STRUCTURAL STUDIES OF THE PROTEINASE INHIBITORS OF GERMINATED HORSEGRAM (*Dolichos biflorus*) SEEDS

A Thesis submitted to the

UNIVERSITY OF MYSORE

for the award of the degree of

DOCTOR OF PHILOSOPHY

in

BIOCHEMISTRY

by

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July, 2004

DECLARATION

I hereby declare that this thesis entitled **"Structural studies of the proteinase inhibitors of germinated horsegram (*Dolichos biflorus*) seeds"**, submitted herewith, for the degree of **Doctor of Philosophy** in **Biochemistry** of the **University of Mysore**, Mysore, is the result of work done by me in the Department of Protein Chemistry and Technology, Central Food Technological Research Institute (CFTRI), Mysore, India, under the guidance and supervision of **Dr. Lalitha R. Gowda**, during the period of November, 2000 - July, 2004.

I further declare that the results of this work have not been previously submitted for any other degree or fellowship.

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July, 2004. PRADEEP KUMAR

CERTIFICATE

I hereby certify that this thesis entitled **"Structural studies of the proteinase inhibitors of germinated horsegram (*Dolichos biflorus*) seeds"** submitted by **Mr. Pradeep Kumar** to the University of Mysore, Mysore, for the degree of **Doctor of Philosophy** in **Biochemistry** is the result of research work carried out by him in the Department of Protein Chemistry and Technology, Central Food Technological Research Institute (CFTRI), Mysore, under my guidance and supervision. This work has not been submitted either partially or fully for any other degree or fellowship.

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ACKNOWLEDGMENTS

The words are inadequate to express my deep sense of gratitude towards Dr. Lalitha Ramakrishna Gowda, for stimulating guidance, unstinting support and encouragement during the course of this investigation. I thank her for providing a inspiring research atmosphere in the laboratory and valuable suggestions during my stay at CFTRI.

I am grateful to Dr. A. G. Appu Rao, Head, Department of Protein Chemistry and Technology for providing all the facilities necessary for carrying out investigations and for his cooperation, timely suggestions that he rendered and keen interest in the work. I would like to thank all the staff and students of the Department of Protein Chemistry and Technology for their cooperation and support.

I wish to record my sincere thanks to Dr. V. Prakash, Director, Central Food Technological Research Institute for giving me an opportunity to work in this institute and providing me the necessary research facilities.

I thank Prof. M. R. N. Murthy, Molecular Biophysics Unit and Prof. H. S. Savithri, Department of Biochemistry, Indian Institute of Science, Bangalore for their valuable suggestions. My sincere thanks are also to Mr. Michael D'Silva and Mr. Sarvanan, DBT facility for protein/peptide sequencing, Indian Institute of Science, Bangalore for technical help.

My thanks are also due to Dr. Nagasuma Chandra and Sridhar Hariharaputran, Bioinformatics Centre, Indian Institute of Science, Bangalore for kind help and expertise in modeling studies.

I acknowledge timely help and cooperation of the staff of FOSTIS-Library, Photography Section, Central Instruments Facility and Services and Department Agri-Horticulture Section.

My heartfelt thanks are due to Dr. D. Rajgopal Rao, Scientist (Ret.), CFTRI, Mysore, Prof. Ramakrishna Gowda, Department of Physics, University of Mysore, Dr. Y. N. Sreerama, Department of Lipid Science and Traditional Foods and Dr. Asha Martin, Department of Food Safety and Analytical Quality Control Laboratory, CFTRI for their constant support and encouragement.

I gratefully acknowledge the Council of Scientific and Industrial Research, New Delhi for the Senior Research Fellowship granted to me.

I specially acknowledge Devaraj Gouda, Beena Prince, Prince Xavier, Subba Rao, Ayyagari Ramesh, Vishu Kumar, Harish Prashant, Santosh, Mallikarjuna, Vivek, Lingaraj, Devavratha, Thippeswamy, Vijay, Aksha,

and Deepa for their help, cooperation and companionship rendered, which made my stay here very pleasant.

I sincerely appreciate the support and encouragement rendered by my mother, brother, sisters and brother-in-laws, which has enabled me to successfully complete this research work.

Mysore **Pradeep Kumar**
July, 2004

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LIST OF ABBREVIATIONS

λ	Wavelength
°C	Degree centigrade
μg	Microgram
[I]	Inhibitor concentration
[S]	Substrate concentration
4-VP	4-Vinylpyridine
Å	Angstrom unit
ANS	8-Anilino-1-napthalenesulfonate
APS	Ammonium persulfate
ATZ	Anilinothiazolinone
BAPNA	$\propto$-N-Benzoyl-DL-arginine-*p*-nitroanilide
BCIP	5-Bromo-4-chloro-3-indolylphosphate
BSA	Bovine serum albumin
BTPNA	N-Benzoyl-L-tyrosine-*p*-nitroanilide
$CaCl_2$	Calcium chloride
CAPS	3-[Cyclohexylamino]-1-propanesulfonic acid
CBB	Coomassie brilliant blue
CCl_4	Carbon tetrachloride
CD	Circular dichroism
CIU	Chymotrypsin inhibitory units
CM	Carboxymethyl
cm	Centimeter
CNBr	Cyanogen bromide
CU	Chymotrypsin unit
DEAE	Diethylaminoethyl
DEPC	Diethylpyrocarbonate
DMSO	Dimethylsulfoxide
DTT	DL-Dithiothreitol

EDTA	Ethylene diamine tetra acetic acid
g	Grams
GuHCl	Guanidine hydrochloride
h	Hour
HCl	Hydrochloric acid
HGFI	Horsegram flower inhibitor
HGGI	Horsegram germinated inhibitor
HGI	Horsegram inhibitor
HGLI	Horsegram leaf inhibitor
HPLC	High performance liquid chromatography
IU	Inhibitory units
kDa/Da	Kilo Daltons/Daltons
K_i	Inhibitory constant
L	Liter
MALDI-MS	Matrix assisted laser desorption ionization - Mass spectrometry
$MgCl_2$	Magnesium chloride
min	Minute
mL	Milliliter
M_r	Molecular weight
mtorr	Millitorr
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBT	Nitroblue tetrazolium
nm	Nanometer
PAGE	Polyacrylamide gel electrophoresis
PE	Pyridylethylated
pI	Isoelectric point
pmole	Picomole
PMSF	Phenylmethyl-sulfonyl fluoride

PTC	Phenylthiocarbamyl
PVDF	Polyvinilidine diflouride membrane
RP	Reverse phase
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid
TEA	Triethylamine
TEMED	N-N, N'-N'-Tetramethyl 1, 2-diaminoethane
TFA	Trifluoroacetic acid
TIU	Trypsin inhibitory unit
TNBS	2, 4, 6-Trinitrobenzenesulfonoic acid
TPCK	Tosyl-phenyalanine chloromethylketone
Tricine	N-Tris (hydroxymethyl) methyl glycine
Tris	Tris (hydroxymethyl) amino methane
TU	Trypsin unit
UV-VIS	Ultraviolet-Visible
v/v	Volume by volume
Vs	Versus
w/v	Weight by volume

Introduction

The existence of protein-proteinase inhibitors (PPIs) in nature was described as early as 1894 when Fermi and Pernossi (1894) noted the “anti-trypsin activity” of serum. The PPIs are prevalent among many plant species, are diverse in number and are specific towards inhibiting proteolytic enzymes that include elastase, thrombin, plasmin, kallikrein, trypsin, chymotrypsin, chymase, tryptase, bacterial enzymes such as 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 isolation and characterization of PPIs and the fundamental concepts associated with protein-inhibitor interactions comprise the pioneering work of Kunitz during 1930s and 1940s (Kunitz and Northrop, 1936; Kunitz, 1945, 1946, 1947a, 1947b) summarized by Birk (1987, 1989). The inhibitors have since been the subjects of research in varied disciplines. Inhibitor proteins have been studied as model systems for elucidation of the mechanism of inhibition of proteinases and protein-protein association. The occurrence in valuable plant foods and their possible involvement in nutritive properties have been investigated by nutrionists. The involvement of plant proteinases inhibitors in prevention of tumerogenesis and unique pharmacological properties has gained considerable interest.

Since 1940, a very large number of inhibitors have been identified and characterized from wide range of organs and species. Seeds are by far the richest source and it is probable that all seeds contain one or more inhibitors, although the levels vary greatly and

their detection is dependent on the selection of the appropriate target enzymes and assay conditions. The vast majority of the inhibitors characterized to date are active against endoproteinases. The high number of known and partially characterized inhibitors of plant origin and confusion in the nomenclature, led to a classification on the basis of sequence homology, nature of reactive or inhibitory site and interaction with proteinases according to the standard mechanism (Reviewed by: Laskowski and Kato, 1980; Laskowski, 1986). Detailed information about the serine proteinase inhibitor families of animal, plant and microbial origin were reported recently (Shewry, 1999; Laskowski *et al.*, 2000). The most recent classification of plant PPIs, adapted from De Leo *et al.*, (2002) is depicted in Table 1. This classification is based on the primary structure of the proteinase. With the exception of the cysteine proteinase and metallocarboxypetidase inhibitor families, all the other reported families of PPIs contain inhibitors of serine proteinases, the most diffused and studied.

Among these families, the Bowman-Birk and Kunitz inhibitor families are most widely distributed, and the most abundant in the seeds of leguminous plants as well as the most intensively studied groups.

Kunitz soybean trypsin inhibitor family

The Kunitz inhibitors are the second major family of inhibitors, which are widely distributed, and abundant, in seeds of leguminous plants. The first plant proteinases inhibitor to be isolated and characterized was Kunitz soybean trypsin inhibitor (KTI). KTI is a 'typical', single headed legume trypsin inhibitor comprising of 121 amino acids with an M_r about 20 kDa, four cysteine residues that form two intra-chain disulfide bonds, primarily inhibiting trypsin, but also

weakly inhibiting chymotrypsin. The inhibitor is inactivated by heat and gastric juice. KTI has played a pivotal role in understanding the standard mechanism of proteinase inhibitor because of the analogous nature to their substrates. The purification, crystallization, kinetics of interaction and complex formation of KTI with trypsin (Kunitz, 1947a, 1947b) is a major landmark in the study of PPIs. The numerous studies on specificity, stability, physical, kinetic and other properties of KTI have been summarized (Kassel 1970; Birk, 1976).

Table 1. **Proteinase inhibitor families of plant origin*.**

Inhibitor family	PI code	InterPro accession number
Bowman-Birk proteinase inhibitors	BBI	IPR000877
Cereal trypsin/α-amylase inhibitors	BRI	IPR001768
Cysteine proteinase inhibitors	CYS	IPR000010
Metallocarboxypeptidase inhibitors	MCI	Not available
Mustard trypsin inhibitor	MSI	Not available
Potato type I inhibitors	PI1	IPR000864
Potato type I proteinase inhibitors	PI2	IPR003465
Serpin	SPI	IPR000215
Soybean trypsin inhibitor (Kunitz)	KNI	IPR002160
Squash inhibitors	SQI	IPR000737

The database is accessible at **http://bighost.area.ba.cnr.it/PLANT-PIs.** *The table is reproduced with the kind permission of the author (De Leo *et al.*, 2002).

Bowman-Birk proteinase inhibitor family

The Bowman-Birk inhibitors (BBI) are one among the most widely distributed groups, being particularly abundant in legume seeds. Soybean BBI, isolated by Bowman (1946) and characterized by Birk *et al*., (1963), serves as a prototype for the BBI family (Birk, 1961, 1985, 1987, 1989).

The common main feature of these small proteins (M_r of 7-9 kDa) is the unusually large content of conserved fourteen ½ cystine residues forming a network of seven disulfide bonds in a single chain molecule. The first covalent structure of soybean BBI, a single polypeptide chain of 71 amino acid residues was elucidated by Odani and Ikenaka (1973). The covalent structure of a 76 amino acids BBI of horsegram seeds (HGI-III, Prakash *et al*., 1996) is depicted in Figure 1.1.

The BBIs consist of two homologous regions (Region 1 and 2), linked together by two polypeptide chains. Each region contains three loops (loop I, II and III for region 1 and I', II' and III' for region 2). The reactive site of each region is contained in the outermost loop (loop I or loop I') of nine amino acid residues held by a disulfide bridge. HGI-III inhibits two proteinases simultaneously and independently. Trypsin generally binds to the first reactive site (loop I) and chymotrypsin to the other reactive site. The reactive site at the amino-terminus is generally called first reactive site and the other at the carboxy-terminus is called second reactive site. In BBIs, the two binding sites inhibit independently and simultaneously two (not necessarily identical) molecules of proteinases and therefore are named as 'double headed inhibitors'. The proteinases inhibited are trypsin, chymotrypsin and elastase.

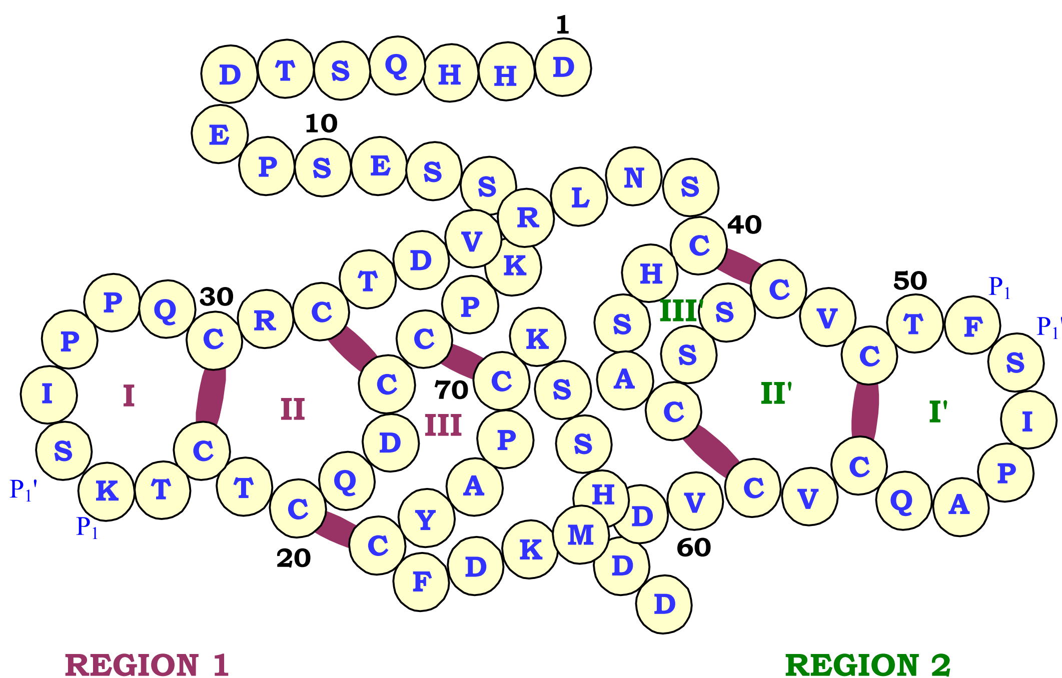


Figure 1.1 **Covalent structure of the major BBI of horsegram (HGI-III).**

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 amino- and carboxy-terminal segments folded in an extended conformation.

The present review will be restricted to BBIs of Leguminosae.

Bowman-Birk inhibitors of Leguminosae

Legume plants are morphologically classified into three families, *Caesalpineae*, *Mimosaceae* and *Fabaceae*, according to Hutchinson (1969). BBIs have been found in the seeds of members of *Fabaceae*, the most advanced family. The *Fabaceae* family can be further sub-divided into 50 tribes. The inhibitors LBI, GBI, ABI and MAI and MBI are from the plant tribe *Phaseoleae*, BBI, SBI-C-II and SBID-II are from *Gramineae*, VAI from *Vicieae* and PI-A-II and PI-B-II from *Stylosantheae*. The above three tribes, *Phaseoleae*, *Gramineae* and

Vicieae are located in the middle of all the tribes of *Fabaceae* and are morphologically close. *Stylosantheae* is one of the most advanced tribes in the family. The two tribes *Phaseoleae* and *Glycineae* in the morphological classification are scarcely distinguishable in the phylogenetic tree, because the inhibitors obtained from seeds of both tribes are classified in group I and II (Ikenaka and Norioka, 1986).

Localization of Bowman-Birk inhibitors

BBIs are ubiquitous and known to be present in all parts of the plant body. Although BBI isoforms are concentrated in the cotyledons, their subcellular localization still remains unsolved. A number of studies utilizing differential or density gradient centrifugation or immunofluorescence microscopy have suggested that the BBIs are localized in the cytosol rather than in the protein bodies (Hobday *et al.*, 1973; Miege *et al.*, 1976; Pusztai *et al.*, 1977; Chrispeels and Baumgartner, 1978). Some of the studies on the mung bean (Wilson, 1988) using ultracentrifugation using glycerol gradients and soybean (Horisberger and Tecchini-Vonlanthen, 1983) by protein A-gold electron microscopy revealed the BBIs are localized in the protein bodies. Godbole *et al.*, (1994b) showed that the BBIs in pigeon pea has neither a storage role nor a role in controlling endogenous proteinase activity on basis of their localization in the cytosol.

Multiplicity of Bowman-Birk inhibitors

Most of the legume seeds studied this far have several forms of BBIs, called isoinhibitors. Many of them have been characterized with respect to their M_r, pI, reactivity and their partial and complete primary structure. The complete amino acid sequence have been reported for isoinhibitors of soybean (BBI: Odani and Ikenaka, 1972; SBC-C-II: Odani and Ikenaka, 1977b; D-II and E-I: Odani and Ikenaka, 1976,

1978a), lima bean (LBI-I, -IV and -IV': Stevens, *et al.*, 1974), garden bean (GBI-II and II': Wilson and Laskowski, 1975), adzuki bean (ABI-I: Ishikawa, *et al.*, 1979; II and II': Yoshikawa, *et al.*, 1979a; I-A and I-A': Kiyohara, *et al.*, 1981; IIa and IIc: Ishikawa, *et al.*, 1985), *Macrotyloma axillare* (MAI-DE-3 and DE-4: Joubert *et al.*, 1979), mung bean (MBI: Zhang *et al.*, 1982; MBI-F: Wilson and Chen, 1983), *Vicia angustifolia* (VAI: Shimokawa *et al.*, 1984) and peanut (PI-A-I, A-II, B-I and B-II: Norioka and Ikenaka, 1983b; B-III: Norioka and Ikenaka, 1983a), horsegram (Sreerama *et al.*, 1997), Winter pea seeds (Quillien *et al.*, 1997) and White Sword bean (Park *et al.*, 2002).

Most of the isoforms have similar amino acid composition and primary sequences but exhibit heterogeneity at the amino- and carboxy-termini. Soybean inhibitor, E-I (Odani and Ikenaka, 1978a) and Hwang's soybean inhibitor II (Hwang, *et al.*, 1977) lack the nine amino-terminal amino acid residues of soybean D-II (Odani and Ikenaka, 1978a), adzuki bean inhibitor II' lacks the amino-terminal nine residues of adzuki bean inhibitor II (Yoshikawa *et al.*, 1979a), lima bean inhibitor I lacks the amino-terminal eight residues of lima bean inhibitor IV (Stevens *et al.*, 1974) and garden bean inhibitor II' lacks amino terminal residues of GBI-II (Wilson and Laskowski, 1975). Cleavages at amino sides of Ser(1), Asp(5) and Val(7) of PI-A-II of peanut seed inhibitor convert PI-A-II to PI-A-I, PI-B-I and PI-B-II respectively (Norioka and Ikenaka, 1983b). The sequences of PsTI-I and PsTI-II are identical with those of PsTI-IVa and PsTI-IVb respectively, with a nine carboxy-terminal residues deleted (Sierra *et al.*, 1999). Weder and Kahelyss (1998) isolated 23 proteinase inhibitors from Syrian local small lentils (*Lens culinaris*) and showed they inhibited bovine trypsin and chymotrypsin.

Origin of multiple inhibitor forms is attributed to either different genes or due to proteolysis (Hwang *et al.*, 1977; Orf and Hymowitz, 1979; Freed and Ryan, 1980; Tan-Wilson *et al.*, 1985; Hartl *et al.*, 1986), but the physiological significance of these inhibitory forms during seed development and germination, still remained unsolved.

The inhibitors characterized so far although are different from each other either at their amino- or carboxy-terminus, they share the 14 ½ cystine residues that are conserved in all the BBIs that help in maintaining an active conformation forming a net work of seven disulfide bridges. In addition to an array of seven disulfide bonds, amino acids common to all inhibitors are 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).

Molecular properties

BBIs are known for their unusual resistance to various proteolytic enzymes (including pepsin and pronase) and stability at acidic, alkaline pH and high temperatures. This remarkable stability of this class of inhibitor proteins is attributed to the high content of disulfide bridges. Several studies were carried out to evaluate the stability of BBIs (Abe *et al.*, 1978; Odani and Ikenaka, 1978a; Mehta and Simlot, 1982; Birk, 1985; Ohtsubo *et al.*, 1985; Babar *et al.*, 1988; Godbole *et al.*, 1994a; Terada *et al.*, 1994a) and substantiate the fact that most of the BBIs are quite stable to extremes of pH and high temperature. However BTCI from *Vigna anguiculata* (da Silva *et al.*, 2001), trypsin/chymotrypsin inhibitors from horsegram (Mehta and Simlot, 1982) and CLTI-I (*Canavalia lineata*) (Terada *et al.*, 1994a) show lower thermostability at alkaline pH than the acidic condition.

The effect of temperature on the stability of BTCI (da Silva *et al.*, 2001) at pH 7.0 using Tyr as an indicator molecule for molecular re-arrangement of the inhibitor, suggested that during heating a considerable part of the polypeptide backbone folding is preserved retaining its inhibitory activity.

Another intriguing and anomalous behavior of Bowman-Birk class inhibitors is self-association under native conditions. Most of BBIs of legumes are single polypeptides of M_r 6-9 kDa. However SDS-PAGE and analytical gel filtration chromatography indicate their M_r to be 16-18 kDa, suggesting that they exist as dimers in solution. Such self-association and anomalous behavior on SDS-PAGE resulting in a large overestimation of M_r been reported for several legume BBIs (Haynes and Feeney, 1967; Pusztai, 1968; Millar *et al.*, 1969; Gennis and Cantor, 1976b; Birk, 1985; Wu and Whitaker, 1990; Bergeron and Neilsen, 1993; Terada *et al.*, 1994a; Godbole *et al.*, 1994a; Sierra *et al.*, 1999). Many of the BBIs tend to undergo self-association to form homo dimers or trimers and more complex oligomers (Odani and Ikenaka, 1978b). The three-dimensional model of black-eyed pea BBI-chymotrypsin complex (De Freitas *et al.*, 1997) and light scattering data (Ventura *et al.*, 1981) suggest that the inhibitor molecules are in continuous equilibrium between monomers and several forms of multimers. In contrast to above BBIs, WSTI-IV (Deshimaru *et al.*, 2002) and SBI-C-II (Odani and Ikenaka, 1977a) (Wild soja, *Glycine max*), PVI-3I (Funk *et al.*, 1993) (Bushbean, *Phaseolus vulgaris var. nanus*), TaTI (Tanaka *et al.*, 1996) and TcTI2 (Tanaka *et al.*, 1997) (Torresea cearensis, *Amburana cearensis*), MSTI (Catalano *et al.*, 2003) (Snail medic seeds, *Medicago scutellata*), WII (Brown and Ryan, 1984) (alfa alfa leaves, *Medicago sativa)* and FBI (Asao *et al.,* 1991) (Faba beans, *Vicia faba L*) exists as monomers. The data available on the protein-

protein interactions responsible for the self-association of BBIs is sparse.

Primary structure of Bowman-Birk inhibitors

The primary structure of Bowman-Birk type inhibitors has been extensively characterized from various leguminous sources specially from seeds which is an abundant source: Soybean (Yamamoto and Ikenaka, 1967; Hwang *et al.*, 1977; Odani and Ikenaka, 1977a), navy bean (Wagner and Riehm, 1967, lima bean (Haynes and Feeney, 1967), mung bean (Chu and Chi, 1965; Chu *et al.*, 1965, Chrispeels and Boumgartner, 1978), kidney bean (Pusztai, 1968), (Tur-Sinai *et al.*, 1972; Norioka *et al.*, 1982), garden bean (Wilson and Laskowski, 1973), adzuki bean (Yoshida and Yoshikawa, 1975; Ishikawa *et al.*, 1979, 1985), peanut chickpea (Belew and Eaker, 1976; Belew *et al.*, 1975), black eyed pea (Gennis and Cantor, 1976a), *Vicia angustifolia* (Abe *et al.*, 1978), *Macrotyloma axillare* (Joubert *et al.*, 1979), faba beans (Asao *et al.*, 1991), *Erythrina variegata* (Kouzuma *et al.*, 1992), *Phaseolus vulgaris* var. nanus (Funk *et al.*, 1993), *Canavalia lineata* (Terada *et al.*, 1994b), *Torresea acreana* (Tanaka *et al.*, 1996), Horsegram (Prakash *et al.*, 1996) (*Medicago scutellata* (Cecialinai, *et al.*, 1997), winter pea seeds (Quillien *et al.*, 1997), *Torresea cearensis* (Tanaka *et al.*, 1997) and Wild soja (Deshimaru *et al.*, 2002).

Classification of Bowman-Birk inhibitors of legumes

Norioka and Ikenaka (1983b) classified the legume BBIs into four groups (Table 1.2) based on the amino acid residues around the putative reactive sites. Sequences around the reactive sites of the inhibitors in each group are very well conserved (Table 1.2).

Table 1.2 **Reactive site residues of BBIs and their classification**

Group	Reactive site residues: Amino-terminal reactive site -P_2'P_1'$P_1P_2P_3P_4$-	Reactive site residues: Carboxy-terminal reactive site -P_2'P_1'$P_1P_2P_3P_4$-	Example
I	-T-X-S-X-P-P-	-T-R-S-X-P-G-	WSTI-IV, SBI-C-II, PVI-3I, MBI, DE-4
II	-T-L-S-X-P-P-	-T(A)-X-S-X-P-A	CLTI-I, -II, PVI-3II, BTCI, HGI-III, BBI, LBI-IV & VI', ABI-I, -II, MAI-DE-3, -4
III*	-T-X-S-X-P-P-	-X-X-S-X-P-P-	VAI, FBI, PsTI-IVb
IV*	-D-R-R-A-P-P-	-T-R-S-X-P-P-	PI-A-II, PIB-III, PI-B-II

*Reactive site residues are derived from only few sequences and are still to be confirmed by comparison of more sequences of these classes.

Comparison of monocot and dicot Bowman-Birk inhibitors

Prakash *et al.*, (1996) aligned and compared 27 domains of BBIs from both monocot and dicots. Ancestral amino acid sequences constructed showed 36 % identity between the monocot and dicots. The ancestral sequence corresponding to dicots has 42-85 % identity with the individual dicot sequences. Despite this close similarity between their sequences, dicot BBI shows a broader specificity for the enzymes inhibited, when compared to monocots. The first site of dicot inhibitors inhibits trypsin while the second reactive site inhibits trypsin, chymotrypsin or elastase.

BBIs from dicots usually have M_r of 7-9 kDa. In contrast, monocots can be divided in to two classes, one of size ~ 9 kDa with one

reactive site and other of size 16 kDa with two reactive sites (Odani *et al.*, 1986; Tashiro *et al.*, 1987; 1990; Nagasue *et al.*, 1988). The 16 kDa proteins have two 8 kDa domains (Prakash *et al.*, 1996). Further difference was observed with the conserved cysteine residues. Monocot BBIs have 10 ½-cystine residues. The four Cys lost in monocots correspond to C^3, C^{10}, C^{11} and C^{13} of dicots (Figure 1.2).

In monocots, the single reactive site situated at the amino-terminal region of the 8-kDa proteins align very well with the first reactive site of the dicots. All monocots inhibit trypsin but seem to have lost their second reactive site. The residues corresponding to P_1 position of this site are not potential targets for trypsin, chymotrypsin or elastase. More importantly, these inhibitors have lost Cys^{10} and Cys^{11}, which form a disulfide linkage in dicot (Figure 1.2). This disulfide holds the second inhibitory loop and presents it in to the enzyme in a favored conformation (Tsunogae, *et al.*, 1986; Chen *et al.*, 1992).

The BBIs sequences from monocots and dicot shows that the monocots 8 kDa inhibitors have lost their second reactive site during the process of evolution (Tashiro *et al.*, 1990; Prakash *et al.*, 1996). It is likely that gene duplication in monocots leading to the evolution of 16 kDa double headed BBIs occurred after the loss of the second reactive site in the monocot 8 kDa proteins. Probably due to the loss of second reactive site, monocots BBIs shows greater variability in the sequence when compared to dicots, these observations also suggest that the gene duplication occurred only in monocots and that dicots are unlikely to have 16 kDa inhibitor (Prakash *et al.*, 1996).

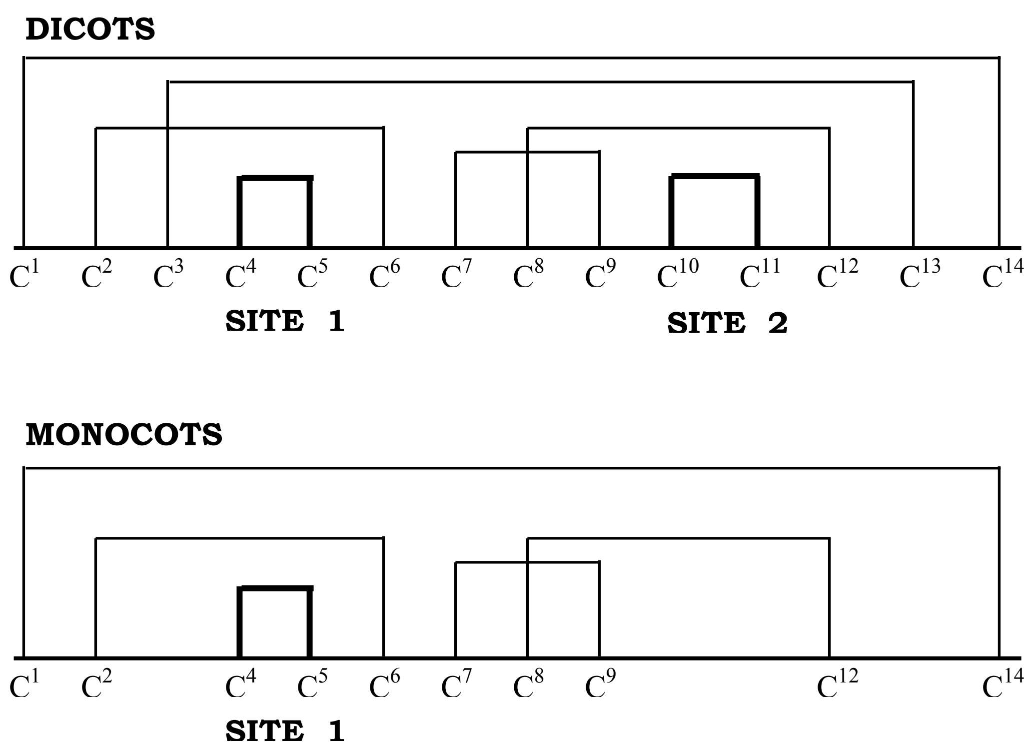


Figure 1.2 **Disulfide connectivity in dicot and monocot BBIs.** (adapted from Prakash *et al.*, 1996)

Reactive sites and inhibitory activities of Bowman-Birk inhibitors

The reactive site of a BBI is defined as the part of the inhibitor molecule that enters into direct molecular contact with the active center of the proteinase upon formation of the proteinase-inhibitor complex.

The inhibitors inhibit their cognate proteinases by varied mechanisms. Inhibition of serine proteinases by inhibitor proteins is often mediated by an exposed reactive site loop that is fixed in a characteristic "canonical" conformation thought to be similar to that of productively bound substrate (Laskowski and Kato, 1980; Bode and Huber, 1992). At or near the reactive site of the inhibitor there exists an amino acid residue that is specifically recognized by the primary

substrate-binding site of the target proteinases. By the notation of Schetchter and Berger (1967), amino acid residues around the reactive site of an inhibitor designated as P_3, P_2, P_1, P_1', P_2', P_3', where P_1-P_1' is reactive site scissile peptide bond is susceptible to hydrolysis during complex formation between inhibitor and its target proteinase (Laskowski, 1986).

The BBI family consists of two subsets that have 9 amino acid residues in the disulfide bridged reactive ring (Cys^4-Cys^5 and Cys^{10}-Cys^{11}), but in peanut inhibitors, the insertion of two amino acid resides, probably Tyr and Phe, in the tryptic reactive disulfide loop results in the eleven membered instead of a nine membered ring. The convex shaped canonical reactive site loop, which is complementary to the concave shape of the proteinase active site (Apostoluk and Ostlewski, 1998), contains inhibitory specificity for serine proteinase inhibitor and is dependent on P_1 residues of the reactive site. This specificity is seen in all the BBIs: Arg and Lys for trypsin; Leu, Tyr and Phe for chymotrypsin and Ala for elastase. Replacement of P_1 Leu^{43} residue of the second reactive site, chymotrypsin reactive site of BBI by Gly, Ala, Val, Met, Phe, Trp and D-Trp showed that the Phe^{43} and Trp^{43}-derivatives were most effective for the inhibition of α-chymotrypsin. The Met^{43} inhibitor interacted some what more weakly with the enzyme, Val^{43}, Ala^{43} and D-Trp^{43} - derivatives had much lower inhibitory activities and the Gly^{43} derivative was inactive (Odani and Ono, 1980). These results indicate that the P_1 residues of the reactive site of BBI are the most important for the inhibitory activity, which is in agreement with the substrate specificity of the target enzyme. Exceptions are the first reactive site (Arg-Arg) of peanut inhibitors (A-II and B-II: Norioka and Ikenaka, 1983b, 1984) and the second reactive site (Arg-Ser) of soybean inhibitor CII (Odani and Ikenaka, 1977b), of

which P_1 Arg residues predict specificity for trypsin, but to which both trypsin and chymotrypsin can be bound. The inhibitory specificity is not only dependent on the P_1 amino acid residue alone, but also on a wider area around that reactive site and fits the area of reactive site of the target proteinases as suggested by X-ray analyses of inhibitor-proteinase complexes (Huber *et al.*, 1974; Sweet *et al.*, 1974; Huber and Bode, 1978; Satow *et al.*, 1978; Mitsui *et al.*, 1979).

With the exceptions of the hyper-exposed P_1 position, some of the other positions in the nine membered loop are conserved and are generally occupied by amino acids with side chain that can act as conformational constraints. These include disulfide-linked Cys, hydrogen bonded amino acids such as Ser or Thr, and backbone constraint Pro. The role of the conserved residues such as Ser (P_1'), Thr (P_2) and Pro (P_3' and P_4') were recently investigated by Brauer and Leatherbarrow (2003), McBride *et al.*, (1998) and Brauer *et al.*, (2002) respectively.

The role of conserved Thr (P_2) was studied by constructing the variants at P_2 position using an 11 amino acid cyclic peptide template (McBride *et al.*, 1996) derived from anti-tryptic loop domain of the BBI MAI-DE4 (Maeder *et al.*, 1992). The 26 variants studied had the consensus SC**X**FSIPPQCY at P_4, P_3, P_2, P_1, P_1', P_2', P_3', P_4', P_5', P_6', P_7' and were cyclized at P_3-P_6' Cys residue. Analysis of variants with respect to K_i and hydrolysis rates data shows that the requirements for inhibiting at the P_2 locus (Thr) are fine-tuned. The kinetic studies with structural data suggest that the β-hydroxyl group and γ-methyl group play a vital role in participating in intramolecular hydrogen bonding and intermolecular contacts with proteinase which inturn leads to

reduced hydrolysis rates and significant increase in K_i values respectively.

The two reactive loops are β-hairpins with a type VI β-turn, which is centered around a *cis* peptide bond for a strictly conserved Pro at the P_3' subsite (Tsunogae *et al.*, 1986; Werner and Wemmer, 1992; Lin *et al.*, 1993; Voss *et al.*, 1996; Song, *et al.*, 1999; Koepke *et al.*, 2000).

The P_4' subsite in the BBIs is also frequently a Pro residue, though this has *trans* peptide geometry. The high content of constraining residues appears to be the basis for the retention of much of the proteins biological activity and three-dimensional structure in fragment (proteins-derived or synthetic) incorporating the sequence of BBI reactive site loops (McBride and Leatherbarrow, 2001; Brauer *et al.*, 2001).

Brauer *et al.*, (2002) examined the *cis-trans* geometry at P_3'-P_4' by inhibitory activity and structure for a series of synthetic fragments where each of these Pro residues was systematically replaced with Ala and indicated that peptides having Pro at P_3' are potent inhibitors and a *cis* peptide bond at this position is necessary for biological activity. Though a P_4' Pro is not essential for activity, it effectively stabilizes the *cis* conformation at P_3' by suppressing alternative conformations.

Like wise Ser at P_1 position is conserved for all the reactive loops of BBI (Laskowski and Kato, 1980). This finding with only a single exception has been confirmed by a more recent review (Apostoluk and Ostleswski, 1998). X-ray crystal structures of BBI proteins (Tsunogae *et al.*, 1986; Chen *et al.*, 1992; Lin *et al.*, 1993; Sierra *et al.*, 1999, Koepke *et al.*, 2000) and SFTI-1 (Luckett *et al.*, 1999) consistently

indicate that the P_1' Ser side chain participates in the intramolecular hydrogen bond network within the reactive site loop. Recently Brauer *et al.*, (2003) by using a combined approach of kinetic and structural analysis of variant proteinomimetic peptides demonstrated that the hydrogen bond potential of the side chain oxygen atom of the P_1' Ser is not essential for the integrity of the reactive site loop and that it provides only a small contribution to the trypsin affinity and no apparent contribution to the stability against tryptic turnover.

Standard Mechanism of inhibition

The protein inhibitors of serine type proteinase confront the key phenomenon of enzyme specificity. These inhibitors, reviewed by Laskowski and others (Laskowski and Kato, 1980; Read and James, 1986; Bode and Huber, 1992; Laskowski and Qasim, 2000), feature peptide sequences that bind in a substrate-like manner to specific proteinases, and based on sequence, would be expected to be rapidly proteolyzed. However, the inhibitors are bound more tightly than good substrates of these enzymes (with association constants up to 10^{14} M^{-1}), yet are hydrolyzed more slowly by factors of 10^6-10^{10}. These are known to form acyl-enzyme intermediate rapidly. Despite this rapid first step, further hydrolysis is slowed dramatically because of tight and oriented binding of the cleaved peptide, preventing acyl-enzyme hydrolysis and favoring the reversal of the reaction. The inhibitors comprise at least 18 convergently evolved families that display a strikingly similar conformation of the peptide backbone surrounding the reactive site, despite an absence of similarity in the sequence topology (Figure 1.3) (Laskowski and Kato, 1980; Hubbard *et al.*, 1991; Bode and Huber, 1992; Apostoluk and Otlewski, 1998; Tyndall and Fairlie, 1999; Jackson and Russel, 2000).

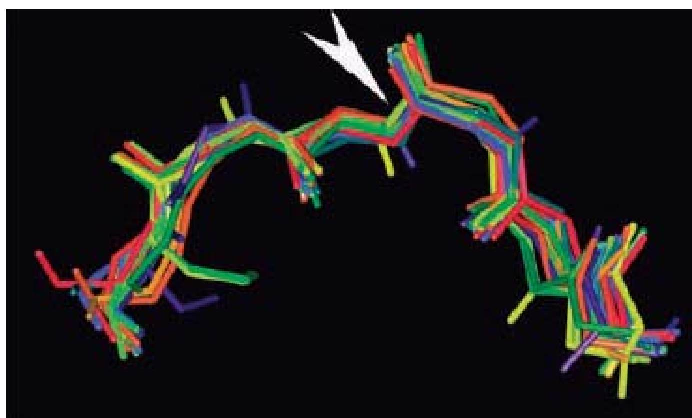
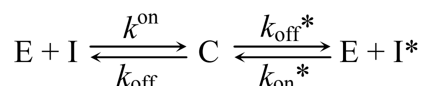


Figure 1.3 **Superposition of reactive site loop backbones of proteinase inhibitors in complex with proteases.** White arrow indicates the cleavage site**.** The figure is reproduced with the kind permission of Radisky and Koshland (2002).

It turns out that many, but not all trypsin, chymotrypsin. elastase, subtilisin etc. inhibitors share the same mechanism of enzyme inhibition association with their target enzymes given by:



$$E + I \underset{k_{off}}{\overset{k^{on}}{\rightleftarrows}} C \underset{k_{on}*}{\overset{k_{off}*}{\rightleftarrows}} E + I*$$

Where ‘C’ is the stable enzyme inhibitor inactive complex, ‘E’ the free enzyme, ‘I’ the virgin free inhibitor and I* the modified inhibitor with peptide bond hydrolyzed.

The above equation implies that the virgin and modified inhibitor are at equilibrium. The equilibrium constant K_{hyd} has been measured for many inhibitor variants and is in the order of unity for most of them (Ardelt and Laskowski, 1991). As C is the same substance whether formed by reacting the enzyme with virgin or with modified inhibitor the equation implies that virgin and modified inhibitors are thermodynamically of equivalent strength (they are equivalent if K_{hyd} =

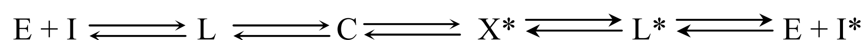
1). On the other hand, for many systems the ratios k_{on}/k_{on}* and k_{off}/k_{off}* are very large.

The inhibitors, which follow the standard mechanism often, exhibit extremely large values for the association equilibrium constant (K_a) given by the relation

K_a= [C]/[E] [I]   Where 'C' is the stable enzyme inhibitor inactive complex, 'E' the free enzyme and 'I' the virgin free inhibitor.

The proof that BBIs obey the standard mechanism (Laskowki and Kato, 1980) is demonstrated not only by showing that a single peptide bond hydrolyzed in it but also by showing that the bond is resynthesized upon complex formation (Ardelt and Laskowski, 1985).

Therefore extension for the above could be written as



Where L and L* are the loose complexes and X* the additional intermediate.

Radisky and Koshland (2002) co-crystallizing the classical serine proteinase subtilisin (EC 3.4.21.62) with the classical inhibitor, chymotrypsin 2 (CI2) and studied the standard mechanism of hydrolysis and religation of the hydrolyzed peptide bond. In this mechanism the equilibrium between the Michaelis complex and the acyl enzyme was quickly established and the Michaelis complex was thermodynamically favored relative to the acyl-enzyme. The rapidity with which the equilibrium was attained indicated the absence of a large energy barrier to acylation. These results disproved the hypotheses that either rigidity or poor orientation prevented productive

nucleophilic attack. Radisky and Koshland (2002) summarized the effect of this class of inhibitor as analogous to the clogging of a gutter drain by a combination of twigs and leaves and called it "*clogged gutter mechanism for proteinase inhibitors*". Jensen *et al.*, (1996) used high performance capillary electrophoresis to monitor the hydrolysis of BBI in the presence of catalytic amounts of bovine trypsin. The rate constant depended on the pH and the final event of hydrolysis revealed by the stability of EI complex, which is a central aspect of proteinase inhibitor mechanism.

Structure of Bowman-Birk inhibitors

Despite the large number of BBIs purified and studied, the three dimensional structure of only a few BBIs have been reported. The X-ray structure of tracy bean inhibitor, PI-II (Chen *et al.*, 1992), peanut inhibitor, A-II (Suzuki *et al.*, 1993), soybean (Voss *et al.*, 1996) and Winter pea seed (Sierra *et al.*, 1999). The crystal structures of two complexes of BBIs with trypsin have been established. Adzuki bean, AB-I (Tsunogae *et al.*, 1986) and that of a synthetic peptide of 22 residues derived from mung bean, MBI-F (Li *et al.*, 1994). Although ternary complex of BBI/trypsin/chymotrypsin and BBIs from horsegram were crystallized, the structures have not been solved (Gaier *et al.*, 1981; Prakash *et al.*, 1996).

Werner and Wemmer, (1992, 1992a) characterized a BBI in solution by two dimensional ^{1}H-NMR. Each of the two distinct binding domains for serine proteinases is comprised of a beta-hairpin with a short segment making a third strand of antiparallel beta-sheet.

The structure of soybean BBI at 2.8 Å resolution (Voss *et al.*, 1996), differed from the NMR structure of Werner and Wemmer (1992)

in the orientation of the two proteinase insertion loops and several amino- side chain. The ternary complex of BBI with bovine trypsin investigated by Koepke *et al.*, (2000) showed the environmental elements – polar or hydrophobic - responsible for the trypsin specificity and indicated their use was adjusted according to different applications.

The X-ray structure of A-II from peanut (Suzuki *et al.*, 1993) showed that the inhibitor molecule had an elongated shape with two reactive sites, one at both ends of the dimension. A model of interaction between BBIs from peanuts based on the structure of Suzuki *et al.*, (1987) is represented by *Tyrannosaurus* and *Stegosaurus* as trypsin and chymotrypsin respectively (Hilder *et al.*, 1990). Sierra *et al.*, (1999) reported the dimeric crystal structure of Winter pea seed inhibitor, PsTI-IVb by molecular replacement at 2.7 Å using the X-ray co-ordinates of soybean as a search model. The inhibitor crystallized as a nearly perfect two-fold symmetric dimer and for the first time, the carboxy-terminal end was discernable. New structural features including interaction between the subunit in the monomer were elucidated.

Wu and Sessa (1994) by circular dichroism studies found the native soybean BBIs had 61 % β-sheet and 38 % unordered form, 1 % β-turn and no α-helical structure. No significant changes in the secondary structure were observed when heated up to 80 °C. However in the presence of metabisulfite there was a decrease in β-turn. The data of Wu and Sessa (1994) support the stable conformation of BBIs. Acetylation of soybean BBI with long chain unsaturated fatty acids decreases its thermostability (Malykh *et al.*, 2001). De Freitas and Ventura (1996) determined the secondary structure of black eyed

cowpea BBI by Fourier-transform infrared spectroscopy and quantitatively estimated 32 % β-structure, 23 % β-turns and unordered structure 28 % and no α-helix was detected. The thermodynamics of binding of chymotrypsin with the black-eyed pea BBI indicated that the binding was entropically driven (De Freitas *et al.*, 1999).

The simultaneous binding of trypsin and chymotrypsin to horsegram BBI was studied by the models of trypsin and chymotrypsin bound to BBI constructed based on the known structure of ABI-inhibitor complex (Prakash *et al.*, 1997). The model showed short contacts between the two proteinase molecules mainly around the residue 174 of trypsin and 95 of chymotrypsin. Most of the atoms involved in the short contact were separated by 2.0 Å. About 10 atoms have distances less than 2.0 Å. This shows that the two enzymes can be simultaneously inhibited by the inhibitor. Competitive binding studies showed that binding of the enzyme in presence of the other is associated at the best with a minor negative cooperativity (Prakash *et al.*, 1997).

The reduction of HGI-III, the major BBI of horsegram resulted in the loss of inhibitory activity accompanied by a loss in structure as reflected by circular dichroism (Ramasarma *et al.*, 1995). Reduction of disulfides affected the tertiary structure but not the secondary structure indicating the disulfide linkages play a predominant role in maintaining three-dimensional structure of the HGIs (Ramasarma *et al.*, 1995). Horsegram BBI followed the “two state” mode of unfolding where in all the seven-disulfide bonds were reduced simultaneously resulting in the fully reduced protein without any accumulation of partially reduced intermediates. Oxidative refolding of the BBI was

possible only at very low inhibitor concentration in a disulfide-thiol buffer (Singh and Rao, 2002).

Physiological significance of proteinase inhibitors

The physiological significance of plant proteinase inhibitors (PIs) has been questioned for a long time. A number of possible functions for these plant PIs have been proposed, they include control of endogenous proteinases, particularly during dormancy and germination of seed and serving as a storage depot, particularly for sulfur containing amino acids (Richardson, 1977). The *in vivo* function of these PIs is not yet shown. In some instances it seems likely that several of these functions may be served simultaneously. A possible storage function is implied by observed decline in trypsin inhibitory activity during germination of *Vigna radiata* (Lorensen *et al.*, 1981), *Phaseolus vulgaris* (Pusztai, 1972; Wilson, 1981) and *Pisum sativum* (Hobday *et al.*, 1973).

Although the physiological functions of these PIs remained poorly understood, these inhibitors are helpful for the host plant in defending against the pest and predators.

In the co-evolving system of plant-insect interactions, plants are able to synthesize a wide range of molecules to defend themselves against insect attack. The PIs are considered to be one of the most effective inhibitors (Koiwa *et al.*, 1997; Falco *et al.*, 2001). The use of PIs in developing insect resistance in transgenic plants is of dual benefit, as they inhibit insect midgut proteinases, there by protecting other defense proteins from proteolytic degradation (Michaud, 1997). PIs block digestive proteinases in insect guts and starve them of essential amino acids (Broadway and Duffey, 1986; Ryan, 1990). In the control of insect pests, the use of genes encoding plant PIs for the

transformation of crop genomes is an alternative to the use of the *B. thuringiensis* endotoxin sequence. Hilder *et al.*, (1987) expressed the cowpea (*Vigna unguiculata*) Ti gene in tobacco to increase its resistance against herbivorous insects. It was followed by many similar studies in various plant species (Johnson *et al.*, 1989; Boulter, 1993; McManus *et al.*, 1994; Schroeder *et al.*, 1995; Urwin *et al.*, 1995; Duan *et al.*, 1996; Michaud *et al.*, 1996; Xu *et al.*, 1996). Plant derived PIs are of particular interest because they are part of the plant natural defense system which plants have evolved against insect predation.

Bowman-Birk inhibitors as cancer chemopreventive agents

BBIs are known for their anticarcinogenic nature and have been extensively studied both in *in vivo* and *in vitro* model studies. Most of the epidemiological evidence indicates that the vegetarian diet containing high amounts of soybean products are associated with low cancer incidence and mortality rates, particularly breast, colon and prostate cancer (Messina *et al.*, 1994; Kennedy, 1993). Although two other agents in soybean (phytic acid and sterol β-sitosterol) suppress carcinogenesis in animals, BBI is far more effective in suppressing cancers in animals than, other known anticarcinogenic agents in soybean (Kennedy, 1995). Several BBIs in their pure form have been shown to suppress carcinogenesis (Billings *et al.*, 1987b; 1989; 1991b).

