or cathepsin E. Aspartic PIs have been also been isolated from sunflower (Park et al. 2000), barley (Kervinen et al. 1999) and cardoon (*Cyanara cardunculus*) flowers named as cardosin A (Frazao et al. 1999). The detailed structural analysis of prophytepsin, a zymogen of barley aspartic proteinase shows a pepsin like bilobe and a plant specific domain. The N terminal has 13 amino acids necessary for inactivation of the mature phytepsin (Kervinen et al. 1999), and the aspartic PI cardosin A from cardoon shows regions of glycolylations at Asn-67 and Asn 257. The Arg-Gly-Asp sequences recogonizes the cardosin receptor which is found in a loop between two-beta strands on the molecular surface (Frazao et al. 1999). The squash (*Cucurbita maxima*) phloem exudates expressed a novel aspartic PI (SQAPI), constituting a fifth family of aspartic PIs. However, a comparison of the SQAPI sequence to the phytocystatin (a cysteine PI) family sequences showed 30% identity (Christeller et al., 2006).

Metallo PIs:

The metallo-proteinase inhibitors in plants are represented by the metallo-carboxypeptidase inhibitor family in tomato and potato plants (Rancour and Ryan, 1968; Graham and Ryan, 1997). The inhibitors of the metallo-carboxypeptidase from tissue of tomato and potato are polypeptides (4 kDa) that strongly and competitively inhibit a broad spectrum of carboxypeptidases from both animals and microorganisms, but not the serine carboxypeptidases from yeast and plants (Havkioja and Neuvonen, 1985). The inhibitor is found in tissues of potato tubers where it accumulates during tuber development along with potato inhibitor I and II families of serine proteinase inhibitor. The inhibitor also accumulates in potato leaf tissues along with inhibitor I and II proteins in response to wounding (Hollander-Czytko et al. 1985). Thus, the inhibitors accumulated in the wounded leaf tissues of potato have the capacity to inhibit all the five major digestive enzymes like, trypsin, chymotrypsin, elastase, carboxypeptidase A and carboxypeptidase B of higher animals and many insects (Hollander-Czytko et al. 1985).

Table 1.1. Classification of PIs (Table adapted from Laing and McManus, 2002).

Proteinase inhibited	Name of the PI family	Molecular weight range	Known distribution
Serine	Bowman-Birk inhibitor	6000-9500 Da (cereals 14000-15000 Da)	Legumes, *Zea mays*, rice, wheat and barley
	Cereal trypsin and α-amylase inhibitor	11500-14000 Da	Barley, wheat, sorghum, ragi, rice and rye
	Kunitz inhibitor	19000-24000 Da	Legumes, potato, cereals and *Arabidopsis*, and in other spp. Usually found in seed
	Mustard trypsin inhibitor	6600-7100 Da	Brassicaceae, including mustard, rape and *Arabidopsis*
	Proteinase inhibitor 1	7200-9100 Da	Solanaceous species, cereals, squash (seed and phloem), *Arabidopsis* and legumes
	Proteinase inhibitor 2	5000-6000 Da	Solanaceous species including potato, tomato, tobacco, aubergine and capsicum. Found in tubers, fruits, seeds, leaves and flowers
	Serpins	42000-44000 Da	Wheat, rice, barley, *Arabidopsis*, squash, soybean and cotton
	Squash inhibitor	3000-3500 Da	Cucurbit seeds

Proteinase inhibited	**Name of the family**	**Molecular weight range**	**Known distribution**
Cysteine	Cystatins	10000-16000 Da	Rice, maize, wheat, potatoes, soybean and *Arabidopsis* Found in seeds, tubers, pollen, flowers and leaves
	Multicystatin	6000-87000 Da	Potato (8 active sites), sunflower (3 active sites) and other species
	Kunitz inhibitor	20089 Da	Potato
	Pineapple bromellain inhibitor	5800-5900 Da	Pineapple
Aspartic	Kunitz	20000-21000 Da	Potato
	SQAPI	10500 Da	Squash
	Wheat inhibitor	58000 Da	Wheat
Metallo	Carboxy-Peptidase inhibitor	4100-4300 Da	Solanum

Physiological role of PIs:

Endogenous function: Initially, it was thought that many plant PIs do not have endogenous functions against plant proteases but show specificities for animal or microbial enzymes (Reeck et al., 1997). Hence they could be applied to combat invasion by pest or pathogen by their action on foreign proteolytic enzymes (Ryan, 1989; Brzin and Kidric, 1995). Such conclusions may have culminated from studies that use commercially available proteases, e.g., trypsin, chymotrypsin, elastase, and subtilisin from animal or microbial sources, as test enzymes in activity assays with plant PIs (Brzin and Kidric, 1995). Subsequently, accumulating evidences which supported the developmental regulation and tissue-specific accumulation of plant PIs suggested for their endogenous functions (Lorberth et al., 1992).

PIs regulate endogenous proteinase levels before and during seed germination for storage protein digestion and to control protein turnover (Ryan, 1981). Arg-1, a proteinase isolated from *Ipomoea batatas* was completely inhibited by sweet potato trypsin inhibitor (Hou and Lin, 2002). The 25 kDa inhibitor from cucumber leaves, which was not showing significant inhibition against proteinases from animals and microorganisms, showed competitive inhibition against glutamyl endopeptidase from the leaves of the cucumber (Yamauchi et al., 2001). Proteinase inhibitor II (PIN2) from *Solanum americanum* not only enhanced resistance to caterpillars when expressed exogenously, but also inhibited endogenous proteases that are expressed during seed development (Chye et al., 2006).

As storage proteins: The PIs serving as storage proteins in plants was identified by the presence of high content of inhibitors in seeds and other storage organs of plants and of their dynamics in the course of seed maturation and germination (Richardson, 1977). Similar to other storage proteins, inhibitors are located in vacuoles and protein bodies (Wingate et al., 1991). It was also demonstrated that certain PIs belong to the same

protein families as storage proteins and thus had common origin. A significant homology was found between barley trypsin inhibitor and castor glutamine-rich storage protein (Odani et al., 1983). Barley and wheat proteinase and α-amylase inhibitors belonged to the same protein superfamily as prolamins of the tribe Triticeae (Kreis et al., 1985).

On the other hand, certain storage proteins of plants exhibit activities of PIs (Genov et al., 1997). In addition, the storage proteins like sporamin of *I. batatas* (Yeh et al., 1997), G2 storage globulin of *Colocasia esculenta* (Shewry, 2003) and dioscorin, a major storage protein of *Dioscorea batatas* (Hou et al., 1999) showed trypsin inhibitory activity. It is generally believed that PIs emerged in the course of evolution of storage proteins (Odani et al., 1983). A 2S storage protein from the seed of *Brassica juncea* is formed via processing of a precursor exhibiting the activity of a trypsin inhibitor (Mandal et al., 2002). The vacuoles of maturating seed of *Cucurbita maxima* contain a high molecular weight protein (100 kDa), which serves as common precursor for a vicilin-like protein and a trypsin inhibitor (Yamada et al., 1999).

Protective agents: The possible role of PIs in plant protection was investigated as early as 1947 when, Mickel and Standish observed that the larvae of certain insects were unable to develop normally on soybean products. Subsequently the trypsin inhibitors present in soybean were shown to be toxic to the larvae of flour beetle, *Tribolium confusum* (Lipke et al. 1954). Following these early studies, there have been many examples of PIs active against certain insect species, proved by both *in vitro* assays against insect gut proteases (McManus and Burgess, 1995; Giri et al., 2003; Oliverira et al., 2007; Pereira et al., 2007) and in *in vivo* artificial diet bioassays ((McManus and Burgess, 1995; Bhattacharyya et al., 2007a; Ramos et al., 2009; Telang et al., 2009). On the other hand, the PI gene expression has been detected in leaves of several species following wounding caused by herbivory. These studies suggest their role in protecting plants from insect attack and microbial infection (Ryan, 1990).

Modulation of apoptosis: A new role for PI in the modulation of apoptosis or programmed cell death (PCD) has been identified. Cysteine proteinase plays an important role in the regulation of programmed cell death leading to hypersensitive (HR) reaction, following pathogen attack. It has been shown that the ectopic expression of cystatin inhibits the induced cysteine proteinase activity, which in turn blocks PCD (Solomon et al., 1999). It was suggested that in plants balancing between the cysteine proteinase and cysteine PI activity regulates the programmed cell death. Sin and Chye (2004) demonstrated that *S. americanum* PI-II proteins, SaPIN2a and SaPIN2b are expressed in floral tissues that are destined to undergo developmental PCD, suggesting possible endogenous roles in inhibiting trypsin- and chymotrypsin-like activities during flower development.

Localization of PIs:

In plants, PIs are produced either as prepro-proteins or pre-proteins (Graham et al., 1985), which are subsequently subjected to *in vivo* processing. Furthermore, they have been subcellularly localized to various compartments in the plant cell. SKTI was reported to be associated with the cell walls and to a lesser extent with protein bodies, cytosol, and nuclei, while the BBI has been localized in protein bodies, nuclei, and cytosol (Horisberger and Tacchini-Vonlanthen, 1983). Trypsin inhibitor from *V. radiata* has been reported to occur in the cytosol of cotyledonary cells (Baumgartner and Chrispeels, 1976). However, the wound-induced inhibitors of tomato (Wingate et al., 1991), potato (Hollaender-Czytko et al., 1985), and *S. americanum* (Sin and Chye, 2004) are located in vacuoles. Xu et al. (2004) described the expression of a PIN2 protein of *S. americanum* in phloem of stems, roots, and leaves suggesting a novel endogenous role for PIN2 in phloem. A chickpea Kunitz trypsin inhibitor is located in the cell wall of different organs (Hernandez-Nistal et al., 2009).

Mechanism of action:

The mechanism of binding of the plant PIs to the insect proteases appears to be similar with all the four classes of inhibitors. The inhibitor binds to the active site on the enzyme to form a complex with a very low dissociation constant (10^{-7} to 10^{-14} M at neutral pH values), thus effectively blocking the active site. A binding loop on the inhibitor, usually "locked" into conformation by a disulphide bond, projects from the surface of the molecule and contains a peptide bond (reactive site) cleavable by the enzyme (Walker et al., 1998; Birk, 2003). This peptide bond will be cleaved in the enzyme inhibitor complex, but cleavage does not affect the interaction. Therefore a hydrolyzed inhibitor molecule is bound similar to an unhydrolyzed one.

The inhibitor thus directly mimics a normal substrate for the enzyme, but does not allow the normal enzyme mechanism of peptide bond cleavage to proceed to completion i.e., dissociation of the product (Walker et al., 1998). PIs inhibit the proteinase activity of insect gut proteolytic enzymes and reduce the quantity of proteins that can be digested (Ramos et al., 2009), and also cause hyper production of the digestive enzymes which enhances the loss of sulfur amino acids (Shulke and Murdock, 1983) as a result of which, the insects become weak with stunted growth and ultimately die. Retardation of insect development, slower rate of growth and reduced fitness for survival would allow a much wider window within which the other pesticides including biopesticides could be successfully employed for the management of insects.

Functions and applications of PIs:

(i) Transgenic plants against insect resistance:

Several transgenic plants expressing PIs have been produced in the last 20 years and tested for enhanced defensive capabilities against insect pests (**Table 1.2**). Since the economically important orders of insect pests namely Lepidoptera, Diptera and

Coleoptera, use serine and cysteine proteinases in their digestive system to degrade proteins in the ingested food, efforts have generally been directed at genes encoding PIs active against these mechanistic classes of proteases for developing transgenic plants. The PI genes have been particularly useful in developing transgenic plants resistant to insect pests, as they require the transfer of a single defensive gene, and can be expressed from the wound-inducible or constitutive promoters of the host (Boulter, 1993). The first PI gene was successfully transferred was that coding for CpTi. The transgenic tobacco produced showed and transgenic tobacco with significant resistance against *Heliothis virescens* (Hilder et al., 1987). The transformation of plant genomes with PI-encoding cDNA clones appears attractive not only for the control of plant pests and pathogens, but also as a means to produce PIs useful in alternative systems, and the use of plants as factories for the production of heterologous proteins (Sardana et al., 1998).

Apart from PIs, the bean α-amylase inhibitor 1 in transgenic peas (*Pisum sativum*) provides complete protection from pea weevil (*Bruchus pisorum*) under field conditions (Morton et al., 2000). When both SKTI and BBI were introduced and expressed in transgenic sugarcane, the growth of neonate larvae of sugar-cane borer (*Diatraea saccharalis*) feeding the leaf tissues was significantly retarded as compared to larvae feeding on leaf tissues from untransformed plants (Falco and Silva-Filho, 2003). Transgenic potato expressing two cystatin genes developed resistance to a nematode, coleopteran insects (Cowgill et al., 2002) and transgenic rape plants expressing rice cystatin 1 were resistant to aphid (Rahbe et al., 2003). Expression of oryzacystatin, the rice cysteine PI, into the tobacco plant induced significant resistance against two important poly viruses, tobacco etch virus (TEV) and potato virus Y (PVY). (Campos et al., 1999). The constitutively expressed NtPI in transgenic tobacco showed tolerance to multiple stresses like sodium chloride, variable pH and sorbitol apart from resistance to insect pests (Srinivasan et al., 2009)

Table 1.2. Transgenic plants expressing proteinase inhibitors

Source gene	Transformed plant	Target insect	Reference
Cowpea trypsin inhibitor (CpTI)	Tobacco	*Heliothis virescens*	Hilder et al., 1987
	Tobacco	*Helicoverpa zea*	Hoffmann et al., 1992
	Tobacco	*Spodoptera litura*	Sane et al., 1997
	Tobacco	*Heliothis virescens, Spodoptera frugiperda*	Pujol et al., 2005
	Strawberry	*Otiorynchus sulcatus*	Graham et al., 1997
	Rice	*Sesamia inferens; Chilo suppressalis*	Xu et al., 1996
	Potato	*Lacanobia oleracea*	Gatehouse et al., 1997
	Cotton	*Helicoverpa armigera*	Li et al., 1998
	Cabbage	*Pieris rapae*	Fang et al., 1997; Hao and Ao, 1997
	Cabbage	*Heliothis armigera*	Hao and Ao, 1997
	Pigeonpea	*Helicoverpa armigera*	Lawrence and Koundal, 2001
CpTI and pea lectin	Tobacco	*Heliothis virescens*	Boulter et al., 1990
CpTI and snowdrop lectin	Sweet potato	*Cyclas formicarius*	Newell et al., 1995
	sweet potato	*Euscepes postfaciatus*	Golmirizaie et al., 1997
Soybean (Kunitz) trypsin inhibitor	Tobacco	*Spodoptera litura*	McManus et al., 1999
	Rice	*Nilaparvata lugens*	Lee et al., 1999
	Sugarcane	*Diatraea saccharalis*	Falco and Silva-Filho, 2003
Soybean serine PI (C-II)	Potato/ tobacco	Coleoptera/Lepidoptera	Shulke and Murdock, 1983
SKTI-4	Sweet potato	*Cyclas* spp.	Cipriani et al., 1999
Soybean Kunitz, C-II and PI-IV inhibitor	Potato/ tobacco	*Spodoptera littoralis*	Marchetti et al., 2000
Corn cystatin	Rice	*Sitophilus zeamais*	Irie et al., 1996
Maize PI (*mpi*)	Rice	*Chilo suppressalis*	Vila et al., 2005

Table 1.2 (continued)

Source gene	Transformed plant	Target insect	Reference
Oryzacystatin I	Poplar	*Chrysomela tremulae*	Leple et al., 1995
	Potato	*Myzus persicae*	Gatehouse et al., 1996
	Potato	*Leptinotarsa decemlineata*	Lecardonnel et al., 1999
	Oilseed rape	Cabbage seed weevil	Girard et al., 1998a
	Oilseed rape	*Myzus persicae*	Rahbe et al., 2003
Barley trypsin inhibitor (*CMe*)	Indica and japonica rice	*Sitophilus oryzae*	Alfonso-Rubi et al., 2003
	Barley	*Agrotis ipsilon*	Carbonero et al., 1993
	Tobacco	Lepidoptera	Carbonero et al., 1993
	Wheat	*Sitotroga cerealella*	Altpeter et al., 1999
Mustard trypsin inhibitor 2	Tobacco and *Arabidopsis*	*Spodoptera littoralis*	De Leo et al., 1998
	Tobacco	*Spodoptera littoralis*	De Leo and Gallerani, 2002
	Tobacco	*Plutella xylostella*	De Leo et al., 2001
	Arabidopsis	*Mamestra brassicae*	
	Oilseed rape	*Spodoptera littoralis*	
Potato PI-II	Tobacco	*Manduca sexta*	Johnson et al., 1989
	Tobacco	*Chrysodeixis eriosoma*	McManus et al., 1994
	Rice	*Sesamia inferens*	Duan et al., 1996
	Poplar	*Plagiodera versicolora*	Klopfenstein et al., 1997
	Sugarcane	*Antitrogus consanguineus*	Nutt et al., 1999
Sweet potato trypsin inhibitor	Tobacco	*Spodoptera litura*	Yeh et al., 1997
	Cauliflower	*Spodoptera litura*, *Plutella xylostella*	Ding et al., 1998
Nicotiana alata protease inhibitor	Tobacco	*Helicoverpa punctigera*	Heath et al., 1997
	Tobacco and pea	*Helicoverpa armigera*	Charity et al., 1999
	Apple	*Epiphyas postvittana*	Maheswaran et al., 2007

Table 1.2 (continued)

Source gene	Transformed plant	Target insect	Reference
Tomato PI- I, II	Tobacco	*Manduca sexta*	Johnson et al., 1989
Tomato PI- I	Nightshade Tobacco Alfalfa	-	Narvaez-Vasquez et al., 1992
Solanum americanum PI (*SaPIN2a*)	Lettuce	-	Xu et al., 2004
	Tobacco	*Helicoverpa armigera*, *Spodoptera litura*	Luo et al., 2009
Arabidopsis thaliana cysteine PI (*Atcys*)	White poplar	*Chrysomela populi*	Delledonne et al., 2001
Alocasia macrorrhiza PI (*GTPI*)	Tobacco	*Helicoverpa armigera*	Wu et al., 1997

(ii) Functional food ingredient:

Diet has shown the ability to affect the onset or progression of chronic diseases, but only recently that the molecular basis of diseases prevention by dietary involvement has been under scientific consideration (Dirsch andVollmar, 2001). Functional foods are foods consumed as part of a normal diet rather than pills. PIs, especially BBIs fit the definition of a functional food. Sound evidence from human studies using acceptable biomarkers is providing the basis for the promotion of BBI as a functional food ingredient (Losso, 2008). Proteinase levels in the body are biomarkers of health and performance. The identification of biomarkers of health and the development of food ingredients and technologies that address the issues of biomarkers will be keys to the success of functional foods.

The role of nutrition in the prevention and possibly the treatment of breast, colon, and prostate cancer is receiving considerable attention in the medical scientific

community. In particular, the role of PIs of food origin such as BBI is being recognized by biomedical researchers as potential chemopreventive agents (Kennedy, 1998; Lippman and Matrisian, 2000; Sawey, 2001; Kennedy and Wan, 2002). The contribution of PIs in the treatment of human chronic diseases has long been recognized. The rationale of providing moderate concentration of inhibitors in the diet to restore normal anti-proteinase level lies on the premises that when most disease erupts or symptoms appear, medical intervention becomes costly. PIs have the advantage of blocking the carcinogenesis process at an early stage (Wattenberg, 1992). Good nutrition is recognized as key to prevention. Food fortification has long been recognized as an effective strategy to overcome nutrient deficiency in affected populations. BBI is available in soymilk and the consumption of soymilk could serve as a means of ingesting BBI. Strategies to consume BBI within its natural matrix include soymilk, tofu, or aqueous extract of soybean. Soymilk contains isoflavones, oligosaccharides, and saponins which are all health enhancing bioactive compounds. After observing that US born descents of Asian immigrants are equally susceptible to develop the common cancers ascribed to Americans, it was suggested that genetic difference may not account for the variation in cancer rate. Rather eating habits may have a significant role to play (Kennedy, 1998).

(iii) Satiety agent:

Digestive proteinases and PIs form complexes in the intestinal tract, which are subsequently excreted. A deficiency of active digestive proteinase in the small intestine induces the further synthesis of proteolytic enzymes by the pancreas, as a result of the suppression of the negative feedback regulation of pancreatic secretion through increased release of the hormone cholecystokinin (CCK) from the intestinal mucosa (Reseland et al., 1996). CCK release has been associated with the feeling of satiety or fullness and reduced hunger in humans and animal models (Campfield, 1997). The size of the pancreas in relation to total body weight is a key factor in determining the extent to which

hypersecretion of digestive enzymes, in response to the action of BBI, results in pancreatic hypertrophy. Pathophysiological changes such as pancreatic enlargement, increased CCK secretion, and growth depression do not occur in all species fed a soybean diet (Rackis, 1974). For instance, pig exhibits pancreatic secretion and growth inhibition but no pancreatic enlargement (Yen et al., 1977). Pig is the animal closer to human as far as the gastrointestinal physiology is concerned. However, if CCK production has to be increased in human as a result of PIs in the diet, without the enlargement of the pancreas, PIs may also be good for food intake and appetite control. BBI may provide the feeling of fullness and satiety and be good for obesity control (Losso, 2008).

(iv) Chemoprevention:

PIs are well established as a class of cancer chemopreventive agents (Kennedy, 1998). While PIs from different families have been shown to prevent the carcinogenic process, the most potent of the known anticarcinogenic PIs are those with the ability to inhibit chymotrypsin-like proteinases. The BBI from soybean is until now the PI that has been most studied as an anticarcinogenic agent. A crude BBI concentrate (BBIC) was developed by Kennedy (1993) to allow clinical studies based on the properties of BBI. BBIC contains BBI, soybean cystatin, STI, isoflavones, saponins (Lipman and Matrisian, 2000). BBIC has undergone preclinical, phase I, and II of clinical trials. BBIC administered in a single oral dose between 25 and 800 chymotrypsin inhibitory units to 24 subjects with oral leukoplakia was well tolerated and showed no sign of toxicity (Armstrong et al., 2000a). *In vitro* studies using BBI have demonstrated that, BBI alone is an anticarcinogen that is effective at nano molar concentrations and has irreversible effects on cancer cells (Kennedy, 1998). Kennedy (1998) suggests that the uniqueness of BBI lies in its ability to reverse the “initiated” state of cells after initiation has occurred, a property ascribed to few anticarcinogens.

Oral cancer has of the lowest survival rates of all cancers. In the US, 30,000 new cases are diagnosed annually, more than 8,000 deaths occur, and the survival rate is about 50% after 5 years (NIDCR, 2006). In other countries, the survival rate is as low as 25% (Jane-Salas et al., 2005). BBI was found to shrink precancerous lesions in the mouth that lead to oral cancer called leukoplakia. BBI, in the form of lozenges, was administered to 32 participants with leukoplakia. BBI helped shrink the precancerous lesions in the mouth in about one-third of the participants (Kennedy, 1993). BBI administered to 24 subjects with oral leukoplakia, as single oral doses for 1 month in a phase I clinical trial, at levels approximately four times those ingested in Japanese diets was very well-tolerated and showed no toxic or allergic reactions (Armstrong et al., 2000a). In a phase II clinical trial, BBI at concentration between 200 and 1066 chymotrypsin units was administered to 32 patients with leukoplakia for 1 month (Armstrong et al., 2000b). Nineteen male subjects with benign prostatic hyperplasia and lower urinary tract symptoms participated in a phase I trial of BBIC (Malkowicz et al., 2001). The clinical data were encouraging to allow a phase II clinical trial. BBI has been shown to reduce the proliferation of MCF7 breast cancer cells through accumulation of MAP kinase phosphatase-1 (Wen et al., 2005).

(v) Other medical applications:

Epidemiological studies have suggested that people who consume soybean or pulses regularly have a reduced rate of some degenerative diseases that strike later in life. BBI had more successful stories in the medical or paramedical scientific community than in the nutrition scientific community. BBI showed the property of prevention of hair and weight loss in animal models (Evans et al., 1992), increased lifespan, ability to potentiate the cytotoxicity of cis-platinum in lung cancer treatment (Kennedy, 1998), ability to prevent and not cause some birth defects (Von Hofe et al., 1990). Kennedy (1998) pointed out that people from Asia who are regularly on a high soybean diet reproduce themselves without any sign of BBI toxicity

Chymase is a chymotrypsin-like serine proteinase found in mast cells of many mammalian species. It is a chemoattractant that may catalyze the accumulation of inflammatory cells in the development of chronic inflammatory response of allergic and non-allergic diseases (Tani et al., 2000). The chemotactic activity of chymase was inhibited by BBI, suggesting that the hydrolytic activity of chymase may be responsible for its chemotactic activity (Tani et al., 2000). Inflammation associated with tumor promotion was inhibited by BBI (Kennedy, 1998). US Patent 6,767,564 (Morris et al., 2005) covers the use of BBI for the treatment of multiple sclerosis and other autoimmune diseases. Serine PIs have also been shown to control abnormal exocytosis and secretion of endogenous proteinases which is characteristic of a number of diseases (Kennedy, 1998). The abnormal exocytosis of elastase, a serine proteinase from human neutrophil, is considered to be the primary source of tissue damage associated with inflammatory diseases as pulmonary emphysema (Ishizawa et al., 2004), adult respiratory distress syndrome (Nakayama et al., 2002), septic shock (Devine, 2003), cystic fibrosis (Greer et al., 2004), Chronic bronchitis (Lai et al., 2004) and rheumatoid arthritis (Tur et al., 2004). Compounds which directly inhibit elastase or its release from human neutrophils are of increasing interest in the development of new anti-inflammatory drugs.

Excessive protein intake has been reported to contribute to the progressive deterioration of renal function in diabetes and other disease states. Excessive protein intake may be associated with increased hyperfiltration, glomerular hypertension, and progressive deterioration of kidney function whereas restriction or use of soy protein may slow the progression of nephropathy (Anderson et al., 1998). BBI at a dose of 20 mg/kg body weight administered i.p. to 3-methylchloranthrene (MCA)-treated A/J mice caused a 15% reduction in total cholesterol (Kennedy et al., 2003). Because the liver is the major site for the production of cholesterol and since cholesterol contributes to heart disease, BBI may also contribute to the beneficial effects of soy protein in the prevention of heart disease.

Chapter 2

Scope, Approach and Objectives

Chapter 2

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Scope of the work:

The seeds of higher plants contain large quantities of biologically active enzyme inhibitors, which comprise phenolic compounds, amylase and proteinase inhibitors (PIs) (Carlini and Grossi-de-sa, 2002; Losso, 2008). PIs are generally found as constitutive components in storage tissues like seeds and tubers and are expressed in plants in response to pest and pathogen attack (Ryan, 1990). They are low molecular mass stable proteins having the ability to inhibit proteolytic enzymes, by competing with substrates for access to the active site of the enzyme. Plant PIs are capable of inhibiting proteinases from different sources, including digestive enzymes of mammals and insects as well as bacterial and fungal enzymes. The presence of PIs in seeds is essential for maintaining physiological processes, such as regulation of endogenous proteinase levels (Chye et al., 2006), as storage proteins (Mandal et al., 2002), stabilization of the enzymes during desiccation phase (Lam et al., 1999). Further, the PIs also protect seeds from pests (Haq et al., 2004) and invading microorganisms (Mosolov and Valueva, 2006). In plants, PIs play a role in controlling programmed cell death (Solomon et al., 1999) and their expression is known to increase in response to various abiotic (Casaretto et al., 2004) and biotic stresses, particularly while defending against insect pests (De Vos et al., 2006).

Majority of the plants examined for PIs, belonged to three main families namely Leguminosae, Solanaceae and Gramineae. The serine PIs isolated from various legume seeds belonged to either Kunitz or Bowman-Birk family. The Kunitz-type inhibitors (around 20 kDa) have two disulfide bridges and possess a single reactive site (Richardson, 1991). They have been shown to inhibit trypsin, chymotrypsin, subtilisin and tissue plasminogen activator, and other functions like inhibiting α-amylase and

aspartic proteinase cathepsin D (Christeller and Laing, 2005). The Kunitz-family inhibitors mainly showed insecticidal (Araujo et al., 2005; Bhattacharyya et al., 2007a; Ramos et al., 2009), antifungal (Wang and Ng, 2006), anti-inflamatory (Fook et al., 2005; Mello et al., 2009) and anti-malarial properties (Bhattacharyya and Babu, 2009) as well as inhibitory activity against blood clotting enzymes like, plasma kallikerin and factor Xa (Oliva et al., 2000).

Bowman-Birk type inhibitors (BBIs) have molecular mass between 6 and 9 kDa with high cysteine content and possess two distinct binding loops responsible for inhibiting two enzyme molecules (Clemente and Domoney, 2006). BBIs from legume seeds contained single polypeptide with a conserved and characteristic pattern of 14 cysteine residues forming 7 intrachain disulphide bridges (Prakash et al., 1996), which are responsible for stability and double headed structure of BBI (Qi et al., 2005). The two heads are located at the opposite sides of the molecule and inhibit trypsin and chymotrypsin independently or simultaneously (Singh and Appu Rao, 2002). BBIs were also shown to be inhibiting serine proteinases like duodenase and enzymes involved in pathogenesis of human diseases like chymase, tryptase, matriptase, cathepsin G and leukocyte elastase (Scarafoni et al., 2007).

The importance of these inhibitory sites differs from each other with respect to biological significance (Clemente and Domoney, 2006). The trypsin inhibitory site was shown to be associated with protection of plants against insects and microorganisms, as well as it exerted negative effect on bioavailability of dietary proteins. Conversely, the chymotrypsin inhibitory site has been implicated in cancer chemopreventive effects (Kennedy, 1998). The anti-carcinogenic properties of BBIs have been clearly demonstrated in a variety of *in vitro* and *in vivo* animal model systems (Kennedy et al., 2003; Clemente et al., 2005). In addition to their natural biological functions, they have been described as useful tools in biochemical and physiological studies to understand the

proteinase functions in human pathologies, such as blood clotting, hemorrhage, inflammation and cancer (Kennedy, 1998; Birk, 2003).

The role of PIs in plant defense against insect pests is well known (Lawrence and Koundal, 2002; Haq et al., 2004). Following the early studies of Mickel and Standish (1947) and Lipke et al. (1954), several of the PIs were tested for their efficacy against gut proteinases using *in vitro* and *in vivo* studies. These studies indicate that the PIs inhibited larval gut proteinase activity, which caused retardation of larval growth and development (Telang et al., 2009) and also a decline in the fertility and fecundity of the adult moths (Tamhane et al., 2005). Based on this several transgenic plants were produced in the last two decades and were tested in laboratory as well as in field conditions for their insecticidal potential (Haq et al., 2004; Mosolov and Valueva, 2008).

Approach of the study undertaken:

Proteinase inhibitors (PIs) are ubiquitous in nature. Plant PIs may arise to regulate the proteinases present endogenously or exogenous proteinases from different sources as part of defense mechanism. The proteinases which were inhibited by plant PIs, are of serine, cysteine, aspartic and metallo-proteinases class. The PIs are easily identified by a simple assay using synthetic substrates specific for each of the proteinases. Each of these different proteinases has specific synthetic (chemical) substrates apart from natural (protein) common substrates (casein and gelatin). For eg., trypsin, which cleaves lysine or arginine (basic residues) from C-terminal side, had a specific substrate BAPNA. On hydrolysis of BAPNA (Erlanger et al., 1961), an yellow coloured product, p-nitroanilide is formed which has maximum absorbance at 410 nm. Similarly GLUPHEPA is the synthetic substrate for chymotrypsin (Mueller and Weder, 1989). The availability of these synthetic substrates made easy to detect the specificity of the PIs.

Apart from spectrophotometric assays, PIs are clearly visualized in gels by activity staining studies using gelatin-PAGE (Felicioli et al., 1997). Copolymerization of polyacrylamide gel with gelatin is a simple method to detect PIs present in the crude extract. After electrophoresis, the gel slabs were incubated in respective proteinase solution to detect the PIs against that particular proteinase. These proteinases hydrolyze the gelatin copolymerized with polyacrylamide gels. However, in the regions where the PIs are located the proteinases can not degrade the gelatin due to the formation of proteinase-PI complex, which appear as dark blue bands on clear back ground.

The presence of PIs in leguminaceous plants is widely reported. Red gram is known to contain several isoforms of trypsin and chymotrypsin inhibitors (Kollipara et al., 1994; Pichare and Kachole, 1994, 1996; Chougule et al., 2003). The PIs from wild types of red gram are good source of inhibitors against gut proteinases of *Helicoverpa armigera* (Chougule et al., 2003). Hence we made an attempt to further screen some of the available wild types along with the cultivars to identify the potential candidates of PIs against *Achaea janata* (major devastating pest on castor) and *Spodoptera litura* (polyphagous pest). There was also an ambiguity regarding the family of the PIs present in red gram and their molecular mass. The PIs from crude extracts showed higher inhibitory activity against *A. janata* gut proteinase. Hence we further aimed to purify and characterize PIs from red gram variety ICP 14770. The presence of serine type PIs in leguminous plants is well acquainted. But reports from literature revealed the presence of cysteine type PIs in black gram. To resolve this ambiguity we further intended to purify PIs also from black gram variety TAU-1.

After purification the PIs (RgPI and BgPI) from red gram and black gram, were subjected to biochemical and structural characterization. Occurrence of isoforms are common in plant PIs (Domoney et al., 1995). The iso-inhibitors of RgPI and BgPI were subjected to in gel tryptic digestion and further analyzed in MALDI-TOF/MALDI-TOF-

TOF. The spectrum obtained was searched in Mascot with available database for homology. BBI type inhibitors are familiar to have high cysteine content which offers greater stability to them. Hence we examined effect of temperature, pH as well as reduction of cysteine residues with DTT on the activity and structural conformation of these PIs. The chemical modification of amino acid residues of RgPI/BgPI was performed to identify the residues responsible for determining the specificity of their binding towards trypsin or chymotrypsin.

The role of PIs in combating the insect pests is well established and is widely demonstrated by using *in vitro* and *in vivo* experiments using purified PIs. Inhibition of digestive gut proteinases by *in vitro* methods is a simple approach to evaluate the PIs for their potential to suppress the growth of insect pests (Christeller and Shaw, 1989). The insecticidal potential of RgPI and BgPI was examined by assessing the inhibitory activity of purified PIs against midgut trypsin-like proteinases isolated from the fifth instar larvae of *H. armigera, S. litura, A. janata, P. demoleus, A. albistriga, C. cephalonica, B. mori* and *D. nerii*. The midgut proteinases of these insect larvae mainly contained trypsin-like activity, when compared to chymotrypsin-like activity. Hence, we examined the inhibitory activity of RgPI and BgPI only against midgut trypsin-like proteinases in all *in vitro* studies.

For the establishment of an efficient novel insect control strategy based on PIs, an understanding on the digestive proteinases of target insect is also necessary apart from knowledge on inhibitor purification (Patanker et al., 2001; Haq et al., 2004; Araujo et al., 2005). Hence the midgut proteinases were characterized by performing the casein-SDS-PAGE (7.5%) (Garcia-Carreno et al., 1993). The midgut proteinases were incubated with proteinase specific chemical inhibitors like PMSF, TPCK and TLCK (irreversible inhibitors, 300 μM each) prior to electrophoresis to identify the various trypsin/chymotrypsin proteinases and their location in the respective zymograms. To

examine the sensitivity of these trypsin/chymotrypsin proteinases to PIs from red gram and black gram, the gel strips after electrophoresis were incubated, either with RgPI or with BgPI along with a positive control BBI (100 µg/ml).

However, the strong inhibitory activity observed in the *in vitro* assays does not necessarily retard the larval growth and development (Edmonds et al., 1996). Hence, we further performed the insect feeding assays to evaluate the antibiosis exerted by RgPI and BgPI on *A. janata*, *S. litura* and *M. sexta*. The *in vivo* effect of RgPI and BgPI on the larval growth and development of *A. janata* was performed by using leaf coating assays. Fresh castor leaves are coated with PIs in different concentrations/leaf area and newly hatched larvae are allowed to grow on them. Similarly, the effect of RgPI or BgPI on growth and development of *S. litura* and *M. sexta* was examined by feeding the larvae on artificial diet supplemented with PIs. The PIs are mixed with artificial diet (% w/v) and newly hatched larvae were allowed to grow on it. The effect of feeding of the larvae was monitored by evaluating the changes in their body weight and mortality rate.

The proposed work was fulfilled through the following **objectives:**

1. Screening of crude seed extracts of cultivars and wild types belonging to red gram for proteinase inhibitors active towards two insects *Achaea janata* and *Spodoptera litura*
2. Purification and characterization of proteinase inhibitor from seeds of red gram cultivar ICP 14770 (ICPL-332)
3. Purification and characterization of proteinase inhibitor from seeds of black gram variety TAU-1
4. Evaluation of insecticidal properties of above purified proteinase inhibitors using *in vitro* and *in vivo* assays.

Chapter 3

Materials and Methods

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Plant material:

Seeds of 14 cultivated accessions of pigeonpea and eight wild types were obtained from Rajendra S Paroda Gene Bank, International Crop Research Institute for Semi Arid Tropics (ICRISAT), Patancheru, Hyderabad, India. Seeds of black gram cultivated variety TAU-1 were obtained from local market. The details of the seed material used in the present study were shown in **Table 3.1**.

Chemicals:

Bovine serum albumin (BSA), bovine pancreatic trypsin (7500 BAEE units/mg), bovine pancreatic α-chymotrypsin (7500 ATEE units/mg) and casein (Hammerstein grade) were obtained from Sisco Research Laboratory (SRL), Mumbai, India. DEAE-Cellulose, Sephadex G-50, Cyanogen Bromide-activated-Sepharose 4B, Coomassie Brilliant Blue R250, *N*-α-benzoyl-DL arginine-*p*-nitroanilide (BAPNA), *n*-glutaryl-L-phenylalanine-*p*-nitroanilide (GLUPHEPA), N-p-Tosyl-L-Phenylalanine Chloromethyl Ketone (TPCK), Nα-p-Tosyl-L-Lysine Chloromethyl Ketone (TLCK), Phenyl methyl sulfonyl fluoride (PMSF), Trypsin inhibitor (SKTI, soybean), trypsin-Chymotrypsin inhibitor (BBI, from soybean), 1,2-Cyclohexanedione, 2,4,6-Trinitrobenzenesulfonic acid (TNBS), *N*-Acetylimidizole, *N*-Bromosuccinimide, Diethyl pyrocarbonate (DEPC), Gelatin and Tricine (N-tris(hydroxymethyl) methyl glycine) were obtained from Sigma (St. Louis, MO, USA). Immobiline dry strips (pH 3-11 non-linear, 11 cm), IPG buffer (pH 3-11 non-linear), 3[(3-Cholamidopropyl) dimethylammonio]-propanesulfonic acid (CHAPS), Dithiothreitol (DTT), Urea and Iodoacetamide were procured from GE Healthcare Bio-Sciences Corp., USA. Sequence grade modified porcine trypsin was obtained from Promega. All other chemicals and reagents were of analytical grade.

Table 3.1. Plant material used in the present study

S. No.	ICP No.	Scientific name	Alternative accession identifier	Cultivar name	Origin
1.	ICP 2376	*Cajanus cajan*	RG 102; P 3888		India
2.	ICP 7118	*Cajanus cajan*		C 11	India
3.	ICP 7182	*Cajanus cajan*		BDN 1	India
4.	ICP 7623	*Cajanus cajan*		BDN 2	India
5.	ICP 11298	*Cajanus cajan*	ICWR-Sel 7867		ICRISAT
6.	ICP 11300	*Cajanus cajan*	IC-BR-Sel. 4765		ICRISAT
7.	ICP 11950	*Cajanus cajan*	2223-1-E8-3E8		ICRISAT
8.	ICP 11957	*Cajanus cajan*	8094-2-S2X		ICRISAT
9.	ICP 11962	*Cajanus cajan*	PPE-36-2		ICRISAT
10.	ICP 11964	*Cajanus cajan*	PPE-45-2-3E		ICRISAT
11.	ICP 11968	*Cajanus cajan*	6840-E1-3EB		ICRISAT
12.	ICP 13198	*Cajanus cajan*	ICP 4070-E2-5EB		ICRISAT
13.	ICP 13210	*Cajanus cajan*	GS-1-4EB		ICRISAT
14.	ICP 14770	*Cajanus cajan*	ICPL 332	Abhaya	ICRISAT
15.	ICP 15625	*Cajanus albicans*	ICPW 024		India
16.	ICP 15639	*Cajanus lanceolatus*	ICPW 038		Australia
17.	ICP 15665	*Cajanus platycarpus*	ICPW 064		India
18.	ICP 15761	*Cajanus sericeus*	ICPW 160		India
19.	ICP 15774	*Cajanus volubilis*	ICPW 173		India
20.	ICP 15815	*Rhynchosia bracteata*	ICPW 214		India
21.	ICP 15859	*Rhynchosia rothii*	ICPW 258		India
22.	ICP 15868	*Rhynchosia sublobata*	ICPW 267		South Africa
23.		*Vigna mungo*		TAU-1	India

Preparation of crude extract:

Mature dry seeds (red gram or black gram) were decorticated and ground to a fine powder, which was depigmented and defatted with several washes of acetone and hexane, respectively. The solvents were filtered off and the seed powder was air-dried. The dried seed powder was extracted with 1:6 (w/v) 50 mM Tris-HCl (pH 8.0) containing 1% PVP, under mild stirring condition for overnight at 4^{o}C. The suspension was centrifuged twice at 10,000 rpm for 20 min under 4^{o}C. The clear supernatant obtained after centrifugation was collected and used as crude extract in further purification of PIs.

Protein estimation:

Protein content was determined by Folin-Ciocalteau method using BSA as a standard (Lowry et al., 1951). The working solution consists of 4% Na_2CO_3 in 0.2 N NaOH: 2% sodium potassium tartarate: 1% $CuSO_4$ which were mixed freshly (23:1:1) before adding to the samples.

Assay of proteinase inhibitors:

Trypsin or chymotrypsin inhibitory activity was determined by using appropriate volumes of crude extract or purified protein that results in 40-60% decrease in corresponding enzyme activity. Assay mixture (1.0 ml) consists of PIs in assay buffer, 50 mM Tris-HCl, containing 20 mM $CaCl_2$ either at pH 8.2 for trypsin or pH 7.8 for chymotrypsin. 10 µg of trypsin or 80 µg of chymotrypsin was added to the assay mixture and incubated for 15 min at 37^{o}C. Residual proteinase activity in the above assay mixture was determined after incubating for 45 min at 37^{o}C using 1 mM BAPNA (1.0 ml) as a substrate for trypsin (Erlanger et al., 1961) and 1 mM GLUPHEPA (1.0 ml) as a substrate for chymotrypsin (Mueller and Weder, 1989). The reaction was terminated by adding 0.2 ml of 30% acetic acid. The activity of PIs was expressed as trypsin inhibitor (TI) units/mg protein or chymotrypsin inhibitor (CI) units/mg protein. One TI or CI unit was

defined as the amount of inhibitor required to inhibit 50% of the corresponding enzyme activity. BAPNA and GLUPHEPA were dissolved in DMSO.

Purification of PIs:

Ammonium sulfate fractionation:

The crude extract was initially subjected to (i) 0 to 25%, (ii) 25 to 60% (in case of *C. cajan*) or 25-80% (in case of *V. mungo*) and (iii) 60 to 90% (in case of *C. cajan*) or 80-100% (in case of *V. mungo*) $(NH_4)_2SO_4$ fractionation. The required amount of dry $(NH_4)_2SO_4$ salt was added slowly to the crude extract or further subsequent fractions (supernatants of 0-25% suspension and further) under mild stirring conditions and the stirring was continued for 1 h at 4°C. The protein precipitated in each step was centrifuged at 12000 rpm for 20 min under 4°C and the pellet formed was dissolved and dialyzed against 50 mM Tris-HCl (pH 8.0). Subsequently, the different $(NH_4)_2SO_4$ precipitated fractions were examined for their protein content and tested for TI activity. The fraction with maximum TI activity was used for further purification.

Anion-exchange chromatography:

Activation of DEAE-Cellulose (Sigma, D3764): DEAE-Cellulose was activated according to the manufacturer's instructions (Sigma Aldrich Chemicals Pvt. Ltd.). About 35 ml of pre-swollen DEAE-Cellulose was suspended in 5 volumes of milliq water and allowed to settle for 30 min. The settled volume is defined as the column volume (CV). The water was removed by filtration and the resin was resuspended in 2 CV of 0.1 M NaOH containing 0.5 M NaCl for 10 min and transferred to Buchner funnel where washing was continued with 2 CV of same solution. Later, the resin was sequentially washed with 2 CV of 0.5 M NaCl, followed by 0.1 M HCl containing 0.5 M NaCl and finally with milliq water until the effluent pH is >5.0. The resin was then suspended in 2 CV of 1 M NaCl and the pH of slurry was adjusted between 7.0-8.0 with NaOH. Finally

the resin was washed with 5 CV of milliq water, followed by 2 CV of 10x and 5 CV of 1x of 50 mM Tris-HCl (pH 8.0) (equilibration buffer). The suspension was degassed until the air bubbles are cleared using vacuum pump. Then the matrix was loaded in to a glass column (2.2 X 20.0 cm), which was closed at lower end with glass wool. After loading the matrix, column was equilibrated with 2 CV of 1x equilibration buffer.

