STUDIES OF SEED STORAGE PROTEINS IN LEGUMES WITH A SPECIAL REFERENCE TO PIGEONPEA

THESIS SUBMITTED TO

THE UNIVERSITY OF POONA

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

(IN CHEMISTRY)

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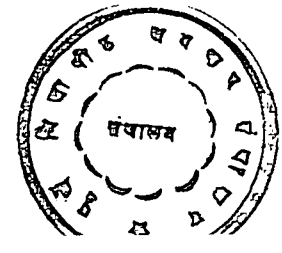

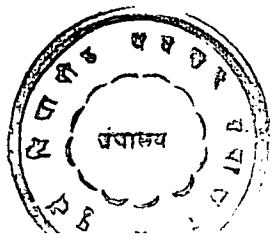

A C K N O W L E D G E M E N T

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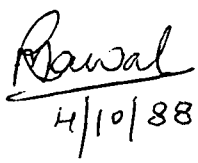

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Dated : October 4, 1988

D E C L A R A T I O N

Certified that the work incorporated in the thesis entitled "**STUDIES OF SEED STORAGE PROTEINS IN LEGUMES WITH A SPECIAL REFERENCE TO PIGEONPEA**" submitted by **MISS MADHUMALTI R. MAWAL** was carried out by the candidate under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

P K Ranjekar

(P.K. Ranjekar)
Research Guide

LIST OF ABBREVIATIONS

AE-Protein	S-2-Aminoethyl-protein
bp	basepairs
BSA	Bovine Serum Albumin
CD	Circular dichroism
cpm	counts per minute
Ci	curie
DAF	Days after flowering
DABITC	Dimethyl aminobenzene isothiocyanate
DVS	Divinyl sulphone
ELISA	Enzyme-linked immunosorbent assay
EDTA	Ethylene diamine tetra acetic acid
Gdn Hcl	Guanidine Hydrochloride
kD	Kilodaltons
nm	nanometer
PITC	Phenyl isothiocyanate
PMSF	Phenyl methyl sulphonyl fluoride
PBS	Phosphate buffered Saline (0.1 Na_2HPO_4, 0.4 M NaH_2PO_4, 0.1 M NaCl, pH 7.2)

PAGE	Polyacrylamide gel electrophoresis
PAD	Polyaldehyde dextran
RIA	Radioimmunoassay
rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TAE	Tris-Acetate-EDTA buffer (40 mM Tris, 20 mM Acetic acid, 2mM EDTA, pH 8.1)
Tris	Tris-hydroxymethyl amino methane

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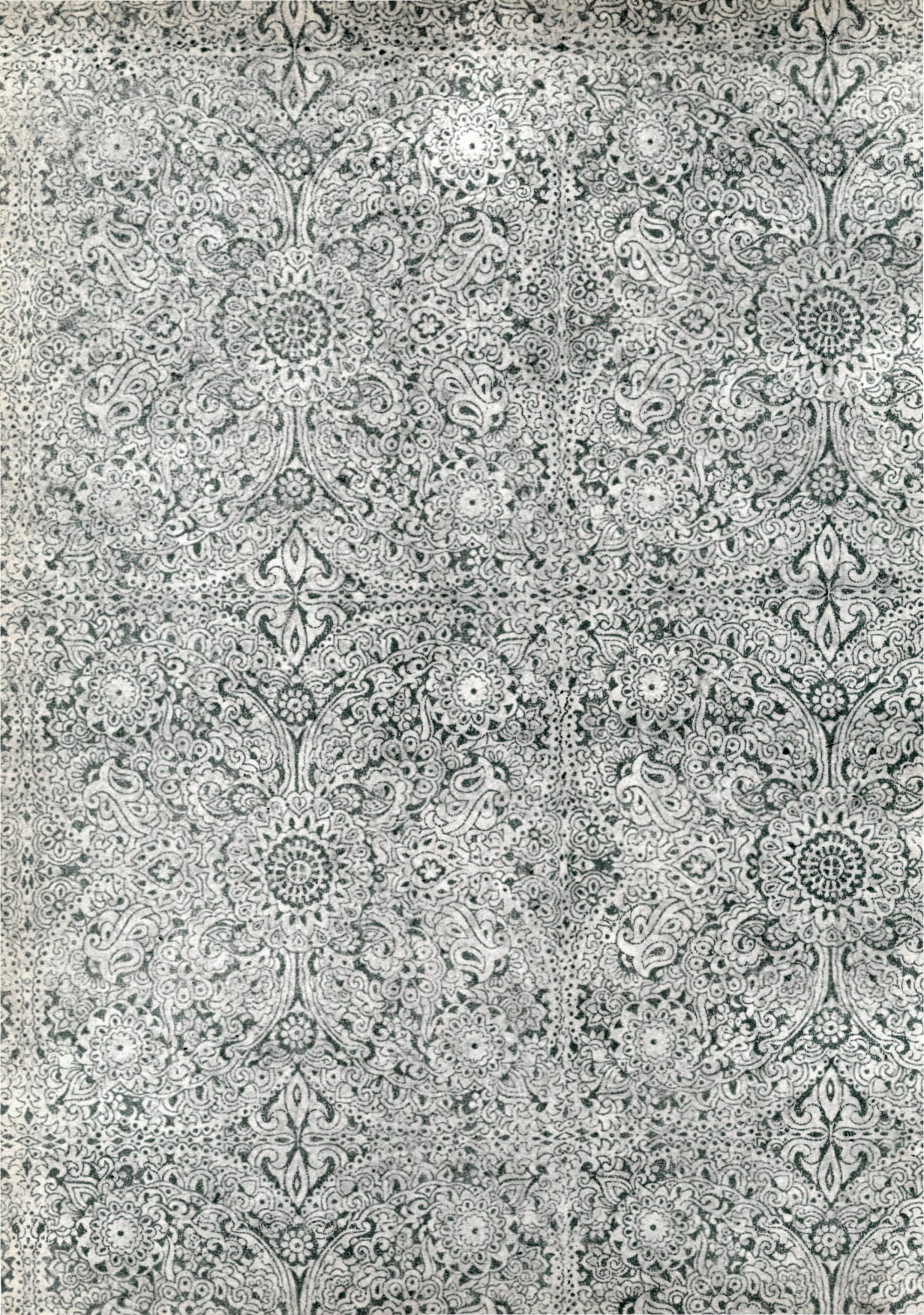
L I S T O F F I G U R E S

L I S T O F T A B L E S

SYNOPSIS



1. Genesis of the problem

Food legumes, particularly the grain legumes, or pulses, are an important source of proteins in all tropical or subtropical countries. In India, they provide the only high protein component of the average diet and over 10 million tonnes are consumed annually.

Although Osborne developed fractionation procedure of seed storage proteins as early as 1924, the actual research on seed storage proteins began only after 1960. During the period of last 20 years, extensive work has been carried out on seed storage proteins in soya bean, french bean, pea and broad bean in family Leguminoseae. Pigeonpea (*Cajanus cajan*) is a major crop of the tropical and subtropical regions and is grown extensively in the Indian sub-continent, Africa and parts of Latin America. Its seeds are an important source of protein in human diet. The protein concentration in the seed varies from 18 to 26%. The seed proteins, like other legume proteins, are deficient in sulphur amino acids. When the present work on pigeonpea was initiated by us in 1984, very little information was available in literature about its seed storage proteins. The present studies were therefore, undertaken to characterise the globulins which are the major storage proteins in the seeds of pigeonpea. It was thought that all this information would help in the site directed mutagenesis of cloned globulin genes in pigeonpea. The latter

work has recently been initiated in our laboratory for improvement of nutritional quality of globulins in pigeonpea.

2. Objectives and Strategies

The main objectives of the work embodied in this thesis were as follows :

(i) To study the molecular heterogeneity of seed globulins.

(ii) To determine the secondary structure of pigeonpea seed globulins.

(iii) To raise antibodies against globulins in pigeonpea and determine their immunological properties.

(iv) To use pigeonpea globulin antibodies to determine the rate of deposition of pigeonpea globulins during seed development.

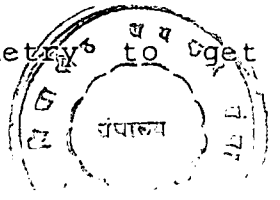
(v) To study relatedness of pigeonpea globulins with globulins from other legumes.

(vi) Computer analysis of known cDNA and genomic sequences to find regions that are conserved among legume storage protein genes.

The above studies were carried out using the following strategies :

(i) Biochemical characterization of seed globulins to assess their molecular heterogeneity.

(ii) Biophysical characterization of seed globulins using Circular Dichroism and Spectrofluorometry to get



an insight into the secondary structures of proteins.

(iii) Radioimmunoassays for quantitation of pigeonpea seed globulins at different stages of development during post anthesis.

(iv) Characterization of pigeonpea seed globulins using various immunochemical approaches to determine antigenicity of the protein.

(v) Relatedness of pigeonpea globulins with globulins from other legumes using various Biochemical, Biophysical and Immunological approaches.

(vi) Homology and relatedness among various legume seed storage proteins using approaches like Codon usage, Hydropathy, Chau-Fasman Rule, Amino Acid analysis and Dot matrix analysis for understanding the molecular organization and structural complexity.

3. Organization of the thesis

The entire thesis has been organized in six chapters.

Chapter I describes the current information of seed storage proteins and their genes.

Chapter II details various methods employed for isolation and purification of pigeonpea globulins, legumin and vicilin. Separation of vicilin subunits on DEAE-Sephacyl is also described.

Chapter III includes biochemical and biophysical characterization of pigeonpea globulin, legumin and vicilin.

Chapter IV deals with various Immunological approaches used for characterization of pigeonpea globulin and legumin. New methodologies like Enzyme-linked Immunosorbent Assay (ELISA) and radioimmunoassay (RIA) which have been developed are also described in detail.

Chapter V describes homology and relatedness of pigeonpea globulins with globulins from ten other legumes using various biophysical, biochemical and immunological methods.

Chapter VI analyzes the known cDNA and genomic sequences of legume seed storage proteins to assess the sequence homology so as to search for conserved and diverged regions in these genes for determining probable sites that could be available for site directed mutagenesis.

4. Summary of the thesis

The salient features of the results obtained in the present work are summarized below :

Isolation and purification of pigeonpea globulin, legumin and vicilin :

Pigeonpea globulin representing about 50% of the total seed storage protein was isolated from the alkaline seed meal extract and was purified on a sepharose CL-6B column to remove the ribonucleic acid contamination associated with the protein. The purified globulin was fractionated into legumin and vicilin by selective precipitation, ammonium sulphate saturation and zonal isoelectric precipitation. The molecular weight of legumin holoprotein was 375,000 with 4 subunits of Mr 72,000; 66,000; 60,000 and 56,000. The vicilin holoprotein had a molecular weight of 190,000 with 2 subunits of Mr 72,000 and 57,000.

Partial biochemical characterization of pigeonpea globulin, legumin and vicilin :

The amino acid composition of globulin, legumin and vicilin showed the presence of high content of glutamic acid and aspartic acid with low levels of sulphur containing amino acids cysteine and methionine. Tryptic digests of legumin and vicilin proteins shared about 7 peptides indicating a partial similarity in their structure.

Pigeonpea vicilin subunits were separated and partial N-terminal sequencing was carried out manually using

DABITC method. The N-terminal amino acid sequence for vicilin subunit 1 (72 kD) was Gly-Ala-Arg-Val-Asp-Gln-Glu while for vicilin subunit 2 was Thr-Thr-Cys-Met-Glu-Ser-Gly.

Secondary structure and stability of pigeonpea legumin and vicilin :

Circular dichroism studies showed that pigeonpea legumin and vicilin existed in a predominantly β-pleated sheet structure. Fluorescence studies revealed that pigeonpea vicilin is unusually stable to both urea and guanidine hydrochloride.

Development of sensitive immunoassays for quantitation of seed globulin :

Antibodies raised against pigeonpea globulin and legumin were used for all the immunological work. Prior to quantitation of pigeonpea globulin, its antigenicity was assessed by Western Blotting and the major antigenic determinant was found to reside on a polypeptide of Mr 60,000 while other subunits of Mr 31,000 and 16,000 were antigenic to a lesser extent. The antigenicity of pigeonpea globulin was further confirmed by immuno-electrophoresis where a single precipitin arc was observed.

A RIA was used for quantitation of pigeonpea globulin at different stages of development. Maximum globulin was detected at 28 days after flowering. This RIA was also used for screening large number of pigeonpea varieties for quantitation of globulin content. A computer programme in Fortran language facilitated the quantitation of pigeonpea globulin. Among the pigeonpea varieties screened, Gwalior-3 was found to contain the highest globulin content. Finally, a sensitive and rapid ELISA for quantitation of pigeonpea globulin was established which could be conveniently adapted for quantitating proteins as low as 0.1 ng. This method could also be used for quantitation of other proteins.

Comparison of pigeonpea globulin with globulin from other legumes :

Analysis of SDS-PAGE data of globulin by Jaccard index and immunochemical comparison of globulin revealed that pigeonpea globulin showed a maximum homology with that of bengalgram, a moderate homology with globulin of cowpea, frenchbean and greengram and least homology with blackgram globulin.

Computer analysis of known cDNA and genomic sequences :

In order to study homology and divergence among

legume seed storage protein genes, all cDNA and genomic sequences reported till March, 1988 were analysed using different approaches like dot-matrix, hydropathy analysis, amino acid sequence comparison, codon usage and homology around (i) post-translational cleavage site (ii) disulphide linkage site and (iii) glycosylation site. The main objective of this work was to assess the sequence homology so as to search for conserved and diverged regions in these genes for determining probable sites that could be available for site directed mutagenesis. The homology around putative post-translational processing site, canonical glycosylation site and postulated disulphide linkage site suggests that these three factors may be playing an important role in stabilization of conformations and compartmentalization of seed storage protein. Secondly, it can be postulated that modification of isoleucine to methionine may not possibly affect the globulin structure and hopefully may not affect accumulation in protein bodies. Finally taking into account all the data together, it could be proposed that genes encoding the 11S and 7S storage protein of legumes, oat globulin, patatin and napin might have evolved from a common ancestral gene.

In our laboratory, a major research programme entitled "Genetic manipulation of pigeonpea for improved protein

quality using recombinant DNA technology" has recently been undertaken. The phase 1 programme of this long range project has been cloning and sequence analysis of genes encoding globulin and structural analysis of globulin protein. It is hoped that the structural information of globulin that is obtained during my work will be of use in this project. For example, the partial N-terminal sequence analysis will enable us to make the oligonucleotide probes for fishing out the genes encoding vicilin protein. Similarly, the antibodies raised against pigeonpea globulin and legumin will be used for immunoscreening the cDNA library of pigeonpea. Furthermore, the biophysical data will prove to be useful in the proposed work of site-directed mutagenesis of cloned genes where it is essential to know the precise regions where mutations can be introduced without altering the overall secondary and tertiary structure of the protein.

5. Publications

1. Novel application of Quantitative immunoassays for screening seed globulins of cowpea varieties.
M.R. Mawal, S.A. Ranade, Y.R. Mawal, S.N. Ranadive A. Bhattacharya and P.K. Ranjekar.
Bioscience Reports, 5, 673 - 681, (1985).

2. Biochemical and Immunological Characterization of Rice Albumin.
Yogesh, R. Mawal, Madhumalti, R. Mawal and P.K. Ranjekar.
Bioscience Reports, 7, 1 - 9, 1987.

3. Novel method for screening pigeonpea varieties for globulin content by Radioimmunoassay.
Madhumalti, R. Mawal, Yogesh, R. Mawal, T.N. Guru Row and P.K. Ranjekar.
Biotechnology techniques, 2, 53 - 56 (1988).

4. A sensitive and rapid immunochemical method for quantitation of proteins.
M.R. Mawal, Y.R. Mawal, S.N. Ranadive and P.K. Ranjekar.
Bioscience Reports (1988) In Press.

5. Characterization of pearl millet (Pennisetum americanum) prolamins.
M.N. Sainani, N.A. Sahasrabudhe, Y.R. Mawal, M.R. Mawal, V.S. Gupta and P.K. Ranjekar
(Communicated).

6. Homology and divergence of rice glutelin gene to fourteen other seed storage protein genes of seven flowering plants.
Yogesh, R. Mawal, Madhumalti, R. Mawal, Ujwal Shinde and P.K. Ranjekar.
(Communicated).

7. Partial characterization of globulin seed storage protein in pigeonpea and its comparison with seed globulins from twelve other legumes.
Madhumalti, R. Mawal, Yogesh, R. Mawal and P.K. Ranjekar.
(Communicated).

Papers to be communicated :

1. Conservation of Secondary Structure of Vicilin Storage Protein from Twelve Legumes.
Madhumalti R. Mawal, Yogesh, R. Mawal and P.K. Ranjekar.

2. Comparison of Alcohol Induced Conformation Changes of Prolamin Storage proteins from Cereals.
Yogesh, R. Mawal, Madhumalti R. Mawal and P.K. Ranjekar.

3. Unusual Secondary Structure of Rice Albumins.
Yogesh, R. Mawal, Madhumalti, R. Mawal and P.K. Ranjekar.

Papers Presented at Scientific Meetings

1. Immunochemical Characterization of Vigna unguiculata Storage Protein.
   Yogesh, R. Mawal and Madhumalti R. Mawal.
   55th Annual Meeting of the Society of Biological Chemists, INDIA, at Trivandrum, 15 - 17 December, 1986.

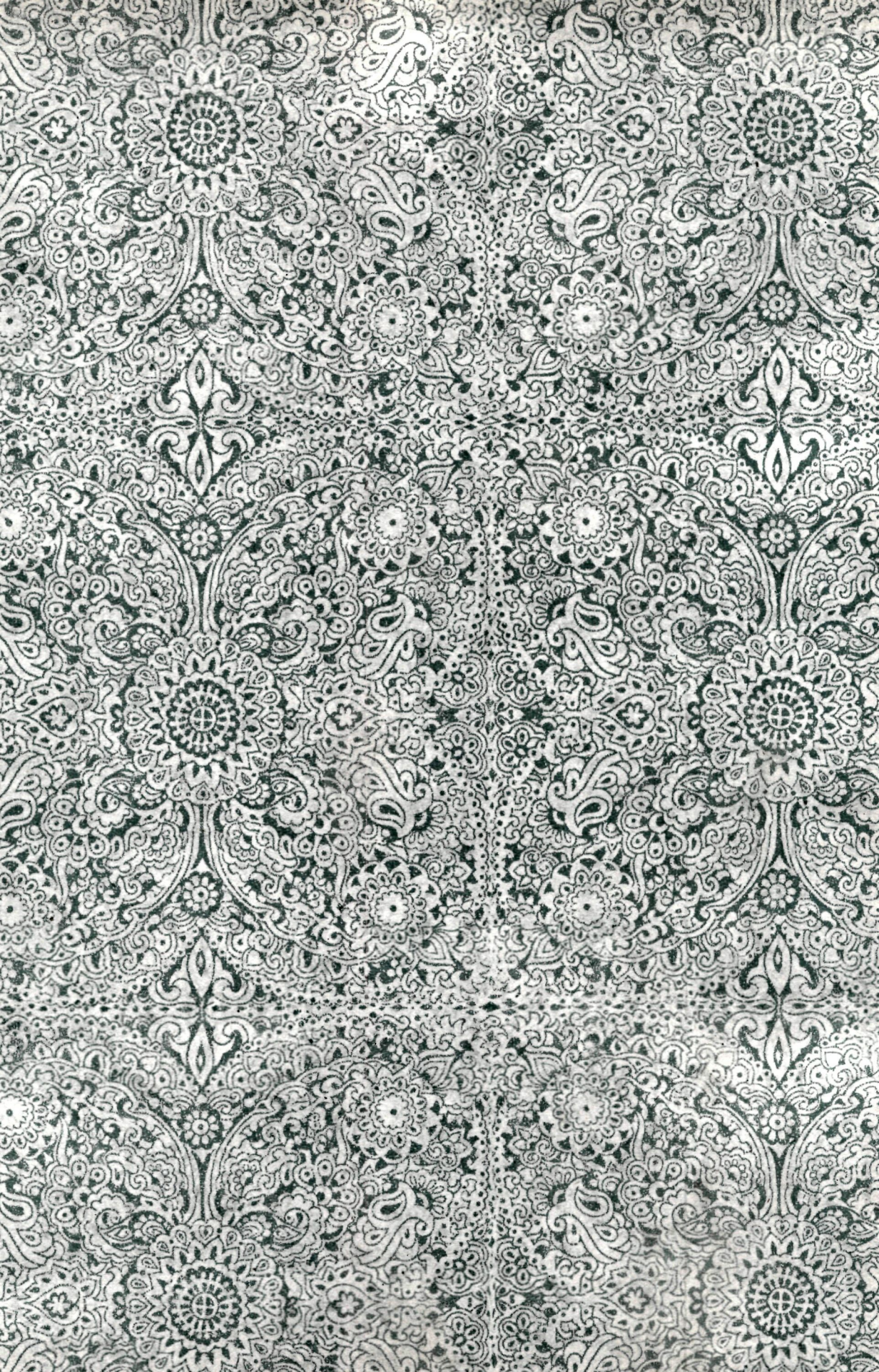
2. Seed Storage Protein Genes.
   Madhumalti R. Mawal
   Lecture delivered during short-term training course on Plant Genetic Engineering : Cloning and Analysis of a Specific Plant DNA Fragment at National Chemical Laboratory, September 10 - 30, 1987.

3. Seed Storage Proteins and their Genes in Plants.
   Yogesh, R. Mawal, Madhumalti R. Mawal, Mohini Sainani and P.K. Ranjekar.
   Presented at the National Workshop on "Advances in Plant Genetics and Gene Manipulations in Higher Plants" at Hyderabad during March 1 - 20, 1988.

4. Homology and Divergence of Rice Glutelin Gene with Fourteen other Seed Storage Protein Genes of Six Flowering Plants.
   Yogesh, R. Mawal, Madhumalti, R. Mawal and P.K. Ranjekar.
   Plenary Lecture during the International Conference on "Research in Plant Sciences with its Relevance to Future" at Delhi during March 7 - 11, 1988.

CHAPTER 1

SEED STORAGE PROTEINS AND THEIR GENES IN LEGUMES



1.1 NUTRITIONAL IMPORTANCE OF LEGUMES :

Pulses, also known as grain legumes or beans, are seeds of leguminous plants and belong to the family leguminoseae. They are 2-3 times richer in protein than cereal grains, and are second only to cereals as a source of human and animal food. Pulses, remain a major food in Latin America (especially *Phaseolus vulgaris*), Indian subcontinent (especially *Lens culinaris*, *Cajanus cajan*, *Cicer arietinum*, *Vigna radiata* and *Vigna mungo*) and Far East (especially *Glycine soja*). They are an important source of proteins in the diet of people who cannot afford to buy animal products or do not consume meat for religious reasons. Even when meat is the source of proteins in human diet, legumes constitute a principal source of protein in the feed of animals which provide meat. While pulses make an important component of vegetarian diet due to their nutritional value, they also carry bacterial colonies in root nodules, enabling them to utilize and fix atmospheric nitrogen. Since all parts of the legume plant are rich in protein, they also form an excellent organic manure when ploughed in the field.

1.2 NOMENCLATURE AND CLASSIFICATION OF LEGUME SEED STORAGE PROTEINS :

Osborne, a pioneer in the field of seed protein fractionation, published in 1924 a major classification

procedure of seed proteins based on their solubility characteristics (1). He classified seed proteins as albumin (soluble in water), globulin (soluble in salt solutions but sparingly soluble in water), prolamin (soluble in 70-80% ethanol) and glutelin (soluble in dilute acids or alkalies but insoluble in water, salt solutions or alcohols). Unlike cereals, where a major part of seed proteins is either prolamin or glutelin, globulin constitutes a major fraction of seed proteins in legumes. Osborne and Campbell (2) showed that the major protein in legumes was salt soluble and that the globulin from pea could be fractionated into two components which they named legumin and vicilin. The basis of this classification was (1) legumin in solution did not coagulate at 100 °C whereas vicilin did at 98-100 °C and (ii) legumin required more salt to remain in solution than vicilin. It was demonstrated later that fractions similar to pea legumin and vicilin could be extracted from other leguminous seeds like Glycine max (2) and Phaseolus vulgaris (3-5). Later, Danielsson (6) examined seed proteins in over 30 species from various genera of the leguminosae and concluded that with a few exceptions (Acacia longifolia, Acacia penninervis, Acacia verticillate and Trifolium rapens), all species contained two globulin components namely vicilin and legumin with sedimentation coefficients of approximately 7S and 11S, respectively.

Storage proteins have been defined on the basis of several criteria (7) which include :

(i) Tissue specificity : The proteins are usually only found in seed tissues.

(ii) Temporal accumulation : The proteins start to accumulate relatively late in the ontogeny of the seed, and this accumulation continues till the seed matures.

(iii) Effect of N nutrition : The accumulation of storage proteins is disproportionately decreased relative to other seed proteins, when nitrogen is severely limiting whereas it is disproportionately increased with excess nitrogen.

(iv) Subcellular location : The proteins are present in discrete deposits within the cell called protein bodies.

(v) Absence of other functions : The storage proteins serve as a source of nitrogen to the growing seedling and are degraded during seed germination. The proteins normally considered as major storage proteins usually have no other function, although in rare cases, certain enzymes also have a storage function.

1.3 SIZE AND SEQUENCE HETEROGENEITY OF GLOBULINS IN LEGUME SEED STORAGE PROTEINS:

During his studies on characterization of legume

storage proteins, Danielsson (6) has shown for the first time that vicilin and legumin from Pisum sativum behave as a single peak in the ultracentrifuge with a $S_{20,w}$ value of 6.5-8.1 and 12.6, respectively. In free flow electrophoresis in the presence of 0.2 M sodium chloride, these proteins again behave as a single component. Derbyshire et al. (8) have summarised the physical characteristics and properties of seed proteins from a variety of dicots. In their studies, they have shown that the 11S fraction (legumin) is a major constituent of seed storage globulins in pea and soybean and generally consists of multiple forms of heterohexamers (9). This fraction usually has a molecular weight in the range of 300,000-400,000. The holoprotein is composed of acidic and basic subunits (so named because of their isoelectric points) with molecular weights around 40,000 and 20,000, respectively. These subunits are cross-linked by disulphide bonds and are always devoid of bound carbohydrate group. The N-terminal sequence of a basic polypeptide of legumin is probably very constant through many genera of leguminosae and even in other plant families, with only discrete point mutations (the N-terminal amino acid seems to be always glycine). Moreover, legumin hexamers cannot associate into dodecamers or possibly superoligomers of larger degrees of polymerization. This has been shown for legumins of Arachis hypogaea (α-arachin), Glycine max (glycinin), Pisum sativum, Vicia faba,

Vigna mungo, Cicer arietinum and Lupinus ungustifolius (δ-conglutin). The peptide mapping experiments (10) and the comparison of the coding nucleotide sequences and amino acid sequences of the 11S globulins reveal a distinct sequence homology between both the $(\alpha\beta)$-monomers building up the hexameric globulin molecule of a species and the $(\alpha\beta)$-monomers of different species (11 - 14). A pronounced sequence homology also occurs within the N-terminal region of the β-chains from dicotyledonous seeds and that from Avena sativa, a monocotyledonous plant. Therefore, it appears that the N-terminal region of either polypeptide types has been conserved during the evolution.

Comparatively less is known about the 7S protein. The latter is a major fraction in Phaseolus where it has been well characterised. The molecular weight for the 7S protein varies from 140,000 - 200,000. It is composed of several subunits which are not linked by disulphide bonds. Contrary to legumin, vicilin always contains a bound carbohydrate but its subunits are devoid of cysteine or cystine (except for β-conglutinin of the lupinus seed). The only common feature of vicilin and legumin is the ability of vicilin to associate into heterotrimers as shown in Glycine max (β-conglycinin), Phaseolus vulgaris (trimers or tetramers) and Vicia faba. Furthermore, β-conglycinin is able to associate to a higher

degree of heterohexamers while the δ-conglutin of Lupinus ungustifolius appears to be homohexamers.

From the above information, it is clear that both legumin and vicilin exhibit a high degree of heterogeneity and polymorphism in legume storage proteins (15). The occurrence of genetic variants which often explains the secondary minor bands of globulin electrophoretic patterns as well as the multiplicity of a few kinds of subunits is the first cause of heterogeneity. Other causes are post-translational, among which one can mention: the occurrence of several degrees of oligomerization (which results in a series of sedimentation coefficients); the possible interchange of some of the subunits, as shown by β-conglycinin which arises into multiple forms and the contingent variation in size or nature of bound carbohydrate groups (of which the exact function remains still unknown).

1.4 SECONDARY STRUCTURE AND PACKAGING OF LEGUME SEED STORAGE PROTEINS :

Many functional properties of globular proteins are determined by their three-dimensional structure. X-ray structural analysis of protein crystals can give a detailed information with atomic resolution. For proteins from which a single crystal diffraction pattern can be obtained, the

molecular shape may be deduced from this pattern. So far, only phaseolin from Phaseolus vulgaris (16) and conavalin from Concanavalin ensiformis (17) have been crystalised. There are, however, a number of limitations and disadvantages to this method (18), the most serious of which is the preparation of a suitable single crystal. Several other alternative procedures have, therefore, been used to get a limited insight into the protein structure. Small angle X-ray scattering (SAXS) is one such method that has been used in case of legumins of Glycine max, Cannabis sativus, Bertholletia excelsa, Nicotina tabacum, Brassica napus, Sesamum indicum, Helianthus annuus, and Vicia faba, and vicilin of Phaseolus vulgaris and has shown that these proteins possessed a spherical shape. Dynamic light scattering is yet another approach that has been applied for 11S globulin from Helianthus annuus, Brassica napus and Vicia faba, and 7S globulin from Phaseolus vulgaris to determine hydrodyanamic properties of these proteins (19). It was found that 11S globulin from sunflower seeds and from rape seeds have a Stroke's radius of 5.7 nm and a molar mass of 3×10^5 g/Mol. However, the 11S globulin from Vicia faba differed from the former two proteins. It has Stroke's radius of 6.3 nm with molar mass of 3.5×10^5 g/Mol, while 7S globulin from Phaseolus vulgaris showed Strokes radius of 4.7 nm and molar mass of 1.45×10^5 g/Mol. The ORD and CD

data of the globulins from Cucumis sativus, cucurbit seeds, Arachis hypogea (20), Lupinus unqustifolius (21), Bertholletia excelsa (22), Glycine max (20, 23, 24), Helianthus annuus, Brassica napus and Vicia faba (25) have shown the presence of a significant amount of β-sheets (40% - 50%) and a small portion (5% - 14%) of α-helical structures in these proteins. It has been further shown in these studies that the 11S globulins belong to the class of B-proteins. Based on the amino acid sequence data, the secondary structures of the α- and β-polypeptide chains of Pisum sativum, Glycine max and Vicia faba was predicted. Here it was found that despite the differences of the amino acid sequences between the species, the main features of the secondary structure were conserved. The predominant secondary structure arrangement of 11S globulin in Pisum sativum, Glycine max and Vicia faba is the alternating pattern of β-strands and β-turns. The concordance of the experimentally determined secondary structure with the predicted secondary structures points to a distinct homology among the 11S globulins in these three species.

According to Pernollet and Mosse (26), the 11S globulin molecule from Pisum sativum, Glycine max, Vicia faba, Arachis hypogeae, Lupinus unqustifolius, Pisum sativum, Brassica napus, Cucumis sativus Sesamum indicum, Bertholletia excelsa,

Helianthus annuus, Nicotiana tabacum and Phaseolus vulgaris is an oligomer formed by the association of six monomers. Each ($\alpha\beta$)-monomer differs slightly in their amino acid sequences from one another. The arrangement of the six ($\alpha\beta$) -monomer was investigated using electron microscopy, X-ray diffraction and X-ray scattering and all the globulins were found to have a similar shape. Apart from the similarity in shape, the quaternary structure of the 11S globulins also seems to be uniform. Such a similarity in shape and quaternary and secondary structure raises the question of the significance of the structural conservation of 11S globulin. One explanation would be the hypothesis of the maximal packing of the proteins within the protein bodies of the seeds put forward by Pernollet and Mosse (27). Here, they presume that either the compact folding of the polypeptide chains allows a maximal amino acid packing or the arrangement of the ($\alpha\beta$)-monomers leads to a high compactness of the globulin structure.

1.5 IMMUNOCHEMICAL RELATEDNESS OF LEGUME SEED GLOBULINS:

Apart from biochemical and biophysical characterization, legume seed storage proteins have also been analyzed using immunological approaches. These studies have given a

valuable information with respect to relatedness among legume proteins and quantitation of these proteins (8, 28 - 30). During their study of seed storage proteins by immuno-electrophoresis in several varieties belonging to the genus Phaseolus, Kloz et al., (30 - 32) have investigated the taxonomic distribution and variability of the main storage proteins and have found that the so-called American endemics belonging to genus Phaseolus formed a relatively closegroup which was distinct from the other species of the genus. When the antibodies raised against 7S component of Phaseolus were compared with vicilins of tribe Viciaceae, no immunological correspondence was detected. Extensive immunological work has been carried out in species belonging to the tribe Lupineae. Here, it was observed that the seed proteins of the tribe Lupineae are considerably different from those of tribe Vicieae (30). According to Nowacki and Prus-Glowack (33), different species of American members belonging to genus Lupinus form a serologically closely related group in which Lupinus luteus, Lupinus albus, Lupinus angustifolius and Lupinus perennis are most distinct. American members, however, do not differ from one another to such a degree as do the mediterranean species. Based on immunoelectrophoretic separation (33, 34), the intercrossing behaviour of different species of the genus Lupinus was found to show a similar picture.

Storage proteins of soybean seeds have been subjected to very extensive immunological studies and are shown to consist of at least four immunologically distinct components i.e. glycinin, γ-conglycinin, β-conglycinin and α-conglycinin (35). Among these four proteins,Glycinin, γ-conglycinin and β-conglycinin are located in the protein bodies of cotyledon cells. Glycinin when subjected to heat treatment showed no loss in immunological activity. Further characterization by complement fixation assays indicated some reduction in antigenic sites in heated glycinin sample which was more pronounced at higher temperatures.

Daussant et al.(36) and Neucere (37, 38) have studied α-arachin and α-conarachin, the major proteins of Arachis seeds and have found a two-fold higher concentration of -arachin in the cotyledons than in the embryonic axis (37). Semiquantitiation of α-arachin during early stages of embryogenesis indicates a progressive synthesis during the course of seed development. α-Arachin appears to be one of the first detectable proteins to undergo catabolism during the very early phases of germination. A comparative immunological survey of the seed proteins in 36 species of the genus Arachis has shown evidence of α-arachin and α-conarachin in some wild species that is comparable to that in cultivated Arachis hypogaea, although both qualitative and quantitative

differences of these proteins have been observed among them. It has been further observed that the cultured Arachis hypogeae contains higher amount of α-arachin as compared to the wild species (38).

Dudman and Millerd (29), using polyclonal antisera to storage proteins, failed to detect the homologies between the legumin and vicilin storage proteins from different species and genera such as Vicia faba, Phaseolus vulgaris, Glycine max and Pisum sativum. Cross immunoelectrophoresis suggests that broadbean and pea cotyledons may contain as many as seven different globulin components and no interaction among any of these precipitated proteins is observed (38 - 41). These workers have further concluded that legumin and vicilin from the above plants have no determinants in common and are completely different proteins (41 -43). In contrast to the findings of Dudman and Millerd (29), the results of Croy (44) indicate that pea legumin has all the determinants of broadbean legumin with an addition of one or more.

Guldager (41) has suggested that the legumin of pea is composed of two slightly different proteins which have a very high degree of common antigenic structure. This could not be demonstrated by Vicia legumin (40). It was found that the antibodies raised against pea legumin holoprotein reacted with both the acidic and basic subunits of broadbean legumin.

There was no immunological correspondence between the two types of subunits. Thus immunologically it has been possible to demonstrate reconstitution of isolated acidic and basic subunits of the pea and broadbean legumin holoprotein (45).

Domoney *et al* (46) have developed a highly sensitive enzyme linked immunosorbent assay (ELISA) for the quantitative detection of legumin in *Pisum*. These workers found that legumin was more stable to heat treatment and acid precipitation than vicilin. Comparison of the legumins of other members of the *Vicieae*, i.e. *Lathyrus*, *lens* and *Cicer* show that they have proteins with similar immunological properties and compositions. Coyler and Luthe (47) employing radioimmunoassay have quantitated the amount of globulin in oat. Similarly, quantitation of quinine in cultured plant tissue (48) was performed using radioimmunoassay.

1.6 BIOSYNTHESIS AND DEPOSITION OF STORAGE PROTEINS :

The most critical property of a storage protein is its synthesis and accumulation within the cell of a developing seed without being subjected to the normal cycle of breakdown and resynthesis that metabolic proteins such as enzymes and membrane proteins undergo. During germination, these stored proteins must then be able to be broken down to their constituent amino acids. The strategy which has evolved

to protect storage proteins during their synthesis and accumulation is the sequestation of these proteins in protein bodies where they are not exposed to the proteinases responsible for the breakdown of metabolic proteins. In legumes, the protein bodies arise by progressive deformation and gradual breaking up of the large central vacuole. Both legumin and vicilin are found in the same protein bodies in pea (49 - 51). In cereals such as wheat, maize and rice, the protein bodies arise from dilations of the endoplasmic reticulum (52 - 54). Rice has interestingly two types of protein bodies, one containing prolamin and the other containing glutelin and globulin (55, 56).

All the evidence to date is consistent with the notion that most major storage proteins are synthesised on endoplasmic reticulum (57) and then are transported via the golgi apparatus (58) to their site of deposition in the protein bodies. The structure of storage proteins must, therefore, contain the specifications that determine their selective transport from the site of synthesis to the site of accumulation. For example, all storage protein polypeptides studied todate are initially synthesised with a typical leader sequence that facilitates the transport of the nascent polypeptides first through the membrane of the endoplasmic reticulum and then into the lumen. However, we understand

very little of the sequence requirements that specify the subsequent steps in the transport of storage proteins and their deposition in the protein bodies.

The accumulation of storage proteins in protein bodies has been reviewed earlier (28,57-59). It is well established that the embryogenesis is remarkably constant from one legume species to another. The deposition of storage proteins is concomitant with the biosynthesis of other storage products during the cell expansion phase of embryo growth which follows the rapid cell division phase during which essentially no storage protein is sythesised. Although variable, depending on species, cultivars, and growth conditions, the onset of legume storage proteins takes place between the ninth and twelth day after flowering and the maximal synthesis occurs between the fifteenth and thirtieth day.

During the deposition of storage proteins, different protein groups are synthesised at different rates. This was first investigated by Bishop in barley (60). Later, Landry and Moureaux (61) have shown in maize that the prolamins (zeins) and glutelins are not synthesised upto two weeks after pollination, while salt soluble proteins are already accumulating. In legumes, the onset and rate of synthesis of both legumins and vicilins are different (62). This has been shown for many species including peas (40,59) and

(63). In pea, Gatehouse et al., (64) have shown that the rate of legumin synthesis remains roughly constant throughout the protein deposition period contrary to the synthesis of vicilins which fluctuates : different components of legumin fraction are present predominantly at different stages of development. On the contrary, within protein groups such as prolamin, the proteins are synchronously synthesised, despite the location of their genes on different chromosomes as is shown for wheat gliadins (65) and maize zeins (66).

1.7 STRUCTURE AND ORGANIZATION OF SEED STORAGE PROTEIN GENES:

Seed storage proteins are encoded by multigene families which exhibit a high degree of conservation and linkage among themselves. The number of genes constituting gene families has been estimated to range from 5 to 100 (67). These genes occur singly or in clusters; and on same chromosome as well as on different chromosomes. The organization of seed storage protein genes is similar to that of other plant genes (68). Briefly, on the 5' end of the genes upstream to the first amino acid codon, typical eukaryotic transcription initiation signals, TATA Box and CAAT Box are located. On the 3' end of the gene downstream to the translation termination codon(s) are consensus polyadenylation and transcription stop signals, AATAA. The open reading frame

TABLE 1.1

CHARACTERISTIC FEATURES OF PLANT STORAGE PROTEIN GENES

Source	Gene family	CAAT/TATA Box (nucloetide upstream of transcription start site)	Polyadenylation signals (nucleotide downstream to translation termination codon)	Introns
1. Maize	Zein	30 nucleotides	Occur twice 30 bp	Absent
2. Barley	Hordein		3 poly (A) signals	
3. Wheat	Gliadin	TATA Box -- - 104 nu	2 Poly (A) signals	Absent
		CAAT Box -- - 141 nu	80 and 127 nu	
4. Frenchbean	Phaseolin	3 TATA Box -- - 28, -37 and -39	Poly (A) 16 nu	5 introns
		2 CAAT Box, -- - 67 and -74 nu		
5. Pea	Legumin	TATA Box -- - 66	3 Poly (A)	3 introns 88, 88 and 88 nbp long
	Vicilin		1 Poly (A) 20 bp	

(a)	(2)	(3)	(4)	(5)
6. Soybean	Glycinin	TATA Box -- - 25bp CCAT Box -- - 105 bp	3 Poly (A)	3 introns 238, 292 and 624 bp long
	Conglycinin		2 Poly (A) 132 bp long	4 introns 85, 115, 132 and 40 bp long
7. Sorghum	Kafinin	nd (not determined)	nd (not determined)	Absent

precursors (73-79). The comparison of sequences of cDNAs and genomic DNAs for zein polypeptides has shown the absence of introns and the presence of transcriptional regulatory sequences that are similar to those in other eukaryotic genes (80-86). However, an unusual feature of some of the zein genes is the presence of multiple promoter regions separated by about 1000 nucleotides (rich in A+T bases) at its 5' end (86-89).

Hordein Genes of Barley :

The barley storage proteins called hordeins are encoded by a multigene family (90). Hordeins are classfied into 3 polypeptide classes called B, C and D-Hordeins which are encoded by loci Hor-2, Hor-1 and Hor-3 respectively. The two genes encoding polypeptides of the B1 sub-family of B-Hordein have been described in detail by Brandt et al (91) and Forde et al. (92). It has been shown by these authors that neither gene possesses introns and each codes for a precursor polypeptide with a probable signal sequence of 19 amino acids followed by two distinct domains. The first domain at the N-terminal consists of degenerate tandem repeats of proline/glutamine rich tracts of the octapeptide (92) or tetrapeptide core sequence (91). The second domain is relatively rich in methionine and cysteine and poor in proline and glutamine. The C-terminal end of 35 amino acids

contains no glutamine. It has been further reported that the two gene sequences of B 1 sub-family are very similar, but that described by Brandt et al (91) lacks 69 bp sequence in domain 1 that is present in the sequence reported by Forde et al (92). This suggests that insertions and deletions of blocks of sequence in the repeat region may be a major source of size polymorphism within the B-Hordein polypeptide family.

Gliadin Genes of Wheat :

Gliadins are the prolamin type wheat storage proteins and are typically characterised by the presence of 30-78 kD monomeric proteins. Based on electrophoretic mobilities, gliadins have been classified into α, β, and γ-gliadins. As in case of zein in maize, the gliadins in wheat are also encoded by a multigene family and are believed to have evolved by gene duplication and divergence from ancestral genes (93). From the genomic DNA library in charon 32, clones encoding presumably the α-gliadin polypeptides have been identified and one of them is sequenced (94). Comparison of the nucleotide sequence of this clone with that of cDNA clones (95, 96) reveals that this gliadin clone/gene lacks introns as in the case of zein genes.

Phaseolin Gene of Frenchbean :

The seed storage proteins in Phaseolus vulgaris are of two types (i) G 1 protein or phaseolin and (ii) G 2 protein.

(coding region) is generally interrupted by introns with the exception of zein genes of maize (67) and α-gliadin genes of wheat (68). A very striking observation about the storage protein genes is the presence of blocks of nucleotide sequence repetitions in the coding regions such that the translated proteins also have a repeating block structure of amino acids. This repetitive block structure is presumed to have evolved in such a way so as to follow folding/aggregation/ packaging of polypeptides into a specific 3-D-structure which can form protein bodies (69). To-date, genes for storage proteins have been characterised in plant species such as maize, barley, wheat, french bean, pea, soybean, cottonseed, sunflower, oat and potato. Table 1.1 gives a list of characteristic features of seed storage protein genes in these plants.

Zein Genes of Maize :

The major storage protein in maize is the alcohol soluble prolamin fraction referred to as zein which is the best characterised nuclear gene family in plants. Zein polypeptides occur predominantly in two size classes of 19 and 22 kD, and 10-20 polypeptides can be identified in each of the size class (70-72). Analysis of mRNAs and *in vitro* translation experiments have accentuated this heterogeneity and have shown that these proteins are synthesised as

The phaseolin protein is a glycoprotein and has been extensively studied. It has three major polypeptides termed as α,B, and γ of molecular weights 51, 48 and 45.5 kD respectively (97).

Phaseolin mRNA is one of the first plant messengers to be isolated and translated *in vitro* (98, 99). In fact, phaseolin gene is the first plant gene where the presence of intron has been reported for the first time by Sun *et al.*, (100). The transfer of phaseolin gene to sunflower via Ti-plasmid vector is one of the earliest reports of transfer of a plant gene to another plant (101). In this experiment, it was found that poly(A)$^+$ RNA from the transformed tissue directed synthesis of a protein that could be immunoprecipitated by antiphaseolin antibodies in a cell free system. No intact phaseolin proteins were, however, detected in the transformed tissue, except for a 26 kD protein suggesting thereby that the phaseolin protein which was synthesised in the transformed tissue was being endogenously degraded. This was confirmed by incubating the phaseolin protein with extracts of tumor tissue where degradation of the protein was observed (102). Later, the phaseolin gene was also transfered and expressed in tobacco (103, 104).

Legumin and Vicilin Genes of Pea :

The storage globulins of pea are of three types, with

legumin (11S, 380 - 410 kD) and vicilin (7S, 150 kD) being the most abundant proteins. The third type of protein has been designated as convicilin. The cDNA from legumin mRNA has been cloned and completely sequenced. The comparison of nucleotide sequence and the amino acid sequence shows that the acidic polypeptide (40 kD) is at the N-terminus of the 60 kD precursor followed by the basic polypeptide (20 kD) (105). In convicilin, analysis of mRNAs and cDNAs has revealed homology of vicilin to pea and other 7S globulins like phaseolin and glycinin (106).

By comparing the codon usage patterns of the sequences of plant storage proteins, it has been observed that codons containing CG are little used. For example, the arginine codons AGA and AGG in pea have been preferred over the other four codons namely CGA, CGC and CGT (106).

Glycinin and Conglycinin Genes of Soybean

The major storage proteins of soybean are the globulins consisting of 2S, 7S (conglycinin) and 11S (glycinin) components (107). Polyribosomes and poly (A)$^+$ mRNA from developing soybean seeds code for the synthesis of the subunits of 7S and 11S storage proteins (108, 109). The glycinin, like the legumin of pea, is synthesised as a 60-62 kD precursor (108-111). It is believed that the removal

of the signal sequence in glycinin is cotranslational while the cleavage between the subunits may occur post-translationally (112).

Kafirin Genes of Sorghum :

The major storage protein in sorghum is the alcohol soluble prolamin fraction referred to as kafirin. Kafirin polypeptides occur predominantly in two size classes of 19 kD and 22 kD and are similar to the 19 and 22 kD zein proteins in their electrophoretic mobilities and sequence studies. Although the kafirin from sorghum appears to be homologous to zein, the number of genes per haploid genome seems to be lower than has been reported for the zein complex in Zea mays (113). Sequence analysis of kafirin genes indicates a close similarity of nucleotide sequence homology, lack of introns and presence of typical eukaryotic promoter and poly-adenylation signal (R. DeRose and T.C. Hall pers. comm.).

1.8 EXPRESSION OF STORAGE PROTEIN GENES :

In recent years, gene cloning coupled with efficient systems for gene transfer has unravelled the complexity of plant gene regulation. The initial gene transfer experiments have primarily made use of undifferentiated calli where it has been demonstrated that foreign genes can be introduced into a plant genome and faithfully expressed. Further

developments in gene transfer technologies have allowed the introduction of foreign genes into cells from which fertile plants containing the foreign gene in all cells could be regenerated. Such transgenic plants provide a much better background for studying gene regulation in its full capacity. Many plant genes are expressed in a highly regulated manner. The gene products of such genes may be present only in certain cell types, at specific stages of development or only following the application of distinct environmental stimuli. A specific gene can be turned on by very different inducers or a single stimulus may have totally different effects on different genes.

The abundance of seed proteins and their mRNAs in the seeds have made it possible to study the precise developmental and tissue-specific regulation of their expression (114,115).

Studies on expression of phaseolin gene in sunflower and tobacco have revealed that phaseolin mRNA and protein products could be detected at high levels in seeds but not in leaves, thereby demonstrating correct organspecific expression. In bean, phaseolin is found exclusively in the protein bodies of the embryo, while in tobacco, seed storage proteins are present both in embryo and endosperm. Interestingly, the transferred phaseolin gene directs the synthesis of

phaseolin only in the tobacco embryo. This suggests that either the transcriptional and translational machinery of the tobacco cells is capable of discriminating between bean and tobacco regulatory sequences or alternatively that the gene may be expressed in the tobacco embryo but the gene products may fail to accumulate in this tissue. The bean-specific expression pattern is retained in tobacco not only with respect to tobacco but also with respect to temporal expression. In tobacco, the phaseolin proteins start to accumulate 15 days after pollination as in bean, whereas the synthesis of endogenous storage proteins begins only after 9 days.

Similar experiments were performed with a soybean gene coding for the alpha' subunit of beta-conglycinin, which was studied in transgenic petunia plants (116). In this case, the soybean gene was found to be expressed predominantly in the seeds of petunia, and the timing of the expression followed the pattern of the endogenous petunia proteins.

In monocots, the endosperm is the tissue containing most of the storage proteins. Expression of a zein genomic clone has been studied in transformed sunflower tissue using an oncogenic vector (117, 118). Here, transcription was found to start at the authentic site, but the level of expression was relatively low (1% of the level in the maize endosperm) and no protein was made. The low level of expression may not

be surprising mainly for two reasons. First, not all monocot genes are well expressed in dicots. Second, zeins are expressed in a highly tissue specific fashion and sunflower tumor cells may not be a suitable tissue for the expression of these genes. A zein gene introduced in transgenic tobacco was not expressed in seeds or in any other tissue. Demonstration of transcriptional activity of monocot genes in the seeds of transgenic dicots may be further complicated by the fact that the endosperm, which is the major storage tissue in monocots, is only a minor component of dicotyledonous seeds (119). Perhaps genes coding for monocot storage proteins that are expressed in both endosperm and embryo, such as globulins may be better expressed in transgenic dicots (120, 121).

