Structural and genetical studies on legumin of pisum sativum L. and vicia faba L.

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STRUCTURAL AND GENETICAL STUDIES ON LEGUMIN OF *PISUM SATIVUM* L. AND *VICIA FABA* L.

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To

My Father

ABSTRACT

Studies have been made on the structure and genetics of the major seed storage protein, legumin, in *Pisum sativum* L. and *Vicia faba* L.. Two dimensional gel electrophoresis, using non-reducing conditions in the first dimension and reducing conditions in the second dimension, used in conjunction with other different combinations of various electrophoretic techniques, has been found to be of great value in elucidating the structure of legumin. In both these legumes, legumin has been shown to be composed of a number of subunit pairs of different molecular weights, *Vicia* legumin (subunit pairs of mol. wt. 37,000-79,000) being more heterogeneous in this respect than the *Pisum* legumin (subunit pairs of mol. wt. 35,000-58,000). The subunit pairs are associated into hexameric molecules by non-covalent interactions; there exist a number of different molecular forms containing the subunit pairs in different combinations. Each subunit pair contains a large, relatively acidic subunit (mol. wt. 22700-58,000 in *Vicia* and 24,500-43,000 in *Pisum*; pI range 4.6-6.1 in *Vicia* and 4.85-6.15 in *Pisum*) and a smaller, relatively basic subunit (mol. wt. 21,000-23,000 in *Vicia* and 20,700-22,700 in *Pisum*; pI range 8.2-8.5 in *Vicia* and 6.2-8.0 in *Pisum*) linked covalently by one or more disulphide bonds.

Genetic studies on *Pisum sativum* have shown that various legumin subunits all followed monogenic inheritance,

the alleles showing co-dominance with respect to each other. The genes for some but not all different types of legumin subunits have been shown to be inherited independently. The gene(s) for the acidic subunits of the major legumin subunit pair(s) is located 15 map units distant from the tl locus on the side away from the r locus on chromosome 7. A further storage protein gene has been mapped in that the gene for the convicilin subunits is strongly linked to the locus k on chromosome 2.

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CONTENTS

Section I INTRODUCTION

A very simple classification of reserve proteins of fruits and seeds is based on the pioneering work of T.B. Osborne with various plant proteins. This classification in spite of subsequent modifications, retains its importance by defining the following four groups of proteins on the basis of their solubility characteristics (Osborne, 1924):

1. Albumins : These proteins are soluble in water and the group is mainly made up by enzymes.

2. Globulins : These are insoluble in water but soluble in salt solution and storage proteins of legumes are the predominant members.

3. Prolamins : These are proteins which are soluble in 70-80% ethanol and insoluble in water and salt solutions. Members of Gramineae e.g. maize, wheat and barley have prolamins as their storage proteins although some cereals e.g. oats and rice do not.

4. Glutelins : These are insoluble in all the above solvents but soluble in dilute acids or dilute alkalies. Cereals like rice are rich in glutelins; they also occur in the other cereals but to a lesser extent than prolamins.

While investigating 'proteids' (a term used previously for proteins) of pea, Osborne and Campbell (1898) separated two types of globulins which they designated as legumin and vicilin. Studies by repeated dilution of salt solutions showed that vicilin is soluble in dilute salt solutions and coagulates when heated to 100^{o}C whereas legumin is less soluble in dilute salt solutions and does not coagulate on heating. Also legumin was found to be richer in sulphur content (0.42%) than vicilin (0.18%). Later, Osborne and Harris (1907) separated these fractions by ammonium sulphate fractionation.

Osborne's separatory techniques have been replaced by the modern superior methods of ultracentrifugation, electrophoresis and chromatography. Ultracentrifugation was introduced by Svedberg and Lysholm (1927) who made some studies on seed proteins. Later, extensive studies of seed proteins by ultracentrifugation were performed by Danielsson (1949) and from his work on 34 leguminous species he concluded that legume seeds generally consist of two types of globulins with sedimentation coefficients approximately 11S and 7S. The mol.wt. of the heavy fractions was calculated to be 331,000 and that of the lighter fraction as 186,000. He also equated the 11S and 7S proteins with Osborne's legumin and vicilin respectively by coagulation studies on these proteins. Later on, with the help of electrophoretic techniques, Danielsson (1950) determined the isoelectric points of legumin and

vicilin to be 4.8 and 5.5 respectively. By carrying out some studies on the chemical composition of the two proteins, Danielsson and Lis (1952) showed that legumin was richer in acidic and basic amino acids than vicilin and vicilin had a higher content of neutral amino acids.

However, in considering the values given above for legumin and vicilin, it is important to realize that they have been obtained using 'impure' preparations, which in the case of vicilin consisted of several different proteins and in the case of legumin contained small amounts, less than 5% of impurities, usually vicilin. However, few results obtained by modern, technologically advanced methods of protein chemistry have been reported which challenge the basic findings of the earlier workers. Rather, use is still made to a considerable extent of the fundamental knowledge gained by way of their experiments.

As mentioned, originally the terms legumin and vicilin were coined for the globulins of Pisum sativum (Osborne and Campbell, 1898) but were extended to similar proteins in other members of the family Leguminosae (Danielsson, 1949). However, further investigations have shown that these terms should perhaps be restricted to the members of the tribe Vicieae e.g. Vicia, Pisum, Cicer, Lens and Lathyrus (Derbyshire et al., 1976). Until comparatively recently, modern techniques of protein separation were mostly employed to study the globulin proteins of legumes of other tribes e.g. glycinin in soyabeans and arachin in peanuts. However, lately,

considerable work has also been done on the globulins of Pisum sativum and Vicia faba of the tribe Vicieae.

Pisum sativum and Vicia faba provide a favourable food for a considerable part of the world's population. Their seed protein content falls in the range of 20-30% and the two crops are thus a rich source of protein. In most varieties, legumin is the dominating protein fraction of their globulins and constitutes as much as 60-80% of the total protein (Gatehouse et al., 1979). Like other legumes, the major seed proteins of pea and broad bean are nutritionally first limiting in the sulphur amino acids, methionine and cysteine. Legumin is the protein fraction richer in these amino acids (Boulter et al., 1973) and thus it was decided to devote the present studies to the legumin of Pisum sativum and Vicia faba.

After the initial separations by Osborne and Campbell (1898) and Osborne and Harris (1907), legumin of Pisum sativum and Vicia faba has been separated from vicilin by various workers employing different methods. Crude legumin was isolated by isoelectric precipitation (Danielsson, 1949), by zonal isoelectric precipitation (Wright and Boulter, 1974), by ion-exchange chromatography (Grant and Lawrence, 1964), by sucrose density gradient centrifugation (Casey 1979a; Utsumi and Mori, 1980) and by hydroxylapatite column chromatography (Gatehouse et al., 1980). Highly purified legumin has recently been prepared by Casey (1979a) by immunoaffinity chromatography using antilegumin antibodies.

Molecular weights of legumin reported so far fit well into the hexameric model given by Wright and Boulter

(1974). Using the Svedberg equation, Danielsson (1949) determined the mol.wt. of legumin to be 331,000 in the ultracentrifuge. Later on, larger values were reported by the same technique for legumin of Pisum sativum (Brand et al., 1955; Brand and Johnson, 1958; Casey, 1979a). These workers showed that the mol. wt. values of pea legumin varied between 390,000 and 410,000. Gel filtration has also been employed by Casey (1979a) and Croy et al. (1979) to determine mol. wt. They reported the mol. wt. value to be 395,000 and 390,000 respectively. Recently, a somewhat lower mol. wt. ($350,000 \pm 10,000$) for pea legumin has been reported by Blagrove et al. (1980) who instead of using the Svedberg equation used the 'meniscus depletion sedimentation equilibrium method' in the ultracentrifuge. The mol. wt. of Vicia faba legumin has been calculated to be lower than that of Pisum sativum. It was determined as 328,000 by sedimentation studies (Wright, 1973), as 347,000 by gel filtration (Croy et al., 1979) and as 320-400,000 on polyacrylamide gradient gels (Utsumi and Mori, 1980). The latter workers obtained four different molecular forms of legumin whose mol. wt. varied from 320,000 to 400,000.