DEAE-Cellulose chromatography: The 25-60% (*C. cajan*) or 25-80% (*V. mungo*) $(NH_4)_2SO_4$ fraction, which showed maximum TI activity was applied to DEAE-cellulose column, equilibrated with 50 mM Tris-HCl (pH 8.0). After reloading the sample thrice, the column was washed with above buffer until all the unbound proteins were washed away completely by monitoring the OD at 280 nm. The proteins bound to the column were eluted with a linear gradient of 0.1 to 1.0 M NaCl in the above buffer at a flow rate of 30 ml/hr. The fractions of 1.0 ml were collected and analyzed for total protein by monitoring the absorbance at 280 nm and total activity by TI assay. The fractions showing inhibitor activity against trypsin were pooled, dialyzed against 50 mM Tris-HCl (pH 8.0) containing 100mM NaCl using 4 kDa cutoff membrane and concentrated with Amicon filters (Millipore, 3 kDa cutoff).

Affinity chromatography:

Addition of ligand (trypsin) to the CNBr activated Sepharose 4B (Sigma, C9142): Trypsin was added to CNBr activated Sepharose 4B according to the manufacturer's instructions (Sigma Aldrich Chemicals Pvt. Ltd.). The dry resin was suspended (1 gm dry resin was swollen to 3.5 ml) and washed with 0.001 N HCl (pH 2-3) (200 ml/gm dry resin) using sintered glass filter (G3 grade). The use of HCl preserves the activity of the reactive groups, which otherwise lead to hydrolysis at high pH. Trypsin was dissolved in coupling buffer (0.1 M $NaHCO_3$ pH 8.3, containing 0.5 M NaCl). The resin was washed with coupling buffer (5 ml/gm dry resin) and immediately transferred to trypsin solution (30 mg trypsin in 5 ml coupling buffer). Trypsin solution and resin were mixed using

rotospin for overnight at 4^{o}C. The remaining active groups were blocked by addition of 5 ml of 1 M ethanolamine (pH 8.0) for 2 hrs at room temperature. The excess protein was removed by 4 alterative washes of coupling buffer and acetate buffer (0.1 M, pH 4.5 containing 0.5 M NaCl). Finally the resin was added to a glass column (1.3 X 15 cm) with lower end closed with glass wool and equilibrated with 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl.

Trypsin-Sepharose 4B chromatography: The active fraction pool from ion exchange column was loaded on to a trypsin-Sepharose 4B column, pre-equilibrated with 50 mM Tris-HCl (pH 8.0) containing 100mM NaCl. After passing the ion exchange fraction pool, the column was washed with above buffer until all the unbound proteins were washed out. The bound protein was eluted with 0.01 N HCl with a flow rate of 30 ml/hr. Subsequently the fractions (1.0 ml) were neutralized with 20 µl of 2M Tris-base and analyzed for total protein at 280 nm and TI activity at 410 nm using BAPNA as substrate. The fractions with inhibitory activity against trypsin were pooled, dialyzed against 50 mM Tris-HCl, pH 8.0 using 4 kDa cutoff membrane and concentrated using Amicon filters (Millipore, 3 kDa cutoff).

Gel-filtration chromatography:

Preparation of Sephadex G-50 (Sigma, G50150): The dry resin was suspended in 50 mM Tris-HCl (pH 8.0) for overnight at room temperature (1gm dry resin was swollen to 11.0 ml). The slurry was degassed and packed in a glass column (1.5 X 100.0 cm) with lower end closed with glass wool and the column was equilibrated with 2 CV of 50 mM Tris-HCl (pH 8.0).

Sephadex G-50 chromatography: The affinity fraction pool with minor contaminants was finally subjected to gel filtration chromatography on a Sephadex G-50 column. The concentrated affinity fraction pool was loaded on to the column and subsequently 1.0 ml

fractions were collected by adding 50 mM Tris-HCl, pH 8.0 to the column at a flow rate of 15 ml/hr. The total protein and inhibitory activity of the collected fractions were determined by monitoring the absorbance at 280 nm and performing TI assay, respectively. The fractions with inhibitory activity against trypsin were pooled and concentrated using Amicon filters (Millipore, 3 kDa cutoff) for further use.

Electrophoresis:

Gelatin-PAGE: TI or CI bands were visualized using Gelatin-PAGE in non-denaturing (in absence of SDS) and denaturing (in presence of SDS) conditions (Felicioli et al., 1997). Gelatin was incorporated (0.1% to final concentration) in to the resolving gel mixture which consists of 375 mM Tris-HCl buffer (pH 8.8), 12.5% acrylamide, 0.1% SDS (w/v), 0.05% APS (w/v) and TEMED. The stacking gel contained 125 mM Tris-HCl (pH 6.8), 4% acrylamide, 0.1% SDS (w/v), 0.04% APS (w/v) and TEMED. The samples were mixed with 4x sample buffer (3:1 ratio) containing 180 mM Tris-HCl (pH 6.8), 4% SDS (w/v), 40% glycerol (w/v) and 0.04% bromophenol blue (BPB) (w/v). Electrophoresis was performed at 60 V in stacking gel and 120 V in resolving gel. Following electrophoresis, the gel was washed thrice with Triton-x-100 (30 min each wash) to remove SDS from the gel. The excess Triton-X-100 was removed by washing the gel with distilled water. Later the gel was incubated in 0.1M Tris-HCl containing trypsin (0.1 mg/ml) at pH 8.2 or chymotrypsin (0.2 mg/ml) at pH 7.8 or gut proteinases of equivalent activity initially for 30 min at 4^{o}C and subsequently for 2 h at 37^{o}C. After hydrolysis of gelatin, the gel was washed with distilled water to remove the excess enzymes and stained with Coomassie Brilliant Blue (CBB) R250. The presence of TI or CI was identified by the appearance of dark blue bands in a clear background due to complex formation of the unhydrolysed gelatin with stain. Commercially available soybean trypsin inhibitor (STI/SKTI) or soybean trypsin chymotrypsin inhibitor (BBI) was used as marker in gelatin-PAGE.

Tricine-SDS-PAGE: Tricine-SDS-PAGE was performed using 4% stacking gel and 18% separating gel as described by Schagger and von Jagow (1987) under reducing and non-reducing conditions. The samples were reduced with 50 mM DTT at 56^{o}C for 1 h followed by alkylation with 2 fold molar excess of iodoacetamide i.e., 100 mM for 45 min in dark at room temperature. After reduction, the samples were mixed with 4x sample buffer (3:1 ratio) which consists of 180 mM Tris-HCl (pH 6.8), 4% SDS (w/v), 40% glycerol (w/v) and 0.04% BPB (w/v). The separating gel contained 1 M Tris-HCl (pH 8.45), 0.1% SDS (w/v) and 10% glycerol (w/v) while stacking gel contained 0.75 M Tris-HCl (pH 8.45), 0.075% SDS (w/v). The anode buffer (lower tank) consisted of 0.2 M Tris-HCl (pH 8.9) and cathode buffer (upper tank) consisted of 0.1 M Tris, 0.1 M Tricine and 0.1% SDS. Electrophoresis was performed at 50V for 1 hr and continued at 150V until the tracking dye migrate completely to the bottom of the gel. The protein molecular mass standard used was PMW-L (Banglore Genei), which contained ovalbumin (43 kDa), carbonic anhydride (29 kDa), trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa), aprotinin (6.5 kDa) and insulin (3 kDa). Gel was stained with either CBB R250 or silver nitrate method.

Native-PAGE: Native-PAGE was carried out in 4% stacking and 12.5% separating gels as described by Laemmli (1970). SDS was excluded in resolving and stacking gels, tank buffer and 4x sample buffer. Resolving gel contained 375 mM Tris-HCl (pH 8.8) and 10% glycerol (w/v), while stacking gel contained 125 mM Tris-HCl (pH 6.8) and 10% glycerol (w/v). Tank buffer consisted of 25 mM Tris and 192 mM glycine. The samples were mixed with 4x sample buffer which contained 180 mM Tris-HCl (pH 6.8), 40% glycerol (w/v) and 0.04% BPB (w/v). Electrophoresis was performed at 4^{o}C with a constant voltage of 100V. Commercially available soybean trypsin inhibitor (STI/SKTI) or soybean trypsin chymotrypsin inhibitor (BBI) was used as marker protein in native-PAGE. Gel was stained with either CBB R250 or silver nitrate method.

Two-Dimensional Electrophoresis: Isoelectric focusing (IEF) was performed with Immobiline dry strips pH 3–11 (Non-linear) using Ettan IPG Phor 3 Isoelectric focusing system (GE Healthcare) following the manufacturer's instructions. The RgPI or BgPI (50 µg) was mixed with rehydration buffer which consists of 0.5% IPG buffer pH 3-11 (Non-linear), 8M Urea, 2% CHAPS and 20 mM freshly prepared DTT and applied on to an IPG strip holder. The IPG strip was gently placed into the strip holder with out air bubbles between strip and buffer. After 1 h rehydration the strips were subjected to isoelectric focusing through sequential steps as follows: (i) 500 V for 12 h; (ii) gradient-6000 V for 2.30 h; (iii) gradient- 6000 V for 30000 Vhrs; (iv) stand by mode at 500 V until removed from IEF system. After first dimension, the strips were equilibrated in a sequential fashion first with DTT (10 mg/ml), followed by iodoacetamide (25 mg/ml) in a buffer consisting of 6M Urea, 75 mM Tris-HCl (pH 8.45), 29.3% Glycerol (v/v), 2% SDS (w/v). Later the strips were placed on the surface of the second-dimension Tricine-SDS-PAGE gels horizontally (4% stacking and 18% separating gel without sample wells) and sealed with 0.5% agarose in upper tank buffer and a trace of BPB, leaving a well for loading marker (PMW-L). Proteins were stained with CBB R250 or silver nitrate method (Handbook of GE Healthcare).

Preparative gel electrophoresis: After electrophoresis, a sample strip from one side of the gel was used for staining to locate the bands. The unstained regions corresponding to the stained regions of the gel were excised and placed in a 4 kDa cutoff dialysis bag containing 50 mM Tris-HCl (pH 8.0). The dialysis bag after sealing from both the ends was placed in a horizontal tank and electrophoresis was performed using above buffer for overnight under 4^{o}C at 50 V. After removing the gel pieces, the buffer with samples was concentrated and used for further studies. The purity of the electroeluted proteins was examined in both Tricine-SDS-PAGE and Native-PAGE.

Gel staining:

Coomassie Brilliant Blue (CBB) R250 staining: Staining solution was prepared by dissolving the CBB R250 (0.1%) in methanol: acetic acid: water (40:10:50) for overnight at room temperature. The staining solution was filtered using Whatman No.1 paper. Freshly prepared CBB R250 staining solution was added to the gel and kept under gentle shaking for 1 h. The staining solution was removed and the gel was destained with methanol: acetic acid: water (40:10:50) under constant shaking until the background blue colour disappears and clear bands are visualized.

Silver staining: After electrophoresis, the gel was fixed in a solution of methanol: acetic acid: formaldehyde: water (50:12:0.05:37.95) for 1 h with gentle agitation. After fixation, the gel was washed thrice in 50% ethanol (20 min each wash). Then the gel was pre-treated with 0.02% sodium thiosulfate for 1 min and rinsed with water thrice (20 sec each wash). Later the gel was incubated in freshly prepared 0.2% silver nitrate solution containing 0.075% formaldehyde for 20 min and rinsed with water thrice (20 sec each). Finally the gel was developed with 6% sodium carbonate containing 0.05% formaldehyde until the protein bands are stained and immediately washed with water. The protein bands are fixed with methanol:acetic acid:water (50:12:38).

Characterization:

Inhibitor constant (K_i) determination: K_i of purified PIs against both trypsin and chymotrypsin was determined by pre-incubating the respective enzyme with increasing concentrations of RgPI or BgPI for 15 min followed by 45 min incubation with different concentrations of (0.125, 0.25, 0.375, 0.5, 0.625 and 0.75 mM) BAPNA and GLUPHEPA, respectively, at 37^{o}C. The K_i value was estimated by using Sigma Plot 10.0, Enzyme Kinetics Module 1.3 (Systat Software Inc., San Jose, California, USA).

Molecular mass analysis of native and reduced PIs by MALDI-TOF: Molecular mass of TI was determined by using a Bruker Daltonics Reflex IV instrument (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser (337 nm) and operated in linear mode with a matrix of α-cyano-4-hydroxy-cinnamic acid in 0.1% TFA/CH_3CN 2:1 ratio, respectively. The protein was reduced with 50 mM DTT for 1 h at 56^oC followed by alkylation with 2 fold molar excess of iodoacetamide for 45 min in dark at room temperature. For MALDI-TOF analysis, ~1.0 µg of RgPI or BgPI was mixed with equal volume of matrix and 2 µl of this mixture was applied on a MALDI sample plate and allowed to crystallize at room temperature. External standards (Bruker's protein calibration standard: Bradykinin, 757.399 Da; Angiotensin-II, 1046.541 Da and ACTH (18-39), 2465.198 Da) were used for calibration.

Ingel digestion of iso-inhibitor bands and MALDI-TOF-MS: The isoinhibitors of RgPI and BgPI separated during 2-D electrophoresis were excised and destained with acetonitrile: 25 mM NH_4HCO_3 (1:1). After destaining, the excised spots were incubated for 5 min in 100% acetonitrile to completely dry them. After discarding the acetonitrile, the gel spots were incubated with 10 mM DTT for 1 h at 56^oC to reduce the protein and subsequently alkylated by incubating with 55 mM iodoacetamide at room temperature in dark for 45 min. After washing with 25 mM NH_4HCO_3, the excised spots were incubated in 100% acetonitrile for drying and digested with 10 ng of trypsin at 37^oC for overnight. Sufficient quantity of acetonitrile: trifluro acetic acid: water (50:1:49) was added to the tubes and vortexed well. The supernatant containing the peptide mixture was concentrated using speed vac and analyzed using matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) on an Autoflex (Bruker, Bremen, Germany).

Temperature and pH stability: The effect of temperature on trypsin or chymotrypsin inhibitory activity of RgPI and BgPI was tested by incubating them at 37^oC, 40^oC, 50^oC,

60°C, 70°C, 80°C, 90°C and 100°C for 30 min. After cooling the samples to room temperature the residual trypsin and chymotrypsin inhibitory activity was assayed at 37°C in presence of BAPNA and GLUPHEPA, respectively. The effect of pH on trypsin or chymotrypsin inhibitory activity of PIs was examined at pH ranging from 2 to 12 using the following buffers at final concentrations of 50 mM: glycine-HCl (pH 2-3), sodium acetate-acetic acid (pH 4-5), sodium phosphate buffer (pH 6), Tris-HCl (pH 7-9) and glycine-NaOH (pH 10-12). After pre-incubation at 37°C for 1 hr in the respective buffers, the residual inhibitor activity was measured at pH 8.2 for trypsin and pH 7.8 for chymotrypsin.

Effect of DTT and 2-Mercaptoethanol on TI activity: RgPI or BgPI in 50 mM Tris–HCl buffer (pH 8.0) was incubated with different concentrations of DTT (0.05 to 1.0 mM) and 2-ME (1.0 to 20 mM) independently in 25 mM NH_4HCO_3 for 15 min at 56°C. In the samples where the effect of DTT was examined, the reaction was terminated by adding iodoacetamide at twice the amount of each DTT concentration. The residual inhibitory activity against trypsin and chymotrypsin was estimated using BAPNA and GLUPHEPA, respectively.

Chemical modification of aminoacids: Arginine residues were modified according to Patthy and Smith (1975) using CHD. RgPI or BgPI in 50 mM borate buffer (pH 9.0) were incubated with 15-fold molar excess of CHD and the reaction tube was flushed with nitrogen and kept at 37 °C for 2 h. The reaction was terminated by the addition of 5% acetic acid. Lysine residues were modified using TNBS according to the method of Haynes et al. (1967). RgPI or BgPI in 50 mM phosphate buffer (pH 7.6) were incubated with 10-fold molar excess of TNBS at 40°C for 2 hrs. The reaction was stopped by adding 10% SDS followed by 0.2 ml of 1 N HCl. Tyrosine residues were modified using NAI (Yu et al., 1991). RgPI or BgPI in 50 mM Tris-HCl (pH 7.5) were incubated with 60-fold molar excess of NAI at 37 °C for 2 h. The reaction was terminated by adding

excess NAI and dialyzed for 5 h at 4°C against 50 mM Tris-HCl (pH 7.5). Modification of tryptophan residues was performed using NBS according to the method of Spande and Witkop (1967). RgPI or BgPI in 50 mM sodium acetate (pH 4.0) were incubated with 60-fold molar excess of NAI at 37°C for 2 h. The residual inhibitory activity against trypsin and chymotrypsin was estimated using BAPNA and GLUPHEPA, respectively. The activity staining gels were done by gelatin-SDS-PAGE (12.5%).

Circular dichroism spectroscopy: Circular dichroism (CD) spectral measurements were performed on a JASCO J810 spectropolarimeter at a scan speed of 50 nm/min with three accumulations. The entire instrument, including the sample chamber, was constantly flushed with Nitrogen gas during the operation. Path length of 0.1 cm for far-UV and 1 cm for near UV was used. Buffer scans were recorded under the same conditions and subtracted from the protein spectra before further analysis. For examining secondary structure at near UV (190-250 nm), RgPI or BgPI at concentration of 0.3 mg/ml in 10 mM phosphate buffer (pH 7.4) was used. For determining tertiary structure at far UV (250-300 nm), RgPI or BgPI was used at a concentration of 5 mg/ml. The secondary structure analysis was performed by CD spectrum deconvolution using CDSSTR method. Effect of temperature on secondary structure was measured by incubating the samples at temperatures from 25°C to 90°C using the peltier (thermostat). Near UV Spectra were recorded at each temperature. Similarly the effect of pH on secondary structure of RgPI and BgPI was monitored at the near UV spectra at corresponding pH. The effect of disulfide bonds reduction and alkylation on secondary structure of RgPI and BgPI was determined in presence of 1 mM DTT and 2 mM iodoacetamide. The PIs were incubated with DTT for 1 h at 56°C followed by iodoacetamide for 45 min in dark at room temperature and spectra at 190-250 nm were recorded.

N-terminal sequencing: PIs resolved in 2-D gel were electroblotted on to PVDF membrane (sequence grade, 0.22 μm from Millipore) using 10 mM CAPS buffer (pH

11.0). The membrane was stained with 0.1% CBB R250 in methanol:acetic acid:water (40:1:59) and destained with 50% methanol in water. The spots of interest were excised from the membrane and transferred into 1.5 ml vials and washed thrice alternatively with milliq water and 50% methanol in water. Sequence of N-terminal region was determined by Edman degradation in an Applied Biosystem, Model 494 Procise protein sequencing system. The sequence was read by analysis software program: model 610. All sequences were confirmed by second operator.

Insects:

Experiments with *Manduca sexta* larvae were done at Department of Animal Physiology, University of Osnabrück, Osnabrück, Germany. Larvae of *Spodoptera litura* and *Achaea janata* were obtained from Directorate of Oil seeds Research (DOR), Hyderabad. *Helicoverpa armigera* larvae were obtained from Central Research Institute for Dryland Agriculture (CRIDA), Santoshnagar, Hyderabad. *Papilio demoleus*, *Amsacta albistriga* and *Daphnis nerii* larvae were collected in Hyderabad University campus. *Corcyra cephalonica* and *Bombyx mori* larvae were obtained from the laboratory of Prof. Aparna Dutta Gupta, Dept. Animal Sciences, University of Hyderabad. The photographs of larval forms of different insects were depicted in **Figure 3.1** and details of their common name, family name and the damage caused to plants by them are shown in **Table 3.2**.

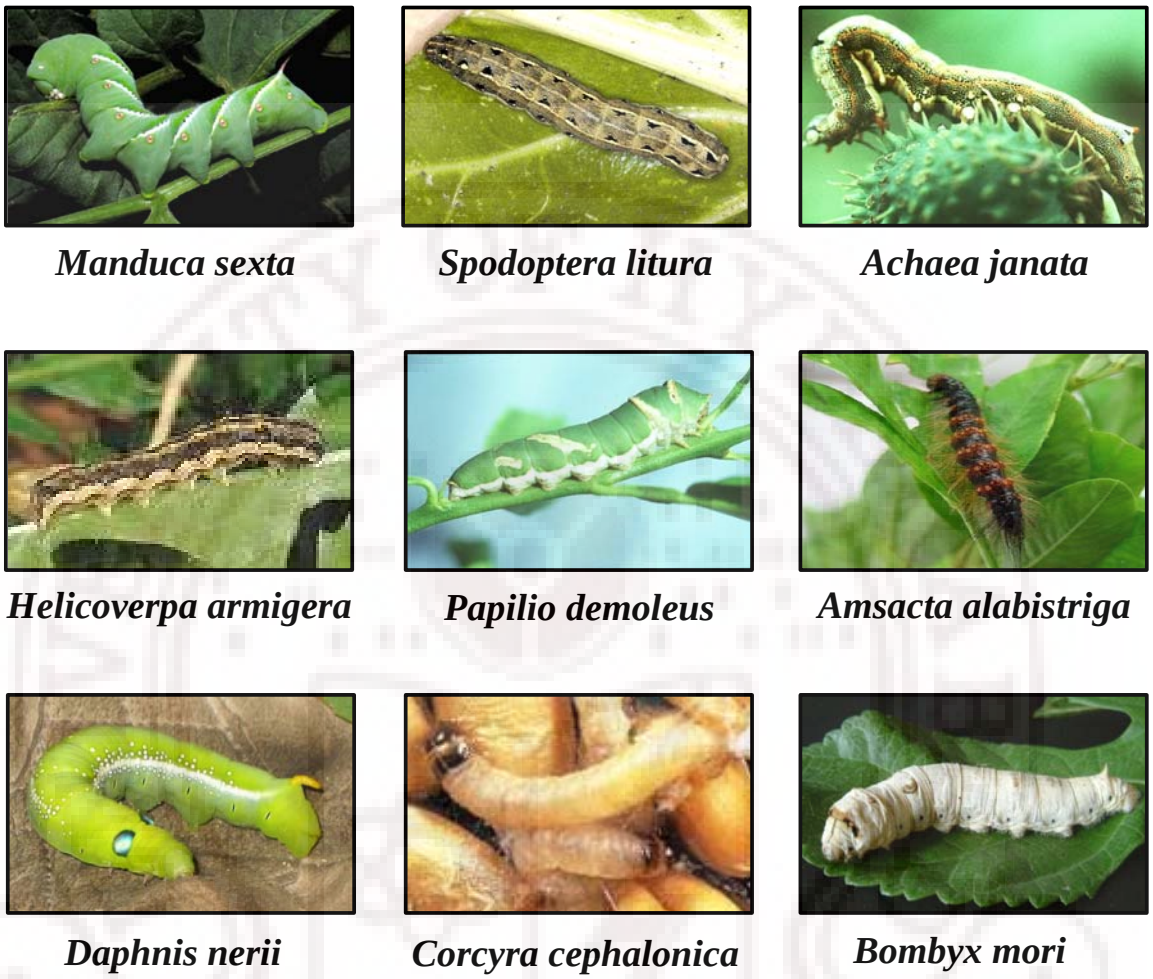

Fig. 3.1. Larvae of lepidopteran insects studied in the present investigation.

Table 3.2. Details of the common name, family name and the damage caused to plants by various lepidopteran insects used in the present study.

S.No.	Scientific name	Common name	Family	Damage caused to plants
1.	*Achaea janata*	Castor semilooper	Noctuidae	Feeds on many different species of plants, but castor bean and croton are preferred hosts.
2.	*Amsacta albistriga*	Red hairy caterpillar	Arctiidae	Feeds on foliage of groundnut, red gram, cow pea and beans.
3.	*Bombyx mori*	Silk moth	Bombycidae	They prefer white mulberry, but not being strictly monophagous. They also attack other species of *Morus* and some other Moraceae members.
4.	*Corcyra cephalonica*	Rice moth	Pyralidae	Infesting rice grains during storage.
5.	*Daphnis nerii*	Oleander Hawk moth	Sphingidae	The caterpillars feed mainly on *Nerium* leaves, a highly toxic plant to which toxicity they are immune. They also may feed on most plants of the *Apocynaceae* family, as *Tabernaemontana divaricata* and *Alstonia scholaris* in India.
6.	*Helicoverpa armigera*	Cotton bollworm	Noctuidae	It is a polyphagous and cosmopolitan pest and the larvae feed on wide range of cultivated plants.
7.	*Manduca sexta*	Tobacco hornworm	Sphingidae	Feeds on foliage of Solanaceaous plants, especially tobacco and tomato leaves.
8.	*Papilio demoleus*	Citrus butter fly	Papilionidae	The larvae feed on the citrus leaves.
9.	*Spodoptera litura*	Tobacco cutworm	Noctuidae	Polyphagous pest affecting many crop plants.

Rearing of *Spodoptera litura* and *Achaea janata*:

The insects were reared in clean plastic tubs at insect culture room in University of Hyderabad. Fresh castor leaves were provided to the larvae everyday. Pupae were allowed to reach adult stage in sand. The adults were fed with 20% commercial honey (Honeyrex) containing 200 mg vitamin E (Evinal-200) provided through a cotton swab. The egg masses were allowed to hatch on a moist filter paper. The cultures were maintained in insect culture room at 26 ± 1^{o}C temperature, 60 ± 5% relative humidity (RH) and 14:10 h light-dark photoperiod.

Extraction of larval gut enzymes:

Larval gut enzymes were extracted from 4th to 5th instar larvae of the following insects: *A. janata*, *A. albistriga*, *B. mori*, *C. cephalonica*, *D. nerii*, *H. armigera*, *M. sexta*, *P. demoleus* and *S. litura* (Girard et al., 1998b). The larvae was narcotized on ice for 15 min and dissected in an insect Ringer solution (0.13 M NaCl, 0.5 M KCl, 0.1 mM $CaCl_2$ and 1 mM PMSF). The midguts were removed and placed on iso-osmotic saline (0.15 M NaCl) solution. Gut tissue was homogenized in 0.15 M NaCl and centrifuged twice at 12,000 rpm for 10 min at 4^{o}C. The supernatant was collected and stored frozen at -20^{o}C for further *in vitro* assays.

Effect of TI on midgut proteolytic activity of lepidopteron insects:

The effect of PIs on the midgut trypsin-like or chymotrypsin-like proteinases was determined by incubating the RgPI or BgPI (an aliquot which gives 40-60% inhibition) in assay buffer (1.0 ml), 50 mM glycine-NaOH (pH 10.5) (for *A. janata*, 50 mM Tris-HCl containing 0.02 M $CaCl_2$, pH 8.2), with midgut extracts (an aliquot of midgut extract which gives 1 OD/45 min with 1 mM BAPNA or GLUPHEPA) at 37°C for 15 min. The residual trypsin-like or chymotrypsin-like activity of the above samples was measured in

presence of 1.0ml of 1 mM BAPNA or GLUPHEPA, as a substrate, respectively. The reaction was terminated by adding 0.2 ml of 30% acetic acid (v/v).

Zymogram of insect gut extract:

Zymogram of insect gut extracts was done by the method of Garcia-Carreno et al. (1993). An aliquot of (aliquot volume which gives maximum number and resolution of isofroms of proteinases) insect gut extracts were separated in SDS-PAGE (7.5%) at 100V in 4°C. After electrophoresis, the gel was washed with 2.5% Triton-X-100 (v/v) thrice (30 min each wash) and incubated with 2% casein (w/v) in 0.1 M glycine-NaOH (pH 10.5) for 30 min at 4°C with gentle agitation. The gel was washed with water and incubated in 0.1 M glycine-NaOH for 2 h at 37°C without agitation. Commercially available irreversible PIs: TPCK, TLCK (dissolved in ethanol) and PMSF (dissolved in isopropanol) were added (300 μM each) to the gut extract before loading on to SDS-PAGE. Reversible PIs (RgPI, BgPI and BBI, 100 μg/ml) were added to 0.1 M glycine-NaOH (pH 10.5) and the gels were incubated in this solution immediately after electrophoresis for 30 min before incubating with casein solution. Finally the gel was washed with water and stained with CBB R250. Destaining of the gels result in the appearance of clear bands in blue background, due to hydrolysis of casein in the region of gut proteinases.

In vivo effect of RgPI and BgPI on growth and development of lepidopteran insects:

To examine the effect of PIs on *S. litura* and *M. sexta* larval growth and development, an artificial diet was supplemented with different concentrations (0.025% and 0.050% for *S. litura*, 0.001% and 0.01% for *M. sexta*) of RgPI and BgPI . Two grams of agar powder (2%) was mixed with 100 ml of boiling water, followed by addition of 7 gm of yeast extract, 1.5 gm of wheat germ, 3 gm of casein, 2.6 gm of sucrose, 0.2 gm of sorbic acid, 0.3 gm of cholesterol and 1 gm of salt mixture. The mixture was allowed to cool down to 50^{o}C. To this 0.35 gm of ascorbic acid, 0.2 gm of sodium benzoate, 4.3 ml of 4%

formaldehyde, 1 ml of vitamin mixture and 24 mg of chloramphenicol (suspended in 0.1 ml of ethanol) were added. Chloramphenicol was added basically to block bacterial growth on the diet. After cooling down the diet mixture to room temperature, the RgPI and BgPI in different concentrations were added to diet and mixed thoroughly before adding to trays. The trays are stored at 4^{o}C until further use. To test the effect of PIs on *A. janata* growth and development leaf coating assays were done. Fresh castor leaves was coated with PIs at required concentration (2, 4 and 8 µg/cm^2 leaf area) and allowed to dry at room temperature. The control leaf was coated with 50 mM Tris-HCl, pH 8.0. Newly-hatched larvae were allowed to grow either on the feed or leaf with and without PIs and the difference in the average weight of the larvae was examined.

Statistical methods:

All experiments were done in triplicate and data represented in results are the mean of three or four independent experiments (mean±SE).

Chapter 4

Screening of cultivars and wild types of red gram for proteinase inhibitors active towards *Achaea janata* and *Spodoptera litura*

Chapter 4

Screening of cultivars and wild types of red gram for proteinase inhibitors active towards *Achaea janata* and *Spodoptera litura*

Red gram (pigeonpea) (*Cajanus cajan* (L.) Millsp.) is an important pulse crop in semi-arid tropical and sub tropical areas of the world. Various reports indicated the presence of several isoforms (iso-inhibitors) of trypsin and chymotrypsin inhibitors from the seed extracts of red gram (Kollipara et al., 1994; Pichare and Kachole, 1994, 1996; Chougule et al., 2003). Pichare and Kachole (1994) reported the presence of nine trypsin and seven chymotrypsin inhibitors in pigeonpea seed extracts based on the X-ray film contact print technique. Positive correlation existed between the trypsin inhibitor and chymotrypsin inhibitor activities in pigeonpea (Kollipara et al., 1994). Further, the pattern of these trypsin and chymotrypsin inhibitors were known to change during seed germination and seed development (Godbole et al., 1994c; Ambekar et al., 1996). Chougule et al. (2003) reported that PIs from some cultivars and wild types of pigeonpea were active against *Helicoverpa armigera* gut proteinases.

To further strengthen the insecticidal potential of pigeonpea PIs, we screened the cultivars and wild types against gut proteinases of lepidopteran insect pests like, *Achaea janata*, which causes severe damage and loss to castor crop in large areas of Andhra Pradesh in India (Budatha et al., 2008) and *Spodoptera litura*, a serious polyphagous pest of several economically important crops (Bhattacharyya et al., 2007a). In the present investigation, we also identified the inhibitory activity of cultivars as well as wild types against bovine pancreatic trypsin and chymotrypsin. These PIs from cultivars and wild types showed differences in activity against trypsin-like proteinases of midgut enzymes from *A. janata* and *S. litura*.

Results and Discussion

Activity of PIs in cultivars and wild types of pigeonpea:

Crude extracts prepared from cultivars and wild types of pigeonpea seeds were examined for inhibitor activity against bovine pancreatic trypsin and chymotrypsin (**Fig. 4.1A and B**). Among 14 cultivars, six of them: ICP 2376, ICP 11950, ICP 11957, ICP 11968, ICP 13198 and ICP 14770 had TI activity of 24.1-25.9 TI units/mg protein and other eight cultivars: ICP 7118, ICP 7182, ICP 7623, ICP 11298, ICP 11300, ICP 11962, ICP 11964 and ICP 13210 had TI activity of 14.5-20.2 TI units/mg protein **(Fig. 4.1A**). The TI activity was more pronounced than CI activity in cultivars. The CI activity of the cultivars was 4.2-10.4 units/mg protein (**Fig. 4.1A**). The TI activity is at least two-fold higher than CI activity in cultivars.

Among wild types, TI activity (27.6-30.9 TI units/mg protein) or CI activity (23.1-23.9 CI units/mg protein) was highest in *R. bracteata* ICP 15815, *R. rothii* ICP 15859 and *R. sublobata* ICP 15868 (**Fig. 4.1B**). The other wild types: ICP 15625, ICP 15639, ICP 15665, ICP 15761 and ICP 15774 had TI activity between 3.1-12.3 TI units/mg protein (**Fig. 4.1B**). The CI activity of wild types ICP 15625 and ICP 15761 were 11.3 and 6.6 CI units/mg protein, respectively. Wild types ICP 15639 had negligible CI activity (0.5 CI units/mg protein), while ICP 15665 and ICP 15774 had no CI activity (**Fig. 4.1B**). In wild types, the variation between TI and CI activity was less, when compared with cultivars. Similar observations were reported in some cultivars and wild types of *C. cajan* (Singh and Jambunathan, 1981; Kollipara et al., 1994).

Wild genotypes had greater variation in TI and CI activity when compared with cultivars, which is evident through activity profiles (**Figs. 4.2 and 4.3**). In gelatin-PAGE under non-denaturing condition, the cultivars exhibited homomorphism in terms of TI and CI isoforms when the gels were incubated with bovine pancreatic trypsin or chymotrypsin, respectively. In all cultivars at least five TI and CI bands were observed

(**Fig. 4.2A and B**). The uniform banding pattern of inhibitor profiles among cultivars suggest that these inhibitors are strongly conserved, which makes them very useful as markers in evolutionary studies (Weder, 1985; Kollipara et al., 1993).

Wild types showed variation in number of TI and CI isoforms and their banding pattern (**Fig. 4.3A and B**). The wild types *R. bracteata* ICP 15815, *R. rothii* ICP 15859 and *R. sublobata* ICP 15868 showed greatest number of TI and CI bands. The wild types, *C. albicans* ICP 15625 and *C. volubilis* ICP 15774 showed one or two TI and CI isoforms. In contrast, the wild types *C. lanceolatus* ICP 15639, *C. platycarpus* ICP 15665 and *C. sericeus* ICP 15761 had not shown any TI or CI isoforms on gelatin-PAGE (**Fig. 4.3A and B**). The mobility of the TI or CI isoforms of cultivars is different from those of wild types. In wild types the inhibitors exhibited highly species specific banding pattern. However, TI profile of cultivars and wild types overlapped with their CI profile (**Figs. 4.2 and 4.3**). These findings indicate that same protein had TI and CI activities, which is a characteristic feature of Bowman-Birk type of PIs (Clemente and Domoney, 2006; Losso, 2008). The presence of Bowman-Birk type of PIs was reported earlier in pigeonpea (Godbole et al., 1994a; Kollipara et al., 1994). Wide differences in activities of PIs in wild types were due to genetic variation among them and cultivated varieties could have lost such genetic diversity during the course of domestication (Chougule et al., 2003).

In agreement with Chougule et al. (2003), the PIs of pigeonpea showed wide genetic diversity among wild types, while cultivars were homogeneous (**Figs. 4.2 and 4.3**). In contrast, the cultivars of pigeonpea showed variation in inhibitor isoforms when they were separated by IEF and detected by agarose trypsin overlay method (Pichare and Kachole, 1996). However, the same cultivars showed similar number of inhibitor isoforms when separated in gelatin-PAGE under non-denaturing condition and visualized by X-ray film contact print method (Pichare and Kachole, 1994). This variation in results could be due to variation in the techniques used for separation and visualization of PIs.

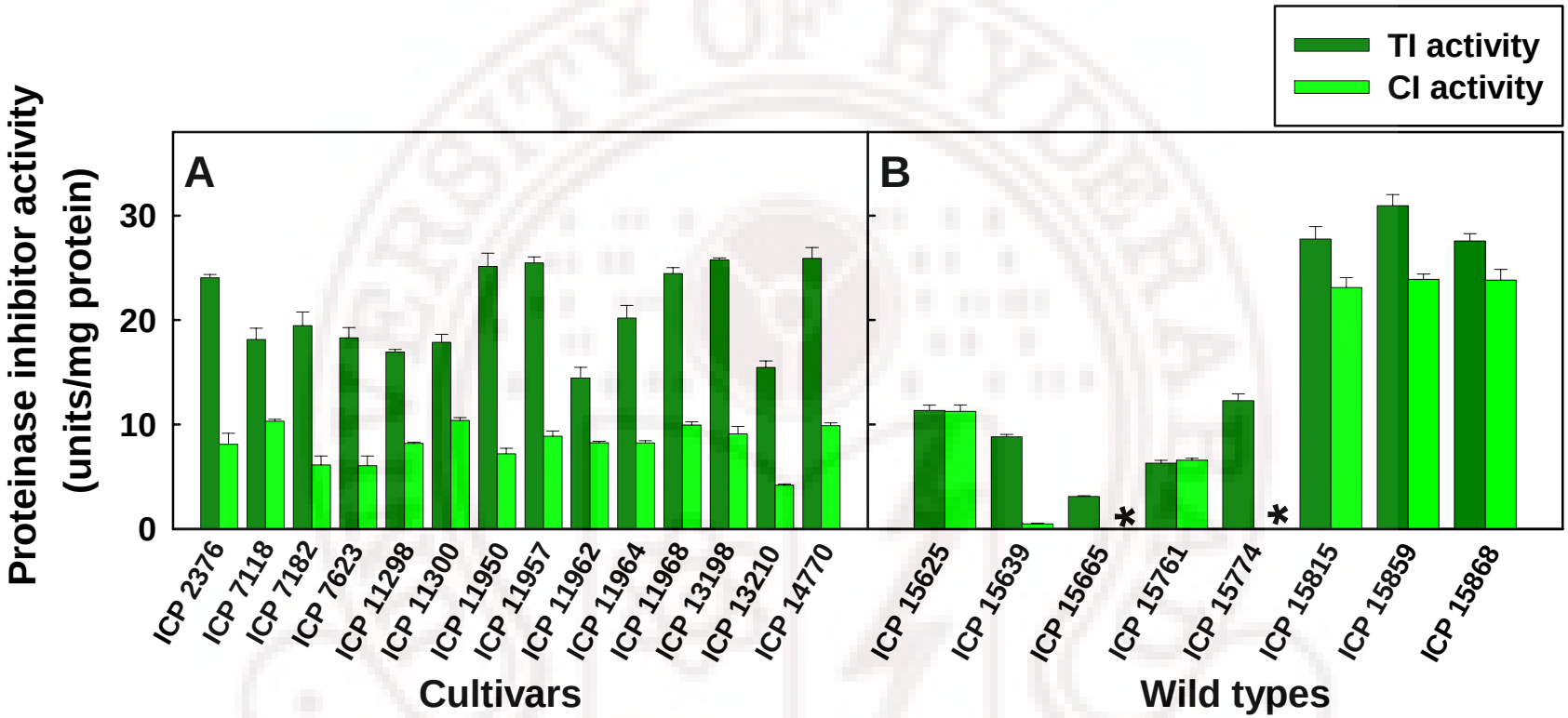

Fig. 4.1. Inhibition of bovine pancreatic trypsin and chymotrypsin by proteinase inhibitors from crude extracts of cultivars (A) and wild types (B) of pigeonpea. Values are mean ± S.E. from three independent experiments each with three replicates. *****CI activity was not observed.

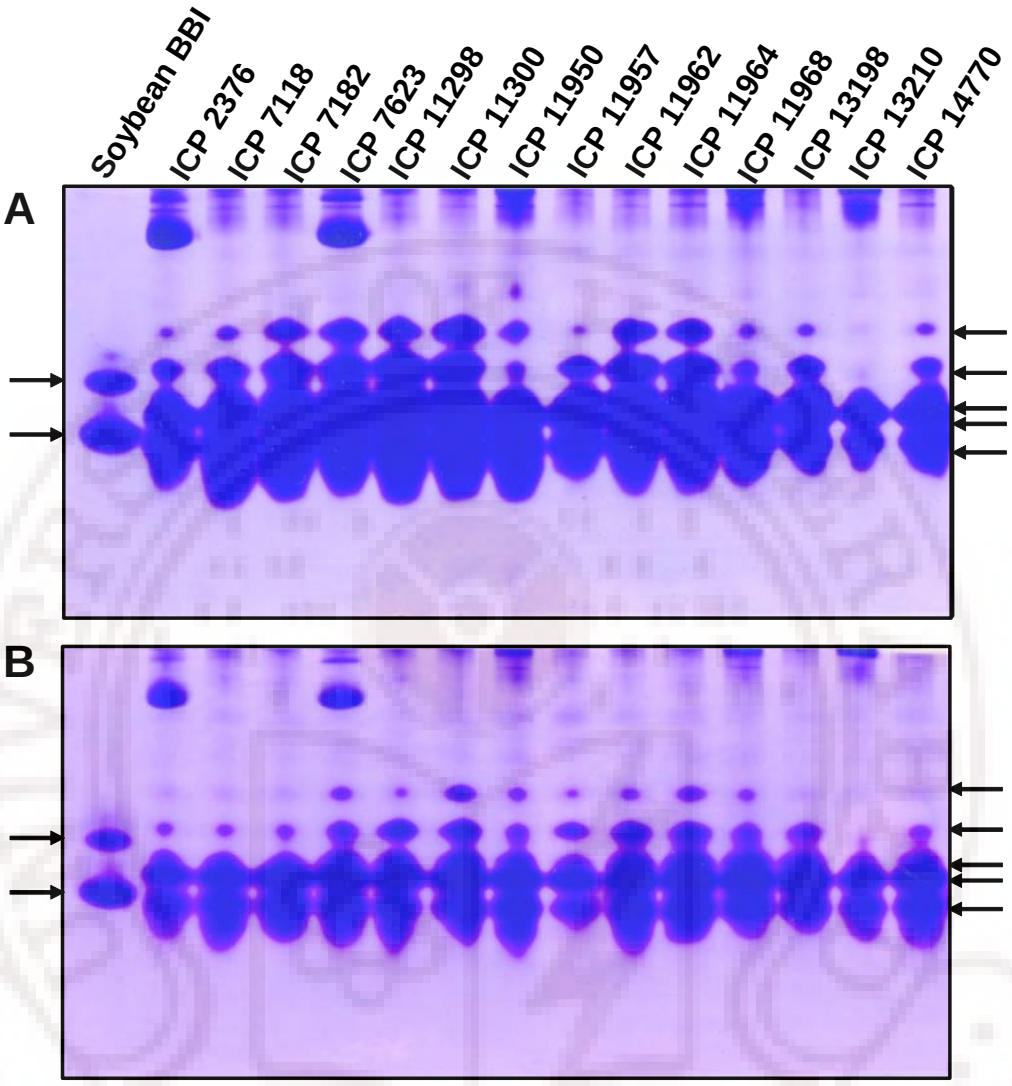

Fig. 4.2. Inhibitor profiles of pigeonpea cultivars against bovine pancreatic trypsin (A) and chymotrypsin (B). Proteins (50 µg) from crude extracts were separated on gelatin-PAGE (12.5%) in non-denaturing condition. In lane 1, 5 µg of soybean BBI (8 kDa) was loaded. Other details were as described in materials and methods.

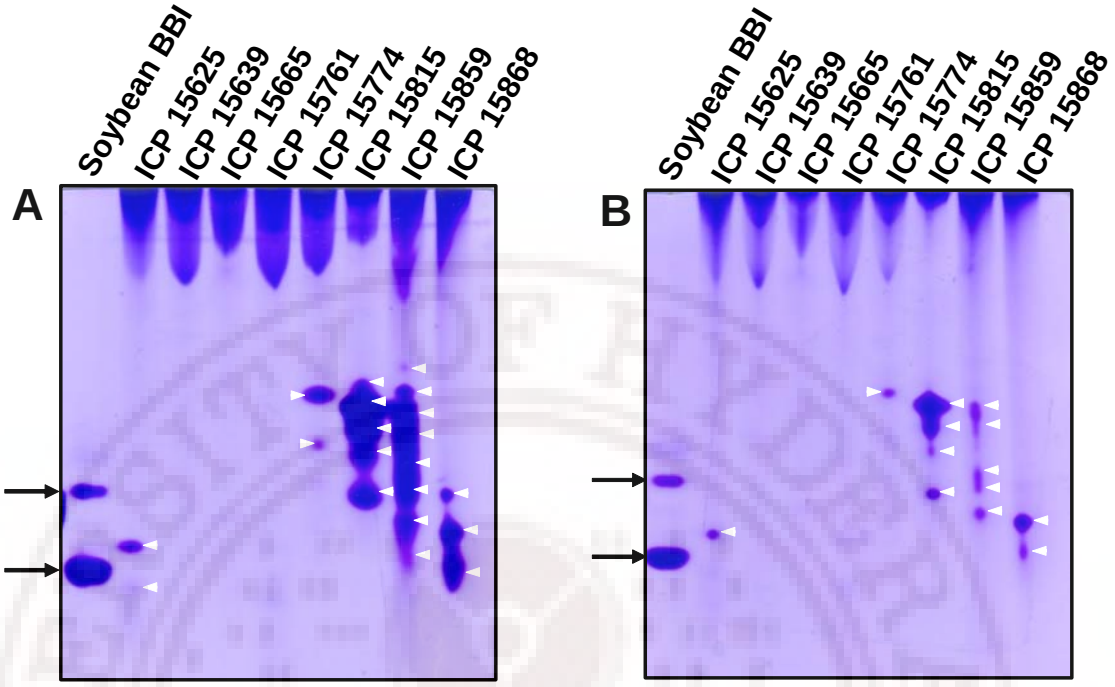

Fig. 4.3. Inhibitor profiles of pigeonpea wild types against bovine pancreatic trypsin (A) and chymotrypsin (B). Proteins (50 µg) from crude extracts were separated on gelatin-PAGE (12.5%) in non-denaturing condition. In lane 1, 5 µg of soybean BBI (8 kDa) was loaded. Arrow heads indicate the number of inhibitor bands in each lane.

