STUDIES ON MOLECULAR ASPECTS OF SEED STORAGE PROTEINS IN HORSE GRAM *{Dotichos bifiorus L.)*

THESIS SUBMITTED TO THE

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IN

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BY

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... Dedicated to

My parents

and

Dr. Sailaja Koduri

DECLARATION

I hereby declare that this thesis entitled " **Studies on molecular aspects of seed storage proteins in horse gram *{Dolichos biflorus* Z..)",** which is submitted to the University of Mysore, Mysore, for the degree of **Doctor of Philosophy in Biotechnology,** is the result of research work carried out by me in the **Department of Food Microbiology, Central Food Technological Research Institute, Mysore, India,** under the guidance of **Dr. N. K. Singh** during the period 1993-1999.

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

R.Venkatesha 8/4/99

(R.T.VENKATESHA)

CERTIFICATE

I hereby certify that the thesis entitled **"STUDIES ON MOLECULAR ASPECTS OF SEED STORAGE PROTEINS IN HORSE GRAM *(Dolichos biflorus* L.)"** submitted by **Mr. R.T. Venkatesha** for the degree of **Doctor of Philosophy in Biotechnology** of the University of Mysore, Mysore, is the result of research work carried out by him in the Department of Food Microbiology, Central Food Technological Research Institute, Mysore, under my guidance during the period July 1993 to January 1999.

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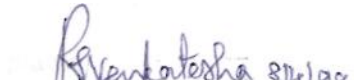


(R.T. VENKATESHA)

CONTENTS

LIST OF ABBREVIATIONS

BBI	-Bowman-Birk inhibitor
BCIP	-5-Bromo-4-chloro-3-indolyl phosphate.
Bisacrylamide	-N, N` methylenebisacrylamide
bp	-Base pairs
BSA	-Bovine serum albumin
C	-Chymotrypsin
C^0	-Centigrade
cDNA	-Complementary deoxyribonucleic acid
cm	-Centimeter
DAI	-Days after imbibition
DAF	-Days after flowering
dNTPs	-Deoxyribonucleoside triphosphates
E	-Elastase
e. g	-For example
EDTA	-Ethylene diamine tetra acetate.
Fig	-Figure
g	-Gram
h	-Hour
HGI	-Horse gram inhibitor
i.e.,	-That is
IPTG	-Isopropylthio-β-D-Galactoside
kDa	-Kilo Dalton
LB	-Luria – Bertani
M	-Molar
2-me	-2-mercaptoethanol
Ml	-Milliliter
µl	-Microliter
µm	-Micrometer
NBT	-Nitro blue Tetrazolium
PAGE	-Polyacrylamide gel electrophoresis
PCR	-Polymerase chain reaction
%	-Per cent
rpm	-Revolutions per minute
SDS	-Sodium dodecyl sulphate
SEM	-Scanning electron microscope
SSC	- Saline sodium citrate.
T	-Trypsin
TBS	-Tris buffered saline.
TBST	-Tris buffered saline with Tween-20
TEMED	-N, N, N', N',-Tetramethylethylenediamine
Tris	-Tris (hydroxy methyl) amino methane
um	-Micrometer
V	-Volts
V/V	-Volume per volume.
Vs	-Versus
W/V	-Weight per volume.
WII	-Wound induced inhibitor
X-Gal	-5-bromo-4-chloro-3-indolyl-β-D-galactoside.

INTRODUCTION

CHAPTER-1

INTRODUCTION

The plant seed is not only an organ of propagation and dispersal of the species but also a major plant part harvested by humankind for food. Grain legumes are one of the world's most important sources of food supply in terms of food energy as well as nutrients especially in the developing countries. Sometimes these are the main source of protein in the diet because animal foods are not available, expensive nor are they readily accepted in India. In addition to protein, the legumes are also a rich source of carbohydrates, vitamins and minerals. India is a major country involved in the production and consumption of grain legumes. In certain regions of India legumes constitute 30-50% of the total diet (Deshpande and Damodaran, 1990).

Horse gram *{Dolichos biflorus* L.) is considered to be the poor man's pulse particularly in South India where it is cultivated in an area of around 50,000 ha (Yadava and Vyas, 1994). Its maximum acreage is in Andhra Pradesh, Karnataka and Tamil Nadu but it is also grown in Maharastra and Madhya Pradesh to some extent. The crop has wide adaptability and thrives well under adverse climatic conditions and in poor soils.

Horse gram seed contains about twenty three per cent protein and has a higher lysine content than pigeon pea and chick pea making it a good complement to a cereal based diet. The horse gram seed is consumed after cooking, frying or as sprouts in rural parts of India by the poorer section of the population. As the name implies, it is also used as horse and cattle feed. The seed and its extracts are used to treat kidney stones, urinary diseases and piles in traditional medicine (Yadava and Vyas, 1994). Horse gram seeds also contain important anti-nutritional proteins like trypsin inhibitors and lectins (Liener and Kakade, 1980). In terms of their abundance and function, one of the most important groups of storage proteins found, in the seeds are the enzyme inhibitors. These proteins form stoichiometric complexes with various hydrolytic enzymes and cause complete inhibition of catalytic function. In legumes, serine proteinase inhibitors form an important family of inhibitors, which are of two main types viz., the Bowman-Birk type and the Kunitz type. The former is distributed extensively in legumes and has been well studied (Richardson, 1991). In addition to its

antinutritional role, it also plays beneficial role in plants by regulation of endogenous proteinases as well as in protection against plant pests and pathogens (Ryan, 1990).

As primary gene products, Bowman-Birk inhibitors have been recognized as an ideal candidate for the transfer of resistance against insect pests to crop plants by genetic engineering (Ryan, 1990). The primary structure of Bowman-Birk inhibitors from horse gram and several other legumes have already been elucidated by protein sequencing (Prakash *et ah,* 1996). This provides an opportunity for molecular cloning of their gene by polymerase chain reaction. The cloned inhibitor gene could be used to introduce insect resistance in the crop plants by genetic engineering. Similarly, horse gram lectins have been studied at both protein and DNA level and their genes could also be used in developing insect resistance (Schnell *et al.,* 1987). In addition to lectins and trypsin inhibitors which are minor proteins, horse gram seed also contains major storage proteins which have not been studied in any detail. However, the trypsin inhibitors have been studied at the protein level only and no attempt has been made to clone the genes coding for proteinase inhibitors. Similarly, no work has been reported on the major storage proteins of horse gram either at the protein or DNA level. Studies on these proteins will provide an insight into their variation, distribution and relationship with other legume seed storage proteins.

With the above background, the present study was initiated with the aim of generating more information on the seed proteins of horse gram. Particular emphasis was given on the immunochemical characterization, developmental studies and cloning of Bowman-Birk type proteinase inhibitors by polymerase chain reaction. In addition, other classes of seed proteins namely vicilin, legumin and narbonin of horse gram seeds were also studied using electrophoretic and immunological techniques.

REVIEW OF LITERATURE

CHAPTER-2

REVIEW OF LITERATURE

2.1 IMPORTANCE OF GRAIN LEGUMES IN HUMAN NUTRITION

Legumes are one of the world's most important sources of food supplies, especially in developing countries in terms of food energy as well as nutrients. The grain legumes, including soybean and groundnut, are ranked fifth in terms of annual world grain production, at around 171 million metric tons (Deshpande and Damodaran, 1990). Developing nations produce over 60% of the pulses which are a major source of food protein in these countries. The protein content of the dry weight of legumes ranges from 20 to 40 per cent (De Lumen, 1992). Food legumes are the main source and some times the only source of protein in the diets because animal foods are not available, expensive or not readily accepted as in India (Reddy *et* α/.,1982; Toenniessen, 1985). In addition to protein, legumes are a good source of carbohydrates, B-complex vitamins and minerals (Deshpande and Damodaran, 1990). India is a major developing country involved in the production and consumption of legumes (Uebersax *et ah,* 1991).

The major importance of legumes lies in their actual and potential value as a source of plant protein for human nutrition. Legumes are deficient in sulphur containing amino acids, threonine and tryptophan. The poor quality of legumes is complemented when consumed with cereals, which are rich in sulphur containing amino acids. In some parts of India, legumes constitute 30-50% of the total diet. Although the per capita availability of legumes is about 50 g/day, the daily intake in various regions of India ranges from 14 to 140 g per capita per day (Salunkhe, 1983).

The digestibility of cereal seed protein is generally 75-90% whereas that of raw and cooked legume seed is 15-80% and 50-90%, respectively (Eggum and Beames, 1983). The albumins and globulins of legumes are often found to be considerably less digestible than cereal proteins. The amino acid profiles of individual storage protein fractions may vary from the overall profile for a given source. The minor albumin and globulin fractions of cereals have fair to very good amino acid balance. Within legumes, there is considerable difference between 7S and US globulins as the former is lower in sulphur containing amino acids but generally higher in lysine (Phillips, 1997). Thus, grain legumes provide a good complement to the mainly cereal-based diets in the developing countries. In addition to their nutritional significance, grain legumes are used

to produce a variety of food products in these countries. The seed proteins also play an important role in determining their processing quality.

2.2 PHYLOGENY OF GRAIN LEGUMES AND CLASSIFICATION OF THEIR SEED STORAGE PROTEINS

The domesticated food legumes are all members of the subfamily Papilionoideae of the family Leguminosae and belong to three tribes namely Vicieae, Genisteae and Phaseoleae (Fig. 2.1). The common and scientific names of major grain legumes used as food source are listed in Table 2.1.

Table 2.1 Common grain legumes used as food source

Common name	Botanical name
Soybean	*Glycine max*
Pea	*Pisum sativum*
Common bean, Kidney bean, French bean	*Phaseolus vulgaris*
Cowpea	*Vigna unguiculata, Vigna sinensis*
Pigeon pea, Red gram	*Cajanus cajan*
Chick pea, Bengal gram	*Cicer arietinwn*
Hyacinth bean, Lablab bean	*Dolichos lablab, Lablab niger, Lablab purpureus*
Horse gram	*Dolichos biflorus, Macrotyloma uniflorum*
Lupins	*Lupinus* sps.
Mung bean, Green gram.	*Phaseolus aureus, V. radiata,*
Black gram, Urd bean	*Phaseolus mungo, V. mungo*
Groundnut, Peanut	*Arachís hypogaea*
Jack bean, Horse bean	*Canavalia ensíformis*
Kesari dal, Grass pea	*Lathyrus sativus*
Lentils, Masur dhal	*Lens esculenta, Lens culinaris*
Adzuki bean	*Phaseolus angularis*
Broad bean, Faba bean	*Viciafaba*
Vetch	*Vicía sativa*
Narbon bean	*Vicia narbonensis*
Moth bean	*Vigna aconitifolia*
Wild pea	*Macrotyloma axillare, Dolichos axillaris*

Systematic study of seed storage proteins was carried out at the turn of this century when they were classified based on their solubility forms into four groups (Osborne, 1924). These groups are (i) Albumins : water soluble (ii) Globulins : dilute

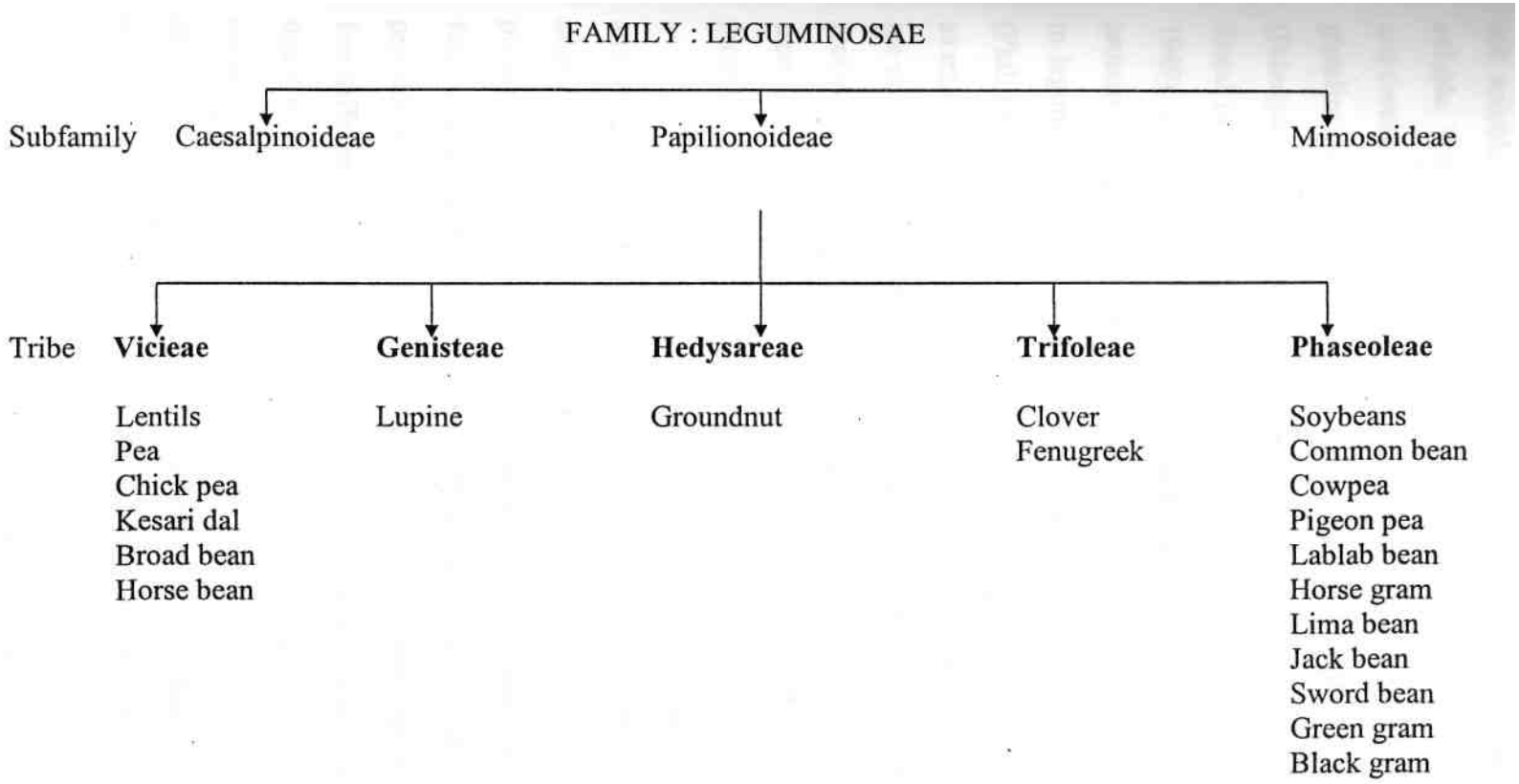


Fig. 2.1 Botanical classification of major food legumes (from Deshpande and Damodaran, 1990)

salt soluble, (iii) Prolamins : alcohol soluble and (iv) Glutelins : dilute acid or alkali soluble. The protein content and percentage of different solubility fractions of legumes and cereals vary considerably. The principal storage proteins of cereals are prolamins and glutelins except for rice and oat where the IIS globulins are the major storage proteins (Brinegar and Peterson, 1982 ; Okita *et al.,* 1989). Albumins and globulins are mainly found in dicots and globulins are the major storage proteins of legumes (Danielsson, 1949). Legumes do not contain prolamins but a small percentage of alcohol soluble protein has been often reported (Phillips, 1997). Globulins seem to always predominate in legume seeds but in mung bean glutelins comprise almost 30% of the total protein (Phillips, 1997). Albumins are sometimes considered to have a storage role and comprise as much as 20% of the total proteins (Youle and Huang, 1981). The seed storage proteins are rich in amides (glutamine and asparagine) which constitute upto 40% of the total seed proteins. The storage proteins are further classified into 2S, 7S and 1 IS proteins based on their sedimentation coefficient (Danielsson, 1949). The distribution of major storage protein groups in food plants is quite variable (Shewry, 1995).

Based on their function, the seed proteins are also classified into house keeping and storage proteins (Higgins, 1984). The house keeping proteins are responsible for normal cell metabolism of plants and constitute relatively small amounts of numerous protein species. Some of these proteins are enzymes or enzyme inhibitors whose functions are concerned with seed protection, viability and germination. The storage proteins account for the major part of the seed proteins and are represented by relatively few different species of proteins. The main characteristics of seed storage proteins are that (i) they accumulate in seeds in large quantities, (ii) they are tissue specific and occur only in the seed, (iii) they are synthesized during seed development, (iv) are often sequestered in membrane-bound organelles (protein bodies) and (v) are hydrolyzed to release its constituent amino acids during germination (Deshpande and Damodaran, 1990).

In general, storage proteins lack enzyme activity but in few cases, they themselves have been shown to exhibit metabolic activity (Shewry, 1995). The storage proteins are of particular importance because they determine not only the amount of protein in the seed but also their functional quality for various end uses. Because of their abundance and economic importance, a large volume of literature is available on the seed storage proteins as reviewed by Derbyshire *et al.* (1976), Mosse and Pemollet (1982),

Higgins (1984), Murray (1984), Wright (1987, 1988), Chrispeels (1984), Casey *et al.* (1986, 1993), Shotwell and Larkins (1989), Utsumi (1992), Shewry (1995), and Shewry *et al,* (1995). A detailed review of the major classes of seed storage proteins present in the grain legumes is presented below.

2.3 2S PROTEINS

The 2S proteins are present in a diverse range of species of different families of plants (Youle and Huang, 1981). They consist of both albumins and globulins. The albumins are mainly metabolic proteins involved in cellular activities including the synthesis and degradation of the storage proteins (Bash'a and Beevers, 1975). The proportion of albumins varied from 20% of the total protein in sunflower to 30% in Brazil nut (Youle and Huang, 1981; Kortt and Caldwell, 1990). In *Brassica,* 13% of the total salt extractable proteins are albumins (Monsalve and Rodriguez, 1990). Some albumins, e.g., 2S protein from Brazil nut, are rich in sulphur containing amino acids. Similar proteins are present in sunflower, *Medicago* and cotton (Kortt and Caldwell, 1990; Coulter and Bewley, 1990; Galau *et al,* 1992).

The 2S albumins of Cruciferae, called napins consist of two polypeptide chains of about 9 kDa and 4 kDa which are held together by disulphide bonds (Ericson *et al,* 1986). The two polypeptides are the products of a single precursor molecule cleaved with a loss of linker peptide and small peptides at N- and C-termini (Ericson *et al,* 1986). This type of albumins are also present in *Arabidopsis,* raddish, kohlrabi, mustard, pumpkin, cotton, Brazil nut, lupin and castor bean (Sharief and Li, 1982; Shewry, 1995). The 2S albumin precursors of pea are cleaved but no inter-chain disulphide bonds are formed (Higgins *et al,* 1986). No proteolytic processing of the precursor takes place however in sunflower (Anisimova *et al,* 1994).

In addition to the 2S albumins, smaller globulins of 2S-4S are also found in several dicots and monocots accounting for more than 5% of the total protein (Derbyshire *et al,* 1976). The amino acid composition of 2S globulins generally show high concentrations of amides and arginine. A 2S globulin, conglutin δ from *Lupinus angustifolius* (Gayler *et al,* 1990), a 2S globulin with some trypsin and chymotrypsin inhibitor activities from soybean (Koshiyama *et al,* 1981) and a 2S globulin (narbonin) from *Vicia narbonensis* (Schlesier *et al,* 1978) have been studied in detail.

Among the 2S globulins of legumes, narbonin has been well characterized at biochemical and molecular levels (Hennig *et ah,* 1995; Nong *et al.,* 1995). The narbonin is a monomer of 290 amino acid and its structure closely resembles the enzyme triose phosphate isomerase but lacks in enzymatic activity. Narbonin is also found in *V. pannonica* whose amino terminal sequence is identical to narbonin of narbon bean but differs in internal structure as detected by peptide mapping (Schlesier *et al.,* 1993). Narbonin gene and cDNA has been cloned from narbon bean and genomic DNA fragments encoding narbonin-like proteins were amplified from narbon bean, faba bean, vetch, *V. pannonica,* soybean and jack bean by PCR and it was found that there is more than one narbonin gene in the above legumes (Nong *et al;* 1995). They also speculated that narbonin genes are common or universal and may exist in other families as well. But the expression of these genes has not been studied in legumes other than narbon bean and *V. pannonica.* Thus, 2S proteins of legume include a variety of protein species including albumins, globulins and enzyme inhibitors and some of these have been studied at the molecular level.

2.4 7S GLOBULINS

Globulins are the most prevalent group of seed storage proteins in dicotyledonous plants and accounts for 50-90% of the seed proteins in legumes (Utsumi, 1992). Three main groups have been recognized namely, 2S globulins, 7S globulins (vicilin-like) and US globulins (legumin-like) (Danielsson, 1949). Of these, the 2S globulins are a minor group as described above, and the 7S and US globulins are the predominant groups (Derbyshire *et al.,* 1976). The US globulin predominates in certain legumes like *Vicia* while 7S globulins predominates in pea, *Phaseolus* and winged bean (Derbyshire *et al.,* 1976; Utsumi, 1992). Even within a species, the ratio of US to 7S globulin varies, e.g., from 0.5 to 1.7 in soybean (Saio *et al,* 1969), 0.2 to 1.5 in pea (Casey *et al,* 1982) and 0.3 to 0.5 in field bean (Gatehouse *et al.,* 1980). Both genetic and environmental factors affect this ratio (Blagrove *et al,* 1976; Gayler and Sykes, 1981).

The 7S globulins are typically trimeric in nature having a molecular weight of 150-190 kDa of glycosylated subunits of 50-75 kDa. The well known 7S globulins are vicilins of pea, conglycinins of soybean and phaseolin of beans. They are highly polymorphic in nature due to combination of multiple structural genes and extensive post-translational processing. Due to the lack of cysteine residues, non-covalent forces stabilize the mature proteins. The presence of vicilin-like proteins has been reported from

large number of legumes (Danielsson, 1949; Derbyshire *et al,* 1976). These globulins are of two types based on the occurrence of post-translational proteolysis as in case of pea and faba bean, and absence of such proteolysis as in soybean and *Phaseolus* (Shewry, 1995). The vicilins of legumes are glycosylated in nature with varied subunit size (Table 2.2).

The genes and cDNAs for vicilin-like proteins have been cloned from several species (Table 2.3). All the 7S globulin genes determined except convicilin have six exons and five introns (Utsumi, 1992). The size of each exon and intron exhibits a rough homology among 7S globulin genes. There is also homology in the nucleotide sequences of the exon/intron junctions. All genes possess the TATA box 30-40 bp upstream to the transcription initiation site and DNA sequence homology with animal enhancer core sequence. Some genes have GAAT box and all genes except the β-conglycinin β-subunit gene have a vicilin box 120-1.40 bp upstream of the transcription initiation site (Gatehouse *et al.,* 1986). The vicilin box, highly conserved among 7S globulin genes, may be involved in the regulation of the 7S globulin genes (Gatehouse *et al.,* 1986). A homologous sequence of CACA box element occurs in many 7S globulin genes (Utsumi, 1992). This sequence occurs in other seed protein genes and may have a role in expression of these genes (Goldberg, 1986). The 7S and US globulins are suggested to have evolved from a duplication of single ancestral gene (Gibbs *et al.,* 1989) which is consistent with X-ray analysis of phaseolin (Lawrence *et al,* 1990).

The β-conglycinin of soybean is composed of three kinds of subunits α, α and β (Thanh and Shibasaki, 1976) while phaseolin of French bean is composed of α, β or γ subunits with different molecular weights (Brown *et ah,* 1981) (Table 2.3). Wright (1987, 1988) observed 46% homology between phaseolin and pea vicilin and 53% homology between pea vicilin and α' subunit of soybean β-conglycinin. The β-fractions of pigeon pea protein corresponds to vicilin based on their solubility and acid precipitation (Krishna *et al.,* 1977). In addition to vicilin, a second class of 7S globulins called convicilin are present in faba bean and pea with a subunit size of 64 and 68 kDa, respectively (Croy *et al.,* 1980; Newbigin *et al,* 1990). The soybean convicilin has an additional 121 amino acid residues close to the N-terminus as compared to the vicilins (Bown *et al.,* 1988). The 7S globulins of chick pea are similar in amino acid composition and N-terminal amino acid sequence to the vicilins of pea and broad bean (Jackson *et al.,* 1969). In mung bean and cowpea, vicilin is most abundant fraction (Ericson, 1975; Carasco *et al.,* 1978). The

lablab bean has been shown to contain three 7S proteins which can be separated by hydroxyapatite chromatography (Derbyshire *et al.,* 1976).

Table 2.2 Nomenclature and subunit size of 7S globulins of some legume seeds

Plant	Protein	Subunit size (kDa)	Reference
Pea	Vicilin	50, 33, 19, 16, 13.5 and 12.5	Gatehouse *et al.* (1984)
	Convicilin	68	Newbigin *et al.* (1990)
Soybean	β-conglycinin	α : 57-83	Iibuchi and Imahori (1978)
		α': 57-76	Beachy *et al.* (1981)
		β : 42-53	Thanh and Shibasaki (1976)
Broad bean	Vicilin	50, 35, 31, 19	Scholz *et al.* (1983)
	Convicilin	64	Matta *et al.* (1981b)
French bean	Phaseolin	α: 51-53	Hall *et al.* (1977)
		β : 47-50	Bollini and Chrispeels (1978)
		γ : 43-47	
Adzuki bean	7S protein	25-55	Sakakibara *et al.* (1979)
Lupinus albus	γ-conglutin	40	Melo *et al.* (1994)
	β-conglutin	15-72	
Lupinus mutabilis	γ-conglutin	42-43	Santos *et al.* (1997)
	β-conglutin	15-65	
Cowpea	Vicilin	52-56	Carasco *et al.* (1978)
Green gram	Vicilin	24 - 63	Ericson (1975)
Grass pea	Vicilin	18.5 - 78	Chandana (1992)

Table 2.3 7S globulins of some plant species for which cDNA and genes have been sequenced

Plant	Protein	Subunit	Reference	
			cDNA	Gene
Field bean	Phaseolin	α	Slightom *et al.* (1985)	Anthony *et al.* (1990)
		β	Slightom *et al.* (1985)	Slightom *et al.* (1983)
Soybean	β-conglycinin	α	Schuler *et al.* (1982a,b) Sebastiani *et al.* (1990) Coates *et al.* (1985)	Schuler *et al.* (1982a)
		α	Schuler *et al.* (1982a,b)	Schuler *et al.* (1982a) Doyle *et al.* (1986)
		β		Harada *et al.* (1989)
Pea	Vicilin	47 kDa	Lycett *et al.* (1983)	
		50 kDa	Lycett *et al.* (1983) Spencer *et al.* (1983)	Higgins *et al.* (1988)
	Convicilin	68 kDa	Domoney and Casey (1990) Casey *et al.* (1984); Newbigin *et al.* (1990)	Bown *et al.* (1988) Newbigin *et al.* (1990)
Field bean	Vicilin		Bassüner *et al.* (1987)	Weschke *et al.* (1987)
Cotton	α-globulin		Chlan *et al.* (1987)	Chlan *et al.* (1987)

Source: Utsumi (1992)

2.5 11S GLOBULINS

The 11S globulins (legumin-like proteins) are one of the major storage proteins of dicots but are also found in a few monocots, gymnosperms and spores of ferns (Shewry, 1995). The proportion of 11S globulin varies considerably ranging from almost all the proteins in some pumpkin seeds to an apparent absence in the seeds of cereals like barley and rye (Danielsson, 1949; Debryshire *et al.,* 1976). The legumin-like proteins are hexameric with subunits interacting non-covalently. Each subunit has an acidic and a basic polypeptide linked by a single disulfide bond (Nielsen, 1984). Each subunit pair is synthesized as a precursor protein that is proteolytically cleaved after disulphide bond formation. The basic polypeptides are well conserved with only a small amount of variation close to the C-terminus. In contrast, the acidic polypeptides have considerable variation in sequence within and between species notably in the hypervariable region (Wright, 1987). These regions are highly charged indicating a location at the protein surface. These regions have small sequence repeats, with poor conservation (Shewry, 1995).

Some regions of the legumin-like proteins are highly conserved including those at the junction of the acidic and basic polypeptides. The cleavage of the precursor into acidic and basic polypeptides occurs at an evolutionarily conserved asparagine-glycine bond by specific asparaginyl endopeptidase (Scott *et al.,* 1992; Hara-Nishimura *et al.,* 1995), and is a pre-requisite for the formation of hexamers (Jung *et al,* 1997). The cysteine residues, which form the disulphide bond and the amino acids on either side of the cystein residue, are also conserved. Legumins are non-glycosylated in nature except the 12S globulins of *Lupin* seeds (Duranti *et al,* 1988). Although there is a considerable variation in the size of the individual acidic and basic polypeptides, generally their sizes are 40 and 20 kDa respectively (Shewry, 1995). However, larger as well as smaller pairs of subunits occurs in different legume species (Table 2.4). In pea legumin preparations, as many as 22 different α and 11 different β subunits have been found (Matta *et al.,* 1981b). Glycinin of soybean are also heterogeneous (Casey *et al.,* 1986). In green gram, the legumin fraction consists of polypeptide chains of 37, 34 and 20 kDa (Derbyshire and Boulter, 1976).

Table 2.4. Nomenclature and subunit size of 11S globulins in some legume seeds

Plant	Name of protein	Subunit size (kDa)		Reference
Pea	Legumin	Acidic	37.6 - 40.4	Matta *et al.* (1981a)
		Basic	20.7 - 21.9	
Soybean	Glycinin	Acidic	10.0 - 42.0	Moriera *et al.* (1979)
		Basic	20.0	Staswick *et al.* (1983)
				Staswick *et al.* (1984)
				Momma *et al.* (1985a)
Broad bean	Legumin	Acidic	23.0 - 58.0	Matta *et al.* (1981b)
		Basic	21.0 - 23.0	
French bean	Legumin		20.0 - 37.0	Derbyshire and Boulter (1976)
Groundnut	Arachin		21.4 - 47.5	Krishna and Mitra (1987)
Lupinus albus	α-conglutin		19.0 - 46.0	Melo *et al.* (1994)
			19.0 - 60.0	Santos *et al.* (1997)
Lupinus mutabilis	α-conglutin		40.0 - 65.0	Santos *et al.* (1997)
Lentil	G1 fraction		14.5 - 61.0	Naves and Lourenco (1995)
Grass pea	Legumin		32.0 – 89.0	Chandana (1992)

The cDNA and genes for the 11S globulins have been cloned from several species (Table 2.5). Most of the 11S globulin genes cloned so far have four exons and three introns except β-type subunit of pea and field bean which lack intron I, while the genes of rape seed and sunflower lack intron III (Table 2.6). But there is a rough homology in the structure among these genes (Table 2.6) and a homology in the nucleotide sequences around the exon/intron junctions. The characteristic TATA, CAAT and CACA boxes and the animal enhancer core sequence in the 5'upstream regions of the US globulin genes are similar to those of 7S globulin genes (Utsumi, 1992). Sequence comparison of the US globulin genes revealed a highly conserved sequence of legumin box 100-130 bp upstream of the transcription initiation site (Utsumi, 1992). The legumin box is observed only in the 11S globulin genes and all the US globulin genes of legume seeds examined so far have this box. It has been speculated that the legumin box may regulate the expression of these genes.

Table 2.5 11 S globulins of legumes for which cDNAs/genes have been sequenced

Plant	Protein	Subunit	Reference	
			CDNA	Gene
Soybean	Glycinin	$A_{1a}B_{1b}$	Negoro *et al.* (1985); Utsumi *et al.* (1987b)	Sims and Goldberg (1989)
		$A_{1b}B_2$	Marco *et al.* (1984); Momma *et al.* (1985b); Utsumi *et al.* (1987a)	Marco *et al.* (1984); Fukazawa *et al.* (1987); Kitamura *et al.* (1990).
		A_3B_4	Fukazawa *et al.* (1985); Scallon *et al.* (1985)	Nielsen *et al.* (1989)
		$A_5A_4B_3$	Momma *et al.* (1985a); Scallon *et al.* (1985)	Scallon *et al.* (1987); Nielsen *et al.* (1989)
Pea	Legumin	-	Croy *et al.* (1982); Lycett *et al.* (1984b); Domoney *et al.* (1986)	
		A		Lycett *et al.* (1984a); Rerie *et al.* (1990)
		B,C		Lycett *et al.*(1985)
		J, K		Gatehouse *et al.* (1988)
		(Pseudo)		Bown *et al.* (1985)
Field bean	Legumin	A	Wobus *et al.* (1986); Schlesier *et al.* (1990)	
		B	Wobus *et al.* (1986)	
		-	Heim *et al.* (1994)	
Chickpea	Legumin	-	Saha *et al.* (1996)	

Source : Adopted and modified : Utsumi (1992)

Table 2.6 Structure of 11S globulin genes from different plant species

Protein	Legumin box[a] (bp)	TATA box[a] (bp)	Signal peptide (residues)	Exon I[b]	Intron I	Exon II	Intron II	Exon III	Intron III	Exon IV	Reference
Glycinin $A_{1a}B_{1b}$	-117	-32	(19)	286	328	254	291	558	381	387	Sims and Goldberg (1989)
Glycinin A_2B_{1a}	-116	-32	(18)	277	238	254	292	537	624	387	Thanh *et al.* (1989)
Glycinin $A_{1b}B_2$	-117	-32	(19)	286	617	245	312	525	439	387	Cho and Nielsen (1989)
Pea legumin A	-117	-33	(21)	286	88	251	88	627	99	387	Lycett *et al.* (1984a)
Pea legumin A type	-127	-32	(22)	279	88	251	89	633	85	387	Rerie *et al.* (1990)
Arabidopsis CRA1	?[c]	-56[d]	?	301	126	266	96	447	115	402	Pang *et al.* (1988)
Arabidopsis CRB	X[e]	-62[d]	?	283	210	263	311	429	239	390	Pang *et al.* (1988)
Glutelin 1-2	X	-32	(24)	331	89	275	103	480	83	411	Takaiwa *et al.* (1987)
Oat 12 S globulin	X	-32	?	328	117	271	122	512	100	435	Schubert *et al.* (1990)
	X	-28	(24)	328	117	275	126	516	100	435	Shotwell *et al.* (1990)
Pea legumin J	-102	-27	(22)	549	-	-	138	585	99	375	Gatehouse *et al.* (1988)
Field bean legumin B4	-107	-32	(22)	549	-	-	95	528	100	375	Bäumlein *et al.* (1986)
Cruciferin	-248	-36	(23)	282	228	362	467	819	-	-	Ryan *et al.* (1989)
Helianthinin	X	-27	(20)	288	99	258	80	933	-	-	Vonder Haar *et al.* (1988)

[a] Position from the transcription initiation site. [b] Values are given in base pairs for all exons and introns.
[c] Not determined; [d] Position from the translation initiation site. [e] Not present.
Source : Utsumi (1992)

2.6 PROTEINASE INHIBITORS

One of the most important groups of storage proteins found in seeds are the enzyme inhibitors (Richardson, 1991). These proteins are capable of forming stoichiometric complexes with various hydrolytic enzymes and cause the competitive inhibition of catalytic function. Although seed is the major source of proteinase inhibitors, they are also present in bulbs, tubers and fruits. In addition, their presence is reported in gum secretions of leguminous and other trees, rubber latex as well as leaf tissues (Xavier-Filho and Campos, 1989). They are also expressed in flowers (Pena-Cortes *et al.,* 1991; Atkinson *et al.,* 1993), in response to mechanical wounding or microbial infection in the leaves (Pautot *et al,* 1991; Ryan, 1992; McGurl *et al,* 1995).