There have been conflicting reports in the literature as to whether legumin is glycosylated or not. Reports by Basha and Beevers (1976) suggested that legumin was a glycoprotein where amino sugars and neutral sugars were associated with this protein. These reports were confirmed by Browder and Beevers (1978) who claimed

to have demonstrated the occurrence of glucosaminyl asparagine in glycopeptides isolated from legumin. On the other hand, other authors considered that legumin is not associated with carbohydrates (Bailey and Boulter, 1970; Casey 1979a; Gatehouse *et al.*, 1980). A fluorescent labelling technique and a sensitive radiochemical labelling technique both failed to show any carbohydrate bound to *Pisum* legumin and it was concluded by Gatehouse *et al.* (1980) that legumin was not a glycoprotein. This controversy has been ended by Hurkman and Beevers (1981) who showed that the earlier reports of legumin glycosylation by their group were actually due to the presence of some low mol. wt. glycopolypeptides which co-purified with legumin and not to legumin as such. Thus legumin of *Vicia faba* and *Pisum sativum* is not a glycoprotein.

Legumin synthesis starts quite late during seed development (Wright and Boulter, 1972). They showed that legumin synthesis in *Vicia faba* starts after vicilin synthesis but that the rate of its synthesis is faster so that by the time the seed completes its development, legumin content is usually found to be much higher in the mature seed. However, using *in vitro* developmental studies of the cotyledons, Millerd *et al.* (1975) and Guldager (1978) showed that legumin synthesis starts in very young embryos as soon as cell division has ceased. These workers measured the amount of legumin synthesized by immunoelectrophoresis

which is a more sensitive method than that used by Wright and Boulter (1972). However, using the highly sensitive technique of 'enzyme-linked immunosorbent assay' (ELISA), Domoney et al. (1980) have shown that legumin synthesis starts from the initial stages of embryo development. They could detect legumin in embryos weighing as little as 2×10^{-3}g. According to these authors, legumin may be synthesized in the embryo when DNA endo-reduplication is still occurring but only in cells which have stopped dividing.

The overall amino acid composition of Pisum and Vicia legumin shows a clear similarity between the two (Jackson et al., 1969; Bailey and Boulter, 1970; Casey and Short, 1981). The storage role of legumin is characterized by the presence of very high percentage of amides i.e. asparagine, arginine and glutamic acid. These findings are similar to the amino acid composition of legumin like proteins in many other leguminous and non-leguminous seeds (Derbyshire et al., 1976).

Resemblance between the legumin of Pisum sativum and Vicia faba has also been shown by immunological studies performed by a number of workers. Using antiserum prepared against Pisum legumin, Danielsson (1949) showed that legumin of Vicia faba cross-reacted with that of Pisum sativum. Later on with the help of a semiquantitative serological method, Kloz and Turkova (1963) showed that the intensity of the Vicia

legumin cross-reaction was about 74% of the homologous reaction to Pisum legumin. Antigenic identity between the two legumins using antilegumin antibodies prepared against Vicia legumin was also shown to occur by Dudman and Millerd (1975) and Croy et al.(1979); the latter workers showed that Pisum legumin had one or more antigenic determinants in addition to those of Vicia legumin.

The very high mol. wt. of Pisum and Vicia legumin is self-indicative of the subunit structure of this protein. Vaintraub and Gofman (1961) isolated legumin of Pisum sativum by isoelectric precipitation and proposed that it consisted of 12 subunits of mol. wt. 33,000 each. They also found the N-terminals to be glycine and leucine. However, Jackson et al.(1969) showed the presence of N-terminal threonine in addition to the glycine and leucine reported by Vaintraub and Gofman (1961). The occurrence of these three N-terminals has been recently confirmed by Casey (1979a). Bailey and Boulter (1970) determined the N-terminals of Vicia legumin to be glycine, leucine and threonine also. They proposed that Vicia legumin was composed of three types of subunits of mol. wt. 56,000, 42,000 and 23,000. By using densitometric scanning and in vivo radiochemical labelling of these legumin subunits, they further showed that these subunits were present in the ratio of 1:3:6. Thus Pisum and Vicia legumin were shown to be heterogeneous.

The well known hexameric model of legumin was proposed by Wright and Boulter (1974) using Vicia faba. They showed that legumin consists of two types of subunits, (i) α , acidic subunits of mol. wt. about 40,000 and (ii) β , basic subunits of mol. wt. about 20,000. It was shown that the two types of subunits (one each) were joined by disulphide bonds to form an intermediary of about 60,000 mol. wt.. Six subunit pairs were proposed to comprise the legumin molecule. They determined the acidic subunits to be of mol. wt. 37,000 and found three different basic subunits of mol. wt. 23,800, 20,900 and 20,100. It was also shown that the acidic subunits were richer in aspartic acid, glutamine and arginine than the basic subunits which were richer in alanine, valine and leucine.

It has been demonstrated that the higher mol. wt. subunits are acidic and the lower mol. wt. subunits basic, by electrophoresis in urea (Thomson et al,, 1978; Croy et al.,1979) and by isoelectric focusing (Krishna et al 1979; Casey, 1979b). Electrophoresis on urea-polyacrylamide gels (Thomson et al., 1979) and on cellulose acetate membranes (Croy et al.,1979) has confirmed these findings by showing that in alkaline pH large subunits move towards anode and the small subunits move towards cathode.

Heterogeneity in subunit molecular weight and charge has been studied in detail in Pisum sativum (Thomson and Schroeder, 1978; Croy et al.,1979; Krishna

et al., 1979; Gatehouse et al, 1980). Thomson and Schroeder (1978) reported a complex system of classification in which they differentiated legumin into five groups (LA, LB, LC, LD and LE). Groups LA, LB and LC correspondended to acidic subunits of mol. wt. about 40,000, LD to the basic subunits of mol. wt. 20,000 and LE was constituted by subunits of mol. wt. 25-27,000 of undecided identity. Similar conclusions were reached by Casey (1979b) who divided acidic subunits of different concentrations into major (corresponding to LA and LC of Thomson and Schroeder) and minor subunits (equivalent to LB of Thomson and Schroeder). Isoelectric points of the major subunits were in the range of 6.0-6.5 and those of minor subunits 5.6-5.8. These values for isoelectric points for acidic subunits are very different from those determined by Krishna et al. (1979) as 4.55-4.9 and by Gatehouse et al. (1980) as 5.1-5.3 for acidic subunits. Use of 6M urea (Krishna et al., 1979) and 50% formamide (Gatehouse et al., 1980) did not cause a significant difference in the pI values of basic subunits which were determined to be 8.45 - 8.8 and 8.3 - 8.7 respectively by these workers.

In addition to the heterogeneity found at the subunit level, charge and molecular weight heterogeneity at the molecular level has been observed in Vicia faba. This has been recently shown by Utsumi and Mori (1980) who suggested 7 molecular forms of molecular weight

320,000, 350,000, 380,000 and 400,000 as measured by gradient gel electrophoresis. Acidic subunits of mol. wt. 61,700, 59,800, 48,000 and basic subunits of mol. wt. 23,000, 20,500 and 19,000 were observed.

Heterogeneity in the primary structure has been revealed by amino acid sequence studies of basic subunits carried out by Gilroy and Boulter (1979) in Vicia faba and Casey et al. (1981) in Pisum sativum. A comparison of these results shows the occurrence of sequence microheterogeneity at three of the same positions in both the molecules. Thus, this heterogeneity seems to have arisen as a result of point mutations in the nucleotide sequence.

Few studies have been done regarding the inheritance of Pisum legumin. Thomson and Schroeder (1978) found that acidic subunits (classified by them as LA and LC legumin groups) were each controlled by a single pair of genes. The genes responsible for these subunits were co-dominant and segregated independently of each other. Basic subunits represented by LD group were found to be under multigenic control.