As an anticarcinogenic agent, BBI has been studied in the purified as well as in the form of a soybean extract in which BBI has been concentrated, termed as BBI concentrate (BBIC); 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.*, 1990b; Kennedy *et al.*, 1993) and this

BBI appears to be a universal cancer preventive agent. Because the use of purified BBI in human trial would be prohibitive in cost, BBIC was developed for use in large-scale human cancer-prevention trials. BBIC has achieved Investigational New Drug Status (IND No. 34671; Sponsor, Ann. R. Kennedy) from the US Food and Drug Administration (FDA). In BBIs, the active anticarcinogenic activity has been shown to be the chymotrypsin inhibitor activity. The purified BBIs and BBIC suppress carcinogenesis induced by different types of carcinogens in different animal species, several organ system and tissue types, cells of epithelial and connective tissue when administered by several different routes, including the diet leading to different types of cancer and induced by various chemical and physical carcinogens.

Studies with MSTI, a BBI from snail medic seeds (*Medicago scutellata*), (Catalano *et al.*, 2003) revealed that antichymotryptic activity is not a strict requirement for the antitumoral effect. Wherein the inhibitory activity and antitumoral activity are not correlated. Cleavage of BBI-I and linearization of the two resulting peptides, carrying the chymotrypsin and trypsin inhibitory sites, lack inhibitory activity, act-radio protectively (Gueven *et al.*, 1998). A recent analysis of a few nona-peptides mimicking the second active loop of BBI-I, suggested that radio-protective and inhibitory properties are inherent to different sites (Dittmann *et al.*, 2001). Although BBI and other PIs do not function as free radical scavengers (St Clair *et al.*, 1991), they can achieve the same final result as antioxidants in that they keep free radicals from being produced in cells and thereby decrease the amount of oxidative damage (Frenkel *et al.*, 1987).

The anti-inflammatory activity of BBIs are due to the direct and potent inhibitory effect on the catalytic activities of the major

proteinases involved in inflammatory process, such as cathepsin G, elastase (Larionova *et al.*, 1993), chymase (Ware *et al.*, 1997) and several unidentified proteinases (Kennedy, 1998). Kennedy (1998) hypothesized that proteinase inhibitors suppress carcinogenesis by affecting the amounts of certain proteolytic activities (Long *et al.*, 1981; Messadi *et al.*, 1986; Billings *et al.*, 1987a, 1988, 1989, 1990, 1991a; Carew and Kennedy, 1990; Oreffo *et al.*, 1991; Billings and Habres, 1992; Billings, 1993; Habres and Billings, 1993) or the expression of certain proto-oncogenes (Chang *et al.*, 1985, 1990; Chang and Kennedy, 1988; Caggana and Kennedy, 1989; St. Clair *et al.*, 1990a; Li *et al.*, 1992), both of which are thought to play an important role in carcinogenesis (Kennedy, 1993a, Kennedy, 1994(S); Kennedy and Billings, 1987; Kennedy, 1993b).

Other applications of Bowman-Birk inhibitors

Other applications in pharmacology include co-administration of BBIs along with the carrier matrix. Several studies were conducted on oral bio-avaibility of insulin, which is co-administered with these auxiliary agents like trypsin inhibitors. (Fujji *et al.*, 1985; Morishita *et al.*, 1992; Yamamoto *et al.*, 1994). Recently Marschutz and Bernkop-Schnurch (2000) developed a drug carrier matrix containing BBI, which protects embedded insulin from degradation by luminally secreted serine-proteinase trypsin, chymotrypsin and elastase *in vitro*.

Proteolysis of Bowman-Birk inhibitors during germination

Germination has been suggested as an inexpensive and effective technology for improving the quality of legumes, by enhancing their digestibility (Reddy *et al.*, 1989), increasing the levels of amino acids (Chang and Harrold, 1988) and reducing the contents of antinutritinal factors (Vidal-Valverde and Frias, 1992) such as trypsin inhibitors. A

decline in the trypsin inhibitor and the protein content in the legume seed cotyledon is commonly observed during germination and the subsequent growth of the seedling (Hobday, *et al.*, 1973; Baumgartner and Chrispeels, 1976; Chrispeels and Baumgartner, 1978; Wilson, 1981; Lorensen *et al.*, 1981; Wilson and Tan-Wilson, 1983; Godbole *et al.*, 1994b; Sreerama and Gowda, 1998). It is generally assumed that bulk of this decrease is due to proteolysis of the inhibitor by seed proteinases. During the process of germination some inhibitors present in the dry seed disappear completely with concomitant appearance of new active species that are not present in the dry seed (Lorensen *et al.*, 1981; Tan-Wilson *et al.*, 1982; Wilson and Chen, 1983; Sreerama and Gowda, 1998).

Most of the studies on the fate of seed inhibitors during germination are on quantitation of the inhibitor levels. Initial observations on conversion of inhibitors were from the work on kidney bean by Pusztai (1972). Much of the evidence on the specific partial proteolytic cleavage of the BBIs was obtained from the work of Tan-Wilson *et al.*, (1982) and Wilson and Chen (1983) on soybean and mung bean respectively.

Several mung bean isoforms from dry seed and germinating seed have been purified and characterized (Wilson and Chen, 1983). MBTI-F (80 amino acids) is the major isoform in dry seed and the inhibitors that appear during germination are MBTI-E, MBTI-C and MBTI-A. Structural studies confirmed that MBTI-E was identical to MBTI-F with the exception of a deleted tetrapeptide at the carboxy-terminal (-Lys-Asp-Asp-Asp). The proteinase responsible for this cleavage was identified and named, proteinase F. The conversion of MBTI-E to MBTI-C, consists of a minimum three proteolytic events, the loss of an

additional two carboxy-terminal residues (Asp and Met), amino-terminal residues from S^1–S^8 (SSHHHDSS-) and an internal cleavage at Ala^{35}-Asp^{36}. The deduced pathway for the degradation of MBTIs is F→E→C→A.

A similar proteolysis of was observed in soybean in the conversion of BBSTI-E to BBSTI-D (Madden *et al.*, 1985). A similar modification by limited proteolysis during germination was noticed in adzuki bean (*Vigna angularis*) (Yoshikawa *et al.*, 1979). In spite of differences in the rates of modification of BBIs in different species, the proteolysis of Bowman-Birk inhibitors during seed germination and early seedling growth would appear to be a general phenomenon in the legumes (Madden *et al.*, 1985). Sreerama and Gowda (1998) observed the disappearance of the BBIs of horsegram during germination, with concomitant appearance of new forms.

The specific inhibitor activities of germinated soybeans, chickpeas. lentils, mung bean, fenugreek and alfalfa seeds against human and bovine trypsin and chymotrypsin decreased considerably during germination (Weder and Link, 1993).

Botanical description of horsegram (*Dolichos biflorus*)

Horsegram (*Dolichos biflorus*), a protein rich leguminous pulse, is a crop native to South-East Asia and tropical Africa. Horsegram is a slender, sub-erect annual herb with slightly twining, downy stems and branches. The leaves are trifoliate with pale yellow flowers. The pod is linear and flattened, which contains 5-7 seeds. The seeds are small, 3-6 mm, and flattened. The seed color ranges from light red, brown, black or mottled (Purseglove, 1974).

Dolichos biflorus has recently been classified as *Macrotyloma uniflorum* and seems to have features similar to *Macrotyloma axillare* (formerly *Dolichos axillaris*) (Verdcourt, 1970).

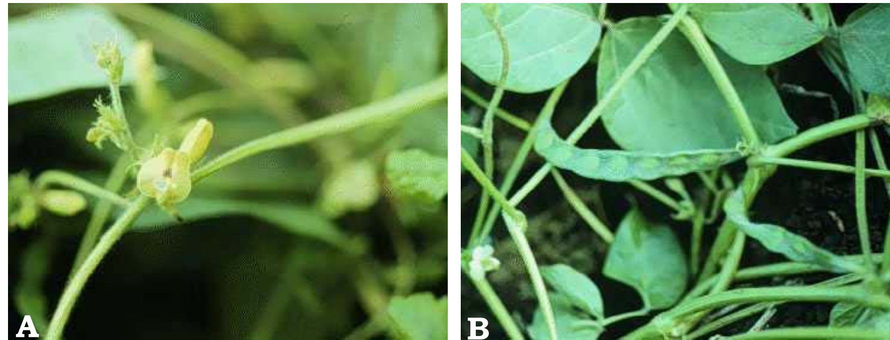


Figure 1.4 **The horsegram (*Dolichos bilflorus*) plant.** A. Horsegram plant with flower and B. Horsegram plant with flower and pod.

Aim and Scope of present investigation

Legume seeds are noted for the large amount of reserve protein in their cotyledons and also for their levels of protein-proteinase inhibitors specially the BBIs, active against mammalian pancreatic proteinases. In addition to proteinase inhibitory activity, the anticarcinogenic activity and radio-protective activity of BBIs from legumes have been widely studied (Sorsa *et al.*, 1997; Kennedy, 1998; Billings *et al.*, 1988; Miyata *et al.*, 1998; Ware *et al.*, 1999; Miyata, *et al.*, 2000; Catalano, *et al.*, 2003). Immune stimulating properties of these inhibitors have also been reported (Harms-Ringdahl *et al.*, 1979). The BBIs have been implicated to play a vital role in the arsenal defense mechanism that plants use to protect against insect predators and against environment hazards during germination and seedling growth. Transgenic plants expressing the BBI gene are known to enhance resistance to predation (Hilder, *et al.*, 1987).

Horsegram (*Dolichos biflorus,* Phaseolae; family Leguminosae), is a pulse crop native to South East Asia and Tropical Africa and used mainly for animal feed, however, it is also consumed by the poorer classes of population after cooking, frying or roasting. Previously a group of double-headed Bowman-Birk type of proteinase inhibitors active against both trypsin and chymotrypsin from horsegram were reported (Ramasarma and Rao, 1991). Subsequently, four isoinhibitors were purified to homogeneity and characterized (Sreerama *et al*, 1997; Sreerama and Gowda, 1997). All four were double headed, inhibiting both trypsin and chymotrypsin simultaneously and independently. Further, the complete primary structure of the major isoinhibitor (HGI-III) was elucidated (Prakash *et al.*, 1996) and shown to be a single polypeptide of seventy-six amino acids containing seven disulfide bridges.

During the germination of horsegram (*Dolichos biflorus*), the BBIs present in the dormant seed rapidly disappeared with the concomitant appearance of new active species (Sreerama and Gowda, 1998). These inhibitor species were electrophoretically similar to the BBIs found in the flower, leaf and early stages of seed development. Preliminary investigations in our laboratory involving the partial purification of the inhibitors present after complete germination indicate them to be of a smaller size and to be less charged than the inhibitors of the dormant seed. Yet the inhibitors retain the double-headed characteristics. It is not clear whether these forms and those that occur in the dormant seed are products of the same gene or the products of different genes that undergo post-translational modifications. Determining the complete primary structure of the inhibitors from the germinated seeds would establish the structural similarities of these inhibitors with those of the isoinhibitors present in the dormant seed, flower and leaf and assess the evolution of these proteins and assess whether the new inhibitors in the germinated seed are products of stored mRNA or are products of proteolytic degradation of the dormant seed inhibitors and if they are the products of proteolysis, is this a specific or non-specific degradation.

The main objectives of the present investigation are

- Purification and characterization of the inhibitors from the flower, leaf and those which appear upon germination of horsegram (*Dolichos biflorus*) seeds.
- Determination of the complete primary structure of the inhibitors which appear upon germination by using newer methods of protein sequencing and prediction of their higher levels of protein structure by comparison.

- In vitro proteolysis of the dormant seed isoinhibitors with proteinase that appears upon germination of horsegram seeds.

The primary structure and comparison among the BBIs would not only generate information at the molecular level on the evolution of multiple isoforms, but also provide insights in designing small potent inhibitors for developing insect resistance transgenic plants and effective anticarcinogenic agents.

Materials and Methods

2.1 MATERIALS

2.1.1 Plant material

Horsegram seeds (*Dolichos biflorus*) were procured from the local market, which served as starting material. Horsegram flowers were harvested from plants grown in local fields and the cotyledons of different developmental stages and tender leaves were harvested from seedlings grown in lab under dark at room temperature (25 ± 2 °C).

2.1.2 Chemicals

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

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, endoproteinase Asp-N (EC 3.4.24.33), carboxypeptidase Y (EC 3.4.16.1), bovine serum albumin (BSA), cyanogen bromide (CNBr), acrylamide, N-N'-methylene bis-acrylamide, tris (hydroxymethyl) amino methane (Trizma base), coomassie brilliant blue R-250, triethylamine (TEA), trifluoroacetic acid (TFA), ∝-N-benzoyl-DL-arginine-*p*-nitroanilide HCl (BAPNA), N-benzoyl-L-tyrosine-*p*-nitroanilide (BTPNA), pepstatin-A, 4-vinylpyridine (4-VP), guanidine hydrochloride (GuHCl), N-N, N'-N'-tetramethyl 1, 2-diaminoethane (TEMED), DL-dithiothreitol (DTT), 2, 4, 6-trinitrobenzenesulfonoic acid (TNBS), diethylpyrocarbonate (DEPC), 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), phenylmethyl-sulfonyl fluoride (PMSF) and iodoacetamide were purchased from Sigma Chemical Co., St. Louis. MO, USA.

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

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, West Germany. Polyvinilidine difluoride membrane (PVDF, 0.45 μm) was from Pierce, USA.

Ammonium sulfate, 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 E. Merck (India) Ltd., Mumbai, India.

Amino acid standards (Pierce H.), sodium dodecyl sulfate (SDS), phenylisothiocynate (PITC), 5-bromo-4-chloro-3-indolylphosphate (BCIP), nitroblue tetrazolium (NBT) and citraconic anhydride were from Pierce Chemical Company, Rockford, Illinois, USA.

Purification materials, Sephadex G-50, CM-Sephadex C-25, CM-Sepharose, DEAE-Sephadex A-25, DEAE-Sephacel and Sepharose-4B were from Pharmacia LKB, Uppsala, Sweden.

Pyrex™ brand hydrolysis and derivatization tubes were from Corning, NY, USA. The vacuum vials and reusable enclosures were from Waters Associate, Milford, MA, USA.

SDS-PAGE medium and low range molecular weight markers were from Bangalore Genie, Bangalore, India.

High performance liquid chromatography (HPLC) grade solvents were obtained from Qualigens Fine Chemicals and Ranbaxy Fine Chemicals Ltd., India.

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

2.1.3 HPLC columns

BIOSEP-SEC-S 2000 (300 × 7.8 mm, 5 μ, manufacturer exclusion limit: 1-300 kDa) and BIOSEP-SEC-S-3000 (300 × 7.8 mm, 5 μ, manufacturer exclusion limit: 5-700 kDa) and Phenomenex ODS column (250 × 4.6 mm, 5 μ) were from Phenomenex, Torrance, USA. Aquapore C-8 column (220 x 2.11 mm) from Pierce Chemical Company, Rockford, Illinois, USA, Pico-Tag amino acid analysis column (150 × 3.9 mm, 4 μm) and Symmetry Shield RP_{18} (150 × 4.6 mm, 5 μ) were from Waters Associate, Milford, MA, USA.

2.2 METHODS

2.2.1 Germination of horsegram seeds (*Dolichos biflorus*)

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. Two hundred g seeds were washed with triple distilled water and then imbibed in distilled water for 24 h at 25 ± 2 °C. These seeds were germinated on moist filter paper in the dark for 120 h at 25 ± 2 °C. After 120 h of germination, the cotyledons were dissected free of the seed coats and axes and frozen at –20 °C until used.

2.2.2 Preparation of defatted horsegram flour

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.

2.2.3 Assay methods

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

2.2.3.1 Assay of trypsin and trypsin inhibitor activity

Trypsin was assayed according to the modified photometric method of Kakade *et al.*, (1969) using the substrate BAPNA. 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 consisted of 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 the reaction arrested by adding 0.25 mL of 30 % acetic acid. The absorbance of *p*-nitroanilide liberated was measured at 410 nm against an appropriate blank in which the reaction was arrested by adding 30 % acetic acid prior to the addition of BAPNA.

Assay of trypsin inhibitory activity

The trypsin solution was incubated with an aliquot of inhibitor solution for 10 min at 37 °C and the reaction started by the addition of 1.25 mL BAPNA diluted in buffer 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).

2.2.3.2 Assay of chymotrypsin and chymotrypsin inhibitory activity

The reaction velocity was determined by measuring the absorbance of *p*-nitroanilide at 410 nm resulting from the hydrolysis of BTPNA. The stock of 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 stopped by adding 0.25 mL of 30 % acetic acid and the liberated product, *p*-nitroanilide measured at 410 nm against an appropriate blank.

Assay of chymotrypsin inhibitory activity

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.

2.2.4 Preparation of purification matrices

2.2.4.1 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 are 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. Occasionally the beads were stirred slowly with a glass rod, allowed to settle and fines removed by decantation. 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 30 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.

2.2.4.2. *Preparation of CM - Sephadex C-25* and DEAE-Sephadex A-25

The Sephadex ion-exchangers are provided by introducing functional groups on to Sephadex, a cross-linked dextran matrix. These groups are attached to glucose units in the matrix by stable ether linkages. Sephadex ion-exchangers are supplied as dry powders and the swelling properties of Sephadex-based ion exchangers are related to those of the parent Sephadex G types.

Fifteen g of CM-Sephadex C-25 or DEAE-Sephadex A-25 were allowed to swell in 0.05 M sodium acetate (pH 5.0) and 0.1 M ammonium hydrogen carbonate (pH 8.2) respectively for 24 h. The gel matrices were washed 4-5 times to remove fines and degassed. The slurry of CM-Sephadex C-25/DEAE-Sephadex A-25 was packed in a glass column (14 × 3.4 cm and 7.2 × 2.7 cm) at a flow rate of 40 mL/h and equilibrated at a flow rate of 20 mL/h.

The two Sephadex based ion-exchangers were regenerated by washing the matrix with 2 M NaCl containing their counter ions. Washing with high salt buffers removes any macromolecules bound by ionic forces. The column was finally re-equilibrated with buffer and stored in 0.02 % sodium azide.

2.2.4.3 *Preparation of DEAE-Sephacel*

DEAE-Sephacel was provided as a suspension in 20 % ethanol, which has a wet bead size of 40-160 μ. The gel matrix was washed several times in distilled water and 100 mM ammonium hydrogen carbonate buffer (pH 8.2) and equilibrated with the same buffer for 6 h before packing.

The DEAE-Sephacel suspension in 0.1 M ammonium bicarbonate buffer was packed in a glass column (7.2 × 2.7 cm) at a flow rate of 50 mL/h. The column matrix was equilibrated in buffer until the pH of the eluent was the same as the equilibrating buffer.

Regeneration of DEAE-Sephacel

The bound contaminants were removed using 1 M NaCl in the equilibrating buffer. More strongly bound substances were removed by washing with 0.5 M NaOH on a Buchner funnel. The ion-exchanger

resin was washed with distilled water until the pH of the eluent was the same as that of the water. The gel matrix was further washed with 0.5 M HCl, which was followed by washing with distilled water. The gel matrix was finally washed with 0.1 M ammonium bicarbonate buffer (pH 8.2), packed in a column and equilibrated in the same buffer until the pH of the eluent was 8.2 and stored in equilibrating buffer containing 0.02 % sodium azide.

2.2.4.4 Preparation of trypsin affinity column

The ligand, trypsin was immobilized on Sepharose-4B in two steps. The first step involved the activation of Sepharose-4B by cyanogen bromide (CNBr) and in the second step the ligand, trypsin was coupled to the activated Sepharose-4B at alkaline pH.

Cyanogen bromide activation

The activation process was carried out using the method of March *et al.*, (1974). The washed slurry of Sepharose-4B beads, consisting of an equal volume of gel and water (20 mL of beads + 20 mL of water) was added to 40 mL of 2 M sodium carbonate and mixed by stirring slowly and allowed to cool on an ice bath to 4 °C. The rate of stirring was then increased and 2 mL of an acetonitrile solution containing CNBr (2 g CNBr/mL of acetonitrile) was added all at once. The slurry was stirred vigorously for 1-2 min (not more than 3 min) until small clumps that appeared following the addition of CNBr were dissolved. The slurry was poured on to a sintered glass funnel, washed with 10 volumes each of 0.1 M sodium bicarbonate, pH 9.5, water and coupling buffer (0.1 M sodium hydrogen carbonate, pH 9.0 containing 0.5 M NaCl). After the final wash, the slurry was filtered under vacuum to obtain a moist compact cake.

Coupling of trypsin to activated Sepharose-4B

The activated moist compact cake was immediately 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. After coupling, the supernatant was decanted carefully and residual active groups were blocked by adding excess of a small primary amine, 0.2 M glycine (pH 8.0) for 2 h at 25 ± 2 °C. 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.

2.2.5 Protein estimation

Protein concentration was determined by the dye binding method of Bradford (1976) and Zor and Selinger (1996). Bovine serum albumin (BSA) was used as the standard.

2.2.6 Polyacrylamide gel electrophoresis (PAGE)

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

2.2.6.1 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 adjusted to 8.8 with HCl (6N), 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 (6N), solution made up to 100 mL and stored at 4 °C.

D. *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).

I. *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).

Table 2.1 **Preparation of separating gel and stacking gel**

Solution	**Resolving gel (mL)**		**Stacking gel (mL)**
	(15% T, 2.7% C)	**(17.5% T, 2.7% C)**	**(5% T, 2.7% C)**
Solution A	3.00	3.50	0.83
Solution B	1.50	1.50	0
Solution C	0.00	0.00	1.25
Distilled Water	1.40	0.90	2.83
Solution D	0.06	0.06	0.05
TEMED	0.01	0.01	0.01
Solution E	0.03	0.03	0.03

Initially contents of the separating gel were mixed, 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 a boiling water bath 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. 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 in a boiling water bath 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'.

2.2.6.2 *Native PAGE (Non-denaturing gel for acidic proteins)*

Polyacrylamide gel electrophoresis under native conditions was carried out to evaluate the purity of the inhibitors and also to check the

presence of isoforms. Separating gels (10 % T, 2.7 % C) were prepared as described in section (2.2.6.1) by mixing solution 'A' (2.0 mL) and 'B' (1.5 mL) with water (2.46 mL) without addition of 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 minus 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.

2.2.6.3 Acid –PAGE (Non-denaturing for basic proteins)

Non-denaturing gels for basic proteins were prepared by a modified procedure of Reisfeld *et al*. (1962). All the solutions were similar to that of electrophoresis of acidic proteins except solutions 'B', ' C' and 'I'.

B. Potassium acetate (9.81 g) was dissolved in water, the pH of the solution was adjusted to 4.3 with acetic acid, made up to 100 mL in water and stored at 4 °C.

C. Potassium acetate (9.81 g) was dissolved in water, the pH of the solution was adjusted to 6.8 with acetic acid, made up to 100 mL in water and stored at 4 °C.

I. Sample buffer was prepared in solution C (diluted 1:4), containing glycerol (20 %, v/v) and bromocresol green (0.1 %, w/v) as the tracking dye.

Separating gel (10 % T, 2.7 % C) was prepared as described earlier (Section 2.2.6.2) substituting 'A' (2.0 mL) and 'B' (1.5 mL) and water (2.30 mL). TEMED 50 μL and solution 'E' 150 μL was added. The gel was layered with buffer saturated with n-butanol.

Stacking gel (5 % T, 2.7 % C) was prepared similar to that for native PAGE for acidic proteins, substituting solution 'C'.

Tank buffer was prepared by dissolving 1.42 g of β-alanine in water, pH was adjusted to 4.5 with glacial acetic acid and made up to 100 mL. Samples were diluted with sample buffer (1:1, v/v) and loaded on to gel, with the lower electrode of the apparatus connected to the negative power supply lead. After the electrophoresis run, proteins were visualized using CBB R-250.

2.2.6.4 Gelatin embedded PAGE for trypsin and chymotrypsin 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 2.2.6.2 and 2.2.6.3. 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 7.8 for chymotrypsin) containing either trypsin or chymotrypsin (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.

2.2.6.5 Protease activity staining

Following electrophoresis, the gel was washed with water and equilibrated with 0.1 M Tris-HCl, pH 7.5 for 10 min at 25 °C. The gel was overlaid on to a second polyacrylamide gel prepared in 0.1 M Tris-HCl buffer (pH 7.5), containing (0.5 % w/v) gelatin, carefully preventing formation of air bubble between the gels. The sandwich was incubated at 37 °C for 2 h. The protease bands were visualized in the gelatin gel after staining with CBB R-250 as clear bands against a blue background, owing to the hydrolysis of gelatin by the protease.

2.2.7 Kinetic studies of trypsin and chymotrypsin

Kinetics of two serine proteases, trypsin and chymotrypsin were studied by estimating the liberated product, *p*-nitroanilide from the chromogenic substrates BAPNA and BTPNA 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 Double-reciprocal (Lineweaver and Burk, 1934) and Dixon plots (Dixon, 1953) were used to determine the type of inhibition and inhibitory constants respectively.

2.2.8 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 2.2.3.1 and 2.2.3.2). The plot of I/E Vs % residual activity was used to evaluate the stoichiometry.

2.2.9 Molecular weight by analytical gel filtration chromatography

The apparent molecular weight of the purified inhibitors were examined by analytical gel filtration using a BIOSEP-SEC-S 2000 (300 × 7.8 mm, 5 μ) column. The column was equilibrated in 0.1 M sodium phosphate buffer (pH 7.25) at 0.5 mL/min. The column was calibrated with alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome C (14.4 kDa) and HGGI-III (6.4 kDa).

2.2.10 Molecular weight by Matrix Assisted Laser Desorption Ionization-Mass Spectroscopy (MALDI-MS)

The exact molecular mass of the inhibitors were obtained from MALDI-MS, Ketos Analytical (UK) Kompact Seq model which uses a 337 nm nitrogen laser desorption and 1.7 nm linear flight path. The instrument was calibrated over the low mass range of 399-5000 Da.

2.2.11 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/chymotrypsin inhibitor activity was assayed as described earlier (2.2.3.1 and 2.2.3.2)

2.2.12 Reduction and Pyridylethylation

Reduction and alkylation with 4-vinylpyridine (4-VP) was carried out following the method of Hermodson *et al.*, (1973). Five mg of inhibitor was dissolved in 1 mL Tris-HCl buffer (0.1 M, pH 7.6) containing 6 M GuHCl and 0.1 mg of EDTA in a stoppered vial. A 20-fold molar excess of DTT was added and flushed with nitrogen. After 3 h at 25 °C, the mixture was treated with a 3 fold molar excess of 4-VP over DTT for 90 min. The solution was then acidified to pH 2.0 with 98 % formic acid. The alkylated protein was separated from the reagents and native protein by RP-HPLC using an Aquapore C-8 column (220 x 2.11 mm, Pierce) with a linear gradient of 0-70 %, 0.1 % TFA and 70 % acetonitrile containing 0.05 % TFA. The protein was further repurified using a Phenomenex C-18 (250 x 4.6 mm, 5 μ) column employing the same solvent system.

2.2.13 Western Blotting (Semi Dry blot) of inhibitors

Preparation of PVDF membrane: The PVDF membrane cut to the required size (slightly larger than the gel) was soaked in methanol for 1-2 min before use. The membranes were then immersed in transfer buffer (10 mM CAPS, pH 11.0) containing 10 % (v/v) methanol and equilibrated for 10 min (Matsudaira, 1987; Speicher, 1989).

Blotting: Following electrophoresis, the gel was immediately rinsed 2-3 times in water to remove the buffer ions (Tris and Gly) and equilibrated in CAPS buffer (10 mM, pH 11 containing 10 % v/v HPLC grade methanol) for 10 min. Semi-dry electroblotting was carried out using a semidry blotting apparatus (Towbin *et al.*, 1979). The transfer was carried out for 120 min using a current of 0.8 mA/cm^2 of the filter paper.

Staining of the electroblotted proteins on PVDF membrane: Although CBB R-250 is a very sensitive method for staining, Ponceau S staining was used for detecting the proteins.

a. *Staining solution*: 0.1 % Ponceau S (w/v) in 5 % acetic acid (v/v)

b. *Destaining solution*: Triple distilled water

The PVDF membrane was immersed in staining solution for 5 min and destained immediately with water after detecting the bands. For amino acid analysis and amino terminal sequence, the bands were cut from membrane, dried and used.

2.2.14 DOT-BLOT analysis of inhibitors

About 100 μg of the purified inhibitors were immobilized on a nitrocellulose membrane by repeated application employing a current of hot dry air to accelerate the drying until the required protein was immobilized.

Immunodetection of inhibitors: Following immobilization, the nitrocellulose membrane was washed several times in immunoblot buffer (5 % skimmed milk powder in phosphate buffered saline, pH 7.0). The membrane was incubated overnight in immunoblot buffer containing antibodies raised against HGI-III (1:100 dilution). After repeated washes in the immunoblot buffer, the membrane was incubated with the secondary antibody alkaline phosphatase conjugated goat anti-rabbit immunoglobulins for 1-2 h at 25 ± 2 °C. After several washes in immunoblot buffer and finally in substrate buffer (100 mM Tris, 0.5 M NaCl, 5 mM $MgCl_2$, pH 9.5), the alkaline phosphatase activity was detected with a mixture of BCIP and NBT in substrate buffer.

2.2.15 Modification of amino group by 2, 4, 6–trinitrobenzene sulfonic acid (TNBS)

Free amino groups present in the inhibitors were modified with TNBS using the procedure of Haynes *et al.*, (1967). To different aliquots of inhibitor solution (0.16 mg/mL), 1 mL of 4 % sodium hydrogen carbonate, pH 8.5 and 1 mL of TNBS in water was added and incubated at 25 °C. Aliquots (0.2 mL) of the solution were removed from the reaction mixture at regular time interval, diluted with ice-cold water to stop the reaction and assayed for the residual inhibitor activity.

2.2.16 Citraconylation of Lys residues by citraconic anahydride

Lys residues of HGI-III were modified at 25 ± 2 °C with citraconic anhydride by the method of Dixon and Perham (1968). The protein (1 mg/mL) was dissolved in water and the pH adjusted to 8.0. Citraconic anhydride (1 μL/ mg of protein) was added to the solution, while maintaining pH 8 by the addition of 5 M NaOH. Once the pH of the reaction mixture was stable, protein was desalted and analyzed SDS-PAGE.

2.2.17 Modification of Arg residues

Arg residues were modified by the reviewed method of Smith (1977) using 1, 2-cyclohexanedione. The protein was dissolved in 0.2 M sodium borate buffer, pH 9.0 (0.1 mg/mL) and reacted with 0.15 M 1, 2-cyclohexanedione at 35 °C for 2 h. The reaction mixture was acidified by 30 % acetic acid and dialysed against 10 mM acetic acid to remove excess reagents. The inhibitory activity was assayed as described earlier (Section 2.2.3.1)

2.2.18 Carbethoxylation of His residues

His residues were modified to N-carbethoxyhistidine using diethylpyrocarbonate (DEPC) (Miles, 1977). DEPC solutions were freshly prepared. The concentration of stock DEPC solution was determined by reaction with 10 mM imidazole (Miles, 1977). The protein (0.1 mg/mL) was solubilised in 0.1 M phosphate buffer (pH 7.5) and at a fixed time intervals, aliquots of DEPC (from stock of 10 mM) were added to the reaction mixture and the reaction monitored by the increase of absorbance at 240 nm due to the formation of N-carbethoxyhistidine against an appropriate blank. The final concentration of DEPC ranged from 0.01 to 0.40 mM.

2.2.19 Circular Dichroism spectral measurements

Differences in the secondary and tertiary structures of horsegram trypsin inhibitors were determined by CD-spectral studies in 0.1 M Tris-HCL buffer, pH 7.5 at 25 °C. The CD measurements were carried out in a Jasco-810 Spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) using a path length of 10 mm and 1 mm cuvettes with 1.25 mg/mL and 0.3 mg/mL of protein for near-UV and far-UV respectively.

The molar ellipticity (θ) was calculated according to the equation

$$[\theta] = \theta \times 100 \times M_r \; l \; c$$

Where 'θ' the measured ellipticity, 'M_r' the molecular weight of protein, 'c' the concentration of protein in mg/mL and 'l' the pathlength in centimeters.

2.2.20 Fluorescence studies

Fluorescence measurements of horsegram BBIs were carried out at 27°C using a Shimadzu (Model RF 5000) recording spectrofluorimeter in a 3 mL cuvette with a path length of 1 cm.

Intrinsic fluorescence

Protein was dissolved in 25 mM Tris-HCl buffer, pH 7.5 (0.1 mg/mL), excited at 275 nm and emission recorded between 300 and 400 nm.

8-anilino-1-napthalenesulfonate (ANS) binding

ANS binding was monitored by measuring the fluorescence between 440 nm and 540 nm after excitation at 375 nm. The protein (0.1 mg/mL) dissolved in 25 mM Tris-HCl buffer (pH 7.5) was titrated with ANS to a final concentration of 80 μM. The samples were excited at 375 nm and the binding monitored by measuring fluorescence at 472 nm. The readings were converted to relative fluorescence and plotted against ANS concentration.

2.2.21 Capillary electrophoresis

The purified inhibitor isoforms were electrophoresed on a Prince Technologies capillary electrophoresis system equipped with a coated capillary (Prince Technologies B.V., Netherlands) (id = 75 μm, length = 100 cm), at 28 °C by applying a voltage of 30 KV. Prior to analysis, the capillary was equilibrated with Tris-Gly buffer (25 mM Tris and 192 mM Gly at pH 8.3). The protein samples (1 mg/mL in running buffer) were injected at 20-mBar pressure for 10 sec. The inhibitors were detected at 230 nm with an online lambda detector 1010 Bishoff set at 230 nm.

2.2.22 Amino acid analysis

Amino acid analysis was performed according to the method of Bidlingmeyer *et al.*, (1984) using a Waters Associate Pico-Tag amino acid analysis system.

The amino acid analysis was carried out using a three-step procedure. In first step the protein samples were acid hydrolyzed to free amino acid residues, amino acid were modified by PITC in the second step and last step includes the separation of the modified amino acids by RP-HPLC.

Hydrolysis of Protein: An aliquot of 50 μL of reverse phase purified samples were pipetted in to a tube (6 × 50 mm Pyrex ™) and placed in the special vacuum vial. The vial was then attached to the Waters Associates Pico-Tag workstation manifold and the samples were dried under vacuum to 50-60 mtorr. After drying, the vacuum was released and 200 μL of constant boiling HCL (6 N) containing phenol 1 % (v/v) was pipetted in to bottom of the vacuum vial. The vacuum vial was then reattached to the manifold, evacuated and sealed under vacuum. Samples were hydrolyzed in the workstation at 110 °C for 24 h. After hydrolysis the residual HCl inside the vacuum vial was removed under vacuum. Standard free amino acids as a mixture (Pierce H) containing up to 25 nmol of each amino acid were placed in the tubes (6 × 50 mm) and dried under vacuum. Free amino acids and hydrolyzed samples were dried down under vacuum after adding re-drying solution (10-20 μL) containing ethanol:water:triethylamine (TEA) (2:2:1) to each tube. When the vacuum reached 50-60 mtorr, the samples were ready for derivatization.

Derivatization of hydrolyzed amino acids: The derivatization reagent was made fresh each time and consisted of ethanol:triethylamine:water :PITC (7:1:1:1). The PITC was stored at –20 °C under nitrogen to prevent the formation of breakdown products. To make 300 μL reagent, sufficient for 12 samples, 210 μL of ethanol was mixed thoroughly with 30 μL of each PITC, TEA and water. PTC amino acids were formed by adding 20 μL of the reagent to the dried samples and sealing them in the vacuum vials for 20 min at 25 ± 2 °C. The excess of reagents were then removed under vacuum using the workstation. When the vacuum reached 50-60 mtorr, the samples were ready for analysis by RP-HPLC.

Chromatography: The HPLC was carried out using a Waters Associate HPLC system consisting of binary gradient pumping system and photodiode array detector (Model 2996) with a Millennium data processor. The temperature was controlled at 38 ± 1 °C with a column heater. Samples were injected in volumes ranging from 5-50 μL. The column was an application specific Pico-Tag column (150 × 3.9 mm).
Solvent systems: Solvent system consisted of two eluents, (A) an aqueous buffer (0.14 M Sodium acetate containing 0.5 mL/L of TEA, titrated to pH 6.4 with glacial acetic acid:acetonitrile (94:6) and (B) 60 % acetonitrile in water. The gradient run for the separation consisted of % B traversing to 46 % B in 10 min. After the run a washing step in 100 % B was included so that the residual sample components were removed.

The PTC amino acids were detected at 254 nm. The gradient elution programme is shown in Table 2.2 and the separation of a standard amino acid mixture is shown in the Figure 2.1.

Table 2.2: The gradient programme for amino acid analysis

Time (min)	Flow (mL)	% A	% B	Curve
00.01	1.0	100	0	
10.00	1.0	54	46	7
11.00	1.0	0	100	6
13.00	1.0	0	100	6
14.00	1.0	100	0	6
25.00	1.0	100	0	6

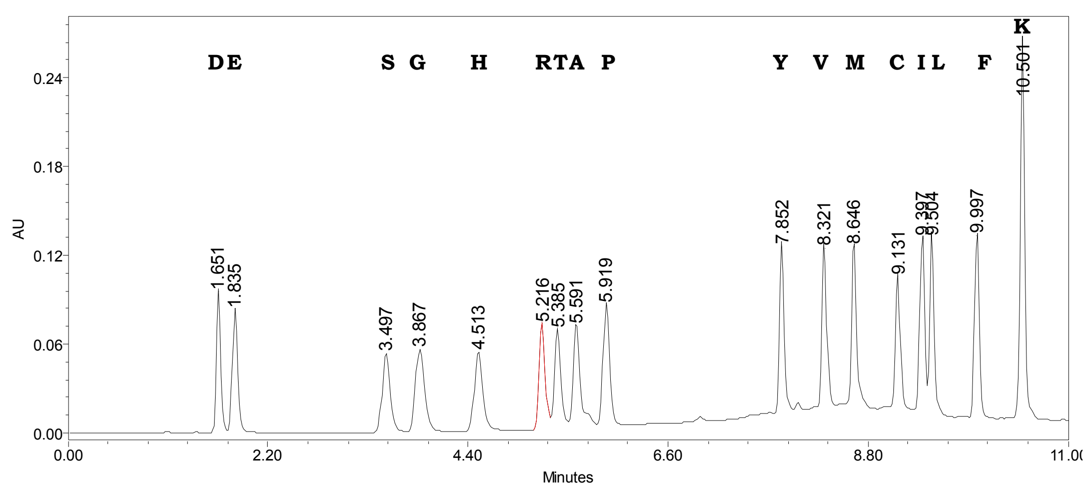


Figure 2.1 **Elution profile of PTC-amino acids using a Pico-Tag amino acid analysis system.** The amino acids are represented by the single letter code. Column: Pico-Tag™ (150×3.9 mm, 4 μm); flow rate: 1.0 mL/min; wave length: 254 nm; temperature: 38 °C; injection volume: 5 μL (312.5 pmole of each amino acid); solvent A:140 mM Na acetate, 3.6 mM triethylamine (TEA), pH 6.4/acetonitrile (94/6); solvent B: 60% acetonitrile.

2.2.23 Automated gas phase protein sequencing

Automated gas phase sequencing was carried out on the protein sequenator PSQ-1 (Shimadzu). This sequenator carries out Edman-degradation by supplying gaseous reagents for the coupling and cleavage reactions. A flow diagram of the steps involved in automated

sequencing is shown in Figure 2.2. The protein or peptide was spotted on a glass fiber disc previously coated with polybrene and washed for three cycles. Alternatively, the cut PVDF membrane which contains the electrotransferred protein band was directly placed on the glass fiber disc. The coupling reaction is carried out with phenyl isothiocynate (R1) in presence of gaseous trimethylamine (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 gaseous TFA to form an anilino thiazolinone (ATZ) derivative. Both the coupling and cleavage reactions are performed in a temperature controlled reaction chamber. The free ATZ-amino acid is extracted from conversion flask by n-butyl chloride (S3). The ATZ-amino acid is converted to more stable PTH-amino acid by reaction with 25 % TFA (R4). The PTH-amino acid is dissolved in acetonitrile (S4) and automatically injected into the HPLC. The PTH-amino acids are separated by RP-HPLC. Figure 2.3 depicts the separation of the PTH-amino acids using an isocratic elution. The PTH-amino acid in each cycle identified, quantified and recovery percentage calculated using an online CR4A system. The results are displayed and recorded.

2.2.24 Enzymatic cleavage of pyridylethylated HGGIs:

2.2.24.1 a-TPCK trypsin cleavage:

The three HGGIs were cleaved at R-X and K-X bond by digesting with TPCK-trypsin at 1:100 enzyme to protein ratio in 0.1 M ammonium hydrogen carbonate, pH 8.2 for 1 h at 37 °C with continuous stirring. The trypsin was added from a stock of 10 mg/mL and the reaction was terminated by reducing the pH of the reaction mixture to 2.0 by adding 98 % formic acid. The sample was concentrated to dryness and re-dissolved in 0.5 mL of 0.1 % TFA (Leavis *et al.*, 1978).

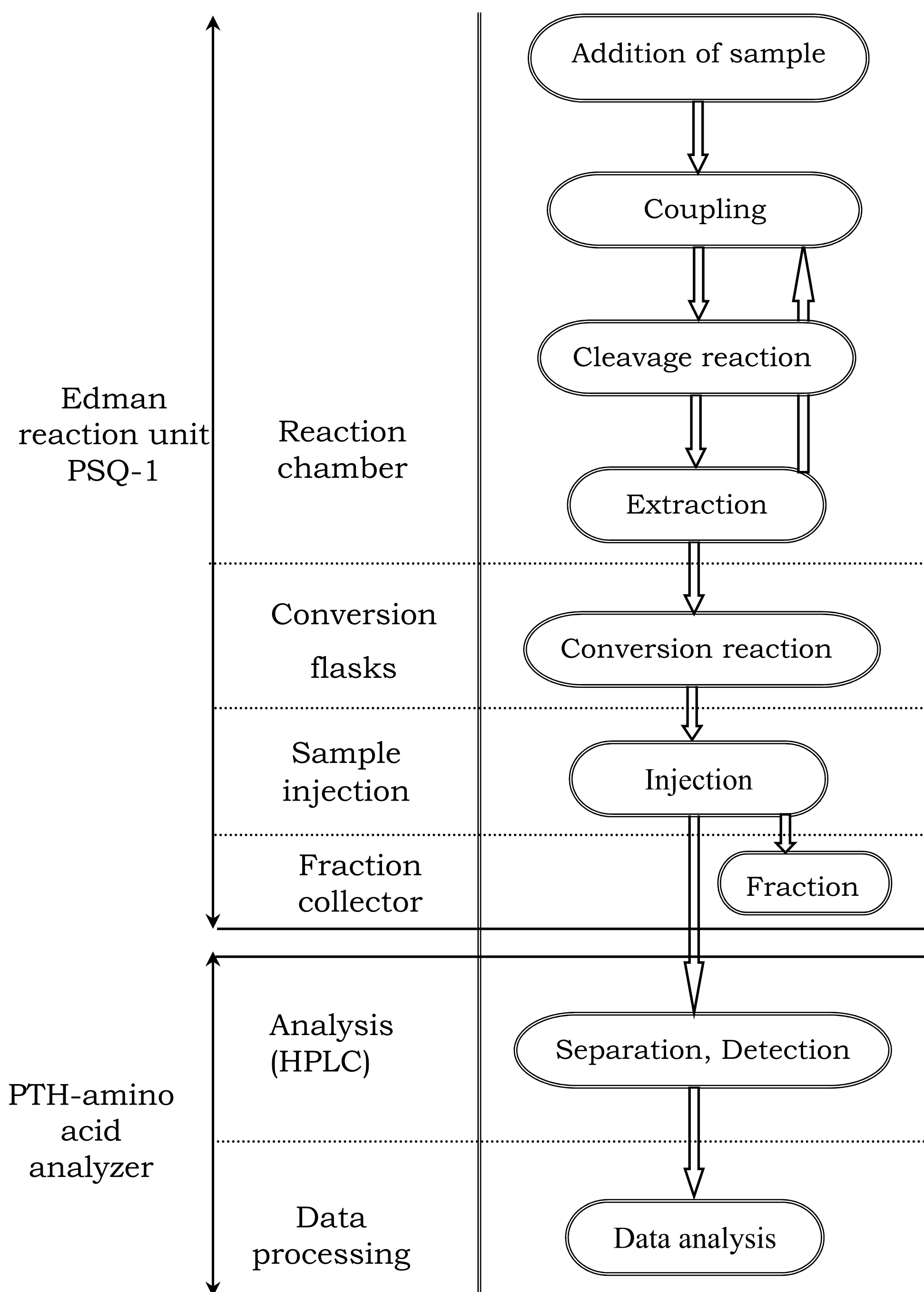


Figure. 2.2 **Flow diagram of the reactions that occur during gas phase sequencing of protein or peptide on PSQ-1 (Shimadzu) sequenator**.

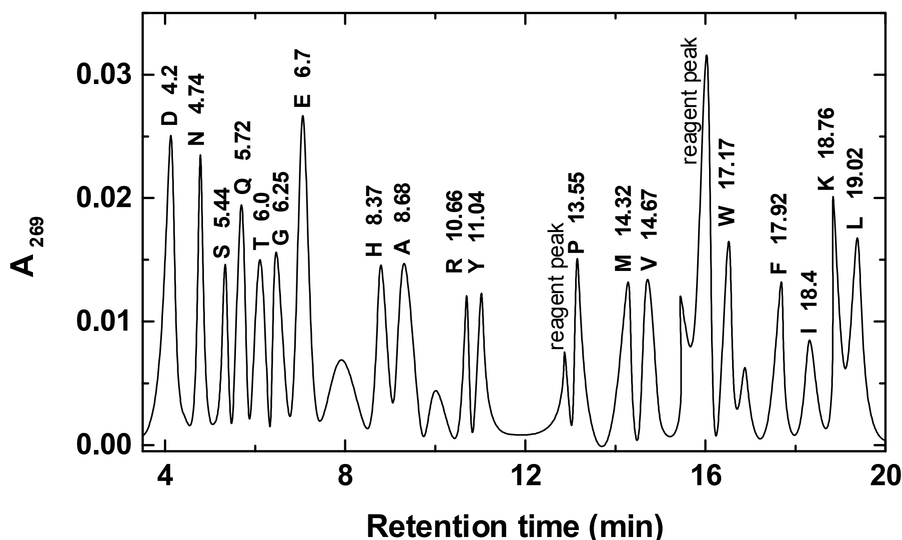


Figure 2.3 **RP-HPLC separation of PTH-amino acids standards on the automated protein sequenator**

2.2.24.2 Endoproteinase Asp-N cleavage:

Endoproteinase Asp-N (1:100 w/w enzyme to substrate ratio) was used to cleave at X-Asp linkages (Drapeau, 1980; Guild and Strominger, 1984). The incubation was carried out in 0.25 mL of 0.1 M Tris-HCl (pH 8.5) at 37 °C for 18 h and the reaction was stopped by concentrating the sample to dryness. The mixture of peptides obtained was separated as described in Section 2.2.12.

2.2.25 Chemical cleavage with cyanogen bromide

Pyridylethylated HGGIs were dissolved in 0.5 mL of 70 % formic acid. A 50 fold molar excess of CNBr over Met residues was added from the stock solution in 70 % formic acid with continuous stirring. The reaction mixture was flushed with nitrogen and incubated for 24 h in the dark at 25 ± 1 °C in sealed vials. Excess of reagents were removed from the digestion mixture by diluting with 15 volumes of water and freeze-drying. This step was repeated two times and finally the mixture was freeze-dried (Gross, 1976; Allen, 1989).

2.2.26 Purification of peptides for amino acid sequencing

The peptides obtained after the enzymatic and chemical cleavage were purified and repurified by RP-HPLC using the following solvent system.

Solvent system:

Solvent A: 0.1 % TFA in water

Solvent B: 0.05 % TFA in 70 % acetonitrile and 30 % water (Mahoney and Hermodson, 1980 and Hermodson and Mahoney, 1983)

The water used in the preparation of the above solvents was Milli Q water having a conductance of 18.2 ohms. The aqueous solvents were degassed for 15 min and 2 min for organic solvents using an oil pump before addition of TFA.

The freeze-dried samples of peptide mixtures were dissolved in a minimal amount of solvent A. About 100-200 μg of sample protein digest was loaded on to Phenomenex ODS column (250 × 4.6 mm, 5μ) pre-equilibrated with solvent A. The column washed for 5 or 10 min in solvent A to remove unbound peptides and salt present in the sample. The bound peptides were eluted by a linear gradient of solvent B at a flow rate of 0.7 mL/min. The elution of the peptide was monitored at 230 nm and the peak fractions were collected individually. The column was washed with 100 % solvent B for 10 min and allowed to re-equilibrate in solvent A for 15 min prior to the next injection.

The peak fraction collected over several runs were concentrated and repurified as described above. The peak fractions collected during repurification were concentrated to 100 μL and stored at –20 °C and used for the sequence determination and amino acid analysis.

2.2.27 Preparation of starting material from horsegram tissues

2.2.27.1 Horsegram flower

The horsegram flowers collected from the plants grown in the fields were ground to a fine paste in a pestle and mortar. The paste was cooled to 4 °C and ice-cold acetone was added, stirred gently for 1-2 min and acetone removed by filtration. The residue obtained was air dried to remove the excess of acetone at 25 ± 2 °C for 24 h. The dried flower acetone powder was stored at -20 °C until used.

2.2.27.2 Horsegram leaves

The horsegram seeds were germinated as described earlier (Section 2.2.1). After 120 h of germination, the tender leaves were dissected free of axis and an acetone powder was prepared.

2.2.28 Methods used for characterization of proteinase

2.2.28.1 Preparation of horsegram storage protein

Defatted horsegram flour (100 g) in 0.025 mM Tris-HCl, pH 7.5 was stirred at 4 °C for 12 h and filtered through cheesecloth. The filtrate was centrifuged at 10,000 × g for 30 min. The pH of the supernatant was adjusted to pH 4.5 by addition of 6 N HCl and the solution was allowed to stand overnight at 4 °C. The precipitate was collected by centrifugation at 10,000 × g for 30 min. The precipitate was dissolved in a minimal volume of water and heated to 60 °C for 20 min to denature and inactivate any endogenous proteinases and dialyzed extensively (4 L × 5) against water. The dialysate was concentrated by lyophilization and used as substrate.

