Chapter 1

INTRODUCTION

Protease inhibitors are molecules, which inhibit or antagonize the actions of proteases, thereby inhibiting the splitting of proteins into smaller peptide fractions and amino acids by a process known as proteolysis. The protein PI is a protein which combines reversibly with one or more proteases to form complexes of discrete stoichiometry in which all of the catalytic functions of the protease are competitively inhibited (Laskowski and Sealock, 1971). PIs are small proteins which represent a class of well studied compounds of plant defence and are abundant in storage organs such as seeds and tubers. Majority of PIs in plant kingdom occur in three main families; Fabaceae, Solanaceae and Poaceae (Richardson, 1991). The PIs are specific towards inhibiting proteolytic enzymes such as elastase, thrombin, plasmin, kallikrein, trypsin, chymotrypsin, chymase, tryptase, bacterial enzymes like subtilisin, fungal enzymes, endogenous plant proteinases and insect digestive enzymes (Garcia-Olmedo *et al.,* 1987; Hilder *et al.,* 1990; Richardson, 1991; Belozersky *et al.,* 1995). The natural PIs constitute a final regulatory step in the control of protease activities by inhibiting the proteases. During homeostasis there is a biological balance between proteases and their natural inhibitors (Losso, 2008). An imbalance between proteases and their endogenous inhibitors in the body may trigger uncontrolled proteolysis which leads to irreversible tissue destruction such as inflammation processes, rheumatoid arthritis, periodontitis, hypertension, gastric ulcer, muscular dystrophy, pathological angiogenesis, or tumor growth and metastasis (Noel et al., 1997; von der Helm et al., 2000). They are predominant in many plant species.

Protein-proteinase inhibitors were isolated first from seeds in 1940s; with the characterization of trypsin inhibitors from soybean and α-amylase inhibitor from cereal grains. Legume seeds are noted for their large reserve of protein in their cotyledons and also for their levels of PIs, active against

mammalian pancreatic proteases (Ryan, 1973). In plants, these inhibitors act as anti-metabolic proteins, which interfere with the digestive process of insects (Bown et al., 2004). PIs are widely classified into five families on the basis of the catalytic mechanisms and conditions of action of the proteases inhibited by them: serine, cysteine, metallo, aspartic, and threonine protease inhibitors. The most extensively studied of the PIs are those that inhibit the serine proteases, trypsin and chymotrypsin. The role of serine protease inhibitors as defensive compounds against predators is particularly well established (Reeck et al., 1997). Because of their ability to inhibit the enzymes involved in the digestive processes of humans and animals, protease inhibitors have been referred to as ‘‘antinutritional compounds’’ so far. Although these proteins are antinutritional factors they are beneficial to plants serving as protective agents against insects, fungi or microbial predators (Chilosi et al., 2000). Some of the PIs have now been favourably reconsidered in view of their biological behaviour in pharmacological and medical applications (Scarafoni et al., 2007). The new era of PIs is the transition from antinutritional status to the drug status.

On the basis of sequence homologies of their inhibitor domains, PIs have been classified into 48 families (Rawlings et al., 2004). Most of these interact with the proteinases according to a common mechanism. In each inhibitor molecule, there exists on the surface one peptide bond, the reactive site, which combines with the cognate enzyme mimicking the substrate of the enzyme. Such inhibitors are designated as standard mechanism inhibitors or canonical inhibitors. The plant PIs with their families and some typical examples are listed in Table 1.1.

Table 1.1 **Families of plant PIs with some examples.**

(Adapted from Habib and Fazili, 2007)

Common name	**Type example**	**Target Protease**	**References**
Kunitz (plant)	Soybean Kunitz trypsin inhibitor	Trypsin, Chymotrypsin	Laskowski and Kato (1980)
	Barley subtilisin inhibitor	Subtilisin, α -amylase	Vallee et al., (1998)
	Winged-bean chymotrypsin inhibitor	α -chymotrypsin	Habu et al., (1992)
	Kunitz cysteine peptidase inhibitor 1	Cysteine proteases	Gruden et al., (1997)
	Proteinase inhibitor A inhibitor unit	Trypsin, Chymotrypsin, Kallikerin	Laskowski and Kato(1980)
	Kunitz subtilisin inhibitor	Subtilisin-type microbial serine proteases	Terada et al., (1994)
	Cathepsin D inhibitor	Cathepsin D, Trypsin	Strukelj et al., (1992)
	Trypsin inhibitor	Trypsin and α-chymotrypsin	Lin et al. (1991)
Cereal	Ragi seed trypsin/ α-amylase inhibitor	α -amylase	Hojima et al., (1980)
	Barley trypsin/factor XIIa inhibitor	α -amylase, Trypsin	Lazaro et al., (1988)
	Wheat trypsin/α-amylase inhibitor	α -amylase, Trypsin	Shewry et al., (1984)

	Maize trypsin/factor XIIa inhibitor	Mammalian trypsin, activated hageman factor	Mahoney et al., (1984)
Squash	Trypsin inhibitor MCTI-1	Pancreatic elastase	Wieczorek et al., (1985)
	Trypsin inhibitor MCTI-II	Trypsin	Huang et al., (1992)
	Macrocyclic squash trypsin inhibitor	Trypsin	Hernandez et al., (2000)
	Trypsin inhibitor CSTI-IV	Trypsin	Wieczorek et al., (1985)
Potato type I	Chymotrypsin inhibitor I	Chymotrypsin, Trypsin	Richardson (1974)
	Glutamyl peptidase II inhibitor	Glu *S.griseus* protease , Subtilisin	Ogata et al., (1991)
	Subtilisin-chymotrypsin inhibitor CI-1A	Subtilisin , Chymotrypsin	Greagg et al., (1994)
	Wheat subtilisin/ chymotrypsin inhibitor	*B.lichenoformis* subtilisin, α-chymotrypsin	Poerio et al., (2003)
Mustard	Mustard trypsin inhibitor	β-trypsin	Menengatti et al., (1992)
	Mustard trypsin inhibitor-2	Bovine β-trypsin, α-chymotrypsin	Ceci et al., (1995)
	Rape seed trypsin inhibitor	Trypsin, Chymotrypsin	Ceciliani et al., (1994)
Cystatin	Onchocystatin	Cysteine proteinase	Lustigman et al., (1992)
	Ovocystatin	Thiol proteases	Laber et al., (1989)

	Oryzacystatin II	Cysteine proteinases	Ohtsubo et al., (2005)
Kininogen	Metalloprotease inhibitor	Atrolysin C, Jararhagin	Cornwall et al., (2003)
	Sarcocystatin	Cysteine proteinase	Saito et al., (1989)
Bowman-Birk	Bowman–Birk plant trypsin inhibitor unit I	Trypsin, Chymotrypsin	Odani and Ikenaka (1976)
	Bowman-Birk trypsin/chymotrypsin inhibitor	Trypsin, Chymotrypsin	Suzuki et al., (1987)
	Sunflower cyclic trypsin inhibitor	Trypsin, Cathepsin G, Elastase, Chymotrypsin and thrombin	Mulvenna et al., (2005)
Potato type II	Proteinase inhibitor II	Trypsin, Chymotrypsin	Greenblatt et al., (1989)
	Potato peptidase inhibitor II inhibitor unit 1	Trypsin, Chymotrypsin	Keil et al., (1986)
	Tomato peptidase inhibitor II inhibitor unit 1	Trypsin, Chymotrypsin	Graham et al., (1985)
	Tomato peptidase inhibitor II inhibitor unit 2	Trypsin, Chymotrypsin	Barrette-Ng et al., (2003)

An inhibitor domain is defined as the segment of the amino acid sequence containing a single reactive site after removal of any parts that are not directly involved in the inhibitor activity. Among the plant PI families, the Bowman-Birk (BBI) and Kunitz inhibitor families are the most widely distributed, and most abundant in the seeds of leguminous plants in addition to being the most intensively studied inhibitors.

Kunitz inhibitor family

The first plant PI to be isolated and characterized was the Kunitz trypsin inhibitor (KTI) found in the seeds of soybean (*Glycine max)* and is inhibitory to trypsin from a wide variety of sources. The inhibitors in this family are widespread in plants and have been described in legumes, cereals and in solanaceous species (Ishikawa et al., 1994; Laskowski and Kato, 1980). Soybean seeds generally have a higher content of Kunitz inhibitors than BBIs. KTIs are proteins with one or two polypeptide chains and low Cys content, generally with four Cys residues connected by two intra chain disulfide bridges, each comprising 170-180 amino acids and a single reactive site. The inhibitor is inactivated by heat and gastric juice. The purification, crystallization, kinetics of interaction and complex formation of KTI with trypsin (Kunitz, 1947a, 1947b) is a major landmark in the study of plant proteinases inhibitors. The studies on specificity, stability, physical, kinetic and other properties of KTI have been documented (Kassel 1970; Birk, 1976). KTI inactivates the anionic form of human trypsin, and therefore considered to be an antinutritional factor. The members of KTI family inhibit serine proteases, but may also inhibit other proteases (Ritonja et al., 1990; Laing and McManus, 2002). KTIs are also produced under stress, as has been reported in potato tubers (*S. tuberosum*) (Plunkett et al., 1982; Park et al., 2005; Ledoigt et al., 2006).

The literature on Bowman-Birk inhibitor (BBIs) will be reviewed in detail.

Bowman-Birk inhibitor (BBI)

The BBIs characteristically are single polypeptides with molecular masses in the range of 6-9 kDa and comprise of a binary arrangement of two subdomains with a conserved array of seven disulphide linkages that play a

vital role in stabilising the kinetically independent configuration of the reactive site on the outer most exposed loop and a well-characterized ability to inhibit trypsin and chymotrypsin. (Birk 1985; Ikenaka et al., 1986). BBIs, novel bicyclic and bifunctional inhibitors, have been extensively studied and their mechanism of inhibition very well established (Steiner, 1972).

Occurrence of the BBI

Sources

There are several sources of BBIs. BBIs are distributed among both dicots and monocots. Among dicots majority of the inhibitors are concentrated in legumes. The BBI is one of the major serine protease inhibitors commonly found in leguminous plants. Soybean BBI, isolated by Bowman (1946) and characterized by Birk (1961), and named after the two scientists, serves as a prototype for the BBI family (Birk, 1961, 1985, 1987, 1989). While the soybean BBI is the classical representative of the BBI family and has been extensively studied, related BBIs from other monocotyledonous and dicotyledonous seeds have been identified and characterized (Tashiro and Maki, 1979; Norioka et al., 1982; Tashiro et al., 1987; Weder and Haussner 1991; Prakash et al., 1994; De Freitas et al., 1997; Song et al., 1999; Rao et al., 1999; Luckett et al., 1999; Rakwal et al., 2001; Zablotna et al., 2002; Zhang et al., 2008; Sun et al., 2010; Fang et al., 2011; Rocco et al., 2011). BBIs are mainly found in the seeds of Fabaceae family of Leguminuous plants.

BBIs of Leguminosae

In Leguminosae, the suborders Caesalpinieae and Mimosaceae are considered to be primitive whereas Fabaceae is considered to have evolved. Tropical trees or shrubs belonging to Caesalpinieae and Mimosaceae contain

the KTI but temperate herbs of new tribes contain only BBI family. Herbs are thought to be more evolved than trees. The leguminous plants which had only the KTI, gradually acquired the BBI family during evolution from trees to herbs, as they adapted to temperate zone. This view was supported by the advanced biochemical nature of BBI family of inhibitors. Some of the tropical trees and shrubs have acquired the BBIs and this acquisition was more frequent in tropical herbs. The tendency to lose KTI and acquire BBI as the evolution of legumes proceeds was clearly observed in temperate plants, which had completely lost KTI and comprised of only BBIs (Konarev et al., 2002).

Legume seeds contain multiple BBIs differing in the number of amino acid residues and hydrophobicity (Wu and Whitaker, 1991). The isoinhibitors of these BBIs vary due to differences in genetic polymorphism or to post-translational modification (Quillien et al., 1997). In soybean, the soybean cultivar determines the number of BBI isoforms present with trypsin inhibitory activity (Gladysheva et al., 2000). BBI have been isolated from the tribe Vicieae, which includes adzuki beans (*Phaseolus angularis*), peanut (*Arachis hypogaea*), red kidney bean (*Phaseolus vulgaris*), brazilian pink bean (*Phaseolus vulgaris*), lima bean (*Phaseolus lunatus*), pigeon pea (*Cajanus cajan*, syn*. Cajanus indicus*), horsegram *(Dolichos biflorus*), field bean (*Dolichos lablab),* rice bean (*Vigna umbellata* T), *Lupinus albus,* redgram (*Cajanus cajan*), fava beans (*Vicia faba* cv. Egypt 1), grass pea (*Lathyrus sativus* L) and faba bean (*Vicia faba* cv. Giza 843) (Kiyohara et al., 1981; Norioka et al., 1982; Wu and Whitaker, 1991; Funk et al., 1993; Godebole et al., 1994; Sreerama et al., 1997; Devaraj et al., 1999; Maggo et al., 1999; Scarafoni et al., 2008; Prasad et al., 2010; Fang et al., 2010; Rocco et al., 2011 and Fang et al., 2011). The PIs from pea (*Pisum sativum)*, are similar in structure to the soybean BBI. The molecular mass being 6.8–7.9

kDa (Ferrasson et al., 1995), similar to that of BBI. However, inhibitors with a molecular mass up to 12 kDa in peas have been reported. cDNA (Domoney et al., 1993) and protein (Ferrasson et al., 1995) sequencing of inhibitors indicated that they belong to BBI family.

BBIs of Compositeae

Sunflower trypsin inhibitor-1 (SFTI-1)1 is one of the smallest, naturally occurring plant protein inhibitors reported to date, with a molecular mass of 1.513 kDa (Luckett et al., 1999). This peptide is shorter than other members of the BBI family, exhibits a novel cyclic structure, and has considerably enhanced potency relative to other peptides of similar length. SFTI-1 was partially characterized by classical techniques, and its structure confirmed. The crystal structure of this novel 14 amino acid cyclic peptide has been determined both in complex with trypsin (Luckett et al., 1999) and free in solution (Korsinczky et al., 2001). SFTI-1 is one of a number of head-to-tail cyclized peptides that have been discovered in recent years from bacteria, plants, and animals (Trabi and Craik, 2002), and there is increasing interest in backbone cyclization for the stabilization of peptides and proteins (Craik et al., 2002).

SFTI-1 appears to be a natural peptide mimic of BBI as it shares strong sequence and structural homology with the trypsin inhibitory loop of BBI family. Despite strong sequence and structural conservation, SFTI-1 has only been found in the seeds of sunflower, which, as a member of Compositae is phylogenetically removed from other plant families containing BBIs. Furthermore no other protein sharing similarities with BBI have been identified in Compositae, suggesting that SFTI-1 was restricted to a single, phylogenetically isolated species (Konarev et al., 2002).

BBIs of monocots

In contrast to BBIs from dicots, BBIs from monocots, including cereal grains, have received relatively little attention. 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 (Tashiro et al., 1987; Prakash et al., 1997). It has been suggested that larger inhibitors have arisen from smaller ones by gene duplication (Odani et al., 1986). The BBI from barley seeds consists of 125 amino acid residues with two separate inhibitory loops. It folds into two compact domains (N and C domain) with tertiary structures similar to that of the 8 kDa BBI from dicots (Park et al., 2004). The barley inhibitor structure consists of 11 beta-strands and the loops connecting these beta-strands but it lacks alpha-helices. The five disulfide bridges in each domain are a subset of the seven disulfide bridges in 8 kDa dicotyledonous BBIs (Song et al., 1999).The existence of only a single inhibitory loop within the 8 kDa inhibitors of BBIs from monocots attributed to the second inhibitory loop found in dicots becoming non-functional during evolution. Subsequent to this second loop becoming non-functional, 16 kDa inhibitors such as the BBI from barley may have evolved in monocots by gene duplication. It was therefore thought that the 16-kDa inhibitors evolved from the monocotyledonous 8-kDa single-headed inhibitors (Prakash et al., 1996). Qu et al., 2003 identified seven BBI genes from japonica rice (*Oryza sativa* subsp. *japonica* var Teqing). Four of the seven BBI genes have two repetitive cysteine-rich domains, whereas one has a truncated domain with only one reactive site. Rice BBI exhibited only trypsin inhibitory activity. The accumulation of rice BBI transcripts was differentially regulated in germinating embryos and also in the leaves, roots,

and flower organs at later developmental stages. No experimental data on the anti-carcinogenic activity of BBIs from monocots have been reported.

Biosynthesis of BBI

Hammond et al. (1984) isolated and analyzed the gene encoding soybean BBI and determined its nucleotide sequence by constructing cDNA clones from mRNA of developing soybean seeds. The mRNA for BBI was shown to accumulate early during embryogeny and to be about 450 nucleotides long. BBI genes have no introns. Galasso et al., (2009) constructed a BAC library from common bean in order to isolate the entire multigene BBI family and to study its genome organization. BBI was synthesized as a preinhibitor containing a sixteen residue extension at the N-terminus (Joudrier et al., 1987; Hilder et al., 1989; Domoney et al., 1995). The pro-BBI was converted to BBI following cleavage of a Ser-Asp bond. BBI was found in the intracellular spaces of cotyledons of ungerminated seeds and was rapidly released from the seed within the first eight hours of imbibition (Horisberger and Tacchini-Voulanthen, 1983). The studies on the germinated BBIs from horsegram revealed that germinated inhibitors are derived from the isoinhibitors of the dormant seed by a limited proteolysis and not by de novo synthesis. These inhibitors differ from each other at their N-terminus (Kumar et al., 2002). de Barros et al., (2011) characterized in silico and analyzed the expression of the members of BBI family in soybean. Eleven potential BBI genes were identified and each one of them contained at least one characteristic BBI conserved domain, in addition to a potential signal peptide. The expression profiles of the three genes identified are similar along seed development. Their expressions reached a maximum in the intermediate stages and decrease as the soybean seed matured.

Deshimaru et al., (2004) suggested that genes coding for BBIs of wild soja (wBBI) and soybean comprise a multigene family. cDNA sequences for wBBIs were highly homologous to those for respective soya bean homologs. Phylogenetic analysis of these cDNAs demonstrated the evolutional proximity between the two leguminae strains. The sequences of cDNA clones encoding a soybean BBI and its isoinhibitors (D-II and C-II) have been identified. Nucleotide sequence homologies among these clones and between the two reactive domains in each clone were very high. It was suggested that the high nucleotide sequence homologies among these clones and between the two reactive domains was indicative of an evolution from a common ancestral inhibitor (Baek et al., 1994).

Screening/Identification

The key characteristic features of BBI, i.e. low molecular weight (about 8.0 kDa) during electrophoresis, and its inhibition towards serine proteases like trypsin and chymotrypsin are used to identify BBIs. Monoclonal antibodies (mAb) against soybean BBI have been generated and used to detect and quantify BBI in foods, soybean germplasm, and animal tissues and fluids. Brandon et al. (1989, 1991) developed and patented monoclonal antibodies and immunoassay methods to detect BBI in processed foods. Arentoft et al. (1993) used high-performance capillary electrophoresis (HPCE) to characterize the association complexes between soybean and pea BBI and mAb against BBI. Polyclonal antibodies of low specificities against BBI have also been reported (Hwang et al., 1977; Sreerama and Gowda, 1997). Mao et al., (2005) developed monoclonal antibodies against soybean BBI which can recognize the protease-reactive loops. Monoclonal antibodies differentially reactive with native and

reductively modified BBI was developed which can be useful in detecting BBI metabolites in human body fluids (Wan et al., 1995).

Quantitative Assays

Immunoassays, specifically ELISA using polyclonal or monoclonal antibodies to BBI are ideal methods for BBI quantitative determination in a protein matrix (Brandon et al., 1989, 1991; Gladysheva et al., 2000). Wan et al., (2002) used an enzyme-linked immunosorbent assay was used to measure BBI concentrations in serum samples collected from human subjects and animals treated with BBIC. Gladysheva et al. (2000) raised antibodies against BBI polymerized with glutaraldehyde to quantitate BBI in soybean preparations. The lack of commercially available antibodies against BBI makes it difficult for investigators to measure BBI quantitatively in soybean products that claim the health enhancing properties of BBI.

Caesin was used as a substrate for measuring trypsin inhibitory activity of natural trypsin inhibitors (Kunitz 1947a). Most investigators have used active site titration with trypsin or chymotrypsin to determine BBI activity (Larionova et al., 1993; Ware et al., 1997). The assays for trypsin or chymotrypsin inhibition using chromogenic substrates, benzoyl-DL-arginine-p-nitroanilide (BAPNA) (Kakade et al., 1969) and benzoyl-L-tyrosine-p-nitroanilide (BTPNA) (Tan et al., 1984) are used commonly to determine the BBI. Esterolitic activity of trypsin and chymotrypsin are assayed by the method of Hummel (1959) using N-benzoyl-L-arginine ethyl ester (BAEE) and N-benzoyl-L-tyrosine ethyl ester (BTEE) as substrates. Yakoby and Raskin (2004) used a modified method for trypsin and chymotrypsin inhibition to evaluate the BBI levels using N-acetyl-DL-phenylalanine β-naphthylester (APNE) as substrate and o-dianisidine tetrazotized as the dye. Weder et al., (1993) developed fluorogenic substrates for the determination of trypsin

and chymotrypsin inhibitors. N α-benzyloxycarbonyl-L-arginine-4-methylcoumarinyl-7-amide (Z-Arg-MCA) and benzyloxycarbonyl-glycyl-glycyl-L-arginine-4-trifluoromethylcoumariny l-7-amide (Z-Gly-Gly-Arg-TFMCA) were found to be suitable for trypsin, and L-alanyl-L-alanyl-L-phenylalanine-4-methylcoumarinyl-7-amide (Ala-Ala-Phe-MCA) was suitable for chymotrypsin. Dark blue inhibitor bands on a light-blue-fluorescent background were obtained with Z-Arg-MCA/trypsin and Ala-Ala-Phe-MCA/chymotrypsin, whereas Z-Gly-Gly-Arg-TFMCA/trypsin resulted in dark inhibitor bands on a fluorescent green background. The rapid determination of BBI using perfusion RP-HPLC followed by UV detection has been developed recently (Anta et al., 2010). The expressed BBIs in transformants have been detected using GUS (GUS: β-glucuronidase) expression system and PCR analysis (Livingstone et al., 2007). RT-PCR (Real Time-PCR) is also used as a method to quantitate the expression of BBIs (de Barros et al., 2011).

Molecular properties

BBIs are double-headed inhibitors with a low molecular mass of 8–20 kDa and a high Cys content (Odani and Ikenaka, 1976), the cysteines being important in maintaining the active conformation of the inhibitor with the disulphide link between two cysteines holding the inhibitory loops of the inhibitor open, thus allowing the target enzyme to gain access (Prakash et al., 1996). Most BBIs exist in various isoforms. A particular interest is attached to BBI due to its unusual property of possessing strong inhibitory activity against two different proteolytic enzymes. The loops (or reactive sites) are known to inhibit trypsin and chymotrypsin in monocotyledonous plants and trypsin, chymotrypsin, and elastase in dicotyledonous plants and are able to form a 1:1:1 stoichiometric enzyme-inhibitor complex with trypsin and chymotrypsin. The two independent heads, located at opposite

sides of the molecule, are made of a tricyclic domain in which the binding site is located (Odani and Ikenaka 1973).

Three-dimensional structure of peanut, soybean, and barley BBIs (Song et al., 1999; Suzuki et al., 1987; Werner and Wemmer, 1992) revealed that disulphide bridges occur mostly between two conserved cysteine residues (e.g., C1–C14, C2–C6, C3–C13, C4–C5, C7–C9, C8–C12, C10–C11) in all three studied inhibitors. It makes them much conserved residues during evolution.The disulfide connectivity pattern in dicot and monocot BBIs are given in Figure 1.1.

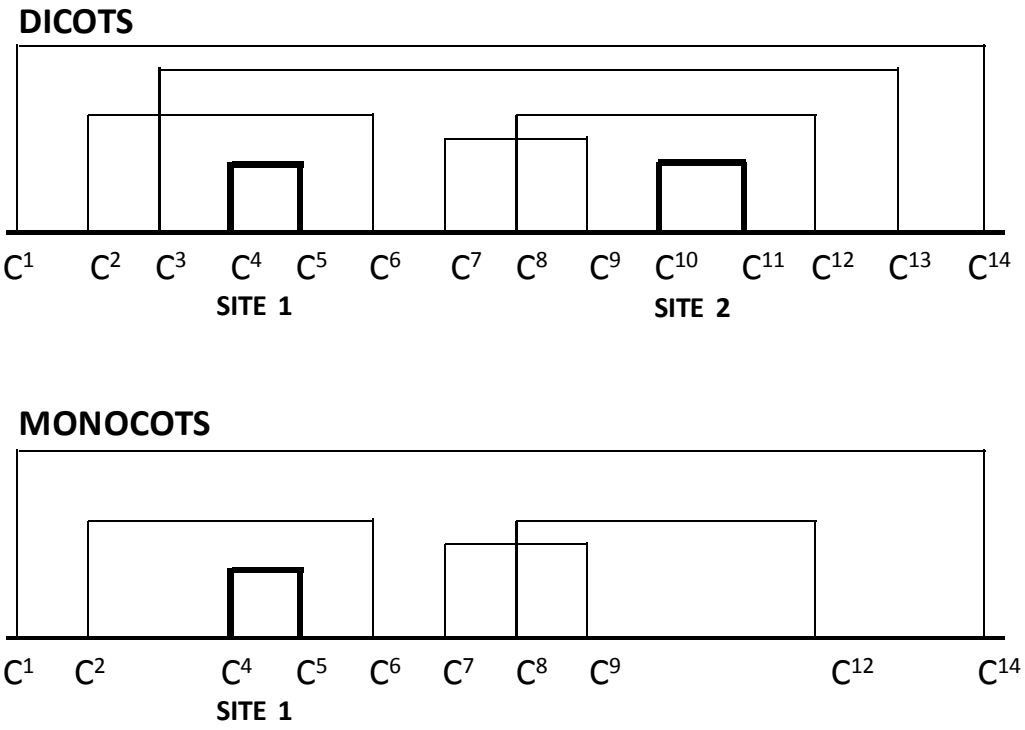


Fig. 1.1 **Disulfide connectivity in dicot and monocot BBIs** (The figure is reproduced with the kind permission of Prakash et al*.,* 1996).

Most of the legume seeds studied this far have several forms of BBIs, called isoinhibitors (Odani and Ikenaka, 1972). Many of them have been characterized with respect to their M_r, pI, reactivity and their partial and complete primary structure. Most of the isoforms have similar amino acid composition and primary sequences but exhibit heterogeneity at the N-and C-termini (Odani and Ikenaka, 1978a). The isoelectric point of BBI is pH 4.0 to pH 4.2. For various legumes, the pI values vary between pH 4.0 and pH 9.77 (Weder and Haussner, 1991). The variation was due to the difference in amino acid residues of various BBI. BBI is rich in cystine amino acid residues (about 20%). The covalent structure of BBI was elucidated by Odani and Ikenaka (1973).

Isolation and purification of BBIs

Bowman (1946) pioneered the isolation procedure of BBI while Birk and associates purified the protein (Birk, 1961; Birk et al., 1963). The method described by Bowman involved the precipitation of an acetone-insoluble powder. Defatted soybean was often the best starting material. Fratali and Steiner (1969) used ion exchange chromatography on DEAE cellulose to improve the isolation procedure. The method of Odani and Ikenaka (1977) remains the classical method of BBI isolation. This involves extraction of soybeans with 60% ethanol at room temperature followed by addition of two volumes of cold acetone, filtration, pH adjustment of the filtrate to pH 5.3, and acetone precipitation. The acetone precipitate was dissolved in water, dialyzed against distilled water and ion exchanged through 5 mM sodium acetate equilibrated CM-cellulose at pH 4.0. Elution of BBI was carried with a salt gradient from 0 M NaCl to 0.5 M NaCl. Additional purification using DEAE in ammonium acetate at pH 6.5 was required to produce a purified BBI. BBI obtained was homogenous but the

yield was very low. Yeboah et al., (1996) isolated BBI from defatted soy bean flour by extraction at acid pH, 40% ammonium sulfate precipitation, hydrophobic interaction chromatography, and gel filtration chromatography. Trypsin-sepharose affinity purification was used as a key step in the purification of BBIs (Swartz et al., 1977). Kumar et al., (2002) purified BBI from germinated horsegram seeds (HGGI) using size exclusion chromatography followed by ion exchange chromatography. Two BBI type trypsin inhibitors (CmTI1 and CmTI2) were purified from *Cratylia mollis* seeds by acetone precipitation, ion exchange, gel filtration and reverse-phase chromatography (Paiva et al., 2006). Cheung and Ng (2007) isolated a BBI from green lentil (*Lens culinaris*) using affinity chromatography on Affi-gel blue gel followed by ion exchange chromatography on Q-Sepharose, ion exchange chromatography by FPLC on MonoQ and Mono S. These steps were followed by gel fitration by FPLC on Superdex 75. A protease inhibitor (BgPI) was purified from black gram, *Vigna mungo* (cv. TAU-1) seeds by using ammonium sulfate fractionation, followed by ion-exchange, affinity and gel-filtration chromatography (Prasad et al., 2010 a). Fang et al., (2011) purified a 15 kDa BBI from the seeds of Faba bean (*Vicia faba* cv. Giza 843) using cation exchange chromatography on an SP-Sepharose column, anion exchange chromatography on Q-Sepharose and Mono Q columns, and finally size exclusion chromatography on a Superdex 75 column. Chitosan resin-trypsin was prepared for one-step affinity purification of BBI from a wild-type soybean (Zhang et al., 2011).

Inhibitory properties

BBI contain two active sites, which primarily inhibit the proteolytic enzymes, trypsin and chymotrypsin. Almost all serine inhibitor families from plants are competitive inhibitors and all of them inhibit proteinases with a

similar standard mechanism (Laskowski and Kato, 1980). SFTI-1, the BBI from sunflower inhibited β-trypsin with a subnanomolar K_i of 0.1 nM, and also inhibits cathepsin G. SFTI-1 has considerable selectivity; it proved to be 74-fold less inhibitory towards chymotrypsin, and was found to be 3 orders of magnitude less inhibitory towards elastase and thrombin. In contrast, it had no effect on Factor Xa (Luckett et al., 1999). The inhibitors isolated from horsegram seed and after germination are potent competitive inhibitors of trypsin and chymotrypsin (Sreerama et al., 1997; Kumar et al., 2002). Scarafoni et al., (2008) isolated BBI from *Lupinus albus* which was capable of inhibiting two molecules of trypsin simultaneously (K_i 4.2±0.4 nM) but not chymotrypsin. BBI isolated from red gram (RgPI) exhibited a noncompetitive type inhibitory activity against bovine pancreatic trypsin and chymotrypsin, with inhibition constants of 292 and 2265 nM (Prasad et al., 2010). The BBI isolated by Prasad et al., (2010 a) from black gram (BgPI) also exhibited noncompetitive mode of inhibition against bovine pancreatic trypsin and chymotrypsin. A 15 kDa rice BBI displays competitive inhibition towards trypsin with K_i of 4.0×10^{-7} M and non-competitive inhibition towards chymotrypsin with K_i of 9.3×10^{-6} M (Yan et al., 2009). BBIs isolated from *Lathyrus sativus* L., LSI-1/4 were active towards trypsin and α-chymotrypsin, with IC_{50} values for 12.6 nM for trypsin whereas a lower activity was observed against bovine α-chymotrypsin. Among these inhibitors, LSI-1 showed the presence of an Ala residue in the second reactive site, thus explaining the low anti-chymotrypsin activity and was endowed with anti-elastase activity, being able to inhibit human leukocyte elastase (Rocco et al., 2011). Fang et al., (2011) isolated a BBI from Faba bean which manifested significant antiproteolytic activity against trypsin (5761 BAEE units/mg, K_i 20.4×10^{-9} M), but only a slight chymotrypsin inhibitory activity (< 10 BTEE units/mg).

Chemistry and structure of BBIs

Primary, Secondary, and Tertiary structure

The primary structure of BBIs has been extensively characterized from various leguminous sources. The molecular weight of soybean BBI from the amino acid sequence of 71 residues is 7.975 kDa (Birk, 1985). BBI isolated from *Lupinus albus* was 63 amino acid long (Mr 6.858 kDa; pI 8.22) (Scarafoni et al., 2008). ES-MS of pea BBI isoinhibitors gave molecular values of 6.916, 6.807, 7.676, 7.844, 7.848, and 7.944 kDa (Ferrasson et al., 1995). Two BBIs (CmTI1 and CmTI2) were purified from *Cratylia mollis* seeds with 77 and 78 amino acid residues, respectively. Comparison of the molecular mass of CmTI (Mr 17 kDa determined by SDS–PAGE) with the molecular masses determined by MALDI-TOF of CmTI1 (8.5545 kDa) and CmTI2 (8.6244 kDa) (Paiva et al., 2006). Horsegram (*Dolichos biflorus*) inhibitor was 8.321 kDa by amino acid sequence analysis and 8.625 kDa by ES-MS (Prakash et al., 1996). The molecular weight determined by SDS-PAGE showed an anomalous migration (~ 16 kDa), in which the horsegram BBI existed as a dimer (Ramasarma et al., 1994; Sreerama et al., 1997). Molecular mass of AATI (*Apios americana* trypsin inhibitor) was 6.347 kDa by MALDI-TOF-MS (Zhang et al., 2008). Molecular weight obtained by MALDI-TOF-MS of soybean BBI was 9.857 kDa (Losso et al., 2004). The alignment of 35 domains of BBIs from both monocots and dicots is shown in Figure 1.2.

Stability and inhibitory activity of all members of BBI family has been attributed to the disulfide bridge and extensive hydrogen bond networks. The anti-trypsin and chymotrypsin canonical loops are contained within a nonapeptide region joined via a disulphide bond between flanking cysteine residues (McBride et al, 2002).

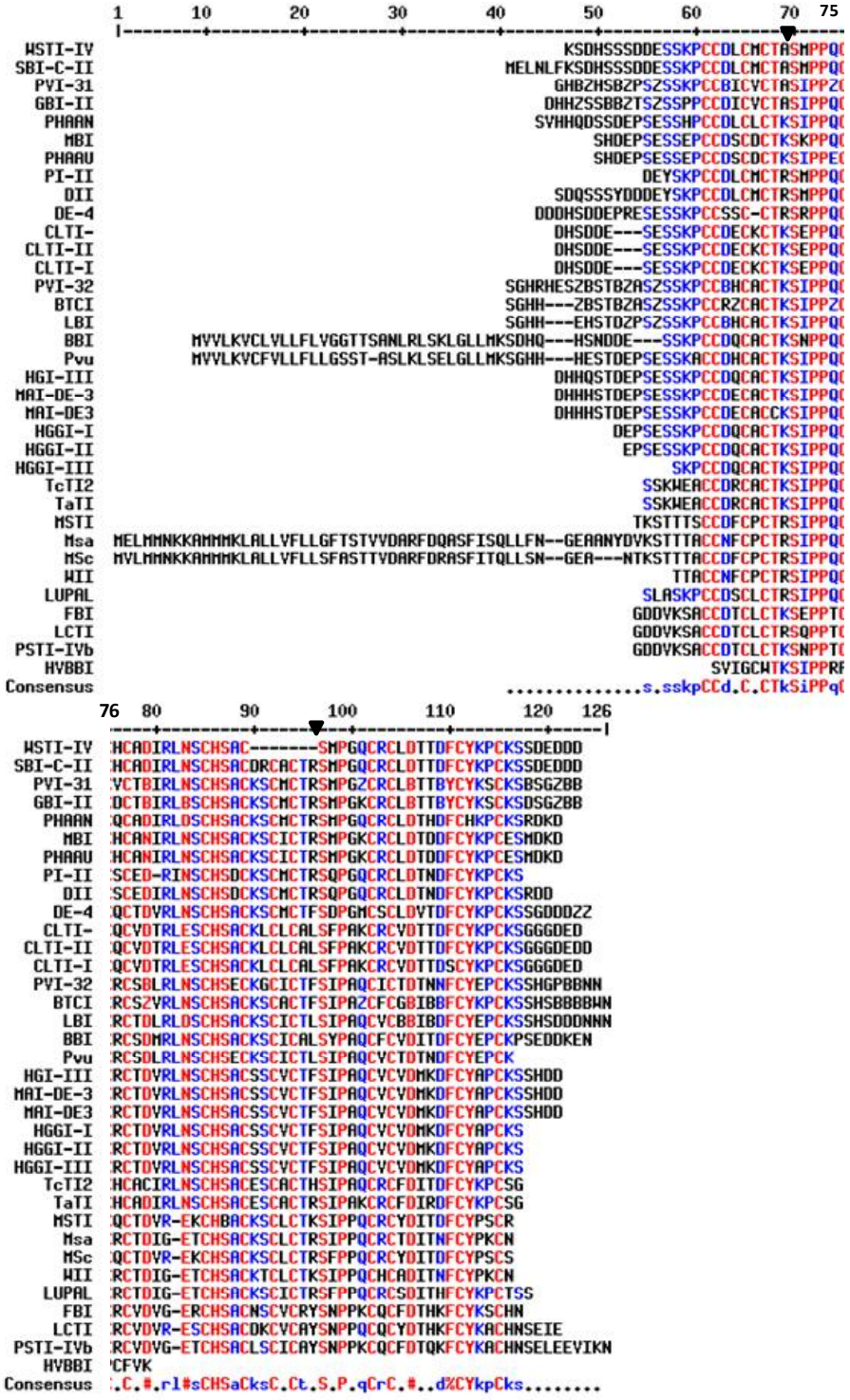


Fig 1.2 **Alignment of 35 Bowman-Birk inhibitor domains.** The arrowheads are between the PI and PI' residues of the reactive sites.

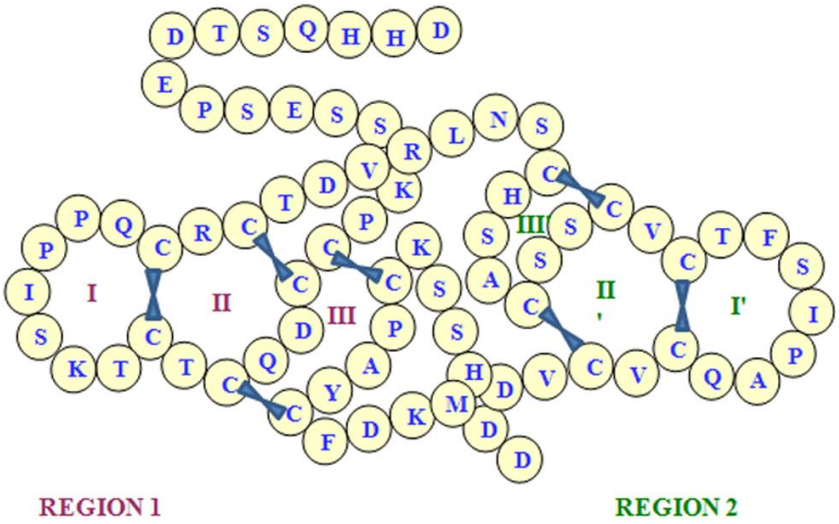


Fig. 1.3 **Schematic representation of the major BBI from horsegram which consists of two domains, each consisting of three peptide chain rings made by disulphide bridges.**

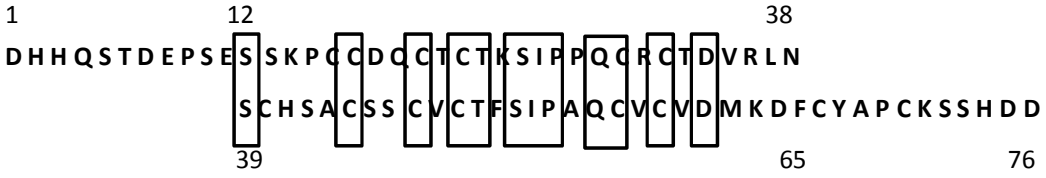


Fig. 1.4 **The internal homology in horsegram BBI, HGI-III.** The N-terminal and C-terminal regions are aligned and the identical amino acids residues are boxed.

All members of the BBI family have two tandem homology regions inhibiting trypsin and chymotrypsin on the same polypeptide chain, each comprising a consensus motif of three β-strands with a kinetically independent reactive site on the outermost loop (Ikenaka and Norioka, 1986). The schematic representation of horsegram BBI (HGI-III) is given in Figure 1.3. and the internal homology of HGI-III is depicted in Figure 1.4.

The secondary structures of BBIs have been reported by a number of investigators. Steiner and Frattali (1969) concluded from circular dichroism study that BBI had low α-helical content. Ikeda et al. (1968) found that BBI was non-helical from circular dichroism and optical rotatory dispersion study. Kay (1976) reported the circular dichroism spectrum of BBI without any structural calculation such as α-helix. Bewley and Birk (1978) found that BBI did not contain appreciable amounts of α-helix from its circular dichroism spectrum. Secondary structure was dominantly aperiodic with relatively small degree of ordered structures in the form of two regions of antiparallel β-sheets one in each of the subdomains. Werner and Wemmer (1991, 1992) used two-dimensional proton NMR spectroscopy to determine the three-dimensional structure of BBI and found that residues Q^{11}-T^{15} form an antiparallel, β-sheet with residues Q^{21}-S^{25} in the tryptic inhibitory domain and an analogous region of antiparallel, β-sheet between residues S^{38}-A^{42} and Q^{48}-V^{52} in the chymotrypic inhibitory domain. The structure of SFTI-1 consists of two β-strands connected at each end by turns and was braced by a single disulfide bond that creates two distinct loops. One loop, known as the binding loop, contains the reactive site sequence Lys^{5}-Ser^{6}, while the "secondary" loop contains a β-hairpin turn that essentially cyclizes the binding loop (Luckett et al., 1999; Korsinczky et al., 2001). The absence of α-helix conformation appears to be a characteristic of BBI and analogs. The studies on the inhibitory activity and conformation changes of soybean trypsin inhibitors induced by ultrasound revealed that BBI was more stable than Kunitz type of inhibitors because of its stable secondary structure (Huang et al., 2008). The reduction of the disulfide linkages with DTT of horsegram BBI affected the tertiary structure significantly and secondary structure was not affected considerably (Ramasarma et al., 1995).