Casey (1979b) used two-dimensional electrophoretic analysis to study the inheritance of legumin subunits and showed that the major acidic subunits of legumin followed a 1:2:1 segregation in F_2 generation i.e. were controlled by a single pair of co-dominant genes.

Improvement of a character by breeding can be

expedited if the genetics of the character in question is well studied. Furthermore it would appear that qualitative and quantitative improvement of pea protein could be effected through changes in the legumin fraction. The legumin fraction is important due to it constituting a comparatively high proportion of the seed proteins and due to its higher content of sulphur-amino acids than other storage proteins. For these reasons, studies on the 'structure and genetics of legumin' was selected as the topic for this thesis.

Section II MATERIALS AND METHODS

I. MATERIALS

A. Biological materials

The various studies made use of the following lines of (a) *Vicia faba* L. and (b) *Pisum sativum* L.

(a) *Vicia faba* L.

Seeds of the lines 'Wierboon', 'Felix' and 'Minica' were kindly provided by Dr. G. Dantuma, Centre for Agrobiological Research, Wageningen, The Netherlands.

(b) *Pisum sativum* L.

Seeds of the varieties English Wonder, Kelvedon Wonder, Peter Pan, Pioneer, Little Marvel, Meteor, Mangtout and Feltham First were purchased from Tyneside Seed Stores, Gateshead, Co. Durham. Lines 110, 180, 360, 611, 806, 807, 808, 851, 1238, 1249, 1256, 1263, 1293, 1552 and 2162 were kindly provided by Dr. S. Blixt, Weibullsholm Plant Breeding Institute, Sweden.

B. Chemicals

Most of the chemicals used were from BDH Ltd., Poole, Dorset, U.K. and were of the purest grade available. Trizma base [Tris (Hydroxymethyl) amino methane] and Coomassie brilliant blue R250 were from Sigma Chemical Company, Poole, Dorset, U.K. Carrier ampholytes for isoelectric focusing were manufactured by Pharmacia Fine Chemicals, Uppsala, Sweden and LKB-Produkter A.B.,

Bromma-Stockholm, Sweden. Hydroxylapatite was from BioRad Laboratories Ltd., Watford, Herts, U.K.; DEAE-cellulose was manufactured by Whatman Ltd., Springfield Mill, Maidstone, Kent.

Visking tubing was obtained from Medical International, 49 Queen Victoria St., London.

Cellulose acetate membranes were supplied by Beckman Instruments Inc., Fullerton.

Uncoated cellophane for drying gels was obtained from W.E. Cannings, Avonmouth way, Avonmouth, Bristol.

II. METHODS

A. Growth of biological materials

Seeds of pea varieties were germinated in Alkathene polyethylene beads in a dark spray room at 28°C with water misting at regular intervals for 5-6 days. Seedlings were then transferred to plastic pots containing compost (Levington : John Innes No.2 = 1:3 parts) in the Botanic Gardens, University of Durham. Plants were grown under 12h light per day and at a minimum temperature of 14°C. Plants were also grown in culture bottles of nutrient solution in a 'Warren Sherer model CEL 511-38' growth cabinet under controlled environmental conditions of light, temperature and humidity, as described by Evans *et al.* (1979). Pods were harvested when dry-ripe and the seeds stored in a cold room at 4°C.

B. Preparation of single seed extracts

The testa was removed from a part of the seed and a quarter of seed (half of the cotyledon) was cut off taking care that the radicle and the other parts of embryo were not injured. The cotyledon was ground and a known weight of seed meal (assumed to have 20% globulins on dry weight basis) was finely suspended by whirlmixing and sonication, in a known volume of extraction buffer to get a final protein concentration of 10 mg/ml of extract. The extraction buffer consisted of 0.125M Tris-HCl pH 6.8 with 2% (w/v) Sodium dodecyl sulphate (SDS) and 1% (v/v) 2-Mercaptoethanol. Suspended seed meal was heated in 1.5 ml centrifuge tubes with frequent shaking in a boiling water bath for 30 to 40 min. The extract was clarified by centrifugation in a Haematocrit centrifuge at full speed and 10% (v/v) glycerol was added to the supernatant to make it dense for subsequent loading on to the gels. For electrophoresis under non-reducing conditions, 2-mercaptoethanol was omitted from the extraction buffer and for electrophoresis under non-dissociating conditions, extraction buffer consisted of only 0.125M Tris-HCl pH 6.8 and the suspended meal instead of being heated, was left at room temperature for 3-4h.

C. Extraction of Globulins

Salt soluble proteins (globulins) were extracted by the method of Croy (1977). The testas were removed from dry seeds and the cotyledons were ball-milled to a fine powder. All subsequent operations were carried out at 7°C. Seed meal was defatted twice with hexane (30ml g^{-1}) and then extracted with 50mM sodium borate buffer pH 8.0 (20ml g^{-1}). After 2 h of stirring, the extract was centrifuged at 16,000 g for 1h. The supernatant which contained dissolved globulins and albumins was dialysed overnight against 33mM sodium acetate buffer pH 5.0. The precipitated globulins were collected by centrifugation at 16,000g at 4°C for 30 min. The pellet was washed and suspended in distilled water and then freeze-dried to give the globulin preparation. Albumins left in the supernatant were dialysed against water at 4°C and freeze-dried.

D. Hydroxylapatite column chromatography

Legumin was purified from the globulins or the total protein extract by hydroxylapatite column chromatography following the method given by Gatehouse et al. (1980). The column (3.2cm x 20cm) was equilibrated with 50mM potassium phosphate buffer pH 8.0. The total extract prepared by stirring seed meal in equilibrating buffer, or globulins dissolved in the equilibrating buffer, were loaded on top of the column and the column

washed with equilibrating buffer until the non-bound materials were fully eluted as the first peak. Further elution was effected by either of the following methods : i) Stepwise elution - using 0.3M-phosphate buffer for the second peak and 1M-phosphate buffer for the third peak, ii) Gradient elution by using a linear gradient of phosphate buffer pH 8.0 with molarity increasing from 50mM to 1M. 300 ml each of 50mM and 1.0M phosphate buffer was used for the purpose. The eluant was monitored automatically at 280nm by the detector unit Type 8303A of an LKB UVICORD II UV Absorptiometer. The flow of solutions was regulated by using an LKB Varioperspex 12000 peristaltic pump and the salt concentration of fractions was measured on a refractometer. Fractions representing various peaks were pooled, dialysed against distilled water for two days and then freeze-dried on an Edwards Modulyo freeze dryer. All buffers used in this column chromatography contained 0.05% sodium azide and were sterilized by filtration through a 0.45 μm pore size cellulose acetate filter.

E. Ion-exchange chromatography

Ion-exchange chromatography was performed on a DEAE-cellulose column (23 x 1.6cm) equilibrated with 50mM-Tris HCl, pH 8.0 containing 0.05% sodium azide. Legumin dissolved in the equilibrating buffer was loaded on top of the column and the column washed with the same buffer till the non-bound materials were eluted

under the first peak. Further elution was carried out by using a linear concentration gradient of sodium chloride from 0.0M to 0.3M. 5ml fractions were collected at 280nm using an LKB fraction collector at a flow rate of 50ml h^{-1}. The required fractions were pooled together, dialysed against distilled water and freeze dried for analysis by various electrophoretic techniques. Molar concentration at which a particular protein was eluted was calculated from the refractive index of that fraction using a calibration curve.

F. Ultrogel column chromatography

Globulins were chromatographed on a column of ultrogel AcA22 (70 x 22cm) equilibrated with 0.1M Tris-HCl pH8.0 containing 0.25M-sodium chloride and 0.05% sodium azide. The column was calibrated for mol. wt. by using the following standard proteins: rabbit immunoglobulins (150,000), catalase (232,000), glutamate dehydrogenase (340,000), ferritin (440,000) and thyroglobulin (669,000). The sample, in about 2ml of buffer, was applied to the column and eluted by upward flow at the rate of 6ml h^{-1}. The effluent was monitored automatically at 280nm as described for hydroxylapatite column chromatography. The apparent mol. wt. of a protein was calculated by measuring the elution volume of protein peak relative to the void volume.