Thus it can be seen that the seed storage protein genes are expressed in a very highly regulated manner. How is the very specific expression of the seed storage protein genes (122-124) brought about? Obviously, cis-acting elements present in the genes or their flanking regions in Vicia faba, Pisum sativum and Phaseolus vulgaris are likely to be important determinants. A sequence comparison between legumin 5' flanking regions has led to the identification of short ($<$ 100 bp) consensus sequences with a putative regulatory role (125). The functional significance of such

sequences, however, needs to be established by mutational analysis in conjunction with gene transfer methods.In a limited number of cases, it has been shown by gene transfer experiments that all the information needed for regulated expression is contained within the gene and the flanking stretches of DNA. Initially the presence of a protein binding site in the 5' region of a zein gene of maize was identified. The 15 bp sequence was found to be conserved and was presumed to play a role in the regulation of zein gene expression. This is the first example of a specific interaction involving a plant gene upstream element (126). Later, preliminary studies with a wheat gliadin gene have identified the trans-acting factors that bind to putative regulatory elements. When wheat nuclear extract was incubated with a fragment from the upstream region of this gliadin gene, specific binding to a conserved sequence was detected. Interestingly, this binding activity was only present in nuclear extracts derived from endosperm and not from other tissues.

1.9 HORMONAL REGULATION OF SEED PROTEIN EXPRESSION:

It is well known that hormones like abscisic acid (ABA), gibberellic acid (GA), indole acetic acid (IAA) and cytokinin have an effect on the growth of plants. However, not much is known about the effect of these hormones on the

expression of storage proteins. It is known that in higher plants, the process of embryogenesis is usually preceded by the period of dormancy. This quiescent period can be bypassed by excising the embryo's and culturing them under appropriate conditions, which suggests that the surrounding tissues are involved in the maintenance of dormancy (127-129). A number of studies have shown that the growth regulator abscisic acid (ABA) prevents precocious germination (130, 131). ABA is known to induce the synthesis of specific proteins, among which are certain seed storage proteins (132-135). The inducing effects of ABA on the expression of beta-conglycinin genes are specific for the alpha' subunit; the genes for other subunits are not affected (135). This provides an interesting example of differential regulation of related genes. Other ABA-inducible proteins such as lectins and agglutinins may be involved in maintaining dormancy, in protecting seeds against desiccation, and in preventing the deleterious effects of hydrolytic enzymes (133, 134, 136).

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Whereas ABA is involved in dormancy, the plant hormone gibberellic acid (GA) is produced upon the onset of germination. This compound induces the synthesis of a number of enzymes involved in the breakdown of seed storage products (120).

1.10 EVOLUTION OF LEGUME SEED STORAGE PROTEINS :

From nucleic acid and protein sequencing studies it is becoming increasingly clear that storage proteins in a given legume may have homologous counterparts in other legume species. Sequences corresponding to Pisum sativum vicilin, for example, are found in Phaseolus vulgaris (phaseolin) and Glycine max (conglycinin), while the subunit sequences of Pisum sativum legumin and Glycine max glycinin are extremely similar. It is reasonable to suggest that such homologous sequences have evolved from a common ancestor and have been conserved during evolution because they are in some way important to the synthesis, structure or stability of storage proteins. Schuler et al., (137, 138), for instance, have postulated that certain conserved conglycinin sequences may be necessary for mRNA stability because similar sequences are also found in the 3' noncoding regions of mRNAs for other Glycine max polypeptides. Lycett et al., (139) suggest that such sequences which also have homologous counterparts in Pisum sativum vicilin, might equally well have been conserved because they are important to the structure or function of proteins. If one ascribes to a storage protein the "functions" of being transported, sequestered and stored in a dry state and recognised by proteolytic enzymes on seed germination, it might be assumed that there have been limitations to change these proteins during their evolution. The

fact that legumin like proteins are found in families as diverse as cucurbits (140, 141) and cereals (142-144) suggests a need to conserve atleast certain elements of the genes for 11S proteins; alternatively it is possible that convergent evolution to an 11S-like protein has taken place in response to the requirement of the "functions" outlined above.

It has been suggested that the ancestors of storage proteins may have had some clear function such as enzymic activity. The observed oxaloacetate decarboxylase activity of cucurbitin (145), for example, may give a clue to the origins of 11S storage proteins. The discovery of small regions of internal homology in the amino acid sequence of glycinin acidic subunits (146) raises the possibility that these repeated domains could be the products of a series of gene duplications within the eventual structural loci; the glycinin family of proteins may have evolved as a consequence of a complicated series of gene duplications to give regions of homology both within and between glycinin polypeptides.

In comparing different legumes, e.g. soybean to peas and peas to faba beans, one is struck by the fact that the pattern of differential gene expression of the storage protein genes has also been conserved, i.e. 7S proteins

appear demonstrably earlier in the developmental process than the 11S. This also can apply where there is differential expression among different genes of the same gene family. i.e. one constituent polypeptide of a 7S vicilin appearing during development before another.

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CHAPTER 2

ISOLATION OF PIGEONPEA GLOBULIN AND ITS FRACTIONATION

ABSTRACT

Globulin accounts for about 50 % of the total seed proteins in pigeonpea. It was extracted from the defatted seed meal by the alkaline extraction method and was purified on a Sepharose CL-6B column to remove the RNA contamination associated with the protein. This globulin preparation was fractionated into legumin and vicilin using a combination of three procedures namely selective precipitation, ammonium sulphate saturation and zonal isoelectric precipitation. Complete purification of legumin and vicilin required 2 and 6 cycles, respectively of the above three procedures. The purified legumin and vicilin holoprotein had molecular weights of 375 kD and 190 kD, respectively. The purified protein fractions served as the starting material in all the further characterization work.

2.1 INTRODUCTION

As described in the review of literature, seed storage proteins have been classified into four main fractions namely, water soluble, salt soluble, alcohol soluble and alkali or acid soluble. In the work involving characterization of a specific seed storage protein fraction, the first step, therefore, is to isolate this fraction in pure form free from other classes of proteins. After achieving this, the next step is to fractionate this protein further according to specific criteria. In globulins, for example, legumin and vicilin proteins are present with sedimentation coefficient of 11S and 7S, respectively and can be isolated by taking advantage of their differential salt solubility.

In pigeonpea, very little work has been carried out on seed storage proteins except for a preliminary report by Krishna et al (1,2). The latter have partially characterised pigeonpea seed proteins and have shown that about 78 % of seed proteins are salt soluble out of which 61 % are globulins. They have further reported the presence of three fractions (α, β and γ) in the globulin protein from pigeonpea seeds. The α and β-fractions correspond in their properties to legumin and vicilin respectively from other legumes. It is further reported that the γ-protein contains

more sulphur containing amino acids than legumin and vicilin, and vicilin holoprotein has a molecular weight of 180 kD.

In this chapter, I describe the isolation and purification of pigeonpea globulins and its fractions namely, legumin and vicilin.

2.2 MATERIALS AND METHODS :

2.2.1 Seed Material :

Seeds of pigeonpea (variety T-21) were procured locally from Parekh Traders, Pune. Mutated pigeonpea varieties TT-5, TT-6 and TAT-10 (by neutron bombardment) were obtained from Bhabha Atomic Research Centre, Trombay, Bombay. Cultivated varieties of pigeonpea (listed in Table 2.1) were a kind gift from Dr. Remanandan, ICRISAT, India. For developmental studies, pigeonpea pods from 0 to 50 days after flowering (DAF) were used.

2.2.2 Chemicals :

All the chemicals were obtained from SD's (India), E. Merck (India) or Glaxo (India) and were always of 'Analar' or 'Guaranteed Reagent' specifications. Sephadex G-50, Sepharose CL-6B, standard proteins (Human thyroglobulin, Lactate dehydrogenase, Malate dehydrogenase, Ovalbumin and β-lactoglobulin) as molecular weight markers were from

TABLE 2.1

LIST OF PIGEONPEA ACCESSIONS USED

Sr. No.	ICP No. Species name	Pedigree	Origin
1.	26	T-21	Uttar Pradesh
2.	28	Pusa Ageti	New Delhi
3.	32	P-230	Uttar Pradesh
4.	657	P-672	Uttar Pradesh
5.	828	P-2710	Andra Pradesh
6.	1105	P-4989	Uttar Pradesh
7.	1140	P-4-110-3-1	Uttar Pradesh
8.	1191	P-4655	Madhya Pradesh
9.	1822	P-1923	Andra Pradesh
10.	2073	P-1685	Andra Pradesh
11.	2624	ST-1	Andra Pradesh
12.	2629	Granada-1	Peru
13.	3341	P-4769-2	Uttar Pradesh
14.	3534	P-1880	Andra Pradesh
15.	4008	P-793/1	Bihar
16.	4779	NP-69	New Delhi
17.	5357	P-3328-3	Uttar Pradesh
18.	6392	JA-276	Madhya Pradesh
19.	6394	JA-277-1	Madhya Pradesh
20.	6407	P-130-4	Uttar Pradesh

Sr. No.	ICP No/ Species name	Pedigree	Origin
21.	6443	NR(WR)-5	New Delhi
22.	6889	EC-107634	Puerto Rico
23.	6896	EC-107641	Puerto Rico
24.	6925	Code No. 13	West Indies
25.	6997	DSLR-17	Madhya Pradesh
26.	7018	DSLR-38 (Baigani)	Madhya Pradesh
27.	7035	DSLR-55	Madhya Pradesh
28.	7065	DSLR-85	Madhya Pradesh
29.	7118	C-11	Maharashtra
30.	7183	PS-41	New Delhi
31.	7201	HY-3A	Andra Pradesh
32.	7220	Prabhat	New Delhi
33.	7221	Gwalior-3	Madhya Pradesh
34.	7332	ANM-11	Madhya Pradesh
35.	7365	ANM-37	Madhya Pradesh
36.	7375	ANM-44	Madhya Pradesh
37.	7385	ANM-55	Madhya Pradesh
38.	7403	ANM-73	Madhya Pradesh
39.	7484	ANM-136	Madhya Pradesh
40.	7579	ANM-231	Madhya Pradesh
41.	7599	BS-5	New Delhi

Sr. No.	ICP No./ Species name	Pedigree	Origin
42	8504	A.C.314 (white)	Guadeloupe
43.	9387	PI-395307	Andra Pradesh
44.	9467	PI-396055	Andra Pradesh
45	-	TT-5	BARC, Bombay
46.	-	TT-6	Barc, Bombay
47.	-	TAT-10	BARC,Bombay
48.	-	BDN-1	Maharashtra
49.	-	BDN-5	Maharashtra
50.	Atylosia acunitifolia	IBS-2419	Australia
51	A. albicans	JM-2337	Karnataka
52.	A. cajanifolia	PR-4876	Orissa
53.	A. goensis	JM-3501	Kerala
54.	A. grandifolia	EC-124363	Papua New Guinea
55.	A. lineata	JM-3366	Tamil Nadu
56.	A. lanceolata	EC-137220	Australia
57.	A. mollis	JM-4311	Uttar Pradesh
58.	A. platycarpa	PR-4557	Maharashtra
59.	A. scarabaeoides	PR-4516	Maharashtra
60.	A. sericea	JM-1961	Maharashtra
61.	A. volubilis	PR-4877	Orissa

Pharmacia, Sweden. Trizma base was from Sigma Chemical Co., (USA). Solvents like n-hexane and acetone were obtained locally and were always distilled prior to use.

2.2.3 Preparation of seed meal :

A seed is a storage organ of plants accumulating considerable amounts of reserve food material including lipids and fats. The fatty material must be eliminated before any of the seed storage proteins can be isolated on solubility basis. For this purpose, seed coats of all the seeds were first removed mechanically. These seeds were then finely powdered in a Remi make blender at a maximum speed for 5-15 min. and this fine powder was then packed into a column over a bed of anhydrous sodium sulfate. All lipids/fats were extracted by percolating n-hexane (approximately 5 volumes of the packed volume) according to Kartha and Sethi (3). After passing n-hexane, acetone was allowed to percolate through the column for removing all traces of n-hexane and moisture and the defatted seed powder was finally allowed to dry at 60 ^{o}C to remove the traces of acetone.

2.2.4 Fractionation of seed proteins :

To get an idea about the proportions of different seed storage protein fractions in pigeonpea, the defatted seed meal was fractionated on the basis of differential solubility as determined by Osborne (4). In this procedure,

the seed meal was successively extracted with 0.1M potassium phosphate buffer, pH 8.0, containing 1 mM PMSF (Phenyl methyl sulphonyl fluoride), 70% ethanol and 0.1N NaOH to separate the total seed proteins into salt soluble, prolamin and glutelin fractions, respectively.

For obtaining the salt soluble fraction, the pigeonpea seed meal (20 g) was extracted with 0.1M potassium phosphate buffer, pH 8.0 (200 ml) containing 0.5 M NaCl and 1 mM PMSF for 3 h with continuous agitation. It was later centrifuged at 12,000 x g for 15 min and the residue was re-extracted with the same buffer. The two supernatants containing albumin and globulin from both the extractions were pooled and designated as salt soluble protein. The globulin from this salt soluble fraction was precipitated by dialysis against 25 mM sodium acetate buffer, pH 4.7 leaving albumin in the solution. The residue obtained after extraction of the seed meal with potassium phosphate buffer, pH 8.0 contained prolamin and glutelin fractions which were further separated with 70 % ethanol and 0.1N NaOH in a similar manner. All the four protein fractions were suspended in distilled water, frozen and lyophilized. Nitrogen estimation for these fractions was done by Microkjeldahl analysis.

2.2.5 Extraction of pigeonpea globulin from seed meal :

In the initial work of extraction, an attempt was

made to isolate pigeonpea globulin from the seed meal by the acidic extraction method. In this method, the seed meal was suspended in 0.5 M NaCl (meal to solvent ratio 1:16 w/v) and the pH was adjusted to 3.5 with 6 N HCl. The extraction was continued for 4 h in cold with stirring. The slurry was centrifuged at 10,000 x g for 30 min and the supernatant was referred to as an acidic extract. This acidic extract was initially dialysed against running tap water for 2-3 h to decrease the salt concentration. Later, it was dialysed overnight against 25 mM sodium acetate buffer, pH 4.7. The precipitated globulin protein was collected by centrifugation of the dialysate at 12,000 x g for 15 min and the protein pellet was dissolved in 0.15 M sodium phosphate buffer, pH 7.2. The globulin prepared by this method, however, showed a low solubility when dissolved in 0.15 M sodium phosphate buffer, pH 7.2. It was felt that long exposure of the protein to the acidic pH was probably responsible for its poor solubility. Hence, an alternate method where the exposure of the protein to the acidic pH would be minimal was adopted.

In the alternate method, the defatted meal was blended in cold with 0.1 M potassium phosphate buffer, pH 8.0 containing 0.5 M NaCl and 1 mM PMSF for 2-5 min in a Remi mixer at top speed. The ratio of seed meal to buffer was

1:10 (w/v). The slurry was kept on a stirrer for 3 h for maximum extraction of seed globulin and was then passed through four layers of cheese cloth. The filtrate was centrifuged at 10,000 x g for 30 min and the supernatant was passed through glass wool to remove traces of low density material that floated during centrifugation. The filtrate was referred to as an alkaline extract and contained both albumin and globulin proteins. For separation of these two proteins, the alkaline extract was diluted with water so that the buffer concentration was equal to or less than 0.05 M. The pH of the solution was brought down to 4.8 with slow addition of 6 N HCl and the solution was continuously stirred. Since the isoelectric point of globulin is 4.7-4.8, it precipitates at this pH leaving albumin in solution. The precipitated globulin protein was collected by centrifugation at 12,000 x g for 15 min in cold.

2.2.6. Purification of pigeonpea globulin :

Prior to use, the globulin preparation obtained by the alkaline procedure was dissolved in 0.15 M sodium phosphate buffer, pH 7.2 and was dialyzed overnight against the same buffer. When this globulin preparation was scanned in the usual ultraviolet range, the absorption at 260 nm was higher than that at 280 nm. It was, therefore, checked for the presence of nucleic acids by agarose slab gel

electrophoresis using 1 % agarose gels in TAE (0.04 M Tris, 0.02 M acetic acid, 0.002 M EDTA) buffer, pH 8.0 at a constant current of 40 mA. During the sample preparation for electrophoresis, pigeonpea globulin (25 ug) was dispersed in three tube as follows : Tube 1 served as a control and contained only the protein; to tube 2 and 3, RNase and DNase, respectively were added and the tubes were incubated at 37 °C for 1 h; after one hour, 10 X reaction terminating buffer (50 % glycerol, 100 mM EDTA, 0.25 % bromophenol blue) was added to a final concentration of 1 X. After electrophoresis, the gels were stained with ethidium bromide (0.5 µg/ml) in dark for 10 min, visualized on a long wavelength 302 nm UV transilluminator (UV products, San Gabriel, California) and photographed with a 35 mm SLR camera (Minolta X 700 with macrophotography and zoom lens system) using a red filter. The globulin preparations showed the presence of ribonucleic acid while deoxyribonucleic acid was found to be absent. The RNA contamination was removed by passing the globulin protein through a Sepharose CL-6B (2.4 cm x 56 cm) column equilibrated with 0.05 M Tris - HCl buffer, pH 8.0 containing 0.5 M NaCl. The column was first packed with Sepharose CL-6B at a flow rate of 30 ml/h using a peristaltic pump. The globulin pellet dissolved in 0.05 M Tris-HCl buffer, pH 8.0 containing 0.5M NaCl was then loaded onto the column. The

elution was carried out at a flow rate of 24 ml/h and 12 ml fractions were collected for measurement of their absorbance at 280 nm. The void volume fractions having a molecular weight equal to or greater than 4×10^6 daltons and a maximum absorption at 260 nm were collected and the rest of the 280 nm absorbing fractions containing globulins were pooled. Globulin protein was precipitated from the pooled fraction by overnight dialysis against 25 mM sodium acetate buffer, pH 4.7. The precipitated globulin was collected by centrifugation at 12,000 x g for 15 min. The protein pellet was dissolved in 0.15 M sodium phosphate buffer, pH 7.2 and kept at 0 ^{o}C till further use. The flow sheet for purification of globulin is depicted in Fig. 2.1.

2.2.7 Fractionation of pigeonpea globulin:

After the removal of ribonucleic acid contamination, the pigeonpea globulin was fractionated into legumin and vicilin components using a combination of the following three different procedures.

Selective precipitation method :

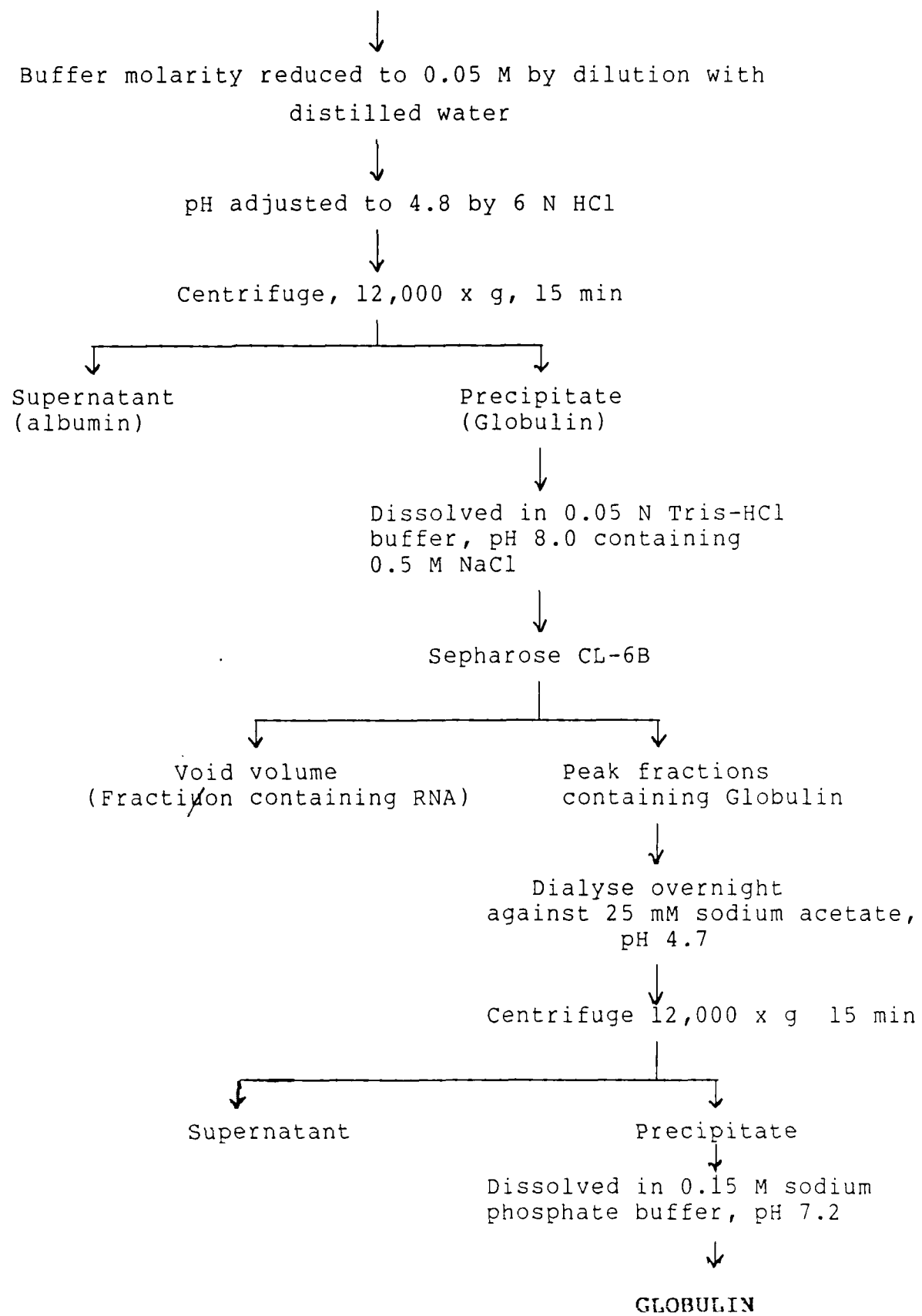
The globulins were first fractionated into legumin and vicilin by the method of Danielsson (5) and Bailey and Boulter (6) with some modifications. In this method, the protein solution was dialysed overnight against 33 mM sodium

Figure 2.1

Flow sheet for purification of globulin from defatted seed meal

FIGURE 2.1

PURIFICATION OF PIGEONPEA GLOBULIN FROM SALT SOLUBLE PROTEIN



acetate buffer, pH 4.7 containing 0.2 M NaCl. Under these conditions, the legumin fraction precipitated (since legumin requires high salt concentration to remain in solution) leaving the vicilin fraction in solution. The precipitated legumin was collected by centrifugation at 12,000 x g for 15 min and the pellet was dissolved in 0.15 M sodium phosphate buffer, pH 7.2. The supernatant containing vicilin was dialysed against 0.15 M sodium phosphate buffer, pH 7.2 for further analysis. Although the selective precipitation method yielded enriched legumin and vicilin fraction, these two fractions were always found to be cross-contaminated when checked by electrophoresis on cellulose acetate membrane (CAM).

Electrophoresis on CAM was carried out in a Beckman microzone electrophoretic apparatus essentially according to the method of Blagrove and Gillespie (7). In this method, about 2 - 4 μg protein in 0.15 M sodium phosphate buffer, pH 7.2 was applied on the membrane with the help of a sample applicator provided by Beckman Instruments Co. Electrophoresis was carried out at room temperature and at a constant voltage of 150 volt for 20 min. During electrophoresis, the current usually increased from about 4 - 5 mA to about 7 - 8 mA at the end of 20 min and was never allowed to exceed 9 mA. After electrophoresis, the membranes

were stained for about 5 min in 0.2 % (w/v) coomassie brilliant blue R-250 dissolved in methanol:acetic acid:water (3:1:6 v/v) and destained in the same solvent without the dye.

Ammonium sulphate saturation :

Since the legumin and vicilin proteins precipitate at different salt concentrations, further purification was achieved by fractionating with different ammonium sulphate saturation. Here, the protein concentrations were usually maintained around 2-3 mg/ml and fractionation was attempted by precipitations at definite concentrations of ammonium sulphate between 0-40 %, 40-65 % and 65-85 % saturation. A required amount of finely ground ammonium sulphate was added slowly to a protein solution with constant stirring at room temperature. After dissolution of ammonium sulphate, the solution was left standing for 1-2 h in cold. The precipitated protein was collected by centrifugation at 12,000 x g for 15 min. The protein pellet was dissolved in a minimum quantity of 0.15 M sodium phosphate buffer, pH 7.2 and excess ammonium sulphate was removed by dialysis against the same buffer for atleast 2 days in cold. After dialysis, the contents of the dialysis bag were kept at 0 ^{o}C till further analysis. By cellulose acetate membrane electrophoresis, it was observed that legumin and vicilin

holoproteins precipitated at 65 % and 85 % ammonium sulphate saturation, respectively. It was further noticed that these two proteins were cross contaminated with each other.

Zonal isoelectric precipitation:

In view of the cross contamination problem, further purification of legumin and vicilin was achieved by zonal isoelectric precipitation on a Sephadex G-50 column by the method of Scholz et al (8) with minor modifications. The following two buffers were used in this procedure :

Phosphate Buffer, pH 7.0

824 ml of 0.2 M disodium hydrogen phosphate and 176 ml of 0.1 M citric acid, pH 7.0

Citrate Buffer, pH 4.8

493 ml of 0.2 M disodium hydrogen phosphate and 507 ml of 0.1 M citric acid, pH 4.8.

Both buffers contained 0.02 % sodium azide.

Sephadex G-50 column (2.4 cm x 75 cm) was washed and equilibrated with citrate buffer prior to its use. The protein sample (less than 10 ml) in 0.15 M sodium phosphate buffer, pH 7.2 was placed at the top of the column. The column was eluted with the phosphate buffer, pH 7.0 and 5-6 ml fractions were collected. The column eluant was monitored continuously at 280 nm. Since the legumin and vicilin are

known to have differences in their isoelectric point, their separation on the sephadex G-50 column with phosphate buffer, pH 7.0 caused a change in the initial pH of 4.8 to a final pH 7.0. Hence pH of all the fractions was measured in a Toshniwal digital pH meter.

After each run, the column was washed with 20 ml of 0.2 N NaOH and then with 20 ml of 0.2 % sodium dodecyl sulphate (SDS) in order to remove the proteins retained on the column. The column was then equilibrated with citrate buffer before reusing.

For partial N-terminal sequence analysis , purified pigeonpea vicilin was next loaded on DEAE-Sephacel column (2 x 12 cm) in order to separate the subunits which were later used. Ion exchange fractionation of purified pigeonpea vicilin was performed on a DEAE-Sephacel column equilibrated with 50 mM Tris-HCl buffer, pH 8.6 containing 8 M urea and 0.01 M B-Mercaptoethanol at room temperature. Elution of the vicilin subunits was carried out using a 0-0.5 M NaCl gradient at a flow rate of 24 ml/hour and 4 ml fractions were collected for measurement of their absorbance at 280 nm. Peak fractions containing the subunits were pooled, dialysed extensively against distilled water and used for partial N-terminal sequencing as described in chapter 3.

2.2.8 Molecular weight determination :

For molecular weight determination of purified legumin and vicilin holoproteins, gel filtration was performed on a Sepharose CL-6B column (1.8 x 60 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 8.0 containing 0.5 M NaCl. Prior to determination of the molecular weight of legumin and vicilin, the column was first calibrated using standard molecular weight markers (Human thyroglobulin, 669 kD; Lactate dehydrogenase, 140 kD; Malate dehydrogenase, 90 kD; Ovalbumin, 44 kD; and β-lactoglobulin, 18.4 kD). The column was eluted at a flow rate of 12 ml/h and 1.2 ml fractions were collected. The absorbance of all the column fractions was measured at 280 nm. The elution volume (Ve) for each of the standard protein was determined by measuring the volume of fractions from the first tube to the peak fraction. The void volume (Vo) was determined using blue dextran 2000. A calibration graph was obtained by plotting elution volume against the molecular weight of the protein.

Molecular weights of legumin and vicilin holoproteins were determined by loading separately 5 mg of each protein on the pre-calibrated column. The elution volume was determined as described above and the exact molecular weights were calculated from the standard graph.

2.3 RESULTS :

2.3.1 Relative percentage of pigeonpea seed fractions :

The result of fractionation of pigeonpea seed meal into different solubility fractions are summarised in Table 2.2. The seed meal has 3.6 % nitrogen corresponding to 22 % of the total protein (% nitrogen x 6.25). Out of the total seed protein (i.e. 22 %), about 69 % is extracted in 0.1 M phosphate buffer, pH 8.0 containing 0.5 M NaCl and 1 mM PMSF. This fraction includes both the albumin and globulin proteins. From the buffer extracted protein, which is also designated as salt soluble protein, the globulin protein is specifically precipitated by dialysis against sodium acetate buffer, pH 4.7 at a low ionic strength of 25 mM. Under such conditions, the albumin protein remains soluble and is easily separated from the precipitated globulin protein by centrifugation. The salt soluble fraction consists of 75 % globulin and 25 % albumin protein. When the amount is expressed on total seed protein basis, globulin and albumin account for about 53 % and 17 %, respectively. The glutelin protein, soluble in dilute alkali, is about 23 % of the total seed protein while about 2 % of the seed protein is soluble in alcohol, representing the prolamin fraction.

2.3.2 Fractionation of pigeonpea globulin into legumin and

TABLE 2.2

ANALYSIS OF PIGEONPEA SEED FRACTIONS

Fraction	Solubility	Amount of protein Extracted (mg)	Relative Proportion (%)
Albumin	Water soluble	80.0 ± 5.0	16.8 ± 0.15
Globulin	Salt solubble	250.0 ± 15.0	52.5 ± 0.46
Prolamine	Alcohol soluble	10.0 ± 0.04	2.11 ± 0.01
Glutelin	Alkali soluble	110.0 ± 9.4	23.15 ± 0.29
Residue	-	25.0 ± 2.5	5.26 ± 0.16

The values represent averages of three determinations.

vicilin :

As described in materials and methods and in the flow sheet (Fig. 2. 1), the pigeonpea globulin was isolated by alkaline extraction procedure and was freed from RNA contamination by sepharose CL-6B column chromatography. This globulin was initially fractionated into legumin and vicilin by selective precipitation method. Here, dialysis of globulin against 33 mM sodium acetate buffer, pH 4.7 containing 0.2 M NaCl led to the precipitation of legumin, leaving vicilin fraction in the supernatant. Although this procedure gave enriched legumin and vicilin fractions in the precipitate and the supernatant respectively, it did not yield pure legumin or vicilin fractions as judged by cellulose acetate membrane electrophoresis. The two fractions were always cross-contaminated even after three or four cycles of selective precipitation method. Another problem with the selective precipitation procedure was that the solubility of the legumin and vicilin proteins was very poor in sodium phosphate buffer, pH 7.2. Hence these two proteins were further purified by ammonium sulphate precipitation. Here, it was found that the legumin fraction precipitated at 65 % saturation while vicilin at 85 % saturation. However, as in the previous case, this procedure also did not yield pure legumin and vicilin fractions free from each other. Hence further purification was achieved

using zonal isoelectric precipitation. The elution pattern of pigeonpea legumin on a zonal isoelectric precipitation column is shown in Fig. 2.2. Since the pI of vicilin is less than that of legumin, the first peak in this figure is of vicilin while the second peak is that of legumin. Legumin and vicilin proteins were purified to homogeneity by repeated cycles of zonal isoelectric precipitation. When the vicilin was subjected to zonal isoelectric precipitation and the elution pattern was monitored at 280 nm, there were two peaks of almost equal size (Fig. 2.3). Very little protein was detected in the second peak containing legumin when measured by Folin-Lowry method. The first peak, containing vicilin when rechromatographed on the zonal isoelectric precipitation column gave a single peak without any trace of the retarded legumin peak (Fig. 2.3). Purification of vicilin usually required two cycles of zonal isoelectric precipitation column chromatography which is summarised in Fig. 2.4. The method followed for purification of legumin is outlined in Fig. 2.5. The legumin protein was enriched by initial dialysis against sodium acetate buffer, pH 4.7 containing 0.2 M NaCl since legumin can be precipitated at low salt concentration of 0.2 M. Under these conditions, the precipitated legumin had vicilin contamination (Fig. 2.6, bold line) which could be removed by repeated zonal

Figure 2.2

Elution profile of pigeonpea seed globulin on a zonal isoelectric precipitation column. The column (2.4 x 75 cm) was packed with Sephadex G-50 in citrate buffer, pH 4.8 to a bed height of 67 cm. The elution was carried out with phosphate buffer, pH 8.0 at a flow rate of 55 ml/hr. Fractions of 5.5 ml were collected and absorbance at 280 nm (●----●) as well as pH (o----o) were measured. Peak I represents vicilin fraction while peak II is legumin fraction.

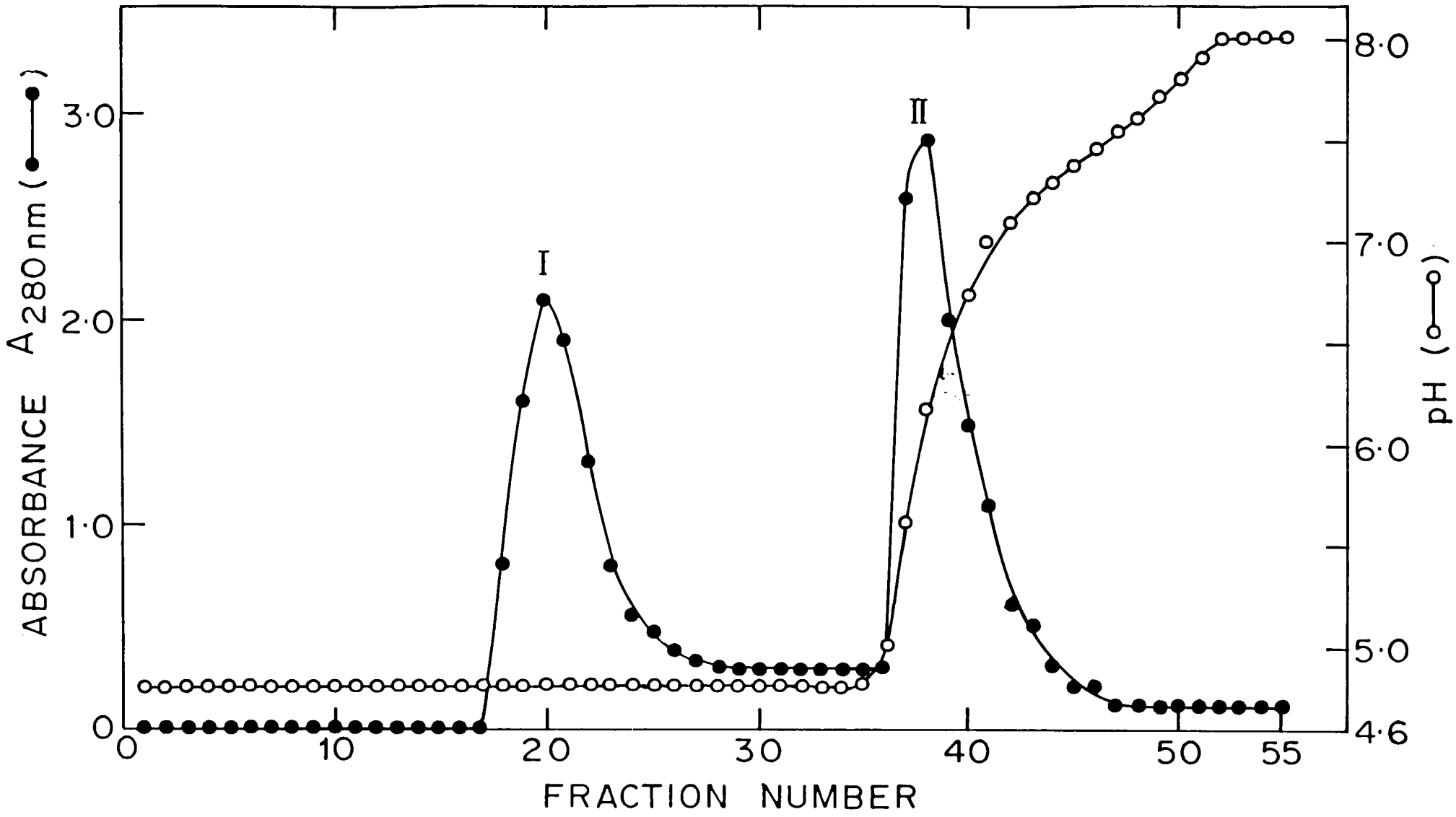

ABSORBANCE A280nm (●——●)
pH (○——○)
I
II
3·0
2·0
1·0
0
8·0
7·0
6·0
5·0
4·6
0
10
20
30
40
50
55
FRACTION NUMBER

FIG . 2·2

Figure 2.3

Elution profile of pigeonpea legumin (enriched in vicilin fraction by isoelectric precipitation) from pigeonpea seed meal on a zonal isoelectric precipitation column (2.4 x 75 cm). Other details are as in Fig. 2.3. The % transmission at 280 nm was monitored in a Uvicord. The broken line indicates the elution pattern when the first peak was rechromatographed. The horizontal bar indicates the fractions pooled to recover the protein for further analysis. Peak I indicates vicilin fraction while peak II indicates legumin fraction.

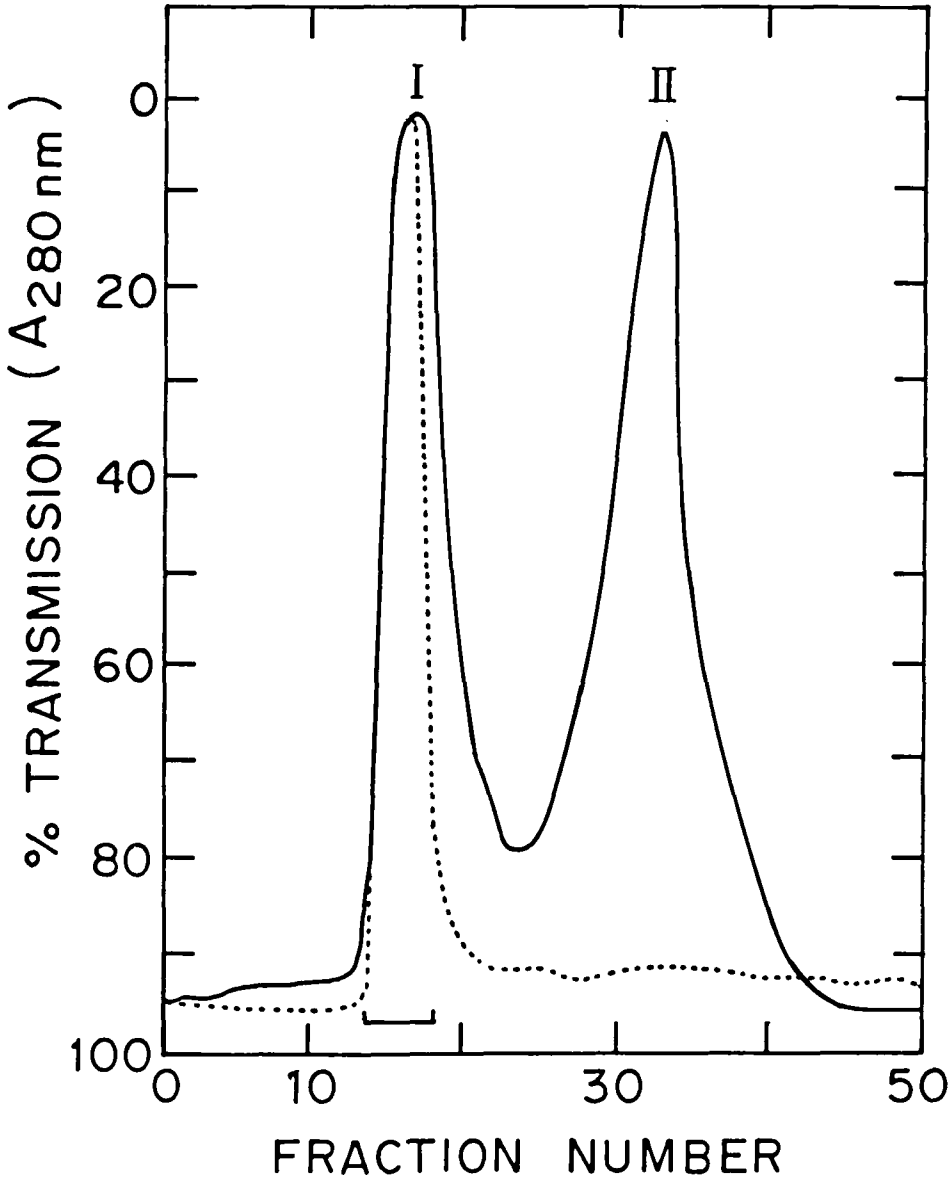

I
II
0
20
40
60
80
100
% TRANSMISSION (A280 nm)
0
10
30
50
FRACTION NUMBER

FIG . 2·3

Figure 2.4

Flow sheet for purification of vicilin from purified globulin.

FIGURE 2.4

PURIFICATION OF VICILIN FROM GLOBULIN

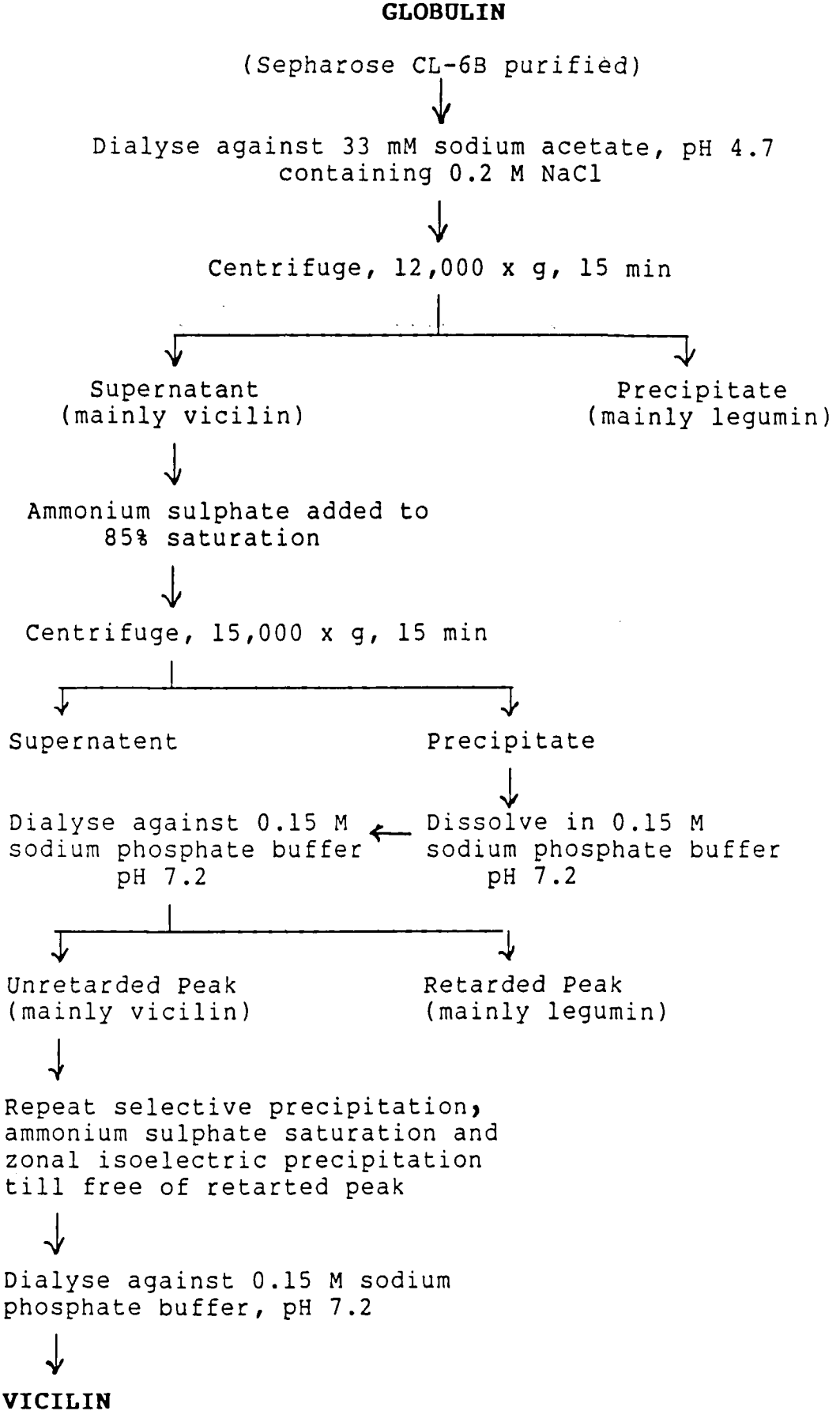
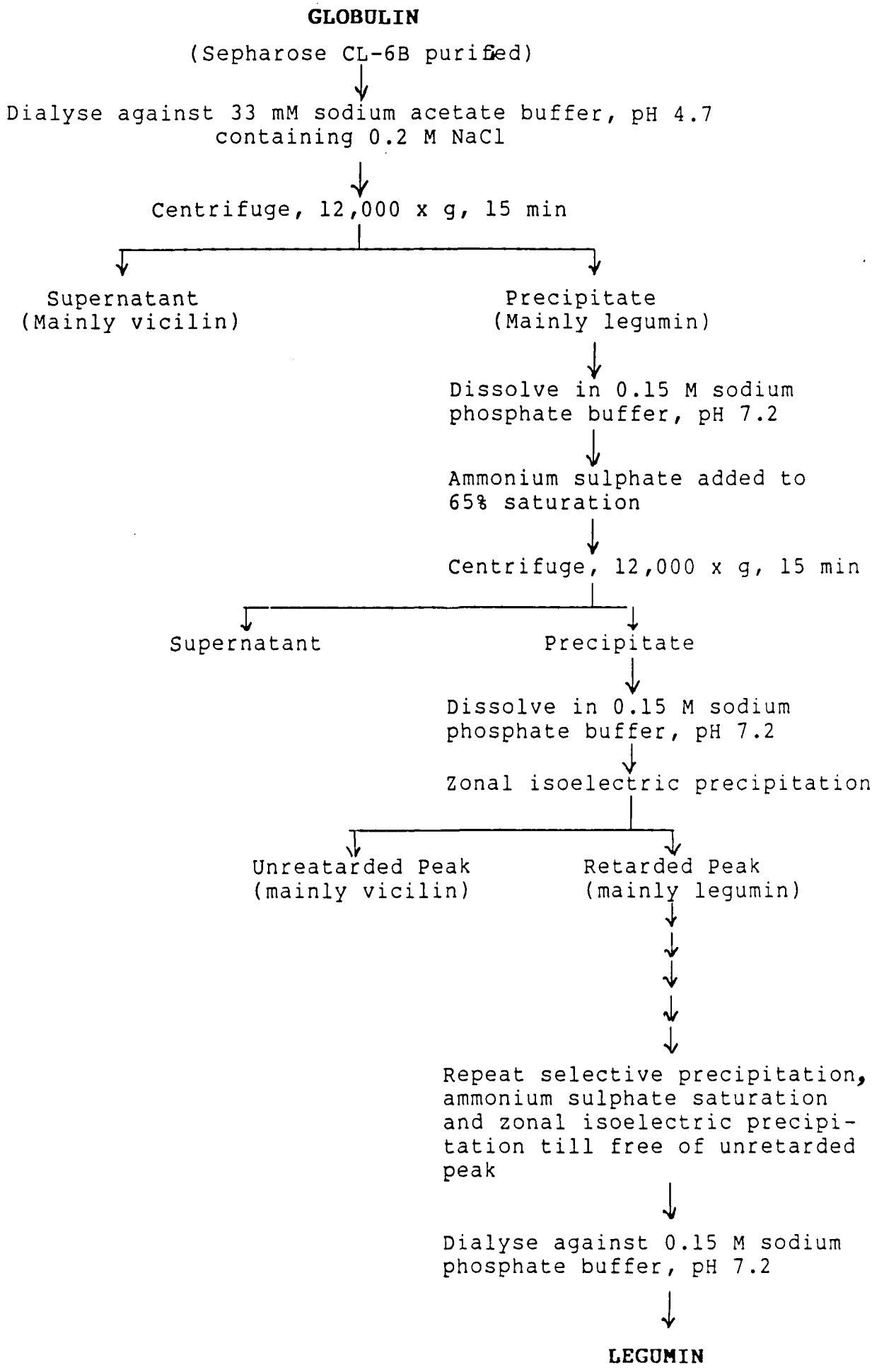


Figure 2.5

Flow sheet for purification of legumin from purified globulin.

FIGURE 2.5

PURIFICATION OF LEGUMIN FROM GLOBULIN



isoelectric precipitation and selective precipitation of the retarded legumin peak at 65 % ammonium sulphate saturation. The zonal isoelectric precipitation was repeated till there was no unretarded vicilin peak (Fig. 2.6, dotted line). This usually required about 5 - 6 cycles of zonal isoelectric precipitation column chromatography. The legumin prepared by this method was readily soluble in 0.15 M sodium phosphate buffer, pH 7.2, unlike the legumin prepared by selective precipitation method.

2.3.3 Molecular weight of legumin and vicilin holoproteins:

Molecular weight of the legumin and vicilin holoproteins was estimated by gel filtration chromatography on a Sepharose CL-6B column. The column was previously calibrated using standard proteins of known molecular weight and the standard graph for the sepharose CL-6B column is shown in Fig. 2.7. Here, a single symmetrical peak is observed for each of the purified legumin and vicilin fraction. The elution volumes for legumin (Fig. 2.8) and vicilin (Fig. 2.9) are 52 and 58 ml, respectively. Based on these volumes, the molecular weights of the proteins with respect to the standard proteins used are estimated to be 375,000 and 190,000, for legumin and vicilin respectively.

Figure 2.6

Elution profile of globulin (enriched in the legumin fraction by isoelectric precipitation) from pigeonpea seed meal on a zonal isoelectric precipitation column (2.4 x 75 cm). Other details are as in figure 2.2. The % transmission at 280 nm was monitored in a Uvicord. The broken line indicates the elution pattern after six cycles of chromatography of the second peak. The horizontal bar indicates the fractions pooled for further analysis. Peak I indicates vicilin fraction while peak II indicates legumin fraction.