The tertiary structure of BBI has been studied in considerable details using X-ray crystallography and NMR (Zhu et al., 2001; Chen et al., 1992; Werner and Wemmer, 1991). The structure has two domains namely the antitryptic and the anti-chymotryptic domain, respectively (Werner and Wemmer, 1991). X-ray crystallography revealed that the inhibitory domains of BBI are exposed and easily accessible to proteolytic enzymes. The distribution of polar and non-polar residues throughout the BBI molecules and the distribution of chymotrypsin inhibitory sites are highly conserved. X-ray crystal structure of BBI revealed exposed hydrophobic patches and a polar domain interface solvated by internal water molecules (Philipp et al., 1998). As a result of exposed hydrophobic patches and buried charged residues and water molecules, BBI has a pronounced tendency to self-associate (Voss et al., 1996).

Apart from soybean BBI, the internal water molecules were found in pea, peanut and mungbean BBIs, indicating that they are an invariant part of the tertiary structures of these inhibitors. The most significant feature of BBIs was the absence of a 'hydrophobic core', which was characteristically present in majority of the globular proteins and acts as a major force in folding and stability (Dill, 1990). These structural peculiarities in BBIs resulted in a 'constrained' conformation held together to a large extent by covalent 'locks' in the form of the conserved disulfide bonds (Fig. 1.1). In the absence of a 'hydrophobic core' and elaborate secondary structural elements, the remarkable stability exhibited by the BBIs was by virtue of the conserved disulfide bonds. BBIs are good models for folding studies specifically to deduce the efficiency of folding in the absence of the 'hydrophobic effect' and the importance of the disulfide bond formation and breakage during folding (Dill et al., 1995; Singh & Rao, 2002).

In addition to the disulfide bonds, the comparison of the reported amino acid sequences of BBIs from legumes indicated the presence of a minimum of four and some as high as seven Pro residues (Prakash et al., 1996). The cis-prolines in the reactive site loops were conserved in most of the BBIs from legumes. Refolding was done in the presence of peptidyl-prolyl cis–trans isomerase (PPI), which is known to catalyze the cis–trans isomerization of the prolyl imidic peptide bonds (Singh and Rao, 2002).

Crystal structure proposed by Koepke et al., (2000) revealed that polar residues, hydrophilic bridges and weak hydrophobic contacts were predominant in subdomain 1, interacting specifically with trypsin. However, close hydrophobic contacts across the interface were characteristic of subdomain 2 reacting with both trypsin and chymotrypsin.

The two homologous regions of the BBI structure form the so-called "Bow tie" motif (Chen et al., 1992) and are made up of four antiparallel β-strands and four connecting loops with N and C-terminal segments folded in an extended conformation. The structure of the ternary complex of the BBI purified from snail medic (*Medicago scutellata*) seeds (MSTI) and two molecules of bovine trypsin has been solved by X-ray diffraction (Capaldi et al., 2007). The two binding loops of the MSTI differ in only one amino acid and have in both cases an arginine in position P1. When compared to the NMR model of the uncomplexed MSTI, the inhibitor in the functional assembly with trypsin shows the largest differences in the two P2`residues. Compared with the similar ternary complex of the soybean trypsin inhibitor, this model shows very small differences in the polypeptide chain of the trypsin binding sites and its largest difference in the area between Asp ^{26} and His ^{32} of the MSTI which in the soybean inhibitor has an extra Leu inserted in position 29 (Capaldi et al., 2007). Catalano et al., (2003) determined the high-resolution three-dimensional structure MSTI in

solution by ^{1}H NMR spectroscopy. The structure of MSTI comprises two distinct symmetric domains each composed of a three-stranded β-sheet containing a VIb type loop, where the active sites are located. The two active domains exhibit different conformational features. A higher flexibility and hydrophobicity is displayed by the second domain in comparison to the first domain, and these properties have been correlated to a lower trypsin inhibitory specificity in the case of MSTI.

NMR structure of lentil BBI (LCTI) exhibited a canonical BBI conformation with two antipodal β-hairpins containing the inhibitory domains. The ion pairs and hydrogen bonds stabilized the tertiary structure which involves the side chain and backbone of Asp^{10}-Asp^{26}-Arg^{28} and Asp^{36}-Asp^{52} residues (Ragg et al., 2006). Barbosa et al., (2007) studied the structure of BBI from *Vigna unguiculata* seeds (BTCI) in complex with β-trypsin. The studies pointed that the trypsin inhibitory domain of the reactive loop has conformational rigidity while antichymotryptic subdomain has hydrophobicity and conformational mobility which was responsible for the self-association tendency.

Homology

Naturally occurring BBIs constitute a major PI family in cereal and legume seeds. These proteins have the capacity to inhibit one or more of a range of serine proteases, including the digestive enzymes trypsin and chymotrypsin. BBIs interact with the active sites of serine proteases in a 'canonical', i.e. substrate-like, manner via exposed reactive site loops of conserved conformation within the inhibitor. Multiple BBI variants can be found within and among species. Four double headed BBI type inhibitors were isolated from peanuts by Norioka et al., (1983). All five isotypes showed two reactive sites for trypsin with different K_i and one reactive site

for chymotrypsin. Unlike the soybean BBI, peanut BBI type showed weak inhibitory activity towards chymotrypsin and a preferential inhibitory activity against trypsin. The primary structures of 23 BBI sequences, isolated from genomic DNA of eight Italian landraces of common bean *(Phaseolus vulgaris* L.) were studied. The similarity among the analysed sequences ranged from 92 to 99%. The sequences were highly similar to common bean (Piergiovanni and Galasso, 2004). Rice bran contained BBI analog which showed strong inhibitory activity against trypsin and weak against chymotrypsin (Tashiro and Maki, 1979). Wheat germ trypsin inhibitors I (MW = 14.5 kDa) and II (MW = 7 kDa) were highly homologous to BBI of leguminous plants (Odani et al., 1986). Lima bean BBI showed 70% sequence homology to BBI (Prakash et al., 1996). BBI and lima bean BBI shared several similar chemical and physical properties (Seidl and Leiner, 1971). BBI isolated from *Lupinus albus* showed homology to other BBIs. The 14 cysteine residues were in the conserved position previously described for other BBIs (Qi et al., 2005) and the residue at the P1 position of the N-terminally located reactive site was arginine (Arg15), conferring the specificity for the trypsin inhibition (Scarafoni et al., 2008). A 15 kDa BBI from faba beans (*Vicia faba cv*. Egypt 1) exhibited substantial homology in N-terminal amino acid sequence to other protease inhibitors and showed antiproteolytic activity against trypsin but hardly any activity against chymotrypsin (Fang et al., 2010).

The 14 ½ cystine residues were common and were conserved in all the BBIs which are responsible for maintaining an active conformation forming a net work of seven disulfide bridges. In addition to an array of seven disulfide bonds, Pro (P_3'), Pro (P_4') at the tryptic reactive domain, Pro (P_3'), Ser (P_1') at chymotrypsin reactive site (Prakash *et al.*, 1996) were the common amino acids to all inhibitors.

Heterogeneity and isoforms of BBI

BBIs, the products of multi-gene families and the derivation of multiple isoforms has been associated with protein processing at both the N- and C-terminal ends (Domoney et al., 1993, 1995). The existence of a large number of BBI isoinhibitors are reported to be due to the presence of multiple genes (Kalume et al., 1995). A multitude of BBI isoforms have been reported to exist in a single soybean cultivar. A cultivar of soybean has upto twelve isoinhibitors (Odani and Ikenaka, 1977; Hwang et al., 1977; Tan-Wilson et al., 1985; Larionova et al., 1993; Gladysheva et al., 2000). Four isoforms differing in N- and C- terminal ends were reported in horsegram seeds (Sreerama et al., 1997). The germinated seeds shared three isoinhibitors varying in electrophoretic mobility. These inhibitors were derived from the isoinhibitors of the dormant seed by a limited proteolysis and not by de novo synthesis (Kumar et al., 2002). Trypsin inhibitors from winter pea seeds consist of six protease inhibitors (PSTI I, II, III, IVa, IVb, and V) which all belong to the BBI family of inhibitors (Quillien et al., 1997; Ferrasson et al., 1995). Clemente et al., (2004) studied the effect of variation within inhibitory domains on the activity of pea protease inhibitors from the BBI family. Limited post translational processing had occurred at the N-terminal ends, which were responsible for the variation of the proteins.

Most of the inhibitors characterized are different from each other either at their N- or C-terminus. The existence of several BBI analogs across the plant kingdom brings about several differences in terms of amino acid sequence and amino acid residues at active sites of different BBI analogs although in most cases the reactive site loop residues are conserved.

Two BBI isoforms were isolated from tepary bean *(Phaseolus acutifolius* G.) seed proteins. In many isoforms several regions with amino acid microheterogeneity are found, corroborating the possible presence of

isoforms. This inhibitor existed in dimer and trimer forms and had an unusual character, the ability to bind metals (Campos et al., 2004).

Four BBIs, named LSI-1/4, were isolated and purified from *Lathyrus sativus* L. seeds. N-terminal sequences (up to 30 residues) of LSI-1/4 inhibitors were identical with the exception of sequence positions 21, 27 and 28 and highly similar to those of other BBIs isolated from Leguminosae plants. Inhibitors LSI-1/4 were active against trypsin and α-chymotrypsin. LSI-1 sequence showed the presence of an Ala residue in the second reactive site, thus explaining the low anti-chymotrypsin activity of this inhibitor. In addition, LSI-1 was endowed with anti-elastase activity, being able to inhibit human leukocyte elastase (Rocco et al., 2011).

Oligomeric status of BBIs

Another captivating and unusual behaviour of BBIs is self-association under native conditions. Most of the BBIs of legumes are single polypeptides of molecular weight 6-9 kDa. However, SDS-PAGE and analytical gel filtration chromatography indicate their molecular weight to be 16-18 kDa, suggesting that they existed as dimers in solution. BBI is a mixture of monomer, dimer, trimer, and /or tetramers, a property which was concentration-dependent as a result of self-association (Birk, 1985). Self-association of BBI monomers into dimers and trimers have also been reported with lima and kidney beans (Haynes and Feeney, 1967; Pusztai, 1968). The self association of BBI by membrane osmometry was established and the association was found to be the monomer-dimer type (Harry et al., 1969). Self-association of BBI to form homodimers or trimers or more complex oligomers in solution were also reported by others (Odani and Ikenaka, 1978 a and Silva et al., 2005). The three-dimensional model of black-eyed pea BBI-chymotrypsin complex (de Freitas et al., 1997) and light scattering data (Ventura et al., 1981) suggested

that the inhibitor molecules were in continuous equilibrium between monomers and several forms of multimers. Terada *et al., (*1994a) observed that the molecular mass of BBIs from *Canavalia lineata* were greater than expected when estimated by the method of Laemmli (1970).

Sierra *et al*., (1999) demonstrated that the C-terminal tail of PsTI-IVb, the BBI of winter pea seeds made no contact with its own subunit yet was held by interactions with the other subunit. These included (a) a hydrogen bond between the guanidium group of Arg^{23} of one subunit and the polar group of the side chain of Glu^{68} and (b) an ion-pair between Lys^{16} of one subunit and carboxyl group of Glu^{69} of the other subunit.

The two-dimensional ^{1}H NMR structure revealed of soybean BBI the antichymotryptic domain at the C-terminus was fully exposed and presumed to be the location of the self-association surface (Werner and Wemmer, 1992). Catalano et al., (2003) showed that BBI from *Medicago scutellata* (MSTI) undergoes self-association and the residues involved in this mechanism are localized at opposite faces of the molecule, having the highest positive and negative potential, respectively, thus indicating that electrostatic intermolecular interactions were the driving forces for self association.

In the NMR structure of lentil (*Lens culinaris, L)* BBI at physiological pH, there exists an asymmetric distribution of opposite charges with a negative electrostatic potential centred on the C-terminus, and a highly positive potential surrounding the antitryptic domain (Ragg et al., 2006). In WSTI-IV, an elastase inhibitor of Wild Soja contained an alanine at the first reactive site and was of a monomer clearly indicating a role for the first reactive site in oligomerisation (Deshimaru et al., 2003).

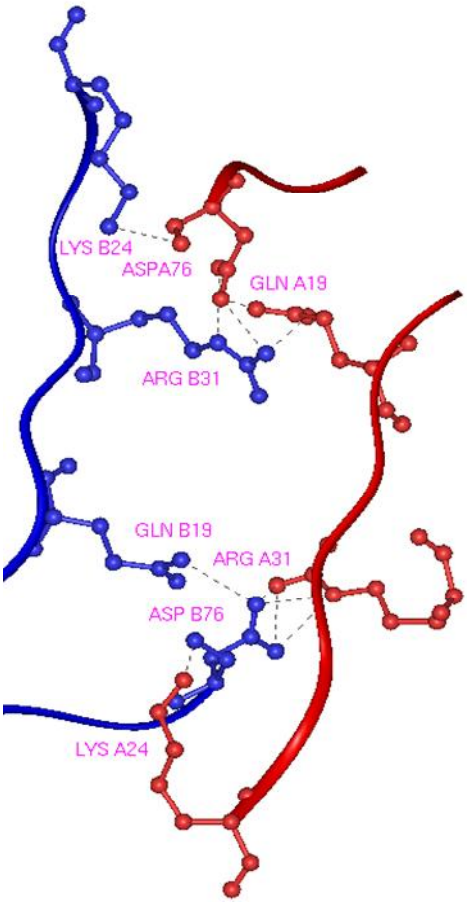


Fig. 1.5 **Interactions at the dimer interface in HGI-III responsible for self association.** (The figure is reproduced with the kind permission of Kumar *et al.*, 2004)

BBI purified from redgram (*Cajanus cajan*) seeds exhibited two protein bands with molecular masses of ~ 8.5 and ~ 16.5 kDa under nonreducing condition corresponding to the monomeric and dimeric forms. MALDI-ToF mass spectrometry confirmed the presence of dimer as well as other oligomeric forms: trimer, tetramer, and pentamer (Prasad et al., 2010).

The BBIs of horsegram exhibit molecular masses of ~16.0 kDa by SDS-PAGE and analytical gel filtration, although ESMS analysis and amino acid analysis revealed a mass of ~ 8.0 kDa (Sreerama *et al.,* 1997). In contrast, the

inhibitors of the germinated horsegram seeds (HGGI) were single polypeptides of 6.5-7.2 kDa and therefore existed as monomers. Kumar et al., (2004) from their studies conclude that interactions involving the C-terminus played a unique role in dimerization of BBIs. The involvement of C-terminal end or subdomain II in the oligomerisation was also demonstrated for other BBIs (Werner et al., 1992, Barbosa et al., 2007, Voss et al., 1996, Catalano et al., 2003 and Koepke et al, 2000). Kumar et al., (2004) using techniques like chemical modification and homology modelling demonstrated that an interaction between Lys^{24} and C-terminal Asp^{76} reckon the dimerisation (Figure 1.5).

Reactive site peptide bond of BBI

The sequence of the reactive site and the identity of P1 residue (Schechter and Berger, 1967) of the BBI inhibitors reflect the substrate preference or enzyme specificity. Classical soybean BBI simultaneously inhibits trypsin via one reactive site, Lys^{16}-Ser^{17}, and α chymotrypsin via another, Leu^{43}-Ser^{44}. The amino acids present in the primary contact region of the inhibitor with the enzyme were usually labelled P1, P2 and P3 on one side of the scissile bond of the inhibitor and P1`, P2`and P3`on the other. The P1 residue of the inhibitor and the corresponding S1 enzyme pocket together with few adjacent residues are primarily responsible for the recognition phenomenon. The presence of an Arg or Lys at P1 recognized the active site of trypsin; the presence of a hydrophobic residue, Leu, Phe, Trp or Tyr that of chymotrypsin while the presence of an Ala was selective for the active site of elastase (Table 1.2).

Comparisons among BBIs from different species showed that the first N-terminally located reactive site was more conserved than the second C-terminally located one: Lys is normally the P1 residue of the first site,

whereas at the second P1 site, there may be amino acids of different structures present (Tanaka et al., 1997). Alignment analysis showed that the PI` amino acid of these inhibitors is usually Ser (Wu and Whitaker, 1991 and Prakash et al., 1996). The P1 residue of BBI results in large changes in specificity. At the P2 position Thr was found to be optimal (McBride et al., 1998) and P2` position is usually confined to isoleucine (Gariani et al., 1999).

The conformation of the canonical loop required to bind the target enzyme is stabilised by several intra-molecular hydrogen bonds. Nishino et al., (1977) showed that synthetic cyclic peptides having the nonapeptide motif retain inhibitory activity and structural features of the full-length BBI protein. Multiplicity has been generated by incorporating all the natural amino acids in some positions of binding loop. By screening these libraries, the active peptides were identified and the inhibitory activity was evaluated in relation to the residues at the positions P2, P1, P2` and P3`. In this way, the best residue for each position was identified and it was estimated how much of the substitution reduced the inhibitory activity. These studies also showed that the susceptibility of peptides to hydrolysis were sequence dependent and did not necessarily correlate with inhibitory activity (McBride et al., 2002).

The *cis-trans* geometry at P_3'-P_4' position was examined (Brauer *et al*., 2002) by inhibitory activity and the structure for a series of synthetic fragments indicated that peptides having Pro at P_3' were potent inhibitors and a *cis* peptide bond at this position was necessary for biological activity. The P_4' Pro even though not essential for activity, effectively stabilized the *cis* conformation at P_3' by suppressing alternative conformations. Studies on synthetic BBI variants also show that Phe is the optimal P1 residue and that the chymotrypsin inhibition decrease following the series Phe > Trp > Leu > Met > Val > Ala > dTrp > Gly > desLeu (Odani and Ono, 1980).

Table 1.2 **Residues P1 and P`1 and the reactive peptide bond of a few BBIs and analogs** (Adapted from Losso, 2008)

Source	Active site	
	Trypsin	**Chymotrypsin**
Barley *(Hordeum Vulgare)*	$R-^{17}S-^{18}$	$R-^{75}S-^{76}$
Rice *(Oryza sativa)*	$K^{76}-T^{77}$	No inhibition
Peanut A-II *(Arachis hypogaea)*	$R^{19}-S^{20}$	$R^{47}-S^{48}$
Job's Tears(*Coix lachryma jobi*)	$R^{17}-S^{18}$	No inhibition
Azuki bean (*Phaseolus angularis)*	$K^{26}-K^{27}$	$Y^{53}-S^{54}$
Soybean BBI precursor (*Glycine max)*	$K^{55}-S^{56}$	$L^{82}-S^{83}$
Wheat (*Triticum aestivum)*	$R^{17}-S^{18}$	$V^{43}-G^{43}$
Azuki bean I-A (*Phaseolus angularis)*	$K^{26}-S^{27}$	$R^{53}-S^{54}$
Peanut B-II (*Arachis hypogaea)*	$R^{13}-R^{14}$	$R^{41}-S^{42}$
Kidney bean*(Phaseolus vulgaris)*	$A^{25}-S^{26}$	$R^{52}-S^{53}$
Foxtail millet FMTI-II (*Setaria italica)*	$K^{16}-S^{17}$	No inhibition
Wheat II-4 (*Triticum aestivum)*	$K^{15}-S^{16}$	$T^{41}-S^{42}$
P.V.Kidney bean (French bean) PVI-3 *(Phaseolus vulgaris)*	$K^{29}-S^{30}$	$F^{56}-S^{57}$
Foxtail millet FMTI-III (*Setaria italica)*	$K^{16}-S^{17}$	No inhibition
Soybean-D II (precursor) (*Glycine max)*	$S^{32}-M^{33}$	No inhibition
Kidney bean PVI-4 *(Phaseolus vulgaris)*	$K^{30}-S^{31}$	$F^{57}-S^{58}$
Mung bean (*Vigna mungo)*	$K^{20}-S^{21}$	$R^{47}-S^{48}$
Lima bean (*Phaseolus lunatus)*	$K^{26}-S^{27}$	$L^{53}-S^{54}$
Cow pea (*Vigna unguiculata)*	$K^{26}-S^{27}$	$F^{53}-S^{54}$
Broad bean *(Vicia fava)*	$K^{16}-S^{17}$	$Y^{42}-S^{43}$
Horsegram *(Dolichos biflorus)*	$K^{24}-S^{25}$	$F^{51}-S^{52}$
Sunflower *(Helianthus annum)*	$K^{5}-S^{6}$	No inhibition

Whilst the dual headed BBI proteins are able to inhibit two proteases at the separate reactive sites, there are exceptions and measured alteration in the relative affinity when one site is already occupied. Peanut inhibitor was found one such BBI to be devoid of antichymotrypsin activity when pre-complexed with trypsin and vice versa (Tur Sinal et al., 1972).

Norioka and Ikenaka (1983) classified the legume BBIs into four groups based on the amino acid residues around the putative reactive sites. Sequences around the reactive sites of the inhibitors in each group were very well conserved. Group I has -T-X-S-X-P-P- as -P_2'P_1'$P_1P_2P_3P_4$- residues at the first reactive site whereas -T-R-S-X-P-G- consists the second reactive site. Group II consists of -T-L-S-X-P-P- at first reactive site and -T(A)-X-S-X-P-A for the second reactive site. For the group III BBIs the first reactive site has residues -T-X-S-X-P-P- and -X-X-S-X-P-P- at the second reactive site. Group IV consists of -D-R-R-A-P-P- and -T-R-S-X-P-P- respectively for the first and second reactive sites. X can be any aminoacid among the twenty.

Chemical modification

Guanidination of BBI does not affect the inhibitory activity. β-Mercaptoethanol reduces BBI and performic acid oxidizes BBI and these reagents abolish BBI inhibitory activity against both trypsin and chymotrypsin (Birk, 1976). CNBr treatment cleaved BBI into two active fragments (Birk et al., 1986). A modified BBI retained the capacity for simultaneous binding of trypsin and human leukocyte elastase (Larionova et al., 1995). A nanopeptide containing amino acid residues 41–49 has a significantly reduced antichymotryptic activity compared to the entire BBI molecule (Dittmann et al., 2001). A $Ser^{44}\rightarrow Gly^{44}$ mutation using limited enzymatic hydrolysis abolished the reactivity of BBI at the chymotrypsin inhibitory site (Odani and Ikenaka, 1978 b). Enzymatic modification of P`1

Arg residues in the tryspin subdomain active site of peanut BBI (Arg^{16}-Arg^{17}) to citrulline was associated with the disappearance of BBI trypsin-inhibitory activity (Kurokawa et al., 1987). The deiminated BBI preserved its chymotrypsin inhibitory activity. The anti-chymotryptic activity of lima beans was lost when lima bean BBI was incubated with trypsin at pH 3.5 as a result of the cleavage of a single leucyl-serine bond (Krahn and Stevens, 1970). Pancreatic elastase hydrolyzes the Leu^{43}-Ser^{44} bond and abolishes most of the chymotrypsin inhibitory activity (Getler and Birk, 1970). Epitope mapping of horsegram BBI, HGI-III was performed using carboxamidomethylated-HGI-III or pyridylethylated HGI-III by digestion with TPCK-trypsin, lysyl endoproteinase C and cyanogen bromide (Sreerama and Gowda, 1997).

Enzymatic fragmentation

The realisation that classical BBI could form a 1:1:1 complex with two separate proteinases led early workers to determine if they could dissect these two independent activities. Hogle and Liener (1973) first demonstrated that activity was dependent upon the disulphide content. Controlled reduction resulted in complete loss of activity when an average of 4 bonds was broken (Gariani, et al., 1997). The chemical and enzymatic scission of BBI, the divalent protease inhibitor into two active fragments showed the anti-chymotryptic activity of the resulting fragment was decreased by 60% of the original inhibitor, but the anti- tryptic activity remained unchanged Odani and Ikenaka (1973). The chymotrypsin inhibitory fragment of the BBI from Adzuki bean subjected to peptic digestion remained active unlike the fragments obtained from soybean BBI. The trypsin inhibitory fragment had a lower antitryptic activity than the

original inhibitor and was gradually inactivated by trypsin (Yoshikawa, et al., 1980).

The fragments obtained after the peptic cleavage in *Macrotyloma axillare* BBI were not as active as the intact inhibitor. The trypsin inhibitory fragment retained 63% of its activity whereas the chymotryptic inhibitory fragment had lost almost complete activity. The reconstituted fragment mixtures showed enhanced activity therefore Townshend et al., (1982) proposed that noncovalent interdomain interactions enhance the inhibitory efficiency.

Stability of BBIs

BBIs are stable at cooking temperatures and also towards the acidic pH found in the digestive systems of humans and animals, most probably due to their large number of conserved disulfide bonds. BBIs exhibited extreme resistance to various proteolytic enzymes (including pepsin and pronase) and stability at acidic and alkaline pH and high temperatures. The major BBI from horsegram, HGI-III, also exhibited extreme thermal stability up to 6 h after heating at 90 ^{o}C. Although the stability of the germinated horsegram BBI (HGGI-III) was comparable to HGI-III up to 3 h, HGGI-III lost 50% of its activity after 6 h incubation at 95 °C. The decrease in the thermal stability led to the suggestion that the dimeric form of the inhibitor is more stable than the monomer (Kumar et al., 2004). Marin-Manzano et al., (2009) showed that BBIs survive faecal fermentation in their active form and do not affect the microbiota composition in vitro. This pointed towards the extreme stability of BBI towards various proteolytic enzymes. *Apios Americana* trypsin inhibitor (AATI) belonging to BBI family retained the inhibitory activity even after heating at and incubating at a wide range of pH indicating that they have extreme heat stability and pH stability (Zhang et al.

2008). A redgram proteinase inhibitor exhibited noncompetitive type inhibitory activity against bovine pancreatic trypsin and chymotrypsin. It was stable up to a temperature of 80 ^{o} C and was active over a wide pH range between 2 and 12. It also decreased the activity of larval midgut trypsin-like proteinases in *Manduca sexta* (Prasad et al., 2010). Dietary BBI has been demonstrated to slow growth and increased insect mortality in the case of drosophila by inhibiting the digestive enzymes trypsin and chymotrypsin, resulting in a reduced supply of amino acids. Dietary BBI affected energy utilization pathways in the midgut cells of *D. melanogaster* (Li et al., 2010). An ELISA method was used to measure the urinary levels of BBI metabolites in nine human subjects after consumption of 36-oz or 60-oz soymilk (containing 105 or 175 mg of BBI) at two time points 36 h apart. The results demonstrated that urinary BBI excretion rates peaked within 6 h and decreased to baseline levels within 12-24 h after soymilk ingestion. These data suggested that BBI ingested was absorbed and could be bioavailable for cancer chemoprevention in other organs in addition to those in the gastrointestinal tract (Wan et al., 2000).

Standard Mechanism of action of BBIs

The only explicit molecular mechanism which has thus far been proposed was that of Laskowski and co-workers (Laskowski & Sealock, 1971, Finkenstadt & Laskowski, 1967). According to this model, the formation of the stable complex involved the splitting of a susceptible peptide bond in the inhibitor and the formation of an acyl bond between the new *α*-carboxyl and the serine component of the catalytic site of the enzyme. The reaction of trypsin with BBI is biphasic, an initial second-order combination being followed by a slower first-order change. The rate constant for dissociation of the complex increased at acid pH. This dissociation has high activation

energy. BBI, which has been converted to the modified form by prolonged incubation with catalytic amounts of trypsin, forms a complex with trypsin, which has the same rate constant and activation energy for dissociation as the complex formed by the native inhibitor. The second-order kinetics of combination observed at extreme dilutions can be reconciled with the first-order process found at high concentrations, if it is postulated that the initial loose complex undergoes a subsequent intramolecular rearrangement to form the stable species.

The simplest model for the complete process has the form:

$$T + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C' \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C$$

where T, I, C', and C represent trypsin, inhibitor, initial complex, and final complex, respectively. The slow unimolecular step might be associated with the formation of an acyl bond.

The interaction of inhibitor with enzyme has been very extensively studied. In the BBI-trypsin inhibition complex, the most important interactions are salt bridges and hydrogen bonds, whereas in the BBI-chymotrypsin inhibition complex, the most important interactions are hydrophobic (Fernandez et al., 2007).

The enzyme inhibitory activity of BBI was associated with two subdomains of the BBI molecule, Ser^{17}-Lys^{16} and Leu^{43}-Ser^{44}. Lysine residue was involved in the mechanism of trypsin inhibition but not in the chymotrypsin inhibition (Sreerama and Gowda, 1997). During trypsin inhibition, the Lys^{16} of the BBI molecule extends into the primary pocket of trypsin forming a direct network of two hydrogen bonds to Asp^{189} therefore blocking the active site of trypsin (Flecker, 1987). Removal of the N- or C-

terminal amino acid did not affect BBI inhibitory activity toward trypsin or chymotrypsin. Chymotrypsin inhibition involved the interaction of Leu^{43} or Ser^{44} with chymotrypsin active site.

Evolution of inhibitors

BBIs were the products of multi-gene families. The presence of multiple genes and the possibility of hydrolysis have been cited as evidence of the existence of a large number of BBI isoinhibitors (Kalume et al., 1995). The gene size and coding regions of the inhibitors are generally small with no introns (Boulter, 1993) and many of these inhibitors are products of multigene families (Ryan, 1990). BBI type double-headed protease inhibitors were assumed to have arisen by duplication of an ancestral single headed inhibitor gene and subsequently diverged into different classes *i.e.* trypsin/trypsin (T/T), trypsin/chymotrypsin (T/C) and trypsin/elastase (T/E) inhibitors (Odani et al., 1983). The mature proteins consist of a readily identifiable core region, covering the invariant cysteine residues and active centre serines, which are bound by highly variable N and C-terminal regions. The amino acid replacements in this region from all pair-wise comparisons showed that the differences between the different classes of inhibitor within a species (around 16.5/62 residues) are much greater than the differences within a class between different species (around 11/62 residues). Considering that 18 of the residues in this region are obligatorily invariant for proteins to be classified as BBI type inhibitors. Corrected divergence between pair-wise combinations of sequences calculated according to the method of Perler et al., (1980) revealed that the average divergence between trypsin-specific and chymotrypsin-specific second domains (about 36%) was very similar to that between the first and second domains (about 40%). The gene duplication leading to T/T and T/C families

occurred very close to the duplication, leading to the appearance of the double-headed inhibitors and that the number of silent substitutions had reached saturation in all these genes (Hilder et al., 1989).

Mello et al., (2003), studying the primary structure of BBI, reported that inhibitors from dicotyledonous species accumulated slight differences during their evolution. The phylogenetic analysis of BBI family revealed that inhibitors from peanut may be regarded as an intermediate type between monocotyledonous and dicotyledonous species. The invariant residues of BBI were instrumental in forming the intra-molecular bonds required to confer to the binding loop the rigidity necessary to be biologically active. This explains why the nine residue disulphide-linked motif (P3- P6`) of both trypsin and chymotrypsin binding loops was highly conserved among the legume species. As concerns the trypsin binding loop, variation is detected only at P1, P2` and P5` positions within and among the legume species. The P2` position plays an important role in the inhibition because it fits into an apolar S2 enzyme pocket. Ile or Arg are detected mostly at P2` position, while no variation is observed at P1 and P5` (Piergiovanni, and Galasso, 2004).

The cysteine residues of BBI also play an important role being necessary to create disulphide bonds within the protein. Even though the BBI sequences have slightly changed during the evolution of dicotyledonous genera, the number of cysteine residues are invariable and equal to 14 suggesting that they have functional similarities (Mello et al., 2003). BBIs from monocotyledonous plants also have conserved cysteine residues although their number varies even within a specific genus and may present 10–26 cysteines forming 5–13 disulphide bonds. These structural dissimilarities are not likely to reflect functional differences; rather it might suggest duplications of conserved sequences without affecting the

properties of the inhibitor. The pattern of evolution of BBIs in monocotyledonous and dicotyledonous plants are different, especially in respect to duplication events. Tashiro et al., (1987) suggested that duplicated molecules would consist of tandemly repeated sequences and the connecting peptides between domains could be hydrolysed by a proteinase to yield different types of BBIs. Legume seeds, such as *Erythrina variegate,* do not have the N and C-terminal segments of their BBI, and may have lost these terminals during evolution (Kimura et al., 1994).

BBI derived synthetic inhibitors

One goal of inhibitor research has been the reduction of size and simplification of BBIs to their minimal structural elements. In additional to the conserved disulphide bridge network, the majority of BBIs have a core reactive site loop of 9 residues except for the 11 residue loop found at one reactive site loop of peanut inhibitors A-II, B-II and B-III (Norioka and Ikenaka, 1983). This 9 residue loop is a short type VIb β-turn. This inhibitory loop has been described as a two stranded antiparallel β-sheet comprising two regions separated by cis Pro at P3`. All contacts with proteases are through the front side of the loop (P3-P2`) (Tsunogae et al., 1986). Of the interacting residues P1 serine is well conserved, possibly due to its involvement in the intramolecular hydrogen bonding within the inhibitor loop and the P3 cysteine residue was essential for disulphide bond formation (McBride et al., 2002).

Nishino et al., (1977) showed that synthetic cyclic peptides with an incorporated nonapeptide motif retain inhibitory activity and structural features of the full-length BBI protein. This result brought in the creation of combinatorial peptide libraries using the native sequence as a template (McBride et al., 1996, McBride et al., 1998, Gariani et al., 1999 and McBride

et al., 2002). The activity was found to be dependent on the ends of the loop being disulphide linked as in the parent protein. Non cyclic peptides have little or no activity. It appears that having an additional residue each side of the 9-mer core improves inhibition properties and stability of the peptide. The small synthetic peptides based on the core reactive site loop of 9 residues retain the same structure as the corresponding part of a full-sized protein and also retain inhibitory activity (Brauer et al., 2002).

Brauer et al., (2002), isolated the antiproteinase activity of small (approximately 11 residues), cyclic, synthetic peptides, which display most of the functional aspects of the protein. The interaction between BBIs and their target enzymes follows a tight-binding inhibitory mechanism. Synthetic peptides derived from the BBI reactive site loop retain the inhibitory ability and were used to study interaction between BBIs and enzymes (Scarpi et al., 2002), stability towards proteolytic hydrolysis (Gariani and Leatherbarrow, 1997) and as tools for pharmacological studies (Dittmann et al., 2001). Analysis of the BBI-chymase interaction showed that the complex did not dissociate in SDS– PAGE under non-reducing conditions (Ware et al., 1997). BBI fails to inhibit tryptase (Ware et al., 1997) but BBI derived peptides are reported to have the tryptase inhibitory activity (Scarpi et al., 2004).

SFTI, a 14 aminoacid back bone cyclised protein (Luckett et al, 1999) appears to be a natural peptide mimic of the BBIs as it shares sequence and structural homology with the trypsin inhibitory loop of the BBI family. This peptide is widely exploited now as a back bone in mutational studies (Korsinczky et al., 2005, Daly et al, 2006) and also in combinatorial chemistry (Zablotna et al., 2007). More generally, there is a growing interest in both protein mimicry and the use of natural scaffolds as templates for altered activity (Fear et al., 2007). Short peptides, although binding to the active site of the enzyme, are more rapidly hydrolyzed (as compared with the whole

inhibitor). Their behaviour as substrates and the inhibition observed is temporary. The N-terminal segment of CMTI (Squash inhibitor), which is directly involved in the interaction with bovine β-trypsin, was used as a possible template to design low molecular mass proteinase inhibitors (Kazmierczak et al., 2003).

A novel bifunctional bicyclic inhibitor has been created by Jaulent and Leatherbarrow (2004) that combines features both from the BBI proteins, which have two distinct inhibitory sites, and from sunflower trypsin inhibitor-1 (SFTI-1), which has a compact bicyclic structure. The inhibitor was designed by fusing together a pair of reactive loops based on a sequence derived from SFTI-1 to create a backbone-cyclized disulfide-bridged 16-mer peptide. This peptide has two symmetrically spaced trypsin binding sites.

A series of trypsin inhibitor of SFTI-1 modified in substrate-specific P1 position have been synthesized by the solid-phase method. Lys^5 present in the wild type inhibitor was replaced by Phe derivatives substituted in para position of the phenyl ring, L-pyridylalanine and N-4-nitrobenzylgycine yielded potent chymotrypsin and cathepsin G inhibitors (Łegowska et al., 2009).

Austin et al., (2010) carried out the in vivo biosynthesis of wild type sunflower trypsin inhibitor 1 (SFTI-1) inside *E. coli* cells using an intra-molecular native chemical ligation in combination with a modified protein splicing unit. A small library containing multiple Ala mutants was also biosynthesized. This study demonstrated the exciting possibility of generating large cyclic peptide libraries in live *E. coli* cells, and is a critical first step for developing in vivo screening and directed evolution technologies using the cyclic peptide SFTI-1 as a molecular scaffold.

Heterologous expression of BBIs

In addition to a general intrinsic interest in sequences of plant genes, especially at a stage when sequences for relatively few genes have been collected, the sequences of genes for inhibitors of the BBI type find added importance for the following reasons: (A) these are genes for the sulphur-amino acid rich storage proteins of the soybean seed (B) the possibility that the soya BBI serve or, through gene transfer to other species, may be made to serve a defensive function is of great interest and (C) sequence data are important for studies of site-directed mutagenesis, which could be of importance in adapting the molecules for broad usage in certain agricultural and medical applications (Foard et al., 1983).

Flecker (1987) achieved the cloning of a gene coding for a BBI type protease inhibitor by chemically synthesizing the cDNA and expressing it in *E. coli* as a fusion protein with a β-galactosidase fragment. To reveal the inhibitory activities towards proteases and tumour cells, the open-reading frame of the buckwheat trypsin inhibitor gene was chemically synthesized and expressed in *E. coli* M15. Inhibitory activity assays suggested that the recombinant protein strongly inhibited the activity of trypsin and inhibited the proliferation of multiple myeloma IM-9 cell lines in a dose-dependent manner (Zhang et al., 2007).

A fusion protein based expression system was developed in the gram-positive bacterium *Bacillus subtilis* to produce the soybean BBI (sBBI). This system makes use of *B. subtilis* as a useful host for the production of therapeutic proteins (Vogtentanz et al., 2007). Attempts have been made to replace the chymotrypsin inhibitory loop of soybean BBI (sBBI) with a VEGF (vascular endothelial growth factor) binding peptide (BBI-AV) and have significantly reduced the overall purification yield when BBI-AV is produced

as a fusion protein in a *B. subtilis* expression system. Site-saturation libraries were generated at 39 out of the 66 amino acid residues of BBI-AV in which F50R yielded nearly the same level as wild-type sBBI (Collier et al., 2009).

A putative rice trypsin/chymotrypsin inhibitor of the BBI family, of about 20 kDa, was expressed in *E. coli* as a fusion protein bearing an N-terminal $(His)_6$ purification tag (Li et al., 1999). This inhibitor exhibited in vitro trypsin-inhibiting activity but no chymotrypsin-inhibiting activity. Over expression of the inhibitor in transgenic rice plants resulted in resistance to the fungal pathogen *Pyricularia oryzae*, indicating that proteinase inhibitors confer resistance against the fungal pathogen in vivo and that they might play a role in the defense system of the rice plant (Qu et al., 2003).

Fernandez et al., (2007) described that in the phagemid expressed TryBBI-trypsin complex the most important interactions are salt bridges and hydrogen bonds, and the central residue in the interface is the Lys^{16} at the P1 loop position. At the same time, "*in silico*" experiments showed that in the ChyBBI-chymotrypsin complex, the most important interactions are hydrophobic.

Qi et al., (2010), cloned the cDNA of the mung bean trypsin inhibitor, one of the most studied BBIs. A modified peptide, Lys33GP, with 33 residues derived from the long chain of the Lys active fragment of mung bean trypsin inhibitor, was successfully expressed in *E. coli* as a glutathione-S-transferase fusion protein. Small peptides derived from the Lys active fragment of this trypsin inhibitor were fully active against trypsin.

Application of BBIs

Physiological role in seeds

BBI is to seeds what α1-antitrypsin is to humans. The physiological significance of BBI-type proteins is associated with their three major roles in

plants: regulation of endogenous seed proteinases, storage of sulphur amino acids, and defence against pathogens and insect attack (Flecker, 1987; Ryan, 1990; Azzouz et al., 2005). Many trypsin inhibitors from diverse classes are implicated in the protection of plants against insects and fungi. In the latter case, they appear to have an antidigestive effect, through proteolysis inhibition. During seed germination, the concentration of BBI inhibitors decreases. The result was evaluated by the studies on germination in which protease K1 initiates the degradation of BBI followed by extensive proteolysis by protease B2 (McGain et al., 1989). Wound-induced leaf expression of BBI was reported as an example of anti-pest function for BBI (Brown et al., 1985).

The biotechnological potential of BBIs has been intensely investigated. These inhibitors are thought to play important roles in plant defence mechanisms against insects and can be used for biological pest control. PIs with insecticidal activities have emerged as an interesting strategy for insect pest control (Azzouz et al., 2005). Pea and soybean BBI induced significant mortality and growth depression on pea aphid *Acyrthosiphon pisum* (Rahbe et al., 2003; Ceci et al., 2003). BBI from wheat kernel was reported to exhibit antifungal activity (Chilosi et al., 2000). Soybean BBI has a broader range of inhibitory activity against gut proteases in *Helicoverpa armigera* (Bown et al., 2004). Wound-induced leaf expression of trypsin inhibitors in tobacco leaves (McMunnus, 1994) and alfalfa (Brown et al., 1985) indicated that BBI may have an anti-pest function. Shan et al., (2008) reported that BBI from wheat showed tolerance to salt stress. In vitro inhibition assays by Pereira et al., (2007) revealed that BBI from *Phaseolus coccineus* L. seeds was found to inhibit trypsin-like enzymes, bovine pancreatic chymotrypsin, and trypsin found in *Hypothenemus hampei* larval midgut. Kim et al., (2011) reported a successful strategy to increase the

cysteine content of soybean seed through the overexpression of a key sulfur assimilatory enzyme. BBI, rich in cysteine content is a sulphur rich protein. Several transgenic soybean plants that overexpress a cytosolic isoform of O-acetylserine sulfhydrylase have been generated. These transgenic soybean plants exhibit a marked increase in the accumulation of BBI.