Possible functions of these inhibitors have been proposed, including the regulation of endogenous proteinases during seed germination, storage of sulphur amino acids during dormancy and the protection of plant from insects and microorganisms (Ryan, 1973). Apart from their anti-nutritional nature, proteinase inhibitors have created considerable interest due to their defensive role against pests and pathogens, biomedical applications (Kennedy *et al.,* 1993) and as food additive to prevent unwanted proteolysis in fishery products (Izquierdo *et al.,* 1994). The proteinase inhibitors have also been used for biosystematic studies especially in legumes (Weder,1985). Hence, it is of interest to define these proteinase inhibitors in terms of their beneficial as well as anti-nutritional properties and study these at the molecular level.

As many as ten families of proteinase inhibitors have been distinguished in plants (Table 2.7). The first 8 families belong to serine proteinase inhibitors while 9^{th} and 10^{th} belong to metallo and cysteine inhibitor families, respectively. The inhibitors, which inhibit cysteine proteinases, are called cystatins and these have been studied in detail from rice and corn. A number of α-amylase inhibitors, which inhibits both proteinases and amylases, have been reported from wheat (Garcia-Olmedo *et al.,* 1987) and ragi (Shivaraj and Pattabiraman, 1981). Some proteinase inhibitors also act as endochitinase as in the case of Job's tears while maize bifunctional inhibitor is related to thaumatin-like antifungal proteins (Richardson, 1991).

Table 2.7 Proteinase inhibitor families

Sl.No.	Family	Monomer		Enzymes Inhibited	Distribution
		Size (kDa)	½ Cys		
1	Bowman-Birk	8-9 (14)	14(18)	Trypsin* Chymotrypsin Elastase	Leguminosae
2	Kunitz	21-22	4	Trypsin[b] Chymotrypsin Subtilisin Kallikrein Amylase[b]	Leguminosae Gramineae Araceae Alismataceae
3	Potato I	8-9	0-2	Chymotrypsin Trypsin Subtilisin	Solanaceae Gramineae Leguminosae Polygonaceae Curcurbitaceae
4	Potato II	6 (12)	8	Trypsin Chymotrypsin	Solanaceae
5	Cucurbit	3	6	Trypsin Hageman factor	Cucurbitaceae
6	Cereal super family	12-13	10	Amylase[b] Trypsin Hageman factor	Gramineae (CM, 2S storage proteins in Cruciferae Euphorbiaceae Lecythidaceae Leguminosae)
7	Ragi A12/ barley, rice PAPI	12-13	7-8	Amylase[b] Protease?	Gramineae
8	Thaumatin-PR like	22-23	16	Amylase[b] Trypsin	Gramineae Solanaceae
9	Carboxy-peptidase	4	6	Carboxy-peptidase	Solanaceae
10	Cystatin-like	12	0	Cysteine-proteinases	Gramineae

* Double-headed; [b] Bifunctional (α-amylase/protease) . Source : Richardson (1991)

The best studied groups of inhibitors are serine proteinase inhibitors which are widely distributed (Belitz and Weder, 1990; Richardson, 1991). Legume seeds generally contain two groups of serine proteinase inhibitors, the Kunitz type and the Bowman-Birk

type (Table 2.7). Among the wound inducible serine proteinase inhibitors, the potato I and II families of inhibitors have been studied in detail (Bryant *et. al,* 1976; Plunkett *et. al.,* 1982). The work on these families of inhibitors is reviewed below in more detail.

2.6.1 Kunitz type inhibitors

The first plant proteinase inhibitor to be isolated was Kunitz inhibitor from soybean (Kunitz, 1945, 1946) and later its complete primary structure was elucidated (Koide and Ikenaka, 1973a,b; Koide *et al.,* 1973). The soybean Kunitz inhibitors are single chain inhibitors of about 21 kDa and include only four cysteins forming two disulphide bridges. This is an inhibitor of trypsin but also inhibits chymotrypsin weakly. The inhibitors which are clearly homologous to soybean type have been reported from a number of legumes e.g., winged bean (Yamamoto *et al.,* 1983; Habu *et al,* 1992), *Erythrina latíssima* (Joubert *et al.,* 1985), *Canavalia lineata* (Terada *et al.,* 1994a), *Schizolobium parahyba* (Souza *et al.,* 1995) and potato (Mitsumori *et* α/.,1994). These inhibitors are mainly distributed among Papillionoidea, Caesalpineae and Solanaceae. Although most of these inhibitors are single chain molecules the inhibitors of Mimosoideae are composed of a large α chain (16 kDa) and a small β chain (5 kDa) linked together by a disulphide bridge. Such two chain inhibitors presumably arise via post-translational proteolytic processing (Odani *et al,* 1979). This type of inhibitors have been characterized from *Albizzia julibrissa* (Odani *et al.,* 1979), *Acacia elata* (Kortt and Jermyn, 1981), *Adenanthera pavonina* (Negreiras *et al.,* 1991), *Prosopis juliflora* (Richardson, 1991) and *Enterolobíum contortisiliqum* (Batista *et al,* 1996) The members of legume Kunitz inhibitor family have also shown homology (20-30%) with α-amylase/subtilisin inhibitors of barley (Hejgaard *et al.,* 1983; Svendsen *et al.,* 1986), wheat and rice (Richardson, 1991). A partial list of the cloned cDNAs and genes of Kunitz type inhibitors is presented in Table 2.8.

Table 2.8 List of some Kunitz proteinase inhibitor genes

Source	Target enzyme *	Reference
Soybean	T	Goldberg *et al.* (1981)
	T	Jofuku and Goldberg (1989)
Acacia confusa	T	Hung *et al.* (1992)
Giant taro	T & C	Mathews *et al.* (1996)
Sweet potato	T	Yeh *et al.* (1997)
Winged bean	C	Peyachoknagul *et al.* (1989)
	C	Habu *et al.* (1992)

*T -Trypsin C -Chymotrypsin

2.6.2 Potato I and II family of inhibitors

Wounding or mechanical damage to potato and tomato initiates synthesis of two non-homologous inhibitors of serine proteinases, Inhibitor I and Inhibitor II, throughout the plant (Bryant *et al,* 1976; Plunkett *et* α/.,1982). The type I (PI-I) family includes all those sequences which show high levels of homology with the chymotryptic inhibitor I proteins isolated from potato tubers (Melville and Ryan, 1970). These inhibitors are small monomeric proteins of 8.1 kDa and inhibit chymotrypsin at a single reactive site (Melville and Ryan, 1970; Plunkett *et al,* 1982). The PI-I can accumulate to levels of 2% of the soluble protein within 48 h of insect attack or wounding (Brown and Ryan, 1984; Graham *et al.,* 1986). The type Π (PI-II) inhibitors are monomeric of 12.3 kDa and contain two reactive sites, one of which inhibits chymotrypsin and the other trypsin (Bryant *et al.,* 1976; Plunkett *et al.,* 1982).These inhibitor molecules have an internal sequence homology of two domains suggestive of gene duplication and elongation (Richardson, 1991). Several cDNA and genomic clones have been isolated for the PI-I and PI-II types of proteinase inhibitors (Table 2.9).

Table 2.9 List of genes of some proteinase inhibitors I and II families

Source	Type of inhibitor	Target enzyme*	Reference
Potato	PI-II	T & C	Keil *et al.* (1986)
	PI-II	T & C	Sanches-Serrano *et al.* (1986)
	PI-I	C	Cleveland *et al.* (1987)
Tomato	PI-I	C	Graham *et al.* (1985a)
	PI-II	T & C	Graham *et al.* (1985b)
	PI-II	T	Taylor *et al.* (1993)
Tobacco	PI-II	T & C	Atkinson *et al.* (1993)
	PI-II	T	Balandin *et al.* (1995)

*T -Trypsin C -Chymotrypsin

2.6.3 Bowman-Birk type inhibitors

The Bowman-Birk inhibitors (BBIs) were first recognized in soybean and navy bean (Bowman, 1944) and the inhibitor of soybean was subsequently purified and characterized (Birk, 1961; Birk *et al,* 1963). The first complete amino acid sequence of BBI to be determined was that of Lima bean (Tan and Stevens, 1971) followed by that of soybean (Odani and Ikenaka, 1972). These inhibitors are single chain polypeptides with a molecular weight of 8 to 10 kDa. Most BBIs are double headed with two homologous reactive sites, specific for different proteinases (usually trypsin, chymotrypsin and some times elastase) and form a 1:1 binary complex with either trypsin or chymotrypsin and a ternary complex with both the enzymes; hence termed double headed. Although, the BBIs are characterized in legumes, homologous inhibitors are present in wheat and some cereals suggesting an ancient origin for this family of proteins (Odani *et al,* 1986; Richardson, 1991).

The BBIs generally exhibit relatively high contents of aspartic acid + asparagine and serine and cystein. Methionine, valine, tyrosine and phenylalanine are low in abundance while tryptophan is usually absent. These inhibitors are relatively stable to heat, acidic pH and have an unusual resistance to various proteolytic enzymes including even those they do not inhibit (Ryan, 1981). Their stability is attributed in part to their high degree of cross linking through seven disulphide bridges (Mossor *et al.,* 1984) although other non-covalent interactions also contribute significantly to their stability

(Ryan, 1981). Although BBIs are non-glycosylated in nature and are relatively small proteins, they have a tendency to self-associate forming dimers, trimers and tetramers in solution (Gennis and Cantor, 1976; Wu and Whitaker, 1990; Frokiaer *et al,* 1994).

The BBIs of many legumes are found to occur in multiple iso-forms exhibiting different specificities towards proteinases in the same material (Richardson, 1991). The origin of these multiple iso-forms may be attributed either to the expression of multiple genes, allelic variation and or post-translational proteolytic cleavage of a few amino acids at N- or C-terminal end of the inhibitors during seed maturation. The cleavage is generally not complete resulting in a mixture of parental and truncated inhibitor forms found in the dry seeds (Wilson, 1988). The number of isoforms varies from 2 in wild pea and *Canavalia lineata* (Joubert *et al,* 1979; Terada *et al,* 1994b), 4 in cowpea and horse gram (Hilder *et al,* 1989; Sreerama *et al,* 1997), 5 in chickpea (Harsulkar *et al,* 1997), 9 in pigeon pea (Ambekar *et al,* 1996) to 11 in great northern beans (Ryas-Duarte *et al,* 1992).

The double headed BBIs are assumed to have arisen by duplication of an ancestral single headed inhibitor gene and subsequently diverged into different classes i.e., trypsin/trypsin (T/T), trypsin/chymotrypsin (T/C), trypsin/elastase (T/E) inhibitors (Tan and Stevens, 1971; Odani and Ikenaka, 1976). The first reactive site of dicot BBIs inhibits trypsin with the exception of first reactive site of peanut inhibitors (A-II and B-II; Norioka and Ikenaka, 1983) and second reactive site of soybean inhibitor C-II (Odani and Ikenaka, 1977), which bind both trypsin and chymotrypsin.

The BBIs of legumes are seed specific except for the alfalfa inhibitor which is an wound induced inhibitor (WII) and also smallest of all reported BBIs (Table 2.10).

The complete amino acid sequences have been obtained for several of the BBIs and they show extensive homology within tribes (Prakash *et al,* 1996).

Table 2.10 List of legume BBIs for which primary structure is determined

Name of species	Name of the inhibitor	Number of amino acids	Enzyme inhibitory site*	Reference
Lima bean	LB I	83	T/C	Tan and Stevens (1971)
Soybean	BBI	71	T/C	Odani and Ikenaka (1972)
	C-II	83	E/T & C	Odani and Ikenaka (1977)
	D-II	83	T/T	Odani and Ikenaka (1978)
Common vetch	VAI	72	T/C	Shimokawa *et al.* (1984)
Garden bean	GB II & II'	71	E/T	Wilson and Laskowski (1975)
Wild pea	DE-3, DE-4	76	T/C	Joubert *et al.* (1979)
Adzuki bean	1-A,1-B	78	T/C	Ishikawa *et al.* (1985) Kiyohara *et al.* (1981)
	1-A'	82	T/C	Yoshikawa *et al.* (1979)
Green gram	MB I	72	T/T	Zhang *et al.* (1982)
	MBI-F	80	T/T	Wilson and Chen (1983)
Horse gram	HG I	76	T/C	Prakash *et al.* (1996)
Faba bean	FB I	63	T/C	Asao *et al.* (1991)
Erythrina variegata	EB I	61	T/C	Kimura *et al.* (1994)
Canavalia lineata	CLTI-I	75	T/C	Terada *et al.* (1994b)
	CLTI-II	76	T/C	
Medicago scutellata	MsTi	62	T/T	Ceciliani *et al.* (1997)
Medicago sativa	WI I	58	T/T	Brown *et al.* (1985)
Peanut	A-II	70	T/C	Norioka & Ikenaka (1983)
	B-II	63	T/T & C	
Cowpea	BTCI	80	T/C	Morphy *et al.* (1987)

* T = Trypsin; C : Chymotrypsin; E - Elastase

The BBIs had received considerable attention during earlier days due to their anti-nutritional nature but lately these have become important due to their defensive role against insect pests and pathogens. As primary gene products, they have been recognized as an ideal candidate for the transfer of protection against insect pests to crop plants by genetic engineering (Hilder *et al.,* 1987; Ryan, 1990). The isolation of genes encoding these inhibitors will provide information on their number and organization as well as probes to study their regulation of expression in addition to their use in developing insect resistant transgenic plants.

The BBI genes are generally cloned from a cDNA or genomic library. But with the advent of polymerase chain reaction (PCR), it has also been used extensively for the cloning of seed storage protein genes, although some prior knowledge of amino acid sequence of the encoded protein is essential (Nong *et al.,* 1995; Hager *et al.,* 1996). The Bowman-Birk inhibitor genes from legumes and wound inducible BBI from maize have been cloned using all of the above three methods (Table 2.11). In contrast to the availability of numerous protein sequences, only a limited number of genes for the BBIs have been isolated (Table 2.11). A brief account of these clones is given below.

A cDNA clone (pB38) coding for BBI from developing seeds of soybean and a genomic clone (λBB 13.10) encoding a similar BBI were the first BBI clones to be isolated (Hammond *et al.,* 1984). The genomic clone was devoid of introns. The differences between cDNA and genomic clones and Southern analysis indicated presence of more than one BBI gene in soybean. Later, two nearly full length cDNA clones (pB3 and pBl1) of soybean encoding two proteinase inhibitors (PI IV and C-II) were shown to be synthesized as precursors with a short signal peptide (Joudrier *et al.,* 1987). The coding and 5'non-coding regions of the two clones showed 80 and 90% homo logy, respectively. Baek *et al.* (1994) cloned and characterized three soybean cDNAs (pB2, pB24 and pB26) encoding the BBIs. The deduced amino acid sequences of pB2, pB24 and pB26 had 21, 18 and 35 amino acid residues as their leader peptides, respectively. The sequence homology between the two reactive domains in clone pB2, pB24 and pB26 was 86, 71 and 52%, respectively, indicating that clone pB2 was most conservative in nucleotide sequence.

Table 2.11 List of cloned Bowman-Birk inhibitor genes

Name of plant	Method of cloning			Enzyme inhibitory site*	Reference
	cDNA	Genomic DNA	PCR		
Soybean	pB38	-	-	T/C	Hammond *et al.* (1984)
	pB3	-	-	T/T	Joudrier *et al.* (1987)
	pB11	-	-	T/C & E	
	-	λ BB 13.10	-	T/C	Hammond *et al.* (1984, 1985)
	pB2	-	-	T/T	Baek *et al.* (1994)
	pB24	-	-	T/C	
	pB26				
Cowpea	pUSSR c 3/2	-	-	T/T	Hilder *et al.* (1989)
	pUSSR d 4/6	-	-	T/C	
	-	-	pTIVO	T/T	Bijola *et al.* (1994)
Pea	pT15-72	-	-	T/C	Domoney *et al.* (1995)
	pT 112-36	-	-	T/C	
Alfalfa	ATI 18	-	-	T/T	McGurl *et al.* (1995)
	ATI 21	-	-	T/T	
Maize	pTR 3	-	pTR 20	C/C	Rohrmeier and Lehle (1993)

*T =Trypsin; C - Chymotrypsin; E-Elastase

Two cDNA clones (pUSSR d4/6 and pUSSR c3/2) encoding BBI from cowpea seeds have been cloned (Hilder *et al.,* 1989). The pUSSR d4/6, a partial cDNA clone encodes for entire coding sequence for a trypsin/chyrnotrypsin inhibitor but lacks an in-frame ATG translation-initiation codon. But pUSSR c3/2 is a full length cDNA clone coding for a 146 amino acid protein from which an 80 amino acid mature trypsin/trypsin inhibitor is produced after removal of a 66 amino acid unusually long 5' leader sequence.

A BBI gene of cowpea was amplified using both genomic DNA and seed RNA as template for PCR and RT-PCR, respectively (Bijola *et al,* 1994). The size of both the amplicons were 270 bp inclusive of restriction sites of the primers. The similar size for both reveals that the genomic clone was devoid of introns. The genomic clone (pTIVO) coding sequence was similar to the published cowpea trypsin inhibitor clone (Hilder *et ah,* 1989). The Southern analysis of cowpea genomic DNA indicated the presence of more than one gene copy per haploid cowpea genome.

Two pea cDNA clones (pT-15-72 and pT-112-36) show a high degree of similarity both in their coding and non-coding 3' regions suggesting a relatively recent duplication event (Domoney *et al.,* 1995). The predicted mature inhibitors are very similar to each other with only five substitutions among seventy two predicted amino acid residues. Both inhibitors have two active sites with the first site predicted to be active against trypsin while the second for chymotrypsin.

The BBI cDNAs which are expressed developmentally and in response to wounding and soil microorganisms have been cloned from alfalfa (McGurl *et al.,* 1995). The ATI 18, a longer clone encodes a 113 amino acid protein, of which 58 amino acids represented alfalfa trypsin inhibitors preceded by a relatively long putative leader sequence (44-55 amino acids). The Southern blot analysis of alfalfa genomic DNA showed that ATI gene family contains between three and ten members (McGurl *et al,* 1995).

A wound-inducible proteinase inhibitor (WIP1) [pTR3] homologous to BBI was cloned from wound induced maize coleoptiles (Rohrmeier and Lehle, 1993). The deduced amino acid sequence predicts a secretory, cysteine-rich protein of 102 residues (11 kDa) with a potential N-glycosylation site and a typical N-terminal signal sequence of 15 residues. The predicted inhibitor was double headed and active against only chymotrypsin. A corresponding genomic clone (pTR20) was cloned using oligonucleotides primers bordering the cDNA coding region by PCR technique. The

sequence revealed that it has an intron of 90 nucleotides with a typical consensus motifs of the splice junction.

2.6.4 Role of proteinase inhibitors

The presence of proteinase inhibitors in high amount in the seeds, tubers and fruits of many plant species upto 10% of the stored protein has led to speculation that they may act as storage proteins, as regulators of endogenous enzymes or as a defence against pests and pathogens. It seems likely that in certain species these inhibitors may fulfill a combination of these functions. Proteinase inhibitors found in seeds possess many features which argue strongly for their role as storage form of proteins which are immune to digestion until required during germination (Richardson, 1991). Unusually high content of cysteine residues found in many proteinase inhibitors has led workers to point out their value as a source of sulphur (Haiti *et al,* 1986). The biosynthesis and degradation of these inhibitors during seed development and germination run in parallel with that of the better known reserve proteins in several plant species (Wilson, 1988). In general, their presence in high quantity and utilization during germination suggests their role as storage proteins.

In nature, plants have been endowed with many defensive mechanisms to ward off pests and pathogens. But due to the process of crop improvement in order to meet human needs, many crops have lost resistance to certain insect pests and pathogens causing a substantial crop loss (Shah *et al,* 1995). Even with the massive use of insecticides atleast 15% of the world crop yields are lost due to insect predation, with further losses attributable to plant diseases for which insects serve as the transmission vectors (Heinrichs *et al.,* 1985). The increasing pressure to use non-hazardous, environment friendly pest control measures has spurred an interest in natural insecticides such as *Bacillus thuringíensis* insecticidal crystal proteins (Dulmage, 1981; Fischoff *et al.,* 1987) and plant derived proteins like proteinase inhibitors, lectins and amylase inhibitors (Boulter *et al,* 1989; Ryan, 1990; Chrispeels and Raikhel, 1991; Hilder *et al,* 1995; Schroeder *et al,* 1995; Schuler *et al,* 1998).

Plant derived serine proteinase inhibitors are of particular importance because they are part of the plant's natural defense system against insect predation. Many insect species possess serine and cysteine class of digestive enzymes, which predominate in lepidopteran and coleopteran insects respectively (Orr *et al,* 1994). Earlier studies on the effects of proteinase inhibitors, either artificially incorporated into defined diets, or

already present in plant tissues, have shown that these proteinase inhibitors are detrimental to the growth and development of a wide range of insects (Hilder *et al,* 1989; Gatehouse *et al,* 1990).

Insect damage to plant leaves had led to rapid increase in protease inhibitors in various plant parts apparently as a defensive response of the plants. The wounded tomato accumulates potato inhibitor I and II and reduces the growth of larvae of *Spodoptera exigua* (Broadway *et al.,* 1986).The feeding of wounded tomato leaves by *Spodoptera* caused a change in feeding pattern and inhibition (Edwards *et al.,* 1985). The defoliation of gray alder *(Alnus incana)* resulted in the reduction of soluble protein with concomitant increase in trypsin inhibitor which has antifeeding properties causing retarded growth and low survival of predator beetle *{Galerucella lineola)* (Seldal *et al.,* 1994).

The development of transgenic plants with foreign plant proteinase inhibitor genes with elevated expression has made the transgenics resistant to host insect pests. Many transgenic field crops with proteinase inhibitor as transgene resistant to insect pests have been developed (Table 2.12). These studies have demonstrated the feasibility of using proteinase inhibitors in developing insect resistant transgenic crops. These genes have also been used along with lectin in pyramiding genes for durable resistance in tobacco plants and have shown additive protective effect against tobacco bud worm caterpillars (Boulter, 1990).

Evidence also suggests that insects can adapt to the ingestion of proteinase inhibitors by over expressing gut proteases or induce the production of new types that are insensitive to the introduced proteinase inhibitors and overcome the deleterious effects of proteinase inhibitors ingestion (Jongsma and Botter, 1997). Since insects possess complex pool of proteases and inhibition of these major proteases may be achieved by expressing proteinase inhibitors of different types, and/or by improving the affinity of the introduced proteinase inhibitors for the target insect proteases (Urwin *et al,* 1995). Hence, there is a need to isolate trypsin inhibitor genes from different sources for their potential use in the development of insect resistant transgenic plants.

Table 2.12 List of transgenic plants with enhanced resistance towards some insects/pest by expression of seríne proteinase inhibitors (PI)

Transgenic plant	Type of inhibitors	Target insect/pest	Reference
Tobacco	Cowpea PI	*Manduca sexta*	Hilder *et al.* (1987) Gatehouse *et al.* (1992)
Rice	Cowpea PI	*Chilo suppresalis*	Xu *et al.* (1996)
Tobacco	Cowpea PI	*Spodoptera litura*	Sane *et al.* (1997)
Potato	Cowpea PI	*Meloidogyne incongnita* *Globodera pallida*	Hepher and Atkinson (1992)
Potato	Cowpea PI	*Lacanobia oleracea*	Gatehouse *et al.* (1997)
Tobacco	Soybean PI	*Spodoptera littoralis*	Jouanin *et al.* (1990)
Tobacco	Soybean PI	*Heliothis virescens*	Boulter (1993)
Tobacco	Potato PI	*Manduca sexta*	Johnson *et al.* (1989)
Rice	Potato PI	*Sesamia inferens*	Duan *et al.* (1996)
Tobacco	Tomato PI	*Manduca sexta*	Narvaez and Ryan (1992)
Tobacco	Cowpea PI	*Chrysodeixis eriosoma*	McManus *et al.* (1994)
Tobacco	Giant taro PI	*Helicoverpa armigera*	Wu *et al.* (1997)
Cauliflower	Sweet potato PI	*Spodoptera litura*	Ding *et al.* (1998)

2.7 Biosynthesis and degradation of seed storage proteins

The accumulation of proteins during seed development has been studied and reviewed by many workers (Dure, 1975; Millerd, 1975; Higgins, 1984; Boulter, 1984; Shotwell and Larkins,1989; Shewry *et al,* 1995). The embryogenesis in legume is constant from one legume species to another. During the development of seed from the fertilized ovule, protein is laid down at a variable rate. Usually the rate of protein deposition increases dramatically about one third of the way through the seed development (Wright and Boulter, 1972). Usually the synthesis of storage proteins begins on the 9^{th} and 12^{th} day after flowering, with most of the accumulation occurring between 15^{th} and 30^{th} day after anthesis (Mosse and Pernollet, 1982). The pattern of synthesis and deposition of legumin and vicilin are variable. In species, which accumulate both these proteins, vicilin always precedes that of legumin. Temporal expression of protein has been reported in pea (Millerd *et al,* 1978), faba bean (Graham and Gunning, 1970), soybean (Hill and Bridenbach, 1974) and groundnut (Basha *et al,* 1976). The constituent subunits of β-conglycinin and glycinin are also differentially regulated. The α and α' subunits of β-conglycinin are synthesized faster than is the β-sub-unit (Meinke *et al,* 1981, Naito *et al,* 1988), the A_3B_4 sub-unit is the last among the glycinins to be synthesized (Meinke *et al,* 1981). In pea, legumin and vicilin were detected as early as four days after fertilization (Domoney *et al,* 1980) and both legumin and vicilin are synthesized atleast in small amounts throughout seed development (Boulter, 1984). The vicilin, legumin and convicilin accumulate fastest in that order although there is a considerable overlap in the periods of their synthesis. Cessation of synthesis is also probably sequential (Boulter, 1984).

The biosynthesis of proteinase inhibitors follows similar trend as observed for other storage proteins. These proteins are also synthesized as precursor polypeptides (Hammond *et al,* 1984; Hilder *et al,* 1989). BBIs also undergo a second form of post-translational modification during seed maturation which involves the removal of short segments of the inhibitor polypeptide at the amino terminus.

In winged bean seeds, the Kunitz chymotrypsin inhibitor accumulated 30 days after flowering (DAP) and increased continuously till 50 DAP (Habu *et al,* 1992). In pigeon pea, the trypsin inhibitor activity was not detected upto 24 DAP (Ambekar *et al,* 1996) which is contrary to the earlier report in which it was detected as early as 7 DAP (Godbole *et al,* 1994). In chick pea, trypsin inhibitor activity was not detectable at 20

DAF but showed maximum activity at 40 DAF (Harsulkar *et al.,* 1997). They also observed changes in trypsin inhibitor form and varietal differences during seed development.

During seed germination, storage proteins are hydrolyzed into small peptides and amino acids which are utilized by the embryo to synthesize metabolic intermediates. In legume seeds, proteolysis usually occurs after third day and is maximum after five to six days of seed germination. In pea, the vicilin is hydrolyzed more rapidly than legumin at the beginning while the legumin are more rapidly degraded later (Konopska, 1978, 1979). This is in contrast to the earlier report that the legumin degradation is always higher than vicilin (Basha and Beevers, 1975). In French bean and groundnut, peptides smaller than vicilin subunits are evident to be the breakdown product of vicilin prior to complete breakdown during germination (Bollini and Chrispeels, 1978; Basha and Cherry, 1978). In narbon bean, the narbonin protein degraded very slowly compared to vicilin and legumin during germination as detected by immunoblotting (Nong *et al,* 1995). The β-conglycinin of soybean is subjected to limited proteolysis at exposed regions on the molecular surface like domain junctions generating 30 kDa intermediate before nonspecific proteolysis (Kawai *et al.,* 1997).

The location of proteins in the grains has also been studied at microstructure level. Most legume seeds are starch rich but are also rich in protein as compared to cereals. The Scanning electron microscope (SEM) has been employed to study microstructure of food grains. Microstructural variations can be related to textural, chemical and physical variations in bean cultivars (Sefa-Dedeh and Stanley, 1979b) and identification of different cultivars of soybean (Wolf *et al.,* 1981), adzuki bean (Enquist and Swanson, 1992). The SEM examination of black bean (Hughes and Swanson, 1985), adzuki cotyledon and other bean cotyledon cells (Sefa-Dedeh and Stanley, 1979b) revealed spherical starch granules embedded in a protein matrix. Protein matrix is generally composed of small spherical to oval shaped individual protein bodies. The microstructure of soybean cotyledon cells revealed that protein bodies are surrounded by a protein network in which spherosomes were embedded. The adzuki bean, black bean and Mexican red bean cotyledon revealed spherical to oval shaped starch granules embedded in protein matrix composed of small protein bodies (Chilkuri and Swanson, 1991). They also found characteristic pitting of starch granules in adzuki beans but not in black beans or Mexican red beans.

2.8 Evolutionary relationship among the seed storage proteins

The 2S albumins of dicots have sequence similarities with three conserved regions of the prolamins of tribe Triticeae. These albumins are also similar to in sequence to that of Bowman-Birk proteinase inhibitors of legume seeds and sweet protein thaumatin although the level of homology is quite low (Sharief and Li, 1982; Kreis *et ah,* 1985b; Higgins *et ai,* 1986). Thus, 2S albumins and the inhibitors of α-amylase and trypsin of cereals are evolutionarily related to the cereal prolamins (Kreis *et ai,* 1985a,b). The prolamins, the 2S albumins and the cereal inhibitors form a protein super family with limited sequence homology are often referred to as the prolamin super family of proteins (Dayhoff, 1978).

Although, the 7S and 11S globulins show no obvious sequence similarities, they do have broad structural relationship (Shewry, 1995). The mature protein of 7S globulin is trimeric but it may undergo reversible aggregation into hexamers depending on the ionic strength (Thanh and Shibasaki, 1979). In contrast, the 11S globulin is hexameric but is assembled and transported through the secretory system as an intermediate trimer (Muntz *et al,* 1993). Comparison of 7S and 11S globulins by sequence alignment, structure predictions and the canonical 7S globulin X-ray structure have demonstrated that the acidic and basic subunits of 11S globulins are related to the N- and C-terminal regions, respectively of the 7S subunits, indicating their origin from a common ancestral gene (Argos *et al,* 1985; Plietz *et al,* 1987; Wright, 1987, 1988; Gibbs *et al,* 1989; Lawrence *et al,* 1994). This implies that, both 7S and 11S globulins have similar tertiary and quaternary structures (Shewry, 1995).

Sequence homology between Kunitz type inhibitors and a crystalline seed albumin from winged bean and various 7S storage globulin has been reported (Richardson, 1991). A trypsin inhibitor of buckwheat has been reported to have considerable homology to the N-terminus region of the first exon of cotton vicilin gene sharing fourteen identical amino acid residues suggesting a possible evolutionary relationship of this inhibitor with the cotton vicilin gene (Park *et al,* 1997).

Serological relationships have been studied for legumins and vicilins of pea and broad bean (Kloz and Turkova, 1963; Dudman and Millerd, 1975; Croy *et al,* 1979) but no significant cross reactivity was established between 7S globulins of broad bean and pea and any globulin present in pea or kidney bean, although the available sequence data show that significant homology exists among these proteins. This inconsistency could be

due to the presence of relevant immunogenic sites in the variable regions of these proteins (Wright, 1987). The presence of immunologically different conglycinins (α, β and γ) differing in number and subunit pattern has been reported (Catsimpoolas, 1970). Immunological similarity among wheat, oat globulin, millets, rice glutelin and pea or soybean legumin has also been reported (Robert *et al,* 1985a,b; Fabijanski *et al.,* 1985; Okita *et al,* 1988; Luthe, 1991; Srinivas *et al,* 1994).

Immunologically related legumin-like proteins have been detected from several dicots (Alexenko *et al,* 1988). Similarly, antibodies raised against degraded glycinin reacted strongly with storage proteins of pumpkin but no reaction was observed with sesame, Brazil nut or oat protein (Carter *et al,* 1992). An interesting finding was that these antibodies cross-reacted with the 7S protein of soybean and pea. This confirms the existence of homologies between 7S and 11S globulins which is in concurrence with earlier reports based on the sequence comparisons (Wright, 1988). The polyclonal antibodies raised against pea legumin reacted strongly with faba bean and soybean but weakly with sesame and pumpkin 11S globulins (Quillien *et al,* 1995).

The 'vignin', a 7S globulin diversity was surveyed in 81 cultivated and 55 wild accessions of cowpea by electrophoretic and immunoblotting and 27 unique banding patterns were observed for the vignin fraction (Leonard *et al,* 1993). Antibodies have also been utilized to probe the biosynthesis and degradation of 2S globulins, during seed development and germination in narbon bean (Nong *et al,* 1995).