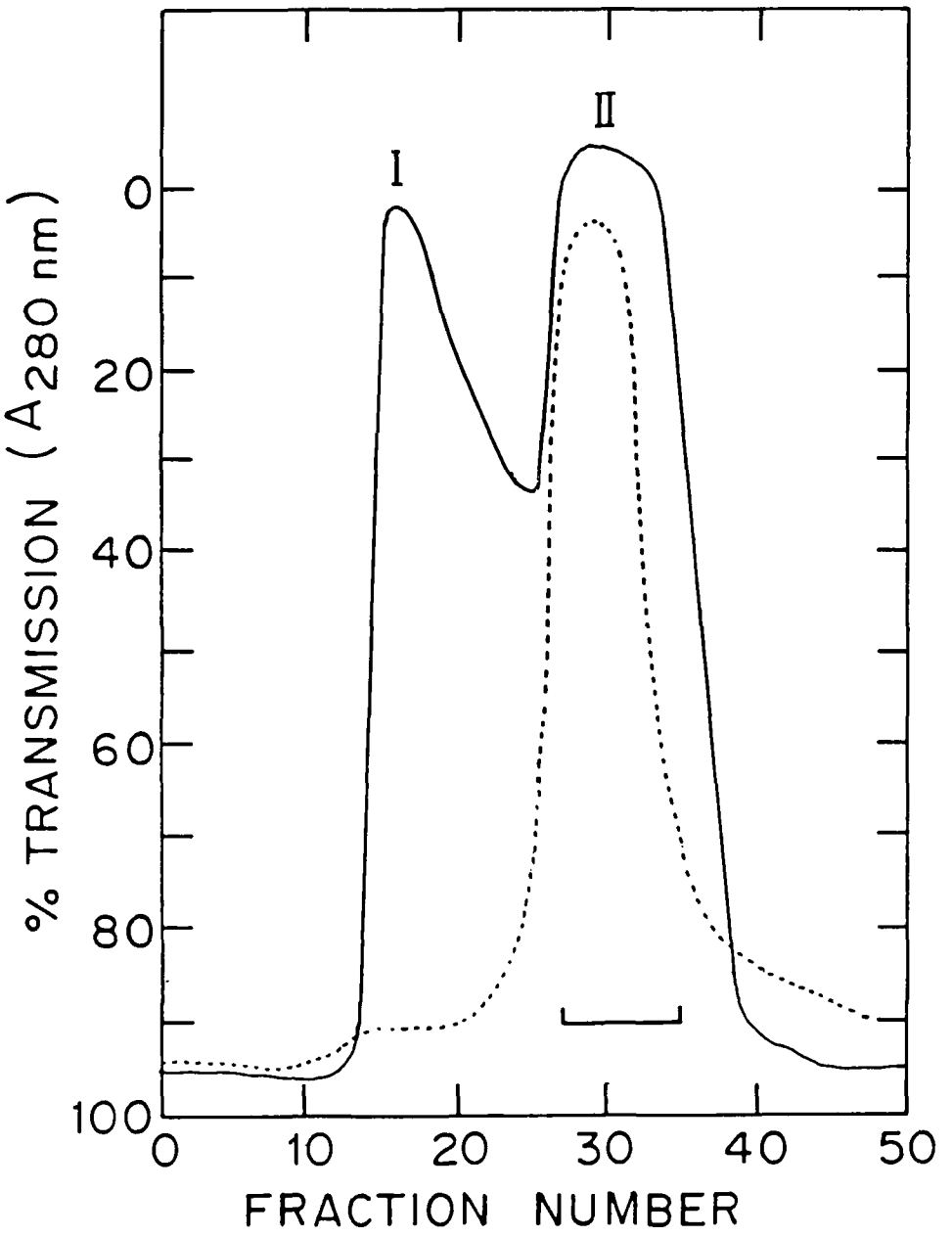

I
II
0
20
40
60
80
100
% TRANSMISSION (A280 nm)
0
10
20
30
40
50
FRACTION NUMBER

FIG. 2·6

Figure 2.7

Calibration of a Sepharose CL-6B gel filtration column (1.8 x 60 cm) for molecular weight estimations. Gel chromatography was performed in 0.05 M Tris-HCl buffer, pH 8.0 containing 0.5 M NaCl. The column was eluted at a flow-rate of 12 ml/hr and 1.2 ml fractions were used for calibrating the column. The numbers indicate (1) Blue dextran 2000 (2000 kD) (2) Human thyroglobulin (669 kD) (3) lactate dehydrogenase (140 kD) (4) Malate dehydrogenase (80 kD) (5) Ovalbumin (44 kD) and (6) B-lactoglobulin (18.4 kD).

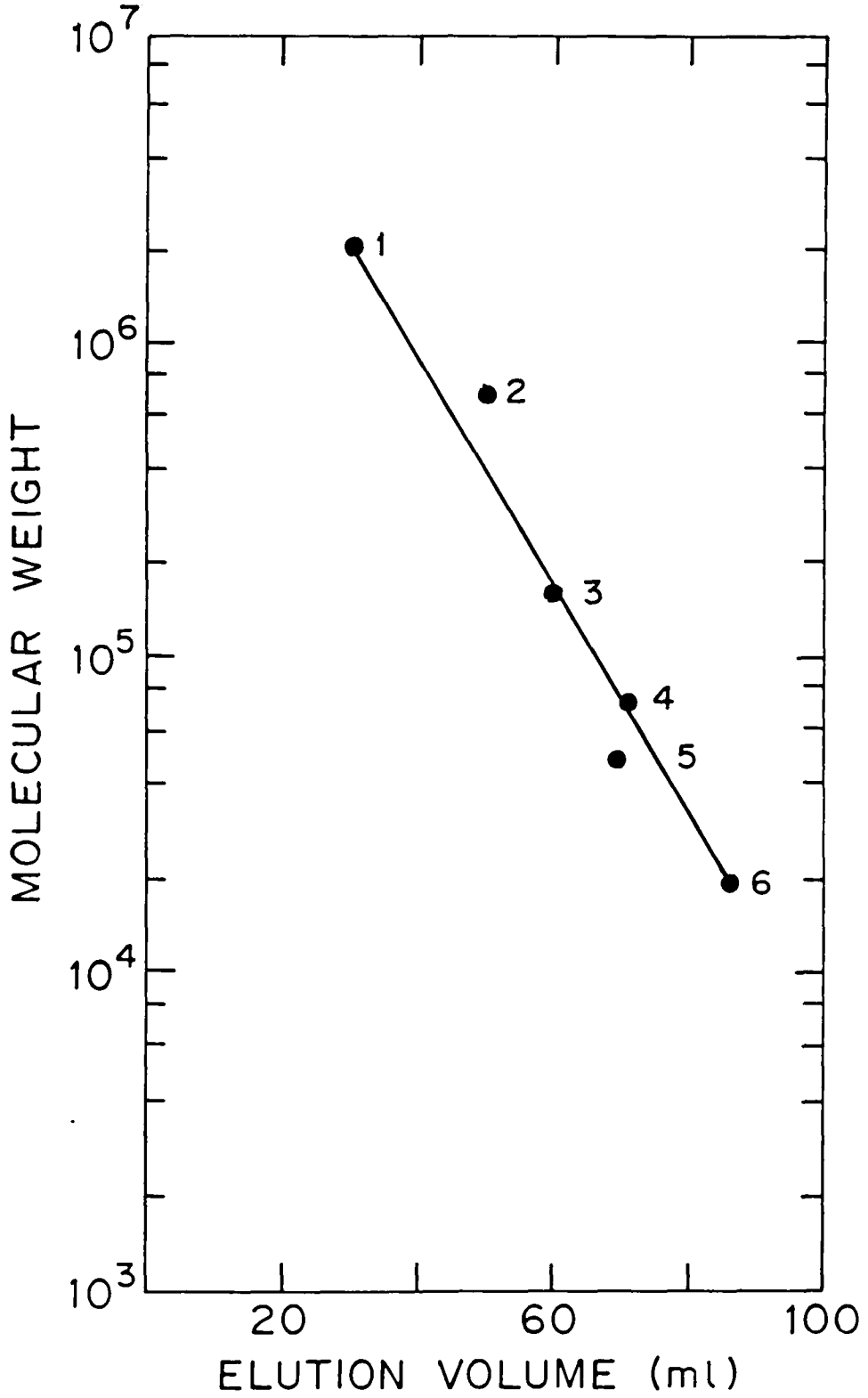

10^7
10^6
10^5
10^4
10^3
MOLECULAR WEIGHT
1
2
3
4
5
6
20
60
100
ELUTION VOLUME (ml)

FIG . 2·7

Figure 2.8

Elution profile of purified legumin from a Sepharose CL-6B column (1.8 x 60 cm). The column was equilibrated and eluted with 0.05 M Tris-HCl buffer, pH 8.0 containing 0.5 M NaCl. The column was eluted at a flow rate of 12 ml/h and 1.2 ml fractions were collected. Protein in the fractions was monitored at 280 nm.

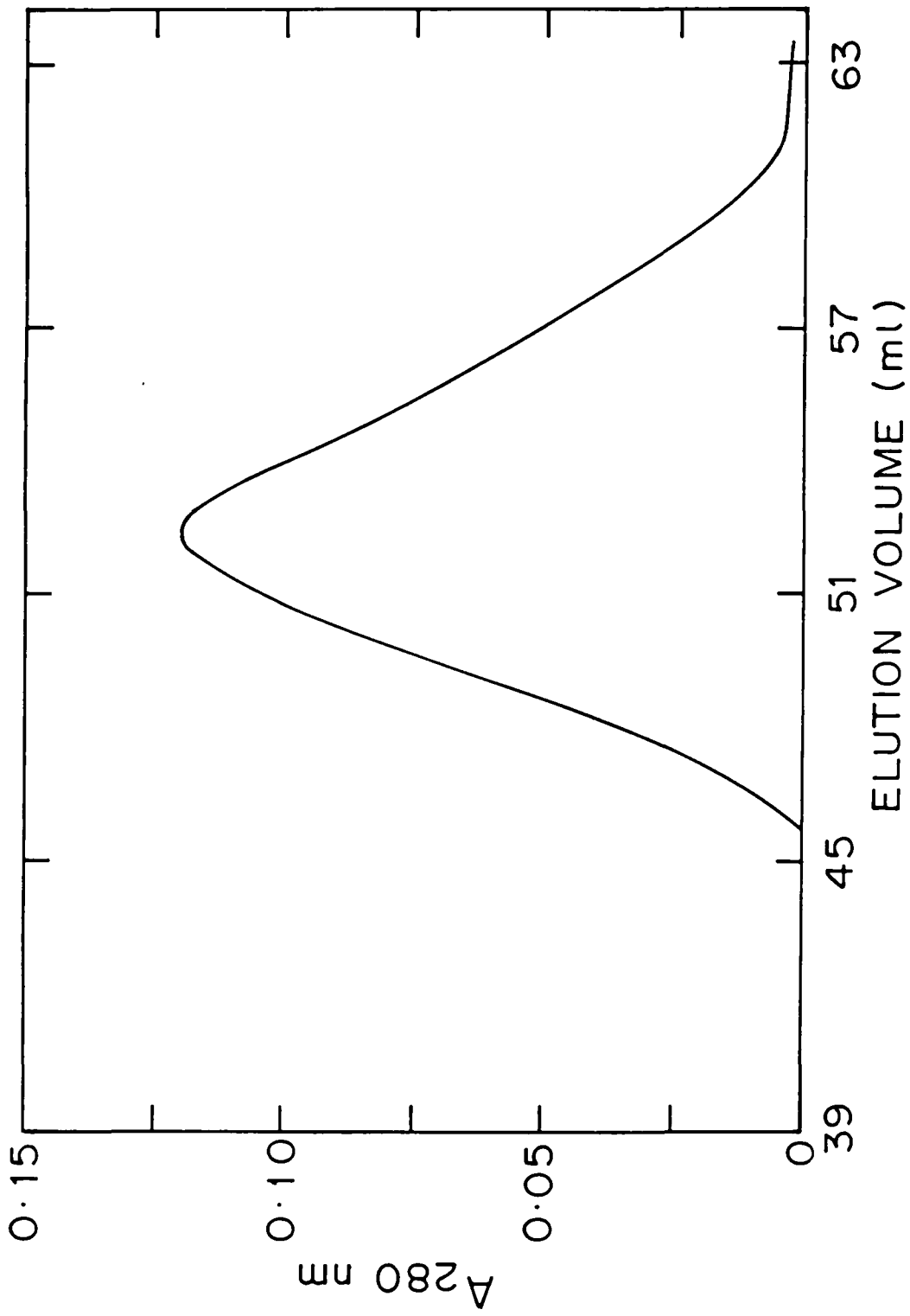


FIG. 2·8

Figure 2.9

Elution pattern of purified vicilin from a Sepharose CL-6B column (1.8 x 60 cm). The column was equilibrated and eluted with 0.05 M Tris-HCl buffer, pH 8.0 containing 0.5 M NaCl. The column was eluted at a flow rate of 12 ml/hr and 1.2 ml fractions were collected. Protein in the fractions was measured at 280 nm.

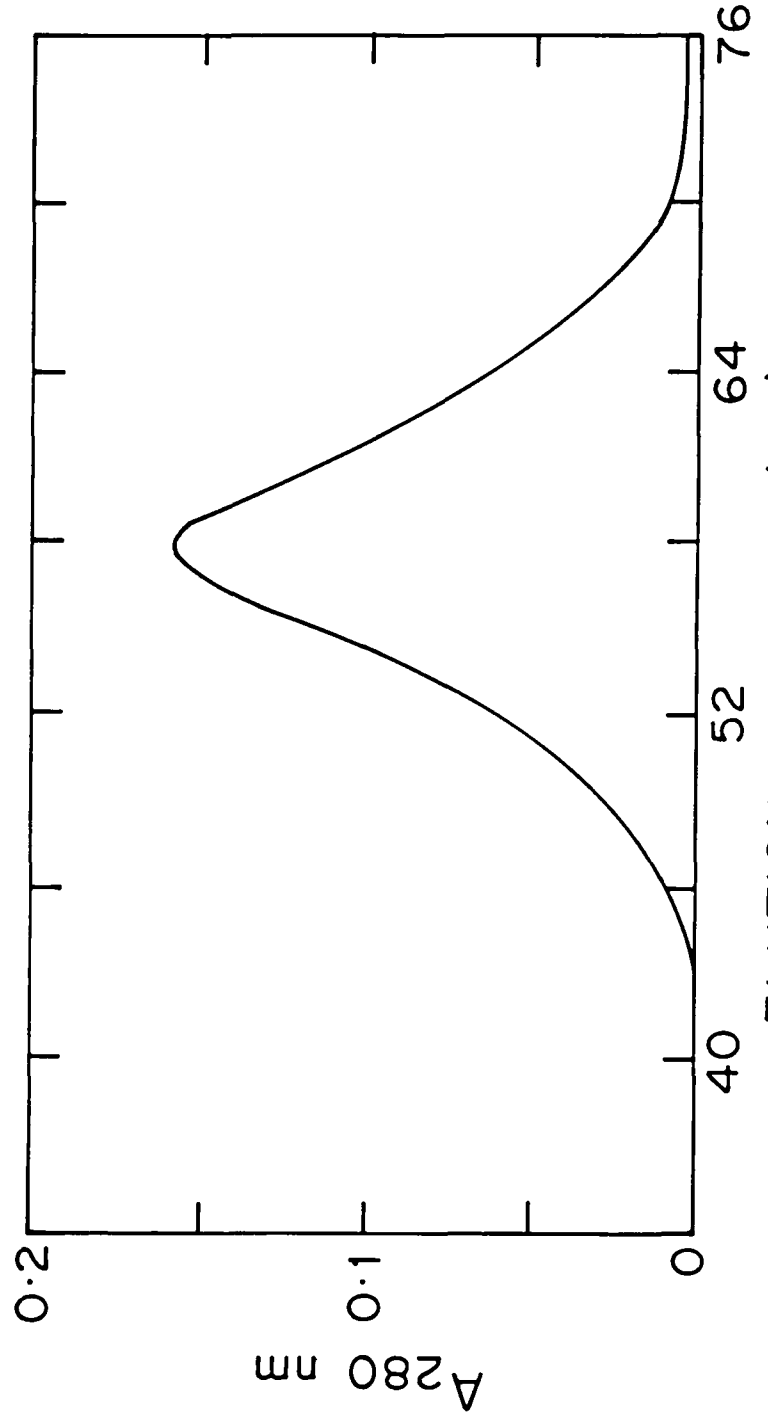


FIG. 2·9

2.4 DISCUSSION :

In the earlier work on extraction of seed globulin, Osborn (9) used first water to isolate albumin and then dilute salt solutions or buffers at alkaline pH containing salts to isolate globulin. Later, Danielsson (10) extracted seed proteins with buffered sodium chloride and these were precipitated at 70 % saturation of ammonium sulphate. When the protein solution was dialyzed, seed globulin precipitated leaving albumin in the supernantent. When the pigeonpea seed meal was extracted with water, appreciable amount of globulin was also solubilised. This could be because of the high mineral content in the seeds. The minerals with water could form buffers enabling the solubilisation of the globulin protein. A similar observation has been made in pea (11) where 75 % to 85 % of total nitrogen was solubilised when 1 part of seed meal was extracted for 30 min. with 10 to 12 parts of water at room temperature. Here, it was inferred that the seed contained the naturally occuring salts in sufficient amounts to promote initial solubilization of the globulin along with albumin. In the present study, proteins which are soluble in alkaline buffers but insoluble betwen pH 4.7 to 4.8 under low ionic strength (25 - 30 mM) were isolated as globulins.

The isolation of globulin and its fractionation into

pure legumin and vicilin are the two important steps before these proteins are characterized. Based on specific differences in the properties of these two proteins, specific fractionation and purification procedures have been designed. Table 2.3 lists the techniques commonly used for the purification of some of the major native storage proteins in legumes.

In the present work, pure legumin and vicilin components are isolated from pigeonpea globulin by using a combination of three procedures namely : selective salt precipitation, ammonium sulphate saturation and zonal isoelectric precipitation. The pigeonpea globulin was fractionated into legumin and vicilin first by selective salt precipitation. This method, however, did not give pure fractions. The precipitated legumin had very poor solubility. A similar observation is reported for soybean globulin (12). One of the reasons for poor solubility of the precipitated protein could be the formation of polymers with disulphide bridges (13). Derbyshire et al (14) have suggested that this could be due to the dissociation of globulin brought about by high acidity in certain local areas. Further purification of legumin and vicilin was therefore achieved by using ammonium sulphate saturation procedure. Here, advantage is taken of the fact that both

TABLE 2.3

METHODS FOR PURIFICATION OF SOME STORAGE PROTEINS

	Protein	Species	Methods	References
1.	Legumin	11S protein from *Pisum sativum*, *Pisum fulvum*, *Vicia faba*, *Vicia sativa*, *Phaseolus vulgaris*, *Vigna radiata* and *Vigna unguiculata*.	Ammonium sulphate fractionation, density gradient centrifugation, gel filtration, immunoaffinity chromatography, ion-exchange chromatography, preparative gel electrophoresis, zonal isoelectric precipitation.	17, 18 19 - 24
2.	Glycinin	11S protein from *Glycine max*	Con A chromatography, cryoprecipitation, density gradient centrifugation, fractional isoelectric precipitation gel filtration, ion-exchange chromatrography	25 - 31
3.	Vicilin	7S protein from *Pisum sativum* *Vicia faba*, *Vigna radiata*, *Conavalia ensiformis*, also used for Phaseolin	Ammonium sulphate fractionation, Con A chromatography, gel filtration, ion-exchange chromatography, isoelectric precipitation.	16, 32 - 36

..2..

(1)	(2)	(3)	(4)
4. Convicilin	7S protein from Pisum and Vicia faba.	Ammonium sulphate fractionation, gel filtration, ion-echange chromatography, zonal isoelectric precipitation	37, 38
5. β-Conglycinin	7S protein from Glycine max that dimerizes at low ionic strength	Ammonium sulphate fractionation, density gradient centrifugation, differential ionic solubility, gel filtration, ion-exchange chromatography	26, 29, 39 - 41
6. Phaseolin	7S protein from Phaseolus vulgaris that associates or dissociates in response to changes in pH or ionic strength	Affinity chromatography, ammonium sulphate fractionation, gel filtration, ion-exchange chromatography, ionic precipitation, preparative electrophoresis.	42 48

legumin and vicilin precipitate at different saturation levels of ammonium sulphate. The main advantage of this method is that the protein pellet obtained had a good solubility, unlike the selective salt precipitation method. The ammonium sulphate concentrations required to precipitate legumin (65 % saturation) and vicilin (85 % saturation) agree well with those reported for other legume proteins. Ammonium sulphate fractionation of globulin also did not yield pure fractions of legumin and vicilin as they were cross-contaminated. Hence further purification was carried out using zonal isoelectric precipitation. Here, legumin was found to be cross-contaminated with vicilin (as evident from Fig. 2.3). In order to obtain pure preparations, repeated cycles of zonal isoelectric precipitation and precipitation of legumin at 65 % ammonium sulphate saturation were used till the unretarded peak containing vicilin eluting from the zonal isoelectric precipitation column was minimal. This took about 6 cycles of zonal isoelectric precipitation (Fig. 2.6).

After obtaining the holoproteins in pure form, their molecular weights were determined by Sepharose CL-6B. The molecular weight of legumin holoprotein of pigeonpea is estimated to be about 375,000 and agrees well with those reported for other legumes (14). The molecular weight of

vicilin holoprotein from pigeonpea was estimated to be 190,000 and this value agrees well with those of vicilins from other legumes which fall in the range of 105,000 - 204,000 (14). The G1 or phaseolin protein from Phaseolus vulgaris at neutral pH had a molecular weight of 105,000 and formed a tetramer of molecular weight 560,000 between pH 3.4-6.6 (15). On a gel filtration column, the pigeonpea vicilin was eluted as a single symmetrical peak (section 2.9). Usually in other legumes, vicilin has been shown to be a very heterogenous protein made up of a number of different proteins. For example, in Pisum the vicilin fraction on a gel filtration column of sephacryl S-200 has been separated into three holoproteins of molecular weight 190,000; 175,000 and 160,000 (16). It thus appears that pigeonpea vicilin is rather a simple protein.

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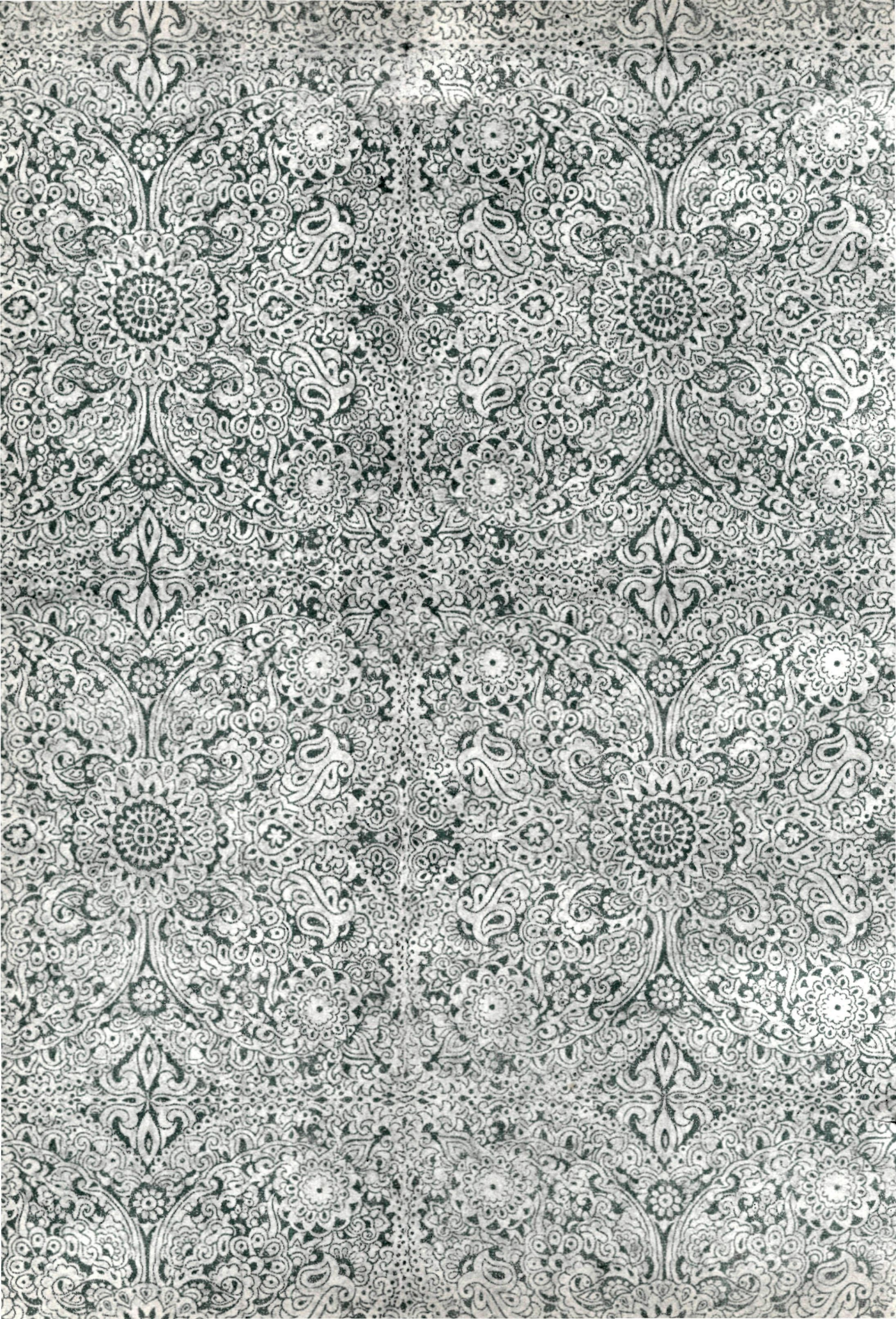
CHAPTER 3

PARTIAL BIOCHEMICAL AND BIOPHYSICAL CHARACTERIZATION OF PIGEONPEA GLOBULIN



ABSTRACT

Th. 7335

Pigeonpea legumin and vicilin holoproteins consisted of 4 subunits (Mr 72,400, 66,000, 60,000 and 56,000) and 2 subunits (Mr 72,000 and 57,000), respectively and their amino acid composition showed the presence of high amounts of glutamic acid and aspartic acid as compared to other amino acids. When these proteins were digested with trypsin, they showed the presence of 19, 16 and 14 peptides as against 12, 14 and 11 peptides which were estimated from their amino acid composition. This probably may be due to incomplete or nonspecific fragmentation. The N-terminal amino acid sequence was Gly-Ala-Arg-Val-Asp-Gln-Glu for purified vicilin subunit 1 (72 kD) and Thr-Thr-Cys-Met-Glu-Ser-Gly for purified vicilin subunit 2 (57 kD). Fluorescence studies showed that pigeonpea vicilin was unusually stable to both urea (8M) and guanidine hydrochloride (6M). Circular dichroism spectra of legumin and vicilin indicated the occurrence of a predominant B-pleated sheet structure in these protein molecules. Th. 7335

3.1 INTRODUCTION :

During the period of last 20 years, extensive work has been carried out on seed storage proteins in legumes and cereals. As mentioned earlier, globulin is the major storage protein in legumes. This protein fraction in turn consists of two components namely legumin and vicilin. These two proteins have been characterized in detail in a few legumes such as soybean, frenchbean, pea and broadbean. For example, information is available about the complete amino acid sequence, secondary structure and crystal structure of phaseolin which is a major vicilin type of protein in frenchbean.

In case of pigeonpea, very little detailed work was carried out on its storage protein except for the two reports by Gopalkrishna et al. (1) and Krishna and Bhatia (2) who have shown that pigeonpea vicilin consists of two subunits of molecular weight of 72 kD and 57 kD and that the γ-protein has a molecular weight of 90 kD. I, therefore, undertook the further work of characterization of legumin and vicilin components of pigeonpea. In the previous chapter, I have already described the isolation and purification of legumin and vicilin components. In this chapter, I describe SDS-PAGE analysis, amino acid composition and peptide mapping, partial NH_2 - terminal sequencing, fluorometric analysis and circular dichroism studies of these two proteins

3.2 MATERIALS AND METHODS:

3.2.1. Chemicals :

All the chemicals were obtained from SD's (India), E. Merck (India) or Glaxo (India) and were always of 'Analar' or 'Guaranteed Reagent's specifications. Acrylamide, obtained from SD's, was recrystallized prior to use. Certain chemicals like N,N'-methylene-bisacrylamide,TEMED (N,N,N',N'-tetramethyl ethylene diamine), ammonium per sulphate, Trizma base, Glycine, Coomassie brilliant blue R-250, trypsin, ethyleneimine, ninhydrin, DABITC (Dimethyl aminobenzene isothiocyanate), PITC (Phenyl isothiocyanate) and Guanidine hydrochloride were obtained from Sigma Chemical Co., (USA). Standard proteins as molecular weight markers i.e. α-lactalbumin, soybean trypsin inhibitor, carbonic anhydrase, phosphorylase b, bovine serum albumin, and ovalbumin were from Pharmacia (Sweden). β-Mercaptoethanol and trifluoroacetic acid (TFA) were from Fluka (Switzerland). Standard amino acid mixture was from Beckman (England). Whatman paper number 3 was from Whatman (England). For sequencing studies, pyridine was distilled three times over : KOH (10 g/l), ninhydrin (1 g/l) and KOH (10 g/l). TFA was redistilled over $CaSO_4 . 5H_2O$ (10 g/l). Polyamide sheets were obtained from Scheicher and Schuell, (Germany).

3.2.2 SDS-Polyacrylamide gel electrophoresis :

For subunit analysis, total globulins, legumin and vicilin were electrophoresed in 12.5% polyacrylamide gels according to Laemmli (3). In these experiments, Tris - glycine buffer, pH 8.3 (0.025 M Tris, 0.192 M glycine containing 0.1% SDS) was used as electrophoresis buffer and 0.125 M Tris-HCl buffer, pH 6.8, containing 0.1% 2-mercaptoethanol, 0.1% SDS, 10% glycerol (v/v) and 0.05% bromophenol blue was used as sample buffer. About 25 ug of pigeonpea globulin, legumin and vicilin were loaded on polyacrylamide gels and electrophoresed at a constant voltage (40 v) for 16 h till the tracking dye reached 1 cm from the bottom of the gel. After electrophoresis, the gels were fixed in 20% TCA (trichloroacetic acid) and the protein bands visualized by staining with coomassie brilliant blue R-250 (0.25% in ethanol : acetic acid:water, 4:1:5 v/v). The excess stain was removed with the destaining solution (ethanol:acetic acid:water, 4:1:5 v/v). α-Lactalbumin (14.4 kD), soybean trypsin inhibitor (20.1 kD), carbonic anhydrase (30 kD) ovalbumin (44 kD), BSA (Bovine serum albumin, 67 kD) and phosphorylase (94 kD) were used as molecular weight markers in these electrophoresis experiments.

3.2.3 Amino Acid Composition Analysis :

Globulin, legumin and vicilin were next analysed

for their amino acid composition. The first step in this experiment was the hydrolysis of purified proteins (1.5 mg each) in 6M HCl at 110 °C for 24, 48 and 72 h in evacuated sealed pyrex tubes in a block heater so as to hydrolyse proteins into a mixture of free amino acids as described by Moore and Stein (4). The hydrolysate was then evaporated to dryness in a vacuum desiccator. The residual HCl in the hydrolysate was removed by dissolving the residue in about 1.0 ml of deionized water and then drying in a vacuum desiccator. This step was repeated twice to ensure complete removal of HCl. The residue was then dissolved in citrate buffer, pH 2.2 and aliquots were analysed using Beckman automatic amino acid analyser (model 120 B) by the method of Spackman et al (5).

Since proline, threonine, serine and tyrosine are partially destroyed during acid hydrolysis a correction was applied by extrapolating to zero hydrolysis time from the data for 24, 48 and 72 h hydrolysis. Half cystine content was determined as cysteic acid after oxidation with performic acid (6) followed by hydrolysis and amino acid analysis as above.

Tryptophan is completely destroyed during the acid hydrolysis and thus could not be detected in the amino acid analysis run. The quantity of tryptophan was, therefore,

determined according to Goodwin and Morton (7) by measuring the absorbance of the protein at 294 nm, 257 nm and 280 nm.

3.2.4 Peptide Mapping :

Prior to tryptic digestion, the free sulfhydryl and disulfide bonds in globulin, legumin and vicilin were first aminoethylated according to the procedure of Jones (8) to facilitate more digestion by trypsin. During aminoethylation, 100 mg of protein was mixed with 2.6 g of deionized urea, 0.3 ml of 5% EDTA and 4.5 ml of 1 M Tris-HCl buffer, pH 8.5. This mixture was diluted to 7.5 ml with water and was treated with 0.1M of β-mercaptoethanol. After 30 min, 0.2 ml of ethyleneimine was added and the solution was incubated for another 30 min. The aminoethylated protein was thoroughly dialyzed first against tap water for 48 h and then against distilled water for 24 h with 5-6 changes to remove urea. During dialysis, the modified protein precipitated and the precipitate was collected by centrifugation, dried in vacuum over NaOH pellets and stored at -20 ^{o}C.

The aminoethylated protein was digested with trypsin to hydrolyse the total protein into smaller fragments. Here, 10 mg S-2-AE-protein was suspended in 5 ml of water and the pH of the solution was adjusted to 8.1. The protein was completely denatured by heating at 80 ^{o}C for 8 min and the

solution was kept in water bath at 37°C with constant stirring. The enzyme trypsin was then added to the solution in an enzyme to protein ratio of 1:50 by weight and the pH of the reaction mixture was maintained at 8.1 (optimum pH for trypsin) by adding 0.01N NaOH. The reaction was stopped after three hours by lowering the pH to 6.4 with 0.05 N HCl and heating the hydrolysate in a boiling water bath for two minutes to completely denature the residual trypsin. The solution was centrifuged and the clear supernatant (hydrolysate) was preserved at -20°C.

Finger printing of peptides generated from AE-globulin, legumin and vicilin was carried out according to the procedure described by Ingram (9). The technique involved high voltage paper electrophoresis in one direction followed by ascending chromatography in a perpendicular direction. The paper electrophoresis was carried out in Michl's buffers (10) i.e. pyridine : glacial acetic acid : water in the ratio of 10:0.4:90 v/v at 1000 volts for 2 h. After electrophoresis, the Whatman 3 paper was allowed to saturate in a chromatographic chamber over pyridine:n-butanol:acetic acid:water solvent (20:30:6:24) (8) for 2 h and then dipped in the solvent system. Ascending chromatography was then carried out for 12 h and the peptide spots on the chromatogram were identified by spraying the

dried paper with 0.3% ninhydrin solution in acetone. Colour of the spots was intensified by heating the paper in an oven at 60-65 ^{o}C for 15-20 min and the developed colour was fixed by dipping the paper in a fixing reagent (1% cupric nitrate and 0.05% nitric acid in acetone).

3.2.5 Partial N-terminal sequencing of vicilin subunits using DABITC method :

The sequence of first 7 amino acids from the NH_2-terminal end of the two vicilin subunits was determined by using DABITC-PITC double coupling method of Chang (10). This analysis was also useful in proving criteria of purity of the protein.

Each pure vicilin subunit (0.5 to 2 nmoles), separated on DEAE-sephacel, was thoroughly dialysed against glass distilled water and was lyophilised in an acid washed reduction tube specially made for the purpose. To the sample, dissolved in 50% pyridine (v/v), 20 µl dimethyl aminobenzene isothiocyanate (DABITC) (NH_2-terminal determination reagent, 10 µmole/µl in pyridine) was added and the tube was flushed with oxygen free dry nitrogen to remove moisture and oxygen. The tube was sealed with a glass stopper and parafilm, and incubated at 54 ^{o}C for 50 min. After the reaction, the tube was opened under nitrogen and 5

µl of phenylisothiocyanate (PITC) was added for coupling with DABITC. The coupling reaction was done at 70 °C for 60-70 min. After coupling, the excess reagent and the by-products were extracted by mixing the reaction mixture with four portions of 250 µl of heptane-ethyl acetate (2:1 v/v) on a vortex mixer and by centrifuging at 2000 x g for 10 min. The supernatant i.e. organic extract was removed with a Hamilton syringe. The aqueous phase was dried under vacuum and redissolved in 50 µl of trifluoroacetic acid (TFA) for cleavage reaction which was performed at 54 °C for 50 min. After the reaction, the sample was dried under vacuum and redissolved in 50 µl of water. The released DABTH was extracted with 200 µl of butyl acetate for NH_2-terminal analysis. Here, complete extraction of the released DABTH was ensured by washing second time with 100 µl of butyl acetate. The pooled butyl acetate extract was evaporated and redissolved in 10-20 µl of ethanol for thin layer chromatography (TLC) of DABTH. The aqueous phase containing the pepetide was dried under vacuum and subjected to determination of the next amino acid from the NH_2-terminal end. DABITC-diethylamine (20-30 pmoles) was cochromatographed with the experimental DABITH samples as an internal standard for reference. The latter was prepared by adding 10 µl of diethylamine and 100 µl of water to 1 ml of DABITC (1 nmol/µl) in ethanol. Two dimentional TLC was carried out

using 2.5 x 2.5 cm polyamide sheets by ascending solvent flow system. Solvents used were acetic acid:water (1:2 v/v) in phase I and toluene : n-hexane: acetic acid (2:1:1 v/v) in phase II where yellow spots appeared. Each sheet was then dried and exposed to HCl vapours when yellow spots turned red/blue.

Identification of DABITH derivatives was made with the help of the diagram as given in the original text of Chang (11).

3.2.6 Fluorescence studies of pigeonpea vicilin :

The fluorescence spectra of pigeonpea vicilin was determined under nondenaturing and denaturing conditions. For this purpose, the sample (0.125 mg protein), was dissolved in PBS (Phosphate Buffered Saline, i.e. 0.1 M disodium hydrogen orthophosphate, 0.4 M monosodium dihydrogen orthophosphate, 0.14 M sodium chloride, pH 7.2). Fluroscence spectra were recorded on a Hitachi 650-60 model which was operated in the energy mode. Whenever necessary, the controlled temperature sample cell holder accessory was used. Depending on the sample concentration, either 1 cm path length rectangular cuvettes or 0.3 cm cylindrical cuvettes were used.

3.2.7 Circular Dichroism (CD) studies of pigeonpea legumin and vicilin :

A preliminary insight into the secondary structure of legumin and vicilin was obtained by measurement of circular dichroism of these proteins. The CD spectra of the samples (0.125 mg) dissolved in PBS, were recorded on a JASCO I-500A spectropolarimeter having JASCO DP-501 N data processor attachment. Cells of varying pathlengths (0.1 mm or 0.5 mm) were used depending on the sample, so as to give a maximum absorbance of about 0.8 at any wavelength over the range scanned.

The molar ellioticity was calculated from the following relation,

$$(\theta)_M = \frac{\theta_{obs} \cdot M}{10.C.L} \text{ deg.cm}^2\text{.decimol}^{-1}$$

where θ_{obs} is observed ellipticity in degrees, C is concentration in moles per liter and L is pathlength in cm.

3.3 RESULTS :

3.3.1 Molecular heterogeneity and sizing of pigeonpea globulins, Legumin and Vicilin:

Prior to further characterization, purified pigeonpea globulin and its subfractions were first analyzed by SDS-PAGE and by determination of their amino acid composition. A

calibration curve, obtained from the electrophoretic mobilities of standard protein molecular weight markers, is depicted in Fig. 3.1. The electrophoregrams of globulin, legumin and vicilin are shown in Fig. 3.2. As is clear from Fig. 3.2, the globulin is a heterogeneous class of protein showing 12 bands while legumin and vicilin show the presence of 4 subunits (Mr 72,400, 66,000, 60,000 and 56,000) and 2 subunits (Mr 72,000 and 57,000) respectively. These data are in close agreement to that reported by Krishna and Bhatia (1).

The amino acid composition of globulin and its purified fractions are shown in Table 3.1. The amounts of glutamic acid and aspartic acid are high as compared to the other amino acids. From the relatively high values of ammonia, it appears that many of the acidic amino acid residues may be amidated. The levels of sulphur containing amino acids in all the three fractions are very low (less than one residue/100 residues). The level of sulfur containing amino acids in legumin of other legumes is relatively higher (greater than one residue/100 residues) than that in pigeonpea legumin. The number of amino acid residues in legumin varies slightly from that reported by Bhatia (12).

3.3.2 Fingerprinting of pigeonpea globulin, legumin and

Figure 3.1

Calibration graph of SDS-polyacrylamide gel for determining the molecular weight of protein subunits. Electrophoresis was performed in Tris-Glycine buffer, pH 8.3. The following proteins with their molecular weight in parenthesis were used to calibrate the SDS-gels; (1) Phosphorylase (94 kD); (2) Bovine serum albumin (67 kD); (3) Ovalbumin (43 kD); (4) Carbonic anhydrase (30 kD); (5) soybean tryosin inhibitor (20.1 kD); (6) α-lactalbumin (14.4 kD). The mobilities were measured relative to the bromophenol blue marker dye front.

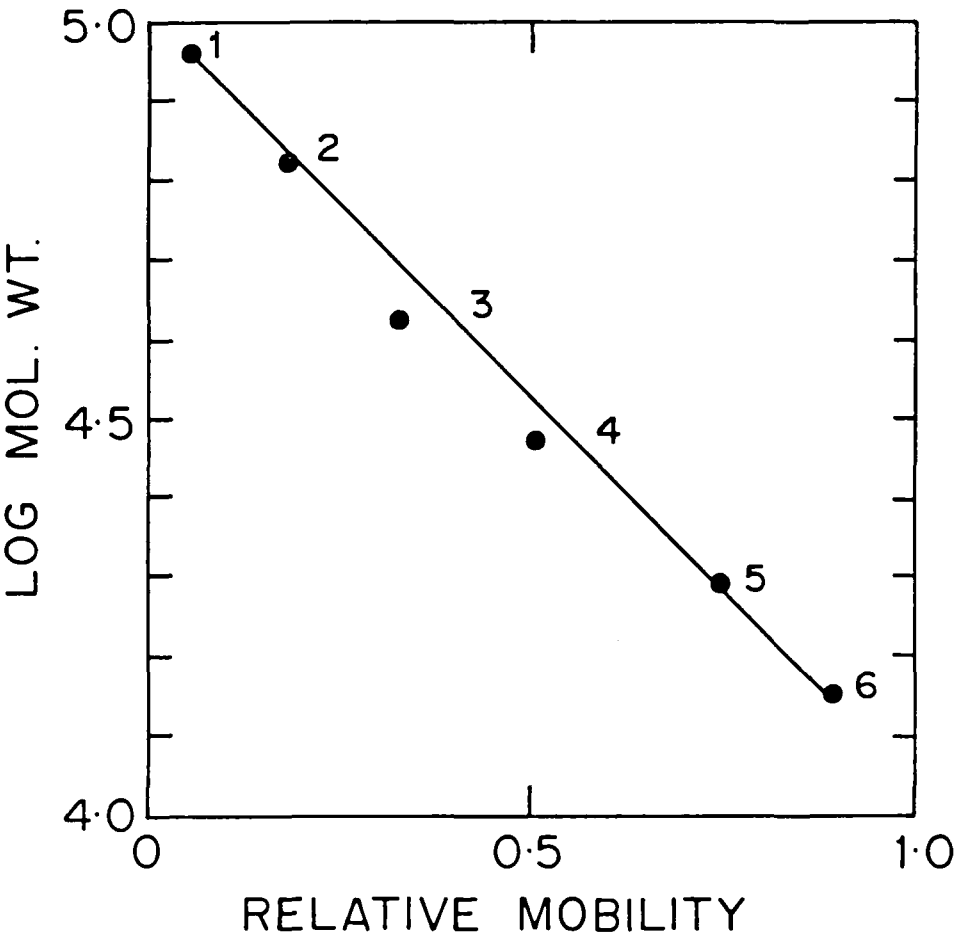


FIG . 3·1

Figure 3.2

SDS-PAGE (12.5 %) of purified (a) globulin (b) legumin and (c) vicilin. Electrophoresis was performed in Tris-Glycine buffer pH 8.3.

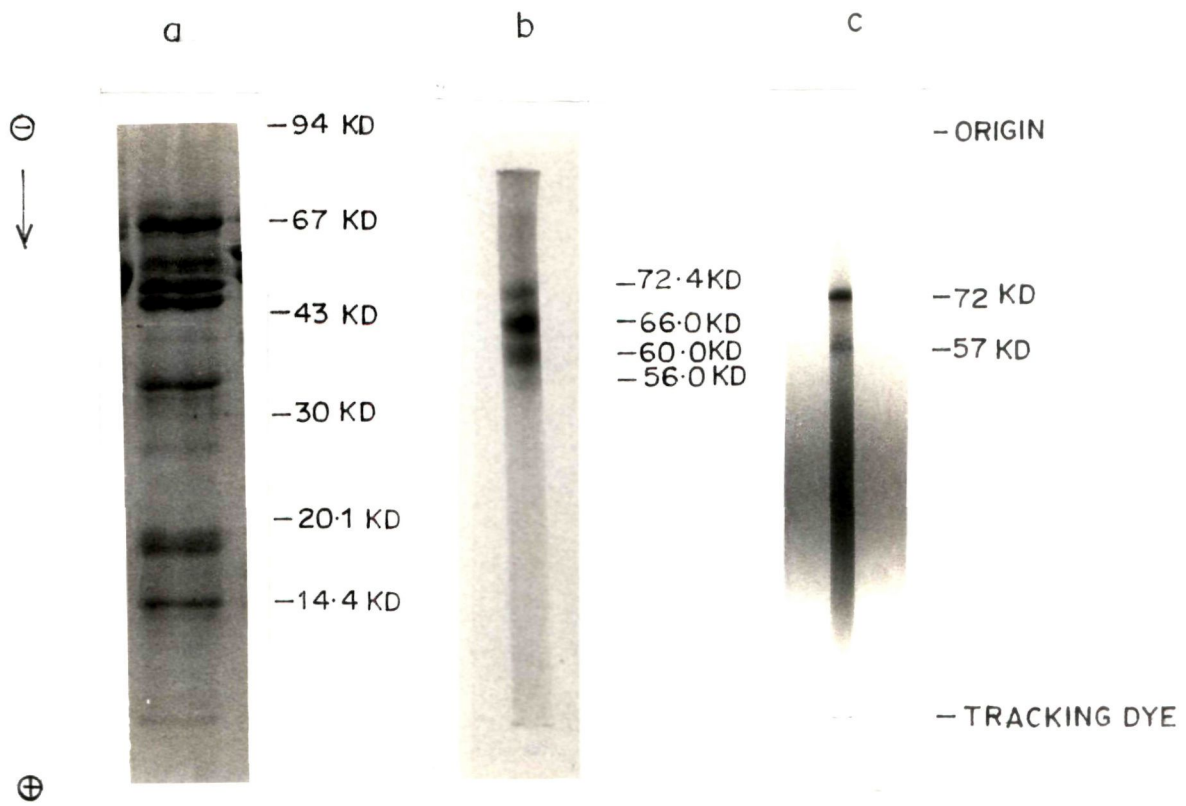

a
b
c
⊖
⊕
–94 KD
–67 KD
–43 KD
–30 KD
–20·1 KD
–14·4 KD
–72·4KD
–66·0KD
–60·0KD
–56·0 KD
– ORIGIN
–72 KD
–57 KD
– TRACKING DYE

FIG. 3·2

Table 3.1

AMINO ACID COMPOSITION (g AMINO ACID/16g N) OF PIGEONPEA GLOBULINS, LEGUMIN AND VICILIN

Amino Acid	Defatted seed meal Total protein	Globulin	Legumin	Vicilin
Lysine	6.76	4.10	4.93	4.50
Histidine	3.84	2.56	2.71	1.99
Arginine	6.18	6.10	7.15	4.26
Aspartic acid	9.12	6.09	8.02	6.15
Threonine	3.01	2.88	3.05	2.42
Serine[a]	4.22	3.40	4.59	3.87
Glutamic acid	18.67	11.01	14.76	9.58
Proline	3.59	3.23	3.71	3.17
Glycine	4.11	2.85	2.18	2.42
Alanine	3.18	3.15	3.29	3.12
Cysteine[b]	0.58	0.52	0.86	0.59
Valine	2.99	2.91	3.22	2.97
Methionine[c]	0.78	0.65	0.71	0.86
Isoleucine	3.09	2.55	3.10	2.55
Leucine	6.92	5.25	7.17	6.41
Tyrosine	2.16	2.17	2.21	2.29
Phenylalanine	8.3	5.15	7.69	4.99
Tryptophan	2.08	2.20	2.10	1.80
Ammonia	7.92	5.98	7.16	4.81

Table 3.1.(Contd)

The values represent averages of three determinations.

a = Extrapolated to zero time.

b = Determined as methionine sulphone after performic acid oxidation.

c = Determined as cysteic acid after performic acid oxidation.

vicilin :

In order to gain a preliminary insight into the amino acid sequences, tryptic digests of pigeonpea globulin, legumin and vicilin were analysed by finger printing (Fig. 3.3). From this figure, it can be seen that most of the peptides appear in the neutral and cathodic region and only a few (one or two) peptides are present in the anodic region. It is further found that globulin, legumin and vicilin yield a total of 19, 16 and 14 cleavage products respectively (Fig. 3.3) whereas 12, 14 and 11 cleavage products for globulin, legumin and vicilin, respectively would have been expected from the number of arginine, lysine and cysteine residues present in these proteins (as calculated from the amino acid data in Table 3.1). Globulin shows the presence of $\gtrsim 4$ residues of lysine, $\gtrsim 6$ residues of arginine and $\gtrsim 1$ residue of cysteine (total 11 residue i.e. 12 cleavage products). Legumin shows the presence of $\gtrsim 5$ residues of lysine, $\gtrsim 7$ residues of arginine and $\gtrsim 1$ residue of cysteine (total 13 residues i.e. 14 cleavage products) while vicilin shows presence of $\gtrsim 5$ residues of lysine, $\gtrsim 4$ residues of arginine and $\gtrsim 1$ residue of cysteine (total 10 residues i.e. 11 cleavage products). The formation of additional peptides may be due to incomplete or nonspecific fragmentation. A similar view has been expressed by Bayer *et al* (13) where formation of additional cleavage products in case of acyl

Figure 3.3

Peptide mapping of pigeonpea globulins and its components. Approximately 2 mg of aminoethylated protein was digested with trypsin (chymotrypsin free) and was used in each experiment. During peptide mapping, high voltage electrophores was first carried out in Michl's buffer for 2 h and then ascending chromatography was carried out in n-butanol:pyridine:acetic acid:water (20: 30: 0.6: 24 v/v).