Inhibition potential of PIs from cultivars and wild types against gut proteinases of *A. janata* and *S. litura*:

Trypsin and chymotrypsin are major digestive enzymes in the midgut of lepidopteran insects (Srinivasan et al., 2006; Karumbaiah et al., 2007). However, in *A. janata* and *S. litura* (Fig. 4.4) trypsin-like activity was more prominent than chymotrypsin-like activity (Budatha et al., 2008). Therefore, we restricted to examine the inhibitory potential of pigeonpea PIs against trypsin-like proteinase activity from midgut preparation of *A. janata* and *S. litura* (Figs. 4.5 and 4.6).

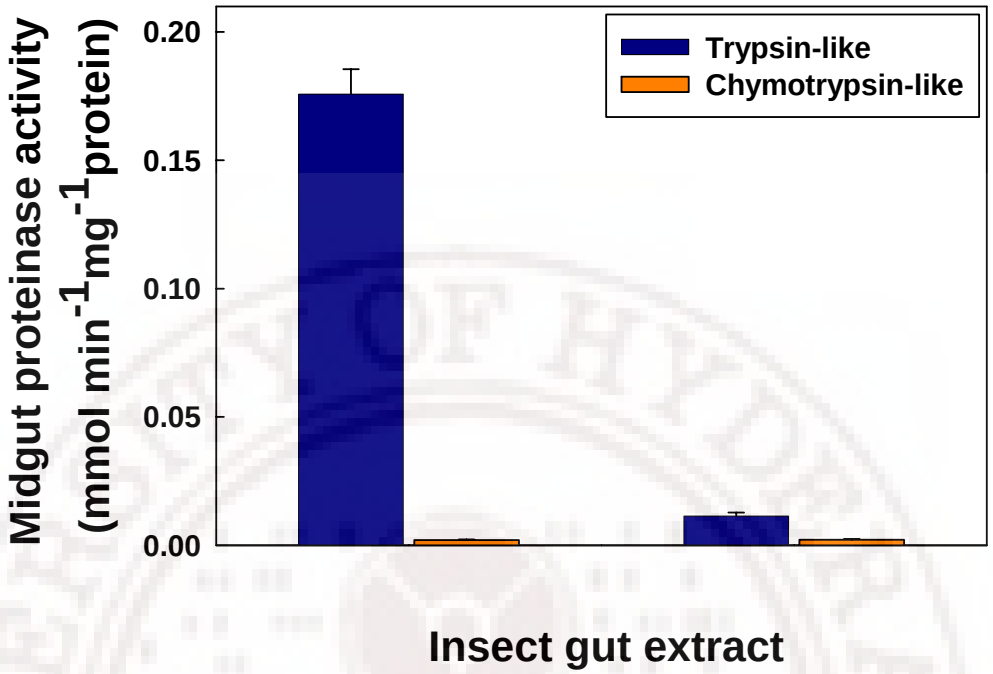

Fig. 4.4. Activity of midgut proteinases from *A. janata* and *S. litura*. Trypsin-like and chymotrypsin-like activities were measured as described in materials and methods. Values are mean ± S.E. from three independent experiments each with three replicates.

PI activity of different cultivars varied between 658 and 1351 inhibitor units/mg protein when incubated with midgut digestive enzymes of *A. janata* (**Fig. 4.5A**). Wild types *R. bracteata* ICP 15815, *R. rothii* ICP 15859, *R. sublobata* ICP 15868, *C. albicans* ICP 15625 and *C. volubilis* ICP 15774 showed PI activity ranging between 244 to 575 inhibitor units/mg protein against midgut digestive enzymes of *A. janata* (**Fig. 4.5B**). The inhibitory activity of PIs from cultivars and wild types against midgut trypsin-like proteinases of *A. janata* was several fold (10 to 50) higher than that observed in presence of bovine pancreatic trypsin (**Figs. 4.1 and 4.5**). Wild types *R. rothii* ICP 15859 and *R. sublobata* ICP 15868 had PI activity of 6.8-8.9 inhibitor units/mg protein when incubated with midgut digestive enzymes of *S. litura*. PI activity of other wild types and

cultivars was 0.9-3.3 inhibitor units/mg protein (**Fig. 4.6A and B**). The inhibitory activity of PIs from cultivars and wild types against trypsin-like proteinases of *S. litura* was 3 to 9 fold less than that observed in presence of bovine pancreatic trypsin (**Figs. 4.1 and 4.6**).

The PIs of all cultivars showed minute differences in their activity, although their activity range varied depending on the source of the trypsin, whether it was from bovine pancreas or insect midgut (**Figs. 4.1A, 4.5A and 4.6A**). The PIs from wild types showed greater variation in their activity, even against insect midgut trypsin-like proteinases as observed in presence of bovine pancreatic trypsin (**Figs. 4.1B, 4.5B and 4.6B**).

The cultivars ICP 7118, ICP 11300 and ICP 14770 showed two distinct PI bands on gelatin-PAGE against *A. janata* midgut proteinases (**Fig. 4.7A**). However, the cultivar ICP 14770 showed these bands with high intensity when compared with other cultivars ICP 7118 and ICP 11300. The wild type *C. volubilis* ICP 15774 showed three PI bands, while *R. bracteata* ICP 15815 and *R. rothii* ICP 15859 showed two PI bands and *R. sublobata* ICP 15868 showed single PI band against *A. janata* midgut proteinases. Thus, the activity profiles of PIs in wild types are different from those of cultivars in presence of *A. janata* midgut proteinases (**Fig. 4.7A**). Interestingly the wild type, *C. volubilis* ICP 15774, which had marginal TI activity but no CI activity, showed higher inhibitor activity against *A. janata* midgut trypsin-like proteinases (**Figs. 4.1B and 4.5B**). The activity profile of PIs from wild types, but not cultivars, showed the presence of specific bands against *S. litura* midgut proteinases (**Fig. 4.7B**). The wild types *C. volubilis* ICP 15774 and *R. bracteata* ICP 15815 showed faint PI bands while *R. rothii* ICP 15859 and *R. sublobata* ICP 15868 showed PI bands with high intensity.

Thus activity staining studies also revealed that PIs from cultivars and wild types had more potential to inhibit the activity of midgut trypsin-like proteinases from *A. janata* when compared with *S. litura* (**Fig. 4.7A and B**). This may be due to non-exposure of *A. janata* to pigeonpea PIs, possibly because pigeonpea is a non-host plant to this

lepidopteran pest. In case of *S. litura*, PIs from cultivars and wild types showed less inhibitory potential. This could be due to low trypsin-like activity in the midgut of *S. litura* when compared with *A. janata* (**Fig. 4.4**) or due to less affinity of these PIs towards the trypsin-like midgut proteinases of *S. litura*.

Our results are in agreement with activity staining studies of Chougule et al. (2003). In their experiment gelatin-PAGE showed maximum number of PI bands with high intensity in wild types but not cultivars of pigeonpea when the gel was treated with midgut proteinases from *H. armigera*. Their study also indicated that cultivars showed less inhibitory potential against *H. armigera* midgut proteinases when compared with wild types of pigeonpea (Chougule et al., 2003). However, from present study it is evident that the PIs from cultivars showed high inhibitory potential against *A. janata* midgut trypsin-like proteinases when compared to wild types of pigeonpea (**Fig. 4.5A and B**).

During host-pest interactions insects adapt to host defense by producing either inhibitor insensitive or inhibitor degrading proteinases (Bown et al., 1997; Giri et al., 1998; Patankar et al., 2001). As the larvae of *A. janata* do not feed on pigeonpea, a non-host plant, the chances of producing inhibitor insensitive or inhibitor degrading proteinases in their midgut is very limited. Therefore, it would be useful to exploit the insecticidal potential of these PIs into crop plants which are destroyed by *A. janata*. Further the non-host plant PIs were shown to be effective against *H. armigera* midgut proteinases (Harsulkar et al., 1999). Our results suggest that the genes of PIs with strong inhibitory activity from cultivars and wild types of pigeonpea could be potential candidates for developing insect resistant transgenic plants against *A. janata*.

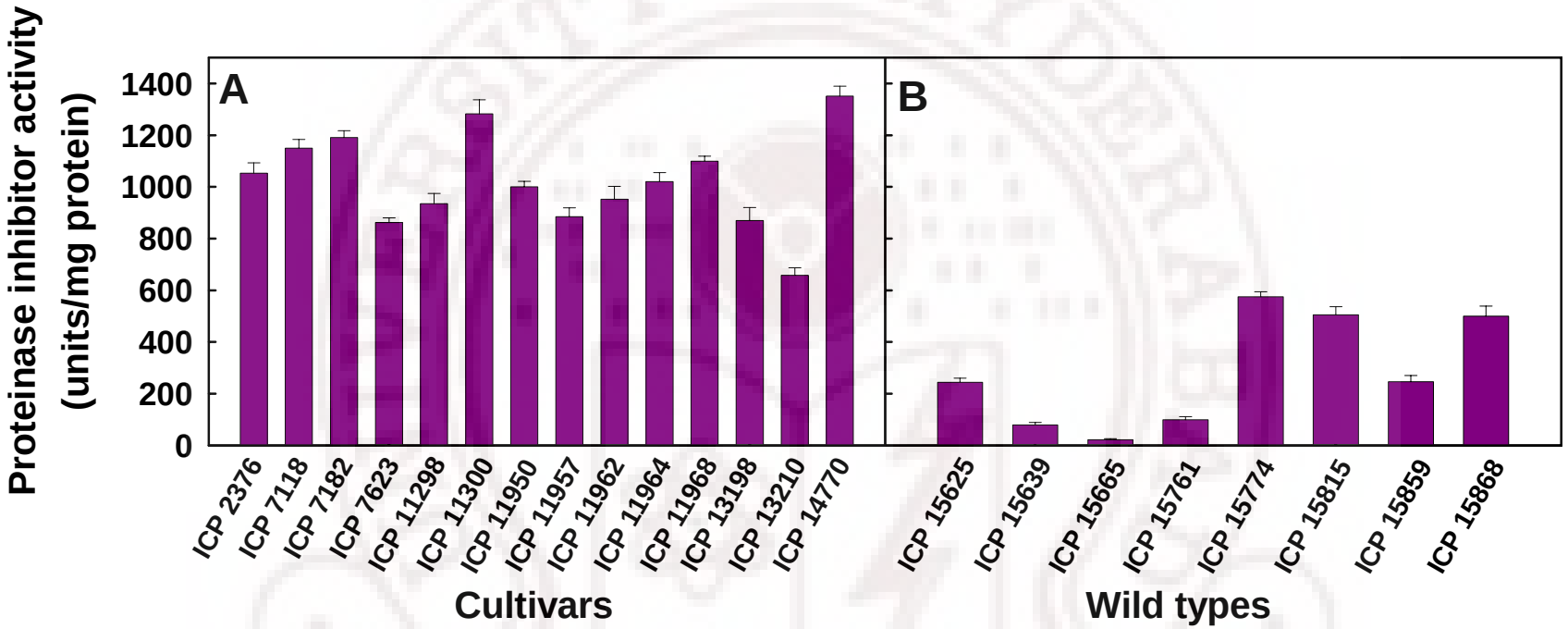

Fig. 4.5. Inhibition of midgut trypsin-like proteinases of *A. janata* by crude extracts of proteinase inhibitors from cultivars (A) and wild types (B) of pigeonpea. The results are shown as mean ± S.E. of three independent experiments done in triplicates.

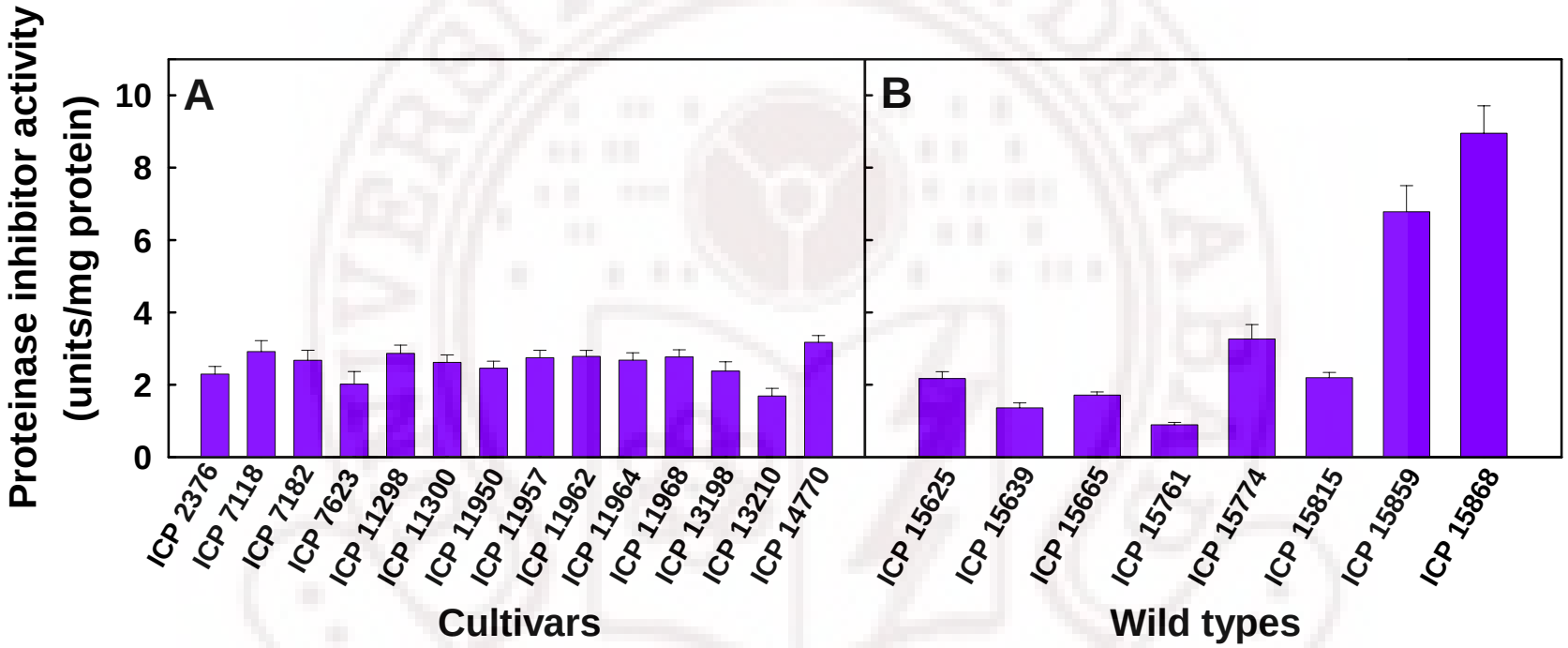

Fig. 4.6. Inhibition of midgut trypsin-like proteinases of *S. litura* by crude extracts of proteinase inhibitors from cultivars (A) and wild types (B) of pigeonpea. The results are shown as mean ± S.E. of three independent experiments done in triplicates.

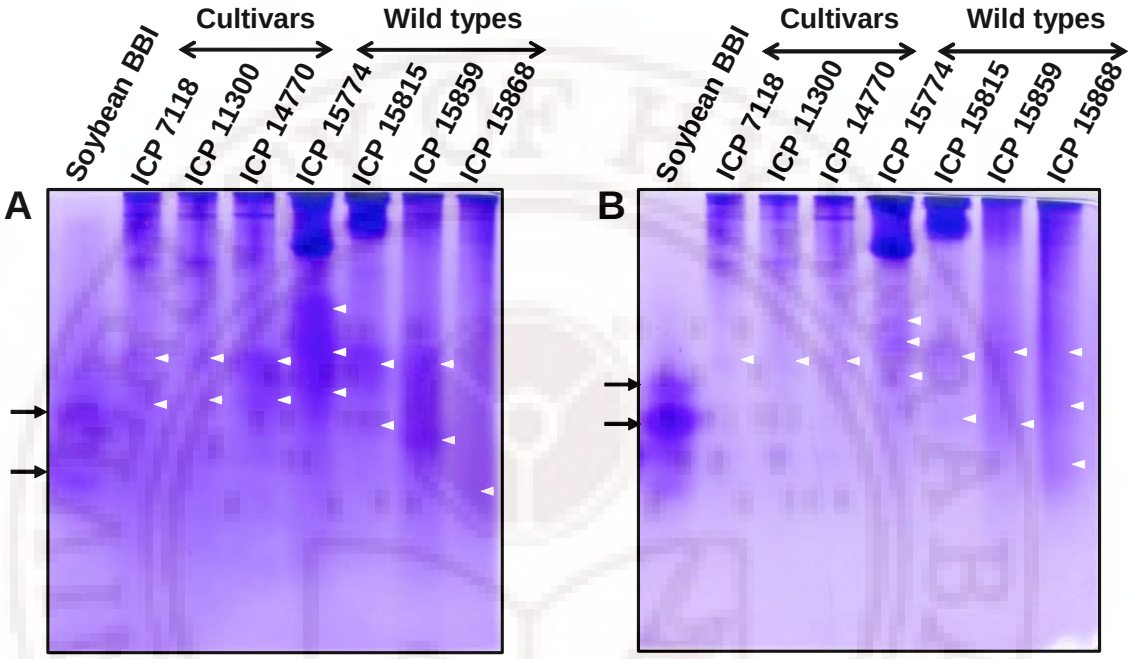

Fig. 4.7. Inhibitor profiles of pigeonpea cultivars and wild types in presence of *A. janata* (A) and *S. litura* (B) midgut proteinases. Equal inhibitor units of different seed extracts were separated on gelatin-PAGE (12.5%) in non-denaturing condition and the gels were incubated in respective midgut proteinases. In lane 1, 5 μg of soybean BBI (8 kDa) was loaded. Arrow heads indicate the number of inhibitor bands in each lane.

Chapter 5

Purification and characterization of proteinase inhibitor from the seeds of red gram (*Cajanus cajan*, cultivar ICP 14770)

Chapter 5

Purification and characterization of proteinase inhibitor from the seeds of red gram (*Cajanus cajan*, cultivar ICP 14770)

The presence of trypsin, chymotrypsin and amylase inhibitors was reported in the mature seeds of *C. cajan* (Pichare and Kachole, 1994, 1996; Giri and Kachole, 1998). Although the PIs from red gram were purified by several research groups, the reports show discrepancy in the molecular mass and the family of inhibitor to which they belong i.e., Bowman-Birk inhibitor and Kunitz inhibitor family (Osowole et al., 1992; Godbole et al., 1994a; Haq and Khan, 2003).

BBI type inhibitors, which possess multiple cysteine residues (~14) are small proteins with molecular mass around 6-9 kDa. They exhibit two reactive sites with inhibitory activity against both trypsin and chymotrypsin (Clemente and Domoney, 2006). Kunitz-type inhibitors (20 kDa) possess four cysteine residues, forming two disulfide bridges and exhibit a single reactive site which can bind to either trypsin or chymotrypsin (Richardson, 1991). The serine PIs isolated from various legume seeds mostly belonged to either Bowman-Birk or Kunitz family (Bhattacharyya et al., 2006, 2007b; Pereira et al., 2007; Lingaraju and Gowda, 2008; Ramos et al., 2008; Zhang et al., 2008; Scarafoni et al., 2008; Mello et al., 2009; Bhattacharyya and Babu, 2009).

Norioka et al. (1988) reported the presence of BBI type of PIs in *C. cajan* based on the gel filtration peaks. Godbole et al. (1994a) purified two different BBIs from the seeds of *C. cajan* cultivar TAT-10. One of them had molecular mass around 15 kDa and inhibited both trypsin as well as chymotrypsin activities. However, the second one which had molecular mass around 10.5 kDa inhibited only trypsin activity. On the other hand, examination of the N-terminal sequence of PI (14 kDa) purified from PUSA-33 variety of *C. cajan* suggested that it belongs to Kunitz inhibitor family (Haq and Khan, 2003). The

PI (18.2 kDa) purified by Osowole et al. (1992) in *C. cajan* not only inhibited the trypsin activity but also showed antimicrobial activity.

To unveil the discrepancy in molecular mass and family of inhibitor, in the present study we have chosen *C. cajan* variety ICP 14770 (also called as ICPL-332 or Abhaya) to purify the PI. The cultivar ICP 14770 is one among the six cultivars which showed higher inhibitory activity against bovine pancreatic trypsin (reported in **Chapter 4**). The purified inhibitor from ICP 14770 was extensively examined for its biochemical properties before confirming that it belong to BBI type family. Further, both *in vitro* and *in vivo* studies indicated that the PI purified from ICP 14770 also possessed insecticidal properties, particularly against midgut trypsin-like proteinases of *Achaea janata* and *Manduca sexta* (reported in **Chapter 6**).

Results and discussion

Purification of proteinase inhibitor:

The crude protein obtained from the mature dry seeds of *C. cajan* was initially subjected to $(NH_4)_2SO_4$ fractionation: 0-25%, 25-60% and 60-90%. The different fractions obtained were assayed for inhibitory activity against trypsin (TI activity). The 25-60% $(NH_4)_2SO_4$ fraction which showed maximum TI activity was loaded onto a DEAE-Cellulose column. The protein bound to the matrix was eluted with a linear gradient of 0.1 to 1.0 M NaCl in 50 mM Tris-HCl (pH 8.0). Based on the absorbance at 280 nm, the proteins were eluted into two peaks: I and II (**Fig. 5.1**). However, the assay for TI activity indicated the presence of active fractions at peak I of A_{280}. The fractions showing TI activity at peak I were pooled and further applied on to a trypsin-Sepharose 4B column. The protein bound to the matrix was eluted with 0.01 N HCl. The fractions eluted from the trypsin-Sepharose 4B column showed single peak for both total protein at 280 nm and total TI activity (**Fig. 5.2**). The active fractions from affinity column, which

contained some high molecular weight contaminants as shown in Tricine-SDS-PAGE (**Fig. 5.4, lane 5**) were further separated by gel-filtration chromatography using Sephadex G-50 column. The column was equilibrated and eluted with 50 mM Tris-HCl (pH 8.0). Two peaks (I and II) related to protein absorbance at 280 nm were observed (**Fig. 5.3**). The peak II fractions which showed TI activity were pooled, named as RgPI and used in further characterization studies. The protocol used in the present study resulted in 55% yield recovery of RgPI with 66.5-fold purification (**Table 5.1**), which was much higher than that reported by Haq and Khan (2003) in PUSA-33 variety of *C. cajan*.

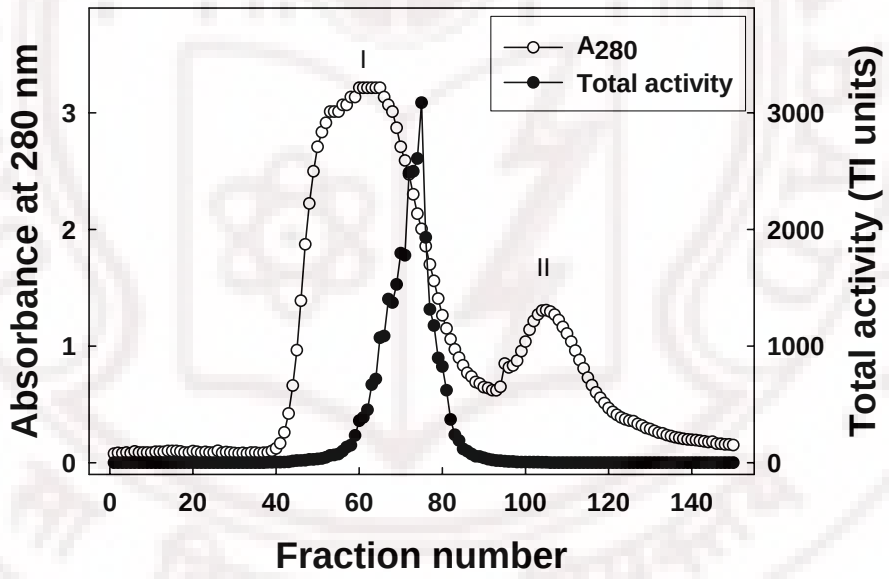

Fig. 5.1. Elution profile of the PI from 25-60% $(NH_4)_2SO_4$ fraction on DEAE-Cellulose column equilibrated with 50 mM Tris-HCl (pH 8.0). The column was eluted in 1.0 ml fractions with a linear gradient of 0.1 to 1.0 M NaCl in the above buffer at a flow rate of 30 ml/h. The fractions were analyzed for total protein at 280 nm and TI activity as described in materials and methods.

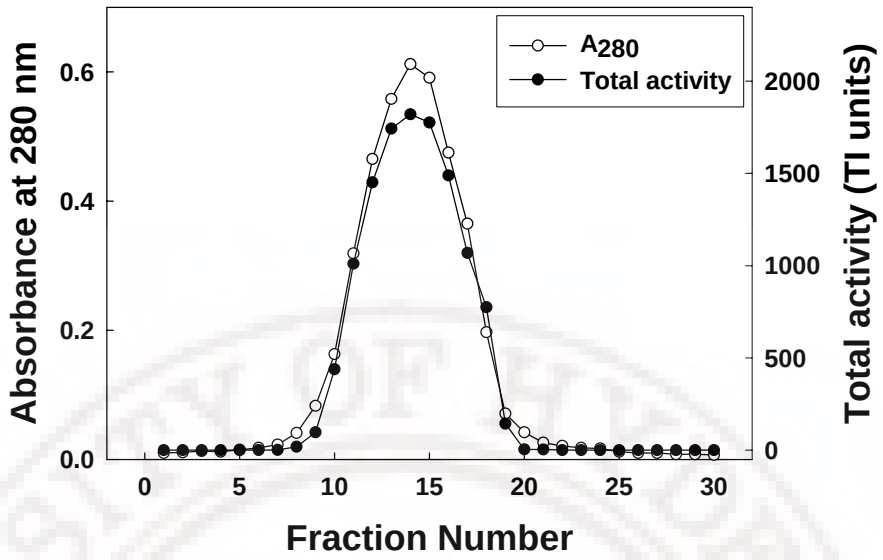

Fig. 5.2. Affinity chromatography of active peak I fractions from DEAE-Cellulose column on a trypsin-Sepharose 4B column equilibrated with 50 mM Tris-HCl (pH 8.0), containing 100 mM NaCl. The protein bound to the column was eluted in 1.0 ml fractions with 0.01 N HCl at a flow rate of 30 ml/h and analyzed for total protein at 280 nm and TI activity as described in materials and methods.

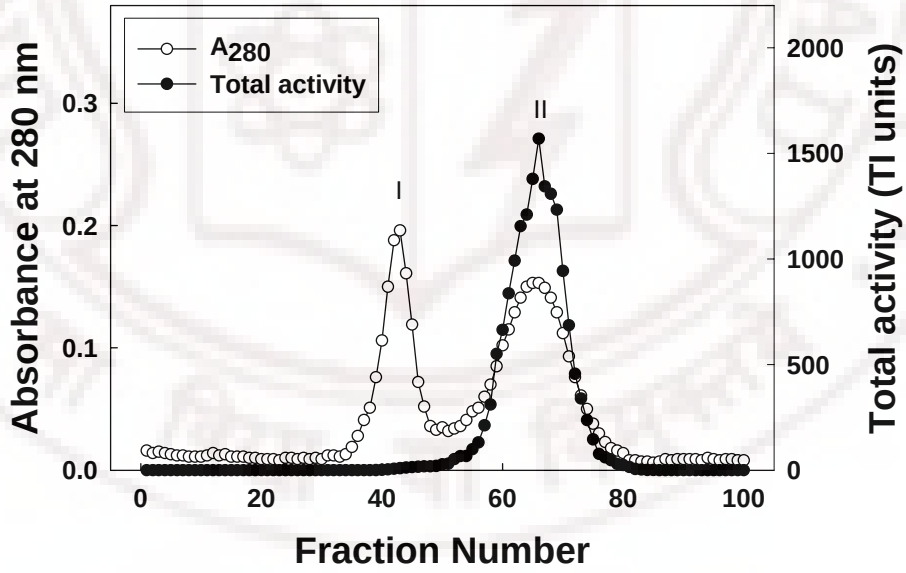

Fig. 5.3. Gel-filtration chromatography on Sephadex G-50 column loaded with affinity fraction pool. Equilibration of the column and elution of 1.0 ml fractions was performed with 50 mM Tris-HCl (pH 8.0) at a flow rate of 15 ml/h. The separated fractions were analyzed for total protein at 280 nm and TI activity as described in materials and methods. Peak II fractions which showed TI activity were pooled and named as "RgPI".

Table 5.1. Purification of proteinase inhibitor (RgPI) from red gram seeds.

Purification step	Total protein (mg)	Total activity (TI units)[a]	Yield recovery (%)	Specific activity[b] (TI units/ mg protein)	Purification (fold)
Crude extract	11426	217962	100	19	1
$(NH_4)_2SO_4$ fraction (25-60%)	3548	144773	66	41	2.16
DEAE-Cellulose column	1933	134052	62	69	3.63
Trypsin-Sepharose 4B column	118	126430	58	1071	56.37
Sephadex G-50 column	95	120118	55	1264	66.52

[a]One TI unit is defined as the amount of RgPI required to inhibit 50% of BAPNA hydrolysis by trypsin. [b]Specific activity is defined as the number of TI units per mg protein.

Molecular mass analysis:

The presence of two bands with approximate molecular masses of 8.5 kDa and 16.5 kDa in Tricine-SDS-PAGE under non-reducing condition indicate that RgPI exists in both monomeric and dimeric states (**Fig. 5.4**). The multimeric nature of RgPI was further evident in MALDI-TOF spectrum, which showed several peaks with molecular masses of 8376.8 Da, 16745.9 Da, 24529.4 Da, 32844.7 Da and 41193.3 Da, respectively, under native condition (**Fig. 5.5A and B**). However, reduction and alkylation of RgPI with DTT and iodoacetamide resulted in the appearance of only one peak at 9189.4 Da in MALDI-TOF spectrum (**Fig. 5.6**). Disappearance of oligomeric peaks in MALDI-TOF spectrum under reducing condition suggests that native structural conformation is essential for self association of RgPI and retaining its multimeric

structure. Further, the increase in mass of RgPI by 812.8 Da on reduction and alkylation demonstrate that RgPI possess 14 cysteine residues by assuming that alkylation of each cysteine residue leads to an increase of 57 Da (**Figs. 5.5A and 5.6**). These 14 cysteine residues might participate in forming 7 disulfide bonds, a characteristic feature of BBI type PIs (Qi et al., 2005), which is also evident in other BBIs isolated from seeds of *Cratylia mollis* (Paiva et al., 2006), *Dolichos biflorus* (Singh and Appu Rao, 2002), *Glycine max* (Werner and Wemmer, 1991; Voss et al., 1996), *Lupinus albus* (Scarafoni et al., 2008), *Medicago scutellata* (Catalano et al., 2003), *Phaseolus acutifolius* (Campos et al., 2004), *P. coccineus* (Pereira et al., 2007) and *Vigna unguiculata* (Barbosa et al., 2007).

BBIs are well known to undergo self-association in solution to form homodimers or trimers or more complex oligomers (Losso, 2008). Such self-association of protein molecules might result in over estimation of molecular mass in SDS-PAGE and gel-filtration chromatography, as reported for several legume BBIs (Wu and Whitakar, 1990; Bergeron and Nielson, 1993; Godbole et al., 1994a; Terada et al., 1994; Sreerama et al., 1997). The presence of (i) hydrophobic surface patches in the monomer which is an unusual structural feature, but common among BBIs and (ii) strong hydrogen bonded network in the dimer structure explains for the existence of BBIs in higher ordered multimeric conformations (Li de la Sierra et al., 1999; Rao and Suresh, 2007). NMR analysis of *M. scutellata* indicated that BBI exists as monomer in solution but can undergo self-association at >2 mM concentration. The residues involved in self-association are localized in opposite faces of the molecule and the electrostatic interaction between these faces is responsible for self-association (Catalano et al., 2003). Kumar et al. (2004) reported that Lys^{24} (P_1 residue) at amino-terminal of one monomer and Asp^{76} at carboxy-terminal of other monomer are responsible for self-association and formation of dimers in horse gram BBI (HGI-III).

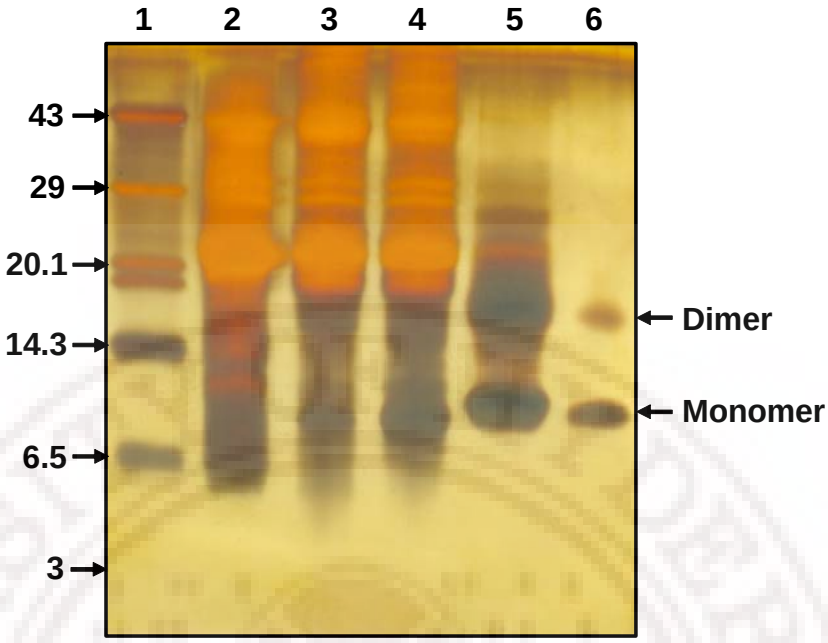

Fig. 5.4. Tricine-SDS-PAGE (18%) showing the purification pattern of RgPI. Lanes 1-6 are loaded with molecular markers (Banglore genei, PMW-L), protein from crude extract (25 µg), 25-60% $(NH_4)_2SO_4$ fraction (25 µg), active fraction pool from anion exchange column (25 µg), active fraction pool from affinity column (10 µg) and active fraction pool from gel-filtration column (5 µg of RgPI), respectively.

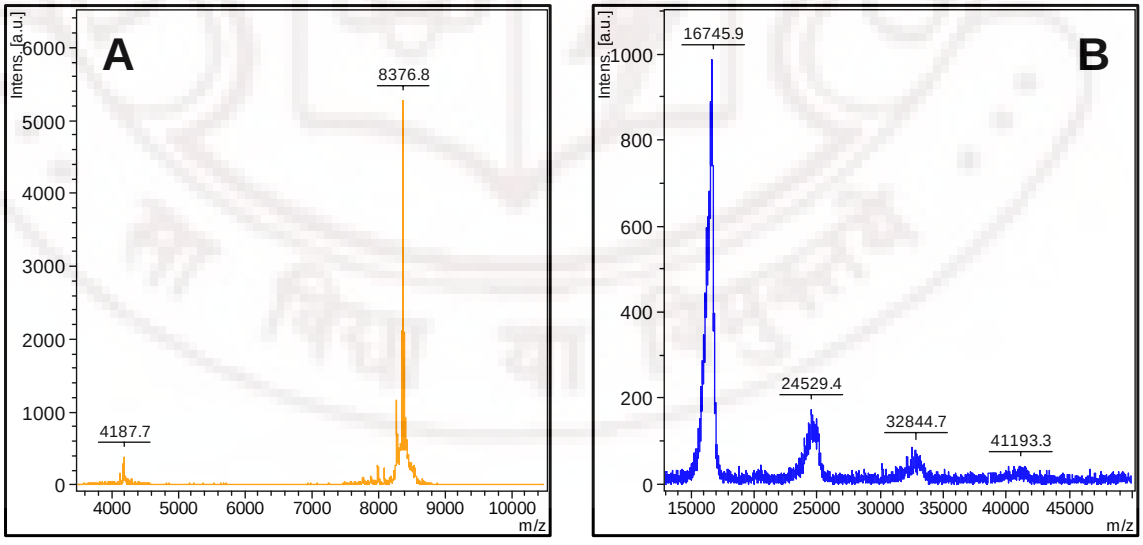

Fig. 5.5. MALDI-TOF mass spectrum of RgPI in native condition. (A) 0-10,000 m/z range (B) 10,000-50,000 m/z range.

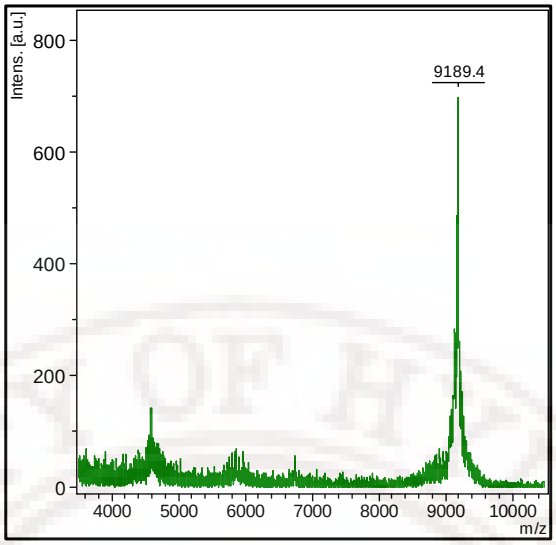

Fig. 5.6. MALDI-TOF spectrum of RgPI after reduction and alkylation with DTT and iodoacetamide, respectively.

Iso-inhibitors of RgPI:

The RgPI showed five different iso-inhibitors in native-PAGE (**Fig. 5.7**). These five different iso-inhibitors of RgPI (1-5) in native-PAGE were further separated by electroelution. These individual iso-inhibitors, $RgPI_1$ to $RgPI_5$, showed inhibitory activity against both trypsin and chymotrypsin (data not shown). These iso-inhibitors clearly resolved with a minute difference in molecular masses in Tricine-SDS-PAGE (**Fig. 5.8A**). However, in native-PAGE individual iso-inhibitors (**Fig. 5.8B, lanes 3-7**) resolved in same pattern as with RgPI pool (**Fig. 5.8B, lane 2**). The differential resolution of iso-inhibitors in native-PAGE could be due to difference in charge of individual iso-inhibitors, as they are separated based on mass to charge ratio (Pichare and Kachole, 1994). The BBI iso-inhibitors isolated from some leguminous seeds like *C. cajan* (Pichare and Kachole, 1994), *D. biflorus* (Ramasarma et al., 1994; Kumar et al., 2002), *G. soja* (Deshimaru et al., 2002), *P. vulgaris* (Wu and Whitaker, 1990), *V. radiata* (Wilson and Chen, 1983) and *V. unguiculata* (Sammour, 2006) also showed similar pattern of separation either in native-PAGE or in SDS-PAGE.

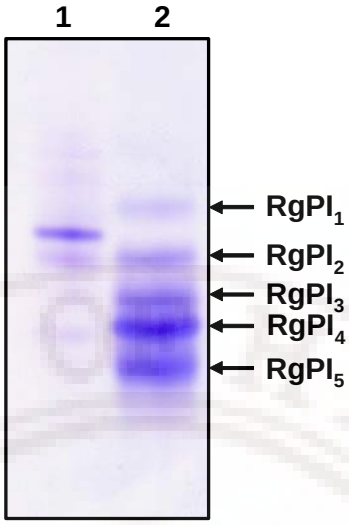

Fig. 5.7. Iso-inhibitors of RgPI separated on native-PAGE (12.5%). The samples in the lane 1 and lane 2 are soybean BBI and RgPI, respectively.

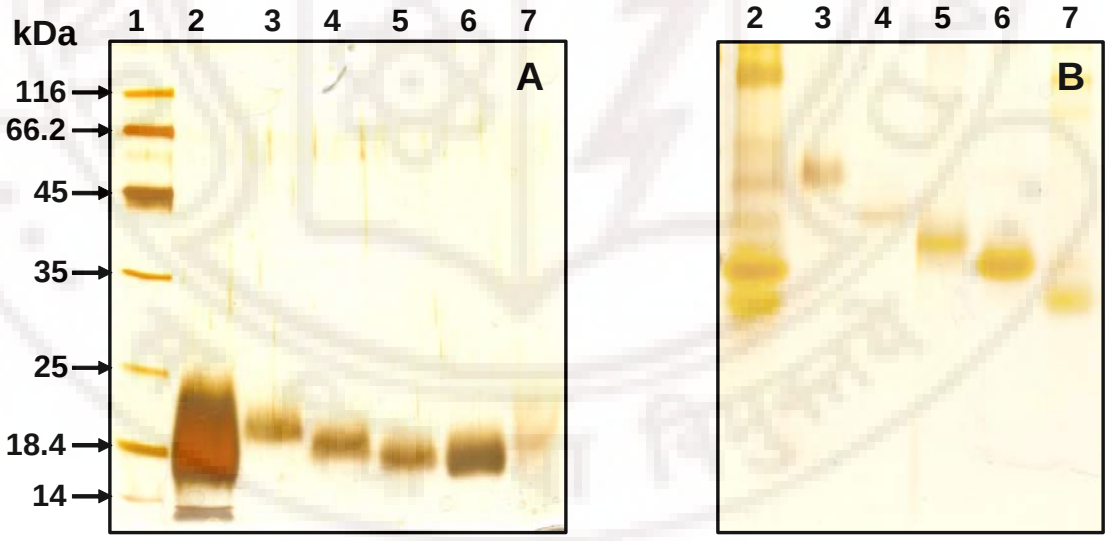

Fig. 5.8. Resolution pattern of individual iso-inhibitors of RgPI in (A) Tricine-SDS-PAGE (12.5%) and (B) native-PAGE (12.5%). Lane 1: molecular mass standards, lane 2: RgPI pool (20 µg) and lanes 3-7: $RgPI_1$ to $RgPI_5$ (10 µg in each). In SDS-PAGE protein was loaded after reduction with 2-mercaptoethanol.

Two-dimensional (2-D) electrophoresis revealed that the pI values of five iso-inhibitors were 5.95, 6.25, 6.50, 6.90 and 7.15, respectively (**Fig. 5.9**). The reason for the existence of several iso-inhibitors of PIs in the same plant and their physiological roles is still not very clear. BBIs are the products of a multigene families and the derivation of multiple isoforms has been associated with protein processing at both the amino and carboxylic ends (Domoney et al., 1993, 1995; Kalume et al., 1995). Harsulkar et al. (1999) proposed that in a co-evolving system, plants and insects had evolved with new forms of PIs and proteinases in order to combat against each other's defense mechanism. Hence, in this context, it is not surprising that the plants are capable of producing numerous iso-inhibitors.

Despite their pronounced micro heterogeneity, the iso-inhibitors of BBIs share a relatively high degree of sequence homology, especially in the inhibitory domains as well as in highly conserved disulphide bridge network (Ragg et al., 2006). The BBIs isolated from various leguminous plants like *C. mollis* (Paiva et al., 2006), *D. biflorus* (Sreerama et al., 1997; Kumar et al., 2002), *Inga umbratica* (Calderon et al., 2005), *Lens culinaris* (Ragg et al., 2006), *P. acutifolis* (Campos et al., 2004), *P. vulgaris* (Bergeron and Nielsen, 1993), *Pisum sativum* (Domoney et al., 1995; Morrison et al., 2007), *Psophocarpus tetragonolobus* (Giri et al., 2003) and *V. unguiculata* (Gennis and Cantor, 1976; Sammour, 2006; Rao and Suresh, 2007) also revealed the presence of iso-inhibitors.