Clinical applications of BBI

BBI has been demonstrated to be effective in preventing or suppressing radiation- and chemical carcinogen-induced transformation in a wide variety of in vitro assays (Kennedy, 1998). In vivo, BBI has also been found to inhibit carcinogenesis in the colon, oesophagus, liver, lung and the oral cavity (Losso, 2008). BBIC prevented atrophy, weakness, and oxidative stress in soleus muscle of hind limb-unloaded mice (Arbogast et al., 2007).

BBIC exhibits very favourable safety profile in pre-clinical studies and in clinical trials in patients with benign prostatic hyperplasia, pre-cancerous conditions, such as oral leukoplakia, and ulcerative colitis (Lichtenstein et al., 2008, Safdi et al., 1997). The lack of toxicity and the potent anti-inflammatory effect of BBIC in animals when assessed in an acute colitis model (Ware et al., 1999a) conferred the potential for BBIC to benefit patients with active ulcerative colitis.

In experimental autoimmune encephalomyelitis (EAE), significant disease suppression of clinical and histological EAE has been reported. Therefore BBIC acts as an excellent candidate for oral therapy in multiple sclerosis patients (Gran et al., 2006). A potent anti-inflammatory effect had been associated with BBI ingestion in several animal studies (Kennedy 1998, Billings et al., 1991, Ware et al., 1999). BBI has been shown to be an effective inhibitor of several human proteases associated with inflammation-mediating cells, including elastase (Tikhonova et al., 1994 and

Larionova et al., 1993), cathepsin G (Larionova et al., 1993 and Gladysheva et al., 1994) and mast cell chymase (Ware et al., 1997). BBI had an immunoregulation effect through inhibition of proteases released from inflammation-mediating cells. BBI reduced autoimmune inflammation and attenuated neuronal loss in a mouse model of multiple sclerosis (Touil et al., 2008).

Dittmann et al. (2001) reported that pre-incubation of normal human skin fibroblasts with BBI, time-dependently blocked radiation-induced activation of tyrosine kinase and inhibited the activation of epidermal growth factor by enhancing specific tyrosine phosphatases. BBI was able to protect cells with functional TP53 from ultra violet B radiation-induced DNA damage. This protective effect was most likely achieved via the activation of the TP53 signalling cascade resulting in the activation of nucleotide excision repair (Dittmann et al., 2001). BBI was found to act as a potent selective normal-tissue radioprotector in vitro and in vivo, apparently without protecting tumors, and thus has the potential to improve clinical radiotherapy (Dittmann et al., 2005).

Li et al., (2011) summarised that BBI has the ability to inhibit lipopolysaccharide-mediated macrophage activation, reducing the release of pro-inflammatory cytokines and subsequent neurotoxicity in primary cortical neural cultures. Thus BBI, through its anti-inflammatory properties, protects neurons from neurotoxicity mediated by activated macrophages.

BBIs have attracted much interest due to their anti-carcinogenic activity, although the exact mechanism of this activity has yet to be established. Human populations consuming large quantities of BBIs in their diet exhibit lower rates of colon, breast, prostate, and skin cancers. The purified BBI works as well as BBIC, an anticarcinogenic agent over a range of doses in both *in vitro* transformation systems and *in vivo* carcinogenesis

assay systems (St. Clair *et al*., 1990a; Kennedy *et al*., 1993). The soybean derived BBI was particularly effective in suppressing carcinogenesis in different species (mice, rats and hamsters), in several organ systems and tissue types and in cells of hematopoietic, epithelial and connective tissue origin when given to animals by several different routes of administration, including in the diet. Kennedy et al., (1996) showed that BBI suppresses carcinogenesis in animals known to have a genetic susceptibility to cancer. Animal carcinogenesis studies had shown that dietary amounts as low as 0.01% BBI can suppress liver carcinogenesis in mice (St. Clair et al., 1990b). Analysis of tissue from rat colitic lesions indicated that activated tissue proteases were potently inhibited by BBI (Hawkins et al., 1996).

Clemente et al., (2005) reported BBIs from recombinant pea (*Pisum sativum* L.) seed protease inhibitors, rTI1B and rTI2B differ in their inhibitory activity, on the growth of human colorectal adenocarcinoma HT29 cells in vitro. A significant and dose-dependent decrease in the growth of HT29 cells was observed using all protease inhibitors, with rTI1B showing the largest decrease. The relative effectiveness of rTI1B and rTI2B correlate to the variant amino acid sequence within their respective chymotrypsin inhibitory domain, in agreement with a chymotrypsin-like protease as a potential target. But the studies with MSTI, a BBI from snail medic seeds (*Medicago scutellata*), (Catalano *et al*., 2003) revealed that antichymotryptic activity was not a strict requirement for the antitumoral effect wherein the inhibitory activity and antitumoral activity are not correlated.

It is thought that the strength of the BBI as a cancer preventive agent lies in its ability to reverse the initiation cells. It is hypothesized that BBI suppressed carcinogenesis by its ability to inhibit serine proteases or the expression of certain proto-oncogenes (Chang *et al*., 1985; Chang and

Kennedy, 1988; Caggana and Kennedy, 1989; St. Clair *et al*., 1990 b; Li *et al*., 1992). BBI was also reported to regulate the cell cycle arrest at G1\S phase (Chen et al., 2005) and immune stimulating properties. Proteinase inhibitors suppress carcinogenesis by affecting proteolytic activities (Long *et al*., 1981; Messadi *et al*., 1986; Oreffo *et al*., 1991; Billings and Habres, 1992; Billings, 1993). All these factors are thought to play an important role in carcinogenesis.

BBI isolated from the seeds of Faba bean (*Vicia faba* cv. Giza 843) inhibited HIV-1 reverse transcriptase activity with an IC_{50} of about 0.76 µM. Furthermore, this inhibitor showed specific antiproliferative activity toward HepG2 hepatoma cells by inducing chromatin condensation and cell apoptosis (Fang et al., 2011).

Dietary supplements with BBIC or the antioxidant formulation significantly reduced the prevalence and severity of the lens opacifications in the mice exposed to iron-ion radiation. Treatment with BBIC or the antioxidant formulation also decreased the severity of the lens opacifications in the mice exposed to proton radiation. These results indicated that BBIC and the antioxidant formulation evaluated could be useful for protecting astronauts against space radiation-induced cataracts during or after long-term manned space missions (Davis et al., 2010).

Park et al., (2007) demonstrated the stabilities of lunasin and BBI to digestion in vitro by simulated intestinal fluid and simulated gastric fluid. The studies concluded the extreme stability of BBI in one hand and the stability provided by the BBI to lunasin in the other suggesting that BBI plays a role in protecting lunasin from digestion when soy protein is consumed orally.

Owing to the positive aspects, BBI has been incorporated in many commercial soy foods (Blanca et al., 2009), such as soymilk, soy-based infant formula, tofu and bean curd.

Horsegram (*Dolichos biflorus*)

Horsegram (*Dolichos biflorus* or *Macrotyloma uniflorum* (Lam.) Verdc.) is a pulse crop native to the south-east Asian subcontinent and tropical Africa. They are legumes of the tropics and subtropics grown mostly under dry-land agriculture. It is extensively cultivated, especially in dry areas of Australia, Burma, India, and Sri Lanka (Duke and Reed, 1981). The use of dry seeds of horsegram is limited due to their poor cooking quality. Horsegram pods and seeds are depicted in Figure 1.6. It is consumed as whole seed, sprouts, or whole meal by a large population in rural areas of Southern India. US National Academy of Sciences identified this legume as a potential food source for the future (1978). It is also known as Gahat, Kulath or Kulthi and Hurali in India and is grown for use as food and fodder. The chemical composition is comparable with commonly cultivated legumes. Like other legumes, the protein is deficient in methionine and tryptophan. Horsegram is an excellent source of iron and molybdenum (Kadam and Salunkhe, 1985).

Comparatively, horsegram seeds have higher trypsin inhibitor and hemagglutinin activities and polyphenols. Dehusking, germination, cooking, and roasting have been shown to produce beneficial effects on nutritional quality. Horsegram is also a good source of protein (Borhade et al., 1984) and appears to be a fairly good source of calcium. Also horsegram strengthens food for people with iron deficiencies. It keeps body warm in winter season as well (Kadam and Salunkhe, 1985). The botanical description of horsegram is listed in Table 1.3.

Table 1.3 **Botanical description of horsegram**

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Fabales
Family:	Fabaceae
Subfamily:	Faboideae
Tribe:	Phaseoleae
Genus:	*Macrotyloma*
Species:	*M. uniflorum*
Binomial name	
Dolichos biflorus or Macrotyloma uniflorum	

Fig. 1.6 **Horsegram pods and seeds**

Aim and Scope of present investigation

BBI are small protease inhibitors found in the seeds of legumes in particular (Laskowski, 1980). Their molecular masses are in the range of 6-9 kDa. They comprise of a binary arrangement of two sub domains with a conserved array of seven disulphide bridges, which play a pivotal role in the stability of the inhibitors. These inhibitors interact simultaneously and independently with two molecules of proteinases. In addition to the protease inhibitory activity, BBI is reported to have anticarcinogenic and radio protective activity (Kennedy, 1998), regulates the cell cycle arrest at G1\ S phase (Chen et.al., 2005) and immune stimulating properties. BBIs have also been implicated to play a vital role in plant defense mechanism.

Horsegram (*Dolichos biflorus)* is a pulse crop native to South East Asia and Tropical Africa. Four isoforms of BBIs, from horsegram seeds (Sreerama et.al., 1997) have been isolated. The complete primary structure of major isoform HGI-III has been determined (Prakash, et.al., 1996). It contains one chymotrypsin inhibitory site and one trypsin inhibitory site. The role of disulphide linkages in maintaining structural integrity of horsegram BBI has been established (Singh & Rao, 2002). Three new isoforms appear upon germination of horsegram seeds that are derived from limited proteolysis during germination and not by *de novo* synthesis (Kumar et. al., 2002). The inhibitors of horsegram (HGIs) are single polypeptides with a molecular mass of 8.5 kDa. However SDS-PAGE and analytical gel filtration indicate the molecular mass to be 16 kDa, suggesting that they exist as dimers in solution. In contrast, inhibitors of germinated horsegram seeds (HGGI) derived from dry seed inhibitors, are single polypeptides of 6.5-7.2 kDa and exist as monomers. The observation that HGGIs, which lack the C-terminal Asp residues, lose the ability to form dimers suggest that these interactions

play a unique role in dimerization of BBIs. Chemical modification of the Lys\Arg residue, a comparative evaluation of the of the amino acid sequences of several BBIs that exist either as monomers or dimers and homology modeling of the dimer led to the identification of an interaction between Lys^{24} (trypsin reactive site) and Asp^{76} in HGI-III that characterizes the dimer formation (Kumar et.al., 2004)

Poor bioavailability, and high susceptibility to proteolysis and fairly large size are significant limitations of BBI for use as an efficient and potent cancer chemopreventive agent. Cloning and expression of small cyclic peptide protease inhibitors based on the DNA sequence of horsegram BBI is an attractive alternative to the isolation and purification of these peptides from plant sources. Such engineered potent cyclic inhibitor of a much smaller size and higher stability could increase their bioavailability and efficacy. Structural studies of these inhibitors would be useful in the design of stable and bioavailable pharmaceuticals and pesticides.

The main objective of the proposed study is to genetically engineer the protein-protease inhibitor of horsegram (*Dolichos biflorus*) towards altering the efficacy, bioavailability and self-association properties.

OBJECTIVES

- Cloning of HGI-III, the major BBI of horsegram (*Dolichos biflorus*).
- Expression of HGI-III in *E.coli* and comparison with native HGI-III.
- Site directed mutagenesis of HGI-III toward altering its inhibitory properties and self association status.
- Expression of cyclic peptide inhibitors based on the inhibitory loop structure of HGI-III using genetic engineering techniques.

The cloning and site directed mutagenesis of HGI-III and its derived proteins provides a platform for studying the residues involved in the dimerisation of HGI-III and also to study the inhibitory properties of these HGI-III derived proteins with respect to their pharmaceutical value.

CHAPTER 2

MATERIALS AND METHODS

2. Materials and Methods

2. 1. Materials

Plant Material

Horsegram seeds (*Dolichos biflorus*) were procured from the local market, which served as starting material.

Bacterial strains and vectors

The bacterial strain *E. coli* DH5α and pRSETC vector were from Invitrogen Corporation, Carlsbad, CA. BL21 (DE3) pLysS and Origami (DE3) pLysS strains and the vector pET20b were obtained from Novagen, Merck Specialities Private Ltd, Mumbai, India.

Chemicals

The chemicals used in the present investigation were procured from the following sources:

Tryptone-Type1, yeast extract, bacto-agar, ampicillin sodium salt and isopropyl thiogalactopyranoside (IPTG) were from Hi-Media Laboratories, Mumbai, India, Genomic DNA extraction kit, gel extraction kit, *Taq* DNA polymerase and Dye Ex ^{TM} 2.0 spin kit, were from Qiagen, GmbH, Hilden,

Germany. *Pfu* DNA polymerase for gene amplification, *Dpn*I for gene manipulation, DNA ladder and 6× DNA loading dye were purchased Fermentas Life Sciences, GmbH, Hilden, Germany, Restriction enzymes *BamH*I, *EcoR*V, *Nde*I, *Nhe*I, *Pvu*II, *Xho*I and *T4* DNA ligase for gene manipulation and expression were purchased from New England Biolabs, Beverly, MA, USA. The oligonucleotides were synthesised by Sigma-Genosys, Sigma-Aldrich Chemical Private Limited, Bangalore India. Agarose was from USB Corporation, Cleveland, USA. The Big Dye^{R} terminator v3.1 cycle sequencing kit, was from Applied Biosystems, USA.

Ethidium bromide (EtBr), RNase, formamide, formaldehyde, deoxynucleotide mixture (dNTPs), porcine pancreatic elastase (2× crystallized, pancreopeptidase E, EC 3. 4. 21. 36), bovine pancreatic trypsin (2× crystallized, type III, EC 3. 4. 21. 4), bovine pancreatic ∝-chymotrypsin (3× crystallized, type II, EC 3. 4. 21. 1), chloromethylketone (TPCK) - treated trypsin, chloromethylketone (TLCK) - treated chymotrypsin, bovine serum albumin (BSA), lysozyme, acrylamide, N-N'-methylene bis-acrylamide, tris (hydroxymethyl) amino methane (Trizma base), coomassie brilliant blue R-250, α-N-benzoyl-DL-arginine-*p*-nitroanilide HCl (BAPNA), N-benzoyl-L-tyrosine-*p*-nitroanilide (BTPNA), N-succinyl Ala-Ala-Ala-*p*-nitroanilide (NAPNA), N-acetyl-DL-phenylalanine-β-napthyl ester (APNE), tetra azotized *o*-dianisidine, N-N, N'-N'-tetramethyl 1, 2-diaminoethane (TEMED), DL-dithiothreitol (DTT), diaminobenzidine, horseradish peroxidase conjugated secondary antibody, imidazole and tryptase from human lung were procured from Sigma-Aldrich, St. Louis, Missouri, USA.

Coomassie brilliant blue G-250 was from Eastman Kodak Co., Rochester, NY, USA.

SDS-PAGE medium and low range molecular weight markers were from Bangalore Genie, Bangalore, India. β-Mercaptoethanol was purchased from Fluka, Switzerland and ammonium persulfate (APS) was procured from ICN Biomedicals Inc., Aurora, Ohio.

Nitrocellulose (0.45 μm) membrane was from Schleicher and Schuell, Germany. Immobilon-P (polyvinyldiflouride membrane PVDF; 0.45 μm) was obtained from Millipore Corporation, USA.

Sodium chloride, sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate, sodium hydroxide, ethylene diamine tetra acetic acid (EDTA) and potassium acetate were from Qualigens Fine Chemicals, Mumbai, India. Glycine and sodium acetate were from Merck (India) Ltd., Mumbai, India.

Purification materials, Sephadex G-50, CNBr activated Sepharose4B, Ni-Sepharose™ 6 Fast Flow and Glutathione sepharose were from Pharmacia LKB, Uppsala, Sweden.

Antibodies used for the experiments were raised in New Zealand white rabbit against horsegram inhibitor HGI-III (Sreerama and Gowda, 1997).

Sodium dodecyl sulfate (SDS), 5-bromo-4-chloro-3-indolylphosphate (BCIP), and nitroblue tetrazolium (NBT) were from Pierce Chemical Company, Rockford, Illinois, USA.

Colon luminal content of the patients was collected from Karnataka Cardiac Diagonostics Centre, Mysore (waste procured after colonoscopy).

High Performance Liquid Chromatography (HPLC) grade solvents were obtained from Qualigens Fine Chemicals and Ranbaxy Fine Chemicals Ltd., India.

HPLC columns

RP-HPLC columns C_{18} Waters Symmetry Shield^{TM} RP18 150 mm × 4.6 mm (i.d), 5 μ, BIOSEP-SEC-S-3000 (300 mm × 7.8 mm (i.d), 5μ, manufacturer exclusion limit: 5-700 kDa) was from Phenomenex, Torrance, USA.

2.2 Methods

2.2.1 Preparation of defatted horsegram flour

Horsegram seeds were first cleaned manually to remove immature and infected seeds. The adhered dust particles were removed by washing the seeds under running tap water and air dried on a filter paper at 25 ± 2 °C. The cleaned seeds were ground to a fine powder and defatted with CCl_4 (1:5 w/v) for 14-16 h at 25 ± 2 °C with occasional stirring. The slurry was filtered and residual CCl_4 was removed by air drying at 25 ± 2 °C. The defatted fine powder obtained was stored at 4 °C until used.

2.2.2 Assay methods

The trypsin, chymotrypsin, elastase and tryptase activity and their inhibitory activity were spectrophotometrically measured by assaying amidolytic activity for trypsin, chymotrypsin, elastase and tryptase in the absence and presence of a known quantity of inhibitor using the chromogenic substrates BAPNA, BTPNA and NAPNA respectively. All the spectrophotometric measurements were measured on a Shimadzu UV-Visible recording spectrophotometer (Model UV-1601).

2.2.2.1 Assay of trypsin and trypsin inhibitor activity

Trypsin was assayed according to the modified photometric method of Kakade *et al*., (1969) using BAPNA as substrate. Forty mg of BAPNA was dissolved in 2 mL dimethylsulfoxide (DMSO) and then diluted (1:100) in 50 mM Tris-HCl buffer, pH 8.2 containing 20 mM $CaCl_2$, prior to enzyme assay. The assay reaction contained 0.5 mL of trypsin solution (40-50 μg of trypsin in 1 mM HCl), 0.5 mL of water and 1.25 mL of the substrate. The reaction was carried out at 37 °C for 10 min and stopped by addition of 0.25 mL of 30 % acetic acid. Absorbance of the liberated *p*-nitroanilide was measured at 410 nm against an appropriate blank in which the reaction was arrested by adding 30 % acetic acid prior to BAPNA addition.

The trypsin solution was incubated with an aliquot of inhibitor for 10 min at 37 °C and reaction started by the addition of 1.25 mL BAPNA diluted (1:100) in 50 mM Tris-HCl buffer, pH 8.2 containing 20 mM $CaCl_2$ and incubated at 37 °C for 10 min. The reaction was arrested by addition of 30 % acetic acid and the residual trypsin activity was measured by recording the absorbance at 410 nm.

Trypsin and trypsin inhibitory unit: One trypsin (TU) unit is arbitrarily defined as an increase in absorbance of 0.01 at 410 nm under conditions of assay. The trypsin inhibitory unit (TIU) is defined as the number of trypsin units inhibited under the same conditions (Kakade *et al.*, 1969).

2.2.2.2 Assay of chymotrypsin and chymotrypsin inhibitory activity

Chymotrypsin activity was assayed by measuring the absorbance of *p*-nitroanilide at 410 nm resulting from the hydrolysis of BTPNA. Stock BTPNA (20 mM) was prepared by dissolving 16.2 mg in 2 mL of DMSO and then made up to 100 mL with 80 mM Tris-HCl buffer, pH 7.8 containing 100 mM $CaCl_2$ and 20 % DMSO (v/v). Chymotrypsin solution (40-50 μg in 0.5 mL of 1

mM HCl) was added to 0.5 mL of distilled water and incubated with 1.25 mL of substrate at 37 °C for 10 min. The reaction was arrested by adding 0.25 mL of 30 % acetic acid and the liberated product, *p*-nitroanilide measured at 410 nm against an appropriate blank.

Chymotrypsin inhibitory assay was performed similar to that of trypsin inhibitor assay and the residual activity of chymotrypsin was calculated by measuring the absorbance at 410 nm.

Chymotrypsin and chymotrypsin inhibitory unit: One chymotrypsin (CU) unit is arbitrarily defined as an increase in absorbance of 0.01 at 410 nm under assay conditions. The chymotrypsin inhibitory unit (CIU) is defined as the number of chymotrypsin units inhibited under the same conditions.

2.2.2.3 Assay of elastase and elastase inhibitor activity

The reaction velocity was determined by measuring the absorbance of *p*-nitroanilide resulting from the hydrolysis of NAPNA. A stock solution of NAPNA (10 mM) was prepared by dissolving 10 mg in 2 mL of DMSO and then made up to 100 mL with 50 mM Tris-HCl buffer, pH 8.2 containing 20 % DMSO (v/v). Elastase (40-50 μg in 0.5 mL of 1 mM HCl) was added to 0.5 mL of distilled water and incubated with 1.25 mL of substrate at 37 °C for 10 min. The reaction was stopped by adding 0.25 mL of 30 % acetic acid and the liberated product, *p*-nitroanilide measured at 410 nm.

Elastase was incubated with an aliquot of the inhibitor for 10 min at 37 °C and the reaction started by the addition of 1.25 mL NAPNA diluted in buffer and incubated at 37 °C for 10 min. The reaction was arrested by addition of 30 % acetic acid and residual elastase activity measured by recording the absorbance at 410 nm.

Elastase and elastase inhibitory unit: One elastase activity unit (EU) is arbitrarily defined as an increase in absorbance by 0.01 at 410 nm under assay conditions. The elastase inhibitory unit (EIU) is defined as the number of elastase units inhibited under the same conditions.

2.2.2.4 Tryptase inhibitory assay

The amidase activity of tryptase and the inhibition was assayed using the chromogenic substrate BAPNA at pH 8.2 in 0.05 M Tris-HCl containing 20 mM $CaCl_2$ at 37 ^{o}C for 10 min. The reaction was arrested by addition of 30 % acetic acid and the residual tryptase activity measured at 410 nm similar to that of trypsin. One tryptase inhibitory unit (TIU) is defined as the amount of inhibitor which reduces the enzyme activity by one unit.

2.2.3 Protein estimation

Protein concentration was determined by the dye binding method of Bradford (1976). Bovine serum albumin (BSA) was used as the standard.

2.2.4 Polyacrylamide gel electrophoresis (PAGE)

Vertical slab gel electrophoresis was carried out using a BROVIGA mini model electrophoresis unit, at 25 ± 2 °C.

2.2.5 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE at alkaline pH (8.3) was carried out according to the method of Laemmli (1970) in a discontinuous buffer system.

Reagents:

A. Acrylamide (29.2 g) and bis-acrylamaide (0.8 g) were dissolved separately in a minimum amount of water and mixed and made up to 100 mL, filtered and stored in a dark brown bottle at 4 °C.
B. *Separating gel buffer:* Tris (18.15 g) was dissolved in water and pH was adjusted to 8.8 with HCl (6 N), solution made up to 100 mL and stored at 4 °C.
C. *Stacking gel buffer:* Tris (3 g) was dissolved in water and pH adjusted to 6.8 with HCl (6 N), solution made up to 100 mL and stored at 4 °C.
D. *10 % Sodium dodecyl sulfate:* SDS (10 g) was dissolved in water (100 mL)
E. Ammonium persulfate was freshly prepared by dissolving 50 mg in 0.5 mL of distilled water
F. *Tank Buffer:* Tris (0.3 g), glycine (1.44 g) and SDS (0.1 g) were dissolved in 100 mL of distilled water.
G. *Staining solution:* Coomassie brilliant blue (CBB) R-250 (0.1 g) was dissolved in a mixture of methanol: acetic acid: water (25:10:65, v/v). The reagent was filtered and stored at 25 ± 2 °C.
H. *Destaining solution:* Methanol: acetic acid: water (25:10:65, v/v).

Sample buffer: Prepared in solution C diluted 1:4, containing SDS (4 % w/v), β-mercaptoethanol (10 % v/v), glycerol (20 % v/v) and bromophenol blue (0.1 % w/v).

Initially contents of the separating gel were mixed (Table 2.1), degassed and poured between the assembled glass plates, the bottom edge sealed with agar (1 % w/v). The gels were layered with 0.5 mL of distilled water and allowed to polymerize at 25 ± 2 °C for 30 min.

After polymerization of the separating gel, contents of stacking gel were mixed and layered above the polymerized separating gel. The gels thus prepared were of the size 10.5× 9 cm and thickness 0.8 mm.

Samples were prepared by dissolving protein (10-25 μg) in solution 'I' (50 μL). The samples were heated in dry blocks at 100 ^{o}C for 5 min. Cooled samples were then layered in the wells immersed in solution 'F" (Tank buffer) and were run at constant voltage (50 V) until the tracking dye, bromophenol blue entered the lower tank buffer.

Table 2.1 Preparation of separating gel and stacking gel.

Solution	**Separating gel (mL)**		**Stacking gel (mL)**
	(12.5% T, 2.7% C)	**(10% T, 2.7% C)**	**(5% T, 2.7% C)**
Solution A	3.33	2.66	0.83
Solution B	2.00	2.00	-
Solution C	-	-	1.25
Distilled water	2.55	3.22	3.03
Solution D	0.08	0.06	0.05
TEMED	0.01	0.01	0.01
Solution E	0.03	0.03	0.03

Total	8.00	8.00	5.00

Medium range protein M_r markers (Phosphorylase b, 97.7 kDa; BSA, 66.3 kDa; ovalbumin, 43.0 kDa; carbonic anhydrase, 29 kDa; soyabean Kunitz inhibitor, 20 kDa and lysozyme, 14.3 kDa and low range protein M_r markers (Ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; soyabean Kunitz inhibitor, 20 kDa and lysozyme, 14.3 kDa, aprotinin, 6.5 kDa and insulin 3.0 kDa) were used. The markers were supplied as a solution having a total protein concentration of 3 mg/mL. The markers were diluted 1:1 with the solution 'I' and boiled for 5 min.

Staining: The gels were stained for protein with reagent 'G' for 3-4 h at 25 ± 2 °C and destained in the reagent 'H'.

2.2.6 Native PAGE

Polyacrylamide gel electrophoresis under native conditions was carried out to evaluate the purity of the inhibitors. Separating gels (10 % T, 2.7 % C) were prepared as described in section (2.2.5) by mixing solution 'A' (2.0 mL) and 'B' (1.5 mL) with water (2.46 mL) excluding SDS.

Stacking gel (5 % T, 2.7 % C) was prepared similar to that for SDS-PAGE excluding SDS from the gel.

Sample buffer (Solution I) was prepared without the addition of SDS and β-mercaptoethanol. Similarly tank buffer was also prepared just as in SDS-PAGE excluding SDS. Protein was dissolved in 20 μL of the sample buffer and layered on the gel. The gel apparatus was connected to the power supply with the lower electrode connected to the positive power supply lead. After

the electrophoresis at constant current (10 mA), proteins were visualized using CBB R-250.

2.2.7 Gelatin embedded PAGE for trypsin and elastase inhibitory activity staining:

Gelatin–PAGE (Felicioli et al., 1997) was performed by adding gelatin (1 %, w/v final concentration) to the acrylamide gel as described in section 2.2.6. Following electrophoresis, the gel was washed with distilled water three times and then incubated at 37 °C in 0.1 M Tris-HCl buffer (pH 8.0 for trypsin and pH 8.2 for elastase) containing either trypsin or elastase (40 μg/mL) for 1 h. After gelatin hydrolysis, the gel was washed with distilled water and stained with CBB R-250 and destained. The presence of the proteinase inhibitor was detected as a dark blue band in a clear background due to the complex of the unhydrolyzed gelatin with the stain.

2.2.8 Inhibitory activity staining

Following electrophoresis (Section 2.2.6), the gel was washed with distilled water and immersed in 0.1 M Tris-HCl, pH 8.2 containing trypsin (40 μg/mL) or chymotrypsin (pH 7.8) for 60 min at 25 °C. The gel was rinsed thrice in distilled water. APNE reagent was prepared as mentioned below. (a) Seven mg of tetra azotized *o*-dianisidine in 10 mL of 0.1 M phosphate buffer pH 7.4 was prepared. (b) APNE 2 mg was dissolved in 0.2 mL DMSO. (a) and (b) were mixed just before use and the gel was stained using the above buffer. Clear zone was visualized in the gel against a pink background after staining the gel. The clear bands indicated the presence of inhibitor.

2.2.9 Size exclusion chromatography

Size-exclusion chromatography measurements were performed using a BIOSEP-SEC-S 3000 (300×8 mm, exclusion limit: 700 kDa for globular proteins) column on a Waters Associate HPLC equipped with a binary gradient pumping system and Waters Model 1296 photodiode array detector set at 230 nm. The column was pre-equilibrated with the corresponding buffers at a flow rate of 1 mL per min prior to sample loading. The column was calibrated using a mixture of standard proteins, bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome c (14.4 kDa), and aprotinin (6.4 kDa).

2.2.10 Molecular weight (mass) determination using MALDI-TOF

The electrospray ionisation-mass spectrometry (ESI-MS) was carried out in the ionisation mode on an Acquity SYNAPT HRMS (Waters Associate). Purified protein suspended in 50 % CH_3CN containing 0.1 % HCOOH was directly infused and analysed.

2. 2. 11 Electroblotting of proteins

Preparation of PVDF membrane: The PVDF membrane cut to the required size (slightly larger than the gel) was soaked in methanol for 5 min before transferring.

Blotting: The gel after SDS-PAGE electrophoresis was rinsed immediately in transfer buffer (10 mM CAPS, pH 11.0 containing 10 % methanol (v/v) and 0.1 % SDS (w/v) for 15 min. Semi-dry electro-blotting was performed using a semi-dry blotting apparatus. The proteins were transferred for 2 h using a current of 0.8 mA/cm^2 of the blotting paper. The membranes were stained with coomassie blue R-250 and destained for protein sequencing or probed with antibodies as described later (Section 2. 2. 12).

Destaining: The PVDF membrane was destained in 50 % methanol. For N-terminal sequence the corresponding bands were excised, washed with 100 % methanol and dried.

2. 2. 12 Western blotting

Following electro-transfer, the membrane was washed with immunoblot buffer (5 % skimmed milk powder in phosphate buffered saline, pH 7.0) four times (4× 30 min). The membrane was incubated overnight at 4 °C in immunoblot buffer containing antibodies raised against HGI-III (1:500 dilutions). After repeated washes (4× 30 min) in the immunoblot buffer, the membrane was incubated with the secondary antibody, alkaline phosphatase conjugated goat anti-rabbit immunoglobulins, for 2 h at 25±2 °C. After four washes (4× 10 min) in immunoblot buffer and a final wash with the reaction buffer (0.1 M Tris, 0.5 M NaCl, 5 mM $MgCl_2$, pH 9.5), alkaline phosphatase activity was detected with a mixture of BCIP and NBT in the reaction buffer. Alternatively when horse-radish peroxidase conjugate was used the HRP activity was detected using diaminobenzidine.

For dot blot analysis, about 100 μg of the proteins were immobilized on a nitrocellulose membrane by repeated application. The membrane was kept at 37 ^{o}C to accelerate drying until the required protein was immobilized. Following immobilization the membrane was sequentially treated with primary and secondary antibodies and immunodetected using alkaline phosphatase activity or HRP activity as described above.

2. 2. 13 Automated gas phase protein sequencing

The N-terminal sequence of rHGI was determined by Edman degradation using an Applied Biosystems 491A automated gas phase protein

sequencer (Procise 491A). Edman-degradation was performed by supplying gaseous reagents for the coupling and cleavage reactions. The flow diagram for the sequence of events is shown in Figure 2.1.

The electroblotted protein (PVDF membrane) was detected by coomassie brilliant blue staining. The band was excised, destained thoroughly with methanol, and subjected to several alternate washes with water and methanol. The membrane was carefully loaded on to a quartz holder, dried over a stream of argon and directly used for the pulsed gas phase sequencing. The coupling reaction is carried out with phenyl isothiocynate (R1) in the presence of gaseous methyl piperidine (R2). Excess of reagents and by products are washed with *n*-heptane (S1) and ethyl acetate (S2). The cleavage reaction is carried out with the 100% TFA to form an aniline-thiazolinone (ATZ) derivative. A temperature controlled reaction chamber was used to carry out the coupling and cleavage reactions.

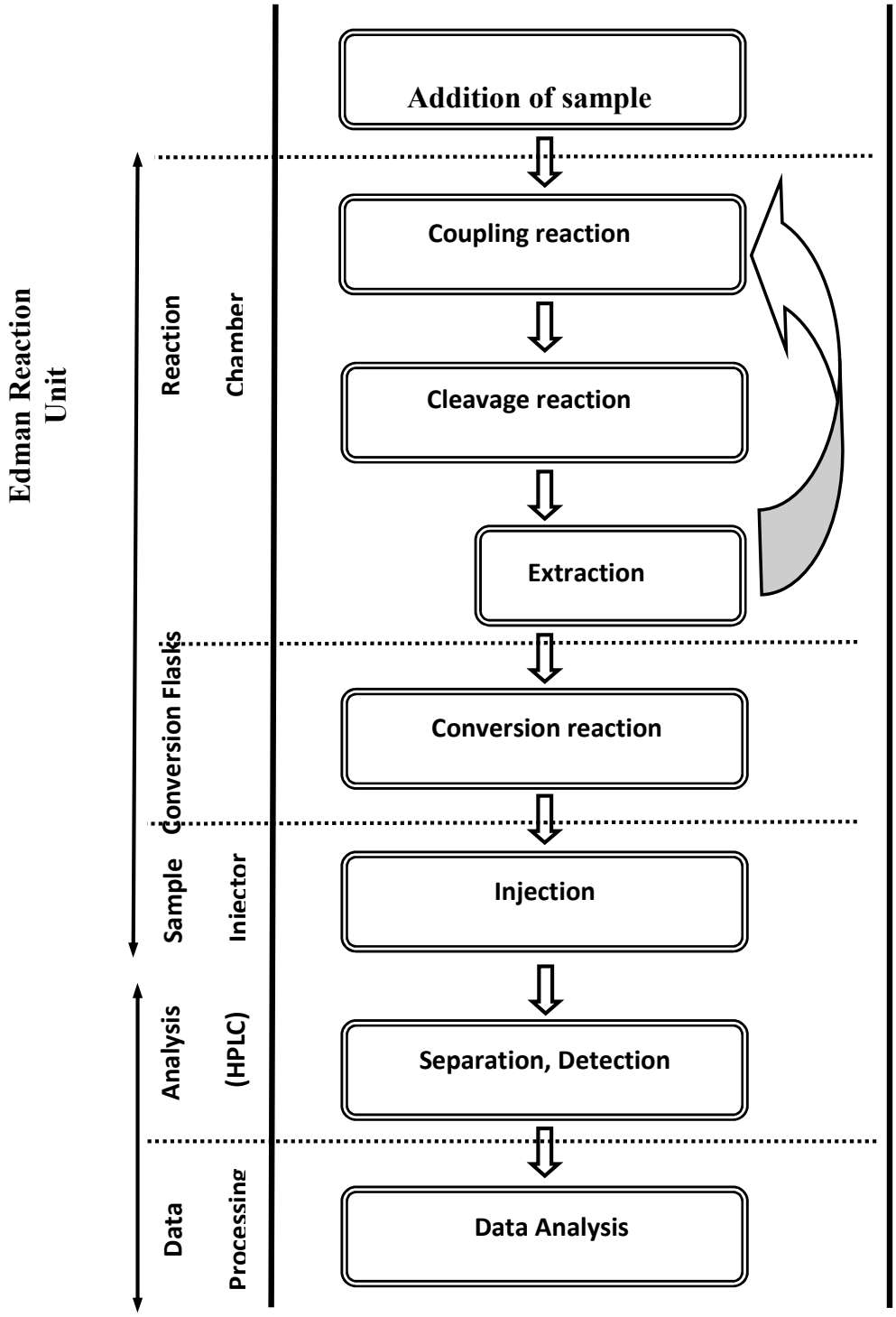


Fig. 2. 1. **Flow diagram of the reactions that occur during gas phase sequencing of protein or peptide on ABI-477A (Applied Biosystem) sequenator.**

The free ATZ-amino acid extracted from conversion flask by n-butyl chloride (S3) is converted to the more stable PTH-amino acid by reaction with 25 % TFA (R4). The PTH-amino acid dissolved in acetonitrile (S4) is automatically injected into the HPLC. The PTH-amino acids are separated by RP-HPLC and detected at 269 nm. The PTH-amino acid in each cycle is identified, quantified and recovery percentage calculated using the Seq. 2. 2 programme. The results displayed and recorded representing the separation profile are compared with the standard PTH-amino acid mixture profile. β-lactoglobulin was used as a standard protein for the performance check of the instrument.

2.2.14 Preparation of reagents for cloning and expression of proteins

All laboratory chemical reagents used were of molecular biology or AR grade or higher grade. All stock solutions and media were sterilized by autoclaving at 121 °C for 15 min at 15 lb pressure. Reagents and buffers not suitable for autoclaving were sterilized by filter sterilization using Millipore disposable sterile filters.

The following reagents were prepared according to Sambrook and Russel (2001), and sterilized at 121 °C, for 15 min at 15 lb pressure unless otherwise indicated.

A. 50× TAE: Tris-base (242 g), 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA dissolved in 1 L distilled water.

B. TE: Tris-HCl (10 mM), pH 7.5 and 1 mM EDTA.

C. 1 M Tris: Tris (12.1 g) was dissolved in autoclaved water and pH adjusted to 8.0 with 6 N HCl.

D. EDTA Solution (250 mM): Dipotassium salt of EDTA (9.3 g) was dissolved in 100 mL RNase free water.

E. Sodium acetate: (3 M pH 4.6 and pH 5.5) Sodium acetate trihydrate (40.81 g) was dissolved in water and adjusted to pH 4.6 or 5.5 as required with glacial acetic acid. Volume was made up to 100 mL and sterilized as described.

F. SDS (10 %): SDS (10 g) dissolved in 100 mL of RNase free water, was heated to 60 °C to completely dissolve SDS. Cooled and pH adjusted to 7.2 with 6N HCl. Stored at room temperature.

G. Luria-Bertani *(LB) medium:* Sodium chloride (5g), tryptone (10 g), yeast extract (5 g) dissolved in deionized water and pH adjusted to 7.0 with 5 N NaOH. Final volume was made up to 1 L with deionized water and sterilized.

H. 2× LB Media: Sodium chloride (1 g), tryptone (2 g), yeast extract (1 g) was dissolved in water, and pH adjusted to 7.0 with 5 N NaOH. Final volume was made up to 200 mL with deionized water and sterilized. Filter sterilized glucose solution was added to a final concentration of 0.2 %.

I. 2× YT Media: Sodium chloride (5 g), tryptone (16 g), yeast extract (10 g) dissolved in water, and pH adjusted to 7.0 with 5 N NaOH. Final volume was made up to 1 L with deionized water and sterilized.

J. LB agar: Tryptone (10 g) and yeast extract (5 g) sodium chloride (5g) were dissolved in water, and pH adjusted to 7.0 with 5 N NaOH. Final volume was made up to 1 L with deionized water then added agar (20 g), sterilized and poured into sterile petri dishes (25 mL/10 cm diameter plate).

K. Preparation of antibiotic: A stock solution of the sodium salt of ampicillin (100 mg/mL) was prepared in sterile water, filter sterilized and stored in aliquots at –20 ^{o}C.

L. LB plates with ampicillin: LB-agar medium was sterilized and cooled to 55 ^{o}C. Ampicillin was added to a final concentration of 100 µg/mL prior to plating.

M. Acid salt buffer: (100 mM $CaCl_2$, 70 mM $MnCl_2$, 40 mM sodium acetate) The pH of the sodium acetate solution was adjusted to 5.5 with acetic acid, the salts were added and final volume made to 100 mL and filter sterilized.

N. Preparation of SOB: Bacto-tryptone (20.0 g), bacto-yeast extract (5.0 g), sodium chloride (0.6 g), potassium chloride (0.19 g) dissolved in 1 L water. Magnesium sulphate and magnesium chloride (10.0 mM), added from 1.0 M stock. The magnesium salts were autoclaved individually before addition of SOB medium.

O. SOC: Filter sterilized glucose (1 M) solution was added to the SOB media to obtain a final concentration of 20 mM.

P. Preparation of IPTG: A stock solution of IPTG (1M) was prepared in sterile water, filter sterilized and stored in aliquots at –20 ^{o}C.

2.2.15 Isolation of genomic DNA

Genomic DNA was isolated from the defatted horsegram seed flour (Section 2.2.1) using a genomic DNA extraction kit following manufacturer`s protocol (Qiagen). Five hundred mg of defatted flour was used to isolate genomic DNA. The yield of purified genomic DNA was 1.65 $\pm$ 0.31 µg. This genomic DNA was used as the template for amplification of the target gene. DNA purity and quantity was determined as described below.

2. 2. 16 Quantification of DNA

The yield of DNA was determined spectrophotometrically at 260 nm, where 1 AU (A_{260}) equals 50 μg of double stranded DNA/mL. The purity was estimated from the relative absorbance at 260 and 280 nm. An A_{260} /A_{280} ratio of 1.8 for DNA is acceptable.

2. 2. 17 DNA agarose gel electrophoresis

The extracted DNA was evaluated by agarose gel electrophoresis. Depending on the percentage of the gel, agarose was weighed and added to required volume of 1× TAE, cooked in a microwave oven and cooled to 55 °C. EtBr was added to a final concentration of 0.5 μg/mL, mixed well and poured into the gel trough and allowed to solidify.