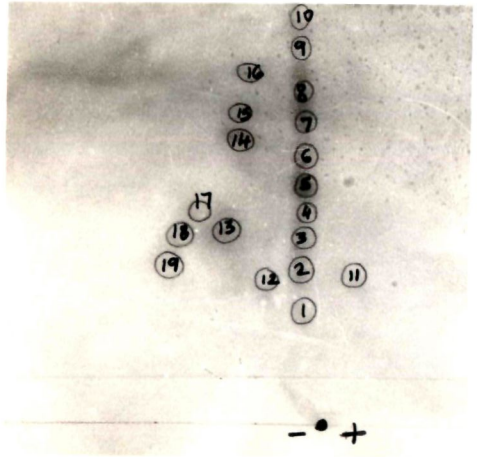
(a) Globulin

(b) Legumin

(c) Vicilin

The tryptic peptide have been numbered for comparative purpose. Arrows indicate similar spots.

a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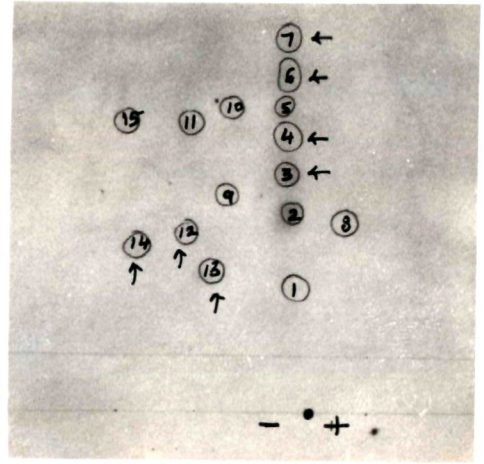
b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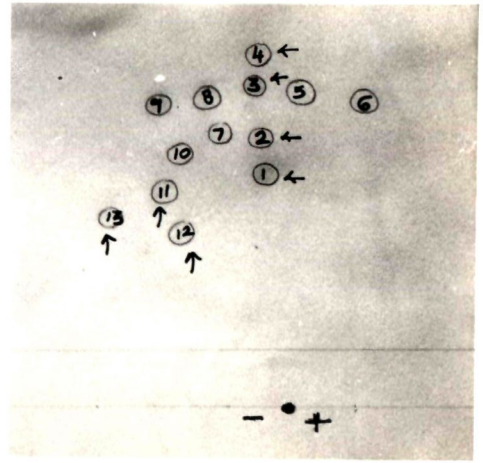
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FIG. 3·3

carrier proteins of citrate lyase from *K. aerogenes* and in fatty acid synthetase from *E. coli* and *K. aerogenes*, has been reported.

3.3.3 Partial N-terminal sequencing of vicilin subunits:

Since pigeonpea vicilin showed only 2 subunits as against legumin which consisted of 4 subunits, these two subunits were separated and purified. Figure 3.4 clearly depicts the separated vicilin subunits of 72 kD and 57 kD on SDS-PAGE. These subunits were then partially sequenced from the N-terminal end using DABITC as an NH_2-terminal determination reagent. This method has several advantages over the widely used Dansyl-chloride (5-dimethylaminonaphthalene-1-sulfonyl chloride) method. In this method, for example, it is possible to recover acid-labile amino acids asparagine, glutamine and tryptophan in nearly quantitative yields and the NH_2-terminal amino acid can be released as a brightly coloured DABTH (dimethylaminoazobenzene thiohydantoin) derivative that can be visualised directly in TLC or identified by HPLC in the visible region. Also, the detection limit of DABTH derivative is about 10 pmole by polyamide TLC (qualitative) as against 0.5 pmole by HPLC (quantitative) and hence it is possible to analyse the NH_2-terminal amino acid with less than 100 pmole of polypeptide.

Figure 3.4

SDS-PAGE of vicilin subunits in pigeonpea. About 30 ug of purified vicilin subunits was loaded on 12.5 % SDS-polyacrylamide gel and the electrophoresis was performed in Tris-Glycine buffer pH 8.3.

(a) Vicilin subunit 2 (57 kD)

(b) Total purified vicilin

(c) Vicilin subunit 1 (72 kD)

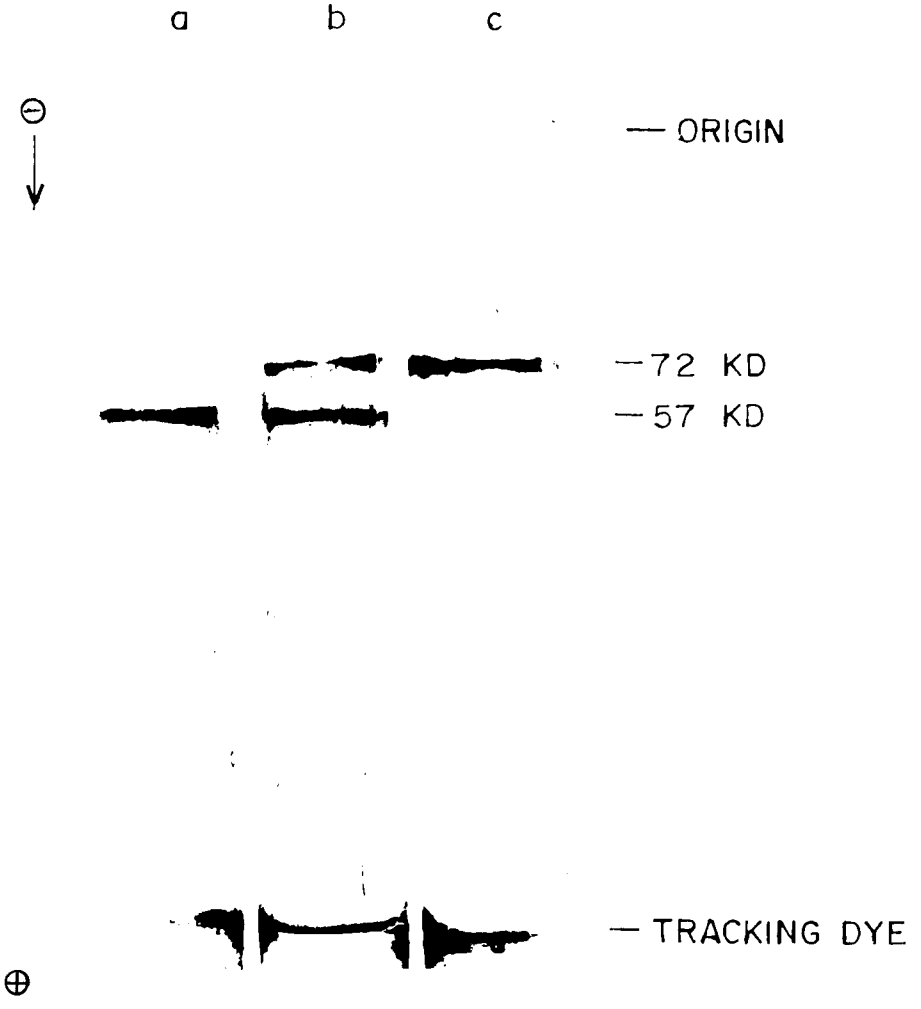

FIG. 3·4

The NH_2-terminal amino acid sequence ascribed to each of the two vicilin subunits is :

Subunit 1 (72 kD) : Gly-Ala-Arg-Val-Asp-Gln-Glu and

Subunit 2 (57 kD) : Thr-Thr-Cys-Met-Glu-Ser-Gly.

Using the manual DABITC-PITC double coupling method, it was possible to sequence only the first seven amino acids from the NH_2-terminal. Later, as the spots became too faint, it was difficult to identify the further amino acid residues. From the sequence, it is clear that there is a heterogeneity among the two vicilin subunits which is in contrast to that reported for other legumes where conservation of these sequences is observed. The lack of homology between the two vicilin subunits in pigeonpea may be due to their differences in amino acid composition. Similar differences in the amino acid residues were reported by Lilley *et al* (14) in case of δ-conglutin from *Lupinus angustifolius*.

The NH_2-terminal residue for vicilin subunit 1 is glycine which is also the amino acid at NH_2-terminal of all basic subunits (15) of legumes. The sequence analysis of the vicilin subunit 2 shows the presence of sulphur containing amino acids cysteine and methionine which are not to be observed in the first seven residues of subunit 1. The presence of one cysteine and one methionine in vicilin (as is

evident from the amino acid composition in Table 3.1), may therefore, be located on the subunit 2. The presence of these sulphur containing residues can be ascertained by sequencing of the CNBr digested fragments of purified vicilin subunits. Since methionine and cysteine are the limiting essential amino acids in pigeonpea, a large proportion of protein fractions containing this amino acid would be advantageous from the nutritional view point. Thus the selection of cultivars containing this fraction would result in improved methionine content in the whole seed.

3.3.4 Effect of urea and Guanidine hydrochloride on the secondary structure of pigeonpea vicilin:

Fluorescence spectroscopy provides a powerful approach to the study of the structure of proteins and peptides. A variety of biological molecules contain naturally occurring or intrinsic fluorophores. Some examples of such fluorophores are the amino acids tryptophan and tyrosine in proteins; and cofactors like NADH, riboflavin and FAD. The ease with which tryptophan fluorescence can be excited in proteins has led to the widespread use of tryptophan emission as a tool for probing protein structure and conformation and is particularly useful for monitoring changes in protein structure.

In the present work, the fluorescence study of pigeonpea vicilin is performed. Figure 3.5 depicts the excitation of native vicilin samples at 274, 281 and 295 nm. Here, a maximum relative fluorescence intensity is observed when vicilin is excited at 281 nm. The latter excitation wavelength was, therefore, used for all the further experiments. Excitation of the native vicilin samples at 281 nm results in an emission spectrum which contains a single emission peak at 329 nm (Fig. 3.5). This spectrum is typical of a protein containing tyrosine but no tryptophan. Similar spectra have been reported for other proteins such as insulin, ribonuclease (16, 17) and Histones H1 and H5 (18) which are devoid of tryptophan. In the presence of even a single tryptophan residue, the predominant emission peak has been reported to occur at 331-342 nm (16, 19). This has been attributed to a transfer of energy from the excited tyrosine to tryptophan (19) and quenching of tyrosine fluorescence by protein environment (16). With the vicilin samples, however, there is no evidence of this typical tryptophan emission peak despite of a tryptophan content of 1.80 g/16N and tyrosine content of 2.29 g/16 N (Table 3.1).

The fluoroscence of tryptophan without interference from tyrosine was investigated by exciting the protein at 295 nm, a wavelength at which only tryptophan absorbs light.

Figure 3.5

Fluorescence emission spectra of pigeonpea native vicilin excited at 274, 281 and 295 nm.

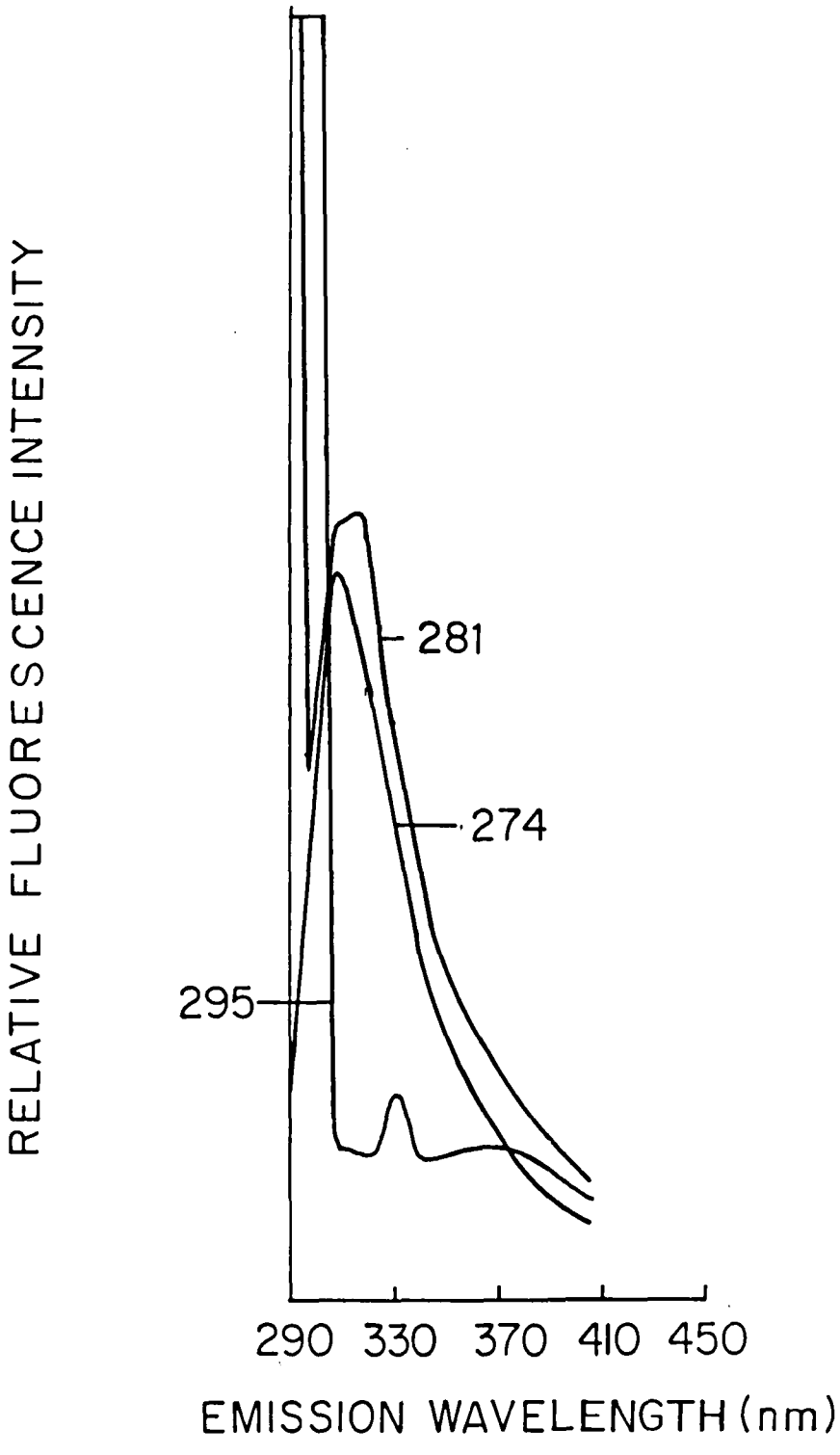
RELATIVE FLUORESCENCE INTENSITY
281
274
295
290 330 370 410 450
EMISSION WAVELENGTH (nm)

FIG . 3·5

Under these conditions, the emission spectrum obtained was characterized by two emission peaks at appromixately 331 and 371 nm (Fig. 3.5). In order to investigate further the two peak phenomenon, the emission spectra of the phosphate buffer and distilled water were measured under similar conditions of excitation wavelength and sensitivity. The resulting emission spectra were characterized by a single peak at 329 nm. Based on this observation, it was felt that the peak at 331 nm represented the Raman band of water. The peak at 371 nm was, therefore, attributed to tryptophan fluorescence. Thus it may be evident that the location of tyrosine did not favour transfer of energy from the excited tyrosine to tryptophan and quenching of the tyrosine fluorescence by the protein environment.

After determination of fluorescence spectra of native vicilin, the effect of denaturants like urea and guanidine hydrochloride (Gdn HCl) on vicilin was carried out and these data are shown in Fig. 3.6 and 3.7 (a-shows spectra at varying concentration and b-is cumulative spectra). As is clear from Fig. 3.6b, no appreciable decrease in fluorescence is observed in the cumulative spectra of vicilin. Here, the effect of increasing concentration of urea (2-8M) on the fluorescence emission spectra of vicilin was determined. In the presence of 4 M urea, a slight decrease in the relative

Figure 3.6

Effect of urea concentration on the fluorescence emission of pigeonpea vicilin excited at 281 nm (a) Native, 1 M, 4 M and 8 M urea (b) cumulative fluorescence.

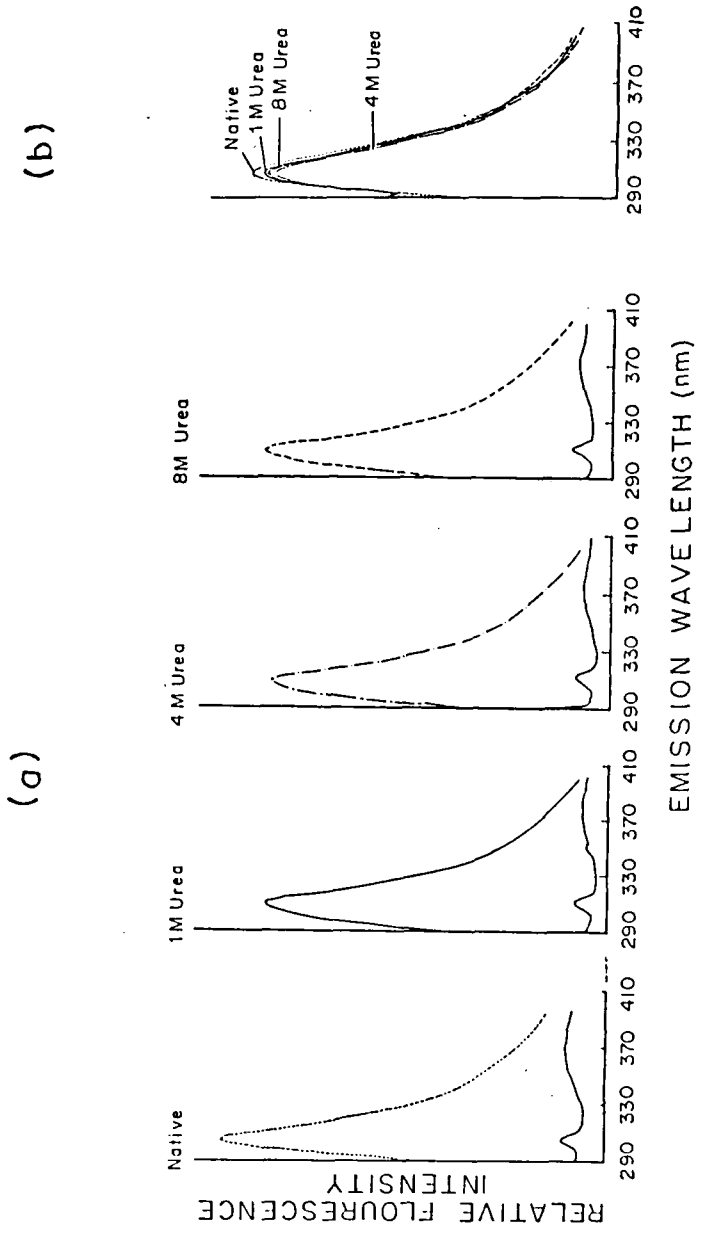
(a)
(b)
Native
1M Urea
4M Urea
8M Urea
290 330 370 410
RELATIVE FLOURESCENCE INTENSITY
EMISSION WAVELENGTH (nm)

FIG. 3·6

Figure 3.7

Effect of guanidine-hydrochloride concentration on the fluorescence emission of pigeonpea vicilin excited at 281 nm

(a) Native, 1 M, 3 M and 6 M Gdn-HCl

(b) Cumulative fluorescence.

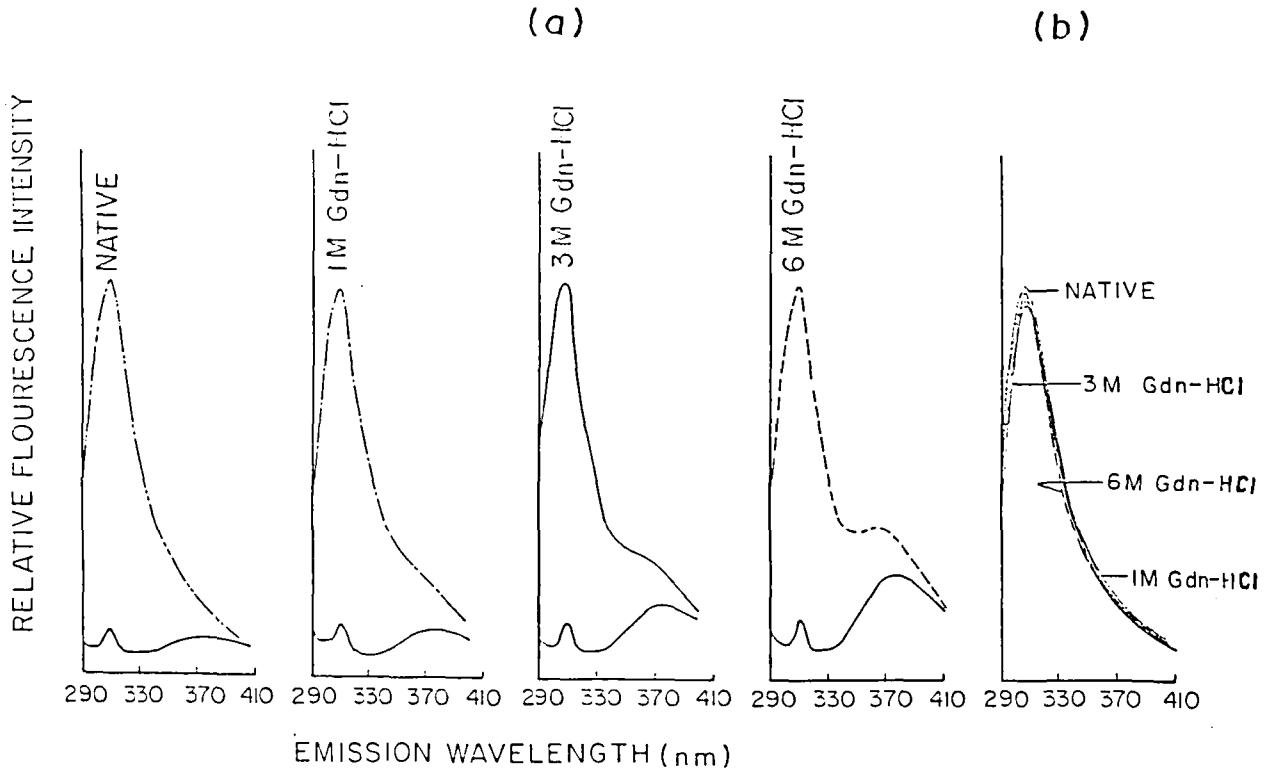
(a)
(b)
RELATIVE FLOURESCENCE INTENSITY
NATIVE
1M Gdn-HCl
3M Gdn-HCl
6M Gdn-HCl
290 330 370 410
290 330 370 410
290 330 370 410
290 330 370 410
290 330 370 410
NATIVE
3M Gdn-HCl
6M Gdn-HCl
1M Gdn-HCl
EMISSION WAVELENGTH (nm)

FIG . 3·7

fluorescence is observed. At high urea concentration, however, this trend is reversed. In the emission spectrum obtained using an excitation wavelength at 281 nm, the changes observed in the presence of urea are shown in Fig. 3.6a. Here, the fluorescence spectra of native vicilin and those obtained under different concentrations of urea are found to be similar.

Since vicilin spectra do not show any appreciable difference in relative fluorescence on denaturation with urea, a denaturation effect with Gdn HCl was studied (Fig. 3.7a). Gdn HCl has been identified as a preferred denaturant for fluorescence studies as it is less subjected to errors arising from exaltation of tryptophan quantum yield (30). From Fig. 3.7, it is clear that the denaturating effects of Gdn HCl are similar to the fluorescence responses observed with urea, except for the shoulder which is observed in relative fluorescence spectra. In the presence of 3M Gdn HCl, a distinct additional peak appears and the shape of this peak increases significantly with the increase in concentration of Gdn HCl to 6 M. Surprisingly, however, the cumulative emission spectra (Fig. 3.7b) in Gdn HCl (background substracted) are more or less identical.

The above results indicate that there is very little change in protein conformation with addition of denaturants

like urea and Gdn HCl.

3.3.5 Secondary structure analysis of pigeonpea proteins :

After determination of the fluorescence emission spectra of total vicilin under native as well as denaturing conditions, the secondary structure of vicilin was studied by CD measurements along with legumin(Fig. 3.8). From Fig. 3.8, it is evident that a major band is observed at 216 nm for both legumin and vicilin while a small negative band at 209 nm for legumin and 207.5 nm for vicilin is observed. A positive band between 195 and 200 nm is observed for both legumin and vicilin. Such spectra is a characteristic feature of a B-sheet predominant structure protein molecule. Since globulins require a high salt solution, the spectra below wavelength 195 nm cannot be taken due to the high background noise.

3.4 DISCUSSION

The studies incorporated into this chapter have given some information with respect to subunit composition of pigeonpea vicilin and legumin holoprotein, similarity of these proteins in their amino acid composition, and tryptic peptide maps; and secondary structure of legumin and vicilin as well as the stability of vicilin to denaturants like urea

Figure 3.8

Circular Dichroism spectra of pigeonpea (a) legumin and (b) vicilin.

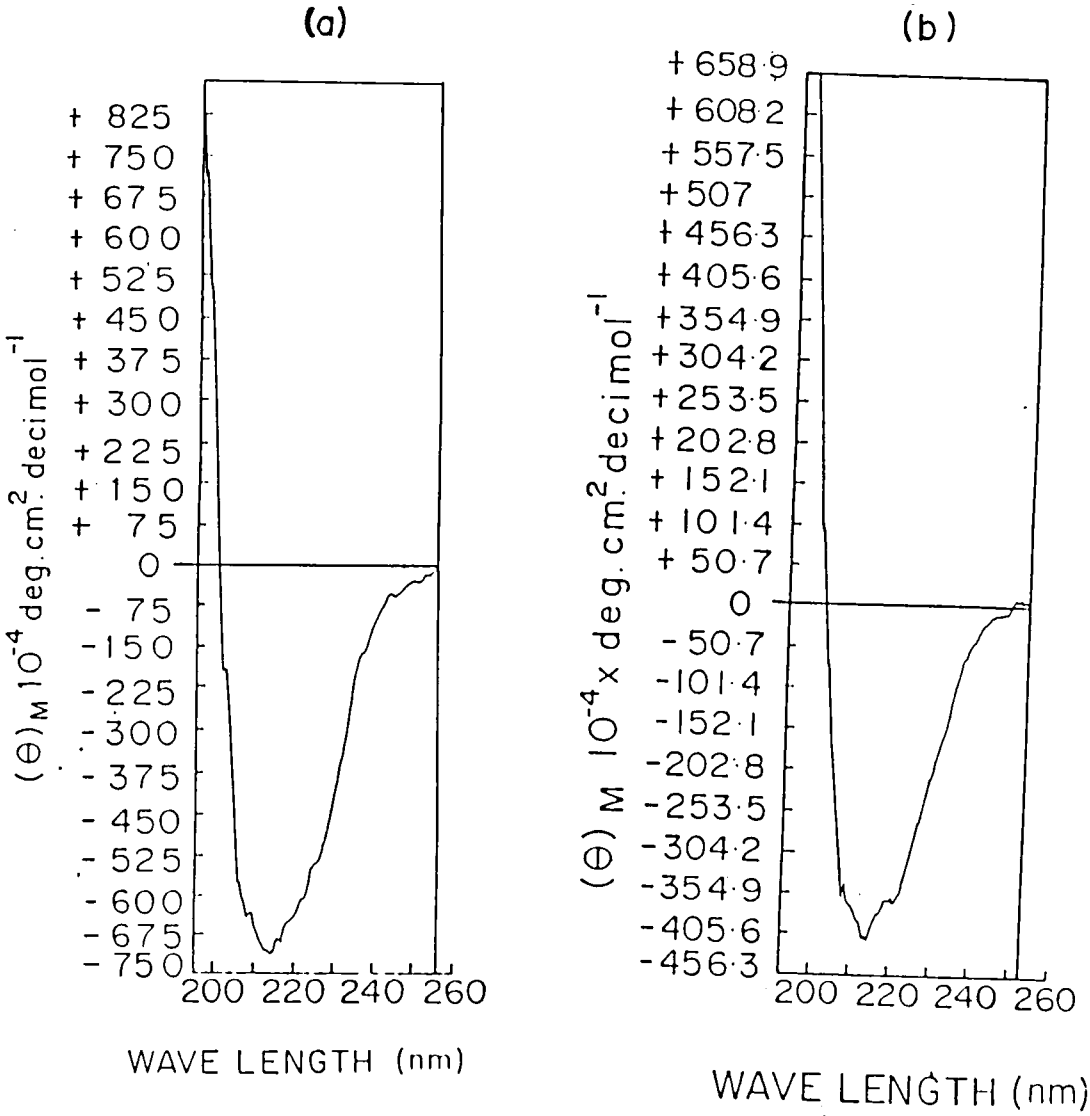
(a)
+ 825
+ 750
+ 675
+ 600
+ 525
+ 450
+ 375
+ 300
+ 225
+ 150
+ 75
0
- 75
-150
-225
-300
-375
-450
-525
-600
-675
-750
200 220 240 260
WAVE LENGTH (nm)
$(\Theta)_M 10^{-4}$ deg.cm^2.decimol^{-1}
(b)
+658·9
+608·2
+557·5
+507
+456·3
+405·6
+354·9
+304·2
+253·5
+202·8
+152·1
+101·4
+50·7
0
-50·7
-101·4
-152·1
-202·8
-253·5
-304·2
-354·9
-405·6
-456·3
200 220 240 260
WAVE LENGTH (nm)
$(\Theta)_M 10^{-4}$ x deg.cm^2.decimol^{-1}

FIG . 3·8

and guanidine hydrochloride.

3.4.1 Microheterogeneity in subunit structure of legumin and vicilin :

The earlier work on storage proteins of grain legumes has given the impression that these proteins are very simple. However, with the availability of sophisticated analytical techniques, the picture of storage proteins that is emerging today is one of increasing complexity and reveals the presence of extensive microheterogeneity (20). The legumin fraction in pea (21, 22), for example, is mainly composed of 40,000 and 20,000 molecular weight subunits with varying amounts of 37,000; 27,000; 25,000 and 18,000 molecular weight subunits. In pigeonpea, as described in results, the legumin protein reveals the presence of four subunits under nondenaturing conditions. However, under denaturing conditions, the 60,000 molecular weight protein was found to consist of two subunits of molecular weight 41,600 and 22,000. Similarly, the 55,900 molecular weight protein showed the presence of a subunit of molecular weight 27,200 indicating that it could be a homodimer (data not shown). Vairinhos and Murray (23) have proposed a model of "processing" to explain the observed variation in the sizes of the larger subunits. According to them a legumin precursor is initially made up of a single polypeptide chain,

then a portion is excised by the hydrolysis of two susceptible bonds which could then give rise to subunits of altered size thus leading to size hetrogeneity. This is a common feature observed in legumin as is clear from the review by Casey and Domoney (24). Most studies on subunit structure and heterogeneity have been carried out for legumin and not much is known about the vicilin protein. Pigeonpea vicilin appears to be a simple protein consisting of only two types of subunits as compared to Pisum vicilin (25) which shows multiple subunits on SDS gels with Mr ranging from 50,000 to 12,500. It has been shown that Pisum vicilin polypeptides in the lower Mr range are produced as a result of post-translational modification (26). A similar proteolytic post-translational modification has also been reported for the vicilin from Vicia faba (27). These lower Mr subunits are absent in phaseolin and conglycinin, the vicilin proteins from Phaseolus vulgaris and Glycine max respectively. By vicilin cDNA comparison, it has been shown that there is a considerable evolutionary divergence around the post-translational processing site in P. vulgaris and G. max as compared to Pisum (28). Since the smaller Mr subunits are absent in pigeonpea vicilin, it is likely that the potential processing sites in pigeonpea vicilins might resemble that of P. vulgaris or G. max (29).

3.4.2 Partial structural similarity between legumin and vicilin in pigeonppea :

The peptide mapping analysis has been carried out to assess the structural relationships between the pigeonpea legumin and vicilin.Using a similar approach, Jackson et al (30) have indicated that there is a considerable overlap in the peptide patterns of legumin and vicilin, ranging from about 80% with Pisum sativum to 40% with Cicer arietinum. They have further interpreted the substantial similarities between the finger printing patterns as indicative of some degree of common structure between legumin and vicilin. Later, Casey and Horstmann (31) have used finger printing to obtain information on the sequence relationships between the α-and β-chains of legumins from Pisum sativum, Vicia faba and Pisum fulvum. They have found that the peptide maps of β-chains are different in Pisum sativum and Pisum fulvum, thus suggesting that the sequence of the B-chains of legumins from Pisum fulvum is not very similar to that from Pisum sativum, inspite of the close serological relatedness of the two Pisum legumins. From the finger printing patterns in Fig. 3.4, pigeonpea legumin and vicilin appear to share about seven peptides (as shown by an arrow) indicating a partial similarity in the structure of these two proteins.

Legume storage proteins, when analysed for their NH -
2

terminal amino acid sequence, show that the amino acid of the basic subunits from all classes of Pisum and Vicia legumin, glycinins, and Phaseolus legumins is invariably glycine, suggesting that the proteolysis of the acidic - basic precursor is very precise. Pigeonpea vicilin also shows the presence of glycine at the N-terminal of vicilin subunit suggesting that it may be the basic subunit. The acidic subunits of the above legumes show several N-terminal groups such as leucine (Pisum, Vicia, glycinin A2), threonine (Pisum, Vicia), isoleucine (glycinin A3, A5), phenylalanine (glycinin A_{la}, A_{lb}), arginine (glycinin A4), methionine (Phaseolus vulgaris) and glycine (glycinin A6). A similar observation has been found in case of pigeonpea vicilin subunit 2 where the N-terminal residue is threonine.

3.4.3 Unusual stability of the secondary structure of pigeonpea vicilin in urea and Gdn HCl :

The first report on vicilin denaturation studies in urea and Gdn HCl has been in Vicia faba (32). Ours is the second such report describing the denaturation profiles of vicilin from pigeonpea. The presence of a predominant shoulder around 250 nm is observed in both Vicia faba and pigeonpea. In the vicilin of Vicia faba, this shoulder has been interpreted as an increased exposure of the tryptophan residue. However, a careful examination of Gdn HCl solution

under identical condition shows a significant background fluorescence centered at 250 nm (Fig. 3.7). Indeed substraction of background results in protein spectra which are almost identical in the presence and absence of Gdn HCl. This observation suggests that the earlier interpretation of the behaviour of vicilin from Vicia faba in presence of Gdn HCl may require reexamination. The absence of any change in the presence of denaturant could be interpreted in 2 possible ways :

(1) the environment of tyrosine fluorophores is identical in both native and unfolded vicilin

(ii) vicilin possesses an unusually stable secondary and tertiary structure which is not readily denatured under these conditions.

3.4.4 The secondary structure of legumin and vicilin is predominantly β sheet in structure :

The CD spectra of pigeonpea legumin and vicilin show peak patterns which are similar to that of B-class. Phaseolin, a vicilin from Phaseolus vulgaris whose sequence is known (33), has been crystallized (34), and is observed to be made up of three subunits arranged in a trimeric trigonal antiprism of about 150 kD. Each of the subunit structure has been found to have two very similar structural domains. Conavalin, another vicilin from jackbean plant, has been

shown to be composed of two trimers facing each other resulting in a hexameric pattern (35). Just like phaseolin, conavalin monomers are made of two domains which are virtually identical in structure. Concerning the secondary structure, conavalin monomer is almost entirely β-type (36). Glycinin, a legumin from Glycine max has been reported to be showing predominantly β-turn in both acidic and basic subunits. Acidic subunits are characterised by successive B-turns and relatively low content of aperiodic structure, while the basic subunit begins with α-helix followed by successive β-turns (36, 37). Pigeonpea vicilin is having a predominantly β-sheet structure and hence resembles other vicilins such as those from phaseolin, conavalin and glycinin. The extent of α- helical and β-sheet in pigeonpea is yet to be ascertained.

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CHAPTER 4

QUANTITATION OF SEED GLOBULINS BY RIA AND MODIFIED ELISA

ABSTRACT

Antibodies raised against globulin and legumin were affinity purified and were used to quantitate the globulin and legumin levels during post anthesis by RIA. These studies reveal that globulin and legumin levels were maximum between 24 to 28 days after flowering (DAF) and 24 to 32 DAF, respectively.

A rapid and sensitive radioimmunoassay (RIA) is described for screening a large number of pigeonpea varieties for quantitation of globulin content. This method employs precipitation of iodine-labelled antigen-antibody complex. Among the 61 pigeonpea varieties screened, Gwalior-3 is found to contain the highest globulin content. The globulin content was also quantitated by modifying the conventional enzyme-linked immunosorbent assay (ELISA). In this method conjugation of pigeonpea globulin antibodies and the enzyme peroxidase together to dextran was employed and the proteins as low as 0.1 ng were detected.

4.1 INTRODUCTION :

Immunochemical procedures provide an important supplement to the battery of available biochemical and biophysical methods for protein characterization and can often yield new information that is not readily obtainable in other ways. Here antibodies serve as extraordinary analytical reagents since they can have specificity for macromolecules (proteins, nucleic acids, and polysaccharides) as well as for small molecules belonging to almost every chemical class. In case of seed storage proteins, extensive immunological work has been reported during last two decades. In earlier years, these proteins were compared by immunological approaches such as double diffusion, rocket electrophoresis and immunoelectrophoresis to assess the taxonomic variability and distribution of different plant species. In recent years quantitative immunoassays like RIA and ELISA have been used to quantitate seed storage proteins during development as well as in different varieties. Such applications are limited to quantitation of globulins of oat (1) and determination of quinine in cultured plant tissues (2) by RIA, use of monoclonal antibodies as probes for gluten detection (3) and for diagnosis of plant diseases (4, 5), use of monoclonal antibodies to examine cereal grain protein homologies (6), and detection/quantitation of low molecular weight compounds such as growth hormones and

terpenoids (7-9).

In the previous two chapters, I have described purification of pigeonpea globulin, its fractionation into legumin and vicilin and characterization of these proteins using a few biochemical and biophysical approaches. In this chapter, I describe the isolation and purification of antibodies against pigeonpea globulin and legumin. These antibodies were used for quantitation of the corresponding proteins during seed formation. For quantitation, standard RIA as well as a modified ELISA procedure were used.

4.2 MATERIALS AND METHODS:

4.2.1 Chemicals:

All the chemicals were obtained from SD's (India), E. Merck (India) or Glaxo (India) and were always of 'Analar' or 'Guaranteed Reagent specifications. Complete and Incomplete Freunds Adjuvant and agar were from Difco (USA). Sephadex G-25 and Sepharose CL-6B were from Pharmacia, (Uppsala, Sweden). Trizma base, Glycine, Iodogen (1,3,4,6-tetrachloro-3a, 6a-diphenyl glycoluril), Tyrosine, Bovine Serum Albumin, Goat anti-Rabbit IgG-Horseradish peroxidase conjugate, 5-Amino salicylic acid, β-Mercaptoethanol, Agarose, Coomassie brilliant blue R-250, Polyethylene glycol (M. W. 8000) and Dextran T-40 were from Sigma Chemical Co.

(USA). (^{125}I) NaI (carrier free) was from Bhabha Atomic Research Centre, Bombay (India). Nitrocellulose paper was from Schleicher and Schuell (Germany). Microtitre plates were obtained locally from Laxbro Co., Pune (India). Collodion bag (10,000 cut off) was from Sartorius (W. Germany). Kodak X-O-Mat (AR5) X-ray film for autoradiography was from Kodak (USA). Divinyl Sulphone (DVS) was from Aldrich (USA).

4.2.2 Raising antiserum to pigeonpea globulins and legumins:

A variety of animals like rabbit, guineapig, horse, goat and mouse are employed as hosts for immunization. However, for most of the immunological work, rabbits are the preferred hosts on account of their moderate size, well known immune response and the ease with which enough serum can be obtained by small bleedings to carry out preliminary characterization of antibodies.

In the present studies, one to three month old New Zealand white rabbits were used and antibodies were prepared for total isolated globulins and legumin of pigeonpea. For immunization,these two proteins were individually dissolved in PBS (Phosphate Buffered Saline, ie. 0.1 M disodium hydrogen orthophosphate, 0.4 M monosodium dihydrogen

orthophosphate, 0.14 M sodium chloride, pH 7.2) at 2 mg/ml concentration and were injected into rabbits, 1 mg at each time in complete Freund's Adjuvant (10). The route of immunization was at multiple subcutaneous sites. Six to eight booster doses of 1 mg protein in Incomplete Freund's Adjuvant were administered at fortnightly intervals and the animals were bled a week after the final booster dose. The blood was allowed to clot and serum was collected by centrifugation. The serum was divided into 1 ml aliquots and stored at -70 ^{o}C. Generally greater than 1:16 titre of the antibodies in the serum was obtained.

4.2.3 Purification of antibodies from rabbit serum by antigen-affinity chromatography:

Prior to purification of antibodies from the serum, the antibodies were precipitated by 40 % ammonium sulphate at 4 ^{o}C for 30 minutes. The precipitated antibodies were obtained by centrifugation at 8000 x g for 10 minutes at 4 ^{o}C. The pellet was resuspended in 2 ml PBS and the ammonium sulphate was removed by extensive dialysis against PBS.

Since the serum contains total antibodies, it is essential to purify specific antibodies. This is accomplished by antigen-affinity chromatography using specific antigen as a affinity ligand. The chromatographic procedure is as follows:

Sepharose CL-4B, activated with divinyl sulphone (DVS), serves as a useful matrix to which a number of proteins can be attached as affinity ligands. In the present work, 10 g of Sepharose CL-4B beads were activated with 2 ml of DVS for 70 minutes at room temperature (9). The beads were washed with water to remove unreacted DVS and resuspended in 0.3 M sodium bicarbonate, pH 9.0. These are the DVS activated beads to which suitable proteins may be coupled. To these activated Sepharose beads (9.5 ml volume), 5 mg each of pigeonpea globulins and legumin (for antigen affinity matrix) were added individually to two beakers and the mixture incubated for six hours at room temperature with very gentle rotating of the beads intermittently. The unbound antigens were washed with 0.3 M sodium chloride by decanting the supernatant. Free activated sites (ie. the sites not bound by the antigens) on beads were blocked with 0.3 M glycine to prevent nonspecific binding. As determined by absorbance at 280 nm, generally 85-95 % of the proteins were bound to the beads.

For antibody-affinity matrix which was later used for radioimmunoprecipitation, pigeonpea globulin antibodies were bound to DVS-activated Sepharose CL-4B beads as described above for the antigen affinity matrix. Both the affinity matrices were stored in PBS containing 0.02 % sodium azide as a preservative.

Affinity beads (5 ml) were packed in a 5 ml syringe column and washed thoroughly with PBS till no absorbance could be measured at 280 nm. One ml of pigeonpea globulin antiserum was passed repeatedly (3 to 5 times) through the above column and the unbound proteins were washed off completely till no absorbance could be measured at 280 nm. The bound antibodies were eluted with 0.3 M Glycine-HCl, pH 3.0 and immediately neutralised with solid Tris to pH 7.0 to 7.4. The purity of these eluted antibody fractions was checked by Ouchterlony double diffusion (11) as described latter in section 4.2.6. The IgG fractions were pooled and dialysed against PBS. These purified antibodies were used for all the further immunological work.

4.2.4 Radioactive (^{125}I) labelling of proteins:

Labelling of the antigen was essential since labelled antigens were used for quantitative and qualitative immunological estimation of proteins. Labelling of proteins *in vitro* is generally achieved by labelling tyrosine residues of proteins with ^{125}I. The reaction is quantitative and labelled proteins of high specific activity can be obtained. The labelling reaction was carried out essentially according to Fraker and Speck (12). Protein (50 to 100 µg) was usually labelled with 0.1 to 0.2 mCi of (^{125}I) NaI (obtained carrier free, specific activity 13-17 mCi/µg) in the presence of

Iodogen for 10 minutes on ice in a total volume of 100 µl. The reaction was terminated by addition of 20 µl tyrosine (0.4 mg/ml in 0.2 M Borate buffer pH 8.4, containing 0.075 M sodium chloride), which binds to the excess ^{125}I. 1 % BSA (0.4 ml) in 0.01 M Tris-HCl buffer, pH 7.4 containing 0.14 M sodium chloride and 1 mg/ml potassium iodide were then added to the reaction mixture to prevent nonspecific binding. The iodinated proteins were freed of unincorporated (^{125}I) NaI by extensive dialysis against PBS. Radioactivity due to ^{125}I was measured in a gamma counter (LKB,Clinigamma model number 1272) and the labelled protein was stored at -70 ^{o}C till further use.

4.2.5 Antigenicity of pigeonpea globulins:

Since total globulins were injected into rabbits, it was essential to ascertain whether all the proteins were antigenic or not, especially when the antigen and the antibodies are to be used in sensitive quantitative immunoassays like Enzyme Linked-Immunosorbent Assay (ELISA). To determine the antigenicity of pigeonpea globulins, two approaches were used:

(i) Western blotting of proteins resolved on gels and their enzymatic immunoassays and

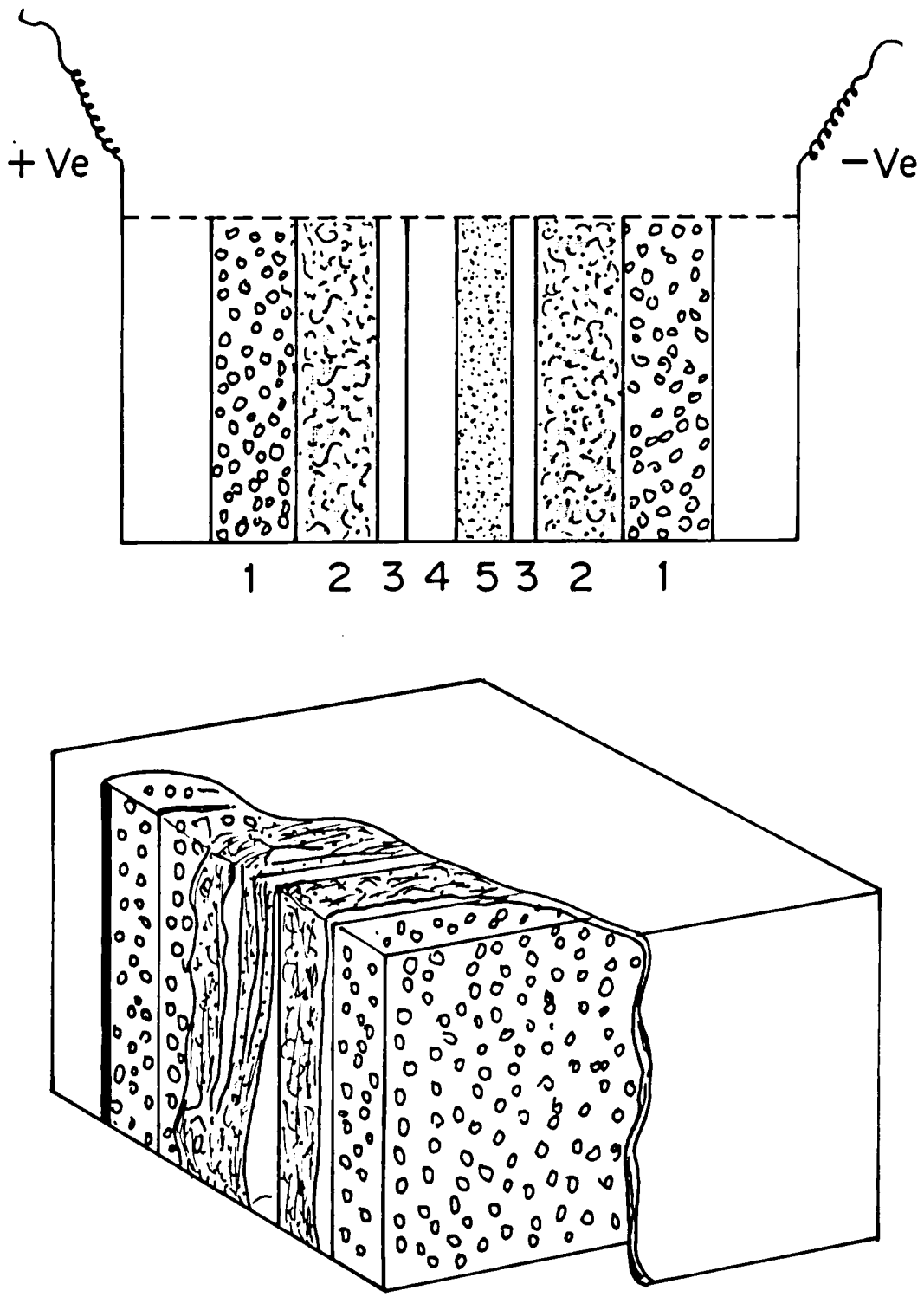
(ii) Immunoprecipitation analysis.

Western blotting and enzymatic immunoassay:

Pigeonpea globulins from SDS-polyacrylamide gels were electrophoretically transferred to nitrocellulose paper according to the method of Towbin et al (13). The apparatus for western blotting fabricated in our laboratory (Fig. 4.1) consisted of a perspex box in which two platinum electrodes were placed. The gel sandwich was made between the two perforated perspex sheets. The nitrocellulose sheet and the gel were packed with care between foam pads while submerged in the buffer so as to ensure that no air bubbles were trapped between the two perspex sheets. The buffer for western blotting consisted of 0.02 M Tris, 0.05 M glycine, pH 7.8, containing 5 % methanol. The electrophoresis was carried out for 36 hours at 5 V/cm. Following the transfer of proteins, the nitrocellulose paper was incubated with 2 % BSA in PBS at 37 °C for 2 hours to block free sites on nitrocellulose paper. Excess BSA was washed off with PBS and the paper was further incubated with 1:300 dilution of pigeonpea globulin antiserum for 1 hour at 37 °C. The antiserum was then washed off with PBS and the paper incubated at 37 °C for 1 hour with 1:1000 dilution of goat antirabbit IgG conjugated with horseradish peroxidase. The blot was washed 2 to 3 times with PBS and the enzyme was assayed with 0.03 % hydrogen peroxide and 2 mg/ml of 5-amino

Figure 4.1

Schematic representation of western blotting apparatus fabricated in our laboratory.



1) POROUS SHEET 2) SPONGE 3) WHATMAN No. 3
4) NITROCELLULOSE PAPER 5) GEL

FIG . 4·1

salicylic acid in 0.5 M sodium acetate buffer, pH 5.0.

Radioimmunoprecipitation analysis:

The antibody-affinity matrix (prepared as earlier) was used for immunoprecipitation of the labelled proteins. To a fixed quantity of beads (100 µl volume usually), labelled pigeonpea globulins (≈1,000,000 cpm) were allowed to adsorb for 2 hours at 4°C. Unbound antigens were removed by washing the beads 3 times with 0.01 M Tris-HCl, pH 7.4, containing 0.5 M sodium chloride. The bound labelled antigen was eluted with SDS-sample buffer (0.5 M Tris-HCl, pH 6.8 containing 10% glycerol (v/v), 0.1 % SDS, 1 % β-mercaptoethanol and 0.05 % bromophenol blue) by incubating the beads in a boiling water bath for 5 minutes. The eluted proteins were then electrophoresed in 12.5 % SDS-polyacrylamide as described in section 3.2.2. Following electrophoresis, the gel was dried and autoradiographed using Kodak X-O-Mat (AR-5) X-ray film at -70°C for 7 days.