Antibodies have been raised against BBIs of horse gram, soybean, pea, and cowpea and used for immunodetection, immuno-affinity purification and epitope mapping of BBIs (Brandon *et al,* 1989; Bijola *et al,* 1994; Frokiaer *et al,* 1994; Sreerama, 1996). The amino acid sequence homology, structural relationship and immunological studies suggest that all the major seed storage proteins like albumins, globulins, prolamins and inhibitors are related to each other to a variable extent suggesting their common evolutionary origin.

2.9 Seed storage proteins of horse gram

Horse gram *{Dolichos biflorus* L.) also described as *Macrotyloma uniflorum* (Gillet *et al,* 1973), belongs to the tribe Phaseoleae of the family Leguminoceae. The former botanical name of horse gram is widely used in the literature. Of the eight species belonging to genus *Dolichos* in India, *Dolichos lablab* and *Dolichos biflorus* are most

extensively cultivated and used as food and fodder (Yadava and Vyas, 1994). The horse gram probably originated in South-East Asian subcontinent and its diploid chromosome number (2n) is 24 (Smart, 1976). In addition to India, horse gram is also cultivated on a limited scale in parts of Asia particularly Burma, in tropical Africa, West Indies and Australia.

Horse gram plant is an erect, trailing annual, 30-35 cm in height, with many slender branches with trifoliate leaves (Fig. 2.2A). The flowers are borne on axillary racemes and are creamy yellow in colour (Fig. 2.2B). The pods are linear, 4 to 5 cm long, dehiscent and five to seven seeded. The seeds are flattened, rhomboidal, 3 to 6 mm long, light red, brown, black, gray or mottled with shining testa (Fig. 2 .3). The horse gram being a crop of dry tropics is drought resistant and hardy. The improved varieties yield upto 2 tons/ha.

The proximate composition of horse gram is comparable with other commonly cultivated legumes. As in other legumes, a large variability exists for horse gram seed protein content ranging from 18.5 to 28.5 per cent (Mushtari-Begum *et al,* 1977; Savithramma and Shambulingappa, 1996). The amino acid profile of horse gram shows primary limitation in methionine and tryptophan.

The presence of anti-nutritional factors like proteinase inhibitors, hemagglutinins (lectins) and amylase inhibitors in horse gram and their growth retardation effect on animals has been studied (Ray, 1968, 1969; Subbulakhsmi *et al,* 1976). An acid stable trypsin/chymotrypsin inhibitor of 13.5 kDa purified from horse gram was stable over a wide range of pH (3-11) and temperature (upto 80°C). A subtilisin inhibitor and an associated trypsin inhibitor purified from horse gram were stable to heat and pH range of 2.5 to 10.5 (Bodhe, 1991). The molecular weights of subtilisin inhibitor and the associated trypsin inhibitor were 7.5 and 8.2 kDa, respectively. The subtilisin with 73 amino acid residues is rich in glutamic acid and valine but contains no half cysteine

Fig. 2.2 Morphological appearance of the horse gram plant.

A. Fully-grown horse gram plant.

B. Close up view of horse gram plant.

The arrow indicates flower and arrowhead indicates immature pod.

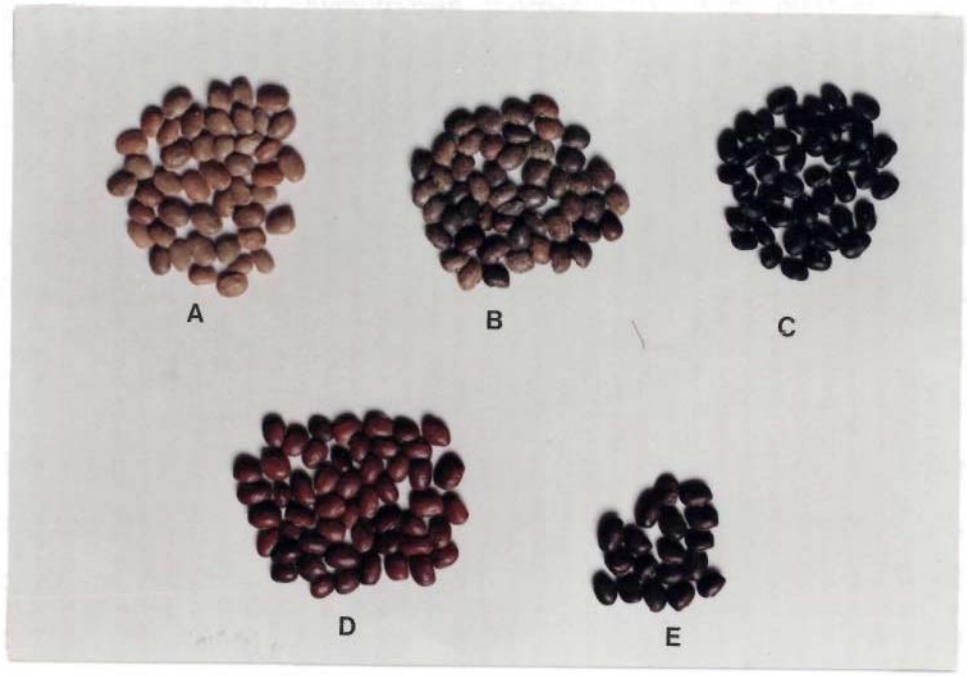


Fig. 2.3. Variation in seed coat colour among horse gram genotypes.

A. R-ΠI-22-110 B. No. 84 C. No. 47

D. D-2-2-2 E. No. 2

while the associated trypsin inhibitor had seven cysteine residues out of a total of 64 amino acid residues (Bodhe, 1991).

Recently, four double headed Bowman-Birk type proteinase inhibitors which inhibits trypsin and chymotrypsin independently, were purified and characterized (Ramasarma and Rajagopal Rao, 1991). Further characterization by X-ray diffraction was carried out and the amino acid content and sequence was determined (Prakash *et al.,* 1994, 1996; Sreerama *et al,* 1997). The molecular weights of the four horse gram inhibitors were found to be 8300, 8625, 8625 and 9625 Daltons for HGI-I, HGI-II, HGI-III and HGI-IV, respectively. A notable feature was the absence of glycine and low content of methionine (Sreerama *et al,* 1997). The HGI-III, a major iso-form was the most potent inhibitor of trypsin, while HGI-II had greater affinity towards chymotrypsin. The amino terminal sequence of horse gram inhibitors showed a high degree of homology with amino termini of other known BBIs (Sreerama *et al,* 1997). The complete primary structure of HGI-III was elucidated which contains 76 amino acid residues. This sequence has homology with Bowman-Birk inhibitors of other legumes and showed maximum homologies of 96 and 76% with DE-3 inhibitor of *Macrotyloma axillare* and soybean BBI respectively (Prakash *et al,* 1996).

In addition to proteinase inhibitors, horse gram also contains two iso-lectins of 113 and 109 kDa with four subunits each. A cDNA clone encoding horse gram lectin has been isolated and characterized (Schnell *et al,* 1987). Horse gram seed contains higher hemagglutinin activity than moth bean, which decreases considerably during seed germination (Subbulakshmi *et al,* 1976).

A perusal of the literature reveals that extensive work on the seed storage proteins of grain legumes has been carried out primarily on soybean, pea, faba bean and French bean, mainly because these are the major legume crops in the developed countries. At the same time the review highlights the lack of information on seed storage proteins of horse gram except for the lectins and proteinase inhibitors. The horse gram being a localized crop in small pockets in the developing countries and consumed mainly by poorer section of the population has received much less attention. The major seed storage proteins of horse gram have not been studied in any detail. Since this crop is grown and consumed extensively in rural parts of Southern India, it will be of immense use to study the type of

storage proteins, their distribution and relationship with storage proteins of other grain legumes.

Since the BBIs are known to play an important role as defense against insect pests, as well as for their nutritional significance, it would be useful to clone the genes for this protein for their possible use in genetic engineering.

2.10 Objectives

With the above background the specific objectives of the present study were as follows:

1. Electrophoretic, developmental and immunochemical studies on horse gram Bowman-Birk inhibitors (BBIs) and cloning of their DNA fragments using polymerase chain reaction (PCR).
2. Electrophoretic and immunochemical characterization of vicilin-like proteins of horse gram seeds.
3. Electrophoretic and immunochemical characterization of legumin-like proteins of horse gram seeds.
4. Studies on the narbonin-like proteins of horse gram seeds.

MATERIALS AND METHODS

CHAPTER-3

MATERIALS AND METHODS

3.1 Plant material

The seeds of 16 different horse gram *(Dolichos biflorus* L.) genotypes of diverse agronomic characters were obtained from Dr. D. L. Savithramma, Assistant Professor, University of Agricultural Sciences, Bangalore, India. These were C-6-3-4, D-6-3-4, Macintosh, A-4-9-5, No.2, S-35, No. 85, BGM-1, D-2-2-2, IC11095, PHG-9, K-42, No. 47, C-7-5-5, R-IΠ 31-49, R-III 90A-1-10-3. Similarly, seeds of 11 different legume species were also obtained from the same source (Table 3.1). In addition, a rice variety (Pusa Basmati) was obtained from the G. B. Pant University of Agriculture and Technology, Pantnagar, India.

3.2 Source of Chemicals

All the chemicals used in the present study were of analytical or molecular biology grade. Trypsin, nitroblue tetrazolium (NBT), X-phosphate, Freund's complete and incomplete adjuvant, glutaraldehyde, 2-N-morpholino ethane sulphonic acid (MES), acetyl-DL- phenylalanine β-naphthyl ester (APNE), O-dianisidine, hexamine cobalt chloride, calf thymus DNA and polyethylene glycol (PEG 8000) were from Sigma Chemical Company. Goat anti-rabbit IgG-alkaline phosphatase conjugate, rabbit anti-mouse IgG-alkaline phosphatase conjugate, protein molecular weight markers, λ Hind III and λ Hind III/Eco RI DNA markers were from Bangalore Genei Pvt. Ltd., Bangalore, India. The λ Hind ΠI/Eco RI DNA markers were also from MBI Fermentas, Lithuania. Bisbenzimide was from Hoefer Pharmacia Biotech. Taq DNA polymerase, dNTPs, restriction enzymes and 100 bp DNA ladder were from Promega Corporation, USA. Nitrocellulose and nylon membranes were from Schleicher and Schuell and Boehringer Manheim, respectively. Tryptone, yeast extract, ampicillin and chloramphenicol were from Hi-Media, India. All other chemicals were of analytical or molecular biology grade and were purchased from Sisco Research Laboratories, Mumbai.

Table 3.1 List of grain legumes used in this study

Sl. No.	Common name	Botanical name	Variety
1	Soybean	*Glycine max*	KHSB-2
2	Lablab bean	*Dolichos lablab*	Hebbal Avare
3	Chick pea	*Cicer arietinum*	Avarodhi
4	Pea	*Pisum sativum*	Boneville
5	Lima bean	*Phaseolus lunatus*	Kbsp-1
6	Green gram	*Vigna radiata*	ML-131
7	Black gram	*Vigna mungo*	T-9
8	Cowpea	*Vigna unguiculata*	Arka garim
9	Sword bean	*Canavalia gladiata*	Unknown
10	French bean	*Phaseolus vulgaris*	Arka komal
11	Pigeon pea	*Cajanus cajan*	Unknown

3.3 Germination and rearing of horse gram plants

The C-6-3-4, a photo-insensitive genotype was grown in pots. The fully opened flowers were tagged periodically and seeds and pod peels were collected at different intervals during seed development. Flowers were also harvested and separated into sepals, petals, androecium and gynoecium (stigma and style only). All the harvested materials were freeze dried and stored at -20°C till use for extraction of proteins to study the protein accumulation and trypsin inhibitors.

The seeds of C-6-3-4 were sown on moist filter papers in petri dishes and kept at room temperature. The filter papers were moistened periodically by adding water. The cotyledons were harvested every 24 h from first to tenth day. The harvested cotyledons were freeze dried and stored at -20°C till their use for studying the degradation of proteins during germination.

3.4 Extraction of total seed proteins

For SDS-PAGE (sodium dodecyl polyacrylamide gel electrophoresis), total proteins were extracted in the sample buffer [2% w/v SDS, 15% (w/v) glycerol, 60 mM Tris-Cl (pH

6.8), 0.02% (w/v) bromophenol dye] from 50 mg of freeze dried samples of seeds. The freeze-dried materials were crushed into fine powder in eppendorf tubes using a knitting needle. To this 600 µl of sample buffer having *2%* (v/v) 2-mercaptoethanol was added, vortexed thoroughly and extracted for 1 h at 65°C. After centrifugation at 10,000 x g for 10 min, 10 µl of supernatants were loaded into each well for electrophoresis.

For the extraction of proteins from the mature seeds, cutting and scrapping, respectively, with a sharp blade removed the germ portion and the testa of all the seeds. The cotyledons free from testa and germ were ground to fine powder by crushing them between butter paper using a hammer. Fifty mg of flour was taken and total proteins were extracted in 600 µl of sample buffer as mentioned above.

For the native gel, freeze dried samples of horse gram flower parts, pod peels and seeds were ground to fine powder with a knitting needle. To this 600 µl of the sample buffer without SDS and 2-mercaptoethanol was added. The sample tubes were vortexed thoroughly and extracted at room temperature for 1 h and then centrifuged at 10,000 x g for 10 min. Ten µl of the supernatants were loaded into the wells for electrophoresis.

3.5 SDS-PAGE

SDS-PAGE was done on a vertical slab gel electrophoresis system according to the method of Lawrence and Shepherd (1980) and as modified by Singh *et al.* (1991).

3.5.1 Stock solutions

2X separating gel buffer : One gram of SDS and 45.4 g Tris-base were dissolved in 450 ml of double distilled water and pH was adjusted to 8.88 with cone. HCl, the final volume was made upto 500 ml and stored at 4°C.

2X stacking gel buffer : 0.4 g SDS and 6.03 g Tris-base were dissolved in 180 ml of double distilled water and pH adjusted to 6.8 with 1 N HCl, the final volume was made upto 200ml and stored at 4°C.

10X Electrode buffer: 10 g SDS, 144.4 g glycine and 30.03g Tris-base were dissolved in 950 ml of distilled water and made upto 1 litre. The pH of the solution was 8.6. Before use, the stock solution was diluted 10 times to a final pH of about 8.3.

Stock acrylamide for separating gel: 75 g of acrylamide and 1.1 g bis-acrylamide were dissolved in 180 ml of distilled water and made upto 250 ml. The solution was filtered through Whatman No.l filter paper and stored in a dark brown bottle at 4°C.

Stock acrylamide for stacking gel: 15 g of acrylamide and 0.4 g of bis-acrylamide were dissolved in 30 ml of distilled water and the volume was made upto 50 ml. The solution was filtered through Whatman No.l filter paper and stored at 4°C in a dark brown bottle.

Ammonium persulfate (APS): 100 mg of ammonium persulfate was dissolved in 1 ml of distilled water. This solution was prepared fresh every time.

3.5.2 Procedure

The separating gels of two different concentrations (10% and 12%) were made by mixing the above stock solutions in different proportions with 100 µl of TEMED. The APS was added just before pouring the gel. The contents were mixed by swirling and poured in between glass plates (150 x 150 x 1.5 mm) and overlaid with 0.2 ml of distilled water and allowed to polymerize for atleast 1 h.

The stacking gel of 9 ml was prepared by mixing 4.5 ml of 2X stacking gel buffer, 3.5 ml water and 1 ml of stock acrylamide. A 25 µl of TEMED and 50 µl of APS was added just before pouring of the gel. After polymerization of the separating gel, excess water was removed with a syringe and a comb was inserted in place and then the above contents were mixed and poured and left to polymerize for 30 min.

After polymerization of the stacking gel, the comb was removed and the wells were washed in IX electrode buffer. The tank was filled with IX electrode buffer after fixing the glass plates to the vertical gel electrophoresis apparatus. Then 10 µl of protein samples were loaded into each well using a microsyringe.

Gels were run at a constant current of 40 mA/gel until the tracking dye (bromophenol blue) reached the bottom of the gel (about 3 h). The voltage started at 150 V and gradually increased upto 250 V at the end of the run.

The gels were stained overnight in 0.025% (w/v) Coomassie brilliant blue R-250 in acetic acid/methanol/water (7:18:75 v/v) containing 6% (w/v) TCA and destained either in acetic acid, methanol and water (10:40:50) or a 3% sodium chloride solution (Sreeramulu and Singh, 1995).

3.6 Casein-substrate gel electrophoresis (Garcia-Carreno *et al.,* 1993)

The protein sample preparation for native gel and casting of the gel was done as in Section 3.4 and 3.5, respectively except that SDS was omitted in all buffers. The protein samples were neither reduced nor heat denatured before loading into wells. Electrophoresis was conducted at a constant current of 40 mA per gel till the bromophenol blue dye front reached the bottom of the gel. After electrophoresis, the gel was washed once in distilled water and incubated in 50 mM Tris-Cl buffer (pH 7.5) containing 0.1 mg/ml of porcine trypsin for 30 min at 5°C. The gel was washed once each in distilled water and then in Tris-Cl buffer (pH 7.5) before incubating in the same buffer containing 2% (w/v) casein for 30 min at 5°C. Then the temperature of the gel was increased to 25°C for 90 min. The gel was washed twice in distilled water and stained with 0.025% (w/v) Coomassie brilliant blue R-250 in acetic acid/methanol/water (10:40:50 v/v) for 2 h and destained in acetic acid/methanol/water (10:40:50).

3.7 Detection of trypsin inhibitor activity with synthetic substrate

After the native gel electrophoresis as in Section 3.6, the trypsin inhibitory activity was detected according to the procedure of Uriel and Berges (1968) with some modifications. The gel was immersed in trypsin (5 mg/100 ml in 0.1 M sodium phosphate buffer, pH 7.4) for 20 min at 37°C. Then the gel was rinsed with distilled water and stained in a solution containing 0.7 mM acetyl-DL-phenylalanine-β-naphthyl ester (APNE) and tetrazotized O-dianisidine (0.5 mg/ml) in 0.1 M sodium phosphate buffer, pH 7.4 and allowed to develop. The presence of trypsin inhibitors was visualized as clear bands with a dark violet or pink background and the gels were photographed.

3.8 Production and characterization of antibodies against horse gram Bowman-Birk inhibitor (BBI)

One mg of purified horse gram BBI (HGI-III) protein was a gift from Dr. Sreerama (Sreerama *et al.,* 1997). Before injecting the protein in the New Zealand white rabbit, 5 ml of blood was drawn from the ear for the preparation of pre-immune serum. A 500 µl of BBI protein in solution (200 µg) was mixed with 500 µl of Freund's complete adjuvant and the emulsified suspension was injected intramuscularly and intraperitonially. For subsequent injections at weekly intervals, the same amount of protein was mixed with Freund's incomplete adjuvant. A total of five injections were given and at the end of fifth injection, the ear veins of rabbit were dilated with the help of xylene and 25 ml blood was drawn to check the antibody titre. This blood was allowed to clot in a refrigerator for 24 h and the serum was collected by centrifugation at 5000 rpm for 8 min and stored at -20°C after adding sodium azide (0.1% w/v).

The immunoglobulins were purified from this serum according to Meyer and Walker (1987) as described below.

Twenty five ml of the antiserum was mixed with 6.25 g of ammonium sulfate and incubated at 4°C for 24 h, centrifuged at 4000 rpm for 20 min, and the supernatant was discarded. The precipitate was washed twice with 1.75 M ammonium sulfate to remove albumins, transferrin, α-protein and free hemoglobin. After this the precipitate was re-suspended in water to the original volume of serum and dialyzed extensively against water containing sodium azide at 4°C for 24 h with frequent changes of water. The purified immunoglobulins were stored at -20°C in small aliquots of 100 µl.

3.9 Other antibodies used in this study

Apart from the above polyclonal antibodies raised against the horse gram BBI, five more antibodies raised against different seed storage proteins were used inihis study. These were obtained from various sources as described below.

Four soybean Bowman-Birk inhibitor monoclonal antibodies were obtained from

Dr. A. Van Amerongen, ATO-DLO, The Netherlands. Ten ml of freeze-dried culture supernatant of four different monoclonal antibodies were diluted each in 1 ml water and each were used at 1:500 dilutions in immunoblots.

The cowpea vicilin polyclonal antibodies were obtained from Dr. Jose Xavier Filho, Universidade Estadual do Norte Fluminense, Brazil. This antibody was raised against vicilin purified from cowpea variety IT 8ID-1045 in white rabbits. The freeze dried antibodies were dissolved in 100 µl saline (150 mM) and used at 1:5,000 dilutions in Western blotting experiments (Yunes *etal,* 1998).

The pea legumin polyclonal antibodies were obtained from Dr. L. Quillien, Laboratorie de Biochimie and Technologie des proteines, INRA, France. The antibodies were raised against native pea legumin. The freeze dried antibodies were diluted to 500 µl distilled water, and used at 1:5,000 dilution (Quillien *et al,* 1995).

The wheat triticin anti-δ antibodies were available in the laboratory. It was raised against the purified basic polypeptide of wheat triticin (legumin-like protein) in rabbits (Singh *et al,* 1993). The antibodies were used at 1:5,000 dilution.

The narbonin polyclonal antibodies were obtained from Dr. Bernhard Schlesier, Institut fur Pflanzengenetik und Kulturpflanzenforschung, Gaterslaben, Germany. These antibodies were raised in rabbit against the narbonin protein from narbon bean (Nong *et al.,* 1995). The freeze dried antibodies were diluted to the original volume of 100 µl and used at 1:1,00,000 dilution in Western blotting.

3.10 Western blotting

3.10.1 Stock solutions

Transfer buffer (per litre) : 20 mM Tris-buffer, 150 mM glycine, 0.0375% (w/v) SDS and 20% (v/v) methanol.

Tris-buffered saline (TBS) : 20 mM Tris-Cl (pH 7.4), 0.9% (w/v) NaCl

Tris-buffered saline containing Tween-20 (TBST): 0.05% (v/v) Tween-20 in the TBS.

Alkaline phosphatase buffer : 100 mM NaCl, 5 mM $MgCl_2$,100 mM Tris-Cl (pH 9.5).

Nitro-blue tetrazolium chloride (NBT): 25 mg was dissolved in 500 µl of 70% dimethyl formamide.

5-bromo-4-chIoro-3-indolyl phosphate (BCIP) : 25 mg was dissolved in 500 µl of 100% dimethyl formamide.

TE buffer: 10 mM Tris-Cl (pH 8.0), 1 mM EDTA.

3.10.2 Electroblotting

Electroblotting of the proteins onto the nitrocellulose membrane was performed according to Towbin *et al.* (1979). After SDS-PAGE, the gels were equilibrated for 30 min in the transfer buffer. The gel dimensions were noted and Whatman 3 mm filter paper sheets and nitrocellulose membrane were cut to the gel size. A filter paper pad containing 8 sheets of Whatman 3 mm paper was soaked in the transfer buffer and placed in an LKB Novablot Cell. The bubbles were removed by rolling a clean glass rod. The nitrocellulose membrane was soaked in the transfer buffer and placed exactly on the filter paper sheets, over which the gel was placed. The gel was overlaid with 8 sheets of pre-soaked Whatman 3 mm filter paper over which the top graphite electrode of the unit was placed and transfer was performed at the rate of 0.8 mA/cm^2 of the membrane for 1 h and 15 min. After the transfer, the membrane was lifted and air-dried to remove the excess buffer before development.

The electroblotted nitrocellulose membrane was rinsed in TBS thrice for 5 min each and incubated in 3% bovine serum albumin (BSA) in TBS overnight with gentle rocking for blocking. After blocking, the membrane was washed with TBST thrice for 10 min each. The membrane was incubated in different antibodies with appropriate dilutions in TBST for 2 h at 37°C with gentle shaking. After washing thrice, 10 min each in TBST, the membranes were incubated with shaking in the secondary antibody i.e., goat anti-rabbit IgG-alkaline phosphatase conjugate (for polyclonals) and rabbit anti-mouse IgG- alkaline phosphatase conjugate (for monoclonals) diluted 1:5000 in TBST for 1 h. After washing thrice 10 min each in TBST, the membrane was incubated in the substrate solution until the desired colour was developed. The reaction was stopped by the addition of TE buffer (pH 8.0).

3.11 Tissue printing

The nitrocellulose membrane was cut into appropriate size and 1 cm square grid lines were drawn. Fully developed green seeds of legumes (horse gram, green gram and chickpea) were harvested and cut into two halves and the exposed section was pressed firmly against the dry nitrocellulose membrane (in grids) and allowed for one min. The seed halves were removed without disturbing the membrane and the tissue printed filters were processed by using legumin and vicilin antibodies using the procedure described above.

3.12 Seed sample preparation for Scanning electron microscopy (SEM)

Two per cent glutaraldehyde was prepared in 50 mM phosphate buffer (pH 7.0). Immature and germinated horse gram seeds were cut in glutaraldehyde buffer into pieces using a sharp scalpel. The cut seed pieces were transferred into eppendorf tubes having 500 µl of 2% glutaraldehyde. After half-an-hour incubation, it was replaced with fresh 2% glutaraldehyde and incubated at 4°C overnight. Then the seeds were passed through increasing concentrations of alcohol (10, 20, 40, 60 and 80%) at an hourly intervals and then finally twice through absolute alcohol. The samples were then dried at 40°C. The mature, dried horse gram seeds were directly cut into pieces with sharp scalpel. The glutaraldehyde treated and dried seed sections were placed in the stubs and gold coated with sputter coater (Polaron Sputter coat system, England, Model 5001). The gold coated samples were viewed under SEM (Leo Electron Microscopy Ltd., UK).

3.13 Design of PCR primers for the amplification of horse gram BBIs

Complete amino acid sequence of Bowman-Birk type proteinase inhibitors are available for several legume species (Richardson, 1991; Prakash *et ah,* 1996). However, only three of these sequences, namely, soybean (Hammond *et al,* 1984; Joudrier *et al,* 1987), cow pea (Hilder *et al,* 1989) and pea (Domoney *et al,* 1995) are known at the nucleotide level also. After comparison of all the BBI amino acid sequences, highly conserved regions across the species were identified and PCR primers were designed with the aim of amplifying BBI DNA fragments from the horse gram. Three different strategies were used for the designing a total of six forward and six reverse primers as described below:

1. In the first case, while the protein sequence of horse gram BBI was not available, the nucleotide sequences of soybean BBIs (Hammond *et al.,* 1984) representing absolutely conserved regions between species were taken directly as primers since the soybean is most closely related to horse gram out of the above three species. One forward (designated as 'N') and two reverse primers ('Cl' and 'C2') were designed in this way (Fig. 3.1, Table 3.2).
2. After the publication of complete protein sequence of horse gram BBI (Prakash *et al,* 1996), three degenerate forward primers (RTV-1, RTV-2 and RTV-3) and two degenerate reverse primers (RTV-R1 and RTV-R2) were designed based on the horse gram protein sequence (Fig.3.1, Table 3.2).
3. Finally, two more forward primers (BBI-Fl and BBI-F2) and two more reverse primers (BBI-R1 and BBI-R2) were designed based on the above strategies except that these were significantly longer. The BBI-Fl and BBI-R1 were non-degenerate soybean sequence modified in some places to fit the horse gram protein sequence (Fig. 3.1, Table 3.2).

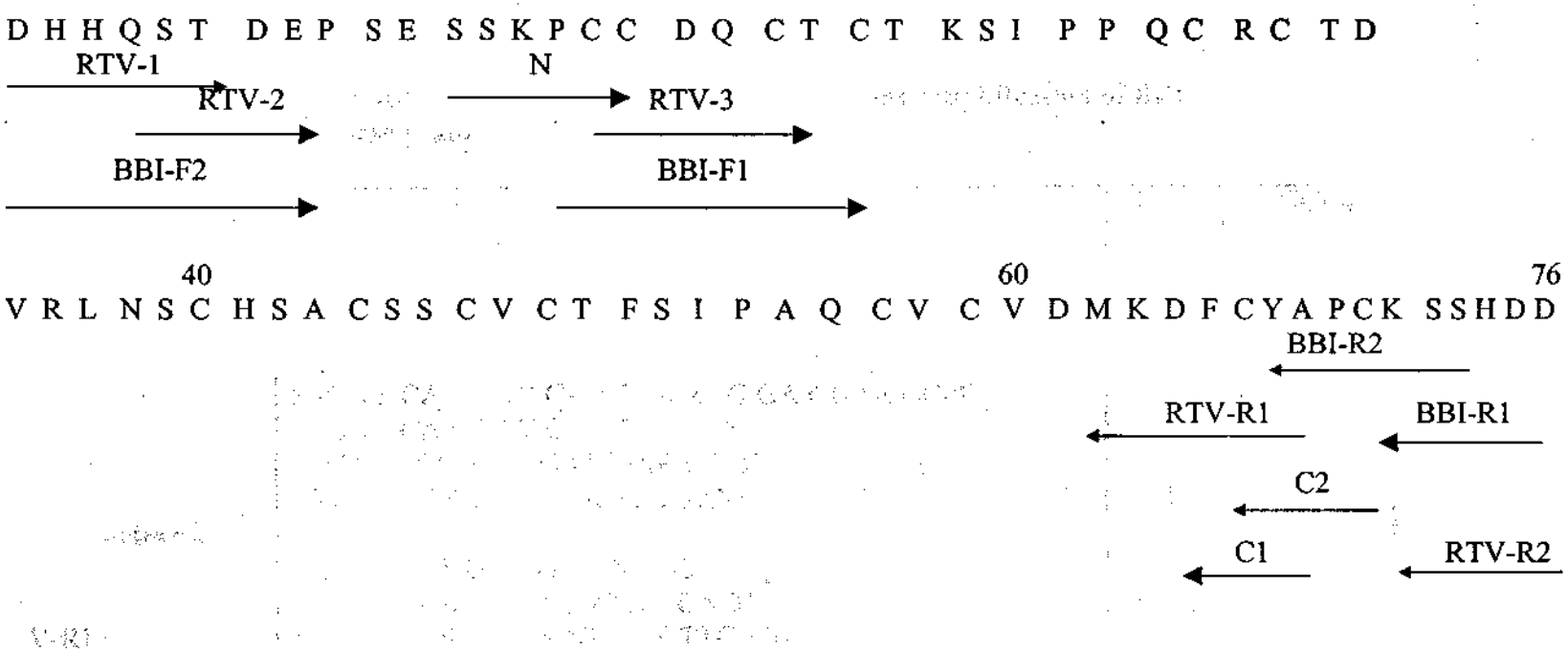


Fig. 3.1 Amino acid sequence of horse gram Bowman-Birk inhibitor (HGI-III) showing the location of amino acid regions for which primers were designed using different strategies. The position of amino acids in the mature protein is numbered on top.

Table 3.2 List of oligonucleotide primers designed and used for the amplification of BBI genes from horse gram

Name	Nucleotide sequence	Length (bp)	GC(%)
Forward Primers			
N	5'-TCT TCA AAA CCA TGC TG -3'	17	41.18
BBI-F1	5'-CCA TGC TGT GAT CAA TGT ACG TGC AC-3'	26	50.00
BBI-F2	5'-GAY CAY CAY CAR TCA ACG GAY GAR CC-3'	26	52.27
RTV-1	5'-GAY CAY CAY CAR TCI AC-3'	17	47.00
RTV-2	5'-CAR TCG ACN GAY GAR CC-3'	17	58.00
RTV-3	5'-TGY TGY GAY CAR TGY AC-3'	17	50.00
Reverse Primers			
C1	5'-TTC GTA GCA GAA ATC-3'	15	40.00
C2	5'-TTG CAA GGT TCG TAG CA-3'	17	47.06
RTV-R1	5'-GCR TAR CAR AAR TCY TTC AT-3'	20	37.00
RTV-R2	5'-TCR TCR TGG GAG GAY TTR CA-3'	20	50.00
BBI-R1	5'-CTT GCA AGG CGC GTA GCA GAA AT-3'	23	52.17
BBI-R2	5'-T/AGA T/AGA YTT RCA GGG NGC RTA RCA-3'	24	49.02

N = ACGT; R = AG; Y = CT; I = Inosine

3.14 PCR Primers for the amplification of narbonin-like genes

One forward primer (NP10) and a reverse primer (NP11), which were based on narbonin cDNA sequences of narbon bean *(Vicia narbonensis* L.) were obtained from Dr. Bernhard Schlesier, Gatersleben, Germany. The NP 10 primer (5'-CCA TAT GCC TAA G/ACC TAT CTT TCG GG-3') has a Ndel site and the NP11 (5'- CGG ATC CTC ATC G/ ATT TGG CGA GGAG-3') has a Bam HI site at their 5'ends for convenient subcloning (Nong et αl., 1995).

3.15 Plant genomic DNA isolation

Plant genomic DNA was isolated from the leaves of horse gram and other legumes using a modified CTAB procedure as follows: Stock solutions

CTAB buffer : 2% CTAB (Cetyl trimethyl ammonium bromide), 1.4 M NaCl, 0.2 M EDTA, 0.1 M Tris-Cl, pH 8.0 and 1% (w/v) polyvinylpyrolidone (PVP 300). Before use, 0.2% (v/v) 2-mercaptoethanol was added to the above buffer.

2 M sodium acetate (pH 5.0)

Phenol:chloroform (50:50) : Phenol was redistilled and equilibrated with Tris-buffer to pH 8.0 and then mixed with equal volume of chloroform. TE: 10 mM Tris-Cl, pH 8.0 1 mM EDTA Procedure

One gram of freshly harvested leaves from ten day old seedlings were ground to a fine powder in liquid nitrogen using pestle and mortar and scrapped directly into a 7.5 ml of pre-heated (60°C) CTAB buffer in an oakridge tube. The tube was swirled regularly for 30 min to ensure efficient DNA extraction. Then 5 ml of chloroform was added and mixed gently but thoroughly, centrifuged at 1600 x g for 10 min at 4°C to separate phases. The upper aqueous phase was removed with a wide bore pipette tip and transferred to a clean tube and mixed gently with 5 ml of cold isopropanol. The DNA was recovered by centrifugation at 1600 x g for 10 min at 4°C. The DNA pellet was washed with 70% ethanol, air dried and dissolved in 500 µl of TE in an eppendorf tube and extracted with 500 µl of phenol/chloroform, followed by a chloroform extraction. After centrifugation, the aqueous phase was transferred to a fresh tube and ethanol precipitated in presence of 50 µl 2 M Na acetate (pH 5.0) and 2 volume ethanol. The DNA was finally dissolved in 500 µl of TE and stored in -20°C freezer till use.