Samples (50 ng DNA) were mixed with sample buffer (6× DNA loading dye) and loaded in the well. The gel was electrophoresed for 45-60 min in 1× TAE buffer at 100V. DNA was visualized under a UV-transilluminator (Jencons, Scientific Ltd. Upland,CA).

2. 2. 18 Primer design

The oligonucleotide primers were designed based on the horsegram BBI protein sequence from our laboratory (Swiss-Prot Acc.No. Q9S9E3) and (GenBank Acc.No. AY049042). The primers synthesized were diluted to a final concentration of 1 nmol/μL with nuclease free water and stored at -20 ^{o}C and used for the further work. Primers were designed for the N-terminal and C-terminal sequence of HGI-III. (HGI-F 5` GAT CAT CAT CAG TCA ACT GAT GAG 3`and HGI-R 5` ATC ATC ATG TGA AGA TTT GCA AGG 3`). A second set of primers were designed for the HGI-III with restriction enzyme sites at 5` and 3` ends (HGI-*Nde*-F 5`CTA GCT AGC CAT ATG GAT CAT CAT CAG TCA3`

and HGI-*Bam*H1-R 5`CGC GGA TCC TTA ATC ATC ATG TGA AG3`). This adaptor with restriction sites helps in cloning of HGI into suitable vectors and finally the proper functional expression of recombinant HGI. The parameters considered during primer design were: the primer length ~ 18-30 nucleotides and a G/C content of 30-50 %. T_m was calculated using the formula $T_m = 2\ ^oC \times (A+T) + 4\ ^oC \times (G+C)$. Optimal annealing temperature was calculated as 5 ^oC below the estimated melting temperature. Complementary sequences within a primer sequence were avoided to reduce hairpin formation. Primers with A or T at 3' end were avoided.

2.2.19 Preparation of chemically competent cells

A single colony of the required *E.coli* strain (described in 2.1) was picked up and inoculated into 2 mL of 2× LB media. The culture was allowed to grow overnight at 37 ^oC. For inoculum, 0.5 mL of pre-inoculum was inoculated into 50 mL of 2× LB and allowed to grow until A_{600} was 0.5. The cells chilled on ice for 30 min were centrifuged gently at 4 ^oC. Supernatant removed and resuspended in half the culture volume with acid salt buffer. The cell suspension was incubated on ice for 45 min, centrifuged at 3000 rpm at 4 ^oC. The pellet was resuspended in 4 mL of acid salt buffer by gently mixing and 15 % ice cold glycerol (final volume) is added to this cell suspension. Cells were aliquoted in different tubes and stored at -80 ^oC.

2.2.20 Transformation in *E. coli* cells

For transformation, either plasmid DNA (50-100 ng) or the ligation mix [Plasmid (1 µg), PCR product (6 µg), 1 mM ATP, 10 units T_4 DNA ligase and assay buffer] was added to 100 µL of competent *E. coli* cells and incubated on ice for 45 min. Subsequently the cells were subjected to heat

shock at 37 ^{o}C for 5 min and then cooled on ice for 5 min. One mL of SOC medium was added to it. The cells were grown at 37 ^{o}C for 1 h and then plated on LB-agar plates containing 100 µg/mL of ampicillin for the selection of transformants and incubated at 37 ^{o}C for 12 to 18 h. Individual colonies were picked and screened.

2.2.21 PCR and cloning of HGI

Genomic DNA isolated from defatted horsegram flour was used as template for the PCR amplification of HGI-III gene using specific primers, HGI-F and HGI-R. PCR reactants used for the amplification are listed in Table 2.2.

The reaction mixture was heated to 94 ^{o}C for 1 min. Subsequently touchdown PCR was performed. Twenty cycles of denaturation at 94 ^{o}C for 15 sec, annealing at 54* ^{o}C for 30 sec whereby there will be a reduction of 0.5 ^{o}C during each cycle and extension at 72 ^{o}C for 20 sec. The next step was followed by 10 cycles of denaturation at 94 ^{o}C for 15 sec, annealing at 44 ^{o}C for 30 sec and extension at 72 ^{o}C for 20 sec was carried out. The product was identified by agarose gel electrophoresis. The obtained PCR product was purified. Restriction enzyme sites were engineered using HGI-*Nde*-F and HGI-*Bam*H1-R as primers and the purified PCR product as template. The reaction mixture was heated to 94 ^{o}C for 5 min, subsequently 30 cycles of denaturation at 94 ^{o}C for 40 sec, annealing at 52 ^{o}C for 40 sec and extension at 72 ^{o}C for 20 sec followed by a single cycle of final extension reaction at 72^{o}C for 10 min was carried out.

Table 2.2 The PCR reaction mixture for the amplification of HGI-III gene.

Reagents	Vol. (µL)
10× Taq polymerase buffer (with 7 mM $MgCl_2$)	2.0
25 mM $MgCl_2$	2.0
10 mM dNTPs	0.75
10 pMoles/µL HGI-F	1.0
10 pMoles/µL HGI-R	1.0
Taq polymerase	0.5
Genomic DNA (150 ng)	1.0
Water (sterile double distilled)	makeup to 20 µL

The 260 base pair PCR product obtained was sequenced and used for further studies. The PCR amplicon was ligated to pRSET C vector digested with *Pvu*II. The ligated product was transformed using competent *E. coli* DH5α cells to generate a plasmid designated as pRSET-rHGI. Restriction enzyme digestion and ligation of the DNA was performed as per the manufacturer's instructions.

2.2.22 Isolation of plasmid DNA

Discrete colonies were inoculated in 2 mL of 2× YT media containing 100 µg/mL ampicillin and grown at 37 ^{o}C for 20 h. The cells were harvested by centrifugation at 8000 rpm for 5 min. The cell pellet was resuspended in 200 µL P1 solution (50 mM Tris-HCl pH 8.0, 10 mM EDTA and 100 µg/mL RNase). The cells were lysed in 200 µL P2 solution (0.2 N NaOH and 1% SDS). Following lysis 200 µL P3 solution (3 M potassium acetate, pH 5.5) was added and centrifuged to remove the debris. DNA present in the

supernatant was precipitated by adding 0.8 volumes of isopropanol at 25 $^{o}\pm$ 2 ^{o}C, incubated for 45 min and centrifuged at 12000 rpm for 10 min to obtain the plasmid DNA. The pellet obtained was washed with 70 % ethanol to remove excess salts and air dried. The DNA was resuspended in sterile double distilled water and analyzed by agarose gel electrophoresis.

2.2.23 DNA sequencing reaction- Di-deoxy method

The PCR products and the plasmid DNA isolated were subjected to DNA sequencing**.** The PCR conditions for the DNA sequencing reaction are as described below (Table 2.3). The DNA sequencing reaction was carried out using the Big Dye^{R} terminator v3.1 cycle sequencing kit, (Applied Biosystems, USA) following the manufacturer`s instructions. Total reaction volume was 20 µL. Primer extension PCR was performed for the sequencing reaction. The reaction mixture was heated to 96 ^{o}C for 10 sec, annealing at 50 ^{o}C for 5 sec and extension at 60 ^{o}C for 4 min for 25 cycles. The reaction was cooled to 4 ^{o}C.

2.2.23.1 PCR cleanup for primer extension reaction

For each sequencing reaction, a 1.5 mL microcentrifuge tube containing the following: 2.0 µL of 3M sodium acetate pH 4.6, 50.0 µL of 95 % ethanol was used. The entire contents of each extension reaction were pipeted into the 1.5 mL microcentrifuge tube and mixed thoroughly. The tubes were vortexed and incubated at 25 $\pm$2 ^{o}C for 20 min and then centrifuged for 20 min at 13000 rpm. The supernatant was discarded and the pellet was rinsed in 70 % ethanol. The pellet was air dried and dissolved in 20 µL formamide, heat denatured and snap chilled. The reaction contents were transferred to the tube with septa for sequencing reaction. The

sequence of the inserted fragment was sequenced using an ABI 310 DNA Genetic Analyser (Applied Biosystems, Foster City, USA).

Table 2.3 The PCR reaction mixture used for Di-deoxy DNA sequencing reaction.

Reagents	Vol (µL)	Vol (µL)	Vol (µL)	Vol (µL)
Template DNA(500 ng of plasmid and 10 ng of PCR product)	1	1	1	1
Forward primer (10 pmol/µL)	0.5	0.5	-	-
Reverse primer (10 pmol/µL)	-	-	0.5	0.5
Ready reaction mix	4	4	4	4
2.5 × buffer	4	4	4	4
Sterilized water	10.5	10.5	10.5	10.5

2.2.24 Expression of rHGI

For bacterial expression of rHGI, the *E.coli* strains BL21 (DE3) pLysS and Origami (DE3) pLysS carrying an inducible T7 RNA polymerase gene were transformed with the plasmid construct PRSET C-rHGI. Bacterial culture of 250 mL 2× YT medium, containing 100 µg/mL of ampicillin was grown at 37 ^{o}C to an absorbance of 1.75 at 600 nm. IPTG was added to a final concentration of 0.3 mM and the culture further incubated at 37 ^{o}C for 4 h.

2.2.25 Preparation of purification matrices

2.2.25.1 Preparation of Sephadex G-50

The Sephadex based resins are prepared by cross-linking dextran with epichlorohydrin. The dextran based Sephadex types were supplied as dry powders. The Sephadex G-50 resins of 20-80 μm dry bead size have a swelling factor 9-11 mL/g of dry beads and fractionation range of 1.5 kDa to 30.0 kDa. Twenty-five grams of Sephadex G-50 dry powder was allowed to swell in 500 mL of 25 mM Tris-HCl buffer (pH 7.5) for 24 h. The beads were stirred slowly with a glass rod, allowed to settle and fines were decanted. The slurry was degassed to remove entrapped air bubbles prior to packing in a glass column (100× 2.1 cm) at a flow rate of 20 mL/h. Further all experiments on this column were performed at 12 mL/h flow rate and when not in use, the column was stored in buffer containing 0.02 % sodium azide.

2.2.25.2 Preparation of Trypsin-Sepharose

The ligand, trypsin was immobilized on Sepharose-4B. CNBr activated Sepharose-4B was used. Trypsin (TPCK) was coupled to the activated Sepharose-4B at alkaline pH.

Coupling of trypsin to activated Sepharose-4B: Ten g CNBr activated Sepharose-4B was transferred to 40 mL of coupling buffer containing trypsin (8 mg trypsin/mL of activated Sepharose beads). Coupling was initially done at 25 ± 2 °C for 2 h with constant shaking and later continued for 20 h at 4 °C. The supernatant was decanted carefully and residual active groups blocked by adding excess of a small primary amine, 0.2 M glycine (pH 8.0) for 2 h at 25 ± 2 °C after the coupling reaction. Uncoupled ligand and glycine were removed by washing alternatively with coupling buffer and 0.1 M sodium acetate (pH 4.0) containing 0.5 M NaCl.

Trypsin-Sepharose was stored in equilibrating buffer (0.05 M Tris-HCl (pH 8.2) containing 20 mM $CaCl_2$, 0.5 M NaCl and 0.02 % sodium azide as an antimicrobial agent.

2.2.26 Purification of rHGI

The supernatant (cell free extract) of expressed rHGI was applied to a Trypsin-sepharose column (12× 3.4 cm) pre-equilibrated with 0.1 M Tris-HCl pH 8.2 containing 0.1 M NaCl at a flow rate of 10 mL/h. The column was thoroughly washed with the same buffer until the A_{230} was zero. The bound rHGI was eluted with 0.2 M glycine-HCl pH 3.0 containing 0.1 M NaCl. Two mL fractions were collected and assayed for trypsin inhibitory activity (Section 2.2.2.1). The pH of the pooled trypsin inhibitor fraction was adjusted to 7.5 and dialysed against water and lyophilised.

2.2.27 Refolding of purified rHGI in solution

Refolding of the purified rHGI and mutants in solution were carried out as reported for CNBr cleaved recombinant soybean BBI (Flecker, 1987). Purified protein (15 mg) was unfolded in 0.24 mL of unfolding buffer (2M Tris/HCl pH 8.0, 0.2% EDTA, 6 M GuHCl and 2 M β-mercaptoethanol) for 18 h at 37 ^{o}C. Refolding was performed by adding the unfolded inhibitor dropwise into 240 mL of refolding buffer (0.08 M Tris-HCL, pH 8.0, 0.1 mM EDTA and 0.2 mM oxidised glutathione with vigorous stirring, for 18 h at 37 ^{o}C. The refolded sample was concentrated by ultrafiltration with a 5 kDa cut off cellulose acetate flat membrane on a Mini Labscale™ TFF system (Millipore Systems, Bedford, Massachusetts, USA), dialysed and lyophilized.

2.2.28 Site directed mutagenesis of rHGI

rHGI mutants K24A and D75A were constructed using QuikChange PCR-based mutagenesis procedure (Fisher and Pei (1997) with pET-rHGI (rHGI ligated to *EcoR*V site of pET20b vector) plasmid as template. K24A was constructed also with pRSET-rHGI as template and primers used are listed (Table 2.4)

Table 2.4 Oligonucleotide sequences used for cloning and site directed mutagenesis of rHGI

Primer	Oligonucleotide sequence
K24A-F	5`TGCGCATGCACAGCGTCAATCCCTCCTCAATGC3
K24A-R	5`GCATTGAGGAGGGATTGACGCTGTGCATGCGCA3`
D75A-F	5` AAATCTTCACATGCTGATCTCGAGCACCAC3`
D75A-R	3`TTTAGAAGTGTACGACTAGAGCTCGTGGTG5
HGI-*Xho*-R	5`CGCGGATCCTTA*CTCGAG*ATCATCATGTGAAG3’
Δ76-*Xho*-R	5`*CTCGAG*TTAATCATGTGAAGATTTGCAAGGTGC3`.

Plasmid DNA was used as the template (10 ng) in amplification reactions of 20 µl containing 1× *Pfu* DNA polymerase buffer, 20 pmoles of each primer, 10mM dNTP mix, 8mM MgSO4 and 1.5 U *Pfu* DNA polymerase. PCR conditions consisted of 5 min at 95 ^{o}C followed by 20 cycles of 95 ^{o}C for 30s, 65 ^{o}C for 50s, and 72 ^{o}C for 6 min. Methylated (parental) DNA was then degraded by addition of *Dpn*I (10 U) to each PCR reaction and incubating at 37 ^{o}C for 1 h, followed by transformation of *E.coli* strain DH5α. After transformant screening, selected recombinant plasmids were extracted by

alkaline lysis method and *E.coli* strain BL21 (DE3) pLysS was transformed for the expression of the mutant proteins. The mutants were further screened using the inhibitory assays.

Table 2.5 The PCR reaction mixture for the amplification of trypsin domain from rHGI.

Reactants	Vol. µL
10× Taq polymerase buffer (with 7mMMgCl_2)	2.0
25mM $MgCl_2$	1.0
10mM dNTPs	0.75
10pMoles/µL HGI-F	1.0
10pMoles/µL HGIT-R	1.0
Taq polymerase	0.5
Plasmid DNA	1.0
Water	Final volume 20

2.2.29 Amplification of trypsin inhibitory domain DNA from horsegram BBI

The trypsin inhibitory loop from horsegram BBI was amplified using rHGI as template. The contents of the final reaction volume are listed in the Table 2.5**.** The reaction mixture was initially denatured at 94 ^{o}C for 5 min. Subsequently 25 cycles of denaturation at 94 ^{o}C for 30 sec, annealing at 60 ^{o}C for 30 sec and extension at 72 ^{o}C for 30 sec was carried out. After the cycles final extention was at 72 ^{o}C for 7 min. The product was identified by agarose gel electrophoresis. The obtained PCR product was purified using a gel extraction kit (Qiagen GmbH, Hilden, Germany) following the manufacturer`s instructions and confirmed by sequencing.

2.2.30 Cloning and construction of recombinant plasmids for total prokaryotic expression of trypsin inhibitory domain (TID).

The purified amplicon with a stop codon at C-terminal was blunt end cloned into the *EcoR*V site of pET20b to generate a plasmid designated as rTID. Chemically competent *E. coli* DH5α cells were transformed with rTID. Positive clones harbouring the rTID were identified by a gel shift assay and PCR. The sequence of the clone rTID was confirmed by dideoxy sequencing (Section 2.2.23).

2.2.31 Expression of recombinant trypsin inhibitory domain (rTID)

E. coli BL21 (DE3) pLysS transformed with the expression vector rTID, was grown overnight at 37 ^{o}C in LB medium (10 mL) supplemented with ampicillin (100 µg/mL). One mL of the cells was diluted into 250 mL of 2× YT medium supplemented with ampicillin (100 µg/mL) and incubated at 37 ^{o}C in an orbital shaker (180 rpm) until the optical density at 600 nm reached 1.75±0.1. IPTG was added to a final concentration of 0.3 mM and the culture was further grown at 37 ^{o}C for 4 h in an orbital shaker. The cells were harvested by centrifugation at 8000 rpm for 15 min at 4 ^{o}C. The pellet was lysed in 35 mL 0.1 M Tris-HCl pH 8.2 by ultrasonication (Vibracell™, Sonics and Materials Inc. New Town, CT. USA). Both the supernatant and the pellet were evaluated for trypsin inhibitory activity as described earlier (Section 2.2.2.1). The supernatant containing the rTID was further purified.

2.2.32 Purification of the rTID

The cell free extract of rTID was applied to a Sephadex G-50 size exclusion chromatography column (100× 2.1 cm) pre-equilibrated with 0.025 M Tris-HCl pH 8.2 at a flow rate of 10 mL/h. Each fraction (2 mL) was

assayed for protein and anti-tryptic activity. The fractions exhibiting trypsin inhibitor activity were pooled, dialysed against water and lyophilised.

The lyophilised fraction was dissolved in 0.1 % TFA and purified by RP-HPLC (Waters Symmetry Shield^{TM} RP18 5µ 4.6 (i.d) × 150 mm column) using a binary gradient of 0.1 % TFA and 70 % acetonitrile in water containing 0.05 % TFA.

2.2.33 Determining the IC_{50} value for tryptase inhibition

Different concentrations of rTID were assayed against fixed concentration of human lung tryptase (Section 2.2.2.4). To determine the IC_{50} value log of % residual activity was plotted against varying inhibitor concentrations. The K_i was also deduced from the IC_{50} value following the equation $K_i = IC_{50}/1+ [[S]/Km]$ (Cheng and Prusoff, 1973).

2.2.34 In vitro stability of rTID and HGI-III to gastrointestinal proteases

Stability of pET20b-rTID and HGI-III to gastrointestinal proteases like pepsin and pancreatin was evaluated by incubating the protein with pepsin (1 mg/mL) for 2 h and pancreatin (1 mg/mL) for 4 h. The reaction was stopped by heat inactivation of the proteases. The residual trypsin inhibitory activity was determined as described above (Section 2.2.2.1).

2.2.35 Kinetic studies of trypsin, chymotrypsin and elastase

Kinetics of three serine proteases, trypsin, chymotrypsin and elastase were studied by estimating the liberated product, *p*-nitroanilide from the chromogenic substrates BAPNA, BTPNA and NAPNA respectively.

Varying concentrations of substrate, BAPNA were incubated with trypsin (50 µg) at 37 °C for 10 min. The reaction was stopped by addition of

30 % acetic acid. The absorbance of product, *p*-nitroanilide liberated was measured at 410 nm against an appropriate blank. The assay was repeated with varying inhibitor concentrations.

Similarly, inhibition of chymotrypsin was studied using BTPNA as substrate. The assay was repeated with varying concentrations of inhibitor.

The effect of varying substrate concentration (NAPNA) with elastase in the presence of fixed concentrations of K24A mutant was studied. The modes of inhibition and dissociation constants were evaluated from the double reciprocal (Lineweaver and Burk, 1934) and Dixon plots (Dixon, 1953) of the data.

2.2.36 Stoichiometry

To evaluate the stoichiometry, fixed quantity of trypsin/chymotrypsin was incubated with increasing amount of the inhibitors in presence of either BAPNA or BTPNA respectively. The residual protease activity was measured as described earlier (Section 2.2.2.1 and 2.2.2.2). The plot of I/E Vs % residual activity was used to evaluate the stoichiometry.

2.2.37 Thermal stability studies

Aliquots of the inhibitor solution (0.25 mL) containing 250 μg of the inhibitor (from a stock of 1 mg/mL in water) were incubated at 95 °C in water bath. Aliquots were removed at different intervals of time and suddenly cooled on ice. The trypsin/elastase inhibitor activity was assayed as described earlier (Section 2.2.2.1 and 2.2.2.3).

2.2.38 pH stability studies

The purified rHGI was dissolved in 0.1 M buffers of pH 3, 5, 7 and 8.2 and incubated for 1 h at 37 ^{o}C. The residual trypsin inhibitor activity was assayed with BAPNA as described earlier (Section 2.2.2.1)

2.2.39 Disulphide bond assay using 2-nitro-5-thiosulfobenzoate (NTSB)

The disulphide content of the rHGI was evaluated using NTSB as described earlier (Thunnhauser et. al, 1984). For the disulphide assay 1.27×10^{-8} mols of rHGI in 0.05 mL of buffer was added to 3.0 mL of NTSB assay solution and incubated at 37 ^{o}C. The change in absorbance at 412 nm due to the formation of 2-nitro-5 thiobenzoic acid (NTB) was followed for 60 min. Ribonuclease was used as the reference standard. The concentration of disulphide bonds was calculated using a molar extinction coefficient of 13600 M^{-1} cm^{-1} at 412 nm for NTB (Ellman 1959).

CHAPTER 3

Functional Expression of the major bowman-birk inhibitor of horsegram-HGI-III

The Bowman-Birk inhibitor, is a prototype of a family of small proteinase inhibitors seen in legumes and many aspects of its chemistry and physiology, such as anticarcinogenic properties, highly differential specificity towards human granulocyte elastase and its role as a potent biological insecticide are of potential interest in medicine and agriculture. The symmetrical pattern of disulfide bridges, rigidifies inhibitory domains of BBI type proteinase inhibitors into a polycyclic, clearly arranged and highly conserved structural framework. Despite the vast potential application of BBIC as therapeutics, reports on efficient production systems are currently limited to the expression of BBIs as fusion proteins. The functional expression of a recombinant BBI is therefore required not only for therapeutic applications but also as a promising model to study mechanism, specifically any molecular changes that might improve its efficacy. In this chapter the results of cloning, expression and biochemical characterisation of a pure and fully active recombinant HGI-III (rHGI), the major BBI of horsegram seeds are presented.

RESULTS

Construction of pRSET-rHGI

Horsegram dry seed powder was prepared and defatted using five volumes of carbon tetrachloride and extraction overnight at 25^{o}C ± 2^{o}C. Genomic DNA was extracted from the defatted flour using genomic DNA extraction kit following manufacturer's protocol (Qiagen, GmbH, Hilden, Germany). The extracted genomic DNA was analysed on 0.8 % agarose gel as described on Section 2.2.17 (Fig. 3.1).

Fig. 3.1 **Agarose gel electrophoresis of horsegram genomic DNA.** Lane 1: genomic DNA.

The yield and purity of the purified DNA was calculated. The A_{260}/A_{280} ratio was 1.87. The yield was 1.65 ± 0.31 µg of purified genomic DNA. Primers were designed based on the N-terminal and C-terminal sequence of HGI-III, the major BBI of horsegram and used to amplify the coding sequences of genomic DNA. The amplification was carried out as described in Section 2.2.21. The only product obtained following amplification of genomic DNA with primers HGI-F/R (Table 3.1) annealed at 54*- 44 ^{o}C was a fragment of ~228 bp (Fig 3.2).

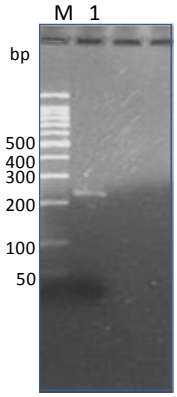


Fig. 3.2 **Agarose gel electrophoresis showing the 230-bp amplified product using the genomic DNA as template.** Lane M: 100 bp gene ruler; Lane 1: Amplification product.

A

5` GAT CAT CAT CAG TCA ACT GAT GAG CCC TCT GAG TCT TCA AAA CCA TGC TGT GAT CAG TGC GCA **TGC ACA AAG TCA ATC CCT CCT CAA TGC** CGC TGT ACG GAC GTT AGG CTG AAT TCG TGC CAT TCA GCT TGC AGT TCT TGT GTT **TGC ACA TTT TCG ATT CCT GCA CAG TGT** GTT TGT GTT GAC ATG AAG GAC TTC TGC TAC GCA CCT TGC AAA TCT TCA CAT GAT GAT 3'

B

D H H Q S T D E P S E S S K P C C D Q C A **C T K S I P P Q C** R C T D V R L N S C H S A C S S C V **C T F S I P A Q C** V C V D M K D F C Y A P C K S S H D D

Fig. 3.3 (A) **The nucleotide sequence of the amplified PCR product of HGI-III gene.** (B) **The translated sequence of the PCR product.** The region in bold indicates the sequence of inhibitory loops.

Table 3.1 **The list of oligonucleotides used for the amplification of HGI-III gene and construction of plasmid pRSET-rHGI**

Primers	Sequences
HGI-F	5`GATCATCATCAGTCAACTGATGAG3'
HGI-R	5`ATCATCATGTGAAGATTTGCAAGG3'
HGI-*Nde*-F	5`CTAGCTAGCCATATGGATCATCATCAGTCA3`
HGI-*BamH*I-R	5`CGCGGATCCTTAATCATCATGTGAAG3'

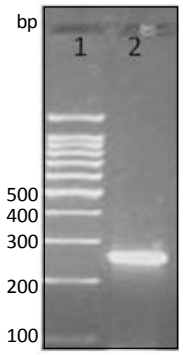


Fig. 3.4 **Agarose gel electrophoresis showing the 260-bp amplicon with the engineered restriction sites.** Lane 1: 100 bp DNA ladder; Lane 2: Amplification product (~260 bp).

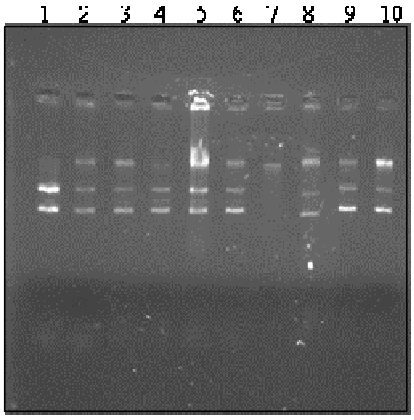


Fig. 3.5 **Agarose gel electrophoresis profile of plasmid DNA isolated from putative clones.** Lane 1: pRSET C vector control, Lanes 2-10: recombinant clones.

Dideoxy DNA sequence analysis of the purified PCR product (Fig. 3.2) revealed that this fragment covered the entire coding sequence of HGI-III indicating the absence of introns (Fig. 3.3 A). The deduced amino acid sequence (Fig. 3.3 B) agreed with that determined for HGI-III of horsegram (Prakash et al., 1996) and differed by a single amino acid from the GenBank sequence (Acc.No. AY049042). It has been reported that genomic clones of BBI isolated from soybean (*Glycine max)* also do not contain any introns

(Hammond et al., 1984). The vector pRSETC was digested with *Pvu*II restriction enzyme and digested product gel eluted. *Nde*I and *BamH*I sites were introduced to the 228 bp amplified product of HGI-III using primers HGI-*Nde*-F and HGI-*BamH*I-R. The 260 base pair PCR product (Fig. 3.4) encoding the 76 amino acids of HGI-III flanked by *Nde*I and *BamH*I was ligated into pRSETC digested with *Pvu*II using *T4* DNA ligase. The ligated product was transformed into chemically competent *E. coli* DH5α cells.

The ampicillin resistant transformants from LB-agar plates containing 100 µg/mL of ampicillin were picked up individually and screened for the presence of the 228 bp HGI gene. The plasmid DNA was isolated as described in Section 2.2.22 and analysed by agarose gel electrophoresis (Fig. 3.5). The plasmid DNA showing a mobility shift with respect to the control pRSET C vector DNA were picked up and screened for the release of HGI-III insert. The recombinants were confirmed by expression analysis, DNA sequencing and insert release by restriction digestion using *BamH*I (Fig. 3.6 A). The resultant vector named pRSET-rHGI habours an extra forty-four aminoacids at the N-terminal region. The forty-four amino acid stretch of the vector including the N-terminal $(His)_6$-tag prior to the coding sequence was removed by digestion with *Nde*I. *Nde*I site being introduced at the 5` end of HGI-III gene, restriction digestion would abet in the removal of the extra sequence. The digested product was allowed to self ligate and transformed into competent *E. coli* DH5α cells (Section 2.2.20) to generate the plasmid pRSET-rHGI (3025 bp) (Fig. 3.7). The transformants were identified by restriction digestion with *BamH*I. The plasmids that had lost the extra amino acids linearise whereas the ones which carried the extra amino acids will have an insert release (Fig. 3.6 B). The nucleotide sequence of the expression vector pRSET-rHGI (3025 bp) was determined, which confirmed the removal of the forty-four amino acids.

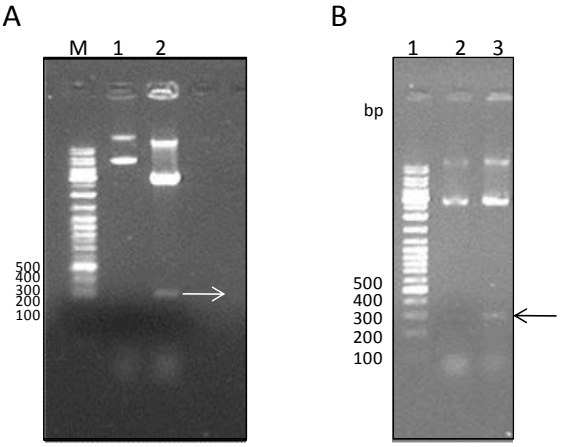


Fig. 3.6 **Agarose gel electrophoresis of the plasmid DNA** digested with *BamH*I. (A) Restriction digestion of pRSET-rHGI (3157 bps) Lane M: 100 bp ladder; Lane 1: pRSET-rHGI (3157); Lane 2: pRSET-rHGI (3157) digested with *BamH*I showing an insert release. (B) Restriction digestion of pRSET-rHGI (3025 bps) Lane 1: 100 bp ladder; Lane 2: pRSET-rHGI (3025); Lane 3: pRSET-rHGI (3157). The arrow indicates the released product.

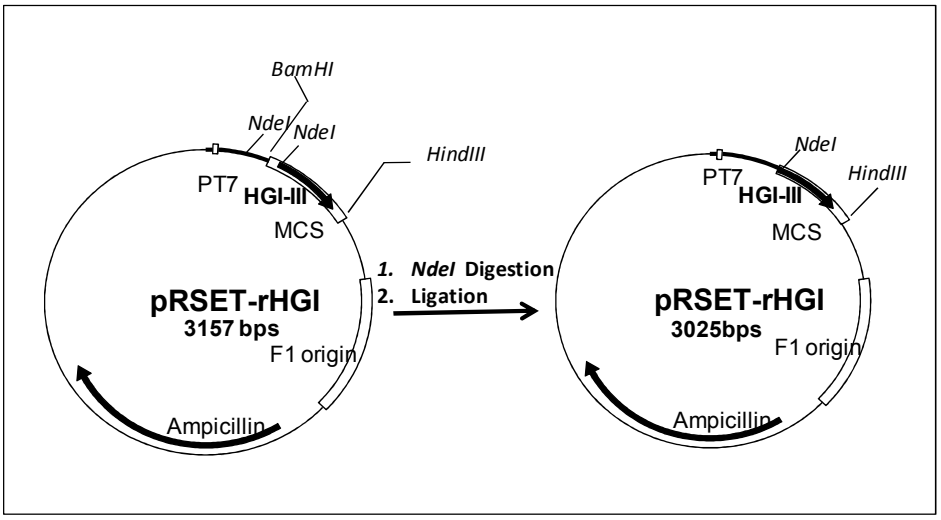


Fig. 3.7 **Schematic representation of the expression cassette in pRSETC vector to produce rHGI protein.**

Functional expression of rHGI

The expression vector pRSET-rHGI was transformed with *E. coli* BL21 (DE3) pLysS cells for the over-expression of rHGI (Fig. 3.8). The cells were harvested at 8000 rpm for 15 min at 4 ^{o}C and then lysed in 0.1 M Tris-HCl pH 8.2 by ultrasonication. The supernatant and pellet were analysed for inhibitory activity. The trypsin inhibitory activity was vested in the supernatant of the cell lysate. The trypsin inhibitory activity of the crude cell lysate from a 1 L culture was $3.5\pm0.14\times10^{5}$ TIU with a specific activity of $1.54\pm0.44\times10^{3}$ TIU/mg protein.

Purification of rHGI

The cell lysate was prepared in 0.05 M Tris-HCl (pH 8.2) containing 0.02 M $CaCl_2$, 0.1 M NaCl. Undissolved particulate matter was separated by centrifugation at 10, 000 rpm for 15 min at 4 °C. The clear supernatant obtained was loaded on to a trypsin-sepharose column (12 × 3.4 cm), pre-equilibrated with 0.1 M Tris-HCl pH 8.2 containing 0.1 M NaCl at a flow rate of 10 mL/h. The column was washed at a flow rate 30 mL/h with the same buffer until the A_{230} was zero. The bound rHGI was eluted with 0.2 M glycine-HCl pH 3.0 containing 0.1 M NaCl. Two mL fractions were collected and assayed for protein as well for the trypsin/chymotrypsin inhibitory activity. The elution profile indicated that rHGI eluted as a single peak when the pH was reduced to pH 3.0 (Fig.3.9). The active fractions were pooled as indicated, dialysed against water to remove the buffer ions and freeze-dried. The specific activity of the purified rHGI was $4.02\pm0.13\times10^{3}$ TIU/mg for anti-trypsin.

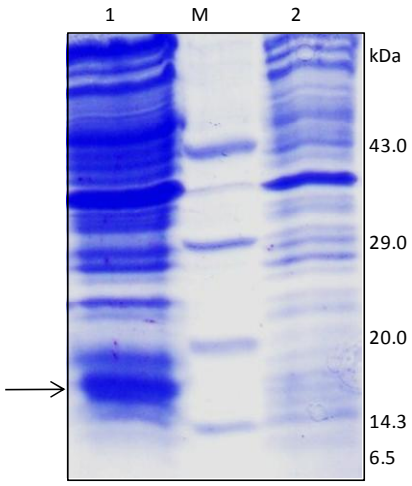


Fig. 3.8 **SDS–PAGE (15% T, 2.7% C) profile of cell free extracts of pRSET-rHGI.** Lane 1: rHGI induced with 0.3mM IPTG; Lane M: Molecular weight markers and Lane 2: uninduced rHGI. Arrow indicates expressed rHGI.

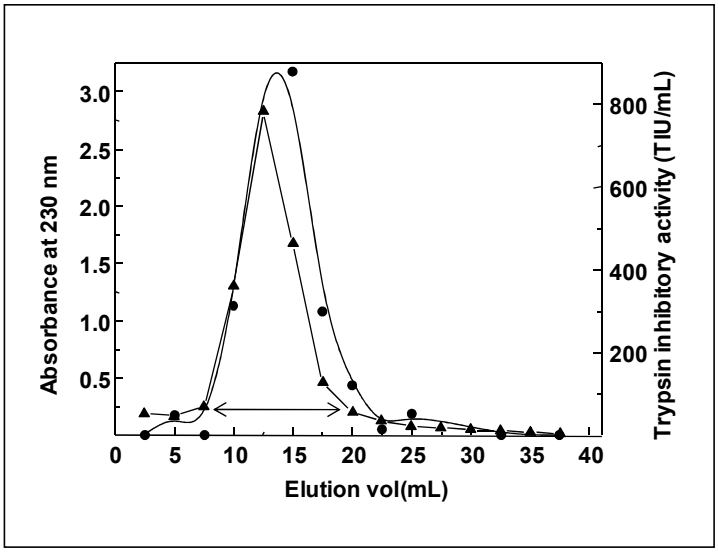


Fig. 3.9 **Trypsin sepharose affinity chromatography profile of rHGI**. The *E. coli* cell lysate was loaded on a trypsin sepharose column equilibrated with 0.1 M Tris-HCl buffer (pH 8.2) containing 0.1 M NaCl at a flow rate 30 mL/h. (–● –) TIU/mL, (–▲–) A_{230}. The bound protein was eluted by altering the pH with 0.2 M glycine-HCl pH 3.0 containing 0.1 M NaCl. The arrow shows the active inhibitor fractions that were pooled.

The chymotrypsin inhibitory activity of rHGI when assayed using the colorimetric substrate BTPNA was 100±0.15 CIU/mg protein, which was much lower than the seed HGI-III (4572 CIU/mg protein). It is plausible that the low chymotrypsin inhibition is due to improper folding. rHGI was therefore over expressed in the Origami strain of BL21 (DE3), a strain in which the mutations of both the thioredoxin reductase and glutathione reductase genes greatly enhance disulfide bond formation in the cytoplasm. The chymotrypsin inhibitory activity of the crude lysate increased to $2.9\pm0.13\times10^3$ CIU/mg protein. The yield of the purified protein was 1.0±0.2 mg/L culture medium, when compared to 9.8±mg/L for rHGI expressed in *E. coli* BL21 (DE3) pLysS. All further expressions were carried out using *E. coli* BL21 (DE3) pLys S.

Biochemical characterization of rHGI

Homogeneity of the purified rHGI was examined by SDS-PAGE, Native-PAGE, RP-HPLC and N-terminal sequence analysis.

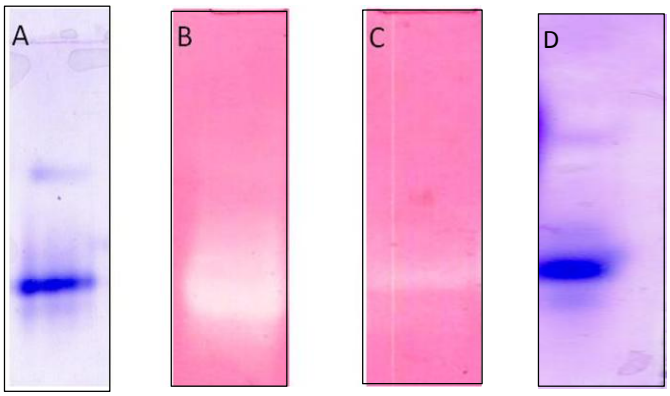


Fig. 3.10 **Native-PAGE (10% T, 2.7% C) profile of rHGI showing trypsin and chymotrypsin inhibitory activity.** (A) Gelatin-embedded native-PAGE. The gels were stained for (B) trypsin inhibitory activity and (C) chymotrypsin inhibitory activity with APNE and (D) protein

The purified inhibitor was electrophoresed on native PAGE (Section 2.2.6), pH 8.3 (10 % T, 2.7 % C) and detected by staining with CBB R-250. Native PAGE followed by incubation independently with either bovine trypsin or chymotrypsin showed that rHGI inhibited both the enzymes (Fig. 3.10 A, B & C). The recombinant inhibitor was homogenous and migrated as single species both by protein staining (Fig. 3.10 D) and by specific staining for trypsin inhibitory activity (Fig. 3.10 B) and chymotrypsin inhibitory activity (Fig. 3.10 C). The purified protein was dissolved in 0.1 % TFA and applied on an RP-HPLC column (Waters Symmetry Shield^{TM} RP18 150 mm × 4.6 mm (i.d), 5 µ column) and eluted using a binary gradient of 0.1 % TFA and 70 % acetonitrile in water containing 0.05 % TFA. RP-HPLC profile of the purified rHGI indicated that the protein was homogenous (Fig. 3.11).