4.2.6 Ouchterlony double diffusion :

This is the simplest of the techniques employed to characterise antigen-antibody reaction. It was first described by Ouchterlony (11), and is most commonly used to (i) estimate titre of antibody and (ii) to compare reactivities of different antigens and antibodies with each

other. In this experiment, double diffusion was carried out on microscopic slides or small sized glass plates (4 cm X 6 cm), in 1 % agarose gel made in PBS containing 0.1 % sodium azide. The glass plates were coated with a thin film of agar which was allowed to dry prior to pouring molten agarose on it. When the agarose had set completely, circular wells (6 mm diameter) were punched out of the gel. Appropriate quantities ($\simeq$ 20 µg) of the antigen and the antibodies were loaded into the wells and these were allowed to diffuse through the gel over a period of 48-72 hours, at 4 °C in a humid chamber. When diffusion was completed, antigen-antibody precipitin lines were observed. The gels were dried in situ and stained with 0.25 % coomassie brilliant blue R-250 in ethanol:acetic acid:water (4:1:5 v/v) and the slides were again dried. The stained bands were then photographed using a visible light source.

To check the antibody titre, various dilutions of antibodies ie. 1:2, 1:4, 1:8, 1:16 and 1:32 were loaded in the peripheral wells. In the central well, pigeonpea proteins (globulin and legumin) were loaded. Diffusion was allowed to take place for 48-72 hours at 4 °C, after which precipitin arc was observed indicating antigen-antibody reaction.

4.2.7 Rocket and Immunoelectrophoresis:

In order to determine the number of antigenic

proteins, rocket and immunoelectrophoresis were carried out as described in LKB application note number 249 (March 1978) (14). Rocket electrophoresis was carried out on a microscopic slide which was precoated with a thin film of agar on 1 % agarose gel containing 0.2 % (v/v) antiserum. After the gel was set completely, circular wells (≃ 6 mm diameter) were punched about 1 cm from the base to load the appropriate quantities of antigen into them. Electrophoresis was carried out at 200V for 18-19 hours at 4 °C. The gels were dried *in situ*, stained and destained as mentioned above in section 4.2.6. Immunoelectrophoresis was carried out on a microscopic slide in 1 % agarose in PBS. Circular wells (≃6 mm diameter) were punched and an appropriate quantity of antigen was loaded into these wells. Proteins were electrophoresed for 200 Volt-hours. After electrophoresis, a central slot was cut in the gel and approximately 100 µl antiserum was loaded into it, followed by incubation at 4 °C in a humid chamber. The gels were dried *in situ*, stained and destained as mentioned in section 4.2.6.

4.2.8 Radioimmunoassay (RIA):

When assaying antibodies or antigen, it is often convenient to have either the antigen or antibody immobilised by attachment to a solid surface. Although,polystyrene beads, red cells, agarose beads etc can be used as a support, it is

more convenient to use a plastic test tube or a microtitre plate as the carrier for the antigen or the antibody. Frequently, the antigen is first bound to the solid phase by addition of the antibody under test. After washing, antibody bound to the antigen is revealed by adding a labelled antibody directed against the first immunoglobulin. This label may be a radioisotope, such as ^{125}I (Radio Immuno Assay), or an enzyme such as horse radish peroxidase (Enzyme Linked Immuno Sorbent Assay). For quantitation of pigeonpea globulins (var. T-21), RIA was established according to Pillai and Mohimen (15) with some modifications. Varying amounts of pigeonpea globulins (0-1000 ng) were mixed with PBS to a final volume of 300 µl. To this solution, 200 µl of PBS, 20 µl of antipigeonpea globulin antibodies and 10 µl of competing labelled pigeonpea globulin (≈ 10,000 cmp) were added. The reaction mixture was incubated at 25°C for 4 hours, for the antigen-antibody reaction to take place. Here, varying amounts of cold or unlabelled antigens are used while the concentration of pigeonpea globulin anitbodies and competing antigen is kept constant. As the concentration of unlabelled antigen increases, there is a decrease in the binding of the labelled antigen. 500 µl of the aqueous solution of 35 % polyethylene glycol (M.W. 8000) was added to precipitate the antigen-antibody complex and the mixture was left at 4°C for 4 hours. After thorough washing of the

unbound antigen by PBS, the precipitated labelled antigen was removed by centrifugation at 2000 x g for 10 minutes at 25°C. The radioactivity in the precipitate was measured in a gamma counter (LKB Clinigamma model number 1272). For RIA of pigeonpea globulin and legumin in developmental stages and in other varieties, unlabelled proteins in various dilutions were used as competing agents and the precipitated labelled antigen-antibody complex was measured as above.

The RIA data were evaluated by means of a linear regression least square fit analysis. A program in FORTRAN computer language was written (Table 4.1), and the analysis was performed using NEC-1000 computer at the National Informatic Centre, Pune.

4.2.9 Conventional and Modified Enzyme-linked Immunosorbent Assay (ELISA):

Quantitation of pigeonpea globulins was further confirmed by (i) conventional ELISA and (ii) modified ELISA.

(i) Conventional ELISA :

It was carried out according to the method of Hudson and Hay (16). In this experiment, varying amounts of antigens and BSA (as a negative control) were used in the range of 0 - 10,000 ng in 100 µl volume. These were then allowed to adsorb overnight onto the wells of ELISA microtitre plate at 4°C.

TABLE 4.1 : APPENDIX

```
C     ****************************************************************
C     LINEAR REGRESSION FIT FOR COMPETATIVE RADIOIMMUNOASSAY - FORTRAN 77
C     ****************************************************************
      DIMENSION X(50),Y(50),Z(50),A(50),W(50),B(50,50)
      DIMENSION Y1(50),Y2(50),Y3(50),Y4(50),YC(50),DIFF(50)
      DIMENSION Y5(50)
      DIMENSION TITLE(18)
C     READ TITLE CARD
      READ(5,1)(TITLE(I),I=1,18)
    1 FORMAT(18A4)
      WRITE( 6,1) (TITLE(I),I=1,18)
C     N=NO. OF INPUT DATA PTS;IJJ=ORDER OF POLYNOMIAL;NABC=NO. OF REPLICATES.
      READ(5,*) N,IIJJ,NABC
    2 FORMAT(3I4)
      DO 32 I=1,N
C     READ CONC. OF COMPETING COLD ANTIGEN AND NABC VALUES OF REPLICATES
      READ(5,*) X(I),Y1(I),Y2(I),Y3(I),Y4(I),Y5(I)
   32 Y(I)=(Y1(I)+Y2(I)+Y3(I)+Y4(I)+Y5(I))/FLOAT(NABC)
      IJK=1
      NN=1
      K=1
      KK=4
   10 LW=2*K+1
      LB=K+2
      LZ=K+1
      DO 11 J=2,LW
   11 W(J)=0.0
      W(1)=N
      DO 12 J=1,LZ
   12 Z(J)=0.0
      DO 14 I=1,N
      P=1.0
      Z(1)=Z(1)+Y(I)
      DO 13 J=2,LZ
      P=X(I)*P
      W(J)=W(J)+P
   13 Z(J)=Z(J)+Y(I)*P
      DO 14 J=LB,LW
      P=X(I)*P
   14 W(J)=W(J)+P
      DO 15 I=1,LZ
      DO 15 K=1,LZ
      J=K+I
   15 B(K,I)=W(J-1)
      DO 16 K=1,LZ
   16 B(K,LB)=Z(K)
      DO 19 L=1,LZ
      DIVB=B(L,L)
      DO 17 J=L,LB
   17 B(L,J)=B(L,J)/DIVB
      IA=L+1
      IF(IA-LB) 18,20,20
   18 DO 19 I=IA,LZ
      FMUL=B(I,L)
      DO 19 J=L,LB
   19 B(I,J)=B(I,J)-B(L,J)*FMUL
   20 A(LZ)=B(LZ,LB)
      I=LZ
   21 SIGMA=0.0
      DO 22 J=I,LZ
   22 SIGMA=SIGMA+B(I-1,J)*A(J)
      I=I-1
      A(I)=B(I,LB)-SIGMA
      IF(I.GT.1) GO TO 21
      IF(M.EQ.IIJJ) THEN
      WRITE( 6,*)N,IIJJ
      WRITE( 6,*)'VALUES OF COEFFS B'
      WRITE( 6,100) (A(I),I=1,LZ)
      WRITE( 6,101)
      ENDIF
  101 FORMAT(//1X,'   X    ',1X,'   Y1   ',1X,'   Y2   ',1X,'   Y3   ',1X,
     1'   Y4   ',1X,'   Y5   ',1X,' YAVG ',1X,' YFIT ',1X,' DEVN ',//)
  100 FORMAT(/4E16.7/)
      DO 23 I=1,10
   23 Z(I)=0.0
      DO 25 II=1,N
      YCAL=0.0
      IF(II.NE.1) GO TO 2460
      YC(1)=100.0
      DVN1=0.0
      WRITE( 6,123) X(1),Y1(1),Y2(1),Y3(1),Y4(1),Y5(1),Y(1)
     1,YC(1),DVN1
      GO TO 25
 2460 CONTINUE
      W(1)=X(II)
      DO 24 I=1,K
      W(I+1)=W(I)*W(1)
   24 YCAL=YCAL+A(I+1)*W(I)
      YCAL=YCAL+A(1)
      IF(M.EQ.IIJJ) THEN
      YC(II)=YCAL
      ENDIF
      DVN=Y(II)-YCAL
      DVN1=DVN
      IF(M.EQ.IIJJ) THEN
      DIFF(II)=DVN1
      ENDIF
      DVNSQ=DVN*DVN
      DVN=DVN*100.0/Y(II)
      ABDVN=ABS(DVN)
      Z(1)=Z(1)+DVN
      Z(2)=Z(2)+ABDVN
      Z(3)=Z(3)+DVNSQ
      IF(M.EQ.IIJJ) THEN
      WRITE( 6,123) X(II),Y1(II),Y2(II),Y3(II),Y4(II),Y5(II),
     1Y(II),YC(II),DVN1
  123 FORMAT(2X,F7.2,8(2X,F6.2))
      ENDIF
   25 CONTINUE
      Z(1)=Z(1)/N
      Z(2)=Z(2)/N
      M=M+1
      IJK=IJK+1
      IF(M.LE.IIJJ) GO TO 10
  170 STOP
      END
```

Unadsorbed proteins were then aspirated out. BSA (2 %) in PBS (100 µl) was added to these wells to prevent nonspecific binding and incubated at 37°C for 2 hours. Excess BSA was then aspirated out and the adsorbed antigens were allowed to react with 1:300 dilution of antipigeonpea globulin serum for one hour at 37°C. Unbound antiserum was aspirated out and the wells were washed once with PBS. To each well, 100 µl of 1:2000 dilution of goat anti-rabbit IgG horseradish peroxidase conjugate was added as a second antibody and was incubated for one hour at 37°C. The peroxidase was then assayed with hydrogen peroxide and 5-amino salicylic acid in sodium acetate buffer, pH 5.0. The intensity of colour was allowed to develop in dark for 30 minutes, following which the reaction was stopped by addition of 50 µl 0.1 N HCl. The intensity of colour in each well was measured at 450 nm and a graph of absorbance at 450 nm was plotted against antigen concentration.

For ELISA of globulins and legumins in developmental stages and of various pigeonpea accessions, various dilutions of globulins were used as competing antigens and peroxidase was measured as above.

(ii) Modified ELISA :

In order to increase its rapidity and sensitivity, the conventional ELISA was modified by conjugation of

pigeonpea globulin antibodies and enzyme peroxidase together to dextran. The details of this proceedure are as follows:

Binding of antibody and peroxidase to dextran :

Dextran T-40 (M.W. 40,000) was oxidised to polyaldehyde dextran (PAD) by Malaprade reaction (17-19). In this reaction, dextran T-40 was mixed with sodium periodate (0.33 g in 200 ml distilled water) for oxidation and was stirred well for 24 hours at 4°C. The resultant PAD was dissolved in a minimum volume of distilled water and extensively dialysed against distilled water and lyophilized. About 100 mg of PAD was dissolved in 10 ml of PBS by stirring the solution for two hours at 4°C. The undissolved PAD was removed by centifugation for 5 minutes at 2000 x g. To the supernatant, 1 mg of horseradish peroxidase and 2 mg of affinity purified pigeonpea globulin antibodies were added, and the mixture incubated overnight at 4°C with slow stirring. The Schiff's bases thus formed were reduced by incubation with 0.3 ml of sodium borohydride solution (5 mg/ml in PBS) for 30 minutes at 4°C (16). Excess sodium borohydride from the reduced schiffs bases was removed by extensive dialysis overnight against PBS. The dialysate was centrifuged at 10,000 x g for 20 minutes at 4°C. The supernatant was concentrated to a 2 ml volume using a collodion bag (Sartorius, 10,000 cut off). Preimmune antibodies (2 mg) were coupled to peroxidase (1 mg)

via dextran by the above method and were used as a negative control in all the experiments. Affinity purified pigeonpea globulin antibodies (2 mg) were coupled to horseradish peroxidase conjugate (1 mg) by periodate oxidation conjugation method according to the procedure of Hurn et al. (20) and the conjugate was concentrated to a 2 ml volume. These conjugates were stored at 4 °C till further use.

ELISA

(i) Comparative ELISA using goat antirabbit IgG conjugate and pigeonpea globulin IgG-dextran conjugate:

The rapidity and sensitivity of our modified ELISA was compared with (a) conventional ELISA using goat anti-rabbit IgG peroxidase conjugate and (b) control ELISA using pigeonpea globulin peroxidase conjugate. BSA and preimmune serum were used as internal negative controls.

ELISA plate was divided into seven parts. To part A, various amounts of BSA (0-1000 ng) were added while in parts B to G various amounts of pigeonpea globulins (0-1000 ng) were added. In the second step, additions were done as follows:

A & F : Pigeonpea globulin antibodies dextran peroxidase conjugate (1:1000 dilution).

B & C : Pigeonpea globulin antibodies (0.2 µg)

D : Preimmune antibodies peroxidase conjugate (1:1000

dilution) (negative control).

E : Pigeonpea globulin antibodies peroxidase conjugate (1:1000 dilution).

G : Preimmune antibodies dextran peroxidase conjugate (1:1000 dilution) (negative control).

In step three , goat antirabbit IgG horseradish peroxidase conjugate (1:1000 dilution) was added to part B, and to the remaining parts A, C to F, 2 % BSA (100 µl) was added. Peroxidase was assayed with 5-amino salicylic acid (0.2 mg) and H_2O_2 (0.01 %). Absorbance of the colour developed in the assay was measured at 450 nm. All the experiments were repeated in triplicates and the best fit value of absorbance at 450 nm was plotted versus antigen amount.

(ii) ELISA for quantitation of pigeonpea globulin :

In order to further confirm the sensitivity of our method, modified ELISA was employed for quantitation of pigeonpea globulins to as low level as possible. In this experiment, various amounts (0-1000 ng) of pigeonpea globulins were added to each well and incubated with various dilutions (1:1000, 1:3000, 1:5000 and 1:7000) of pigeonpea globulin antibodies-dextran-peroxidase conjugate. Preimmune antibodies peroxidase conjugate was used as an internal negative control. The peroxidase was assayed with 5-amino

salicylic acid and H_2O_2 as above.

4.3 RESULTS :

4.3.1 Titre, cross-reactivity and purity of the pigeonpea antibodies :

Antibodies against pigeonpea globulin and legumin were raised in rabbits in order to study their antigenicity and for quantitation of these proteins during days after flowering as well as in various cultivars of pigeonpea. Prior to the use of these antibodies for immunological work, concentration of antibodies was determined by Ouchterlony double diffusion using various dilutions of the antiserum. Antibodies having a titre of 1:32 were used for all immunological work. Since the fractionation of pigeonpea globulin was done on a solubility basis, reactivity of the antiserum for the pigeonpea globulin was checked against various other Osborne fractions along with BSA and ovalbumin (as controls) by Ouchterlony double diffusion. From Fig. 4.2 it can be seen that the antiserum reacts with the pigeonpea globulin alone and no cross contamination with other protein fractions namely, albumin, prolamin and glutelin is observed. After checking the titre and cross reactivity, the purity of the antibodies for pigeonpea globulins was assessed by immunoelectrophoresis (Fig. 4.3 a) where a single precipitin arc is observed, and by rocket electrophoresis (Fig. 4.3 b)

Figure 4.2

Specificity of antibodies raised against pigeonpea globulin. Double diffusion was performed in 1 % agarose gel for 24 - 28 h. The central well contained the antibodies. The peripheral wells contained pigeonpea (1) albumin (2) globulin (3) prolamin (4) glutelin (5) BSA and (6) ovalbumin.

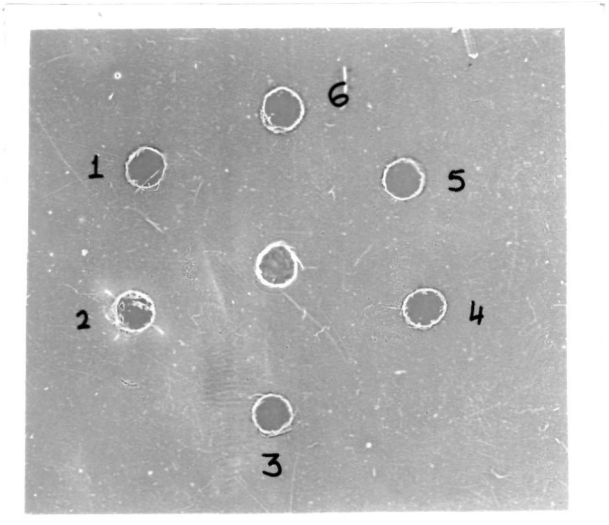


FIG. 4·2

Figure 4.3

(a) Immunoelectrophoresis of pigeonpea globulins in 1 % agarose gel at 200 Volt hour. After electrophoresis, the central slot was cut and 0.2 % antiserum was added. The diffusion was allowed to take place for 16-24 hour at 4 ^{o}C in a moist chamber.

(b) Rocket electrophoresis of pigeonpea globulin in 1 % agarose gel containing 0.2 % antiserum. Electrophoresis was performed in cold at 200 V for 16 - 18 hour.

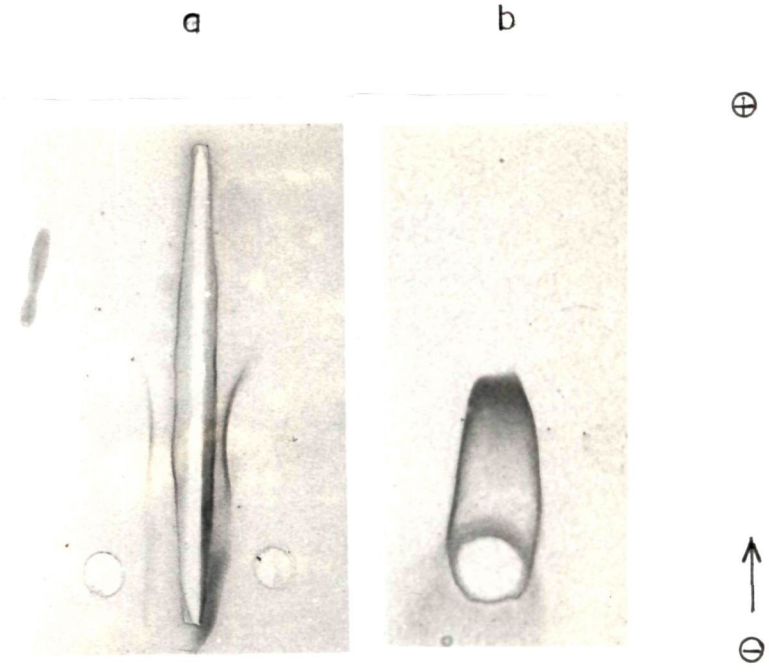


FIG. 4·3

which confirms the presence of single antigenic protein in native pigeonpea globulin. The purity of antibodies for pigeonpea legumin was also similarly assessed. From Fig. 4.4 a, it is clear that immunoelectrophoresis for pigeonpea legumin shows the presence of a single precipitin arc. Rocket electrophoresis (Fig. 4.4 b) further confirms the presence of a single antigenic protein in native pigeonpea legumin,indicating the purity of both antigen and antibody.

4.3.2 Antigenicity of pigeonpea globulins:

As the antibodies against pigeonpea were of polyclonal origin, it was essential to identify the antigenic proteins. As shown earlier in section 3.3.1, it was observed that pigeonpea globulin when electrophoresed on SDS-PAGE revealed the presence of 12 subunits. In order to determine which of the subunits of pigeonpea globulin react with the antibodies, the pigeonpea globulins were electrophoresed in 12.5 % SDS-PAGE, following which they were electrophoretically transferred to nitrocellulose paper. The proteins were then detected by an indirect immunoenzymatic assay and the bands developed after the reaction are shown in Fig. 4.5 a. It is clear from this figure that the major antigenic determinant resides on the polypeptide of molecular weight of 60 kD while the other subunits of molecular weight 31 kD and 16 kD are antigenic to a lesser extent.

Figure 4.4

(a) Immunoelectrophoresis of pigeonpea legumins in 1 % agarose gel at 200 Volt-hours. After electrophoresis, the central slot was cut and 0.2 % antiserum was added. The diffusion was allowed to take place for 16 - 24 h at 4^{o} C in moist chamber.

(b) Rocket electrophoresis of pigeonpea legumin in 1 % agarose gel containing 0.2 % antiserum. Electrophoresis was performed in cold at 200 V for 16 - 18 h.

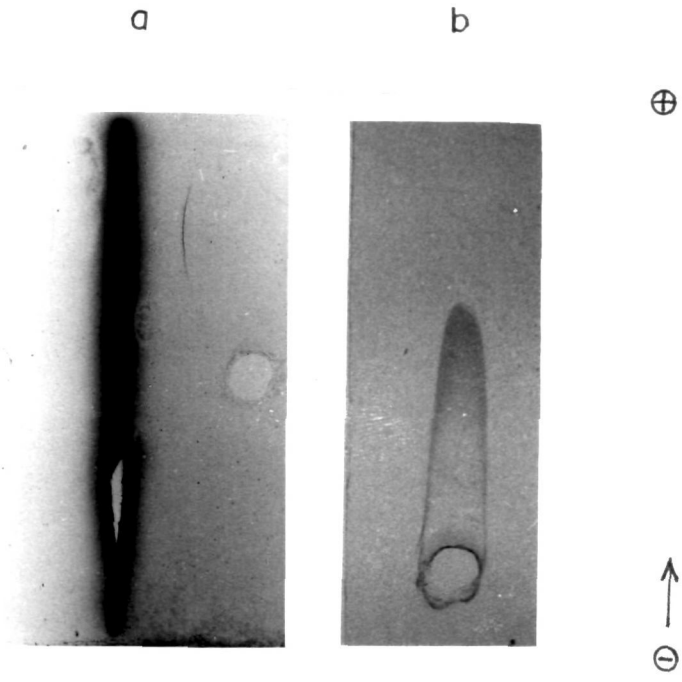


FIG. 4·4

Figure 4.5

(a) Antigenicity of pigeonpea globulins by enzymatic immunoassay of Western blotted SDS-PAGE proteins, using goat antirabbit IgG-peroxidase conjugate.

(b) Antigenicity of pigeonpea globulins by electrophoresis of immunoprecipitate of labelled antigenic subunits in 12.5 % SDS-PAGE followed by autoradiography.

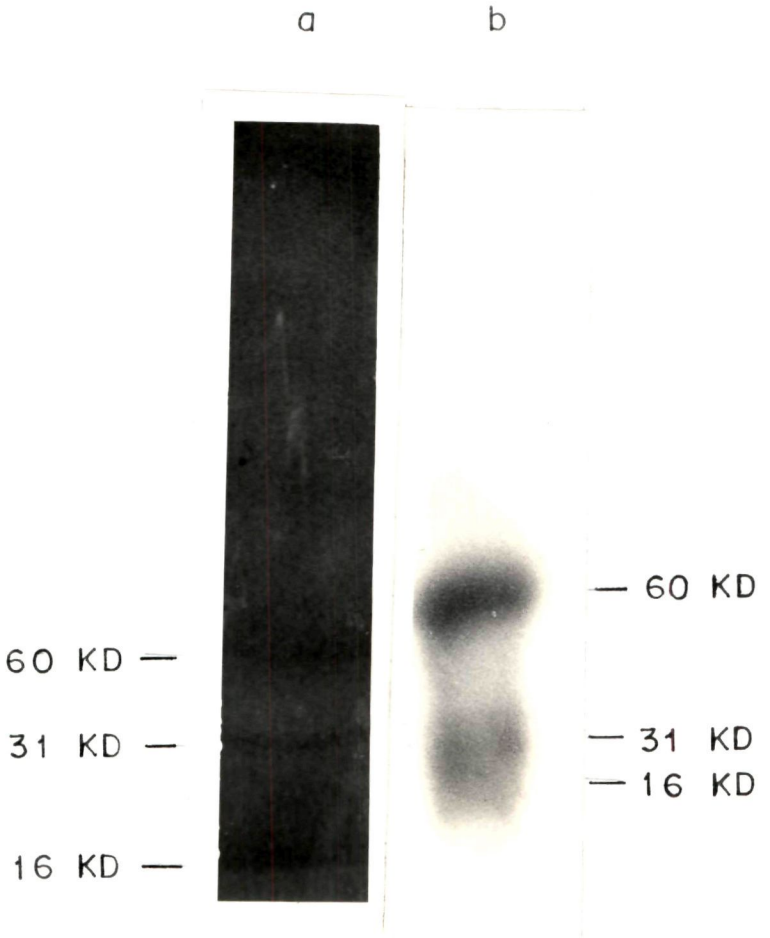


FIG. 4·5

Antigenicity of pigeonpea globulins was further confirmed by radicimmunoprecipitation analysis where first the ^{125}I labelled pigeonpea globulin was allowed to cross-react with the antibodies precoupled to sepharose CL-4B matrix. This immune-complex being very strong, the immuno-precipitated antigen was dissociated and electrophoresed. The autoradiogram of this gel is depicted in Fig. 4.5 b. From the position of the bands in the autoradiogram, the molecular weights are estimated to be 60 kD, 31 kD and 16 kD, with the band of 60 kD being the most intense. These results agree very well with the previously obtained results of enzymatic immunoassay of western blotted proteins (Fig. 4.5 a) and thus clearly indicate that the major determinant of the pigeonpea globulins resides in the 60 kD subunit. The unusual immune response of pigeonpea seed globulins is a rather striking observation. Earlier it has been shown in cotton seed globulin that only two polypeptides of molecular weight 60 kD and 69 kD are antigenic (21) while in cowpea globulin only 52 kD polypeptide posseses major antigenicity (22). Since legume globulins are known to undergo post translational cleavages of the polypeptide chains (23), it is quite possible that some of these cleavage products may not be recognized by antibodies since some of the antigenic domains may have been lost in the process. This may be one of the possible explanations for the unequal immune response

of the above seed proteins.

4.3.3 Quantitation of pigeonpea seed globulins and legumin during seed formation by radioimmunoassay:

During our earlier work of quantitation of seed globulin in cowpea, we had used the solid phase RIA of Pillai and Mohimen with slight modifications (22). In the present work of quantitating pigeonpea globulin, the above RIA was initially used. Although this RIA was sensitive and reliable, it had two major limitations namely: (i) the activation of Whatman paper disk and coupling of antibodies was a laborious process and involved the use of toxic and carcinogenic compounds like divinyl sulphone or cyanogen bromide and (ii) as the disks needed to be stored in 2 % BSA solution at 4 °C, the chances of contamination of this solution were very high. We, therefore, developed the liquid phase RIA which was superior to the solid phase RIA as it overcame the above limitations. This RIA only required the precipitation of antigen-antibody complex by polyethylene glycol which was obtained by centrifugation.

The liquid phase RIA was first used for screening a large number of pigeonpea varieties for their globulin content(24). Prior to this work, the standard RIA was first established for pigeonpea (cv T-21) globulins using their antibodies raised in rabbits. The antigen concentrations were

used in the range of 0-1000 ng, the lower limit of detection being 1 ng. From Fig. 4.6, it is clear that approximately 520 ng of unlabelled pigeonpea (cv T-21) globulins are required to displace 50 % of labelled pigeonpea (cv T-21) globulins in this comparative assay. Since, the RIA can detect upto 1.0 ng of pigeonpea globulins, one seed is enough to quantitate its globulin content.

In pigeonpea, the globulin constitutes a major protein (about 60-80 %), and hence it proves to be a useful indicator for protein estimation. The conventional methods for globulin extraction are laborious, time consuming and not very sensitive. Since varying amounts of globulins are present in various cultivars, it is essential to quantitate protein content prior to selection of varieties of economically important plants.

Taking advantage of the RIA sensitivity, the technique was used to screen seeds of 61 pigeonpea varieties in order to asses the different phenotypic accession with highest globulin content. From Table 4.2, it can be seen that the globulin value ranges between 14 and 20 % of the seed meal. As these germplasm accessions were grown under identical conditions, the genetic variability due to environmental factors could be ruled out. Among the 61 varieties, Gwalior-3 is found to contain the highest globulin

Figure 4.6

Fraction (%) of labelled pigeonpea globulin (cv T-21) precipitated by polyethylene glycol in presence of various amounts of competing pigeonpea globulins.

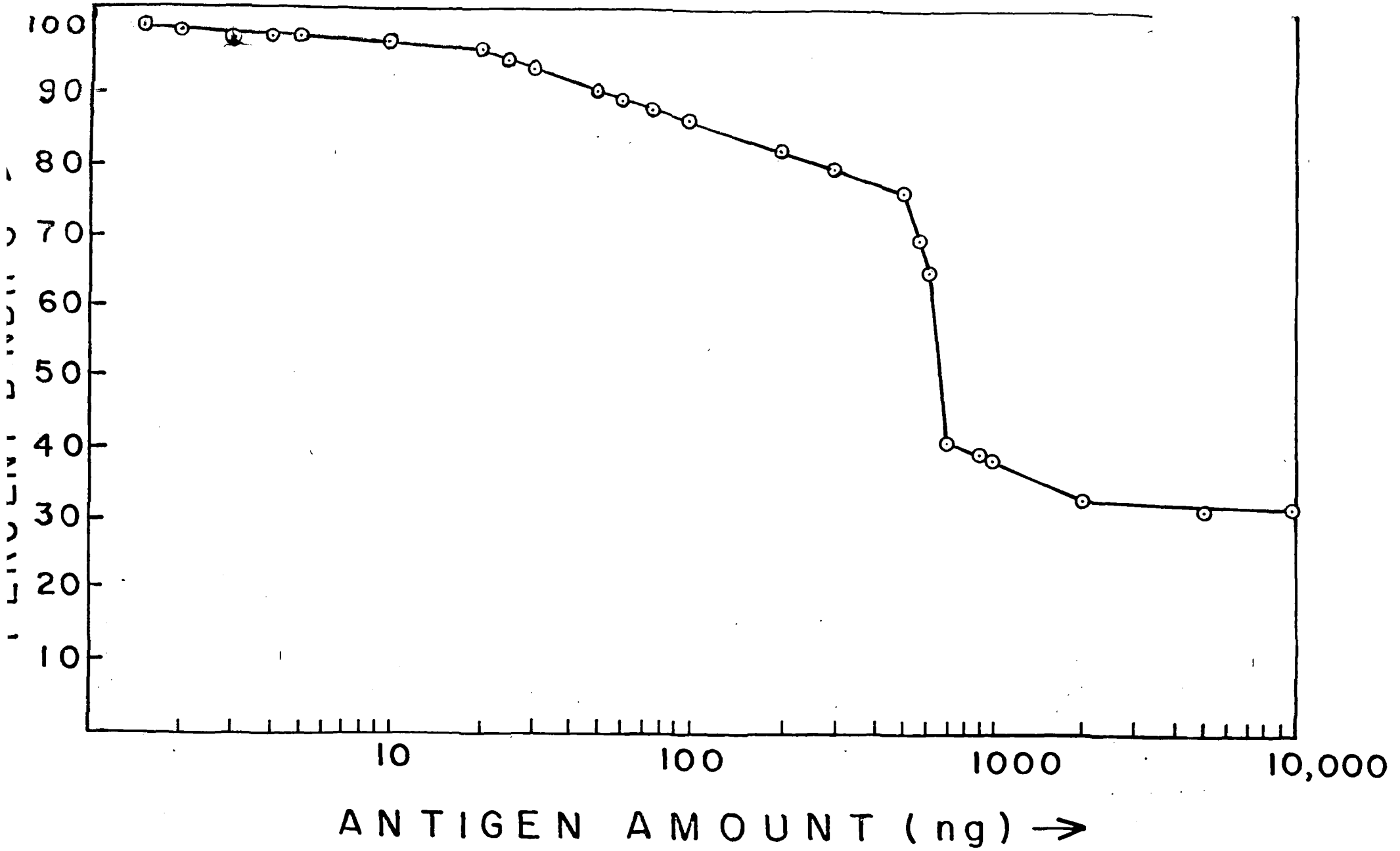

100
90
80
70
60
50
40
30
20
10
10
100
1000
10,000
ANTIGEN AMOUNT (ng) →

FIG. 4.6

TABLE 4.2

RIA FOR PIGEONPEA VARIETIES

Sr. No.	Varieties (Pedigree)	Average weight per seed (mg)	Protein (%)	Globulin detected by RIA (%) seed powder
1.	P-3328-3	127 ± 4.8	19.5 ± 2.6	15.6 ± 1.3
2.	ANM-11	85 ± 2.2	18.4 ± 3.8	14.7 ± 0.9
3.	BS-5	76 ± 1.3	20.1 ± 1.7	16.0 ± 1.2
4.	Prabhat	28.3 ± 2.1	21.4 ± 3.9	16.1 ± 1.1
5.	C-11	68.9 ± 6.4	23.9 ± 2.9	19.1 ± 1.3
6.	Gwalior-3	54.9 ± 5.2	25.9 ± 2.2	19.6 ± 0.9
7.	T-21	52.4 ± 2.1	20.0 ± 3.1	16.0 ± 1.4
8.	P-79311	75.1 ± 4.2	19.3 ± 0.9	15.4 ± 1.1
9.	PI-2419	18.7 ± 1.1	18.8 ± 1.4	15.0 ± 1.3
10.	P-130-4	120.9 ± 8.4	19.2 ± 0.9	14.3 ± 0.9
11.	NR(WR)-5	56.7 ± 2.2	21.8 ± 1.2	17.4 ± 1.4
12.	P-230	39.1 ± 2.1	18.7 ± 2.1	14.9 ± 1.1
13.	P-1880	73.2 ± 4.5	16.5 ± 1.7	13.2 ± 0.6
14.	Granada-1	35.2 ± 2.1	18.9 ± 1.9	15.1 ± 1.4
15.	Code No. 13	115.4 ± 8.4	20.2 ± 0.9	16.1 ± 1.5
16.	DSCR-17	116.3 ± 9.5	19.8 ± 0.7	15.8 ± 0.8
17.	Pusa Ageti	75.4 ± 5.2	23.0 ± 1.1	15.8 ± 1.3
18.	ANM-73	111.7 ± 10.2	21.3 ± 1.5	17.0 ± 0.7
19.	DSLR-55	123.8 ± 10.4	20.0 ± 0.9	16.0 ± 1.1
20.	PS-41	94.5 ± 10.1	20.8 ± 0.6	16.6 ± 1.3

(1)	(2)	(3)	(4)	(5)
21.	EC-107641	104.0 ± 14.1	21.3 ± 1.5	17.0 ± 0.9
22.	ANM-44	16.5 ± 7.1	19.9 ± 1.1	15.9 ± 1.1
23.	DSLR-85	47.5 ± 3.1	20.2 ± 0.8	16.1 ± 1.2
24.	DSLR-38 (Baigani)	66.5 ± 6.1	19.8 ± 2.1	15.8 ± 1.1
25.	A-C-314 (White)	97.3 ± 7.2	20.12 ± 1.8	16.0 ± 1.2
26.	PI-395307	20.1 ± 4.1	19.11 ± 0.9	15.2 ± 1.4
27.	ANM-55	36.9 ± 3.2	21.3 ± 1.7	17.0 ± 1.3
28.	P-673	40.2 ± 6.1	20.1 ± 1.1	16.0 ± 1.2
29.	NP-69	95.5 ± 8.2	18.4 ± 1.0	14.7 ± 1.4
30.	P-4655	80.3 ± 10.3	17.6 ± 1.5	14.0 ± 1.3
31.	P-1923	65.4 ± 7.2	19.8 ± 0.8	15.8 ± 1.5
32.	ANM-37	13.4 ± 2.1	20.1 ± 1.4	16.0 ± 0.8
33.	ST-1	68.7 ± 5.4	18.6 ± 1.2	14.8 ± 1.2
34.	P-1685	28.5 ± 3.1	17.5 ± 1.1	14.0 ± 1.2
35.	JA-276	91.5 ± 9.4	19.3 ± 1.6	15.4 ± 0.9
36.	ANM-136	75.3 ± 10.2	18.4 ± 2.1	16.0 ± 1.7
37.	EC-107634	146.8 ± 4.1	20.1 ± 1.4	15.8 ± 1.1
38.	JA 277-1	45.9 ± 3.2	19.8 ± 1.2	16.0 ± 1.7
39.	P-4769-2	71.1 ± 0.1	21.1 ± 1.6	14.8 ± 0.7
40.	P-4-110-3-1	84.4 ± 6.3	19.8 ± 0.9	13.9 ± 1.1
41.	P-4989	56.9 ± 8.1	17.4 ± 0.9	16.8 ± 1.2

(1)	(2)	(3)	(4)	(5)
42.	HY-3A	45.1 ± 5.1	21.0 ± 1.2	16.8 ± 1.2
43.	TT-5	101.3 ± 9.2	20.3 ± 1.1	16.2 ± 0.6
44.	TT-6	123.2 ± 10.1	21.4 ± 1.5	17.1 ± 0.9
45.	TAT-10	135.7 ± 12.1	22.8 ± 2.1	18.2 ± 1.4
46.	BDN-5	110.8 ± 10.3	21.1 ± 1.6	16.8 ± 0.6
47.	BDN-1	114.9 ± 10.6	20.9 ± 1.2	17.4 ± 1.5
48.	ANM-231	84 ± 9.1	19.5 ± 0.9	18.4 ± 1.2
49.	PI-396055	92 ± 8.1	10.2 ± 0.9	15.2 ± 0.8
50.	A. sericea	29 ± 1.5	28.2 ± 2.1	18.4 ± 1.3
51.	A. grandifolia	48 ± 2.3	27.3 ± 1.8	19.4 ± 1.1
52.	A. lineata	59 ± 5.5	30.2 ± 2.7	16.90 ± 1.6
53.	A. scarabaeoides	61 ± 8.9	26.4 ± 1.9	18.0 ± 0.7
54.	A. albicans	38 ± 4.2	28.9 ± 2.5	17.4 ± 1.5
55.	A. goensis	44 ± 5.6	29.4 ± 2.1	17.8 ± 1.7
56.	A. platycarpa	37 ± 4.1	28.1 ± 1.9	18.0 ± 1.1
57.	A. volubilis	56 ± 6.8	27.6 ± 2.4	17.2 ± 0.9
58.	A. lanceolata	72 ± 10.2	26.5 ± 1.7	17.8 ± 1.2
59.	A. cajanifolia	86 ± 9.9	28.5 ± 2.2	18.6 ± 1.5
60.	A. acutifolia	89 ± 12.6	27.9 ± 1.2	16.8 ± 1.1
61.	A. mollis	51 ± 5.8	27.1 ± 1.8	16.4 ± 1.4

Each value represents an average of three different experiments for pigeonpea varieties.

content of 19.6 % of dry mass seed meal weight.

Attempts were made to study the relationship between size (weight) of a seed and its globulin content which is depicted in Fig. 4.7 a. Here, no significant correlation between these two criteria is found, indicating that the size of the seed cannot be used as a parameter for selection of varieties with high protein content. This is unlike the case Pisum (25) where a linear correlation exists between weight of the seed and the globulin content. The standard RIA was also used for quantitation of legumins from neutron bombarded varieties TT-5, TT-6 and TAT-10. The legumin concentration in these varieties is depicted in Table 4.3. It can be seen that T-21 has a maximum legumin concentration of 1330 ng/mg of seed. Our method, thus provides a satisfactory initial screening technique to select varieties of enhanced intrinsic productivity of proteins.

The liquid phase RIA was next used for quantitation of seed globulins during seed development. For this purpose, pigeonpea pods were collected during 0-50 day after flowering and the specific proteins were isolated. It is clear from Fig. 4.7 b that the level of pigeonpea globulin is low till 6 DAF and it starts increasing thereafter with maximun globulin present at 28 DAF. After this period, there is no appreciable change in the level of globulin till 36 DAF,

Figure 4.7

(a) The relationship between seed weight and globulin content of the seed meal in 61 pigeonpea varieties.

$$\text{Globulin content} = \frac{\text{Globulin detected by RIA/mg seed meal}}{\text{weight of seed}} \times 100$$

These are average values of 5 determinants.

(b) Fraction (%) of labelled pigeonpea globulin (cv T-21) precipitated by polyethylene glycol for globulin at different days after flowering.

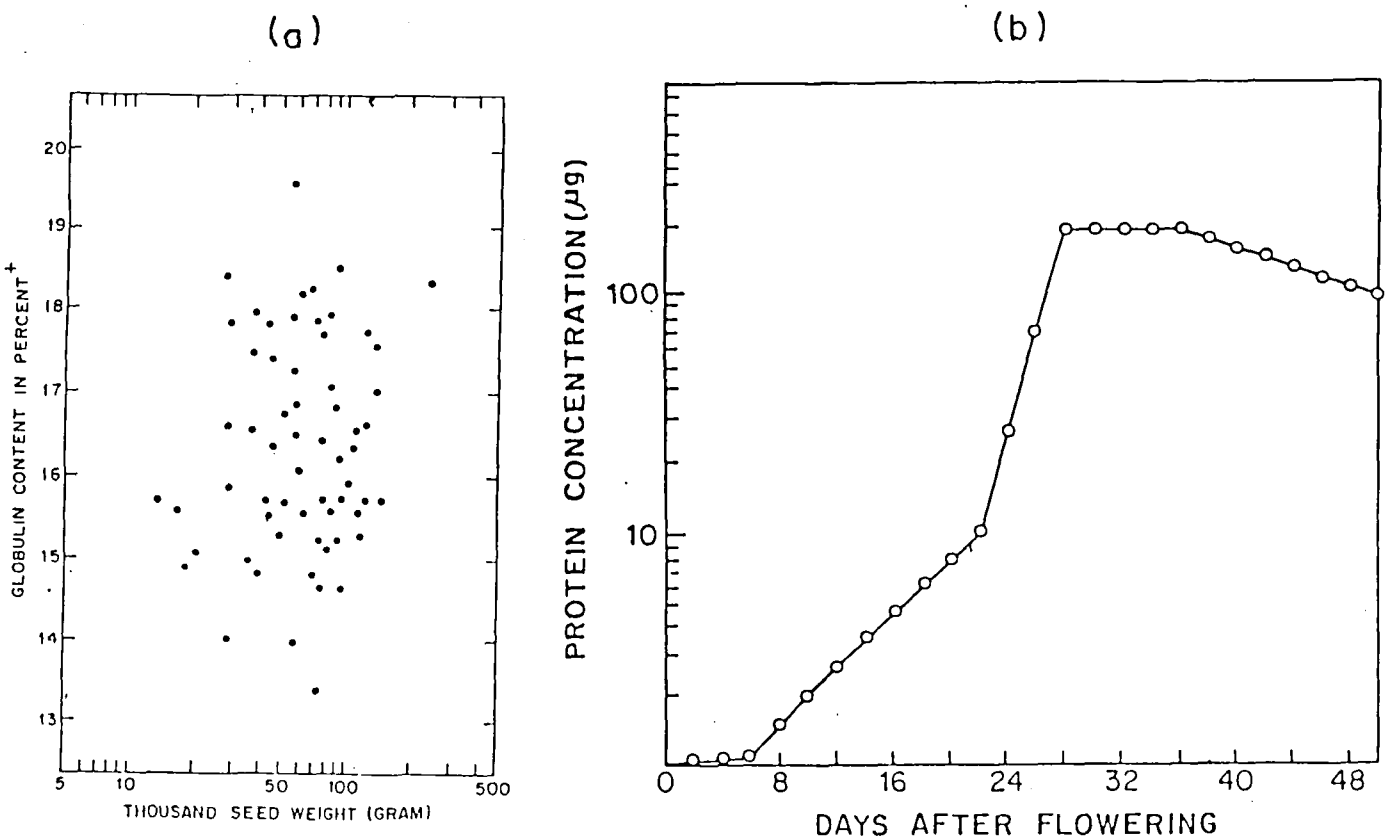

(a)
GLOBULIN CONTENT IN PERCENT+
20
19
18
17
16
15
14
13
5
10
50
100
500
THOUSAND SEED WEIGHT (GRAM)
(b)
PROTEIN CONCENTRATION (μg)
100
10
0
8
16
24
32
40
48
DAYS AFTER FLOWERING

FIG . 4·7

TABLE 4.3

LEGUMIN CONCENTRATION IN DIFFERENT VARIETIES OF PIGEONPEA

Variety	Legumin detected by RIA (ng/mg of seed meal)
T-21	1330 ± 7
TT-5	1280 ± 12
TT-6	1246 ± 9
TAT-10	1195 ± 8

Each value is an average of experiments done in triplicate.

after which it declines. RIA was also used for quantitation of legumins during post anthesis (Fig. 4.8 a). It is clear from Fig. 4.8 b that the level of legumin is low till 8 DAF , it starts increasing thereafter and maximum legumin is present at 24 DAF after which there is no appreciable change till 32 DAF. The legumin level declines after 32 DAF, indicating that the maximum protein is produced around 24 DAF. Since the increase in protein level is associated with the increased synthesis of the corresponding mRNA, the present data is useful in selecting a specific period during development for the isolation of enriched mRNA. Thus in case of pigeonpea legumin mRNA isolation, period between 24 and 32 DAF would be ideal for the isolation of mRNA. This enriched mRNA can then be used for construction of cDNA library to clone the legumin seed storage protein genes.

4.3.4 Quantitation of seed globulin by enzyme-linked immunosorbent assay:

Quantitation of seed globulins was further assessed by ELISA. The conventional ELISA was modified to enhance the rapidity and sensitivity for quantitation of seed proteins. The results of peroxidase assay using goat anti-rabbit IgG peroxidase conjugate and dextran peroxidase conjugate are depicted in Fig. 4.9. It can be seen from this figure that

Figure 4.8

(a) Fraction (%) of labelled pigeonpea legumin precipitated by polyethylene glycol in presence of various amounts of competing pigeonpea legumin.

(b) Fraction (%) of labelled pigeonpea legumin precipitated by polyethylene glycol in presence of various amounts of competing pigeonpea legumin.

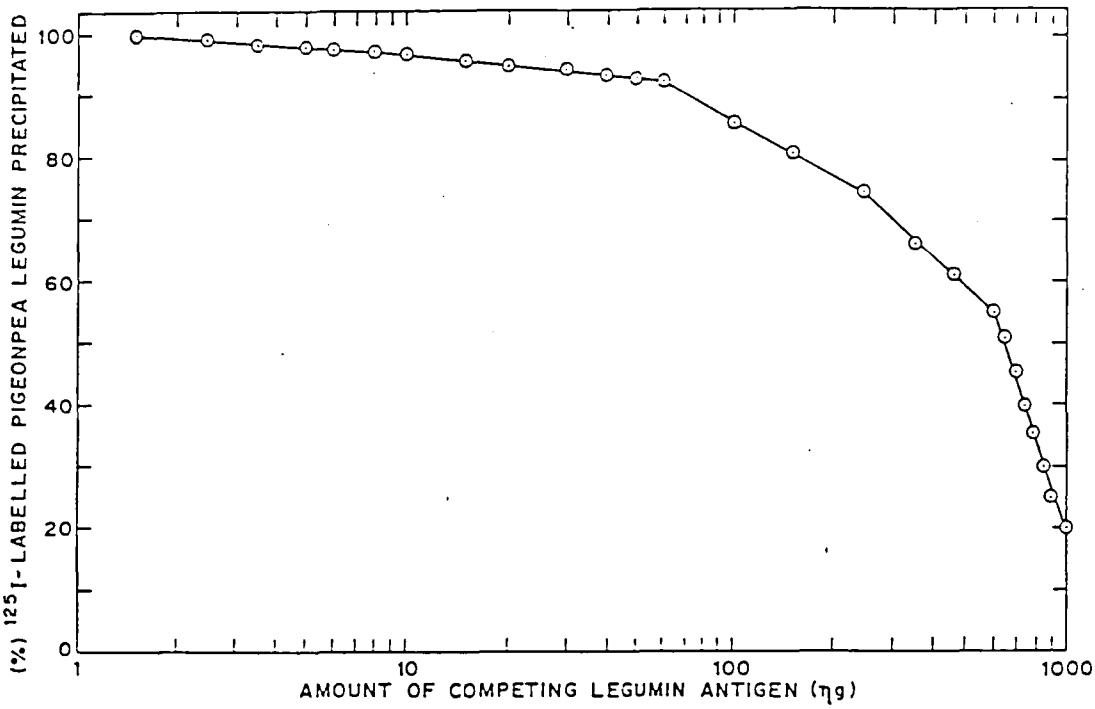


FIG . 4·8 (a)

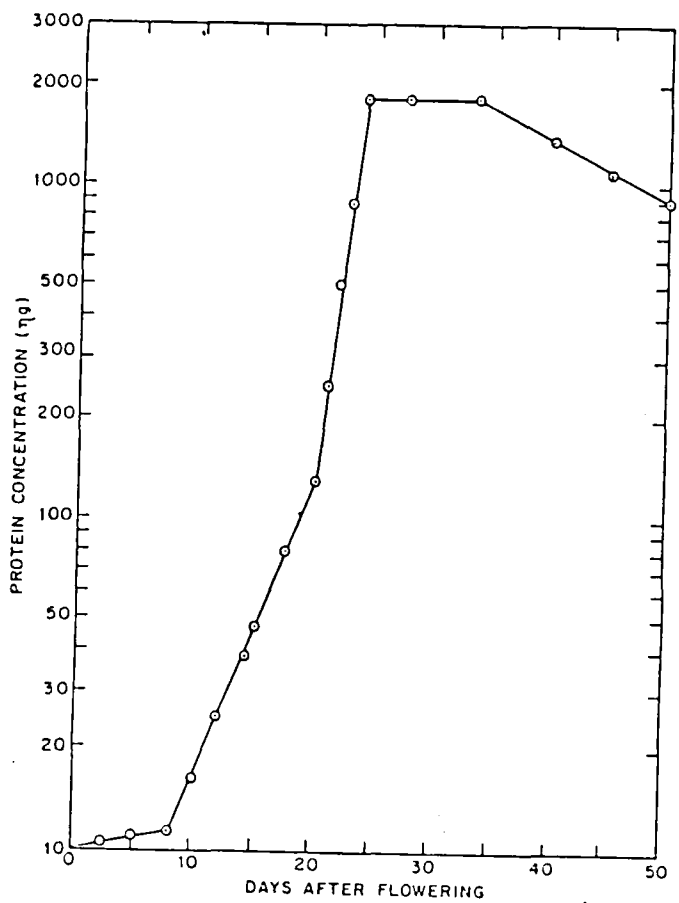


FIG . 4·8 (b)

Figure 4.9

ELISA of pigeonpea globulins using various dilutions of pigeonpea globulin antibodies dextran-peroxidase conjugate. The antigen was adsorbed on the microtiter plate well and ELISA performed as described in materials and methods (section 4.2.9)

(■) 1 : 1000 dilution of dextran conjugate

(⊙) 1 : 3000 dilution of dextran conjugate

(Δ) 1 : 5000 dilution of dextran conjugate

(▲) 1 : 1000 dilution of Goat antirabbit IgG peroxidase conjugate

(⊗) 1 : 7000 dilution of dextran conjugate

(X) 1 : 1000 dilution of pigeonpea globulin antibody peroxidase conjugate

(●) 1 : 1000 dilution of preimmune antibodies peroxidase conjugate

(⊡) bovine serum albumin.