2.2.28.2 Proteinase assay

Total reaction mixture contained 0.1 mL of partially purified proteinase from horsegram germinating seeds, 0.5 mL of substrate (1 % in 0.2 M Gly-HCl, pH 9.25) and 0.4 mL of water. The reaction was started by addition of enzyme and incubated at 37 °C for 30 min. The reaction was stopped by the addition of 1 mL ice-cold TCA (10 % w/v) and allowed to stand at 4 °C for 1 h, centrifuged at 8,000 × g for 20 min. Absorbance of the TCA soluble peptides in the supernatant was read at 280 nm using an appropriate blank.

Proteinase units: One enzyme unit is arbitrarily defined as the increase in absorbance by 0.01 at 280 nm.

2.2.28.3 Effect of pH on protease

Optimum pH of the proteinase activity was determined by carrying out the reaction at various pH as described in previous section. The buffers used were 0.1 M Gly-HCl, pH 3.0; sodium acetate, pH 4.0, 5.0 and 6.0; sodium phosphate, pH 7.0; Tris-HCl, pH 8.0 and Gly-NaOH, pH 9.0 and 10.0.

2.2.28.4 Effect of temperature

The optimum temperature for proteinase activity was studied at temperatures ranging from 0 - 60 °C using 1 % (w/v) substrate in 0.2 M Gly-HCl buffer pH 9.25.

2.2.28.5 Substrate specificity

The substrate specificity of the partially purified proteinase, was determined using gelatin, casein, azocasien, albumin, glycinin and horsegram storage proteins dissolved in 0.2 M Tris-HCl buffer, pH 7.5 and incubated with proteinase for 30 min at 37 °C.

2.2.28.6 pH stability of proteinase

Stability of the partially purified proteinase from germinating horsegram seeds was studied at different pH by incubating the enzyme in different buffers (Gly-HCl, pH 3.0; acetate, pH 5.0; phosphate, pH 7.0 and Gly-NaOH, pH 9.0 and 9.5) for different time intervals and assaying the residual activity at its optimum pH.

2.2.28.7 Effect of class specific inhibitors

The proteinase was incubated with different class specific synthetic protease inhibitors viz. Pepstatin A, EDTA, Iodoacetamide and PMSF for aspartic, metallo, cysteine and serine proteinases respectively and the residual activity determined as described under Section 2.2.28.2.

2.2.29 Comparison of the sequences of HGGIs with other dicot Bowman-Birk inhibitors.

Twenty four dicot BBI sequences were selected from the Protein Data Bank (SWISS PROT) released on 24th OCT, 2003 and reported in literature for comparative studies.

2.2.29.1 Pair wise sequence comparison

Pair-wise alignment of HGGI sequences were carried out to assess the extent of shared similarities and to identify an evolutionary (homologous) relationship with a known protein family. The pair-wise sequence comparisons were carried out using LALIGN program. LALIGN uses the Huang and Miller algorithm (Huang and Miller 1991). The two sequences are compared using a matrix BL50 with gap penalties: -14/-4. The pair-wise comparison was also done using SIM-LALNVIEW using the comparison matrix BLOSUM62, with an open gap penalty of 12 and gap extension penalty of 4.

2.2.29.2 Multiple Alignment and Construction of Phylogenetic Tree

In order to reveal subtle conserved family characteristics of BBI family with the newly sequenced HGGIs, the multiple alignment was carried out. The program MULTALIN (Corpet, 1988) was used to align all the BBI sequences. In this method, all possible pairs of sequences are aligned initially using Lipman and Pearson (1985) FASTP algorithm. The similarity scores are then computed to determine the hierarchial order of clustering sequences. The alignment of sequences that have the highest score are initially accepted and the aligned pair treated as a single sequence. Each further step then combines either two sequences or clusters or a sequence and a cluster. The similarity measures are reevaluated after each combination. Aligned sequences are then analyzed using the PHYLIP program (Felsenstein, 1989). The sequences are analyzed by the maximum parsimony method using the PROTOPARS program. The unrooted phylogenetic tree was drawn.

2.2.30 Hydrophobicity plot

The hydropathy patterns of three inhibitors were established by the method of Kyte and Doolittle (1982), which predicts the interior, and exterior portions of globular proteins remarkably well.

2.2.31 Modeling of HGI-III dimer

The sequences of BBIs from leguminous plants were obtained from the NCBI protein sequence database and aligned using the Clustalw multiple alignment algorithm (Higgins *et al.*, 1994). Of these, a crystal structure for the pea BBI inhibitor was available from the Protein Data Bank (PDB:1PBI). The sequences of the monomeric HGGI-III and dimeric HGI-III were highly similar to that of the pea inhibitor, enabling building their models by standard homology modeling techniques. Models of HGI-III and HGGI-III were built using the Biopolymer module in InsightII (Accelrys Inc.,) and energy minimized

using DISCOVER. Quality of the structure was measured using PROCHECK (Laskowski *et al.*, 1993). A single residue insertion at position 37, that forms part of a loop, was observed in HGI-III and HGGI-III, with respect to the crystal structure template. An analysis of related crystal structures in PDB using DALI (Holm and Sander, 1996) and Insight-II, indicated that the structure of BBI proteinase inhibitor PI-II (1pi2) contained a similar insertion at the same position. The insertion in the loop was therefore modeled based on this structure. Visualization and analysis of the structures were carried out using Insight-II.

The Bowman-Birk inhibitors of germinated horsegram seeds: Purification and characterization

Legumes are notable for their high content of reserve food material and their large content of proteinase inhibitors in their cotyledons (Ryan, 1973; Laskowski and Kato, 1980). Study of these inhibitors in seeds and their metabolism during germination have been hindered due to the multiplicity of inhibitors in these plants (Haynes and Feeney, 1967; Wilson and Laskowski, 1973; Yoshida and Yoshikawa, 1975; Odani and Ikenaka, 1977a). During the germination of horsegram seeds (*Dolichos biflorus*), the BBIs present in the dormant seed rapidly disappeared with the concomitant appearance of eletrophoretically different new active inhibitor species (Sreerama and Gowda, 1998). In attempt to understand the origin of these new forms, the purification and characterization of the three new inhibitor isoforms that appear during the germination of horsegram seeds was undertaken.

RESULTS

Purification of horsegram germinated inhibitors

Extraction

The cotyledons dissected from 120 h of germinated horsegram seeds were ground to fine paste with a mortar and pestle. The meal was extracted overnight in cold with 0.1 M Gly-HCl buffer, pH 2.5 (1:5 w/v). The crude extract was filtered through cheese cloth and the suspended particles in filtrate removed by centrifugation at 10, 000 × g for 30 min at 4 °C. Clear supernatant (pH 3.25) thus obtained was titrated to pH 7.5 with liquor ammonia. The crude extract showed a specific activity of 16.94×10^3 and 39.10×10^3 for trypsin and chymotrypsin inhibition respectively (Table 3.1).

Ammonium sulfate fractionation

Crude extract was saturated to 60 % $(NH_4)_2SO_4$ (36.1 g/100 mL) by addition of finely ground solid $(NH_4)_2SO_4$ at 4 °C. The solution was

allowed to stand overnight in cold for complete precipitation of the proteins. The precipitate was collected by centrifugation at 10, 000 × g for 30 min at 4 °C. The precipitate obtained was dissolved in minimal quantity of water and dialysed extensively against water (5 × 500 mL). The salt free dialysate was concentrated to dryness by freeze-drying. From this fractionation step, a 2.8 fold increase in the specific activity was observed with a yield of 60 % (Table 3.1).

Sephadex G-50 size-exclusion chromatography

The freeze-dried sample was dissolved in a minimal amount of 25 mM Tris-HCl, pH 7.5. Un-dissolved particulate matter was separated by centrifugation at 10, 000 × g for 15 min at 4 °C. The clear fraction obtained was loaded on to a Sephadex G-50 column (100 × 2.1 cm), pre-equilibrated with 25 mM Tris-HCl buffer, pH 7.5 at 25 ± 3 °C at a flow rate of 12 mL/h. Two mL fractions were collected and assayed for protein as well for the trypsin/chymotrypsin inhibitory activity. This step yielded a single major trypsin/chymotrypsin inhibitor peak (Figure 3.1). The active fractions were pooled as indicated, dialysed against water (5 × 500 mL) to remove the buffer ions and freeze-dried. The specific activity of this fraction was 116.25 × 10^3 IU/mg and 273.13 × 10^3 IU/mg for anti-trypsin and anti-chymotrypsin respectively, with a yield of 56 % trypsin inhibitory activity (Table 3.1).

Separation of isoinhibitors

CM-Sephadex C-25 cation-exchange chromatography, was used to resolve the three-inhibitor isoforms present in the partially purified fraction obtained from Sephadex G-50 size-exclusion chromatography step. The sample obtained from previous step was dissolved in 50 mM sodium acetate buffer (pH 5.0) and loaded on a pre-equilibrated CM-Sephadex C-25 column (14 × 3.4 cm) at a flow rate of 10 mL/h.

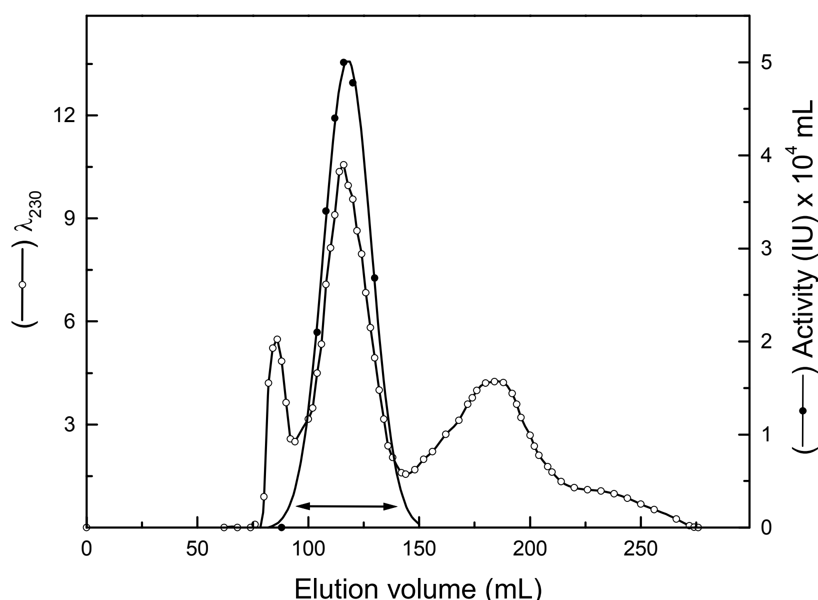


Figure 3.1. **Elution profile of horsegram germinated inhibitors on Sephadex G-50 chromatography.** The crude protein obtained from $(NH_4)_2SO_4$ fractionation was loaded on to Sephadex G-50 column (100 × 2.1 cm) equilibrated with 25 mM Tris-HCl, pH 7.5. The sample was eluted with same buffer at a flow rate 12 mL/h. Fractions of 2 mL were collected and the active fractions were pooled as shown (⟷).

Washing the column at 20 mL/h resulted in the elution of two major protein peaks (Figure 3.2) in the buffer as unbound protein peaks. Upon washing the column with 0.3 M NaCl in the equilibrating buffer during column regeneration, a major protein peak eluted (Figure 3.2) that showed both trypsin and chymotrypsin inhibitory activity.

The three peaks that eluted were designated as HGGI (horsegram germinated inhibitor)-I, HGGI-II and HGGI-III respectively according to their order of their elution. The fractions having inhibitory trypsin/chymotrypsin activity were pooled individually, dialysed against water and concentrated to dryness. Contaminating traces of HGGI-II in the HGGI-I fraction (Figure 3.2) and vice versa were removed in a final step of purification by anion-exchange chromatography.

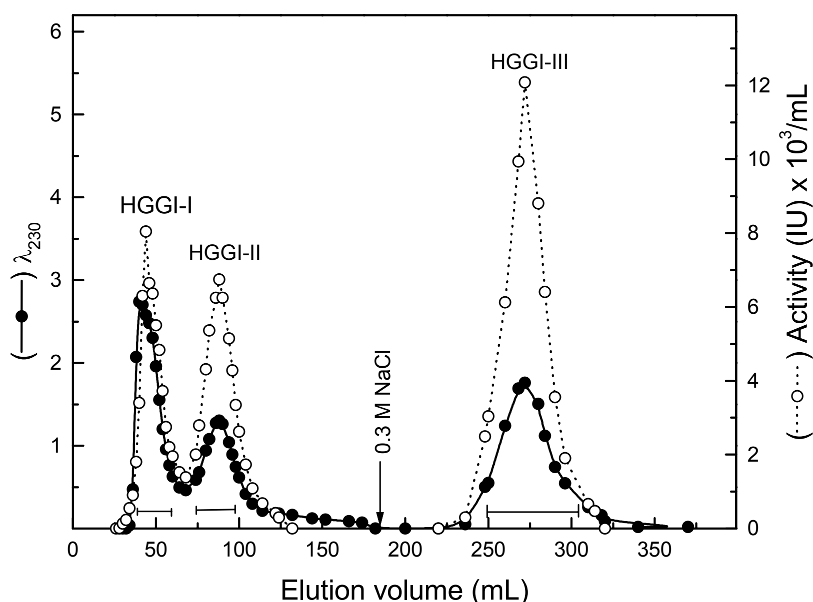


Figure 3.2. **CM-Sephadex C-25 chromatography of horsegram germinated isoinhibitors**. The column was washed with 50 mM sodium acetate buffer, pH 5.0 and inhibitors eluted with 50 mM sodium acetate buffer containing 0.3 M NaCl; flow rate 20 mL/h. The active inhibitor fractions were pooled as shown (——).

Anion-exchange chromatography (DEAE-Sephadex A-25 chromatography)

The two-inhibitor fractions, HGGI-I and HGGI-II were finally purified individually by loading on to a DEAE-Sephadex A-25 column (7.2 × 2.7 cm) (Figure 3.3), pre-equilibrated with 0.1 M ammonium bicarbonate at a flow rate of 20 mL/h at 25 ± 3 °C. During the purification, the ratio of chymotrypsin inhibitory units (CIU) to trypsin inhibitory units (TIU) remained nearly constant, indicating that the same protein was responsible for the both the activities (Table 3.1). The recovery of HGGI-I, HGGI-II and HGGI-III were 4.2 %, 8.3 % and 25 % respectively. The results of the purification are summarized in Table 3.1.

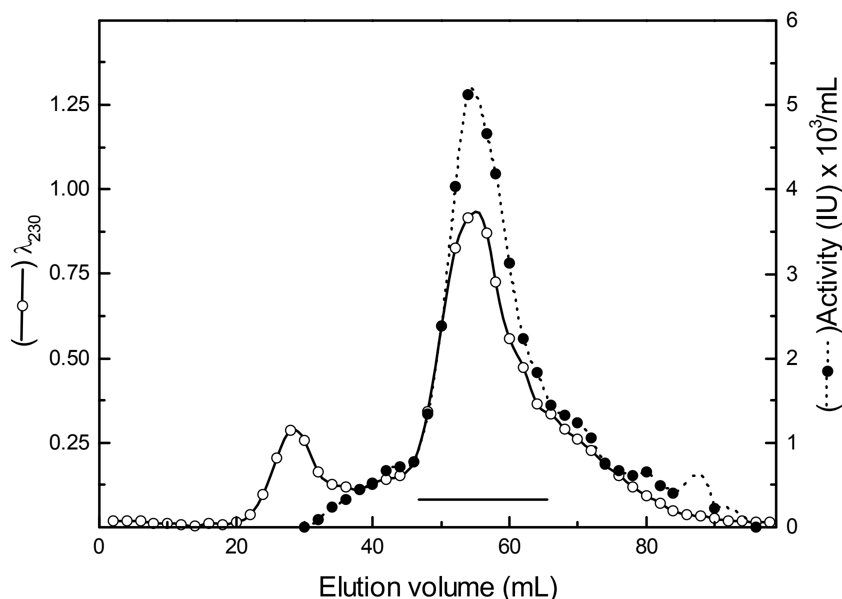


Figure 3.3a. **DEAE-Sephadex A-25 chromatography profile of HGGI-I.** The sample dissolved in 0.1 M ammonium bicarbonate buffer (pH 8.2), loaded and eluted from pre-equilibrated DEAE-Sephadex column with 0.1 M ammonium bicarbonate buffer (pH 8.2) at 20 mL/h. 2 mL fractions were collected and active inhibitor fractions were pooled as indicated (——)

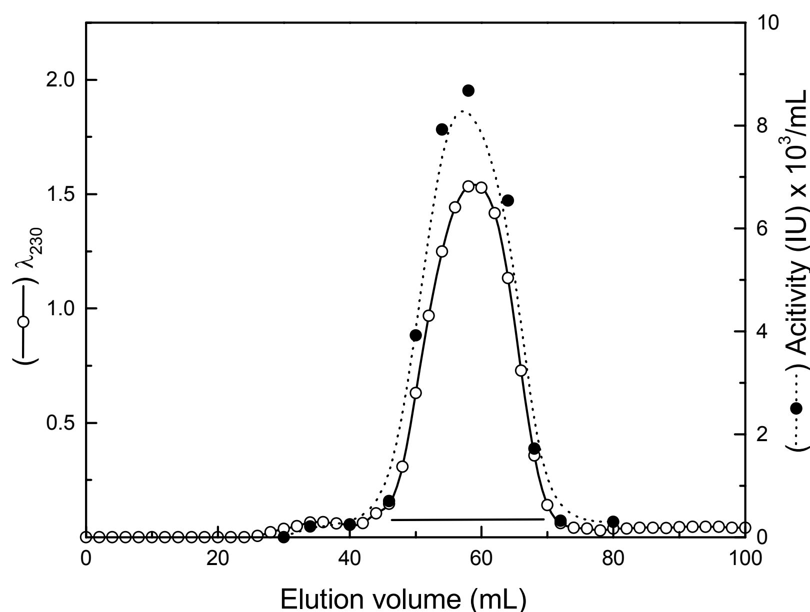


Figure 3.3b. **DEAE-Sephadex A-25 chromatography profile of HGGI-II.** The sample dissolved in 0.1 M ammonium bicarbonate buffer (pH 8.2), loaded and eluted from pre-equilibrated DEAE-Sephadex column with 0.1 M ammonium bicarbonate buffer (pH 8.2) at 20 mL/h. 2 mL fractions were collected and active inhibitor fractions were pooled as indicated (——).

Criteria of homogeneity

Homogeneity of the three germinated horsegram isoinhibitors was examined by acid and basic PAGE, gelatin-PAGE, HPLC analytical gel filtration and capillary electrophoresis.

The purified isoinhibitors were electrophoresed on a (10 % T, 2.7 % C) polyacrylamide gel in β-alanine–acetic acid buffer (pH 4.5) and detected by staining with CBB R-250. Similarly the inhibitory activity was detected by incorporating gelatin (final concentration 1% w/v) and digesting with trypsin/chymotrypsin as described in section 2.2.6.4. The three inhibitors were homogenous and migrate as single species both by protein staining (Figure 3.4A) and by specific staining for trypsin inhibitory activity (Figure 3.4B) and chymotrypsin inhibitory activity (Figure 3.4C).

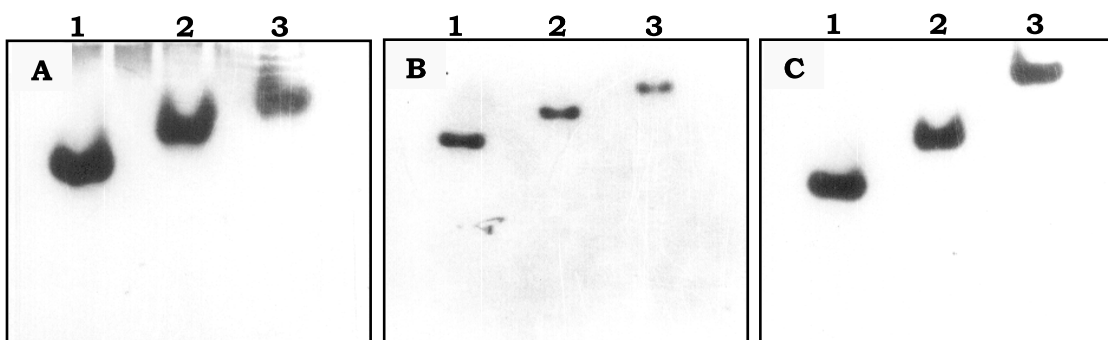


Figure 3.4 **Native PAGE (10 % T, 2.7 % C) of the HGGIs at pH 4.5**. HGGI-III (lane 1); HGGI-II (lane 2) and HGGI-I (lane 3). The gels were stained for (A) protein; (B) trypsin inhibitory activity and (C) chymotrypsin inhibitory activity.

The homogeneity of the HGGIs was further assessed by native PAGE, pH 8.3 and gelatin-PAGE (Figure 3.5). The two inhibitors, HGGI-I and -II were electrophoressed on a 10 % T, 2.7 % C polyacrylamide gel in Tris-HCl buffer, pH 8.8 and located both by protein and enzyme activity as described above. HGGI-I and –II migrated as single species (Figure 3.5, lane 2 and 1) in both the gels indicating their homogeneity.

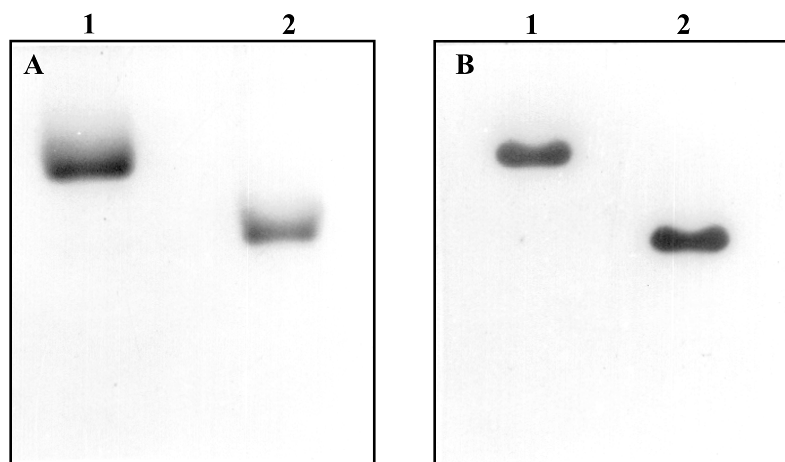


Figure 3.5 **Native-PAGE (10 % T, 2.7 % C) of purified horsegram germinated inhibitors.** HGGI-II (lane 1) and HGGI-I (lane 2). (A) The gel was stained with CBB R-250 for protein and (B) Trypsin inhibitory activity.

The purity of the three inhibitors (HGGIs) was examined by capillary electrophoresis by using Tris-Gly buffer (pH 8.3) at 20-mbar pressure. All the three proteins, HGGI-I, HGGI-II and HGGI-III eluted as sharp single peaks with retention times of 3.188, 3.302 and 3.232 min respectively, showing the homogeneity of the purified inhibitors (Figure 3.6).

Further the release of single amino-terminal amino acid residues Asp (D), Glu (E) and Ser (S) for HGGI-I, HGGI-II and HGGI-III respectively (Figure 3.7) evidenced the purity of the three inhibitors.

Molecular weight

Three different analytical techniques were used to ascertain the apparent molecular weight of the three HGGIs and results obtained are summarized in Table 3.2. The purified inhibitors were loaded on a BIOSEP–SEC-S 2000 analytical gel filtration column equilibrated with 0.1 M sodium phosphate buffer at a flow rate 0.8 mL/min.

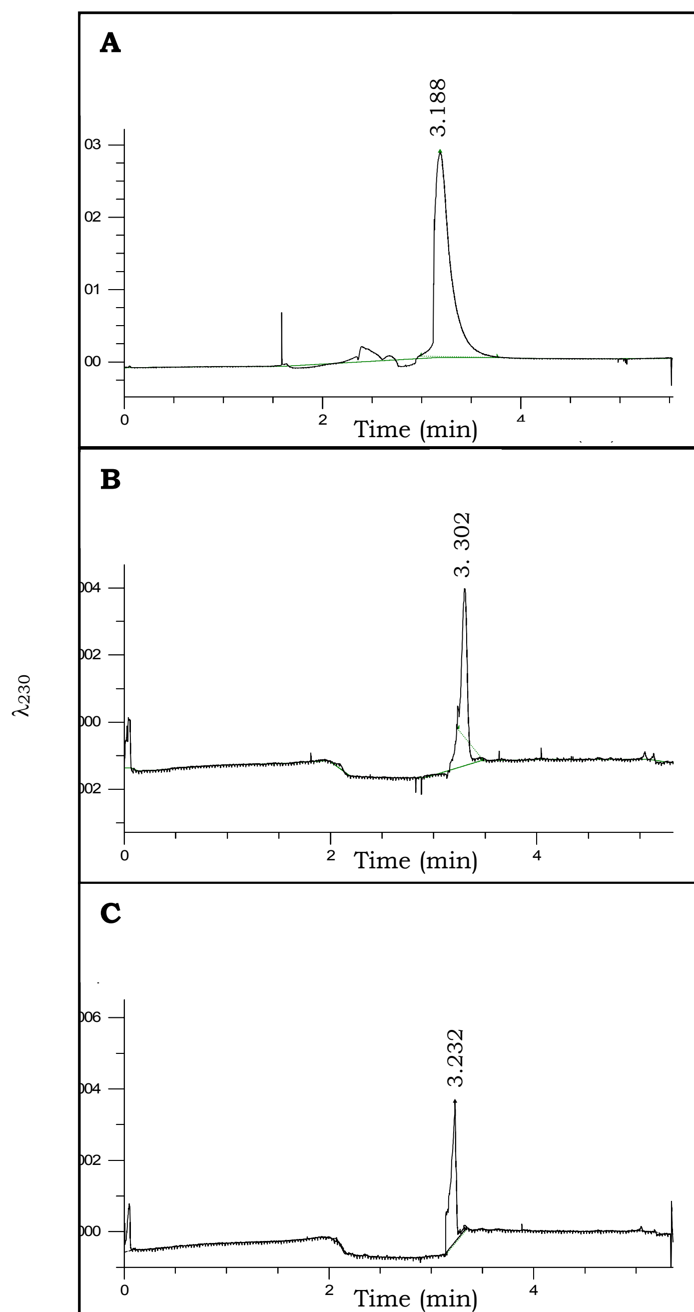


Figure 3.6 **Capillary electrophoresis profile of HGGIs** in Tris-Gly buffer at pH 8.3. The proteins were electrophoresed at 20-mbar pressure and elution monitored at 230 nm. (A) HGGI-I; (B) HGGI-II and (C) HGGI-III.

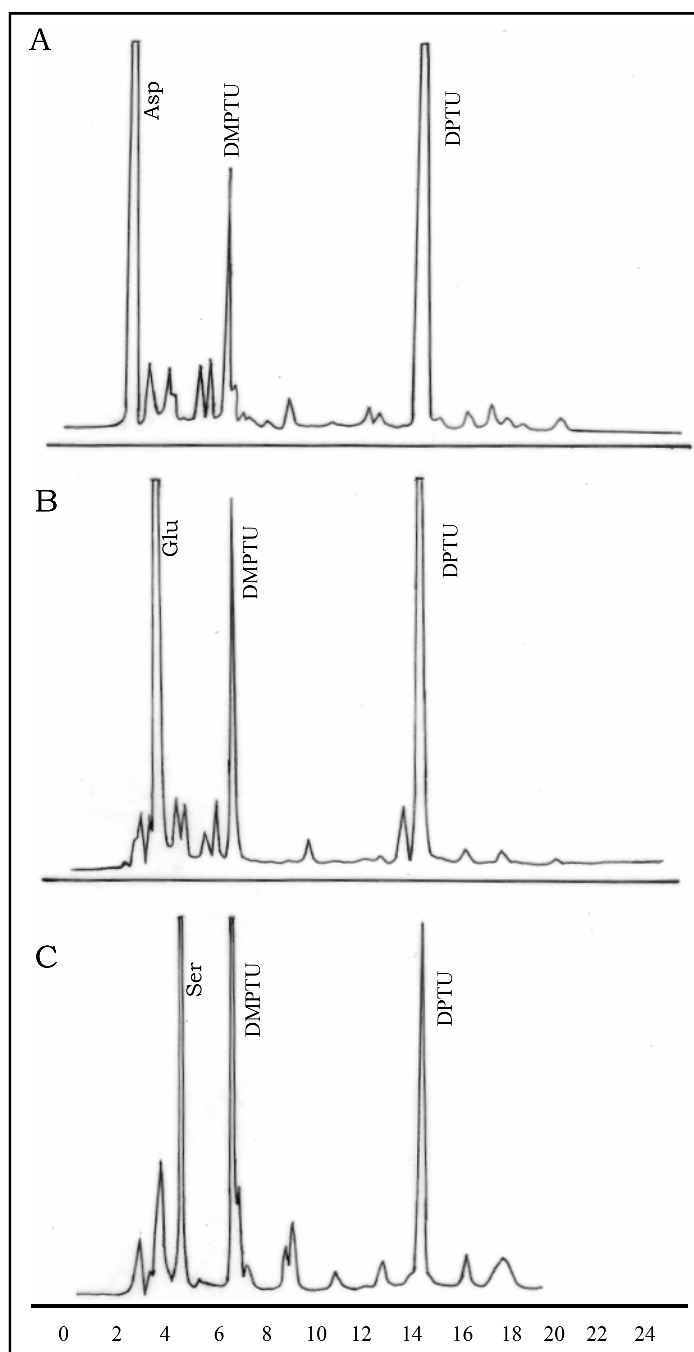


Figure 3.7 **Elution profile of the single amino-terminal amino acid released upon Edman degdration.** (A) HGGI-I; (B) HGGI-II and (C) HGGI-III.

The molecular weights were calculated from a plot of elution time Vs log of M_r (Figure 3.8 and 3.9). The molecular weight determined by SDS-PAGE (Figure 3.10) and analytical gel filtration chromatography on BIOSEP-SEC-S-2000 were 7000±500 Da, indicating them to be single polypeptides. These values are in close agreement with the molecular weights determined by MALDI-MS (Table 3.2).

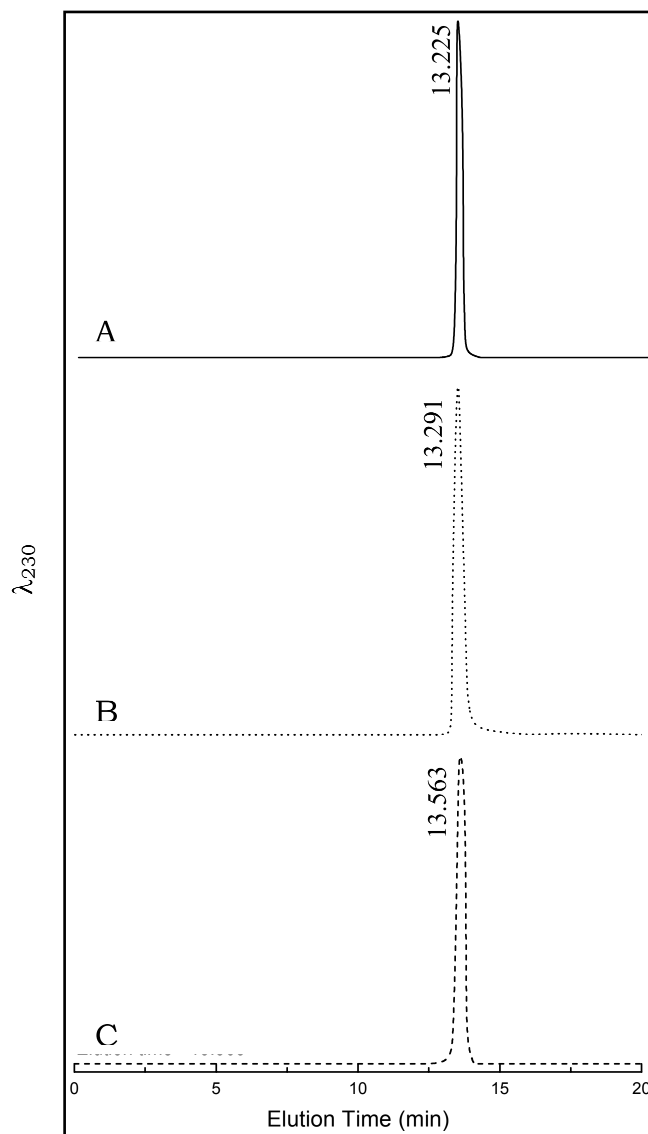


Figure 3.8 **BIOSEP-SEC-S-2000 analytical gel filtration chromatography of purified HGGIs**. The column was equilibrated and calibrated with markers in 0.1 M sodium phosphate buffer pH 7.5. (A) HGGI-I, (B) HGGI-II and (C) HGGI-III were dissolved in above buffer, eluted at a flow rate 0.8 mL/min and monitored at 230 nm.

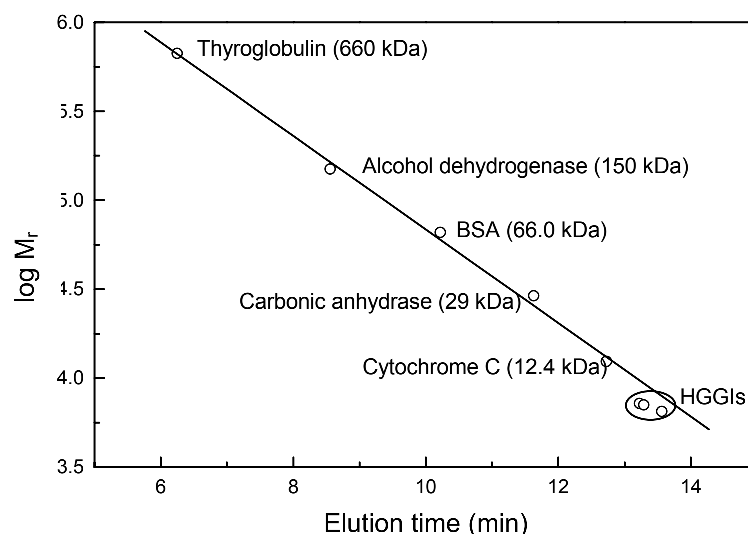


Figure 3.9 **Molecular weight determination of HGGIs by analytical gel filtration chromatography.** Figure shows the plot of retention time Vs log M_r of standard proteins.

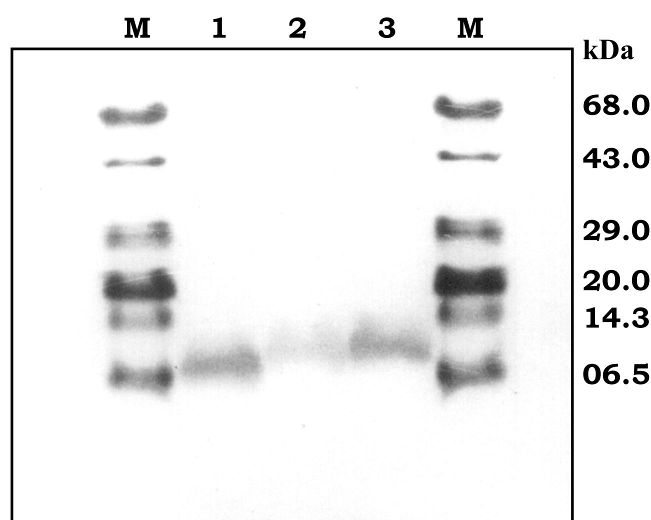


Figure 3.10. **SDS-PAGE (15 % T, 2.7 % C) of purified HGGIs**. Molecular weight standards (lane M): bovine serum albumin (68 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), soyabean trypsin inhibitor (20.0 kDa), lysozyme (14.3 kDa), aprotinin (6.5 kDa); HGGI-III (lane 1); HGGI-II (lane 2) and HGGI-I (lane 3).

Table 3.2. **Apparent molecular weights of germinated horsegram inhibitors.**

Analytical Method	Apparent molecular weight (in Da)		
	HGGI-I	HGGI-II	HGGI-III
SDS-PAGE	7000±500	7000±500	6500±500
Analytical Gel Filtration	7000±500	7000±500	6500±500
MALDI-MS	7216.7	7074.6	6493.5

Isoelectric point (pI)

Isoelectric focusing of the three isoinhibitors was carried out on a Pre-cast Ampholine® PAG Plate using markers having a pI range from 3.5 to 9.5. The pIs of HGGI-I, -II and III were found to be 4.99, 5.09 and 5.28 respectively. Figure 3.11 depicts the distance moved by proteins from cathode Vs pI of marker proteins and HGGIs.

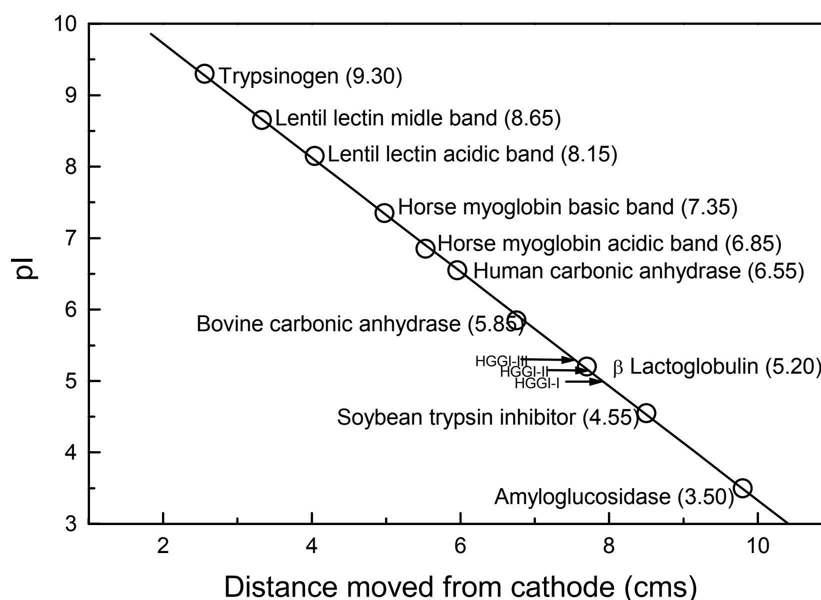


Figure 3.11 **Determination of isoelectric point of HGGIs.** The plot shows the distance moved from cathode vs pI.

Kinetic properties

The type of inhibition exhibited by the HGGIs 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 HGGIs are competitive towards both trypsin and chymotrypsin (Figure 3.12A and 3.12B).

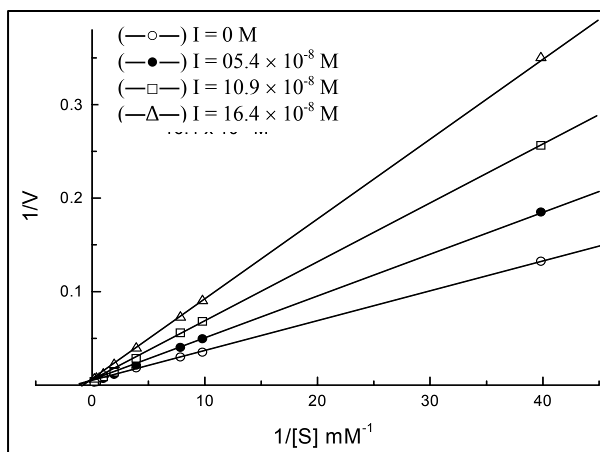


Figure 3.12A **Double reciprocal plot showing the effect of varying substrate concentration (BAPNA) on the activity of trypsin in the presence of various concentration of HGGI-III.** Similar plots were obtained for HGGI-I and HGGI-II.

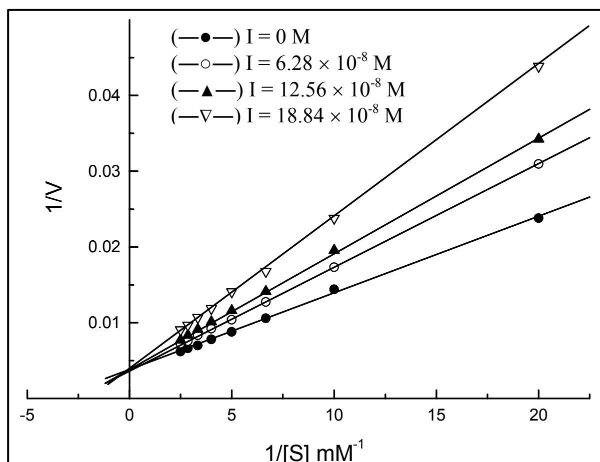


Figure 3.12B **Double reciprocal plot showing effect of varying substrate concentration (BTPNA) on the activity of chymotrypsin in the presence of various concentration of HGGI-II.** Similar plots were obtained for HGGI-I and HGGI-III.

Dissociation constants (K_is)

The inhibitor constants (K_is) (Table 3.3) for HGGIs were deduced from Dixon plots (Figure 3.13A and 3.13B) by plotting [I] vs 1/[V] at various concentrations of the substrate. All the three inhibitors show a higher binding affinity towards trypsin when compared to chymotrypsin.

Stoichiometry of inhibitors

Stoichiometric relation of trypsin and chymotrypsin with the HGGIs was established by evaluating the residual activity after titrating various concentration of the inhibitor with a fixed concentration of trypsin/chymotrypsin. The Figure 3.14 shows the titration curves of bovine trypsin and chymotrypsin with the HGGIs.

Table 3.3 **Kinetic properties of horsegram germinated inhibitors.** The dissociation constant (K_is) for trypsin and chymotrypsin were obtained from Figure 3.13.

Inhibitor	**Dissociation constants (K_is, M)**	
	Trypsin	Chymotrypsin
HGGI-I	1.98×10^{-7}	4.77×10^{-7}
HGGI-II	1.89×10^{-7}	3.64×10^{-7}
HGGI-III	0.98×10^{-7}	7.73×10^{-7}

Modification of free amino groups by 2, 4, 6-trinitrobenzenesulfonic acid

BBIs are known for their double-headed characteristic, which inhibits two molecules of proteases simultaneously and independently. The amino acid (P_1) present at the reactive site determines the protease inhibited.

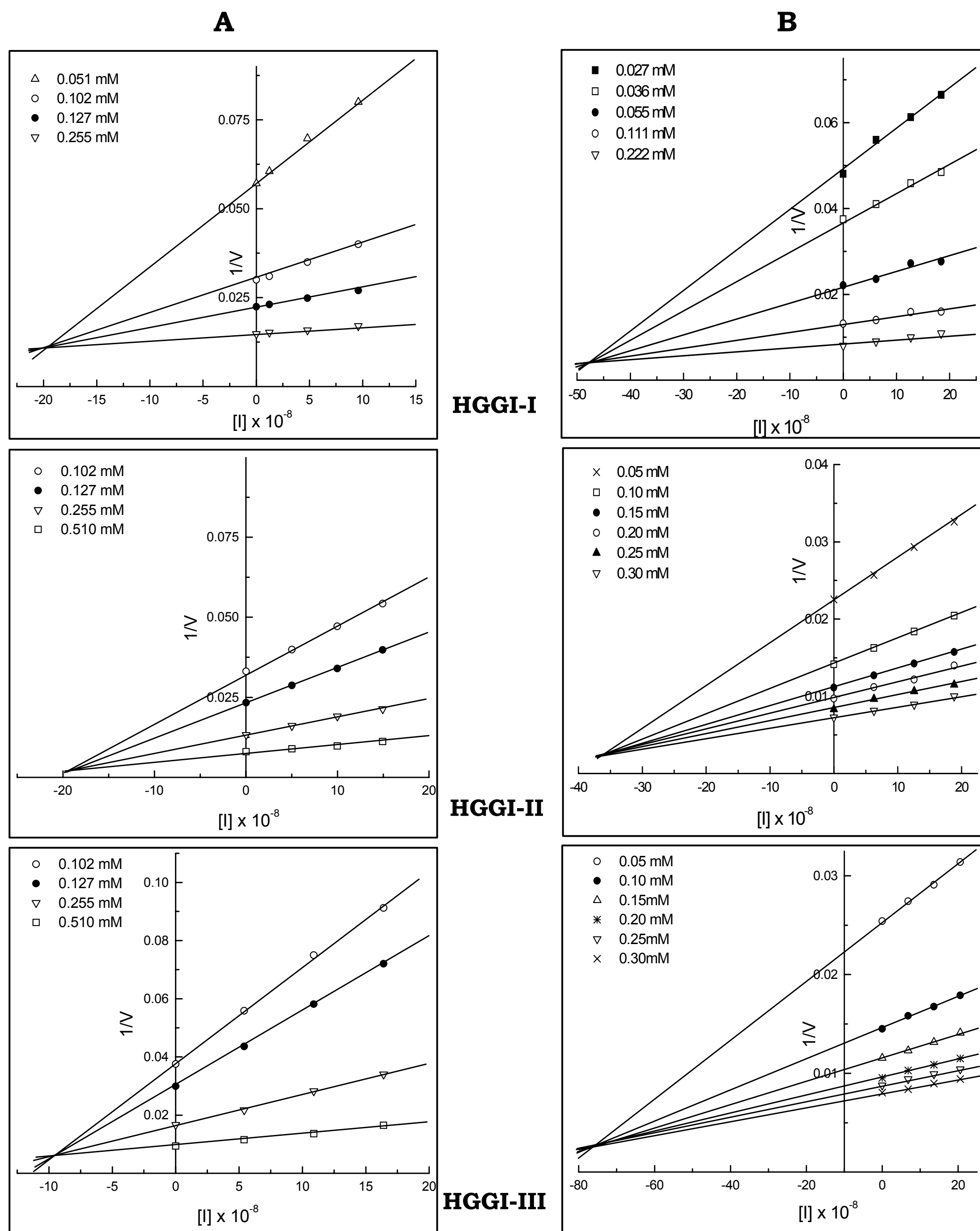


Figure 3.13 **Dixon - plots for determining the dissociation constants (K_is) of HGGIs with trypsin and chymotrypsin.** Panel A: Trypsin and Panel B: Chymotrypsin. Substrate concentrations are as indicated.

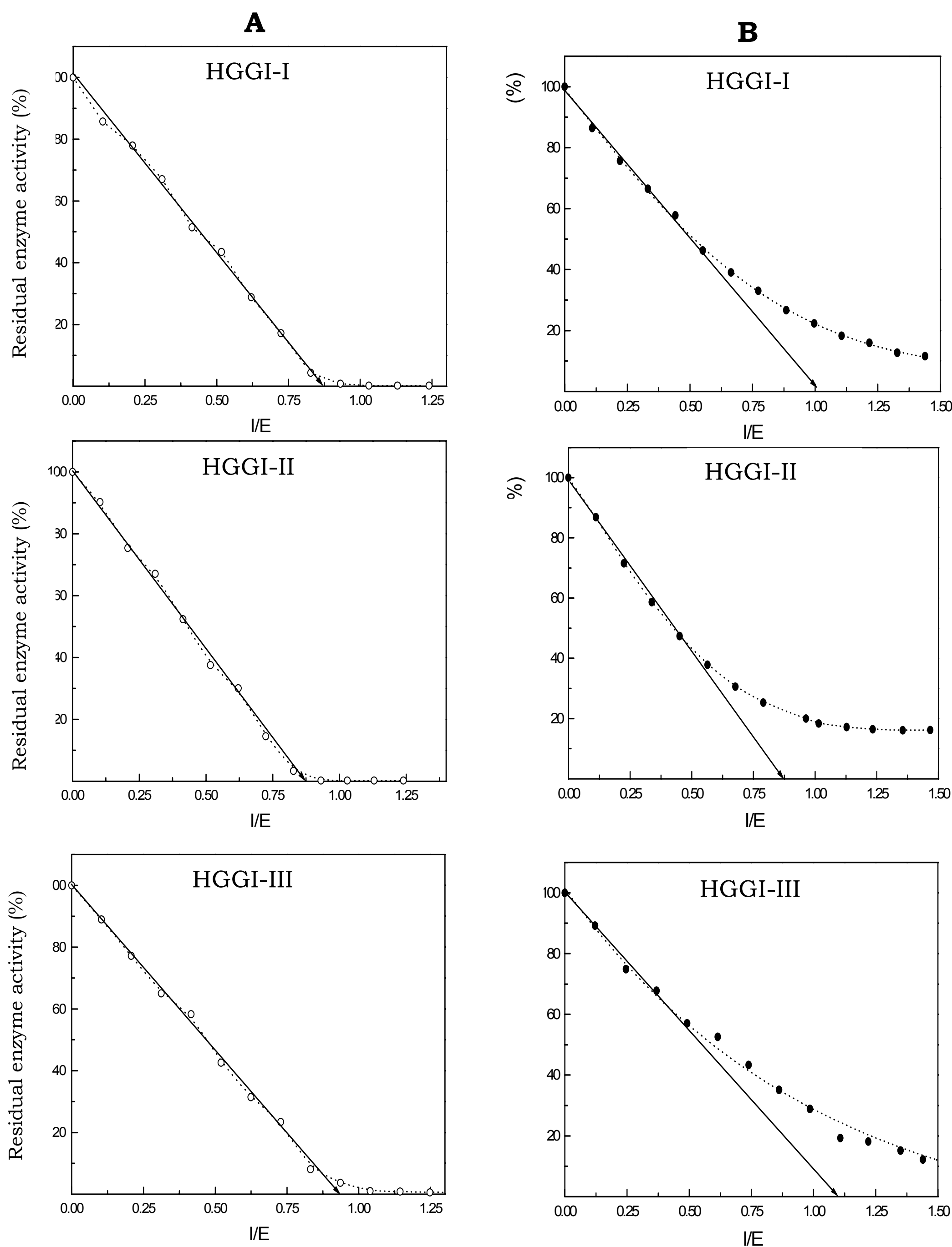


Figure 3.14 **Inhibition of trypsin and chymotrypsin by the HGGIs.** Panel A: Trypsin and Panel B: Chymotrypsin. The residual activity of trypsin/chymotrypsin at various inhibitor to enzyme ratios is shown. Trypsin and chymotrypsin concentrations were 4.830×10^{-7} M and 2.222×10^{-6} M respectively. The x-intercept of the straight line depicts the apparent binding stoichiometry.