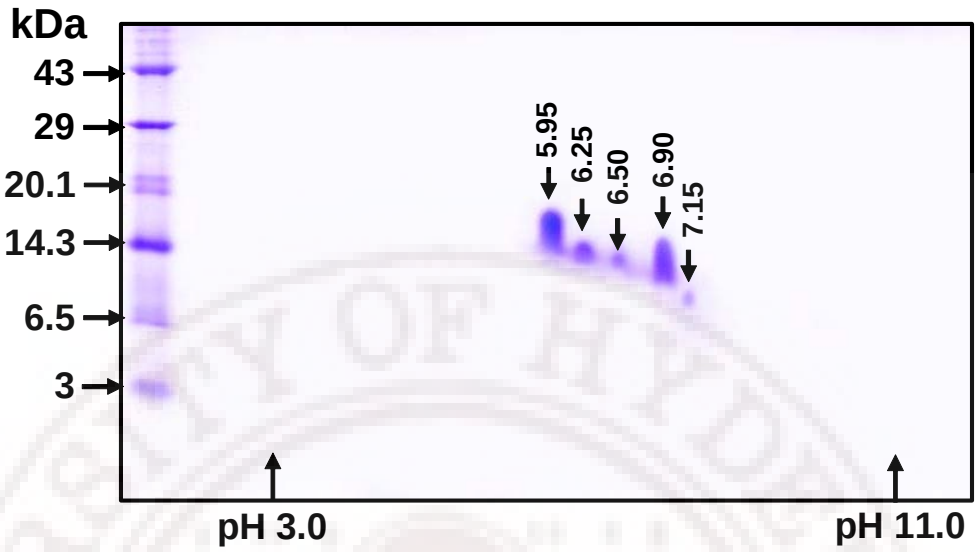

Fig. 5.9. Two-Dimensional gel showing the iso-inhibitors of RgPI. The proteins were separated in IEF using 3.0 – 11.0 pH non-linear strips having maximum resolution at pH 4.0 – 7.0. The second dimension was performed in Tricine-SDS-PAGE (18%) under reducing condition along with marker proteins having molecular mass ranging from 3 to 43 kDa.

Mass spectrum of tryptic digested RgPI iso-inhibitors:

The iso-inhibitors of RgPI with different pI values were excised from 2-D gel (**Fig. 5.9**) and subjected to tryptic digestion. MALDI-TOF analysis of the peptide mixture for all the iso-inhibitors of RgPI showed identical mass spectrum (**Fig. 5.10**). For example, the peaks with m/z values closer to 1638, 1851, 1993, 2150, 2501 and 2705 were found to be common in all the five iso-inhibitors. Further ionization of all these peaks in MALDI-TOF-TOF and Mascot search for MS/MS ion database revealed that peak 1638.8 m/z matched to the following sequence LNSCHSACDRCACTR of BBI type PI from *G. max* (**Figs. 5.11 and 5.12**).

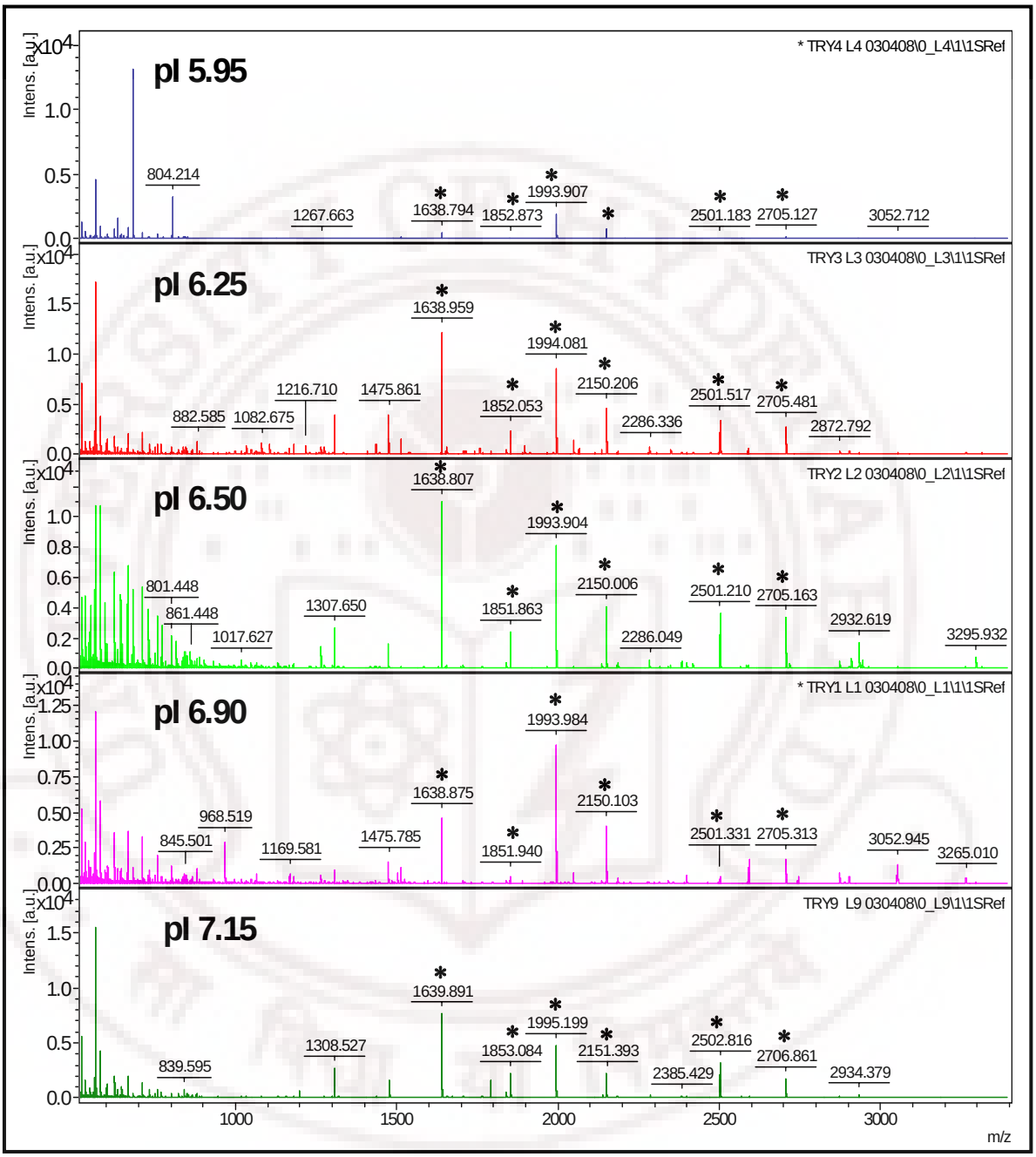

Fig. 5.10. MALDI-TOF mass spectrum of in gel tryptic digested iso-inhibitors of RgPI with different pI values: 5.95, 6.25, 6.50, 6.90 and 7.15.

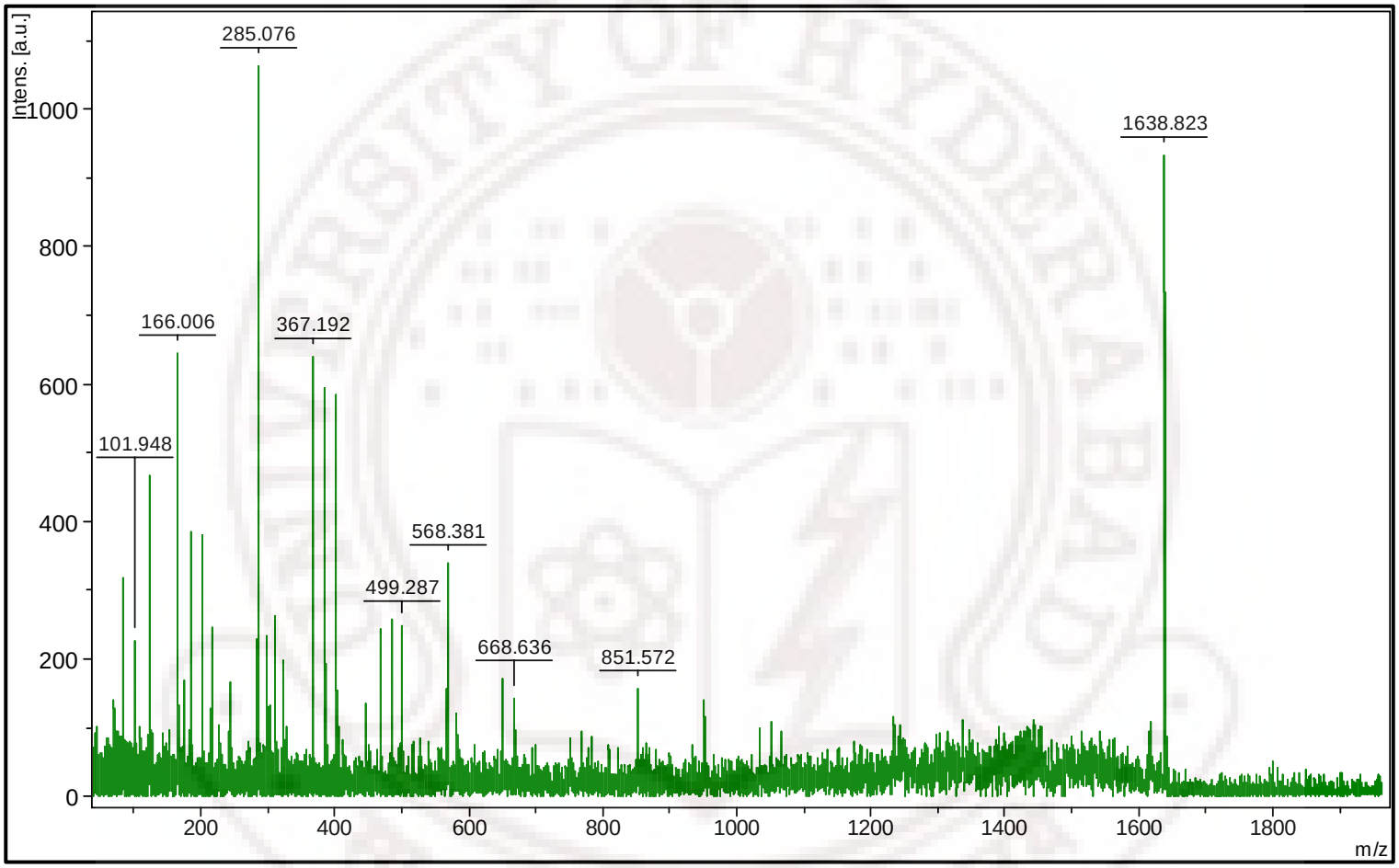

Fig. 5.11. MS/MS fragment spectrum of peak 1638.8 m/z

Match to: IBBC2_SOYBN Score: 18 Expect: 2.7e+02
Bowman-Birk type proteinase inhibitor C-II OS=Glycine max PE=1 SV=2
Found in search of DATA.TXT

Nominal mass (M_r): 9195; Calculated pI value: 4.63
NCBI BLAST search of IBBC2_SOYBN against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Glycine max

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: 18%

Matched peptides shown in Bold Red

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

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	%	Miss	Sequence
42 - 56	1638.8000	1637.7927	1638.6483	-0.05221	1	R.LNSCHSACDRCACTR.S (No match)

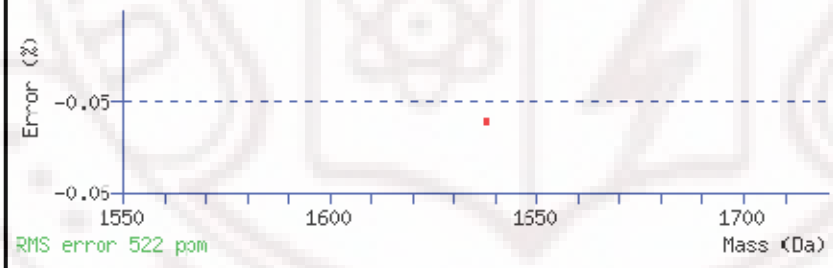

Fig. 5.12. Mascot search for MS/MS ion database of peak 1638.8 m/z.

N-terminal sequencing of RgPI iso-inhibitor with pI value 5.95:

The sequence of the amino acids up to 10 residues in the N-terminal region of RgPI iso-inhibitor with pI value 5.95 was determined by using automated Edman degradation in an Applied Biosystem Model 494 Procise protein sequencing system. The residues from N-terminal regions were as follows, Asp-Gln-His-His-Ser-Ser-Lys-Ala-Cys-Cys (DQHHSSKACC). This sequence showed homology with BBIs from *Phaseolus* spps. (**Fig. 5.13**). Thus taken together the results from the Mascot MS/MS ion search of the 1638.8 m/z peak and N-terminal sequencing of RgPI iso-inhibitor with pI value 5.95 reveals that the PI purified from *C. cajan* in the present study belongs to the BBI family.

```
P.acutifoliusCAL49456.1         MGLKNNNTMVLKVCFMLLFLLGTSTASLKLSELGLLMKSGHHHHDSSDEP 50
P.acutifoliusP83311.1           ------------------------------------SGHHHHDSSDEP 12
P.polyanthusCAQ58092.1          -MGVKNNNTMVLKVCFVLLFLLGTCTASLKLSELGLLMKSGHHHESTDEP 49
P.costaricensisCAL69279.1       -MGLKNNNTMVLKVCFVLLFLLGTCTASLKLSELGLLMKSGDHHESTDEP 49
P.vulgarisCAE77683.1            --------MVVLKVCFVLLFLLGTCTASLKLSELGLLMKSGHHHESTDKP 42
P.vulgarisCAD32699.1            --------MVVLKVCFVLLFLLGTCTASLMLSELGLLMKSGHHHESTDEP 42
P.polyanthusCAQ52821.1          -MGLKNNNTMVLKVCFVLLFLLGTCTASLKLSELGLLMKSGHHHESTDEP 49
P.maculatesCAL51271.1           -MGLKNNNTMVLKVCFVLLFLLGTSTASLKLSELGLLMKSGHHHESTDES 49
P.oligospermusCAL51270.1        -MGLKNNNTMVLKVCFMLLFLLGTSTASLKLSELGLLMKSGHHHESTDEP 49
P.grayanusCAQ52359.1            -MGLKNNNTMVLKVCFRLLFLLGTSTASLKLSELGLLMKSGHHHESTDEP 49
P.hintoniiCAQ52357.1            -MGLKNNNTMVLKVCFMLLFLLGTSTASLKLSELGLLMKSG-HHESTDEP 48
P.glabellusCAQ52825.1           -MGLKNNNTMVLKVCFMLLFLLGTSTASLKLSELGLLMKNGHHHESTDEP 49
P.filiformisCAQ52823.1          -MGLKNNNTMVLKVCFVLLFLLGTSTASLKLSELGQLTKSGHHHESTDEP 49
RgPI                            ----------------------------------------DQHH------ 4
                                                                          **

P.acutifoliusCAL49456.1         SESSKACCDHCACTKSIPPQCRCADLRLNSCHSECRSCICTFSIPAQCVC 100
P.acutifoliusP83311.1           SESSKACCDHCACTKSIPPQCRCA-LRLN-CN-HCRSCICTFSIPAQCVC 59
P.polyanthusCAQ58092.1          SESSKACCDHCACTKSRPPQCRCSDLRLNSCHSECKSCICTLSIPAQCVC 99
P.costaricensisCAL69279.1       SESSKACCDHCACTKSRPPQCRCSDLRLNSCHSECKSCICTLSIPAQCIC 99
P.vulgarisCAE77683.1            SESSKACCDHCACTKSIPPQCRCSDLRLNSCHSECKSCICTLSIPAQCIC 92
P.vulgarisCAD32699.1            SESSKACCDHCACTKSIPPQCRCSDLRLNSCHSECKSCICTLSIPAQCVC 92
P.polyanthusCAQ52821.1          SESSKACCDHCACTKSIPPQCRCSDLRLNSCHSECKSCICTFSIPAQCIC 99
P.maculatesCAL51271.1           SESSKACCDHCACTKSIPPQCRCSDLRLNSCHSACKSCICTLSIPAQCVC 99
P.oligospermusCAL51270.1        SESSKACCDHCACTKSIPPQCRCSDLRLDSCHSACKSCICTLSIPAQCVC 99
P.grayanusCAQ52359.1            SESSKACCDQCACTKSIPPQCRCSDLRLNSCHSACKSCICTFSIPAQCVC 99
P.hintoniiCAQ52357.1            SESSKACCDQCACTKSIPPQCRCSDLRLNSCHSECKSCICTFSIPAQCVC 98
P.glabellusCAQ52825.1           SESSKACCDQCACTKSIPPQCRCLDLRLN-----------PLLRPAQCVC 88
P.filiformisCAQ52823.1          SESSKACCDQCACTKSIPPQCRCSDLRLNSCHSACKSCICTLSIPAQCVC 99
RgPI                            --SSKACC---------------------------------------- 10
                                  ******

P.acutifoliusCAL49456.1         TDTNDFCYEPCKSGHDDDDSGN 122
P.acutifoliusP83311.1           TDTNDFCYEPCKSGHDDDDSG- 80
P.polyanthusCAQ58092.1          TDTNDFCYEPCKPSHDDDSGN- 120
P.costaricensisCAL69279.1       TDTNDFCYEPCKPSHDDDSGN- 120
P.vulgarisCAE77683.1            TDTNDFCYEPCK---------- 104
P.vulgarisCAD32699.1            TDINDFCYEPCK---------- 104
P.polyanthusCAQ52821.1          TDTNDFCYEPCKPSHDDDSGN- 120
P.maculatesCAL51271.1           TDINDFCYEPCKSSHDDDSDN- 120
P.oligospermusCAL51270.1        TDINDFCYKPCKSSHDDDSDN- 120
P.grayanusCAQ52359.1            TDIDDFCYEPCKPSHDDDSDN- 120
P.hintoniiCAQ52357.1            TDINDFCYEPCKPSHDDDSDN- 119
P.glabellusCAQ52825.1           TDIDEFCYEPCKSSHDDDSDNK 110
P.filiformisCAQ52823.1          TDINDFCYEPCKPSHDDDSDN- 120
RgPI                            ----------------------
```

Fig. 5.13. Sequence alignment of RgPI (pI value 5.95) with BBI inhibitors from different *Phaseolus* spp. using ClustalW.

Inhibitor activity and inhibitor constant (K_i) determination:

RgPI showed inhibitory activity against both trypsin and chymotrypsin, a characteristic feature of BBI type PIs. However inhibitory activity against trypsin was more pronounced when compared with chymotrypsin. Most of the BBI type PIs isolated from leguminous seeds are double headed with two different reactive sites for trypsin and chymotrypsin, respectively (Singh and Appu Rao, 2002). A few exceptions of BBI isolated from *M. scutellata* (Ceciliani et al., 1997), *Dioclea glabra* (Bueno et al., 1999), *C. mollis* (Paiva et al., 2006) and *L. albus* (Scarafoni et al., 2008) showed activity against trypsin alone.

Trypsin lost 55% of its activity when the molar ratio of RgPI to trypsin was 0.50. As the ratio of RgPI to trypsin increased from 0.50 to 1.0, trypsin lost ~100% of its activity. A liner extrapolation to obtain 100% inhibition indicated that RgPI binds to trypsin at ~1:1 molar ratio (**Fig. 5.14**), which means that one molecule of RgPI will bind to one molecule of trypsin. Most of the PIs belonging to either Kunitz or BBI family, inhibited trypsin activity preferably when their concentrations are at 1:1 stoichometry (Bueno et al., 1999; Devraj and Manjunatha et al., 1999; Cavalcanti et al., 2002; Macedo et al., 2003; Haq et al., 2005; Bhattacharya et al., 2006, 2007; Lingaraju and Gowda, 2008). However, few PIs also showed the stiochometry ratio with trypsin as 0.5:1.0 i.e., one molecule of PI will bind to two molecules of trypsin (Scarafoni et al., 2008; Zhang et al., 2008). In contrast, RgPI showed no obvious stoichiometry with chymotrypsin (**Fig. 5.14**). Similar pattern of inhibition for chymotrypsin was observed with PIs isolated from *Apios americana* tubers (Zhang et al., 2008) and *Peltophorum dubium* seeds (Macedo et al., 2003).

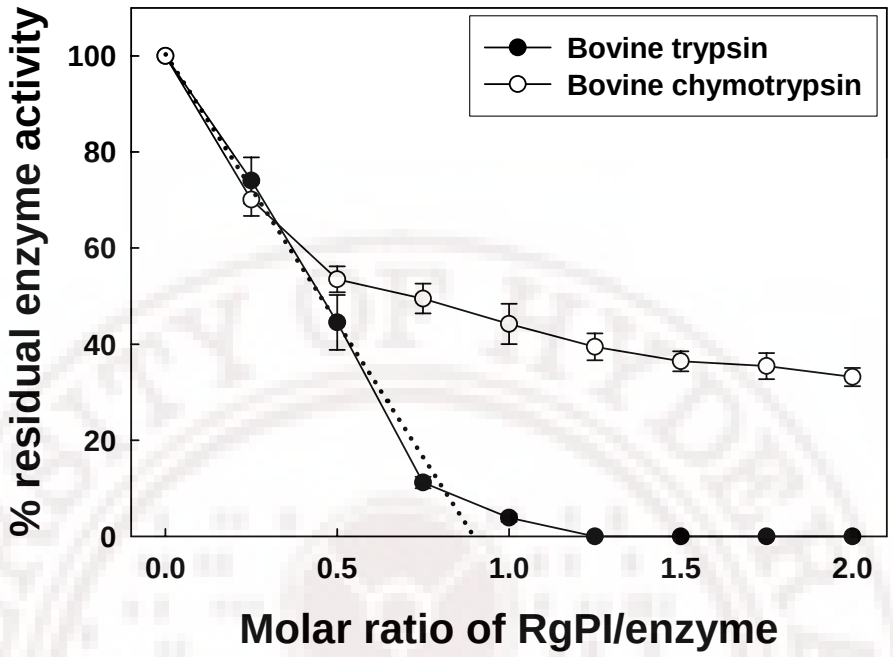

Fig. 5.14. Titration curves of trypsin and chymotrypsin inhibition by RgPI. Increasing concentrations of RgPI was added to a fixed concentration of enzyme. The concentration of trypsin or chymotrypsin in the reaction mixture was 1 x 10^{-6} M. After incubating the reaction mixture for 15 min, residual trypsin or chymotrypsin activity was determined by monitoring the hydrolysis of synthetic substrates BAPNA and GLUPHEPA, respectively. The molar ratio of the inhibitor to the trypsin or chymotrypsin was the intercept of x-coordinate, when the tangent was extrapolated to the zero activity (Knights and Light, 1976).

The K_i for both trypsin and chymotrypsin was examined by increasing the concentration of RgPI with different concentrations of their respective substrates BAPNA and GLUPHEPA. Lineweaver-Burk plot showed non-competitive type of inhibition with both trypsin and chymotrypsin with a K_i of 292.1 nM for trypsin and 2265.0 nM for chymotrypsin (**Fig. 5.15A and B**).

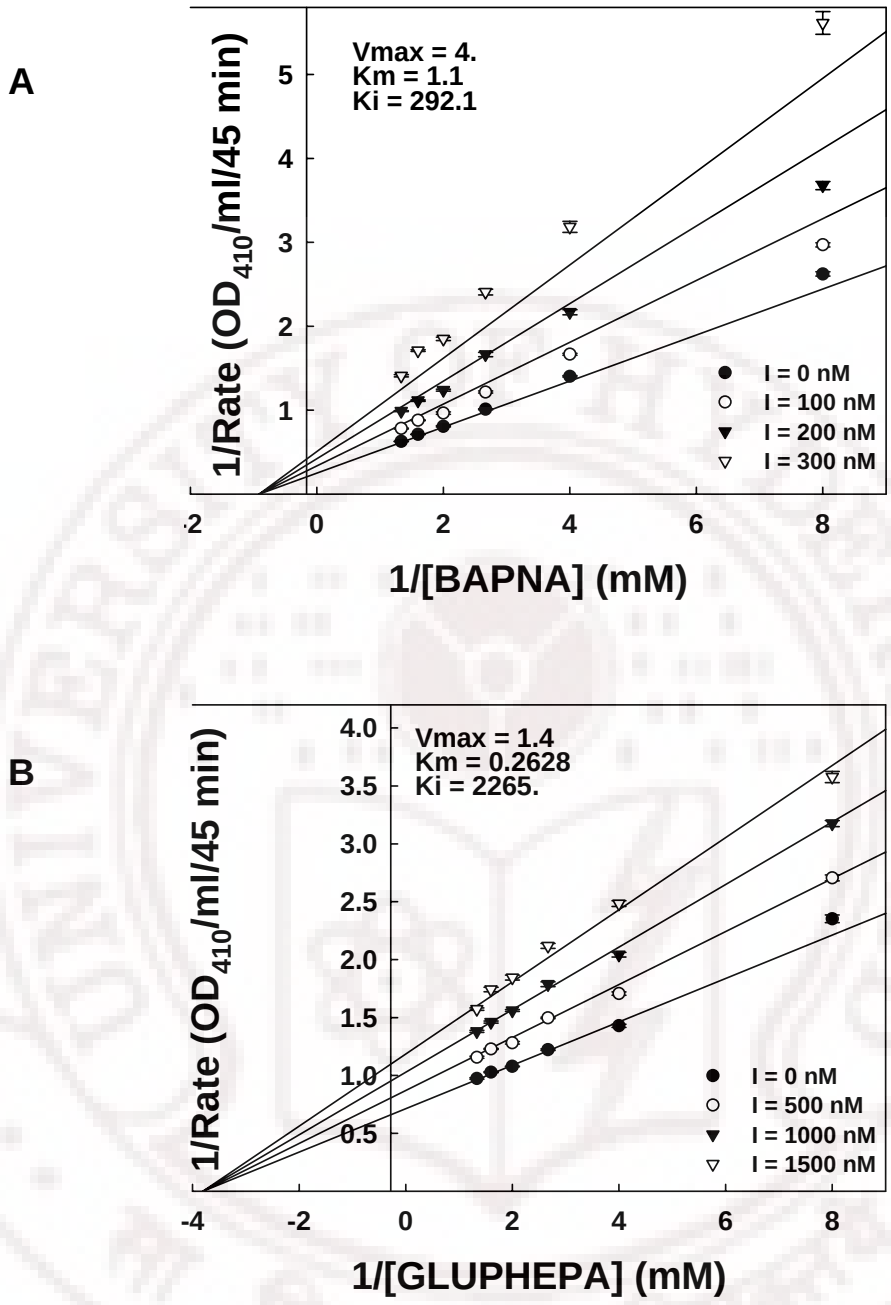

Fig. 5.15. Kinetic analysis of trypsin and chymotrypsin inhibition by RgPI. Lineweaver-Burk plot showing non-competitive nature of trypsin (A) and chymotrypsin (B) inhibition by RgPI at various concentrations of BAPNA and GLUPHEPA (0.125, 0.250, 0.375, 0.500, 0.625 and 0.750 mM).

The lower K_i value observed for trypsin (292.1 nM) when compared with chymotrypsin (2265.0 nM) indicate that the RgPI possess 7.75-fold higher affinity for trypsin over chymotrypsin. This could be due to the difference in the rates of binding of RgPI to the enzyme (**Fig. 5.14**; Haq and Khan, 2003). The K_i value reported for trypsin in *C. cajan* was found to be 153 mM (Osowole et al., 1992). However, BBI type PIs from different Leguminosae plants showed K_i values for trypsin in the range between 0.1 to 5 nM (Terada et al., 1994; Ceciliani et al., 1997; Tanaka et al., 1997; Bueno et al., 1999; Deshimaru et al., 2002; Paiva et al., 2006; Ragg et al., 2006; Scarafoni et al., 2008; Zhang et al., 2008) except a few in which K_i values for trypsin was slightly higher i.e., 0.21 to 52 μM (Ramasarma et al., 1994; Devraj and Manjunatha, 1999). The difference in the K_i value for trypsin and chymotrypsin for RgPI resembled with BBIs isolated from *Lens culinaris* (Ragg et al., 2006). In spite of having a higher K_i for bovine pancreatic trypsin (when compared to other BBIs) RgPI showed significant inhibitory activity against midgut trypsin-like proteinases isolated from larvae of *M. sexta* and *A. janata* (reported in **chapter 7**).

Structural characterization of RgPI:

The secondary and tertiary structure of RgPI was analyzed by Circular dichroism (CD) spectroscopy in the presence of 10 mM phosphate buffer (pH 7.4). Far-UV CD spectrum (190-250 nm) of RgPI which represents its secondary structure was shown in **figure 5.16**. Analysis of the composition of secondary structure by CDSSTR programme showed the predominance of β-sheets (68%) and random coil (25%) over α-helix (11%) (**Table 5.2**). Similar reports were observed for BBIs isolated from *G. max* (Wu and Sessa, 1994), *I. umbratica* (Calderon et al., 2005) and *V. unguiculata* (Rao and Suresh, 2007). However, the distinctive feature of BBI type PIs which tend to have very less composition of ordered structure (α-helix and β-sheets) and high content of aperiodic

structure, offer greater flexibility to the BBI molecule (Ramasarma et al., 1995). The disulfide linkages in the BBIs minimize the conformational entropy and enhance the stability of these proteins. Near-UV CD spectrum (250-300 nm) of RgPI which represent its tertiary structure was shown in **figure 5.17**. The tertiary structure showed the presence of positive CD at 257 nm which is attributed to the presence of disulfide bonds (Ramasarma et al., 1995). It also displayed a minimum at 283 nm, which further confirms the contribution of disulfide bonds and presence of tyrosine residue (Singh and Appu Rao, 2002; Haq et al., 2005).

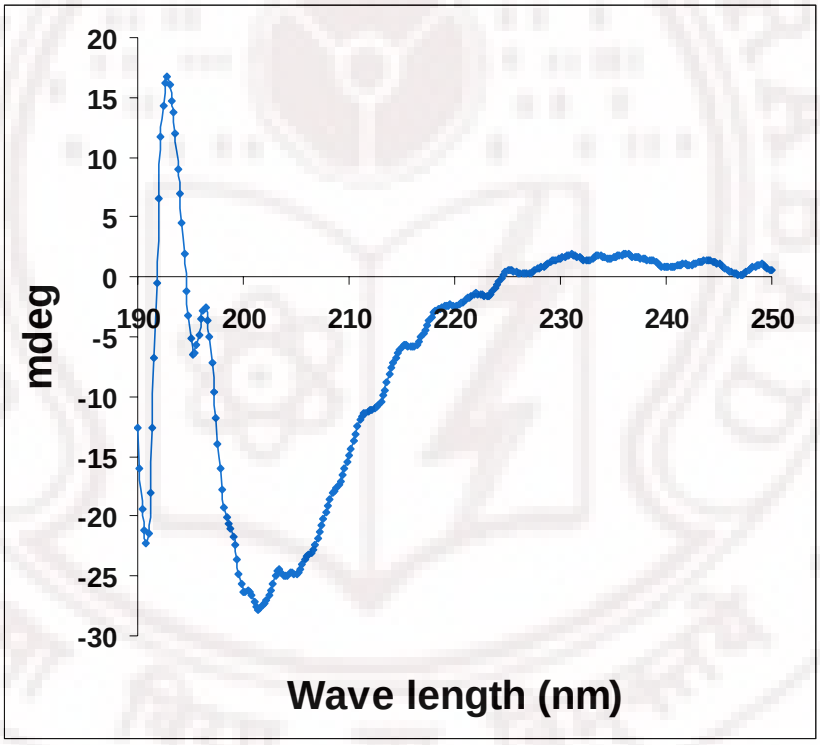

Fig. 5.16. Far-UV CD spectra of RgPI representing secondary structure. The difference spectrum for buffer (10 mM phosphate buffer, pH 7.4) and protein (RgPI) sample was shown. The other details were as described in materials and methods.

Table 5.2. Composition of secondary structure of RgPI analyzed by CDSSTR programme.

Secondary structure	Percent of secondary structure content
α-helix	11.00
Anti-parallel β-sheet	33.60
Parallel β-sheet	14.10
B-turn	20.10
Random coil	25.00

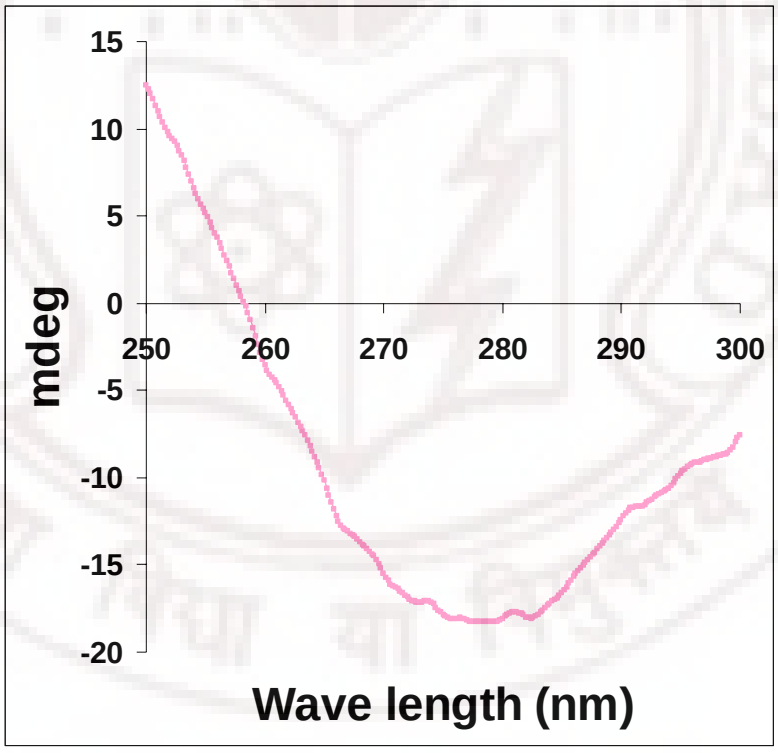

Fig. 5.17. Near-UV CD spectra of RgPI representing tertiary structure. Other details were as described in Fig. 5.16 and materials and methods.

Temperature and pH stability of RgPI:

Inhibitory activity of RgPI against trypsin and chymotrypsin was examined after incubation at different temperatures for 30 and 60 min as shown in **figure 5.18**. Trypsin and chymotrypsin inhibitory activity of RgPI was stable until 30 min even after heating up to 100°C. However, marginal loss in trypsin and chymotrypsin inhibitory activity (10-20%) was observed when heated for 60 min at 100°C. Increase in temperature from ambient to 90°C resulted in marginal changes in the far-UV CD spectra of RgPI (**Fig. 5.19**). The reversal of the temperature induced conformational changes in the far-UV CD spectra (90°C to 25°C) suggests that the PI possessed some degree of flexibility and retained its activity under thermal stress.

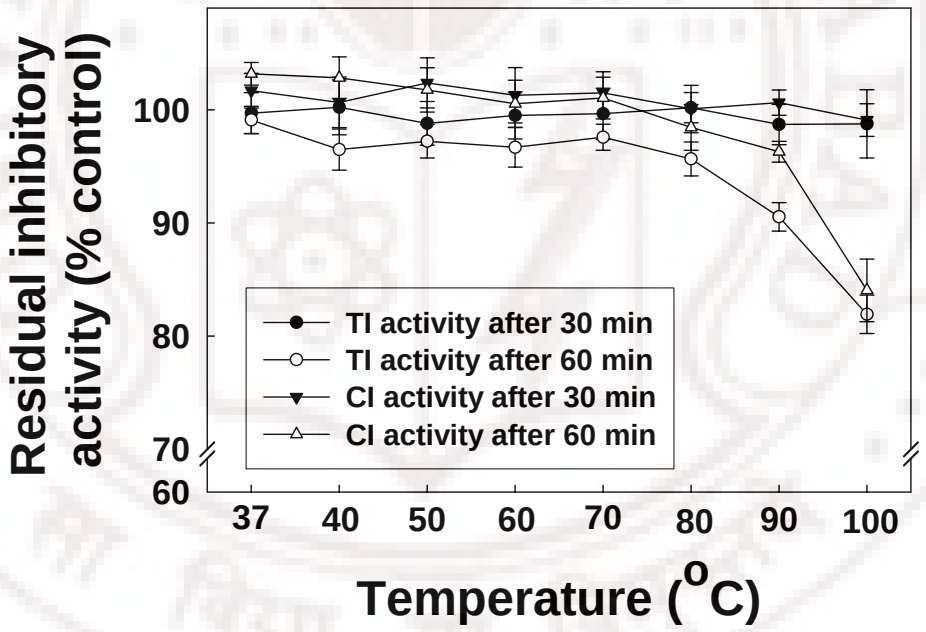

Fig. 5.18. Temperature stability of RgPI after incubation for 30 and 60 min at the indicated temperatures. Residual inhibitory activity of RgPI against trypsin and chymotrypsin was estimated in presence of BAPNA and GLUPHEPA, respectively, as described in materials and methods.

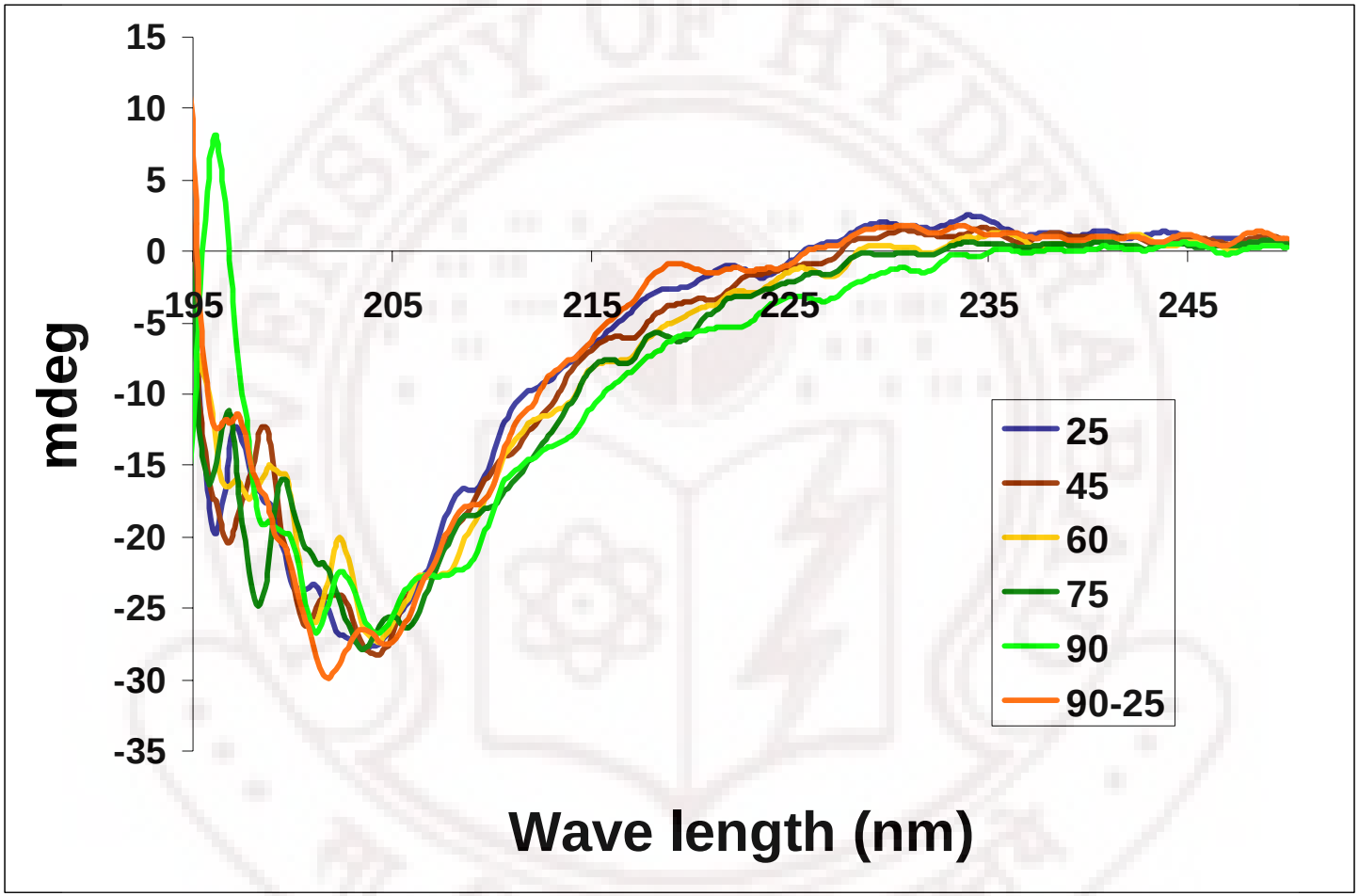

Fig. 5.19. Effect of temperature on far-UV CD spectra (secondary structure) of RgPI. The required temperature (^{o}C) was induced using the thermostat (peltier, Jasco). Other details were as described in Fig. 5.16 and materials and methods.

The inhibitory activity of RgPI against both bovine pancreatic trypsin and chymotrypsin was also stable at different pH tested between 2.0 and 12.0 (**Fig. 5.20**). Except at pH 6.0, negligible loss in trypsin and chymotrypsin inhibitory activity was observed. The marginal loss (10%) in inhibitory activity at pH 6.0 could be due to iso-electric precipitation at that particular pH (**Fig. 5.9**). The far-UV CD spectral studies displayed structural changes at both acidic (pH 2.0 and 4.0) and basic side (pH 10.0 and 12.0) (**Fig. 5.21**). The loss in secondary structure at acidic pH was due to loss of native confirmation caused by electrostatic repulsion at low pH. In activity assays, after pre-incubating the RgPI at respective pH, the assay was performed at pH 8.0, which could revert the loss in secondary structure to active conformation of the PI. This could be the reason for the observed stability in activity assays at different pH. The presence of many cysteine residues which form disulfide bonds may account for this striking stability in structure and activity of BBI type of PIs (Singh and Appu Rao, 2002). The stability of RgPI towards temperature and pH corroborated well with the properties of BBIs isolated from *D. biflorus* (Singh and Appu Rao, 2002), *Dioclea glabra* (Bueno et al., 1999), *G. soja* (Deshimaru et al., 2002) *C. mollis* (Paiva et al., 2006), *L. albus* (Scarafoni et al., 2008), *P. coccineus* (Pereira et al., 2007).

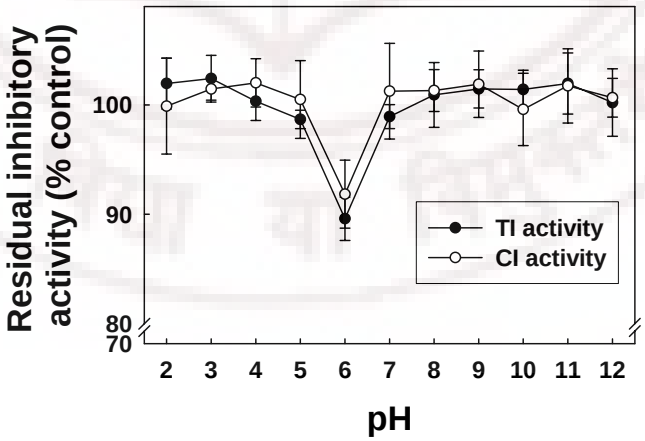

Fig. 5.20. Stability of RgPI after incubation at various pH ranging from 2.0 to 12.0 for 1h. The residual inhibitory activity was determined as described in materials and methods.

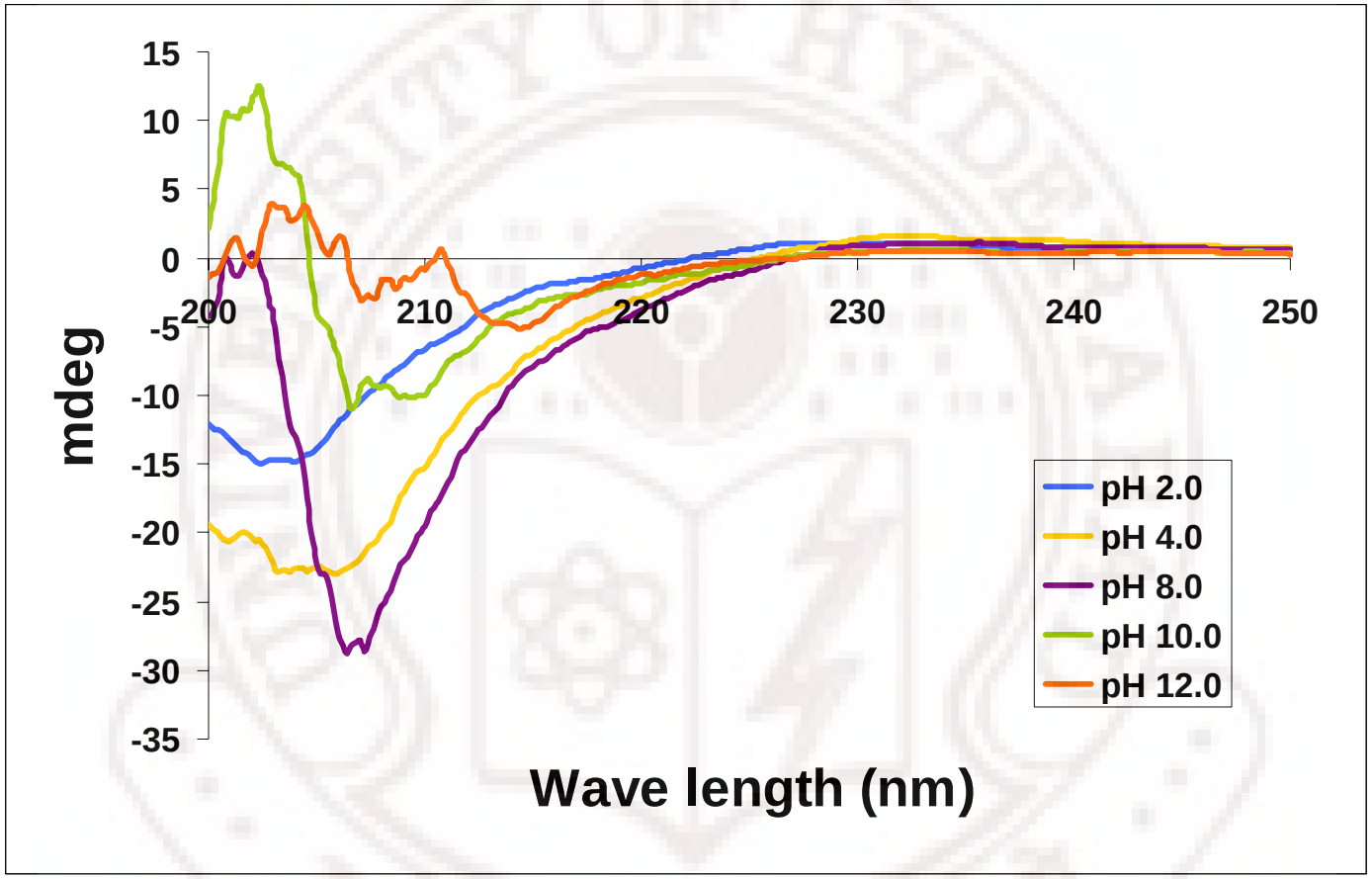

Fig. 5.21. Effect of pH on the far-UV CD spectra (secondary structure) of RgPI. The RgPI was incubated at respective pH for 1 h before taking the spectra. Other details were as described in Fig. 5.16 and materials and methods.