3.16 DNA estimation by spectrofluorimeter

The amount of DNA in the samples was estimated by spectrofluorimeter using Hoefers Dyna Quant-200 as per the manufacturer's protocol as follows: TNE (10 X): 100 mM Tris-Cl (pH 7.4), 10 mM Na_2-EDTA, 2M NaCl. Filtered with 0.45 µm filter and stored at 4°C.

Dye **stock** : 10 mg bisbenzimide (Hoechst No. H-33258) was dissolved in 10 ml of filtered water and stored at 4°C.

DNA standard: One mg of calf thymus DNA was dissolved in one ml of TE (pH 8.0) and stored at 4°C. The working standard was made by diluting 1 µg of this DNA 10 times in TE to get a final concentration of 100 ng/µl.

For DNA estimation, 100 µl of stock dye, 10 ml of 10 X TNE and 90 ml of filtered distilled water were mixed and 2 ml of this solution having different concentrations of calf thymus DNA (50 ng - 2 µg) was used for calibrating the instrument. The estimation of DNA for different samples was done after this calibration. The Dyna Quant-200 gave direct reading of the DNA concentration in the samples.

3.17 Agarose gel electrophoresis

Horizontal agarose gel electrophoresis was done according to the standard protocol described by Sambrook *et al.* (1989) as follows:

Agarose of medium EEO was used at different concentration (0.8 to 2%) in IX TAE for the separation of different size DNA products. Electrophoresis was carried out at a constant voltage of 50 V and the gels were stained in ethidium bromide solution for 10 min, destained in distilled water and visualized on a UV-transilluminator (Photodyne, USA). The gels were photographed using a CCD-camera based gel documentation system using a Gel Base/Gel Blot™ Pro programme package (UVP, UK).

3.18 Polymerase chain reaction (PCR)

The PCR amplification was carried out in a Perkin Elmer thermal cycler using different concentrations of forward and reverse primers for the amplification of DNA fragments of BBIs and narbonin- like genes from horse gram and other control species. All the ingredients of the PCR were added into 0.5 ml thin walled PCR tubes except Taq polymerase enzyme as follows:

Plant genomic DNA	5-10 µl (100-500 ng)
10X Taq polymerase buffer	5 µl
Forward primer	1 µl (1-2 µm)
Reverse primer	1 µl (1-2 µm)
dNTP mix	1 µl (200 µm each)
Taq polymerase enzyme	1 µl (1.5 units)
Sterile distilled water	to 50 µl

After an initial denaturation at 95 °C for 5 min, the temperature was brought down to 85°C and then Taq DNA polymerase was added and a drop of mineral oil was overlaid on the sample. This was done for a hot start of the reaction to avoid non-specific amplification. Then the thermal cycler was programmed for the 40 cycles as follows:

Step 1	Denaturation	94°C for 1 min
Step 2	Annealing	50-60°C for 1 min
Step 3	Extension	72°C for 1-2 min

After the last cycle, one extension step of 72°C for 10 min was included. On completion of the reaction, the samples were chilled on ice and stored at -20°C for further analysis.

3.19 Reamplification of the PCR amplicons

The DNA bands of interest were cut out of the gel after agarose gel analysis of PCR products. The cut band was placed in 400 µl of TE (pH 8.0) in an eppendorf tube and incubated for 3 h at 4°C. Ten µl of the supernatant was taken as template for reamplification by PCR as described above.

3.20 **Cloning** of **PCR products**

Three different methods (cloning kits) were used for the cloning of PCR products as described below. The choice of method depended on the availability of the kit in the lab.

3.20.1 **Sure clone ligation kit** (Pharmacia Biotech)

This was used for the cloning of BBI PCR products amplified by primers N and Cl (Table 3.2) as per the manufacturer's instruction. First, the concentration of the PCR product was estimated by spectrofluorimeter. Then blunting/kinasing reaction was set in

a microcentrifuge tube using 500 ng of PCR DNA in a volume of 20 µl. The blunting/kinasing products were treated with phenol: chloroform and then purified on a Sephacryl S-200 column. Out of a 20 µl of the column effluent, 2 µl was used in a ligation reaction with dephosphorylated pUC-18 vector. The ligation was carried out at 16°C for 2 h and then stored at -20°C until its use in transformation.

3.20.2 pGem T-Easy vector system (Promega, Madison)

This was used for the cloning of PCR products obtained with the narbonin-specific primers according to manufacturer's instructions. The PCR products were first purified using Quiagen tip-20 (Quiagen Inc, USA) and then quantified by spectrofluorimetry as described earlier. The ligation reaction was set using 500 ng of the PCR product and 50 ng of pGem-T easy vector in 10 µl reaction volume at 4°C overnight.

3.20.3 PCR®II-TOPO vector system

Similarly, the PCR products for the horse gram Bowman-Birk proteinase inhibitors in one of the experiments were cloned using PCR®II-TOPO vector system from Invitrogen, USA as per the manufacturer's protocol.

3.21 Transformation

The ligated PCR products were used for the transformation of *E. coli* host cells JM 109 using Hanahan's (1983) procedure as described by Sambrook *et al.* (1989) except for the plating of transformation mix which was performed as follows:

A 5 µl of the ligation mixture was added to 200 µl of competent cells, mixed gently and left on ice for 30 min. After a heat shock for 90 sec at 42°C, 400 µl SOC was added and incubated at 37°C by shaking for 45 min. This transformed mixture was plated on LB plates containing ampicillin (50 µg/ml), IPTG (4 µl/plate) and X-gal (40 µl/plate) for the selection of the recombinants.

3.22 Plasmid preparation

The recombinant white colonies appeared on the ampicillin plates were picked and inoculated into 2 ml of LB broth with ampicillin (50 µg/ml). The tubes were incubated with shaking at 37°C overnight. For the small scale isolation of plasmids,

using the alkaline lysis procedure of Birnboim and Doly (1979) was followed as described by Sambrook *et al.* (1989). The large scale preparation of clones and control plasmids was also done using alkaline-lysis method according to the protocol described by Sambrook *et al.* (1989) from a 500 ml culture. The air dried plasmid pellet was dissolved in 3 ml of TE (pH 8.0) and purified by polyethylene glycol (PEG) precipitation according to Sambrook *et al.* (1989).

3.23 Restriction digestion of plasmids

The plasmid DNAs isolated by small scale or large scale preparations were restriction digested with appropriate restriction enzymes' (Pvu II or Eco RI) using compatible buffers supplied by the manufacturers. The restriction digestion was done to check for the presence and size of the inserts. The double digestion of clone 7 (a cloned BBI PCR product) with Bam HI and Eco RI was performed sequentially for the release of the insert for labelling purpose.

3.24 Electro-elution of DNA fragments from agarose gels

The DNA insert of the clone 7 was isolated from agarose gels for the labelling of insert DNA to be used in Southern blotting. After electrophoresis of clone 7 double digested with enzymes Bam HI and EcoRI, the gel was stained in ethidium bromide and visualized using long wavelength UV light. The DNA insert was excised from the gel and placed into a pre-treated dialysis bag in IX TAE. The DNA was eluted at 40 V for 1 h in IX TAE buffer and current flow was reversed for one min at the end to release the DNA sticking to the sides of the dialysis tubing. The eluted DNA was purified by phenol : chloroform extraction and ethanol precipitation and dissolved in 25 µl TE (pH 8.0).

3.25 DNA labeling and Southern hybridization of horse gram BBI

The insert of clone 7 eluted from agarose gel as above was labeled using DIG DNA labeling and detection kit (Boehringer Mannheim Biochemica, Germany) as per their instructions. The PCR amplicons amplified with primer pair 'N' and 'Cl' using genomic DNA of different legume species were electrophoresed in a 1% agarose gel at a constant volt of 50 V for 3 h. After the run, the gel was stained in ethidium bromide and photographed. The Southern transfer of DNA onto nylon membrane was done under neutral conditions according to Sambrook *et al.* (1989). After overnight capillary transfer,

the blot was dried and the DNA on the blot was cross linked onto the nylon membrane by UV cross linking for 3 min on a UV transilluminator.

The filter was prehybridized in a sealed plastic bag with 10 ml of hybridization buffer at 68°C for 6 h. The solution was replaced with 5 ml of hybridization buffer containing 50 ng of labelled 'clone 7' DNA (freshly denatured by boiling) and incubated at 65°C overnight. After hybridization, the filter was washed twice with 2X SSC (20X SSC = 3 M NaCl, 0.3 M Na-citrate, pH 7.5), 0.5% SDS for 5 min each and then with 0.1X SSC, 0.1% SDS twice for 15 min each at 68°C with shaking. The washed filter was used for the detection of the hybridized probe.

The filter was washed with buffer I (0.1 M maleic acid, 1.5 M NaCl, pH 7.5) for 1 min and then incubated for 30 min with 20 ml of buffer II (1% blocking solution). The antibody, DIG alkaline phosphatase conjugate was diluted 1:5000 in buffer II and the filter was incubated in 5 ml of this solution for 30 min. The unbound antibody conjugate was removed by washing twice with 20 ml of buffer I for 15 min each. After this the filter was equilibrated with buffer III (alkaline phosphatase buffer, pH 9.5) for 2 min and incubated with 10 ml of substrate solution (45 µl NBT and 35 µl X-phsophate in 10 ml buffer III) in a plastic bag in the dark till the desired bands appeared (8 h). Then the reaction was stopped by adding 5 ml of TE (pH 8.0) and the blot was documented by photography.

3.26 Sequencing of clones

Three of the cloned PCR products were sequenced using the plasmid DNA of clones prepared by large scale procedure and PEG purification as described in Section 3.22. The clone 7 was sequenced fully in one direction using M 13 forward primer while the clone 2 with a bigger DNA insert was sequenced from both the directions using Ml3 universal primers by dideoxy chain termination method (Sanger *et ah,* 1977). The clone pRTV-2-2 was sequenced with T7 promoter primer in one direction only using automated DNA sequencer (ABI PRISM, model 377, Version 3.0) at the Department of Biotechnology (DBT) facility for automated DNA sequencing at Indian Institute of Science, Bangalore, India. The DNA sequences were analyzed using PC Gene and DNA Inspector software packages.

RESULTS AND DISCUSSIONS

CHAPTER-4

RESULTS AND DISCUSSIONS

4.1 General studies on horse gram seed proteins

Seed storage proteins accumulate in sub-cellular organelles called protein bodies. These are membrane bound spherical organelles, a few microns in diameter, filled with proteins and phytates. Scanning electron microscopic (SEM) study of these protein bodies have been undertaken in many legume species (Mosse and Pernollet, 1982). The membranes of the protein bodies are generally smooth, though in some cases have granular appearance (Barker *et al.,* 1976; Burr and Burr, 1976)

4.1.1 Scanning electron microscopic study of horse gram seeds

In the present study, the organization of protein bodies in the horse gram seeds was studied at three different stages i.e., immature seeds [18 days after flowering, DAF], mature dry seeds and germinating seeds [3 days after imbibition, DAI]. The samples for SEM study were prepared as described in section 3.13. The Scanning electron micrograph of immature horse gram cotyledon shows presence of smaller size starch granules compared to that present in mature grains and these are covered with a protein matrix (Fig. 4.1). Discrete spherical protein bodies very small in size are also embedded in the protein matrix. Large spaces may be observed within each cell. The SEM of mature cotyledon revealed that these spaces observed in the immature cotyledons are filled with the starch granules and protein matrix (Fig. 4.2A). These starch granules are larger in size than in the immature and are fully covered by a protein matrix. Protein bodies are clearly visible, spherical in shape with 2-3 μm diameter and are embedded in the protein matrix (Fig. 4.2B). In the cotyledons of germinating seeds, the protein matrix becomes thinner and appears to have collapsed over the starch granules (Fig. 4.3). Protein bodies are still visible in the embedded protein matrix. Free spaces reappeared in the cells of germinating grain which are free from the protein matrix. The starch granules, which were clearly visible now, are elliptical in shape and slightly constricted at the center.

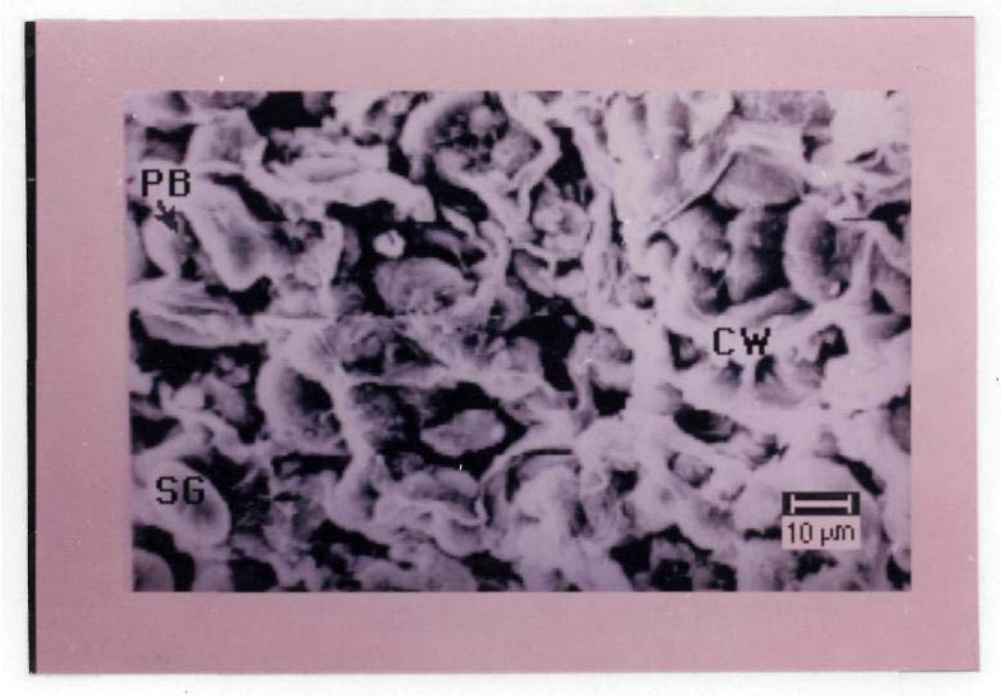


Fig. 4.1 Scanning electron micrograph of an immature (18 days after flowering) horse gram cotyledon.
SG = Starch granule PB = Protein bodies (indicated with a black arrow) CW = Cell Wall

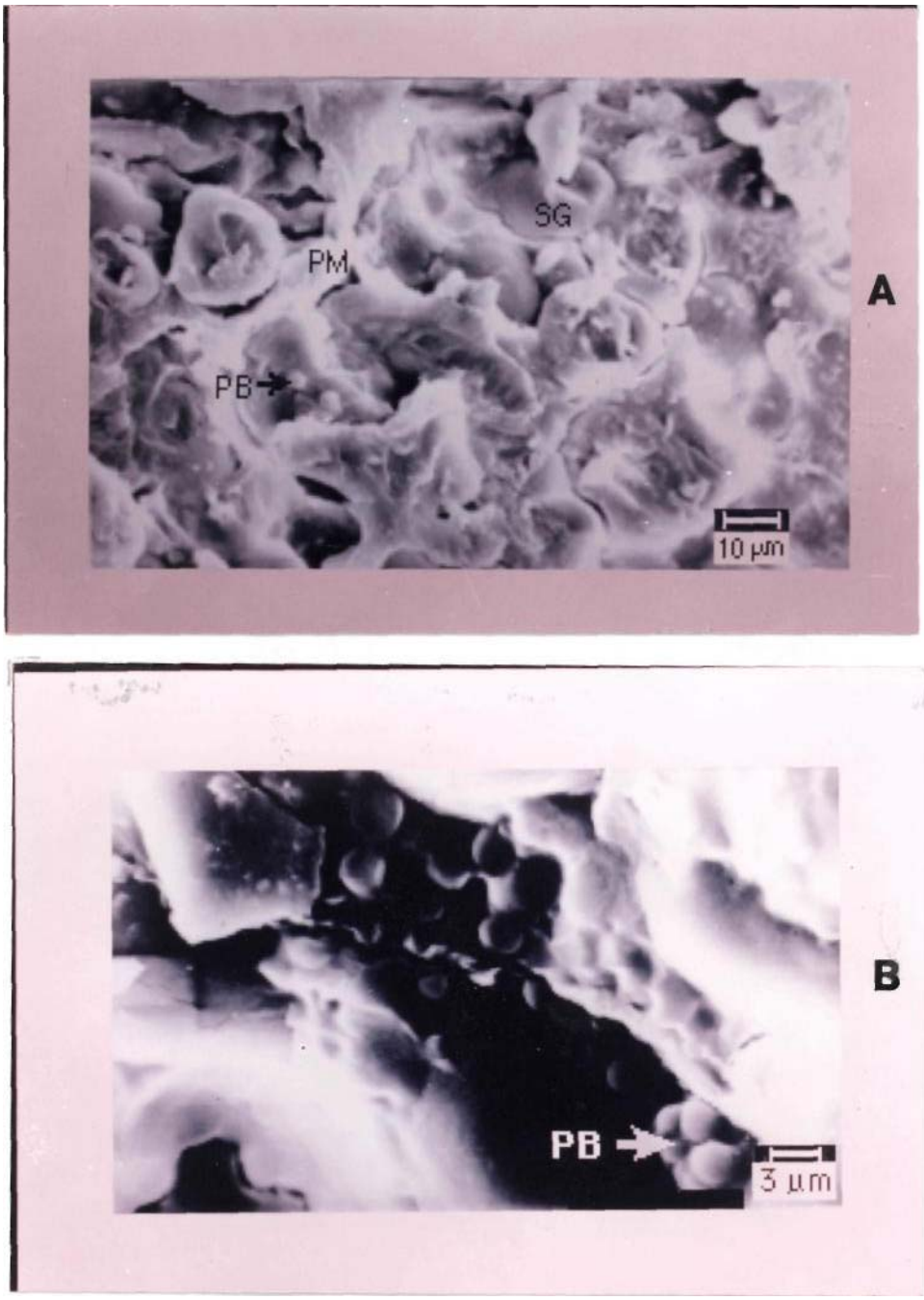


Fig. 4.2 Scanning electron micrograph of a mature dry cotyledon of horse gram. SG = Starch granule PB = Protein bodies PM = Protein matrix.

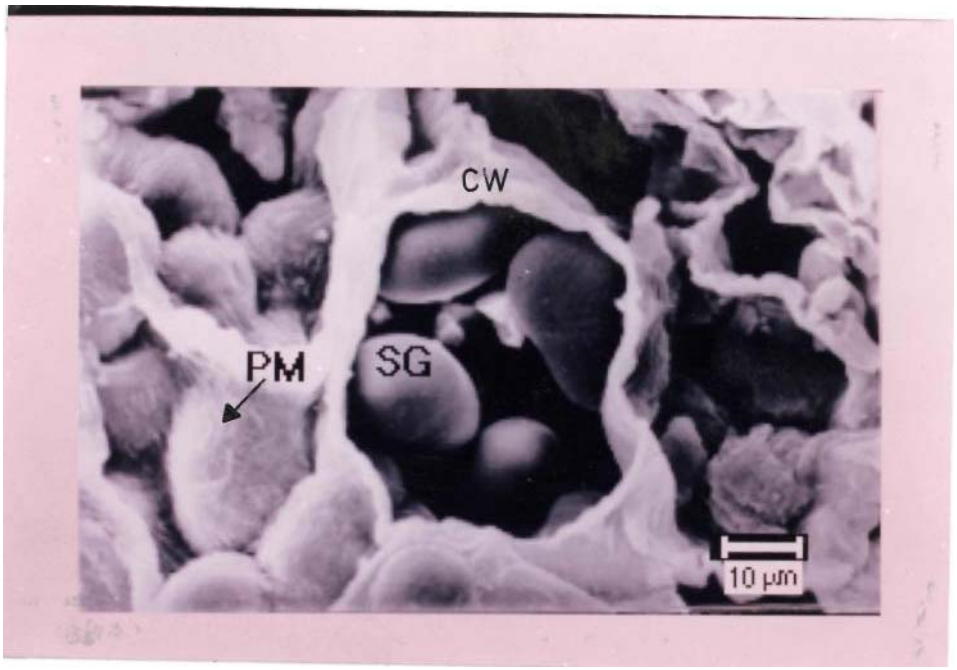


Fig. 4.3 Scanning electron micrograph of cotyledon of germinating horse gram seed (3 days after imbibition).

SG = Starch granule PM = Protein matrix CW = Cell Wall

Discrete protein bodies are 2-3 μm in diameter which are similar in size to that of protein bodies from French bean (Barker *et al,* 1976, Pusztai *et al,* 1978). The surface of the protein bodies are smooth and similar to that of *Vigna radiata* (Thomson, 1979). In the geminating seeds, the collapse of the protein matrix on the starch granules may probably be due to hydration and partial digestion. The protein bodies are still visible on the matrix even three days after imbibition similar to that observed in faba bean (Weber *et al,* 1979).

4.1.2 Electrophoretic studies on horse gram seed proteins

The innovation of ultracentrifugation and electrophoresis as analytical tools has led to the closer investigations of seed storage proteins (Derbyshire, *et al,* 1976). The use of SDS-PAGE has allowed the characterization of a large number of individual polypeptides and their polymorphism between varieties (Mosse and Pernollet, 1982). In the present study, the total seed proteins of sixteen horse gram lines were fractionated under reducing conditions by SDS-PAGE in 12% polyacrylamide gels for protein polymorphism.

The predominant proteins of horse gram resolved into two groups of bands of 52-66 kDa (group A) and 28-34 kDa (group B) in size. No significant polymorphism was observed for the total seed protein profiles among the sixteen genotypes studied except for some minor differences (Fig 4.4). The genotype R-IΠ 90A-1-10-3 lacked a protein of size 76 kDa while carrying an 80 kDa protein of relatively higher intensity than present in other genotypes (Fig. 4.4, lane 5). In contrast, the 80 kDa protein was absent in the genotype IC11095 (lane 11). An unique protein of 20 kDa size was present in genotype K-42 (lane 13).

The study shows the absence of any significant variation in seed storage proteins of horse gram although the lines studied here had diverse agronomic characters (Savithramma and Shambulingappa, 1996). This result is similar to that observed in winged bean where no significant differences were observed in eighty different pure lines of winged bean (Blagrove and Gillespie, 1978). This could be due to the lack of genetic divergence among seed storage proteins of horse gram during evolution. Detailed work on the individual classes of horse gram seed proteins are presented in the following sections.

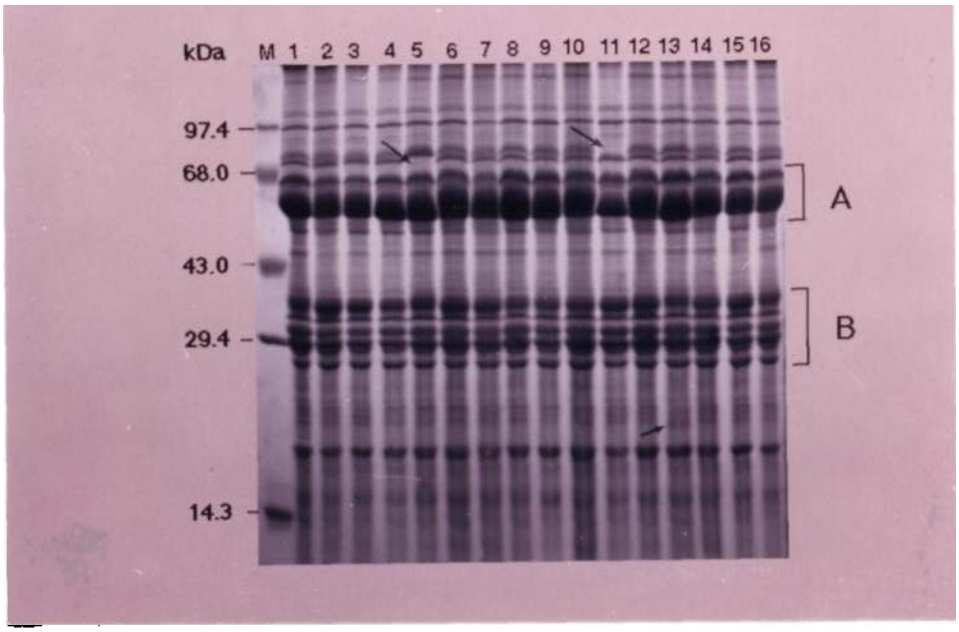


Fig. 4.4 SDS-PAGE analysis of total seed proteins of horse gram genotypes in a 12% polyacrylamide gel.

M. Protein size marker

1. No. 47	2. A–4-9–5	3. D–6–3-4
4. Macintosh	5. R–III 90–A–1–10–3	6. No. 2
7. S–35	8. No. 85	9. BGM–1
10. D–2–2–2	11. IC11095	12. PHG–9
13. K-42	14. C–7–5–5	15.C–6–3–4
16. R–III 31–49		

A and B indicate the predominant storage protein groups. The arrows in lane 5 and 11 indicate absence of a 76 and an 80 kDa protein respectively. Arrow in lane 13 indicates the presence of a 20 kDa unique protein band.

4.2 Bowman-Birk Proteinase inhibitors of horse gram

Four iso-inhibitors of the Bowman-Birk inhibitors (BBIs) of horse gram are double headed and can inhibit both trypsin and chymotrypsin simultaneously (Ramasarma and Rajgopal Rao, 1991). These iso-inhibitors have been characterized by amino acid composition and amino terminal analysis (Sreerama *et al.,* 1997). HGI-III is the major iso -form whose primary structure has been elucidated by amino acid sequencing (Prakash *et al.,* 1996).

4.2.1 Polymorphism and developmental expression of trypsin inhibitors in horse gram seeds and floral organs

4.2.1.1 Variability for trypsin inhibitors in the seeds of different horse gram genotypes

Variation among the iso-inhibitors of trypsin in sixteen horse gram genotypes was studied by fractionating their total seed proteins in 10% polyacrylamide gels and detecting their presence as described in section 3.6. A horse gram seed protein fraction enriched for trypsin inhibitors was included as control (Fig. 4.5). The bands near the origin of the gel (marked I in Fig. 4.5) are actually undigested seed proteins and not the residual casein, used as substrate. This is evident from the absence of these bands in the control sample (lane 8) and their non-hexagonal appearance which is typical of the trypsin inhibitor bands in these gels (Garcia-Carreno *et al.,* 1993). Region II of the gel contains the typical hexagonal shaped bands except for one dark band in the control sample. Each activity spot (hexagonal band) represents an iso-inhibitor of trypsin in horse gram seeds.

Variability in trypsin inhibitor profile among the sixteen horse gram genotypes was minimal except for the slowest moving band found in the control and which was absent in many genotypes (eg. lanes 11 and 13). The absence of significant differences observed for the trypsin inhibitor profile in horse gram was similar to the results obtained in pigeon pea (Pichare, 1992; Pichare and Kachole, 1994). In contrast, significant polymorphism of the trypsin inhibitors has been observed in pea (Domoney *et al,* 1995), winged beans (Kothekar *et al,* 1996) and chick pea (Harsulkar *et al.,* 1997). The absence of such polymorphism in horse gram could be due to their narrow genetic base as also reflected in the overall protein profile described earlier (Fig. 4.4).

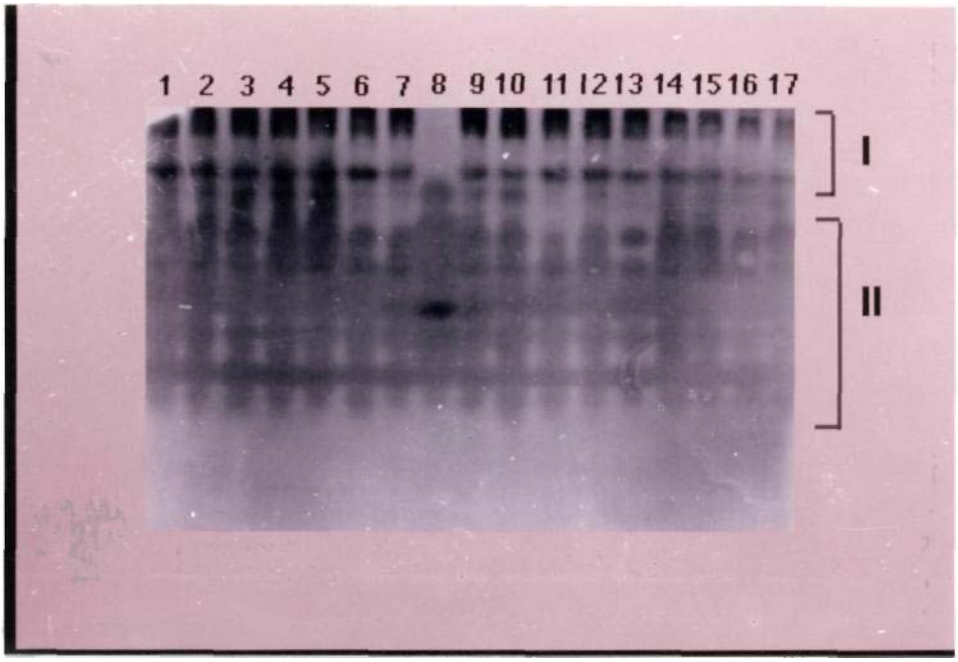


Fig. 4.5 Assay for trypsin inhibitors among 16 horse gram genotypes analysed by casein–substrate gel analysis.

1. No. 47 2. A–4–9–5 3. D–6–3–4 4. Macintosh
5. R–III90–A–1–10–3 6. No. 2 7. S–35
8. Protein enriched for trypsin inhibitors from C–6–3–4
9. No. 85 10. BGM–1 11. D–2–2–2 12. IC11095
13. PHG–9 14. K–42 15. C–7–5–5 16. C–6–3–4
17.R–III 31–49

Group I includes undigested horse gram proteins while group II bands are the trypsin iso–inhibitors.

4.2.1.2 Accumulation of trypsin inhibitors during seed development

The total seed proteins of horse gram extracted during different stages of development were fractionated in a 10% polyacrylamide gel and the trypsin inhibitory activity was detected as above. The trypsin inhibitory activity first appeared on the 15th day after flowering (DAF) as two iso-inhibitors (Fig. 4.6). The pattern remained the same till 18th DAF. The number of iso-inhibitor bands increased from 2 to 6 there after and remained constant till seed maturity.

The simultaneous appearance of two inhibitors during seed development observed in this study is similar to the results obtained in pea (Domoney *et al,* 1995). In pea it was confirmed that these inhibitors were products of two different genes. Our result indicate two additional trypsin iso-inhibitors. The N-terminal sequences of BBIs have been reported by Sreerama *et al.* 1997. Sequence difference indicates that they could be the products of two genes. Furthermore, a glycine rich trypsin inhibitor has been reported from horse gram which differs from the four iso-inhibitors reported by Sreerama *et al.* (1997). Distinct genes coding for different Bowman-Birk iso-inhibitors have been characterized in soybean (Baek *etal.,* 1994).

The increase in the number of iso-inhibitor bands from 2 to 6 during seed development could be due to the post-translational modification, aggregation or late expression of additional genes. The post-translational modifications may involve removal of a few amino acids from the amino or carboxy terminal ends. An incomplete cleavage results in a mixture of parental and truncated inhibitor forms (Wilson, 1988). The presence of such multiple iso-forms have been reported in many legumes (Ryas-Duarte *et al.,* 1992 ; Domoney *et al.,* 1995 ; Ambekar *et al,* 1996). These workers also report the post translational modification of BBIs giving rise to additional forms.

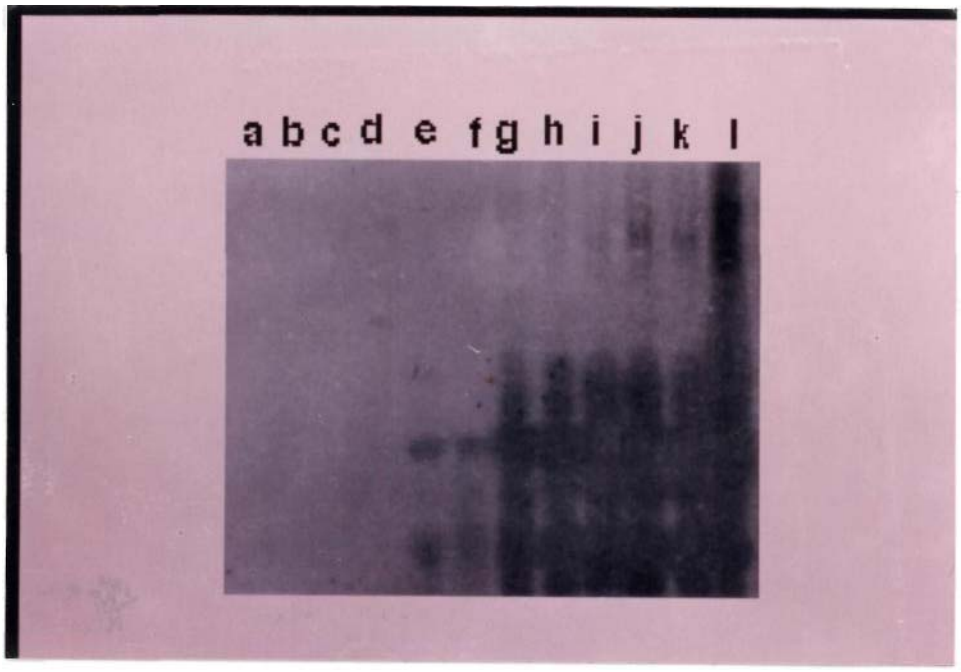


Fig. 4.6 Accumulation of trypsin inhibitors during seed development of horse gram genotype C–6–3–4 analysed by casein-substrate gel electrophoresis.
a. 3 Days after flowering (3 DAF) b. 6 DAF c. 9 DAF
d. 12 DAF e. 15 DAF f. 18 DAF g. 21 DAF
h. 24 DAF i. 27 DAF j. 30 DAF k. 33 DAF
l. 36 DAF

4.2.1.3 Presence of trypsin inhibitors in the flower parts and pod peels of horse gram

During the course of these developmental studies, trypsin inhibitor activities in horse gram flower were assayed and the electrophoretic patterns of these inhibitors in activity gels and their location in specific floral parts were further determined. The total proteins of sepals, petals, gynoecium (stigma and style only), androecium and immature seeds were fractionated on a 10% gel and trypsin inhibitor activity detected using synthetic substrate, APNE as described in section 3.7 (Fig. 4.7). The proteins of pod peels at different developmental stages were also analyzed in a similar manner (Fig. 4.8).