The modification of the HGGIs with 1, 2-cyclohexanedione had no effect on the inhibition, indicating the absence of Arg at the reactive site (Ramasarma and Rao, 1991). The involvement of Lys residue in inhibition of trypsin was studied by modification of free amino groups using 2, 4, 6-trinitrobenzenesulfonic acid (picrylsulfonic acid). Figure 3.15 shows the effect of Lys modification on trypsin and chymotrypsin inhibitory activity. Complete loss of trypsin inhibitory activity was observed after 25-30 min of incubation with 2, 4, 6-trinitrobenzenesulfonic acid indicating the presence of Lys at the reactive site for trypsin inhibition.

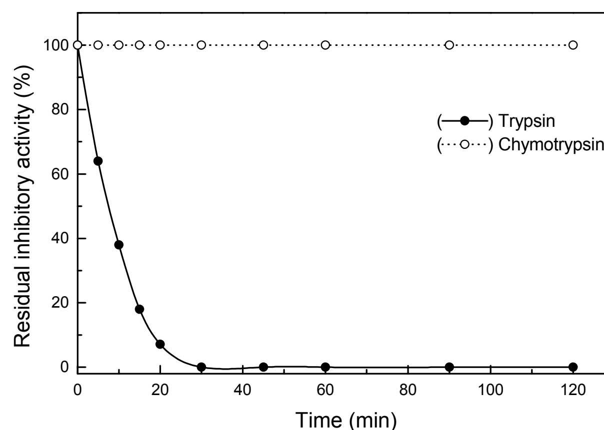


Figure 3.15 **Effect of Lys modification on trypsin inhibitory activity of HGGI-III.** Similar curves for the loss of trypsin inhibitory activity were obtained for HGGI-I and HGGI-II.

Dot-Blot analysis of HGGIs

Cross-reactivity of the three HGGIs was studied using antibodies raised against HGI-III, the major inhibitor present in the dormant horsegram seeds (Sreerama and Gowda 1997). The purified HGGIs were 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 all the three HGGIs (Figure 3.16). HGI-III served as

the positive control and BSA served the negative control, which showed no cross reactivity (Figure 3.16).

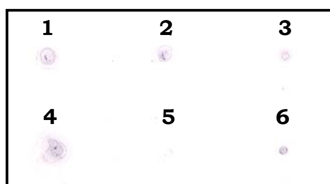


Figure 3.16 **Dot-Blot analysis of germinated horsegram inhibitors using antibodies raised against HGI-III.** (1) HGGI-I (2 μg); (2) HGGI-II (2 μg); (3) HGGI-III (2 μg); (4) Gel filtration fraction (2 μg); (5) BSA (2 μg, as negative control) and (6) HGI-III (2 μg as positive control).

Amino acid composition

Amino acid composition of the HGGIs was determined after pyridylethylation of the inhibitors. All the three HGGIs could be classified as Bowman-Birk type inhibitors, because of their low molecular weight and unusually high content of ½ cystine residues (Table 3.4). All the three isoinhibitors have similar amino acid composition with the exception of acidic amino acids Asp and Glu. The remarkable features of these proteins absence of Gly and low content of Met. Trp was not detected.

Amino-terminal sequence

The three-pyridylethylated HGGIs were subjected to amino-terminal sequence analysis on an automated gas phase sequenator PSQ-1. The amino-terminal sequence of the three germinated and dormant seed inhibitors of horsegram (Sreerama *et al.,* 1997) are summarized in Table 3.5. The amino-terminal sequence of the HGGIs shows a very high degree of homology to the four inhibitors of dormant seed and to other legume BBIs. The three isoinhibitors vary at their amino-terminal but appear to have identical core sequences (Table 3.5).

Table 3.4 **Comparison of amino acid composition of the HGGIs with HGI-III.**

Amino acid	Relative amino acid composition (mol%)*			
	HGGI-I	HGGI-II	HGGI-III	HGI-III
Asp[a]	6.4	4.2	4.8	11.4
Glu[b]	7.5	5.1	4.4	10.5
Ser	8.2	8.5	7.5	15.2
Gly	0	0	0	0
His	0.5	0.6	0.7	5.0
Arg	3.6	2.4	2.5	2.9
Thr	3.3	3.1	3.2	4.0
Ala	4.6	4.6	4.5	5.9
Pro	6.3	7.3	6.5	8.5
Tyr	1.4	0.7	0.6	1.6
Val	1.2	2.8	3.1	5.4
Met	0.2	0.3	0.2	0.7
½ Cys[c]	12.5	16.2	14.2	17.7
Ile	2.1	2.0	2.0	2.8
Leu	1.9	1.0	1.0	1.3
Phe	3.2	1.9	1.7	2.4
Lys	2.4	2.6	2.7	4.5

*Average of duplicates.
HGGI: Horsegram germinated inhibitor; HGI-III: Horsegram inhibitor (Sreerama *et al.,* 1997); [a]Determined as aspartate and aspargine, [b]glutamate and glutamine & [c]pyridylethylated cysteine.

Table 3.5 **Comparison of amino-terminal sequences of the isoinhibitors of horsegram seeds.**

INHIBITOR	SEQUENCE																			
[a]HGGI – I							D	E	P	S	E	S	S	K	P	C	C	D	Q	C
[a]HGGI – II								E	P	S	E	S	S	K	P	C	C	D	Q	C
[a]HGGI – III													S	K	P	C	C	D	Q	C
[b]HGI – I					S	T	D	E	P	S	E	S	S	K	P	C	C	D	Q	C
[b]HGI – II		H	H	E	S	T	D	E	P	S	E	S	S	K	P	C	C	D	Q	C
[b]HGI – III	D	H	H	Q	S	T	D	E	P	S	E	S	S	K	P	C	C	D	Q	C
[b]HGI – IV		H	H	E	S	T	D	E	P	S	E	S	S	K	P	C	C	D	Q	C

[a]Horsegram germinated inhibitors; [b]Horsegram inhibitors from the dormant seed (Sreerama *et al.,* 1997). The highlighted residues are conserved amino acid residues in BBIs of horsegram.

Discussion

Proteolysis of the Bowman-Birk inhibitors during germination and early seedling growth appear to be a general phenomenon in legumes (Madden *et al.,* 1985). The bulk of the decline in the inhibitory activity during germination is generally attributed to the proteolysis of the inhibitor by seed proteinases (Lorensen *et al.,* 1981). Several studies on appearance and disappearance of electrophoretically distinct proteinase inhibitors during germination have been reported (Pusztai, 1972; Freed and Ryan, 1978; Lorensen *et al.,* 1981; Wilson and Chen, 1983; Ambekar *et al.,* 1996; Wilson, 1988). The disappearance of dormant seed inhibitors and appearance of two new active inhibitor species in horsegram (*Dolichos biflorus*) after 120 h of germination was observed by Sreerama and Gowda (1998). The two inhibitors that appear after 120 h of germination are less charged than the inhibitors of the dormant seed and appear to be smaller in size. Yet the inhibitors retain the double-headed characteristics. It is not clear whether these new forms and those that occur in the dormant seed are products of the same gene or the products of different genes that undergo post-translational modifications or are products of stored mRNA. In order to solve this paradox, purification and characterization of the new isoforms that appear during germination was carried out.

The effect of day light, number of rinses and days of germination on trypsin inhibitory activity (TIA) was studied by Frias *et al.,* (1995). Number of rinses had no significant effect in the final TIA, but the activity of protease inhibitors was significantly higher in seeds illuminated for 6 h per day. Maximal TIA decrease was found in seeds germinated in darkness with daily rinses. These optimal conditions were found to be a more effective approach to reduce the concentration of trypsin inhibitors in lentil seeds. Similar conditions, germination

under dark for 120 h with daily rinse were used by Sreerama and Gowda (1998) for germination of horsegram seeds, in which 50 % decrease in TIA and CIA (chymotrypsin inhibitory activity) was observed by two days of germination. The decrease in the most of TIA was due to the disappearance of the four dormant seed inhibitors.

Purification of the three Bowman-Birk isoforms that appear after 120 h of germination of horsegram seeds involved the preparation of an acidic extract of the cotyledons, $(NH_4)_2SO_4$ fractionation, size-exclusion chromatography and a combination of anion- and cation-exchange chromatography on CM-Sephadex C-25 and DEAE-Sephadex A-25 chromatography. The extraction of inhibitors at acidic pH not only provided a higher specific activity in the crude extract but also prevented enzymatic browning (Wu and Whitaker, 1990). The prevention of browning could be due to the inactivation of the polyphenol oxidase in the seeds. The size-exclusion chromatography of the crude inhibitor preparation on Sephadex G-50 at pH 7.5 resulted in a single active trypsin/chymotrypsin inhibitory active fraction (Figure 3.1). This inhibitory peak was well separated from the bulk of other high and low molecular weight proteins (Figure 3.1).

The inhibitors failed to bind to DEAE-Sephadex A-25 at pH 8.2, indicating a decrease in the net negative charge. The cation-exchange chromatography on CM-Sephadex proved to be a successful step in the separation of the three Bowman-Birk isoforms present in size-exclusion chromatography fraction. Among the three active inhibitor species, two eluted as unbound protein fractions and the third inhibitor (HGGI-III) was recovered during the regeneration of column with NaCl (Figure 3.2). The electrophoretic mobility on native PAGE at pH 8.8 (Figure 3.5) of the two unbound inhibitors (HGGI-I and HGGI-II) corresponded to

the two inhibitors detected previously by Sreerama and Gowda (1998). Native PAGE of the third fraction (HGGI-III) at pH 8.8 showed no visible band by either protein or activity staining and could be a reason for its not being detected by Sreerama and Gowda (1998). However PAGE and gelatin-embedded PAGE at pH 4.5 indicated that the protein was a BBI (Figure 3.4, lane 1). Re-examination of gelatin-PAGE gels (pH 8.8) (Figure 3.5) indicated a visible activity band at the origin, previously considered as aggregated protein unable to enter the gel (results not shown).

Traces of dormant seed inhibitor (HGIs) still present in the fractions obtained from CM-Sephadex chromatography C-25 were separated from the HGGIs by anion-exchange chromatography on DEAE-Sephadex A-25. This step had been successfully used for the resolution of the four dormant seed inhibitors of horsegram seeds (Sreerama *et al.,* 1997). All the four inhibitors (HGIs) bound strongly to DEAE-Sephadex and could be resolved using a ammonium bicarbonate gradient. In contrast the inhibitors of the germinated seed (HGGI-I and HGGI-II) did not bind to DEAE-Sephadex at pH 8.2, indicating a decrease in the net negative charge (Figure 3.3 and 3.5) when compared to HGIs, which bound to DEAE-Sephacel (Sreerama *et al.,* 1997).

Among the three isoinhibitors, HGGI-III is the major form (Table 3.1). The specific activity of these new inhibitor species towards the trypsin inhibition is five fold higher (Table 3.1) than that earlier reported for the dormant seed inhibitors (Sreerama *et al.,* 1997).

Reports of self-association of BBIs resulting in a large overestimation of molecular mass by size-exclusion chromatography

and SDS-PAGE are frequent in the literature (Haynes and Feeney, 1967; Millar *et al.,* 1969; Whitley and Bowman, 1975; Gennis and Cantor, 1976b; Bergeron and Neilsen, 1993). The inhibitor of dormant seed exhibited molecular masses of ≈16.0 kDa by SDS-PAGE and analytical gel filtration, although ESMS analysis and amino acid analysis showed them to have a mass of ~ 8.0 kDa (Sreerama *et al.,* 1997). This large overestimation of molecular weights has been attributed to the legume BBIs existing in a state of equilibrium between monomer-dimer-trimer forms (Wu and Whitaker, 1990; Terada *et al.,* 1994b; Godbole *et al.,* 1994a). However such an overestimation of the molecular mass of HGGI-I, -II and -III was not observed by size-exclusion chromatography (Figure 3.8 and 3.9) or SDS-PAGE (Figure 3.10) suggesting that they exist only as monomers. The structural features responsible for the anomalous behavior of the dry seeds and other Bowman-Birk inhibitors has been evaluated and presented in Chapter 5.

The three HGGIs have distinct isoelectric points (Figure 3.11). The pIs of the inhibitors were measured by comparing isoelectric position of each inhibitor with the pI markers. The pIs of HGGIs increase in the order of HGGI-III > HGGI-II > HGGI-I. HGGI-I and II do not bind to the cation-exchanger CM-Sephadex C-25 at pH 5.0 (Figure 3.2) as at this pH their charge is near neutral. This increasing order of pI is also reflected in the electrophoretic mobility of the isoinhibitors at pH 4.5, where in HGGI-III with the highest pI also has the highest R_F (Figure 3.4). These pIs are in close agreement with the reported pIs of *P. vulgaris* trypsin inhibitor 5.0 (Pusztai, 1968), 4.3, 4.5, 4.7 and 4.9 (Mosolov *et al.,* 1976) and 4.46, 4.82, 4.84 and 5.09 (Wu and Whitaker, 1990) for kidney beans, 4.4 and 4.45 for navy beans (Gomes *et al.,*

1979), 4.7 for Kintoki beans (Miyoshi *et al.,* 1978) and 4.6, 4.8, 5.1 and 5.6 for horsegram (Sreerama *et al.,* 1997).

The amino-terminal sequence analysis (Table 3.5) and amino acid analysis (Table 3.4) reveal that the three isoinhibitors vary at the amino-termini but have identical core sequences. The BBIs of horsegram seeds also show a high degree of homology to the other legume BBIs (Prakash *et al.,* 1996). Comparison of the determined amino-terminal sequences of the isoinhibitors of the germinated seed with those of the dormant seed show that, the HGGIs are derived from the dormant seed inhibitors. The amino-terminal sequence analysis of the HGGIs, which appear upon germination, indicated that they are *in situ* products of limited amino-terminal proteolysis of the isoinhibitors present in the dormant seed. Wilson and Chen (1983) demonstrated that two electrophoretically distinct inhibitors (MBI-E and MBI-C) present in the germinating mung bean (*Vigna radiata*) are the products of a carboxy-terminal proteolysis of the major inhibitor MBI-F. The initial event in this degradation of the mung bean trypsin inhibitor during germination was the removal of the carboxy-terminal tetrapeptide Lys^{77}-Asp-Asp-Asp^{80}. Degradation then proceeded through a number of specific cleavages including an internal cleavage and the removal of eight residues of the amino-terminus (Wilson and Chen, 1983). A similar modification by limited proteolysis has been observed during the germination of adzuki bean (*Vigna angularis*) (Yoshikawa *et al.,* 1979). The reduced electrophoretic mobility of the HGGIs at pH 8.8 observed earlier (Sreerama and Gowda, 1998) can now be attributed to the loss of the Asp and Glu residues from the amino-terminus and or at the carboxy-terminus of the dormant seed inhibitors, HGI-I, II, III and IV (Table 3.5). The initial proteolytic event that occurs during germination to generate HGGI-I and II appears to be catalyzed by the

action of an endoproteinase that is specific for the amino-terminal side of an acidic residue. In both the BBIs and the Kunitz type inhibitors of soybean, the initial proteolysis occurs at the amino-terminal side of an acidic residue i.e. between Lys-Glu in BBSTI-E and Leu-Asp of Kunitz soybean trypsin inhibitor (Orf and Hymowitz, 1979). The amino acid composition of the isoinhibitors HGGI-I, II and III are shown in the Table 3.4. The substantially high Cys content of all the three isoinhibitors is characteristic of all BBIs (Liener and Kakade, 1980). A notable feature is the drastic reduction of the Asp, Ser and His content of HGGIs when compared to HGI-III (Sreerama *et al.,* 1997). A part of this loss can be accounted for by the observed amino-terminal proteolysis that occurs during germination (Table 3.5). In addition, proteolysis of the carboxy-terminal tail of isoinhibitors that are also rich in Ser, His and Asp (Prakash *et al.,* 1996; Sreerama and Gowda, 1997) could add to the drastic loss observed. The results of complete primary structure determination of the HGGIs (Chapter 4) confirm the carboxy-terminal proteolysis also occurs upon germination.

The K_is of the inhibitor complexes with trypsin at pH 8.2 and chymotrypsin at pH 7.8 were determined (Table 3.3). The binding affinities for trypsin are of the order HGGI-III > HGGI-II > HGGI-I. All the three inhibitors show higher binding affinity towards trypsin when compared to chymotrypsin. The inhibition constants indicated that binding of the inhibitors to trypsin and chymotrypsin was very tight. The K_i values obtained for HGGIs are comparable to the values of the HGIs (Sreerama *et al.,* 1997) and other BBIs (Terada *et al.,* 1994a). HGGI-III displays a higher binding affinity to trypsin compared to HGGI-I and II despite its missing amino-terminal tail (Table 3.5). This implicates the contribution of the amino-terminal of the inhibitor in trypsin binding possibly via electrostatic interaction. As the negative

charge of the amino-terminal from HGGI-I to III decreases (Figure 3.5), the binding affinity of the inhibitor to trypsin increases (Table 3.3). In the dimeric crystal structure of BBI inhibitor from pea seeds, the carboxy-terminus tail of the inhibitor is shown to make contact with the trypsin molecule (Sierra *et al.,* 1999). However in the X-ray structure of the adzuki bean inhibitor-trypsin complex, no interaction of the carboxy-terminal tail with trypsin was detected (Tsunogae *et al.,* 1986). The involvement of amino-terminus in trypsin needs to be investigated more deeply.

Inhibition of trypsin and chymotrypsin at fixed concentrations were studied by varying the inhibitor concentrations to deduce the stoichiometric relation. The comparison of titration curves (Figure 3.14) reveals that the three HGGIs have a strong affinity to both trypsin and chymotrypsin. The most noteworthy difference is the inhibition pattern of trypsin and chymotrypsin with HGGIs. Inhibition with chymotrypsin for all the three was linear up to 60 % and inhibition was incomplete with excess of inhibitor. In contrast, trypsin inhibition was linear and complete. It was found that the three inhibitors (HGGIs) bind to trypsin and chymotrypsin at 1:1 molar ratio (Figure 3.14). Similar results were obtained with trypsin inhibition by the dormant seed inhibitors HGIs (Sreerama *et al.,* 1997), but such a stoichiometry could not be established with chymotrypsin. Inability to obtain a stoichiometry of 1:1 for chymotrypsin binding has also been observed for the BBIs isolated form the other legumes (Terada *et al.,* 1994a; Norioka *et al.,* 1982, Kimura *et al.,* 1994 and Godbole *et al.,* 1994a).

The BBIs of horsegram have two separate reactive sites for inhibition of trypsin and chymotrypsin and are independent of each

other. However five BBIs of 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. Adzuki bean inhibitor II inhibits trypsin and chymotrypsin at different sites, though its complex with chymotrypsin has no trypsin inhibitory activity (Yoshikawa *et al.,* 1979a). Tur-Sinai *et al.,* (1972) reported that the complex of their peanut inhibitor and trypsin lacked chymotrypsin-inhibiting activity and complex with chymotrypsin no longer had trypsin inhibiting activity.

Modification of Arg residues using 1, 2-cyclohexanedione indicated the amino acid responsible for trypsin inhibition in dry seed BBIs was not Arg (Ramasarma and Rao, 1991). Later Sreerama *et al.,* (1997) studied the effect of Lys modification on the trypsin inhibitory activity in the four dry seed inhibitors of horsegram. The involvement of Lys residue in inhibition of bovine trypsin and chymotrypsin was studied by modification of free amino groups in HGGIs using 2, 4, 6-trinitrobenzene sulfonic acid. A rapid loss (25-30 min) of trypsin inhibitory activity was observed for all the three inhibitors (Figure 3.15). It is evident from the results that there exists a direct relation between the loss of trypsin inhibition and Lys modification. Further it appears that one or more fast reacting amino groups are essential for the inhibitory activity of these inhibitors. Lys modification had no influence on the chymotrypsin inhibitory activity, indicating that Lys had no role to play in the inhibition of chymotrypsin.

Studies with the antibodies raised against, HGI-III revealed the presence of three sequential epitopes Asp^{1}-Lys^{14}, Leu^{37}-Lys^{63} and Asp^{64}-Lys^{71} (Sreerama and Gowda, 1997). Although, HGGIs have

truncated amino-terminal tails, they still retain their cross-reactivity with the antibodies raised against HGI-III, suggesting that the presence of similar antigenic sites and core sequence, still exist in the germinated inhibitors.

The shared similarity in the core sequence of HGGIs with HGI-III and the anomalous behavior of dicot BBIs with respect to their molecular weight is discussed by determining the primary structure of the HGGIs and comparing them with HGI-III and other dicot BBIs inhibitor of legumes in Chapter 4 and 5.

The amino-terminal sequence, M_r and amino acid composition are consistent with the HGGIs being derived from HGIs of the dormant seed by proteolysis during germination and not by *de novo* synthesis, as distinct gene products. The relatively high M_r of BBIs (8-10 kDa) is a limitation for use as cancer chemopreventive agents. A smaller version of soybean BBI obtained by chemical and enzymatic cleavage (Birk, 1976; Odani and Ikenaka, 1978b) inhibited radiation transformation as effectively as whole BBI (Kennedy, 1993). Therefore HGGI-III smaller in size; a potent inhibitor of chymotrypsin and devoid of the highly immunogenic amino-terminus of HGI-III (Sreerama and Gowda, 1997) may serve as an effective cancer chemopreventive agent.

Table 3.1 Purification of germinated horsegram inhibitors*

Step	Total Protein (mg)		Total inhibitory units (IU) × 10^5	Specific activity (Units/mg) × 10^3	Fold purification	Yield (%)	CIU/ TIU
Crude extract	39.21	T	6.64	16.94	----	100	2.3
		C	15.33	39.10	----	100	
$(NH_4)_2SO_4$ precipitation	8.55	T	4.02	47.02	2.8	60.5	2.2
		C	9.05	105.85	2.7	59.0	
Sephadex G–50 chromatography	3.20	T	3.72	116.25	6.9	56.1	2.3
		C	8.74	273.13	7.0	57.0	
CM-Sephadex C-25 ion–exchange chromatography							
HGGI–I	0.35	T	0.38	108.57	6.4	5.7	2.3
		C	0.86	245.70	6.3	5.6	
HGGI–II	0.66	T	0.70	106.06	6.2	10.5	2.3
		C	1.59	240.90	6.2	10.4	
HGGI–III	1.87	T	1.66	88.77	5.2	25.0	2.1
		C	3.48	186.10	4.8	22.7	
DEAE-Sephadex A- 25 ion-exchange							
chromatography	0.21	T	0.28	133.33	7.9	4.2	2.3
HGGI–I		C	0.64	304.76	7.8	4.2	
	0.41	T	0.55	134.15	7.9	8.3	2.4
HGGI – II		C	1.32	321.95	8.2	8.6	

*These are the results of a typical purification starting from 200 g (85 g cotyledons) of horsegram seeds. These values were reproduced in two separate purifications. IU = Inhibitory units (T: trypsin; C: chymotrypsin) are expressed as 1 unit = 0.01 decrease in absorbance at 410 nm under assay conditions. TIU: trypsin inhibitory unit; CIU: chymotrypsin inhibitory unit.

The Bowman-Birk inhibitors of germinated horsegram seeds: Determination of primary structure

The three isoinhibitors purified from germinated horsegram seeds differ from inhibitors of the dry seed with respect to electrophoretic mobility and molecular size. The amino-terminal sequence analysis (Chapter 3) indicates that these inhibitors could be *in situ* products of proteolysis. The complete amino acid sequence of HGGI-I, -II and –III was determined. This chapter describes, the complete amino acid sequence, obtained by enzymatic and chemical cleavage of the inhibitors, isolation and sequencing of the purified peptides followed by overlapping the peptide sequences to obtain the complete primary structure.

RESULTS

Comparison of amino acid composition, amino-terminal sequence and molecular weight of the three germinated horsegram inhibitors (HGGIs) (Chapter 3) suggested that, the three inhibitors were similar, except truncation at the amino-terminus. Therefore the methodology adopted to determine the primary structure of each of these inhibitors was similar.

Identification of amino-terminus

The three HGGIs were subjected to reduction and alkylation in presence of 4-VP as described earlier (Section 2.2.12). The purified PE-HGGIs were subjected to Edman analysis on an automated PSQ-1 sequenator. This resulted in the unambiguous identification of 30, 20 and 19 amino acid residues of HGGI-I, -II and –III from the amino-terminus respectively (Table 4.1). The sequences obtained accounted for 30 % of the amino acid sequence (Figure 4.7, 4.8 and 4.9). The obtained amino-terminal sequences indicate that the HGGI-II and HGGI-III differ from HGGI-I by lack of a single Asp and the hexapeptide –DEPSES respectively.

Table 4.1. **Amino-terminal sequence of HGGIs**

Inhibitor	Sequence
HGGI-I	DEPSESSKPCCDQCACTKSIPPQCRCTDVR.....
HGGI-II	EPSESSKPCCDQCACTKSIP.......
HGGI-III	SKPCCDQCACTKSIPPQCR.....

Enzymatic cleavage of HGGIs

The amino acid composition, similarity at the amino-terminal sequence to HGI-III revealed that TPCK-trypsin and endoproteinase Asp-N could be used to enzymatically cleave the HGGIs.

Cleavage with TPCK-trypsin, separation of peptides and sequence determination.

The cleavage of the PE-HGGIs was carried out using catalytic amounts of TPCK-trypsin as described (Section 2.2.24.1). The enzymatic digest was then loaded on a Phenomenex ODS RP-HPLC column (250 × 4.6 mm, 5 μ) and peptides fractionated using a linear gradient of 0.1 % TFA in water and 70 % acetonitrile containing 0.05 % of TFA. Table 4.2 indicates the linear gradient programme employed. The elution profiles of the tryptic peptides are shown in Figure 4.1. The peptide fractions were designated as T-I - T-V in order of their elution. These peptide fractions were collected over several runs and re-chromatographed on the same column using the same solvent system. The peptide fraction T-II, on re-purification yielded two peptides designated T-2 and T-3. The peptide fractions T-I, -III, -IV and -V on repurification indicated they were homogenous. These fractions were labeled as T-1, T-4, T-5 and T-6 respectively (Figure 4.2).

Table 4.2 **Linear gradient programme for separation of peptides on RP-HPLC column**

Time (min)	(%) A	(%) B
0	100	0
5	100	0
65	0	100
70	0	100
75	100	0
85	100	0
85.01	STOP	

A: 0.1 % TFA in water and B: 70 % CH_3CN containing 0.05 % TFA.

Sequencing of tryptic peptides

The number of peptides expected upon tryptic digestion based on the estimated Lys and Arg residues were seven. However, only six peptides were obtained. The peptide fractions were found to be homogenous by Edman analyses on automated PSQ-1 sequenator. The obtained amino acid sequences of these peptides are listed in Table 4.3.

The peptide sequences of T-2 for all the three HGGIs could be mapped to their respective amino-terminal sequences. (Figure 4.7, 4.8 and 4.9). Peptide T-6 was the largest peptide with 27 amino acid residues and T-1 was the smallest with five residues. The sequences of peptides T-1, T-3, T-4, T-5 and T-6 of HGGI-I, -II and –III were identical. The peptides T-1 and T-3 accounted for the peptides obtained by cleavage at Arg where as T-2, T-4, T-5 and T-6 at Lys. The sum of the amino acid residues of the six tryptic peptide fractions account for over 95 % of the sequences of HGGI-I, HGGI-II and HGGI-III.

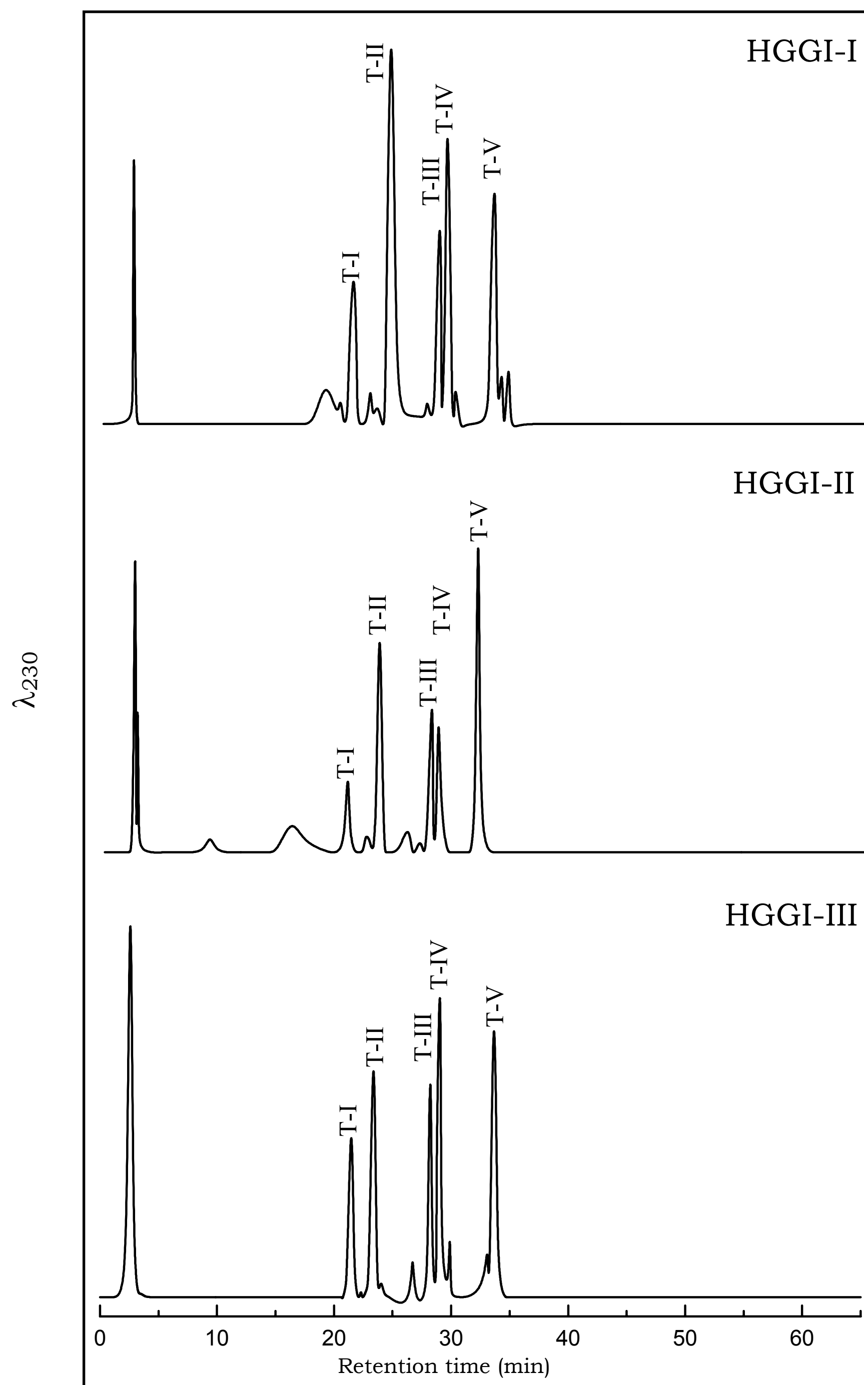


Figure 4.1 **RP-HPLC profile of the tryptic digests of PE-HGGIs.** The digest was chromatographed on a Phenomenex ODS column (250 × 4.6 mm, 5 μ) and peptides were fractionated using a gradient of 0.1 % TFA in water and 70 % acetonitrile in 0.05 % TFA at a flow rate of 0.7 mL/min.

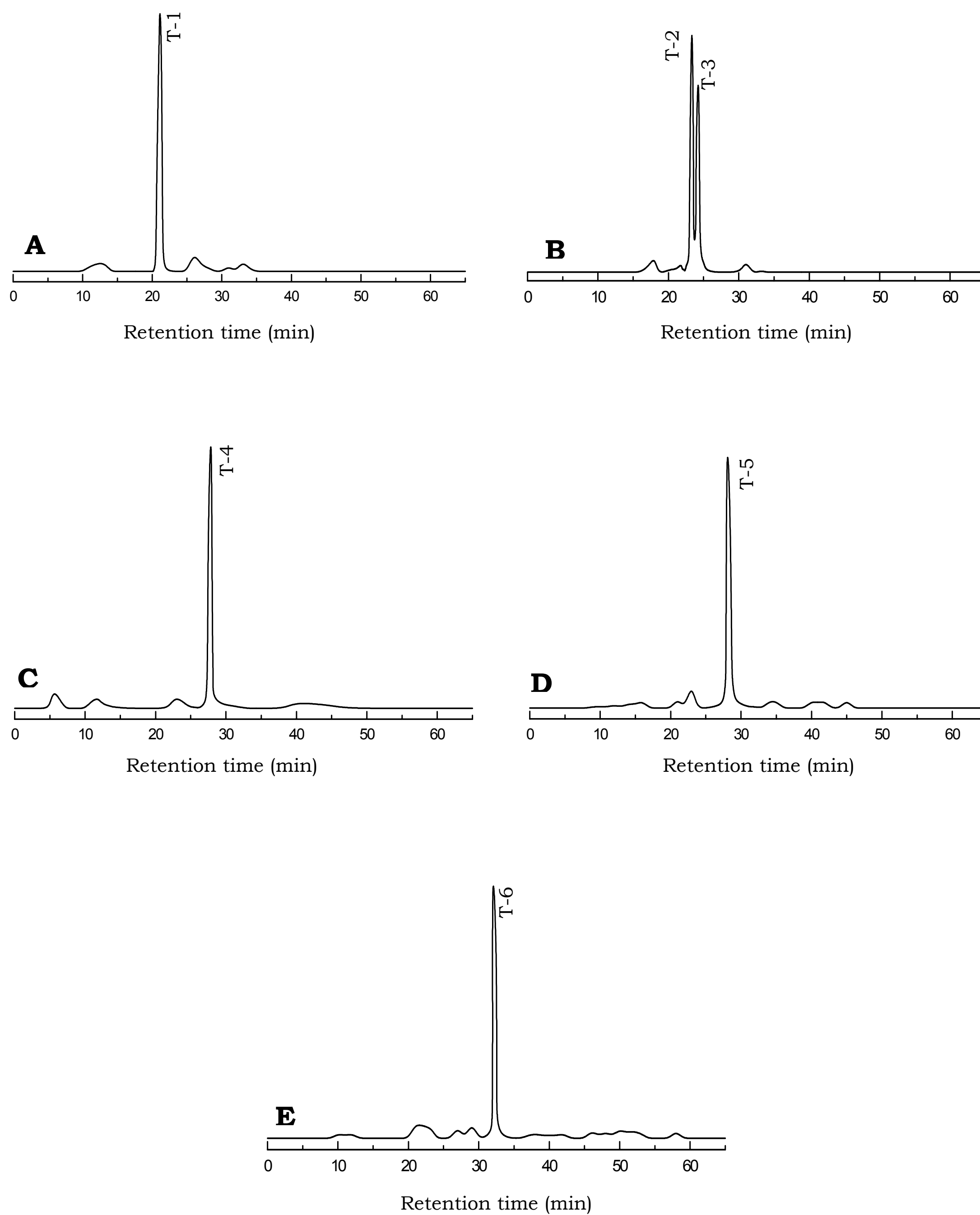


Figure 4.2 **Rechromatography of the tryptic fragments of HGGI-I by RP-HPLC.** Elution of the peptides was monitored at 230 nm. Similar results were obtained for the tryptic peptides of HGGI-II and HGGI-III. (A) T-I; (B) T-II; (C) T-III; (D) T-IV and (E) T-V.

Table 4.3 **Amino-acid sequences of tryptic peptides.**

Inhibitor	Peptides		Sequence	Amino acids
HGGI-I	T-I	T-1	CTDVR	5
	T-II	T-2	DEPSESSKPCCDQCACTK	18
		T-3	SIPPQCR	7
	T-III	T-4	DFCYAPCK	8
	T-IV	T-5	DFCYAPCK	8
	T-V	T-6	LNSCHSACSSCVCTFSIPAQCVCVDMK	27
HGGI-II	T-I	T-1	CTDVR	5
	T-II	T-2	EPSESSKPCCDQCACTK	17
		T-3	SIPPQCR	7
	T-III	T-4	DFCYAPCK	8
	T-IV	T-5	DFCYAPCK	8
	T-V	T-6	LNSCHSACSSCVCTFSIPAQCVCVDMK	27
HGGI-III	T-I	T-1	CTDVR	5
	T-II	T-2	SKPCCDQCACTK	12
		T-3	SIPPQCR	7
	T-III	T-4	DFCYAPCK	8
	T-IV	T-5	DFCYAPCK	8
	T-V	T-6	LNSCHSACSSCVCTFSIPAQCVCVDMK	27

Cleavage with endoproteinase Asp-N, separation of peptides and sequence determination

A highly specific endoproteinase Asp-N, which hydrolyses peptide bonds present at the amino-terminal side of an Asp residue (Section 2.2.24.2) was used to obtain peptides to complete the sequence. The PE-HGGIs were digested individually with endoproteinase Asp-N as described earlier (Section 2.2.24.2). The digests were fractionated and

rechromatographed by RP-HPLC. The HPLC profiles of the digests are shown in Figure 4.3. Each of the peptide fraction was collected manually over several runs and rechromatographed on the same column. The four-peptide fractions obtained for each of the inhibitors appeared to be homogenous (Figure 4.4).

Sequencing of endoproteinase Asp-N peptides of HGGIs

The homogenous endoproteinase Asp-N peptides (A-1 - A-4) were analyzed on an automated PSQ-1 sequenator and sequences obtained are listed in Table 4.4. The sequence of A-1 indicates it was the amino-terminal peptide and a formed a part of T-2 obtained from trypsin cleavage. The sequence of peptide A-2 was similar to that of peptides, T-4 and T-5, which correspond to the carboxy-terminus of HGGIs. With the exception of A-1, the sequences of the other peptides of HGGI-I, -II and –III were identical. The peptide, A-3 with 16 amino acid residues contained the sequence of peptide T-2 and T-3. The 28-residue peptide A-4 contained the peptide sequence of the peptide fragment T-6 (Table 4.4).

Chemical cleavage with cyanogen bromide, separation of peptides and sequence determination

The amino acid composition of HGGIs revealed the presence of single Met residue. The sequences of T-6 indicated the presence of a Met residue at a position similar to that of HGI-III (Prakash *et al.*, 1996). Therefore the PE-HGGIs were cleaved with CNBr in 70 % formic acid (Section 2.2.25). The CNBr digest was lyophilized and peptides purified by RP-HPLC (Figure 4.5 and 4.6). Two peptide fragments were anticipated by cleavage at the Met residue. The two peptides CNBr-1 and CNBr-2 of each of the HGGIs were sequenced. The complete sequence of peptide CNBr-1 (9 amino acids) corresponded to the

carboxy-terminal and was identical to A-2 of the endoproteinase Asp-N digest and also contained the sequence of T-4 and T-5 obtained from trypsin digestion. The sequences of CNBr-1 further evidenced that the HGGIs vary at the amino-terminus. The amino acid sequence further revealed that in the parent molecules CNBr-1 follows CNBr-2.

The complete amino acid sequence of HGGIs

The complete amino acid sequence of the three inhibitors, HGGI-I, -II and –III having 66, 65 and 60 amino acid residues respectively were deduced by overlapping sequences of peptide fragments generated by enzymatic and chemical cleavage. The framework of the peptides of HGGI-I, -II and –III are shown in Figures 4.7, 4.8 and 4.9.

The enzymatic and chemical cleavage pattern for all the three inhibitors were similar. Subtle differences were found in the amino acid sequences of the peptides T-2, A-1 and CNBr-2, which correspond to amino-terminal residues of the inhibitors. The peptide sequences of T-1 to T-6 provided the framework for the complete primary structure. The amino-terminal sequence analyses of HGGI-I, -II and –III led to the identification of residues 1-28, 1-19 and 1-18 respectively. The amino-terminal sequences were further confirmed by the complete sequences of tryptic peptides T-2, Asp-N peptides A-1 and the partial sequence of CNBr-2. Asp-N peptide, A-3 sequences of the three HGGIs overlapped into this amino-terminal sequence. This completed the stretch 1-27, 1-26 and 1-21 of HGGI-I, -II and –III respectively. This stretch was also reconfirmed by the alignment of the tryptic sequences in the order T-2 and T-3. The sequence of the peptide A-3 obtained from Asp-N digestion provides the overlap for peptides T-3 and T-1.

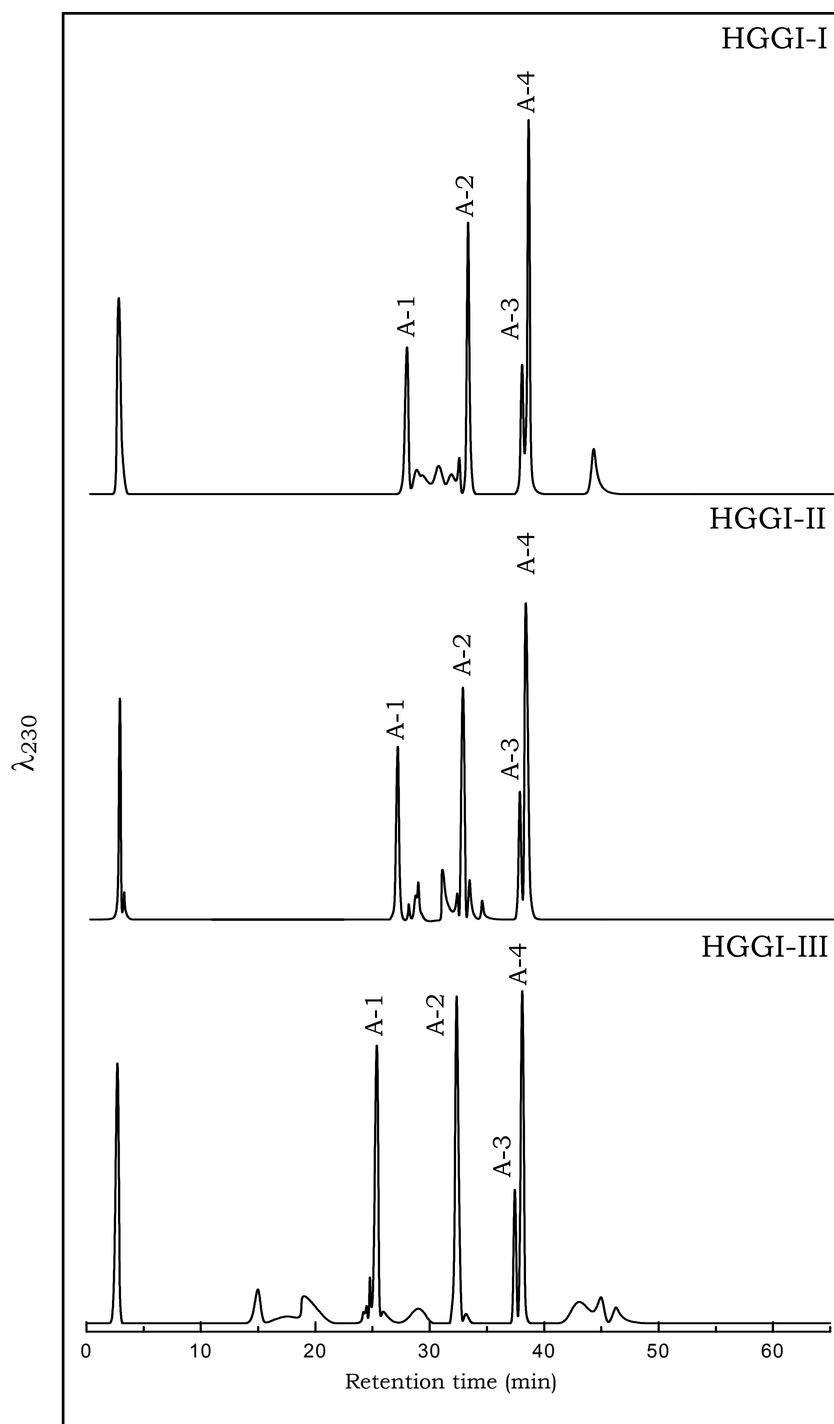


Figure 4.3 **Fractionation of endoproteinase Asp-N digests of PE-HGGIs.** The digest was loaded on a Phenomenex ODS column (250 × 4.6 mm, 5μ) and peptides resolved using a gradient of 0.1 % TFA in water and 70 % acetonitrile in 0.05 % TFA at a flow rate of 0.7 mL/min. The peptides were detected at 230 nm.

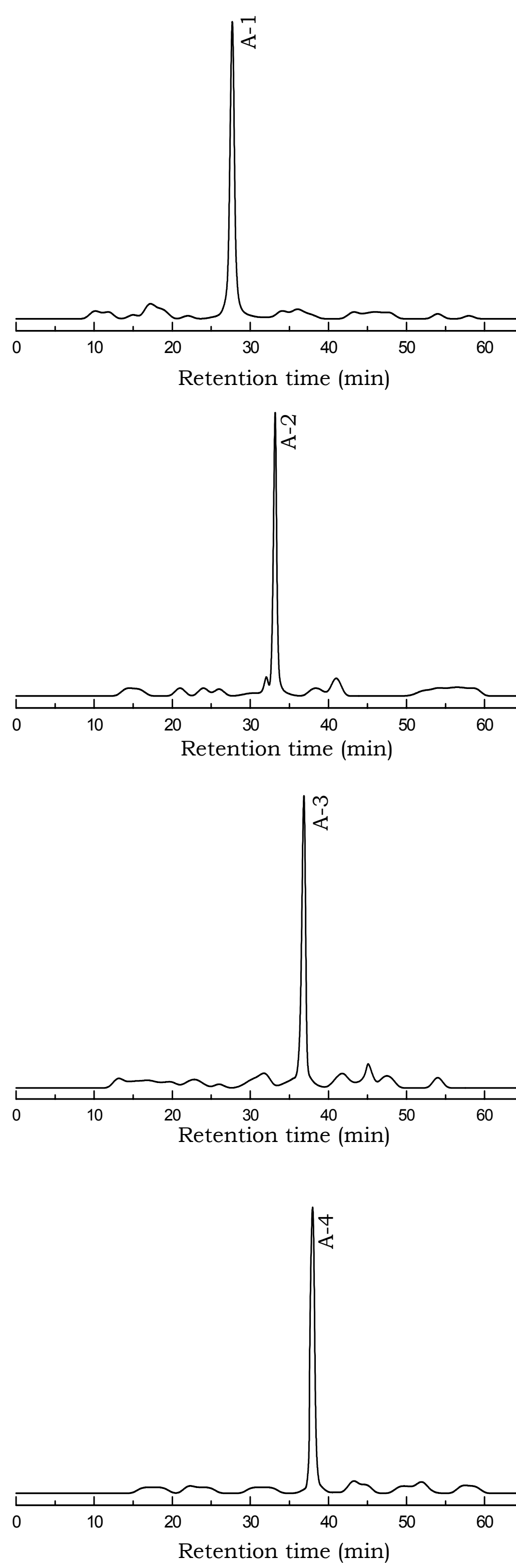


Figure 4.4 **Rechromatography of the endoproteinase Asp-N peptide of HGGI-I by RP-HPLC.** Elution of the peptides was monitored at 230 nm. Similar results were obtained for Asp-N peptides of HGGI-II and HGGI-III**.**

Table 4.4 **Amino-acid sequences of endoproteinase Asp-N peptides.**

Inhibitor	Peptides	Sequence	Amino acids
HGGI-I	A-1	DEPSESSKPCC	11
	A-2	DFCYAPCKS	9
	A-3	DQCACTKSIPPQCRCT	16
	A-4	DVRLNSCHSACSSCVCTFSIPAQCVCV	27
HGGI-II	A-1	EPSESSKPCC	10
	A-2	DFCYAPCKS	9
	A-3	DQCACTKSIPPQCRCT	16
	A-4	DVRLNSCHSACSSCVCTFSIPAQCVCV	27
HGGI-III	A-1	SKPCC	5
	A-2	DFCYAPCKS	9
	A-3	DQCACTKSIPPQCRCT	16
	A-4	DVRLNSCHSACSSCVCTFSIPAQCVCV	27

Chemical cleavage with CNBr has resulted in two peptides from cleavage at a Met residue. The sequence of CNBr-1 peptide has provided the overlap between T-6, T-4 and T-5, which were identical in the three HGGIs. Peptide A-2 and CNBr-1 of all the three inhibitors are found to terminate with a Ser residue. The reported sequence of the carboxy-terminal CNBr-1 peptide of HGI-III (Prakash *et al.*, 1996) indicates that the carboxy-terminus of HGGIs is Ser.

The molecular mass calculated on the basis of the sequence of HGGI-I, -II and –III are 7109, 6993 and 6464 Da respectively. These results are in close agreement to the molecular mass 7216.7, 7074.6 and 6493.5 respectively (Chapter 3, Table 3.2).

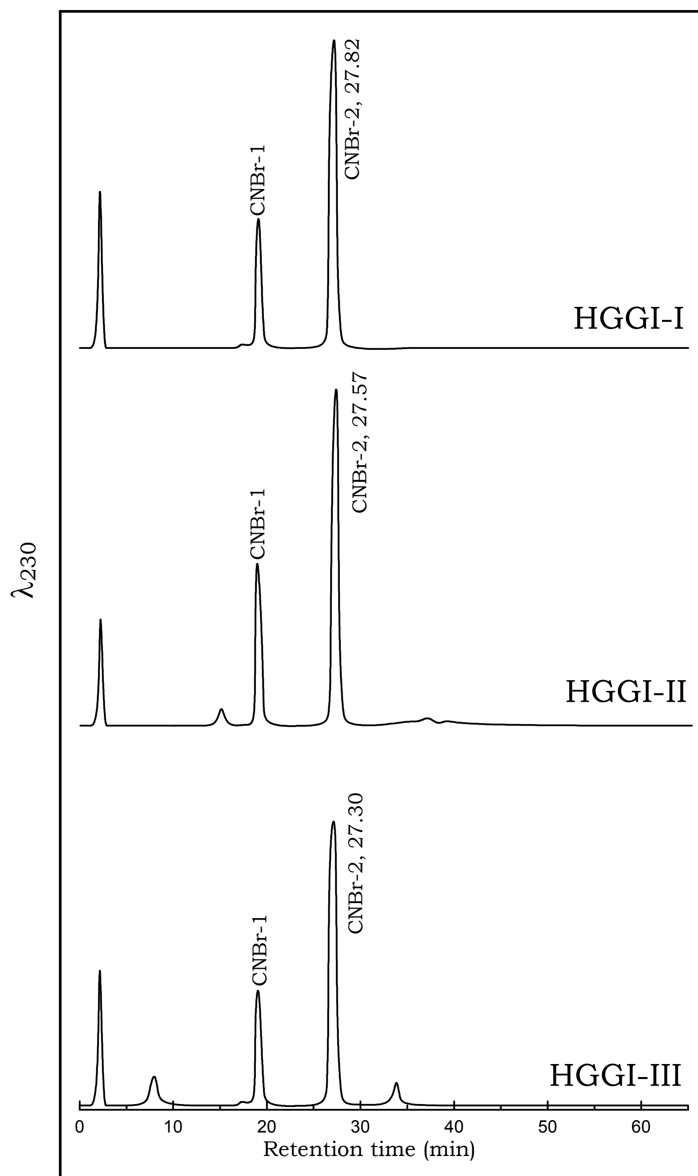


Figure 4.5 **Fractionation of cyanogen bromide peptides of PE-HGGIs by RP-HPLC.** The CNBr digests were fractionated on a Phenomenex ODS column (250 × 4.6 mm, 5μ) using a linear gradient of 0.1 % TFA in water and 70 % acetonitrile in 0.05 % TFA at a flow rate of 0.7 mL/min and detected at 230 nm.