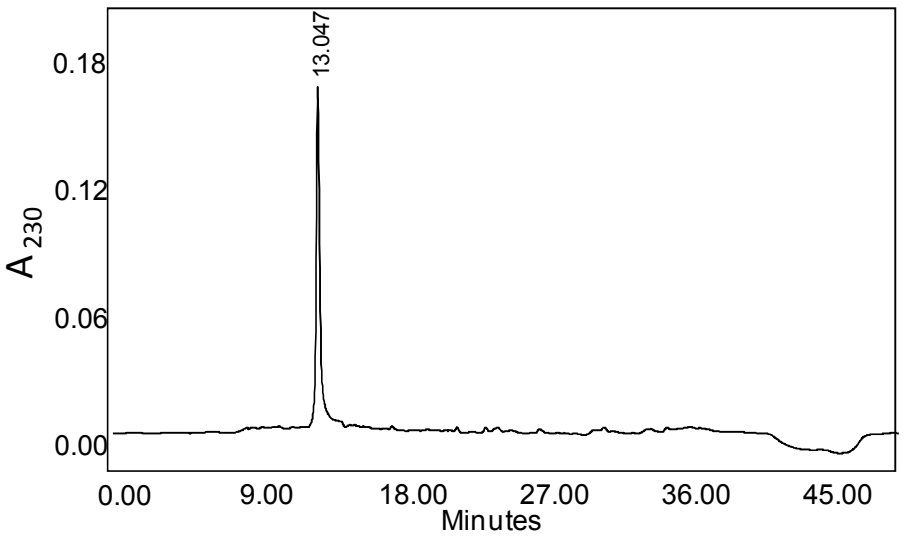


Fig.3.11 **RP-HPLC profile of purified rHGI using a binary gradient of 0.1 % TFA and 70 % acetonitrile in water containing 0.05 % TFA.**

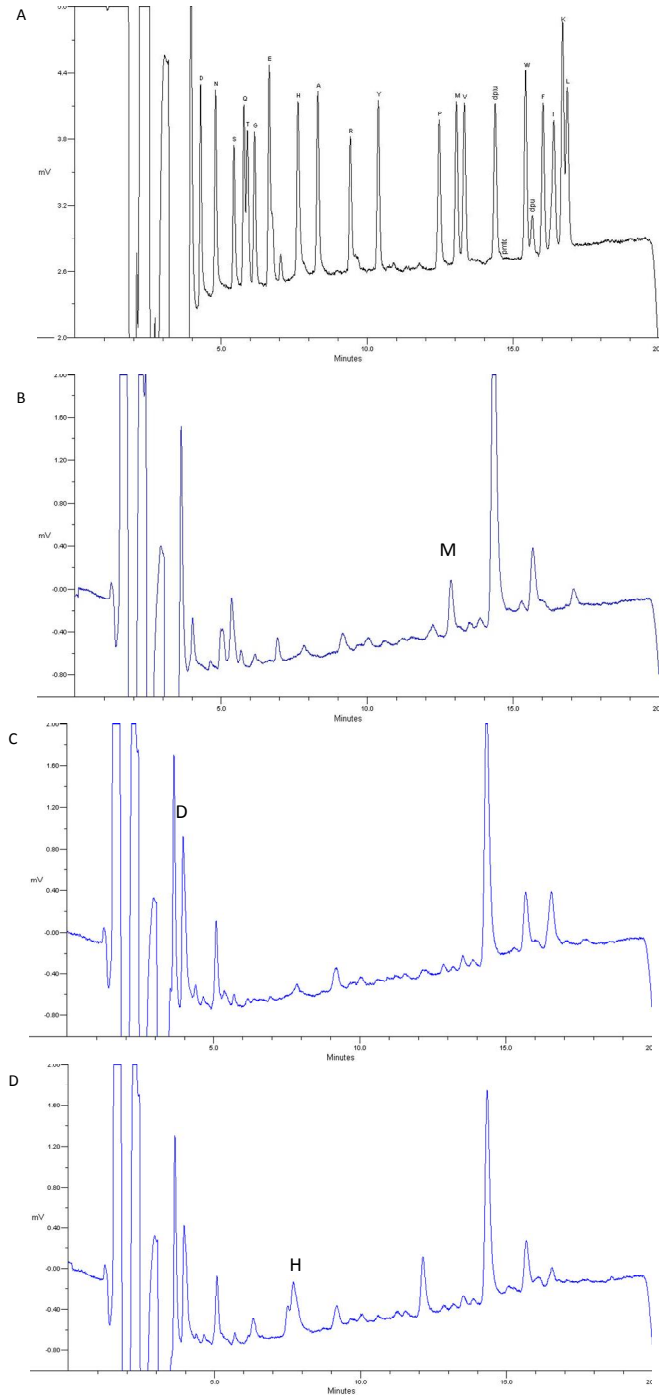


Fig. 3.12 RP-HPLC elution profile of the PTH- amino acids released from rHGI in the first six cycles of Edman degradation by automated gas phase sequencing. The eluted peaks were compared with the standard profile to deduce the sequence. (A) Standard PTH aminoacids; B-D cycles 1-3.

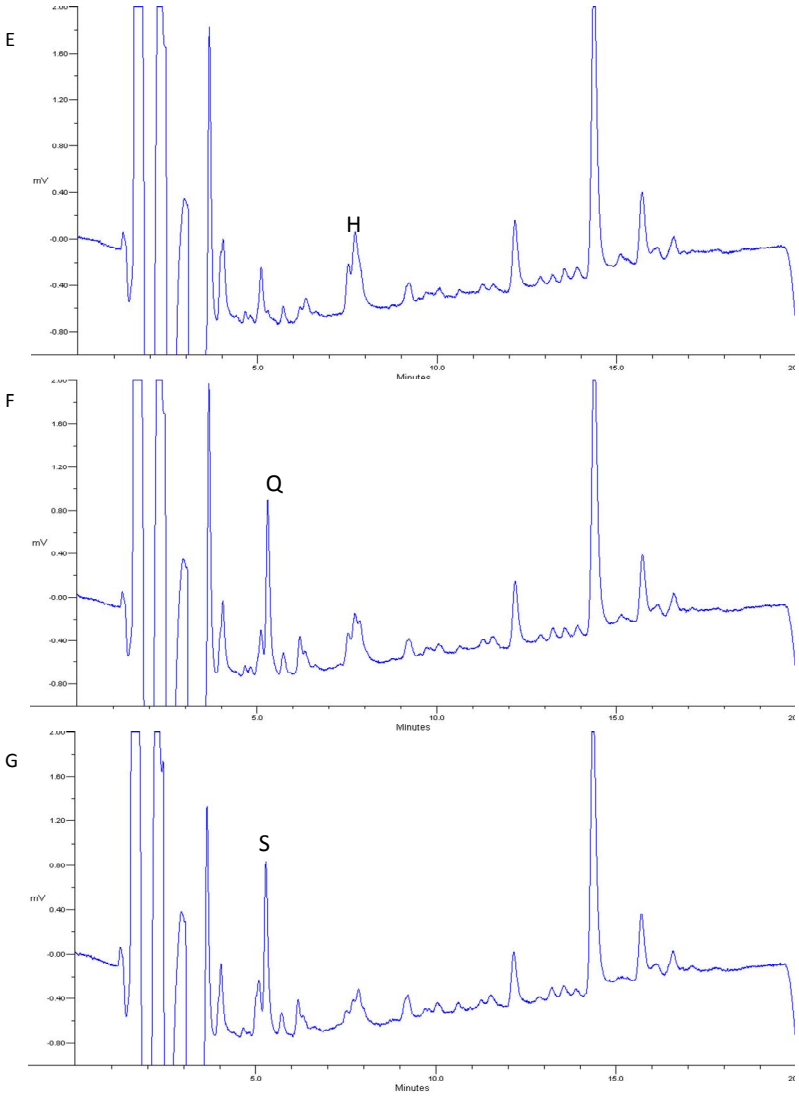


Fig. 3.12 continued

RP-HPLC elution profile of the PTH- amino acids released from rHGI in the first six cycles of Edman degradation by automated gas phase sequencing. The eluted peaks were compared with the standard profile to deduce the sequence. E-G cycles 4-6.

N-terminal sequence analysis

The purified rHGI was subjected to N-terminal sequence analysis on an automated gas phase sequenator. The release of a single N-terminal amino acid residue Met (M) for rHGI evidenced the purity of the inhibitor. N-terminal sequence analysis by Edman degradation showed the sequence to be NH_2- MDHHQSTDEP…….(Fig. 3.12) consistent with that reported for horsegram HGI-III and that of the translated sequence (Fig. 3.3 B).

Molecular weight determination

The apparent size of rHGI was evaluated by SDS-PAGE and size exclusion chromatography. The size of rHGI by SDS-PAGE was ~16 ±1.2 kDa (Fig. 3.13 Lane 3), which is consistent with its dimeric status in solution like the seed inhibitor (Fig. 3.13 Lane 1). Size-exclusion-HPLC on a BIOSEP-SEC-S 3000 column using 0.1 M Tris-HCl, pH 7.25 also revealed that rHGI eluted with a retention time of 9.293 min corresponding to a molecular mass of ~16 kDa, which was in close agreement to horsegram seed HGI-III (Fig 3.14). These results provide further evidence that rHGI like horsegram seed HGI-III in solution associates to form a dimer. This anomalous behaviour of legume BBIs is well documented. ESI-tandem MS indicated that the molecular mass of rHGI was 8676.925 Da (Fig. 3.15) and the protein showed an isotopic pattern of $(M+H)^{8+}$ charge state (Fig. 3.16). These results are in close agreement to that reported for seed HGI-III and consistent with the theoretical value (8.690 kDa) deduced from the translated amino acid sequence (Fig. 3.3 B).

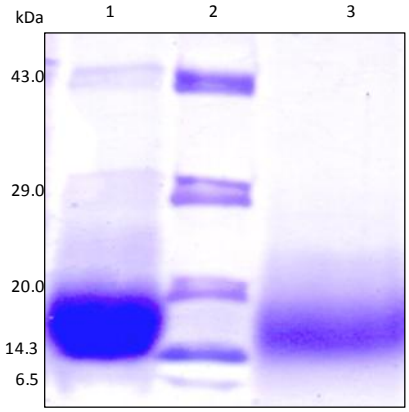


Fig. 3.13 **SDS-PAGE (15%T, 2.7%C) profile of purified HGIs.** Lane 1: Seed HGI-III, Lane 2: Molecular weight markers and Lane 3: purified rHGI.

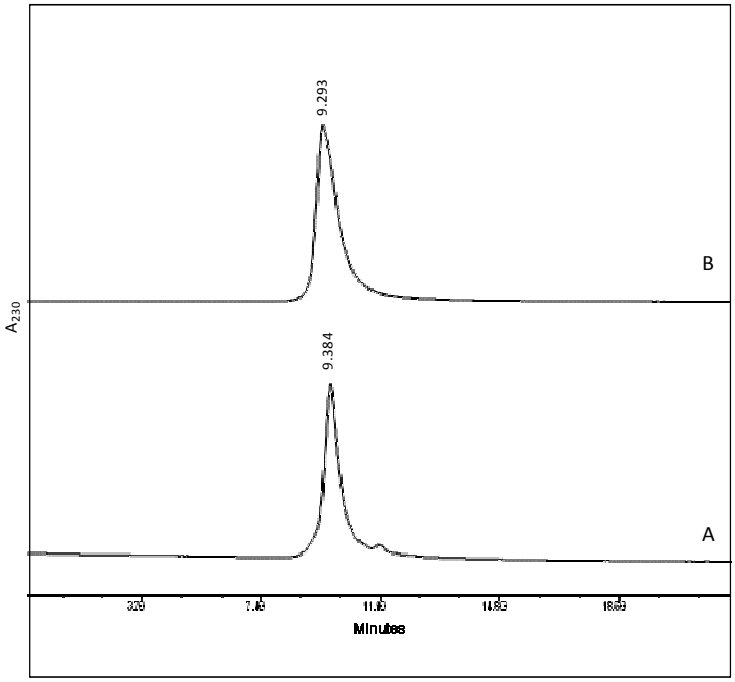


Fig. 3.14 **Size-exclusion chromatography of seed HGI-III and rHGI.** The purified proteins were dissolved in 0.1M Tris-HCl, pH 7.5 and loaded on to a BIOSEP-SEC-S 3000 column pre-equilibrated in the same buffer and eluted at 1 mL/min. (A) HGI-III; (B) rHGI.

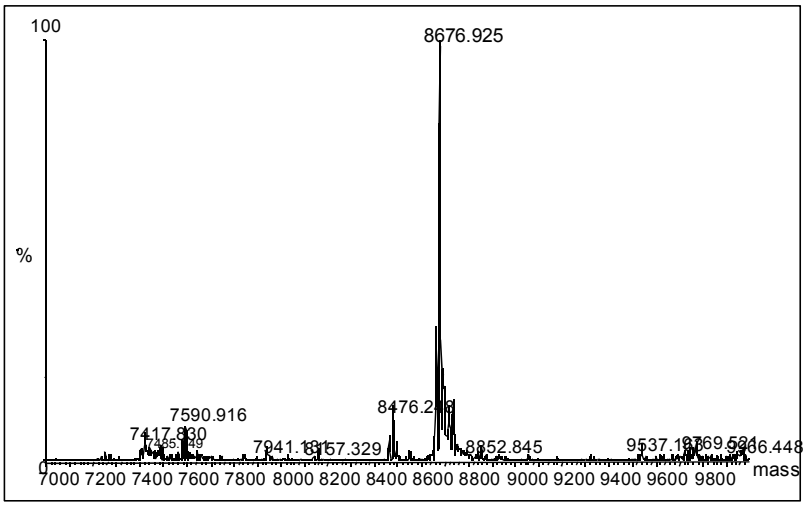


Fig. 3.15 **ESI mass spectrum of rHGI showing the molecular mass of 8676.925 Da.**

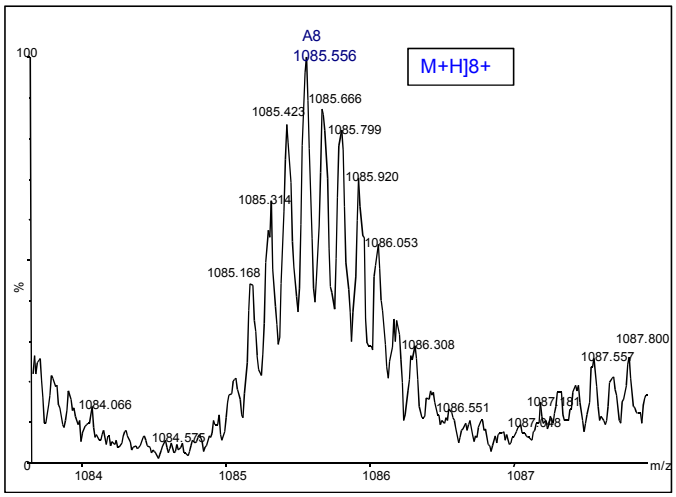


Fig. 3.16 **An expansion of the molecular peak of rHGI of Fig. 3.15 showing an isotopic pattern of $(M+H)^{8+}$ charge state.**

Estimation of disulphide bonds

Disulphide bridges are well conserved in BBIs. Therefore to estimate the number of disulphide bridges in rHGI, these bridges were initially cleaved quantitatively to free thiol using excess sodium sulphite, pH 9.5 at 37 ^{o}C. To denature the rHGI, guanidine hydrochloride (6 M) was added to the inhibitor solution, which makes the disulphide bonds accessible. The determination of free thiol groups released as a result of the denaturation of disulphide bonds was evaluated using NTSB (as described in section 2.2.39) in presence of excess sodium sulphite. The number of disulphide bonds reacted per molecule of the inhibitor, followed by the change in absorbance at 412 nm was calculated to be seven (Fig. 3.17). This corresponds to the presence of fourteen half cystine residues per molecule of rHGI. The number of disulphide bonds of the reference protein Ribonuclease A was also determined to test the validity of the analytical procedure, and found to be four which agrees well with the reported values (Fig. 3.17).

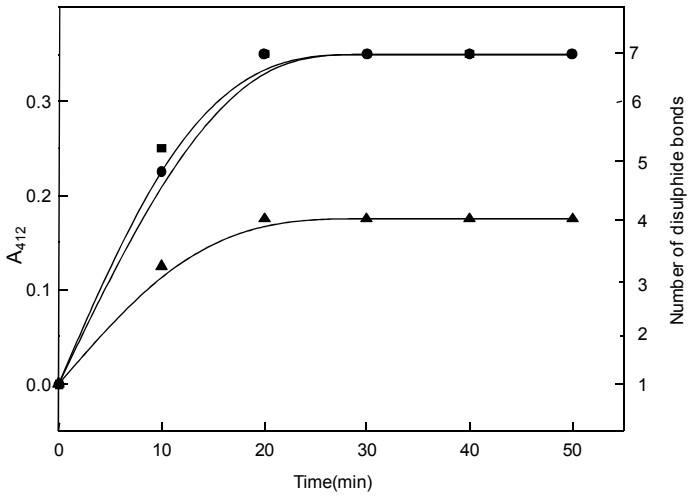


Fig. 3.17 **Assay for disulphide bonds of rHGI**. The assay was carried out by following the change in absorbance at 412 nm due to NTB produced in the reaction. (▲) Ribonuclease A, (●) rHGI, (■) HGI-III.

pH and thermal stability of rHGI

BBI proteins are stable to extreme conditions like heat and can withstand wide range of pH. To understand whether rHGI also retains these properties, pH and thermal stability studies were conducted. Preincubation of rHGI for 60 mins in the pH range of 3.0-9.0 had no effect on the trypsin inhibitor activity. Further 95 % of the inhibitor activity was retained at all pH studied (Fig. 3.18). The thermal stability studies of rHGI showed that heat treatment did not affect the trypsin inhibitory activity at 90 ^{o}C for 120 mins. At 100 ^{o}C, rHGI showed a 20% decrease in the activity after 120 min (Fig. 3.19). These results are in agreement to that reported for other legume seed BBIs (Richardson, 1991, Laskowski et al., 1980).

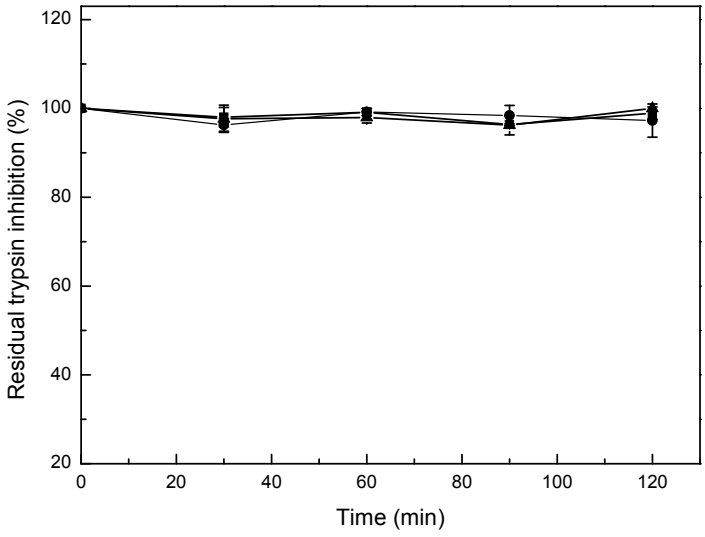


Fig. 3.18 **Effect of pH on the stability of rHGI**. (–■–) pH 3.0; (–● –) pH 5.0; (–▲–) pH 7.0. The residual trypsin inhibitor activity was determined.

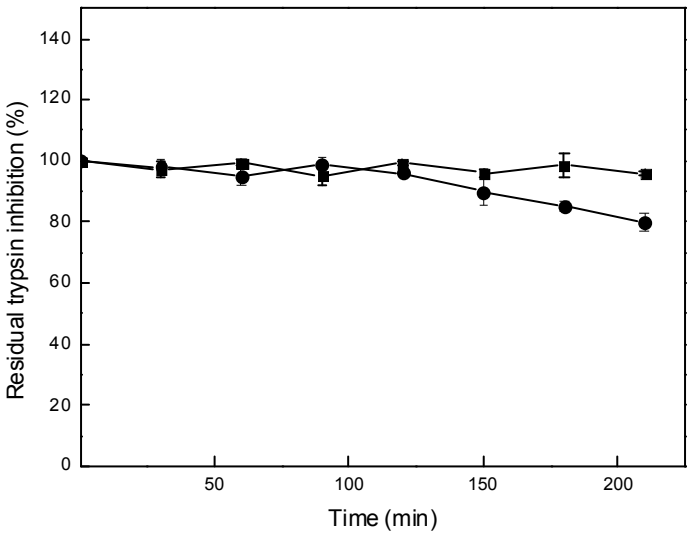


Fig. 3.19 **Thermal stability of rHGI**. (–■–) 90 ^{o}C and (–● –) 100 ^{o}C. rHGI was incubated at 90±1 ^{o}C and 100±1 ^{o}C. At regular time intervals the residual trypsin inhibitor activity was determined.

Inhibitory properties of rHGI

The stoichiometry of inhibition against bovine pancreatic trypsin and chymotrypsin was assessed using BAPNA and BTPNA respectively. Increasing concentrations of rHGI were incubated with a fixed concentration of the enzyme and the residual enzyme activity assayed. A linear extrapolation to obtain 100 % inhibition indicated that rHGI bound to trypsin in a 1:0.9 molar ratio (Fig. 3.20 A), whereas there was no obvious stoichiometry with chymotrypsin (Fig. 3.20 B) from the titration pattern of the inhibitory activity similar to the HGIs isolated from horsegram seed [Sreerama et al., 1997].

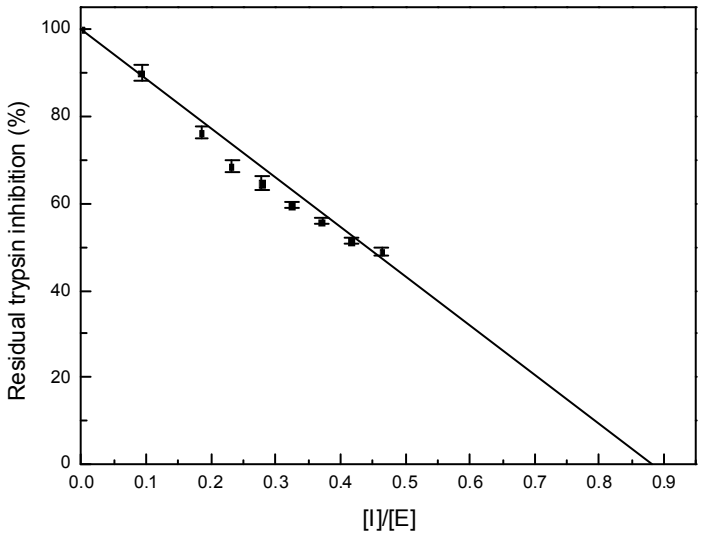


Fig. 3.20 (A) **Stoichiometric titration of bovine trypsin inhibition by rHGI**. Increasing quantities of inhibitor were added to a fixed concentration of enzyme (2.5 nM). Residual enzyme activity was determined using BAPNA. Each point is the average of three assays.

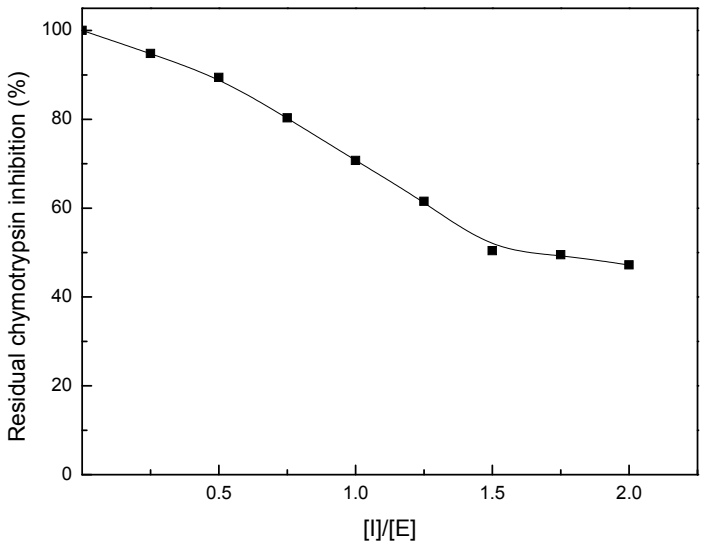


Fig. 3.20 (B) **Stoichiometric titration of bovine chymotrypsin inhibition by rHGI**. Increasing quantities of inhibitor were added to a fixed concentration of enzyme (5 nM). Residual enzyme activity was determined using BTPNA. Each point is the average of three assays.

The initial rates of reaction in the presence and absence of rHGI followed Michaelis Menten kinetics (Fig. 3.21). The mode of rHGI inhibition was evaluated from the double reciprocal plots (Fig. 3.22) of trypsin/chymotrypsin titrated with different concentration of their respective substrates. The results indicate that rHGI is a competitive inhibitor of both trypsin and chymotrypsin (Fig. 3.22). The apparent K_i for trypsin inhibition from Dixon plots of the same data was $5\pm0.15\times 10^{-8}$ M for rHGI (Fig 3.23 A &Table 3.2). The K_i of HGI-III purified from horsegram seed was $8.7\pm0.13\times10^{-8}$ M for trypsin whereas the K_i towards chymotrypsin was $3.9\pm0.16\times10^{-7}$ M. The inhibitory constant of rHGI for chymotrypsin ($3.4\pm0.12\times10^{-6}$ M) (Fig 3.23 B) was one order of magnitude below the activity of the natural inhibitor (Table 3.2). The consideration that this difference could result from some incorrectly folded protein coupled with the reported flexibility of the chymotrypsin domain prompted investigation on unfolding and refolding of rHGI.

Table 3.2: **Dissociation constants (Ki) of HGI-III and rHGI for trypsin, and chymotrypsin inhibition**

Inhibitor	Bovine trypsin	Bovine chymotrypsin
HGI-III	$8.7\pm0.13\times10^{-8}$ M	$3.9\pm0.16\times10^{-7}$M
rHGI	$5.0\pm0.15\times10^{-8}$ M	$3.4\pm0.12\times10^{-6}$ M
rHGI refolded	$6.1\pm0.13\times10^{-8}$ M	$3.0\pm0.15\times10^{-7}$ M

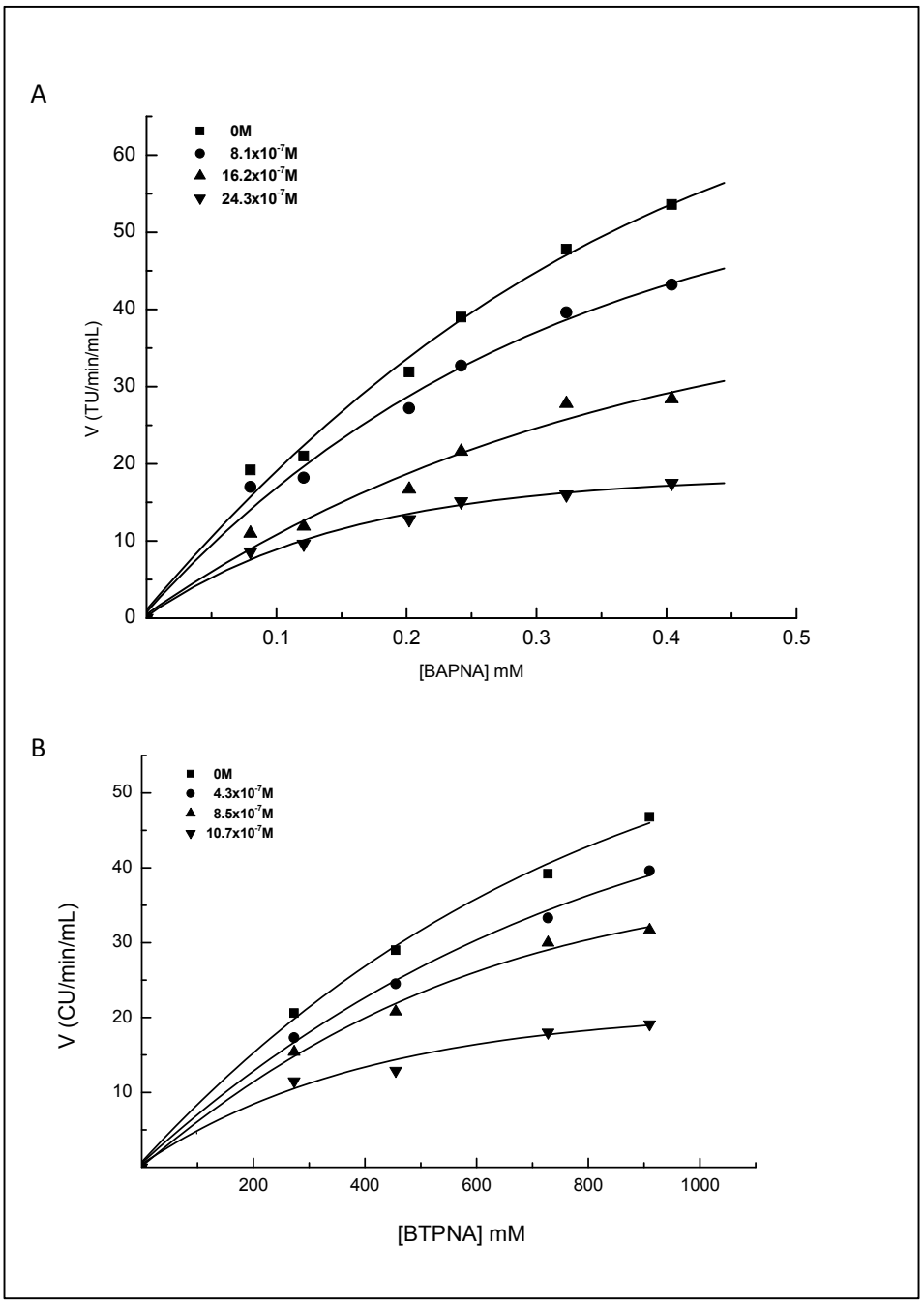


Fig. 3.21 **Michaelis-Menten plot** showing the effect of substrate concentration on the activity of (A) bovine trypsin (B) bovine chymotrypsin in the presence of various concentrations of rHGI. Data points are average of three determinations.

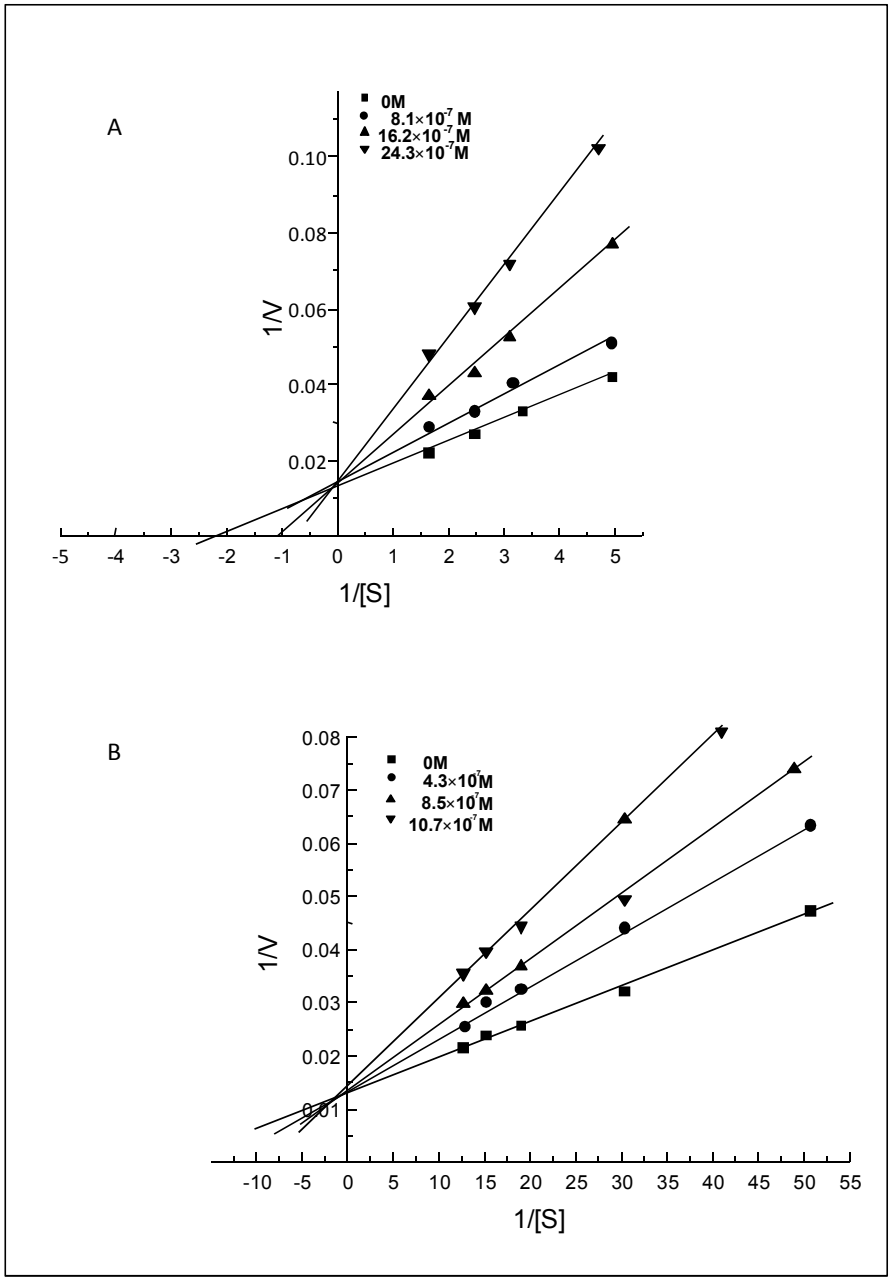


Fig. 3.22 **Lineweaver-Burk plot** of data from Fig. 3.21showing competitive inhibition by rHGI for (A) trypsin (B) chymotrypsin. Data points are average of three determinations.

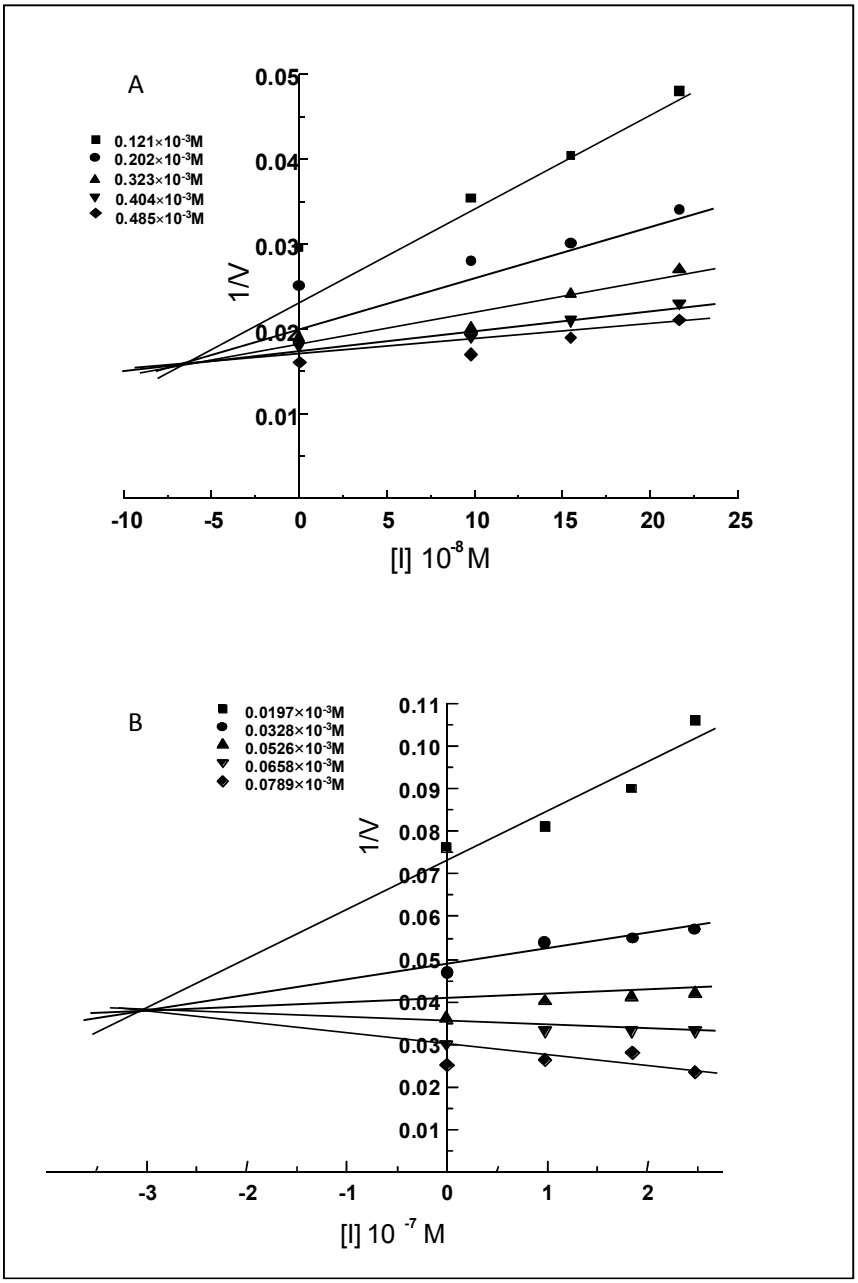


Fig. 3.23 **Dixon plot for determining the dissociation constant (K_i) of rHGI.** (A) bovine trypsin. (B) bovine chymotrypsin. Data points are the average of three determinations.

Refolding of the purified rHGI in solution was carried out as reported for CNBr cleaved recombinant soybean BBI (Flecker, 1987). Refolding of rHGI led to an increase in one order of magnitude for the chymotrypsin inhibitory constant (Table 3.2) in close agreement to the natural inhibitor. In contrast refolding had very marginal effect on the K_i for trypsin inhibition (Table 3.2). These K_i values thus establish a very high affinity between these proteases and rHGI, close in agreement with the K_is reported for other legume BBIs.

Simultaneous and independent inhibition of trypsin/chymotrypsin by rHGI

The simultaneous inhibition of trypsin and chymotrypsin by rHGI were evaluated. Competitive inhibition studies were performed with the purified rHGI. In the first experiment, the chymotrypsin inhibitory activity was measured in the presence of varying amounts of trypsin. Initially rHGI was incubated with various concentrations of trypsin (0.5 µM to 3 µM) for 10 min at 37 °C and inhibition of chymotrypsin measured using BTPNA as substrate. Similarly, after incubating the inhibitor with various concentrations of chymotrypsin (0.5 µM to 3 µM) for 10 min at 37 °C, the effect on the inhibition of trypsin was measured using BAPNA as substate. The competitive studies revealed that rHGI inhibits chymotrypsin even in presence of trypsin (Fig. 3.24) and viceversa (Fig. 3.25). The competitive binding studies point towards the independent and simultaneous binding of rHGI to the cognate enzymes. These results are consistent with that of the HGI-III purified from horsegram seeds, which is double-headed inhibiting trypsin and chymotrypsin independently and simultaneously (Sreerama et al., 1997).

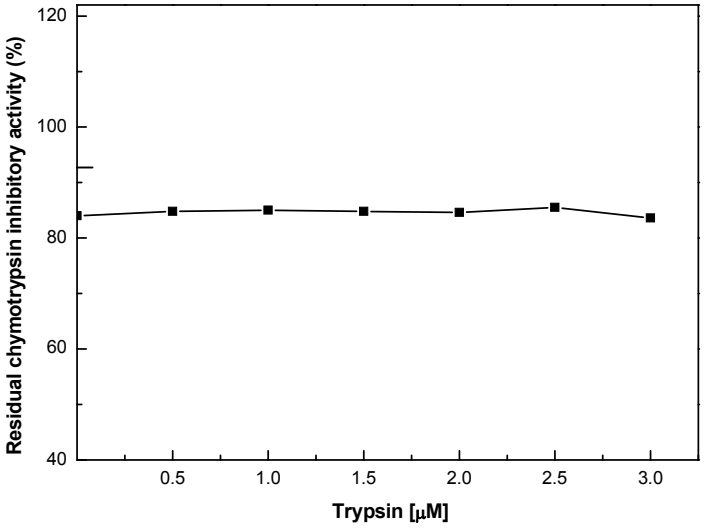


Fig.3.24 **Chymotrypsin inhibition by rHGI in the presence of varying concentrations of trypsin.** Data points are average of three determinations.

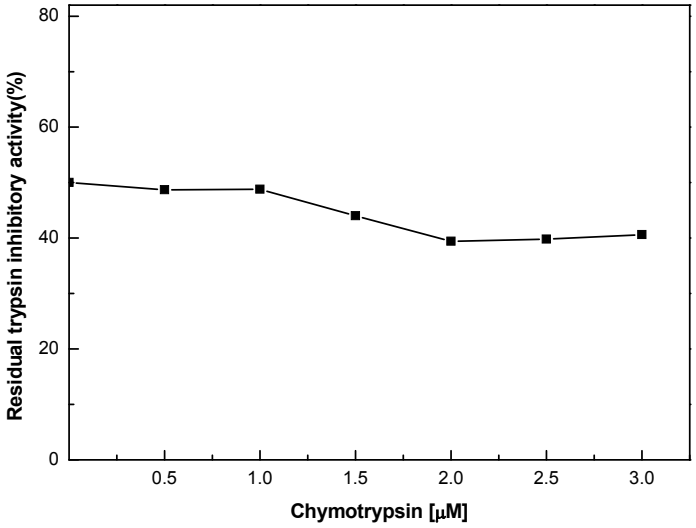


Fig.3.25 **Inhibition of trypsin by rHGI in the presence of varying concentrations of chymotrypsin.** Data points are the average of three determinations.

Cross reactivity of rHGI with anti HGI-III

Cross-reactivity of the rHGI was studied using antibodies raised against HGI-III, the major inhibitor present in the dormant horsegram seeds (Sreerama and Gowda, 1997). The purified rHGI was immobilized on nitrocellulose membrane and subjected to immuno-detection. The blot was developed with a solution of BCIP and NBT to ascertain the cross reactivity. Positive antigen-antibody reaction was detected with rHGI (Fig. 3.26). HGI-III served as the positive control and BSA served the negative control, which showed no cross reactivity (Fig. 3.26).

Fig. 3.26 **Dot-Blot analysis of recombinant horsegram inhibitor using antibodies raised against HGI-III**. (1) rHGI (2) BSA and (3) HGI-III.

Discussion

Protease inhibitors are found in various plants and have been studied in Leguminosae, Gramineae and Solanaceae. The Bowman-Birk family and Kunitz family of proteinase inhibitors discovered first in soybean seeds have been studied extensively. BBIs are classified as double-headed inhibitors because the protein contains two reactive site domains within the same polypeptide. BBI`s are recognised to have a trypsin (Lys-Ser) and a chymotrypsin (Phe/Leu-Ser) inhibitory site (Birk, 1961), or two trypsin (Arg-

Ser) inhibitory sites (Hwang et al., 1977). The occurrence of multiple low molecular weight protease inhibitors among members of the legume family (Chu and Chi, 1965; Wilson and Laskowski, 1973) as well as distantly related plants (Bryant et al., 1976) suggests that the genes may have been conserved throughout the evolution of several plant species. Isolation of the genes encoding these protease inhibitors have provided information on their number and organization as well as probes for studying the regulation of their expression. Hammond et al., (1984) constructed and characterized a cDNA clone corresponding to the BBI. DNA sequence analysis confirmed that this sequence corresponds to the BBI mRNA. Although there is extensive sequence homology between the BBI cDNA and genomic clones, they differ slightly in their deduced amino acid sequences (Hammond et al., 1984). The gene size and coding regions of BBIs are small and devoid of introns (Boulter, 1993). The availability of these inhibitors may allow engineering of transgenic crops inherently resistant to these insect pests. These aspects therefore encourage the identification and characterization of novel protease inhibitor genes from diverse plant species. Besides their natural biological functions, protease inhibitors might also be useful in treating human pathologies such as inflammation, haemorrhage (Oliva et al., 2000) and cancer (Kennedy, 1998).