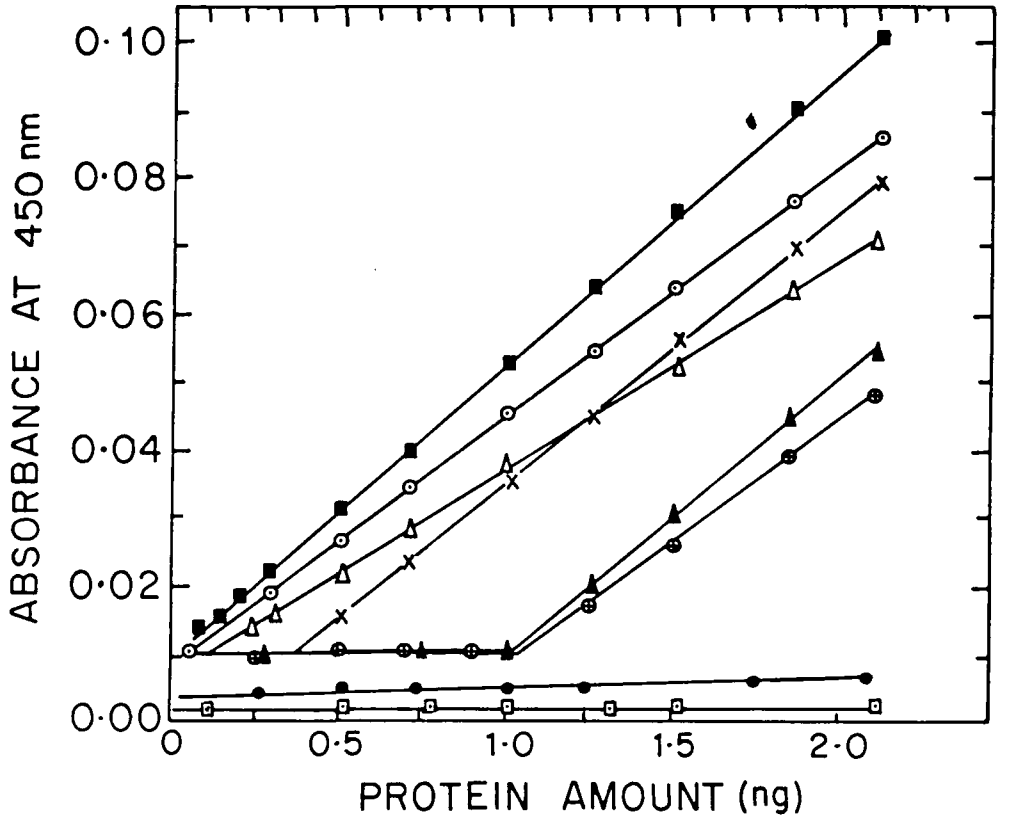

ABSORBANCE AT 450 nm
0·10
0·08
0·06
0·04
0·02
0·00
0
0·5
1·0
1·5
2·0
PROTEIN AMOUNT (ng)

FIG . 4·9

the goat anti-rabbit IgG horseradish peroxidase conjugate can detect pigeonpea globulin upto 1 ng when the dilution of 1:1000 is used. In contrast, at the same dilution, pigeonpea globulin IgG peroxidase conjugate can detect 0.34 ng while the dextran conjugate detects 0.1 ng of pigeonpea globulin. In fact, for measuring 1 ng pigeonpea globulin, 1:7000 dilution of dextran conjugate is sufficient. These results indicate that the dextran conjugate has a ten fold greater efficiency of quantitating pigeonpea globulin than the commercial goat anti rabbit IgG conjugate and a three fold more efficiency than the pigeonpea globulin IgG peroxidase conjugate. From Fig. 4.9, it is further evident that as the dilution of the dextran conjugate increases, the detectable amount of pigeonpea globulin decreases. Based on these studies, we decided that a dilution of 1:7000 was sensitive enough to quantitate pigeonpea globulins in the range of 0 - 1000 ng (Fig. 4.10).

To confirm the efficiency of the dextran conjugate method, we compared the time required to complete one assay. This comparison is schematically represented in Fig. 4.11. It is clear from the figure that while the conventional ELISA using goat anti-rabbit IgG conjugate takes about three hours and forty minutes for completion, the ELISA using dextran conjugate is completed in just one hour and fifty minutes,

Figure 4.10

ELISA of pigeonpea globulins. The antigen was adsorbed on microtiter plate wells and pigeonpea globulin antibodies - dextran conjugate (1:1000) was allowed to bind to these antigens. The bound conjugate was quantitated

(○) 1 : 1000 dilution of dextran conjugate

(□) 1 : 1000 dilution of pigeonpea globulin peroxidase conjugate

(⊗) 1 : 1000 dilution of preimmune antibodies peroxidase conjugate and

(●) bovine serum albumin.

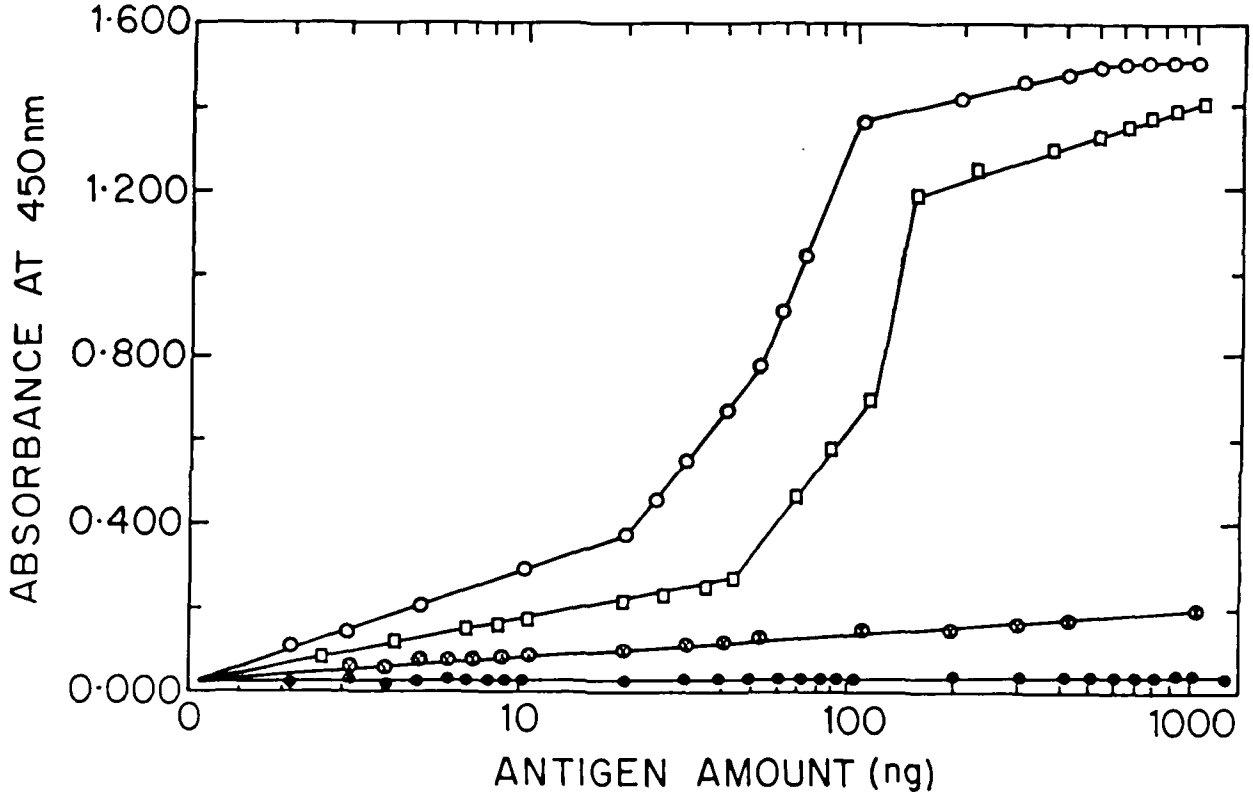

1·600
1·200
0·800
0·400
0·000
ABSORBANCE AT 450nm
0
10
100
1000
ANTIGEN AMOUNT (ng)

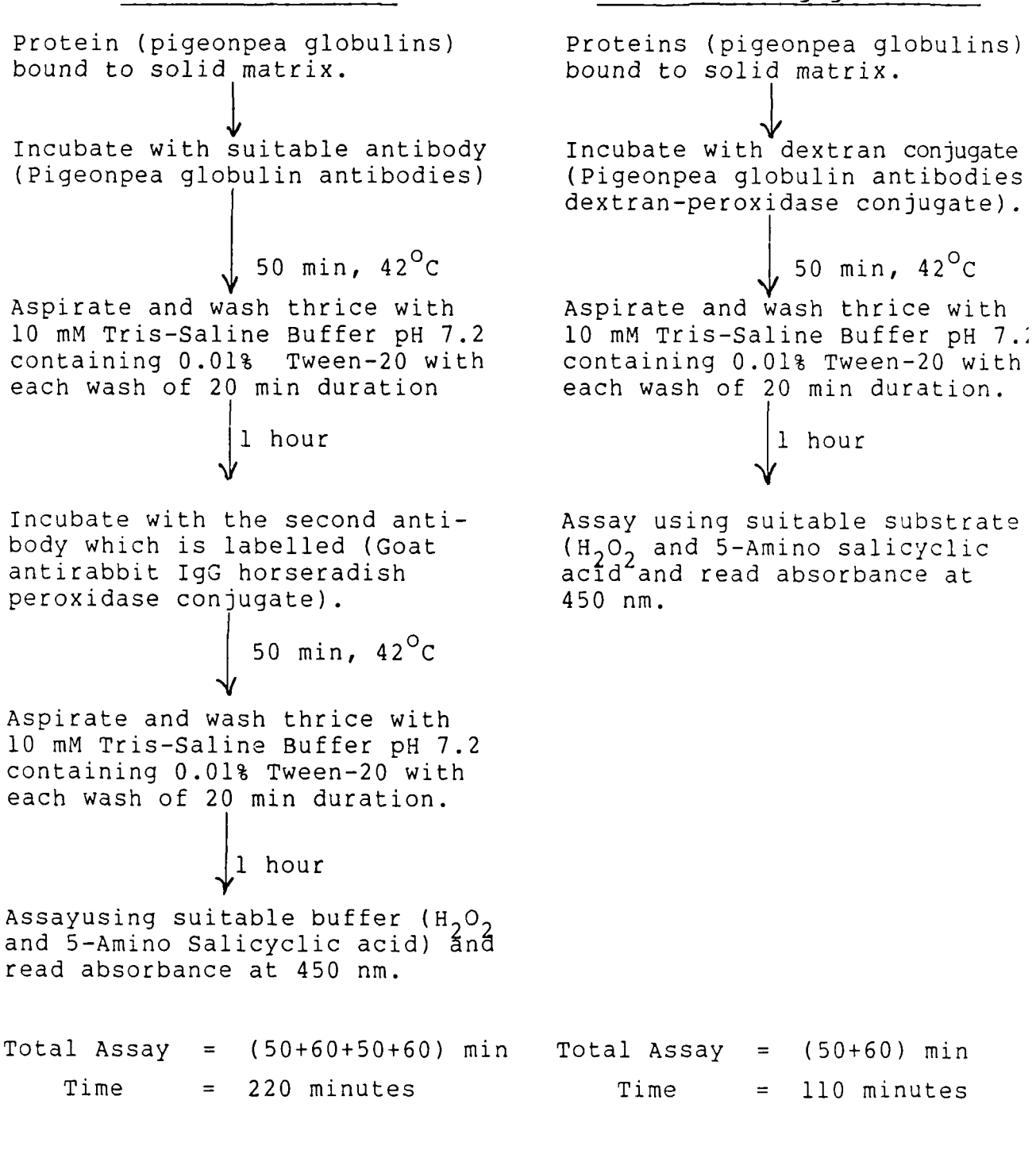
FIG . 4·10

Figure 4.11

Schematic representation for comparison between conventional ELISA and ELISA using pigeonpea globulin antibody-dextran peroxidase conjugate.

Figure 4.11

Schematic representation for comparision between conventional ELISA and ELISA using pigeonpea globulin antibody-dextran-peroxidase conjugate.



thereby, achieving a time saving of half the duration of the conventional ELISA. We further used this method for screening pigeonpea globulins from variety T-21 along with the neutron bombarded varieties TT-5, TT-6 and TAT-10 for globulin content/seed. Table 4.4 shows the amount of globulins required for 50 % binding of the labelled antigen (cv T-21) to dextran-pigeonpea globulin IgG conjugate. It can be seen from the table that 53, 71 and 88 ng of competing TT-5, TT-6 and TAT-10, respectively are required for 50 % binding of T-21 globulins to dextran conjugate globulins. The corresponding value for T-21 globulin is 48 ng.

4.4 DISCUSSION :

In the present work, I have described isolation of polyclonal antibodies against pigeonpea globulin and legumin protein, checking the purity as well as antigenicity of these proteins and development of sensitive immunoassays for quantitation of seed globulins during seed development.

Immunoassays have become an important tool in biomedical research and continuous efforts are being made to increase convenience, specificity and sensitivity of such assay (26). In recent years, seed proteins have also been analysed by immunological methods which are extremely sensitive and specific. The conventional methods that afford

TABLE 4.4

ELISA OF GLOBULINS OF DIFFERENT VARIETIES OF PIGEONPEA USING DEXTRAN-CONJUGATE

Variety	Globulin detected by ELISA (ng/mg of seed meal)	50% Binding (ng)
T-21	1.01 ± 0.05	48 ± 0.82
TT-5	0.84 ± 0.03	53 ± 0.71
TT-6	0.76 ± 0.04	71 ± 0.41
TAT-10	0.56 ± 0.06	81 ± 0.86

Each value is an average of experiments done in triplicate.

the requisite sensitivity include radiolabels, enzyme labels and fluorescence or phosphorescence labels (20, 27-32). Although these assays are elaborate, they are time consuming and require the use of sophisticated instruments. One of the way, to reduce assay time is to use the recognizing antibodies and the marker label in the same step. This would require coupling of marker label to the antibody. However, it has been shown that there is loss and/or reduction in binding of antibodies to antigen when the former is coupled to other protein/marker enzymes (33). An alternative to this would be to have the recognizing antibody and the marker label coupled saparately to a different matrix at the same time so that the whole matrix as such may be used in the assay. In this context, it is interesting to note that coupling of Bleomycin (BLM), a potent anticancer glycopeptide antibiotic and murine monoclonal anti-HLA IgG antibody (H-1) to dextran without a significant loss in antibody activity is reported (19,34). We,therefore, thought of using this approach to couple antibodies and a marker enzyme to dextran and to use this conjugate in an ELISA. Our method employing dextran conjugate is more sensitive and rapid. Furthermore, since the use of a second antibody is avoided, our method will prove to be economical also. An additional advantage of our method is that it is a simple method requiring no elaborate calibrations, no radioactive label or

no expensive equipment, and it uses stable reagents. The only prerequisite for our method is the availability of antibody against the protein to be quantitated. Any unknown protein can therefore be quantitated to as low as an amount as 0.1 ng once antibodies against the same protein are obtained.

Ours is probably the first attempt where partial immunological charaterization has been carried out for pigeonpea globulins. Also RIA and ELISA have been empolyed for the first time for quantitation and screening of seed globulins in different varieties of a given plant species. Furthermore, with the level of automation and sensitivity offered by these RIA and the fact that single seed has enough proteins to be used in a RIA or ELISA experiment, the following important biotechnological applications can be envisaged:

(i) Screening a large number of species/cultivars/ varieties as possible germplasms for desirable traits;

(ii) Quantitation of major constituents of the varieties of interest;

(iii) Understanding the developemental biology of plant proteins and

(iv) Diagnosis of plant diseases.

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CHAPTER 5

HOMOLOGY OF PIGEONPEA GLOBULIN WITH GLOBULIN FROM OTHER LEGUMES

ABSTRACT

Globulins from eleven other legumes namely, groundnut (*Arachis hypogeae*), bengal gram (*Cicer arietinum*), dolicos (*Dolicos biflorus*), cowpea (*Vigna unguiculata*), greengram (*Vigna radiata*), mothbean (*Vigna aconitifolia*), blackgram (*Vigna mungo*), fenugreek (*Trigonella foenumgraecum*), jackbean (*Concanavalin ensiformis*), soybean (*Glycine max*), and frenchbean (*Phaseolus vulgaris*), were compared with pigeonpea globulins to assess their relatedness among each other. Upon SDS-PAGE, all globulins showed the presence of major bands in the molecular weight range of 43-61 kD and around 14 kD. Jaccard index of similarity showed a maximum homology between pigeonpea and bengalgram. The circular dichroism spectra of twelve vicilin storage proteins were similar and belonged to B class proteins. Using antibodies raised against pigeonpea globulin it was found that bengalgram showed maximum homology to pigeonpea as compared to cowpea, soybean, frenchbean while blackgram showed the least homology.

5.1 INTRODUCTION:

In the previous two chapters, I have described the characterization of pigeonpea globulin and its constituent proteins employing a few approaches such as SDS-PAGE, peptide mapping, CD spectra and quantitative immunoassays. These approaches have given basic information about the size heterogeneity, amino acid makeup, secondary structure and rate of synthesis during seed formation. I was next interested in comparing the properties of pigeonpea globulin with those in other legumes in order to assess the relatedness among them. Such comparative studies have earlier been carried out in pea species and in species belonging to subfamily Papilionoideae. For example, it has been shown by Przybylska et al. (1) that Pisum abyssinicum, P. fulvum and P. cinereum are relatively distant taxa, whereas P. eletius, P. syriacum and P. sativum are more closely related. These results were confirmed by Hadcova et al (1) using immunoelectrophoretic techniques. Sammour (3) using electrophoretic and serological studies have compared some members of the subfamily Papilionoideae. In earlier studies, although the electrophoretic studies revealed the presence of legumin and vicilin-like protein outside Vicieae, all the serological studies failed to detect legumin or vicilin-like protein outside Vicieae. However, Sammour et al (3) using

western blotting technique have shown that the representative of the tribes Cajaneae and Phaseoleae except Phaseolus vulgaris and Phaseolus coccineus gave a cross-reaction with pea vicilin antiserum.

Since no detailed information was known about pigeonpea globulin before the present work was undertaken, nobody attempted a comparison of pigeonpea globulins with globulins from other leguminous species. In this chapter, I have compared seed globulins from different legumes with pigeonpea globulin using SDS-PAGE, amino acid composition and various immunological methods.

5.2 MATERIALS AND METHODS :

Seeds of pigeonpea (Cajanus cajan), cowpea (Vigna unguiculata), mothbean (Vigna aconitifolia), greengram (Vigna radiata), blackgram (Vigna mungo), groundnut (Arachis hypogeae), bengalgram (Cicer arietinum), dolicos (Dolicos biflorus), soybean (Glycine max), frenchbean (Phaseolus vulgaris), fenugreek (Trigonella foenumgraecum) and jackbean (Concanavalin ensiformis) were procurred locally from Parekh Traders and Pocha seeds, Pune.

The details of all the methodologies used in comparing legume seed globulins have already been described in earlier chapters.

The polyacrylamide gel data were analyzed using the

Jaccard index of similarity as applied to Pisum (4 ,5), where not only the presence but also the intensity of each band was taken into account to establish relatedness of a protein among different legumes.

$$\text{Similarity Index (S.I.)} = \frac{\text{Number of common bands}}{\text{Number of common band} + \text{Number of uncommon band}} \times 100$$

5.3. RESULTS

5.3.1 SDS-PAGE and Jaccard Index:

Electrophoresis of proteins in polyacrylamide and other gels has been used extensively to characterise crop cultivars (6) and to compare different plant species (7-9). Taxonomical affinities among species have also been established on the basis of electrophoretic data. In order to study the relatedness among legumes, the globulins from pigeonpea and other legumes were electrophoresed on 12.5% SDS-PAGE. From Fig. 5.1, it is clear that globulin subunits show certain similarities as well as species specific patterns. All the globulins, for example, show the presence of major bands in the molecular weight range of 43-61 kD and around 14 kD. These correspond to the most abundant subunits of the globulins. Species specific differences are also discernible as in case of pigeonpea, bengalgram, fenugreek

Figure 5.1

SDS-PAGE (12.5 %) of globulins from various legumes. About 30 ug protein was loaded in each track after treatment with SDS. Lane (a) Marker protein (phosphorylase 94 kD); bovine serum albumin 67 kD); Ovalbumin 43 kD; soybean trypsin inhibitor 20 kD, (b) pigeonpea (c) greengram (d) frenchbean, (e) fenugreek (f) bengalgram (g) mothbean, (h) blackgram (i) soybean (j) dolicos (k) groundnut and (l) cowpea.

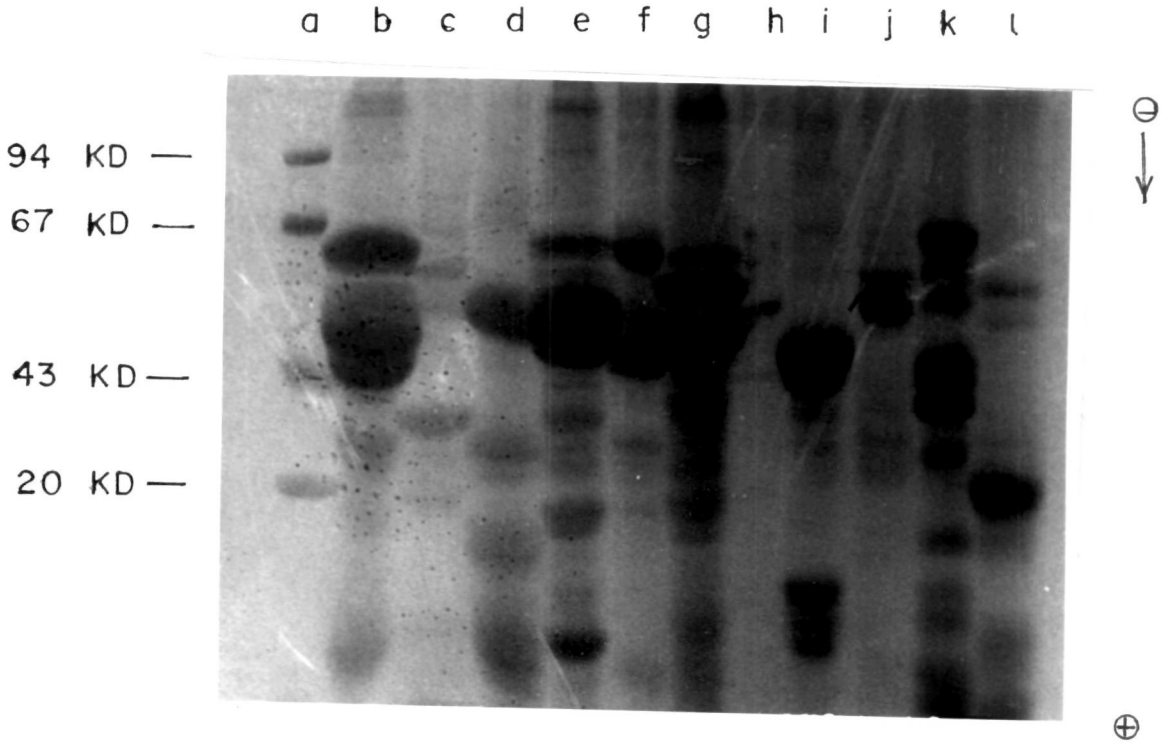


FIG . 5·1

and soybean where the band number and intensity of proteins above molecular weight 80 kD are different. It is further evident that the band number is varying in globulins below molecular weight 14.4 kD. As the globulins were isolated in presence of PMSF (Phenyl Methyl Sulphonyl Fluoride), the possibility of obtaining the proteolytic degradation products may be ruled out.

Based on the SDS-PAGE data, the index of similarity among legume globulins was calculated using Jaccard indices. The affinity matrix prepared after calculation of Jaccard indices for matched pairs is given in Table 5.1. From the table, it is clear that pigeonpea and bengalgram share a maximum homology (index value 66.67), while the next best value is 45.16 for fenugreek.

The Vigna's namely greengram, blackgram, cowpea, and mothbean appear to share more or less similar Jaccard index (around 20-25). From the table, it is further evident that fenugreek is related to greengram, frenchbean, bengalgram, mothbean and soybean to the same extent showing similarity in their index value which falls in the range of 35-37.

5.3.2 Amino acid analysis:

The globulins from legumes were next compared for their

TABLE 5.1

JACCARD INDEX SIMILARITIES IN ELEVEN LEGUMES

	Pigeonpea	Greengram	Frenchbean	Fenugreek	Bengalgram	Mothbean	Blackgram	Soybean	Dolicos	Groundnut	Cowpea
jeonpea	100	24.14	27.27	45.16	66.67	41.18	20.69	42.86	29.63	41.46	27.78
eengram		100	12	34.79	29.17	17.24	5.00	18.18	16.67	12.82	18.52
enchbean			100	37.04	23.34	25.00	8.00	33.33	8.00	19.05	15.15
nugreek				100	37.93	37.38	7.14	34.62	15.39	26.19	24.24
ngalgram					100	26.47	7.14	29.62	20.00	26.19	24.24
thbean						100	10.00	31.04	26.92	24.44	22.22
ackgram							100	19.05	25.00	19.44	14.81
ybean								100	25.00	26.32	20.00
licos									100	19.45	19.23
undnut										100	28.57
vpea											100

amino acid composition. A comparison of the amino acid composition of these globulins from the available data and those calculated by us for pigeonpea and mothbean is depicted in Table 5.2. It can be seen from Table 5.2, that all the globulins contain (i) high amount of asparagine (Asx), glutamine (Glx) and arginine (Arg) as compared to other amino acids and (ii) low amount of cysteine.

For each amino acid, the highest and the lowest content found among these genera were used to calculate the ratio of maximum content/minimum content. It is evident from the table that the amino acids which fall predominantly in the ratio range of 1.2 - 1.9 are Glycine (Gly), Alanine (Ala, Valine (Val), Leucine (Leu), Isoleucine (Ile), Serine (Ser), Threonine (Thr), Tyrosine (Tyr), Proline (Pro), Histidine (His), Arginine (Arg), Asparagine (Asx), Glutamine (Glx), while the amino acids which show ratios from 1.91 - 2.6 are Phenyl alanine (Phe), Tryptophan (Trp), Methionine (Met), Cysteine(Cys) and Lysine (Lys). Maximum variation is observed in the content of lysine (3.45 g/16 g N in groundnut to 7.85 g/16 g N in greengram) while the variation in other amino acids is to a much lesser extent.

The molecular weights of purified vicilin holoproteins were estimated by gel filtration chromatography using a sepharose CL-6B column which was previously

TABLE 5.2

AMINO ACID COMPOSITION OF (g AMINOACID / 16 g N) OF GLOBULINS FROM LEGUMES

Species	Prot. %	Gly	Ala	Val	Leu	Ile	Ser	Thr	Tyr	Phe	Trp	Pro	Met	Cys	Lys	His	Arg	Asx	Glx	Total
Groundnut	25.00	5.90	4.05	4.40	6.55	3.60	4.95	2.70	4.00	5.05	1.05	4.70	1.40	1.45	3.45	2.20	11.60	11.70	18.90	97.60
Pigeonpea	29.00	4.11	3.18	2.99	6.92	3.09	4.22	3.01	2.16	8.30	2.08	3.59	0.78	0.98	6.76	3.84	6.18	9.12	18.67	89.98
Bengalgram	19.40	4.00	4.10	4.60	7.60	4.40	5.20	3.50	3.30	6.60	ND	4.30	1.40	ND	7.20	2.23	8.80	11.70	16.00	95.00
Dolicos	26.40	4.30	4.50	5.10	8.20	4.40	5.50	3.70	3.60	4.80	ND	5.40	0.90	1.10	6.20	2.80	7.00	11.50	16.30	95.30
Soybean	40.00	4.40	4.30	5.15	7.90	5.00	5.15	3.90	3.60	5.10	1.13	5.90	1.55	1.65	6.35	2.75	8.10	11.80	18.00	101.90
Frenchbean	27.00	3.80	4.00	4.85	8.30	4.45	6.00	4.30	3.40	6.10	ND	3.50	1.15	1.10	6.70	2.70	6.70	12.40	15.90	95.05
Fenugreek	33.00	4.20	3.10	3.30	5.65	4.30	4.10	2.90	2.80	4.10	0.80	3.95	0.80	1.10	5.50	2.15	8.50	9.20	15.20	81.65
Mothbean	26.00	3.60	4.14	5.14	8.10	4.03	5.70	3.90	3.50	5.70	1.20	4.40	0.97	1.03	6.90	2.40	8.70	11.90	16.80	98.07
Blackgram	21.00	4.40	4.50	4.65	8.30	3.95	5.30	3.75	3.70	5.60	ND	3.90	1.85	1.05	7.25	2.80	6.35	12.00	16.10	95.40
Greengram	28.00	3.75	4.25	5.10	8.35	4.40	4.10	3.40	3.30	4.90	ND	4.05	1.30	0.85	7.85	2.90	7.35	11.80	17.60	97.25
Cowpea	27.00	4.10	4.20	5.20	7.30	4.10	5.00	3.80	3.20	5.20	ND	3.50	1.90	1.70	6.30	3.10	7.10	11.00	16.60	93.30
Maximum		1.64	1.45	1.74	1.27	1.62	1.46	1.59	1.85	2.02	2.60	1.69	2.44	2.00	2.28	1.79	1.88	1.36	1.24	
Minimum																				

calibrated using standard proteins of known molecular weights. A single symmetrical peak was observed for each of the purified vicilin proteins. The estimated molecular weights for these proteins are depicted in Table 5.3. It is evident from the table that the molecular weights for all the holoproteins fall in the range of 105,000 - 204,000 in all the vicilins of legumes under consideration.

5.3.3. Secondary structure of legume vicilins:

A structural homology among 11S globulins from Glycine max, Vicia faba, Lupinus unqustifolius, Arachis hypogeae, Helianthus annus, Brassica napus and Bertholletia excelsa, has been observed earlier on the basis of encoding nucleotide sequence, amino acid sequences, predicted secondary structure of the polypeptide chains and reconstitution of oligomeric legumin molecule from the polypeptide chains of various species. The experimental results concerning the structure of the 7S vicilins are, however, still scarce (10-13). Hence CD spectra of pigeonpea vicilin along with vicilin from other legume storage proteins were analysed for their secondary structure.

Fig. 5.2 shows the CD spectra for vicilins from different legumes. Since vicilins were dissolved in high salt solutions, the spectra below the wavelength 195 nm could

TABLE 5.3

MOLECULAR WEIGHTS FOR VICILIN HOLOPROTEIN FROM VARIOUS LEGUMES

	Source	Molecular Weight (Dalton)
1.	Pigeonpea	190,000
2.	Cowpea	186,000
3.	Mothbean	175,000
4.	Greengram	165,000
5.	Blackgram	150,000
6.	Groundnut	185,000
7.	Bengalgram	155,000
8.	Dolicos	170,000
9.	Soybean	195,000
10.	Frenchbean	150,000
11.	Fenugreek	160,000
12.	Jackbean	140,000

Figure 5.2

Circular dichroism spectra of vicilins from various legumes

(a) Pigeonpea (b) greengram (c) soybean

(d) frenchbean (e) groundnut (f) blackgram

(g) cowpea (h) bengalgram (i) mothbean

(j) jackbean and (k) dolicos.

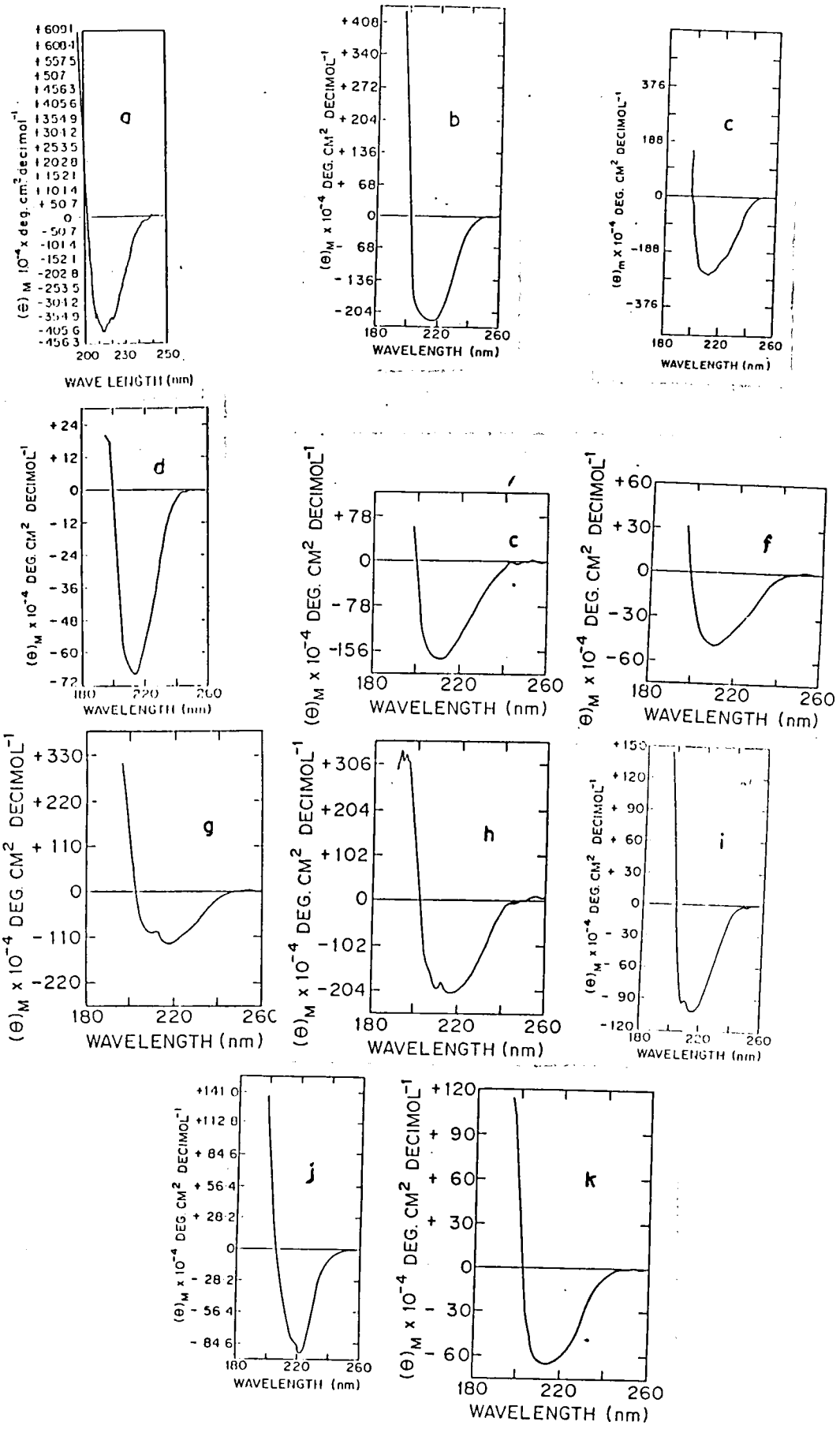

a
b
c
d
e
f
g
h
i
j
k
WAVELENGTH (nm)
WAVE LENGTH (nm)
DECIMOL⁻¹
DEG. CM² DECIMOL⁻¹

FIG. 5·2

not be taken because of high background noise. In all the CD studies, phaseolin was taken as control as its amino acid sequence and CD data were available. The CD spectrum of phaseolin under our experimental conditions compared well with the literature data (14). The absorption minima and molar ellipticity of all the vicilins were derived from Fig. 5.2 and are summarized in Table 5.4. From the data in Fig. 5.2 and Table 5.4, it can be seen that the CD spectra of all the vicilins show a peak pattern similar to B-class containing both α-helices and β-sheet. Furthermore, the peak minima wavelength of all the vicilins are very close to each other.

Since out of 12 vicilins under consideration, the amino acid sequence data of four vicilins were known, we deduced the secondary structure of these vicilins using Chou-Fasman secondary structure prediction and then compared the predicted secondary structure with that obtained from the actual CD data. From Fig. 5.3, it can be clearly seen that there is a considerable similarity between the predicted and the actual CD data of four vicilins. The differences that are observed in the alignment of secondary structure are due to insertions of a hypervariable region.

TABLE 5.4

ABSORPTION MINIMA AND MOLAR ELLIPTICITY

OF VICILIN FROM LEGUMES

Vicilin Source	Peak minima at wavelength (nm)	Molar ellipticity $(\theta)_M \times 10^{-2}$ deg. cm^2. $decimol^{-1}$
Cowpea	217	126.5
Bengal gram	217	209.0
French bean	215	68.4
Dolicos	215	66.0
Green gram	217	224.0
Black gram	215	51.0
Groundnut	215	171.0
Jack bean	217	81.7
Fenugreek	217	73.0
Soybean	215	276.0
Pigeonpea	214	712.0
Mothbean	216	102.0

Figure 5.3

Chou-Fasman secondary structure prediction of amino acid sequence data of four known vicilins from legumes.

a:Pisum sativum vicilin b:Phaseolus vulgaris phaseolin

c:Glycine max α-conglycinin d:Concanavalin ensiformis con A

A-alpha helical β-Beta structure R-random coil structure

FIG . 5·3

5.3.4. Immunochemical relatedness :

The immunological relatedness of pigeonpea globulins with globulins from other legumes was next determined using the antibodies against pigeonpea globulins. As a first step towards this objectives, the double diffusion approach was employed. In this method, antibodies against pigeonpea globulins were allowed to cross-react with globulins from other legumes and the results obtained are depicted in Fig. 5.4. From the data in this figure, it can be seen that a maximum cross-reaction is observed between pigeonpea globulin antibody and globulins from bengalgram. It is further noticed that globulins from other plants show a cross-reaction with pigeonpea antibody to a varying extent. The immunological relatedness among globulin proteins was next assessed by rocket and immunoelectrophoresis. In these methods, the globulins from other legumes were allowed to cross-react in agarose with pigeonpea globulin antibodies. Under the influence of electric charge, the proteins migrate depending on their overall charge. The number and intensity of protein bands observed is characteristic of their antigenicity and is not a gross effect. For rocket electrophoresis, the peak height is directly proportional to the concentration of the protein. Hence keeping the concentration factor of each protein constant (10 μg), rocket intensity was

Figure 5.4

Comparative Ouchterlony double diffusion of seed globulins.

Central well : anti pigeonpea globulin antibodies.

Peripheral wells : Globulin from (a) pigeonpea (b) fenugreek (c) soybean (d) dolicos (e) bengalgram (f) cowpea (g) frenchbean (h) bengalgram (i) groundnut (j) greengram (k) mothbean and (l) bovine serum albumin.

Double diffucison was performed in 1 % agaorse for 48 - 72 h at 4 ^{o}C in a moist chamber.

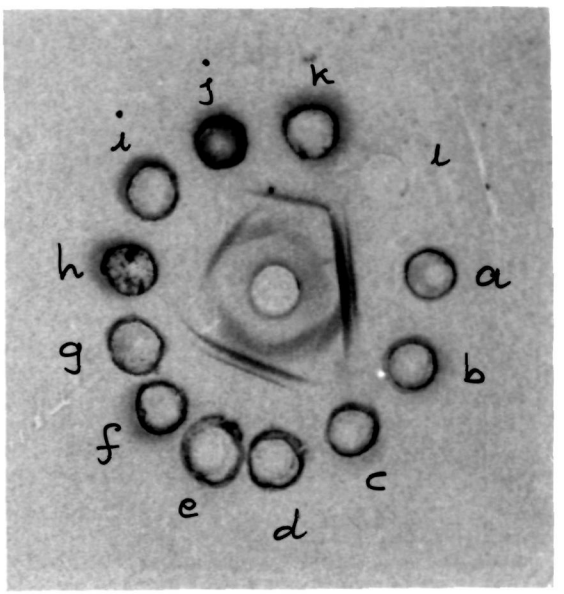


FIG. 5·4

calculated and this is shown in Table 5.5. Similarly, for immunoelectrophoresis, keeping the concentration factor constant, the proteins were electrophoresed in one direction and allowed to diffuse in the other direction. The rocket and immunoelectrophoresis (Fig. 5.5 and Table 5.5) reveal very high immunological relatedness (Intensity value = ++++) with bengalgram, fenugreek, soybean, goundnut and mothbean; moderate homology (Intensity value = +++) with dolicos, cowpea and frenchbean; and little homology with greengram (Intensity value = ++) and blackgram (Intensity value = +).

Since the globulins from legumes were able to react with antipigeonpea globulin, a quantitative measure of relatedness of pigeonpea globulin with globulins from other legumes was performed using RIA and ELISA. Figure 5.6 shows the comparative ELISA for these globulins. The figure clearly indicates that bengalgram, fenugreek and soybean are more closely related to pigeonpea than groundnut, mothbean and dolicos, while blackgram is least related. A quantitative measure obtained by these methods, and the results depicted in Fig. 5.6 and Table 5.5 are in agreeable range showing a standard deviation of $\pm$ 3 %. Table 5.6 shows the amount of globulins required for 50 % binding of the labelled antigen (pigeonpea globulin). It can be calculated from Table 5.6 that 590, 611 and 650 ng of competing

TABLE 5.5.

RELATEDNESS AMONG GLOBULINS FROM LEGUMES

	Immuno[a] electrophoresis	Rocket[a] electrophoresis	% relatedness[b]	Jaccard Index[c]
Pigeonpea	++++	++++	100	100
Bengalgram	++++	++++	88	66.67
Fenugreek	++++	++++	85	45.16
Soybean	++++	++++	80	42.86
Groundnut	++++	++++	79	41.46
Mothbean	+++	++++	73	41.18
Dolicos	+++	+++	71	29.63
Cowpea	+++	++	65	27.78
Frenchbean	+++	++	60	27.27
Greengram	++	++	57	24.14
Blackgram	+	++	50	20.69

(a) ++++ - very high homology; +++ - Substantial homology; ++ - Moderate homology; + - low homology as observed in Fig. 5.5

(b) % relatedness calculated from mean of 50% binding d(from RIA) and 50% absorbance (from ELISA) as described earlier

(c) As depicted from Table 5.1.

Figure 5.5

a) Comparative rocket electrophoresis of globulins from various legumes in 1% agarose gel containing 0.2% antipigeonpea globulin antibodies. Electrophoresis was performed in cold at 200 V for 16-18 h. The different wells show globulins from

(a)	pigeonpea	(b)	bengalgram	(c)	fenugreek
(d)	soybean	(e)	groundnut	(f)	blackgram
(g)	dolicos	(h)	cowpea	(i)	frenchbean
(j)	greengram	(k)	mothbean		

b) Comparative immuno electrophoresis of globulins from various legumes in 1% agarose gel containing 0.2% antipigeonpea globulin antibodies. Electrophoresis was performed in cold at 200 V for 16-18 h. The different wells show globulins from

(a)	pigeonpea	(b)	greengram	(c)	fenugreek
(d)	frenchbean	(e)	bengalgram	(f)	soybean
(g)	mothbean	(h)	blackgram	(i)	groundnut
(j)	dolicos	(k)	cowpea	(l)	BSA

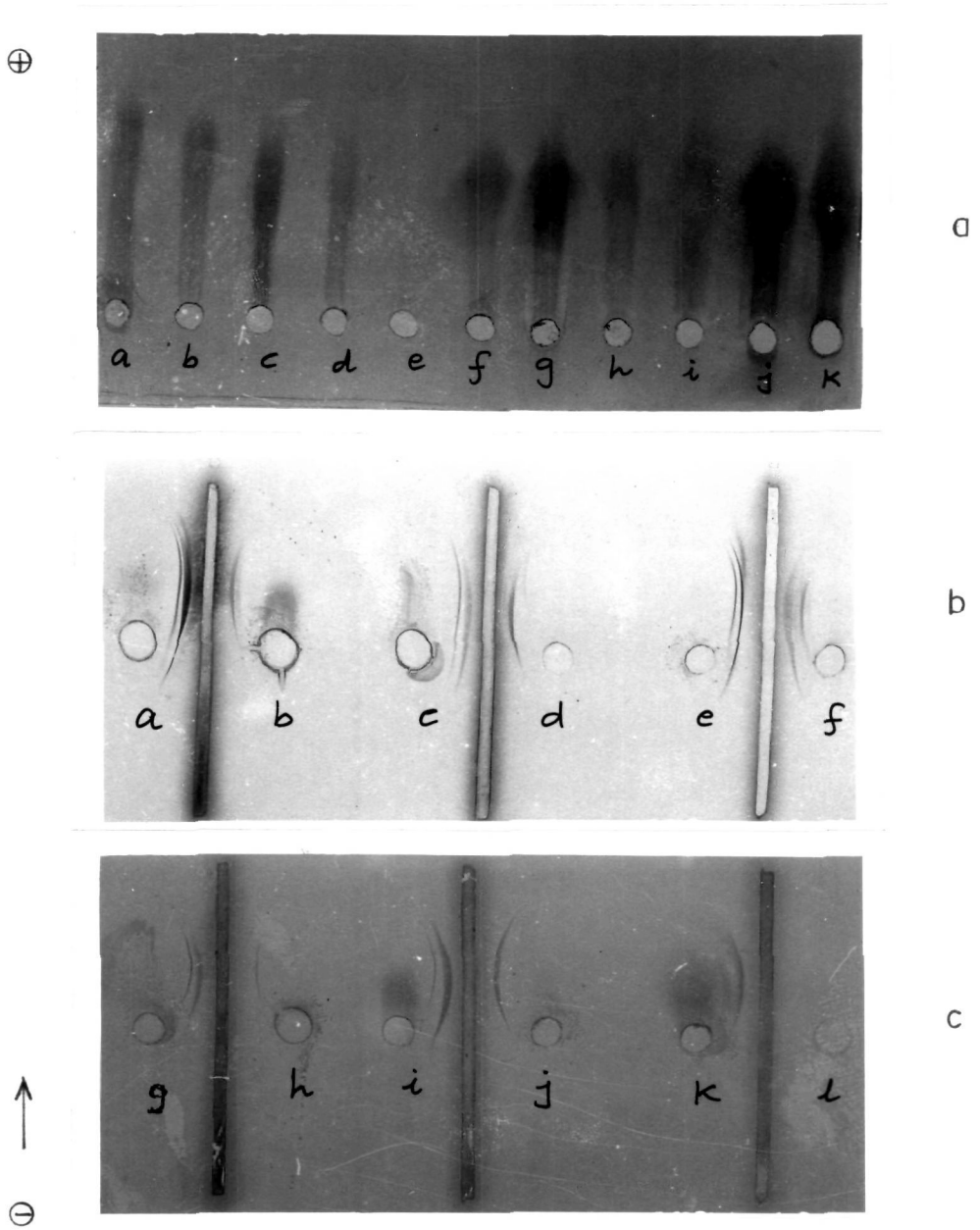
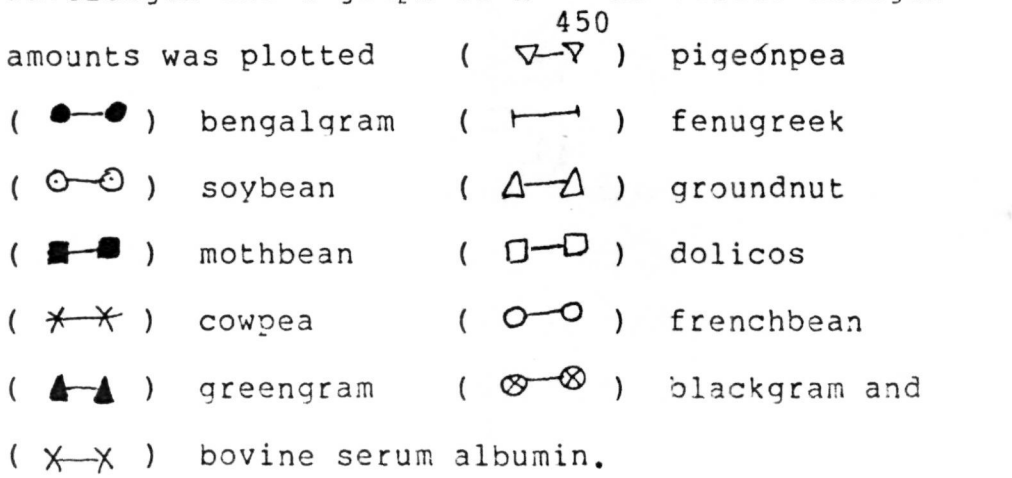


FIG. 5·5

Figure 5.6

Comparative ELISA of seed globulins : ELISA was carried out in polystyrene microtitre plates for varying amounts of antigen (50 - 10,000 ng). The enzyme reaction was assayed at 450 nm wavelength and a graph of A_{450} nm versus antigen amounts was plotted



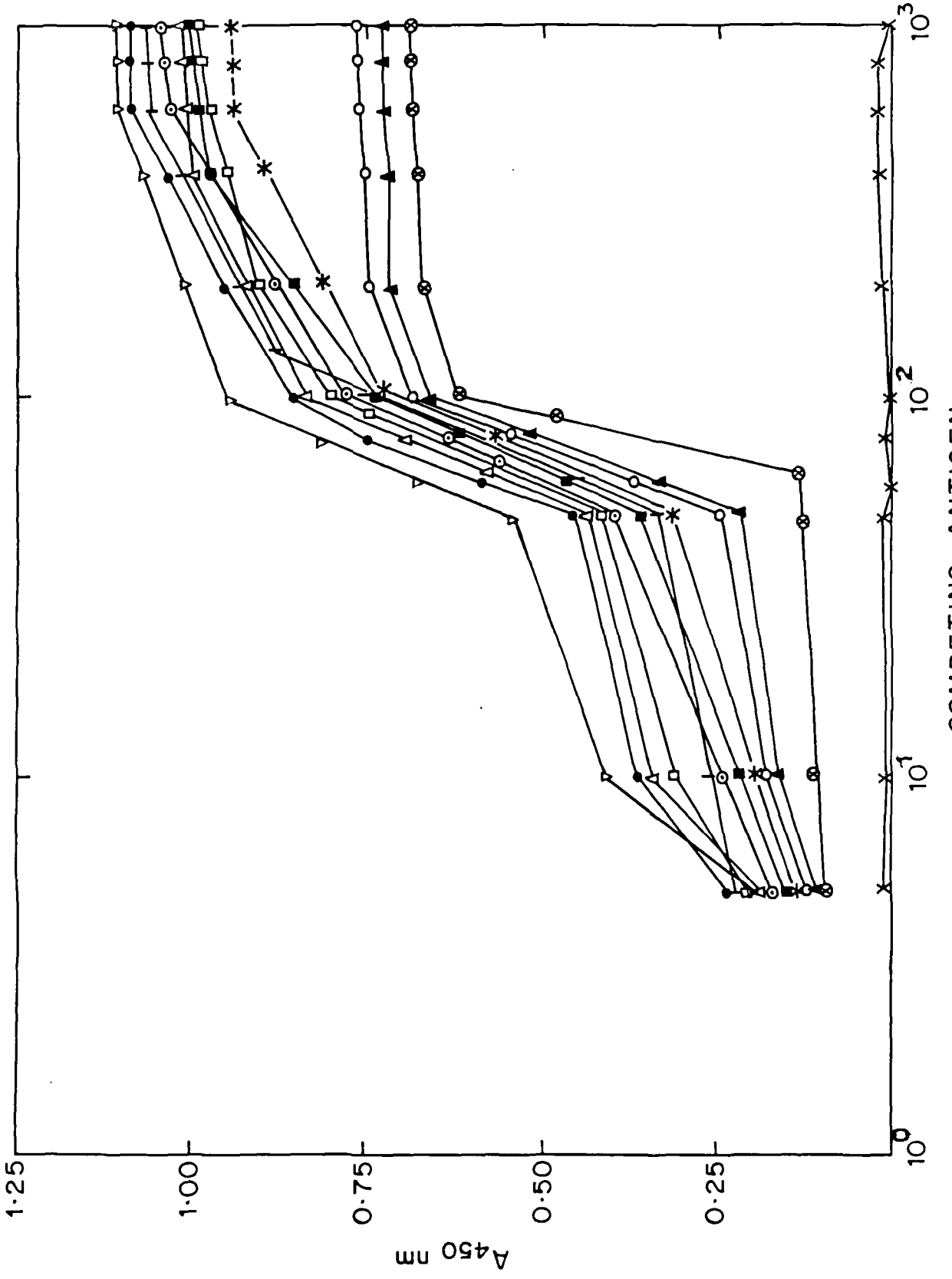

1·25
1·00
0·75
0·50
0·25
A450 nm
10^0
10^1
10^2
10^3
ng COMPETING ANTIGEN

FIG 5·6

TABLE 5.6

RIA OF GLOBULINS FROM VARIOUS LEGUMES

Source	ng of competing antigen for 50%* binding of pigeonpea globulin
Pigeonpea	520 ± 23
Bengalgram	590 ± 18
Fenugreek	611 ± 30
Soybean	650 ± 26
Groundnut	659 ± 32
Mothbean	713 ± 29
Dolicos	734 ± 15
Cowpea	800 ± 21
Frenchbean	868 ± 14
Greengram	914 ± 36
Blackgram	1040 ± 80

All the values represent average of experiments done in triplicate.