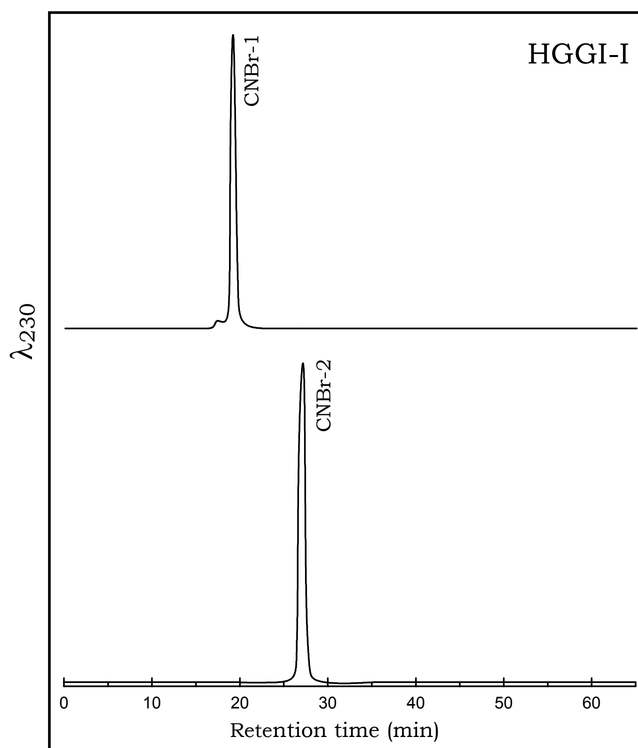
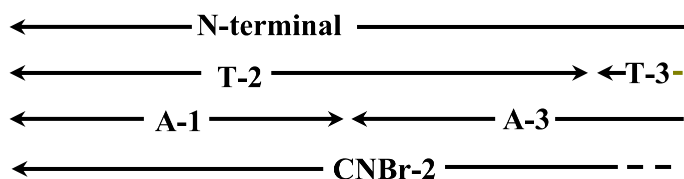


Figure 4.6 **Repurification of cyanogen bromide peptides of HGGI-I.** Similar results were obtained with the HGGI-II and HGGI-III.

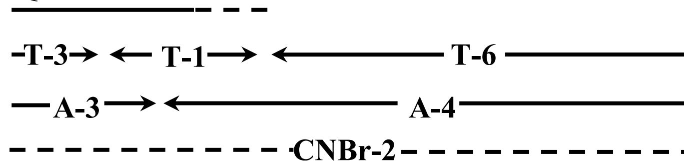
Table 4.5 **Amino-acid sequences of cyanogen bromide peptides.**

Inhibitor	Peptides	Sequence	Amino acids
HGGI-I	CNBr-1	KDFCYAPCKS	10
	CNBr-2	DEPSESSKPCCDQCACTKSIPPQCRCTD….	28
HGGI-II	CNBr-1	KDFCYAPCKS	10
	CNBr-2	EPSESSKPCCDQCACTKSI….	19
HGGI-IIII	CNBr-1	KDFCYAPCKS	10
	CNBr-2	SKPCCDQCACTKSIPPQC….	18

DEPSESSKPCCDQCACTKSIPP



QCRCTDVRLNSCHSACSSCVCT



FSIPAQCVCVDMKDFCYAPCKS

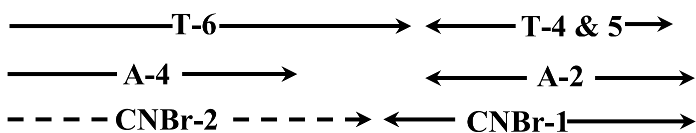
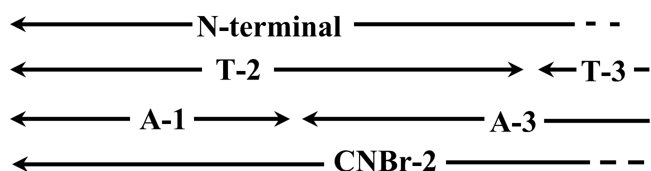
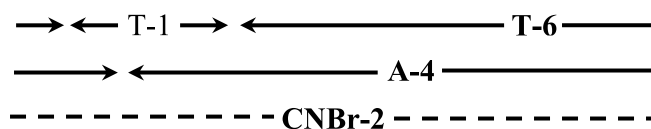


Figure 4.7 **The complete amino acid sequence of HGGI-I.** Peptides obtained from TPCK-Trypsin, Endoproteinase Asp-N and CNBr cleavage are designated as T, A and CNBr respectively**.**

EPSESSKPCCDQCACTKSIPPQ



CRCTDVRLNSCHSACSSCVCT



FSIPAQCVCVDMKDFCYAPCKS

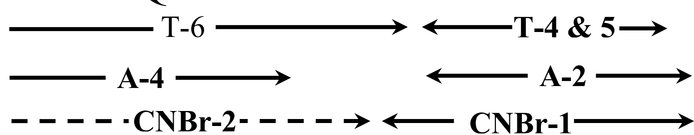


Figure 4.8 **The complete amino acid sequence of HGGI-II.** Peptides obtained from TPCK-Trypsin, Endoproteinase Asp-N and CNBr cleavage are designated as T, A and CNBr respectively.

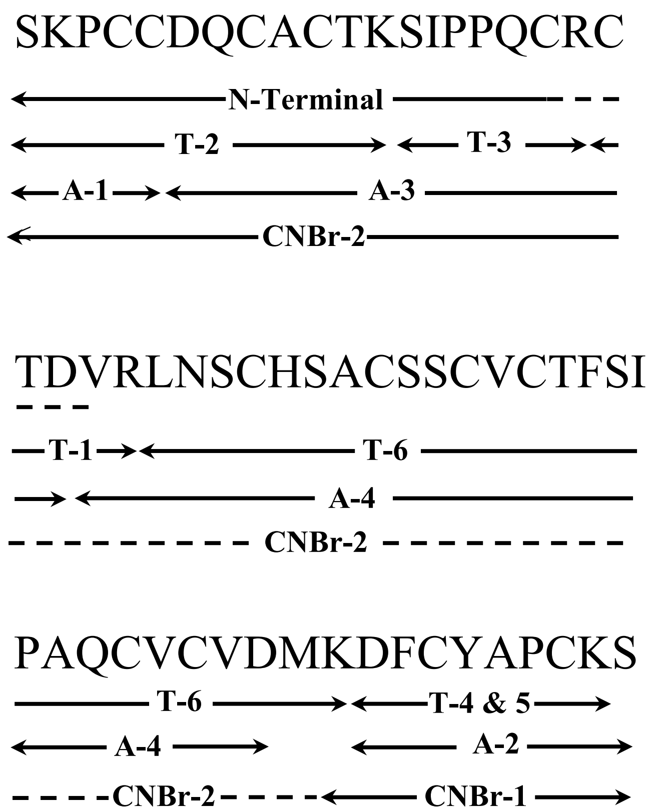


Figure 4.9 **The complete amino acid sequence of HGGI-III.** Peptides obtained from TPCK-Trypsin, Endoproteinase Asp-N and CNBr cleavage are designated as T, A and CNBr respectively.

Comparison of HGGI sequences with the dry seed inhibitor, HGI-III

The amino acid sequences of the HGGIs were aligned with the sequence of HGI-III, the major isoinhibitor present in the dry seed (Figure 4.10). The sequences of the HGGIs are identical to HGI-III sequence, except for the truncation at both, the amino- and carboxy-terminus of the sequence. The three inhibitors from germinated seeds (HGGIs) differ from each other only at the amino-terminus. The absence of the charged tetra peptide, -SHDD at the carboxy-terminus is common to all the three HGGIs. The 14 half-cystine residues are conserved in the HGGIs, as in the case of all legume BBIs sequenced thus far. The trypsin reactive site with Lys and chymotrypsin reactive site with Phe as the P_1 residue in HGI-III remain unaltered in the sequence of the HGGIs.

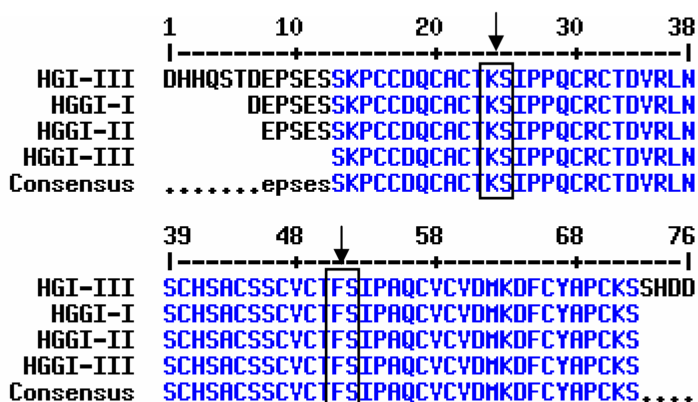


Figure 4.10 **Homology of horsegram dormant seed inhibitor (HGI-III) with horsegram germinated inhibitors (HGGIs).** The two reactive site P_1 of horsegram inhibitors are represented with an arrow.

Comparison of BBI sequences

The sequences of HGGI-III and other legume BBI sequences listed in Table 4.6 were aligned by using SIM-LALNVIEW and LALIGN program to match a pair wise comparison. Table 4.6 shows the alignment scores for all pairs of the HGGI-III sequence. The HGGIs showed 100 % identity with each other and HGI-III. HGGI-III shows low

alignment scores with FBI (*Vicia faba L.*) and PsTI-IVb (Pea seeds) irrespective of the matrix used for pair-wise comparison (Table 4.6). HGGI-III showed maximum identity and similarity to HGI-III, the major isoinhibitor of horsegram dormant seed and MAI-DE-3 (*Macrotyloma axillare*).

Table 4.6. **Pair-wise comparison of HGGI-III with other BBIs of dicot legume plants**

Common name /Botanical name	**Abbreviation**	**SIM-LALNVIEW**		**LALIGN**	
		Percent identity	**Alignment score**	**Percent identity**	**Alignment score**
Wild Soja *Glycine soja*	WSTI-IV	65	212	62	310
Bushbean *Phaseolus vulagris var. nanus*	PVI-3I	61.7	259	61.7	354
	PVI-3II	75	303	75	413
Torresea cearensis *Amburana cearensis*	TcTI2	76.4	268	76.4	366
	TaTI	76.4	374	76.4	375
Black eyed pea or Cowpea	BTCI	75	298	75	404
Tracy bean *Glycine max*	PI-II	68.3	248	68.3	352
Snail medic seeds *Medicago scutellata*	MSTI	66.1	225	66.1	320
Alfa alfa leaves *Medicago sativa*	WII	61.8	213	61.8	304
Faba beans *Vicia faba* L.	FBI	60	196	57.9	284
Pea seeds	PsTI-IVb	58.2	197	56.1	286

Horsegram *Dolichos biflorus*	HGI-III	100	359	100	481
	HGGI-I	100	359	100	481
	HGGI-II	100	359	100	481
	HGGI-III	100	359	100	481
Garden bean *Phaseolus vulgaris*	GBI-II	61.7	253	61.7	349
Soybean Glycine max	SBI-C-II	70	271	70	375
	BBI	79.7	300	79.7	413
Mung bean *Vigna radiata* or *Phaseolus aureus*	MBI	66.7	265	66.7	367
Apple leaf *Lonchocarpus capassa*	DE-4	73.3	267	73.3	369
Macrotyloma axillare	MAI-DE-3	98.3	356	98.3	476
Lima bean *Phaseolus lunatus*	LBI	80	310	80	421
Canavalia lineata	CLTI-I	70	269	70	370
	CLTI-II	70	269	70	370

Hydrophobicity Profile

The variation of hydrophobicity (Kyte and Doolittle, 1982) along the polypeptide is an important characteristic that often reflects the role of a given segment in the structure of the protein. Figure 4.11 shows the hydropathy profile as a function of residue position for each of the HGGIs. Hydrophilic regions occur at the amino- and carboxy-termini with the more hydrophobic regions around the putative reactive

sites. The terminal residues of soybean and peanut are also hydrophilic and appear not to have well defined electron density in the X-ray structures reported (Chen *et al.*, 1992; Suzuki *et al.*, 1993).

Multiple alignment

The sequences of HGGIs were aligned for maximum homology with MULTALIN (Corpet, 1988) programme. More than 60 % of the amino acid residues including the ½ cystine residues are invariant among legume BBIs are conserved in HGGIs (Figure 4.12). The homologous inhibitor other than HGI-III was *Macrotyloma axillare* DE-3 sharing 98.3 % identity (Table 4.6). The sequences of BBIs vary at the amino- and carboxy-termini.

Phylogenetic tree

A phylogenetic tree of legume BBIs (Table 4.7) constructed on the basis of their amino acid sequence relationship (Figure 4.12) is represented in Figure 4.13. The phylogenetic tree constructed from PHYLIP-PROTPARS programme clearly demarcates the two major groups of BBIs, group-I and group-II, which are categorized according to the sequences around putative reactive site (Figure 4.13). The length of the each branch is proportional to the number of residues, which differed between overlapping regions of the compared sequences. The three HGGIs can be classified as a group-II BBIs, based on the homology to group-II BBIs and a characteristic Pro-Ala sequence near second reactive site (Figure 4.12).

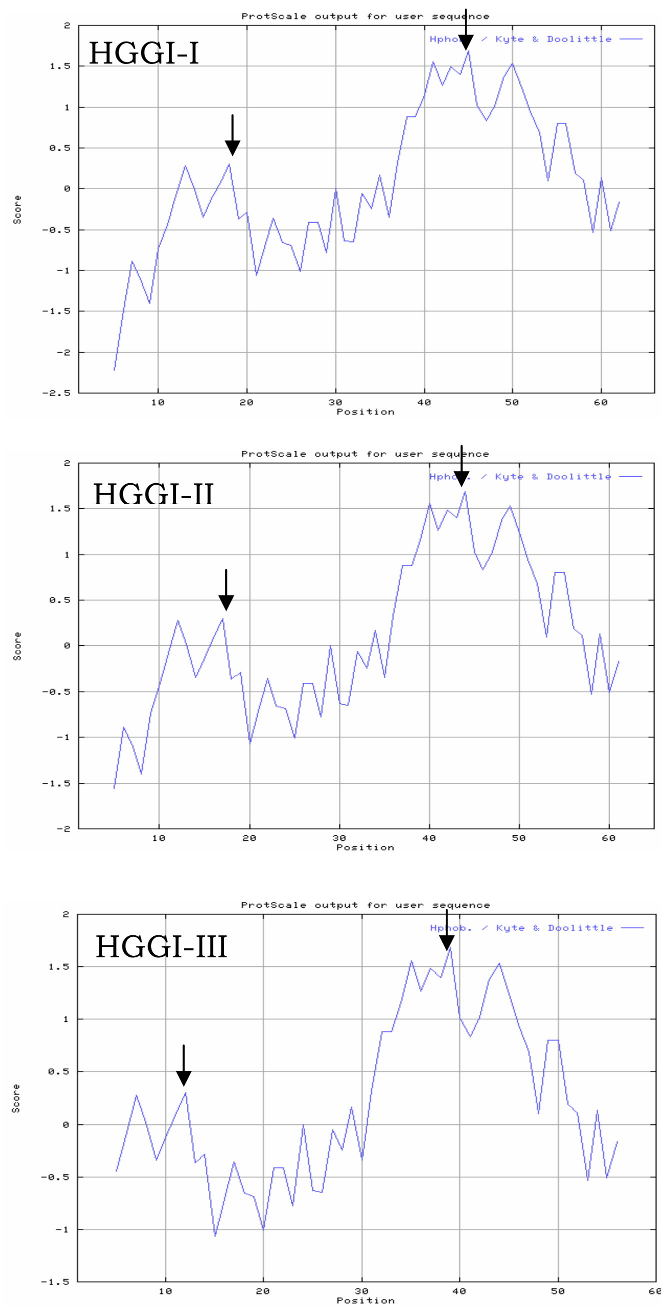


Figure 4.11 **Hydropathy profile of HGGIs.** Hydropathy was calculated by the method of Kyte and Doolittle (1982). Arrows indicate the position of putative reactive sites.

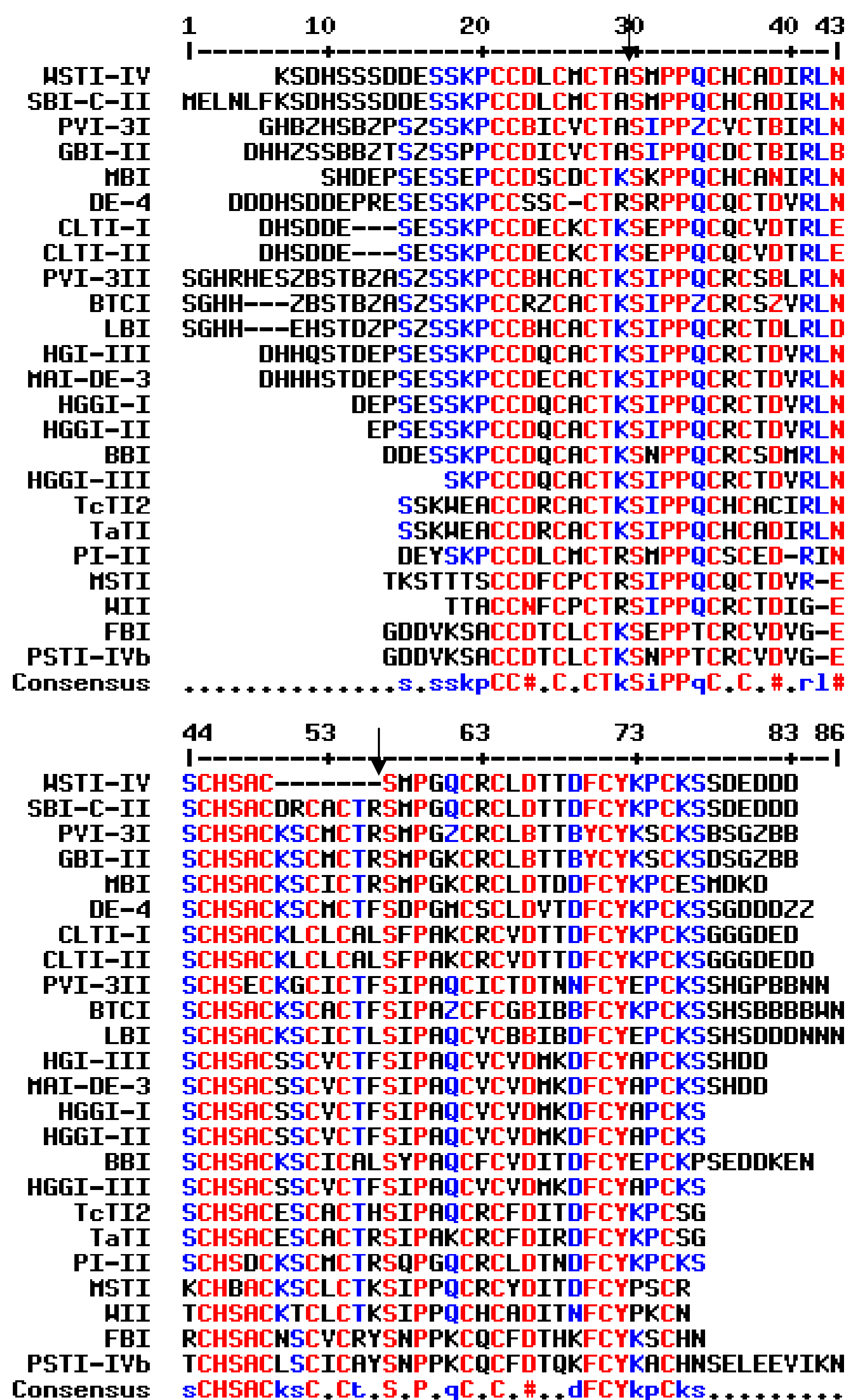


Figure 4.12 **Multiple alignment of legume BBI sequences**. The sequence alignment was performed using MULTALIN. The arrows are between the P_1-P_1' residues of the reactive site (Schechter and Berger, 1967). Abbreviations used are as described in Table 4.6.

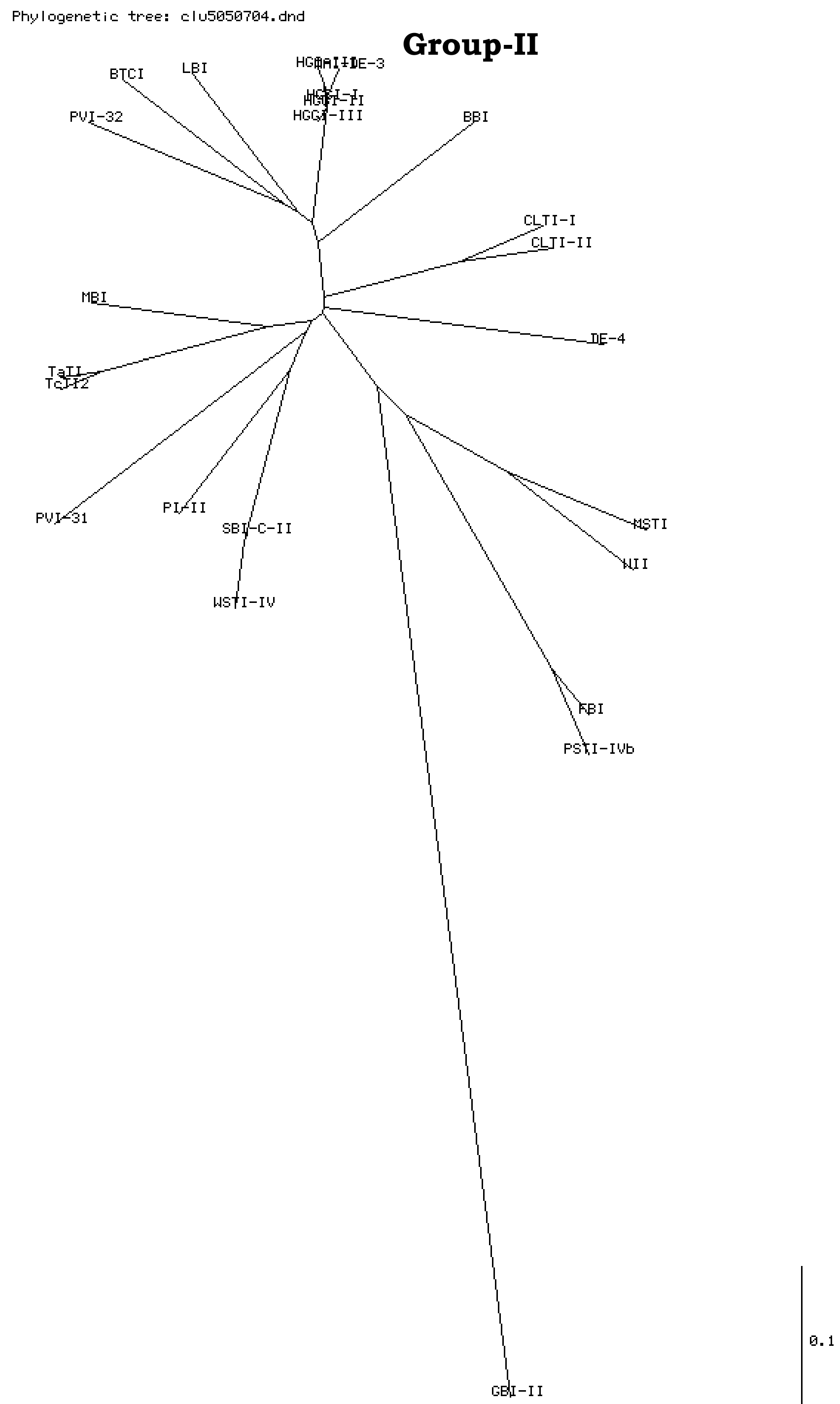


Figure 4.13 **Phylogenetic tree of legume BBIs.**

DISCUSSION

HGI-III, the major BBI of horsegram seeds is a highly stable globular protein consisting of single polypeptide chain of 76 amino acid residues, including 14 ½-cystine residues (Prakash *et al.*, 1996). The determined primary structure of the three HGGIs (Figure 4.10) differs from the major inhibitor HGI-III only at the amino- and carboxy-termini. This fact suggest that these three inhibitors that appear upon germination are not the products of stored mRNA but derived from the dry seed inhibitor by a limited proteolysis at the both the amino- and carboxy-terminus. The cleavages occur between Thr^{6}-Asp^{7}, Asp^{7}-Glu^{8} and Ser^{12}-Ser^{13} at the amino-terminus and Ser^{72}-Ser^{73} at carboxy-terminus in the conversion of HGI-III to HGGI-I, HGGI-II and HGGI-III (Figure 4.10). CNBr-1 and A-2 both being carboxy-terminal fragments of HGGIs, are identical to the C-terminal CNBr fragment of HGI-III, (Prakash *et al.*, 1996) with the loss of the tetrapeptide –SHDD indicating a cleavage at Ser^{72}-Ser^{73} of HGI-III. Cleavage between Ser^{3}-Ser^{4} and Asp^{7}-Asp^{8} has been reported in the conversion of the peanut inhibitor A-II to A-I and A-II to B-I respectively (Norioka and Ikenaka, 1983b). The cleavage appear to be due to the action of some protease(s) that have wide substrate specificities or special substrate specificity. The sequencing study clarified that the HGGIs consists of 66, 65 and 60 residues corresponding to a calculated M_r of 7109, 6993, 6464 respectively, which confirmed our previous report (Kumar *et al.*, 2002).

Absence of free Cys residues, resistant to proteolytic cleavage (results not shown) and multiple alignment of dicot BBIs (Figure 4.12) are in agreement with other BBIs in lacking free Cys residues. Similarity in core sequence among HGI-III and HGGIs and the

conserved 14 ½ cystine residues indicate similar disulfide pattern in HGGIs (C_1-C_{14}, C_2-C_6, C_3-C_{13}, C_4-C_5, C_7-C_9, C_8-C_{12}, C_{10}-C_{11}).

Amino acid sequence comparison together with modification of reactive site residues (Chapter 3) suggested that Lys-Ser and Phe-Ser in HGGIs are the reactive sites for the trypsin and chymotrypsin respectively. HGGIs belong to group-II legume BBI as evidenced by the presence of a characteristic Pro-Ala sequence near the second reactive site. Among all the dicot BBIs compared, the most homologous proteins to HGGIs are HGI-III and MAI-DE-3 of *Macrotyloma axillare* with 100 % and 98.3 % identity respectively. The striking similarities in the primary structure of the BBIs from *Dolichos biflorus* (HGI-III and HGGIs) and from *Macrotyloma axillare* (MAI-DE-3) reflect upon the common evolutionary origin of the two plants. Comparison among the group-II BBIs from leguminous plants showed the variation at amino- and carboxy- terminal tail and most of the positions in the core sequence are occupied by identical amino acids (Sreerama 1996). Close evaluation of amino- (SKP**C^4**C....) and carboxy-terminal (.....P**C^{58}**KS) residues of HGGI-III, indicate that the HGGI-III could be the last isoform during the process of germination. Cleavage at either of the terminal may not be possible as the two Cys residues; Cys^4 and Cys^{58} are bridged by the disulfide bond making nearly a closed compact molecule with help of other 6-disulfide bonds.

Dicot BBIs show broader specificity for enzyme inhibited. Among the 24 dicot BBIs compared, the first reactive site of dicot usually inhibits trypsin or elastase and the second reactive site either trypsin or chymotrypsin. In monocots, the only reactive site situated at the amino-terminal region of the 8 kDa protein aligns well with the first reactive site of the dicots. All monocots inhibit trypsin but seems to have lost their second reactive site (Prakash *et al.*, 1996). The residues

corresponding to the P_1 position of this site are not potential targets for trypsin, chymotrypsin or elastase. More importantly, these inhibitors have lost Cys^{10} and Cys^{11}, which form a disulfide linkage in dicots. This second reactive site although its not active, is strongly hydrophilic. This increased hydrophilicity might have arisen due to the fact that the disulfide is lost in this segment, resulting in higher flexibility and water accessibility of this loop (Prakash *et al.*, 1996).

Although HGGIs and HGI-III were structurally similar, differences were observed in their molecular properties and kinetic properties. HGGIs have 5-fold higher specific activity towards trypsin and exist as monomeric units in nature (Chapter 3). Where as HGI-III, exists as dimer (Sreerama *et al.*, 1997). The comparison of the sequences (Figure 4.10) reveals the role of the charged residues present at both the termini in dimerization. The crucial residues involved in the dimerization of HGI-III are investigated and results presented in the following chapter.

Self-association of Bowman-Birk inhibitors of horsegram

The inhibitors of the dry seed of horsegram (HGIs) are single polypeptides of M_r 8.5 kDa, however SDS-PAGE and analytical gel filtration studies indicate the M_r to be ~ 16.0 kDa (Sreerama *et al.*, 1997) suggesting they self-associate to form dimers in solution. In contrast the HGGIs derived from HGI-III are single polypeptides of M_r 6.5 kDa and exist as monomers in solution (Kumar *et al.*, 2002; Chapter 3). The significant difference between the primary structure of inhibitors from the germinated seed (HGGIs) and the dormant seed (HGI-III) is the varied truncation at the amino-terminus and absence of charged carboxy-terminus. This observation suggests that the structural elements responsible for the self-association occur at either the carboxy- and/or the amino-terminus. In this chapter chemical modification studies, comparative evaluation of several BBIs and homology modeling have been used to demonstrate the role of individual amino acids in the self-association of BBIs.

RESULTS

Purification of horsegram inhibitor, HGI-III

Purification of HGIs was carried out according to method of Sreerama *et al.*, (1997) with some modifications. The crude extract of defatted horsegram flour was directly loaded on a trypsin-Sepharose column pre-equilibrated in 0.05 M Tris-HCl, pH 8.2 containing 0.02 M $CaCl_2$ and 0.5 M NaCl at 10 mL/min. The elution of the bound trypsin inhibitors was carried out at 50 mL/h using 0.2 M Gly-HCl buffer, pH 3.0 containing 0.5 M NaCl (Figure 5.1). The fractions having trypsin inhibitory activity were pooled, titrated to pH 7.5 with liquor ammonia and dialyzed against water extensively to remove NaCl and buffer ions present in the pooled sample. The four isoforms (HGI-I, -II, -III and –IV) were resolved on a DEAE-Sephacel column (Figure 5.2) as reported earlier (Sreerama *et al.*, 1997). The major inhibitor, HGI-III was used for further studies.

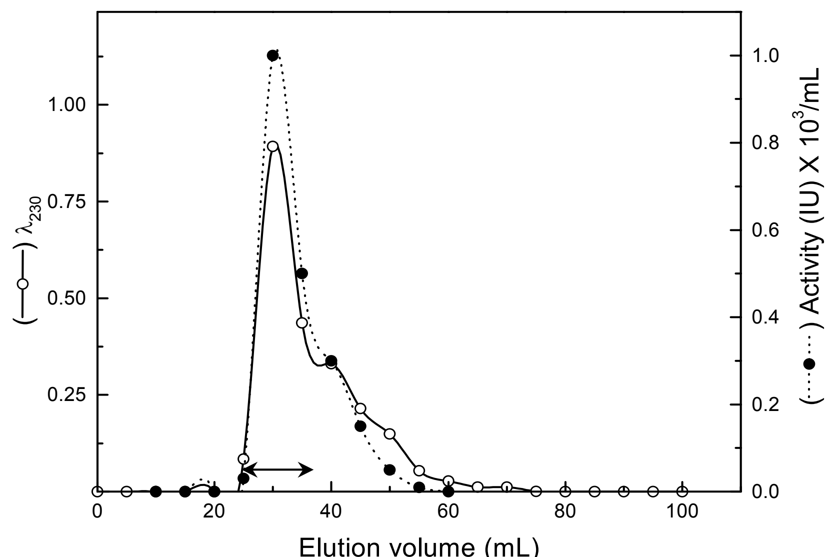


Figure 5.1 **Elution profile of horsegram inhibitors on Trypsin-Sepharose chromatography**. The crude extract was loaded on a Trypsin-Sepharose equilibrated with 0.05 M Tris-HCl, pH 8.2 containing 0.02 M $CaCl_2$ and 0.5 M NaCl at a flow rate 10 mL/h. The sample was eluted with 0.2 M Gly-HCl buffer containing 0.5 M NaCl at a flow rate 50 mL/h. Fractions of 5 mL were collected and the active fractions were pooled as shown (⟷).

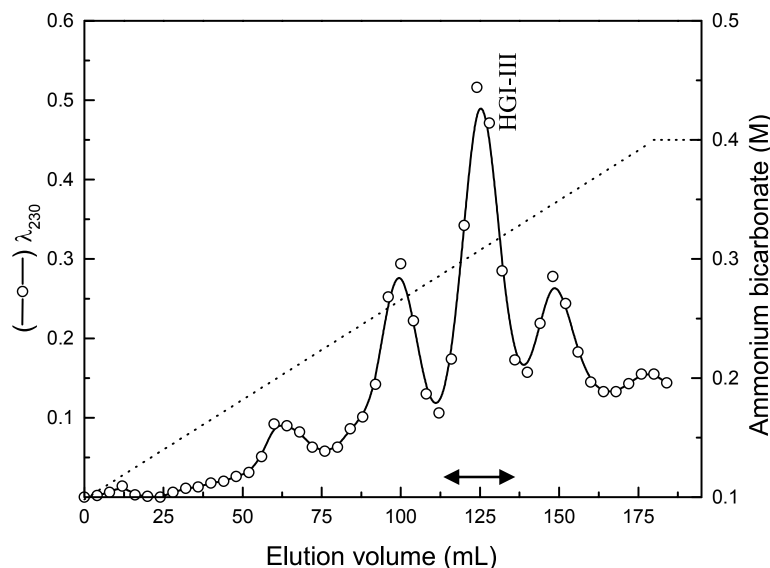


Figure 5.2 **DEAE-Sephacel chromatography profile of HGIs.** Inhibitor pool obtained from Trypsin-Sepharose chromatography dissolved in 0.1 M ammonium bicarbonate buffer (pH 8.2) and subjected to DEAE-Sephacel chromatography. The bound inhibitors were eluted with a linear gradient of 0.1 M to 0.4 ammonium bicarbonate buffer (pH 8.2) at 20 mL/h. 2 mL fractions were collected and active inhibitor fractions pooled as indicated (⟷).

SDS-PAGE and size-exclusion chromatography

SDS-PAGE of HGGI-III on a 15 % T and 2.7 % C gel is shown in Figure 5.3. HGGI-III moves as a single polypeptide of M_r ~ 6.5 kDa. HGGI-I, HGGI-II also move as single polypeptides of M_r ~ 7.0 kDa on SDS-PAGE (Figure 3.10, Chapter 3). In contrast, HGI-III moves as a single polypeptide of M_r ~ 16.0 kDa (calculated from the relative mobility). The exact M_r of HGI-III as determined by electrospray mass spectrometry (Sreerama *et al.*, 1997) and by sequence (Prakash *et al.*, 1996) is ~ 8.0 kDa. These results suggest that the HGI-III in solution undergoes self-association to form a dimer. HGGI-III – an *in situ* degradation product of HGI-III essentially exists as a monomer. The reduced and alkylated HGI-III is a polypeptide of M_r ~ 8.0 kDa (Sreerama *et al.*, 1997).

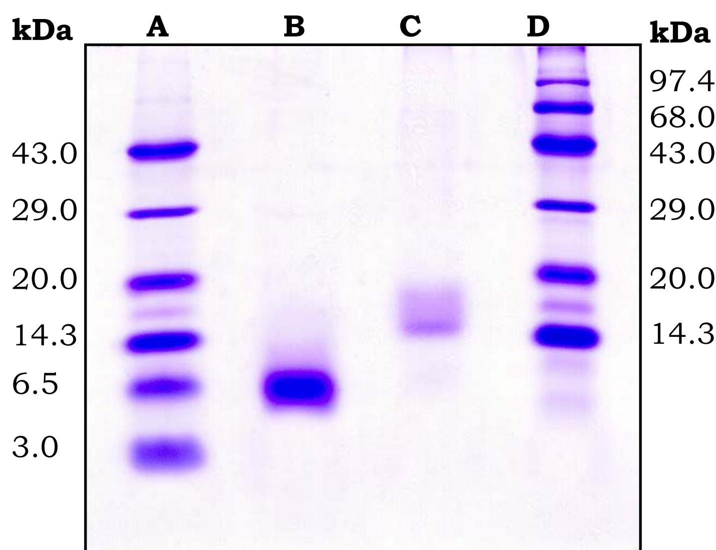


Figure 5.3 **SDS-PAGE (15 % T, 2.7 % C) of HGI-III and HGGI-III.** Low molecular weight markers (lane A), HGGI-III (lane B), HGI-III (lane C) and high molecular weight markers (lane D).

The monomer/dimer status of HGI-III and HGGI-III was further evaluated by size-exclusion HPLC on a BIOSEP-SEC-S 3000 column using 0.025 M, Tris-HCl pH 7.25. HGI-III was well separated from

HGGI-III (Figure 5.4). HGGI-III eluted later with a retention time of 22.92 min corresponding to a M_r of 6.5 kDa. HGI-III elutes at 20.26 min which corresponds to a M_r ~16.0 kDa. These results provide further evidence that HGI-III in solution associates to form a dimer.

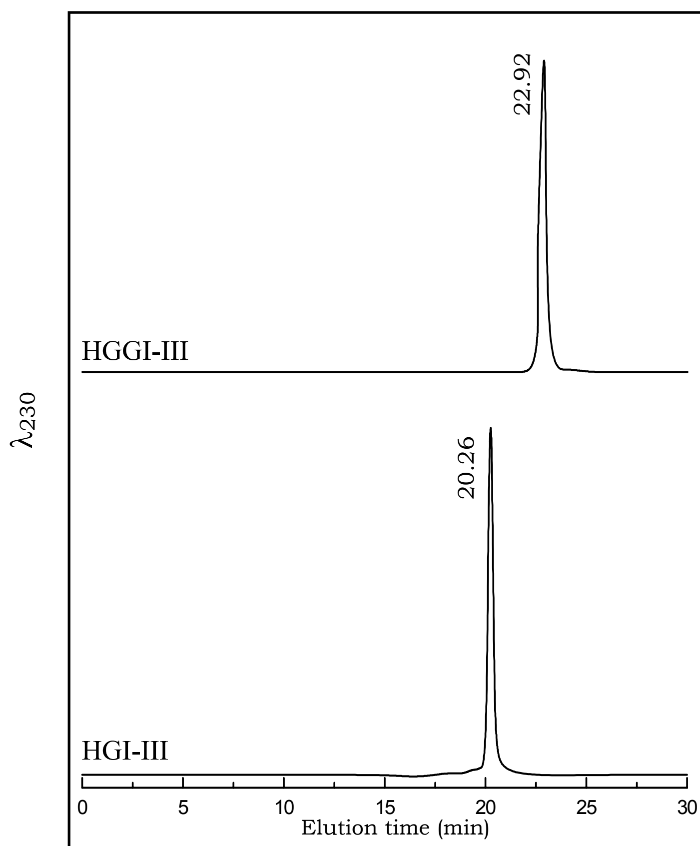


Figure 5.4 **Size-exclusion chromatography of HGGI-III and HGI-III.** The samples were dissolved in 0.025 M, Tris-HCl pH 7.25 buffer and loaded on a BIOSEP-SEC-S 3000 pre-equilibrated with same buffer and eluted at 0.5 mL/min.

Circular Dichroism spectra of HGI-III and HGGI-III

Secondary and tertiary structure of the HGI-III and HGGI-III were analyzed from the Far-UV and Near-UV circular dichroism spectra in 0.1 M Tris-HCl, pH 7.5 buffer at 25 °C. The Far-UV CD spectra of HGI-III and HGGI-III exhibit a deep negative band between 200-206 nm (Figure 5.5A). This is quite similar to polypeptides and proteins in constrained unordered conformation including some protease

inhibitors (Jirgensons, 1973) and confirms that HGI-III and HGGI-III are non-helical proteins. The inhibitors exhibited a minimum at 280 nm (Figure 5.5B), a contribution of both disulfide bonds and the single Tyr residue. The secondary and tertiary structure conformations analyzed from Near- and Far-UV spectral data are summarized in Table 5.1. These results are in close agreement with those reported earlier for BBIs (Steiner and Frattali, 1969; Ikeda *et al.*, 1968; Wu and Sessa, 1994; De Freitas *et al.*, 1997) and the absence of α-helical structure in (Bewly and Birk, 1978; Sierra *et al.*, 1999) in BBIs.

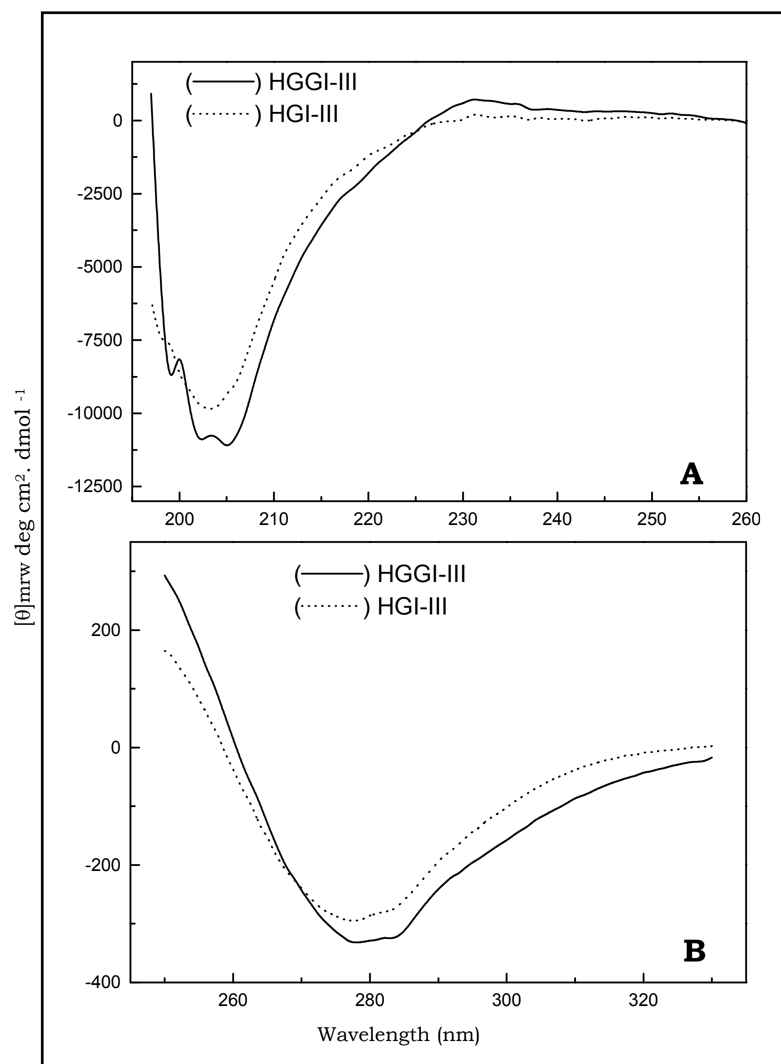


Figure 5.5 **Far-UV (A) and Near-UV (B) circular dichroism spectra of HGI-III and HGGI-III at 25 °C in 0.1 M Tris-HCl buffer, pH 7.5.**

Table 5.1 **Secondary and tertiary structure comparison of HGI-III and HGGI-III*.**

Inhibitor	α-helix (%)	β-sheet (%)	β-turn (%)	Random coil (%)	RMS (%)
HGI-IIII	0	65.9	0	34.1	55.2
HGGI-III	0	47.2	13.6	39.2	42.46

*These spectral results were the average of 3 scans and corrected for the buffer blank.

Fluorescence studies

The structural differences if any between the HGI-III and HGGI-III were further evaluated by measuring the intrinsic fluorescence and extrinsic fluorescence in presence of ANS. HGI-III and HGGI-III were excited at 275 nm in 0.1 M Tris-HCl, pH 7.5 and emission was recorded from 300-400 nm. The emission spectra of the two inhibitors were identical with an emission maximum at around 330 nm (Figure 5.6).

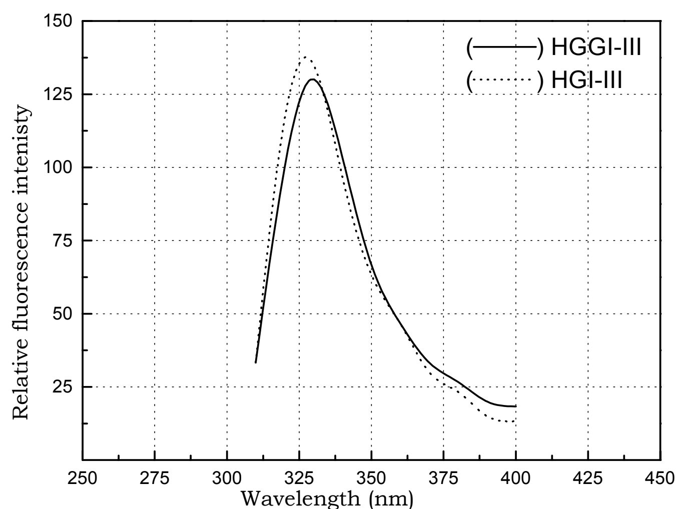


Figure 5.6 **Fluorescence emission spectra of HGI-III and HGGI-III in 0.1 M Tris-HCl buffer at pH 7.5.** Excitation wavelength, 275; Excitation and emission slit width: 5 nm.

Extrinsic fluorescence measurements were carried out with ANS to evaluate the accessibility of the Tyr residue in monomeric HGGI-III and dimeric HGI-III. Neither of the inhibitors was quenched with ANS and the behavior of the two inhibitors was very similar further confirming the two inhibitors are structurally similar.

Rationale for chemical modification

HGI-III exists as a dimer, whereas HGGI-III exists as a monomer. A closer evaluation of the sequences shows that the most significant difference between the two, is the physiological deletion of the peptide -DHHQSTDEPSES and the tetrapeptide –SHDD at the amino- and carboxy- terminus, respectively. Hence, either the deleted amino- and/or the carboxy-termini are involved in the self-association of HGI-III. HGI-I, yet another of the isoinhibitors present in the dry seed of horsegram, although truncated at the amino-terminus, exists as a dimer in solution (Sreerama *et al.*, 1997). These observations implicate that the residues of the deleted tetrapeptide play a major role in the dimerization of HGI-III. PsTI-IVb, a BBI from Winter pea seeds, has been crystallized as a nearly perfect 2 fold symmetric dimer in the asymmetric subunit which includes its carboxy-terminal segment (Sierra *et al.*, 1999). The carboxy-terminal tail, from residues 68 to 70 (-EEV), that constitutes an extended β-strand, makes no contact with its own subunit yet is held by interactions with the other subunit. Two specific interactions that have been discerned between the two subunits are (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 the dyad related carboxyl group of Glu^{69} of the other subunit. This observation, together with the fact that the deleted tetrapeptide contained Asp residues, suggests that such interactions could well be the premise to self-

association in HGI-III. The effect of chemically modifying Arg and Lys residues of HGI-III has been studied. Chemical modification of these residues may disrupt such a subunit interaction in HGI-III leading to the formation of monomers.

Chemical modification of Arg and Lys residues of HGI-III

The Lys residues of HGI-III were chemically modified using citraconic anhydride. Citraconylation resulted in the acetylation of the free ε-amino group of Lys. SDS-PAGE (15 % T, 2.7 % C) of the modified HGI-III revealed an increased relative mobility compared to the unmodified inhibitor (Figure 5.7, Lane D). The M_r calculated on the basis of the relative mobilities of a set of standard proteins, was ~8.5 kDa corresponding to that of a monomer. The conversion of the dimer form of the HGI-III to monomer by citraconylation suggests that a Lys residue is involved in the self-association of HGI-III. The guanidium group of Arg residues were modified using 1, 2-cyclohexanedione, resulting in a heterocyclic condensation product between the guanidium group of Arg and the carbonyl of 1, 2-cyclohexanedione. The adduct formed was further stabilized by carrying out the reaction in borate buffer. SDS-PAGE (15 % T, 2.7 % C) analysis at pH 8.8, led to the understanding that the modification had no effect on the relative mobility of HGI-III (Figure 5.7, Lane C). The relative mobilities of both the unmodified (Figure 5.7, Lane B) and the modified inhibitor were identical (Figure 5.7, Lane C). These results ruled out the essentiality of an interaction involving Arg for self-association of HGI-III.

Effect of Zn^{2+} on the self-association of HGI-III

The changes in the monomer-dimer status of HGI-III, in the presence of Zn^{2+}, were followed by size-exclusion chromatography on a

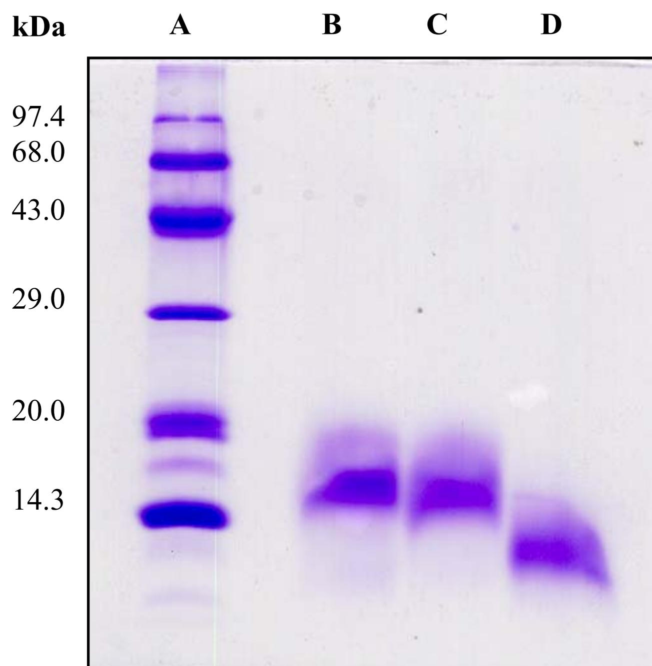


Figure 5.7 **SDS-PAGE (15 % T, 2.7 % C) of chemically modified HGI-III**. High molecular weight markers (lane A); unmodified HGI-III (lane B); Arg modified HGI-III (lane C) and Lys modified HGI-III (lane D).