Recently, with the rapid development of biotechnology many studies on gene cloning and the expression of protease inhibitors have been reported (Li et al., 1999, Chen et al., 1997). A putative rice trypsin/chymotrypsin inhibitor of the Bowman–Birk family, about 20 kDa, was expressed in *E. coli* as a fusion protein bearing an N-terminal (His)6 purification tag (Li et al., 1999). To reveal the inhibitory activities towards proteases and tumour cells, the open-reading frame of the buckwheat trypsin inhibitor gene was chemically synthesized and expressed in *E. coli*

M15 (Zhang et al., 2007). The cDNA cloning of BBI from *Apios americana* tubers also has been reported (Zhang et al., 2008). The reports on the cloning and expression of soybean BBI also demonstrates that in almost all the strategies used, the clones were expressed as fusion proteins and required an activation process for the functional expression. The results presented here demonstrate the functional expression of the major BBI, HGI-III of horsegram seeds and the biochemical characterisation of the recombinant inhibitor. The expression and purification of HGI-III has not been previously reported although the isolation and purification of four BBIs from horsegram seeds and its biochemical characteristics have been documented (Sreerama et al., 1997, Prakash et al., 1996, Sreerama and Gowda, 1997, Kumar et al., 2004). In this study, the amplified DNA fragment produced by PCR reaction using genomic DNA isolated from horsegram seed as template was of expected size (~228 bp) covering the entire HGI-III gene. The DNA sequence analysis of the plasmid (pRSET-rHGI) indicated a coding region of 228 bp devoid of introns. None of the BBI genes sequenced so far has any introns; the isolated sequenced cowpea trypsin inhibitor gene is also devoid of introns (Lawrence et al., 2001). The amino acid sequence deduced for rHGI was in agreement to the amino acid sequence determined earlier (Prakash et al., 1996). rHGI was over expressed in a soluble form in *E. coli* BL21 cells (Fig. 3.8). The target rHGI could be easily purified by a single step trypsin affinity chromatography utilising the strong binding potential to trypsin (Fig. 3.9). Unlike other BBIs that have been expressed as fusion proteins, rHGI was expressed devoid of any fusion tags. The strategy used here in creating rHGI without the extra sequence (His tag fusion) could be used for other small proteins to avoid the interference of the fusion tags in the function, stability and other molecular interactions of the expressed proteins. Fusion tags from the vector have

been reported to interact and interfere with the thermal stability and oligomerisation status in the case of coiled coil proteins (Xu et al., 2002). The purification strategy used in our study yields ~98 % pure recombinant protein (Fig. 3.9) in a single step purification. The requirement of other proteases to cleave the fusion tag proteins, a strategy used in most of the recombinant proteins was avoided in the present study used. This was made possible by utilising the strong binding property between rHGI and trypsin for purification.

The molecular mass of rHGI was 8.676 kDa (Fig. 3.15) and the purity was >98 % (Fig. 3.11). Further the inhibitory activity assays suggested that rHGI like the natural seed HGI-III strongly inhibited both bovine trypsin and chymotrypsin (Fig. 3.10). The BBIs of horsegram have two separate reactive sites for inhibition of trypsin and chymotrypsin and are independent of each other. Soybean C-II inhibits both trypsin and chymotrypsin at a single reactive site (Odani and Ikenaka, 1977). The BBI, A-II isolated from peanut inhibits trypsin at two reactive sites, of which one site can also inhibit chymotrypsin (Norioka et al., 1982). The stoichiometry of inhibition against bovine pancreatic trypsin and chymotrypsin was assessed using BAPNA and BTPNA respectively. Inhibition of trypsin at fixed concentrations was studied by varying the rHGI concentrations to deduce the stoichiometric relation. Although a near 1:1 stoichiometry was observed for trypsin no such stoichiometry was obtained for chymotrypsin. No obvious stoichiometry for chymotrypsin binding was also observed in BBIs isolated from other legumes (Terada et al., 1994a; Norioka et al., 1982; Kimura et al., 1994; Godbole et al., 1994).

The competitive binding studies of rHGI with bovine trypsin and chymotrypsin revealed the simultaneous and independent inhibition of rHGI

towards these proteases similar to that reported for HGI-III (Fig. 3.24 and Fig. 3.25). The Adzuki bean inhibitor II inhibits trypsin and chymotrypsin at different sites, but its complex with chymotrypsin fails to give any trypsin inhibitory activity (Yoshikawa et al., 1979). Five BBIs from peanut (*Arachis hypogaea*) (Norioka et al., 1982) inhibit bovine trypsin and chymotrypsin at molar ratios of 1:2 and 1:1 respectively, yet the inhibition of trypsin and chymotrypsin are not independent of each other. Tur-Sinai et al., (1972) reported that the complex of their peanut inhibitor with trypsin lacked chymotrypsin-inhibiting activity and the complex with chymotrypsin no longer had trypsin inhibiting activity. The dissociation constant of rHGI and refolded rHGI for trypsin were similar to that reported for natural HGI-III (Sreerama et al., 1997). In contrast the inhibition towards chymotrypsin was one order of magnitude below the activity of the natural inhibitor. This difference was due to incorrect folding as reckoned by the decreased K_i values after refolding (Table 3.2). The dissociation constant for chymotrypsin of the refolded rHGI was $3\pm0.15\times10^{-7}$ M in close agreement to the natural HGI-III (Fig. 3.23). A chemically synthesised soybean BBI gene cloned and expressed as a β-galactosidase fusion protein also showed that the dissociation constant of complexes with trypsin are similar to the natural BBI (Flecker, 1987). Prokaryotic expression of a rice BBI shows that the fusion protein has trypsin inhibitory activity but lacks chymotrypsin inhibitory activity (Qu et al., 2003).

The similarity of dissociation constants of rHGI and seed HGI-III accompanied by competitive inhibition of trypsin and chymotrypsin (Fig.3.22) affirms that the intramolecular disulphide bridges are in the correct orientation. In addition the extreme thermal (Fig. 3.19) and pH stability (Fig. 3.18) exhibited by rHGI indicates the correct disulphide formation. Further the experimental measurements of seven disulphide

bonds in rHGI are commensurate with the theoretical values (Fig 3.17). BBIs are extra ordinary proteins which are able to survive the cooking and digestive process and reach the colon in the active stage. The thermal and pH stability study shows that the expressed BBI also retained this capability. Considering the positive therapeutic benefits of BBIs and their relative stability at cooking temperature (Birk, 1985) and the acidic digestive system in humans (Yavelow et al., 1983), BBI levels have been determined in commercial foods (Blanca et al., 2009). Soybean BBIs are reported to survive faecal fermentation and are active anticarcinogens (Marin-Manzano et al., 2009). Polyclonal antibodies raised against the major inhibitor, HGI-III cross reacts with the rHGI (Fig. 3.26) suggesting that the antigenic determinants are similar in both. Data on the cloning and expression of legume BBI are sparse. The gene for Buckwheat trypsin inhibitor was cloned and expressed in *E. coli* and shown to specifically inhibit the proliferation of IM-9 human β lymphoblastoid cells in a dose dependent manner, but the dissociation constants were not determined (Zhang et al., 2007). Using chemical DNA synthesis, a fully active recombinant soybean BBI (Flecker, 1987) was expressed in *E. coli* and a cDNA coding for lentil trypsin/chymotrypsin BBI expressed in the methylotrophic yeast *Pichia pastoris* was functionally active (Caccialupi et al., 2008).

The rHGI inhibitor similar to that of dry seed inhibitor exhibited molecular masses of ~16 kDa by SDS-PAGE and analytical gel filtration, although ESI-MS analysis showed them to have a mass of ~ 8 kDa. This large overestimation of molecular weights has been attributed to the legume BBIs existing in a state of equilibrium between monomer-dimer-trimer forms, the dimer being the predominant form. (Wu and Whitaker, 1990; Terada et al., 1994a; Godbole et al., 1994). However such an overestimation of the molecular mass was not observed in germinated HGIs suggesting that they

exist only as monomers (Kumar et al., 2002). Similarly an in vitro synthesised BBI and related soybean inhibitor also exhibits the phenomenon of self association (Foard et al., 1982). In onion trypsin inhibitor, the molecular mass is 7.6426 kDa with respect to MALDI-TOF analysis but SDS-PAGE analysis shows a molecular mass of 18 kDa and 8 kDa in the absence of reducing agent whereas the inhibitor may exist as a mixture of monomer and dimer (Deshimaru et al., 2003).

The BBIs are promising models for studies on protein-protein interactions and to clearly distinguish between structural and functional aspects using recombinant DNA techniques. Data on the cloning and expression of BBIs is limited. The cloning and heterologous expression of a functional rHGI provides a platform for production and systematic alteration of amino acid residues to expedite the stability, mechanism of action and unveil the fine specificity. The approach presented here has yielded a pure and fully active recombinant protein with seven disulphide bridges. The functional expression of rHGI has paved the way to study mechanisms at the molecular level.

CHAPTER 4

SITE DIRECTED MUTAGENESIS to validate the role of c-terminal tail in the dimerisation of hgi-iii

The major BBI of horsegram, HGI-III, a single polypeptide of ~8.6 kDa exists as dimer in solution. In direct contrast, the three inhibitors of germinated horsegram seeds (HGGIs) are single polypeptides of 6.5-7.2 kDa that exist as monomers and exhibit no self-association (Kumar et al., 2002). Chemical modifications coupled with a comparative evaluation of several legume BBI protein sequences and homology modelling indicated that the dimerisation of HGI-III was characterised by a crucial electrostatic interaction that comprised the C-terminal tail of sub-domain II (Kumar et al., 2004). The results of a site directed mutagenesis approach to validate the involvement of the C-terminal tail of HGI-III in the crucial interaction that is vital for self association to form dimers are presented and discussed.

Results

Primer designing for the site directed mutagenesis

Primers were designed for the region to be mutated. Complimentary primers were designed such that primers were ~ 18-30 nucleotides length and the mismatch region with respect to template DNA was in the middle of the primer. For the K24A mutation, AAG, which codes for `K' was replaced with GCG coding for `A'. For a D75A mutation, GAT was replaced with GCT. Primers for K24A and D75A were designed as shown in Fig. 4.1. Using the DNA of either pET-20bHGI or pRSET-rHGI as the template and the corresponding set of mutant primers (Table 2.4) the mutant plasmids rHGI K24A, pET-20bHGI K24A and pET-20bHGI D75A were constructed by the PCR based Quik Change method.

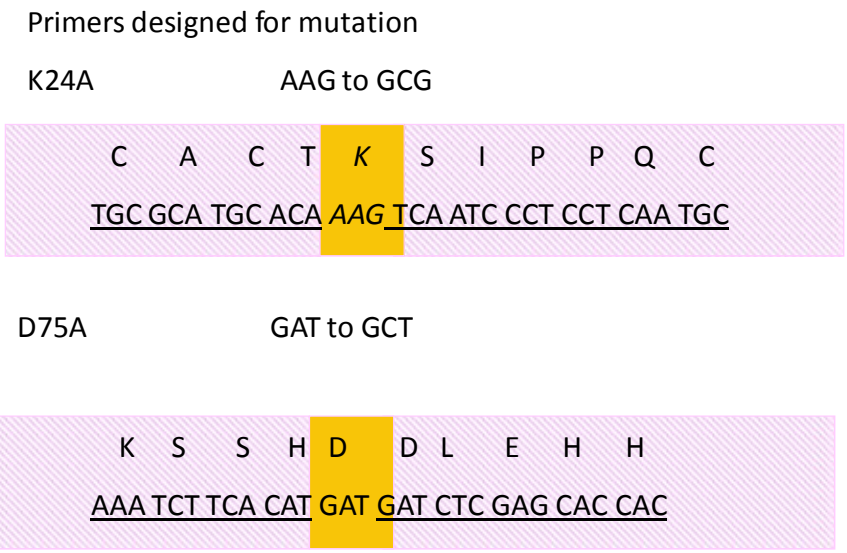


Fig. 4.1 **Primer designing strategy for site directed mutagenesis of rHGI.** The region to be mutated is boxed.

Construction of point mutants of rHGI

Site directed mutagenesis of rHGI was carried out. The primer pairs used for site directed mutagenesis are as described earlier (Table 2.4). Mutants were obtained using the Quik Change PCR-based mutagenesis method (Fisher and Pei, 1997) and plasmid DNA of pRSET-rHGI or pET-20bHGI as template. *Pfu* DNA polymerase was used for amplification. The schematic for the method is represented in Fig. 4.2. The PCR product upon amplification comprises of both mutant and wild type DNA. Selection of mutant DNA is achieved by selective degradation of methylated (parental) DNA using the restriction enzyme *Dpn*I. The digested products are separated by agarose gel electrophoresis. Mutant DNA is then transformed into chemically competent *E. coli* DH5α.

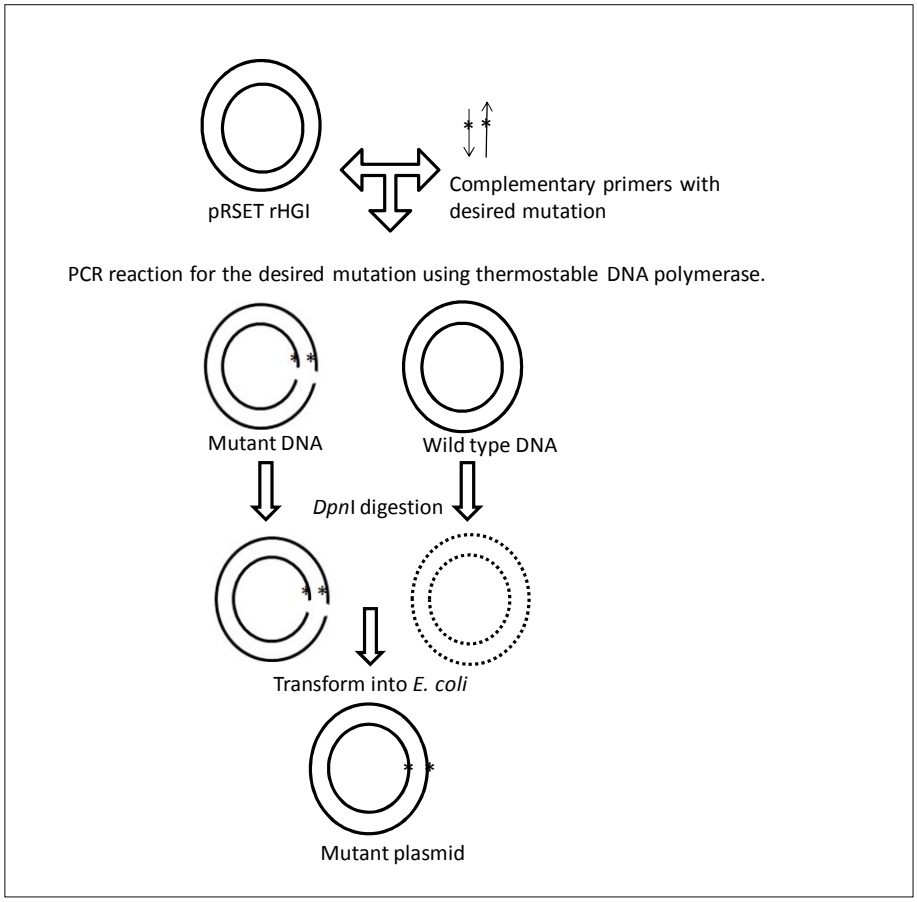


Fig. 4.2 **Schematic representation of site directed mutagenesis using the Quik change PCR method.**

Plasmid DNA of the mutants was isolated by alkaline lysis method and sequenced according to the dideoxy chain termination method to check for random PCR errors. The mutants were then expressed in *E. coli* strain BL21 (DE3) pLysS and purified.

Mutagenesis of Lys^{24} to Ala

According to homology modelling and chemical modification studies (Kumar et al., 2004), the trypsin reactive site residue K^{24} of HGI-III was involved in dimeristion. Therefore K^{24} was initially mutated to A to disrupt

the electrostatic interactions. The mutant rHGI-K24A was expressed as described earlier, in *E. coli* BL21 (DE3) pLysS (Fig. 4.3). The partial purification of the rHGI-K24A was carried out by Sephadex G-100 size exclusion chromatography. The purity of the partially purified protein after size exclusion chromatography was ~ 60%. The purification of the K24A mutant by trypsin-sepharose affinity chromatography was hindered by the fact it had lost its binding affinity to trypsin. `A' is reported to be at the reactive site of BBIs that inhibit elastase. In rHGI-K24A mutant `K' which is responsible for trypsin recognition was replaced by the hydrophobic residue `A' whereby trypsin recognition was replaced by elastase. Therefore an inhibitory study of rHGI-K24A was evaluated against porcine pancreatic elastase. The inhibitory activity was detected by incorporating gelatin to native PAGE (final concentration 1% w/v) and digested with elastase as described in section 2.2.7. The property of rHGI-K24A as an elastase inhibitor was detected as a dark blue band in a clear background due to the complex of the unhydrolyzed gelatin (Fig. 4.4 A). The elastase inhibitory activity with its substrate (NAPNA) was 4.8×10^3 EIU/mg protein. The purified protein however lost its trypsin inhibitory activity but retained its chymotrypsin inhibitory potential as evidenced in gelatin embedded PAGE (Fig.4.4 B&C & Table 4.1). This was not unexpected as the chymotrypsin recognition site of rHGI was F, which remained unchanged.

Size exclusion chromatography

The role of the mutation of K^{24} to A on the oligomerisation status of rHGI-K24A was evaluated by size exclusion chromatography. Size exclusion measurements were performed using a BIOSEP-SEC-S 3000 column on a Waters Associate HPLC. The column was pre-equilibrated with 0.1M Tris-HCl, pH 7.5 at a flow rate of 1 mL per min prior to sample loading.

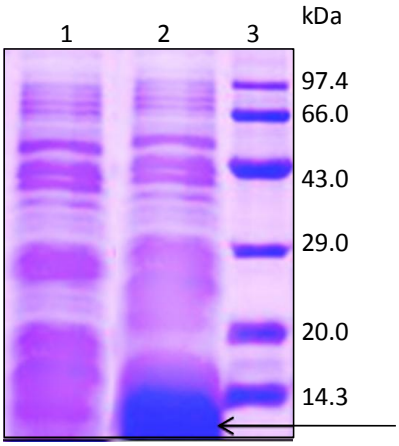


Fig. 4.3 **SDS–PAGE (12.5% T, 2.7% C) profile of cell free extracts of rHGI-K24A.** Lane 1: uninduced rHGI-K24A; Lane 2: rHGI-K24A induced with 0.3mM IPTG and Lane 3: Molecular weight markers. Arrow indicates expressed rHGI-K24A.

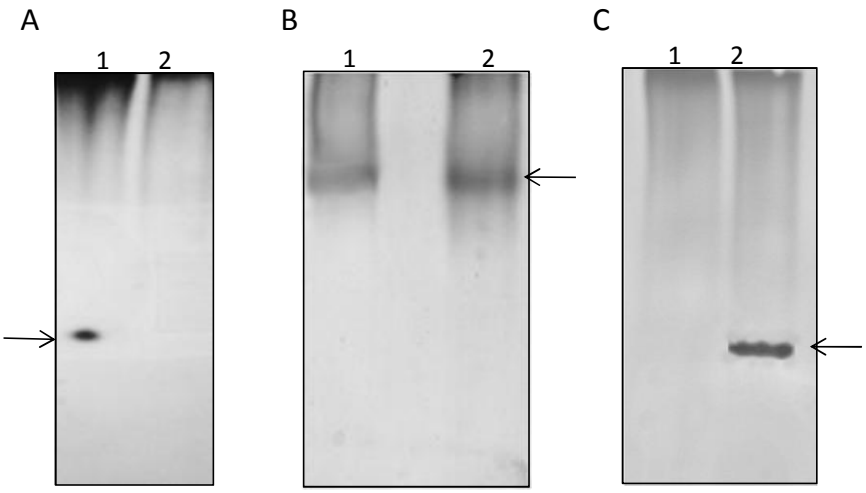


Fig. 4.4 **Gelatin embeded Native-PAGE of K24A mutant**. (A) Elastase inhibition. Lane 1: K24A mutant; Lane 2: Wild type rHGI (B) Chymotrypsin inhibition. Lane 1: Wild type rHGI; Lane 2: K24A mutant (C) Trypsin inhibition. Lane 1: K24A mutant; Lane 2: Wild type rHGI. The arrow indicates the position of the inhibitor.

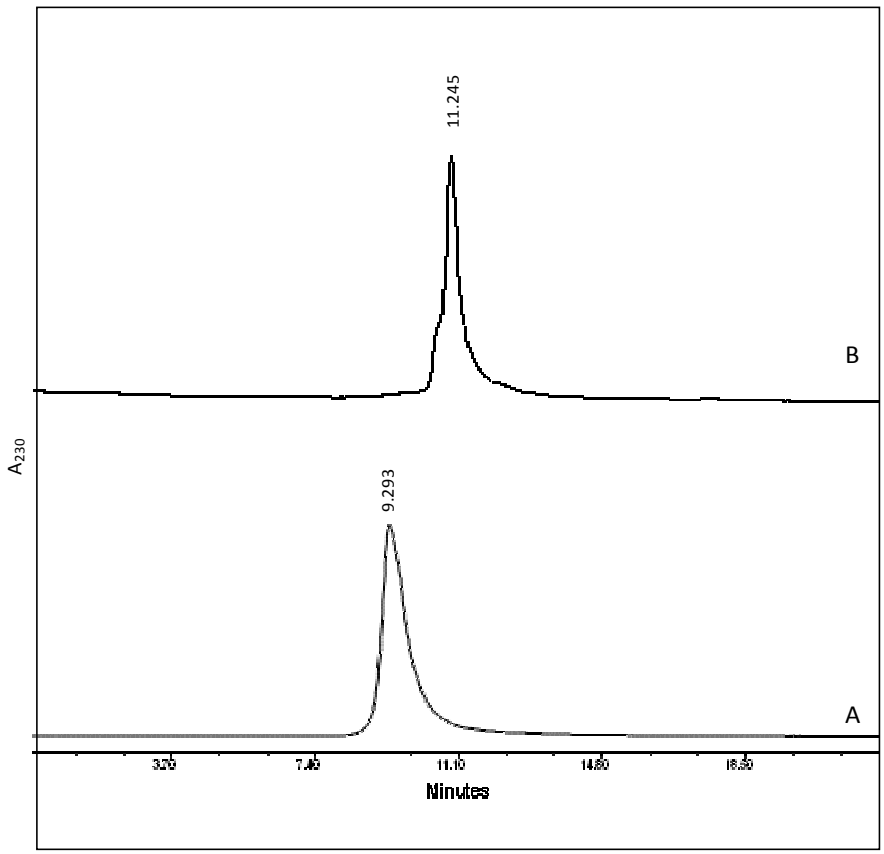


Fig. 4.5 **Size-exclusion chromatography of rHGI and K24A mutant.** The purified proteins were dissolved in 0.1M Tris-HCl, pH 7.5 and loaded on to a BIOSEP-SEC-S 3000 column. (A) rHGI and (B) K24A mutant of rHGI.

rHGI-K24A eluted later than rHGI with a retention time of 11.25 min corresponding to a M_r of ~8 kDa. rHGI elutes at 9.29 min which corresponds to a M_r ~16.0 kDa. These results provide further evidence that rHGI in solution associates to form a dimer whereas rHGI-K24A mutant remains as a monomer (Fig. 4.5).

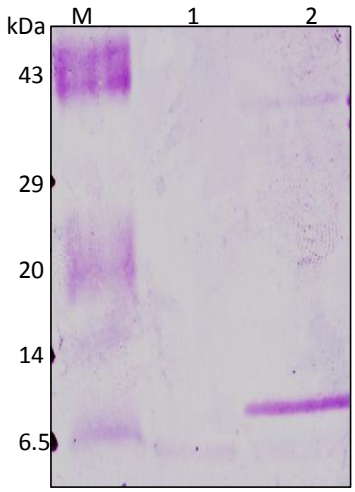


Fig. 4.6 **SDS-PAGE (15%T, 2.7%C) followed by Western blotting and immuno-detection with anti-HGI-III.** Lane M: Rainbow molecular weight markers; Lane 1: Bovine serum albumin and Lane 2: K24A mutant of rHGI.

These results were further confirmed by SDS-PAGE followed by immunodetection with anti-HGI-III. The results clearly evidence that the K24A mutant migrates faster with an apparent M_r of 8.8 kDa, that of a monomer (Fig 4.6).

Thermal stability studies of rHGI-K24A

The thermal stability studies were conducted to study the stability of mutant K24A. Relatively little or no changes in the elastase inhibitory activity were observed up to 2 h for the K24A mutant. Although the stability of K24A mutant was comparable to rHGI up to 2 h, it lost 30 and 50 % of its activity after 3 and 6 h incubation at 90 °C respectively (Fig 4.7). These results indicate that the monomer is less stable than the dimer.

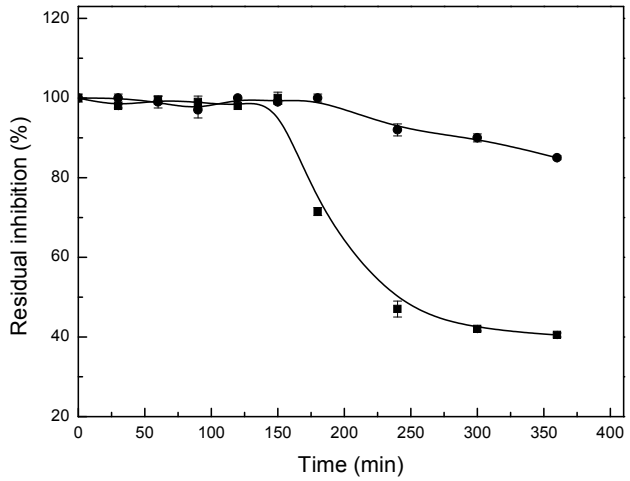


Fig. 4.7 **Thermal stability of rHGI and rHGI-K24A mutant at 90±1 ^{o}C** (●): rHGI and (■): rHGI-K24A. The inhibitors were incubated at 90±1^{o}C in a water bath. At regular time intervals the residual trypsin or elastase inhibitor activity was determined.

Kumar et al., (2004) while studying the germinated horse gram inhibitors showed that monomers are less stable compared to dimer BBIs. Further kinetic studies of the mutants were not carried out due to the purification constraints reckoned by the loss of trypsin affinity. Therefore a C-terminal $(His)_6$ tagged expression of the mutant was constructed to abet in the purification.

Construction of expression plasmids and point mutants of HGI-III as C-terminal $(His)_6$ tagged proteins.

The HGI-III coding region was PCR amplified using the primer pair HGI-F (5`GATCATCATCAGTCAACTGATGAG3') and HGI-*Xho*-R (Table 2.4) from pRSET-rHGI. The amplified product was digested with *Xho*I and ligated into pET-20b digested with *EcoR*V and *Xho*I restriction enzymes.

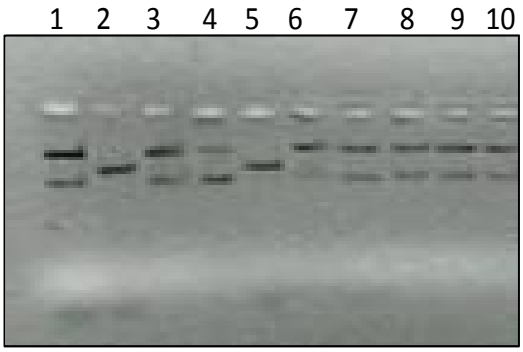


Fig. 4.8 **Analysis of recombinant plasmid DNA using 0.8 % agarose gel electrophoresis**. Lane 1: pET20b vector control; Lanes 2-10: recombinant plasmids.

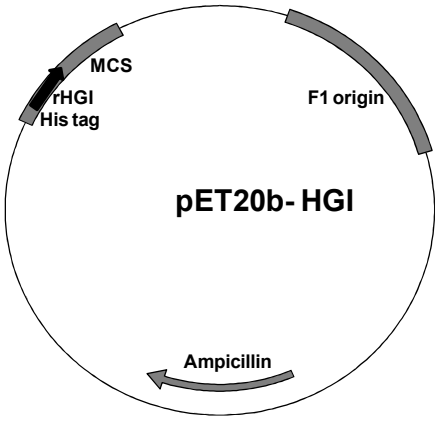


Fig. 4.9 **Schematic representation of pET20b-HGI expression cassette**.

The ligation mixture was transformed with chemically competent *E.coli* DH5α cells and the recombinants were screened using 0.8 % agarose gel electrophoresis. The mobility of the recombinants was compared with respect to the control pET20b vector plasmid. The recombinants were identified by their retarded mobility in comparison to the control (Fig. 4.8). The recombinant DNA was confirmed by subjecting the plasmid DNA to di-deoxy DNA sequencing reaction. The confirmed recombinant plasmid was designated as pET20b-HGI (Fig. 4.9) and was used in the expression studies.

Expression of pET20b-HGI

For bacterial expression of pET20b-HGI, the *E.coli* strain BL21 (DE3) pLysS was transformed with the plasmid construct pET20b-HGI. In this plasmid HGI-III gene is under the control of T7 RNA polymerase gene, which is under the control of lacUV5 promoter and is induced by analogs of lactose like IPTG for the expression of desired rHGI. The cells were grown at 37 ^{o}C and induced after they attained a growth of 1.75 absorbance at 600 nm. After the addition of 0.3 mM IPTG the culture was incubated at 37 ^{o}C for another 4 h. The cells were harvested at 8000 rpm for 15 min at 4 ^{o}C and then lysed in 0.1 M Tris-HCl pH 7.4 by ultrasonication. Trypsin inhibitor assays were carried out for both the supernatant and pellet of the cell lysate. The supernatant which showed the trypsin inhibitory activity was used for further purification of the pET20b-HGI.

One step purification of the $(His)_6$-tagged fusion proteins

The cell free extract was applied to a Ni^{2+}-NTA Sepharose column pre-equilibrated with 0.02 M Tris-HCl containing 0.5 M NaCl and 0.03 M imidazole pH 7.4. The column was washed with the same buffer until A_{280} returned to baseline. Unbound proteins were eluted in this buffer. The bound $(His)_6$-tagged fusion protein was eluted with the above buffer containing 0.5 M imidazole and fractions analysed for trypsin inhibitory activity. The fractions containing the fusion protein were collected and dialysed overnight against water at 4 ^{o}C (5 $\times$ 500 mL) to remove the buffer ions. The $(His)_6$ tagged fusion protein after purification was analysed on SDS-PAGE and was found to be ~98% pure and moved as a dimer in solution (Fig. 4.10).

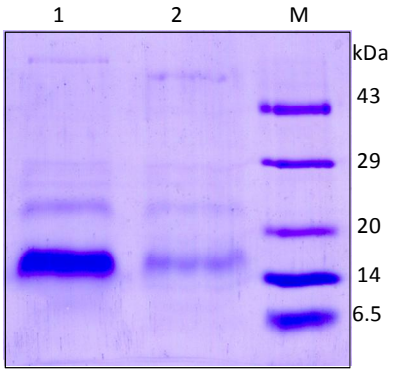


Fig. 4.10 **SDS-PAGE (15%T, 2.7%C) analysis of expressed pET-20bHGI and rHGI.** Lane 1: pET-20bHGI; Lane 2: rHGI; Lane M: Molecular weight markers

The yield of pET-20bHGI was 6.2±0.6 mg/L culture broth. The expressed protein from the recombinant (pET-20bHGI) would have in addition to the 76 residues of HGI-III, two amino acids (L and E) plus the 6 residue His tag ($(His)_6$) at the C-terminus and two amino acids (M and D) at the N-terminus. The expression vector pET20b-HGI was used in all further experiments and mutation studies.

Using pET-20bHGI as the template and the corresponding set of mutant primers (Table 2.4) the mutant clones pET-20bHGI K24A and pET-20bHGI D75A were constructed by the PCR based Quik Change method.

Expression of pET-20bHGI K24A mutant

After transformation into *E. coli* DH5α cells and plasmid DNA isolation, the sequence was validated by the dideoxy chain termination method for random PCR errors. The plasmid named pET-20bHGI K24A was used for the expression of the mutant in *E. coli* strain BL21 (DE3) pLysS cells (Fig. 4.11). The overexpression of protein was carried out similar to that of pET-20bHGI.

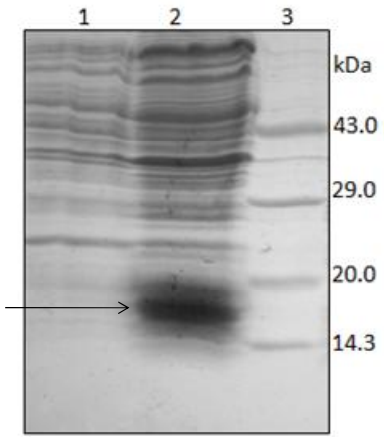


Fig. 4.11 **SDS–PAGE (15% T, 2.7% C) profile of cell free extracts of pET-20bHGI K24A.** Lane 1: uninduced pET-20bHGI K24A; Lane 2: pET-20bHGI K24A induced with 0.3mM IPTG and Lane 3: Molecular weight markers. Arrow indicates expressed pET-20bHGI K24A.

The transformed cells were grown at 37 ^{o}C and induced with 0.3 mM IPTG after reaching an absorbance of 1.75 at 600 nM. The expressed protein was analysed for the elastase inhibitor activity and found to be concentrated in the supernatant of the cell lysate. pET-20bHGI K24A was purified by Ni^{2+}-Sepharose chromatography as mentioned earlier. The purification yielded ~ 98% pure protein, which was used in further studies. The yield of pET-20bHGI K24A was 5.8±0.5 mg per litre of culture.

Construction of pET-20bHGI D75A

The pET-20bHGI D75A mutant was constructed using Quik change mutagenesis as described earlier. The plasmid was transformed in *E. coli* strain BL21 (DE3) pLysS cells for the expression of the recombinant protein (Fig. 4.12). The over expression of protein was carried out similar to that of pET-20bHGI.

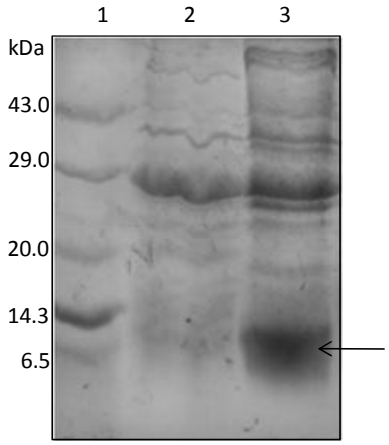


Fig. 4.12 **SDS–PAGE (15% T, 2.7% C) profile of cell free extracts of expressed pET-20bHGI D75A.** Lane 1: Molecular weight markers; Lane 2: uninduced pET-20bHGI D75A and Lane 3: pET-20bHGI D75A induced with 0.3mM IPTG. Arrow indicates expressed pET-20bHGI D75A.

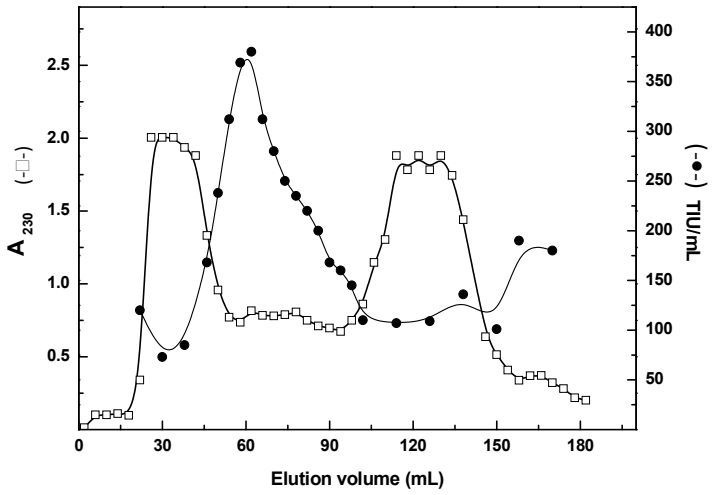


Fig. 4.13 **Purification profile of pET-20bHGI D75A.** The *E. coli* cell lysate was loaded on a Sephadex G-100 column equilibrated with 0.025 M Tris-HCl buffer (pH 8.2) at a flow rate 10 mL/h. (−●−) TIU/mL,(− □ −) A 230.

The trypsin inhibitory activity of the crude cell lysate from a 1 L culture was 4.1±0.17×10^5 TIU with a specific activity of 2.3±0.42×10^3 TIU/mg protein. The pET-20bHGI D75A mutant was purified by Sephadex G-100 size exclusion chromatography (Fig. 4.13). The column was pre-equilibrated with 0.025 M Tris-HCl pH 8.2 at a flow rate of 10 mL/h. Each fraction (2 mL) was assayed for protein and anti-tryptic activity. The fractions exhibiting trypsin inhibitor activity were pooled, dialysed against water and lyophilised. The yield of purified pET-20bHGI D75A was 6.8±0.4 mg/L culture broth. The purified protein was ~95% pure.

Construction of Δ76 mutant of rHGI

Using pET20b-HGI DNA and the primer pair HGI-F and *Δ*76-*Xho*-R (Table 2.4) the deletion mutant pET-20bHGI *Δ*76 was constructed as described above for pET20b-HGI. The pET20b vector was double digested with *EcoR*V and *Xho*I and the PCR product digested with *Xho*I was subjected to ligation. The recombinants were screened and expressed in *E. coli* strain BL21 (DE3) pLysS. Fig. 4.14 shows the expression pattern for pET-20bHGI *Δ*76. The expressed protein was purified by Sephadex G-100 size exclusion chromatography (Fig. 4.15). The Sephadex G-100 column was pre-equilibrated with 0.025 M Tris-HCl pH 8.2. The column was run at a flow rate of 10 mL/h. The cell free supernatant having expressed pET-20bHGI *Δ*76 was loaded on to the column. Each fraction (2 mL) was assayed for protein and trypsin inhibitor activity. The fractions exhibiting trypsin inhibitor activity were pooled, dialysed against water (5×400 mL) and lyophilised. The peak fraction showed an activity of 390 TIU/mg of protein. The recovery of pET-20bHGI *Δ*76 was ~40 %, after the purification, with a specific activity of 3.85±0.13×10^3 TIU/mg.

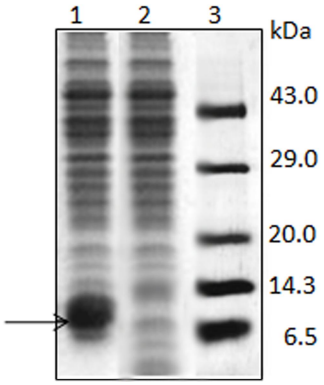


Fig. 4.14 **SDS-PAGE (15%T, 2.7%C) analysis of cell free extracts of pET-20bHGI *Δ*76.** Lane 1: pET-20bHGI*Δ*76 induced with 0.3mM IPTG; Lane 2: uninduced pET-20bHGI*Δ*76; Lane 3: Molecular weight markers. Arrow indicates the expressed protein.

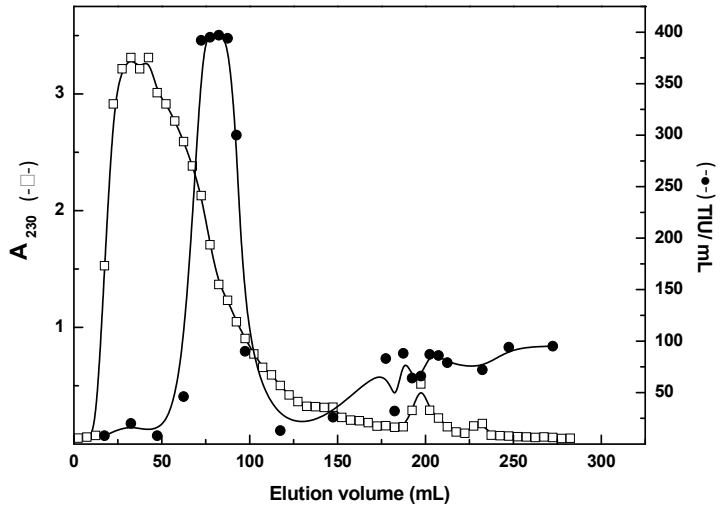


Fig. 4.15 **Purification profile of pET-20bHGI *Δ*76.** The *E. coli* cell lysate was loaded on a Sephadex G-100 column equilibrated with 0.025 M Tris-HCl buffer (pH 8.2) at a flow rate 10 mL/h. (−•−) TIU/mL,(− □ −) A_{230}. The fractions exhibiting trypsin inhibitor activity were pooled.

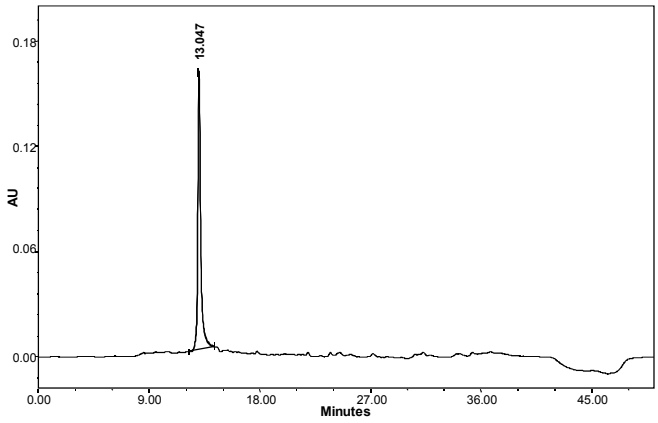


Fig. 4.16 **RP-HPLC purification of pET-20bHGI Δ76.** The pooled trypsin inhibitor fraction of Sephadex G-100 purification step was lyophilised and repurified by RP-HPLC using a binary gradient.

Sephadex G-100 purification was followed by RP-HPLC using a C-18 column (Fig.4.16). The purified protein was ~98 % pure. The yield of purified pET-20bHGI Δ76 was 4±0.9 mg/L culture broth.