Trypsin inhibitory activity was present in all the floral parts tested including sepals, petals, androecium and gynoecium and also in the pod peels while the pattern of these bands differed from those present in the immature and mature seeds (Fig. 4.7). The trypsin inhibitors of floral parts constituted two slow moving bands as compared to the faster moving bands present in seed. Among the iso-inhibitors in floral parts and pod peels, the faster moving band was of weaker intensity in gynoecium and androecium while the intensity of this band was much greater in the sepals, petals and pod peels. The mobility of the pod peels trypsin inhibitor iso-forms was similar to those from floral parts. Pod peels during developmental stages contained two trypsin inhibitor bands from 5-25 DAF, which had disappeared by 30 DAF (Fig. 4.8). The presence of trypsin inhibitors in the horse gram flower is similar to that reported in alfalfa (McGurl *et al,* 1995). The proteinase inhibitors I and II families are expressed in the flowers of solanaceous crops (Pena-Cortes *et al.,* 1991 ; Atkinson *et al,* 1993). But their higher expression in sepals and petals than in androecium and gynoecium of horse gram flower is contrary to the report in tobacco where maximum activity in stigma extracts rather than petals was reported. The presence of such inhibitors could well play a defensive role to protect the important reproductive organ from insect pests and pathogens. The trypsin inhibitors of flowers and pod peels are similar which are in turn different from the inhibitors found in the seeds. It is also of interest that, the inhibitors expressed in flowers continued to be expressed only in pod peels but not in seeds. Further characterization of these flower specific inhibitors is required to elucidate their exact nature. The cloning of their genes may also provide additional genes for control of insect pests through genetic engineering (Ryan, 1990).

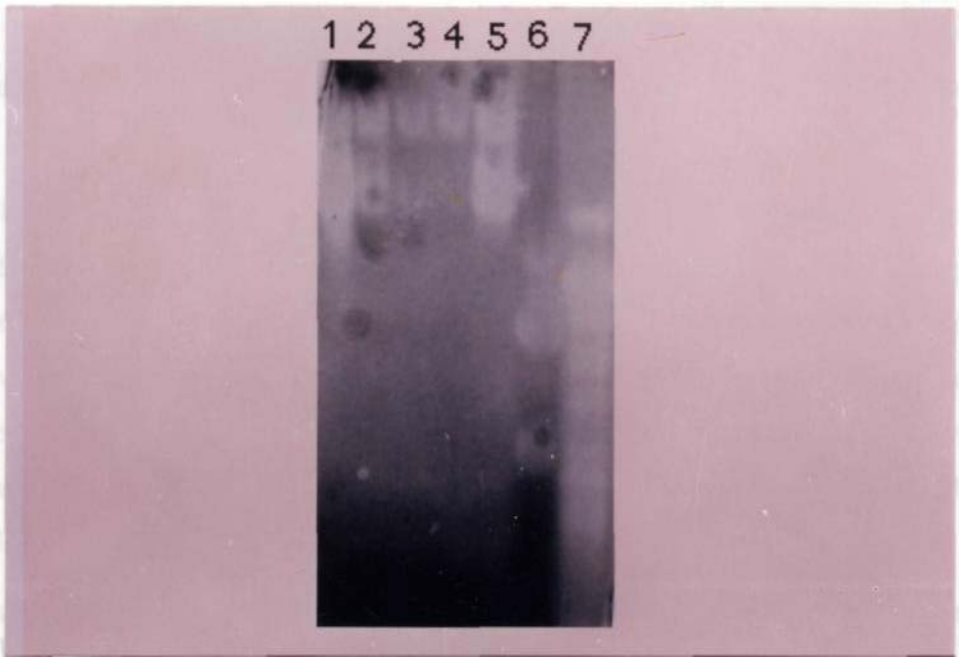


Fig. 4.7 Detection of trypsin inhibitors in different tissues of horse gram by activity analysis using synthetic substrate, APNE.

1. Sepals 2. Petals 3. Gynoecium (Stigma and style only)
4. Androecium 5. Pod peels (18 days after flowering)
6. Immature seed. 7. Mature dry seed.

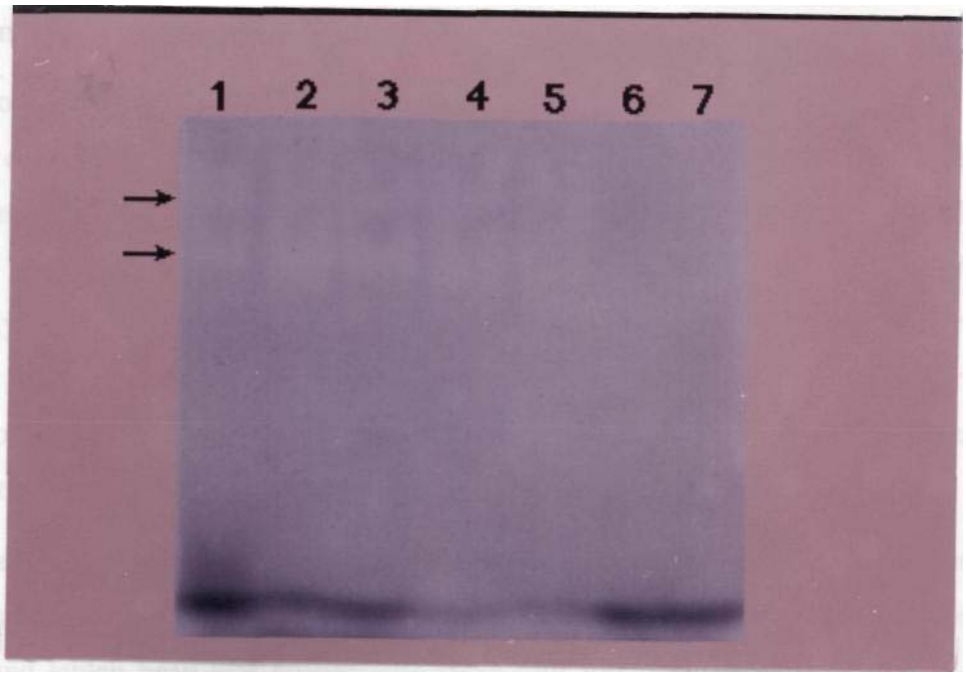


Fig. 4.8 Detection of trypsin inhibitors in horse gram pod peels during development using the synthetic substrate, APNE.

1. 5 Days after flowering (DAF) 2. 10 DAF 3. 15 DAF
4. 20 DAF 5. 25 DAF 6. 30 DAF 7. Mature seeds

4.2.2 Production and characterization of polyclonal antibodies against the horse gram BBI

Polyclonal antibodies were raised against the horse gram BBI as described in section 3.9 checked for the titre and used to detect variability among BBIs in different horse gram genotypes. In order to check the antibody titre, the total seed proteins of horse gram variety "C-6-3-4" were fractionated by SDS-PAGE in a 12% gel and electroblotted onto nitrocellulose membrane. The electroblotted membrane was cut into five strips, each having two protein lanes. These strips were probed with different dilutions of the above antibody in the range of 1:100-1:10,000 (Fig 4.9).

At lower dilutions (1:100 and 1:500) the antibodies reacted strongly with at least 4 bands in the 15 kDa range. At higher dilution the number of reacting bands were sequentially reduced to only one at 1: 10,000 dilution where the antibody reacted specifically with a protein apparently 15kDa in size (Fig. 4.9). Although the exact size of BBIs are 8-10 kDa based on amino acid sequence data, they move anamolously in SDS-PAGE moving in a molecular weight range of around 15 kDa even under reducing conditions (Bijola *et al,* 1994 ; Sreerama *et ai,* 1997).

The total seed proteins of seven horse gram genotypes were fractionated on 12% SDS-PAGE, electroblotted on to nitrocellulose membrane and probed with horse gram BBI polyclonal antibodies (Fig. 4.10). The immunoblot showed no major differences for BBI among those genotypes, except that the genotype C-6-3-4 showed a reactive band of 30 kDa, possibly a dimer of the 15 kDa band. Dimerization of bands was observed occassionally but was not a common feature (Fig 4.9). A relatively poor reaction observed with the genotypes D-6-3^1 and S-35 may be attributed to by these cultivars containing lower amounts of BBI.

The reaction patterns of the polyclonal antibodies was compared with four different monoclonal antibodies raised against native soybean BBIs. Total seed proteins of soybean, horse gram and lablab bean was probed with these four monoclonals as well as with the horse gram polyclonal antibodies. One strip of the gel was stained in Coomassie brilliant blue (Fig. 4.11 A) and the remaining part of the gel with five identical replicates was transferred on to nitrocellulose membrane. The membrane was cut into five identical strips to be probed with the monoclonal and polyclonal antibodies (Fig. 4.1 IB). The reaction

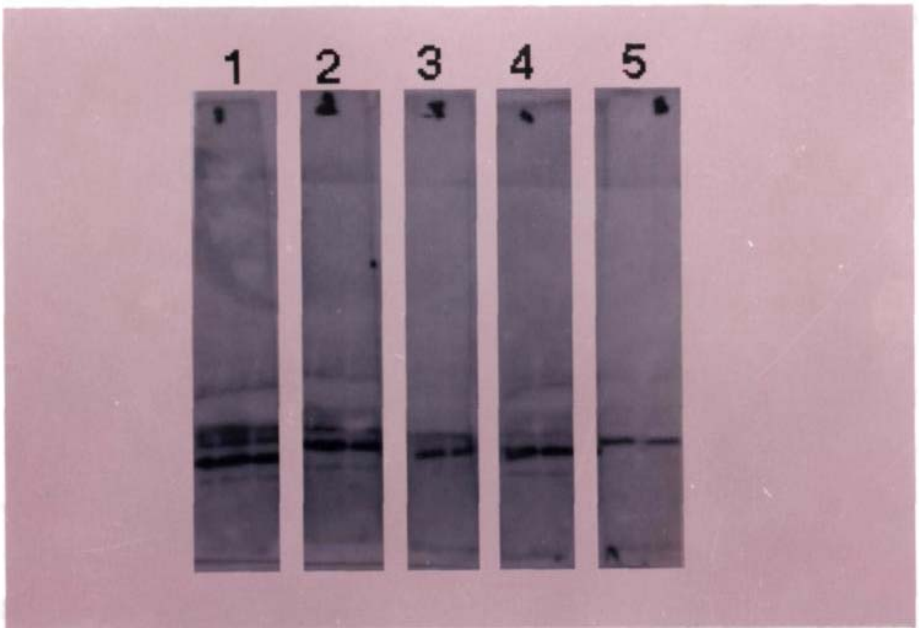


Fig. 4.9 Reaction of anti–horse gram BBI polyclonal antibodies at different dilutions against total horse gram seed proteins.

1. 1: 100 2. 1 : 500 3. 1 : 1000 4. 1 : 5,000 5. 1 : 10,000

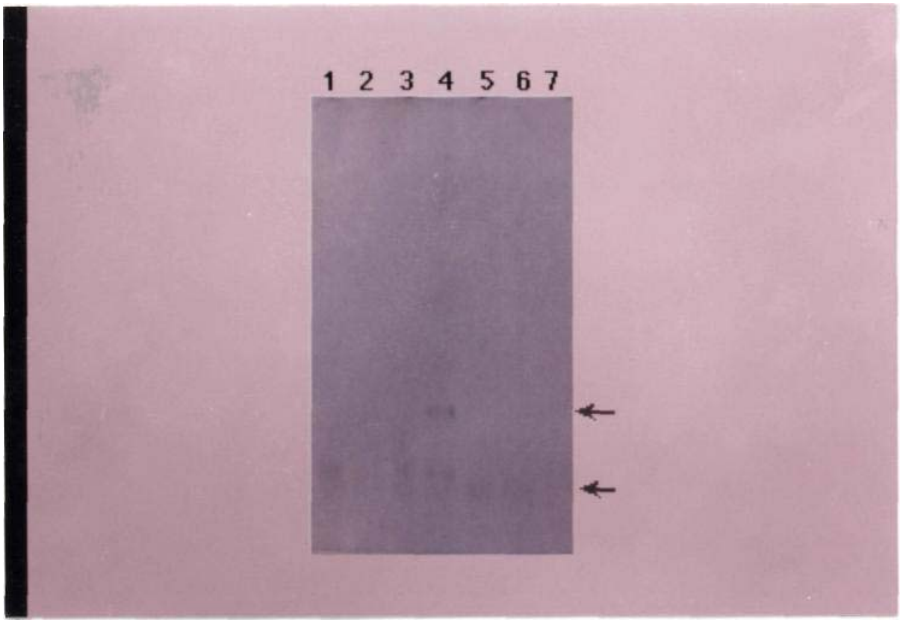


Fig. 4.10 Immunodetection of BBIs of different horse gram genotypes using anti – horse gram BBI polyclonal antibodies.

1. PHG–9 2. D–6–3–4 3. IC11095 4. C–6–3–4
5. Macintosh 6. BGM–1 7. S–35

Arrows indicates cross–reactive BBIs. The upper band indicates a dimeric form of BBIs.

pattern of the four monoclonal antibodies were similar to polyclonal antibodies indicating the highly specific nature of the polyclonal antibodies raised in the present study. All the four monoclonal antibodies reacted strongly with proteins sized 15 kDa in horse gram 31 kDa in soybean and 29 and 31 kDa proteins of lablab bean. Two of the monoclonal antibodies viz., '7E10.D6-67'and '15F7-64' reacted weakly with 15 and 17 kDa proteins in soybean but strongly with 46 and 17 kDa proteins of horse gram and only the 46 kDa protein of lablab bean. The differential reaction of monoclonal antibodies suggests that 7E10.D6-67 and 15F7-64 could belong to one group while the 7E10.C10-C11 and 15F7.F2 belongs to another group. The polyclonal antibodies raised against horse gram BBI also reacted with proteins of 46 kDa in horse gram and lablab bean similar to two of the monoclonals. This shows that the monoclonal antibodies of soybean BBI have reacted with similar epitopes of horse gram BBI . This could be due to the amino acid sequence homology between horse gram and soybean BBI being as high as 76% (Prakash *et al.,* 1996). The weak reaction of monoclonal antibodies with 15 kDa protein of soybean and strong reaction with a 31 kDa protein is intriguing. This protein may likely be a dimeric form of the BBIs since both monoclonal and polyclonal antibodies reacted strongly with it, as well as it's size being exactly double the size of monomeric BBIs as observed on SDS-PAGE gels. In addition, a similar reaction was observed with lablab bean protein where both monoclonal and polyclonal antibodies reacted strongly with proteins, 29 and 31 kDa in size. Importantly, reaction was observed with a 46 kDa protein of horse gram and lablab bean by both monoclonal and polyclonal antibodies. A trimeric form of the BBI may be expected to attain that size. Such a tendency of BBIs to exist in dimeric and trimeric form in solution had been reported (Sreerama *et al,* 1997). The differential reaction of monoclonal antibodies suggests that they belong to two different groups. This might indicate that the antibodies might recognize different epitopes since it is suggested that the 46 kDa protein is trimeric form, these may represent structure rather than sequence epitopes.

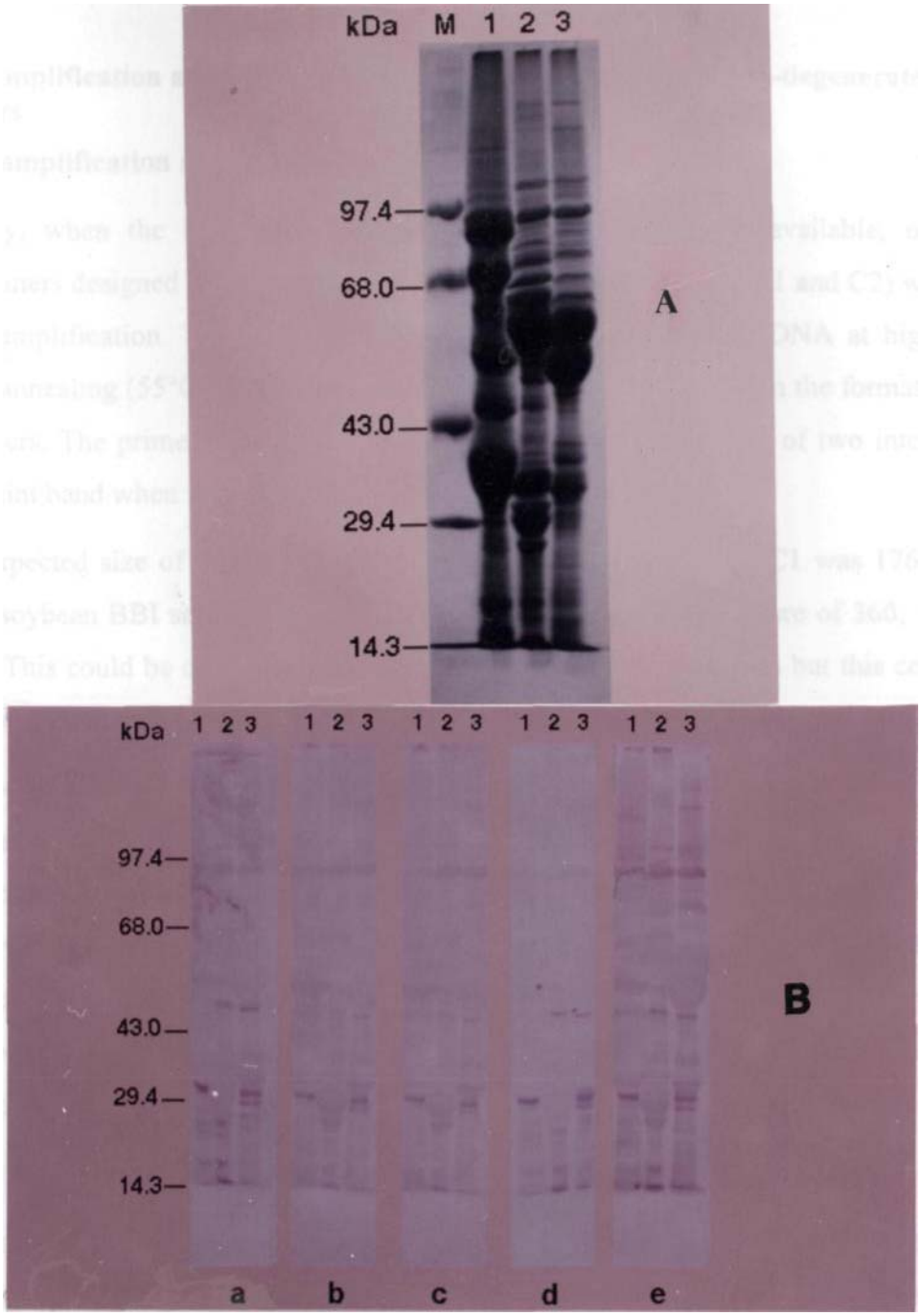


Fig.4.11 Immunoblot analysis of BBIs of three different legumes using monoclonal and polyclonal antibodies.

A. SDS–PAGE of total seed proteins in 10% polyacrylamide gel stained with Coomassie brilliant blue M. Protein size markers

1. Soybean 2. Horse gram 3. Lablab bean

B. Reaction of the above with A–D monoclonal antibodies raised against soybean BBI.

a. 7 E10. D6–67 b. 7E. 10–C10–C11 c. 15F7.F2

d. 15F7–64 e. Anti–horse gram BBI polyclonal antibodies

All the monoclonal antibodies reacted with a protein of 15 kDa in horse gram and 31 kDa in soybean.

4.2.3 PCR amplification and cloning of horse gram BBI DNA using non-degenerate primers

4.2.3.1 PCR amplification and cloning of the amplicons

Initially, when the horse gram BBI protein sequence was not available, non-degenerate primers designed based on the soybean BBI DNA sequence (N, Cl and C2) were used for the amplification. The N and C2 primer pair, failed to amplify DNA at higher stringency of annealing (55°C) while lower annealing temperatures resulted in the formation of primer-dimers. The primer pair N and Cl gave PCR products consisting of two intense bands and a faint band when fractionated in a 1% agarose gel (Fig. 4.12).

The expected size of the BBI amplicon with the primer set N and Cl was 176 bp based on the soybean BBI sequence where as the size of those obtained were of 360, 810 and 1600 bp. This could be due to the annealing of primers to modified sites but this could be confirmed only after cloning and sequencing.

The pooled PCR amplicons were purified and cloned into pUC-18 vector as described in section 3.20.1. After transformation into *E-coli* host strain JM 109, several recombinant clones were selected. Two of these clones, namely clone 7 and clone 2 with different insert size were selected for further analysis. The clone 7 represented the 360 bp amplicon whereas clone 2 represented 810 bp amplicon. The Pvu-II digestion of these clones released inserts of 680 bp and 1120 bp, respectively (Fig 4.13). These included the flanking 322 bp from the pUC-18 vector in addition to the PCR amplicons .

4.2.3.2 Detection of related BBI amplicons in other legumes by Southern blotting

The primer pair N and Cl was used to amplify and check the presence of similar DNA fragments in six legumes viz., lablab bean, green gram, black gram, chick pea, cowpea and soybean in addition to horse gram .The PCR conditions were similar to that used for obtaining clone 7 and clone 2 from horse gram and the amplicons were analysed in a 1% agarose gel (Fig 4.14A).

The lablab bean gave amplicons, which were similar to those of horse gram. The size of the amplicon were ca. 360, 500 and 810 bp in size. The 500 bp band was very faint and was not seen in the earlier experiments with the horse gram DNA. Similarly the 1600 bp

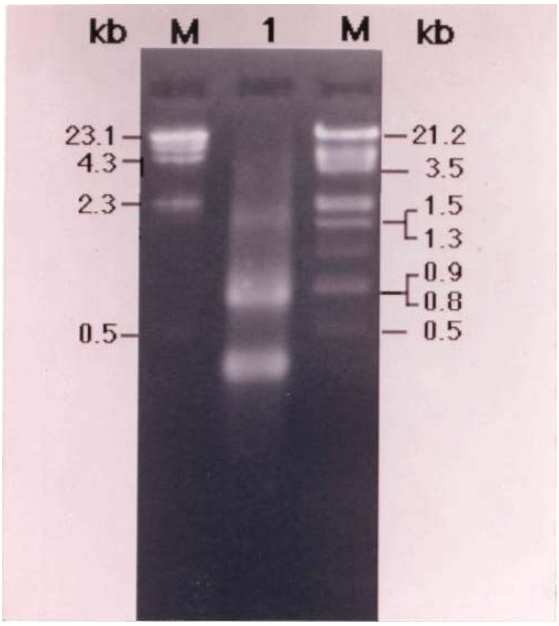


Fig. 4.12 Agarose gel (1%) analysis of BBI PCR amplicons from horse gram genomic DNA using primer pair N and C1 at 55°C annealing temperature .
M. λ Hind III DNA marker 1. PCR amplicons
M. λ Hind III / Eco RI DNA marker.

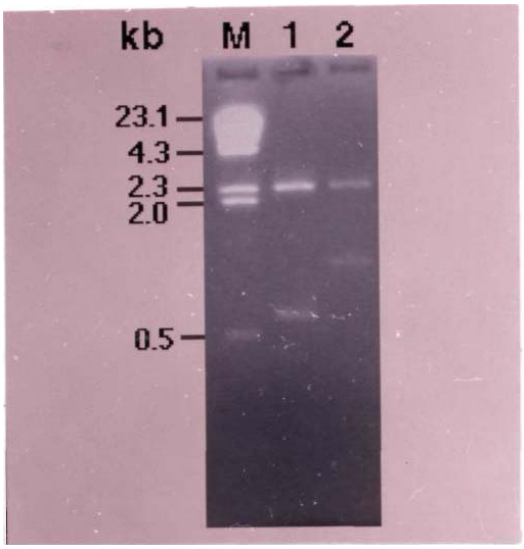


Fig. 4.13 Agarose gel (1%) analysis of horse gram BBI clones obtained with primer pair N and C1. The purified plasmids were digested with Pvu II enzyme.
M. λ Hind III DNA marker 1. Clone 7 2. Clone 2

The inserts include a 322 bp flanking regions of the pUC18 vector .

band shown earlier was not seen here. The PCR amplification of black gram was also quite similar to that of horse gram except that the upper 810 bp band was missing. The amplicons of green gram , chick pea and cowpea were very similar in that all three had a prominent 515 bp band. The first two of these also had a faint band at the 810 bp as in horse gram (Fig 4.14 A, lanes 3, 5 and 6). Soybean gave a single amplicon of 760 bp. in size.

The same gel (in Fig. 4.14 A) was transferred onto nylon membrane by capillary blotting (Section 3.25) and probed with DIG-labelled clone 7 insert (Fig 4.14 B). The Southern blot showed the hybridization of clone 7 insert with 360-380 bp amplicons of horse gram, lablab bean and black gram. There was also an indication of faint reaction with the 810 bp band. The probe did not hybridize with any of the bands in other legumes.

The result shows that, the clone 7 insert sequence is present not only in horse gram but also in lablab bean and black gram revealing it's conserved nature across these species. It is quite interesting to note that the size of the single amplicon observed in the soybean, from which the primer sequence were taken was also of higher size than that expected.

4.2.3.3 Sequence analysis of clone 7 and clone 2 of horse gram

Clone 7 was sequenced completely using M13 forward primer as described in section 3.26, while clone 2 which was bigger in size was sequenced partially from both directions using Ml3 forward and reverse primers. The sequences of clone 7 and clone 2 were compared with the published amino acid and nucleotide sequence of BBIs. The sequence of clone 7 and clone 2 were also compared with each other using the DNA Inspector™ software package.

The sequence of clone 7 revealed that it is 368 bp long. The sequence of N-terminal primer was present at the 5' end of sequence and was repeated twice in the inverse direction, once at 35^{th} nucleotide and than at the 3' end of the sequence (Fig. 4.15). The nucleotide sequence of clone 7 did not show the expected homology with the predicted BBI genes, except for the primer region.

An open reading frame of 50 amino acids appears to be present starting from 5' end (Fig. 4.15). The predicted amino acids sequence of this open reading frame has showed a

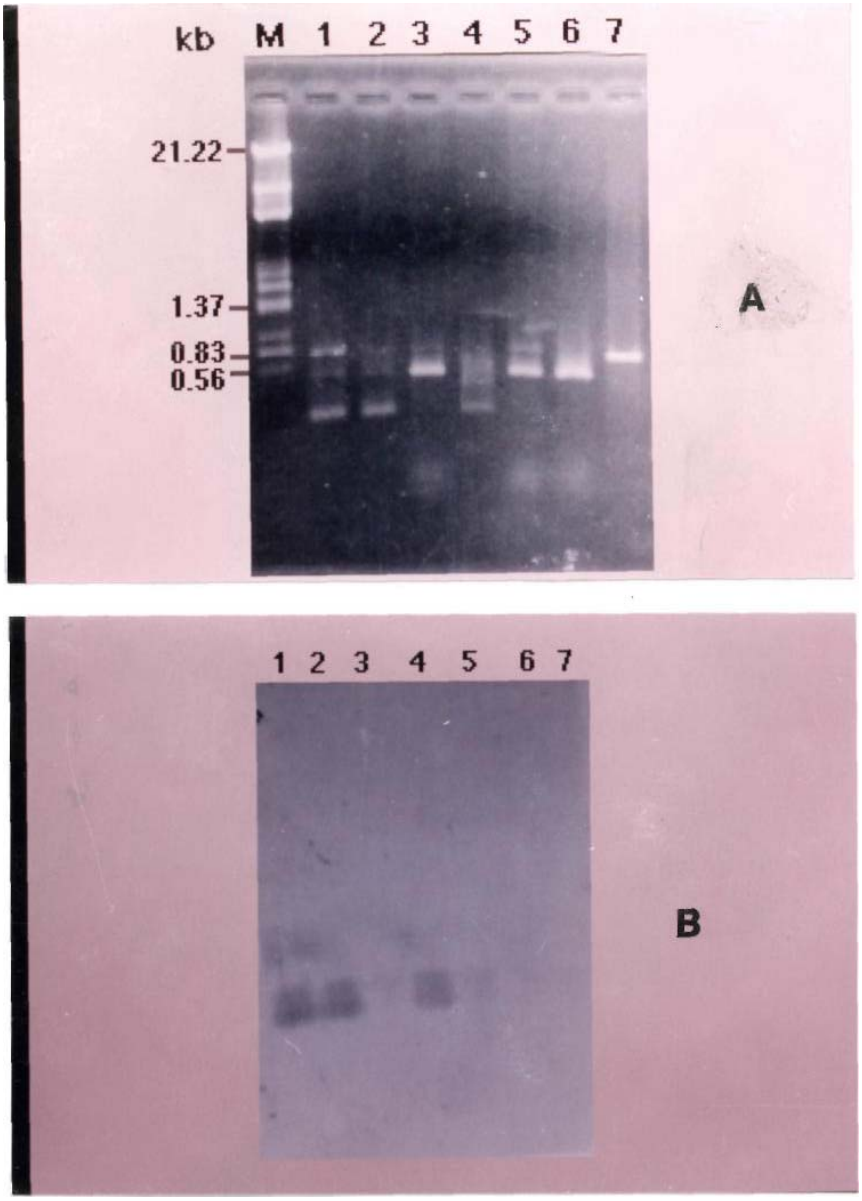


Fig. 4.14 Electrophoretic and Southern blot analysis of PCR amplicons obtained from different legume species with primer pair N and C1 at 55°C annealing temperature.

A. Agarose gel analysis of PCR amplicons.
M. λ Hind III / Eco R1 DNA marker 1. Horse gram 2. Lablab bean
3. Green gram 4. Black gram 5. Chick pea 6. Cowpea 7. Soybean

B. Southern blot analysis of the samples in part A probed with DIG labelled clone 7 insert.

significant homology with the amino-terminal regions of BBI proteins (Fig. 4.16). It is not clear whether such a protein is produced in plant.

The nucleotide sequence of clone 2 was not homologous to that of BBI genes, except for the primer regions. The clone 2 was sequenced with Ml3 forward primer up to 340 bp (designated as clone 2 forward) and contains the 'N' primer sequence twice once at the beginning of the 5'sequence and then at the 133rd nucleotide position. (Fig. 4.17 A). Clone 2 was sequenced upto 357 bp with the M13 reverse primer (designated as clone 2 reverse). The N primer sequence was present thrice, once at the beginning (Nucleotide 1), then at the 31st nucleotide and again at 61st nucleotide from the 5' end (Fig. 17 B). The presence of 'N' primer at both the ends of the clone 2 reveals that it was also an amplicon from the 'N' primers only, like is clone 7.

The nucleotide sequence of clone 7 and clone 2 were compared for the presence of any homology between the two clones since both of them were the products of single primer 'N'. The sequence of both the clones was analysed by comparing the sequence at nucleotide level directly as well as by diagonal homology matrix analysis using the DNA Inspector. The results indicate that both clone 2 and clone 7 contain multiple and inverse repeat sequences of the N primer. The sequence analysis also showed that the 132 nucleotide of clone 7 was identical with that of clone 2 (Fig. 4.18). The rest of clone 2 was different from clone 7. The presence of 132 nucleotide sequence shows their common origin in the horse gram genomic DNA. These clones could be pseudo-genes or heavily modified BBI genes with multiple inversions, insertions and duplications.

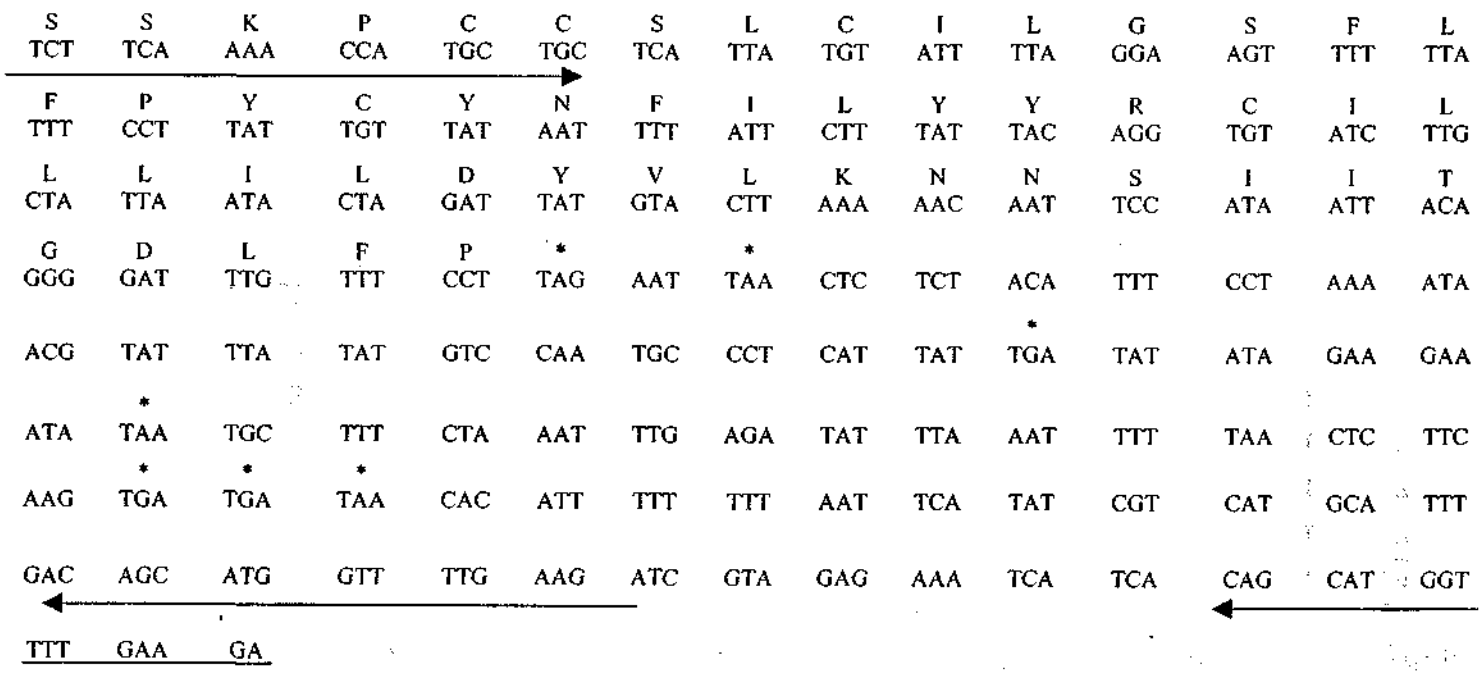


Fig. 4.15 Nucleotide and predicted amino acid sequence of horse gram BBI amplicon of Clone 7. The primer sequences are underlined. Termination codons are indicated by *. Several stop codons and inversions are present in the sequence.