BIOSEP-SEC-S 3000 column and the results summarized in Figure 5.8. HGI-III elutes as a single symmetrical peak with a retention time of 20.26 min (Figure 5.8A). In the presence of either 1 mM $ZnCl_2$ or $ZnSO_4$ at pH 7.25, HGI-III eluted at 22.65 min (Figure 5.8C & 5.8D). This increased retention time pointed to a significantly reduced molecular weight, calculated to be ~ 8.5 kDa. In contrast, when similar experiments were performed in buffer of pH 6.5 containing 1 mM $ZnSO_4$ or $ZnCl_2$, HGI-III eluted at 20.27 min (Figure 5.8E). These results suggested that HGI-III can be converted to its monomeric form in the presence of Zn^{2+} at neutral pH. This dissociation of HGI-III, in the presence of Zn^{2+}, was also followed on SDS-PAGE (Figure 5.9). The treatment of HGI-III with Zn^{2+} increased its relative mobility. The M_r of the Zn^{2+} modified HGI-III was ~ 8.4 kDa. These results concurred with those of size-exclusion chromatography.

The dissociation of HGI-III to monomers in the presence Zn^{2+} at pH 7.25 and the absence of monomer formation in the presence of Zn^{2+} at pH 6.5 (Figure 5.8 E) and below (results not shown) suggested the probable involvement of a His residue also. DEPC was used to modify HGI-III to evaluate the role of His in the self-association phenomenon. DEPC-modified HGI-III eluted at 20.26 min (results not shown), which corresponds to the dimeric form of HGI-III, ruling out the involvement of His in the monomer interaction. His residues in proteins are found to bridge both a Zn^{2+} ion and a carboxylate side chain of nearby Asp (sometimes Glu residues) (Christianson and Alexander, 1989) referred to as an indirect carboxylate-metal co-ordinate (Figure 5.10). The conversion of HGI-III to its monomer in the presence of Zn^{2+} at pH 7.25 probably occurs through this indirect metal co-ordination, involving Asp ($Asp^{75/76}$) residues at the carboxy-terminus of HGI-III. These results indirectly provide evidence that the Asp residues at the carboxy-terminus play a vital role in the dimerization of HGI-III. These results, together with the observation that a modification of Lys and not Arg causes the formation of monomers, support the inference that the interaction between Lys of one monomer and Asp of the other monomer is responsible for the self-association of HGI-III. Pair-wise alignment of the PsTI-IVb with HGI-III indicates that this interaction in the HGI-III dimer should occur between Lys^{24} at the trypsin reactive site and Asp^{76} at the carboxy-terminus.

Comparative evaluation of BBI amino acid sequences in relation to monomer-dimer status

The BBI sequences of legumes were aligned to further evaluate the probability of such a unique interaction, between the first reactive site residue and Asp/Glu at carboxy-terminus dictating the monomer-dimer status of BBIs in solution. The alignment of BBI sequences of

dicots obtained from SWISSPROT (Release 42, October 2003) and those reported in literature is shown in Figure 4.12 (Chapter 4). The program MULTALIN (Corpet, 1988) has been used to align the sequences. Some dicot BBIs exist as monomers in solution despite the close similarity between their sequences, whereas others self-associate to form dimers.

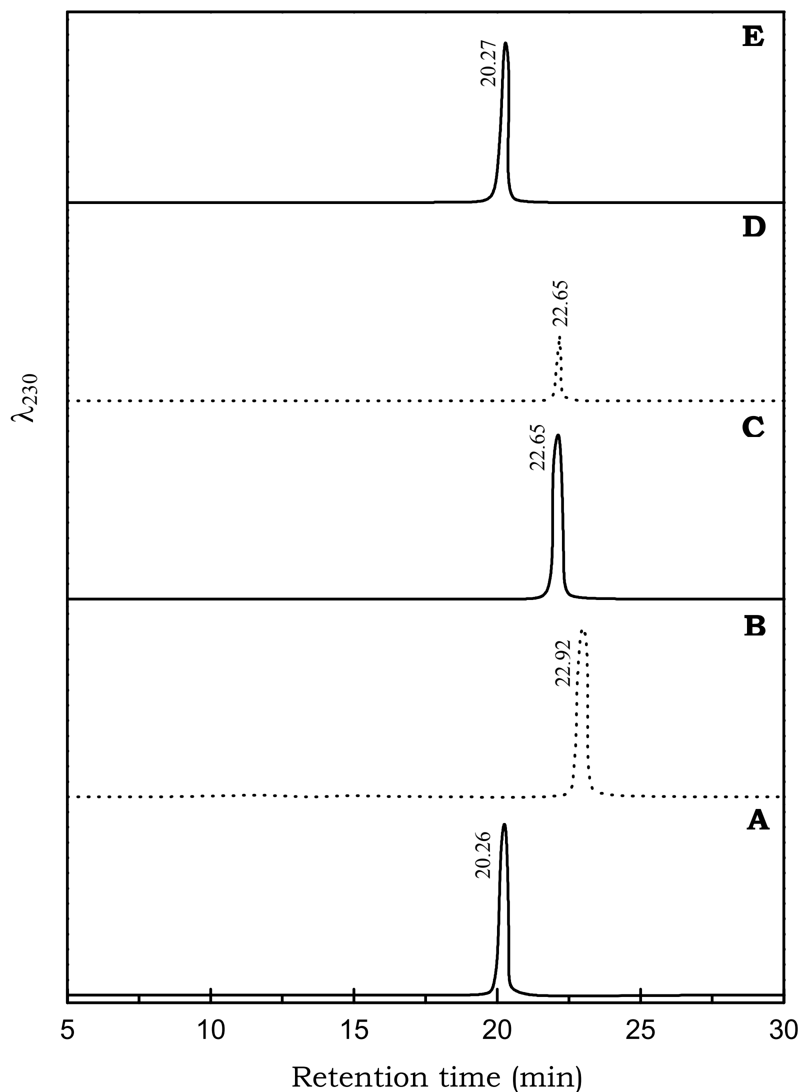


Figure 5.8 **Size-exclusion chromatography of HGI-III and HGGI-III.** The samples were dissolved in different buffers and loaded on a BIOSEP-SEC-S 3000 column pre-equilibrated with respective buffers and eluted at 0.5 mL/min. (A) HGI-III (pH 7.25); (B) HGGI-III (pH 7.25); (C) HGI-III (1 mM $ZnSO_4$, pH 7.25); (D) HGI-III (1 mM $ZnCl_2$, pH 7.25) and (E) HGI-III (1 mM $ZnCl_2$, pH 6.5).

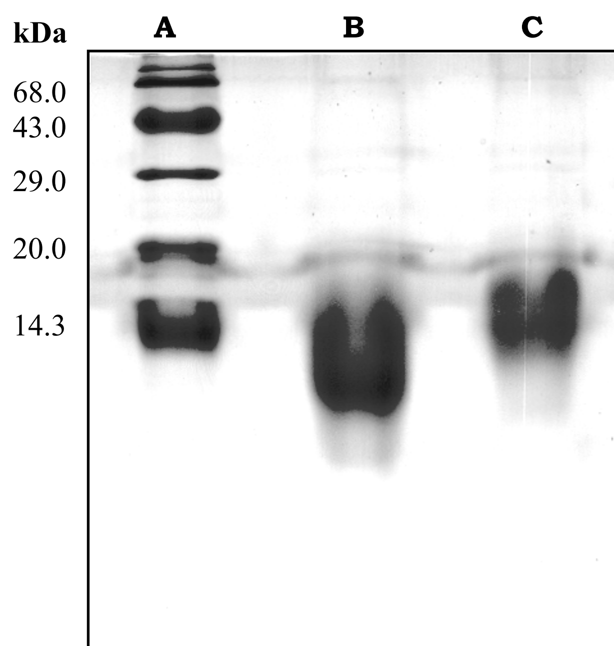


Figure 5.9 **SDS-PAGE (17.5 % T. 2.7 % C) of Zn^{2+} induced monomerization of HGI-III.** Molecular weight markers (lane A); HGI-III incubated in the presence (lane B) and absence (lane C) of 1 mM $ZnSO_4$ for 1 h.

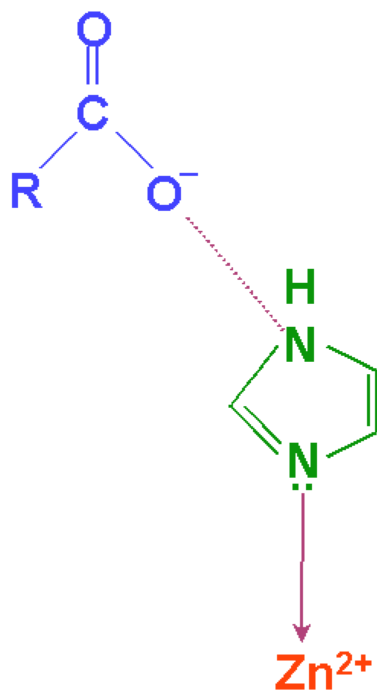


Figure 5.10 **Indirect carboxylate - Zinc coordination across His**

A closer look at the first reactive site residue and the carboxy-terminal sequences in relation to the monomer-dimer status reveals the following:

i. If the first reactive site in the dicot BBIs is Lys/Arg at the P_1 position and the carboxy-terminus contains an Asp/Glu residue, the BBI exhibits self-association to form dimers viz, HGI-III, BTCI, PsTI-IVb, CLTI-I, CLTI-II and BBI [Figure 4.12 and Table 5.2).
ii. If the P_1 position of the first reactive site is occupied by Ala in place of Arg/Lys as in the elastase inhibitors, SBI-C-II, WSTI-IV and PVI-3I, the BBI exists as a monomer in solution
iii. If the first reactive site is occupied by Lys/Arg at the P_1 position but lack Asp/Glu residues at the carboxy-terminal end, the BBI exists as a monomer in solution, e.g., HGGI-I, -II and –III, WII , MSTI, TcTI2 and TaTI and FBI.
iv. BBIs that have the Arg^{23} involved replaced by the other residues such as His or Gln or Ser yet exist as dimers or are self-associated in solution (DE-4, CLTI-I and CLTI-II).

These observations strongly support the fact that the unique interaction between the Arg/Lys at P_1 of the first reactive site of one subunit and Asp/Glu at the carboxyl-terminal of the second subunit is responsible for the observed self-association in BBIs. In HGI-III, this interaction is between Lys^{24} of one subunit and Asp^{76} of the second subunit. The loss of Asp^{76} of the HGI-III, during germination, to form HGGI-III, disrupts this interaction, which leads to HGGIs being monomers.

Table 5.2 **Comparison of self-association status of BBIs of leguminous seeds.**

Abbreviation*	**Monomer/ Dimer status**	**Enzyme inhibited at first reactive site**	**Reference**
WSTI-IV	Monomer	Elastase	Deshimaru *et al.*, 2002
PVI-3I	Monomer	Elastase	Funk *et al.*, 1993
PVI-3II	NR	Tryspin	Funk *et al.*, 1993
TcTI2	Monomer	Trypsin	Tanaka *et al.*, 1997
TaTI	Monomer	Trypsin	Tanaka *et al.*, 1996
BTCI	Dimer	Trypsin	Gennis and Cantor, 1976b
PI-II	NR	Trypsin	Hwang *et al.*, 1977
MSTI	Monomer	Trypsin	Catalano *et al.*, 2003
WII	Monomer	Trypsin	Brown and Ryan, 1984
FBI	Monomer	Trypsin	Asao *et al.*, 1991
PsTI-IVb	Dimer	Trypsin	Sierra *et al.*, 1999
HGI-III	Dimer	Trypsin	Prakash *et al.*, 1996
HGGI-I	Monomer	Trypsin	Kumar *et al.*, 2002
HGGI-II	Monomer	Trypsin	Kumar *et al.*, 2002
HGGI-III	Monomer	Trypsin	Kumar *et al.*, 2002

Table 5.2 contd....

Abbreviation	Monomer/Dimer status	Enzyme inhibited at first reactive site	Reference
GBI-II	NR	Elastase	Wilson and Laskowski, 1973
SBI-C-II	Monomer	Elastase	Odani and Ikenaka, 1977a
MBI	NR	Trypsin	Zhang *et al.*, 1982
DE-4	Dimer	Trypsin	Joubert, 1984
BBI	Dimer	Trypsin	Birk, 1985
MAI-DE-3	NR	Trypsin	Joubert *et al.*, 1979
LBI	Dimer	Trypsin	Haynes and Feeney, 1967.
CLTI-I	Dimer	Trypsin	Terada *et al.*, 1994a.
CLTI-II	Dimer	Trypsin	Terada *et al.*, 1994a.

*Abbreviations used are listed in Table 4.6 (Chapter 4).

Homology modeling of the HGI-III dimer

The sequences of HGI-III and HGGI-III are identical to each other except at the amino- and carboxy-termini, which clearly indicates that both the molecules exhibit the same subunit structure and the changes between them are restricted to the two termini. The models (Figure 5.11) reveal that individual subunits belong to the knottins fold (Murzin, *et al.*, 1995), formed by very few secondary structural elements, stabilized primarily by the seven disulfide bonds, similar to that in the template. HGI-III has an extension of 12 residues at the amino-terminus and an extension of 4 residues at the carboxy-

terminus, as compared to that in HGGI-III. The molecular model of HGI-III dimer indicates that the amino-termini of the subunits are situated at the surface of the dimer and any extension in this region would project into the solvent (Figure 5.11, inset) and this will not influence the stability of the dimer. The carboxy-termini on the other hand are located at the dimer interface and play an important role in the dimer stabilization. The interactions made by the carboxy-terminal segment is infact present in two-fold at the interface, due to the contributions from both the subunits. In particular, the carboxy-terminal Asp from one subunit (Asp-A76 and Asp-B76) forms salt-bridges with Arg from the other subunits (Arg-B31 and Arg-A31 respectively) as illustrated in Figure 5.11. The two Arg and Asp in turn are held in appropriate orientations for interaction by a further network of hydrogen bonds with their neighborhood, involving Gln^{19} and Lys^{24} in both the subunits respectively (Figure 5.11). These observations suggest that only when Lys^{24} of one subunit is hydrogen bonded to Asp^{76} of the second subunit, Arg^{31} (A subunit) and Asp^{76} (B subunit) are in juxtaposition to form the salt bridge resulting in the dimer. In the absence of this critical Lys^{24}-Asp^{76} hydrogen bond, Arg^{31} and Asp^{76} are not in a favored orientation, to form the salt-bridge. The situation is similar in the pea inhibitor and in fact in all the BBI structures known, indicating that the carboxy-terminal segment is the determining factor in dimer formation. In HGGI-III, Asp^{76} is not present due to the truncation of the polypeptide chain during germination (Figure 4.10, Chapter 4) and therefore cannot form the dimer, correlating well with the observation of a monomer from biochemical studies (Chapter 3).

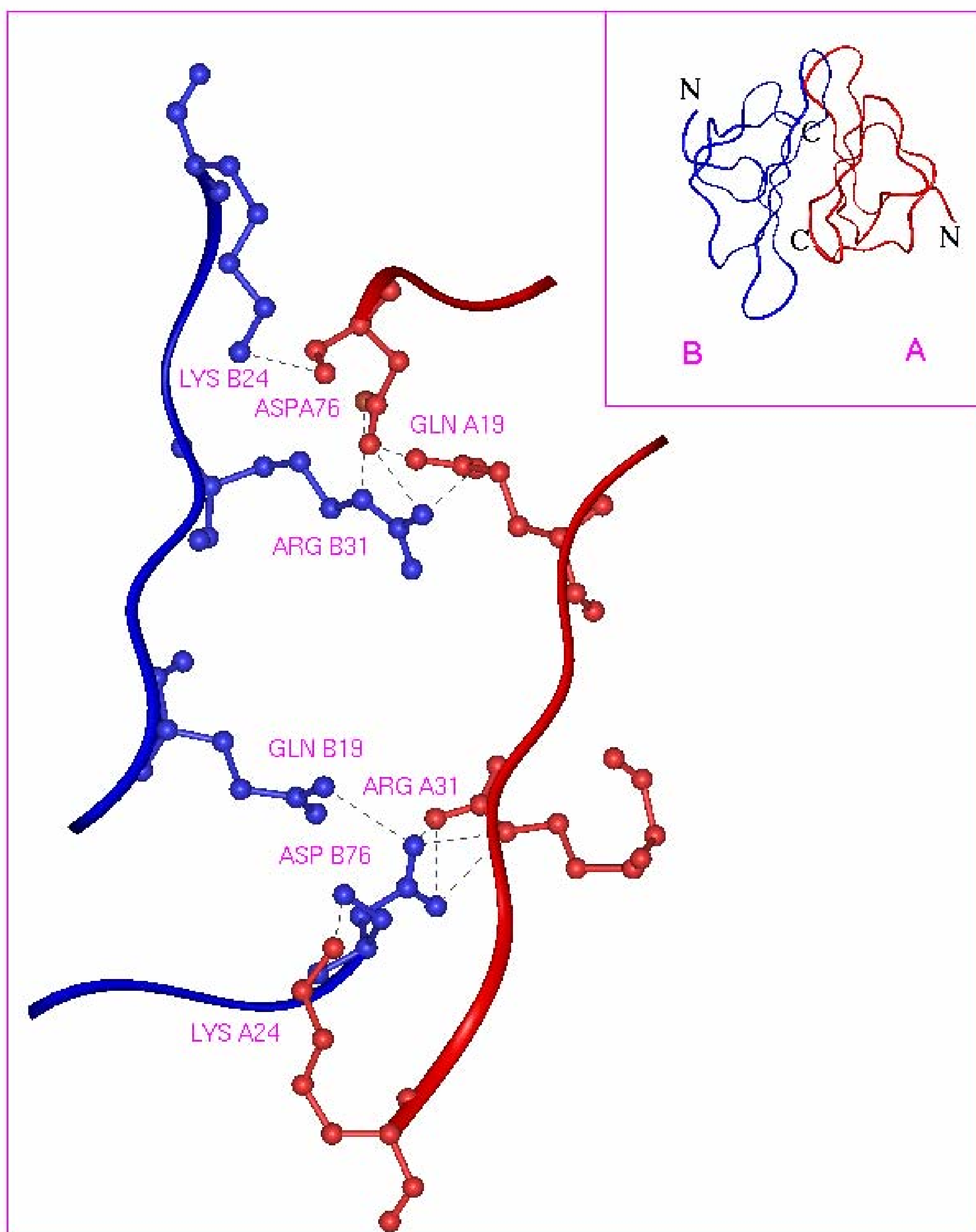


Figure 5.11 **Interactions at the dimer interface in HGI-III**. One monomer is shown in red while the other is in blue. Hydrogen bonds are indicated by dashed lines. The salt-bridges made by the carboxy-terminal Asp (Asp^{76}) in both subunits with Arg (Arg^{31}) from the opposite subunits can be clearly seen. The hydrogen bond between the carboxy-terminal Asp^{76} in both subunits with Lys^{24} from the opposite subunit is also visible. The inset represents the Cα atoms of the entire dimer by a ribbon diagram. The two subunits are labeled A and B and their amino- and carboxy-termini are indicated.

Thermal stability of HGI-III and HGGI-III

To understand the effect of the monomer/dimer status upon the physiological properties the thermal stability of the inhibitors was evaluated. Figure 5.12 represents the changes in trypsin inhibitory property at 95 °C. For HGI-III, relatively little or no changes in the trypsin inhibitory activity was observed upto 6 h (Figure 5.12). Although the stability of HGGI-III was comparable to HGI-III upto 3 h, HGGI-III lost 50 % of its activity after 6 h incubation at 95 °C. The decrease in the thermal stability lead to the suggestion that the dimeric form of the inhibitor is more stable than the monomer.

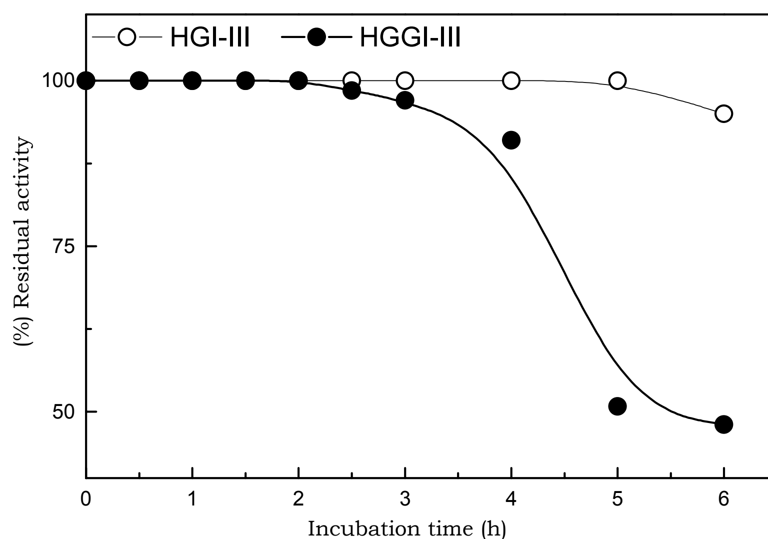


Figure 5.12 **Thermal stability profiles of HGI-III and HGGI-III at 95 °C**.

Discussion

The BBIs from soybean (Birk, 1985), kidney bean (Pusztai, 1968), black-eyed pea (Gennis and Cantor, 1976b) and lima bean (Haynes and Feeney, 1967) exhibit a tendency to self-associate, forming dimers and tetramers (Wu and Whitaker, 1990). Existence of the BBI from horsegram seed (HGI-III) as a dimer is observed on SDS-PAGE (Figure 5.3). The apparent higher M_r, observed for HGI-III, can be attributed to dimerization. The HGI-III, like red kidney bean BBI, exists as a dimer even at lower concentrations (2 μg/ml) (Wu and Whitaker, 1990). The HGGI-III, derived from HGI-III, exhibits no tendency to associate. The relative mobility on SDS-PAGE indicates that it is a monomer with a M_r of 6.5 kDa. A similar behavior is observed in size-exclusion chromatography (Figure 5.4). This distinguishes HGGI-III from HGI-III in its state of association. The amino acid sequence comparison of the two reveals the absence of 12 amino acid residues at the amino-terminus and 4 residues at the carboxy-terminus. The HGI-I, inspite of lacking the amino-terminus exists as a dimer (Sreerama *et. al.*, 1997). These observations point to the existence of a structural element, responsible for the self-association of HGI-III, in the deleted tetrapeptide -SHDD. The *in vitro* synthesized BBI, and related soybean inhibitor exhibit the phenomenon of self-association (Foard *et. al.*, 1982). Structural data obtained with BBIs from pea seed inhibitor have clearly shown the last 11-carboxy terminal residues of the molecule in the near-perfect two fold symmetric dimer (Sierra *et. al.*, 1999). The residues 68-70 constitute an extended β-strand, which makes no contact with its own subunit. The carboxy-terminal tail plays a major role in the dimeric association of PsTI-I and PsTI-II (Sierra *et al.*, 1999). Arg^{23} and Lys^{16} (P_1 residue of the first reactive site) of one sub unit are involved in the inter subunit contacts with Glu^{68} and Glu^{69} at carboxy-terminus of second subunit. The hydrophobic residues that are

exposed in the monomeric BBI of black-eyed pea inhibitors are buried in the multimers. The anomalous distributions of hydrophilic and hydrophobic amino acids do not thermodynamically favor their existence as monomers. The light scattering data also support such self-association of BBI from black-eyed pea (De Freitas *et. al.*, 1997).

SDS-PAGE analysis (Figure 5.9) and size-exclusion studies (Figure 5.8) of HGI-III, in the presence of 1 mM Zn^{2+}, further bring out the role of Asp residues at the carboxy-terminus. The carboxy-terminus of HGI-III, -His^{74} –Asp^{75} –Asp^{76} ligates Zn^{2+} in an indirect carboxylate–metal coordination, disrupting the interactions responsible for self-association. The indirect carboxylate–His–Zn^{2+} interaction occurs within several unrelated proteins. A His residue bridges both Zn^{2+} and carboxylate side chain of a nearby Asp (Christianson and Alexander, 1989).

Multiple sequence alignment of legume BBIs reveal that the P_1 residue and the first reactive site in all the BBIs is one of Arg, Lys or Ala (Chapter 4; Figure 4.12). This is consistent with the inhibition of trypsin or elastase at the first reactive site (Odani and Ikenaka, 1977a). This residue for HGI-III is Lys^{24} (Sreerama *et. al.*, 1997). Arg^{31} of HGI-III, corresponding to Arg^{23} of PsTI-IVb, is conserved in only a few BBIs (Chapter 4; Figure 4.12). In other BBIs, it is replaced by His, Ser, Glu or Val. These observations indicate that the self-association to form dimers involves inter subunit contact of Lys^{24} or Arg^{31} in HGI-III.

The role of Lys^{24} and Arg^{31} of HGI-III in the self-association was probed through a chemical modification approach and further confirmed by homology modeling (Figure 5.11). The modification of Arg using 1, 2-cyclohexanedione residues did not affect the state of

association (Figure 5.7). Modification of Lys residues using citraconic anhydride resulted in the monomeric form of HGI-III (Figure 5.7). These results confirm the involvement of Lys residues in the interaction. The replacement of Arg^{23} which contributes to the dimeric association in PsTI-IVb by Phe in BBI and Ala in A-II did not affect the state of association. All the BBIs that inhibit elastase at the first reactive site wherein Lys/Arg is replaced by Ala exist as monomers in solution (Table 5.2). The BBIs that inhibit trypsin at the first reactive site (having Lys/Arg) but lack the carboxy-terminal Asp/Glu residues exist as monomers. HGGI-I, II and III which inhibit trypsin and are lacking in the carboxy-terminal (Chapter 4, Figure 4.10) exist as monomers in solution (Kumar *et. al.*, 2002). The BBIs that inhibit trypsin at the first reactive site and possess Asp/Glu residues at the carboxy-terminal exist as dimers in solution (Table 5.2). These observations strengthen the premise that the interaction between Lys and Asp is the essence of self-association of HGI-III. This observation can be further extended to other BBIs whose primary structure and state of association are reported (Table 5.2).

The structure of soybean BBI determined by two-dimensional ^1H nuclear magnetic resonance spectroscopy and dynamical simulated annealing (Werner and Wemmer, 1992), also reveals that the antichymotryptic domain at the carboxy-terminus is fully exposed and is presumably the location of the self-association surface of BBI. The second trypsin inhibitory domain of P-II, analogous to the chymotrypsin inhibitory domain has numerous crystal contacts between protein molecules in the trimers (Chen, 1989*) in support of the notion that the antichymotrypsin domain at the carboxy-terminus is the location of the self-association surface. The dimer model (Figure 5.11) clearly discerns the involvement of Asp^{76}, the carboxy-terminal

residue of HGI-III in contacts between the monomers. Of these hydrogen bond between Lys^{24}-Asp^{76} provides the correct orientation for the formation of the Arg-Asp salt-bridge between the subunits.

The association of BBIs caused by this unique interaction between two monomers must be only of the monomer↔dimer type with little or no higher forms present as is observed from the model (Figure 5.11). The self-association of soybean BBIs, found to be of the monomer-dimer type only, increases with ionic strength and is consistent with the theoretical electrostatic free-energy changes (Harry and Steiner, 1970). Considerably high salt concentrations (> 1 M) are required to disrupt the HGI-III dimers (results not shown). NMR analysis indicates that MSTI, - a BBI of Snail Medic seeds, a monomer in solution (Table 5.2) can undergo self-association at concentrations higher than 2 mM. The residues involved in this mechanism are localized at opposite faces of the molecule, having the highest positive and negative potential. It is to be emphasized that the interactions involved in such self-association (Catalano *et al.*, 2003), that are concentration dependent are different from the self-association described here. The hydrogen bond observed between Lys^{24} (of one subunit) and Asp^{76} (of the second subunit) in the modeled dimer (Figure 5.11) is the determinant for self-association of HGI-III. In the presence of denaturing agents like 6 M GuHCl, that disrupts such interactions, HGI-III exhibit a M_r ~8.5 kDa indicating it is a monomer (Sreerama *et al.*, 1997)

The dimer formation may sterically block the chymotrypsin binding site on one monomer. This, probably, accounts for the number of chymotrypsin bound to HGIs determined by activity titration, being less than unity (Sreerama *et al.*, 1997). The HGGIs, which are

monomers, show a 1:1 ratio for both trypsin and chymotrypsin, respectively (Kumar *et al.*, 2002). As already mentioned HGI-III (Chapter 4, Figure 4.10) is highly homologous with HGGI-III, but lacks the carboxy-terminal tetrapeptide –SHDD. The carboxy-terminal portion of BBIs has been suggested to stabilize conformation of the second reactive site and protect it from enzyme attack. The dimer is more thermostable than HGGI-III (Figure 5.12). Most of the exposed hydrophobic residues of the monomeric PsTI-IVb (Sierra *et al.*, 1999) and BTCI (De Freitas *et al.*, 1997) are shielded from the solvent by the other subunit in the dimer. This probably accounts for the increased thermal stability of the HGI-III and is indicative of a functional aspect of the dimerization.

In conclusion using alternate approaches to site directed mutagenesis like producing deletion variants of the inhibitor by germination, can also be used to identify the role of individual amino acid residues in the structure and stability of the inhibitor. The results presented in this study are a clear evidence of the self-association in HGIs being due to a unique interaction between the ε-amino group of Lys^{24} (P_1 of the first reactive site) and the carboxy-side chain of Asp^{76}, at the carboxy-terminus. Furthermore this unique interaction also occurs in all the BBIs that self-associate to form dimers. This self-association is vital to the physiological and functional role of BBIs as it leads to increased thermal stability and greater resistance to enzyme attack by stabilizing the conformation of the second reactive site at the carboxy-terminal domain.

The purification and characterization of a metalloproteinase from germinating horsegram seeds: Role in *in vitro* proteolysis.

It is evident that the new active species HGGI-I, -II and –III in germinated horsegram seeds are proteolytically cleaved products of HGI-III (Chapter 3 and 4). The degradation of HGIs in horsegram seeds during germination and early seedling growth begins with a limited proteolysis as evidenced by the 95 % sequence identity between HGI-III and HGGIs (Chapter 4, Figure 4.10). The differences occur at the amino- and carboxy-termini. The cleavages appear to be characterized by a protease that is specific and inducible during germination. This chapter describes the purification and characterization of a proteinase purified from germinating horsegram seeds and its involvement in the proteolysis of HGIs.

RESULTS

Trypsin/chymotrypsin inhibitory activity profile during germination of horsegram seeds.

Changes in the trypsin and chymotrypsin inhibitory activity and extractable protein during germination of horsegram seeds were studied. Crude extracts were prepared from 5 g of cotyledons at different stages of germination in Gly-HCl buffer, pH 2.5. The imbibed seeds (24 h) are considered as 0 h of germination. A gradual decline in the trypsin and chymotrypsin inhibitory activity was observed (Figure 6.1). The decrease in the two activities paralleled each other. This decrease was accompanied by a concomitant decrease in the total protein. In contrast no change was observed in the specific activity *per se* up to 48 h of germination. A steep increase in the specific activity (Figure 6.2) occurs between 48-72 h germination, with a sharp decrease in the extractable total protein (Figure 6.1).

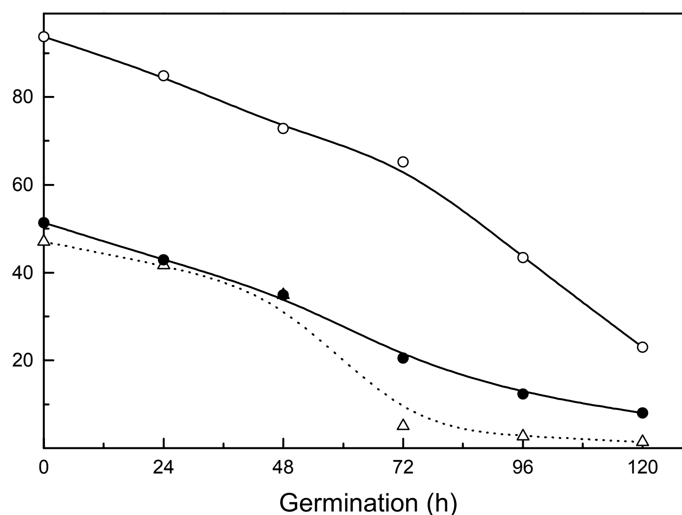


Figure 6.1 **Changes in the protein, trypsin and chymotrypsin inhibitory activity during germination of horsegram seeds.** Total protein in mg (—Δ—); Total trypsin inhibitory units × 10^4 (—•—) and Total chymotrypsin inhibitory units × 10^3 (—○—).

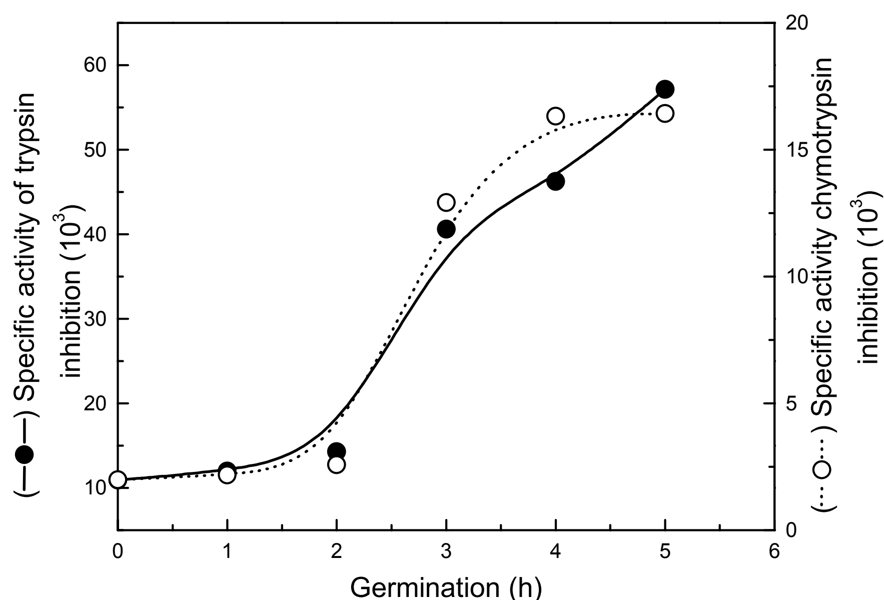


Figure 6.2 **Specific activity profile of trypsin and chymotrypsin inhibition during germination of horsegram seeds.**

The crude extract was analyzed for the inhibitors on native gelatin-PAGE (Figure 6.3). The four inhibitors of the dry seed (HGIs) are still present after 24 h of germination. The four dry seed inhibitors completely disappear from the medium after 96 h of germination. The two inhibitors, HGGI-I and HGGI-II appear after 24 and 72 h germination of horsegram seeds respectively. However HGGI-III appears at 72 h of germination as revealed by native PAGE (results not shown) as described in section (2.2.6.3). HGGI-III was found to be the major inhibitor form present after 120 h of germination.

Changes in the proteinase activity during germination

The total proteolytic activity during germination of horsegram seeds was mapped by assaying the activity of crude extracts obtained from 5 g cotyledons using the storage proteins of horsegram as the substrate.

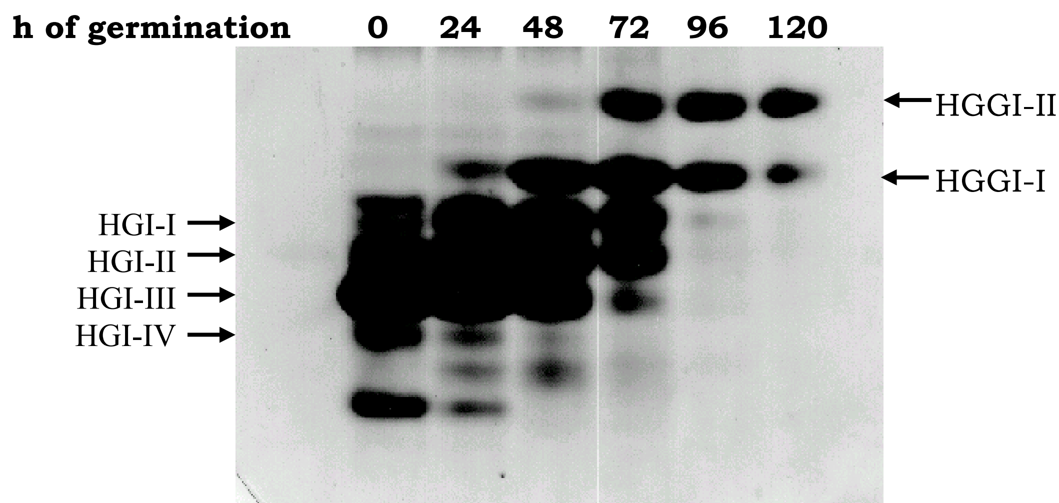


Figure 6.3 **Gelatin-PAGE (10 % T, 2.7 % C) of crude extracts prepared from germinating horsegram seeds.**

The total proteinase activity and specific activity profile of germinating horsegram is shown in Figure 6.4. Proteolytic activity was low at the early stages of germination (0-48 h), but increased sharply from 48 h to 96 h of germination. This was followed by a considerable decline in the proteinase activity. A sharp increase in the specific activity was observed from 48 h to 120 h. The continuous increase in the specific activity can be related to decrease in the protein content of the cotyledons with germination (Figure 6.1).

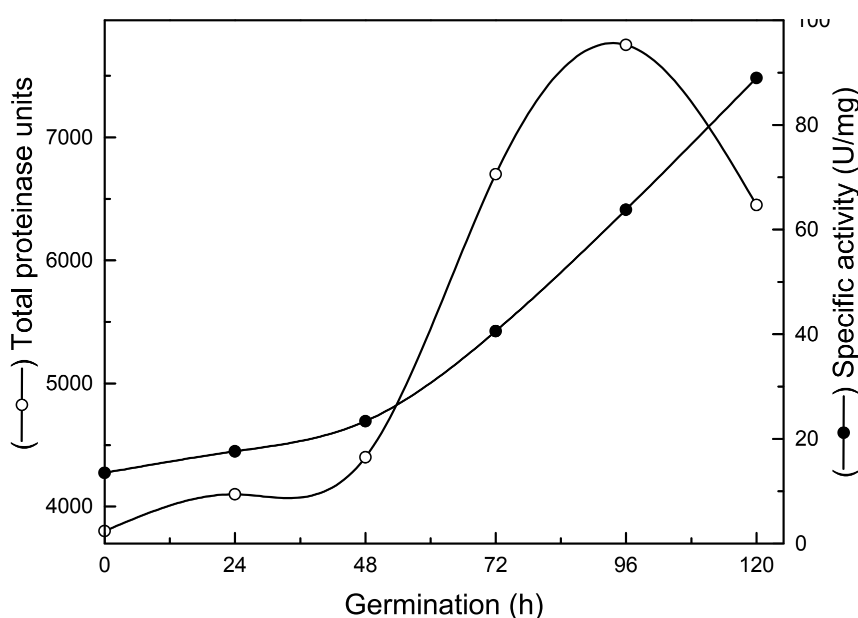


Figure 6.4 **Proteinsae activity and specific activity profile of horsegram seeds during germination**.

To characterize the number of proteinases that appear during germination of horsegram seeds, the crude extracts were analyzed by PAGE followed by an overlapping gel assay (Section 2.2.6.5) and visualized by staining with CBB R-250 (Figure 6.5). The zymogram revealed the presence of a single proteinase in the imbibed seed with a R_f of 0.27 (Figure 6.5). A proteinase that appeared at 24 h of germination disappears rapidly. The two major proteinases that are

detected differ in their temporal expression (Figure 6.5, R_f 0.19 and 0.48). Of the two, the proteinase with a lower R_f (0.19) appears after 48 h of germination whereas the proteinase with higher R_f (0.48) is detected after 72 h of germination. Both continue to be the major proteinases expressed and could be responsible for the degradation of the storage proteins in the cotyledons. The appearance of these proteinases is concomitant with the decline with the total protein (Figure 6.1).

Germination (h)

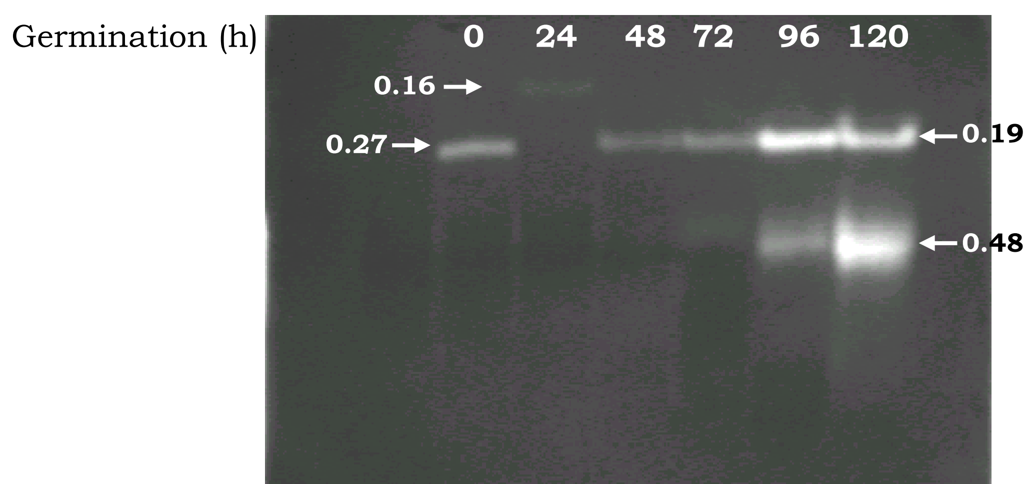


Figure 6.5 **Proteinase activity profile of germinating horsegram seeds.**

Partial purification and characterization of a proteinase from 120 h germinated seeds

The major proteinase (Figure 6.5, R_f 0.48) present in the 120 h germinated horsegram seeds was purified partially by extraction at pH 2.5, $(NH_4)_2SO_4$ fractionation and size-exclusion chromatography on Sephadex G-50. The Sephadex G-50 elution profile is shown in Figure 6.6. Each fraction was assayed for proteinase activity as described earlier (Section 2.2.28.2). The single symmetrical protein peak that eluted immediately after the void was pooled, dialyzed against 5 mM

Tris-HCl, pH 7.5 (5 × 500 mL) and concentrated by lyophilization. This purified proteinase sample was used for further studies.

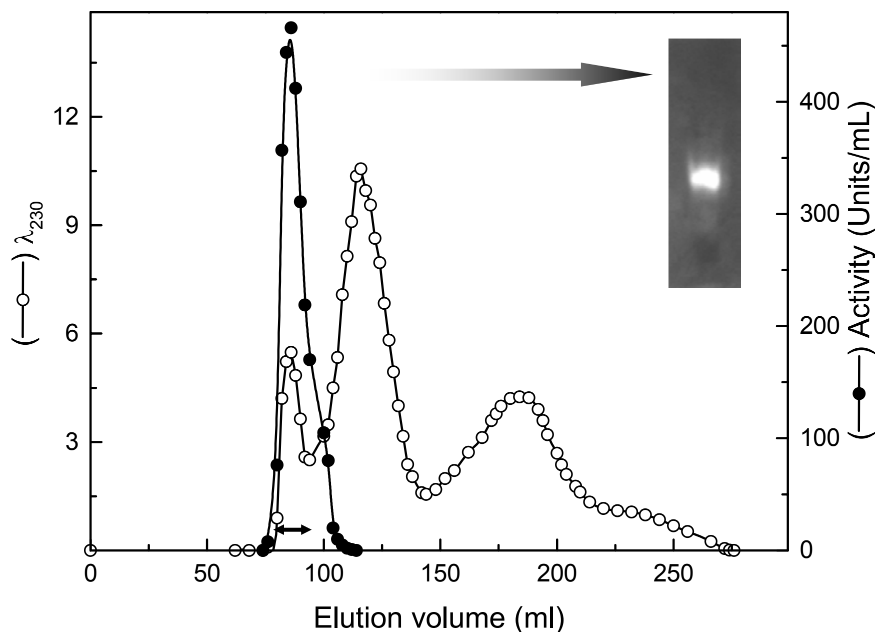


Figure 6.6 **Elution profile of proteinase on Sephadex G-50 chromatography.** The $(NH_4)_2SO_4$ fraction was loaded on a Sephadex G-50 column (100 × 2.1 cm) pre-equilibrated with 25 mM Tris-HCl, pH 7.5. Elution was carried out using the same buffer at a flow rate of 12 mL/h. Two mL fractions were collected. The active fractions were pooled as shown (◄──►). Inset: Overlaid gelatin-PAGE showing the proteinase activity.

The purified proteinase preparation was subjected to native-PAGE followed by detection using the gelatin gel overlay technique (Section 2.2.6.5). The purified enzyme preparation was apparently homogenous. The proteinase moved as a single species, appearing as a clear transparent band against a dark blue background (Figure 6.6, inset).

Substrate specificity

The substrate specificity of the proteinase was investigated. Proteinase substrates, BSA, casein, gelatin, denatured hemoglobin and

ovalbumin of animal origin and glycinin and storage proteins of horsegram seed of plant origin were studied at a final concentration of 1 % (w/v) in 0.1 M Tris-HCl buffer pH 8.0. The highest proteinase activity was observed against horsegram seed proteins followed by glycinin, the major storage protein of soybean (Table 6.1). No measurable activity could be detected with any of the proteins of animal origin. The proteinase appears to be highly specific, exhibiting significant activity towards its natural plant protein substrate with little or no activity towards animal proteins commonly used as substrate. This indicates a role in the mobilization of storage protein(s) during germination. All further studies on the proteinase were carried out using 1 % (w/v) horsegram protein as the substrate.

Table 6.1 **Effect of various proteins as substrates**

Substrate	Enzyme units
BSA	1.0
Casein	0
Gelatin	0
Denatured hemoglobin	1.5
Ovalbumin	1.0
Azo-casien	1
Glycinin	17.2
Horsegram seed proteins	18.5

Effect of pH

The effect of pH on the proteinase activity was studied at various pH, 3.0-10.0 at 0.1 M ionic-strength (Gly-HCl, pH 3.0; sodium acetate, pH 4.0, 5.0 and 6.0; sodium phosphate, pH 7.0; Tris-HCl, pH 8.0 and

Gly-NaOH, pH 9.0 and 10.0) using horsegram seed proteins as substrate at 37 °C. The plot of pH against relative activity is shown in Figure 6.7. The proteinase had a pH optimum of 9.25. The enzyme was relatively inactive between pH 4-8. Half the maximal activity was observed at pH 9.0 and 10.0. This alkaline pH optimum with the natural substrate is an indication of a cytosolic localization.

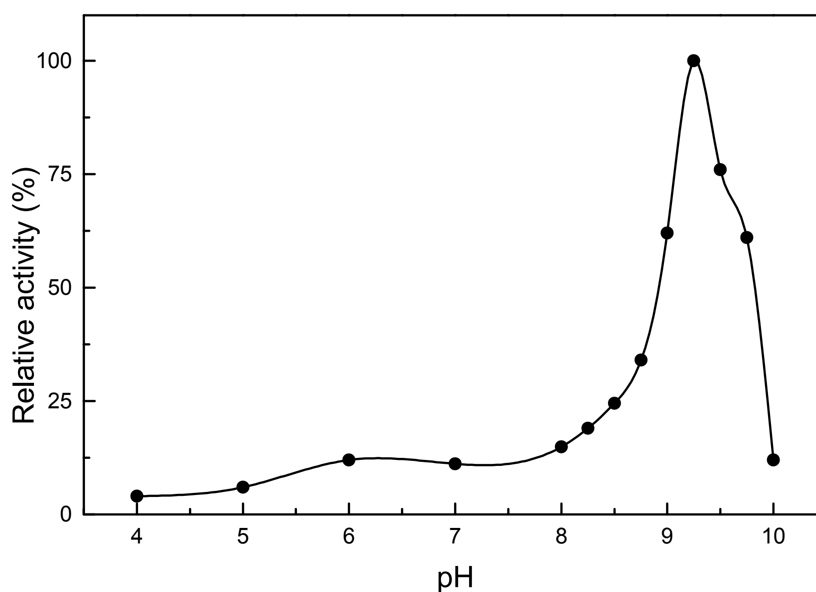


Figure 6.7 **Effect of pH on the proteinase activity.** Buffers used were Gly-HCl (pH 3.0), sodium acetate (pH 4.0 - 6.0), sodium phosphate (pH 7.0), Tris-HCl (pH 8.0) and Gly-NaOH (pH 9.0 - 10.0).

Effect of temperature

The optimum temperature for proteinase activity was evaluated by assaying the enzyme at various temperatures (4 °C - 60 °C) at pH 9.25 using 0.1 M Gly-NaOH buffer. The temperature dependence of the proteolysis of horsegram seed proteins is shown in Figure 6.8. Maximal proteolysis of horsegram seed proteins was observed at 37 °C. The activity decreased markedly, at temperature greater than 37 °C, loosing 50 % of its activity at 45 °C.