Distribution of the subunits in the elution profile was estimated by following the method given by

Croy et al.(1980b). One ml of each fraction was allowed to precipitate after addition of trichloracetic acid to a final concentration of 12%, for 2 h at 4°C. Precipitate was centrifuged, washed twice with ethanol and dried by evaporation. This precipitated protein was dissolved in sample buffer containing 2-ME and analysed by SDS-gel electrophoresis. Quantitative measurement of the amount of protein represented by each band was made by scanning the stained gel strip on a Gilford spectrophotometer and by integrating the areas under the peak corresponding to protein bands.

G. Polyacrylamide gel electrophoresis

a. Under dissociating conditions:

SDS-polyacrylamide gel electrophoresis was carried out in the discontinuous buffer system of Ornstein (1964) and Davis (1964) using the formulations of Laemmli (1970). Twelve and a half percent gel slabs were made in Raven glass plates by using the following stock solutions:

a-1. Separation gel

(i)	Acrylamide stock (containing 30 g acrylamide and 135 mg NN′ -Methylene-bisacrylamide (Bis) to 100ml with water)	25 ml
(ii)	1M Tris-HCl pH 8.8	22.5 ml
(iii)	Water	9.9 ml

(iv)	1% Ammonium persulphate	1.5 ml
	—— degassed ——	
(v)	SDS	0.6 ml
(vi)	TEMED - NNNN′ Tetramethyl ethylene diamine	20 µl

a-2. Stacking gel

(i)	Acrylamide stock (containing 30g acrylamide and 435 mg Bis to 100 ml with water)	3 ml
(ii)	1M Tris-HCl pH 6.8	2.5 ml
(iii)	Water	13.8 ml
(iv)	1% Ammonium persulphate	0.5 ml
	—— degassed ——	
(v)	SDS	0.2 ml
(vi)	TEMED	10 µl

Standard reservoir buffer

(i)	Tris	3.0 g
(ii)	Glycine	14.1 g
(iii)	SDS	1.0 g

made to 1 lt with water.

Electrophoresis was carried out at a current of 15mA for 1h followed by 25mA till the tracking dye (0.1% bromophenol blue) reached the gel bottom. Power was supplied by a Shandon Southern SAE 2761 constant current source. Protein bands were stained overnight with 0.05% Coomassie brilliant blue in methanol, acetic acid and water in the ratio 50:7:43 (v/v). Gels were destained by diffusion in the solvents as given above for the staining solution. Gels, where required, were scanned by using a Gilford 2000 spectrophotometer. Molecular weights of different polypeptides were measured by using the method given by Weber and Osborn (1969). A number of standard proteins were used to calibrate gels by measuring mobility of the protein with respect to the reference marker — bromophenol blue and by plotting these values against the log of mol. wt. Following standard proteins were used for the purpose : myoglobin (17,500), chymotrypsinogen (25,000), ovalbumin (43,000), bovine serum albumin (67,000), transferrin (76,000) and phosphorylase b (97,000).

b. Under non-dissociating conditions

Polyacrylamide gel electrophoresis under non-dissociating conditions was carried out by the method of Gabriel (1971) modified to suit slab gels. Ten percent and 7.5% slabs were formed using the stock solutions and buffers as described for SDS-polyacrylamide gel electrophoresis except that SDS was omitted from all the solutions.

c. SDS-polyacrylamide gradient gel electrophoresis

SDS-polyacrylamide gradient gel electrophoresis was performed according to the method given by O'Farrell (1975). Gradients between 7.5% (light acrylamide solution) and 22.5% (heavy acrylamide solution) concentration were generated by the use of a Pharmacia P-3 peristaltic pump. Heavy acrylamide was poured into the light solution and the light solution with constantly increasing concentration was pumped into the gel assembly from the bottom. Following was the composition of light and heavy acrylamide solutions for the separation gel:

Light acrylamide solution

(i)	Acrylamide stock (29.2g of acrylamide and 0.8g of Bis made to 100ml with water)	6.0 ml
(ii)	1.5M Tris-HCl pH 8.8 containing 0.4% SDS	6.0 ml
(iii)	Water	12.0 ml
(iv)	10% Ammonium persulphate	20 µl
	—— degassed——	
(v)	TEMED	20 µl

Heavy acrylamide solution

(i)	Acrylamide stock (29.2g of acrylamide and 0.8g of Bis made to 100ml in 75% glycerol)	18.0 ml
(ii)	1.5M Tris-HCl pH 8.8 containing 0.4% SDS	6.0 ml
(iii)	10% Ammonium persulphate	10 µl
	—— degassed ——	
(iv)	TEMED	20 µl

Stacking gel constitution and other conditions for electrophoresis were the same as described for SDS-PAGE. Myoglobin, chymotrypsinogen, ovalbumin, bovine serum albumin and transferrin were used for mol. wt. calibration of the gels.

d. Polyacrylamide gradient gel electrophoresis

These gels were prepared by the same method as for the SDS-gradient pore gels except that SDS was omitted from all the solutions and that concentration gradient was from 5% to 15%. Light and heavy acrylamide solutions had the following composition:

Light acrylamide solution

(i)	Acrylamide stock (29.2g of acrylamide and 0.8g of Bis made to 100 ml with water)	4.0 ml

(ii)	1M Tris pH 8.8	9.0 ml
(iii)	Water	11.0 ml
(iv)	10% Ammonium persulphate	20 µl
	—— degassed ——	
(v)	TEMED	20 µl

Heavy acrylamide solution

(i)	Acrylamide stock (23.4g of acrylamide and 0.64g of Bis made to 100 ml with 75% glycerol)	15 ml
(ii)	1M Tris-HCl pH 8.8	9 ml
(iii)	10% Ammonium persulphate	10 µl
	—— degassed ——	
(iv)	TEMED	20 µl

Gels were run at a constant voltage of 100V for 70h till the ferritin marker stopped moving since it had reached the pore size limit. Gels were stained and destained as usual. Thyroglobulin (mol. wt. 669,000), ferritin (440,000) and catalase (232,000) were used as standard proteins for mol. wt. calibration of the gels.

H. Isoelectric focusing

The methods used were based on comments and suggestions made by Vesterberg (1975) and Gatehouse et al. (1980). The technique employed made use of a Pharmacia

flat bed apparatus FBE 3000 and samples were loaded on filter paper pieces (5 x 10mm) after the slab gels were prefocused for half an hour. A constant power of 10 watts was maintained throughout and the completion of electrofocusing was marked by sharp focusing of the standard protein. Gels were fixed overnight in 15% trichloroacetic acid, washed with distilled water and stained in 0.05% Coomassie brilliant blue in methanol, acetic acid and water in the ratio of 25:7:43 (v/v). A solvent mixture in the ratio as given above was used to destain the gels. Urea solution and formamide for electrofocusing of acidic and basic subunits respectively were deionized by stirring for 1h with Amberlite MB-1 mixed bed resin(1g per 100 ml solution). Details regarding the gel systems and other conditions for electrofocusing of different subunits were as follows:

a. Acidic subunits

10 x 20 cm gels of 6% acrylamide concentration with 6M-Urea and 2% (v/v) carrier ampholytes of major buffering capacity in the pH range of 4 - 6.5 were used for acidic subunits of legumin. Lysis buffer for preparing the sample solutions consisted of : (i) 9M Urea (ii) 2% Nonidet P40 and (iii) 1% 2-mercaptoethanol. 0.03M glutamic acid and 1M ethylenediamine were used as anolyte and catholyte respectively. Sample protein and the reference protein (ferritin in this case) were

loaded on the acidic end of the gel. The following constituted the gel recipe made to 35 ml with water :