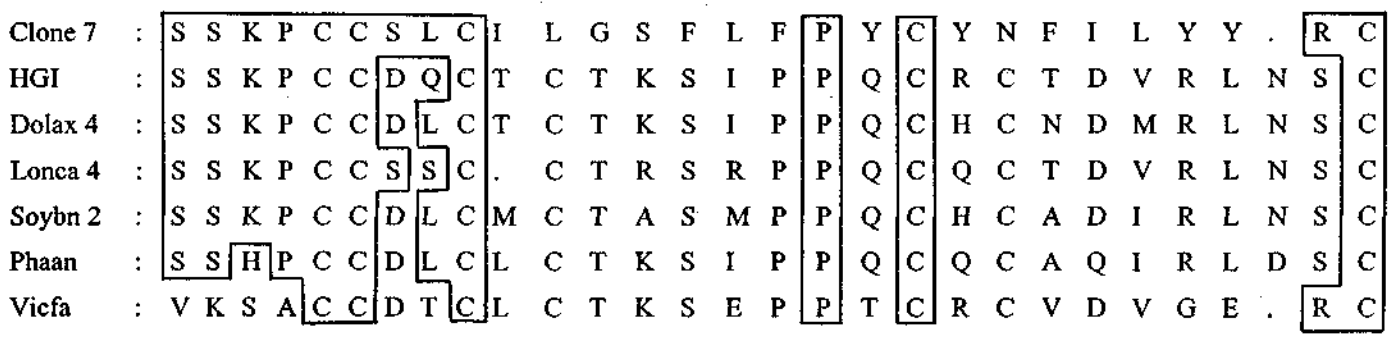


Fig. 4.16 Alignment of predicted amino acid sequence of horse gram BBI amplicon (Clone 7) with the N-terminal domains of Bowman–Birk inhibitor of some legumes. Homologous amino acids are boxed. HGI = Horse gram BBI, Dolax 4 = *Macrotyloma axillaris (DE–4)*, Lonca 4 = *Lonchocarpus capassa*, Soybn 2 = Soybean, Phaan = *Phaseolus angularis*, Vicfa = *Vicia faba*. The amino acid sequence and protein nomenclature were taken from Prakash *et al.*, 1996.

A single gap has been created in clone 7 sequence to improve the alignment.

TCT TCA AAA CCA TGC TGT CAA ATG CAT GAC GAT ATG AAT TAA AAA
AAT GTG TTA TCA TCA CTT GAA GAG TTA AAA ATT TAA ATA TCT CAA
ATT TAG AAA GCA TTA TAT CTC TTC TAT ATA TCA ATA ATG AGG TCT
TCA AAA CCA TGC TGC ATC TTA CCC TTG GCA TAG TGT CAG CTT TAC
TAC AAG TAC TGG TGG AGG GTA TTG ACA AAT CCT CTC CCA AGG NTT
CGC TTA TTA GCT TGG AGC TGA TCT NAG ATA GAA TTT TTT AGG CAN
CNG AAG TGT TTG GGT ACT GAC TTG AGA GCC NCG GGG ATN TTA GGG
GGT AGN AGA ACT TAG GAC CCC CTA CTT G

Fig. 4.17A Nucleotide sequence of BBI amplicon (clone 2) obtained with M13 forward primer. The sequence of the 'N' primer used in PCR is underlined

TCT TCA AAA CCA TGC TGA TTT CTC TAC GAA TCT TCA AAA CCA
TGC TGA TTT CTC TAC GAA TCT TCA AAA CCA TGC TGG TAA TAA
TTT TAC CAA AAG AAT TTG TTA TTG TTA TAT AAA GTC ACA TAT
TTA AAA AAA TGT TTN NGT GAT TTA TAA TAA CTT TTT ACG GCA
TGG AAT AGT AAT ACA GTT CCT TAA TAA ATC TAA AAT TAG GTT
ATA ATG AGT TGG NCC ATT GAA GGT AGA GTG ATT TTA CTA GTG
CAA AAT TTG GAT TTN TCT CAA TGA TGC TAC TGC TAG TTA TAA
TTT CCG NGA ATC TTT NCC TTT NNG GGT TTT AGG TGG ATT TTA
AAA AGT GGN ATC TTG AAA CTG

Fig. 4.17B Nucleotide sequence of horse gram BBI amplicon (clone 2) obtained with M13 reverse primer. The 'N' primer sequence used in PCR is underlined.

```
Clone 7:  TCT TCA AAA CCA TGC TGT GAT GAT TTC TCT ACG ATC TTC AAA
Clone 2:
Clone 7:  TCT TCA AAA CCA TGC TGT CAA ATG CAT GAC GAT ATG AAT TAA AAA
          ||| ||| ||||||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
Clone 2:  TCT TCA AAA CCA TGC TGT CAA ATG CAT GAC GAT ATG AAT TAA AAA
Clone 7:  AAT GTG TTA TCA TCA CTT GAA GAG TTA AAA ATT TAA ATA TCT CAA
          ||| ||| ||||||| ||| ||||||| ||| ||| ||| ||| ||| ||| |||
Clone 2:  AAT GTG TTA TCA TCA CTT GAA GAG TTA AAA ATT TAA ATA TCT CAA
Clone 7:  ATT TAG AAA GCA TTA TAT CTC TTC TAT ATA TCA ATA ATG AGG CCA
          ||| ||| ||| ||||||| ||| ||||||| ||| ||| ||| ||| ||| ||| .|.
Clone 2:  ATT TAG AAA GCA TTA TAT CTC TTC TAT ATA TCA ATA ATG AGG TCT
Clone 7:  TTG GAC ATA
          |. . .|. ..|
Clone 2:  TCA AAA CCA
```

Fig. 4.18 Alignment of nucleotide sequence of horse gram BBI amplicon of clone 7 (inverse sequence shown in Fig. 4.15) and clone 2 (Sequence obtained with M 13 forward primer shown in Fig. 4.17A). The vertical lines indicate sequence identity and the dots indicate mismatches.

4.2.4 **PCR** amplification **and** cloning of horse gram **BBI** DNA using degenerate primers

PCR with heterologous non-degenerate primers produced single primer amplicons with sequences unrepresentative active BBIs. On the availability of a complete horse gram BBI protein sequence during the course of this study (Prakash *et al,* 1996), five new degenerate primers based on the horse gram BBI protein sequence were designed and used for the amplification of BBI gene segments (section 3.13). Since the earlier experiments had shown single primer amplicons, this time both single as well as double primer combinations was used. A representative gel showing the use of second batch of primers is shown in Fig. 4.19.

The use of the five single primers, RTV-2, RTV-R1 and RTV-R2 resulted in amplification while the use of RTV-1 and CI by themselves resulted in no amplification (Fig. 4.19, lanes 6-10). The amplicons of primer pair RTV-1/RTV-R1 were similar to those obtained with RTV-R1 alone (c.f. lanes 1 and 8) and amplicons with primer pair RTV-1/RTV-R2 were similar to those of RTV-R2 alone (c.f. lanes 2 and 9). Similarly, primer pair RTV-2/RTV-R1 amplicons obtained represented a sum of the individual amplicons of primers RTV-2 and RTV-R1 (c.f. lanes 3, 7 and 8). No new bands were amplified with the combination of the two primers. The use of primer pair RTV-2/RTV-R2 resulted in the formation of heavy primer-dimers and the preferential amplification of some bands (lane 4).

The expected size of the horse gram BBI amplicons with the range of primers used here should be between 195-228 bp considering the lack of introns in the BBI genes (Hammond *et al,* 1984). Although the size of some of the amplicons observed were according to the expectation, all were produced even when single primers were used, similar to the results obtained with non-degenerate soybean primers shown previously (section 4.2.3). Another primer, RTV-3 did not amplify genomic DNA when used alone. In combination with RTV-R1 and RTV-R2 no new bands apart from those produced by RTV-Rl and RTV-R2 when used singly were detected (data not shown).

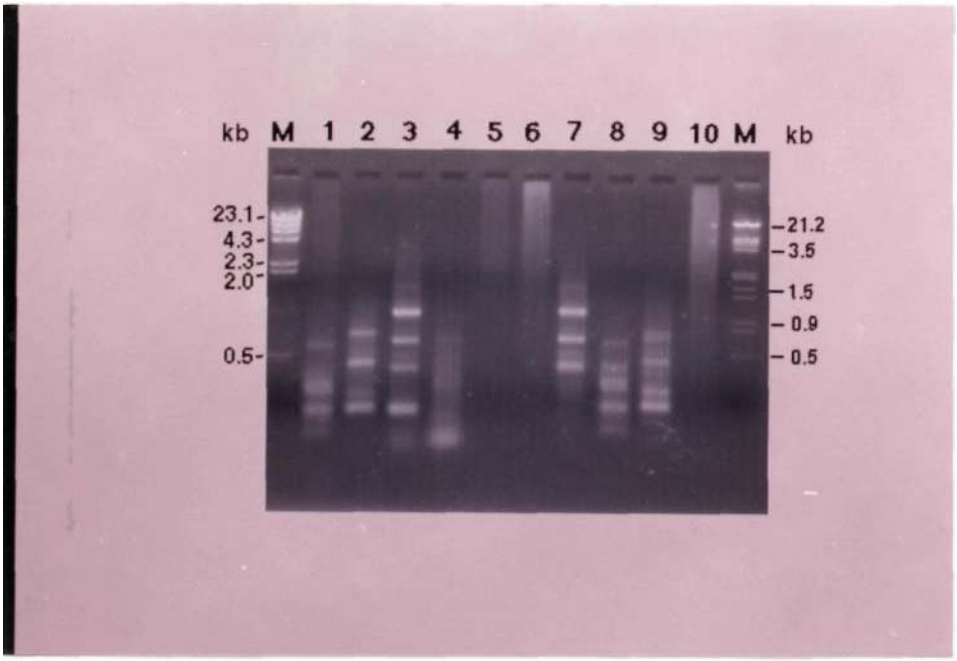


Fig. 4.19 Agarose gel (2%) analysis of horse gram BBI amplicons amplified with different degenerate primers.

M. λ Hind III DNA marker 1. RTV–1 and RTV–R1 2. RTV–1 and RTV–R2 3. RTV–2 and RTV–R1 4. RTV–2 and RTV–R2 5. RTV–1 and CI 6. RTV–1 only 7. RTV-2 only 8. RTV–RI only 9.RTV–R2 only 10. C1 only

M. λ Hind III/Eco R1 DNA markers.

The amplification of multiple DNA fragments and the absence of specific products expected from the combination of forward and reverse primers indicates the amplification of repetitive DNA fragments. Then target sequence must have been duplicated and there must be inversions as noticed with the non-degenerate soybean primers. However, the size of these amplicons obtained with the degenerate primers based on horse gram sequence was different from that of clone 7 and clone 2 described in the previous section. Also due to their degenerate nature it was expected that some of these may be more closely related to the horse gram BBI genes. Hence, amplicons obtained with one of the primers namely RTV-2 was subcloned and sequenced.

The PCR amplification using primer RTV-2 alone was carried out at the higher annealing temperature of 60°C to eliminate any nonspecific amplification and the products were analyzed in a 1% agarose gel (Fig. 4.20). Three bands of size of 457, 720 and 1080 bp similar to that at 55°C annealing temperature were obtained (Fig 4.19). The pooled PCR products were purified and cloned into the PCR II-TOPO vector as described in section 3.20. Out of many recombinants, five clones (numbered pRTV-2-1 to 5) were randomly selected, and the inserts released with Eco RI (Fig. 4.21).

The clones pRTV-2-1, pRTV-2-4 and pRTV-2-5 released an insert of 720 bp in size corresponding to the middle sized amplicon (Fig. 4.20). The clone, pRTV-2-2 had a insert of size 1080 bp representing the larger size amplicon. The smaller size amplicon was not present in these clones. The remaining clone pRTV-2-3 appeared to be a plasmid dimer (Fig. 4.21, lane 3). The larger sized clone pRTV-2-2 was sequenced from one side using the T-7 promoter primer. A sequence of 445 nucleotide was obtained in this way is shown in Fig. 4.22. The nucleotide sequence of this clone was compared with published sequence in the data bank of non-redundant Genbank + EMBL + DDBJ + PDB sequence according to Altschul *et al.* (1997) through an internet search . No homology with the BBI sequence as well to any plant sequence was noticed except for the presence of the RTV-2 primer sequence at one end. Hence the clone represents a unique DNA sequence from the horse gram genome. Manual sequence comparisons with the clone 2 and clone 7 described in section 4.2.3 above showed no similarity with these clones. A short open frame of 53 amino acid at the 5' end of the sequence was observed.

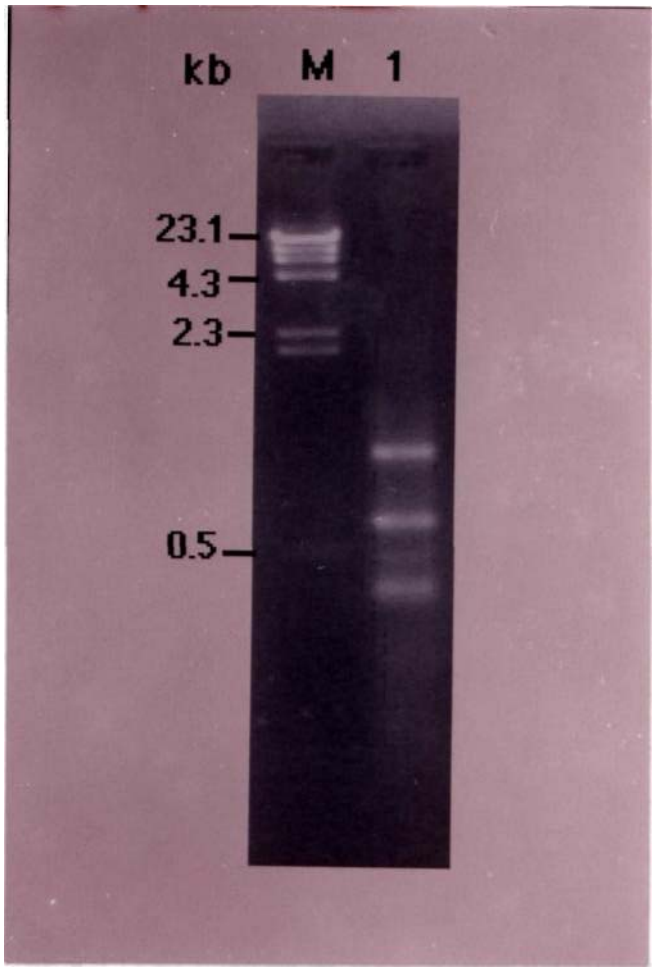


Fig. 4.20 Agarose gel (1%) analysis of horse gram BBI amplified with single primer RTV–2 at 60°C annealing temperature.
M. λ Hind III DNA marker 1. PCR amplicons

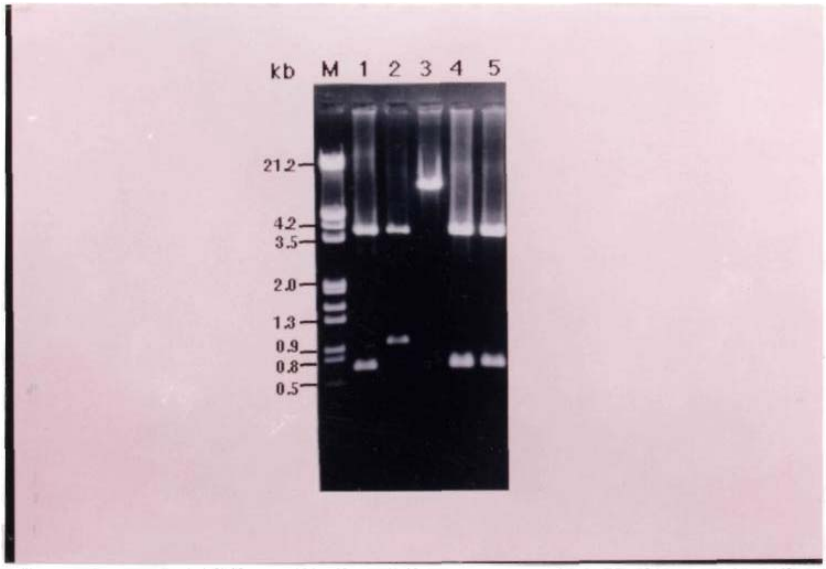


Fig. 4.21 Agarose gel (1%) analysis of horse gram BBI clones obtained with primer RTV–2 (See Fig. 4.20) and cloned in PCR II TOPO vector. The purified plasmid DNA was digested with EcoRI.

M. λ Hind III / EcoRI DNA marker 1. pRTV–2–1
2. pRTV–2–2 3. pRTV–2–3
4. pRTV–2–4 5. pRTV–2–5

CAG TCG ACG GAT GAG CCG CCA CAT CTA GTT AAG AGA AGA AGA GTT TTA
GCG CTT TTT CAA GTG AAT GCG ACA CCG CCA CGT AGA CAT GAA CTT TAC
TTT AGA AAG GCG ATA ACT TTG CTG TCT TCA CGC TCG GAC ATT GTC GCC
AAG TTA ACG CTT CTT TGT CGG CAT TGA ACA TGA CTT GTT GAT ATC CGC
ATG TTC TCG CCT GAC TAT TGC AGT TGA GAA AAA TAT TGT AAA CCA TCG
CGG CAA AGA CGC CAA GCG ACG CTT GGG CGC GCG CGC TAT ACT GAC ATC
TTG GCG ATA GCG AGG CGA CAA CTT TCC GGC ACT AGC GCC GTA GCG TTA
CCT CTC TAA CAA AAA ATC TTT CNA GAG GAG TCA ATC ATA ATT TAT AAA
TGG ACG AGA TAA TTT CAT ATC GCC GAG TTG TCG CCA AGT CCG CGC TGA
AAC ATC NNA TTT T

Fig. 4.22 Nucleotide sequence of horse gram BBI amplicon (clone pRTV–2–2).
The primer sequence used in PCR. is underlined.

After the failure of these homologous degenerate primers to amplify specific horse gram BBI genes, four more degenerate primers (2 forward and 2 reverse) were designed with increased primer length of 23-26 nucleotides for increased specificity (section 3.13) and were used for amplification. The earlier degenerate primers were kept shorter (17-20) to keep the number of degenerate combinations to a minimum. Once again the primers were tried individually and in combination at an annealing temperature of 55°C to 60°C and analysed on 2% agarose gel (Fig 4.23 A,B)

The expected amplicon size of horse gram BBI with primer sets BBI-F2 / BBI-R2, BBI-F2/ BBI-Rl, BBI-F1 / BBI-R2 and BBI-F1 / BBI-Rl was 228, 210 186 and 168 bp respectively. It has to be noted here that at 55°C annealing temperature, the primers BBI-F1, BBI-Rl and BBI-R2, each gave multiple amplicons on their own (Fig.4.23A, lanes 6-8). In addition, all the primer pairs gave multiple amplicons and bye and large the patterns were sum of bands obtained with corresponding single primers (lanes 1-4). However, there was one additional band of the size less than 200 bp in BBI-F2 / BBI-Rl and there was increase in the intensity of the additional band of- 200 bp with the primer pairs BBI-F2 / BBI-Rl and BBI-F2 / BBI-R2 (lanes 3 and 4). To check for the consistency of these additional bands, the annealing temperature was increased to 60°C (Fig. 4.23B). At 60°C annealing temperature, only the BBI-Rl gave multiple bands on it's own, while the amplicons of primer pairs BBI-Fl / BBI-Rl and BBI-F2 / BBI-Rl were similar to the amplicons of BBI-Rl alone, except for an additional band observed with BBI-F2 / BBI-Rl primer pair. Since it's size was similar to the expected size, this band was cut out from the gel and reamplified with individual primers and primer pair BBI-F2 / BBI-Rl at 55°C annealing temperature (Fig. 4.23 C).

The results obtained show that ~ 200 bp amplicon was amplified with the primer pair BBI-F2 / BBI-Rl but also with the primer BBI-Rl alone. However the intensity of the amplicon was much higher when the primer pair BBI-F2 / BBI-Rl was used than when BBI-Rl was used alone. This is consistent with the results in Fig. 23A, lanes 3 and 6. This suggests that there may be an additional overlapping band resulting from the primer pair similar in size, but perhaps different in internal sequence. However, this can only be confirmed after cloning and sequencing of these amplicons.

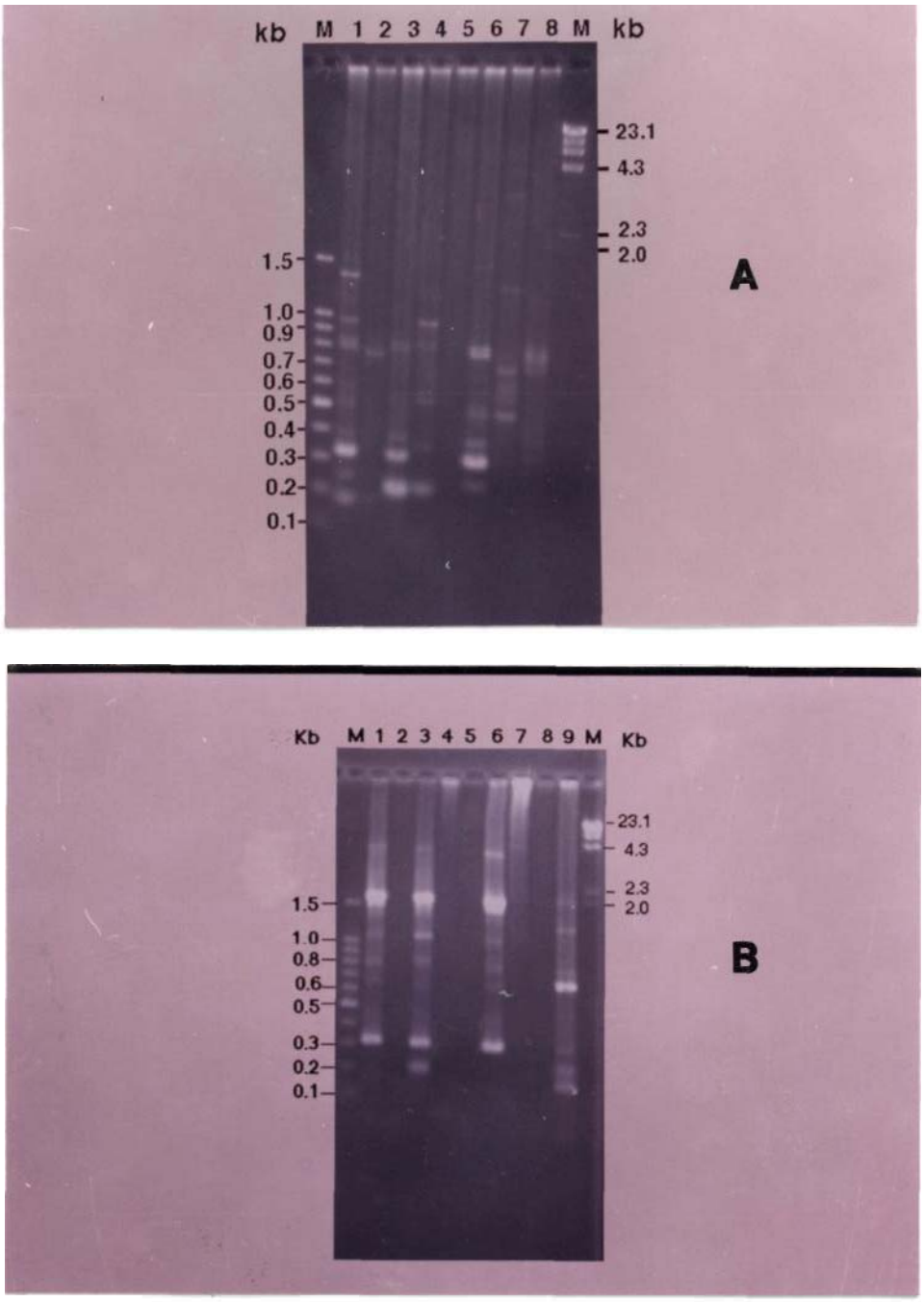


Fig. 4.23 Agarose gel (2%) analysis of horse gram BBI amplicons amplified with degenerate primers.

A. BBI PCR amplicons obtained at 55°C annealing temperature.

M. 100 bp DNA ladder

1. BBI–F1 and BBI–RI 2. BBI–FI and BBI–R2

3. BBI F2 and BBI–RI 4. BBI–F2 and BBI-R2 5. BBI–FI only

6. BBI–RI only 7. BBI–F2 only 8.BBI–R2 only

M. λ Hind III DNA marker.

B. Horse gram BBI PCR amplicons obtained at 60°C annealing temperature.

M. 100 bp DNA ladder, lanes 1–8 are identical to part A.

Lane 9. Soybean amplicons obtained with primer pair BBI–FI and BBI-RI

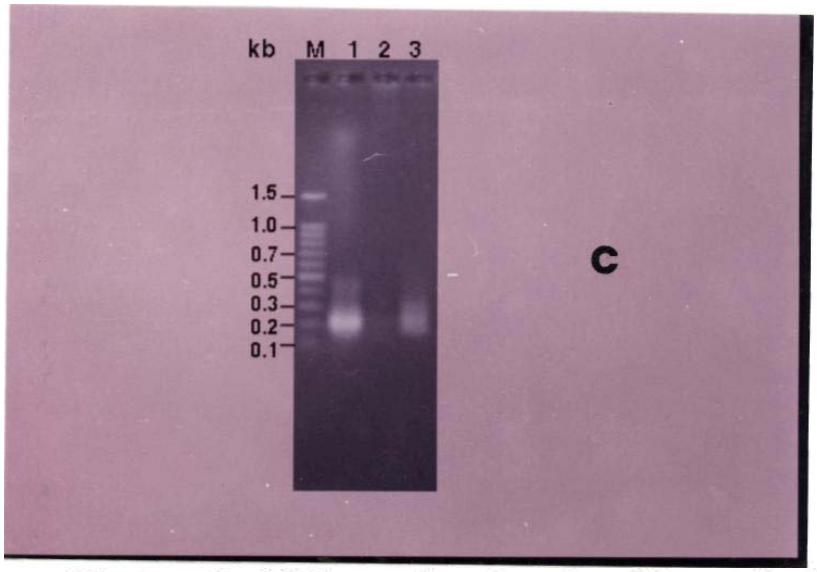


Fig.4.23C. Reamplification of ~ 200 bp amplicon from lane 3 in part B with the same primers.
M. 100 bp DNA ladder 1. BBI–F2 and BBI–RI 2. BBI–F2 only
3. BBI–RI only.

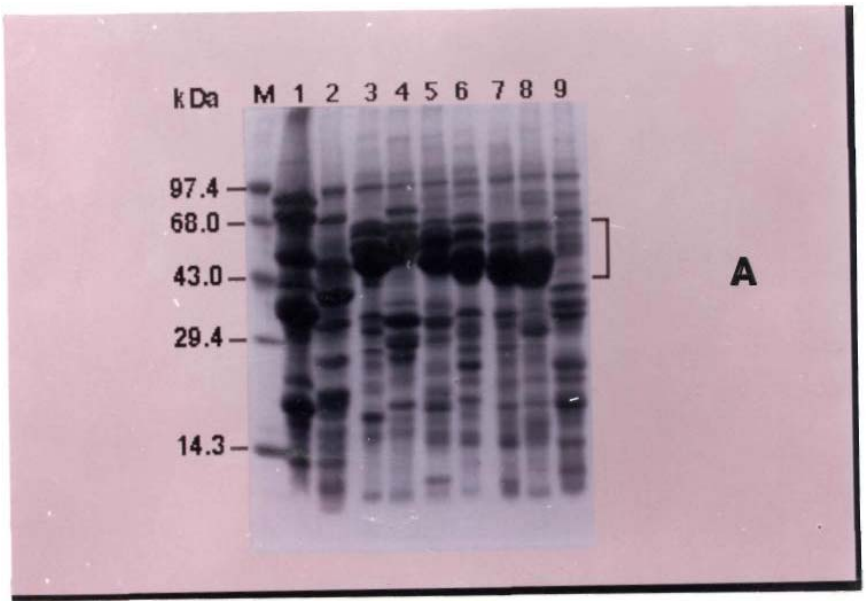
4.3 Vicilin-like proteins

The vicilin-like proteins of legumes are 7S globulins which are typically trimeric in nature with glycosylated subunit size 50-75 kDa in size. However, in some species eg. pea, smaller size vicilin polypeptides have also been reported to be generated by post -translational cleavage of larger vicilin precursor molecules (Shewry, 1995). Vicilins are highly polymorphic in nature. The presence of these proteins has been reported from a large number of legumes (Danielsson, 1949; Debryshire *et al.,* 1976). The well known 7S globulins are vicilins of pea, conglycinins of soybean and phaseolins of beans. The 7S globulins predominate in seeds of pea, French bean and winged bean (Derbyshire *et al.,* 1976; Utsumi, 1992).

4.3.1 Vicilin-like proteins in horse gram and related legumes

The study of vicilins in horse gram and eight other legume species commonly cultivated in India was made by fractionating their total seed proteins by SDS-PAGE under reducing conditions, staining one half of the gel with Coomassie brilliant blue and electroblotting the other half onto nitrocellulose membrane prior to probing with cowpea vicilin antibodies (Fig. 4.24AB). The SDS-PAGE of total seed proteins revealed that the predominant proteins of cowpea, horse gram, lablab bean, green gram, black gram and French bean are present in the size range of 43 to 68 kDa (indicated in Fig. 4.24A, lanes 3-8). The proteins of soybean, pea and chick pea are distributed over a wider size range with no clustering of bands (Fig. 4.24A, lanes 1, 2 and 9).

The immunoblot revealed that the proteins of soybean, pea and chick pea reacted weakly with the vicilin antibodies while that of cowpea, horse gram, lablab bean, green gram, black gram and French bean legumes reacted strongly in the 43-68 kDa size range (Fig. 4.24B, lanes 3 to 8). The antibodies also reacted prominently with proteins of above 100 kDa, as well as smaller size proteins in these legumes. Thus, there were cross reacting protein bands in all the high, medium and low sized range as indicated by brackets in Fig. 4.24B. In soybean, the antibodies did not react strongly with the proteins in the 53 kDa region but reacted with the 33, 17 and 12.5 kDa proteins. In pea, a band of 19 kDa reacted strongly while some other bands weak in reaction in chick pea, proteins of 35, 32 and 16 kDa reacted strongly but also showed other weakly reacting bands. (Fig.4.24B, lanes 2 and 9



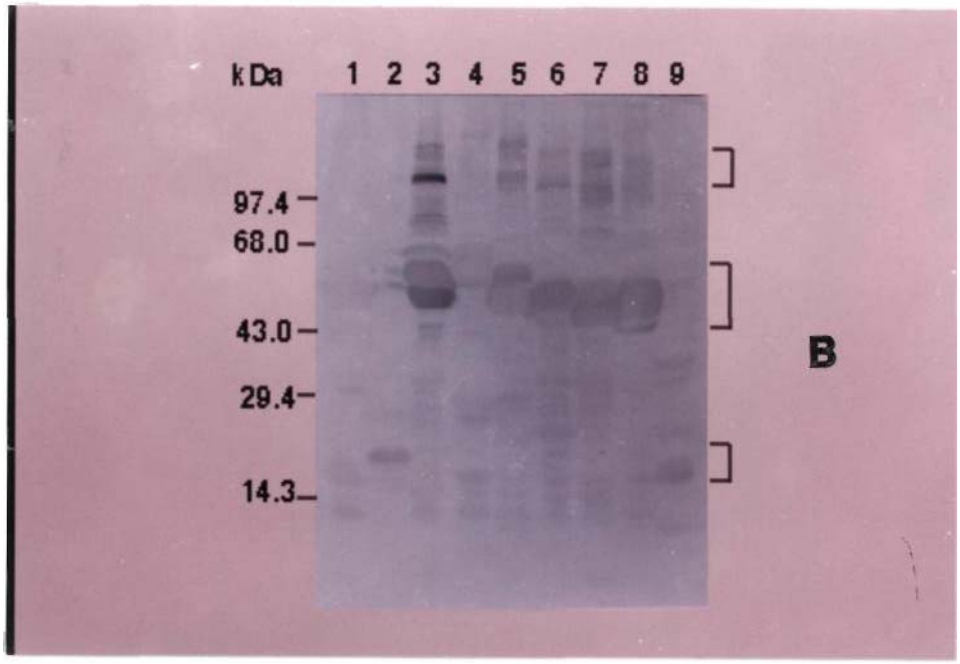


Fig. 4.24 Electrophoretic analysis of total seed proteins and immunodetection of vicilin–like proteins in seeds of horse gram and related legumes.