Effect of reducing agents dithiothreitol (DTT) and 2-mercaptoethanol (2-ME) on inhibitory activity of RgPI:

Disulfide bonds play an important role in maintaining the native conformation of a protein, which in turn provide stability/resistance towards pH and temperature treatments. This type of stability was more evident in BBI type PIs when compared with Kunitz type PIs, as they contain more number of cysteine residues. Therefore, it was of interest to evaluate the effect of reducing agents DTT and 2-ME on the trypsin and chymotrypsin inhibitory activity of RgPI (**Figs. 5.22 and 5.23**). At 0.25 mM concentration of DTT there was a significant decrease in the inhibitory activity of RgPI against trypsin (90%) and chymotrypsin (80%). By increasing the concentration of DTT to 1.0 mM, there was a complete loss in inhibitory activity against both trypsin and chymotrypsin (**Fig. 5.22A**). In contrast, reduction of RgPI with different concentrations of 2-ME resulted in a gradual decrease in trypsin and chymotrypsin inhibitory activity of RgPI (**Fig. 5.22B**). At 1.0 mM concentration of 2-ME there was a negligible loss in inhibitory activity against trypsin or chymotrypsin (**Fig. 5.22B**). Further, increasing the concentration of 2-ME to 10 mM, inhibitory activity against trypsin (74%) and chymotrypsin (48%) was significantly decreased. This difference in inhibition between these two proteinases in presence of 2-ME could be due to presence of more disulfide bonds near trypsin inhibitory site than chymotrypsin inhibitory site (Singh and Appu Rao, 2002). However, this loss in inhibitory activity was more pronounced with DTT than 2-ME.

Activity staining studies showed that RgPI exists as both monomeric and dimeric forms in non-reduced condition (**Fig. 5.23**). Reduction of RgPI with 2-ME showed the loss of dimer formation while retaining the monomer activity. But on reduction of RgPI with DTT, there was a complete loss in both monomer activity and dimer formation (**Fig. 5.23**). This significant decrease in inhibitory activity after treatment with reducing agents

was in contrast to the treatment with temperature or pH. This reduction in inhibitory activity in presence of reducing agents could be due to the loss in conformation of reactive site loop (nine residues) which is formed by disulfide bonds and responsible for maintaining its activity (Qi et al., 2005). RgPI purified in the present study was more susceptible to DTT reduction than the PI isolated from *D. biflorus* (Ramasarma et al., 1995) which was stable even after reduction with 1.0 mM DTT. However, the PI purified from *C. cajan* by Haq and Khan (2003) lost its activity after reduction with 1.0 mM DTT. Thus, it appears that the stability of some of the inhibitors is apparently unrelated to the presence of disulfide bonds.

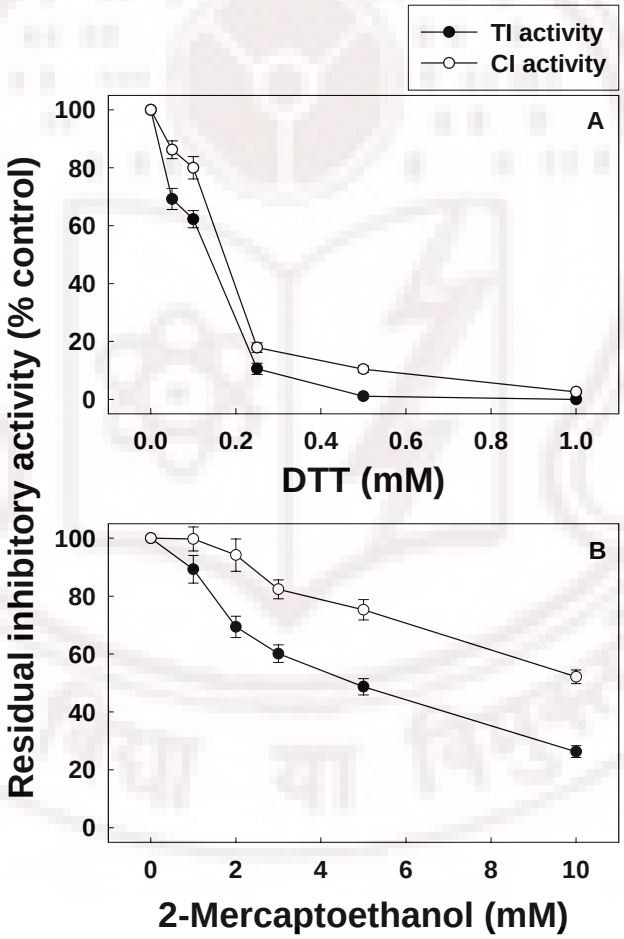

Fig. 5.22. Effect of reducing agents DTT (A) and 2-ME (B) on trypsin and chymotrypsin inhibitory activity of RgPI. The residual inhibitory activity against trypsin and chymotrypsin was determined as described in materials and methods.

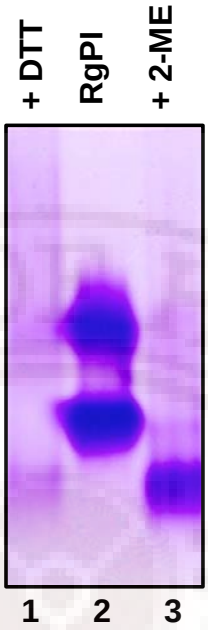

Fig. 5.23. Gelatin-SDS-PAGE (12.5%) showing the activity of reduced RgPI. Lanes 1 and 3 are loaded with RgPI treated with DTT and 2-ME, respectively. Lane 2 was loaded with RgPI untreated with reducing agents. The gel was developed against trypsin as described in materials and methods to visualize the trypsin inhibitor bands.

To evaluate the conformational changes associated with DTT reduction, RgPI was incubated with 1 mM DTT at 56^{o}C for 15 min. Changes in secondary structure before and after reduction with DTT were shown in **figure 5.24**. After reduction of RgPI with DTT, the decreased in ellipticity observed at 203 nm was associated with marginal changes in overall structure of RgPI. However, when the reduction of RgPI with DTT was followed by alkylation with iodoacetamide, the ellipticity at 203 nm in secondary structure was further decreased (**Fig. 5. 24**). These results clearly suggest that breakage of disulfide bonds leads to the loss of reactive site loop which in turn decrease its inhibitory activity.

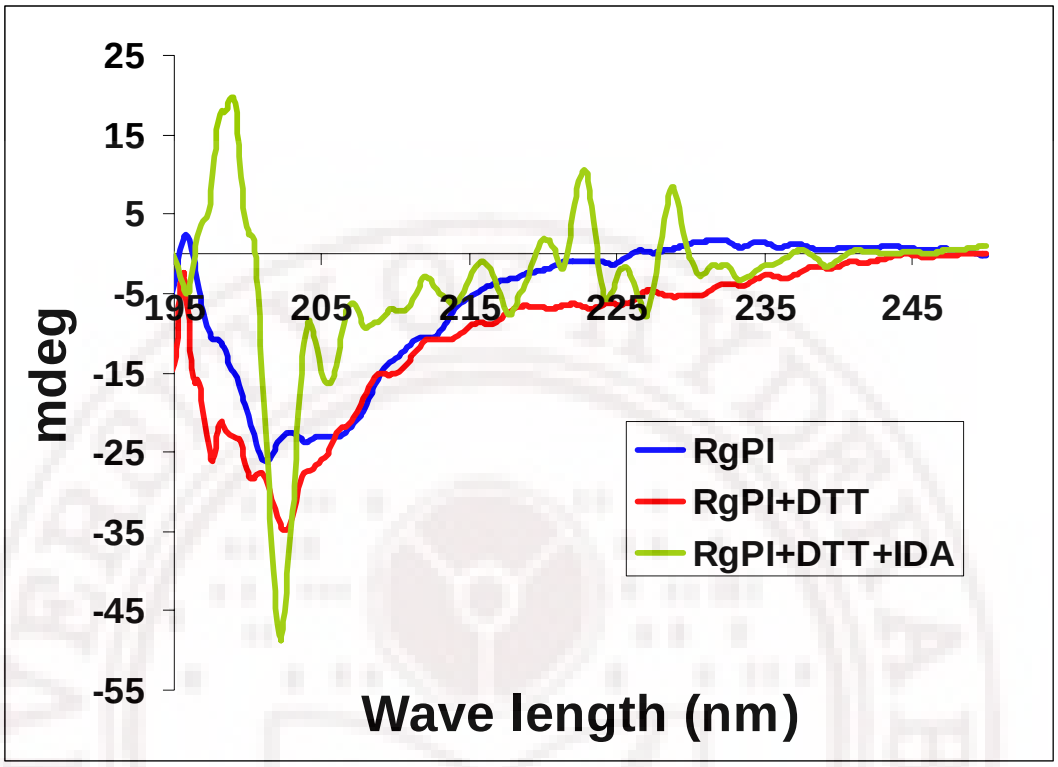

Fig. 5.24. Far-UV CD spectra of native RgPI (—); RgPI reduced with 1mM DTT (—); and reduced RgPI is alkylated with iodoacetamide (—). Other details were as described in Fig. 5.16; materials and methods.

Effect of chemical modification on the inhibitory activity of RgPI:

Chemical modification studies of RgPI were carried out to identify the amino acids responsible for inhibitory activity against trypsin and chymotrypsin. Chemically modified RgPI for specific amino acid was used to assay the inhibitory activity against trypsin and chymotrypsin (**Fig. 5.25**) and activity staining studies (**Fig. 5.26**).

Modification of the guanidium group of arginine residues of RgPI had not shown any effect on the inhibitory activity against both trypsin and chymotrypsin, which is also evident in gelatin-SDS-PAGE showing activity against trypsin and chymotrypsin without

any loss in dimer formation (**Fig. 5.26A and B, lane 3**). The modification of lysine residues of RgPI resulted in the complete loss of inhibitory activity against trypsin and partial loss of inhibitory activity against chymotrypsin. Modified RgPI for lysine when incubated with trypsin showed complete loss in dimer band and a marginal loss in monomer band (**Fig. 5.26A, lane 4**). On the other hand, modified RgPI for lysine when incubated with chymotrypsin, loss in dimer band was observed but not monomer (**Fig. 5.26B, lane 4**). Similar results were observed in horse gram BBI, HGI-III (Kumar et al., 2004), where modification of lysine residues lead to disappearance of dimer. These results suggest that lysine residues are involved in self-association of monomers. The loss in inhibitory activity against trypsin could be due to presence of lysine at P_1 in the reactive site of the inhibitor, which specifies for trypsin binding. The presence of Lysine at the reactive site for trypsin is a characteristic feature of BBI (Qi et al., 2005).

RgPI modified for tyrosine residues did not show any effect on the inhibitory activity against trypsin and chymotrypsin. The activity staining gels for trypsin also did not show any change in the banding pattern when compared with the unmodified RgPI (**Fig. 5.26 A and B, lane 5**). Modification of tryptophan residues in RgPI also lead to complete loss in its inhibitory activity against trypsin and chymotrypsin. Activity staining gels for trypsin/chymotrypsin activity resulted in complete loss of both monomer and dimer bands (**Fig. 5.26A and B, lane 6**). These results suggest the presence of tryptophan residue(s) at the reactive site of the RgPI for both trypsin and chymotrypsin.

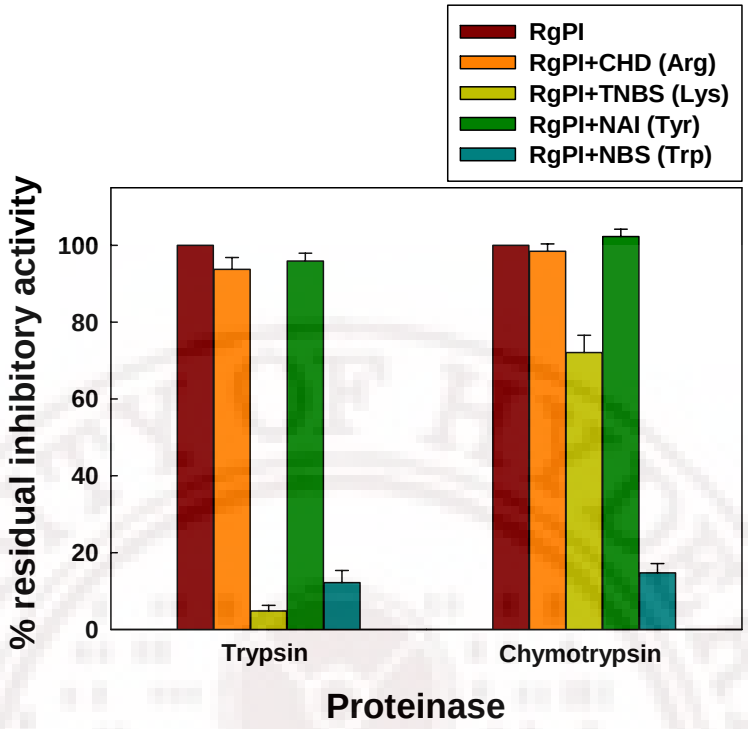

Fig. 5.25. Effect of chemical modification of amino acid residues on inhibitory activity of RgPI against trypsin and chymotrypsin. The residual inhibitory activity was determined as described in materials and methods.

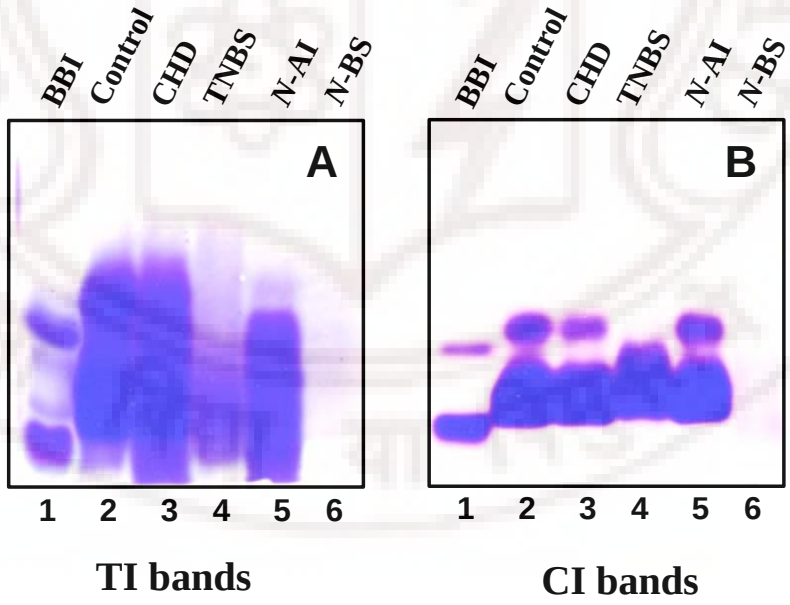

Fig. 5.26. Gelatin-SDS-PAGE (12.5%) showing the trypsin (A) and chymotrypsin inhibitor (B) bands of unmodified (control) and modified RgPI using specific amino acid modifying agents.

Chapter 6

Purification and characterization of proteinase inhibitor from the seeds of black gram (*Vigna mungo*, variety TAU-1)

Chapter 6

Purification and characterization of proteinase inhibitor from the seeds of black gram (*Vigna mungo*, variety TAU-1)

Black gram (*Vigna mungo* (L.) Hepper) belongs to the genus *Vigna* and subgenus *Ceratotropis*. The genus *Vigna* is found to contain trypsin, chymotrypsin, subtilisin and cysteine PIs. Among these PIs, trypsin inhibitors exhibited most polymorphism, and were used as genetic markers in plant diversity and evolutionary studies (Konarev et al., 2002). On the other hand, cysteine PIs with molecular mass of 12 kDa, exhibited extremely high inhibitory activity against papain and cathepsin L. These cysteine PIs were purified from black gram and found to be stable even at high temperatures of 90^{o}C and active in both neutral and alkaline pH ranges (Benjakul et al., 2001).

The seeds of *V. mungo* were explored to study the effect of cysteine PIs on the various physiological processes of *Callosobruchus maculatus* (F.). For eg., adult ovipositional behavior, larval development period, and ability of newly hatched larvae to utilize the host for further growth were examined. Reports of these studies indicated that any delay in developmental period of *C. maculatus* reduced seed loss during storage (Sulehrie et al., 2003). The wild relatives of black gram were known as a good source of PIs for crop improvement against bruchid storage pest, *C. chinensis* (Fujii and Miyazaki, 1987). Dongre et al. (1996) screened cultivated accessions and wild progenitors of black gram for their resistance to infestation by *C. maculatus*. Resistance was evident in a wild progenitor of black gram *V. mungo* var. *silvestris*, which caused reduction in body weight, prolonged developmental period and ultimately affected survival of the *C. maculatus*.

Thus much of the information available from literature indicated the presence of cysteine PIs in black gram, which were effective against *C. maculatus*. To the best of our knowledge there is no information on serine type PIs from black gram, which showed

inhibitory effects against insect pests. Hence we have undertaken the present study to examine the presence of serine PIs active against trypsin and chymotrypsin in black gram variety TAU-1. An attempt was made to purify the trypsin inhibitors from black gram and characterize its biochemical properties. The purified inhibitor also showed insecticidal properties against lepidopteran insects (reported in **Chapter 7**).

Results and discussion

Purification of proteinase inhibitor:

The crude protein obtained from the mature dry seeds of black gram (variety TAU-1) was initially subjected to $(NH_4)_2SO_4$ fractionation. The different fractions obtained were assayed for inhibitory activity against trypsin (TI activity). The 25-80% $(NH_4)_2SO_4$ fraction which showed maximum TI activity was loaded onto a DEAE-Cellulose column. The protein bound to the matrix was eluted with a linear gradient of 0.1 to 1.0 M NaCl in 50 mM Tris-HCl (pH 8.0). The proteins were eluted into two peaks, I and II based on the absorbance at 280 nm (**Fig. 6.1**). The assay for TI activity indicated the presence of active fractions at peak I. These fractions with TI activity were pooled and applied on to a trypsin-Sepharose 4B column. The proteins bound to the affinity matrix trypsin-Sepharose 4B were eluted with 0.01 N HCl. The eluted fractions (1.0 ml) showed single peak for both total protein at 280 nm and total TI activity (**Fig. 6.2**). The active fractions with TI activity from affinity column showed contaminants of high molecular mass in Tricine-SDS-PAGE (**Fig. 6.4, lane 5**). Hence, these fractions were further passed through Sephadex G-50 column, which was equilibrated and eluted with 50 mM Tris-HCl (pH 8.0) in 1.0 ml fractions. Two peaks (I and II) related to protein absorbance at 280 nm were observed (**Fig. 6.3**). The fractions with TI activity which overlapped with peak II were pooled and named as BgPI. **Table 6.1** shows the purification pattern through different steps. BgPI was purified by ~55.6-fold with a recovery of ~42% yield.

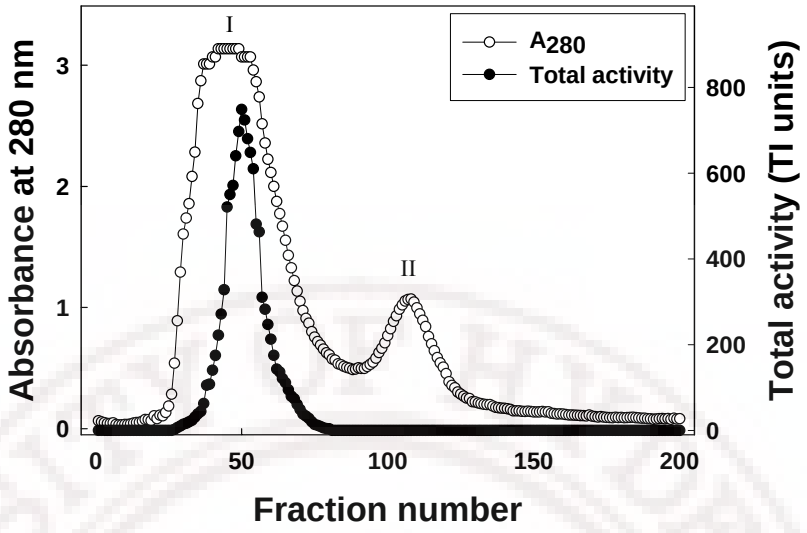

Fig. 6.1. Elution profile of DEAE-Cellulose column loaded with 25-80% $(NH_4)_2SO_4$ fraction. The column was equilibrated and washed with 50 mM Tris-HCl (pH 8.0). The bound protein was eluted into 1.0 ml fractions with a linear gradient of 0.1 to 1.0 M NaCl in the above buffer at a flow rate of 30 ml/h. The fractions were analyzed for total protein at 280 nm and total TI activity, was monitored by following inhibition of BAPNA hydrolysis by trypsin as described in materials and methods.

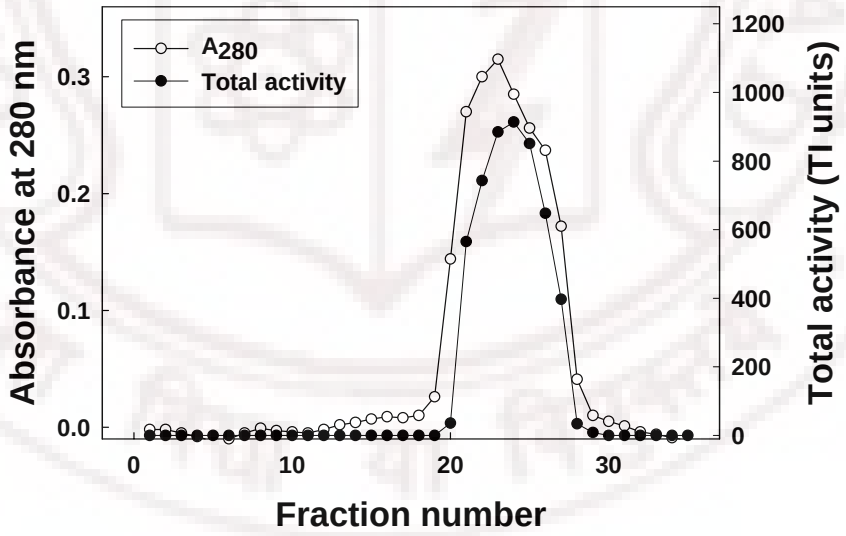

Fig. 6.2. Elution profile of trypsin-Sepharose 4B column loaded with ion exchange column active fraction pool for TI. The column was equilibrated and washed with 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl. The bound protein was eluted in 1.0 ml fractions with 0.01 N HCl at a flow rate of 30 ml/h. The collected fractions were analyzed for total protein and TI activity as described in Fig. 6.1 and materials and methods.

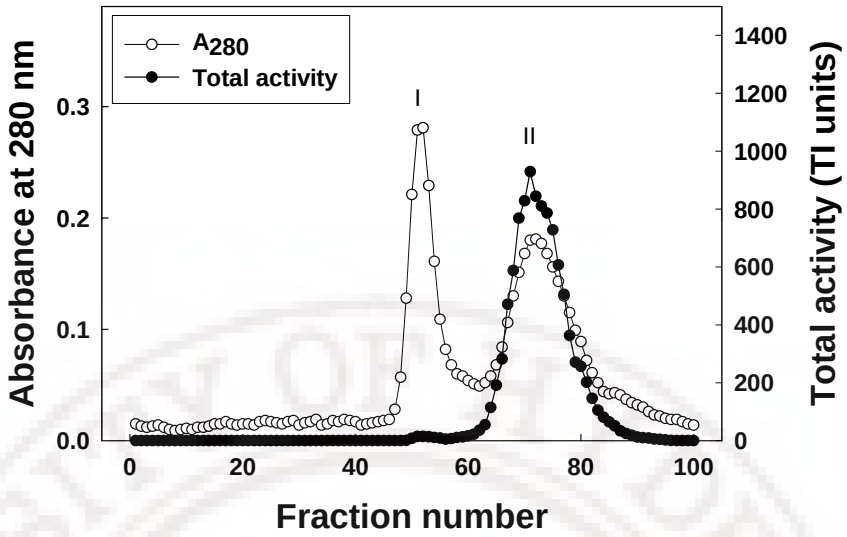

Fig. 6.3. Elution profile of Sephadex G-50 column loaded with affinity column active fraction pool. The column was equilibrated and eluted with 50 mM Tris-HCl (pH 8.0) in 1.0 ml fractions at a flow rate of 15 ml/h. The fractions were analyzed for total protein and TI activity as described in Fig. 6.1 and materials and methods. Peak II fractions which showed TI activity were pooled and named as "BgPI".

Table. 6.1. Purification of proteinase inhibitor (BgPI) from black gram seeds

Step	Total protein (mg)	Total activity (TI units)[a]	Yield recovery (%)	Specific activity[b] (TI units/mg protein)	Purification (fold)
Crude extract	4159	76613	100	18	1
$(NH_4)_2SO_4$ fraction (25-80%)	1240	50842	66	41	2.27
DEAE-Cellulose column	759	43639	57	58	3.22
Trypsin-Sepharose 4B column	38	36208	47	952	52.88
Sephadex G-50 column	32	32035	42	1001	55.61

[a]One TI unit is defined as the amount of BgPI required to inhibit 50% of BAPNA hydrolysis by trypsin. [b]Specific activity is defined as the number of TI units/mg protein.

Electrophoretic and MALDI-TOF analysis of BgPI:

Electrophoretic separation of BgPI showed a single band with an apparent molecular mass of 8 kDa in Tricine-SDS-PAGE under non-reducing condition (**Fig. 6.4**). In agreement with this, MALDI-TOF spectra of native BgPI showed the presence of a peak at 8041.5 Da (**Fig. 6.5**). Previous study on BBI type PIs revealed their molecular mass in the range of 6-9 kDa (Clemente and Domoney, 2006), particularly at ~8 kDa. BBI type PIs have tendency of self-association to form homodimers or trimers or more complex oligomers in solution (Losso, et al., 2008; **Chapter 5** of present study).

Although MALDI-TOF spectrum of native BgPI showed the presence of a second peak at 16082.2 m/z with very limited intensity, self-association tendency might be negligible in BgPI, which was evident through the appearance of single band in Tricine-SDS-PAGE (**Fig. 6.4, lane 6**). The residues lysine/arginine at P_1 position of first reactive site (N-terminal) and aspartic acid/glutamic acid in the carboxy terminus are responsible for self-association of monomers to form stable dimers and any deviation in these residues results in the existence of BBIs as monomers in solution (Kumar et al., 2004). Replacement of lysine/arginine at P_1 position by alanine in the first reactive site of BBI from *Glycine soja* restricted the oligomer formation and BBI were retained as monomers in solution (Deshimaru et al., 2002).

Similarly lack of aspartic acid/glutamic acid in the carboxy terminus of BBIs from *Torresea acreana* (Tanaka et al., 1996), *T. cearensis* (Tanaka et al., 1997), *D. biflorus* (Kumar et al., 2002) and *M. scutellata* (Catalano et al., 2003) also resulted in appearance of only monomers in solution. From the chemical modification studies of BgPI (**Figs. 6.25 and 6.26**), it was observed that lysine is present at the first reactive site of BgPI responsible for trypsin inhibition.

In present study BgPI showed six different iso-inhibitors in native-PAGE (**Fig. 6.6**) and five different iso-inhibitors in 2-D electrophoresis (**Fig. 6.7**). The five iso-

inhibitors had pI values of 4.3, 4.4, 5.0, 5.3 and 6.0, respectively. The presence of multiple genes and the possibility of hydrolysis have been cited as evidence of the existence of a large number of BBI iso-inhibitors (Kalume et al., 1995). The BBIs isolated from various leguminous plants (as mentioned in **Chapter 5** of the present study), and RgPI isolated in the present investigation from red gram (**Chapter 5, Figs. 5.7 and 5.9**) also revealed the presence of iso-inhibitors.

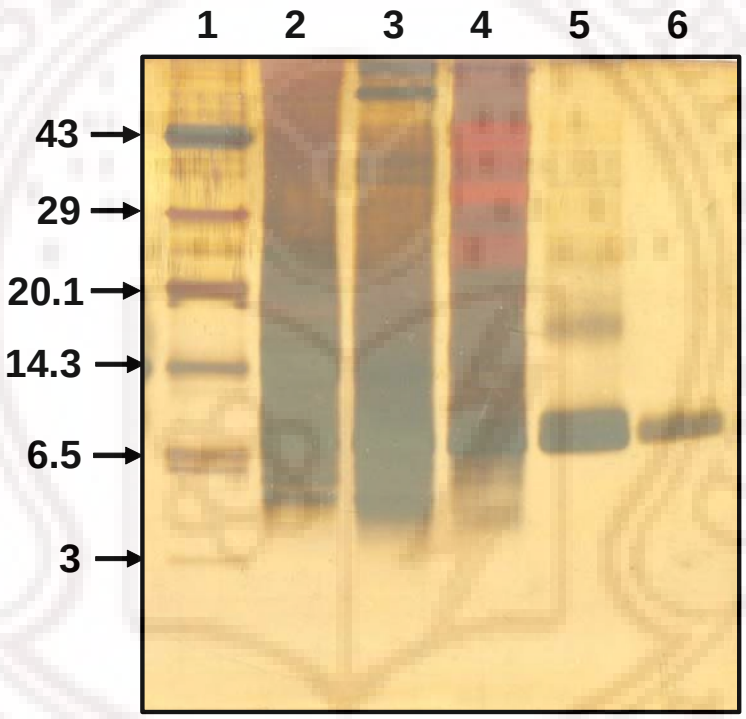

Fig. 6.4. Tricine-SDS-PAGE (18%) in non-reducing condition showing different purification fractions. Lanes 1-6 are loaded with molecular mass standards (Bangalore genei, PMW-L), crude extract (25 µg), 25-80% $(NH_4)_2SO_4$ fraction (25 µg), active fraction pool from anion exchange column (25 µg), active fraction pool from affinity column (10 µg) and active fraction pool from gel-filtration column (5 µg of BgPI), respectively.

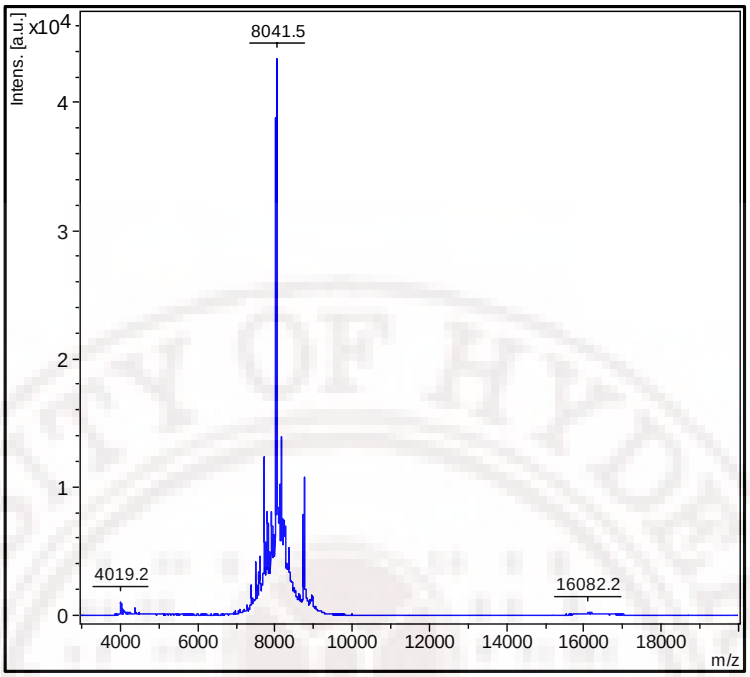

Fig. 6.5. MALDI-TOF mass spectrum of BgPI in native condition showing peaks at 8041.5 and 16082.2 m/z, respectively. The peak at 4019.2 m/z represents doubly charge form of BgPI.

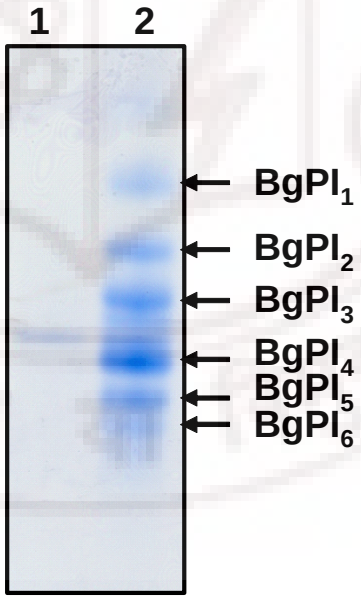

Fig. 6.6. Iso-inhibitors of BgPI separated on native-PAGE (12.5%). The samples in the lane 1 and lane 2 are soybean BBI and BgPI, respectively.

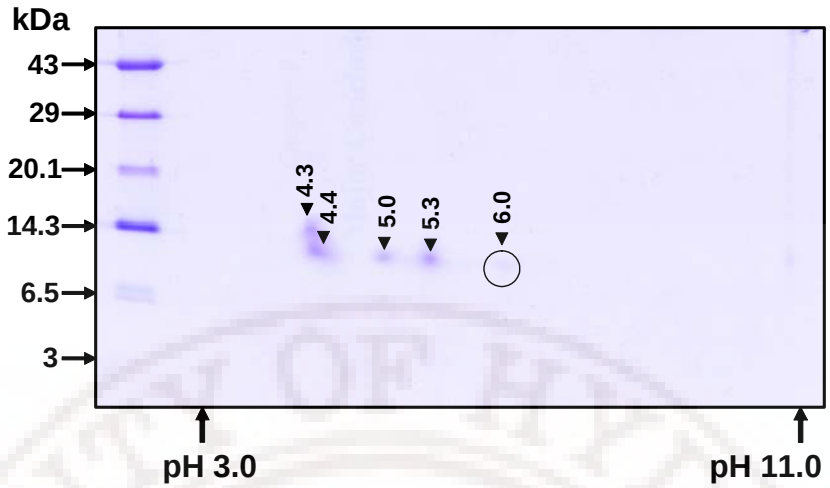

Fig. 6.7. Two-dimensional gel showing the iso-inhibitors of BgPI. The proteins were separated in IEF using 3.0 – 11.0 pH non-linear strips having maximum resolution at pH 4.0 – 7.0. The second dimension was performed in Tricine-SDS-PAGE (18%).

Mass spectrum of tryptic digested iso-inhibitors:

The iso-inhibitor spots observed in 2-D gel with pI values: 5.3, 4.4 and 4.3 (**Fig. 6.7**) were excised and subjected to tryptic digestion. The peptide mixture showed identical mass spectrum for all the three iso-inhibitors of BgPI when analyzed in MALDI-TOF (**Fig. 6.8**). Mascot search results for the mass spectrum of iso-inhibitor with pI value 4.3 showed 55% homology to BBI type PI from *V. unguiculata* subsp. *cylindrica* and several other BBI type PIs (**Fig. 6.9**). In Biotools display the same iso-inhibitor (pI 4.3) showed homology in mass to a peptide fragment consisting of the following sequence: SIPPQCHCADIRLNSCHSACK (**Fig. 6.10**). The peak 1453.7 m/z related to iso-inhibitor (pI 4.4) was ionized in MALDI-TOF-TOF and the resulting lift spectrum showed 74% homology with BBI type PIs isolated from various leguminous species, in Mascot MS/MS ion search (**Figs. 6.11 and 6.12**). In Biotools display, 1453.7 m/z peak of iso-inhibitor (pI 4.4) showed a sequence of 'SIPPQCHCADIR' (**Fig. 6.13**) with a mass accuracy shown in **figure 6.14**. Taken together the results we suggest that the PI purified from *V. mungo* var. TAU-1 in the present study belong to BBI type family.

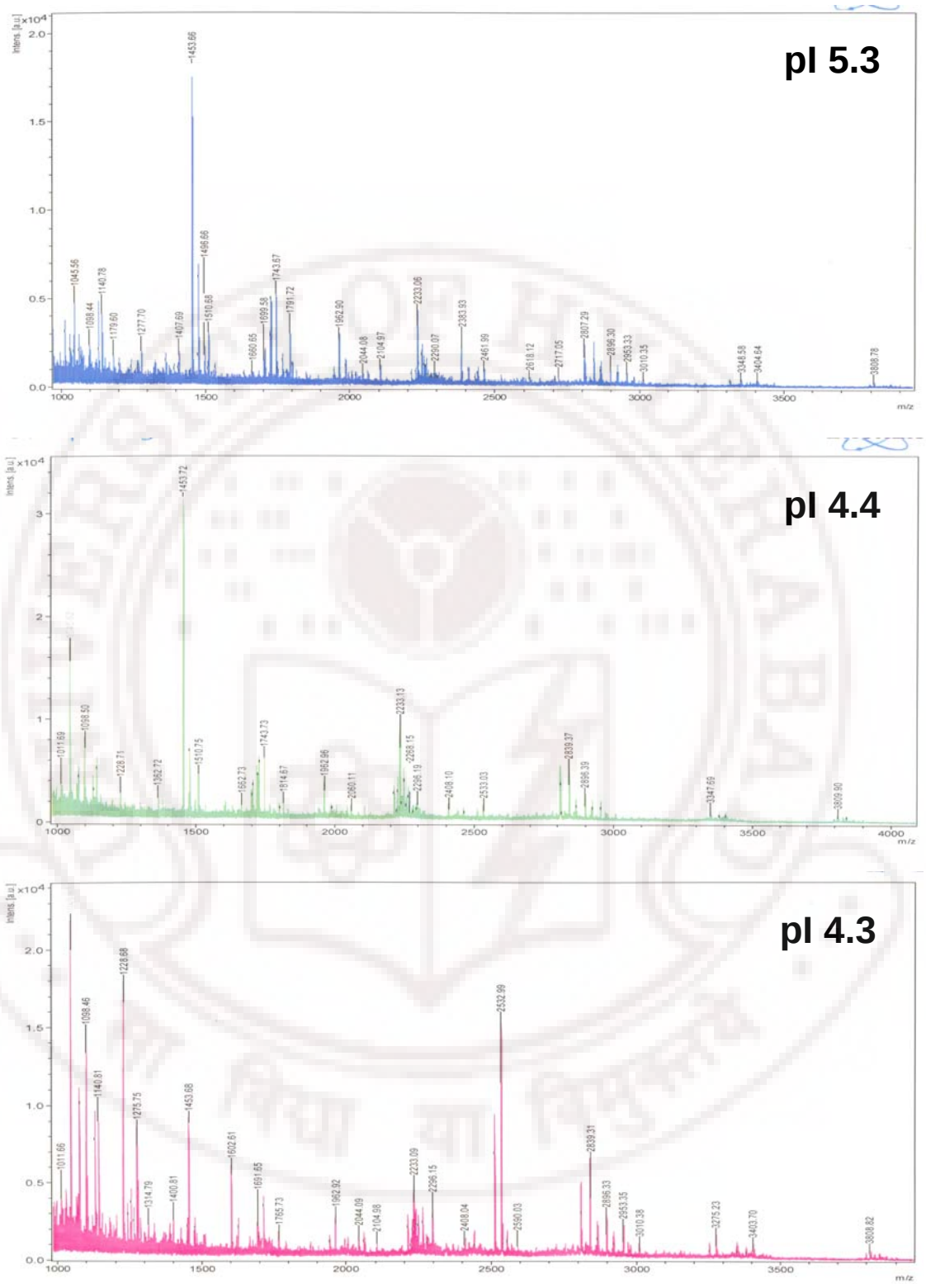

Fig. 6.8. MALDI-TOF mass spectrum of in gel tryptic digested iso-inhibitors of BgPI with pI values of 5.3, 4.4 and 4.3.

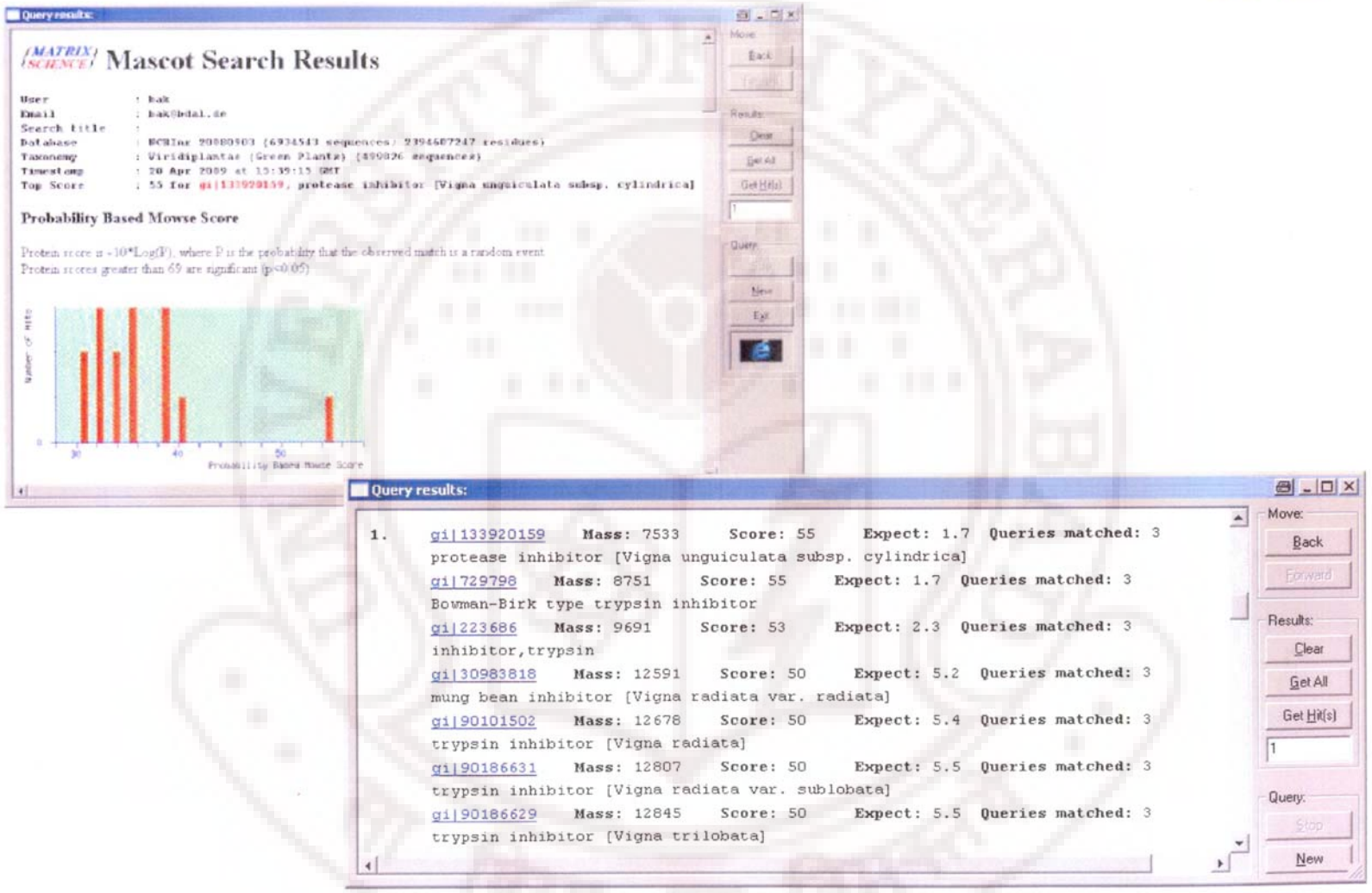

Fig. 6.9. Mascot search results for MALDI-TOF MS of BgPI iso-inhibitor (pI 4.3).