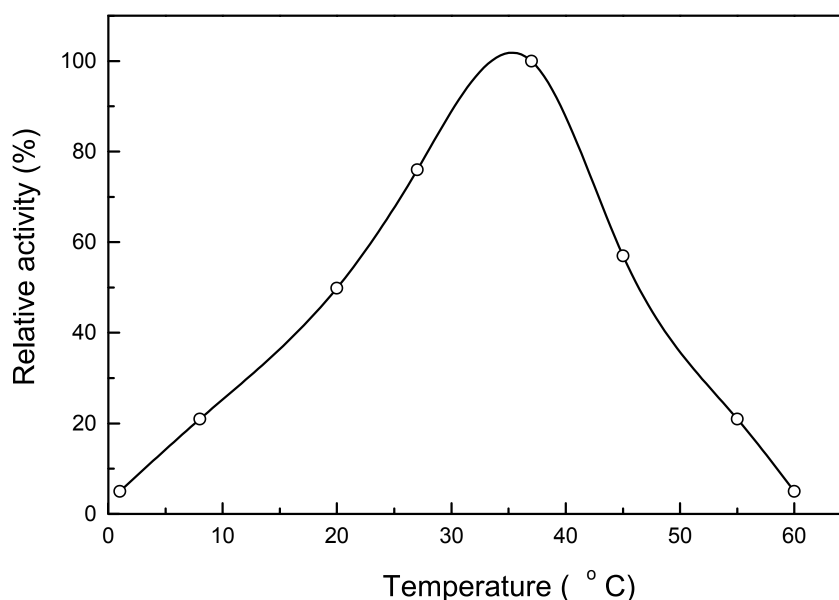


Figure 6.8 **Effect of temperature on the horsegram proteinase activity.** The proteinase was assayed using horsegram seed proteins at indicated temperatures in 0.1 M Gly-NaOH buffer, pH 9.25.

pH stability of proteinase

The effect of pH on the stability of the proteinase was studied by incubating the proteinase in buffers of various pH (0.1 M Gly-HCl, pH 3.0; 0.1 M citrate, pH 5.0; phosphate, pH 7.0 and 0.1 M Gly-NaOH, pH 9.0) at 37 °C. At regular intervals aliquots were removed and assayed for residual proteinase activity. The stability of metalloproteinase at different pH as function of time is shown in Figure 6.9. The proteinase was stable over a period of 24 h (results till 4 h shown) at pH 7.0 retaining 100 % of its activity. Although the proteinase exhibits a pH optima of 9.25, it looses 20-25 % of its activity at pH 9.0. The stability of the enzyme decreased at pH 3 and 5 loosing 30 % of its activity (Figure 6.9). The proteinase was stable at room temperature (25 ± 2 ° C) at pH 7.5 retaining 100 % of its activity over a period of 48 h.

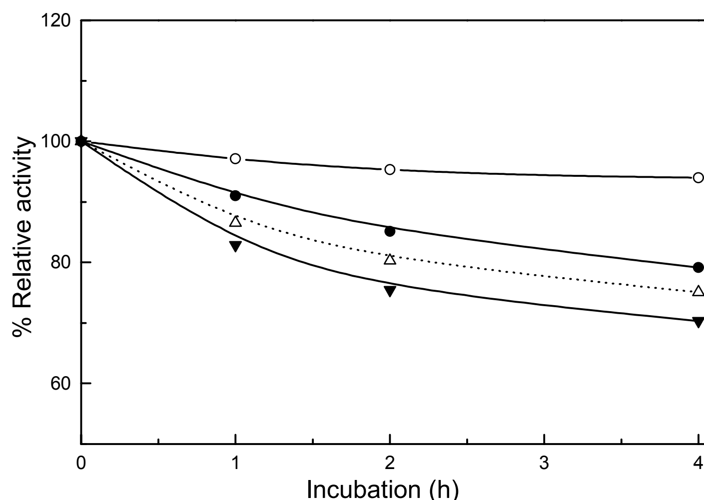


Figure 6.9 **pH stability of proteinase of germinated horsegram seeds**. The proteinase was incubated at 37 °C in different buffers and the residual activity measured at pH 9.25 using horsegram seed storage protein as substrate. (—▼—) Gly-HCl, pH 3.0; (—Δ—) citrate, pH 5.0; (—○—) phosphate, pH 7.0 and (—●—) Gly-NaOH, pH 9.0.

Effect of class specific inhibitors

The effect of acidic, serine, cysteine and metalloprotease class specific inhibitors is summarized in Table 6.2. The proteinase was strongly inhibited by the metal-chelator, EDTA, but the activity was not affected by either pepstatin A, PMSF or iodoacetamide. These data suggest that the enzyme belongs to the class of metalloproteinases. The three inhibitors HGGI-I, -II and -III did not inhibit the proteinase. These results confirm that the proteinase does not belong to the serine proteinase class.

Effect of divalent metal ions on metalloproteinase activity

The data (Table 6.2) are consistent with the proteinase being a metalloproteinase. Hence the effect of divalent metal ions such as Mg^{2+}, Zn^{2+}, Ca^{2+} and Ba^{2+} as effectors, on the activity of the proteinase was evaluated. The proteinase was preincubated at 1 mM concentration of the divalent metal ions and assayed for proteinase activity under

optimum conditions (37 °C and at pH 9.25). The results show that the addition of Mg^{2+} increases the activity about two-fold (Table 6.3). However Ca^{2+} had no effect. In contrast in the presence of Zn^{2+} and Ba^{2+} the enzyme showed lower activity. These results suggest that the protease is a Mg-dependent metalloproteinase.

Table 6.2. Effect of class specific inhibitors on the proteinase activity.

Inhibitor	Protease Specificity	Concentration (mM)	Relative activity (%)
EDTA	Metallo	10.0	0
Pepstatin-A	Acidic	0.0043	87
Iodoacetamide	Cysteine	0.270	92
PMSF	Serine	1.0	93
HGGIs			
HGGI-I	Trypsin	0.0015	100
HGGI-II	Trypsin	0.0015	100
HGGI-III	Trypsin	0.0015	100

Table 6.3 Effect of divalent metal ions on metalloproteinase activity.

Divalent metal ion	Relative activity (%)
Ba^{2+}	67
Zn^{2+}	78
Mg^{2+}	182
Ca^{2+}	107

***In vitro* proteolysis of horsegram inhibitors**

These studies were aimed at understanding the degradation mechanism of the HGIs during germination. *In vitro* cleavage of horsegram inhibitors (HGIs) was performed employing the purified metalloproteinase. Among the four dry seed inhibitors HGI-I, HGI-III and HGI-IV were used for *in vitro* cleavage. One hundred microgram of each of the HGIs was incubated with three units of metalloproteinase in 0.1 M Gly-NaOH buffer, pH 9.25 containing 1 mM $MgCl_2$ for 24 h at 37 °C. The products of the digestion were evaluated by gelatin embedded-PAGE. The degradation products derived from *in vitro* cleavage of HGIs are shown in Figure 6.10. HGI-III, the major isoinhibitor present in the dormant seed on cleavage with metalloproteinase leads to appearance of new electrophoretic forms with a lower R_f. Among the three new active species, the major degradation product corresponds to HGGI-I (Figure 6.10, Lane 5). The digestion of HGI-I with the proteinase led to the formation of a species whose R_f was comparable to that of HGGI-I (Figure 6.10, Lane 3), however cleavage did not occur to same extent as that of HGI-III. Complete disappearance of the HGI-III and HGI-I were not observed. In contrast HGI-IV was degraded completely (Figure 6.10, Lane 7). These results suggest that among the inhibitors the preferred substrate was HGI-IV.

Effect of EDTA on germination of horsegram

The proteinase purified from germinating horsegram is a metalloproteinase requiring Mg^{2+} ions for its activity. The effect of EDTA on the germination of horsegram seeds was studied. The horsegram seeds were imbibed in water containing 10 mM EDTA for 24 h and germinated in the dark for 120 h. The cotyledons were dissected free of seed coat and axis and analyzed on gelatin-PAGE. The differences in

the spatial and temporal appearance of HGGIs during the process of germination of horsegram seeds with and without metal chelator, EDTA is shown in Figure 6.11 and 6.12. Germination in absence of EDTA follows the usual degradation pathway, wherein a gradual decline in the inhibitory activity was observed with formation of new inhibitors (Figure 6.1 and 6.3). However in presence of EDTA, the degradation of HGIs is inhibited. The results show that the presence of EDTA did not inhibit the conversion of HGIs to HGGI-I (Figure 6.11 EDTA: Lane 2 and 4). However further cleavage of HGGI-I to form HGGI-II and -III was inhibited. In contrast in the absence of EDTA, HGGI-II is formed (Figure 6.11, Control: Lane 2 and 4). These results implicate that the Mg^{2+} requiring metalloproteinase may not involved in the initial degradation of HGIs to HGGI-I. In the presence of EDTA marked difference was observed in the trypsin inhibitor activity after 120 h of germination (Figure 6.12). However, in absence of EDTA the trypsin inhibitory activity was ~ 15 % of the original.

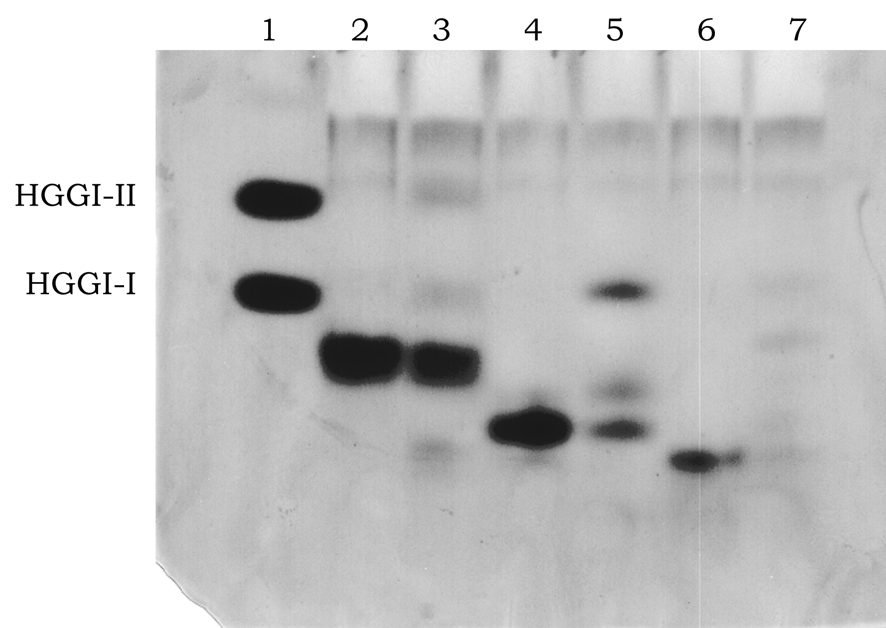


Figure 6.10 ***In vitro* proteolysis of horsegram inhibitors.** HGGI-I and -II (lane 1); HGI-I, -III and -IV (lane 2, 4 and 6) and HGI-I, -III and -IV digested with proteinase for 24 h at 37 °C (lane 3, 5 and 7) respectively.

In the presence of EDTA initial sprouting of the seeds occurred after 24 h and no morphological changes were visible after 120 h of germination. In contrast in the absence of EDTA, germination was normal with appearance of leaves and roots. These results suggest that these inhibitors serve as nitrogen sink source during germination and seedling growth.

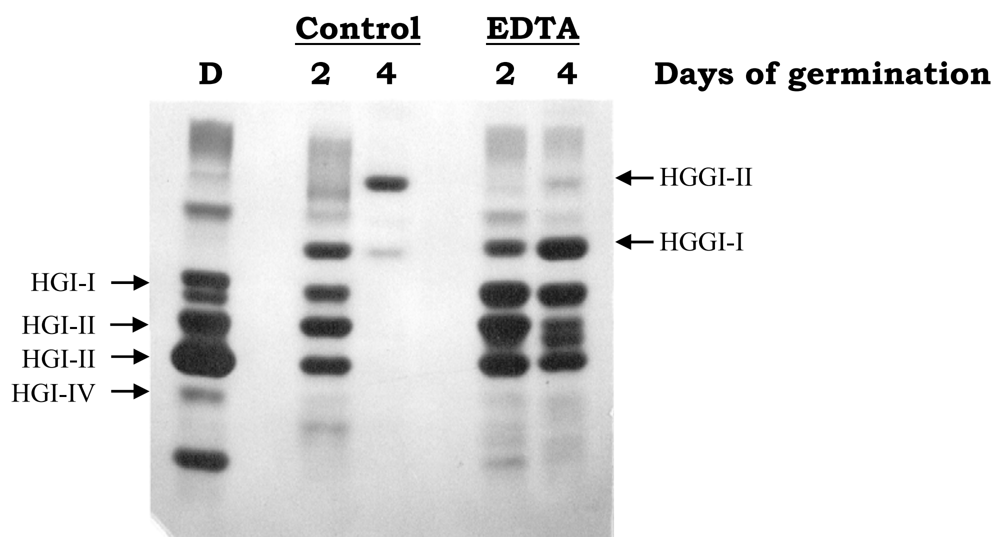


Figure 6.11. **Effect of EDTA on the degrdation of horsegram inhibitors.** D: dry seed inhibitors

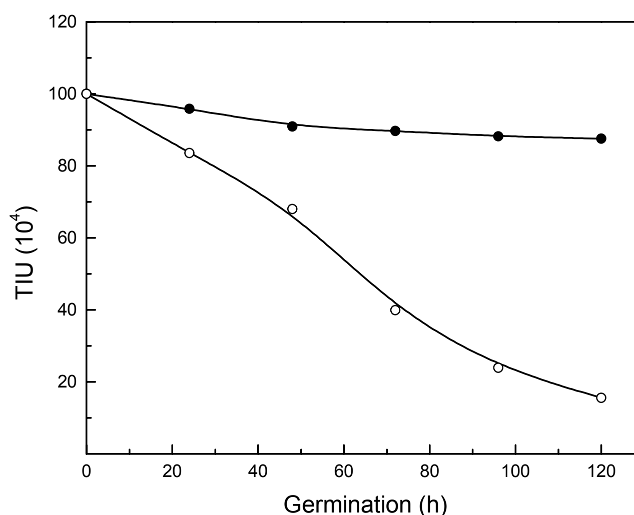


Figure 6.12 **Changes in the total trypsin inhibitory activity during germination of horsegram seeds in presence (—●—) and absence (—○—) of 10 mM EDTA.**

Discussion

During germination and early seedling growth the large amount of reserve protein in legumes are degraded to supply the developing plant with energy and metabolic precursors. The legume seeds are also noted for their large content of proteinase inhibitors (Laskowski and Kato, 1980; Ryan, 1973) and have been suggested to serve as storage depots particularly the sulfur containing amino acids.

Sreerama and Gowda (1998) reported the disappearance of the BBIs of horsegram (HGIs). Concomitant with the disappearance of the HGIs was the appearance of new Bowman-Birk type inhibitors, HGGIs. Earlier results (Chapter 3 and 4) indicate that the HGIs undergo extensive and specific proteolysis during the early stages of germination. The decrease in the inhibitor levels *per se* of horsegram seed is consistent with a steep decrease in the total protein content (Figure 6.1). In contrast as the level of these inhibitors decreased during germination (Figure 6.1), the specific activity of these inhibitors increased five fold. The K_i for the trypsin inhibition (Table 3.3, Chapter 3) of these inhibitors indicates that these are of higher potency. The increased specific activity of these inhibitors is probably highly desirable for the developing plants as they function as allelochemicals against microbial, insect, avian and mammalian predators.

Most of the studies on the fate of seed inhibitors during germination have concentrated on quantitation of inhibitor levels (Wilson, 1981; Gennis and cantor, 1976b; Hobday *et al.*, 1973; Lorensen *et al.*, 1981; Wilson and Tan-Wilson, 1983a). The actual mechanism of inhibitor disappearance has not been studied in detail in legume plants. Initial observations on inter-conversion of inhibitors were first observed from the results of kidney bean (Pusztai, 1972;

Wilson, 1981). Later Tan-Wilson *et al.*, (1982) and Wilson and Chen (1983) studied cleavage of BBI during germination of soybean and mung bean respectively.

A definite and gradual decline in the amount of inhibitors in the cotyledons of soybean during germination was observed. However throughout the period of germination the amount of inhibitor per gram fresh weight remained relatively constant (Tan-Wilson *et al.*, 1982). The conversion of the native horsegram inhibitors, HGIs is essentially complete within 96 – 120 h leading to formation of HGGI-I, -II and –III. No further changes were observed after 120 h as the cotyledons undergo senescence and abscission. The cotyledons of horsegram like mung bean rapidly undergo senescence and abscission therefore serving primarily only as storage organs (Madden *et al.*, 1985).

The decline in the inhibitory activity is accompanied by an increase in the proteinase activity (Figure 6.1 and 6.4). The sharp decline in protein between 48-96 h of germination is accompanied with a ~4.5 fold increase in the specific activity of the total proteinases (Figure 6.4). These results suggest that a temporal and spatial correlation does exist between the synthesis of the proteinases and protein degradation. The germinating horsegram seeds contain at least four proteinase activities (Figure 6.5, R_f 0.27, 0.16, 0.19 and 0.48) of which one proteinase (R_f 0.27) occurs in the imbibed seeds. The major proteinase (R_f 0.48) is synthesized from 72 h of germination and accumulates rapidly thereafter 96 and 120 h (Figure 6.5). Vicilin peptidohydrolase, a major endopetidase, in the mung bean is known to be synthesized form the 3rd day of germination and accumulates rapidly during the 4th day and 5th days (Baumgartner and Chrispeels, 1976). Proteinase F responsible for converting the mung bean

inhibitors, MBTI-F to MBTI-E is present in both dry as well as the germinated seeds (Wilson, 1988). In the period when most of the inhibitory activity was lost in the cotyledons of horsegram seeds (72 h), a considerable amount of endogenous proteinase activity accumulated (Figure 6.1 and 6.4). In adzuki beans an endopeptidase activity increased in the cotyledons (Yoshikawa *et al.*, 1979a) during seedling growth.

The major proteinase (R_f 0.48; Figure 6.5) was partially purified by size-exclusion chromatography. The purified proteinase appears to be remarkably specific towards its own storage protein, exhibiting significant activity (Table 6.1) towards its natural storage protein with little or no activity towards animal protein commonly used as substrates. Reilly *et al.*, (1978) have described an endopeptidase from pumpkin (*Cucurbita moschata*), which cleaves the legumin type globulin of the pumpkin seed but is inactive towards bovine casein, hemoglobin and serum albumin. A similar situation is found in mung bean (*Vigna radiata*) (Wilson and Tan-Wilson, 1983a).

The ability to act on storage protein of ungerminated seeds suggests that the proteinase initiates the mobilization of storage proteins in horsegram seeds. Vicilin peptidohydrolase of mung bean (Shutov and Vaintraub, 1987), proteinase A of Vetch (*Vicia sativa*)(Shutov *et al.*, 1978; Bulmaga and Shutov, 1977; Shutov *et al.*, 1984b) and wheat (Shutov *et al.*, 1984a) and proteinase P-Ia of corn (*Zea mays*) (Fuzimaki *et al.*, 1977; Abe *et al.*, 1977; Abe *et al.*, 1978a), hydrolyze their own storage proteins. These proteinases are not found in the dry seeds and appear after the onset of germination and their activity increases during germination. Evidently the proteinase of

horsegram like these proteinases has a common function i.e. to initiate storage protein mobilization and participate in further hydrolysis.

The decidly alkaline pH optima of 9.25 of the purified proteinase (Figure 6.7) is suggestive of a cytosolic location. A number of studies using differential or density gradient centrifugation provide evidence that the Bowman-Birk inhibitors are localized in the cytosol (Pusztai, *et al.*, 1977; Chrispeels and Baumgartner, 1978; Miege *et al.*, 1976). Ultra centrifugation in glycerol gradient have shown nearly 50 % of the BBIs are present in the cytosol. These observations together with the alkaline pH optima suggest the involvement of this proteinase in the degradation of BBIs during germination. *In vitro* studies with partially purified proteinase indicate its ability to degrade the dry seed inhibitors, HGI-III and HGI-IV (Figure 6.10). Where as no activity was noted towards HGI-I. Proteinase F of mung bean with an acidic pH optimum 4.5 to 5.0 was shown to hydrolyze the Asp^{76}-Lys^{77} bond of MBTI-F to produce MBTI-E. No activity was noted towards MBTI-E.

Complete inhibition by EDTA and significant stimulation by exogenous Mg^{2+} is suggestive of the enzyme being a metalloproteinase (Table 6.2 and 6.3). The enzyme was not affected by other class specific proteinase inhibitors. A temporal and spatial correlation exists between the development of an SH-dependent acid endopeptidase in germinating seeds of cowpea (*Vigna unguiculata*) (Harris *et al.*, 1975). A SH-dependent endopetidase that appears in germinating castor seeds localized in the vacuoles and formed after the fusion of protein bodies, hydrolyzes the 11S protein of the ungerminated castor seed. A metallo-endopeptidase containing Zn^{2+} with an optimum pH of 8-8.2 exists in germinating buckwheat. The enzyme localized in the protein bodies cleaves peptide bonds, which contains Leu, Tyr or Phe in the P_1

position (Belozerski *et al.*, 1990) and cleaves legumin prepared from mature buckwheat as well as soybean seeds. The purified proteinase of germinating horsegram with a alkaline pH optima, able to hydrolyze *in vitro* the storage protein of ungerminated seeds is a metalloproteinase. This proteinase differs from the proteinases reported in germinating seeds of other legumes and cereals.

Trypsin/chymotrypsin inhibitors of horsegram (*Dolichos biflorus*) flowers and leaves: Purification and characterization

Initial studies carried out by Sreerama and Gowda, (1998) indicated that the two inhibitor forms present in flower, leaf, husk and early stages of seed development have apparently similar electrophoretic mobilities to those present in 120 h germinated horsegram seeds. However it is not clear whether the electrophoretically similar forms present in flower and leaf are similar to those present in the germinated horsegram seeds (HGGIs) or are precursor molecules of dormant seed inhibitors (HGIs). Therefore the inhibitors from the horsegram flower and leaf were purified. The results on the purification and characterization of these inhibitors are presented in this chapter.

RESULTS

Purification of horsegram flower and leaf inhibitors

The two inhibitors present in the flowers and the tender leaves were purified from their respective acetone powders (Section 2.2.27) in separate batches.

Extraction of inhibitors

Five grams of flower/tender leaf acetone powders were suspended in a minimal volume of 0.1 M Gly-HCl, pH 2.5 buffer, ground to a fine paste, made up to 50 mL with the same buffer and extracted for 14-16 h at 4 °C. The extract was filtered through cheesecloth and centrifuged at 10, 000 × g at 4 °C for 30 min. The clear supernatant obtained was titrated to pH 7.0 with liquor ammonia and dialyzed extensively against water (6 × 500 mL). The salt free crude extract of flower/leaf was concentrated by lyophilization.

Trypsin-Sepharose chromatography

The freeze dried extracts were dissolved in 0.05 M Tris-HCl, pH 8.2 containing 0.5 M NaCl and 0.02 M $CaCl_2$ and loaded on a trypsin-Sepharose column pre-equilibrated at a flow rate of 10 mL/h. Unbound proteins were removed by washing the column at 40 mL/h for 3 h at 4° C. The bound proteins were eluted from the column by decreasing the pH using 0.2 M Gly-HCl, pH 3.0 containing 0.5 M NaCl at 50 mL/h. Fractions of 3 mL were collected, neutralized and assayed for trypsin inhibitory activity with BAPNA at pH 8.2 (Section 2.2.3.1). The fractions that showed trypsin inhibitory activity were pooled (Figure 7.1), dialyzed against water (6 × 500 mL) and lyophilized. Figure 7.1 shows the elution profile of trypsin inhibitors of horsegram flowers. The elution profile of leaf inhibitors was similar (results not shown). A purification of ≈ 36 fold was achieved for the inhibitors from the flower with a recovery of ~ 60 % trypsin/chymotrypsin inhibitory activity (Table 7.1). Further purification of leaf inhibitors was not possible due to the low protein concentration and poor yield (< 25 %) of the inhibitors. This concentrate was used for further comparison and characterization with respect to M_r, amino acid analysis and cross reactivity studies. PAGE in the presence of gelatin followed by coomassie staining indicates the presence of two inhibitors in the affinity purified flower inhibitors, which are electrophoretically similar to HGGI-I and HGGI-II (Figure 7.3).

CM-Sepharose chromatography of horsegram flower inhibitors (HGFI)

The two trypsin/chymotrypsin inhibitors were fractionated on CM-Sepharose at pH 4.0. The purified inhibitor pool of the previous step was dissolved in 0.05 M sodium acetate buffer, pH 4.0 and loaded on a CM-Sepharose column pre-equilibrated with 0.05 M sodium

acetate buffer, pH 4.0 at a flow rate of 15 mL/h. The unbound proteins were washed with 3-4 bed volumes of buffer. The bound inhibitors were resolved employing a linear gradient of 0-0.3 M NaCl in the same buffer (Total volume: 150 ml) and elution monitored at 230 nm. The fractions were assayed for trypsin inhibitory activity and protein (Figure 7.2). The pooled fractions were dialyzed extensively against water and concentrated to 2 mL by freeze drying. The two inhibitory peaks were designated as HGFI-I and HGFI-II, in order of their relative mobility on native PAGE, which were similar to HGGI-I and HGGI-II. The purification of the HGFIs is summarized in Table 7.1. The yields of HGFI-I and HGFI-II were 40 % and 13 % respectively.

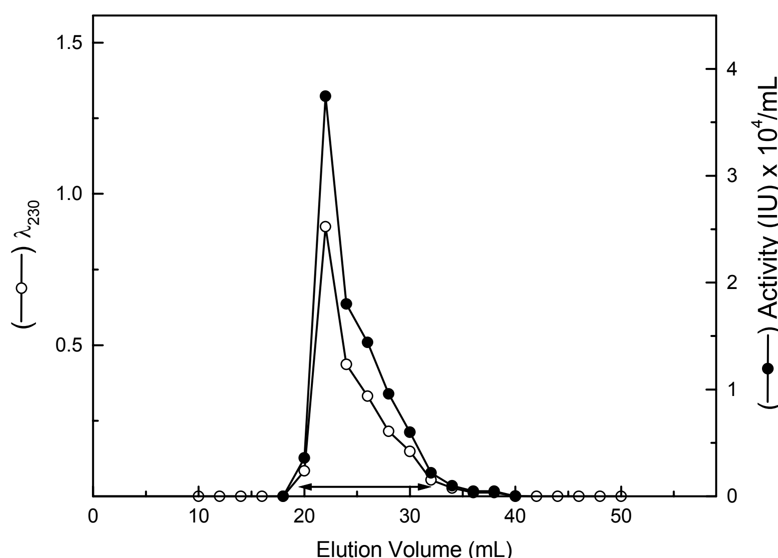


Figure 7.1 **Elution profile of HGFIs on trypsin-Sepharose chromatography.** The crude extract was loaded on a trypsin-Sepharose column equilibrated with 0.05 M Tris-HCl, pH 8.2 containing 0.02 M $CaCl_2$ and 0.5 M NaCl at a flow rate 10 mL/h. The sample was eluted with 0.2 M Gly-HCl buffer, pH 3.0 containing 0.5 M NaCl at a flow rate 50 mL/h. Fractions of 3 mL were collected and the active fractions were pooled as shown (⟷). Similar elution profile was obtained for horsegram tender leaf inhibitors.

Homogeneity of flower and leaf inhibitors

The homogeneity of the HGFI-I, HGFI-II and HGLIs was assessed by native and gelatin-PAGE (Figure 7.3 and 7.4). The inhibitors, HGFI-I, HGFI-II and HGLIs were electrophoressed on a polyacrylamide gel

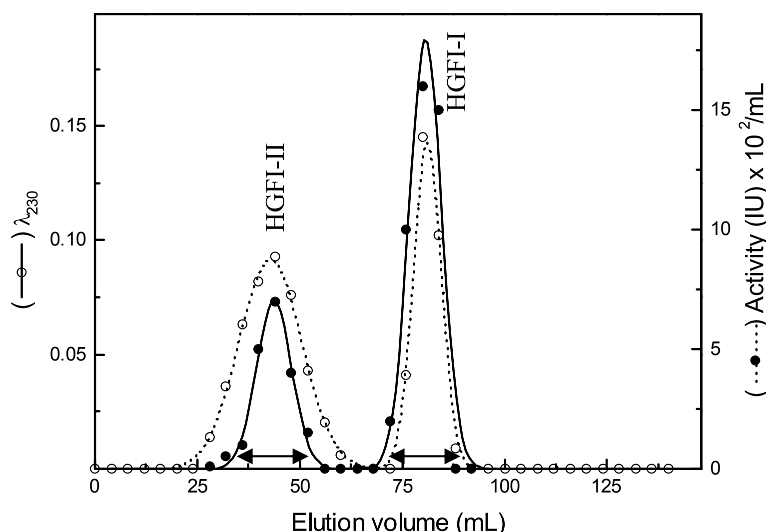


Figure 7.2 **CM-Sepharose chromatography of HGFIs.** The pooled inhibitor fraction of trypsin-Sepharose chromatography dissolved in 0.05 M sodium acetate buffer (pH 4.0) was loaded on a CM-Sepharose (7.2 × 2.7 cm) column. The bound proteins were eluted using a linear gradient of 0-0.3 M NaCl in the above buffer at 20 mL/h. Two mL fractions were collected and active inhibitor fractions were pooled as indicated (←→).

Table 7.1 **Purification of the inhibitors from horsegram flowers***

Step	Total inhibitory units (IU) × 10^3	Total protein (mg)	Specific activity (Units/mg) × 10^3	Yield (%)	Fold purification
Crude	T 18.62	17.08	1.09	100	---
	C 9.24		0.54		
Trypsin-Sepharose chromatography	T 11.07	0.285	38.84	59	35
	C 5.57		19.52	60	36
CM-Sepharose chromatography					
HGFI-I	T 7.47	0.09	83.0	40	76
	C 2.37		26.33	25	48
HGFI-II	T 2.43	0.07	34.71	13	31
	C 0.62		8.85	7	16

* These are the results of a typical purification starting from 5 g of horsegram flower acetone powder. These values were reproduced in two separate purifications. IU = Inhibitory units (T: trypsin; C: chymotrypsin) are expressed as 1 unit = 0.01 decrease in absorbance at 410 nm under assay conditions.

(10 % T, 2.7 % C) in Tris-HCl buffer, pH 8.8 and located both by protein and inhibitory activity staining as described earlier (Section 2.2.6.2 and 2.2.6.4). HGFI-I and -II migrated as a single species (Figure 7.3, lane 2 and 1) both in the inhibitor activity and protein stained gels indicating their homogeneity. In contrast the affinity purified horsegram leaf inhibitor fraction showed the presence of two trypsin inhibitory forms by activity staining and protein staining. The two forms were designated as HGLI-I and HGLI-II, in order of their relative mobility on native PAGE with HGGI-I and HGGI-II (Figure 7.4).

Molecular weight determination of HGFIs and HGLIs

Denaturing SDS-PAGE (17.5 % T, 2.7 % C and 15 % T, 2.7 % C) of purified HGFIs and HGLIs was carried out to estimate the molecular weight in a discontinuous buffer system. The apparent M_r of the HGFIs and HGLIs were estimated from log M_r vs relative mobility of the marker proteins. The M_r was 17.3 kDa and 18.3 kDa for HGFI-I and HGFI-II respectively (Figure 7.5). The M_r of HGLIs were estimated to be 29.6 kDa and 33.3 kDa (Figure 7.6). The HGLIs were named as 'high molecular weight inhibitor (HMWI)' and 'low molecular weight inhibitor (LMWI)' on basis of molecular weight on SDS-PAGE.

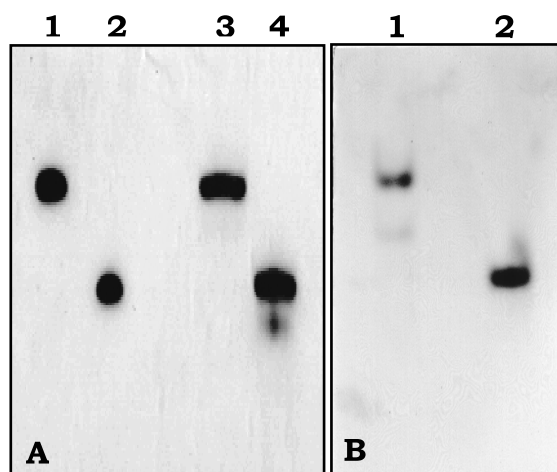


Figure 7.3 **Native-PAGE (10 % T, 2.7 % C) of purified HGFIs.** A: The gel was stained with CBB R-250 for protein and B: Trypsin inhibitory activity. HGFI-II (lane 1); HGFI-I (lane 2); HGGI-II (lane 3) and HGGI-I (lane 4).

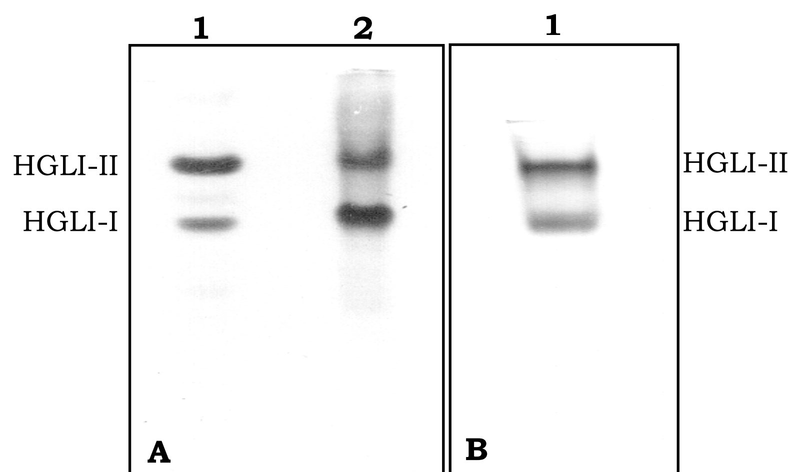


Figure 7.4 **Native-PAGE (10 % T, 2.7 % C) of purified HGLIs.** HGLIs (lane 1); HGGIs (lane 2) A: The gel was stained with CBB R-250 for protein and B: Trypsin inhibitory activity.

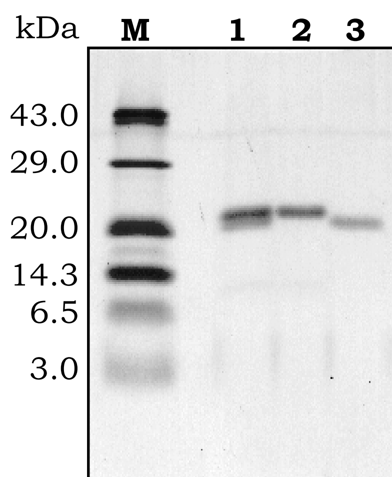


Figure 7.5 **SDS-PAGE (17.5 % T, 2.7 % C) of HGFI-I and HGFI-II.** Low molecular weight markers (lane M), Affinity purified flower inhibitors (lane1), HGFI-II (lane 2) and HGFI-I (lane 3).

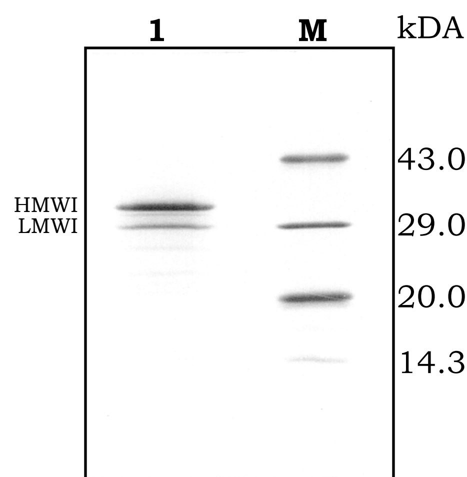


Figure 7.6 **SDS-PAGE (15 % T, 2.7 % C) of HGLIs.** Affinity purified leaf inhibitors (lane 1) and low molecular weight markers (lane M).

Chemical modification of reactive site residues of HGFIs

The amino acid residue present at the P_1 position in the inhibitor reflects the protease inhibited. The flower inhibitors, HGFI-I and -II, inhibit the serine proteases, trypsin and chymotrypsin. The crucial

residue involved in the inhibition of trypsin by HGFIs was evaluated by modification of Lys and Arg residues with 2, 4, 6-trinitrobenzenesulfonic acid and 1, 2-cyclohexanedione respectively (Section 2.2.15 and 2.2.17). The chemically modified inhibitors were analyzed for trypsin inhibitory activity on native gelatin-PAGE and colorimetrically using BAPNA as the substrate. The results are summarized in Table 7.2 and Figure 7.7. The complete loss of inhibitory activity of TNBS modified HGFI-I, indicates that a Lys residue is present at the P_1 position. In contrast, this modification had no effect on the trypsin inhibitory activity of HGFI-II. However the TNBS modified HGFI-II had an increased relative mobility (Figure 7.7, lane 4). The loss of trypsin inhibitory activity by modification with 1, 2-cyclohexanedione indicates that an Arg is present at this active site of HGFI-II.

Table 7.2 **The effect of chemical modification on trypsin inhibitory activity of HGFIs**

Amino acid modified	Inhibitor	Residual inhibitory activity (%)
Lys	HGFI-I	0
	HGFI-II	100
Arg	HGFI-I	100
	HGFI-II	0

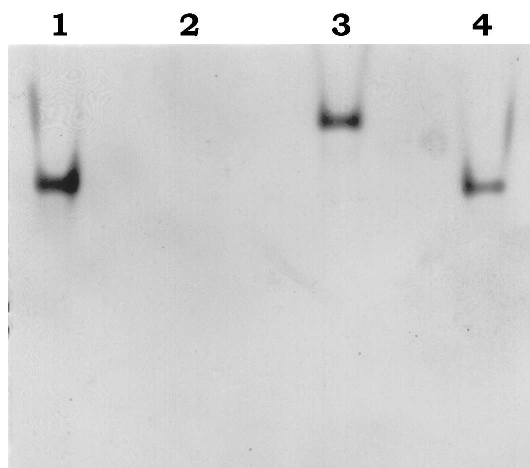


Figure 7.7 **PAGE (10 % T, 2.7 % C) of HGFIs modified using 2, 4, 6-trinitrobenzenesulfonic acid.** HGFI-I (lane 1), modified HGFI-I (lane 2), HGFI-II (lane 3) and TNBS modified HGFI-II (lane 4).

Dot-Blot analysis of HGFIs and HGLIs

Cross-reactivity of purified HGFI-I, HGFI-II and HGLIs were studied using polyclonal antibodies raised against the major dormant seed inhibitor, HGI-III (Sreerama and Gowda, 1997). About 2 μg of each inhibitor was blotted on to nitrocellulose membrane and subjected to immunodetection (Section 2.2.14). The cross-reactivity of the inhibitors was ascertained by color development using BCIP and NBT. Positive antigen-antibody reactions could be detected with HGFI-I, HGFI-II and HGLIs. BSA served as the negative control and showed no cross reactivity (Figure 7.8A and B). HGI-III was used as a positive control.

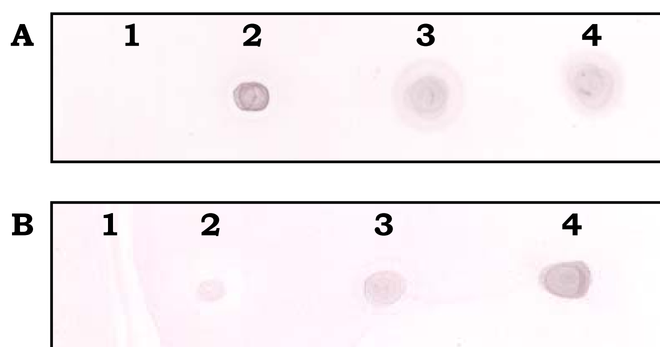


Figure 7.8 **Dot-Blot analysis of HGFIs and HGLIs and the dormant seed inhibitor (HGI-III)** (A) Cross-reactivity with HGFIs: (1) BSA (2 μg); (2) HGI-III (2 μg); (3) HGFI-I (2 μg) and (4) HGFI-II (2 μg). (B) Cross-reactivity with HGLIs: (1) BSA; (2) HGI-III (1 μg); (3) HGLIs (1 μg) and (4) HGLIs (2 μg). HGI-III and BSA served as positive control and negative control respectively.

Amino acid composition

In order to evaluate the total amino acid composition of HGFIs and HGLIs, the inhibitors were transferred to PVDF membrane by semi dry blotting using CAPS buffer. The blotted inhibitors were digested *in situ* with 6 N HCl. The acid hydrolyzed inhibitor proteins were derivatized to phenylthiocarbamyl amino acids by PITC and separated by RP-HPLC. The amino acid composition of HGFIs, HGLIs and HGI-III

are shown in Table 7.3. The amino acid composition of HGFI-I and HGFI-II were similar, with the exception of the acidic amino acid Asp and high content of the imino acid, Pro. Inturn the HGFIs have a substantially higher content of Ser, Gly, Pro, and Val when compared to HGI-III (Table 7.3). The amino acid composition of the two HGLIs (LMWI and HMWI) from horsegram leaves were very similar having a high content of Gly and Arg and low content of His and Pro when compared to HGI-III.

Amino-terminal sequence analysis

The flower inhibitors, HGFI-I and HGFI-II were blotted on to PVDF membrane following native PAGE (10 % T, 2.7 % C) using CAPS buffer at pH 11.00 and subjected to amino-terminal sequence analysis on an automated gas phase sequenator, PSQ-1. The sequence obtained for HGFI-I was unambiguous up to 9 cycles and was found to be Phe-Val-Val-Asp-Asp-Gly-Gly-X-Gly. Amino-terminal analysis of HGFI-II did not provide any sequence owing to the low protein concentrations. The presence of three Gly residues in the sequence further provide evidence that these inhibitors are different from the HGIs in the dormant seed.

RP-HPLC profile of HGGI-III and HGFIs

The RP-HPLC elution profile of HGFIs were compared with HGGI-III of the germinated seed using a linear gradient of 0.1 % TFA and 70 % acetonitrile, 0.05 % TFA in water (Figure 7.9). HGFI-I and HGFI-II had similar retention time but differed from HGGI-III, which eluted earlier. Elution of the HGFIs at a higher acetonitrile concentration further reveals that these are of higher molecular weight and different from the inhibitors of the dry seed.

Table 7.3 **Comparison of amino acid composition of the HGFIs and HGLIs with HGI-III.**

Amino acid	Relative amino acid composition (mol%)*				
	HGFI-I	HGFI-II	(HGLI) LMWI	(HGLI) HMWI	HGI-III
Asp[a]	3.56	7.08	11.56	9.22	11.4
Glu[b]	8.92	10.96	14.54	12.91	10.5
Ser	11.77	10.33	10.35	12.32	15.2
Gly	17.42	21.25	18.22	20.07	0
His	1.07	0.70	1.12	1.15	5.0
Arg	4.20	4.65	9.85	7.46	2.9
Thr	2.40	4.93	5.93	6.80	4.0
Ala	6.95	5.53	4.20	6.17	5.9
Pro	14.10	3.13	2.19	2.67	8.5
Tyr	2.07	3.91	1.15	0.81	1.6
Val	10.71	9.67	4.02	4.34	5.4
Met	1.71	1.44	0.20	0.15	0.7
½ Cys	0.93	0.61	1.49	0.62	17.7[c]
Ile	2.47	4.88	3.28	4.08	2.8
Leu	6.00	8.11	6.01	6.17	1.3
Phe	3.96	3.23	2.51	2.64	2.4
Lys	1.71	2.5	3.30	2.33	4.5

*Average of duplicates.
HGFI: Horsegram flower inhibitor; HGLI: Horsegram leaf inhibitor; LMWI: Low molecular weight inhibitor; HMWI: High molecular weight inhibitor; HGI-III: Horsegram inhibitor (Sreerama *et al.,* 1997); [a]Determined as aspartate and aspargine, [b]glutamate and glutamine & [c]pyridylethylated cysteine.

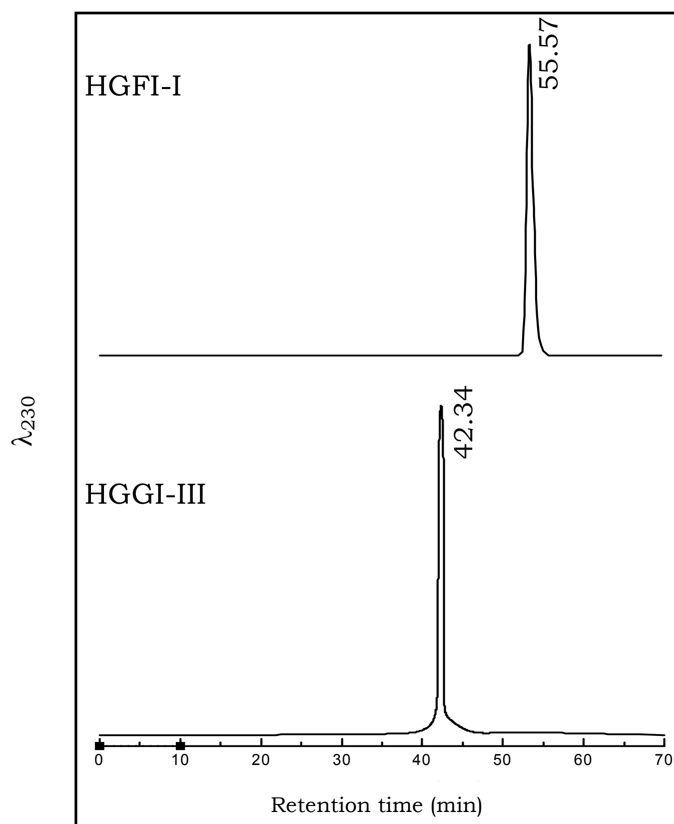


Figure 7.9 **RP-HPLC profile of HGFI-I and HGGI-III.** The purified inhibitors were loaded on a Phenomenex ODS column (250 × 4.6 mm, 5 μ) and eluted using a linear gradient of 0.1 % TFA and 70 % acetonitrile in 0.05 % TFA in water at a flow rate of 0.7 mL/min. Similar elution profile (R_T: 55.75) was observed with purified HGFI-II (Figure not shown).

Discussion

The purification and characterization of the BBIs from the flowers and the leaves of horsegram have been investigated. During seed development a steep increase in the inhibitory activity with a concomitant increase in protein content was observed from 10 days after flowering (Sreerama and Gowda, 1998). The BBIs of the horsegram flowers inhibited trypsin and chymotrypsin simultaneously and independently (Sreerama and Gowda. 1998). The two-inhibitor forms that appear after 120 h germination and the two forms present in the flowers, leaves and husk of *Dolichos biflorus* have apparently similar electrophoretic mobilities and inhibit both trypsin and chymotrypsin (Sreerama and Gowda, 1998). However it was not clear whether these inhibitors were biochemically identical or not. Therefore, the purification and characterization of inhibitor forms from the flowers and leaves were undertaken.

Purification of two inhibitors from an acetone powder of horsegram flowers involved a combination of trypsin-Sepharose and CM-Sepharose chromatography. The trypsin-Sepharose chromatography step resulted in a 36 fold purified inhibitor (Figure 7.1, Table 7.1). The CM-Sepharose chromatography step (Figure 7.2) proved to be a successful step in the resolution of the two flower inhibitors, HGFI-I and HGFI-II. The two inhibitors, HGGI-I and HGGI-II were also resolved by cation exchange chromatography on CM-Sephadex C-25 (Kumar *et al.*, 2002). However the inhibitors of the flower (HGFIs) are apparently different as these inhibitors bind to CM-Sepharose where as HGGI-I and -II elute as unbound fractions. These results also suggest differences in their apparent pI. Among the two inhibitors HGFI-I is more potent with a 2 fold higher specific activity than HGFI-II. The purified HGLIs also had electrophoretic mobilities

similar to the HGGIs (Figure 7.4). The very low yield of protein did not permit further purification on CM-Sepharose.

The inhibitors present in the flowers (HGFIs) and leaves (HGLIs) differ in size from the inhibitors of the dormant seed (HGIs) and from the germinated horsegram seed (HGGIs). These inhibitors of the flower are ~ 18 kDa where as the leaf inhibitors are ~ 29.0 kDa. The amino acid composition of the inhibitors (Table 7.3) also suggest that the inhibitors from the flowers and leaves distinctly different from each other as well as to HGGIs. The remarkably high content of Gly in the HGFIs and HGLIs compared to its absence in the HGIs and HGGIs further support the fact that these inhibitors are different from the seed BBIs. The amino-terminal sequence of HGFIs and RP-HPLC profiles also evidence the difference in the inhibitors. Domoney *et al.*, (2002) characterized three gene classes in *Pisum sativum*, encoding proteinase inhibitor proteins all belonging to BBI family with distinct spatial expression patterns. Among these one class was expressed, exclusively in seeds where as the other two were expressed predominantly in root, endodermal and floral reproductive tissues.

The BBIs of horsegram seed have two independent reactive sites for inhibition of trypsin and chymotrypsin (Sreerama *et al.*, 1997). The HGFIs and HGLIs also inhibit both trypsin and chymotrypsin (Table 7.1) suggesting that they are double-headed. Chemical modification studies indicate that the reactive site P_1 residue for HGFI-I is Lys, where as that of HGFI-II is Arg (Table 7.2 and Figure 7.7). In contrast the reactive site of all the HGGIs for trypsin is Lys (Chapter 3, Kumar *et al.*, 2002). Both the HGFIs and HGLIs when probed with antibodies raised against HGI-III showed cross reactivity. These results suggest common epitopes among the inhibitors.

In conclusion, the HGFIs and HGLIs are members of Bowman-Birk family of proteinase inhibitors, but are distinct from each other and from the seed inhibitors in terms of molecular size, amino acid composition, sequence and active site. To date and the best of our knowledge this is the first and only report that demonstrates the presence of BBIs in the flowers and leaves of legumes.

Summary and Conclusions

Presence of various of isoforms, abundance, low molecular weight, double-headed characteristic, stability towards to extreme pH and temperature of legume Bowman-Birk inhibitors, gained importance in studying protein-protein interactions, protein folding and structure function relationship.

The present investigation entitled **"Structural studies of the proteinase inhibitors of germinated horsegram (*Dolichos biflorus*) seeds"** has been undertaken to understand the evolutionary relationship among various BBI isoforms of horsegram. Several inhibitor isoforms have been purified from seeds, cotyledons, leaves and flowers of horsegram and characterized. The following are the salient features of the present investigation.

- ❖ The three isoforms, HGGI-I, -II and -III from 120 h germinated horsegram seeds were purified by buffer extraction, $(NH_4)_2SO_4$ fractionation, size-exclusion chromatography on Sephadex G-50, cation exchange chromatography on CM-Sephadex C-25 followed by DEAE-Sephadex A-25 anion exchange chromatography.
- ❖ HGGI-I, -II and -III were purified with a yield of 4.2 %, 8.3 % and 25 % respectively (Table 3.1) and HGGI-III was the predominant form.
- ❖ The specific activity of HGGIs towards trypsin inhibition is five fold higher than that of dormant seed inhibitors (HGIs) (Table 3.1, Sreerama *et al.*, 1997).
- ❖ The homogeneity of HGGIs was analyzed on native PAGE (Figure 3.4 and 3.5), capillary electrophoresis (Figure 3.6) and confirmed by the

release of single amino-terminal amino acid residue during Edman analysis of amino-terminal sequence (Figure 3.7).