Evaluation of kinetic properties of the mutants

The kinetic studies of rHGI (chapter 3) revealed that refolding of rHGI gave better inhibition compared to the unfolded recombinant (Table 3.2). Kinetic analysis of pET-20bHGI K24A mutant before refolding indicated that its inhibitory constant towards elastase was $4.0\pm0.13 \times 10^{-7}$ M. Therefore to improve the efficiency of inhibition, refolding of the purified pET-20bHGI K24A mutant in solution was carried out as reported for CNBr cleaved recombinant soybean BBI (Flecker, 1987). The initial rate of reaction in the presence and absence of refolded pET-20bHGI K24A followed Michaelis Menten kinetics. The mode of inhibition was evaluated from the double reciprocal plots of elastase /chymotrypsin (Fig. 4.17) titrated with different concentration of their substrates NAPNA and BTPNA respectively.

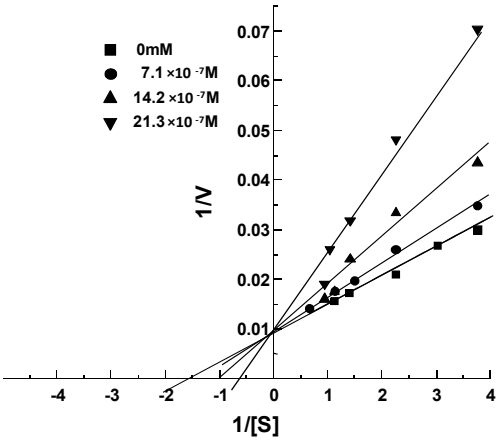


Fig. 4.17 A **Lineweaver-Burk plot showing competitive inhibition by pET-20bHGI K24A for porcine pancreatic elastase.** Data points are average of three determinations.

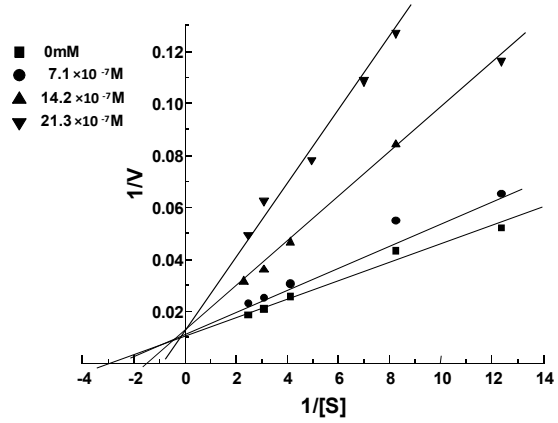


Fig. 4.17 B **Lineweaver-Burk plot showing competitive inhibition by pET-20bHGI K24A for bovine chymotrypsin.** Data points are average of three determinations.

The results indicate that pET-20bHGI K24A mutant was a competitive inhibitor of both elastase and chymotrypsin. Kinetic analysis of the refolded pET-20bHGI K24A protein indicated that it is a potent competitive inhibitor of elastase with a K_i of 1.58 ±0.14×10^{-8} M, (Fig.4.18 A) which was one order of magnitude more potent than the chymotrypsin inhibitory constant. The kinetic constant towards chymotrypsin was 2.6±0.13 ×10^{-7} M (Fig. 4.18 B & Table 4.1).

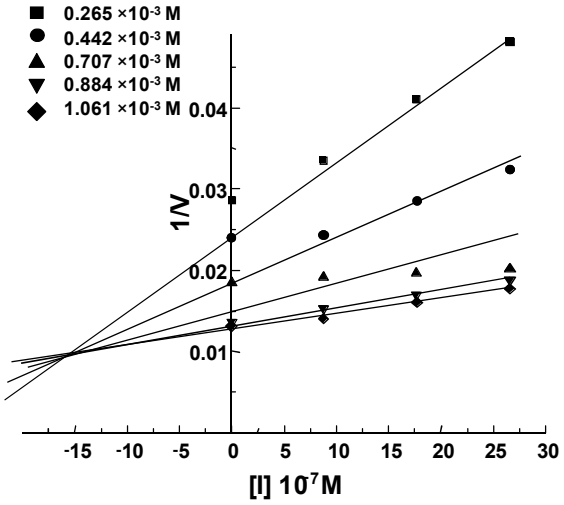


Fig. 4.18 A **Dixon plot for determining the dissociation constant (K_i) of pET-20bHGI K24A mutant against porcine pancreatic elastase.** Data points are the average of three determinations.

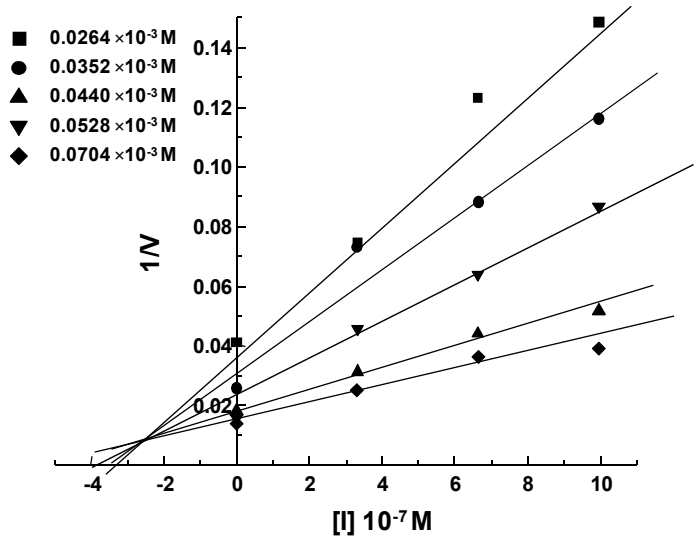


Fig. 4.18 B **Dixon plot for determining the dissociation constant (K_i) of pET-20bHGI K24A mutant against bovine chymotrypsin.** Data points are the average of three determinations.

The type of inhibition exhibited by the pET-20bHGI D75A against trypsin and chymotrypsin was determined by using the chromogenic substrates BAPNA and BTPNA at pH 8.2 and 7.8 respectively. The plot of 1/[S] vs 1/ [V] at different inhibitor concentration indicates that pET-20bHGI D75A is competitive towards both trypsin and chymotrypsin (Fig. 4.19). The inhibitor constants (K_is) for pET-20bHGI D75A were deduced from Dixon plots (Fig. 4.20) by plotting [I] vs 1/ [V] at various concentrations of the substrate. The apparent K_i for trypsin and chymotrypsin inhibition from Dixon plots for pET-20bHGI D75A mutant was 4.8±0.11 $\times 10^{-8}$ M and 3.4±0.18 $\times 10^{-7}$ M respectively. The mutant similar to the wild type rHGI show a higher binding affinity towards trypsin when compared to chymotrypsin.

The inhibitory potential of the pET-20bHGI *Δ*76 mutant towards bovine trypsin and chymotrypsin was evaluated similar to the other mutants. The effect of the pET-20bHGI *Δ*76 on varying substrate concentrations followed Michaelis-Menten kinetics and a Lineweaver-Burk plot (Fig. 4.21) of the data indicated that the mutant was a competitive inhibitor of trypsin and chymotrypsin with respect to the substrates BAPNA and BTPNA respectively. The dissociation constant K_i calculated from the Dixon plot of the data for trypsin was 3.8±0.23 $\times 10^{-8}$ M and 2.2±0.20 $\times 10^{-7}$ M (Fig. 4.22) for chymotrypsin. The binding affinity of the pET-20bHGI *Δ*76 mutant towards trypsin and chymotrypsin were similar to the wild type rHGI. These results suggest that the mutations had no effect on the kinetic properties of the inhibitor.

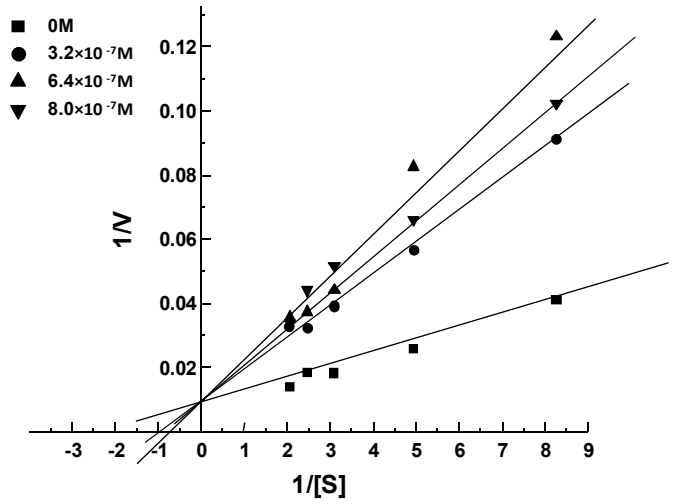


Fig. 4.19 A **Lineweaver-Burk plot of pET-20bHGI D75A mutant showing competitive inhibition for trypsin**. Data points are average of three determinations.

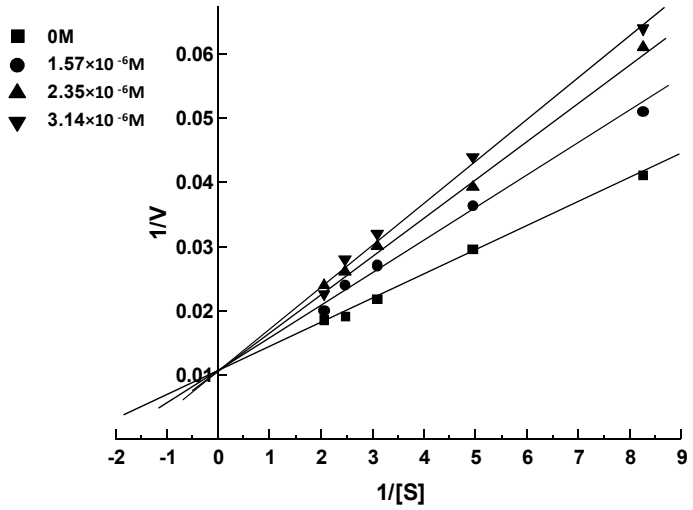


Fig.4.19 B **Lineweaver-Burk plot of pET-20bHGI D75A mutant showing competitive inhibition for chymotrypsin**. Data points are average of three determinations.

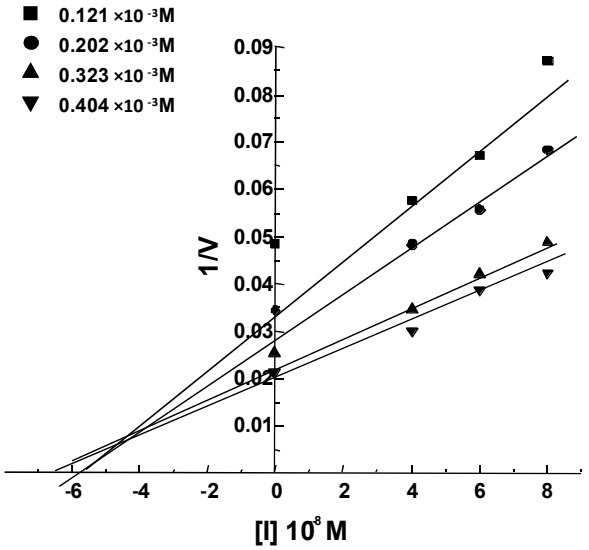


Fig. 4.20 A **Dixon plot for determining the dissociation constant (K_i) of pET-20bHGI D75A mutant bovine trypsin.** Data points are the average of three determinations

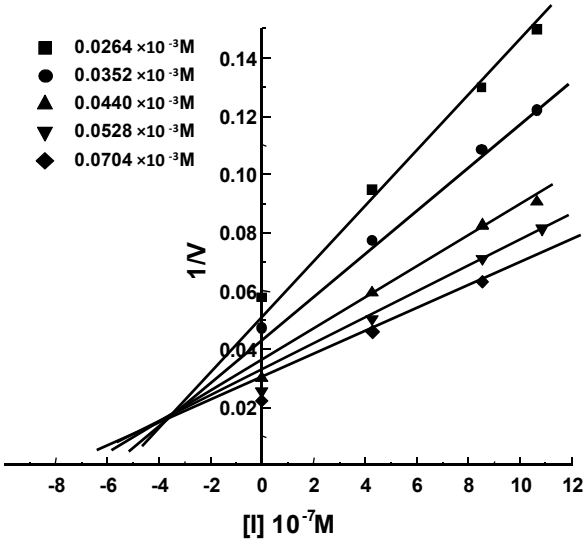


Fig. 4.20 B **Dixon plot for determining the dissociation constant (K_i) of pET-20bHGI D75A mutant against bovine chymotrypsin.** Data points are the average of three determinations.

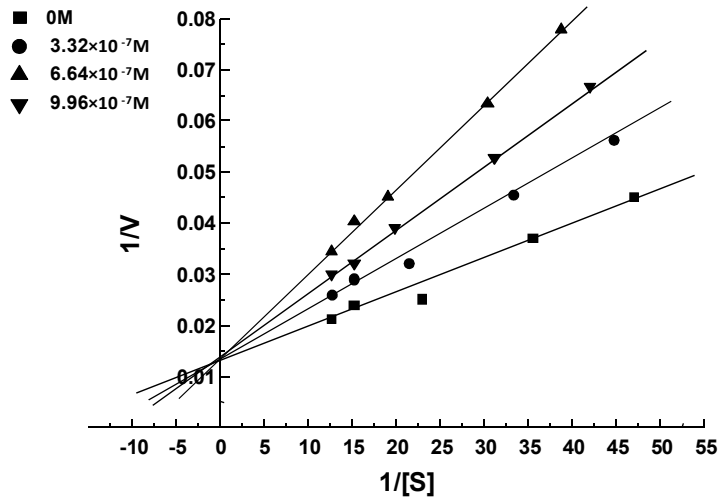


Fig. 4.21 A **Lineweaver-Burk plot showing competitive inhibition by pET-20bHGI *Δ*76 mutant for trypsin.** Data points are average of three determinations.

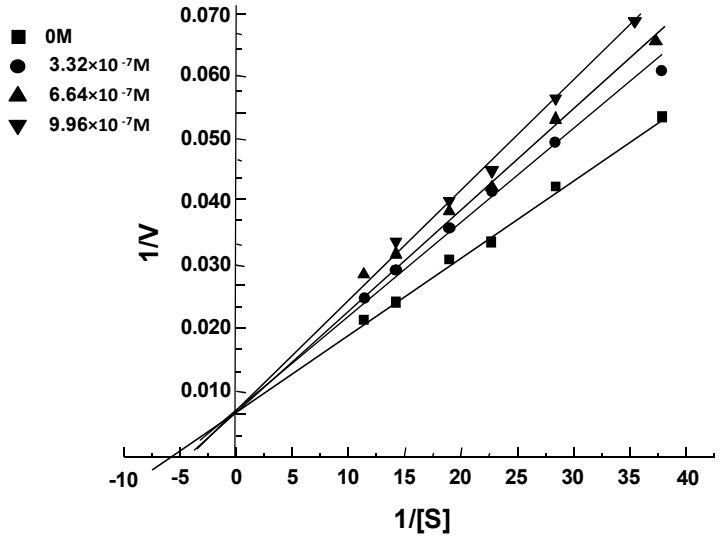


Fig. 4.21 B **Lineweaver-Burk plot showing competitive inhibition by pET-20bHGI *Δ*76 mutant for chymotrypsin.** Data points are average of three determinations.

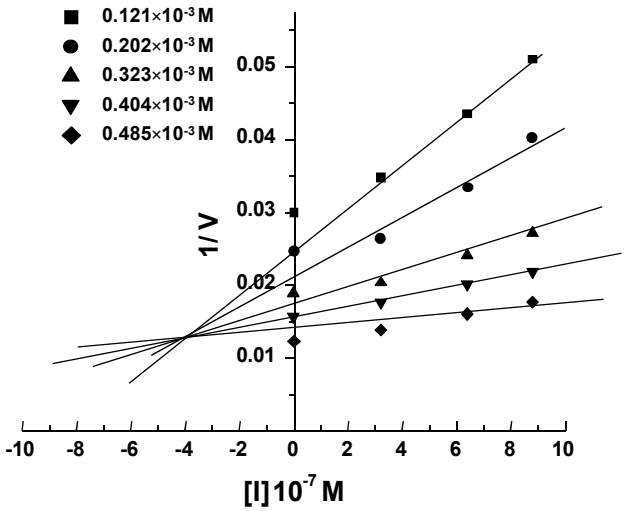


Fig. 4.22 A **Dixon plot for determining the dissociation constant (K_i) of pET-20bHGI Δ76 mutant bovine trypsin**. Data points are the average of three determinations

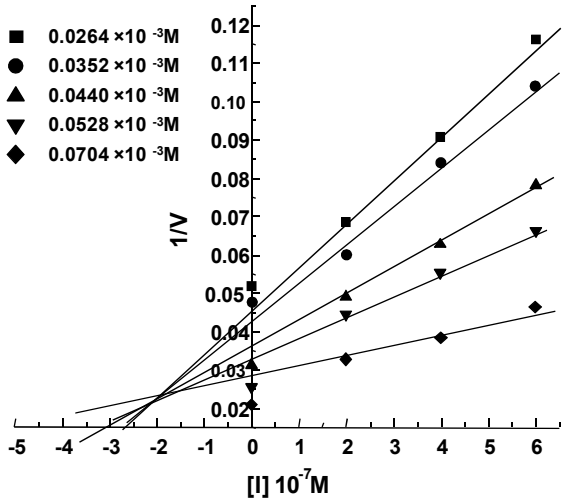


Fig. 4.22 B **Dixon plot for determining the dissociation constant (K_i) of pET-20bHGI Δ76 mutant against bovine chymotrypsin.** Data points are the average of three determinations.

Table 4.1 **Dissociation constants (Ki) of HGI-III and rHGI for trypsin, chymotrypsin and elastase inhibition**

Inhibitor	**Bovine trypsin**	**Bovine chymotrypsin**	**Porcine pancreatic elastase**
pRSET-rHGI	$6.1\pm0.13 \times10^{-8}$ M ($5.0\pm0.15\times10^{-8}$ M)*	$3.0\pm0.15 \times10^{-7}$ M ($3.4\pm0.12\times10^{-6}$ M)*	NA
pET-20bHGI K24A	NA	$2.6\pm0.13 \times10^{-7}$ M	$1.58\pm0.14 \times10^{-8}$ M ($4.0\pm0.13\times10^{-7}$ M)*
pET-20bHGI D75A	$4.8\pm0.11 \times10^{-8}$ M	$3.4\pm0.18 \times10^{-7}$ M	NA
pET-20bHGI Δ76	$3.8\pm0.23 \times10^{-8}$ M	$2.2\pm0.20 \times10^{-7}$ M	NA

NA: Not applicable and * values in parenthesis are the values prior to refolding.

The kinetic constants of rHGI and its mutants for trypsin, chymotrypsin and elastase inhibition are summarised in Table 4.1.

SDS-PAGE analysis of mutants

SDS-PAGE analysis followed by protein staining showed that K24A $(His)_6$ tagged fusion protein moved as a protein of molecular mass ~16±1 kDa (Fig. 4.23 A). This was surprising and not expected. At the same time SDS-PAGE analysis of C-terminal mutants D75A and Δ76 (Fig. 4.23 B&C) indicated that these mutants migrated as monomers of ~8 kDa.

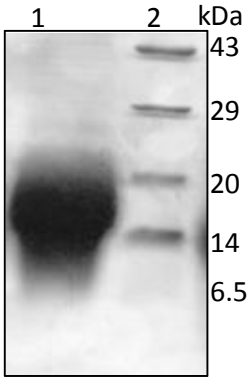


Fig. 4.23 A **SDS-PAGE (15%T, 2.7%C) analysis of purified pET-20bHGI K24A.** Lane 1: pET-20bHGI K24A; Lane 2: Molecular weight markers

Fig. 4.23 A **SDS-PAGE (15%T, 2.7%C) analysis of purified pET-20bHGI K24A.** Lane 1: pET-20bHGI K24A; Lane 2: Molecular weight markers

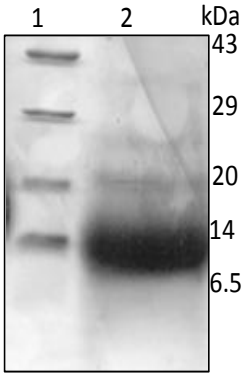


Fig. 4.23 B **SDS-PAGE (15%T, 2.7%C) analysis of expressed pET-20bHGI D75A.** Lane 1: Molecular weight markers; Lane 2: pET-20bHGI D75A

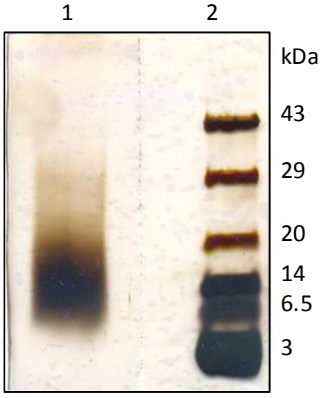


Fig. 4.23 C **SDS-PAGE (15%T, 2.7%C) analysis of expressed pET-20bHGIΔ76.** Lane 1: pET-20bHGI Δ76; Lane 2: Molecular weight markers

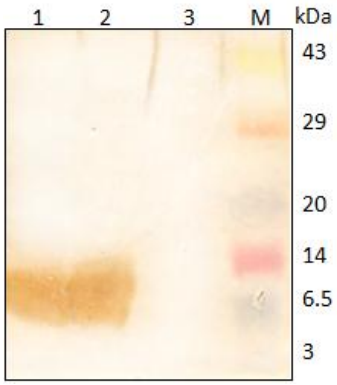


Fig. 4.24 **SDS-PAGE (15%T, 2.7%C) followed by Western blotting and immuno-detection with anti-HGI-III.** Lane 1: pET-20bHGI D75A mutant; Lane 2: pET-20bHGI Δ76 mutant; Lane 3: Bovine serum albumin and Lane M: Rainbow molecular weight markers.

The migration of D75A and Δ76 as monomers were further validated by Western blotting following SDS-PAGE (Fig. 4.24). These mutants of rHGI cross reacted with the anti-HGI-III antibody. This was expected since the epitopes of HGI-III are not affected by the mutations.

Size exclusion chromatography of mutants

rHGI K24A is a monomer (Fig. 4.5), in contrast pET-20bHGI K24A exists as a dimer in solution as revealed by SDS-PAGE (Fig. 4.23A) and size exclusion chromatography (Fig. 4.25A). Size exclusion chromatography, which discriminates between molecules based on their size, was also used as the next step to understand why pET-20bHGI K24A remained as a dimer even after disrupting the K-D ionic interaction. The major difference between rHGI K24A and pET-20bHGI K24A is that the latter has a $(His)_6$

fusion tag. Either the $(His)_6$ through an ionic interaction with the negatively charged N-terminus of the second monomer or some other interaction is responsible for the dimerisation. In direct contrast the size exclusion chromatography profile of the mutants D75A with the $(His)_6$ fusion tag and Δ 76 mutant elutes at ~11.17 mins corresponding to that of a monomer (Fig. 4.25 B & C). Size exclusion studies at different pH were used to understand the role of $(His)_6$ fusion if any. pET-20bHGI K24A eluted as a monomer at pH 5.0 (Fig. 4.25 D). At pH 7.5 (Fig. 4.25A) and 9.5 (Fig.4.25E) the elution time corresponded to that of a dimer. At pH 5, His is protonated, therefore if involved in dimerisation through an ionic interaction the protein should exist as a dimer yet it is a monomer. At pH 7.5 and 9.5, wherein His is unprotonated the protein retains its dimeric status suggesting that the His is probably not responsible for dimerisation.

A previously reported molecular model of HGI-III dimer shows that the C-terminii, are located at the dimer interface and the C-terminal D from one subunit forms a strong salt bridge with R^{31} of the other subunit, which is brought into this appropriate orientation by a hydrogen bond between K^{24} and D^{76} of opposite subunits (Kumar et al., 2004). Therefore it is possible that in pET-20bHGI K24A this salt bridge (R^{31}-D^{76}) is not disrupted and therefore remains a dimer. The role of C-termini D was studied by size exclusion chromatography in the presence of $ZnSO_4$. pET-20bHGI K24A elutes at 11.245 mins in the presence of 1mM $ZnSO_4$ (Fig. 4.25F). This increased retention time reckons a significantly reduced molecular weight. The conversion of pET-20bHGI K24A to a monomer in the presence of $ZnSO_4$ occurs probably through an indirect Zn^{2+} co-ordination with carboxylate side chain of nearby $Asp^{D75/76}$ mediated by the neighbouring His^{74}.

Size exclusion chromatography in the presence of ZnSO4 has been used previously by Kumar et al., (2004) to indirectly provide evidence that the C-terminal residues play a vital role in the dimerization of HGI-III.

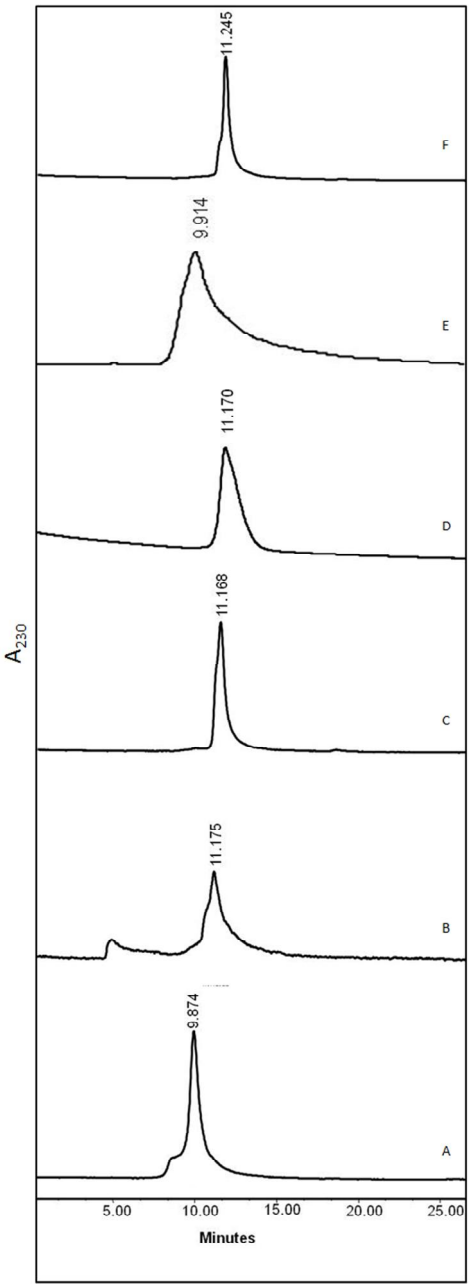


Fig. 4.25 **Size-exclusion chromatography of rHGI mutants expressed with $(His)_6$ fusion protein.** The samples were dissolved in corresponding buffers

and loaded on to a BIOSEP-SEC-S 3000 column pre-equilibrated with respective buffers and eluted at 1 mL/min. A, pET-20bHGI K24A (0.1 M Tris-HCl pH 7.5); B, pET-20bHGI D75A (0.1 M Tris-HCl pH 7.5); C, pET-20bHGI Δ76 (0.1 M Tris-HCl pH 7.5); D, pET-20bHGI K24A (0.1 M Tris-HCl pH 5.0); E, pET-20bHGI K24A (0.1 M Tris-HCl pH 9.5);and *F*, pET-20bHGI K24A (1 mM ZnSO4, in 0.1M Tris-HCl pH 7.5)

It is also possible that there occurs a substantial shift in the pKa of $D^{75/76}$ due to the increase in length at the C-terminus by $(His)_6$ tag fusion reckoning protonation at pH 5.0. Therefore the interactions involving $D^{75/76}$ are disrupted and the protein is dissociated. The observed dissociation of pET-20bHGI K24A to a monomer in the presence ZnSO4 at pH 7.5 (Fig. 4.25 F) in conjunction with the observation that pET-20bHGI D75A with a similar $(His)_6$ fusion tag is a monomer (Fig. 4.25 B&C) points to the involvement of C-terminal $D^{75/76}$ in the dimerisation of this mutant.

Site directed mutations of HGI-III described clearly exemplify the pivotal role of the C-terminal $D^{75/76}$ in the dimerisation previously demonstrated by chemical modifications (Kumar et al., 2004). Table 4.2 summarise the oligomeric status of rHGI and its mutants described.

Table 4.2 **Mutants of HGI showing their oligomeric status**

Mutants	**Oligomeric status**
pRSET-rHGI	Dimer
rHGI-K24A	Monomer
pET-20bHGI D75A	Monomer
pET-20bHGI Δ76	Monomer

Thermal stability studies

The thermal stability of the mutants were evaluated to understand the effect of the monomer/dimer status upon the physiological properties. The mutant proteins were incubated at 90 ^{o}C for different time periods. After the incubation, the proteins were immediately cooled on ice.

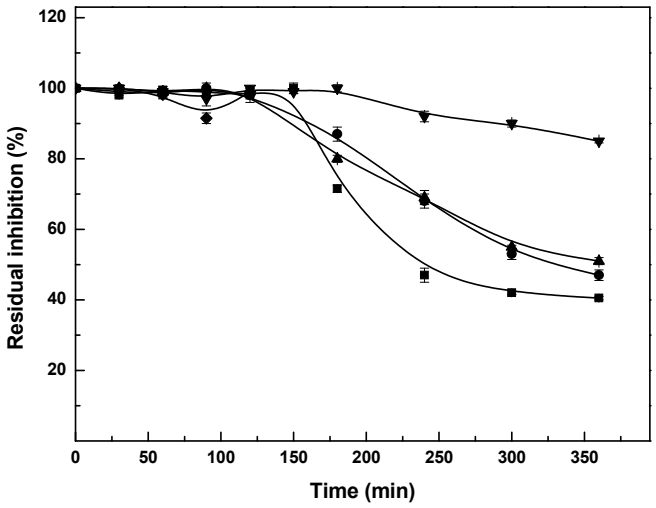


Fig. 4.26 **Thermal stability of rHGI and mutants at 90±1 ^{o}C** (▼): rHGI, (♦) pET-20bHGI K24A, (■): rHGI K24A, (▲): pET-20bHGI ∆76, and (●): pET-20bHGI D75A. The inhibitors were incubated at 90±1^{o}C in a water bath. At regular time intervals the residual inhibitor activity was determined.

The residual inhibitor activity was measured using BAPNA/NAPNA as substrate. The results indicate that for rHGI, relatively little or no changes in the trypsin inhibitory activity was observed upto 6 h at 90 ^{o}C whereas the mutant proteins rHGI K24A, pET-20bHGI ∆76, pET-20bHGI D75A were stable upto 2 h at 90 ^{o}C but started losing ~ 50 % of activity after that (Fig. 4.26). These results were quite similar to that observed in HGI-III and germinated HGGI-III (Kumar et al., 2004). The decrease in the thermal stability of the

mutants lead to the suggestion that mutants, which exists as monomers are less stable compared to the dimer, rHGI.

DISCUSSION

The inhibitors of horsegram are thermally stable single polypeptides with molecular mass in the range ~8.5 kDa (Sreerama et al., 1997). SDS-PAGE and analytical gel filtration indicate the molecular mass to be ~16 kDa (Sreerama et al., 1997) suggesting that they exist as dimers in solution. Kumar et al., (2004) demonstrated that an electrostatic interaction involving the trypsin reactive site (K^{24}) and the C-terminal are the driving forces for HGI-III self association. This self association, together with other structural aspects account for the stability of legume BBIs (Barbosa et al., 2007 & Singh and Rao, 2002). Previously studies other than site-directed mutagenesis were used to deduce the crucial role of an electrostatic interaction between K^{24} and D^{76} in the self association of HGI-III (Kumar et al., 2004). To conclusively prove this premise, a K24A mutation of rHGI was carried out and protein purified. The lack of its binding affinity to trypsin-sepharose and the loss of trypsin inhibitor activity evidenced the mutation. The dissociation constant of the complex with elastase ($K_{i,}$ 1.58±0.14×10^{-8} M) (Fig. 4.18 A) establishes a very high affinity. Size exclusion chromatography, SDS-PAGE of the partially purified protein and immunodetection with anti-HGI-III indicates a mass of ~8.5 kDa clearly establishing the involvement of the reactive site K^{24} in the self association of rHGI. The association of rHGI caused by this unique interaction between two monomers is only of the monomer $\rightarrow$ dimer type with little or no higher forms present as is observed from size exclusion studies (Fig. 4.5). A C-terminal $(His)_6$-tagged fusion

protein of HGI (pETHGI) like rHGI is a dimer. Unexpectedly the K24A mutant of this form was also a dimer. Size exclusion studies either in the presence of ZnSO4 or at pH 5.0 (Fig 4.25 D&F) led to the monomerisation of K24A $(His)_6$ mutant. The dissociation of the pET-20bHGI K24A into a monomer at pH 5.0 and in the presence of 1 mM ZnSO4 together with the monomer status of pET-20bHGI D75A and Δ76 mutant protein strengthen the premise that the C-termini $D^{75/76}$ play a more important role than K^{24} in dimer formation. The dimer model of seed HGI-III clearly discerns the role of the C-termini Asp residues in two contacts between the monomers: 1) a salt bridge (R^{31}–D^{76}) between the monomers and 2) a hydrogen bond with K^{24} to provide the required orientation for the formation of the salt bridge (R^{31}–D^{76}) (Kumar et al., 2004). The reported molecular model of HGI-III dimer indicates that the N-termini of the subunits are situated at the surface of the dimer, and any extension in this region would project into the solvent. This would not influence the stability of the dimer. In contrast the C-termini are located at the dimer interface and play an important role in the dimer stabilization. Therefore any extension of C-termini would have consequences on the dissociation of the dimer as observed with the pET-20bHGI K24A mutant. Xu et al., (2002) report that the attachment of fusion sequences to coiled-coil proteins not only affects the thermal stability but also the oligomerisation state. The monomer status of the pET-20bHGI D75A and Δ76 mutant also point to the involvement of the C-terminal Asp residues in self association. These results in conjunction with the previously published data (Kumar et al., 2004) unequivocally establish the role of an electrostatic interaction between K^{24} of one monomer and $D^{75/76}$ at opposite face of the second monomer, in self association.

Most of the residues involved in self association are highly conserved in BBIs from different seeds. Ragg et al., (2006) while solving the structure of a potent Bowman–Birk protease inhibitor from lentil (*Lens culinaris, L)* seeds showed that at physiological pH, an asymmetric distribution of opposite charges with a negative electrostatic potential centred on the C-terminus, and a highly positive potential surrounding the antitryptic domain. NMR analysis of the self association behaviour of snail medic seeds BBI reveal that the residues involved are localised at opposite faces of the molecule, having the highest positive and negative potential (Catalano et al., 2003). Multiple sequence alignment of legume BBIs show that if the first reactive site is either K or R they inhibit trypsin and tend to self associate. In elastase inhibitors wherein K/R is replaced by A they exist as monomers (Kumar et al., 2004). The K24A mutant of rHGI was converted to an elastase inhibitor (Fig. 4.4) and in solution was a monomer (Fig. 4.5 & 4.6), justifying the previous observation. The self association of legume BBIs relate to their function in storage since such affinity is a requirement for the molecular packing in seeds (Barbosa et al., 2007). The decreased thermal stability of the K24A mutant and C-terminal mutants in comparison to rHGI further advocates that the dimeric form of the inhibitor is more stable than the monomer. The dimer HGI-III was also more thermostable than the monomeric HGGI-III isolated from germinated horsegram seeds (Kumar et al., 2004).

The BBIs are promising models for studies on protein-protein interactions and to clearly distinguish between structural and functional aspects using recombinant DNA techniques. The cloning and heterologous expression of a functional rHGI (Chapter 3) provided a platform for production and systematic alteration of amino acid residues to explore the stability, mechanism of action and unveil the fine specificity of the

interactions involved in the dimeric status. The results presented demonstrate that the self association of HGIs is indeed due to the electrostatic interaction between K^{24} of one monomer and $Asp^{75/76}$ of the second monomer, in agreement with previous data (Kumar et al., 2004) obtained in this laboratory.

CHAPTER 5

Design and molecular engineering of a small trypsin inhibitor based on the binding loop of Horsegram seed trypsin inhibitor

Considerable scientific interest in BBIs is connected with their anticarcinogenic (Kennedy, 1998) and radioprotective effects on normal tissues. The possible mechanisms for these activities have been ascribed and linked to protease inhibition (Ware et al., 1997). It is still unknown whether these activities are correlated to the inhibitory activity of trypsin/ chymotrypsin or other enzymes or to the capability of interacting with other macromolecules (Koepke et al., 2000, Miyata et al., 2000, Sorsa et al., 1997, Ware et al., 1997, Ware at el., 1999). In BBIs the inhibitory activity is vested in the closed nonapeptide loop with residue P1 (Terada et al., 1978). The smallest naturally occurring protease inhibitor that mimics BBI by combining size reduction, yet retaining biological activity is the sunflower trypsin inhibitor (SFTI-I) (Luckett et al., 1999). Only about half of the BBI administered orally is taken up into the blood stream via the intestinal epithelial cells or crosses the intestinal lumen and is distributed through out the body (Billings, 1991). The molecular mass of BBIs (6-9 kDa) is a limitation, as sufficient amounts of BBI do not reach the organs outside the gastrointestinal tract when administered orally, leading to a decreased bioavailability. Engineering BBI to achieve size reduction whilst retaining either the antitryptic or antichymotryptic or both by synthetic and combinatorial chemistry has received considerable attention (Jaulent and

Leatherbarrow 2004, Scarpi et al., 2004, Zabłotna et al., 2007, Fernandez et al., 2007). The cloning and expression of the anti-tryptic domain, a disulfide cyclised loop of 36 aminoacid, its structural and kinetic analysis against bovine trypsin and human tryptase are presented in this chapter.

Results

Design of the stable mini inhibitors

The poor bioavailabilty and high susceptibility to proteolysis are significant limitations of BBIs as efficient and potent cancer chemopreventive agents. These limitations being attributed due to its fairly large size. HGI-III, similar to soybean BBI possesses two domains easily distinguishable (Sreerama et al., 1997). The aim of this study was to obtain a molecularly pruned inhibitor, which was stable, yet exhibited trypsin inhibition. The pictorial representation of trypsin inhibitory domain of horsegram BBI to be cloned is presented in Fig. 5.1. The stable trypsin inhibitor domain (TID) of HGI-III was amplified from the plasmid pRSET-rHGI (described in Section 2.2.29). Primers designed to obtain the open reading frame of 108 bp were HGI-F (5′GAT CAT CAT CAG TCA ACT GAT GAG 3′) and HGIT-R (5′CTC GAG TTA CCT AAC GTC CGT ACA GCG GCA 3′). The 108 bp amplicon included a stop codon at the C-terminal end (Fig. 5.2). The introduction of the stop codon at the C-terminus was to avoid the occurrence of extra aminoacids including the His-tag. Nucleotide sequence analysis of rTID amplicon (Fig. 5.3) revealed the sequence of the complete TID including loop I &II (Fig. 5.1). The amplicon was blunt end ligated into the *EcoR*V site of pET20b using *T4* DNA ligase to generate the plasmid

pET20b-recombinant TID (rTID) (Fig.5.4). The ligated products were transformed into chemically competent *E. coli* DH5α cells. The transformants were subjected to the screening process to confirm the presence of TID. The obtained constructs were evaluated using gel shift assay and PCR. Fig. 5.5 indicates the gel shift assay. Lanes 2, 3 and 5 showing retarded mobility with respect to the control pET20b were selected and used for further confirmation studies like PCR.

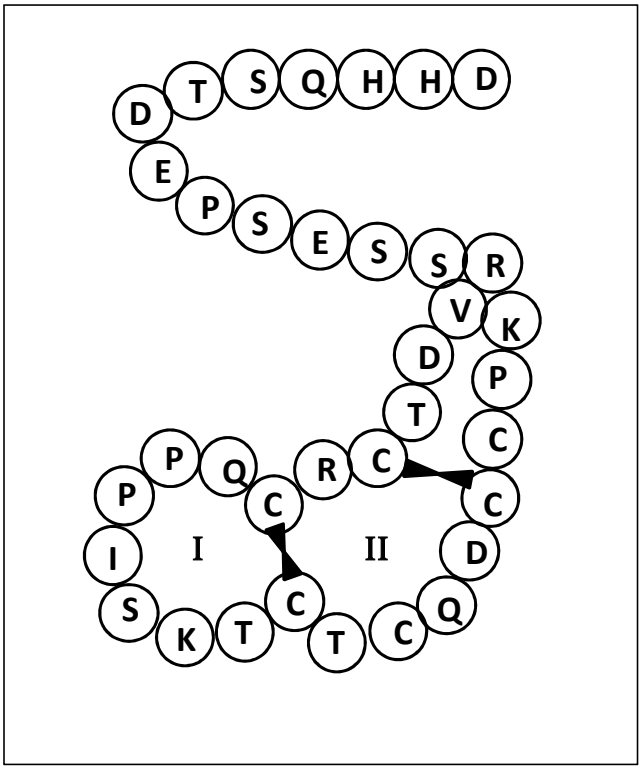


Fig. 5.1 **Pictorial representation of the trypsin inhibitory domain (TID) of the bifunctional horsegram BBI (HGI-III).**

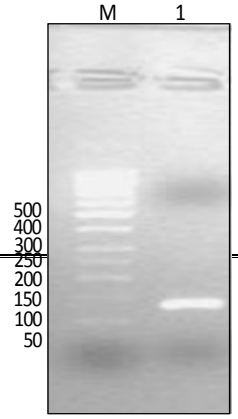


Fig. 5.2 **Agarose gel electrophoresis showing 108 bp amplicon of the TID domain.** Lane 1: 50 bp DNA ladder; Lane 2: PCR amplicon.

5` GAT CAT CAT CAG TCA ACT GAT GAG CCC TCT GAG TCT TCA AAA CCA TGC TGT GAT CAG TGC GCA **TGC ACA AAG TCA ATC CCT CCT CAA TGC** CGC TGT ACG GAC GTT AGG 3'

Fig. 5.3 **The nucleotide sequence of the amplified PCR product of trypsin inhibitory domain.** The region in bold indicates the sequence of trypsin inhibitory loop.