A. SDS-PAGE of total seed proteins in 12% polyacrylamide gel.

M. Protein size markers.

1. Soybean 2. Pea 3. Cowpea 4. Horse gram
5. Lablab bean 6. Green gram 7. Black gram 8. French bean
9. Chick pea.

Bracket indicates the predominant seed storage proteins.

B. Reaction of the above with cowpea vicilin polyclonal antibodies.

Bracket indicates three major regions of cross-reaction with vicilin antibodies.

respectively). The cowpea proteins against which these antibodies were produced reacted strongly as expected. The strongest reacting bands were of 117, 60 and 51 kDa size. In horse gram these antibodies reacted with the protein bands of 161, 118 (higher size), 56-61 (predominant bands) and 27, 26,16, and 13 kDa (Lower size).

The above electrophoretic and immunoblotting studies shows that the predominant proteins of horse gram, cowpea, lablab bean, green gram, black gram and French bean in the size range of 43-68 kDa and these are vicilin-like proteins. This is in agreement with earlier reports that the vicilins are the predominant proteins in French bean (Bollini and Chrispeels, 1978), green gram (Ericson, 1975) and cowpea (Sefa - Dedeh and Stanley, 1979 a).

The reaction of antibodies with a protein of > 100 kDa in the legumes are likely to be due to aggregation of the 43-60 kDa vicilin polypeptides, as there are no reports in the literature on such a larger sized vicilin-like proteins. But the presence of smaller size (< 43 kDa) vicilin-like proteins have been reported earlier and these are the resultant products of post-translational cleavage of the original precursor molecules (Ericson, 1975; libuchi and Imahori, 1978; Hall *etal,* 1977)

However, the reported size of most of the vicilin precursor proteins is around 50 kDa and they are glycosylated and cleaved into smaller fragments after translation (Gatehouse *et al,* 1984, Newbigin *et al,* 1990; Carasco *et al,* 1978; Sakakibara *et al,* 1979). The differential glycosylation of precursor molecules as well as the cleaved smaller polypeptides also results in sub-unit heterogeneity (Shewry, 1995). However, it is clear in horse gram that the concentration of the smaller sized vicilins are much less than those larger in size.

The poor interaction of the cowpea vicilin antibodies with the vicilins of soybean, pea and chick pea could be due to the difference in antigenic sites on the variable regions of vicilin proteins (Wright, 1987). The earlier report on the presence of immunologically differing conglycinins in their number and patterns has also supports the presence of such immunologically distinct vicilins in different legumes (Catsimpoolas, 1970).

4.3.2 Presence of variability for vicilin-like proteins among horse gram genotypes

The extent of variability for vicilin-like proteins was analysed in sixteen horse gram genotypes using the immunoblotting approach. The total seed proteins were fractionated under reduced conditions, one half of the gel was stained in Coomassie brilliant blue, while

the remaining identical half was probed with the cowpea vicilin antibodies (Fig. 4.25AB). No significant protein polymorphism was observed in the SDS-PAGE patterns of these sixteen genotypes even though the selected genotypes were agronomically quite diverse (Fig. 4.25A). All these genotypes showed predominant protein bands in the 52-66 kDa region. The immunoblot shows that, the anti-vicilin antibodies reacted with these major proteins in all the sixteen genotypes and there was no inter-varietal polymorphism (Fig. 4.25B). As discussed earlier, the vicilin is the major predominant protein in horse gram with at least two subunits of size 57 and 59 kDa. The absence of variability for vicilin in horse gram is contrary to the reported extensive variability observed in cowpea (Leonard *et al.,* 1993). This could be due to the diverse material consisting of wild accessions as well as cultivars used in their study.

4.3.3 Studies on horse gram vicilins during seed development and germination

One of the main features of the seed storage proteins is that they are synthesized specifically during seed development and are subsequently hydrolyzed during germination of seed. Their accumulation pattern is differential during seed development (Millerd *et al,* 1978; Higgins, 1984).

Total seed proteins of horse gram extracted from the developing seeds at different intervals after flowering were analysed by SDS-PAGE and then probed with anti-vicilin antibodies (Fig. 4.26AB). At 5 DAF, there were several minor bands but there was no specific accumulation of major storage proteins as observed in the mature seeds (Fig.4.26A). At 10 DAF, the pattern was quite similar to 5 DAF except for the emergence of few discrete bands in the size of 29 and 56 kDa range. As seed development progressed additional protein bands accumulated till seed maturity. The accumulation pattern of vicilin-like protein may clearly be seen in the accompanying immunoblot (Fig. 4.26B). Vicilins appeared at 10 DAF with antibodies reacting strongly with a protein of 57 kDa and weakly with a 55 kDa protein. At 15 DAF, the antibodies detected an additional vicilin of 59 kDa. There was a dramatic increase in the amount of vicilin between 15 and 20 DAF where the reacting bands merged into one another making it difficult to distinguish more than two sub-units. There was no significant change in the accumulation of vicilins from 20 DAF till maturity except that the presumed aggregates of vicilins were clearly visible at maturity (Fig. 4.26B).

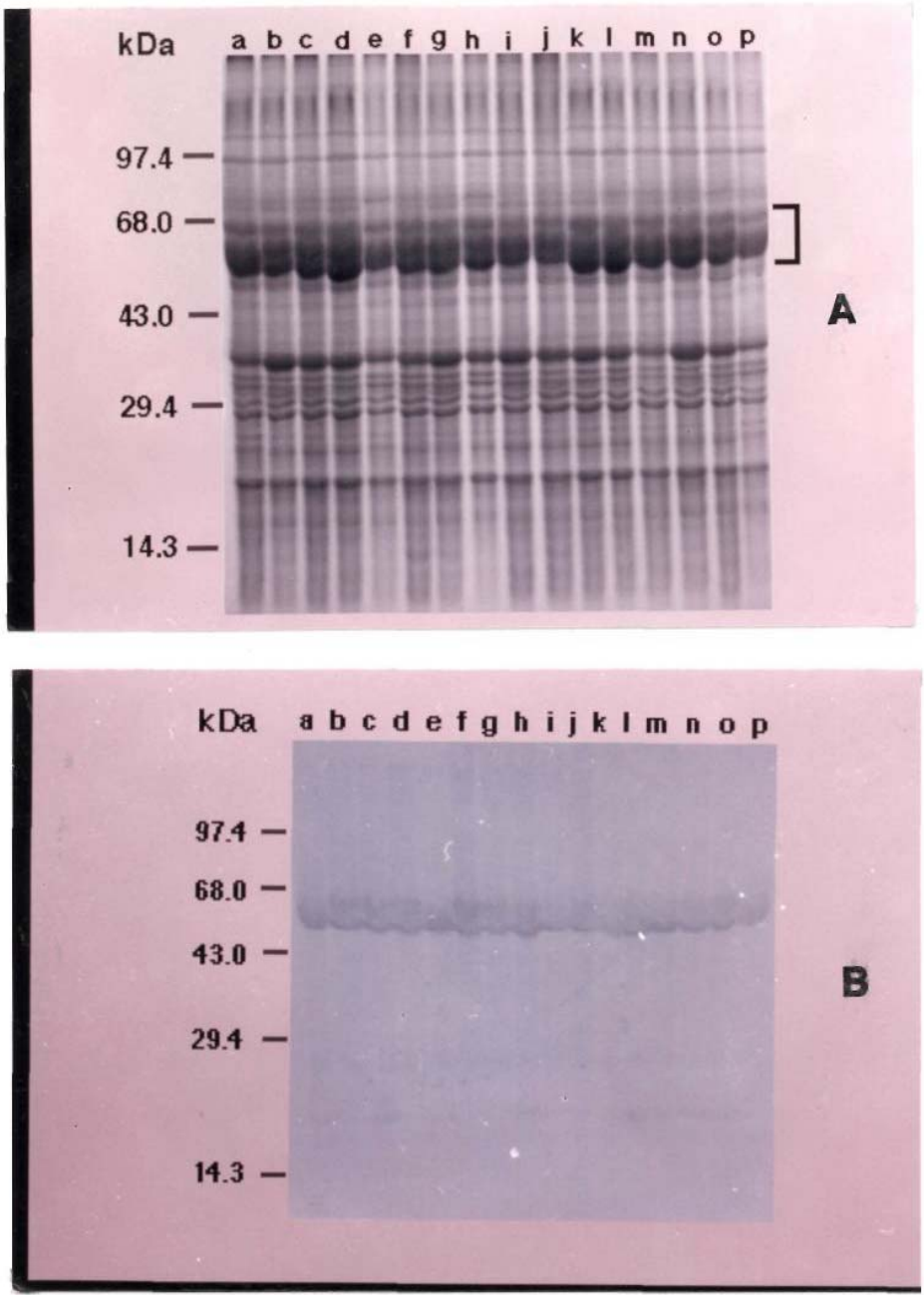


Fig. 4.25 Immunoblot analysis for vicilin–like proteins in 16 different horse gram genotypes.

A. SDS–PAGE of total seed proteins in 12% polyacrylamide gel.

a. No. 47	b. A– 4–9–5	c. D–6–3–4
d. Macintosh	e. R –III 90 A–1–10–3	
f. No. 2	g. S–35	h. No. 85
i. BGM –1	j. D–2–2–2	k. IC11095
l. PHG–9	m. K–42	n. C–7–5–5
o. C–6–3–4	p. R-III 31–49	

The bracket indicates the predominant vicilin–like proteins of horse gram seed.

B. Reaction of the above with cowpea vicilin polyclonal antibodies.

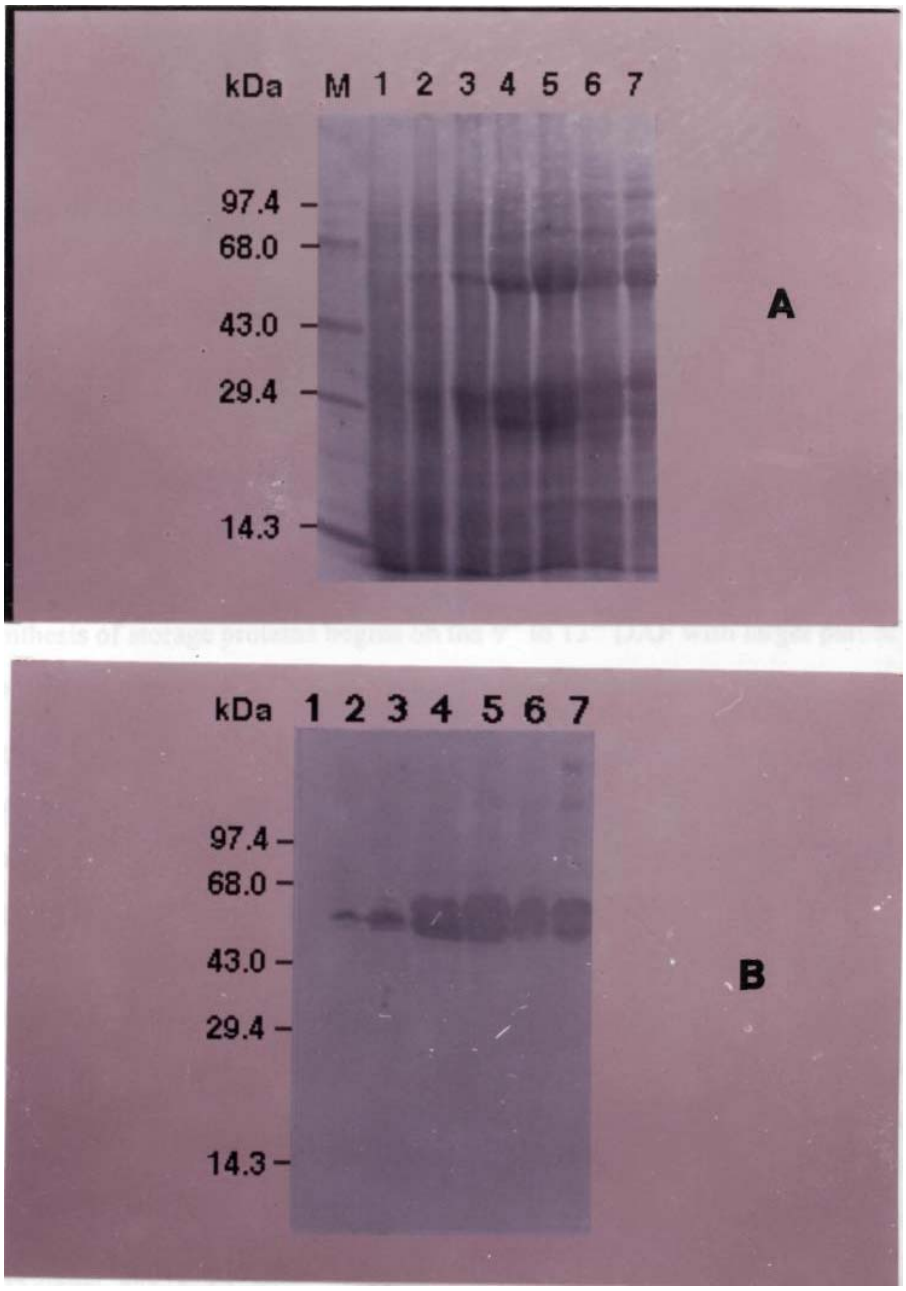


Fig. 4. 26 Accumulation of vicilin–like proteins during horse gram seed development.

A. SDS–PAGE of total seed proteins in 12% polyacrylamide gel.

M. Protein size markers 1. 5 Days after flowering (DAF)
2. 10 DAF 3. 15 DAF 4. 20 DAF
5. 25 DAF 6. 30 DAF 7. Mature seeds.

B. Reaction of the above with cowpea vicilin polyclonal antibodies.

It is clear from SDS-PAGE that the accumulation of seed proteins of different sizes occurred at different stages of horse gram seed development. The immunoblot shows that the major storage protein of horse gram are the three vicilins, 55, 57 and 59 kDa in size. The 57 kDa vicilin appeared early in development while the 59 kDa vicilin accumulated in larger amount only after 20 DAF. The differential accumulation of the horse gram vicilin is similar to that of soybean β-conglycinins where the α and α'sub-units are synthesized earlier than is the β-sub-unit (Meinke *et ai,* 1981; Naito *et ai,* 1988). In faba bean also differential expression of the vicilin sub-units have been reported (Wright and Boulter, 1982). The presence of atleast three subunits of vicilin in horse gram is'similar to the reports in many legumes including the closely related *Dolichos lablab* (Derbyshire *et ai,* 1976). The appearance of vicilins in horse gram is delayed in comparision with that in pea where these proteins appeared as early as 4 days after fertilization (Domoney *et ai,* 1980) and were continuously deposited throughout seed development in small amounts (Boulter, 1984). Usually, the synthesis of storage proteins begins on the 9^{th} to 12^{th} DAF with larger part of the accumulation occurring between 15^{th} and 30^{th} DAF (Mosse and Pernollet, 1982).

The pattern of vicilin degradation during seed germination was also analysed using similar electrophoretic and immunoblotting approaches. Proteins of mature seeds were considered to be from seeds 0 days after imbibition (DAI). The SDS-PAGE of total proteins of germinating seeds revealed that even though some degradation starts at 1 DAI, marked differences were visible only after 2 DAI (Fig.4.27A). At 2 DAI, the amount of the 55-59 kDa vicilins as well as other proteins of low molecular weight particularly that of an 18 kDa band declined rapidly with the concomitant appearance of a 23 kDa protein. At 3 DAI, most of the proteins above 30 kDa in size disappeared or decreased in the intensity. A streak of proteins below 18 kDa position was observed and maintained thereafter. The decline in the intensity of 55-59 kDa vicilins continued till 6 DAI, proteins almost disappearing by 7 DAI. Most of the proteins above 30 kDa disappeared by 7 DAI. The immunoblot results specifically show the degradation pattern of 55-59 kDa vicilin as observed with SDS-PAGE. The 23 kDa protein nor the streak observed below the 18 kDa band reacted with the vicilin antibody (Fig. 4.27B).

These results show that there is a differential degradation of proteins during germination of horse gram seeds where the higher molecular weight proteins are degraded

more rapidly than are proteins of size < 30 kDa. Increased degradation of high molecular weight protein was accompanied with concomitant increase in the amount of a 23 kDa protein band. The 23 kDa protein could well be the degradation product of larger sized proteins. The streak observed below the 18 kDa size proteins at 3 DAI onwards could be due to the smaller and unresolved polypeptides released by hydrolysis of larger proteins. In French bean and peanut peptides smaller in size than the vicilin sub-units are the intermediate breakdown products of vicilin during germination (Bollini and Chrispeels, 1978; Basha and Cherry, 1978). However, the immunoblot results in the present study do not show any intermediate breakdown products of the vicilin. This is contrary to the earlier report in soybean where the β-conglycinin sub-unit α and α' were rapidly degraded generating new β-conglycinin cross reactive intermediate forms (Wilson *et al.,* 1986). They also reported the appearance of at least six polypeptides ranging from 33.4 to 24 kDa in size which the apparent degradation products of β-conglycinin. Kawai *et al.* (1997) also showed limited proteolysis of β-conglycinin at exposed regions of the protein generating 30 kDa single domain fragments before their non-specific proteolysis. The failure of the antibodies to react with intermediates in. the present study could be due to damage of the antigenie epitopes on the intermediate products during hydrolysis. An alternate explanation could be that in horse gram there is a general proteolysis of vicilins instead of the specific proteolysis as reported in soybean. The complete degradation of vicilin at 8 DAI is similar to that reported in soybean (Wilson *et al.,* 1986).

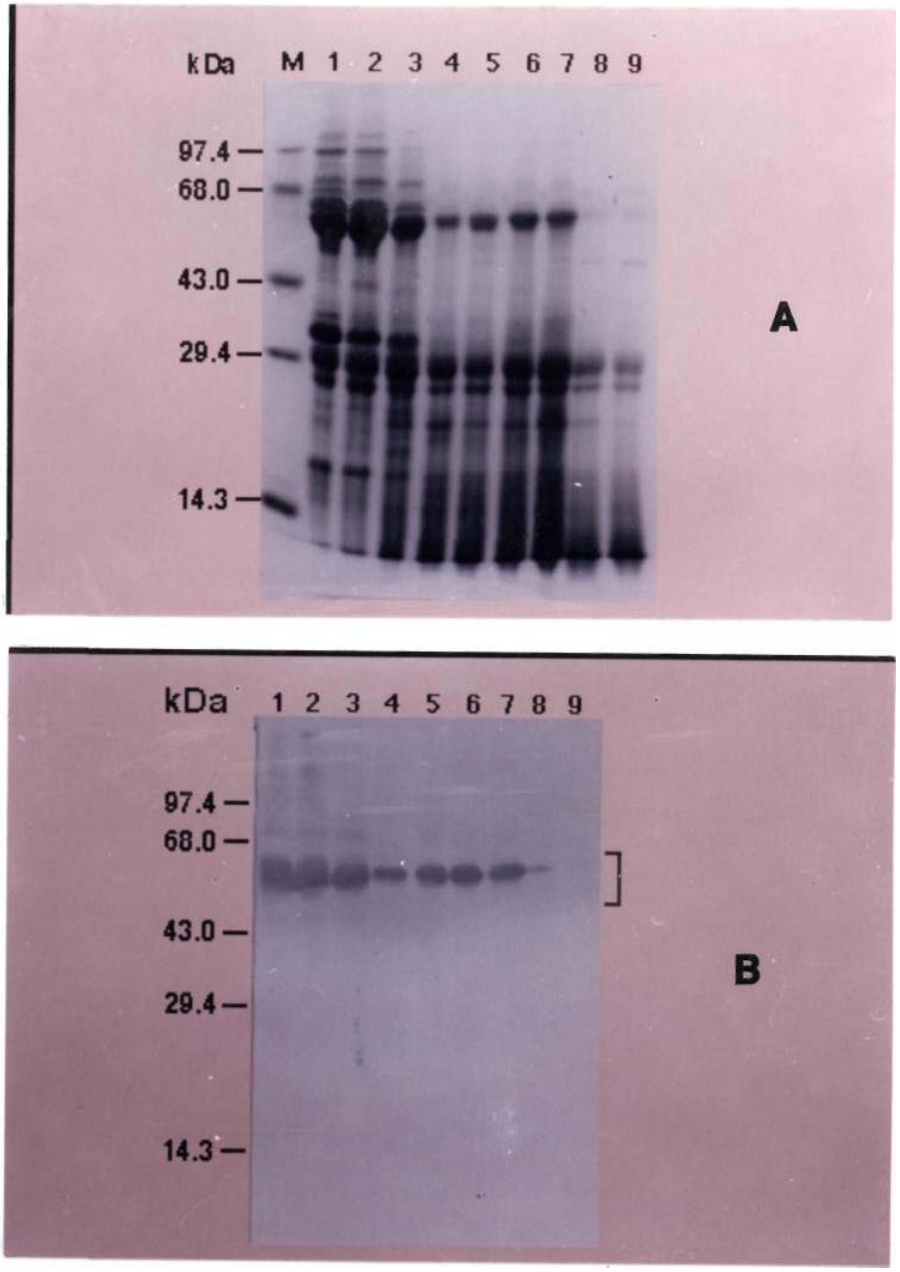


Fig. 4.27 Degradation of vicilin–like proteins during the germination of horse gram seeds.

A. SDS–PAGE of total seed proteins in 12% polyacrylamide gel.

M. Protein size markers. 1. Mature seeds

2. 1Day after imbibition (DAI) 3. 2 DAI 4. 3 DAI

5. 4 DAI 6. 5 DAI 7. 6 DAI

8. 7 DAI 9. 8 DAI

B. Reaction of the above with cowpea polyclonal antibodies.

Bracket indicates the specific reaction of vicilin–like proteins with cow pea vicilin antibodies.

4.4 Legumin-like proteins of horse gram

The legumin-like proteins are one of the most widespread class of storage proteins of dicots. Their presence has also been reported in monocots , including cereals as well as gymnosperms (Brinegaar and Peterson, 1978 ; Jensen and Berthold, 1989 : Singh *et at,* 1993). Each sub-unit pair has a large acidic and small basic polypeptide linked by single disulfide bond (Nielsen, 1984). The sub-unit pair is synthesized as a precursor protein and proteolytically cleaved after disulfide bond formation (Jung *et al,* 1997). Although there is a considerable variation in the size of the individual acidic and basic sub-units, they have a typical size of 40 and 20 kDa, respectively and these are commonly found in seeds (Shewry, 1995).

4.4.1 Immunological studies on legumin-like proteins in horse gram and related legumes with pea legumin antibodies

The presence of legumin-like proteins were studied in horse gram and eight other legume species using the immunoblotting approach. First, the total seed proteins were fractionated by SDS-PAGE in 12% polyacrylamide gel under reducing conditions. One half of the gel was stained with Coomassie brilliant blue and the remaining half of the identical gel was electroblotted onto nitrocellulose membrane and probed with pea legumin polyclonal antibodies.

The SDS-PAGE of total seed proteins of different legumes revealed that except for soybean, pea and chickpea , the predominant proteins are present in the size range of 43-68 kDa while in soybean, pea and chick pea, they are distributed over a wider size range (Fig. 4.28A). These predominant proteins of horse gram and related species are actually vicilin-like proteins as described in the previous section. Pea legumin antibodies reacted with all the legumes tested and strongly with pea and chick pea, the reaction patterns in pea and chick pea being almost similar (Fig. 4.28B). In pea, the proteins of size 68, 41, 28 and 22 kDa reacted strongly and proteins of 90 and 72 kDa also reacted but weakly with the antibodies. There was a faint reaction with 98 kDa proteins from all the legumes except with that from soybean and pea (Fig. 4.28B). Similarly there was a 16 kDa protein reacting in all these legumes, except for French bean (lane 8). In the horse gram, proteins of 49, 31, 28 and 24 kDa reacted in addition to those common bands (lane 3). In soybean, proteins of 56, 32 and 20 kDa, in

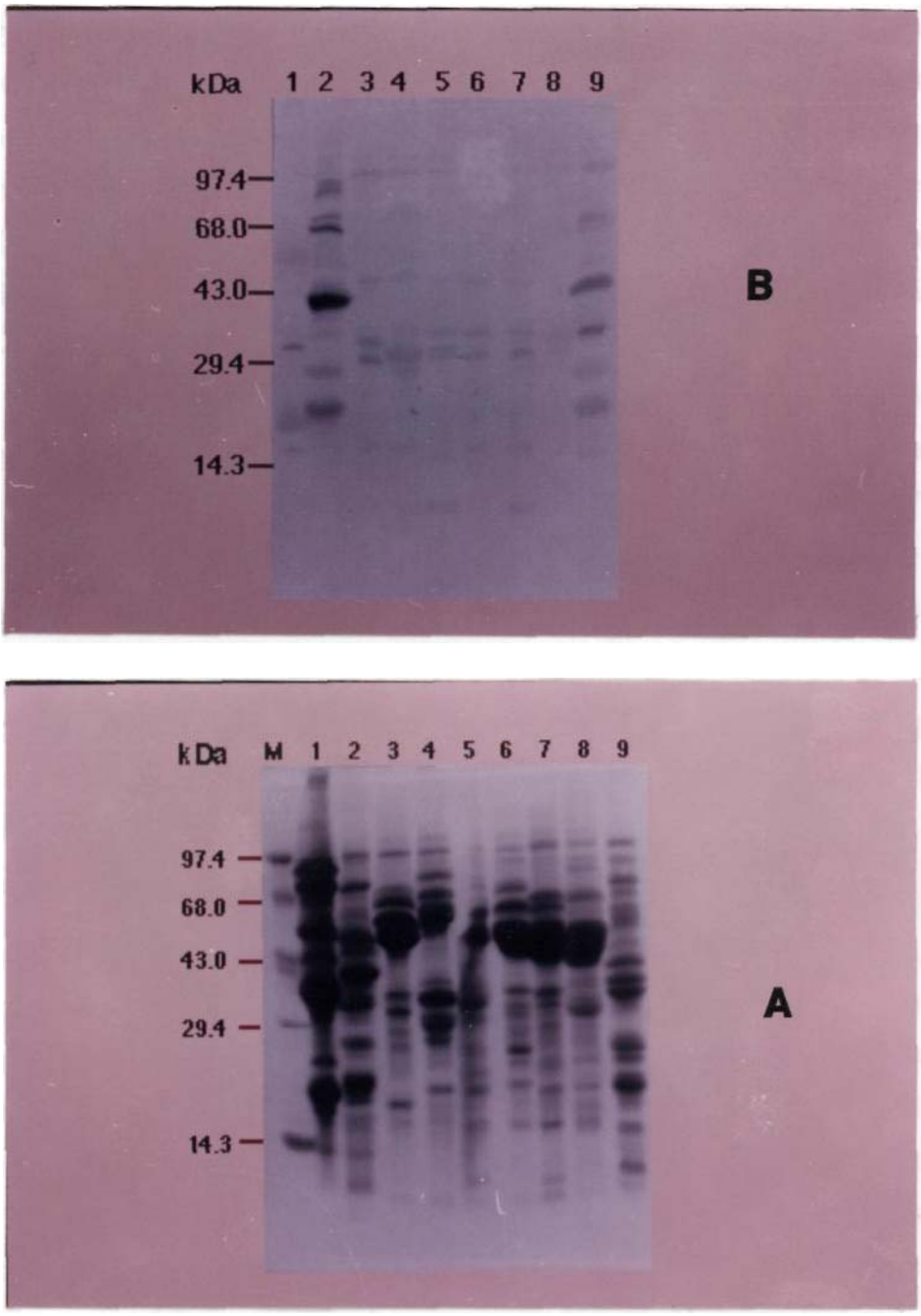


Fig. 4.28 Electrophoresis and immunoblot analysis for legumin-like proteins in the seeds of horse gram and related legumes.

A. SDS-PAGE of total seed proteins in 12% polyacrylamide gel.

M. Protein size markers 1. Soybean 2. Pea
3. Cowpea 4. Horse gram 5. Lablab bean
6. Green gram 7. Black gram 8. French bean
9. Chick pea

B. Reaction of the above with pea legumin polyclonal antibodies.

chick pea proteins of 72, 46, 36, 29 and 22 kDa, in cowpea proteins of 47, 33, 30, 23 kDa, in lablab beans proteins of 47, 35, 32, 31 and 10 kDa reacted. In green gram and black gram, proteins of size 47, 35, 31, 28, and 25 kDa reacted. In addition, a 10 kDa protein of black gram and cowpea reacted with the antibody. French bean showed the poorest reaction with only two bands of 34 and 31 kDa reacting weakly.

Gel analysis of total seed proteins of the nine legumes tested showed that, the predominant proteins of these legumes are present in the size range of 43 to 68 kDa except for soybean, pea and chickpea. The immunoblot revealed that, the legumin antibodies reacted intensely with a number of proteins from pea and cowpea. The intense reaction with 41 and 21 kDa proteins shows that they are the typical large acidic and small basic legumin polypeptides reported in literature (Shewry, 1995). The similar pattern observed for pea and chick pea proteins further confirms the earlier report that chick pea globulins behave like those of pea in terms of their amino acid composition, N-terminal amino acid sequence, peptide and tryptic maps (Jackson *et al,* 1969). The study also reveals the presence of larger sized polypeptides reacting with the antibody ; 51 kDa in soybean, 49 kDa in horse gram, 47 kDa in lablab bean, cowpea, green gram and black gram and 46 kDa protein in chick pea. These could be the variant forms of larger acidic polypeptides which have been reported in faba bean (Matta *et al,* 1981b) and peanut (Krishna and Mitra, 1987). There were small polypeptides in the 28-36 kDa range in all the legumes. Some of these could be the variants of acidic sub-units, as this size class of acidic sub-units have already been reported in pea (Matta *et al,* 1981a), soybean (Momma *et al,* 1985a) and faba bean (Matta *et al,* 1981b). The 16 kDa protein reacting in all the legumes except pea and French bean could be the variant forms of legumin. More work needs to be carried out to confirm the nature of this protein. The proteins of 19.5 kDa in soybean and 22 kDa proteins of pea and chick pea reacted strongly with the antibody. This group of proteins is likely to be the normal basic sub-units of legumin-like proteins. The presence of 10 kDa break down product of the acidic sub-units has been reported in soybean (Momma *et al,* 1985a). This study shows the presence of legumin-like proteins in horse gram, although their amount is limited. The study also confirmed the presence of legumin-like proteins in French bean in a low amount as suggested earlier by Derbyshire *et al.* (1976).

4.4.2 Immunological studies on legumin-like proteins of horse gram with wheat triticin antibody

The presence of basic polypeptides of legumin-like proteins was studied in seeds of horse gram in comparison with that of soybean, chick pea and rice using the immunoblotting technique. One half of the gel was stained in Coomassie brilliant blue while the other identical half was electroblotted onto nitrocellulose membrane and probed with the wheat triticin anti 'δ' antibodies raised against the purified basic polypeptide (Singh *et al,* 1993)

The SDS-PAGE of total seed proteins shows that the amount of protein in rice is very low compared to legumes (Fig. 4.29A). Here equal volume of protein extracted from 50 mg of seed meal of all the grains were taken. The immunoblot showed that the triticin antibodies, reacted strongly and specifically with the 21 kDa protein of soybean and chick pea and 23 kDa protein of rice, as expected. It has even reacted with a 29 kDa protein in horse gram, through the band is barely visible on the photograph (Fig 4.29B). This antibody did not react with any other proteins in these four species, thus highlighting the specificity of these antibodies. The 16 kDa protein reacting with the pea legumin antibodies did not react with this protein indicating that it may not be a basic sub-unit (Section 4.4.1).

Even though the protein content of rice is 2-3 times lesser than that of the legumes, as reflected in the gel, rice protein reacted with the antibody strongly as did than those of soybean and chick pea. This is because rice is much more closely related to wheat than are the legumes. The size of the reacting polypeptides of soybean and rice is as reported earlier (Okita *et al.,* 1989 ; Shewry, 1995). But the reaction of the 29 kDa protein in horse gram indicates that these have unusually larger sized basic legumin polypeptide. This could be a novel variant form of the normal 20-23 kDa basic polypeptides. These results show that the wheat triticin antibodies reacts not only with the basic polypeptides of other monocots but also with that of legumes, the antibody is very specific to the basic polypeptide not interacting with any other polypeptide.

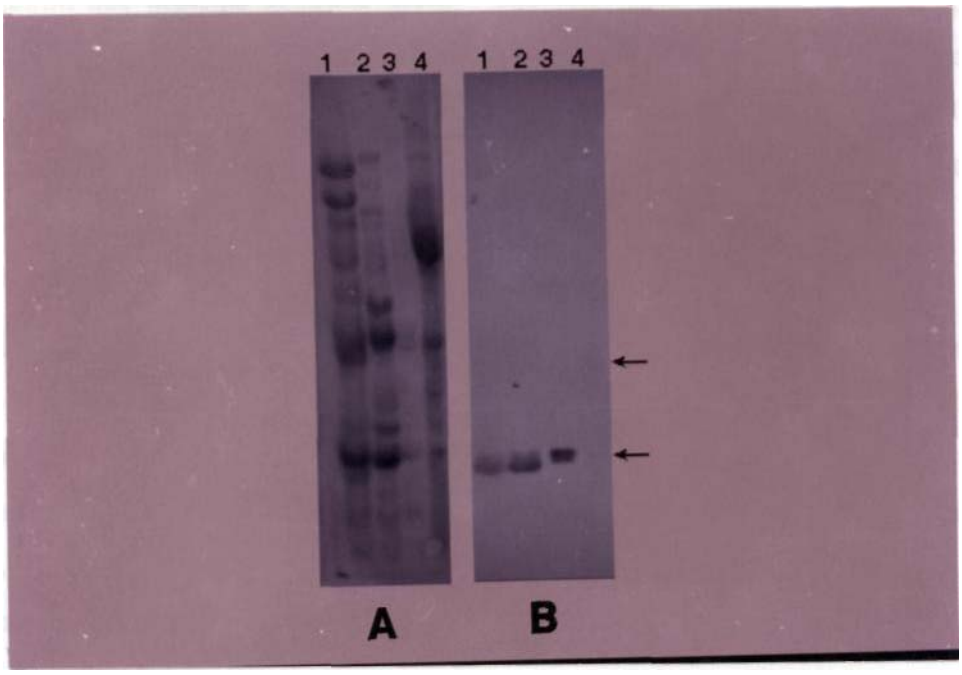


Fig. 4.29 Immunoblot analysis for the presence of small basic polypeptides of legumin-like proteins in horse gram and other species.