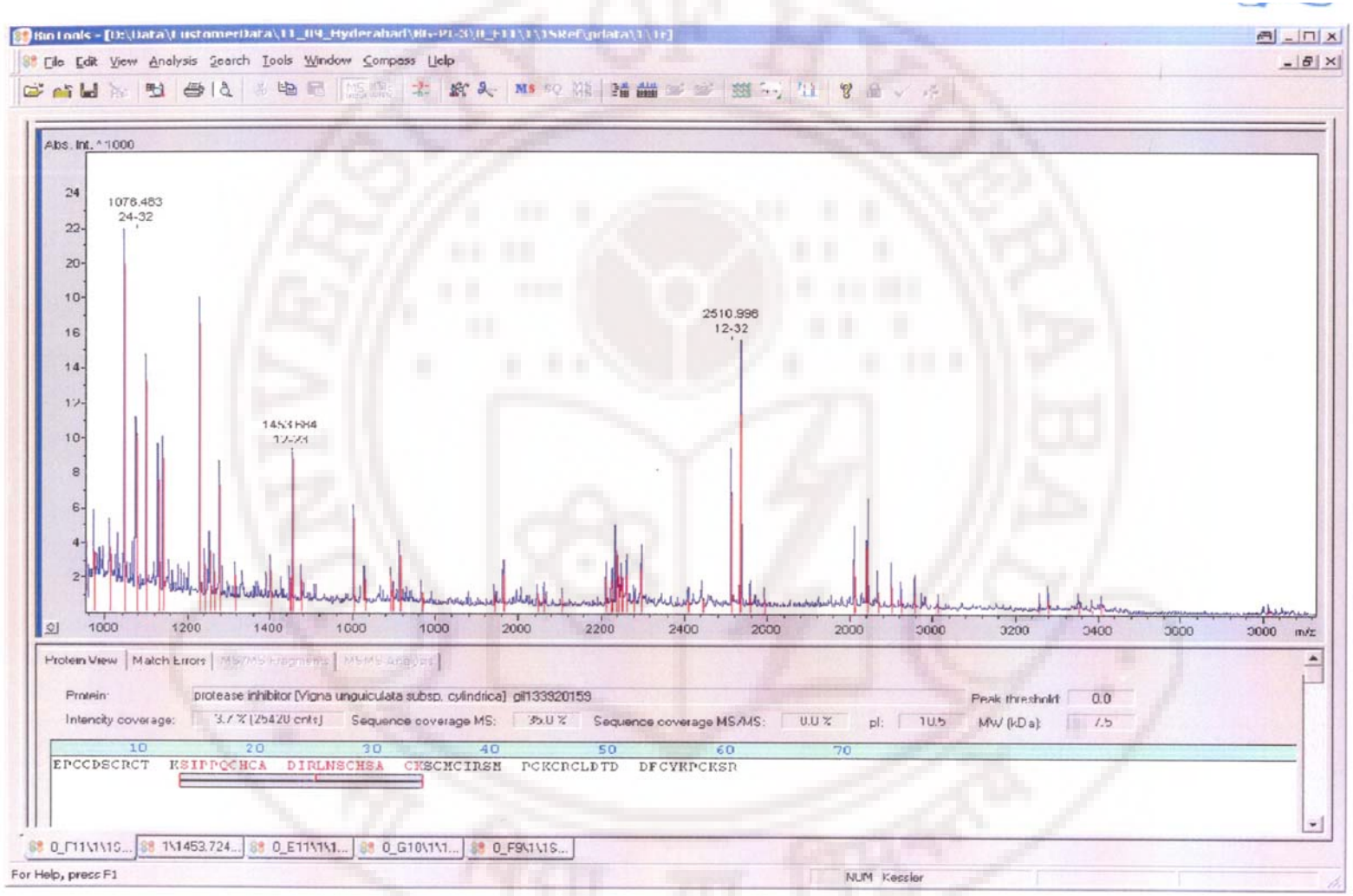

Fig. 6.10. Biotools display of MALDI-TOF MS of BgPI iso-inhibitor (pI 4.3).

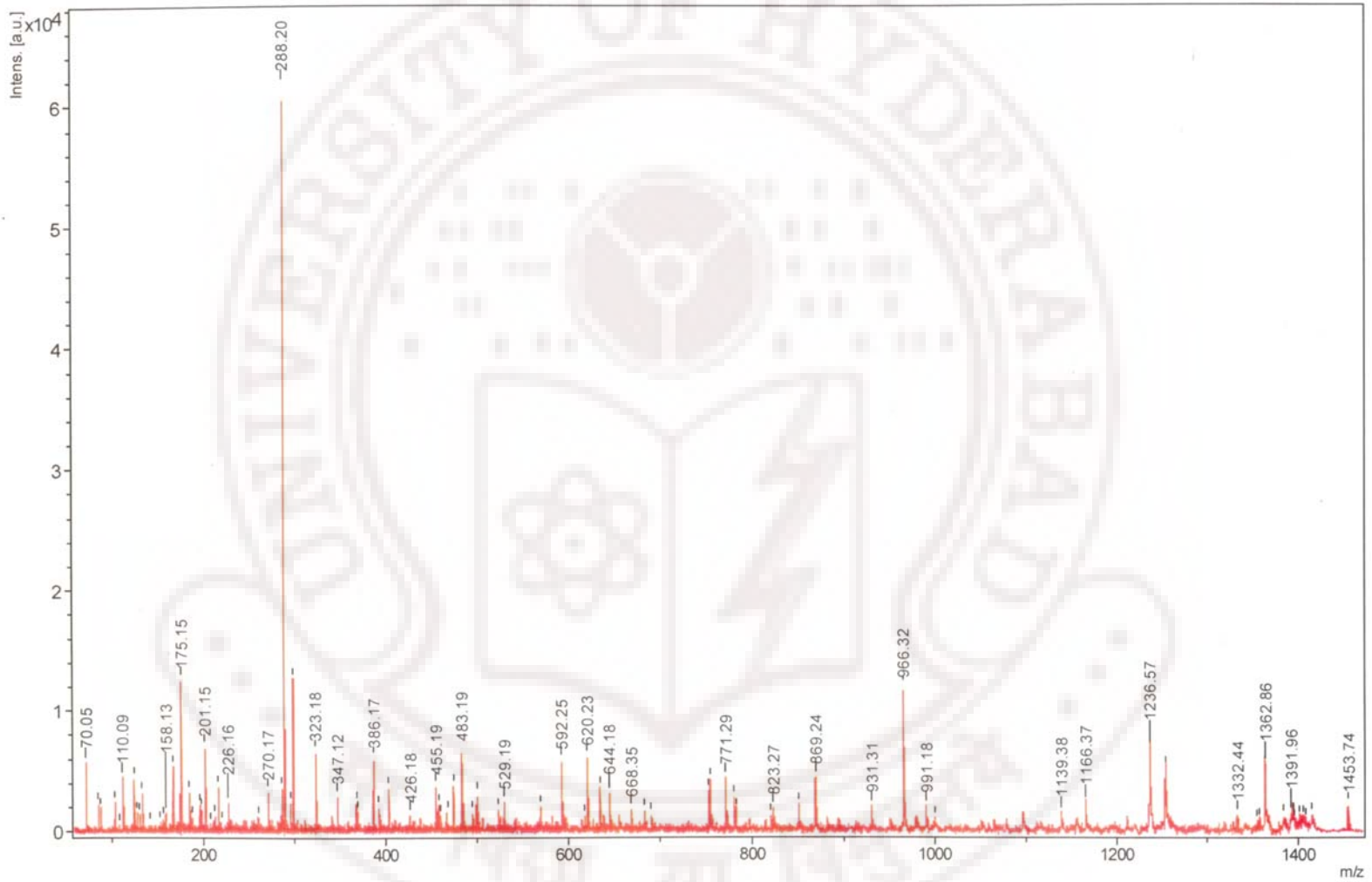

Fig. 6.11. Lift spectrum of peak 1453.7 m/z of BgPI iso-inhibitor (pI 4.4).

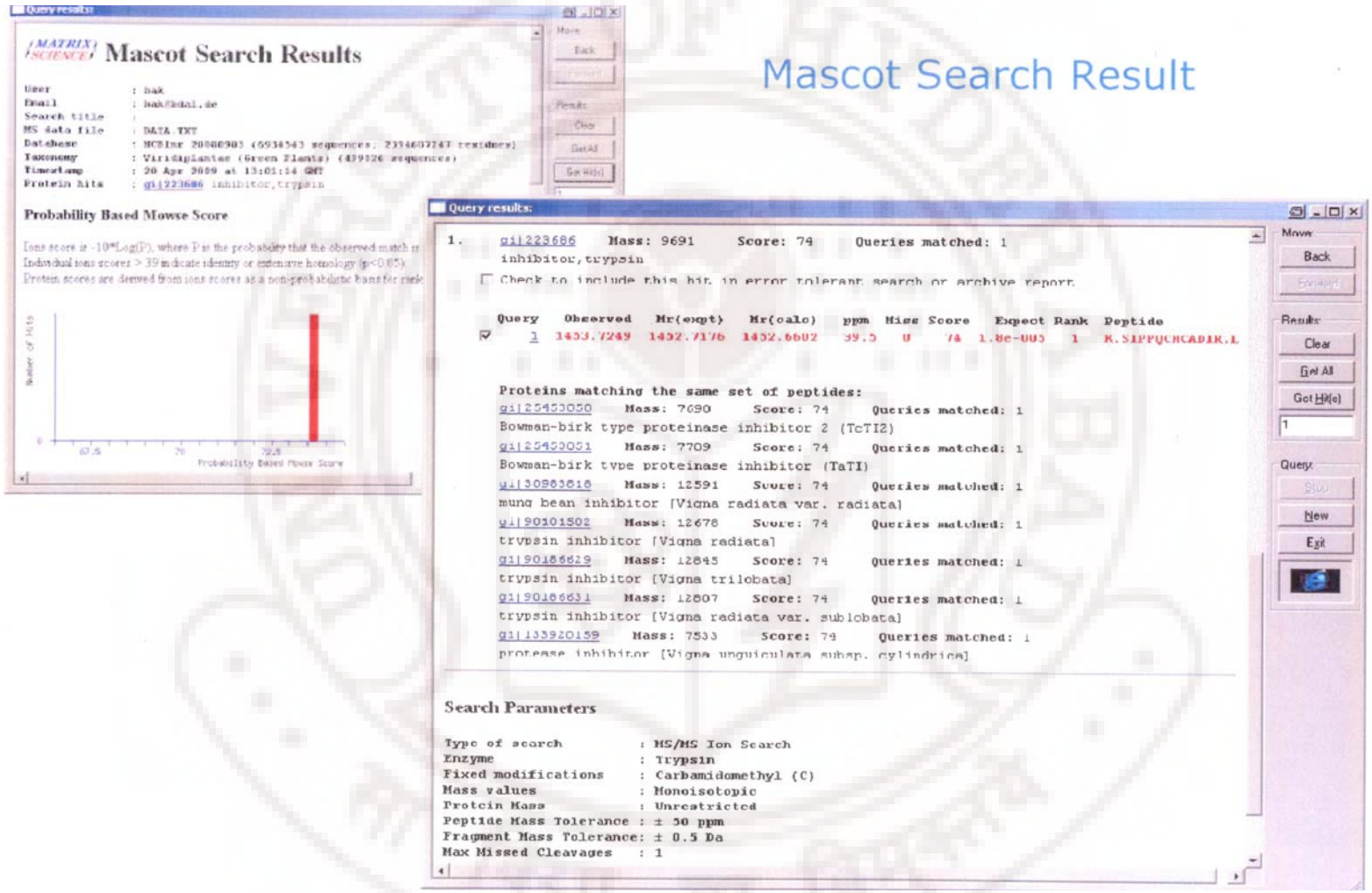

Fig. 6.12. Mascot search results for lift spectrum 1453.7 m/z of BgPI iso-inhibitor (pI 4.4).

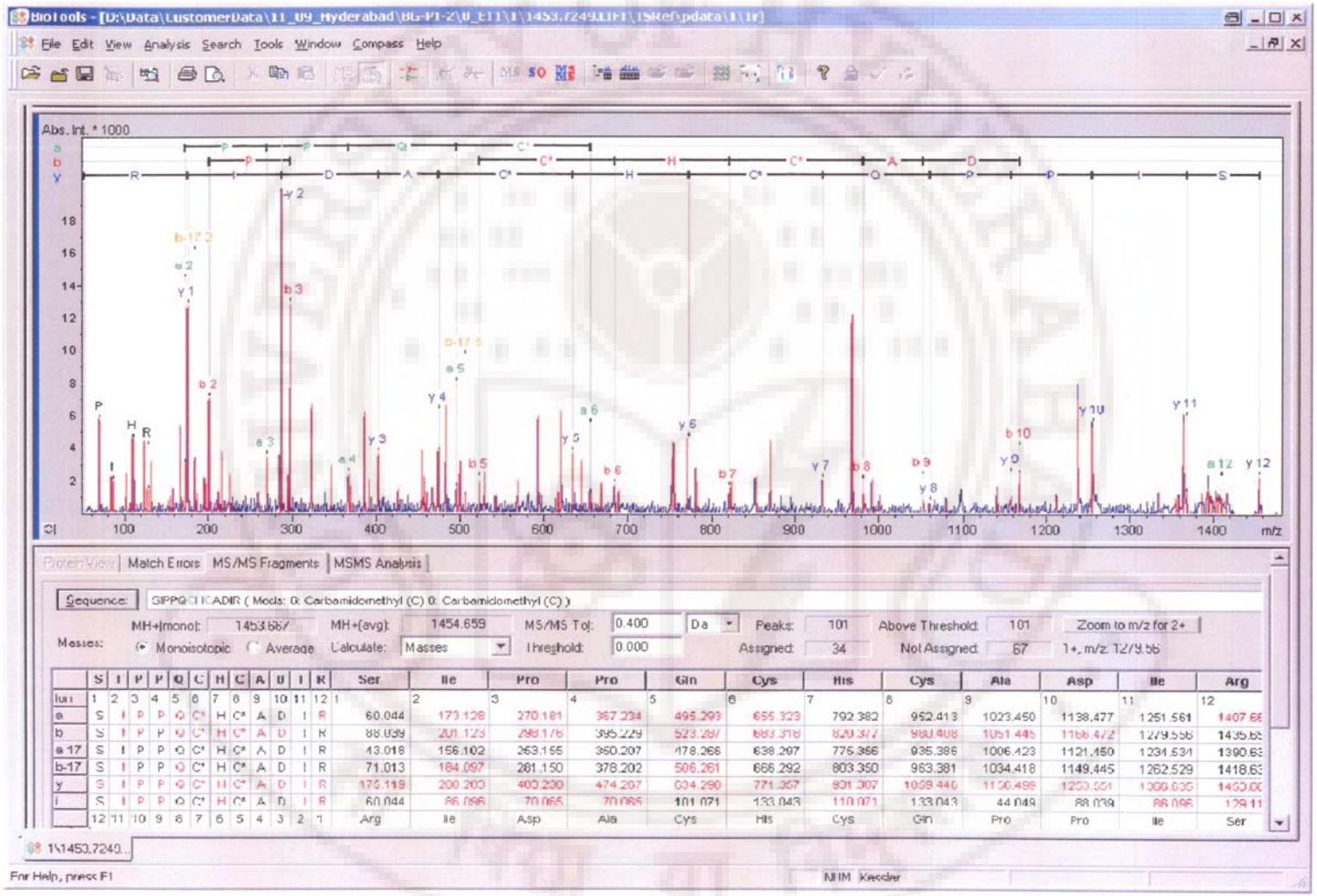

Fig. 6.13. Biotools display of lift spectrum 1453.7 m/z of BgPI iso-inhibitor (pI 4.4).

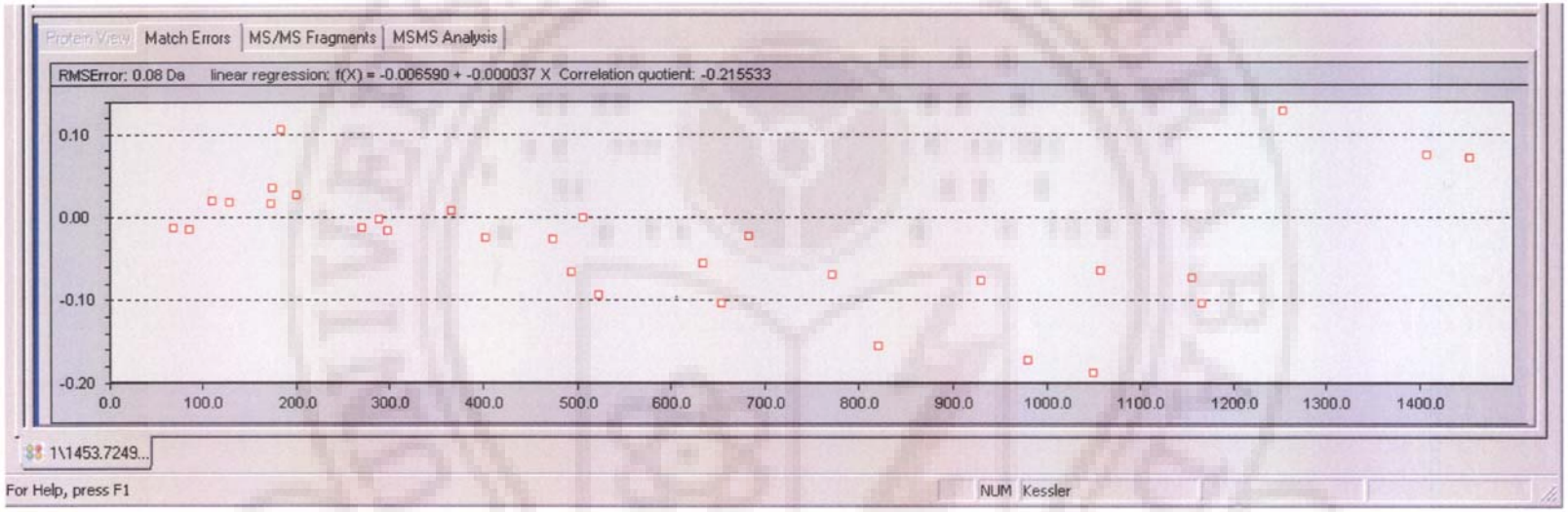

Fig. 6.14. Mass accuracy of Biotools display of lift spectrum 1453.7 m/z of BgPI iso-inhibitor (pI 4.4).

Inhibitory activity and inhibitor constant (K_i) determination:

Similar to RgPI (**Chapter 5, Fig. 5.14**) BgPI also showed inhibitory activity against both trypsin and chymotrypsin, a characteristic feature of BBI type PIs due to presence of two different reactive sites (Singh and Appu Rao, 2002). Nevertheless, inhibition against trypsin activity was more pronounced when compared with chymotrypsin activity. Generally most of the BBIs inhibit both trypsin and chymotrypsin, and have higher affinity towards the trypsin than chymotrypsin and the same was also observed with RgPI (**Chapter 5**).

The stoichiometry of BgPI with trypsin was found to be 1:2, which means that one molecule of BgPI will exhibit TI activity by binding with two molecules of trypsin. On the other hand, BgPI showed less inhibitory effect against chymotrypsin and there was no obvious stoichiometry with chymotrypsin which is evident from the titration pattern of its inhibitory activity (**Fig. 6.15**). The PI isolated from *A. americana* tubers (Zhang et al., 2008) and *L. albus* seeds (Scarafoni et al., 2008) also showed same pattern of stoichiometry with trypsin. The K_i for both trypsin and chymotrypsin were examined by increasing the concentration of BgPI with different concentrations of their respective substrates BAPNA and GLUPHEPA, respectively.

Lineweaver-Burk plot showed non-competitive type of inhibition with both trypsin and chymotrypsin with a K_i of 309.8 nM for trypsin and 10.7 μM for chymotrypsin (**Fig. 6.16A and B**). The lower K_i value observed in the present study for trypsin when compared with chymotrypsin indicates that BgPI has a ~35-fold higher affinity for trypsin, when compared with chymotrypsin. However, BBI type PIs isolated from different leguminous plants showed K_i values for trypsin in the range between 0.1 to 0.5 nM (Terada et al., 1994; Ceciliani et al., 1997; Tanaka et al., 1997; Bueno et al., 1999; Deshimaru et al., 2002; Paiva et al., 2006; Ragg et al., 2006; Scarafoni et al., 2008; Zhang et al., 2008). In spite of having a slightly higher K_i for bovine pancreatic trypsin, the BgPI

showed marked inhibitory activity against midgut trypsin-like proteinases isolated from *A. janata*, *A. albistriga*, *H. armigera* and *P. demoleus* (data presented in **Chapter 7**). The difference between the K_i value for trypsin and chymotrypsin in BgPI resembled with those BBI type PIs isolated from *L. culinaris* (Ragg et al., 2006) and from red gram (RgPI) in the present investigation (**Chapter 5**).

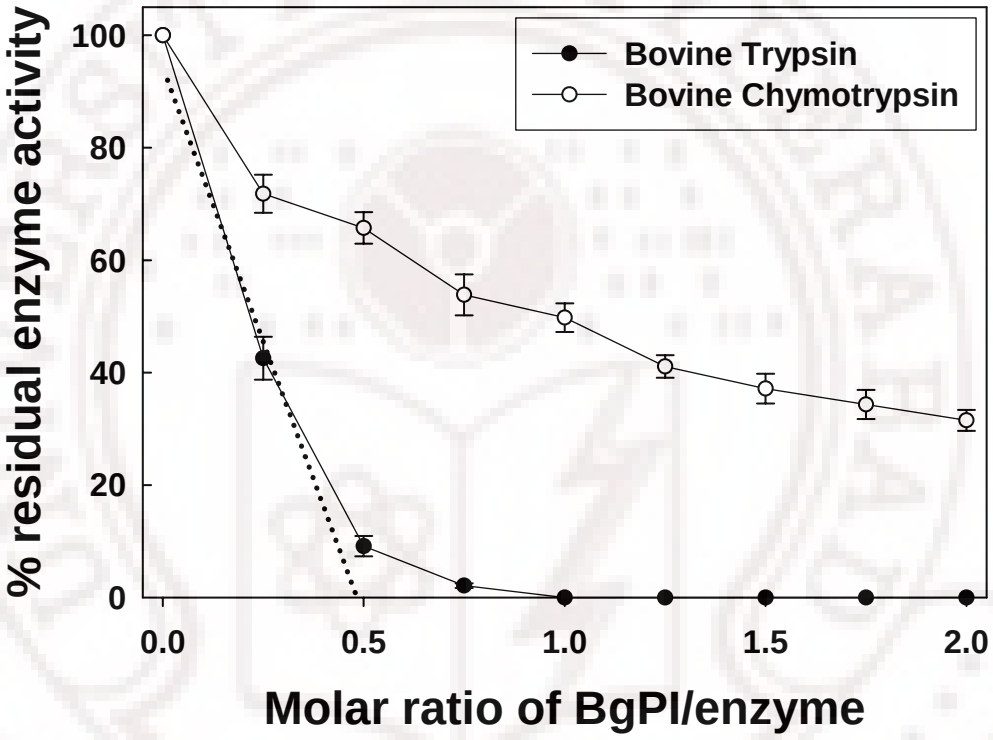

Fig. 6.15. Titration curves of trypsin and chymotrypsin inhibition by BgPI. Increasing concentrations of BgPI was added to a fixed concentration of enzyme. The concentration of trypsin or chymotrypsin in the reaction mixture was 1 x 10^{-6} M. After incubating the reaction mixture for 15 min, residual trypsin or chymotrypsin activity was determined by monitoring the hydrolysis of synthetic substrates BAPNA and GLUPHEPA, respectively. The molar ratio of the inhibitor to the trypsin or chymotrypsin was the intercept of x-coordinate, when the tangent was extrapolated to the zero activity (Knights and Light, 1976).

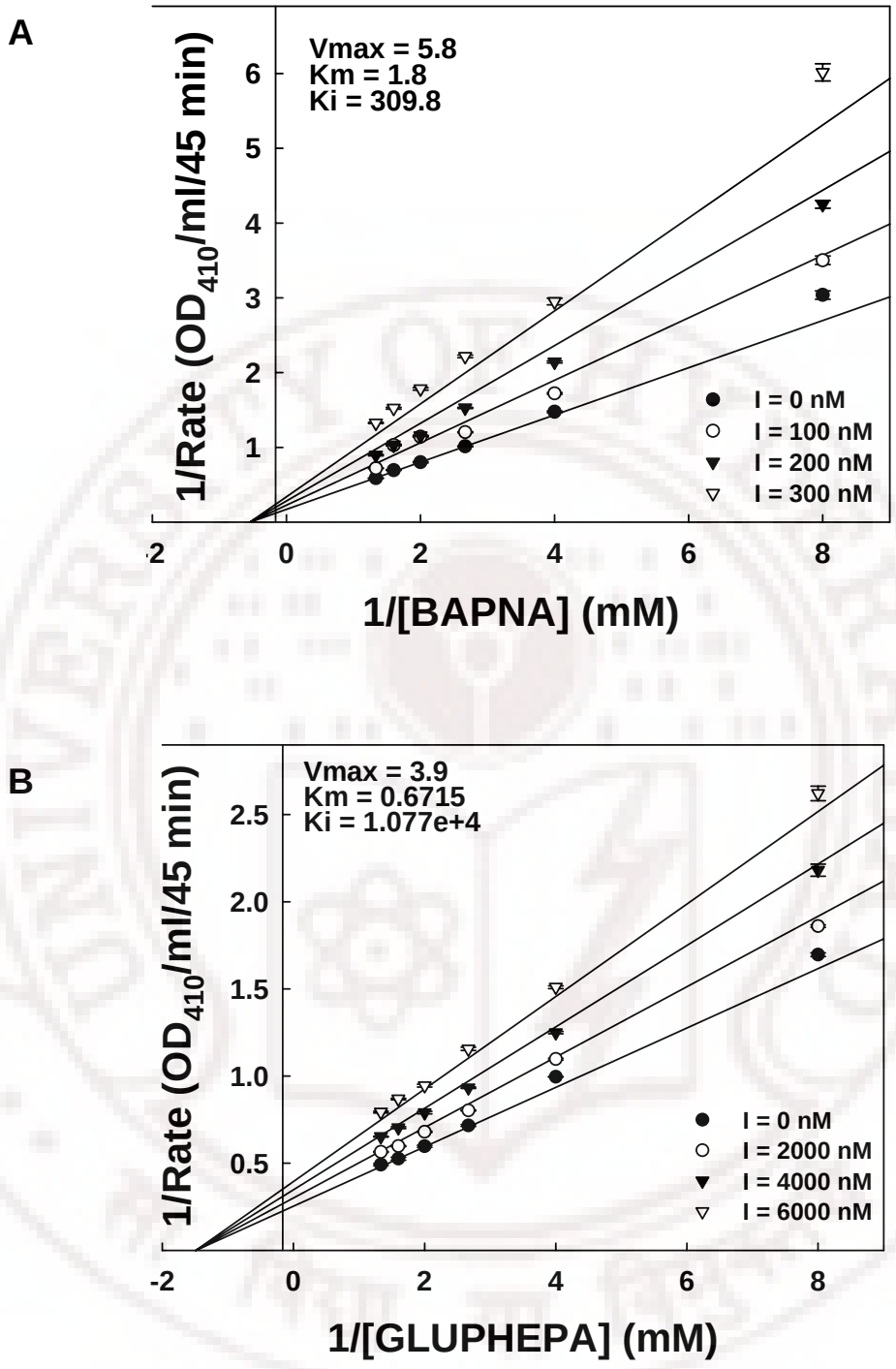

Fig. 6.16. Kinetic analysis of trypsin and chymotrypsin inhibition by BgPI. Lineweaver-Burk plot showing non-competitive nature of trypsin (A) and chymotrypsin (B) inhibition by BgPI at various concentrations of BAPNA and GLUPHEPA (0.125, 0.250, 0.375, 0.500, 0.625 and 0.750 mM).

Structural characterization of BgPI:

The secondary and tertiary structures of BgPI were analyzed using CD spectroscopy in the presence of 10 mM phosphate buffer (pH 7.4). Far-UV (190-250) CD spectrum of BgPI represents the secondary structure and is presented in **figure 6.17**. The composition of secondary structure as assessed by CDSSTR programme were as follows: (i) 11.5% of α-helix; (ii) 64.6% of β-sheet and (iii) 29.2% of random coil (**Table 6.2**). The predominance of β-strands and random coil, when compared with α-helix was also reported in BBI type of PIs isolated from *G. max* (Wu and Sessa, 1994), *I. umbratica* (Calderon et al., 2005), *V. unguiculata* (Rao and Suresh, 2007) and RgPI isolated in the present investigation (**Chapter 5**). Though the aperiodic structures offers greater flexibility to the BBI molecules, the disulfide linkages in them minimize the conformational entropy and enhance the stability of these proteins (Ramasarma et al., 1995).

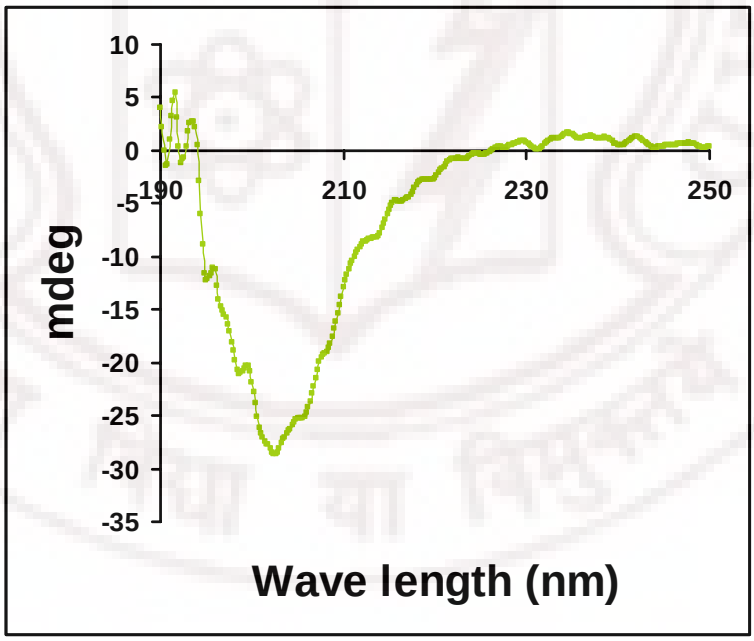

Fig. 6.17. Far-UV CD spectra of BgPI representing secondary structure. The difference spectrum for buffer (10 mM phosphate buffer, pH 7.4) and protein (BgPI) sample was shown. The other details were as described in materials and methods.

Table 6.2. Composition of secondary structure of BgPI analyzed by CDSSTR programme.

Secondary structure	Percent of Secondary structure content
α-helix	11.50
Anti-parallel β-sheet	36.30
Parallel β-sheet	9.70
B-turn	18.60
Random coil	29.20

Near-UV (250-300 nm) CD spectrum of BgPI represents the tertiary structure and is presented in **figure 6.18**. The tertiary structure showed the presence of positive CD at 257 nm (**Fig. 6.18**), which is attributed to the presence of disulfide bonds (Ramasarma et al., 1995). Similar to RgPI in red gram (**Chapter 5, Fig. 5.17**) the tertiary structure of BgPI also displayed a minimum at 283 nm, indicating the contribution of disulfide bonds as well as tyrosine residue (Singh and Appu Rao, 2002; Haq et al., 2005).

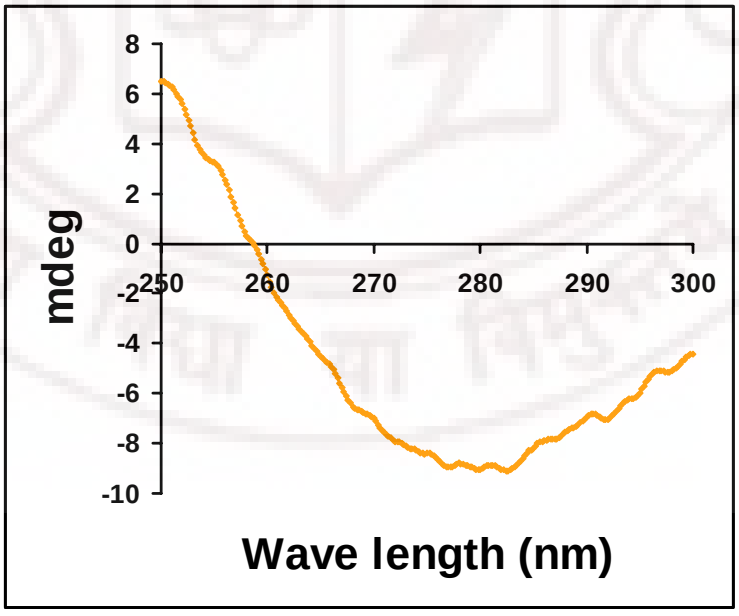

Fig. 6.18. Near-UV CD spectra of BgPI representing tertiary structure. Other details were as described in Fig. 6.17 and materials and methods.

Temperature and pH stability of BgPI:

The effect of temperature on the inhibitory activity of BgPI against trypsin and chymotrypsin was examined after incubating BgPI at a wide range of temperatures (from ambient to boiling) for 30 min as shown in **figure 6.19**. The trypsin and chymotrypsin inhibitory activity of BgPI was quite stable up to 80°C without any reduction in its activity. However, BgPI lost its activity marginally (≤ 15%) against both trypsin and chymotrypsin when incubated at 90°C and 100°C. The far-UV CD spectra taken at different temperatures clearly showed that any increase in ambient temperature resulted in conformational changes in the CD spectra of RgPI (**Fig. 6.20**). These temperature induced conformational changes are reversed, when the inhibitor was cooled down to ambient temperature (25°C) from 90°C, demonstrating that the PI possesses some degree of flexibility and retained its activity under thermal stress, which is also evident with RgPI from red gram (**Chapter 5, Fig. 5.19**)

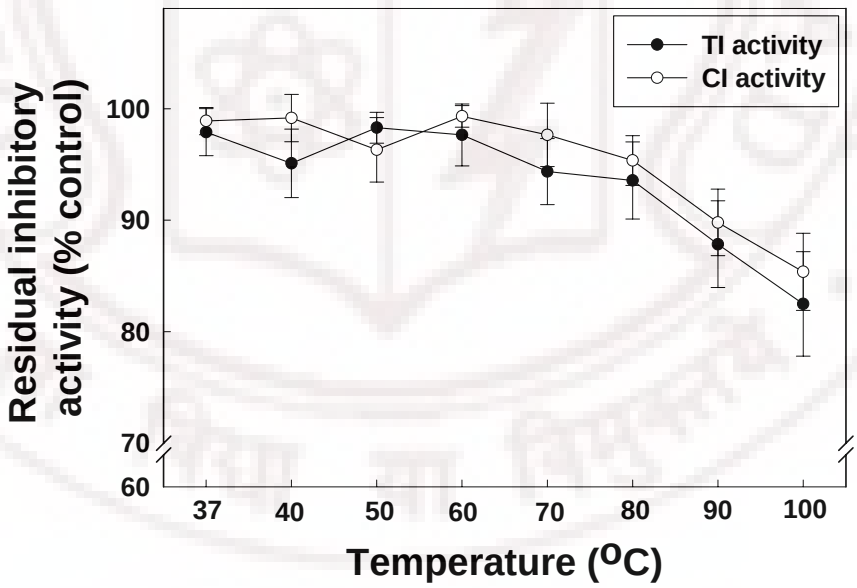

Fig. 6.19. Temperature stability of BgPI after incubation for 30 min at the indicated temperatures. Residual inhibitory activity of BgPI against trypsin and chymotrypsin was estimated in presence of BAPNA and GLUPHEPA, respectively, as described in materials and methods.

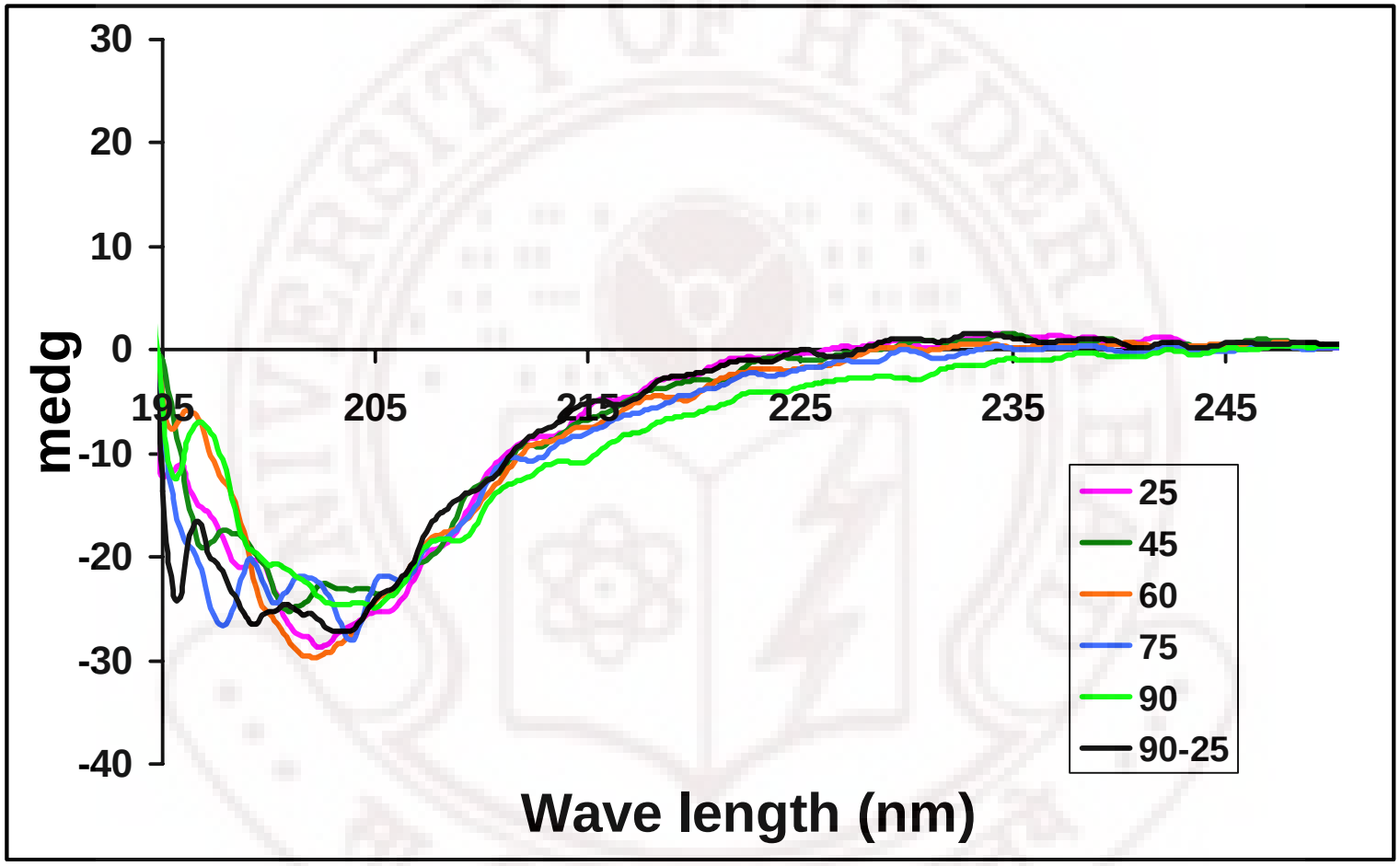

Fig. 6.20. Effect of temperature on far-UV CD spectra (secondary structure) of BgPI. The required temperature (^{o}C) was induced using the thermostat (peltier, Jasco). Other details were as described in Fig. 6.17 and materials and methods.

Further, the inhibitory activity of BgPI against both trypsin and chymotrypsin was also tested at different pH between 2.0 and 12.0 (**Fig. 6.21**). However, only a marginal loss in inhibitory activity (TI or CI) was observed at both acidic and basic pH and the CD spectroscopy studies showed minor structural changes both at acidic pH (2.0 and 4.0) as well as basic pH (10.0 and 12.0) (**Fig. 6.22**). The possible presence of many cysteine residues forming disulfide bonds may account for this striking stability in structure and activity of BgPI (Singh and Appu Rao, 2002). These properties of BgPI obtained in the present study corroborate well with the properties of BBI type PIs isolated from most of the leguminous plants (discussed in **Chapter 5**) and RgPI isolated from red gram in the present investigation (**Chapter 5**).

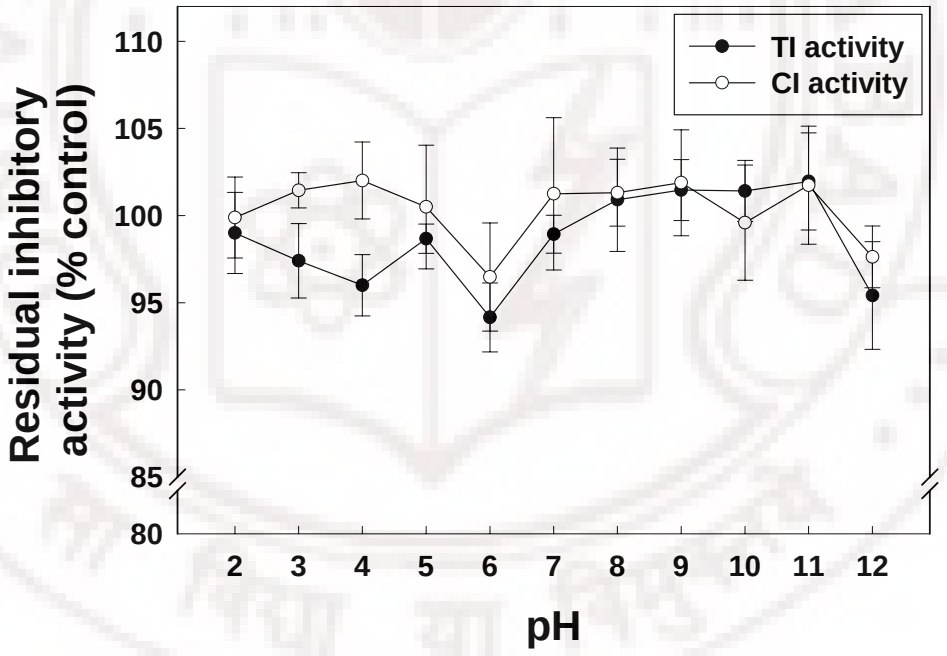

Fig. 6.21. Stability of BgPI after incubation at various pH ranging from 2.0 to 12.0 for 1h. The residual inhibitory activity was determined as described in materials and methods.

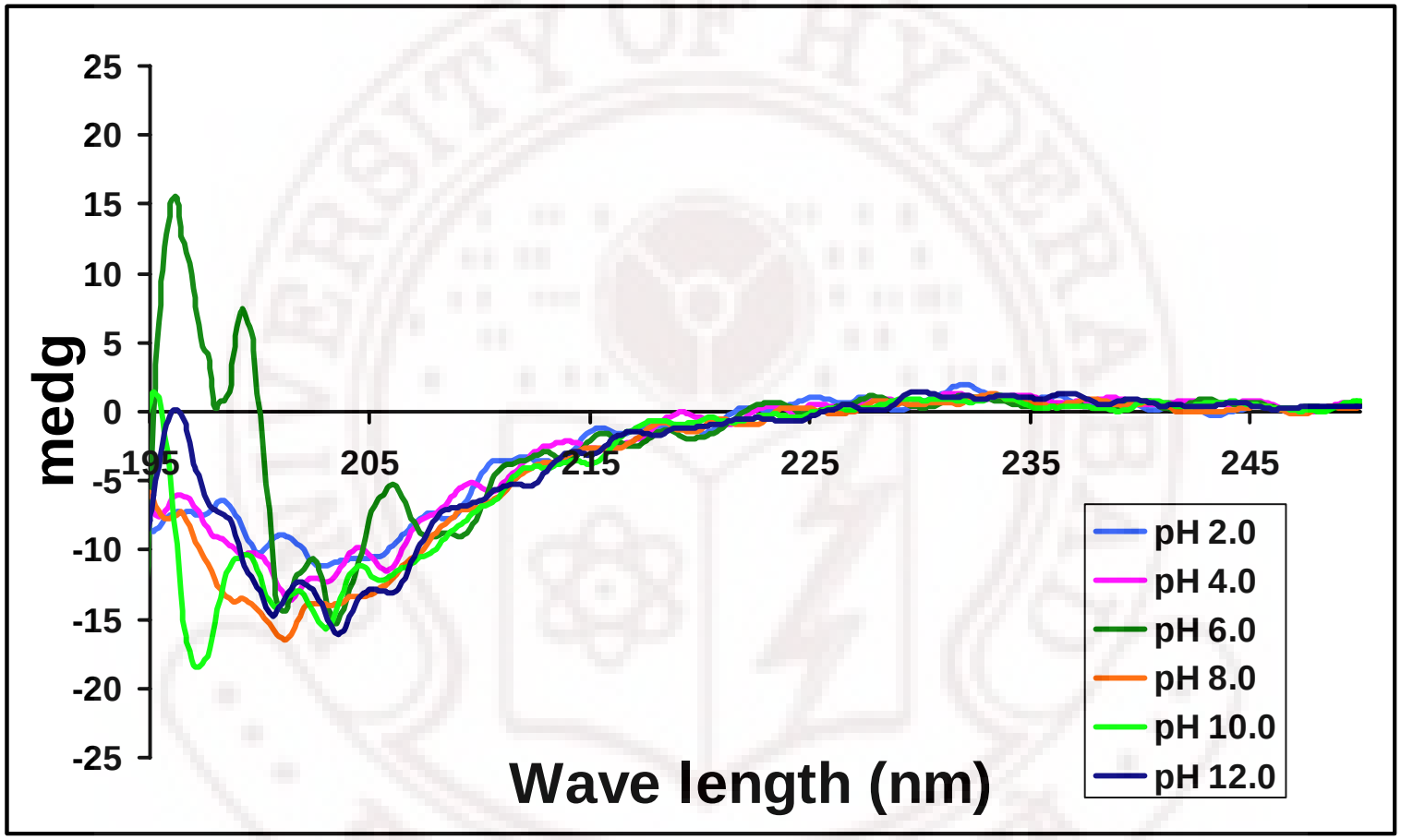

Fig. 6.22. Effect of pH on the far-UV spectra (secondary structure) of BgPI. The BgPI was incubated at respective pH for 1 h before taking the spectra. Other details were as described in Fig. 6.17 and materials and methods.