- The M_r determined by analytical gel filtration chromatography (Figure 3.8 and 3.9) and SDS-PAGE (Figure 3.10) were ~7 kDa, indicating HGGIs to be a single polypeptides and these values are in close agreement with the M_r determined by MALDI-MS (Table 3.2).
- HGGI-I, -II and -III have distinct isoelectric points 4.99, 5.09 and 5.28 respectively.
- HGGIs are competitive in nature, inhibiting trypsin and chymotrypsin simultaneously and independently indicating they are double-headed, with independent sites for trypsin and chymotrypsin inhibition.
- Chemical modification studies revealed that the Lys residue is responsible for the inhibition of trypsin (Figure 3.15) and the trypsin reactive site similar to that of dormant seed inhibitors (HGIs).
- The binding of HGGIs to trypsin and chymotrypsin was very tight. The binding affinities (K_is) for trypsin are of the order HGGI-III > HGGI-II > HGGI-I (Table 3.3) and show higher binding affinity towards trypsin when compared to chymotrypsin.
- HGGIs bind to trypsin and chymotrypsin at 1:1 molar ratio (Figure 3.14) as revealed by stoichiometry studies.
- The cross-reactive studies with the antibodies of HGI-III indicated the presence of similar antigenic epitopes in HGGIs (Figure 3.16).
- The amino acid analysis (Table 3.4) and amino-terminal sequence analysis (Table 3.5) reveal that the HGGIs vary at the amino-termini but have identical core sequence to dormant seed inhibitor, HGI-III.
- Cross-reactive studies, amino acid and amino-terminal sequence analysis indicated that HGGIs are *in situ* products of limited amino-

terminal proteolysis of the isoinhibitors present in the dormant seed (HGIs).

- The complete amino acid sequence of HGGI-I, -II and -III from germinated horsegram seeds was determined by sequential Edman analysis of the intact PE-HGGIs and the peptides derived by digestion with TPCK-trypsin, endoproteinase Asp-N and by chemical cleavage with CNBr. The primary structure of HGGIs was deduced by overlapping the peptide sequences of these various enzymatic and chemically cleaved peptides.
- HGGI-I, -II and -III are single polypeptides of 66, 65 and 60 amino acids with fourteen ½ cystine residues conserved.
- The determined primary structure of HGGIs (Figure 4.10) differs from major dormant seed inhibitor, HGI-III only at the amino- and carboxy-termini. Inturn HGGIs differ from each other only at amino-terminus.
- Comparison of primary structure of HGGIs and HGI-III (Figure 4.10) indicates the specific cleavages occur between Thr^{6}-Asp^{7}, Asp^{7}-Glu^{8} and Ser^{12}-Ser^{13} at amino-terminus and Ser^{72}-Ser^{73} at the carboxy-terminus in the conversion of HGI-III to HGGI-I, -II and -III.
- Amino acid sequence comparison together with modification of reactive site residue confirmed that Lys-Ser and Phe-Ser in HGGIs are the reactive sites for the inhibition of trypsin and chymotrypsin respectively.
- HGGIs like HGI-III, belong to group-II legume BBIs as evidenced by the presence of the characteristic Pro-Ala sequence near the second reactive site.
- Among all the BBIs compared most homologous to HGGIs are HGI-III and MAI-DE-3 of *Macrotyloma axillare* with 100 % and 98.5 %

identity respectively. These striking similarities in the primary structure of BBIs from horsegram (HGI-III and HGGIs) and *Macrotyloma axillare* (MAI-DE-3) reflect upon the common evolutionary origin of the two plants.

- Hydropathy profile of HGGIs were similar to HGI-III (Prakash *et al.*, 1996) in having hydrophilic regions at the amino- and carboxy-termini with more hydrophobic regions around the putative reactive sites.
- The results of SDS-PAGE (Figure 3.10 and 5.3), analytical gel filtration chromatography (Figure 5.4) and exact M_r obtained by ESMS (Sreerama *et al.*, 1997) suggest that the HGI-III in solution undergoes self-association to form a dimer. Where as HGGIs - *in situ* degradation products of HGI-III essentially exist as monomers (Figure 3.10).
- The most significant differences between HGI-III and HGGI-III are the deletion of the peptide -DHHQSTDEPSES and tetrapeptide -SHDD at the amino- and carboxy-terminus respectively. Hence either the deleted amino and/or the carboxy-termini are involved in the self-association of HGI-III.
- HGI-I, inspite of the truncated amino-terminus exists as dimer (Sreerama *et al.*, 1997). This implicates the existence of a structural element responsible for self-association of HGI-III in the deleted tetrapeptide, -SHDD.
- The intermolecular interactions viz. 1. Hydrogen bond between the guanidium group of Arg^{23} of one subunit and the polar group of the side chain of Glu^{68} and 2. An ion-pair between Lys^{16} of one subunit and the dyad related carboxyl group of Glu^{69} of the other subunit in Winter pea seed inhibitor, PsTI-IVb, together with the fact that the

deleted tetrapeptide contained Asp residues, suggest that such interaction could be the premise to self-association in HGI-III.

- Size-exclusion chromatography studies (Figure 5.8) and SDS-PAGE analysis (Figure 5.9) of HGI-III in presence of Zn^{2+}, indirectly confirmed the pivotal role of Asp residues at the carboxy-terminus.
- Chemical modification of Lys and Arg residues of HGI-III further strengthened the involvement of Lys^{24}, the first reactive site residue in self-association of HGI-III.
- Close comparative analysis of various BBI sequences (Figure 4.12, Table 5.2) which exists as monomers and dimers, strongly support the notion that a unique interaction between Arg/Lys at P_1 of the first reactive site of one subunit and the charged residue Asp/Glu at the carboxy-terminal of the second subunit responsible for the observed self-association in all leguminous BBIs.
- The dimeric model (Figure 5.11) clearly depicts the involvement of Asp^{76}. Of all the interactions, the hydrogen bond between Lys^{24} and Asp^{76} provides the correct orientation for formation of the Arg-Asp salt-bridge between the subunits.
- During the process of germination decline in the tryptic/chymotryptic inhibitory activity and protein content was observed (Figure 6.1). This decrease was attributed to the proteolysis of dormant seed inhibitors and seed storage proteins by the proteinases of horsegram.
- Decline in the inhibitory activity (Figure 6.1) accompanied by an increase in the proteinase activity (Figure 6.4) suggest that a temporal and spatial correlation does exist between synthesis of proteinases and protein degradation in cotyledons.

- The major proteinase (Figure 6.5, R_f 0.48) from 120 h germinated horsegram seeds was partially purified by buffer extraction, $(NH_4)_2SO_4$ fractionation followed by size-exclusion chromatography on Sephadex G-50 and judged to be homogenous as revealed by activity staining (Figure 6.6, inset).

- The optimum pH and temperature of the purified proteinase was determined to be 9.25 and 37 °C respectively. The proteinase is remarkably specific towards its own natural storage protein (Table 6.1).

- Complete inhibition by EDTA and significant stimulation by exogenous Mg^{2+} is suggestive of the enzyme being a metalloproteinase (Table 6.2 and 6.3).

- *In vitro* proteolytic studies of HGIs with the metalloproteinase is consistent with the fact that HGGIs are proteolytically cleaved products of dormant seed inhibitors, HGIs (Figure 6.10).

- The possible role of horsegram BBIs as a nitrogen sink source during germination and seedling growth was observed with studies on the effect of EDTA on germination of horsegram (Figure 6.11 and 6.12).

- The trypsin/chymotrypsin inhibitors from flowers and leaves of horsegram were purified by trypsin-Sepharose chromatography at pH 8.2 (Figure 7.1).

- The two horsegram flower inhibitors, HGFI-I and HGFI-II were separated on CM-Sepharose using a linear gradient of NaCl in sodium acetate buffer, pH 4.0 (Figure 7.2).

- HGFIs and HGLIs were electrophoretically similar to HGGI-I and -II (Figure 7.3 and 7.4) and are found to be homogenous both by protein as well as inhibitory activity staining (Figure 7.3 and 7.4).

- HGFIs and HGLIs differ in size from the inhibitors of the dormant seed (HGIs) and germinated horsegram seeds (HGGIs).
- The reactive site P_1 residue for HGFI-I is Lys and HGFI-II is Arg (Table 7.2 and Figure 7.7) as revealed by chemical modification studies.
- Dot-blot analysis (Figure 7.8) and the double headed characteristics (Table 7.1) indicate that HGFIs and HGLIs are members of Bowman-Birk family of proteinase inhibitors. Unusually high content of Gly, higher M_r and distinct amino-terminus sequence support that these inhibitors differ from each other and from the inhibitors present in the dry seed.

The data compiled in this thesis describe the purification and characterization of Bowman-Birk inhibitors from various tissues of horsegram (*Dolichos biflorus*) plant. The self-association, an anomalous behavior of legume BBIs was analyzed by comparing the primary structure of HGGI-III and HGI-III. The crucial interaction between Lys^{24} at P_1 of the first reactive site of one subunit and the charged residue Asp^{76} at the carboxy-terminal of another subunit is the premise of self-association in HGI-III (Kumar *et al.*, 2004). Comparative studies of legume BBI sequences and their self-association behavior indicated that similar interaction could be responsible for the self-association of all reported legume BBIs.

Lack of highly immunogenic amino- and carboxy-terminal residues, low M_r and high potency of HGGIs could be an excellent alternative for use as a cancer chemopreventive agent and developing transgenic insect resistant plants.

References

Abe, M., Arai, S. and Fuzimaki, M. (**1977**), *Agric. Biol. Chem.*, 41, 893.

Abe, O., Ahta, J., Utsumi, Y. and Kuromizu, K. (**1978**), *J. Biochem.*, 83, 1737.

Abe, M., Arai, S. and Fuzimaki, M. (**1978a**), *Agric. Biol. Chem.*, 42, 1813.

Allen, G. (**1989**), In: 'Laboratory techniques in Biochemistry and Molecular Biology: Sequencing of Proteins and Peptides', 73, Elsevier, Amsterdam.

Ambekar, S. S., Patil, S. C., Giri, A. P. and Kachole, M. S. (**1996**), *J. Sci. Food Agric.*, 72, 57.

Apostoluk, W. and Otlewski, J. (**1998**), *Proteins Struct. Funct. Genet.*, 32, 459.

Ardelt, W. and Laskowski, Jr. M. (**1985**), *Biochemistry*, 24, 5313.

Ardelt, W. and Laskowski, Jr. M. (**1991**), *J. Mol. Biol.*, 220, 1041.

Asao, T., Imai, F., Tsuji, I., Tashiro, M., Iwami, K. and Ibuki, F. (**1991**), *Agric. Biol. Chem.*, 55, 707.

Babar, V. S., Chavan, J. K. and Kadam, S. S. (**1988**), *Plant Foods Hum. Nutr.*, 38, 319.

Baumgartner, B. and Chrispeels, M. J. (**1976**), *Plant Physiol.*, 58, 1.

Belew, M. and Eaker, D. (**1976**), *Eur. J. Biochem.*, 62, 499.

Belew, M., Porath, J. and Sundberg, L. (**1975**), *Eur. J. Biochem.*, 60, 247.

Belozersky, M. A., Dunaevsky, Y. E. and Voskoboynikova, E. (**1990**), *Biochem. J.*, 272, 677.

Belozersky, M. A., Dunaevsky, Y. E., Musolyamov, A. X. and Egorov, T. A. (**1995**), *FEBS Lett.*, 371, 2264.

Bergeron, D. and Neilson, S. S. (**1993**), *J. Agric. Food Chem.*, 41, 1544.

Bewly, T. A. and Birk, Y. (**1978**), *Int. J. Pept. Protein Res.*, 12, 249.

Bidlingmeyer, B. A., Cohen, S. A., Tarvin, T. L. (**1984**), *J. Chromatogra.*, 336, 93.

Billings, P. C. (**1993**), Approaches to studying the target enzymes of anticarcinogenic protease inhibitors. In: Troll. W. and Kennedy, A. R. (eds) Protease inhibitors as cancer chemopreventive agents. Plenum Press, New York, 191.

Billings, P. C., Carew, J. A., Keller-McGandy, C. E., Goldberg, A. and Kennedy A. R. (**1987a**), *Proc. Natl. Acad. Sci. USA*, 84, 4801.

Billings, P. C., St. Clair, W., Ryan, C. A. and Kennedy, A. R. (**1987b**), *Carcinogenesis,* 8, 809.

Billings, P. C. and Habres, J. M. (**1992**), *Proc. Natl. Acad. Sci. USA*, 89, 3120.

Billings, P. C., Habres, J. M. and Kennedy A. R. (**1990**), *Carcinogenesis*, 11, 329.

Billings, P. C., Habres, J. M., Liao, D. C., and Tuttle, S. W. (**1991a**), *Cancer Res.*, 51, 5539.

Billings, P. C., Jin, T., Ohnishi, N., Lioa, D. C. and Habres, J. M. (**1991b**), *Carciogenesis*, 12, 655.

Billings, P. C., Morrow, A. R., Ryan, C. A. and Kennedy, A. R. (**1989**), *Carcinogenesis*, 10, 687.

Billings, P. C., St. Clair, W., Owen, A. J. and Kennedy, A. R. (**1988**), *Cancer Res.*, 48, 1798.

Birk, Y. (**1961**), *Biochim. Biophys. Acta,* 54, 378.

Birk, Y. (**1976**), *Methods Enzymol.*, 45, 695.

Birk, Y. (**1985**), *Int. J. Pept. Protein Res.*, 25, 113.

Birk, Y. (**1987**), Proteinase inhibitors. In: Neuberger, A. and Brocklehurst, K. (eds) Hydrolytic enzymes. Elsevier, Amsterdam, 257.

Birk, Y. (**1989**), Protein-Proteinase inhibitors of plant origin and their significance in human nutrition. In: Huisaman, J., van der Poel, T. F. B. and Leiner, I. E. (eds) Proceedings, workshop on antinutritional factors in legume seeds. TNO, Agricultural University Wageningen, Pudoc Wageningen, Netherlands, 83.

Birk, Y., Gertler, A. and Khalef, S. C. (**1963**), *Biochem. J.*, 87, 281.

Bode, W. and Huber, R. (**1992**), *Eur. J. Biochem.*, 204, 433.

Boulter, D. (**1993**), *Phytochemistry*, 34, 1453.

Bowman, D. E. (**1946**), *Proc. Soc. Exp. Biol. Med.*, 63, 547.

Bradford, M. M. (**1976**), *Anal. Biochem.*, 72, 248.

Brauer, A. B. E. and Leatherbarrow, R. J. (**2003**), *Biochem. Biophys. Res. Commun.*, 308, 300.

Brauer, A. B. E., Domongo, G. J., Cooke, R. M., Matthews, S. J. and Leatherbarrow, R. J. (**2002**), *Biochemistry*, 41, 10608.

Brauer, A. B. E., Kelly, G., McBride, J. D., Cooke, R. M., Matthews, S. J. and Leatherbarrow, R. J. (**2001**), *J. Mol. Biol.*, 306, 799.

Broadway, R. M. and Duffey, S. S. (**1986**), *J. Insect. Physiol.*, 32, 827.

Brown, W. E. and Ryan, C. A. (**1984**), *Biochemistry,* 23, 3418.

Bulmaga, V. P. and Shutov, A. D. (**1977**), *Biokhimiya*, 42, 1983.

Caggana, M. and Kennedy, A. R. (**1989**), *Carcinogenesis*, 10, 2145.

Carew, J. A. and Kennedy, A. R. (**1990**), *Cancer Lett.*, 49, 153.

Catalano, M., Ragona, L., Molinari, H., Tava, A. and Zetta, L. (**2003**), *Biochemistry*, 42, 2836.

Cecialinai, F., Tava, A., Iori, R., Mortarino, M., Odoardi, M. and Ronchi, S. (**1997**), *Phytochemistry*, 44, 393.

Chang, J. D. and Kennedy, A. R. (**1988**), *Carcinogenesis*, 9, 17.

Chang, J. D., Billings, P. C. and Kennedy, A. R. (**1985**), *Biochem. Biophys. Res. Commun.*, 133, 830.

Chang, J. D., Li, J. H., Billings, P. C. and Kennedy, A. R. (**1990**), *Mol. Carcinog.*, 3, 226.

Chang, K. C. and Harrold, R. L. (**1988**), *J. Food Sci.*, 53, 783.

Chen, P. (**1989***), Ph. D. thesis, University of Pittsburgh, Pittsburgh, PA. (Cross-reference from Werner and Wemmer, **1992**).

Chen, P., Rose, J., Love, R., Wei, C. H. and Wang, B. C. (**1992**), *J. Biol. Chem.*, 267, 1990.

Chrispeels, M. J. and Baumgartner, B. (**1978**), *Plant Physiol.*, 61, 617.

Christianson, D. W. and Alexander, R. S. (**1989**), *J. Am. Chem. Soc.*, 111, 6412.

Chu, H. M. and Chi, C. W. (**1965**), *Sci. Sinica*, 14, 1441.

Chu, H. M., Lo, S. S., Jen, M. H., Chi, C. W. and Tsao, T. C. (**1965**), *Sci. Sinica*, 14, 1454.

Corpet, F. (**1988**), *Nucleic Acids Res.*, 16, 10881.

da Silva, P. L., Leite, J. R. S. A., Bloch, Jr. C. and De Freitas, S. M. (**2001**), *Protease Peptide Lett.*, 8, 33.

De Freitas, S. M., de Mello, L. V., da Silva, M. C., Vriend, G., Neshich, G. and Ventura, M. (**1997**), *FEBS lett.*, 409, 121.

De Freitas, S. M., Ikemoto, H. and Ventura, M. M. (**1999**), *J. Protein Chem.*, 18, 307.

De Frietas, S. M. and Ventura, M. M. (**1996**), *An. Acad. Bras. Cienc.*, 68, 165.

De Leo, F., Volpcella, M., Licciulli, F., Liuni, S., Gallerani, R. and Ceci, L. R. (**2002**), *Nucleic Acids Res.*, 30, 347.

Deshimaru, M., Hanamoto, R., Kusano, C., Yoshimi, S. and Terada, S. (**2002**), *Biosci. Biotechnol. Biochem.*, 66, 1897.

Dittmann, K. H., Gueven, N., Mayer, C. and Rodemann, H. P. (**2001**), *Protein Eng.*, 14, 157.

Dixon, H. B. F. and Perham, R. N. (**1968**), *Biochem. J.*, 109, 312.

Dixon, M. (**1953**), *Biochem. J.,* 55, 170.

Domoney, C., Welham, T., Ellis, N., Mozzanega, P. and Turner, L. (**2002**), *Plant Mol. Biol.*, 48, 319.

Drapeau, G. R. (**1980**), *J. Biol. Chem.*, 255, 839.

Duan, X., Li, X., Xue, Q., Abo-EI-Saad, M., Xu, O. and We, R. (**1996**), *Nature Biotech.*, 14, 494.

Falco, M. C., Marback, P. A. S., Pompermayer, P., Lopes, F. C. C. and Silva-Filho, M. C. (**2001**), *Genet. Mol. Biol.*, 24, 113.

Felicioli, R., Garzelli, B., Vaccari, L., Melfi, D. and Balestreri, E. (**1997**), *Anal. Biochem.*, 244, 176.

Felsenstein, J. (**1989**), *Evolution*, 39, 783.

Fermi, C. and Pernossi. L. (**1894**), *Z. Hyg. Infektionskr.*, 18, 83.

Foard, D. E., Gutay, P. A., Ladin, B., Beachy, R. N. and Larkins, B. A. (**1982**), *Plant Mol. Biol.*, 1, 227.

Freed, R. C. and Ryan, D. S. (**1978**), *Cereal Chem.*, 55, 534.

Freed, R. C. and Ryan, D. S. (**1980**), *Biochim. Biophys. Acta*, 624, 562.

Frenkel, K., Chrzan, K., Ryan, C. A., Weisner, R. and Troll, W. (**1987**), *Carcinogenesis*, 8, 1207.

Frias, J., Diaz-Dollan, C., Hedley, C. L. and Vial-Vilverde, C. (**1995**), *J. Agric. Food Chem.*, 43, 2231.

Fujji, S., Yokohama, T., Ikegaya, K., Salo, F. and Yakoo, N. (**1985**), *J. Pharma. Pharamcol.*, 37, 545.

Funk, A., Weder, J. K. P. and Belitz H. D. (**1993**), *Z. Lebensm. Unters. Forsch.*, 196, 343.

Fuzimaki, M., Abe, M. and Arai, S. (**1977**), *Agric. Biol. Chem.*, 41, 887.

Gaier, J. R., Tulinsky, A. and Leiner, I. E. (**1981**), *J. Biol. Chem.*, 256, 11417.

Garcia-Olmedo, F., Saliedo, G., Sanchez-Monge, R., Royo, J. and Carbonero, P. (**1987**), Oxford surveys of plant molecular and cell biology, 4, 275.

Gennis, L. S. and Cantor, C. R. (**1976a**), *J. Biol. Chem.*, 251, 734.

Gennis, L. S. and Cantor, C. R. (**1976b**), *J. Biol. Chem.*, 251, 747.

Godbole, S. A., Krishna, T. G. and Bhatia, C. R. (**1994a**), *J. Sci. Food Agric.*, 64, 87.

Godbole, S. A, Krishna, T. G. and Bhatia, C. R. (**1994b**), *J. Sci. Food Agric.*, 66, 497.

Gomes, J. C., Koch, U. and Brunner, J. R. (**1979**), *Cereal Chem.,* 56, 525.

Gross, E. (**1976**), *Methods Enzymol.*, 11, 238.

Gueven, N., Dittmann, K., Mayer, C. and Rodemann, H. P. (**1998**), *Cancer Lett.*, 125, 77.

Guild, B. C. and Strominger, J. L. (**1984**), *J. Biol. Chem.*, 259, 9235.

Habres, J. M. and Billings, P. C. (**1993**), *Cancer Lett.*, 63, 135.

Harms-Ringdahl, M., Forsberg, J., Fedorcsak, I. and Ehrenberg, L. (**1979**), *Biochem. Biophys. Res. Commun.*, 86, 492.

Harris, N., Chrispeels, M. J. and Boulter, D. (**1975**), *J. Exp. Botany*, 26, 544.

Harry, J. B. and Steiner, R. F. (**1970**), *Eur. J. Biochem.*, 16, 174.

Hartl, P. N., Tan-Wilson, A. L. and Wilson, K. A. (**1986**), *Phytochemistry*, 25, 23.

Haynes, R. and Feeney, R. E. (**1967**), *J. Biol. Chem.*, 242, 5378.

Haynes, R., Osuga, T. and Feeny, R. E. (**1967**), *Biochemistry*, 6, 541.

Hermodson, M. A., Ericsson, C. H., Newran, H. and Walsh, K. A. (**1973**), *Biochemistry*, 21, 1346.

Hermodson, M. A. and Mahoney, W. C. (**1983**), *Methods Enzymol.*, 91, 352.

Higgins, D., Thompson, J., Gibson, T., Thompson, J. D., Higgins, D. G. and Gibson, T. J. (**1994**), *Nucleic Acids Res.*, 22, 4673.

Hilder, V. A., Gatehouse, A. M. R. and Boulter, D. (**1990**), Genetic engineering of crop for insect resistance using genes of plant origin. In: Grierson, D. and Lycett, G., Genetic engineering of crop plants (eds) Butterworths, Boston, 551.

Hilder, V. A., Gatehouse, A. M. R., Sheerman, S. E., Barker, R. F. and Boulter, D. (**1987**), *Nature*, 333, 160.

Hobday, S. M., Thurman, D. A. and Barber, D. J. (**1973**), *Phytochemistry*, 12, 1041.

Holm, L. and Sander, C. (**1996**), *J. Mol. Biol.*, 233, 123.

Horisberger, M. and Tecchini-Vonlanthen, M. (**1983**), *Histochemistry*, 77, 313.

Huang, H. and Miller, W. (**1991**), *Adv. Appl. Math.*, 12, 373.

Hubbard, S. J., Campbell, S. F. and Thornton, J. M. (**1991**), *J. Mol. Biol.*, 220, 507.

Huber, R. and Bode, W. (**1978**), *Acc. Chem. Res.*, 11, 114.

Huber, R., Kukla, D., Bode, W., Schwager, P., Bartels, K., Deisenhofer, J. and Steigemann, W. (**1974**), *J. Mol. Biol.*, 89, 73.

Hutchinson, J. (**1969**), Evolution and Phylogeny of Flowering Plants, Academic Press, London, New York, 221.

Hwang, D. L. R., Lin, K. T. D., Yang, W. K. and Foard, D. E. (**1977**), *Biochim. Biophys. Acta*, 495, 369.

Ikeda K., Hanaguchi, K., Yamamoto, M. and Ikenaka, T. (**1968**), *J. Biochem.*, 63, 5521.

Ikenaka, T. and Norioka, S. (**1986**), In: Proteinase inhibitors, Barret, A. J. and Salvensen, G. (eds) 361, Elsevier, Amsterdam.

Ishikawa, C., Nakamura, S., Watanabe, K. and Takahashi, K. (**1979**), *FEBS Lett.*, 99, 97.

Ishikawa, C., Watanabe, K., Sakata, N., Nakagagi, C., Nakamura, S. and Takahashi, K. (**1985**), *J. Biochem.*, 97, 55.

Jackson, R. M. and Russel, R. B. (**2000**), *J. Mol. Biol.*, 296, 325.

Jensen, B., Unger, K. K., Uebe, J., Gey, M., Kim, Y. M. and Flecker, P. (**1996**), *J. Biochem. Biophys. Methods*, 33, 171.

Jirgensons, B. (**1973**), *Biochim. Biophys. Acta,* 328, 314.

Johnson, R., Narvaez, J., An, G. and Ryan, C. (**1989**), *Proc. Natl. Acad. Sci. USA*, 86, 9871.

Joubert, F. J. (**1984**), *Phytochemistry,* 23, 957.

Joubert, F., Kruger, H., Townshend, G. S. and Botes, D. P. (**1979**), *Eur. J. Biochem.*, 97, 85.

Kakade, M. M., Simons, N. and Leiner, I. E. (**1969**), *Cereal Chem.*, 46, 518.

Kassel, B. (**1970**), *Methods Enzymol.*, 19, 839.

Kennedy, A. R. (**1993**), Overview: Anticarcinogenic activity of proteinase inhibitors In: Troll, W. and Kennedy, A. R. (eds) Protease inhibitors as cancer chemopreventive agents, Plenum Press, New York, 9.

Kennedy, A. R. (**1993a**), *In vitro* studies of anticarcinogenic protease inhibitors. In: Troll, W. and Kennedy, A. R. (eds) Protease inhibitors as cancer chemopreventive agents. Plenum Press, New York, 65.

Kennedy, A. R. (**1993b**), *Prev. Med.*, 22, 796.

Kennedy, A. R. (**1994**), *Cancer Res.*, 54 (Suppl.), 1999S.

Kennedy, A. R. (**1995**), *J. Nutr.*, 125, 733.

Kennedy, A. R. (**1998**), *Am. J. Clin. Nutr.*, 68 (Suppl.), 1406S.

Kennedy, A. R. and Billings, P. C. (**1987**), Anticarcinogenic actions of protease inhibitors. In: Cerutti, P. A., Nygaard, O. F. and Simic, M. G. (eds) Anitcarcinogenesis and radiation protection. Plenum Press, New York, 285.

Kennedy, A. R., Billings, P. C., Maki, P. A. and Newberne, P. (**1993**), *Nutr. Cancer*, 19, 191.

Kimura, M., Kousuma, Y., Abe, K. and Yamasaki, N. (**1994**), *J. Biochem.*, 115, 369.

Kiyohara, T., Yakota, K., Masaki, Y., Matsui, O., Iwasaki, T. and Yoshikawa, M. (**1981**), *J. Biochem.*, 90, 721.

Koepke. J., Ermler, U., Warkentin, E., Wenzl, G. and Flecker, P. (**2000**), *J. Mol. Biol.*, 298, 477.

Koiwa, H., Bressan, R. A. and Hasegawa, P. M. (**1997**), *Trends Plant Sci.*, 2, 379.

Kouzuma, Y., Suetake, M., Makoto, K. and Yamasaki, N. (**1992**), *Biosci. Biotech. Biochem.*, 556, 1819.

Kumar, P., Rao, A. G., Hariharaputran, S., Chandra, N. and Gowda, L. R. (**2004**), *J. Biol. Chem.*, 279, 30425.

Kumar, P., Sreerama, Y. N. and Gowda, L. R. (**2002**), *Phytochemistry*, 60, 581.

Kunitz, M. (**1945**), *Science*, 101, 668.

Kunitz, M. (**1946**), *J. Gen. Physiol.*, 29,149.

Kunitz, M. (**1947a**), *J. Gen. Physiol.* 30, 291.

Kunitz, M. (**1947b**), *J. Gen. Physiol.*, 30, 311.

Kunitz, M. and Northrop, J. H. (**1936**), *J. Gen. Physiol.*, 19, 991.

Kyte, J. and Doolittle, R. F. (**1982**), *J. Mol. Biol.*, 151, 105.

Laemmli, U. K. (**1970**), *Nature,* 227, 680.

Larionova, N., Gladysheva, I. P., Tikhonova, T. V. and Kazanskaya, N. F. (**1993**), *Biokhimiia*, 58, 1437.

Laskowski, M. Jr. (**1986**), Protein inhibitors of serine proteinases – mechanism and classification. In: Friedman, M. (eds) Nutritional and toxicological significance of enzyme inhibitors in foods, Plenum Press, New York, 1.

Laskowski, M. Jr. and Kato, I. (**1980**), *Ann. Rev. Biochem.*, 49, 593.

Laskowski, M. Jr. and Quasim, M. A. (**2000**), *Biochim. Biophys. Acta*, 1477, 324.

Laskowski, M. Jr., Qasim, M. A. and Lu, S. M. (**2000**), Interaction of standard mechanism, canonical protein inhibitors with serine proteinases. In: Kleant-hous, C. (eds) Protein-protein recognition: The frontiers in molecular biology series. Oxford University Press, Oxford, 228.

Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (**1993**), *J. Appl. Cryst.*, 26, 283.

Leavis, P. C., Rosenfold, S. S., Gergely, J., Grabarek, Z. and Drabikowski, W. (**1978**), *J. Biol. Chem.*, 253, 5452.

Li, J. H., Billings, P. C. and Kennedy, A. R. (**1992**), *Cancer J.*, 5, 354.

Li, Y., Huang, Q., Zhang, S., Liu, S., Chi, C. and Tang, Y. (**1994**), *J. Biochem.* (Tokyo), 116, 18.

Liener, E., Kakade, M. L. (**1980**), Protease inhibitors. In: Toxic Constituents of Plant Food Stuffs, (II eds) Academic press, New York, 7.

Lin, G. D., Bode, W., Huber, R., Chi, C. W. and Engh, R. A. (**1993**), *Eur. J. Biochem.*, 212, 549.

Lineweaver, H. and Burk, D. (**1934**), *J. Amer. Chem. Soc.*, 56, 658.

Lipman, D. J. and Pearson, W. R. (**1985**), *Science*, 27, 1435.

Long, S., Quigley, J., Troll, W. and Kennedy, A. R. (**1981**), *Carcinogenesis*, 2, 933.

Lorensen, E., Prevasto, R. and Wilson, K. A. (**1981**), *Plant Physiol.*, 68, 88.

Luckett, S., Garcia, R. S., Barker, J. J., Konarev, A. V., Shewry, P. R., Clarke, A. R. and Brady, R. L. (**1999**), *J. Mol. Biol.*, 290, 525.

Madden, M. A., Tan-Wilson, A. L. and Wilson, K. A. (**1985**), *Phytochemistry*, 24, 2811.

Maeder, D. L., Sunde, M. and Botes, D. P. (**1992**), *Int. J. Prot. Res.,* 40, 97.

Mahoney, W. C. and Hermodson, M. A. (**1980**), *J. Biol. Chem.*, 255, 11199.

Malykh, E. V., Tiourina, O. P. and Larionova, N. I. (**2001**), *Biochemistry (Mascow)*, 66, 444.

March, S. C., Parikh, I. and Cuatrecasas, P. (**1974**), *Anal. Biochem.,* 60, 149.

Marschutz, M. K. and Bernkop-Shnurch, A. (**2000**), *Biomaterials*, 21, 1499.

Matsudaira, P. (**1987**), *J. Biol. Chem.*, 262, 10035.

McBride, J. D., Brauer, A. B. E., Nievo, M. and Leatherbarrow, R. J. (**1998**), *J. Mol. Biol.*, 282, 447.

McBride, J. D., Freeman, N., Domingo, G. J. and Leatherbarrow, R. J. (**1996**), *J. Mol. Biol.*, 259, 819.

McBride, J. D. and Leatherbarrow, R. J. (**2001**), *Curr. Med. Chem.*, 8, 909.

McManus, M. T., White, D. W. R. and McGregor, P. G. (**1994**), *Transgen. Res.*, 3, 50.

Mehta, S. L. and Simlot, M. M. (**1982**), *J. Biosci.*, 4, 295.

Miege, M. N., Mascherpa, J. M., Royer-Spierer, A., Grange, A. and Miege, J. (**1976**), *Planta*, 131, 81.

Messadi. P. V., Billings, P. C., Shklar, G. and Kennedy, A. R. (**1986**), *J. Natl. Cancer Inst.*, 76, 4447.

Messina, M. J., Persky, V., Setchell, K. D. R. and Barnes, S. (**1994**), *Nutr. Cancer.*, 21, 113.

Michaud, D. (**1997**), *Trends Biotech.*, 15, 4.

Michaud, D., Nguyen-Quoc, B., Vrain, T. C., Fong, D. and Yelle, S. (**1996**), *Arch. Insect. Biochem. Physiol.*, 31, 451.

Miles E. W. (**1977**), *Methods Enzymol.*, 47, 431.

Millar, D. B. S., Willick, G. E., Steiner, R. F. and Fratalli, V. (**1969**), *J. Biol. Chem.*, 244, 281.

Mitsui, Y., Satow, Y., Watanabe, Y., Hirono, S. and Iitaka, Y. (**1979**), *Nature*, 277, 447.

Miyata, S., Koshikawa, N., Yasumitsu, H. and Miyazaki, K. (**2000**), *J. Biol. Chem.*, 275, 4592.

Miyata, S., Miyagi, Y., Koshokawa, N., Nagashima, Y., Kato, Y., Yasumitsu, H., Hirahara, F., Misugi, K. and Miyazaki, K. (**1998**), *Clin. Exp. Metastasis*, 16, 613.

Miyoshi, M., Hamaguchi, Y., Matsumoto, K. and Muzuno, I. (**1978**), *J. Nutri. Sci. Vitaminol.*, 24, 195.

Morishita, I., Morishita, M., Takayama, K., Machida, Y. and Nagai, T. (**1992**), *J. Pharma.*, 78, 9.

Mosolov, V. V., Fedurkina, N. V., Valueva, T. A. and Sokolova, E. V. (**1976**), *Appl. Biochem. Microbiol.* (Mascow), 12, 26.

Murzin, A. G., Brenner, S. E., Hubbard, T. and Chothia, C. (**1995**), *J. Mol. Biol.*, 247, 536.

Nagasue, A., Fukamachi, H., Ikenaga, H. and Funatsu, G. (**1988**), *Agric. Biol. Chem.*, 52, 1505.

Norioka, S. and Ikenaka, T. (**1983a**), *J. Biochem.*, 93, 479.

Norioka, S. and Ikenaka, T. (**1983b**), *J. Biochem.*, 94, 589.

Norioka, S. and Ikenaka, T. (**1984**), *J. Biochem.*, 91, 1427.

Norioka, S., Omichi, K. and Ikenaka, T. (**1982**), *J. Biochem.*, 91, 1427.

Odani, S. and Ikenaka, T. (**1972**), *J. Biochem.*, 71, 839.

Odani, S. and Ikenaka, T. (**1973**), *J. Biochem.* (Tokyo), 74, 857.

Odani, S. and Ikenaka, T. (**1976**), *J. Biochem.*, 80, 641.

Odani, S. and Ikenaka, T. (**1977a**), *J. Biochem.*, 82, 1513.

Odani, S. and Ikenaka, T. (**1977b**), *J. Biochem.*, 82, 1523.

Odani, S. and Ikenaka, T. (**1978a**), *J. Biochem.*, 83, 737.

Odani, S. and Ikenaka, T. (**1978b**), *J. Biochem.*, 83, 747.

Odani, S., Koide, T. and Ono, T. (**1986**), *J. Biochem.*, 100, 975.

Odani, S. and Ono, T. (**1980**), *J. Biochem.*, 88, 1555.

Ohtsubo, K., Ochiai, Y. S. and Yanase, H. (**1985**), *Agric. Biol. Chem.*, 49, 1895.

Oreffo, V. I. C., Billings, P. C., Kennedy, A. R. and Witschi, H. (**1991**), *Toxicology*, 69, 165.

Orf, J. H. and Hymowitz, T. (**1979**), *J. Am. Oil Chem. Soc.*, 56, 722.

Park, S. S., Sumi, T., Ohba, H., Nakamura, O. and Kimura, M. (**2002**), *Biosci. Biotech. Biochem.*, 64, 2272.

Prakash, B., Murthy, M. R. N., Sreerama, Y. N., Rao, D. R. and Gowda, L. R. (**1997**), *J. Biosci.*, 22, 545.

Prakash, B., Selvaraj, S., Murthy, M. R. N., Sreerama, Y. N., Rao, D. R. and Gowda, L. R. (**1996**), *J. Mol. Evol.*, 42, 560.

Purseglove, J. W. (**1974**), *Dolichos uniflorus,* In: Tropical Crops: Dicotyledons, London, Longman, 263.

Pusztai, A. (**1968**), *Eur. J. Biochem.*, 5, 252.

Pusztai, A. (**1972**), *Planta*, 107, 121.

Pusztai, A., Croy, R. R. D., Grant, G. and Watt, W. B. (**1977**), *New Phytol.*, 79, 61.

Quillien, L., Ferrasson, E., Molle, D. and Gueguen, J. (**1997**), *J. Protein Chem.*, 16, 195.

Radisky, E. S. and Koshland Jr. E. (**2002**), *Proc. Natl. Acad. Sci.*, 10316.

Ramasarma, P. R. and Rao, D. R. (**1991**), *Ind. J. Biochem. Biophys.*, 28, 418.

Ramasarma, P. R., Rao, A. G. and Rao, D. R. (**1995**), *Biochim. Biophys. Acta* , 1248, 35.

Read, R. J. and James, M. N. G. (**1986**), In: Proteinase inhibitors, (eds) Barret, A. J. and Salvesen, Elsevier, Amsterdam, 301.

Reddy, N. R., Sathe, S. K., Salunkhe, D. K. (**1989**), Carbohydrates. In: CRC Handbook of Word Food legumes: Nutritional Chemistry, Processing Technology and Utilization. Salunkhe, D. K. and Kadam, S. S. (eds) CRC Press, Boca Raton, FL, 1, 65.

Reilly, C. C., O'Kennedy, B. T., Titus, J. S. and Splittstoesser, W. E. (**1978**), *Plant Cell Physiol.*, 19, 1235.

Reisfeld, R. A., Lewis, U. J. and Williams, D. E. (**1962**), *Nature*, 4838, 281.

Richardson, M. (**1977**), *Phytochemistry*, 16, 157.

Richardson, M. (**1991**), *Methods Plant Biochem.*, 5, 259.

Ryan, C. A. (**1973**), *Ann. Rev. Plant Physiol.*, 24, 173.

Ryan, C. A. (**1990**), *Ann. Rev. Physiol. Pathol.*, 28, 425.

Satow, Y., Mitsui, Y. and Iitaka, Y. (**1978**), *J. Biochem.*, 84, 897.

Schechter, I. and Berger, A. (**1967**), *Biochim. Biophys. Res. Commun.*, 27, 157.

Schroeder, H. E., Gollasch, S., Moore, A., Tabe, L. M., Craig, S., Hardie, D. C., Chrispeels, M. J., Spencer, D. and Higgins, T. J. U. (**1995**), *Plant Physiol.,* 107, 1233.

Shewry, P. R. (**1999**), Enzyme inhibitors of seeds: Types and properties. In: Shewry, P. R. and Casey, R. (eds) Seed Protein. Kluwer Academy Publishers, Amsterdam, The Netherlands, 587.

Shimokawa, Y., Kuromizu K., Araki, T., Ohata, J. and Abe, O. (**1984**), *Eur. J. Biochem.*, 143, 677.

Shutov, A. D., Beltey, N. K. and Vaintraub, I. A. (**1984a**), *Biokhimiya*, 49, 1171.

Shutov, A. D., Bulmaga, V. P. and Vaintraub, I. A. (**1984b**), *Biochem. Physiol. Pflanzen*, 179, 191.

Shutov, A. D., Korolyova, T. N. and Vaintraub, I. A. (**1978**), *Fiziol. Rast.*, 25, 735.

Shutov, A. D. and Vaintraub, I. A. (**1987**), *Phytochemistry*, 26, 1557.

Sierra, I. Li de La, Quillien, L., Flecker, P., Gueduen, J. and Brunie, S. (**1999**), *J. Mol. Biol.*, 285, 1195.

Singh, R. R. and Rao, A. G. (**2002**), *Biochim. Biophys. Acta*, 1597, 280.

Smith, E. L. (**1977**), *Methods Enzymol.*, 47, 156.

Song, H. K., Kim, Y. S., Yang, J. K., Moon, J., Lee, J. Y. and Suh, S. W. (**1999**), *J. Mol. Biol.*, 293, 1133.

Sorsa, T., Salo, T., Koivunen, E., Tyynela, J., Konttinen, Y. T., Bergmann, U., Tuuttila, A., Neimi, E., Teronen, O., Heikkila, P., Tschesche, H., Leinonen, J., Osman, S. and Stenman, U. H. (**1997**), *J. Biol. Chem.*, 272, 21067.

Speicher, D. W. (**1989**), In: *Techniques in Protein Chemistry*. Hugli T. E. (eds) Academic Press, New York, 24.

Sreerama, Y. N. (**1996**), Ph. D thesis, University of Mysore, Mysore, India.

Sreerama, Y. N. and Gowda, L. R. (**1997**), *Biochim. Biophys. Acta*, 1343, 235.

Sreerama, Y. N. and Gowda, L. R. (**1998**), *J. Agric. Food Chem.,* 46, 2596.

Sreerama, Y. N., Das, J. R., Rao, D. R. and Gowda, L. R. (**1997**), *J. Food Biochem.*, 21, 461.

St. Clair, W. H., Billings, P. C. and Kennedy, A. R. (**1990a**), *Cancer Lett.*, 52, 145.

St. Clair, W. H., Billings, P. C., Carew, J. A., Keller-McGandy, C. E., Newberne, P. and Kennedy, A. R. (**1990b**), *Cancer Res.*, 50, 580.

St. Clair, W. H., St. Clair, D. K. and Kennedy, A. R. (**1991**), *Cancer J.*, 4, 278.

Steiner, R. F. and Frattali, V. (**1969**), *J. Agric. Food Chem.*, 17, 153.

Stevens, F. C., Wuery, S. and Krahu, J. (**1974**), Structure function relationship in lima bean proteinase inhibitors. In: Fritz, H., Tschesche, H., Greene, L. J. and Truscheit, E. (eds) Proteinase inhibitors, Bayer Symposium V, Springer Verlag, Berlin, Heidelberg, New York, 344.

Suzuki, A., Tsunogae, Y., Tanaka, I., Yamane, T., Ashida, T., Norioka, S., Hara, S. and Ikenaka, T. (**1987**), *J. Biochem. (Tokyo)*, 101, 267.

Suzuki, A., Yamane , T., Ashida, T., Norioka, S., Hara, S. and Ikenaka, T. (**1993**), *J. Mol. Biol.*, 234, 722.

Sweet, R. M., Wright, H. T., Janin, J., Chothia, C. H. and Blow, D. M. (**1974**), *Biochemistry*, 13, 4212.

Tanaka, A. S., Sampaio, M. U., Marangoni, S., de Oliveira, B., Novello, J. C., Oliva, M. L., Fink, E. and Sampaio, C. A. (**1996**), *J. Biol. Chem.*, 378, 273.

Tanaka, A. S., Sampaio, M. U., Mentele, R., Aureswald, E. A. and Sampaio, C. A. (**1997**), *J. Prot. Chem.*, 15, 553.

Tan-Wilson, A. L., Cosgiff, S. E., Duggan, M. C., Obach, R. S. and Wilson, K. A. (**1985**), *J. Agric. Food Chem.*, 33, 389.

Tan-Wilson, A. L., Rightmire, B. R. and Wilson, K. A. (**1982**), *Plant Physiol.*, 70, 493.

Tashiro, M., Asao, T., Hirata, C., Takahashi, K. and Kanamori, M. (**1990**), *J. Biochem.*, 108, 669.

Tashiro, M., Hashiro, K., Shiozaki, M., Ibuki, F. and Maki, Z. (**1987**), *J. Biochem.*, 102, 297.

Terada, S., Fujimura, S., Kino, S. and Kimato, E. (**1994a**), *Biosci. Biotech. Biochem.*, 58, 371.

Terada, S., Fujimura, S., Kino, S. and Kimato, E. (**1994b**), *Biosci. Biotech. Biochem.*, 58, 376.

Towbin, H., Staehelin, T. and Gordon, J. (**1979**), *Proc. Natl. Acad. Sci., USA*, 76, 4350.

Tsunogae, Y., Tanaka, I., Yamane, T., Kikkawa, J., Ashida, T., Ishikawa, C., Watanabe, K., Nakamura, S. and Takahashi, K. (**1986**), *J. Biochem. (Tokyo)*, 100, 1637.

Tur-Sinai, A., Birk, Y., Gertler, A. and Rigbi, M. (**1972**), *Biochim. Biophys. Acta*, 263, 666.

Tyndall, J. D. A. and Fairlie, D. P. (**1999**), *J. Mol. Recognit.*, 12, 363.

Urwin, P. E., Atkinson, H. J., Waller, D. A. and McPherson, M. J. (**1995**), *Plant J.*, 8, 121.

Ventura, M. M., Mizuta, K. and Ikemoto, H. (**1981**), *An. Acad. Brasil. Cienc.*, 53, 195.

Verdcourt, B. (**1970**), *Kew Bull.*, 24, 379.

Vidal-Valverde, C. and Frias, J. (**1992**), *Z. Lebensm. Unters. Forsch.*, 194, 461.

Voss, R. H., Ermler, U., Essen, L. O., Wenzl, G., Kim, Y. M. and Flecker, P. (**1996**), *Eur. J. Biochem.*, 242, 122.

Wagner, J. P. and Riehm, J. P. (**1967**), *Arch. Biochem. Biophys.*, 121, 672.

Ware, J. H., Wan, X. S. and Kennedy, A. R. (**1999**) *Nutr. Cancer*, 33, 174.

Ware, J. H., Wan, X. S., Rubin, H., Schechter, N. M. and Kennedy, A. R. (**1997**), *Arch. Biochem. Biophys.*, 344, 133.

Weder, J. K. P. and Kahelyss, R. (**1998**), *J. Sci. Food Agric.*, 78, 429.

Weder, J. K. P. and Link, I. (**1993**), Effect of treatments on legume inhibitor activity against human proteinases. In: Recent advances of research in antinutritinal factors in legume seeds, Wegeningen press, Wegeningen, The Netherlands, 481.

Werner, M. H. and Wemmer, D. E. (**1992**), *Biochemistry*, 31, 999.

Werner, M. H. and Wemmer, D. E. (**1992a**), *J. Mol. Biol.*, 225, 873.

Whitley, E. J. and Bowman, D. E. (**1975**), *Arch. Biochem. Biophys.*, 169, 42.

Wilson, K. A. (**1981**), The structure, function and evolution of legume proteinase inhibitors. In: *Antinutrients and Natural Toxicants in Foods*,

Ory, R. L. (eds) Food and Nutrition Press, Westport, Conn, Chap. 11, 11.

Wilson, K. A. (**1988**), *Crit. Rev. Biotech.*, 8, 197.

Wilson, K. A. and Chen, J. C. (**1983**), *Plant Physiol.*, 71, 341.

Wilson, K. A. and Laskowski, M. Sr. (**1973**), *J. Biol. Chem.*, 248, 756.

Wilson, K. A. and Laskowski, M. Sr. (**1975**), *J. Biol. Chem.*, 250, 4261.

Wilson, K. A. and Tan-Wilson, A. L. (**1983**), *Acta Biochim.*, 30, 139.

Wilson, K. A. and Tan-Wilson, A. L. (**1983a**), *Plant Physiol.*, 71, 268.

Wu, C. and Whitaker, R. (**1990**), *J. Agric. Food Chem.*, 38, 1523.

Wu, Y. V. and Sessa, D. J. (**1994**), *J. Agric. Food. Chem.*, 42, 2136.

Xu, D., Xue, Q., McElory, D., Mawal, Y., Hilder, V. A. and Wu, R. (**1996**), *Mol. Breed.*, 2, 167.

Yamamoto, A., Taniguchi, T., Rikyun, K., Tsuji, T., Fujita, T., Murakami, M. and Muranishi, S. (**1994**), *Pharma. Res.*, 11, 1496.

Yamamoto, M. and Ikenaka, T. (**1967**), *J. Biochem.*, 62, 141.

Yoshida, C. and Yoshikawa, M. (**1975**), *J. Biochem.*, 78, 935.

Yoshikawa, M., Kiyohara, T. and Yoshida, I. (**1979**), *Agric. Biol. Chem.*, 43, 1989.

Yoshikawa, M., Kiyohara, T., Iwasaki, T., Ishii, Y. and Kimura, N. (**1979a**), *Agric. Biol. Chem.*, 43, 787.

Zhang, Y. S., Lo, S. S., Tan, F. L., Chi, C. W., Xu, L. X. and Zhang, A. L. (**1982**), *Sci. Sinica*, 25, 268.

Zor, T. and Selinger, Z. (**1996**), *Anal. Biochem.*, 236, 302.