(i)	Urea	12.6 g
(ii)	Acrylamide	1.96g
(iii)	Bis	0.14g
(iv)	Carrier ampholytes	
	pH range 4-6.5	1.4 ml
	pH range 3-10	0.35ml
	—— degassed ——	
(v)	10% Ammonium persulphate	50 µl
(vi)	TEMED	25 µl

b. Basic subunits

Isoelectric focusing for basic subunits was performed in gels of 8% acrylamide concentration containing 75% formamide, 10% glycerol and 3% (v/v) carrier ampholytes. Protein samples in the lysis buffer (75% formamide, 2% NP40, 1% 2-mercaptoethanol) along with the marker protein (cytochrome C) were loaded on the acidic end of the gel. Electrode buffers used were 0.1M sulphuric acid for anode and 1M Ethylenediamine for cathode. The Following were used to make a 10 x 20cm gel:

(i)	Acrylamide	2.626 g
(ii)	Bis	84 mg
(iii)	Glycerol	3.5 ml
(iv)	Formamide	26.25 ml

(v)	Water	1.4 ml
(vi)	Carrier ampholytes	
	pH range 6-8	0.7 ml
	pH range 8-10	0.7 ml
	—— degassed ——	
(vii)	3.6% Ammonium persulphate	1.75ml

I. Two dimensional electrophoreses

These experiments involved the combined use of polyacrylamide gel electrophoresis (PAGE), SDS-polyacrylamide gel electrophoresis (SDS-PAGE), polyacrylamide gradient gel electrophoresis (PAGGE) and isoelectric focusing (IEF) under the conditions already described. These were performed in different combinations as follows:

a. Polyacrylamide gel electrophoresis ⟶ SDS-polyacrylamide gel electrophoresis

Ten percent or 7.5% gels were run under non-dissociating conditions in the first dimension. Gel strips were incubated separately in SDS-sample buffer with or without 1% 2-ME. A number of washings were given during the 2h period of incubation. Gel strips were slid, with the help of a thin spatula, onto the top of a single or separate SDS-polyacrylamide gels of the dimensions (including thickness) similar to that of the gel for first dimension. The second dimension gels were then run under the conditions as described before for SDS-polyacrylamide gel electrophoresis.

b. SDS-polyacrylamide gel electrophoresis under non-reducing conditions → SDS-polyacrylamide gel electrophoresis under reducing conditions

Gel dimensions and electrophoretic conditions for both the runs were essentially the same. The only difference was that the unreduced samples were run in the first dimension on 10% or 12.5% gels and then gel strips were incubated in sample buffer containing 1% 2-ME to reduce the protein subunits before loading onto the second dimension gel and re-electrophoresed.

c. Polyacrylamide gradient gel electrophoresis → SDS polyacrylamide gel electrophoresis

The first dimension gel was run as described under gradient pore gel electrophoresis. Incubation in sample buffer with and without 2-ME and electrophoresis in the second dimension were similar to that described for PAGE → SDS-PAGE.

d. SDS-polyacrylamide gel electrophoresis → Isoelectric focusing

SDS-polyacrylamide gel electrophoresis in the first dimension was followed by isoelectric focusing in order to study the isoelectric points of polypeptides representing the large and small subunits of legumin. Gel pieces cut from the first dimension gels of reduced and unreduced samples were incubated in respective lysis buffers as given before for isoelectric focusing of the

acidic and the basic subunits. After incubation, gel pieces were placed on the acidic end of the prefocused gel and electrofocusing carried out as described earlier.

e. Isoelectric focusing ⟶ SDS-polyacrylamide gel electrophoresis

Isoelectric focusing performed in the first dimension was followed by SDS-polyacrylamide gel electrophoresis. The gel strip was incubated in the sample buffer containing 1% 2-ME and run in the second dimension as usual.

J. Three dimensional electrophoresis

The various electrophoretic techniques with incubation buffer and conditions differing in each dimension were used in the combinations as summarised below:

a. PAGE —Sample buffer - 2-ME→ SDS-PAGE

SDS-PAGE ←Sample buffer+2% 2-ME— ↓

b. PAGE

↓

Sample buffer + 2-ME

↓

SDS-PAGE IEF → 9.5M Urea+2%NP40+ 2%2-ME → Acidic subunits

IEF → 75% Formamide+2% NP40+2% 2-ME → Basic subunits

K. Cellulose acetate membrane electrophoresis

Cellulose acetate membrane electrophoresis was carried out using 50mM-phosphate buffer pH 7.0 on a Beckman microzone apparatus. Cellulose acetate membranes were presoaked in the phosphate buffer and dried in filter paper folds by applying even pressure. About 2.5 µl of the sample (10mg ml^{-1}) was applied to the membrane with the help of an applicator and a current of 8mA applied to the membrane for 15 min. Membranes were stained and destained like acrylamide gels. Dried membranes were scanned in the reflectance mode on a Joyce Loebl Chromoscan.

L. Immunodiffusion

Immunoprecipitation by double diffusion was carried out by standard methods (Ouchterlony and Nilsson, 1978). Antibodies were prepared by precipitation with ammonium sulphate and chromatography on QAE-A50 (Quaternary amino ethyl) Sephadex following the method of Joustra and Lundgren (1969) or were affinity purified by immunoaffinity chromatography by the methods given by Bokhout and Van Tiggele (1977) and March *et al.* (1974). Precipitin arcs were cut off and washed in 50mM-sodium borate buffer pH 7.2 (containing 0.15M NaCl) to remove soluble proteins and analysed by SDS-polyacrylamide gel electrophoresis following the method of Croy *et al.* (1980b). For this, samples after washing were dissolved in the SDS-sample buffer without 2-ME.

M. Laurell 'rocket' immunoelectrophoresis

This immunoelectrophoresis was performed in 1% agarose gels (10 x 20 x 0.15cm) using a Shandon flat-bed electrophoresis apparatus according to the method of Laurell (1966) as modified by Weeke (1973). 0.3g of agarose was dissolved in 15 ml water by boiling for 2 min and then was kept in a water bath at 60°C. 15 ml of 0.5M Tris-EDTA borate buffer (0.5M Tris, 1.62mM EDTA, 0.075M borate, pH 8.6) equilibrated to 60°C was mixed with the agarose solution and the appropriate antiserum (400 µl of antilegumin or 600 µl of antivicilin antibodies). The mixture was poured into the gel plate assembly and left for 1h to set. Samples were prepared by extracting seed meal (2.5 mg/ml) in 0.5M Tris-EDTA borate buffer for 2h at room temperature. These samples were loaded along with standards of the appropriate protein (legumin or vicilin) into wells made in the gel. The gel was run overnight at a constant voltage of 100V. Gels were pressed onto agarose coated glass plates and washed extensively in 0.02M saline phosphate buffer followed by washing in water. The gels were then pressed, dried, stained in Coomassie brilliant blue (2 min) and destained. The amounts of legumin and vicilin were determined from their rocket heights by comparison with the calibration curves prepared by plotting the rocket heights against amounts of the standard proteins.

N. Statistical methods

a. Chi-square values were calculated by following the formula given by Fisher (1925)

$$\chi^2 = \Sigma \frac{d}{e}^2$$

Where 'd' stands for deviation of observed value from the expected value, 'e' represents the corresponding expected value and 'Σ' is the symbol for 'summation of'.

b. Percentage of crossing over and the probable error was calculated by the 'product ratio' method of Immer (1930). One of the co-dominant factors in the allelic pair was considered as dominant so that in a dihybrid cross involving 'dominant and co-dominant' factors, factors were in a coupling phase.

Nomenclature

The various types of legumin and legumin subunits are represented by the following abbreviations in this thesis :

a. 'Conventional' legumin:- Legumin subunit pairs of conventional legumin model (Wright and Boulter, 1974). Lg-C

b. 'Big' legumin:- legumin subunit pairs of mol. wt. higher than that of Lg-C. Lg-B

c. Small legumin:- legumin subunit pairs of mol. wt. smaller than that of Lg-C. Lg-S

d. Acidic subunits of legumin. Lg-1

e. Basic subunits of legumin. Lg-2

Thus acidic subunits of 'conventional', 'big' and 'small' legumin are called as Lg-1C, Lg-1B and Lg-1S respectively and the basic subunits as Lg-2C, Lg-2B and Lg-2S respectively.