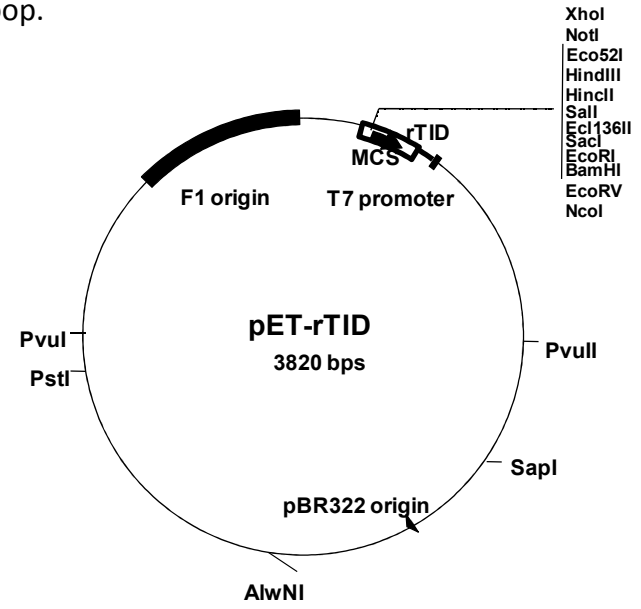


Fig. 5.4 **Schematic representation of the expression cassette in pET20b vector to produce rTID protein.**

PCR was performed using the primers HGI-F and HGIT-R and pET20b-rTID DNA as template. The amplification product of ~110 bp indicates the presence of TID (Fig. 5.6). The dideoxy sequence analysis of pET20b-rTID DNA confirmed the occurrence of TID in pET20b vector. The deduced amino acid sequence of the amplicon was commensurate with that of residue 1-36 of HGI-III in addition to that derived from the vector.

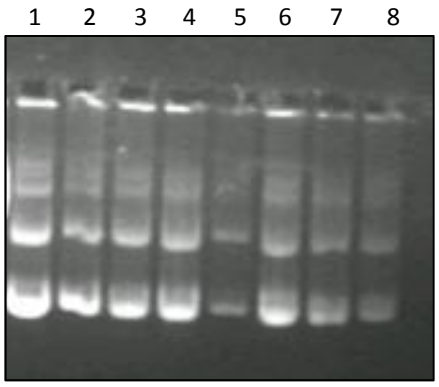


Fig. 5.5 **Agarose gel electrophoresis profile of plasmid DNA isolated from putative clones.** Lane 1: pET20b vector control; Lanes 2-8: recombinant clones.

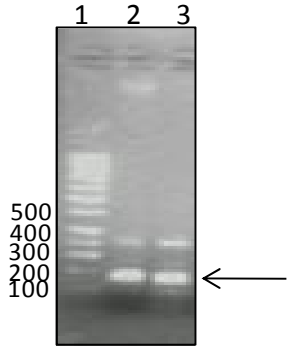


Fig. 5.6 **Agarose gel electrophoresis for the confirmation of rTID.** Lane 1: 100 bp DNA ladder; Lane 2 and Lane 3: rTID amplicon. Arrow indicates the rTID amplicon.

Purification and characterisation of recombinant trypsin inhibitory domain (rTID)

pET20b-rTID was expressed in *E. coli* BL21 (DE3) pLysS and solubilised by sonication to obtain the cell lysate. Fig. 5.7 indicates the SDS-PAGE profile of over expressed pET20b-rTID. The expressed protein pET20b-rTID constituted ~96 % of the total protein. The approximate molecular weight of pET20b-rTID with reference to the standard molecular weight marker was 4±1 kDa. The uninduced rTID does not show any significant band at that particular region. The cell free extract was collected by centrifugation at 10000 rpm for 10 min at 4 ^{o}C after the removal of cell debris. The supernatant and pellet were evaluated for trypsin inhibitory activity. The trypsin inhibitory activity was accumulated in the supernatant. The trypsin inhibitory activity of the crude cell free extract was $4.7\pm0.2\times10^{5}$ TIU/L culture. Partial purification of pET20b-rTID was achieved using size exclusion chromatography, which discriminates between molecules based on their size. The cell free extract was loaded on to a Sephadex G-50 column (1.5×90 cm) equilibrated with 0.025 M Tris-HCl buffer, pH 8.2. Elution was carried out at a flow rate of 10 mL/h. The fractions exhibiting trypsin inhibition were pooled and lyophilised and used for further purification (Fig. 5.8 A). Further purification by RP-HPLC (Waters Symmetry Shield^{TM} RP18 5µ 4.6 (i.d) × 150 mm column) using a binary gradient of 0.1 % TFA and 70 % acetonitrile in water containing 0.05 % TFA (Fig 5.8 B) afforded the rTID in an apparently homogenous form. The specific activity of the purified rTID was $4.6\pm0.5\times10^{3}$ TIU\ mg. The purified protein was ~98 % pure.

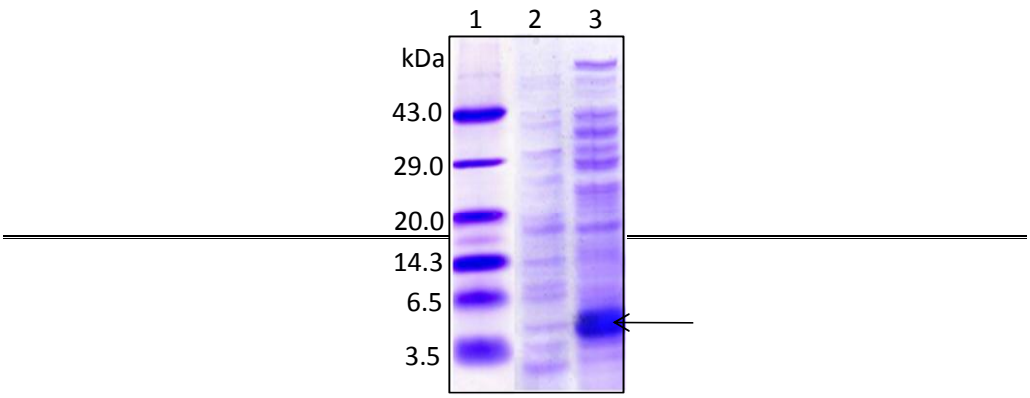


Fig. 5.7 **SDS–PAGE (12.5% T, 2.7% C) profile of cell free extracts of rTID.** Lane 1: Molecular weight markers; Lane 2: uninduced rTID and Lane 3: rTID induced with 0.3mM IPTG. Arrow indicates expressed rTID.

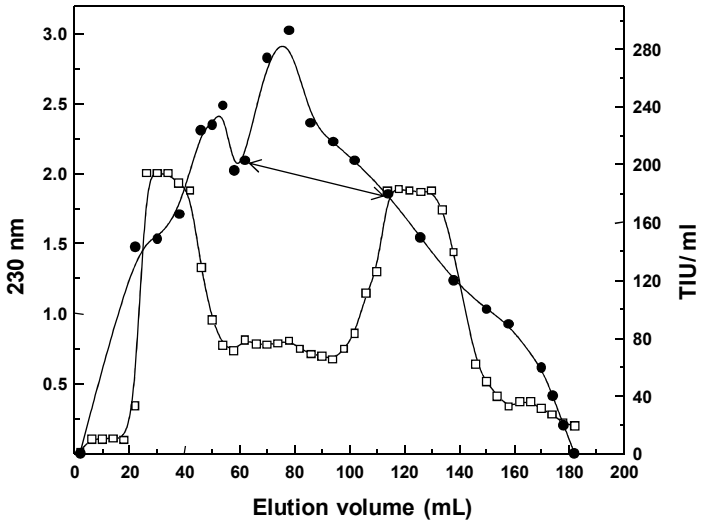


Fig. 5.8 A **Size exclusion chromatography profile of rTID.** The *E. coli* cell lysate was loaded on a Sephadex G-50 column equilibrated with 0.025 M Tris-HCl buffer (pH 8.2) at a flow rate 10 mL/h. (−•−) TIU/mL,(− □ −) A $_{230}$. The arrow shows the active inhibitor fractions that were pooled.

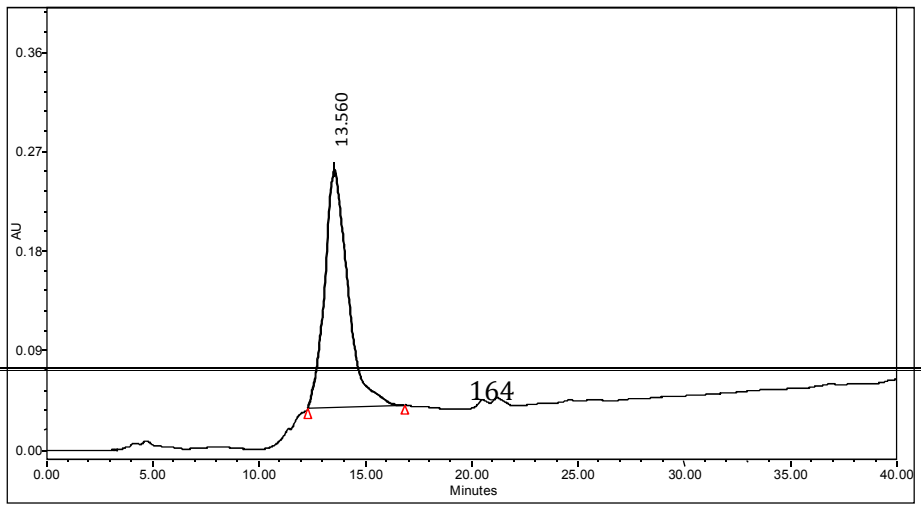


Fig. 5.8 B **RP-HPLC purification of rTID.** The pooled trypsin inhibitor fraction of Sephadex G-50 purification step was lyophilised and repurified by RP-HPLC using a binary gradient.

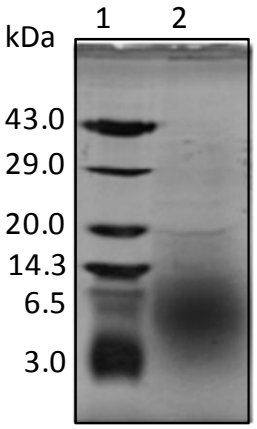


Fig.5.9 **SDS-PAGE (15% T, 2.7% C) profile of rTID.** Lane 1: Molecular weight marker; Lane 2: purified rTID.

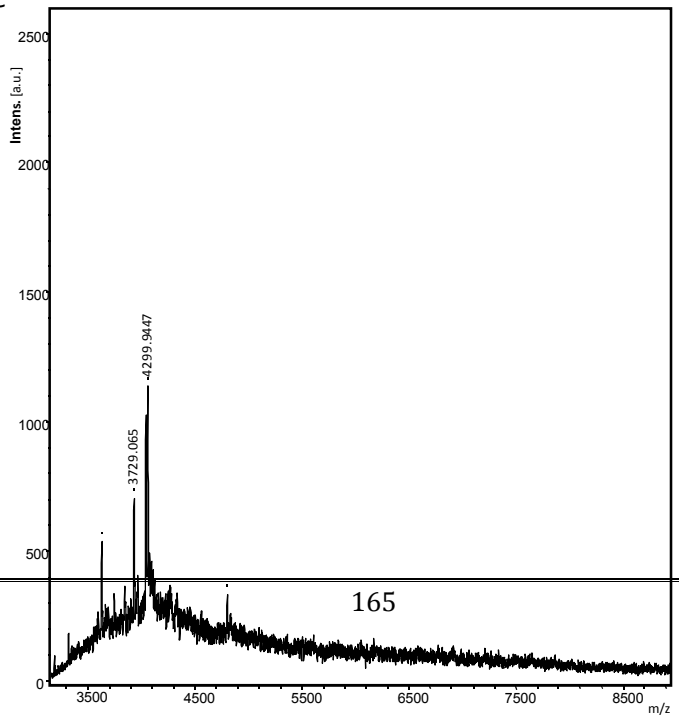


Fig. 5.10 **ESI mass spectrum of rTID showing a molecular mass of 4299.944 Da.**

The apparent molecular mass of pET20b-rTID estimated by SDS-PAGE was ~4±1 kDa (Fig. 5.9), in close agreement to the theoretical molecular mass. The rTID contains in addition to residues 1–36 of HGI-III, two additional residues M&D from the vector at the N-terminal end. ESI tandem MS indicated that the molecular mass of pET20b-rTID was 4.299 kDa (Fig. 5.10).

Size exclusion chromatography

For the determination of molecular weight, size exclusion chromatography was used. Size exclusion chromatography measurements were performed using a BIOSEP-SEC-S 3000 column on a Waters Associate HPLC. The column was pre-equilibrated with 0.1 M Tris-HCl pH 8.0 at a flow rate of 1 mL per min prior to sample loading.

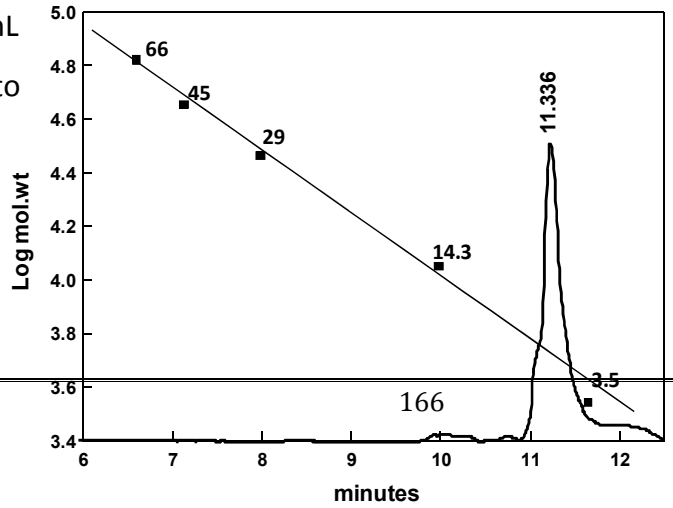
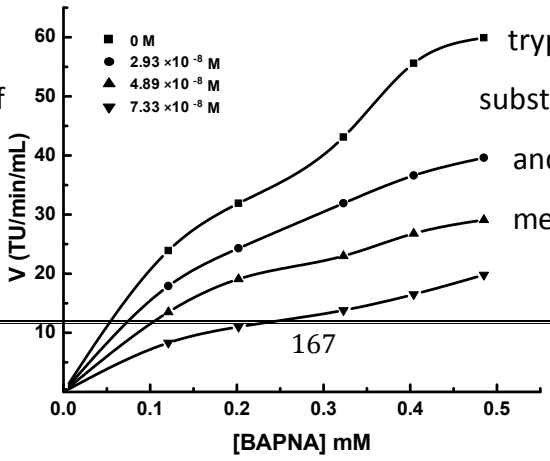


Fig 5.11 **Size-exclusion chromatography of rTID.** The purified rTID was dissolved in 0.1 M Tris-HCl, pH 8.0 and loaded on to a BIOSEP-SEC-S 3000 column pre-equilibrated in the same buffer and eluted at 1 mL/min. Retention time vs log molecular weight standard proteins is also shown.

A mixture of standard proteins, bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), lysozyme (14.4 kDa), and insulin β chain (3.5 kDa) were used for column calibration. The molecular weights were calculated from a plot of elution time vs log of molecular weight (Fig. 5.11). The apparent molecular weight determined by analytical gel filtration chromatography on BIOSEP-SEC-S-3000 was ~4±1 kDa.

Inhibitory properties of pET20b-rTID

The initial rates of reaction in the presence and absence of pET20b-rTID followed Michaelis-Menten kinetics (Fig. 5.12). pET20b-rTID was pre-incubated with trypsin and varying concentrations of substrate, BAPNA for 10 min at 37 ^{o}C and inhibitory activity was measured. The



mode of pET20b-rTID inhibition was evaluated from the double reciprocal plots of trypsin. The plot of 1/[S] vs 1/ [V] at different inhibitor concentration indicates that pET20b-rTID is a non-competitive inhibitor of trypsin (Fig. 5.13). The inhibitor constant (K_i) for pET20b-rTID was deduced from Dixon plot by plotting [I] vs 1/ [V] at various concentrations of the substrate.

Fig. 5.12 **Michaelis-Menten plot showing the effect of substrate concentration on the activity of bovine trypsin in the presence of various concentrations of rTID.** Data points are average of three determinations.

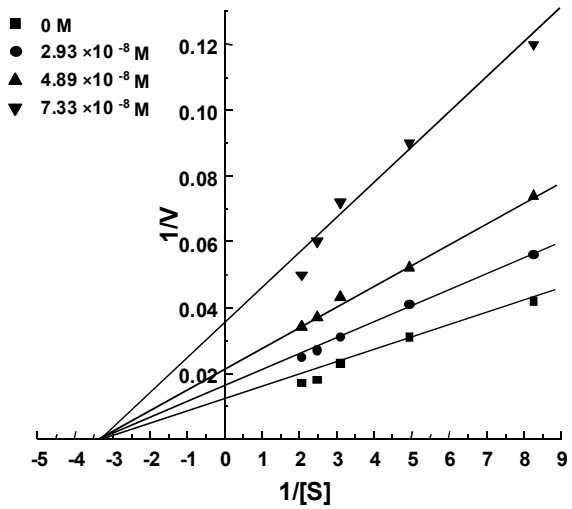


Fig.5.13 **Lineweaver-Burk plot determining mode of inhibition of rTID.** Data points are the average of three determinations.

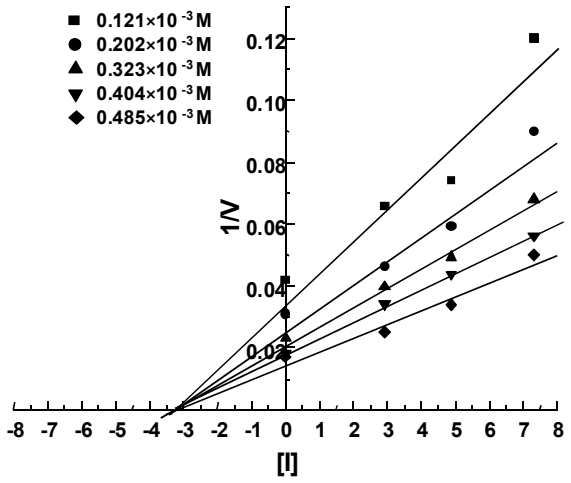


Fig.5.14 **Dixon plot for determining the equilibrium dissociation constant (Ki) of rTID**. Data points are the average of three determinations.

The apparent K_i for trypsin inhibition from Dixon plot for pET20b-rTID was $3.2\pm0.17\times10^{-8}$ M (Fig. 5.14). The K_i thus establishes a very high affinity between trypsin and pET20b-rTID, close in agreement with the K_is reported for other legume BBIs, but one order lower than the cyclic SFTI (Luckett et al., 1999).

Since rTID acts as a strong inhibitor of trypsin, the next task was to check whether it can inhibit any other proteases. Preliminary examination was conducted to detect whether it has any effect on the blood clotting time and was shown to be negative. Tryptase is the predominant neutral protease of human pulmonary mast cell and possesses a trypsin like

specificity. Although the function of these enzymes remains obscure, they could be involved in various processes including chemotaxis, endocytosis, exocytosis, protein turnover, angiogenesis, and fertilization (Woodbury and Neurath, 1980). The inhibition of rTID towards human lung tryptase was evaluated. Different concentrations of rTID were assayed against fixed concentration of human lung tryptase using BAPNA as substrate. To determine the IC_{50} value log of % residual activity was plotted against varying inhibitor concentrations. The K_i was also deduced from the IC_{50} value following the equation $K_i = IC_{50}/1+ [[S]/Km]$ (Cheng and Prusoff, 1973). The purified rTID inhibited human lung tryptase with an IC_{50} value $3.78 \pm 0.23 \times 10^{-7}$ M (Fig. 5.15) and the deduced K_i was 2.2×10^{-7} M.

Studies pointed that BBIC is used as a therapeutic in inflammatory bowel diseases (IBD- including ulcerative colitis). In case of inflammatory bowel disease patients show an increased trypsin activity in their colonic luminal content, suggesting that the balance between tryptic proteolytic activity and protease inhibitors is broken in this particular pathophysiological situation.

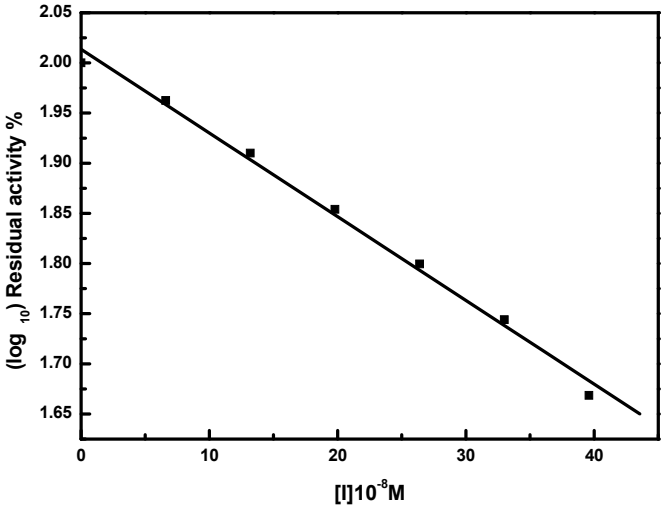


Fig. 5.15 **Determination of IC_{50} value for human tryptase.** Data points are the average of three determinations.

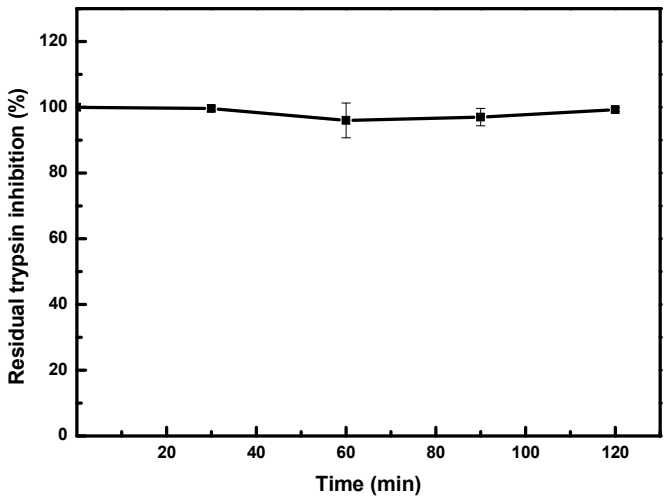


Fig. 5.16 **Thermal stability of rTID at 90 ± 1°C.** rTID was incubated at 90°C. At regular time intervals the residual trypsin inhibitor activity was determined.

The colonal luminal content (waste procured after colonoscopy) from ulcerative colitis was preincubated with pET20b-rTID and titrated against BAPNA as substrate. The studies indicate that pET20b-rTID inhibits trypsin like proteases in the colonal lumen of ulcerative colitis.

Stability studies

Thermal stability of rTID

Thermal stability of rTID was assessed to check whether it can with stand high temperatures including the cooking processes similar to other

BBIs. The purified pET20b-rTID was subjected to pre-incubation for different time periods upto 120 mins at 90 ^{o}C. The residual trypsin inhibition was measured and confirmed that heat treatment had no effect on the trypsin inhibitor activity (Fig. 5.16). These results are in agreement to that reported for the legume seed BBIs and also for the seed inhibitor HGI-III. The remarkable stability of BBIs has been ascribed to the conserved array of seven disulfide bonds. In rTID two of these conserved disulfides are still intact, which plausibly explains the high heat stability.

Invitro stability of rTID to gastrointestinal proteases.

BBIs for therapeutic use must be resistant to gastrointestinal proteolysis once they are ingested orally in order to reach the target tissue in active form. The stability of purified rTID was assessed *in vitro. In vitro* stability against gastric proteases was assessed by incubating rTID and HGI-III with pepsin for 2 h at 37 °C and pancreatin for 4 h at 37 °C. The reaction was stopped by heat inactivation of the proteases, and residual trypsin inhibitor activity determined as described earlier. rTID was resistant to the digestion of gastrointestinal proteases like pepsin and pancreatin or the combination of both. HGI-III, the seed inhibitor was used as a control also showed similar results (Table 5.1). These results are in agreement to the other BBI proteins which reach the colon in their active form.

Table 5.1. *Invitro* stability of rTID to gastrointestinal proteases.

Inhibitor	Protease	Residual trypsin inhibitor activity (%)
rTID	Control	100
	Pepsin	100±1

	Pancreatin Pepsin+Pancreatin	98.3±4 100±2.8
HGI-III	Control Pepsin Pancreatin Pepsin+Pancreatin	100 100±3.2 100±2.3 89.5±1.8

Discussion

Physiological proteinase inhibitors are required for the precise spatial and temporal regulation of proteinase activity. It is known that protease inhibitors have multiple functions, including the regulation of endogenous proteases during germination and protection of the plant from insects and microorganisms. Recent interest has focused on pharmaceutical applications of protease inhibitors. Naturally occurring protein-proteinase inhibitors like BBI therefore find use in therapeutic intervention to limit the activity of proteinases associated with disease. Following the recognition that legume BBIs are effective in suppressing carcinogenesis in several *in vitro* and *in vivo* models (Kennedy, 1998), there has been renewed scientific interest on these molecules. Regarding therapeutic applications, BBIs are given only orally as an extract from soybeans. Recently it has been reported that BBI through its anti-inflammatory properties, protects neurons from neurotoxicity mediated by activated macrophages (Li et al., 2011). A goal in BBI research has been to reduce their size to minimal structural elements yet retaining inhibitor potency and specificity. Since they were first discovered, the BBIs have been extensively studied and one of the first

issues addressed was to try and isolate the active part of the inhibitor. It was shown that the inhibitory properties are largely unaffected by removal of the main protein core following partial digestion (Odani and Ikenaka, 1973; Nishino et al., 1977) which indicated to researchers that synthetic BBI mimics would most probably be biologically active. Each domain in BBI presents a loop that projects out of the main protein core, thus making the protein resemble ''bow ties''. The projecting loops incorporate the reactive site of the inhibitor, and are the only part in direct contact with the protease upon binding. Each reactive site is a 9-mer loop delimited by a disulfide bridge. X-ray crystallographic studies of BBIs in complex with their target proteases then revealed that only part of the protein is in direct contact with the enzyme (Lin et al., 1993). The growing interest of protein mimicking coupled with use of natural scaffolds as templates and capacity for diversity in chemical synthesis has resulted in an enormous number of synthetic peptide mimics (McBride & Leatherbarrow 2001, McBride et al., 2002, Jaulent and Leatherbarrow 2004, Fear et al., 2007, Zablotna et al., 2007). It has been possible to isolate the antiproteinase activity as small (approximately 11 residues), cyclic, synthetic peptides, which display most of the functional aspects of the protein (Brauer et al., 2002).

Horsegram seed HGI-III consists of two similar domains each displaying heterologous anti-proteinase activity (Sreerama et al., 1997). The seed HGI-III inhibits trypsin with a K_i of 8.7×10^{-8} M. A functional form of the inhibitor expressed in *E. coli* is equally active against bovine trypsin with a K_i of $6.1 \pm 0.13 \times 10^{-8}$ M (Chapter 3). The larger size of BBI inhibitor limits its bioavailability, rendering the requirement for higher doses when ingested orally (Kennedy, 1998).

Designing smaller versions of this inhibitor, comprising only the domain that displays either trypsin or chymotrypsin inhibitory activity would increase the bioavailability. We therefore designed and expressed rTID, a potent inhibitor of trypsin with a K_i of $3.2 \pm 0.17 \times 10^{-8}$ M (Fig. 5.14), and of a much smaller size (~4.2 kDa). This inhibitor was not only smaller but was about three fold more potent than the seed inhibitor. Very recently a similar study on the expression of a 33 residue peptide derived from mung bean trypsin inhibitor in *E. coli* as a glutathione-S- transferase fusion protein was reported and the equilibrium dissociation constant for trypsin was $2.12 \pm 0.24 \times 10^{-8}$ M (Qi et al., 2010), very close to that reported for rTID. However the anti-tryptase activity had not been studied. The synthetic SFTI-1 is also a very potent inhibitor of epithelial serine protease, termed 'matriptase' (Long et al., 2001). The phagemid expression of TryBBI and ChyBBI fragments was validated by modeling procedure and molecular dynamics simulation but the biochemical characterisation of the active fragments was not determined (Fernandez et al., 2007). rTID not only inhibited trypsin but also inhibited human lung tryptase with a dissociation constant $K_i = 2.2 \times 10^{-7}$M (Fig. 5.15).

Chymase and tryptase are chymotrypsin and trypsin like proteases produced and stored in human mast cells and released upon degranulation as in inflammation. Tryptase is involved in inflammatory and allergic disorders, among them asthma, rhinitis, multiple sclerosis, psoriasis and rheumatoid arthritis (Scarpi et al., 2004). Although it shares high sequence homology with trypsin and chymotrypsin (~37% sequence identity), human tryptase is unusual among the serine proteinases in that its functional unit seems to be a tetramer of Mr 120000. Elevated levels of tryptase have been considered as a reliable indicator of mast cell activation in systemic anaphylaxis and mastocytosis. In several breast cancer cell lines tryptase-

like enzyme activity has been detected (McIlroy, et al., 1994). Tryptase-like enzymes have also been described as taking part in the activation and internalization of pathogenic viruses such as influenza virus, Sendai virus and human immunodeficiency virus (HIV) (Kido, 1993) and Sakai, et al., 1994). Mast cell tryptase has also been implicated in tumorgenesis and angiogenesis due to its potential to activate enzymes involved in matrix degradation. Unlike almost all other serine proteinases, tryptase is fully active in plasma and in the extracellular space, as there are no known natural inhibitors of tryptase in humans. Owing to these aspects there is much interest in obtaining inhibitors of tryptase. Soybean BBI is a highly effective inhibitor of chymase but not human tryptase. It is opined that the active site of tryptase is restricted in some manner so that it is not accessible to large protein inhibitors normally effective against trypsin-like proteases (Johnson and Barton, 1992). Previously it has been reported that BBI cannot inhibit tryptase (Ware et al., 1997) possibly due to its bigger size preventing it to enter the active tetramer pocket. HGI-III which inhibits both trypsin and chymotrypsin did not inhibit tryptase. The tryptase inhibition by rTID is consistent with the reported structural constrains of tryptase (Marco and Priestle 1997, Stubbs et al., 1997). The smaller size of rTID probably allows it to access the restricted active site of tryptase, which has an unusual tetrameric arrangement with a central pore flanked by active sites. Although tryptase is inhibited by the general synthetic inhibitors of trypsin like proteases viz di-isopropyl fluorophosphate, phenyl methyl sulfonyl fluoride, these compounds are unsuitable for human applications due to their toxicity and low stability. The N-terminal residue of the larger inhibitors such as bovine pancreatic trypsin inhibitor and ovomucoid inhibitor accord a steric clash with insertion in the restricted active site of tryptase. The Leech-derived tryptase inhibitor (LDTI), a small protein of 46 aminoacid residues is

the only known natural inhibitor (Ki = 1.4 nM) of tryptase (Stubbs et al., 1997). Structure of the complex of leech-derived tryptase inhibitor (LDTI) with trypsin and modeling of the LDTI–tryptase system has also been reported **(**Marco et al., 1997**).** The observation that rTID inhibits tryptase at the nM level bolsters the proposition that insertion of rTID occurs after residue 174 of tryptase, and thereby inactivating it.

Preliminary data suggest that rTID inhibits trypsin like enzymes of human colon lumen of patients with ulcerative colitis. This result suggests a potential application as a therapeutic agent for ulcerative colitis. BBIC is efficacious with high trypsin and chymotrypsin inhibitor activity without any apparent toxicity for both achieving clinical response and induction of remission in patient with active ulcerative colitis (Lichtenstein et al., 2008). The rTID like the legume BBIs is stable at cooking temperature and towards the digestive enzymes found in humans and animals. Soybean BBIs are reported to survive faecal fermentation and are active anticarcinogens (Marin-Manzano et al, 2009). BBIs have been reported in the colonic epithelial cells and found to be effective suppressors of induced colon carcinogenesis in mice (Billings et al., 1992). A potent anti-inflammatory effect has been associated with BBI ingestion in several animal studies (Ware et al., 1999). Analysis of tissue from rat colitis lesions indicated that activated tissue proteases are potently inhibited by BBI (Hawkins et al., 1996). These properties propagate their use as therapeutics in the diet for treatment of cancer and other related inflammatory diseases. Human populations whose diet comprises of high concentration of BBI are reported to have lower incidences of colon, breast, prostrate and skin cancer (Yavelow et al., 1983). rTID consisting of the nine residue loop remains active and is a potent inhibitor of trypsin and human tryptase. The K_i values indicate that it is as potent as the horsegram seed inhibitor and other BBIs.

It will be desirable to have a small and stable peptide drug that is capable of inhibiting chymotrypsin or trypsin, or even elastase. The successful expression of rTID in conjunction with it being smaller and stable demonstrates the feasibility of producing a BBI-derived pharmaceutical for therapeutic interventions.

CHAPTER 6

SUMMARY AND CONCLUSIONS

The Bowman-Birk inhibitors (BBIs) are small serine protease inhibitors found in seeds of legumes and in many other plants (Laskowski and Kato 1980). Typically, their molecular masses range between 6 and 9 KDa but most of the BBIs exists as dimers in solution. Usually BBIs inhibit trypsin at the first reactive site (N-terminal) and chymotrypsin at the second reactive site (C-

terminal). BBIs have gained considerable interest in the field of protein science due to their anticarcinogenic activity. The ability to inhibit different proteases found in cancer tissues may account for this remarkable therapeutic property.

In the present investigation entitled, **"Cloning and site directed mutagenesis of horsegram *(Dolichos biflorus)* BBI,''** attempts have been made to identify and understand the role of residues involved in the dimerisation of HGI-III, the major BBI of horsegram seeds through a site directed mutagenesis approach. Attempts were also made to increase the bioavailability of the BBI. The recombinant HGI was pruned such that it retained its trypsin inhibitory potential and attained a new status of inhibition with respect to tryptase and thereby customized as a peptide of great therapeutic value. Following are the salient features of the present investigation.

- The gene coding for HGI-III, the major BBI from horsegram was amplified by PCR from genomic DNA isolated from defatted flour.
- The nucleotide sequence analysis of the HGI-III gene accedes with the reported protein sequence of HGI-III indicating the absence of introns.
- The HGI-III gene was cloned into pRSET C vector to generate the plasmid designated pRSET-rHGI (Fig. 3.7).

- pRSET-rHGI was functionally overexpressed in *E. coli* BL21 (DE3) pLysS cells with a specific activity of $1.54\pm0.44\times10^3$ TIU/mg protein. The protein was purified to homogeneity using trypsin-sepharose affinity column chromatography.

- The homogeneity of rHGI was analyzed by native PAGE (Fig. 3.10 D) and RP-HPLC (Fig. 3.11) and confirmed by the release of a single N-terminal amino acid residue (Fig. 3.12).
- N-terminal sequence analysis revealed that the aminoacid sequence of rHGI was the same as that of seed HGI-III with the exception of the starting Met residue.
- The molecular weight determined by SDS-PAGE (Fig. 3.13 Lane 3) and size-exclusion-HPLC (Fig 3.14) was ~16 kDa. The molecular weight determined by ESI-Tandem MS indicated that the molecular mass of rHGI was 8676.925 Da (Fig. 3.15) and the protein showed an isotopic pattern of $(M+H)^{8+}$ charge state (Fig. 3.16). These results indicated that rHGI existed as a dimer in solution like the dry seed inhibitor.
- The number of disulphide bonds of rHGI was seven and rHGI was extremely stable towards a wide range of pH and temperature.
- The stoichiometry of inhibition of rHGI against trypsin indicated that rHGI bound to trypsin in a 1:0.9 molar ratio (Fig. 3.20 A).
- rHGI competitively inhibited trypsin and chymotrypsin simultaneously and independently indicating it was double-headed, with independent sites for trypsin and chymotrypsin inhibition. The dissociation constant of rHGI against trypsin was $6.1\pm0.13 \times10^{-8}$ M and towards chymotrypsin was $3.0\pm0.15 \times10^{-7}$ M (Table 3.2).
- The cross-reactive studies with the antibodies of HGI-III indicated the presence of similar antigenic epitopes in rHGI (Fig. 3.26).
- Point mutations of rHGI were used to validate the involvement of active site residue Lys^{24} and C-terminal aspartates in the dimerisation of HGI-III.

- The K24A mutant of rHGI was converted to an elastase inhibitor thereby loosing its trypsin inhibitor activity yet retaining its chymotrypsin inhibitor activity (Fig. 4.4). The elastase inhibitory activity was 4.8×10^3 EIU/mg protein.
- SDS-PAGE followed by immuno-blotting and size exclusion chromatography indicated that the rHGI-K24A mutant existed as a monomer (Fig. 4.5 & 4.6) in accordance with the homology modelling studies previously reported.
- The purification of rHGI-K24A mutant was hindered by the lack of the trypsin binding site. For the ease of purification, point mutants of HGI-III were constructed with a C-terminal $(His)_6$ tagged protein in pET20b vector.
- The expressed pET20b-HGI was purified on a Ni^{2+}-NTA Sepharose affinity chromatography which yielded ~98% pure protein. The yield of pET-20bHGI was 6.2±0.6 mg/L culture broth.
- Using pET-20bHGI the following mutants pET-20bHGI K24A and pET-20bHGI D75A were constructed.
- The expressed pET-20bHGI K24A was purified by Ni^{2+}-Sepharose chromatography, which yielded ~ 98% pure protein. The yield of pET-20bHGI K24A was 5.8±0.5 mg per litre of culture.
- The functionally expressed pET-20bHGI D75A retained its trypsin inhibition even after its mutation since its active site residues were intact even after mutation with a specific activity of $2.3\pm0.42\times10^3$ TIU/mg protein.
- The involvement of Asp^{76} in dimerisation was evaluated by a deletion mutant of Asp^{76} construct in pET20b vector designated as pET-20bHGI Δ76. The expressed protein was purified by Sephadex G-100 size exclusion chromatography (Fig. 4.15) followed by RP-HPLC.

- Evaluation of the kinetic properties revealed that all the mutants followed Michaelis Menten kinetics. The mutant pET-20bHGI K24A inhibited elastase. The mutants pET-20bHGI D75A and pET-20bHGI Δ76 inhibited trypsin at the first reactive site and chymotrypsin at the second reactive site. The kinetic constants were similar to the seed HGI-III.
- Comparative analysis of the results of SDS-PAGE (Fig. 4.23) revealed that pET-20bHGI D75A and pET-20bHGI Δ76 mutants existed as monomers whereas pET-20bHGI K24A existed as a dimer, which was unexpected. SDS-PAGE followed by western blotting of pET-20bHGI D75A and pET-20bHGI Δ76 mutants indicated the monomeric status of these mutants (Fig. 4.24).
- Size exclusion studies at different pH and in presence of $ZnSO_4$ for the pET-20bHGI K24A led to the conclusion that the $(His)_6$ tag was not involved in dimerisation. It is plausible that the stronger (R^{31}-D^{76}) salt bridge was not disrupted and therefore this mutant remained as a dimer.
- Size exclusion chromatography studies of the mutants of HGI divulge the pivotal role of C-terminal aspartates in the dimerisation of the molecule (Fig. 4.25).
- The decrease in the thermal stability of the mutants rHGI K24A, pET-20bHGI D75A and pET-20bHGI Δ76 leads to the conclusion that monomeric forms of BBI are less stable than dimer (Fig. 4.26).
- A stable small inhibitor was designed based on the trypsin inhibitory domain (TID) of horse gram HGI-III.
- TID was amplified using the plasmid pRSET-rHGI and cloned into pET20b vector.

- pET20b-rTID was functionally expressed in *E.coli* with an activity of $4.7\pm0.2\times10^5$ TIU/L culture. The protein was purified to homogeneity using Sephadex G-50 size exclusion chromatography followed by RP-HPLC chromatography (Fig. 5.8). The specific activity of the purified rTID was $4.6\pm0.5\times10^3$ TIU\ mg.

- The molecular weight of pET20b-rTID determined by SDS-PAGE (Fig. 5.9) and analytical gel filtration chromatography (Fig. 5.11) were ~4±1 kDa. These values are in close agreement to the molecular weight determined by MALDI-MS (Fig. 5.10).

- pET20b-rTID followed Michaelis Menten kinetics. pET20b-rTID inhibited trypsin through a non-competitive mode. The dissociation constant K_i for the pET20b-rTID is $3.2\pm0.17\times10^{-8}$ M (Fig. 5.14).
- pET20b-rTID inhibited human lung tryptase with an IC_{50} value $3.78\pm0.23\times10^{-7}$ M (Fig. 5.15). The K_i was 2.2×10^{-7} M.
- Preliminary studies revealed that pET20b-rTID inhibited the proteases found in the colonal lumen of ulcer colitic patients.
- pET20b-rTID was extremely stable exhibiting thermal stability similar to other BBIs and also stable towards gastrointestinal proteases like pepsin and pancreatin suggesting that it can withstand high cooking temperature and reach the colon in an active form.

The data compiled in this thesis describes the cloning, functional expression, purification and characterization of recombinant BBI from horsegram (*Dolichos biflorus*). Through a site directed mutagenesis approach the residues involved in the self-association of HGI-III, an anomalous behavior of legume BBIs was analysed. The studies revealed the crucial role of negatively charged C-terminal end in the dimerisation of HGI-III (**Muricken, D. G. & Gowda, L. R.** (2010). Functional expression of

Horsegram (*Dolichos biflorus*) Bowman-Birk inhibitor and its self association. *Biochim. Biophys. Acta.* 1804, 1413-1423). The functional expression of pET20b-rTID, a pruned inhibitor from HGI-III, due to its smaller size, ability ihhibit trypsin and tryptase and increased stability may increase the bioavailability of the protein, thereby can be used in therapeutic applications. The strong inhibition towards trypsin and tryptase may account for this (**Muricken, D. G. & Gowda, L. R.** (2011). Molecular engineering of a small trypsin inhibitor based on the binding loop of horsegram seed Bowman-Birk inhibitor. *J. Enz. Inhib. Med. Chem.*, 1-8.).

The expressed proteins derived from HGI-III inhibiting proteases like trypsin, chymotrypsin, elastase and tryptase and lack of highly immunogenic N- and C-terminal residues, high stability and low molecular weight could be an attractive feature for the use as a therapeutic agent in different diseases like cancer, ulcerative colitis, asthma, rhinitis, multiple sclerosis, psoriasis and rheumatoid arthritis.

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