* Value calculated from the RIA graph (as described earlier).

bengalgram, fenugreek and soybean globulin, respectively are required for 50 % binding of pigeonpea globulin. The corresponding value for pigeonpea globulins is 520 ng.

5.4 DISCUSSION

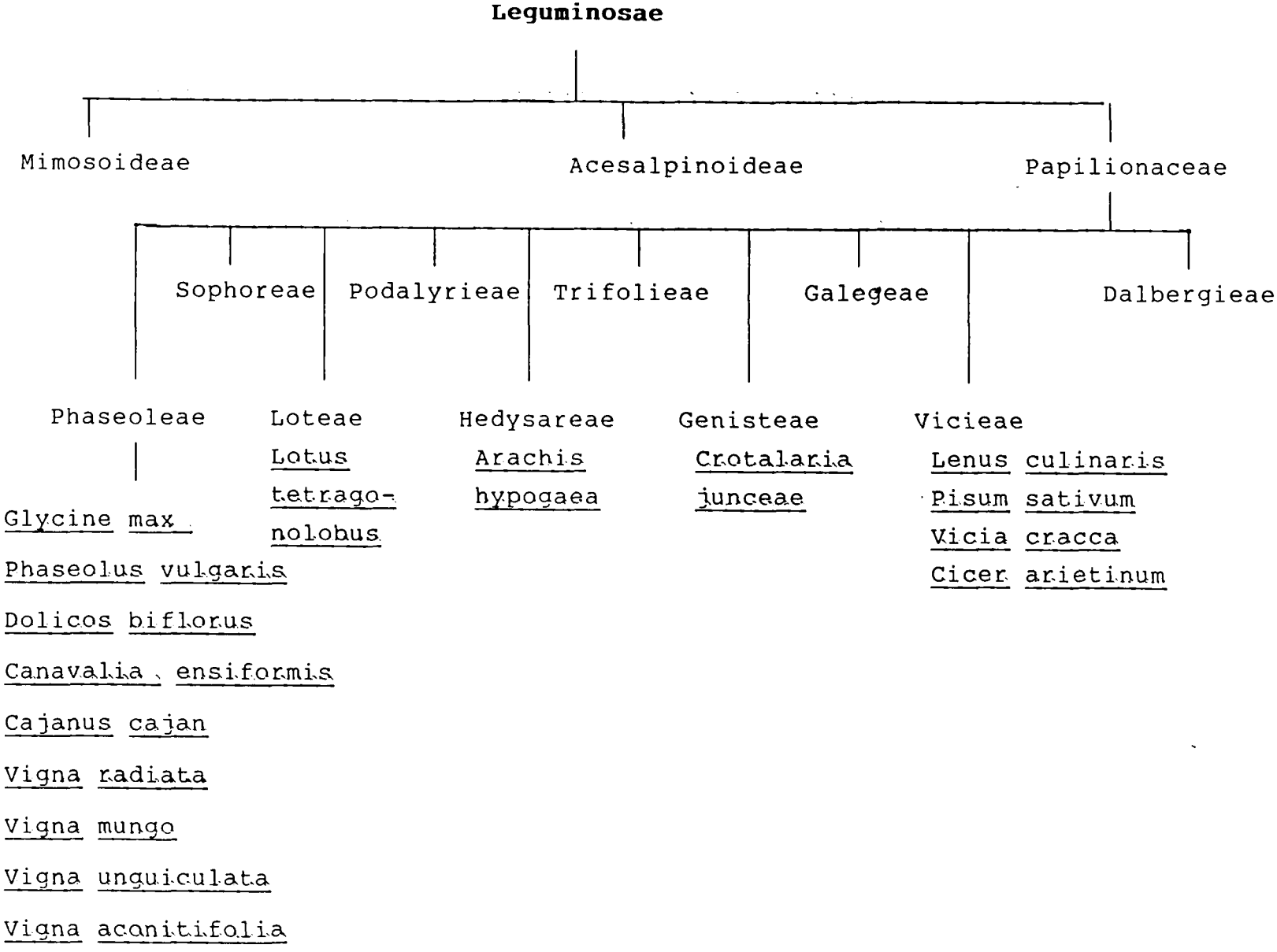
In the earlier work on comparison of seed proteins in legumes, a series of electrophoretic (15,16), immunochemical (17,18), morphological (19), and genetic studies (20) have established the extent of relatedness in a few legumes. These methods have also been used to establish the validity of the taxonomic revision of genera Phaseolus and Vigna. In recent years, several revisions of the tribe Phaseoleae have been suggested (21). For example, one of the genera of this tribe, Phaseolus, was reorganised and many of its species were transferred to other genera like Vigna, Azukia and Macroptilium.

In our work on comparison of pigeonpea globulins with globulins from other legumes using independent methods such as SDS-PAGE, Jaccard index and various immunological techniques (Table 5.5), it is observed that bengalgram globulin shows a maximum homology to pigeonpea globulin. From Fig. 5.7, it can be seen that pigeonpea and bengalgram belong to separate tribes namely Phaseoleae and Vicieae,

Figure 5.7

Taxnomic distribution of legumes

FIG . 5·7


Leguminosae
Mimosoideae
Acesalpinoideae
Papilionaceae
Sophoreae
Podalyrieae
Trifolieae
Galegeae
Dalbergieae
Phaseoleae
Loteae
Hedysareae
Genisteae
Vicieae
Glycine max
Phaseolus vulgaris
Dolicos biflorus
Canavalia ensiformis
Cajanus cajan
Vigna radiata
Vigna mungo
Vigna unguiculata
Vigna aconitifolia
Lotus tetragonolobus
Arachis hypogaea
Crotalaria junceae
Lenus culinaris
Pisum sativum
Vicia cracca
Cicer arietinum

respectively. In spite of this classification, however, pigeonpea shares a maximum homology to bengalgram as compared to Glycine max, Phaseolus vulgaris, Dolicos biflorus, Vigna radiata, Vigna mungo, Vigna unguiculata and Vigna aconitifolia which are members of the same tribe Phaseoleae. This is a very curious finding and more light can be thrown on this observation when the nucleotide and amino acid sequence data of these globulins becomes available.

In our study on the comparison of the observed secondary structures of the legumes, conservation in secondary structure was observed where all vicilins were found to be present in B pleat structure. Hence an attempt was made to compare the known vicilin sequences in legumes using Chou-Fasman approach to assess the sequence homology and conservation of secondary structure among these proteins. The biochemical data of 12 vicilins from present investigations are available and these indicate several variations in their specific properties (11, 12). The observed secondary structure similarity among these 12 vicilins strongly suggests some conservation for the known moiety of the polytpeptide chain inspite of their general microheterogeneity.

If storage proteins were only copolymerized amino acids devoted mainly to the nutrition of young seedlings, a very high mutability in their amino acid sequence would be expected. The secondary structure of these proteins, however, is highly homologous and this phenomenon needs a suitable explanation. Could it be that the storage protein folding has been maintained during evolution in order to fit active site of peculiar endoproteases which hydrolyse seed proteins in the course of germination? Then the regions susceptible to these proteases would be the random coil nature of proteins which are called loops. However, there is no evidence of loops being ready targets for indiscriminate proteolysis and thus the above assumption may not be valid.

According to Pernollet and Mosse (22, 23), the severe conservation of predicted secondary structure of storage proteins is mainly adapted for maximal packaging within the protein bodies. They further stated that the shared common structural features of these proteins could be related to a common ancestral gene. Our CD data where a significant conservation is observed among twelve vicilins also supports the maximal packaging hypothesis of Pernollet and Mosse (22, 23). Apart from supporting the packaging hypothesis, our CD data will also be useful in site directed mutagenesis work of these protein after their amino acid sequence data becomes

available. It is important that the changes that will be introduced in the codons of the specific genes should not affect their secondary structure as well as their normal deposition during post anthesis.

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CHAPTER 6

COMPUTER ANALYSIS OF LEGUME SEED STORAGE PROTEIN GENES

ABSTRACT

In the present work, the cDNA and the genomic sequences of as many as twelve legume storage protein genes have been compared using different approaches like dot matrix, hydropathy analysis, amino acid sequence comparison, codon usage and homology around (i) post-translational cleavage site (ii)disulphide linkage site and (iii) glycosylation site. The main objective of this work was to assess the sequence homology so as to search for conserved and diverged regions in these genes for determining probable sites that could be available for site directed mutagenesis. The homology around putative post translational processing site, canonical glycosylation site and postulated disulphide linkage site suggests that these three factors may be playing an important role in stabilization of conformations and compartmentalization of seed storage proteins. Secondly, it can be postulated that modification of isoleucine to methionine may not possibly affect the globulin structure and hopefully may not affect accumulation in protein bodies. Finally, taking into account all the data together, it could be proposed that genes encoding the 11S and 7S storage protein of legumes, oat globulin, patatin and napin might have evolved from a common ancestral gene.

6.1 INTRODUCTION

Molecular investigations of plant seed storage proteins and their genes are interesting as they provide an excellent system which shows both tissue specific and developmental regulation. The overall goal in studying seed storage protein genes is to have an understanding of these genes with respect to their structural organization, the mechanism which regulates their expression and their diversity among the plant species. With the availability of transformation and regeneration schemes for crop species, storage protein genes can be engineered so as to overcome the inherent deficiency of essential amino acids such as sulphur containing amino acids in legumes, and lysine, threonine and tryptophan in cereals. This can be achieved by adding codons for these amino acids using approaches like site directed mutagenesis.

In recent years while a number of studies have provided information about the primary structures of the legumins and vicilins (1-7), little is known about their primary, secondary and tertiary structural relationships. It has been recognized for some time that the legumins of various species of leguminous plants share antigenic determinants as do the vicilins (8). As cDNA sequences became known, nucleotide and amino acid homologies within these two groups were noted

among leguminous plants (9-12). It was Crouch (13) who observed that a globulin from rape seed (Brassicaceae family) showed homology in amino acid sequence with a legumin from pea (Fabaceae family) suggesting a longer phylogenetic history for storage protein genes than recognized. She also noted that globulins from a range of flowering plants contained the sequence Gly-leu/Ile-Glu/Asp-Glu-Thr-Cys. The probability of such a sequence occuring at random is 1 in 10^6 sets of 6 amino acids using 'synonym' amino acids. It was further observed that the cleavage site for processing the preprotein legumin of pea (14) and soybeans (15) was between ASN and GLY that begins this region of homology above. From these results, it appeared that what was conserved among these proteins was an endoprotease recognition site. This fact reinforced the possibility that the genes for atleast the legumin group of globulin storage proteins from diverse plant families were derived from a single ancestral gene.

In recent years, computer programmes have been developed that allow the comparison of sequences and prediction of protein secondary conformation from primary structures. For example, dot matrix and hydropathy analysis have been used to compare legume storage proteins. Casey et al (16), using dot matrix analysis, have shown regions of homology between convicilin of pea and phaseolin from

frenchbean. In this study, the regions showing similarity and differences were clearly observed from the diagonal lines present. A structural similarity between legumin and vicilin storage proteins from legumes was observed by Argos et al (9) where a maximum homology was noticed between the two groups of proteins, namely, glycinin 2 from Glycine max, and phaseolin from Phaseolus vulgaris. Amino acid alignment of six proteins (vicilin and legumin from Pisum sativum, Phaseolin from Phaseolus vulgaris, β-conglycinin, Glycinin 2 and Glycinin 4 from Glycine max) showed that the degree of sequence homology exceeded 90 % within groups, and 50- 60 % among the groups. Borroto and Dure (17) have compared cDNA and genomic sequences of 13 globulin storage proteins from flowering plants using dot matrix and hydropathy analysis. From the above literature data, it is clear that the comparative information of seed storage proteins in legumes is somewhat scattered and deals with an aspect or two. We, therefore, undertook a comparative study of available cDNA and genomic sequences of storage proteins in legumes using as many as five approaches namely dot matrix, hydropathy, codon usage, secondary structure prediction and amino acid composition. Another aim of the present study was to find out the probable regions in legumes where suitable modifications could be introduced by site directed mutagenesis in order to obtain a gene that will code for

globulin with a better nutritive value.

6.2 MATERIALS AND METHODS

In order to visualize sequence relationships between the cDNA or genomic DNA for various storage proteins, only the coding nucleotides (exons) were compared. Table 6.1 gives the list of storage protein genes that were analysed and the abbreviations adopted for the names of the proteins compared in this study, as well as primary references to their amino acid sequence. Dot matrix for protein and nucleic acid was developed on the principle of Novotny (24) and Jagdeeswaran and McGuire (25). Hydropathy was done according to Kyte and Doolittle (26). Secondary structure prediction was made according to Chou and Fasman (27, 29). Programmes for translation of nucleic acid sequences and codon usage were developed by us.

6.3 RESULTS

6.3.1. Dot matrix analysis :

In attempting to visualize sequence relationships between cDNA and genomic sequences of seed storage proteins/ genes, dot matrix was generated using sequences coding for a legume storage protein gene on X-axis (X-sequence) and another legume storage protein gene on Y-axis (Y-sequence).

TABLE 6.1

LIST OF PROTEINS WHOSE SEQUENCES WERE STUDIED

Protein	Species	Abbreviations	Sequences	References
Legumin	*Pisum sativum*	PSLEGCD.PMB	cDNA	(14)
Legumin	*Pisum sativum*	LEGAN.R84	Genomic	(18)
Legumin	*Pisum sativum*	LEGBAS.CDN	cDNA	(19)
Vicilin	*Pisum sativum*	VICNAR.83	cDNA	(2)
Convicilin	*Pisum sativum*	CONVICIL.BJ	cDNA	(16)
Phaseolin	*Phaseolus vulgaris*	PHASCD.83	Genomic	(5)
Phaseolin	*Phaseolus vulgaris*	CDPHAS81.NAT	cDNA	(20)
$A_{1a}B_x$	*Glycine max*	DA1ABX.GLY	cDNA	(6)
A_2B_{1a}	*Glycine max*	DA2B1A11.GLY	cDNA	(3)
Conglycinin	*Glycine max*	DGM7SA.JBC	cDNA	(7)
$A_5A_4B_3$	*Glycine max*	DGLYA5A4.B3G	cDNA	(4)
Patatin	*Solanum tuberosum*	DPATAT.NAR	Genomic	(21)
Napin	*Brassica napus*	NBAPJMAG.DNA	cDNA	(22)
Globulin (12S)	*Avena sativa*	OATDNA.PMB	cDNA	(23)

This was repeated for all other storage protein genes using various other permutation combinations. To reduce the background noise, inevitably present in comparisons, it was found useful not to compare small fragments as done by Dure (30) but 21 bases in case of DNA and 7 amino acids in case of proteins. This procedure eliminated most of the random fortuitous similarity while preserving the features of true homology. When the segment of this length from both the sequences is equal to a minimal match (12 base in case of DNA and 4 amino acids in case of proteins), a dot is placed in the matrix at the coordinates corresponding to the location of nucleotide in the DNA sequence or amino acid in the protein. The horizontal continuous dots show the homology between two different sequences. Homology with deletions and insertions will give rise to a series of short, parallel diagonal lines. Faint lines with some degree of parallelism to a main diagonal line indicate weak and imperfect homology.

Figure 6.1 shows the dot-matrix analysis of storage protein genes among legumes and the results are tabulated in Table 6.2. From Fig. 6.1 and Table 6.2, it can be seen that the diagonal boundry is very strong running vertically between the subunits of Glycine max i.e. DAIA3X.GLY, DA2B1A11.GLY, DGLYA5A4.B3G and DGM7SA.JBC, revealing very clearly a high homology among them. When the above subunits

FIGURE 6.1

Dot matrix analysis of nucleotide and amino acid sequences of legume storage proteins. The sequence coding for a legume storage protein gene on X-axis was compared with a legume storage protein gene on Y-axis. The window of comparison was 21 bases in case of DNA and 7 in case of proteins with 57.14 % identity required for a positive result to be recorded. The abbreviation details are listed in Table 6.1.

I. X-axis : PHASCD.83

Y-axis :

1 -	DGLYA5A4.B3G	2.	LEGAN.R84
3.	PSLEGCD.PMB	4.	DA2B1A11.GLY
5.	DA1ABX.GLY	6.	DGM7SA.JBC
7.	VICNAR.83	8.	DPATAT.NAR
9.	OATDNA.PMB	10.	LGFBP1
11.	BNAPJMAG.DNA	12.	LEGBAS.CDN
13.	CONVICIL.BI		

II. X-axis : VICNAR.83

Y-axis :

1.	DA1ABX.GLY	2.	LEGAN.R84
3.	PSLEGCD.PMB	4.	PHASCD.83
5.	CDPHAS81.NAT	6.	DPATAT.NAR
7.	DGM7SA.JBC	8.	DGLYA5A4.B3G
9.	CONVICIL.BJ	10.	LGFBP1
111.	LEGBAS.CDN	12.	DPATAT.NAR

III X-axis : DA1ABX.GLy

Y-axis :

1. OATDNA.PMB
2. DPATAT.NAR
3. VICNAR-83
4. DA2B1A11.GLY
5. PHASCD.83
6. LEGAN.R84
7. DGM7SA.JBC
8. BNAPJMAG.DNA
9. CONVICIL.BJ
10. LGFBP1

IV X-axis : DPATAT.NAR

Y-axis :

1. BNAPJMAG.DNA
2. CDPHAS81.NAT
3. LEGBAS.CDN
4. CONVICIL.BJ
5. LGFBP1
6. OATDNA.PMB
7. DGM7SA.JBC
8. DGLYA5A4.B3G

V X-axis : LEGAN.R84

Y-axis :

1. LEGBAS.CDN
2. CONVICIL.BJ
3. BNAPJMAG.DNA
4. DA2B1A11.GLY
5. PSLEGCD.PMB
6. DGLYA5A4.B3G
7. DGM7SA.JBC
8. OATDNA.PMB
9. LGFBP1

VI X-axis : PSLEGCD.PMB

Y-axis :

1. BNAPJMAG.DNA
2. LEGBAS.CDN
3. CONVICIL.BJ
4. LGFBP1
5. CDPHAS81.NAT
6. OATDNA.PMB
7. DA2B1A11.GLY
8. DGLYA5A4.B3G

VII	X-axis	:	DA2B1A11.GLY		
	Y-axis	:	1. CDPHAS81.NAT	2.	CONVICIL.BJ
			3. LEGBAS.CDN	4.	NBAPJMAG.DNA
			5. LGFBP1	6.	PSLEGCD.PMB
			7. DGLYA5A4.B3G		
VIII	X-axis	:	DGLYA5A4.B3G		
	Y-axis	:	1. CONVICIL.BJ	2.	BNAPJMAG.DNA
			3. CDPHAS81.NAT	4.	OATDNA.PMB
			5. LGFBP1	6.	LEGBAS.CDN
			7. DA2B1A11.GLY		
IX	X-axis	:	PSLEGCD.PMB		
	Y-axis	:	1. CONVICIL.BJ	2.	BNAPJMAG.DNA
			3. LEGBAS.CDN	4.	DGLYA5A4.B3G
			5. DPATAT.NAR	6.	OATDNA.PMB
X	X-axis	:	OATDNA.PMB		
	Y-axis	:	1. LEGBAS.CDN	2.	CDPHAS81.NAT
			3. BNAPJMAG.DNA	4.	CONVICIL.BJ
			5. LGFBP1		
XI	X-axis	:	BNAPJMAG.DNA		
	Y-axis	:	1. CDPHAS81.NAT	2.	LEGBAS.CDN
			3. LGFBP1	4.	CONVICIL.BJ

XII	X-axis	:	LGFBP1
	Y-axis	:	1. CDPHA$81.NAT 2. LEGBAS.CDN
			3. CONVICIL.BJ
XIII	X-axis	:	LEGBAS.CDN
	Y-axis	:	1. CONVICIL.BJ 2. CDPHAS81.NAT
XIV	X-axis	:	CONVICIL.BJ
	Y-axis	:	CDPHAS81.NAT

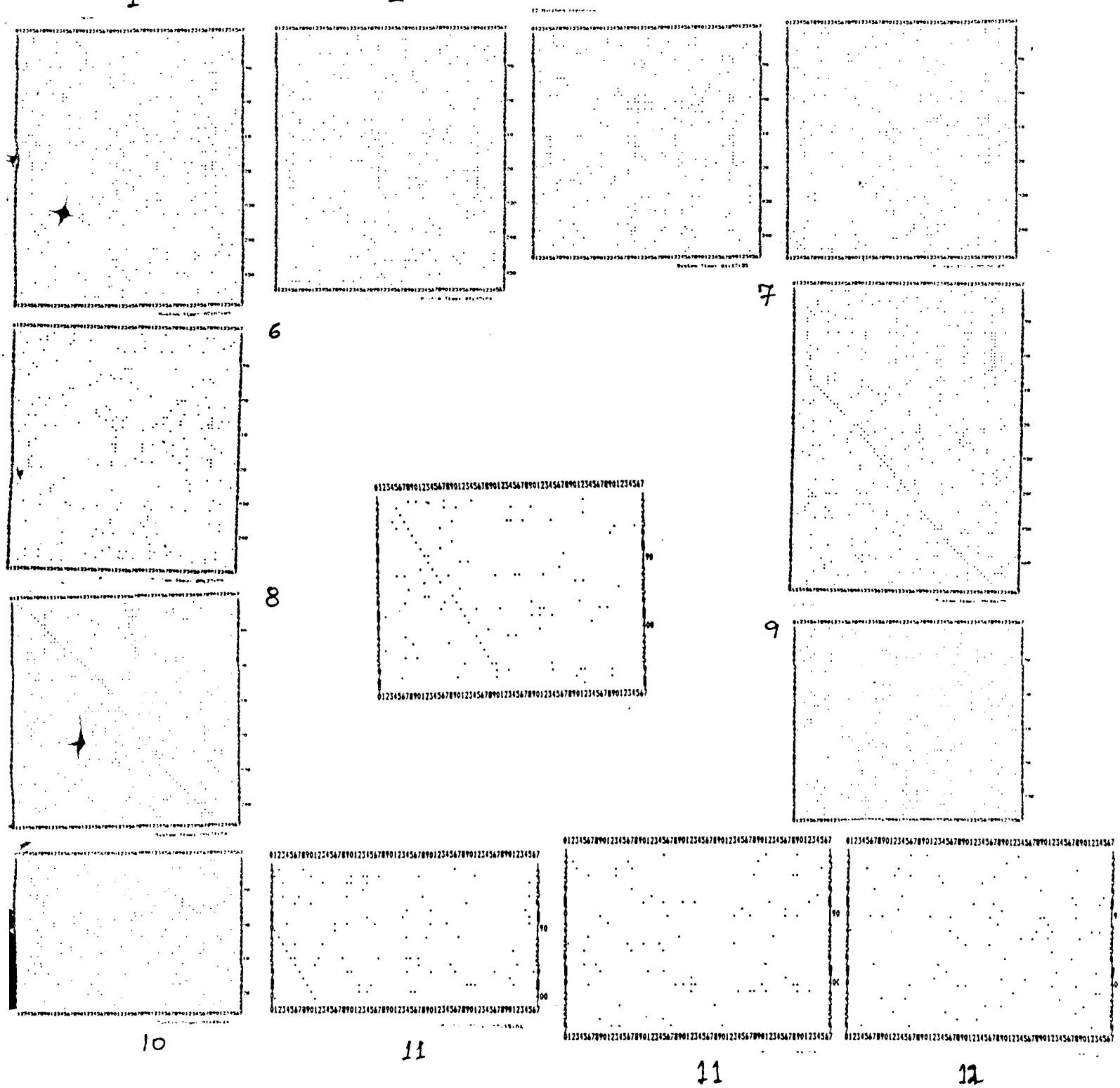
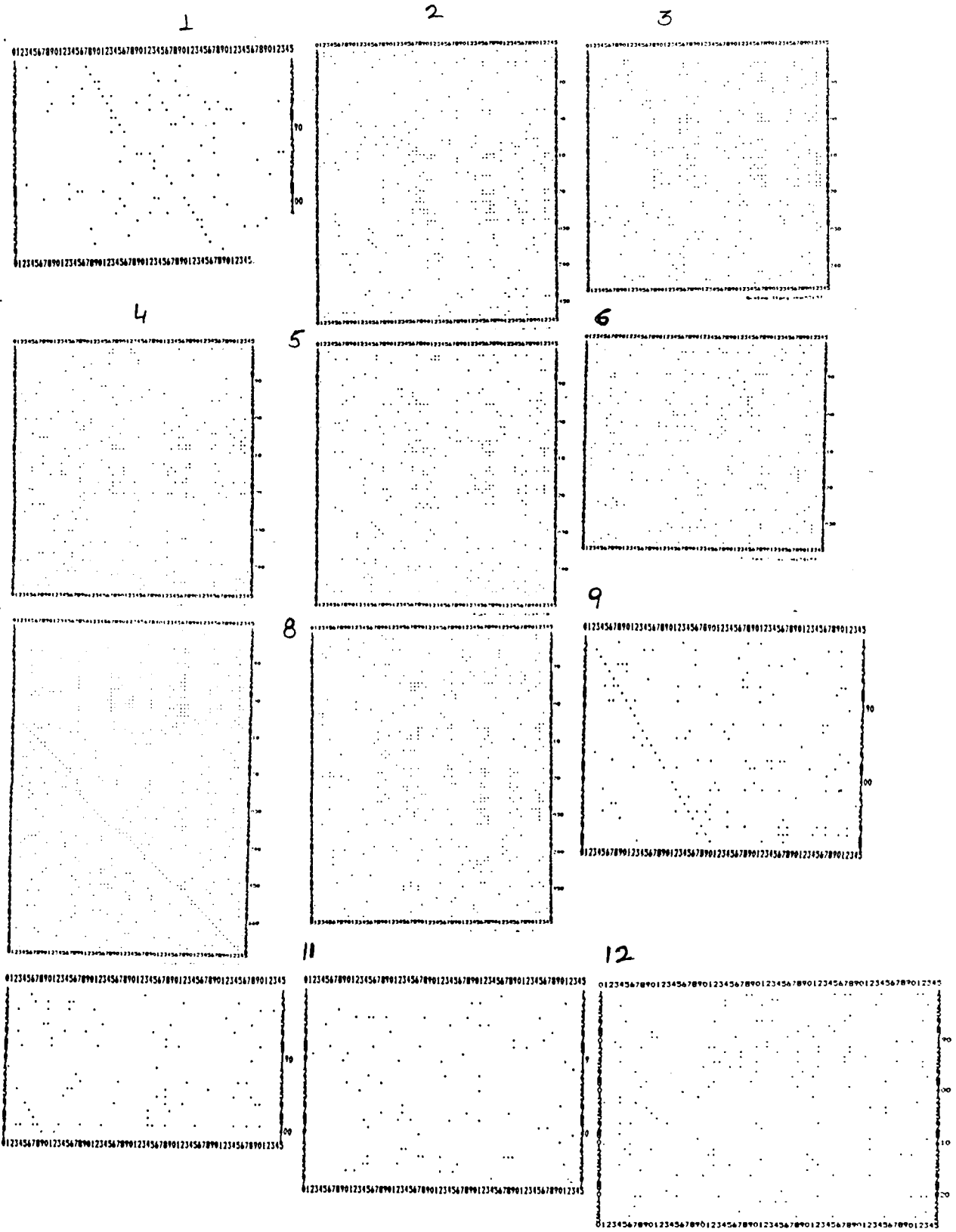


FIG. 6·1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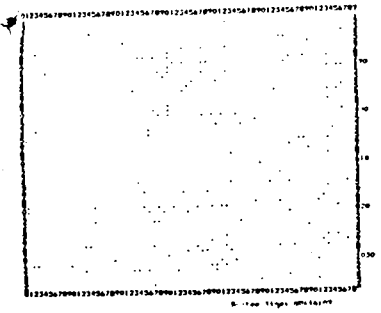

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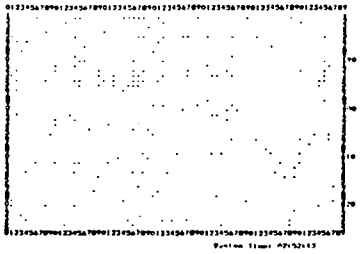

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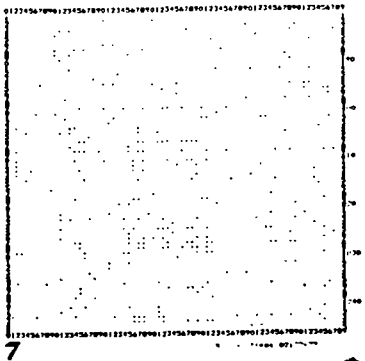

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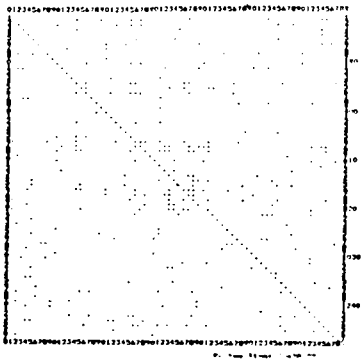

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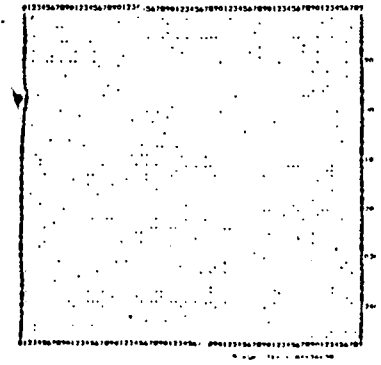

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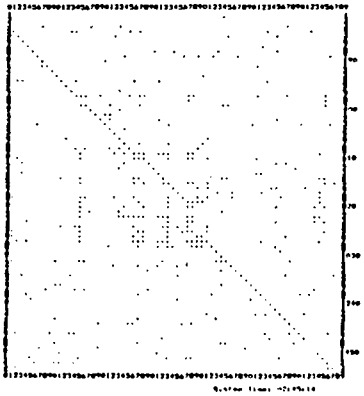

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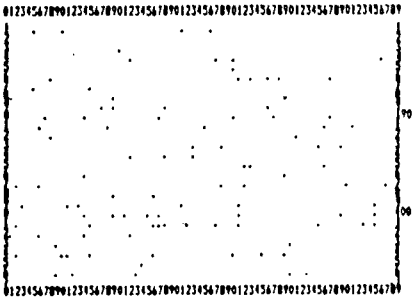

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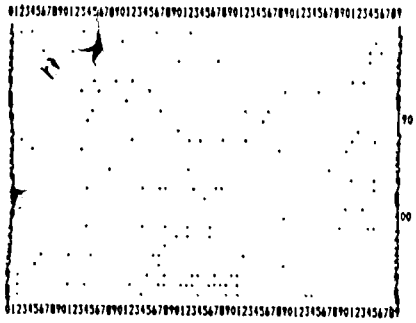

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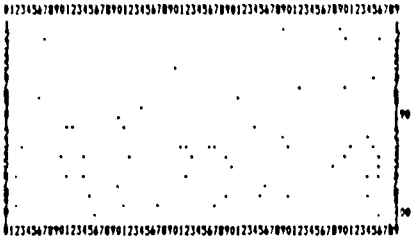

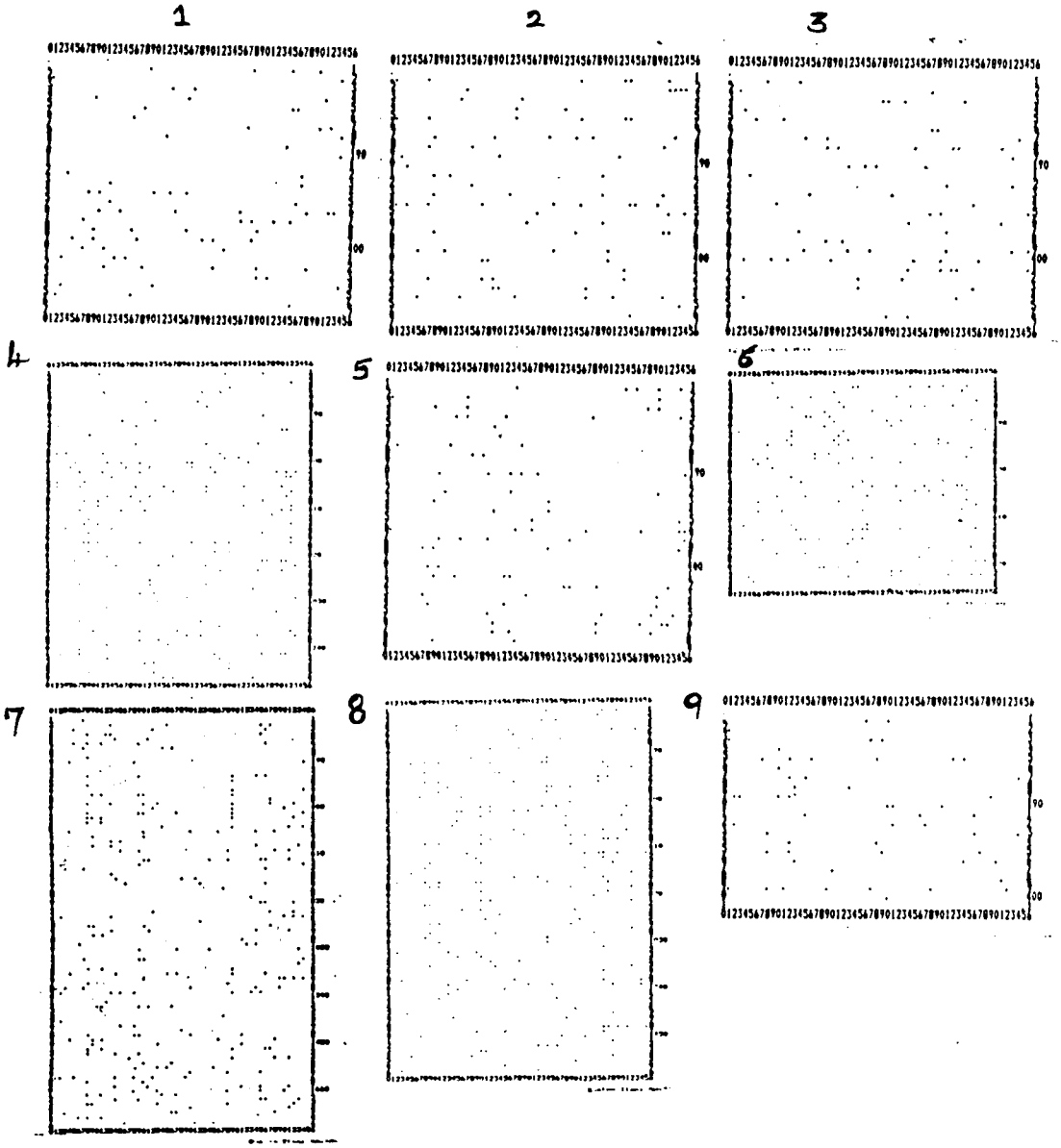
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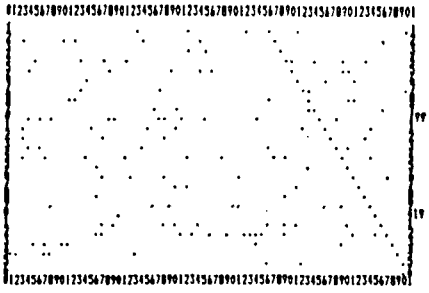

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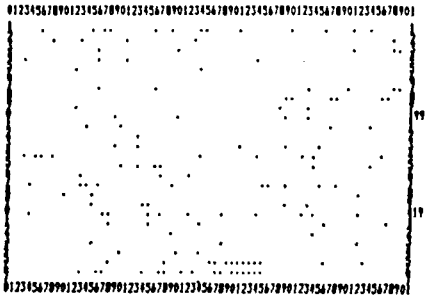

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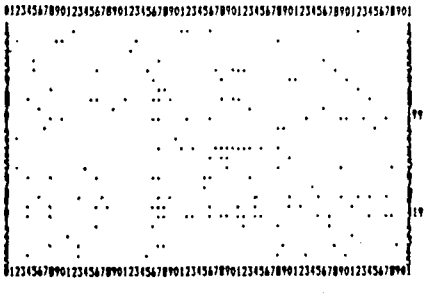

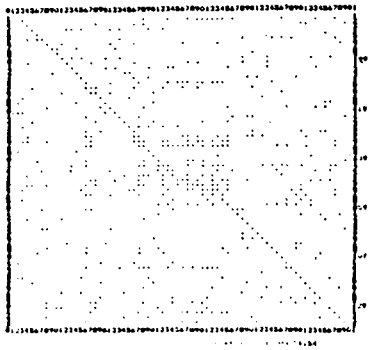

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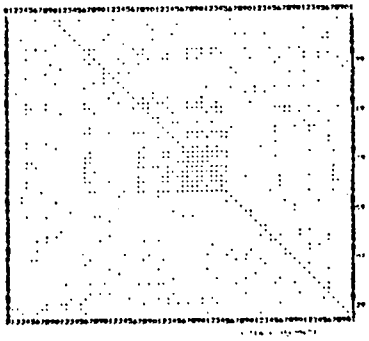

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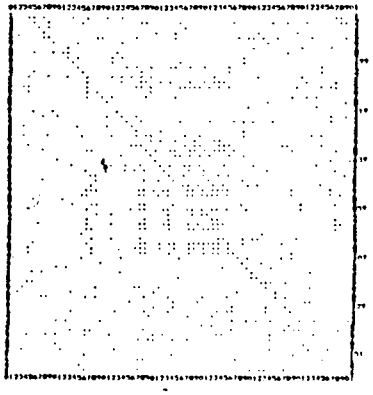

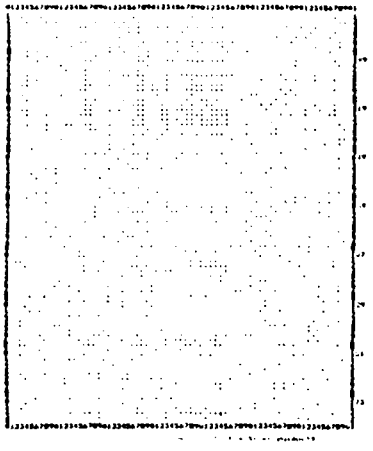

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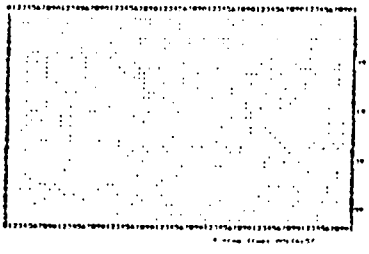

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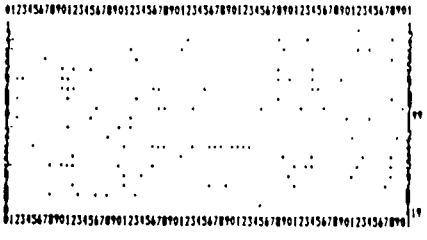

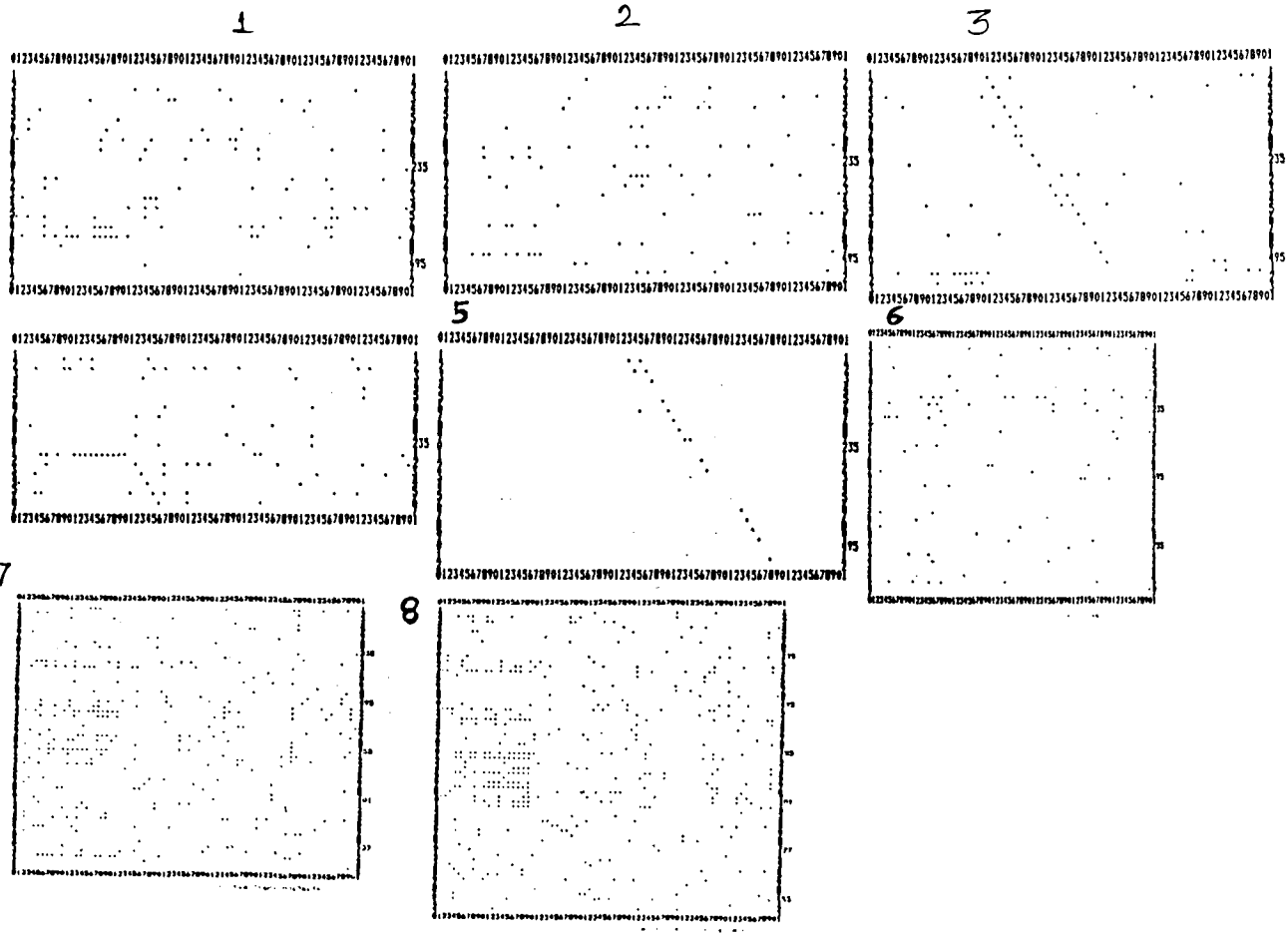

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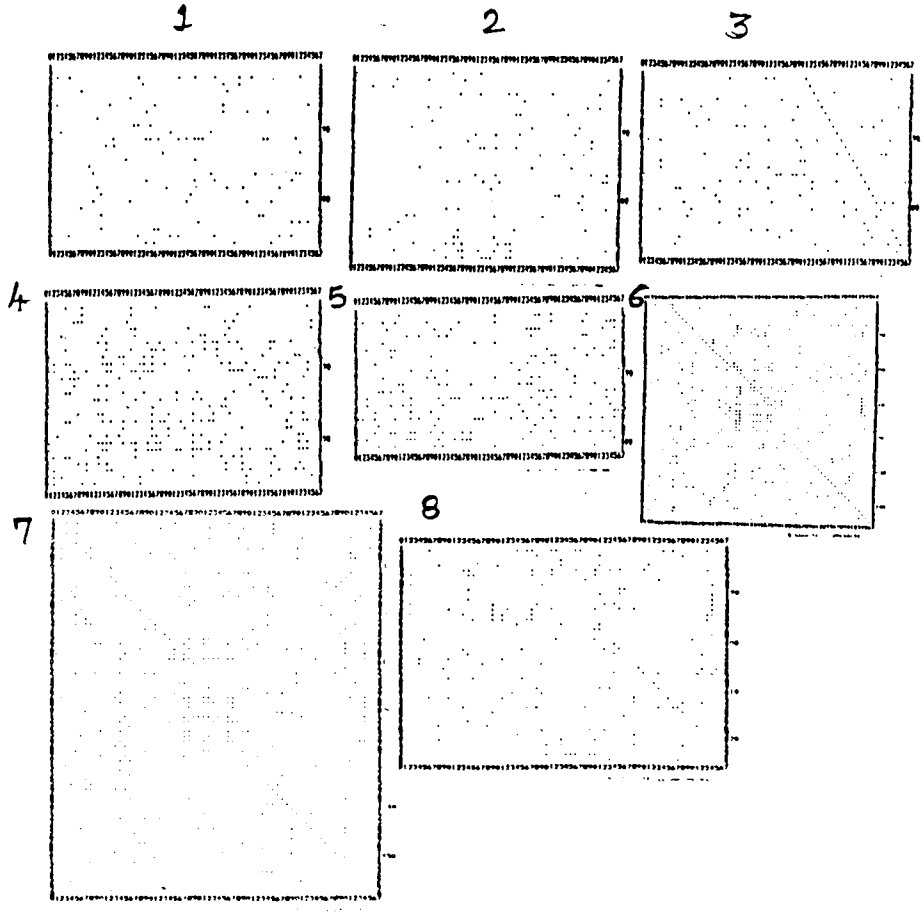