A. SDS-PAGE of total seed proteins in 12% polyacrylamide gel.
1. Soybean 2. Chick pea 3. Rice 4. Horse gram

B. Reaction of the above with triticin anti-δ antibodies.

Arrows indicate the position of cross-reacting proteins.

4.4.3. Immunological studies of legumins during seed development and germination with wheat triticin antibody.

The pea legumin antibodies reacted to a number of bands in the horse gram while the triticin antibodies reacted with a single band of 29 kDa. The latter was used to probe the pattern of legumin-like proteins in horse gram. The total seed proteins of horse gram extracted during seed development were fractionated on 12% polyacrylamide gel under reducing conditions. One half of the gel was stained with Coomassie brilliant blue and the identical half was transferred onto nitrocellulose membrane and probed with triticin antibodies. Seed protein extracts of horse gram during germination were fractionated and probed with the triticin antibodies in a similar way.

The SDS-PAGE of total seed proteins during development shows that the maximum accumulation of protein occurred on 15-25 DAF (Fig. 4.30A). The immunoblot with triticin antibodies showed that the 29 kDa protein started appearing at 10 DAF and was pronounced at 15 DAF onwards (Fig. 4.30B). An additional protein of 35 kDa was observed at later stages (30 DAF). The reaction that observed in the 57-71 kDa region is an artifact due to 2-mercaptoethanol (Tasheva and Dessev, 1983).

The SDS-PAGE gel of germinating horse gram seeds showed that, the general degradation of proteins starts at 1st DAI, increases markedly at 2nd DAI and declines rapidly from the 3rd DAI onwards (Fig. 4.31 A). The 29 kDa band reactive with the triticin antibody did not decrease in intensity till 6 DAI, after which a slight decline in the intensity of this band occurred (Fig. 4.3 IB).

The largest accumulation of the 29 kDa protein during 15 to 25 DAF is consistent with the properties of legumin-like proteins from other legume species (Mosse and Pernollet, 1982). The degradation of acidic and basic polypeptides during seed germination occurs at different rates, with the acidic sub-units of soybean glycinin being degraded faster than the basic sub-units. These appeared even at 8 DAI (Wilson *et al,* 1986). The slow degradation of the horse gram 29 kDa legumin-like protein is concomitant with the observations on soybean glycinin basic polypeptides. However, since the size of the 29 kDa band is different from the normal range of 20-23 kDa for the legumin basic polypeptides, and has to be characterized further to investigate it's true identity and reasons for the size difference.

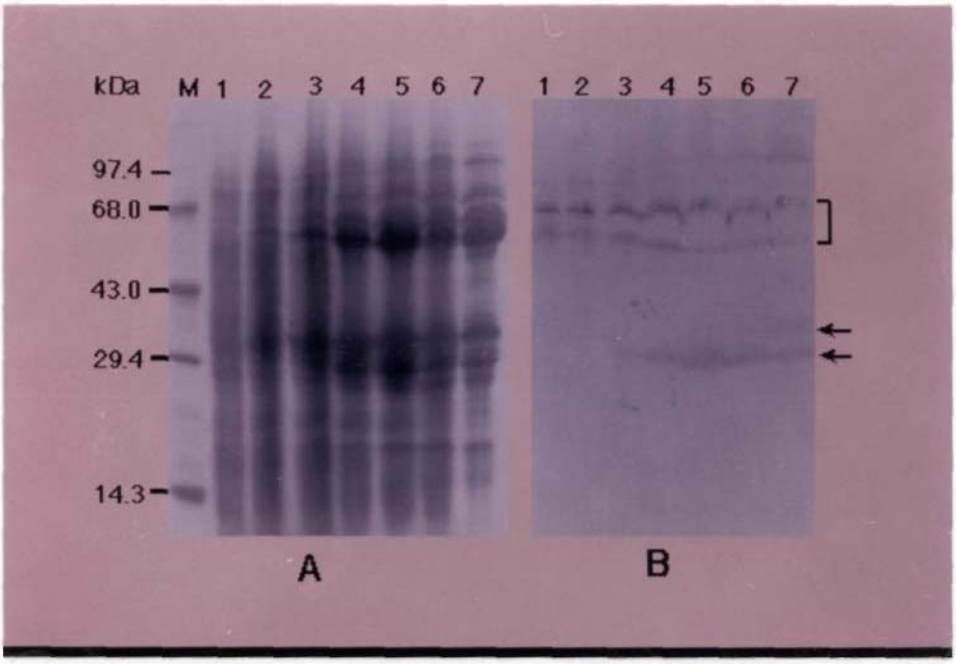


Fig. 4.30 Accumulation of legumin-like proteins during horse gram seed development.

A. SDS-PAGE of total seed proteins in 12% polyacrylamide gel.

1. 5 Days after flowering (DAF) 2. 10 DAF 3. 15 DAF
4. 20 DAF 5. 5 DAF 6. 30 DAF
7. Mature seeds

B. Reaction of the above with triticin anti-δ antibodies. The arrow indicates the increasing amount of legumin basic polypeptides during seed development.

Bracket indicates the artifacts due to 2-mercaptoethanol effect (Tashev and Dessev, 1983).

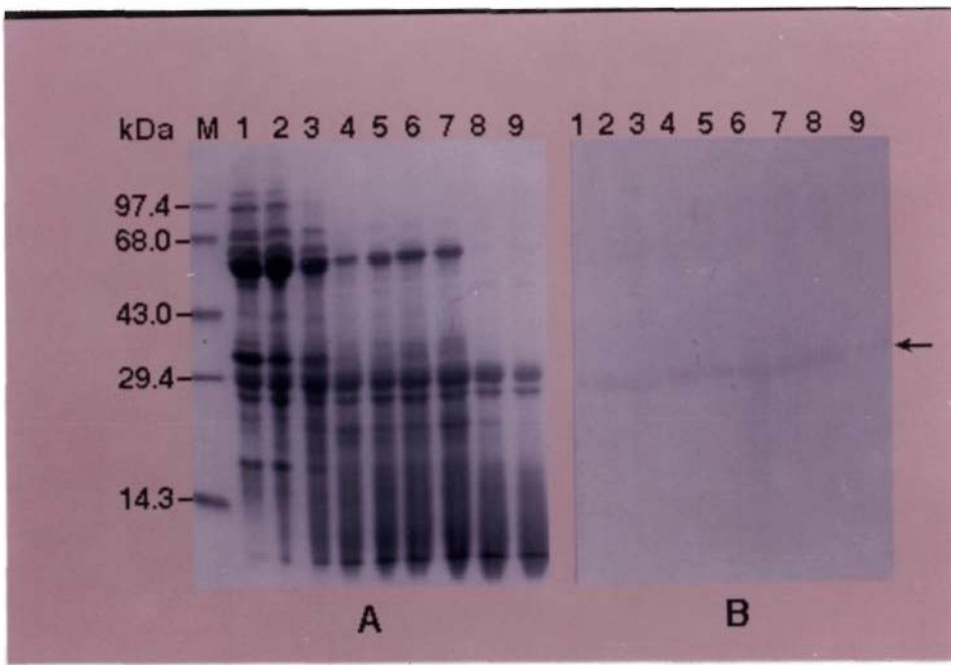


Fig. 4.31 Degradation of basic polypeptides of legumin-like proteins during horse gram seed germination .

A. SDS-PAGE of total seed proteins in 12% polyacrylamide gel.

M. Protein size marker 1. Mature seeds

2. 1 Day after imbibition (DAI) 3. 2DAI 4. 3DAI

5. 4 DAI 6. 5DAI 7. 6DAI

8. DAI 9. 8DAI

B. Reaction of the above with triticin anti-δ antibodies. Arrows indicate the cross reaction of antibodies with a 29 kDa protein.

4.4.4 Tissue print-immunoblot analysis of vicilin and legumin proteins of horse gram seeds.

The vicilin and legumin proteins of horse gram, green gram and chick pea were studied for their distribution in the seed by tissue print-immunoblot technique. One set of tissue printed nitrocellulose filters were probed with pea legumin antibodies (Fig 4.32A) and other filter set was probed with the cow pea vicilin antibodies (Fig. 4.32B). The legumin antibodies reacted most intensely with chick pea, less so with green gram and only weakly with horse gram. The cow pea vicilin antibodies reacted more strongly with the green gram and horse gram tissue prints than chick pea (Fig. 4.32B). This confirms the earlier findings that the horse gram is rich in vicilins (Section 4.3.3) so also the green gram (Ericson, 1975). This study also shows the predominance of legumin-like proteins in chick pea. These results with chick pea are in agreement with the earlier report by Ganesh kumar and Venkataraman (1978) who have shown the predominance of legumins over vicilins in chick pea in ultracentrifugation studies. The distribution of vicilin and legumin protein is uniform rather than restricted to particular portion of these seeds.

4.5 Narbonin-like proteins

The narbonin, a 2S globulin accounts for upto 2.6% of the total globulin in the narbon bean *(Vicia narbonensis).* This protein has been studied intensively both at the protein and the DNA level. (Schlesier *et al,* 1978; Hennig *et al,* 1995 ; Nong *et al,* 1995). The presence of such protein has also been reported in *Vicia pannonica* (Schlesier *et al.,* 1993). The DNAs coding for this proteins in *Vicia* has been isolated and sequenced (Nong *et al.,* 1995). Based on the cDNA sequence, PCR primers were designed to amplify narbonin-like gene fragments from four *Vicia* sps, Jack bean and soybean. The expression of proteins has not been studied in legume species other than *Vicia narbonensis* and *V. pannonica.*

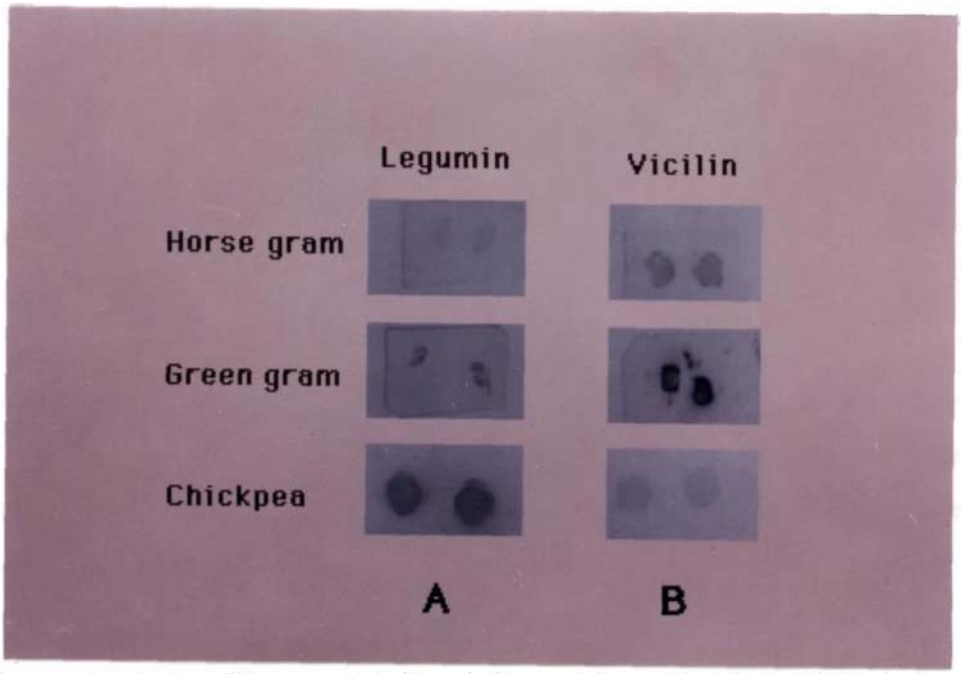


Fig. 4.32 Analysis of tissue prints for vicilin and legumin-like proteins in horse gram, green gram and chick pea with

A. Pea legumin polyclonal antibodies.

B. Cowpea vicilin polyclonal antibodies.

4.5.1 Presence of narbonin-like proteins in horse gram and other legumes

Presence of narbonin-like proteins in horse gram and seven other different legume species were studied by fractionating their total seed proteins in 12% SDS-PAGE under reducing conditions. The purified narbonin protein of narbon bean was also included in this gel as control. One half of the gel was stained in Coomassie brilliant blue (Fig.4.33 A) and the remaining identical half was electroblotted onto nitrocellulose membrane. The filter was probed with anti-narbonin polyclonal antibodies at 1:100,000 dilution (Fig. 4.33B). Out of the eight species examined in this way, three legumes viz, pigeon pea, horse gram and soybean did not show reaction significantly (Fig.4.32B, lanes 5, 7 and 8). The control protein of 33 kDa which was barely visible in the gel reacted strongly as expected in the immunoblot. In addition, reaction was observed with a 78 kDa protein (Fig.4.33B, lane 9). The chick pea showed strong reaction with three major protein of size 65, 42 and 37 kDa and the remaining four species had a single major band of variable sizes reacting with the narbonin antibodies. The size of the protein was 52 kDa in green gram, 46 kDa in black gram, 48 kDa in French bean and 52 kDa in sword bean (Fig 4.33B). In addition, there were minor bands barely visible in the photograph with a size of 29.5 and 19 kDa in green gram, 29.5 kDa in black gram, 16 kDa in chick pea and 20 kDa in French bean.

The results indicate that the narbonin-like proteins are expressed in the seeds of different legume species. However, in horse gram, pigeon pea and soybean their amount is not significant. The results with soybean are in contrast with the earlier published work where DNA fragments coding for narbonin-like proteins have been PCR amplified and cloned from soybean. This may be due to presence of pseudo-genes for narbonin in soybean or due to the failure of the narbonin antibodies to pick up their soybean counterpart. The 78 kDa protein in purified narbonin sample with which the antibodies reacted is a dimeric form of narbonin protein (personal communication by Dr. Bernhard-Schlesier). The strong interaction observed with chick pea proteins could also be due to the fact that narbon bean and chick pea are taxonomically closely related belonging to the same tribe Vicieae while all other legumes tested belonged to Phaseoleae. The sword bean used in this study is related to jack bean from which narbonin-like gene fragments have been cloned (Nong *et al,* 1995). The narbonin antibodies reacted strongly with the sword bean protein in this study.

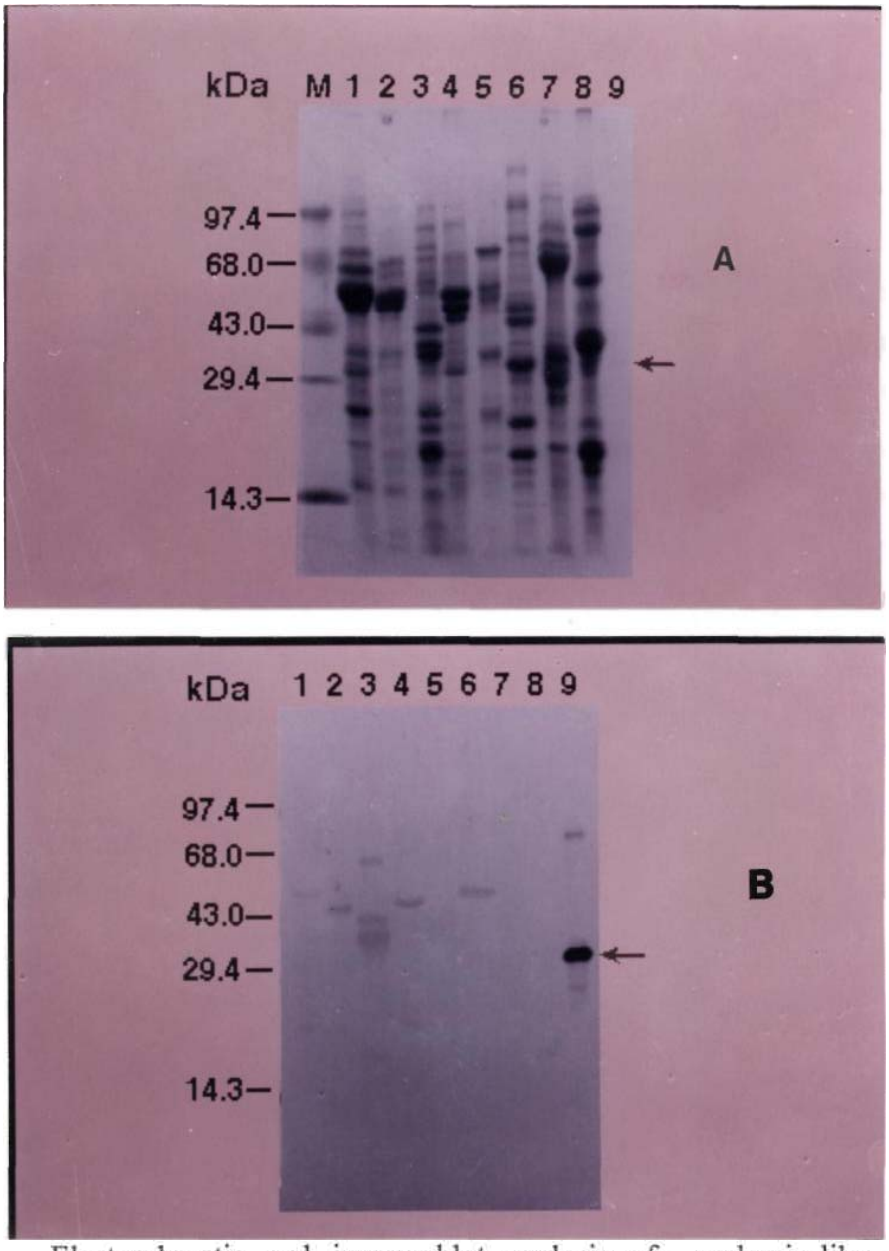


Fig. 4.33 Electrophoretic and immunoblot analysis of narbonin-like proteins in different legumes.

A. SDS-PAGE of total seed proteins in 12% polyacrylamide gel.
M. Protein size markers 1. Green gram 2. Black gram
3. Chick pea 4. French bean 5. Pigeon pea
6. Sword bean 7. Horse gram 8. Soybean
9. Purified narbonin protein of narbon bean.

B. Reaction of the above with narbonin polyclonal antibodies. Arrow indicates the strong reaction with the purified narbonin from narbon bean.

4.5.2. Cloning of PCR products from horse gram using narbonin-specific primers

The primer pair designed for the amplification of narbonin genes (NP10 and NP11, Nong *et al,* 1995) were used to amplify these genes from horse gram. The amplicons were analysed on agarose gel (Fig 4.34A). An amplicon of size 760 bp was produced. This amplicon was purified by Quiagen column and cloned in pGem T-easy vector as described in section 3.20.3. Many recombinants were selected and one of them namely, pHGN-2 was used for further analysis. The plasmid DNA was prepared for this clone, restriction digested with Eco Rl enzyme and electrophoresed in 2% agarose gel (Fig. 4.34B). To check further, the same primers were also used in six other legume species for the amplification of narbonin-like gene fragments, narbonin gene (pNAR2-15) which is similar to the clone NARF6 coding for narbonin gene was used as positive control in the PCR reactions (Nong *et al,* 1995). The analysis of PCR products on agarose gel shows that, five of the seven species including horse gram gave a single amplicon of 760 bp. No amplification was observed with green gram and lima bean (Fig.4.35B). The observed size of the narbonin-like PCR amplicon from horse gram was 760 bp while that from narbonin control gene used in the experiment was 890 bp (Nong *et al,* 1995). Thus, a size difference of around 130 bp exists between the size of narbonin genes amplified from different legumes as compared to that of the control. The similarity in size of amplicons observed in our study may indicate conservation of these genes across the plant species. Sequencing of the gene will be of importance in further understanding the nature of this gene.

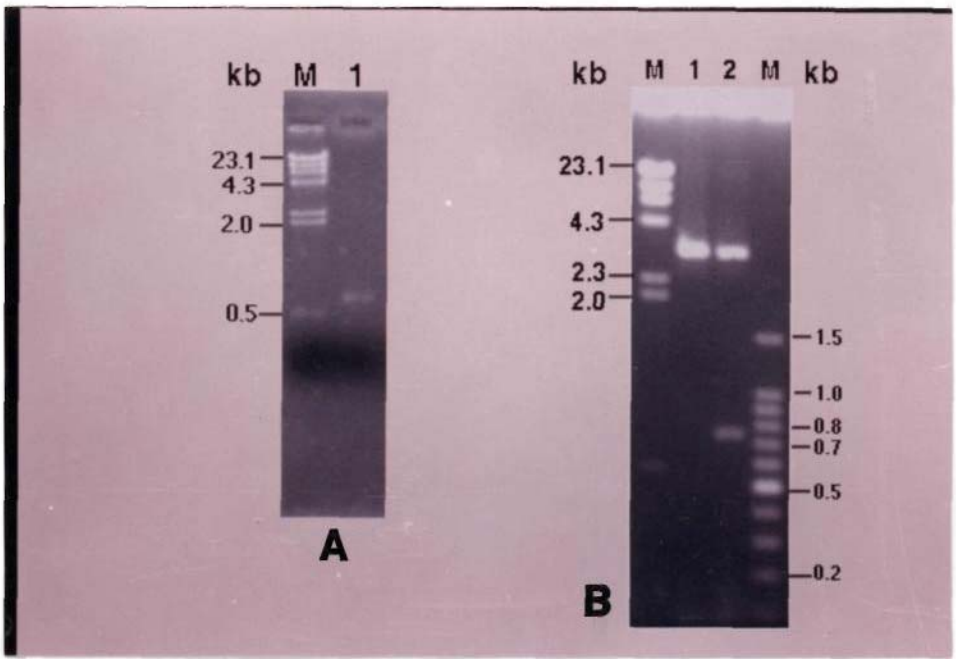


Fig. 4.34 Agarose gel analysis of narbonin-like PCR amplicons of horse gram and it's clone.

A. Horse gram PCR amplicons

M. λ Hind III DNA marker 1.PCR amplicon amplified with NP10/NP11 primers at 55°C annealing temperature.

B. Agarose gel analysis of clones amplified in part A digested with Eco R1.

M. λ-Hind III DNA marker 1.PGem T-easy vector

2. Horse gram narbonin clone (pHGN-2) M. 100 bp DNA ladder.

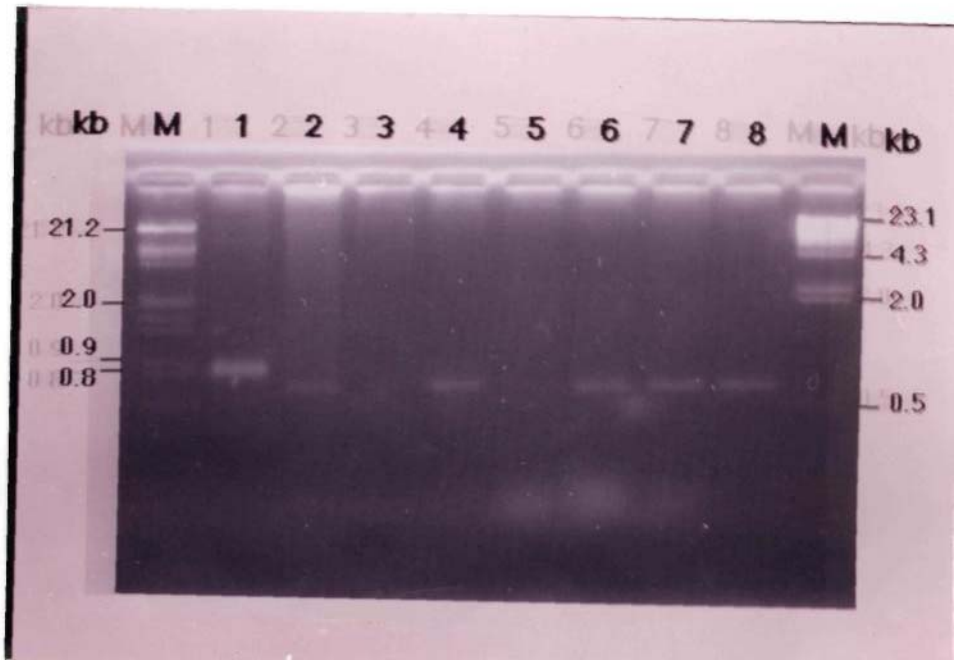


Fig. 4.35 Agarose gel analysis of narbonin-like PCR amplicons obtained from the genomic DNA of different legumes amplified with primer pair NP10 and NP11.

M. λ Hind III/EcoRI DNA markers (pNA2-15, Nong *et al.*, 1995)
1. Narbonin clone of narbon bean
2. Horse gram
3. Green gram
4. Black gram
5. Lima bean
6. Cow pea
7. Chick pea
8. Lablab bean
M. λ Hind III DNA markers

SUMMARY AND CONCLUSIONS

CHAPTER-5

SUMMARY AND CONCLUSIONS

1. Horse gram is one of the important food legumes grown extensively in the Indian peninsular region. The present work was focussed on the molecular aspects of the Bowman-Birk proteinase inhibitor and the other major seed storage proteins of horse gram seed.

2. Scanning electron microscopic studies of horse gram seeds during development, germination and at maturity suggest that the deposition of storage protein occurs in protein bodies and protein matrix. The protein bodies are spherical in shape with 2-3 μm in diameter and have smooth membrane.

3. Electrophoretic analysis of total seed proteins of sixteen horse gram genotypes did not show any significant variation except for some minor differences. The genotypes R-IΠ90A-1-10-3 and IC11095 lacked a protein band of 76 and 80 kDa, respectively, while k-42 had an additional protein of 20 kDa as compared to the remaining genotypes.

4. No significant variability was observed for trypsin inhibitor profile among the sixteen horse gram genotypes tested by activity gels.

5. The synthesis of trypsin inhibitors starts at 15 days after flowering (DAF) as two trypsin inhibitor bands. But the number of bands increases upto 6 at 21 DAF and remains constant till maturity as seen in the activity gels.

6. A differential expression of trypsin inhibitors was observed between flower parts, pod peels and seeds. The flower specific inhibitors had slower electrophoretic mobility compared to seed inhibitors. The inhibitors of flowers and pod peels had similar pattern. The inhibitors of pod peel continued to express till 25 DAF but disappeared at 30 DAF probably due to their degradation.

7. Polyclonal antibodies were raised against purified horse gram Bowman-Birk inhibitor (HGI-III) and these reacted specifically with a single band and showed a high titre. The immunoblots probed with these antibodies did not reveal any significant difference among horse gram genotypes. The reaction pattern and specificities observed in the immunoblots probed with four different anti-soybean BBI monoclonal antibodies were similar to that of the polyclonal antibodies which reacted with the monomer size BBIs of

horse gram, but they also reacted with higher size proteins in soybean and lablab bean, probably the dimeric form of BBIs.

8. Two non-degenerate DNA primers N and Cl were synthesized based on the soybean BBI DNA sequence representing highly conserved regions. These were used for the amplification of BBI sequences of the horse gram genomic DNA. The PCR reaction produced three bands of 360, 810 and 1600 bp, the first two of these were of high intensity. The PCR products were subcloned into plasmid vector pUC-18 and two of the clones namely clone 2 and 7, representing 810 and 360 bp amplicons respectively, were sequenced.

9. The N and Cl primer were also used for the amplification of BBIs from the genomic DNAs of six different legume species and amplicons of different size were obtained. These amplicons were probed with DIG-labeled horse gram BBI clone 7 in Southern blots. Out of the six legumes only horse gram, lablab bean and black gram DNA hybridized with the clone 7.

10. A large part (132 out of 368 bp) of the clone 7 sequence was identical to a part of clone 2 sequence suggesting their common origin. Furthermore, the sequence revealed that both the clones were product of a single primer 'N' which was repeated and inverted a number of times in both clone 2 and clone 7. The clone 2 sequence did not show any clear resemblance with the reported BBIs apart from the presence of primer sequence. The predicted amino acid sequence of clone 7, however showed a long open reading frame of 50 amino acids after which there were several inframe stop codons. The 50 amino acid sequence showed significant homology with the BBI sequences of horse gram and other legumes. Despite clone 7 being clearly related to the horse gram BBI, it did not represent a known BBI gene.

11. Degenerate primers designed based on horse gram BBI protein sequence produced multiple amplicons but once again from single primer only. No additional band was produced by the primer pairs suggesting that they may be amplifying the repetitive DNA fragments of horse gram genomic DNA. One of these amplicons, a 1080 bp fragment obtained with primer RTV-2 was cloned and sequenced from one end upto 445 nucleotides. However, this sequence did not show any homology with the BBI except for the presence of RTV-2 primer sequence. Further, there was no long open reading frame in this sequence.

12. Four additional degenerate primers of significantly longer lengths were used to reduce nonspecific amplification. Three of these primers gave amplification when used alone suggesting the presence of inversions in this region of the genome. The primer combinations did not produce any additional bands other than the sum of the total number of bands with the individual primers, except for a 200 bp band of high intensity with primer pair, BBI-F2/BBI-RI. Reamplification of this band with primer pair BBI-F2/BBI-RI resulted in intense amplification, the BBI-RI primer alone was also able to amplify this band though weakly. This suggests that there may be two amplicons of similar size, one of these must be due to the primer pair combination while other could be a product of single primer BBI-RI. Their exact identity requires further confirmation by cloning and sequencing of these products.

13. Electrophoretic study of total seed proteins of nine different legumes including horse gram revealed that the predominant proteins of horse gram, cowpea, lablab bean, green gram, black gram and French bean are present in the size range of 43-68 kDa while that of pea, soybean and chick pea are distributed over a wider size range. The immunoblot of total seed proteins of these legumes probed with cowpea vicilin antibodies revealed that the predominant proteins of horse gram are vicilin-like. No significant variability in size and number was observed for the vicilin-like proteins in sixteen horse gram genotypes studied by using cowpea vicilin antibodies.

14. The electrophoretic and immunoblotting studies of total seed proteins during development and germination of horse gram seed revealed that two major vicilin-like proteins (55 and 57 kDa) start accumulating at 10 DAF. An additional vicilin protein of 59 kDa appear on 15 DAF. This shows the presence of atleast three different vicilin subunits with differential expression during seed development. These vicilin polypeptides were degraded during seed germination over eight days after imbibition (DAI).

15. Immunoblots of total seed proteins of nine legumes including horse gram probed with pea legumin antibodies revealed the presence of legumin-like proteins in all the legumes tested. Legumin-like proteins are appear to present in lower amounts than are the vicilins. In horse gram, proteins of size 49, 31, 28, 24 and 16 kDa reacted. All other legumes also had similar reacting proteins except soybean, pea and chick pea. The 49 kDa protein

observed in horse gram could be a larger size variant of the acidic subunits of legumin-like proteins. Some of the 28-36 kDa protein could be the smaller size variant of acidic subunits.

16. The immunoblot probed with wheat triticin anti "δ" (a legumin-like basic polypeptide) antibodies reacted strongly and specifically with 21-23 kDa proteins of chick pea, soybean and rice as expected, but reacted weakly with a protein of 29 kDa in horse gram suggesting that this protein may be a larger size variant of the basic polypeptide in horse gram. The immunoblotting results with the developing and germinating horse gram seeds showed that this 29 kDa protein appeared at 10 DAF although weakly but its accumulation increased on 15 DAF and continued to accumulate rapidly upto 25 DAF. This 29 kDa protein did not show any significant decline upto 6 DAI during germination while a slight decline was observed on 7DAI. This is similar to the observation with the basic polypeptides of soybean which also degrade slowly during germination.

17. Immunoblot analysis of seed tissue prints of horse gram, green gram and chick pea seed proteins probed with pea legumin and cowpea vicilin antibodies revealed the predominant nature of vicilin-like proteins in horse gram and green gram and that of legumin-like proteins in chick pea. Further, the distribution of legumin and vicilin-like proteins was uniform throughout the cotyledons.

18. Immunoblot analysis of total seed proteins probed with anti-narbonin antibodies revealed the presence the narbonin-like proteins in cowpea, black gram, green gram, French bean and sword bean. But there was no significant reaction with these antibodies in horse gram, soybean and pigeon pea. The narbonin-specific primers based on the narbon bean DNA sequence gave a single band of 760 bp in horse gram, green gram, black gram, cowpea, chick pea and lablab bean. But there was a difference between the amplicon size obtained in this study and the size of narbonin gene of narbon bean. The PCR amplicon of horse gram was cloned. Further characterization by sequencing of this clone is necessary to know the nature of these gene fragments.

19. To conclude, the results of the present work show that the biologically active BBIs of horse gram seeds are synthesized specifically during seed development and different isoforms accumulate between 15 and 21 DAF. There are alternate forms of trypsin inhibitors in the flower parts and pod peels. Cloning of horse gram BBIs was tried using a PCR based approach. This resulted into PCR products, which were generally products of a single primer

only as confirmed by DNA sequencing, suggesting extensive duplication and inversion in the horse gram genome. One of these clones (clone 7) had significant homology with the horse gram BBI protein sequence but this gene fragment was abnormal in that it had multiple stop codons after an open reading frame of 50 amino acids. The sequence of other amplicons did not show any resemblance to the BBI sequence, except for the presence of primer sequences, even through several primer combinations were used. The difficulties in obtaining a normal BBI gene using PCR approach shows that a cDNA or genomic library based approach may be more suitable for cloning of a functional BBI gene from horse gram.

Electrophoretic and immunoblotting studies using antibodies specific to vicilin, legumin and narbonin-like proteins revealed that vicilin-like proteins are the most abundant seed storage proteins in horse gram. Legumins are a minor protein with an unusally larger sized basic polyeptide and there was no evidence for narbonin-like proteins. The vicilin and legumin like protein of horse gram showed a normal pattern of accumulation during seed development and degradation during germination.

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