Effect of DTT reduction on inhibitory activity and structural conformation of BgPI:

As we already observed that DTT reduction causes complete loss in the inhibitory activity and structural conformation of RgPI (**Chapter 5**), it was of interest to further examine the effect of DTT on the trypsin and chymotrypsin inhibitory activity of BgPI. There was a significant decline in the trypsin inhibitory activity (70%) and chymotrypsin inhibitory activity (62%) at a 0.25 mM concentration of DTT. Further, with increase in DTT concentration from 0.25 to 1.0 mM, the activity of BgPI against both trypsin and chymotrypsin was completely lost within 15 min at 56^{o}C (**Fig. 6.23**). This significant loss in inhibitory activity after DTT reduction was in contrast to that of treatments with temperature and pH. The loss in activity is attributed to the fact that the reactive site loops of inhibitor consists of disulfide bonds which are responsible for maintaining the inhibitory activity (Qi et al., 2005). The BBI type of PI purified from black gram was less susceptible to DTT reduction (**Fig. 6.23**) than PI isolated from red gram (**Chapter 5, Fig. 5.22A**).

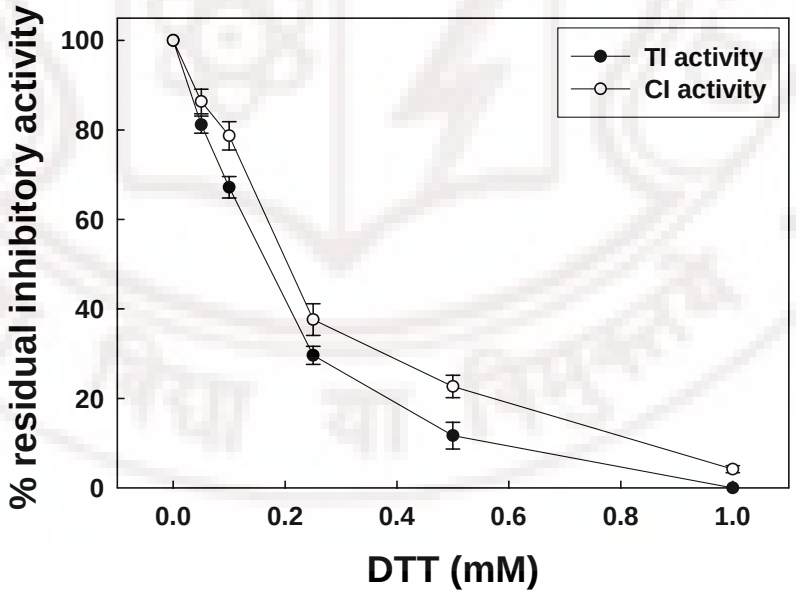

Fig. 6.23. Effect of DTT reduction on trypsin and chymotrypsin inhibitory activity of BgPI. The residual inhibitory activity against trypsin and chymotrypsin was determined as described in materials and methods.

We also examined the conformational changes associated with DTT reduction. BgPI when incubated with 1 mM DTT at 56°C for 15 min the changes in secondary structure were marginal. However, when the reduction of BgPI with DTT was followed by alkylation with iodoacetamide, the ellipticity at 203 nm was increased and complete loss in secondary structural conformation was observed (**Fig. 6.24**). Taken together the results suggest that loss in activity of BgPI could be due to loss of reactive site loop rather than disulfide bond disassociation.

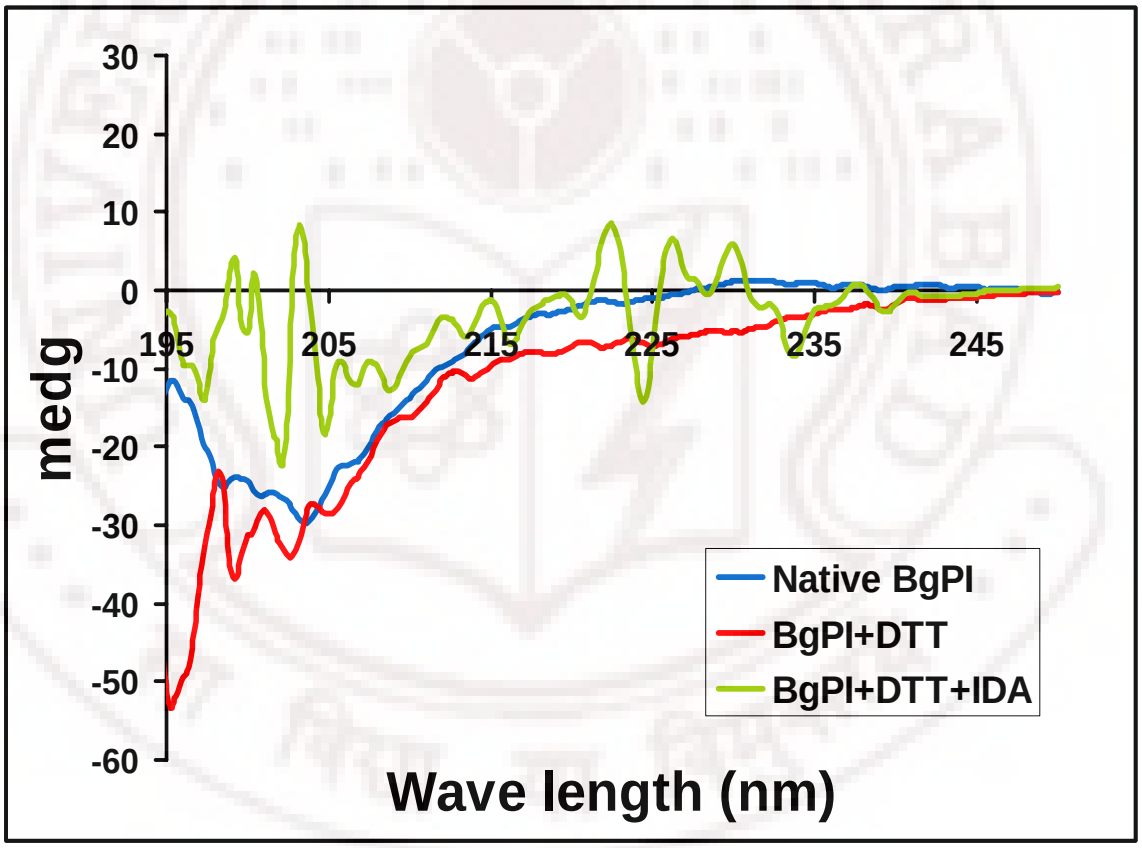

Fig. 6.24. Far-UV CD spectra of native BgPI (—); BgPI reduced with 1mM DTT (—); and reduced BgPI alkylated with iodoacetamide (—). Other details were as described in Fig. 5.17 and materials and methods.

Effect of chemical modification on the inhibitory activity of BgPI:

As we have observed the effects of chemical modification of specific amino acid residues on inhibitory activity of RgPI (**Chapter 5**), we also were interested to identify the specific amino acids that are responsible for inhibitory activity against trypsin and chymotrypsin. BgPI was chemically modified using amino acid specific reagents as described in materials and methods along with controls. The modified BgPI was tested for its inhibitory activity against bovine pancreatic trypsin and chymotrypsin (**Figs. 6.25 and 6.26**).

The modification of lysine or tryptophan residues of BgPI significantly affected its inhibitory activity against bovine pancreatic trypsin by 68% and 100%, respectively. On the other hand, modification of arginine or tyrosine residues of BgPI had no effect on its inhibitory activity against trypsin (**Fig. 6.25**). Modification of tryptophan residues of BgPI also caused complete loss of inhibitory activity against chymotrypsin. However, modification of lysine or arginine or tyrosine residues of BgPI did not cause any serious effect (<11%) on inhibitory activity against chymotrypsin.

The importance of lysine or tryptophan residues of BgPI in forming a stable complex with trypsin/chymotrypsin proteinases was further strengthened by the activity staining studies using gelatin-SDS-PAGE (**Fig. 6.26A and B**). In activity staining studies modification of lysine residues showed faint bands against trypsin but not chymotrypsin indicating the importance of lysine residue at the reactive site for trypsin inhibition, which is a characteristic feature of BBI (Qi et al., 2005). We also observed in the present study that modification of tryptophan residues of BgPI resulted in complete disappearance of bands against trypsin (**Fig. 6.26A, lane 6**) and chymotrypsin (**Fig. 6.26B, lane 6**), which suggests the presence of tryptophan at both trypsin and chymotrypsin reactive sites of BgPI. Corroborating with the inhibitory activity studies

(**Fig. 6.25**), we did not observe any changes in trypsin or chymotrypsin inhibitor bands when the BgPI was modified for arginine or tyrosine (**Fig. 6.26 A and B, lanes 3 and 5**).

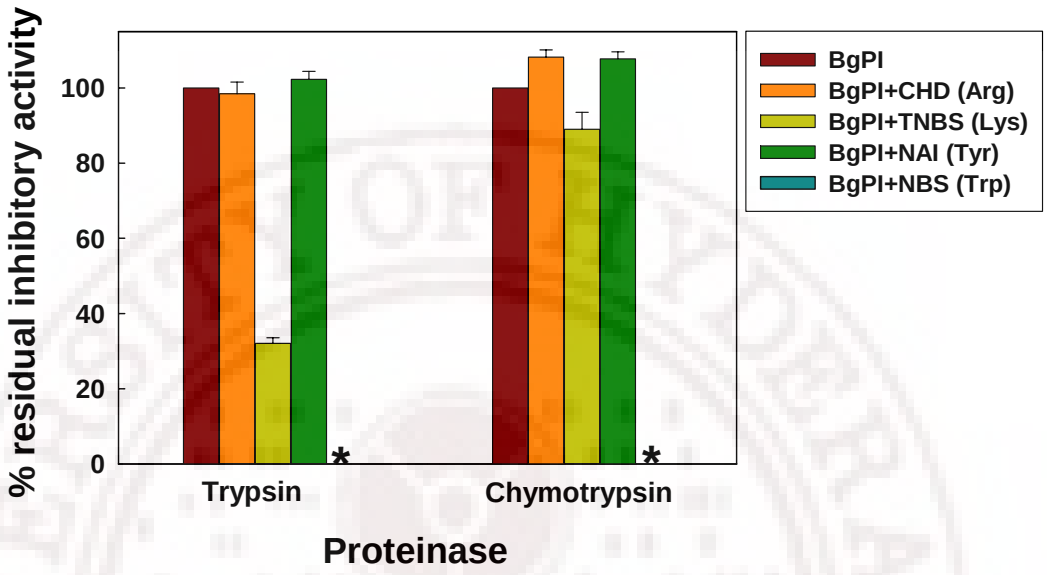

Fig. 6.25. Effect of chemical modification of amino acid residues on inhibitory activity of BgPI against trypsin and chymotrypsin. The residual inhibitory activity was determined as described in materials and methods. *BgPI lost complete inhibitory activity against trypsin and chymotrypsin after modifying tryptophan residues using NBS.

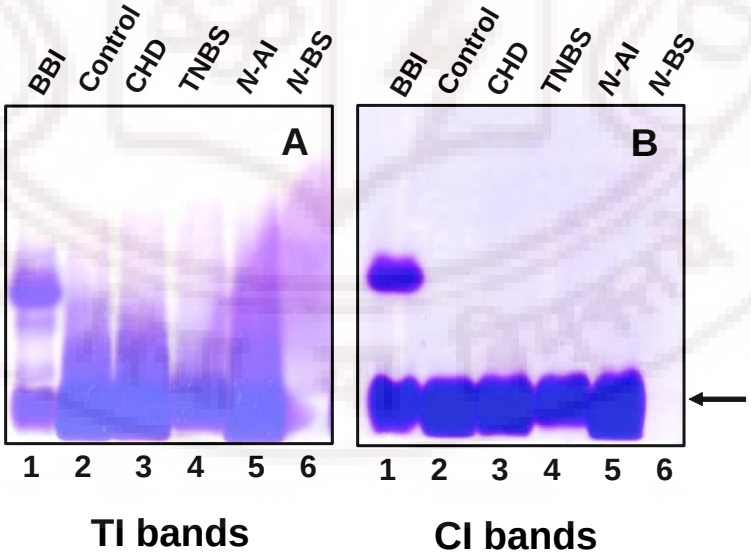

Fig. 6.26. Gelatin-SDS-PAGE (12.5%) showing the trypsin (A) and chymotrypsin inhibitor (B) bands of unmodified (control) and modified BgPI using amino acid specific modifying agents.

Chapter 7

Evaluation of insecticidal properties of purified proteinase inhibitors from red gram (ICP 14770) and black gram (TAU-1)

Chapter 7

Evaluation of insecticidal properties of purified proteinase inhibitors from red gram (ICP 14770) and black gram (TAU-1)

The role of PIs in combating against insect pests is well proven (Fan and Wu, 2005; Mosolov and Valueva, 2008). The use of PIs in insect control strategies primarily depend on inhibition of digestive gut proteinases. Most of the lepidopteran insects, which feed on the economically important crop plants have alkaline midgut fluids with pH optima between 9-11. Serine proteinases: trypsin, chymotrypsin and exopeptidases are the major digestive enzymes responsible for proteolytic activity in their midgut fluid (Christeller et al., 1992; Srinivasan et al., 2006; Karumbaiah et al., 2007). Hence, several research groups have purified PIs against trypsin and chymotrypsin from different plant sources and tested their *in vitro* effect on digestive enzymes and *in vivo* effect towards growth and development of lepidopteran larvae (Macedo et al., 2003; Bhattacharyya et al., 2007a; Oliveira et al., 2007; Chougule et al., 2008). PIs inhibit larval gut proteinase activity, which in turn impairs digestion and absorption of amino acids leading to retardation of larval growth and development (Srinivasan et al., 2005b; Telang et al., 2009). They also cause a decline in the fertility and fecundity of the adult moths (De Leo and Gallerani, 2002; Tamhane et al., 2005).

The crude extracts of red gram cultivar ICP 14770, showed strong inhibitory activity against *A. janata* larval mid gut trypsin like proteinases (**Chapter 4**). The PI purified from this cultivar ICP 14770 belonged to BBI type family (**Chapter 5**). Interestingly the PI purified in the present study from black gram variety TAU-1 also belonged to BBI type family (**Chapter 6**). Extensive literature survey reveals that the information on BBI type PIs possessing insecticidal activity towards the lepidopteran insects was very limited (Giri et al., 2003; Aguirre et al., 2004; Vila et al., 2005). Therefore, in the present study, we evaluated the inhibitory properties of purified BBI

type PIs: RgPI and BgPI from red gram (ICP 14770) and black gram (TAU-1), respectively against larval midgut proteinases of lepidopteran insects by i*n vitro* and *in vivo* methods.

Results and Discussion

In vitro effect of RgPI and BgPI towards midgut trypsin-like proteinases of lepidopteran insects:

Larval midgut trypsin-like proteinases from the following insects: *H. armigera*, *S. litura*, *A. janata*, *P. demoleus*, *A. albistriga*, *C. cephalonica*, *B. mori* and *D. nerii* exhibited diverse susceptibility to inhibition by RgPI and BgPI (**Fig. 7.1**). The inhibitory activity of RgPI or BgPI was greatest (>93%) on midgut trypsin-like proteinases of *A. janata* (**Fig. 7.1**) The inhibitory activity of RgPI was marginal on midgut trypsin-like proteinases belonging to *H. armigera* (21%), *S. litura* (14%), *P. demoleus* (12%), *A. albistriga* (10%) and *C. cephalonica* (10%), while its inhibitory activity on midgut trypsin-like proteinases of *B. mori* (3%) and *D. nerii* (4%) was negligible.

On the other hand, while the inhibitory activity of BgPI was moderate on midgut trypsin-like proteinases belonging to *H. armigera* (48%), *P. demoleus* (66%) and *A. albistriga* (45%), its inhibitory effect on the midgut trypsin-like proteinases of *S. litura* (28%), *C. cephalonica* (22%), *B. mori* (20%) and *D. nerii* (20%) was marginal. However, the inhibitory activity of soybean BBI was moderate (35-40%) on midgut trypsin-like proteinases of all these insects except in *B. mori* (25%) and *D. nerii* (19%) (**Fig. 7.1**). In an earlier study the trypsin inhibitor isolated from *Prosophis juliflora* showed only 45% of inhibition against midgut trypsin-like digestive enzymes from *A. janata*, while the same inhibitor showed 83% inhibition against midgut trypsin-like digestive enzymes from *H. armigera* (Sivakumar et al., 2005).

The inhibitory activity exerted by 1 μg concentration of RgPI or BgPI towards *A. janata* midgut proteinases was greater than that observed in presence of similar concentration of soybean BBI (**Fig. 7.1**). However, while the inhibitory property of RgPI (20 μg) against other larval midgut trypsin-like proteinases was always less when compared with the inhibitory properties of soybean BBI (20 μg), the inhibitory property of BgPI (20 μg) was variable. In fact, the inhibitory potential of BgPI is greater than soybean BBI against trypsin-like midgut proteinases of *H. armigera*, *P. demoleus* and *A. albistriga* (**Fig. 7.1**).

Further, we compared the inhibitory potential of both RgPI and BgPI (non-host PIs) against *A. janata* midgut trypsin-like proteinases at much lower concentrations, i.e., from 50 to 250 ng. At 100 ng concentration, while RgPI inhibited 70% of *A. janata* midgut trypsin-like proteinase activity, BgPI inhibited 40% of its activity (**Fig. 7.2**). Thus, these results suggest that RgPI has more inhibitory potential against midgut trypsin-like proteinases of *A. janata* when compared with BgPI.

Although RgPI and BgPI are known as non-host PIs to *P. demoleus*, *A. albistriga*, *C. cephalonica*, *B. mori* and *D. nerii*, along with *A. janata*, they did not cause any remarkable inhibition in the activity of midgut trypsin-like proteinases in these insects as observed in *A. janata*. Further, the non-host plant PIs effective against *H. armigera* gut proteinases have also been reported from winged bean (Harsulkar et al., 1999; Giri et al., 2003; Telang et al., 2009). The results from the present study suggest that all the non-host PIs may not be necessarily effective against insect pests.

In general, the host plant PIs are either ineffective or respond moderately to pest infestation, as was observed with RgPI and BgPI against *H. armigera* and *S. litura* (**Fig. 7.1**). This could be due to a co-evolutionary interaction between the two organisms (Giri et al., 1998). In plant-insect interaction, insects have evolved and adapted to overcome the effect of host plant defense by producing proteinases that are either insensitive to

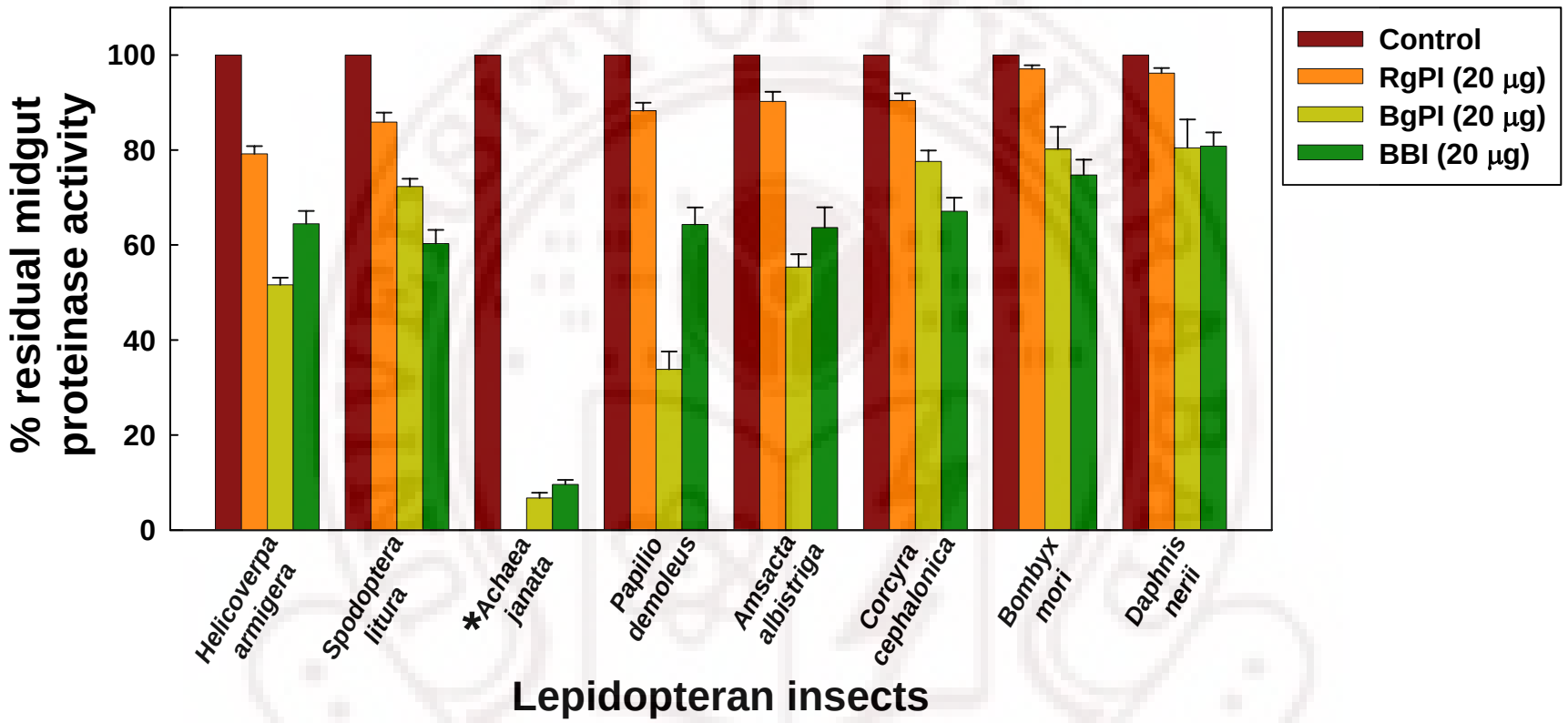

Fig. 7.1. Inhibitory activity of RgPI and BgPI against larval midgut trypsin-like proteinases of Lepidopteran insects. BBI from soybean was used as a positive control. The digestive proteinases were isolated from the midgut of fifth instar larvae and residual trypsin-like proteolytic activity was examined in presence of RgPI/BgPI/BBI (20 µg each) as described in materials and methods. *for *A. janata*, 1 µg of each of RgPI/BgPI/BBI was incubated with midgut proteinases. 100% activity corresponds to the OD at 410 nm of respective midgut proteinases, after 45 min incubation with BAPNA at 37^{o}C, without any inhibitor.

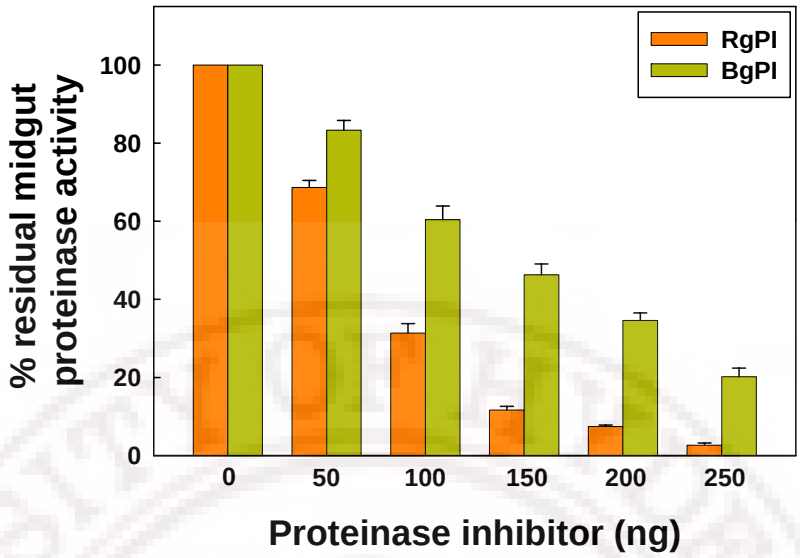

Fig. 7.2. Inhibitory activity of RgPI and BgPI against midgut trypsin-like proteinases of *A. janata*. RgPI and BgPI at 0 to 250 ng were incubated with midgut proteinases and residual trypsin-like proteinase activity was measured using BAPNA as described in Fig. 7.1 and materials and methods.

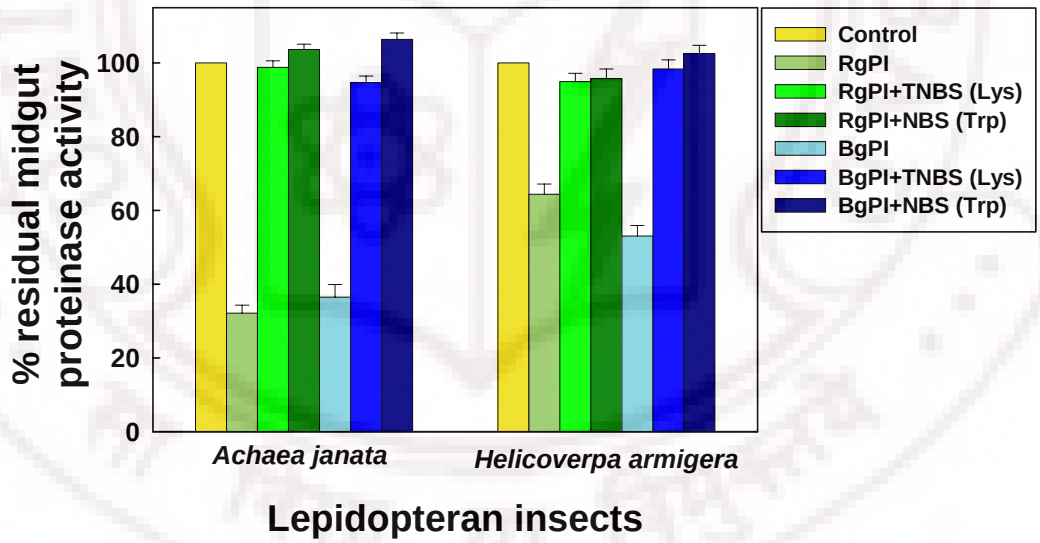

Fig. 7.3. Effect of chemical modification of amino acid residues on the inhibitory activity of RgPI and BgPI against midgut trypsin-like proteinases of *A. janata* and *H. armigera*. The lysine and tryptophan residues were modified using TNBS and NBS, respectively, as described in materials and methods. The midgut proteinases were incubated with modified and unmodified inhibitors for 15 min and residual trypsin-like proteinase activity was measured in presence of BAPNA as described in Fig. 7.1.

inhibitors (Jongsma et al., 1995; Broadway, 1995, 1997; Bown et al., 1997; Paulillo et al., 2000; Brito et al., 2001; Volpicella et al., 2003; Brioschi et al., 2007) or have the capacity to degrade them (Michaud, 1997; Girard et al., 1998b; Giri et al., 1998b; Moon et al., 2004; Telang et al., 2005, Yang et al., 2009).

Interestingly, on the other hand, PIs from host plants if expressed in appropriate amounts could confer resistance to insect pests (Zavala et al., 2004; Srinivasan et al., 2005b; Damle et al., 2005). The BBI type PIs of chickpea were not effective against *H. armigera* gut proteinases due to the production of inhibitor-insensitive proteinases (Giri et al., 1998; Harsulkar et al., 1999). However in *H. armigera*, proteinases inhibited by Kunitz type inhibitors did not show any specific adaptation (Srinivasan et al., 2005a), hence, they exhibited strong inhibitory activity against *H. armigera* gut proteinases (Srinivasan et al., 2005b). Therefore it is necessary to continuously screen for novel plant PIs and identify PIs from non-host plants effective against insect pests.

Plant PIs act as specific substrates for the digestive proteinases, forming a stable complex in which proteolysis is limited and extremely slow (Tiffin and Gaut, 2001; Mello and Silva-Filho, 2002). In general, serine PIs are attributed in the defense of plants against invading organisms. But, the efficacy of a specific inhibitor is dependent on the structural compatibility of the reactive site of the plant proteinase inhibitor with the substrate-binding site of the proteinases in the larval midgut Jongsma et al., 1995).

In the present work amino acid modification studies with both RgPI (**Chapter 5, Figs. 5.25 and 5.26**) and BgPI (**Chapter 6, Fig. 6.25 and 6.26**) clearly demonstrate that lysine and typtophan are responsible in the reactive site for inhibiting the activity of bovine pancreatic trypsin. Hence, we examined the effect of chemical modification of lysine and tryptophan residues on the inhibitory activity of PIs against midgut trypsin-like proteinases of *A. janata* and *H. armigera*. The modification of lysine and tryptophan residues by TNBS and NBS, respectively, lead to significant loss in inhibitory activity of

both RgPI and BgPI against midgut trypsin-like proteinases of *A. janata* and *H. armigera* (**Fig. 7.3**). These results suggest that lysine and tryptophan residues probably play an important role in binding of the PIs to trypsin-like proteinases even in larval midguts of *A. janata* and *H. armigera*. Minor variations in the amino acid sequences near the binding or active site were shown to render the proteinases entirely insensitive towards the PIs (Bown et al., 1998).

Visualization of iso-inhibitors of RgPI and BgPI in presence of insect midgut proteinases:

RgPI and BgPI showed the presence of five to six different iso-inhibitors in native-PAGE (**Chapter 5, Fig. 5.7 and Chapter 6, Fig. 6.6**). Harsulkar et al. (1999) suggested that these iso-inhibitors have arisen due to co-evolution of the plants and insects to combat each others defense mechanisms. In *C. cajan*, using crude extracts it was clearly demonstrated that these iso-inhibitors varied quantitatively in the seeds of different cultivars and wild types and qualitatively against proteinases from different sources (**Chapter 4**). Hence, it was of interest to examine the inhibitory profile of iso-inhibitors of RgPI and BgPI against bovine pancreatic trypsin as well as chymotrypsin and midgut proteinases of *A. janata*, *H. armigera* and *S. litura* using gelatin-native-PAGE.

In activity staining studies, RgPI showed six different iso-inhibitor bands against trypsin and chymotrypsin of bovine pancreatic origin (**Fig. 7.4A, lanes 1 and 2**) and midgut proteinases of *A. janata*, *H. armigera* and *S. litura* (**Fig. 7.4A, lanes 3-5**). In contrast, BgPI showed the presence of seven different iso-inhibitors bands having inhibitory activity against bovine pancreatic trypsin and chymotrypsin (**Fig. 7.4B, lanes 1 and 2**). Interestingly, BgPI showed one or more extra slow moving band(s) in presence of larval midgut proteinases (**Fig. 7.4B, lanes 3-5**) which were not detected in presence of

both trypsin and chymotrypsin (**Fig. 7.4B, lanes 1 and 2**). Both RgPI and BgPI showed marginally high intensity inhibitor bands in presence of *A. janata* midgut proteinases (**Fig. 7.4 A and B, lane 3**) when compared with inhibitor bands in presence of *H. armigera* and *S. litura* (**Fig. 7.4 A and B, lanes 4 and 5**). These results corroborate well with *in vitro* assays, where RgPI and BgPI showed approximately 400-fold higher inhibitory activity against gut trypsin-like proteinases of *A. janata* when compared with *S. litura* and *H. armigera* (**Figs. 7.1 and 7.2**).

In the present study, the mobility pattern of most of the iso-inhibitors of RgPI and BgPI against trypsin or chymotrypsin of bovine pancreatic origin overlapped with larval midgut proteinases (**Fig. 7.4**). These results correlated well with the reports from ground nut, winged bean and potato (Harsulkar et al., 1999), bitter gourd (Telang et al., 2003), and wild types of pigeon pea (Chougule et al., 2003), which also showed correlation between TI and *H. armigera* gut proteinase inhibitor (HGPI) profiles. However, differential TI and HGPI profiles were observed with chickpea seed extracts, where HGPIs are fast moving bands having inhibitory activity against gut proteinases of *H. armigera*, but not against bovine pancreatic trypsin (Srinivasan et al., 2005b).

Identification of proteinases sensitive to RgPI and BgPI from midgut extracts of *A. janata, H. armigera* and *S. litura*:

The proteinase profile of midgut extracts of *A. janata*, *H. armigera* and *S. litura* were examined using casein-SDS-PAGE to identify, which one among them were sensitive to RgPI and BgPI. Midgut proteinases were allowed to react with RgPI, BgPI or BBI (standard PI) at a concentration of 100 µg/ml in 0.1 M glycine-NaOH (pH 10.5) after electrophoretic separation of proteinases on casein-SDS-PAGE. Simultaneously the midgut extract was incubated with irreversible chemical inhibitors like PMSF (inhibitor of serine proteinases), TLCK (inhibitor of trypsin) and TPCK (inhibitor of chymotrypsin)

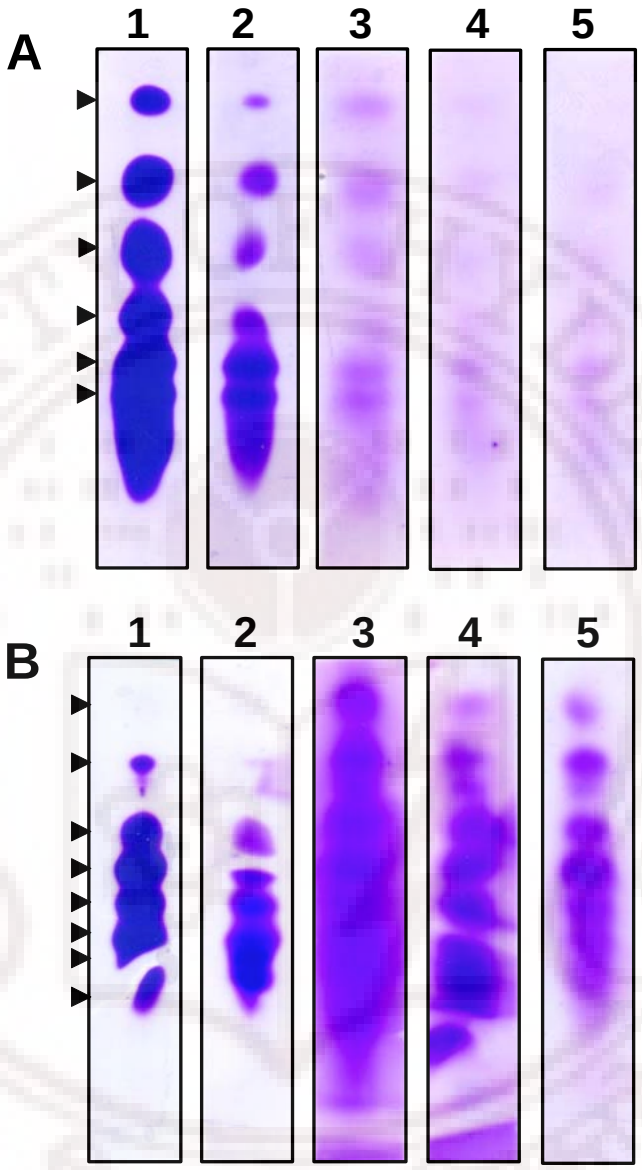

Fig. 7.4. Visualization of iso-inhibitors of RgPI (A) and BgPI (B) on 12.5% gelatin-native-PAGE. Equal TI units (~ 60 TI units) of RgPI and BgPI were loaded in each lane. The gel strips were incubated with trypsin (lane 1), chymotrypsin (lane 2) of bovine pancreas, and larval midgut proteinases of *A. janata* (lane 3), *H. armigera* (lane 4) and *S. litura* (lane 5). Other details were as described in materials and methods.

at 300 μM concentration, prior to electrophoresis to identify the various trypsin- and chymotrypsin-like proteinases and their location in the respective zymogram (**Fig. 7.5 A-C**).

Midgut extract of *A. janata* showed the presence of 8-9 proteinases in controls in the absence of chemical inhibitors or PIs (**Fig. 7.5A, lane 2**). In presence of PMSF, two low molecular weight bands disappeared confirming the presence of serine type proteinases (trypsin and chymotrypsin) in the midgut extracts of *A. janata* (**Fig. 7.5A, lane 3**). In presence of TLCK, two high molecular mass bands and two low molecular mass bands were abolished, confirming the presence of two high molecular mass and two low molecular mass trypsin-like proteinases in the midgut extracts of *A. janata* (**Fig. 7.5A, lane 4**). However in presence of TPCK, there was no change in the proteinase profile of *A. janata*, which suggests that chymotrypsin-like proteinases were either totally absent or present at negligible concentration (**Fig. 7.5A, lane 5**). These results are in agreement with our proteinase assays where midgut extracts of *A. janata* showed higher trypsin-like activity, when compared with chymotrypsin-like activity (**Chapter 4, Fig. 4.4**). The gel strips containing midgut extracts of *A. janata* when incubated with RgPI or BgPI, trypsin-like proteinases of high molecular mass disappeared, which suggest that only high molecular mass proteinases were sensitive to both RgPI and BgPI but not low molecular weight proteinases (**Fig. 7.5A, lanes 7 and 8**). The sensitivity of midgut proteinases of *A. janata* to RgPI or BgPI were comparable to that observed with soybean BBI (**Fig. 7.5A, lane 6**).

Midgut extract of *H. armigera* showed the presence of 6-7 proteinases in the absence of chemical inhibitors or PIs (**Fig. 7.5B, lane 2**). In the midgut extract incubated with PMSF, a lower molecular mass proteinase with higher intensity disappeared confirming the presence of serine proteinase other than trypsin- and chymotrypsin-like (**Fig. 7.5B, lane 3**). In presence of TLCK, two high molecular mass and three low

molecular mass proteinases disappeared, confirming the presence of trypsin-like proteinases in the midgut extracts of *H. armigera* (**Fig. 7.5B lane 4**). Similar to *A. janata*, the midgut extract of *H. armigera* also did not show any changes in proteinase profile in presence of TPCK (**Fig. 7.5B, lane 5**). The gel strips containing midgut extracts of *H. armigera* when incubated with RgPI or BgPI, two high molecular mass and one low molecular mass trypsin-like proteinases disappeared (**Fig. 7.5B, lanes 7 and 8**). Interestingly, incubation with BgPI also showed the maximum inhibition of low molecular mass serine proteinase (**Fig. 7.5B, lanes 3 and 8**). The sensitivity of midgut proteinases of *H. armigera* towards RgPI and BgPI was comparable to soybean BBI (**Fig. 7.5B, lane 6**).

Midgut extract of *S. litura* also showed the presence of 6-7 proteinases (**Fig. 7.5C, lane 2**). Among these, the proteinases with trypsin-like activity were found to be three in number and the proteinases with trypsin- and chymotrypsin-like activity were found to be three in number (**Fig. 7.5C, lanes 4 and 5**). The gel strips containing midgut extracts of *S. litura* when incubated with RgPI and BgPI as well as soybean BBI, two high molecular mass trypsin-like and one low molecular mass trypsin and chymotrypsin-like proteinase completely disappeared (**Fig. 7.5C, lanes 6-8**).

In Lepidoptera, the complex multigene families encode for digestive proteinases (Srinivasan et al., 2006). Hence, it is possible that trypsins occur in various isoforms. High molecular weight trypsin-like proteinases are often aggregates of trypsin with lower molecular weight (Novillo et al., 1999; Wagner et al., 2002). By corroborating these reports with the results from our studies we suggest that RgPI or BgPI inhibit the trypsin-like proteinases by preventing the formation of high molecular mass aggregates of trypsin, by forming stable complexes with low molecular mass trypsin-like proteinases.

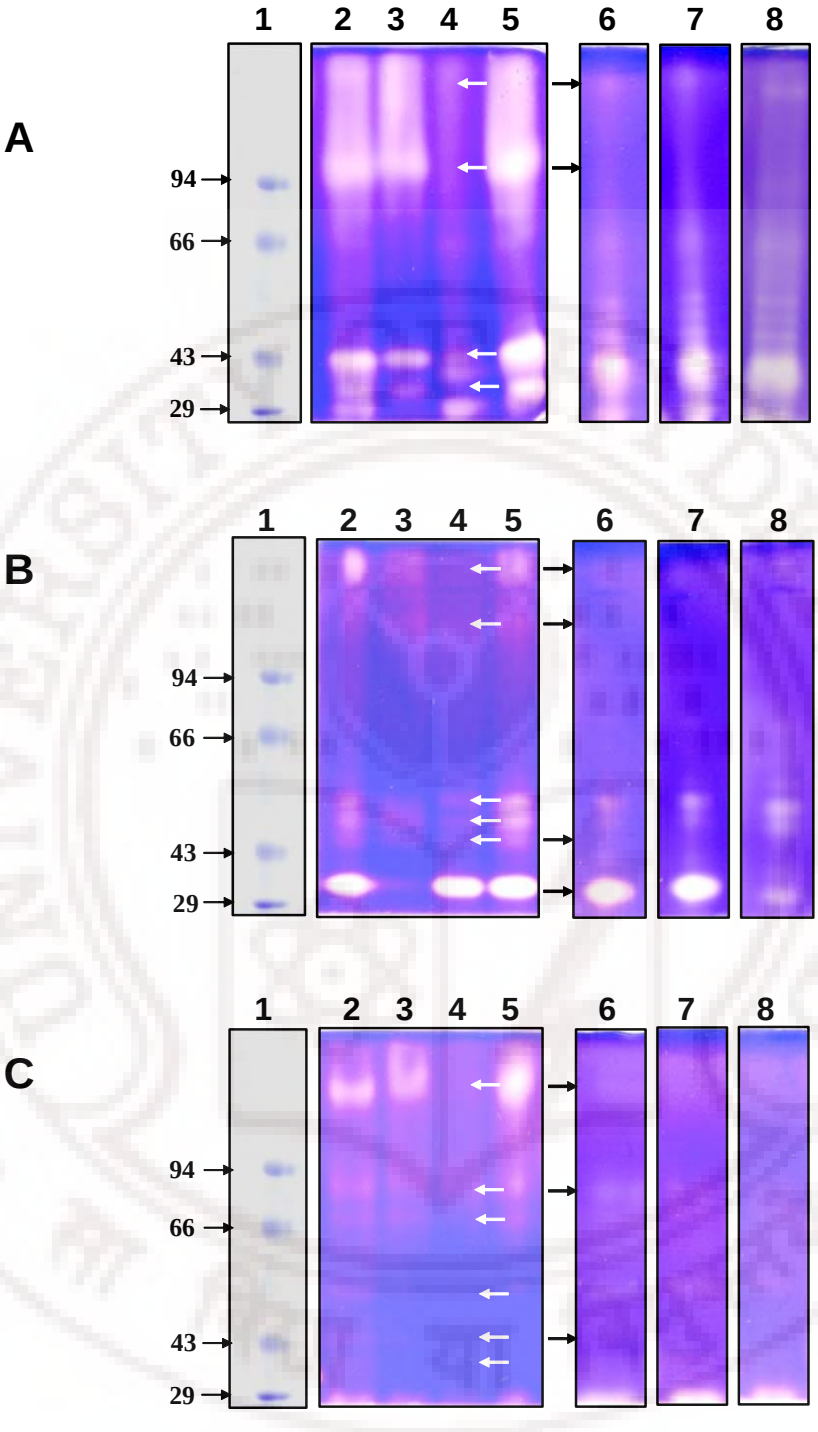

Fig. 7.5. Zymogram of larval midgut proteinase profiles of *A. janata* (A), *H. armigera* (B) and *S. litura* (C) in casein-SDS-PAGE (7.5%) in presence and absence of inhibitors. Lane 1, markers; lane 2, midgut proteinases (control); midgut proteinases incubated with PMSF (lane 3); TLCK (lane 4); TPCK (lane 5); BBI (lane 6); RgPI (lane 7); and BgPI (lane 8).

Effect of RgPI and BgPI on larval growth and development of *A. janata* and *S. litura:*

Although, the *in vitro* studies with RgPI and BgPI demonstrated differential inhibitory activity against *A. janata* and *S. litura* midgut trypsin-like proteinases (**Figs. 7.1 and 7.2**), the ambiguity of whether or not RgPI and BgPI retard the larval growth and development exists (Edmonds et al., 1996). Hence, we further performed the insect feeding assays to evaluate the antibiosis exerted on *A. janata* and *S. litura* by RgPI and BgPI.