Section III RESULTS

Part I PRELIMINARY INVESTIGATIONS

A. Molecular weight calibration of gel systems

Molecular weights of the polypeptides on SDS-polyacrylamide gels were calculated from a calibration curve prepared by running a number of standard proteins on 12.5% single pore and 7-22% gradient pore gels (Figs. 1.1 and 1.2). The mobilities of the proteins relative to the bromophenol blue dye front, run on two types of gels, were as follows:

S.No.	Protein	Mol.Wt.	Relative mobilities	
			Single pore gel	Gradient pore gel
a	Myoglobin	17,500	0.984	0.790
b	Chymotrypsinogen	25,000	0.715	0.615
c	Ovalbumin	43,000	0.520	0.470
d	Bovine serum albumin	67,000	0.350	0.330
e	Transferrin	76,000	0.309	0.310
f	Phosphorylase b	97,000	0.260	-

A linear separation according to mol. wt. was observed in the region of mol. wt. 25,000 and 76,000 in both the systems whereas at lower values, relative mobilities were not linear. Also relative mobilities of the standard proteins on gradient pore gels were lower as compared to those on the single pore gels.

Fig. 1.1 : Calibration curve for mol.wt. of standard proteins (a = myoglobin, b = chymotrypsinogen, c = ovalbumin, d = bovine serum albumin, e = transferrin, f = phosphorylase b) on single pore 12.5% SDS-gels.

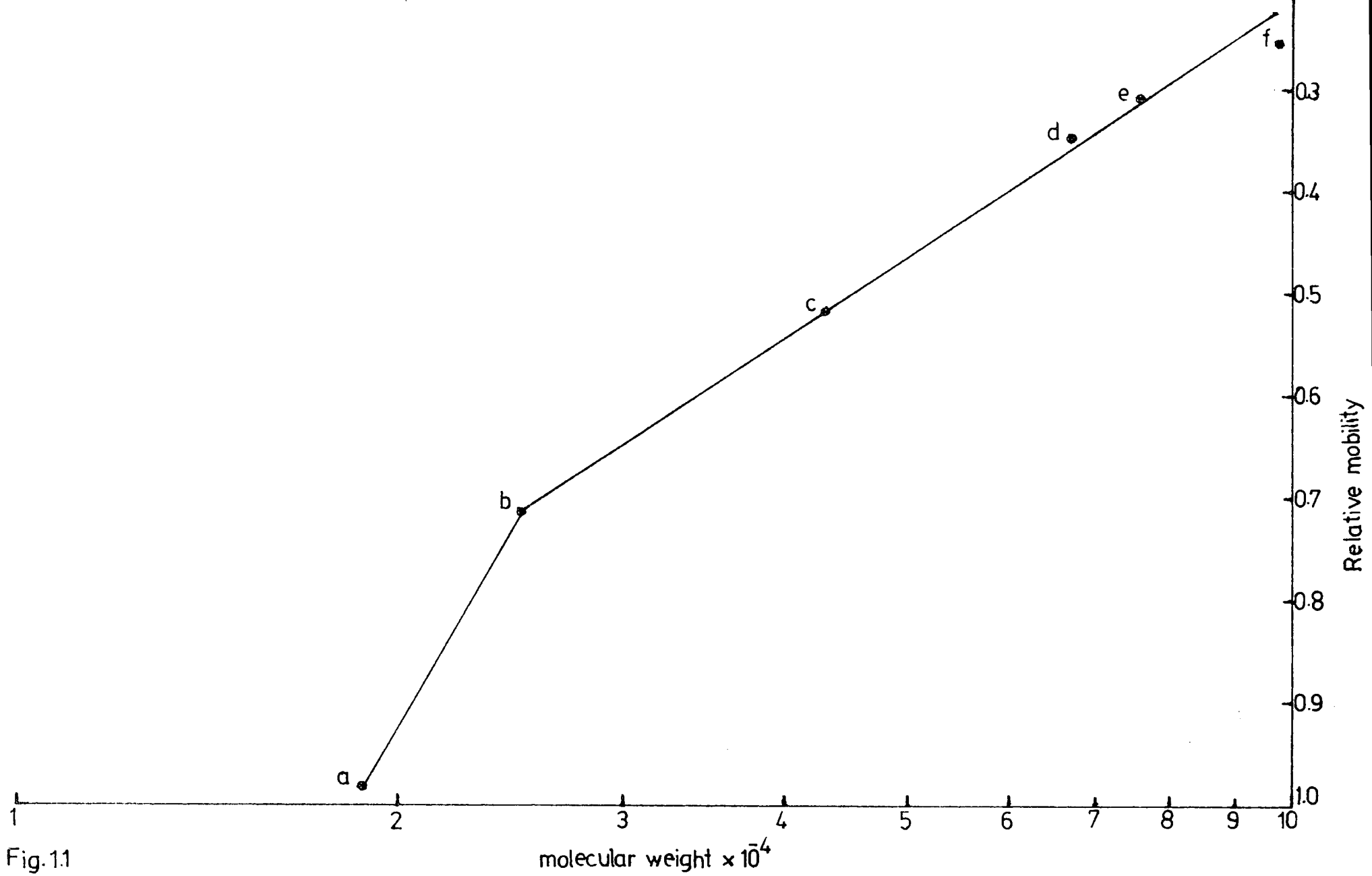

a
b
c
d
e
f
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Relative mobility
1
2
3
4
5
6
7
8
9
10
molecular weight x 10⁻⁴

Fig. 1.1

Fig. 1.2 : Calibration curve for mol. wt. of standard proteins (a = myoglobin, b = chymotrypsinogen, c = ovalbumin, d = bovine serum albumin, e = transferrin) on gradient pore gels.