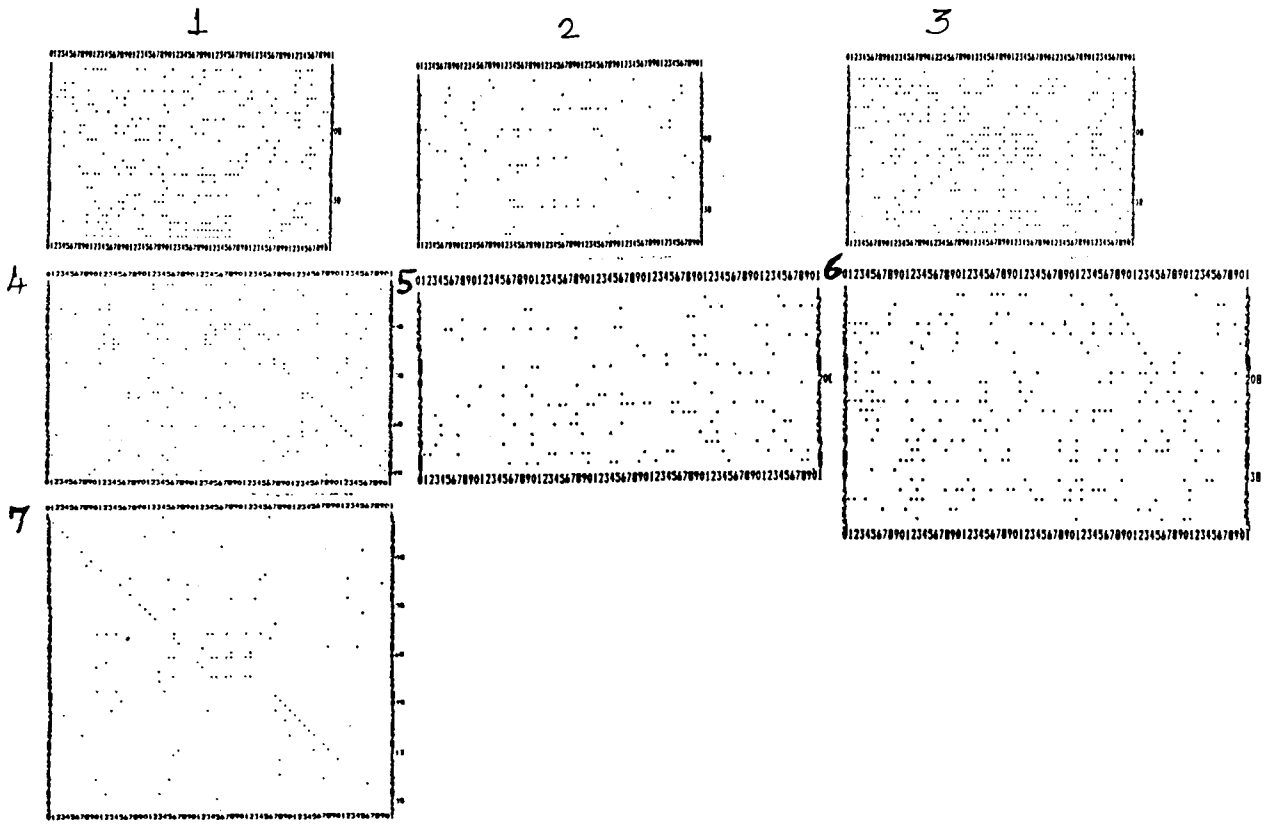

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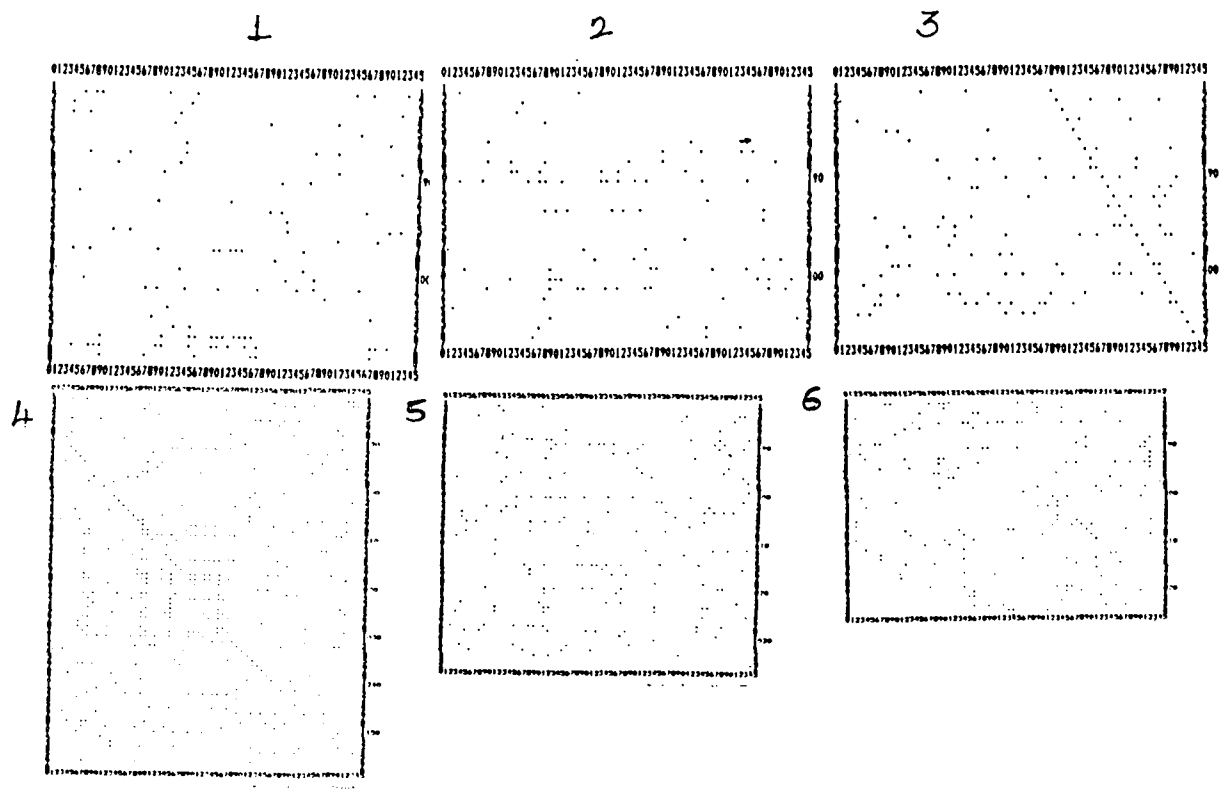

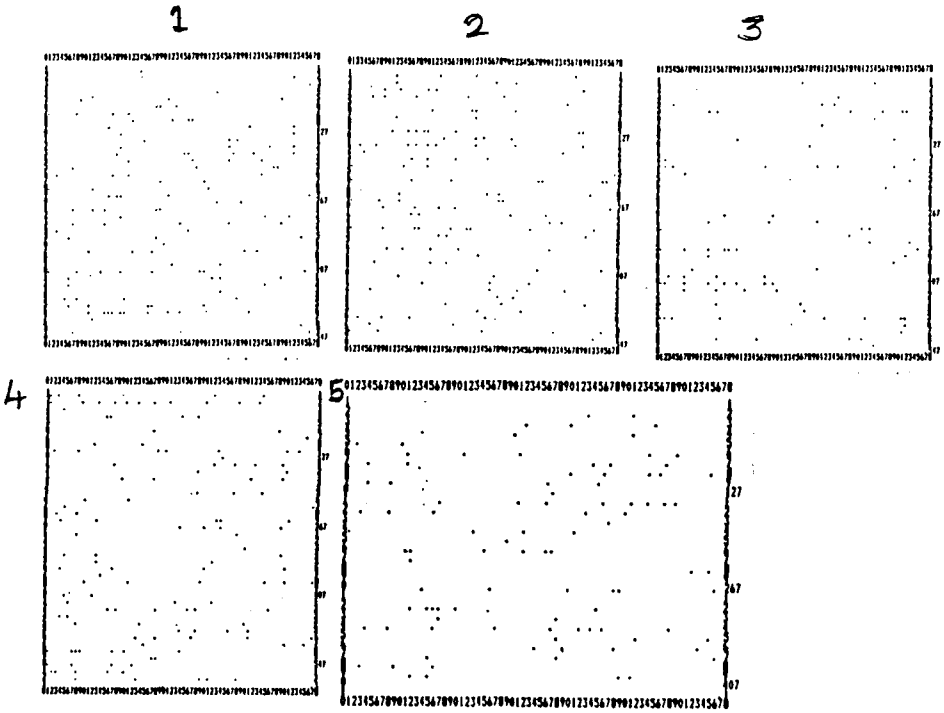

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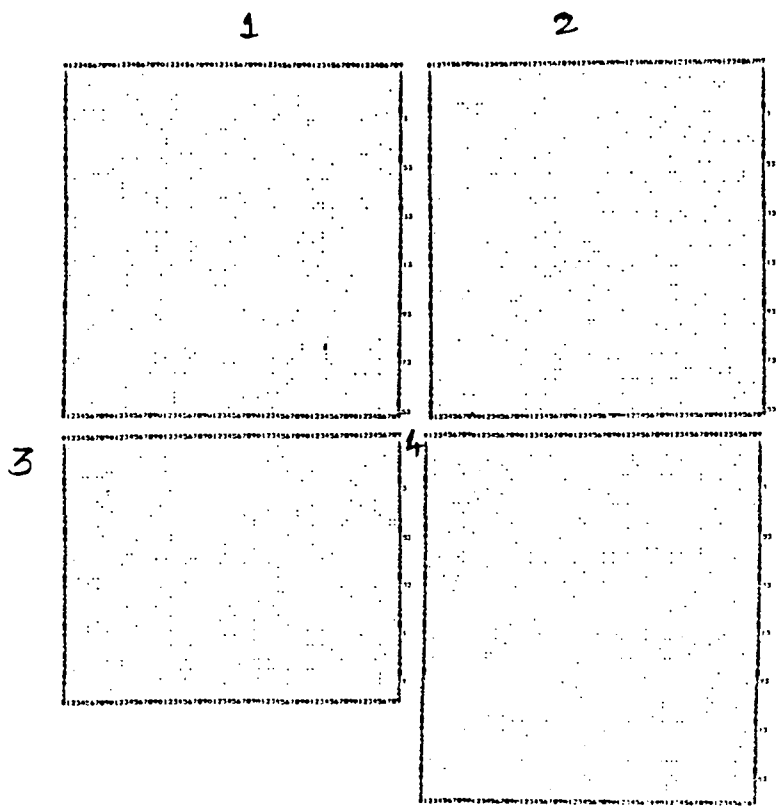
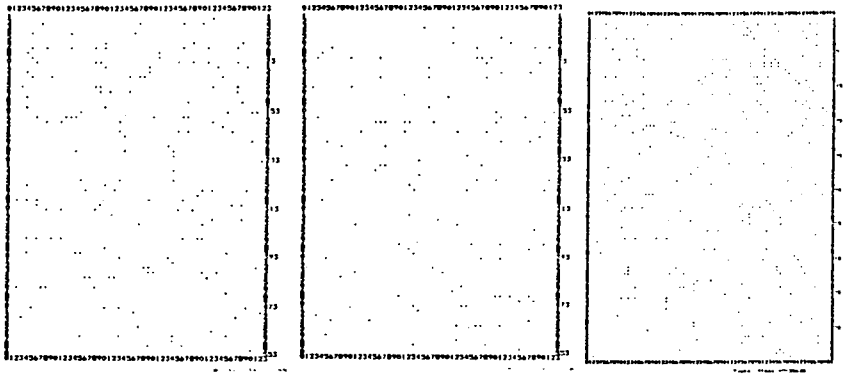


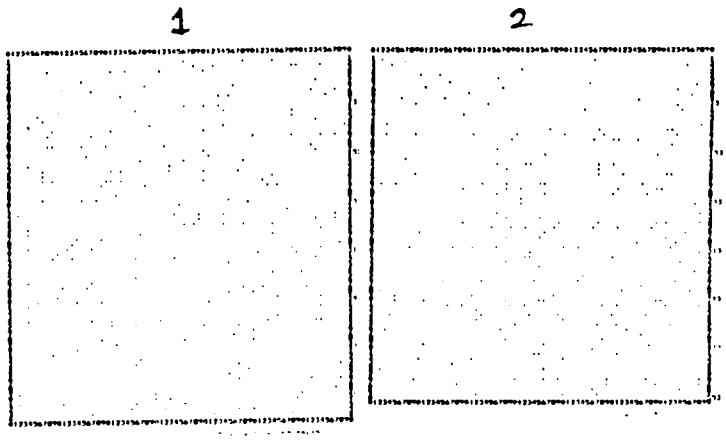
XII

1 2 3



XIII

1 2



XIV

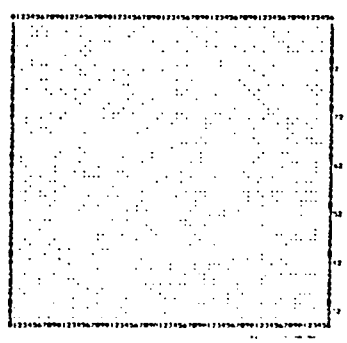


TABLE 6.2

DOT MATRIX ANALYSIS OF LEGUME SEED STORAGE PROTEINS

	PHASCD.83	DAIABX. GLY	VICNAR. 83	DGM7SA. JBC	DA2BIALL. GLY	DPATAT. NAR	PSLEGCD. PMB	LEGBAS. CDN	BNAPJMAG. DNA	LEGAN. R84	OAT DNA. PMB	DGLYA5A4 B3G	CONVICIL. BJ	CDPHAS 81 NAT	LGFBP1
PHASCD.83		++	++++	++++	++	+	+++	+	+	++	++	++	++++	++++	++++
DA1ABX.GLY			++	+++	++++	+	++++	++++	++	++++	++	++++	+	+	+
VICNAR.83				++++	+	+	++	+	+	++	+	++	++++	+++	++
DGM7SA.JBC					+++	+	++	++	++	++++	+	+++	++++	++++	++
DA2BIALL.GLY						+	++++	++++	+	++++	++	++++	+	+++	+
DPATAT.NAR							+	+	++	++	+	+	+	++	+
PSLEGCD.PMB								++++	+	++++	+	++++	+	++	+
LEGBAS.CDN									+	++++	++	+++	++	++	+
BNAPJMAG.DNA										++	+	+	+	+	+
LEGAN.R84											+++	++++	++	++	++
OATDNA.PMB												+++	+	+	+
DGLYA5A4.B3G													++	++	++
CONVICIL.BJ														+	+++
CDPHAS81.NAT															++
LGFBP1															

are compared to other legume storage proteins, a substantial homology is observed with storage proteins from Pisum sativum (PSLEGCD.PMB; LEGAN.R84, CONVICIL.BJ, LEGBAS.CDN and VICNAR.83) and Phaseolus vulgaris (PHASCD.83) and to a lesser extent with Phaseolus vulgaris legumin (LGFBP1) as can be interpreted from the discontinuous diagonal boundary. The homology with Brassica napus BNAPJMAG.DNA and Solanum tuberosum DPATAT.NAR with Glycine max subunits is very weak which can be visualized by very faint lines. The measure of homology has been indicated in Table 6.2. When comparison among various legume 7S and 11S proteins was carried out, the best intragroup results were obtained between Pisum sativum legumin, LEGAN.R84 and LEGBAS.CDN. Phaseolus vulgaris phaseolin PHASCD.83 and Pisum sativum vicilin VICNAR.83; Pisum sativum vicilin VICNAR.83 and Pisum sativum convicilin CONVICIL.BJ; thus indicating a clear relationship among them.

6.3.2 Hydropathy analysis :

After comparing the storage proteins/genes by dot matrix method, hydropathy character of legume storage protein was next investigated. The main purpose of this approach was to track the hydrophilic and hydrophobic regions in storage protein molecules using the method of Kyte and Doolittle (26). In this method, each amino acid has been assigned a

value X reflecting its relative hydrophilicity and hydrophobicity and the programe uses a moving segment approach that continuously determines the average hydropathy within a segment of predetermined length as it advances through the sequence. The consecutive score of hydropathy is plotted from the amino to carboxy terminal end by this method.

Figure 6.2 depicts a graphic visualization of hydropathy character of polypeptide chains of different storage proteins from one end to the other. Hydrophobic domain and hydrophilic domains are indicated by a positive index and a negative index, respectively. From Fig. 6.2, it is evident that the amino region of all the legume storage protein is more hydrophilic than the central and the C-terminal region. The central region, although it contains short interspersed hydrophobic and hydrophilic regions, is predominantly hydrophilic. The carboxy region consists of alternating hydrophobic and hydrophilic packets. Napin from Brassica napus and patatin from Solanum tuberosum differ from other legume storage proteins in their hydropathy plots. In case of napin, the amino terminal region is hydrophobic but the central region is predominantly hydrophilic. The carboxy region contains short interspersed hydrophobic region in the predominantly hydrophilic segment. While in case of patatin,

Figure 6.2

Comparison of the hydropathy analysis of storage proteins. The horizontal axis represents the amino acid position in each precursor form while the vertical axis indicates the hydropathic index.

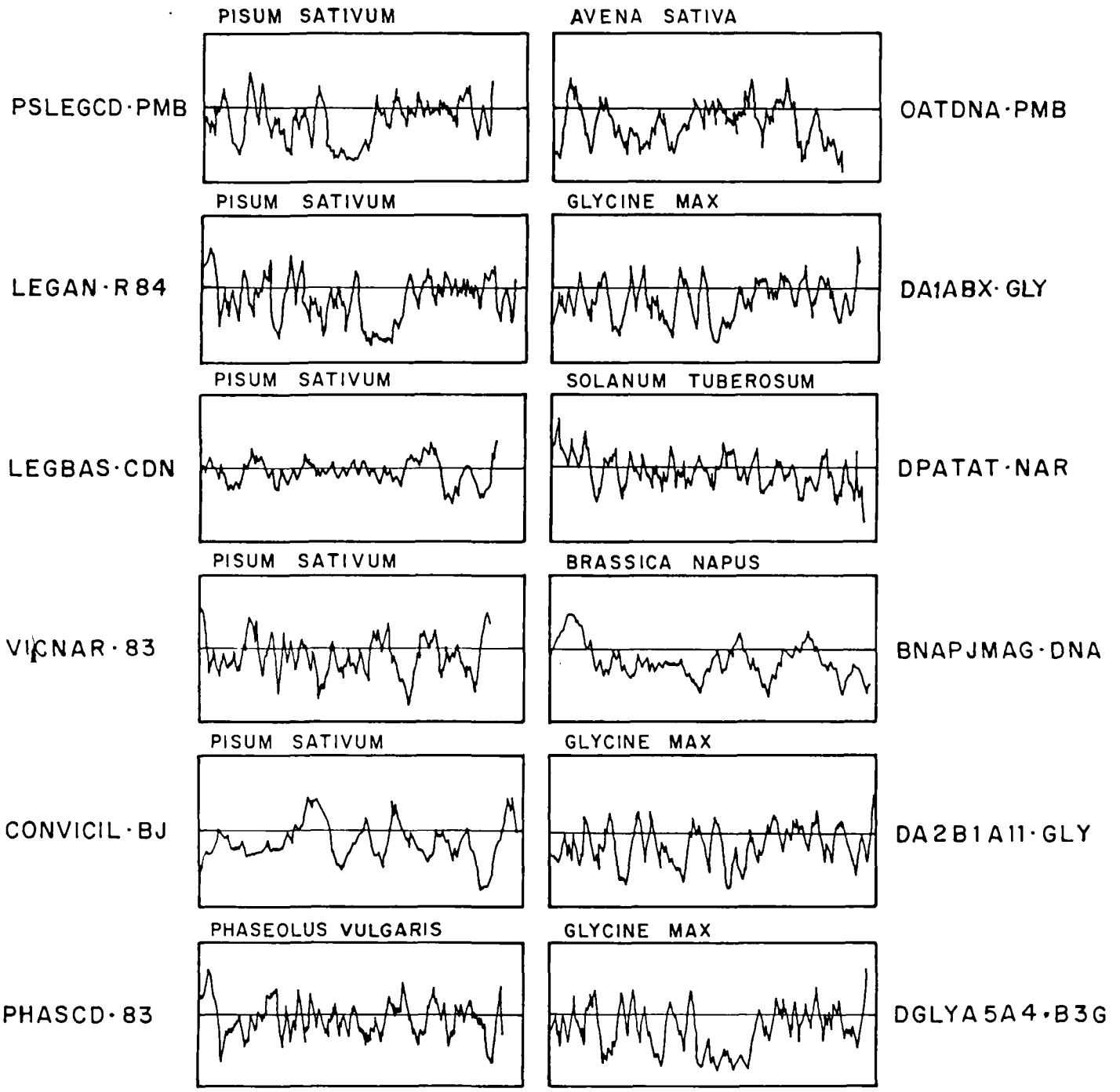

PISUM SATIVUM
AVENA SATIVA
PSLEGCD·PMB
OATDNA·PMB
PISUM SATIVUM
GLYCINE MAX
LEGAN·R84
DA1ABX·GLY
PISUM SATIVUM
SOLANUM TUBEROSUM
LEGBAS·CDN
DPATAT·NAR
PISUM SATIVUM
BRASSICA NAPUS
VICNAR·83
BNAPJMAG·DNA
PISUM SATIVUM
GLYCINE MAX
CONVICIL·BJ
DA2B1A11·GLY
PHASEOLUS VULGARIS
GLYCINE MAX
PHASCD·83
DGLYA5A4·B3G

FIG. 6·2

the amino region is hydrophobic. From the above information, it is thus clear that except patatin and napin, the hydropathy plots of all the other legume storage proteins are grossly similar.

6.3.3. Amino acid sequence comparison :

In the present work, we have compared the deduced amino acid sequences of 9 legume globulins, patatin, napin and oat globulin storage proteins in order to examine the extent of sequence homology. For comparison purpose, only the coding sequence (exons) were compared. The alignment was carried out to maximize sequence homology by introducing gaps manually and the data is depicted in Table 6.3. Alignment position numbers are assigned as shown in the same Table (every match column is counted regardless of deletions or insertions in some of the proteins) and the regions of homology are indicated by a block. From Table 6.3, it is observed that the homologous sequences are interspersed with diverged regions in all the 12 storage proteins. The degree of homology found between the exons varies considerably. Even within individual exons, a high homology is observed separated by regions which show complete divergence or loss of genetic information (due to deletion or insertion). Here, one of the important facts to be noted is that the divergence observed is high in the acidic subunit while in the basic

TABLE 6.3

AMINO ACID SEQUENCE HOMOLOGY AMONG SEED STORAGE PROTEINS

subunit domain III at the C-terminal high conservation is observed. The classification of domain is according to Argos et al (9) and is depicted in Fig. 6.3.

Table 6.4 describes the percentage basematch homology observed when storage protein genes are aligned. The alignment value indicates the percentage basematch of storage protein gene, which is calculated as follows:

$$\% \text{ BASEMATCH} = \frac{\text{TOTAL IDENTICAL BASES DURING COMPARISION}}{\text{CODING NUCLEOTIDE}^{*}} \times 100$$

During comparison, since the two genes compared have unequal nucleotides, the lowest nucleotide number is taken for calculation.

As is clear from Table 6.4, a striking similarity is observed with Glycine max subunits DGLYA5A4.B3G and DA2B1A11.GLY, legumin of Pisum sativum PSLEGCD.PMB and LEGAN.R84 and legumin of Avena sativa OATDNA.PMB. Patatin of Solanum tuberosum and napin of Brassica napus are less homologous to other legumes.

6.3.4 Post-translational events in legume storage proteins:

So far, I have compared the legume storage proteins/ genes by dot matrix analysis, hydropathy analysis and amino

Figure 6.3

Illustration of the predicted domain relationships and differences between the legumin and vicilin - like subunits from legumes. Domain I is referred to as span 1 or the NH_2-terminal region in the text. Similarly, domains II and III correspond to span 2 (central region) and 4 (COOH-terminal region), respectively. Residue inserts (span 3) are shown as loops.

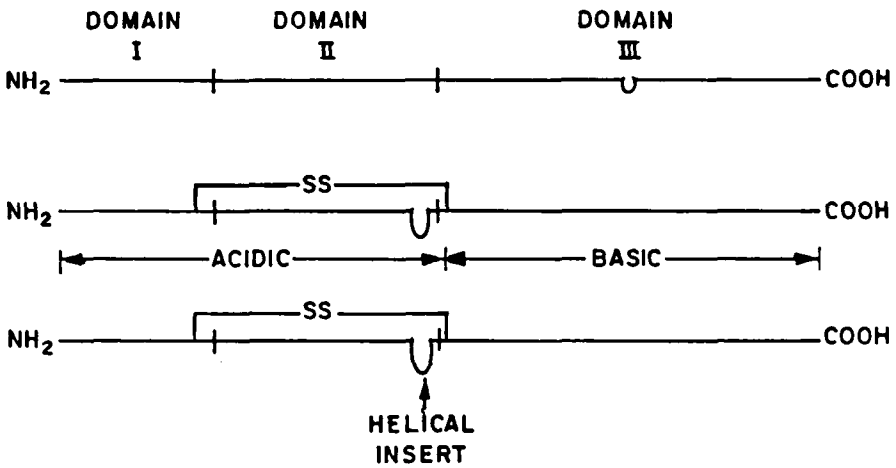

DOMAIN I
DOMAIN II
DOMAIN III
NH2
COOH
SS
NH2
COOH
ACIDIC
BASIC
SS
NH2
COOH
HELICAL INSERT

FIG . 6·3

TABLE 6.4

PERCENTAGE NUCLEOTIDE BASEMATCH OF VARIOUS STORAGE PROTEINS OF ALIGNED RESIDUES

	(1356) VICNAR.83 (1)	(1353) PSLEGCD.PMB (2)	(1551) LEGAN.R84 (3)	(558) LEGBAS.CDN (4)	(558) CONVICIL.BJ (5)	(1398) PHASCD.83 (6)	(1845) DGM7SA.JBC (7)	(1431) DAIABX.GLY (8)	(1404) DA2B1AII.GLY (9)	(1620) DGLYA5A4.B3G (10)	(1161) DPATAT.NAR (11)	(546) BNAPJMAG. DNA (12)	(942) OATDNA PMB (13)
VICNAR.83 (1356)	100												
PSLEGCD.PMB (1353)	19.4	100											
LEGAN. R84 (1551)	26.4	99.6	100										
LEGBAS.CDN (558)	32.4	98.6	99.8	100									
CONVICIL. BJ (588)	69.2	31.1	31.2	21.3	100								
PHASCD. 83 (1398)	41.7	20.62	24.3	25.6	54.3	100							
DGM7SA. JBC (1845)	62.3	22.2	19.6	27.8	59.8	58.4	100						
DAIABX. GLY (1431)	18.28	66.0	64.8	72.9	26.2	15.1	17.0	100					
DA2B1A11. GLY (1404)	19.76	60.9	66.7	72.9	21.4	20.7	26.6	88.4	100				
DGLYA5A4. B3G (1620)	23.8	47.2	46.1	49.7	25.5	24.0	24.9	53.6	54.1	100			

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
ATAT. NAR 1161)	20.76	19.5	26.9	16.6	26.2	20.9	27.6	14.2	18.0	20.6	100	
APJMAG. DNA 546)	30.77	25.5	27.5	17.6	22.4	26.0	27.1	24.9	32.96	30.8	26.2	100
DNA. PMB 942)	19.1	39.6	39.5	49.9	21.1	27.3	19.2	39.49	38.0	24.4	24.3	14.0

acid comparison. Another approach that is used for this purpose is to examine the sequences around the regions where events such as post- translational cleavage, glycosylation and disulphide linkage formation takes place. In legumes, it has been shown that the storage proteins are synthesised in the lumen of the rough endoplasmic reticulum and are then transported to the golgi apparatus where post-translational events occur. One of the such events is post-translational cleavage between asparagine and glycine residues as shown in Glycine max, Phaseolus vulgaris, Pisum sativum and Brassica napus (Table 6.5). When the sequences located near the post-translational cleavage site were aligned, homology is observed around putative processing site, the only exception being pea vicilin where the processing site is N↓D instead of N↓G.

Glycosylation is another post-translational event where the carbohydrate moeity is added to the processed protein. It is well Known that vicilins of legumes are glycosylated while the legumins show absence of carbohydrate moeity . The literature information regarding the glycosylation sites in legume vicilin proteins is summarised in Table 6.6. From Table 6.6, it can be seen that the amino acid sequences of all legume vicilins under comparison

TABLE 6.5

BLE-4 : AMINOACID HOMOLOGY AROUND PUTATIVE PROCESSING SITE

PSLEGCD.PMB	N ↓ G L E E T V C T L
BNAPJMAG.DNA	N G L E E T I C S R
OATDNA.PMB	N G L E E N P C S E
LEGBAS.CDN	N G L E E T V C T A
LEGAN.R84	N G L E E T V C T A
DGLYA5A4.B3G	N G V E E N I C T L
DA1ABX.GLY	N G I D E T I C T M
VICNAR.83	N D K E E E E E E E

↓ INDICATES PUTATIVE PROCESSING SITE

TABLE 6.6

POTENTIAL GLYCOSYLATION SITE

PHASEOLUS VULGARIS (PHASEOLIN)			
PVU	α	N L T	N F T
PVU	β	N L T	N F T
GLYCINE MAX (CONGLYCININ)			
GMA	α'	N G T	N A T
	α		N A T
	β		N A T
PISUM SATIVUM (VICILIN)			
PSA	1		N A S
	2		R A S

POTATO (PATATIN)

NO SITE IN GENOMIC SEQUENCE
BUT 1 SITE IN cDNA SEQUENCE.

$$N \; X \begin{matrix} S \\ T \end{matrix}$$

contain at least one N-glycosyl recognition site ASN - X - THR/SER with exception of Pisum sativum vicilin. It is puzzling that the amino acid sequence of Psa 2 does not contain a N-glycosyl recognition site which could be due to an error in the data or Psa 2 could represent a pea vicilin variant that has lost the site (31).

It is known that legumin storage proteins from legumes consist of an acidic (α) subunit of 20 kD and a basic (β) subunit of 40 kD Mr linked by disulphide bonds. The sequences which form disulphide linkage around these acidic and basic subunit were aligned to study the homology pattern (Table 6.7). As can be seen from the table, the two cysteine residues which participate in the formation of a single disulphide bond that links the subunits together after post-translational cleavage (32, 33) are located just outside either end of the variable region. From Table 6.7, it is further evident that a high homology of sequences in the disulphide bond formation is observed. Here the sequence in acidic subunit is X G C X X T and in basic subunit it is

$$\begin{array}{l} \downarrow \\ \text{N G X E X X C X R,} \end{array} \qquad \begin{array}{l} \text{E} \\ \text{D} \end{array} \quad \begin{array}{l} * \\ \text{T} \end{array} \quad \begin{array}{l} \text{S} \\ \text{RI} \end{array} \quad \begin{array}{l} \text{KL} \end{array}$$

where X is any amino acid. The star indicates the site of disulphide linkage and arrow indicates post-translation cleavage site.

TABLE 6.7

SEQUENCE AROUND DISULPHIDE BONDS IN LEGUMES

	ACIDIC											BASIC										
						*						↓						*				
LEGUMIN A	M	V	F	P	G	C	P	E	T	F	E	N	G	L	E	E	T	V	C	T	A	K	L	R
LEB4 (Vicia faba)	L	T	L	P	G	C	P	Q	T	Y	Q	N	G	L	E	E	T	I	C	S	L	K	I	R
A1aB1b (SOYBEAN)	M	I	Y	P	G	C	S	S	T	F	E	N	G	I	D	E	T	I	C	T	M	R	L	R
A3B4 (SOYBEAN)	F	A	F	P	G	C	P	E	T	F	E	N	G	V	E	E	N	I	C	T	M	K	L	R
A2B1a (SOYBEAN)	M	I	F	D	D/G	C	P	S	T	Y	Q	N	G	I	D	E	T	I	C	S	L	K	I	R
A5A4B3 (SOYBEAN)	P	R	Q	R	G	C	E	T	R	N		N	G	V	E	E	N	I	C	T	L	K	L	H

↓ INDICATE SITE OF CLEAVAGE

* INDICATE DISULPHIDE LINKAGE

6.3.5 Codon usage :

Sequence analysis of genes from several different organisms has revealed a species specific bias in the usage of several degenerate codons which code for an amino acid (10, 34). In yeast, for instance, abundant transcripts use a relatively restricted set of codons which in many cases correspond to the abundant isoaccepting tRNA species (10, 35). Genes that correspond to the least abundant mRNA's use a less restricted, although still biased, set of codons (10). Presumbaly, the extreme codon bias of abundant mRNAs is due to the selective pressue to allow efficient translation of these sequences (36).

In order to gain an insight into the codon bias in seed storage protein genes, coding sequences were compared for preference of the usage of codons. Fig. 6.4 depicts the codon usage for the storage protein genes under study. From this figure, the following results can be summarized.

1. All storage protein genes show that among the codons, ATA, ATC and ATT coding for isoleucine are not predominantly used. Thus the modification of these codons by site directed mutagenesis would lead to codon ATG which codes for methionine. Since both isoleucine and methionine occur predominantly in β-pleat secondary structure, modification of isoleucine to methionine may

Figure 6.4

Summary of codon usage in seed storage proteins. The sequence of the codons is as follows:

1	AAA	ACA	AGA	ATA	AAC	ACC	AGC	ATC	8
9	AAG	ACG	AGG	ATG	AAT	ACT	AGT	ATT	16
17	CAA	CCA	CGA	CTA	CAC	CCC	CGC	CTC	24
25	CAG	CCG	CGG	CTG	CAT	CCT	CGT	CTT	32
33	GAA	GCA	GGA	GTA	GAC	GCC	GGC	GTC	40
41	GAG	GCG	GGG	GTC	GAT	GCT	GGT	GTT	48
49	TAA	TCA	TGA	TTA	TAC	TCC	TGC	TTC	54
55	TAG	TCG	TGG	TTG	TAT	TCT	TGT	TTT	64

The abbreviation details are presented in Table 6.1.

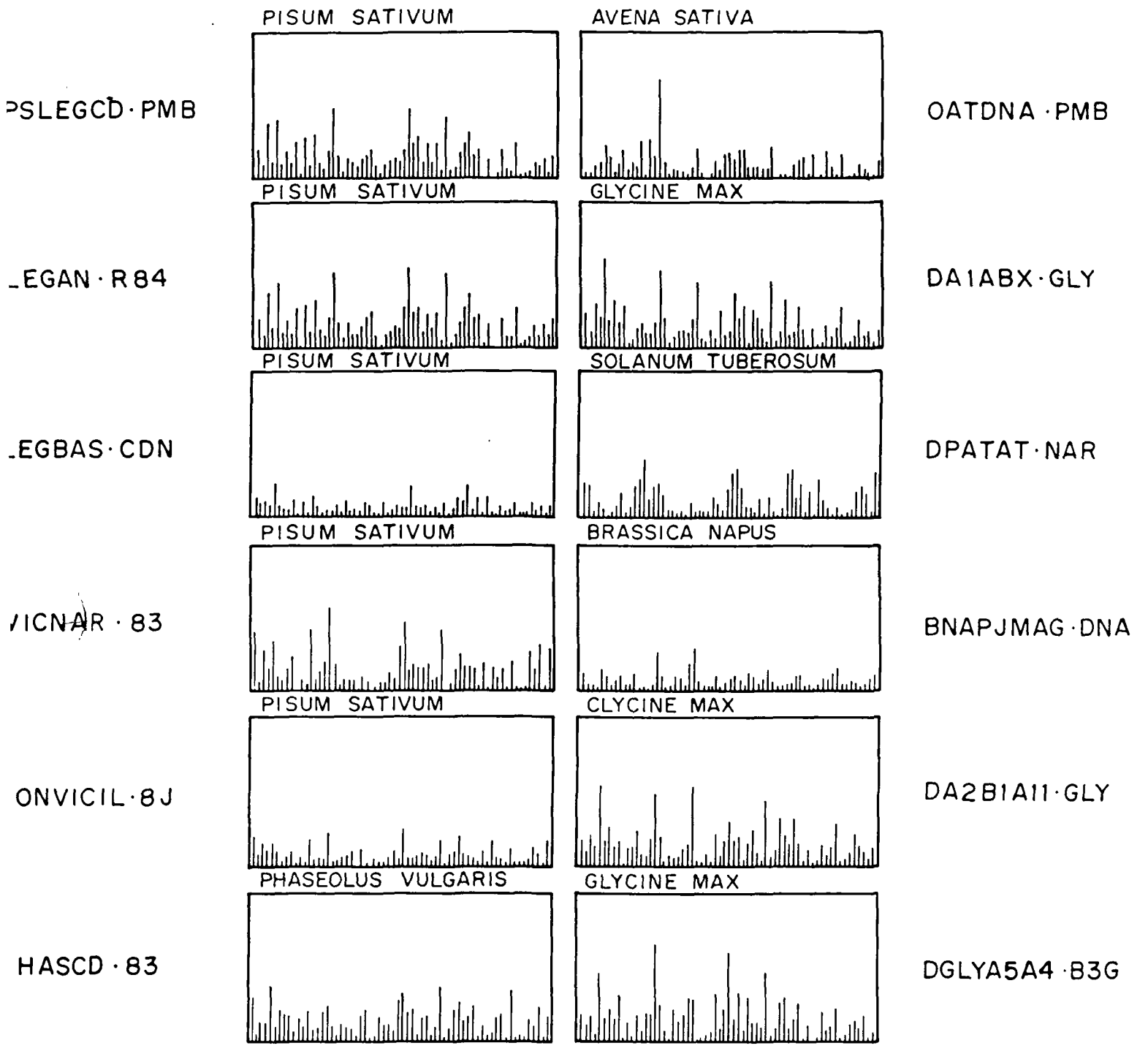

PISUM SATIVUM
AVENA SATIVA
PSLEGCD·PMB
OATDNA·PMB
PISUM SATIVUM
GLYCINE MAX
_EGAN·R84
DA1ABX·GLY
PISUM SATIVUM
SOLANUM TUBEROSUM
_EGBAS·CDN
DPATAT·NAR
PISUM SATIVUM
BRASSICA NAPUS
/ICNAR·83
BNAPJMAG·DNA
PISUM SATIVUM
CLYCINE MAX
ONVICIL·8J
DA2B1A11·GLY
PHASEOLUS VULGARIS
GLYCINE MAX
HASCD·83
DGLYA5A4·B3G

FIG. 6·4

not lead to much alteration in globulin structure and also may not affect accumulation of storage proteins in protein bodies.

2. The three codons ATA, ATC and ATT are not predominantly used.
3. No bias in preference for proline codons is observed.
4. There is no evidence for specific avoidance of codons that contain the dinucleotide CG.
5. Codon TTC is strongly preferred to codon TTT for phenyl alanine.
6. In repeat sequence of Pisum, both GGC and GGA codons are used for glycine. For glutamine, the repeat sequence starts with the codon GAG and ends with codon GAA, thus both codons are used. In repeat sequence of phaseolin, GAG codon is used for glutamic acid.

The functional significance of the usage of different codons for the same amino acid in repeat sequence is unclear. They might simply reflect the situation in the prototype repeats before they were amplified.

6.4 DISCUSSION

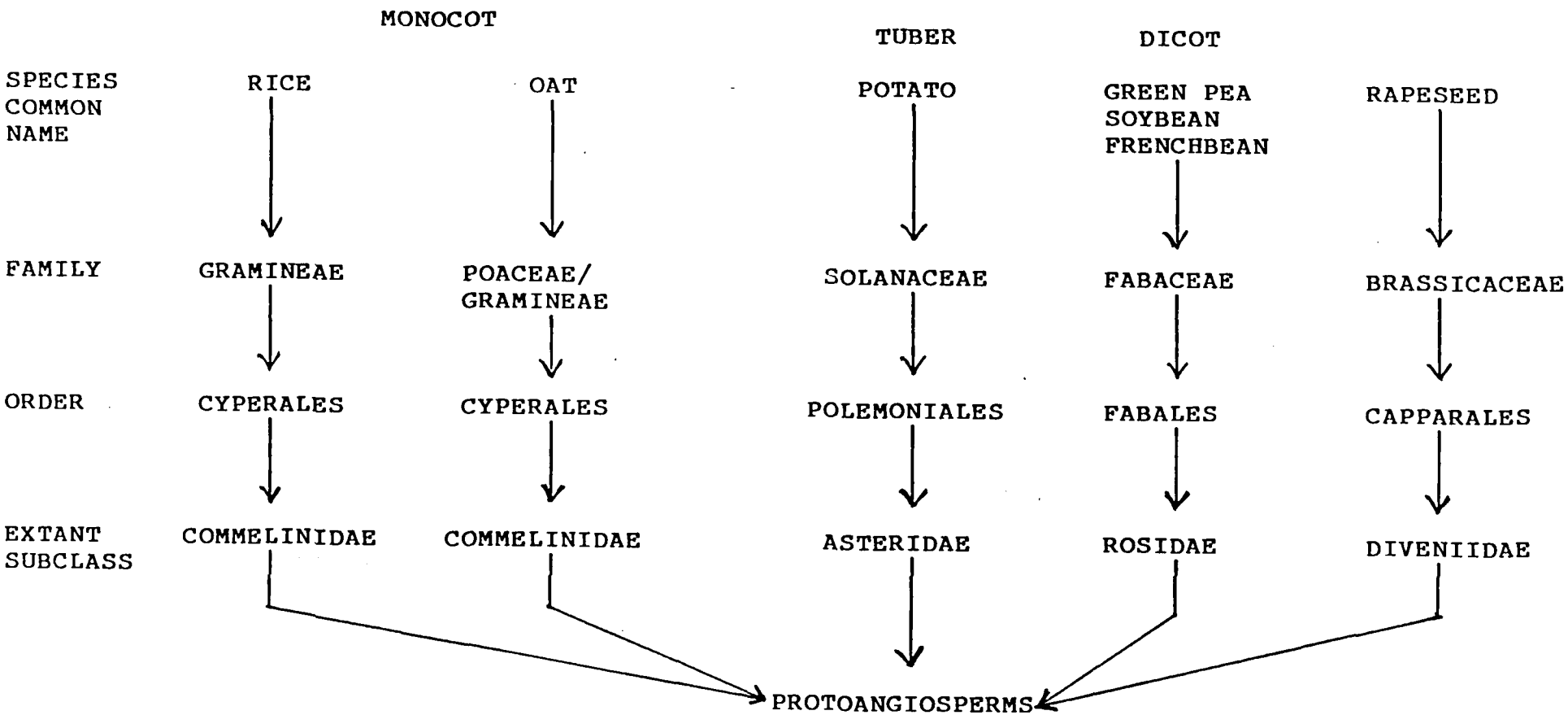
Our basic interest in analysis of the legume storage protein genes stems from the available data which showed some biochemical, immunological and genetic similarities among

legume proteins (8, 11, 37 - 41). Although, reports on comparison of seed storage proteins and their genes are available (12, 14, 17, 18, 19, 26, 31, 39, 42, 43) it is for the first time, to our knowledge that all the available cDNA and genomic sequences of legume storage proteins have been compiled and analyzed by as many as five independent approaches and the data are compared so as to assess for homology and divergence among storage protein genes. Comparison of these sequences by dot matrix analysis, hydropathy and amino acid sequence alignment have demonstrated for the first time that not only the legumins and vicilins from legumes share homology, but also the napin from Brassica napus, Avenin from Avena sativa and patatin from Solanum tuberosum share homology with other storage proteins namely vicilin, convicilin, LEGBAS.CDN of Pisum sativum, phaseolin of Phaseolus vulgaris and A1ABX, A2B1A and A5A4B3 subunit of Glycine max. The globulin seed storage proteins of flowering plants which have been used in our study are known to be members of but 2 gene families that are traceable to the beginning of angiosperm evolution (Fig. 6.5). In fact, some apparent commonality of structural domains have been noted between a legumin and vicilin representative (9). The vicilin family of genes has been lost in evolution of many angiosperms. No vicilins have been found in Brassicaceae family (rapeseed, Arabidopsis) nor in

Figure 6.5

Phylogenetic relationships between organisms providing sequenced globulin storage proteins.

FIGURE 6.5


MONOCOT
TUBER
DICOT
SPECIES COMMON NAME
RICE
OAT
POTATO
GREEN PEA
SOYBEAN
FRENCHBEAN
RAPESEED
FAMILY
GRAMINEAE
POACEAE/ GRAMINEAE
SOLANACEAE
FABACEAE
BRASSICACEAE
ORDER
CYPERALES
CYPERALES
POLEMONIALES
FABALES
CAPPARALES
EXTANT SUBCLASS
COMMELINIDAE
COMMELINIDAE
ASTERIDAE
ROSIDAE
DIVENIIDAE
PROTOANGIOSPERMS

found in Brassicaceae family (rapeseed, Arabidopsis) nor in the representative of the Asteraceae (sunflower). Little is known about their occurrence in monocotyledons plants. Vicilins are major storage proteins in cotton and in leguminous plants, although these families are thought to be only distantly related. All dicotyledonous plants have legumins as do many of the cereals (monocots) like oat and rice. Among the legumin sequences, the cotton proteins have more homology with the sequences of the leguminous plants than does the more closely related sunflower protein (17). From this data, it could be proposed that the genes encoding the 11S and 7S storage proteins of legumes, oat globulin, patatin and napin might have evolved from a common ancestral gene. The weak homology of patatin and napin could further indicate that these two genes might have diverged earlier during the course of evolution.

Apart from the sequence homology, it has also been reported that the legumin and vicilin from legumes show homology to the storage proteins under study on the basis of putative post-translational processing site, disulphide linkage region and probable glycosylation site. It is observed that the post translational processing site N↓G in storage proteins occurs near the C-terminal of variable region. From Table 6.5, it can be suggested that the

secondary structure as well as the ASN and GLY specific sequences may play an important role in recognizing signals for processing enzyme, since the produced exposed region would permit easy accessibility to form a disulphide linkage. This gives stability to these storage protein structure, helping them to be stored in a mannered fashion in protein bodies.

The seed storage proteins are also characterized by the high proportion of aspartate and glutamate and the overall percentage of these amino acid is given in Table 6.8. Since storage protein are stored in protein bodies, where there is protein-protein interaction among them, it is difficult to imagine a structurally integral helix composed largely of negatively charged residues which are likely to repel each other at neutral pH. Therefore, it is, proposed that the disulphide bond formation and post-translational cleavage processess together would be giving some sort of stability to storage proteins so as to be localized in protein bodies with minimal entropy.

As observed by West (44), glycosylation of extra-cellular and cell surface proteins and proteins from certain organelles may reflect a premitive, nonspecific function supporting protein structure and protection. He has further stated that glycosylation presumably has afforded the cell,

TABLE 6.8

AMINO ACID COMPOSITION (MOLE %) AS CALCULATED FROM DEDUCED AMINO ACID SEQUENCE.

AMINO ACID	BNAPJMAG.DNA	CDPHASB1.NAT	DA1ABX.GLY	DAZB1A11.GLY	DGLYA5A4.BJG	PHASCD.83	LEGBAS.CDN	PSLEGCD.PMB	RICEGLU.GEN	VICNAR.83	CONVICIL.BJ	DPATAT.NAR	LEGAN.R84	DATDNA.PMB
ALANINE	6.63	4.35	5.67	6.64	4.08	5.94	11.35	7.11	6.32	5.33	4.10	9.84	7.17	5.43
CYSTEINE	4.42	0.00	1.68	1.71	1.11	0.00	1.08	0.67	1.68	0.22	0.00	0.52	1.36	0.64
ASPARTIC ACID	3.87	3.26	3.36	3.64	5.57	4.99	4.32	4.89	3.16	4.44	6.67	5.18	4.65	1.60
GLUTAMIC ACID	4.97	14.13	8.40	7.92	10.20	8.55	3.78	9.56	5.68	9.78	9.23	5.96	10.08	5.43
PHENYLALANINE	5.52	5.98	3.99	4.07	2.60	5.94	3.78	4.00	4.21	5.33	5.64	4.92	4.26	4.15
GLYCINE	5.52	4.89	7.77	7.49	6.86	5.46	5.41	7.56	7.16	4.89	4.10	5.96	7.56	6.39
HISTIDINE	2.76	2.72	1.68	0.86	2.78	2.38	1.62	2.22	1.89	1.33	1.54	2.07	1.94	2.56
ISOLEUCINE	2.76	7.07	5.46	4.93	3.90	5.94	3.78	4.22	4.00	5.78	5.13	5.44	4.07	5.75
LYSINE	5.52	6.52	5.04	3.85	5.01	5.70	5.95	4.89	2.53	6.89	7.18	5.70	4.46	3.51
LEUCINE	7.73	7.07	6.93	7.07	6.86	10.69	11.35	6.89	7.37	10.44	12.31	10.36	8.33	7.03
METHIONINE	2.76	1.09	1.26	1.50	0.37	1.19	0.54	0.89	0.42	0.22	0.00	2.85	0.97	1.28
ASPARAGINE	3.87	7.07	7.77	8.57	6.12	7.13	8.65	7.33	6.53	8.44	7.69	4.40	7.36	7.67
PROLINE	7.73	4.89	5.88	5.57	6.86	3.56	4.86	4.67	4.00	4.00	3.59	4.15	4.65	4.47
GLUTAMINE	14.36	5.43	10.08	10.92	8.91	5.46	2.70	7.33	11.37	6.89	5.13	3.63	7.17	13.74
ARGININE	3.87	2.72	5.67	6.21	6.68	4.51	7.03	9.78	8.42	5.56	8.21	2.85	9.30	7.03
SERINE	4.42	10.33	7.14	6.42	7.98	9.26	7.57	6.22	8.42	9.33	6.15	7.51	6.01	9.27
THREONINE	5.52	2.72	4.20	3.85	3.71	3.33	5.41	3.11	4.21	2.67	4.62	9.07	2.91	4.15
VALINE	4.97	6.52	4.83	5.57	6.49	6.65	7.57	5.33	8.00	6.00	5.64	4.92	4.84	5.43
TRYPTOPHAN	1.10	0.00	0.84	0.86	1.11	0.24	0.54	0.44	0.63	0.00	0.00	0.52	0.58	0.64
TYROSINE	1.66	3.26	2.31	2.36	2.78	3.09	2.70	2.89	4.00	2.44	3.59	4.15	2.52	3.83
TOTAL RESIDUE	181	184	476	467	539	421	185	450	475	450	195	386	516	313
MOL. WT.	20526	20927	53428	52391	61206	47570	20256	51267	53701	51093	22897	42509	58811	35717

during the evolution of developmental mechanism, an economy in allowing the structural modification of many preexisting proteins using the same enzymatic mechanism. Secondly, according to him, distinctive carbohydrate structure plays a compartmentalization role separate from the enzyme function in catalytic degradation. Post-translational modification such as glycosylation is well suited to the demands of a mechanism for compartmentalization. Storage proteins are also well known for tissue-specificity and localization in protein bodies. In general, they have structure which is conserved so as to pack maximally as put forth by Pernollet and Mosse (45, 46). Thus considering the homology around post-translational cleavage site, disulphide linkage and glycosylation site it can be proposed that all these factors together may play an important role in compartmentalization and stabilization of seed storage proteins.

It is well-known that legumes are deficient in sulphur containing amino acids like methionine and cysteine. In order to increase the nutritional value of these proteins, one of the approaches to be employed would be of site directed mutagenesis where the less preferentially used codons could be modified to codons coding for methionine or cysteine. From our study on the preferential usage of codons, it is found that the codons ATA, ATC and ATT coding for isoleucine are

not predominantly used. Thus the modification of these codons by site directed mutagenesis would lead to codon ATG which codes for methionine. Since both isoleucine and methionine occur predominantly in β pleat structure, this modification may not affect the structure and accumulation of storage proteins in protein bodies. Modification of termination codons TGA to codons TGT or TGC coding for cysteine would not be feasible for the cell as this would affect the termination of protein synthesis. Hence the only feasible modification would be of isoleucine to methionine.

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