The *in vivo* effect of RgPI from red gram was examined on the larval growth and development of *A. janata* using leaf coating assays. Newly hatched larvae of *A. janata* were allowed to feed on fresh castor leaves coated with (2, 4 and 8 μg of RgPI/cm^2 leaf area) or without RgPI. After 6 days of feeding, larvae showed 30%, 49% and 92% retardation in their growth, respectively (**Table 7.1**). While body weight of the larvae fed on control diet was around 171 mg, there was approximately 50% reduction in the body weight (87 mg) of the larvae grown on leaf coated with 4 μg of RgPI/cm^2 leaf area. Further the survival rate of these larvae was found to be reduced to 88%, 52% and 27% when compared with their respective control (**Table 7.1**).

Thus the dose-dependent response between the concentration of RgPI and larval growth or survival of larvae was linear (**Fig. 7.6, and Table 7.1**). The stunted growth of larvae observed with increasing concentrations of RgPI on the castor leaf is represented in the **figure 7.7**. However when these RgPI fed larvae were allowed to grow on the fresh castor leaves without any inhibitor up to 20 days, all of them showed 100% mortality irrespective of their earlier feeding conditions (i.e., RgPI concentration) (**Table 7.1**).

Similar to RgPI, feeding of newly hatched *A. janata* larvae for six days on castor leaf coated with 2, 4 and 8 μg of BgPI/cm^2 leaf area, also showed 22%, 41% and 67% reduction in their body weight, when compared with larvae grown on leaf without any

BgPI. The survival rate of these larvae after 6 days was found to be 93%, 88% and 67%, respectively (**Table 7.2**). Thus the dose-dependent response between the concentration of BgPI and larval growth or survival rate of larvae was found to be linear (**Fig. 7.8**). The stunted growth of larvae with increasing concentrations of BgPI on castor leaf was represented in **figure 7.9**. When these larvae were allowed to grow on fresh castor leaves without any coating of PIs for 20 days, 100% mortality was observed only with respect to larvae which were previously fed on leaf coated with 8 μg of BgPI/cm^2 leaf area.

These results reveal that the larvae fed on the leaf coated with RgPI/BgPI grow slowly due to feeding inhibition associated with lower consumption of leaf, which finally resulted in severe retardation of their growth, when compared with larvae fed on control leaves. Though few larvae survived, they did not undergo normal development and transformed either into defective larval-pupal intermediates which failed to molt or smaller pupae, which ecloped into adults, which were short lived and infertile (**Fig. 7.10**).

This is the first report showing the inhibitory activity of trypsin PIs against *A. janata* larval growth and development. This remarkable *in vitro* and *in vivo* effect of RgPI and BgPI against *A. janata* could be possible, due to the presence of large number of trypsin-like proteinases in their midgut. These results further suggest that both RgPI and BgPI could be used to develop insect resistant transgenic plants against *A. janata*.

Table 7.1. Effect of RgPI on growth and development of newly hatched larvae of *A. janata*. RgPI was coated on castor leaf as described in materials and methods.

Concentration of RgPI (*μg/cm² leaf area*)	Average weight of each larva (mg) [*% control*]	Reduction in growth (*% control*)	Survival rate after 6 days (*% control*)	Mortality after 20 days (*% control*)
0	171 ± 6 (100)	0	100	0
2	119 ± 5 (70)	30	88	100
4	87 ± 6 (51)	49	52	100
8	14 ± 2 (8)	92	27	100

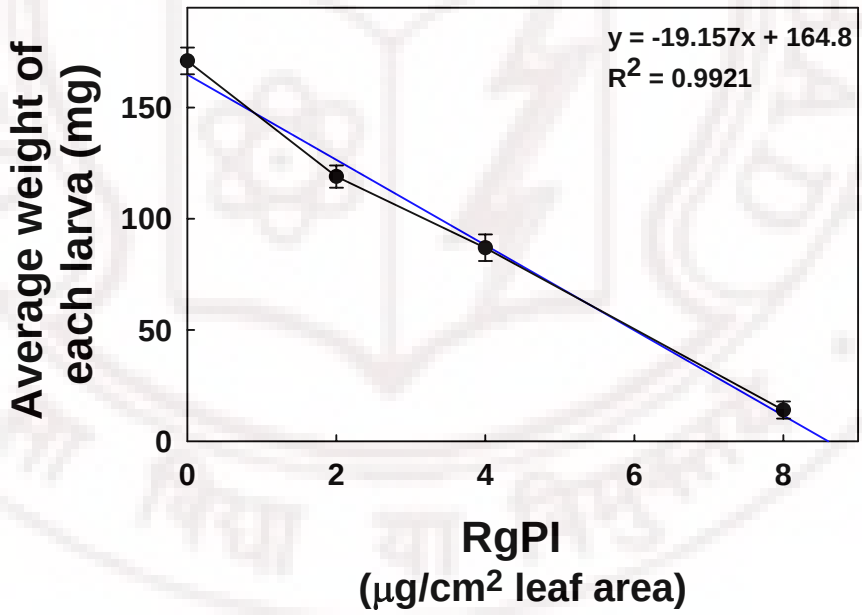

Fig. 7.6. Inhibitory activity of RgPI on growth and development of *A. janata* larva. Newly hatched larvae were allowed to feed on the castor leaf coated with different concentration of RgPI as described in materials and methods. Each point is the mean ± S.E. of 20 larvae.

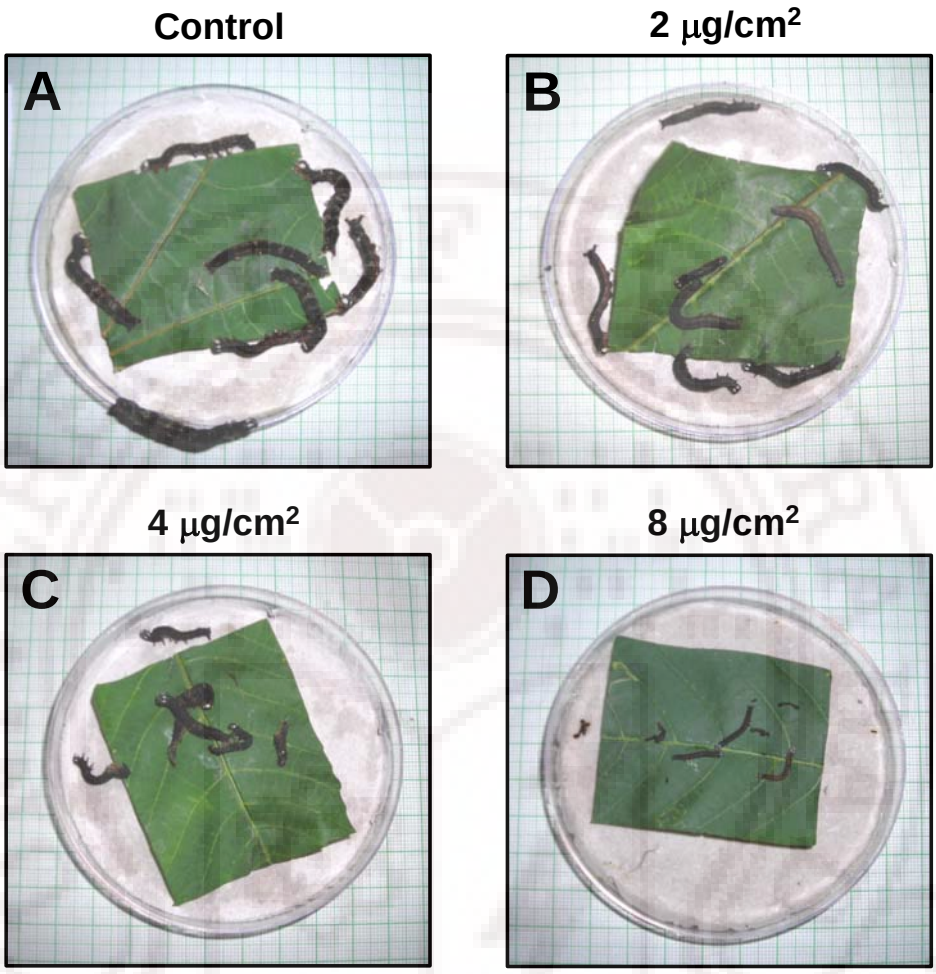

Fig. 7.7. Development of *A. janata* reared on castor leaf coated with RgPI in different concentrations as indicated above. The photographs of the larvae after 6 days of feeding on castor leaves coated without RgPI (A) and with RgPI of 2 μg/cm^2 (B), 4 μg/cm^2 (C) and 8 μg/cm^2 (D) leaf area.

Table 7.2. Effect of BgPI on growth and development of newly hatched larvae of *A. janata*. BgPI was coated on castor leaf as described in materials and methods.

Concentration of RgPI (*μg/cm² leaf area*)	**Average weight of each larva (mg)** [*% control*]	**Reduction in growth** (*% control*)	**Survival rate after 6 days** (*% control*)	**Mortality after 20 days** (*% control*)
0	171 ± 6 (100)	0	100	0
2	133 ± 8 (78)	22	93	80
4	100 ± 5 (59)	41	88	80
8	56 ± 4 (33)	67	67	100

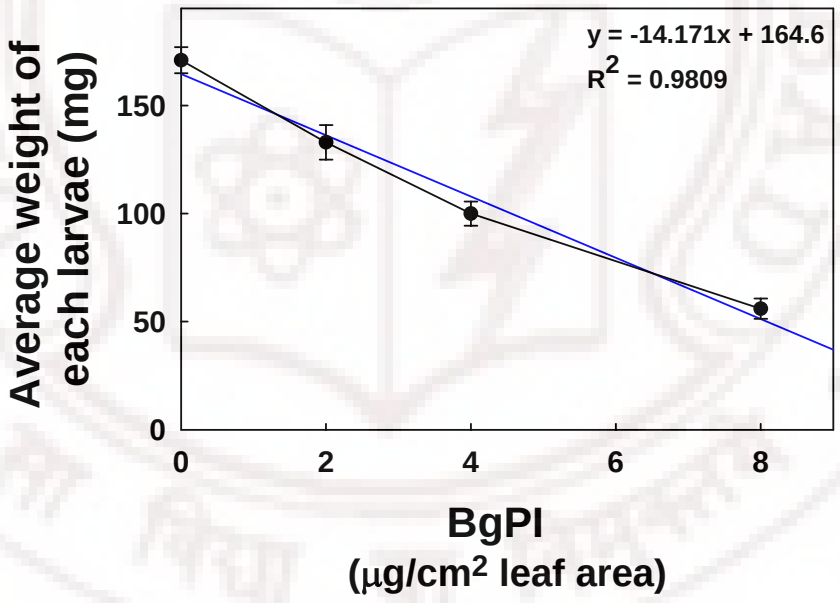

Fig. 7.8. Inhibitory activity of BgPI on growth and development of *A. janata* larva. Newly hatched larvae were allowed to feed on the castor leaf coated with different concentration of BgPI as described in materials and methods. Each point is the mean ± S.E. of 20 larvae.

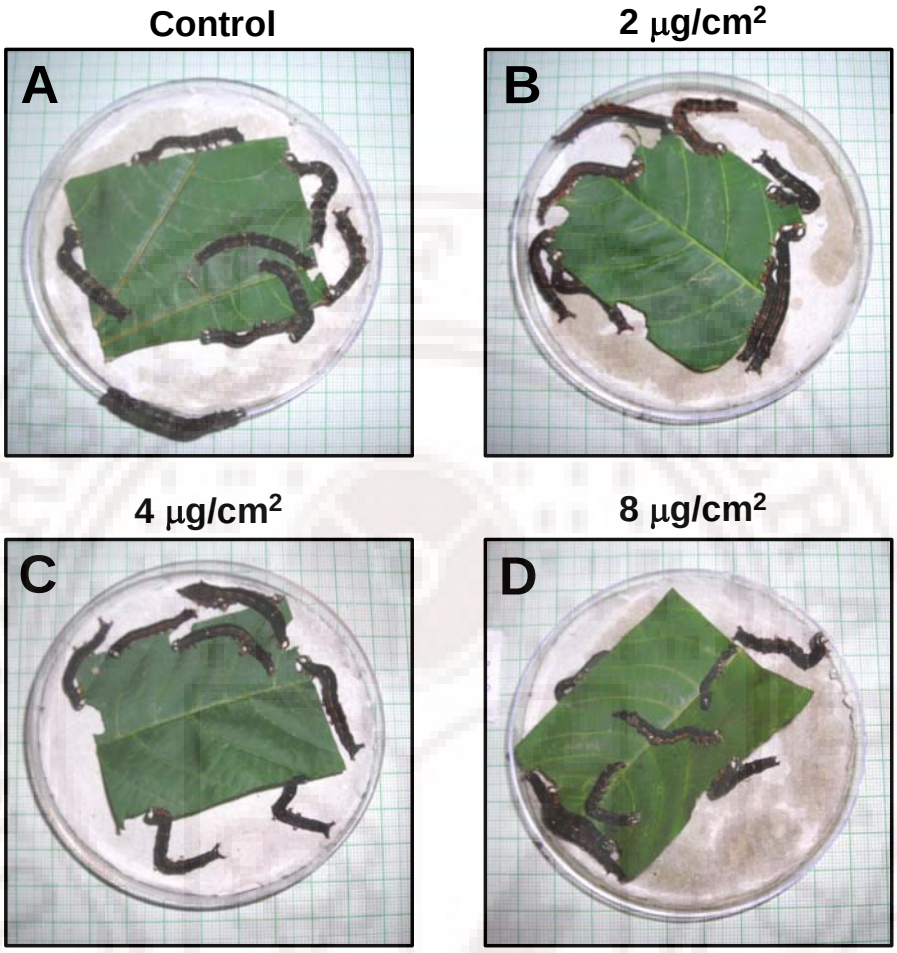

Fig. 7.9. Development of *A. janata* reared on castor leaf coated with BgPI in different concentrations as indicated above. The photographs of the larvae after 6 days of feeding on castor leaves coated without BgPI (A) and with BgPI of 2 μg/cm^2 (B), 4 μg/cm^2 (C) and 8 μg/cm^2 (D) leaf area.

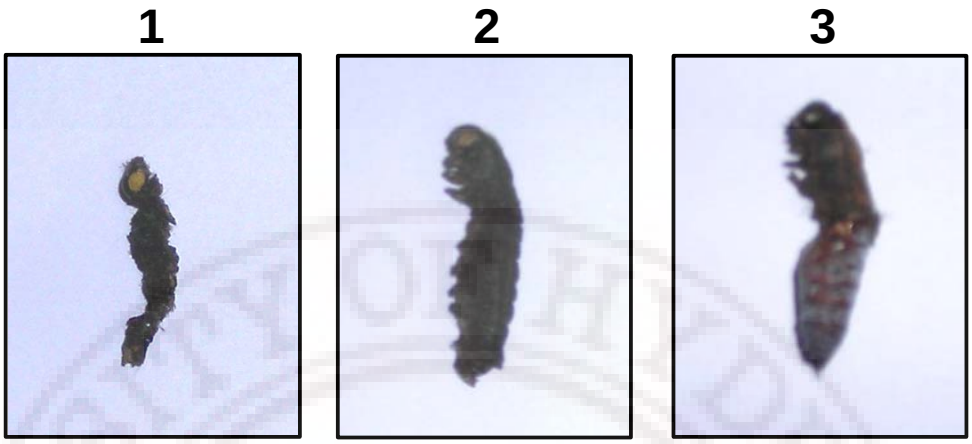

Fig. 7.10. Defective larval-pupal development of *A. janata* in presence of RgPI or BgPI. (1) Larva died in early instar, (2) larva died in late instar, (3) larval-pupal intermediate.

The effect of RgPI and BgPI on growth and development of *S. litura* was assessed by feeding newly hatched larvae on artificial diet supplemented with PIs for seven days. At the end of seventh day, while larvae fed on diet supplemented with RgPI at 0.025% and 0.050% showed 13% and 21%, reduction in the body weight of larvae, BgPI showed 28% and 39% reduction in body weight of larvae, respectively (**Tables 7.3 and 7.4**). A dose dependent response was observed also between the concentration of RgPI or BgPI and larval growth of *S. litura*. However, greatest reduction in the body weight of *S. litura* was observed with BgPI when compared with RgPI. These results corroborate well with the *in vitro* assays, which revealed higher inhibitory effect of BgPI over RgPI on midgut trypsin-like proteinases of *S. litura* (**Fig. 7.1**). The photographs showing stunted growth of *S. litura* larvae with increasing concentrations of RgPI and BgPI, when compared with the larvae grown on control diet were represented in **figures 7.11 and 7.12**. When these

larvae were allowed to further grow on the same diet until pupation, neither RgPI nor BgPI had shown any change in mortality on the larvae of *S. litura*, but delayed developmental period of larvae, when compared with larvae grown on control diet. Although RgPI (14%) and BgPI (28%) showed less inhibitory activity against midgut trypsin-like proteinases of *S. litura* (**Fig. 6.1**), the *in vivo* assays showed moderate effect on growth and development of the larvae. This could be due to presence of either low levels of trypsin-like proteinases or insensitive proteinases in the midgut of *S. litura* (**Chapter 4, Fig. 4.4**). The factors other than trypsin-like proteinases might be susceptible to inhibition by RgPI and BgPI in the *in vivo* assays leading to retardation of larval growth and development in *S. litura*.

RgPI and BgPI were more effective when compared with soybean BBI in retarding larval growth and development in *S. litura* (**Tables 7.3 and 7.4, Figs. 7.11 and 7.12**). However, soybean BBI showed higher inhibitory effect on midgut trypsin-like proteinases of *S. litura*, when compared with RgPI and BgPI (**Fig. 7.1**). Furthermore, *in vivo* assays, which showed only 10% inhibition in body weight of larvae at 0.05% BBI in diet suggest that trypsin-like proteinases, which are moderately inhibited by BBI might not be solely responsible for regulating growth and development of *S. litura*. Our results on the growth and development of *S. litura* corroborate well with the studies of Telang et al. (2003) and Bhattacharyya et al. (2007a).

Table 7.3. Effect of RgPI on growth and development of *S. litura*. RgPI was added to the artificial diet as described in materials and methods.

Concentration of PI in the diet (*% w/v*)	**Average weight of larvae (mg)** (*% control*)	**Reduction in growth** (*% control*)	**Survival rate after 20 days** (*% control*)
Control (No inhibitor)	298 ± 13 (100)	0	100
BBI (0.050%)	269 ± 9 (90)	10	100
RgPI (0.025%)	259 ± 11 (87)	13	100
RgPI (0.050%)	235 ± 8 (79)	21	100

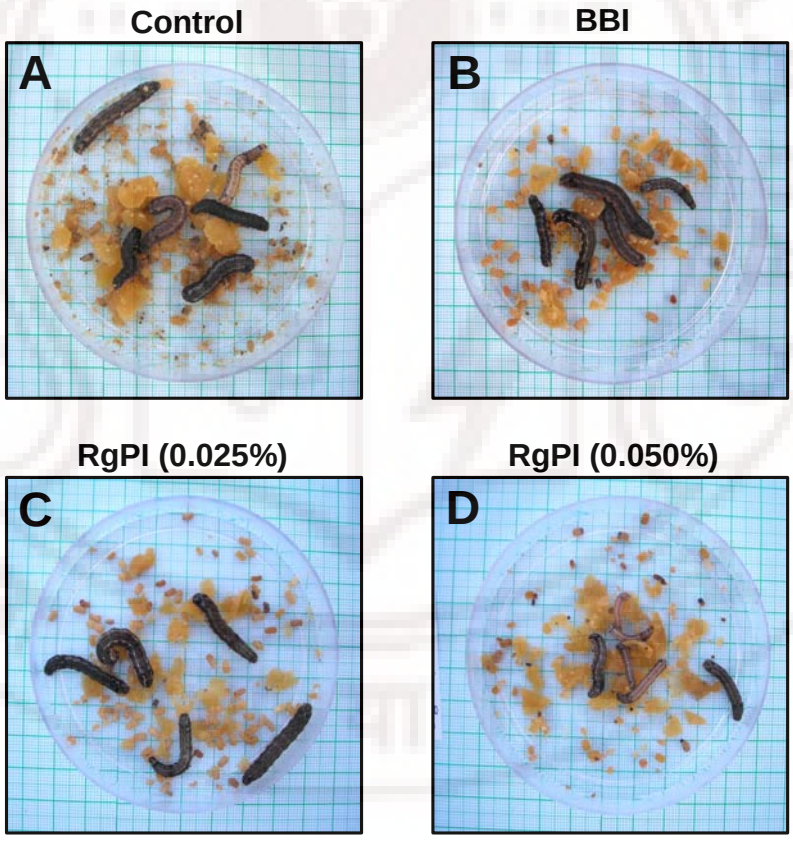

Fig. 7.11. Development of *S. litura* reared on artificial diet containing RgPI in different concentrations. The photographs of the larvae after 7 days feeding on artificial diet containing (A) control (without RgPI), (B) soybean BBI, (C) & (D) RgPI at 0.025% and 0.050% respectively.

Table 7.4. Effect of BgPI on growth and development of *S. litura*. BgPI was added to the artificial diet as described in materials and methods.

Concentration of PI in the diet (*% w/v*)	**Average weight of larvae (mg)** (*% control*)	**Reduction in growth** (*% control*)	**Survival rate after 20 days** (*% control*)
Control (No inhibitor)	298 ± 13 (100)	0	100
BBI (0.050%)	269 ± 9 (90)	10	100
BgPI (0.025%)	215 ± 10 (72)	28	100
BgPI (0.050%)	183 ± 10 (61)	39	100

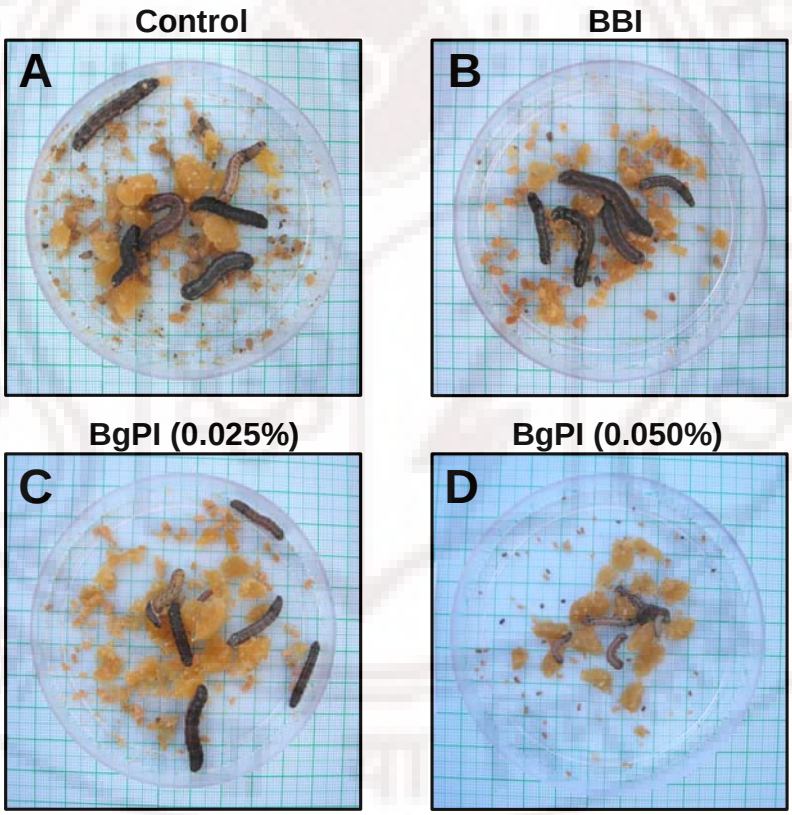

Fig. 7.12. Development of *S. litura* reared on artificial diet containing BgPI in different concentrations. The photographs of the larvae after 7 days feeding on artificial diet containing (A) control (without BgPI), (B) soybean BBI, (C) & (D) BgPI at 0.025% and 0.050% respectively.

In vitro and _in vivo_ effect of RgPI on midgut proteinase activity, growth and development of _M. sexta_:

The inhibitory effect of RgPI against the activity of midgut trypsin-like proteinases isolated from the fifth instar larvae of *M. sexta* was examined. The residual trypsin-like activity of midgut proteinases was monitored in the presence of increasing concentrations of RgPI (**Fig. 7.13**). RgPI showed significant reduction in the activity of midgut trypsin-like proteinases. At 4 µg of RgPI, 85% reduction in the activity was observed and the effect of inhibitor was comparable to that of well known soybean Kunitz trypsin inhibitor, which showed 90% reduction in activity of *M. sexta* midgut proteinases (**Fig. 7.13**). Trypsin inhibitor isolated from *Hyptis suaveolens* moderately inhibited the gut trypsin-like proteases of *M. sexta* (Aguirre et al., 2004). The purified PI from *C. cajan* variety, TAT-10 showed less inhibition against the *H. armigera* gut proteinases (Godbole et al., 1994b), when compared with the crude extracts from wild relatives of *C. cajan*, which showed remarkable inhibition against gut proteinases of *H. armigera* (Chougule et al., 2003). The differences in inhibitory potential observed in above studies could be either due to the allelic variations, which exist in the varieties (Chougule et al., 2003) or alteration in insect gut proteinase profile upon continuous exposure to host plant PIs (Jongsma and Bolter, 1997; Harsulkar et al., 1999). For eg., cultivated varieties are more exposed to insects, when compared with wild types. Hence it is necessary to screen the non-host PIs to combat against insect pests.

However for some insects, though *in vitro* assays provided strong evidence for inhibitory potential of PIs towards the midgut digestive enzymes, concurrent strong reduction in the growth and development was not detected through *in vivo* bioassays (Jongsma and Bolter, 1997). Hence, we further evaluated the *in vivo* effects of RgPI on the growth and development of *M. sexta* larva by feeding experiments. While feeding of

newly hatched larvae for five days on the diet containing 0.001% (w/v) of RgPI decreased their body weight by 15%, feeding of these larvae on diet containing 0.01% (w/v) of the inhibitor decreased their body weight by 50% when compared with the control (**Fig. 7.14A**). The changes in the body size of larvae fed on diet containing RgPI is represented in **figure 7.14B**. These *in vivo* feeding experiments showed similarity to the effects observed with potato proteinase inhibitor II expressed in tobacco (Johnson et al., 1989) and trypsin inhibitor from *Nicotiana attenuate* (Zavala et al., 2008) on *M. sexta*. The differences in the results observed for *in vitro* and *in vivo* experiments could be due to differential expression of numerous trypsin and chymotrypsin isoforms in the *Manduca* gut, as observed at different larval stages (Broehan et al., 2008).

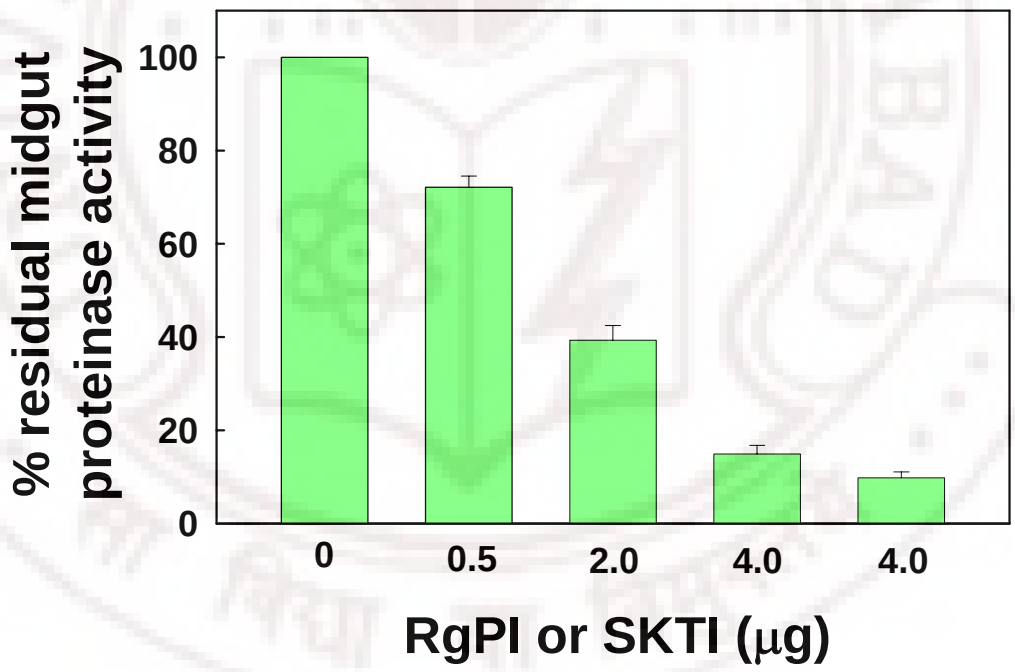

Fig. 7.13. Effect of RgPI on midgut trypsin-like proteinases of *M. sexta*. The digestive enzymes were isolated from fifth instar larvae and trypsin-like activity was examined in presence of RgPI as described in materials and methods. Bars 1 to 4 represent RgPI (µg) and bar 5 represent SKTI (4 µg).

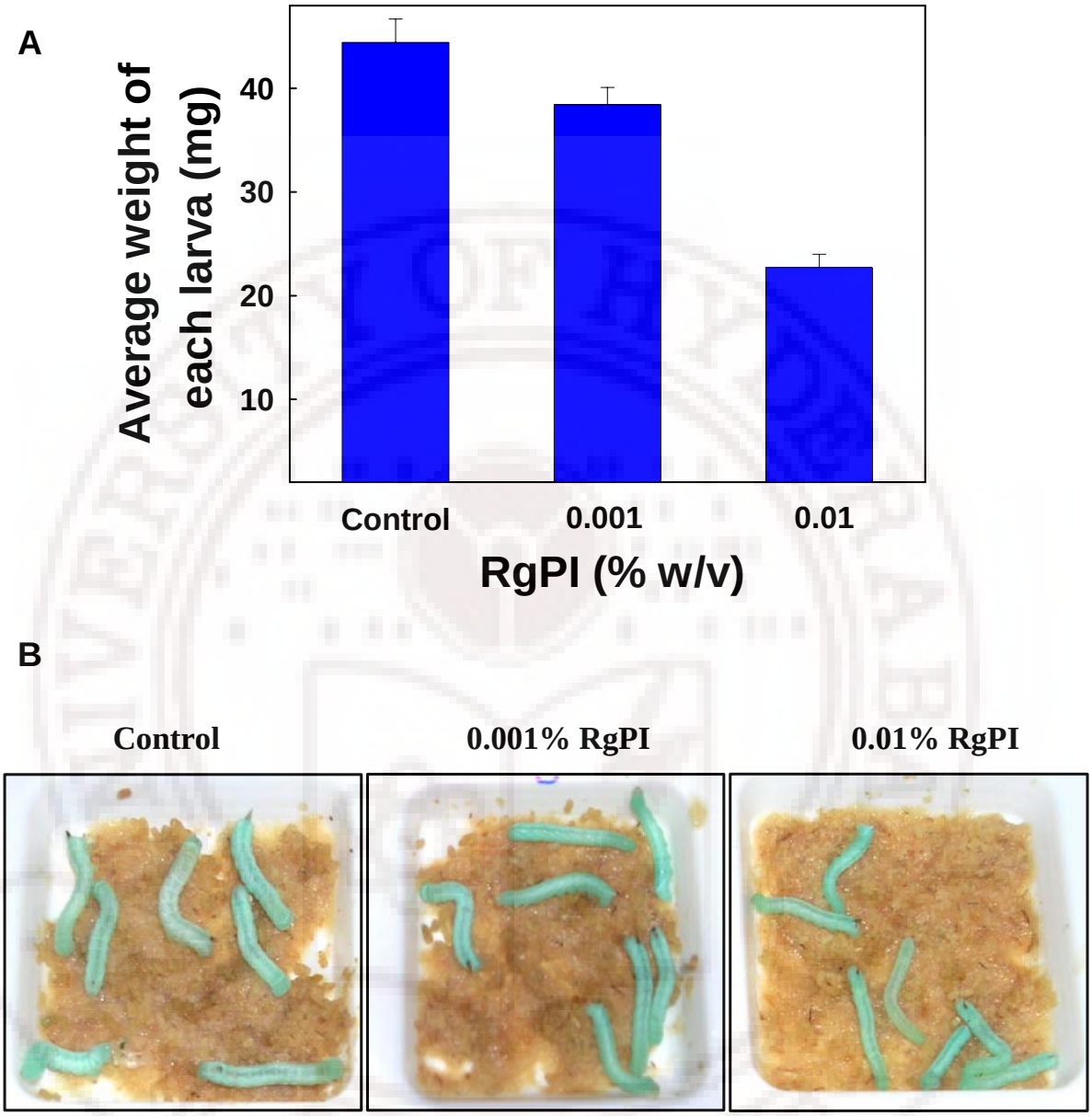

Fig. 7.14. Growth and development of third instar larvae of *M. sexta* reared on artificial diet containing RgPI. (A) Average weight of each larva grown on different concentrations of RgPI. RgPI was added to the artificial diet as described in materials and methods. (B) Photographs of larvae grown on artificial diet, showing stunted growth with increasing concentration of RgPI.

Chapter 8

Summary and Conclusions

Chapter 8

Summary and Conclusions

The occurrence of PIs, which were active against *H. armigera* gut proteinases were reported in some cultivars and wild types of pigeonpea by Chougule et al. (2003). The results from **Chapter 4** in the present study provided detailed information on the differential activity of PIs from crude extracts of various red gram cultivars and wild types against *A. janata* and *S. litura*. The inhibitory potential of these PIs against midgut trypsin-like proteinases were compared with bovine pancreatic trypsin and chymotrypsin.

We have identified inhibitory activity against both trypsin and chymotrypsin of bovine pancreatic origin in cultivars as well as wild types (**Fig. 4.1A and B**). Among the cultivars, highest TI activity was observed in ICP 14770 and CI activity in ICP 11300. The TI activity was more pronounced (two-fold higher) than CI activity in cultivars. In case of wild types highest TI activity and CI activity was observed in ICP 15859 *Rhynchosia rothii*. In wild types, the variation between TI and CI activity was less when compared with cultivars. In gelatin-PAGE, while the cultivars exhibited homomorphism in terms of TI and CI profiles (**Fig. 4. A and B**), wild types showed variation in number of TI or CI bands and exhibited species specific banding pattern (**Fig. 4.3A and B**). Interestingly, the TI profile of cultivars and wild types overlapped with their CI profile, which clearly indicates that the same protein possess both TI and CI activities, which is a characteristic feature of BBI type of PIs (Clemente and Domoney, 2006).

The inhibitory activity of PIs from cultivars and wild types against midgut trypsin-like proteinases of *A. janata* was several fold (10 to 50) higher than that observed in presence of bovine pancreatic trypsin (**Figs. 4.1 and 4.5**). This remarkable inhibitory potential could be due to non-exposure of *A. janata* to the identified PIs, possibly because pigeonpea is a non-host plant to this lepidopteran pest. On the other hand, the inhibitory activity of PIs from cultivars and wild types against trypsin-like proteinases of *S. litura*

was 3 to 9 fold less than that observed in presence of bovine pancreatic trypsin (**Figs. 4.1 and 4.6**). The low inhibitor potential of these PIs towards *S. litura* gut proteinases could be possibly due to presence of low trypsin-like activity in the midgut of *S. litura* (**Fig. 4.4**) or due to less affinity of these PIs with gut proteinases of *S. litura*.

Chapter 5 describes the purification and characterization of PI from red gram cultivar ICP 14770, which is one among several cultivars with higher inhibitory activity against *A. janata* gut proteinases. The PI was purified in sequential steps of $(NH_4)_2SO_4$ fractionation followed by separation in ion exchange, affinity and gel filtration chromatography. The protocol used resulted in ~66.5 fold purification of PI with a recovery of ~55% yield, and the PI was named as RgPI (**Table 5.1**).

The RgPI appeared as monomer and dimer with an apparent molecular mass of 8.5 kDa and 16.5 kDa in SDS-PAGE under non-reducing condition (**Fig. 5.4**). Interestingly MALDI-TOF spectrum of RgPI showed the presence of multiple forms with molecular masses of 8376.8 Da (monomer), 16745.9 Da (Dimer), 24529.4 Da (trimer), 32844.7 Da (tetramer) and 41193.3 Da (pentamer) under native condition (**Fig. 5.5A and B**). However, sequentially reduced and alkylated RgPI showed the presence of only one peak corresponding to 9189.4 Da (**Fig. 5.6**). The increase in mass of 812.6 Da under reduced and alkylated condition indicates the presence of 14 cysteine residues, which is a characteristic feature of BBI (Qi et al., 2005). Many earlier reported BBIs were shown to undergo self association to form homodimers, trimers or more complex oligomers in solution (Kumar et al., 2004). Further more, RgPI showed five different iso-inhibitors in native-PAGE as well as in 2-D electrophoresis with pI values ranging between 5.95 - 7.15 (**Figs. 5.7 and 5.9**). Tryptic digestion of these iso-inhibitors of RgPI showed identical mass spectrum in MALDI-TOF (**Fig. 5.10**). The peak m/z 1638.8 when further ionized in MALDI-TOF-TOF, the resulting MS/MS spectrum showed homology with BBI from *G. max* in Mascot MS/MS ion search (**Fig. 5.12**). The N-terminal sequence

DQHHSSKACC, for 10 residues of iso-inhibitor with pI value 5.95 showed remarkable homology when aligned with BBIs from *Phaseolus* spp. in ClustalW alignment (**Fig. 5.13**).

Chapter 6 describes the purification and characterization of PI from black gram variety TAU-1. The PI was purified by ~55.6-fold and recovered by ~42% and named as BgPI (**Table 6.1**). BgPI showed a single band under non-reducing condition with an apparent molecular mass of 8 kDa (**Fig. 6.4**). On the other hand MALDI-TOF spectrum of BgPI showed the presence of two peaks at 8072.3 Da (monomer) and 16082.2 Da (dimer) (**Fig. 6.5**). However the peak intensity of dimer was very low and hence negligible. Native-PAGE and 2-D electrophoresis of BgPI showed the presence of 5-6 iso-inhibitors with pI value ranging between 4.3 - 6.0 (**Figs. 6.6 and 6.7**). Similar to RgPI, tryptic digested iso-inhibitors of BgPI showed identical mass spectrum in MALDI-TOF (**Fig. 6.8**). The mass spectrum of iso-inhibitor with pI value 4.3 showed 55% homology with BBI type PI from *Vigna unguiculata* subsp. *cylindrica* and several other BBI type PIs (**Fig. 6.9**). The same iso-inhibitor (pI 4.3) showed homology in mass to a peptide fragment consisting of the following sequence:'SIPPQCHCADIRLNSCHSACK' (**Fig. 6.10**). The lift spectrum obtained after ionization of peak 1453.7 m/z of iso-inhibitor with pI value 4.4 showed 74% homology with BBI of *Torresea* spp. and *V. radiata* (green gram) (**Fig. 6.12**). The *de novo* sequence 'SIPPQCHCADIR' obtained from lift spectrum of peak 1453.7 m/z using BioTools over lapped with above sequence for iso-inhibitor of pI 4.3 (**Fig. 6.13**).

RgPI and BgPI showed inhibitory activity against both trypsin and chymotrypsin, which is a characteristic of BBIs as it has two different reactive sites. Nevertheless, inhibition of trypsin activity was more pronounced than that of chymotrypsin activity (**Figs. 5.14 and 6.15**). They showed non-competitive type of inhibition with both trypsin (K_i= 0.29 and 0.3 μM) and chymotrypsin (K_i= 2.27 and 10.7 μM), respectively (**Figs. 5.15**

and 6.16). The secondary structures showed predominance of β-strands and random coil over α-helix (**Tables 5.2 and 6.2**), a characteristic feature of BBI (Wu and Sessa, 1994). The tertiary structure showed the presence of positive CD at 257 nm which is attributed to the presence of disulfide bonds in them (**Figs. 5.17 and 6.18**; Ramasarma et al., 1995). Their inhibitory activities were stable up to 80^{o}C with marginal loss in activity at 90^{o}C-100^{o}C (**Figs. 5.18 and 6.19**). Further, the inhibitory activities were also stable at different pH tested between 2 and 12 (**Figs. 5.20 and 6.21**). Marginal loss in inhibitory activity at pH 6.0 could be due to iso-electric precipitation. Temperature induced conformational changes are reversed when the inhibitors were cooled down to 25^{o}C (Figs. 5.19 and 6.20). The possible presence of many cysteine residues forming disulfide bonds as commonly found in BBIs may account for this remarkable stability in structure and activity (Singh and Appu Rao, 2002).

We observed a loss in the inhibitory activity of both RgPI and BgPI after reduction with DTT (**Figs. 5.22 and 6.23**). However, the loss in activity was more pronounced with DTT than 2-ME, which is also evident from activity staining studies (**Fig. 5.23**). The structural conformation was completely lost after reduction and alkylation with DTT and iodoacetamide, respectively (**Figs. 5.24 and 6.24**). The loss in inhibitory activity could be due to the fact that the reactive site loops of PI are formed by disulfide bonds which are responsible for maintaining the inhibitory activity (Qi et al., 2005). Modification of lysine and tryptophan residues in RgPI and BgPI lead to loss in inhibitory activity against trypsin, suggesting that these residues are present in reactive site of PIs that determines the specificity for trypsin. Similarly modification of tryptophan residues resulted in loss of chymotrypsin activity which implies that tryptophan plays a role in determining specificity for chymotrypsin (**Figs. 5.25 and 6.25**). Based on these biochemical properties exhibited by RgPI and BgPI, we suggest that they belong to BBI family.

.**Chapter 7** demonstrates the insecticidal properties of purified PIs from red gram (RgPI) and black gram (BgPI). Among the lepidopteran insects chosen in the present study, *A. janata* and *S. litura* showed higher trypsin-like activity than chymotrypsin-like activity in their midgut extracts (**Fig. 4.4**). Moreover PIs from both red gram and black gram also showed pronounced inhibitory activity against trypsin, when compared with chymotrypsin (**Figs. 5.14 and 6.15**). As the reports on the BBI type inhibitors active towards the lepidopteran insects are limited, in the present study we examined the inhibitory properties of RgPI and BgPI against trypsin-like midgut proteinases of *H. armigera*, *S. litura*, *A. janata*, *P. demoleus*, *A. albistriga*, *C. cephalonica*, *B. mori*, *D. nerii* and *M. sexta*. Both RgPI and BgPI showed remarkable inhibitory effect on *A. janata* gut proteinases, while only a marginal to moderate inhibition was observed against other lepidopteran insects (**Fig. 7.1**). Studies with RgPI showed 6 different iso-inhibitors bands, while BgPI showed 7 different iso-inhibitor bands active in presence of *A. janata*, *H. armigera* and *S. litura* gut proteinases (**Fig. 7.4A and B**). Zymograms of gut extracts from the larvae of these insects showed that trypsin-like proteinases were inhibited by both RgPI and BgPI (**Fig. 7.5 A-C**).

In vivo effect of PIs from red gram and black gram on growth and development of *A. janata* was performed by using leaf coating assays (**Figs. 7.7 and 7.9**) and in *S. litura* by using artificial diet assays (**Figs. 7.11 and 7.12**). Newly hatched larvae were allowed to grow on the feed containing PIs. RgPI showed higher effect on the growth and development of *A. janata* when compared to BgPI (**Tables 7.1 and 7.2**) and vice-versa with *S. litura* (**Tables 7.3 and 7.4**). However both the PIs showed 100% mortality in larvae of *A. janata* after 20 days (**Tables 7.1 and 7.2**). The midgut trypsin-like proteinase activity and the body weight of *M. sexta* larvae was reduced remarkably in presence of RgPI (**Figs. 7.13 and 7.14**).

Major Conclusions:

1. Proteinase inhibitors from crude extracts of cultivars and few wild types showed higher inhibitory activity against midgut trypsin-like proteinases of *A. janata* when compared with *S. litura*.
2. Proteinase inhibitors purified from red gram and black gram possessed both trypsin as well as chymotrypsin inhibitory activity, which indicates that they belonged to BBI family and subsequently were named as RgPI and BgPI.
3. Both RgPI and BgPI showed remarkable insecticidal activity against larval forms of *A. janata*. They have a great potential to be used as insecticidal agent.
4. Studies with *M. sexta* further confirmed their insecticidal activity against non-host insects.

Chapter 9

Literature Cited

Chapter 9

Literature Cited

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Annexure 1

Research Papers:

E.R. Prasad, Aparna Dutta Gupta and K. Padmasree (2009) Inhibitors of pigeonpea active against lepidopteran gut proteinases. (**under revision** in Journal of Economic Entomology).

E.R. Prasad, Aparna Dutta Gupta and K. Padmasree (2009) Purification and characterization of Bowman-Birk type inhibitor from pigeonpea (under preparation).

E.R. Prasad, Aparna Dutta Gupta and K. Padmasree (2009) Effect of Bowman-Birk type inhibitor from pigeonpea (*Cajanus cajan*) on *Manduca sexta* (tobacco hornworm; Lepidoptera: Sphingidae) by in vitro and in vivo methods. (under preparation).

E.R. Prasad, Aparna Dutta Gupta and K. Padmasree (2009) Purification and characterization of proteinase inhibitor from black gram (under preparation).

Conferences attended:

E.R. Prasad, K. Padmasree and Aparna Dutta Gupta (2006) Identification, purification and characterization of serine proteinase inhibitors from cultivars and wild relatives of chick pea and pigeon pea. *93rd Indian Science Congress,* Acharya N. G. Ranga Agricultural University, Hyderabad, 3-7 January (Poster presentation)