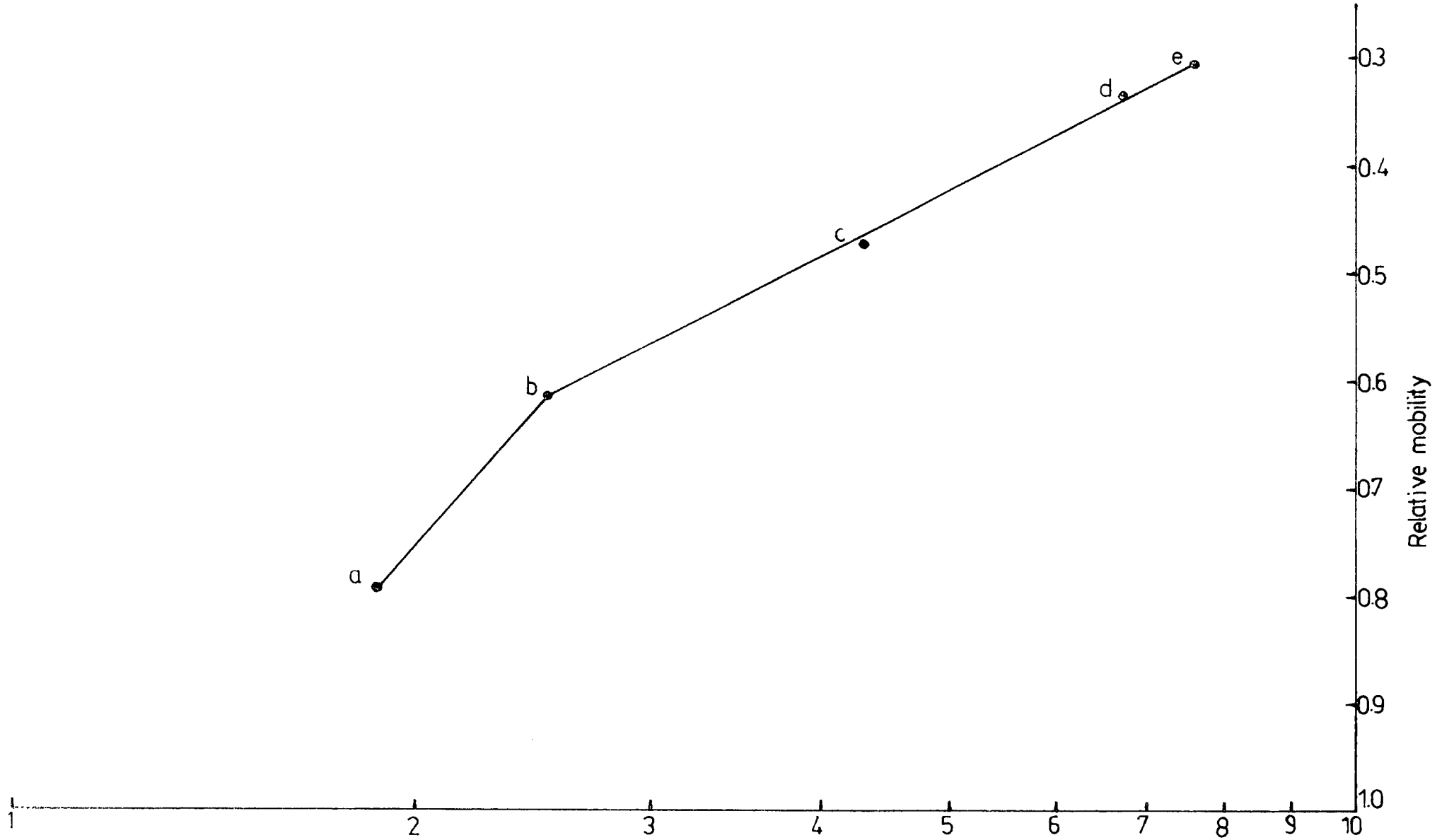

a
b
c
d
e
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Relative mobility
1
2
3
4
5
6
7
8
9
10
molecular weight × 10^{-4}

Fig.1.2

B. Analysis and screening for variation of storage proteins

A large number of Pisum lines (see materials and methods section) were examined using single pore and gradient pore gel electrophoresis by investigating total protein extracts or globulin preparations (Fig.1.3). Based on the results of this analysis, the following storage protein bands were identified : (i) bands of mol. wt. 70,000 and 65,000 representing convicilin (Croy et al., 1980b), (ii) bands in the region of mol. wt. 50,000, 30,000 and 18,000 representing vicilin and (iii) legumin bands in the region of mol. wt. 40,000 and 20,000. Considerable variation among different lines was observed for acidic subunits of conventional legumin (Lg-1C) but very little variation was seen for vicilin, convicilin and basic subunits of legumin.

When globulins of different lines of Vicia faba were examined, the mol. wts. of all these components were found to be slightly lower except legumin basic subunits (Lg-2) which had a mol. wt. somewhat higher than similar components in Pisum. More variation with respect to the number of bands was seen in Pisum. A few bands lying between mol. wt. 50,000 and 70,000, later identified as bands of heterogeneous legumin which were found in Vicia, were absent in Pisum (Fig.1.4). In contrast to the considerable variation of Lg-1C subunits in Pisum, only one type of pattern represented

Fig. 1.3 : SDS-polyacrylamide gel electrophoresis of total protein extracts prepared from single seeds of various lines (180, 360,------2162) of Pisum sativum. CV stands for convicilin, V for vicilin, Lg-1 and Lg-2 for acidic and basic subunits of legumin and Lg-1S for acidic subunits of small legumin subunit pair.

Fig. 1.4 : SDS-polyacrylamide gradient gel electrophoresis of globulins of lines a) Kelvedon Wonder, b) English Wonder and c) Feltham First of Pisum sativum and lines d) Minica and e) Wierboon of Vicia faba. CV stands for convicilin, V for vicilin, Lg-1C for acidic subunits of conventional legumin, and Lg-2 for basic subunits of legumin.

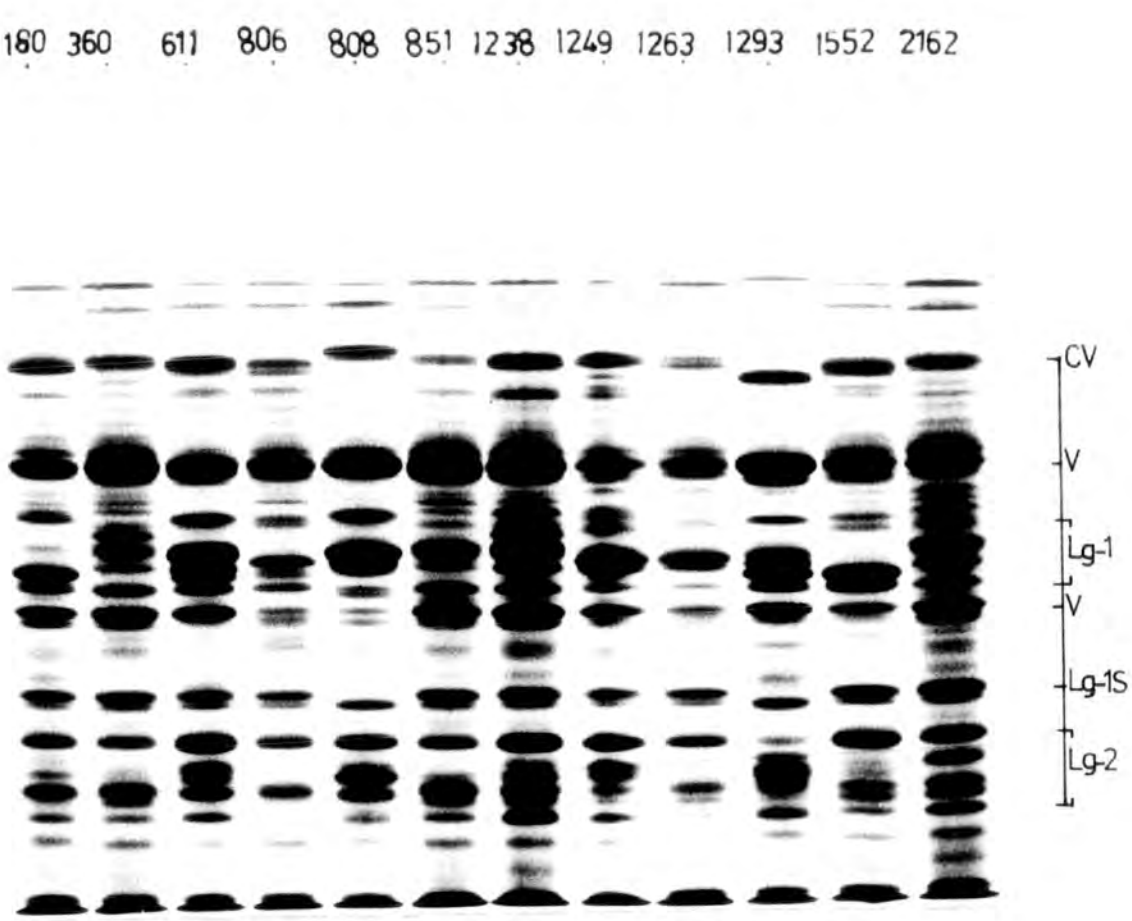

180 360 611 806 808 851 1238 1249 1263 1293 1552 2162
CV
V
Lg-1
V
Lg-1S
Lg-2

Fig.1.3

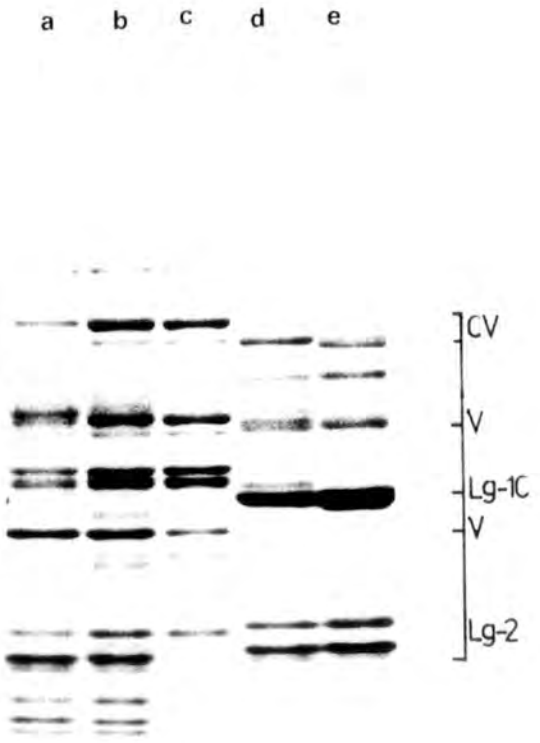

a b c d e
CV
V
Lg-1C
V
Lg-2

Fig.1.4

by a band of mol. wt. 35,000 was seen in Vicia faba.

The pattern of Lg-1 subunits in Vicia and Lg-2 subunits in Pisum in a given line was not constant always. Single seeds of any given generation of a Vicia line had the same pattern for legumin acidic subunits, but when selfed and analysed in the next generation, although all the single seeds examined showed the same pattern, this was different to that of the previous generation. However, these differences only involved quantitative differences in the bands. Two possibilities were thought to be responsible for this effect, either the developmental stage at which seeds were harvested or some storage effects.

C. Purification of storage proteins

a. Globulin preparation

The globulin fraction of Pisum sativum and Vicia faba seeds prepared by the procedure given in the materials and methods section, consisted of vicilin, convicilin and legumin as described previously.

b. Hydroxylapatite column chromatography

Various fractions of seed proteins were separated by hydroxylapatite column chromatography using the following types of elution techniques.

(i) Step-wise elution : This method was used to separate legumin and vicilin fractions of the globulins. The

first peak was obtained by elution with equilibrating buffer (0.05M phosphate buffer, pH 8.0), the second peak was eluted by using a buffer of higher concentration (0.3M) and the third peak by using a still higher concentration (1M) of phosphate buffer pH 8.0. Analysis by SDS-gel electrophoresis showed that no proteins were eluted in the first peak, legumin was obtained in the second and vicilin in the third peak. However, this method did not prove effective enough because it did not give a pure separation of any of the protein fractions.

(ii) Linear gradient elution : Hydroxylapatite column chromatography by linear gradient elution was found to be a better method for fractionation of the proteins in the total protein extracts. All the proteins were eluted in the fractions obtained using a gradient between 0.05M to 1M phosphate buffer (molar concentrations were determined by measuring the refractive indices of various fractions; these values were used to ascertain the molar concentration from a calibration curve already prepared). Molar concentrations and the proteins eluted under different peaks are given in Table 1.1 and Fig. 1.5.

Fig.1.5 : Elution profile from chromatography on hydroxylapatite column using phosphate-gradient elution. For details of the peaks see table 1.1.

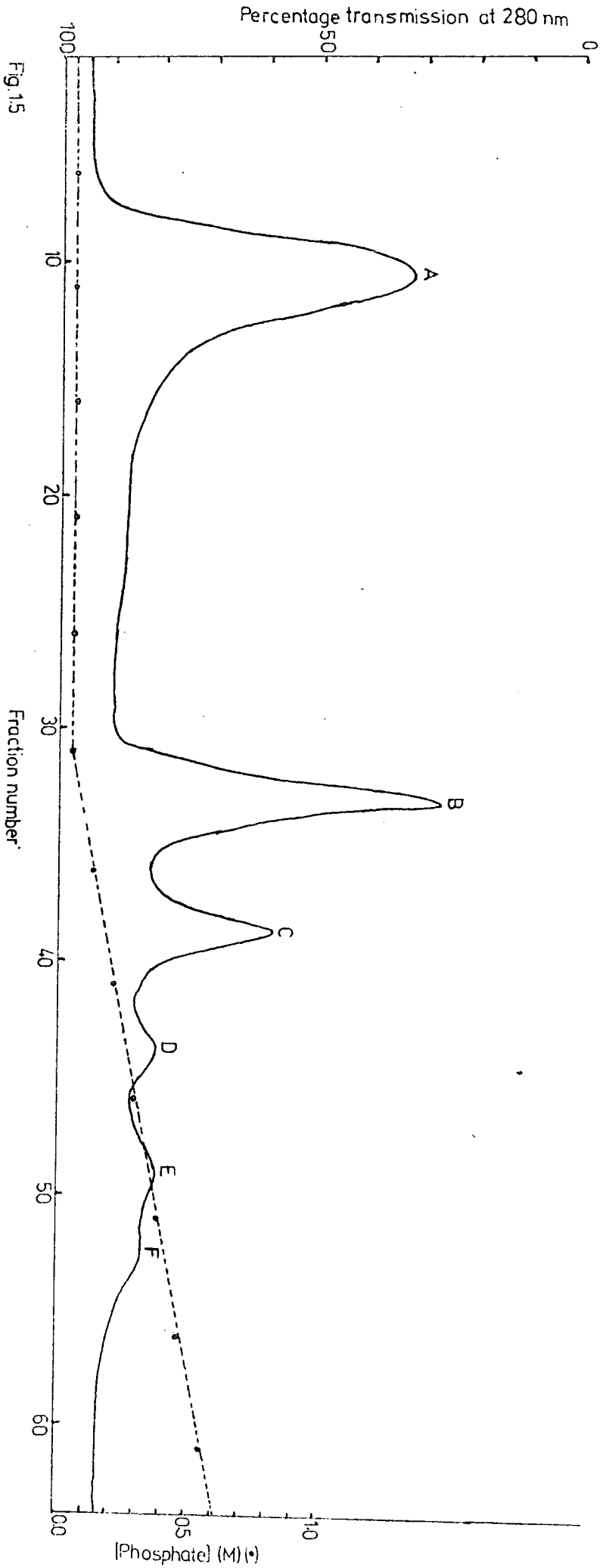

Percentage transmission at 280 nm
100
50
0
10
20
30
40
50
60
A
B
C
D
E
F
Fraction number
0·0
0·5
1·0
[Phosphate] (M) (•)

Fig. 15

Table 1.1 Major proteins and the molar concentration at which they were eluted using hydroxylapatite column chromatography

Peak	Molar Concent-ration	Major proteins (mol. wt.)
A	0.05	Nil
B	0.05-0.1	Albumins (80,000, 50,000, 30,000, 25,000, 22,000 and 18,000).
C	0.13-0.21	Legumin (40,000, 25,000, 20-22,000)
D	0.21-0.28	Legumin, vicilin and convicilin
E	0.32-0.37	Convicilin (70,000, 65,000) and vicilin
F	0.4 -0.45	Vicilin (50,000, 30,000, 18,000).

D. Quantitative determination of legumin and vicilin

Estimations of the legumin and vicilin fractions in globulins were carried out by various electrophoretic and chromatographic techniques as follows:

a) Quantitation by electrophoresis

(i) Gel electrophoresis and scanning : The globulins from bulked seeds of seven commercial lines (English Wonder, Kelvedon Wonder, Little Marvel, Meteor, Peter Pan, Pioneer and Feltham First) grown in different years were extracted and studied on SDS-gradient pore gels. All the

lines were observed to be quite similar in the qualitative pattern of legumin and vicilin. However, the ratio of the concentration of legumin and vicilin on the basis of intensity of staining of subunits showed variation amongst lines for seeds harvested in the same year and variation for the same line in seeds harvested in different years (Table 1.2). As there was overlapping of different polypeptide bands, actual amounts of the legumin subunits was found by running the globulins under reducing and non-reducing conditions. Densitometric measurements of these gels confirmed that although the total concentration of legumin in these lines showed variation from year to year, the ratio of the acidic and basic legumin subunits in all the lines was the same i.e. 1:1 (after correction is made for the difference in the size of legumin subunits). 'Feltham first' and 'Meteor' were found to have a greater legumin to vicilin ratio in harvests of both the years whereas other five lines had lower legumin contents relative to vicilin. Similar variations were also observed in the seed extracts of these lines.

Table 1.2 Densitometric analysis of legumin, vicilin and their subunit contents in pea seeds of different harvests

Pea variety	Harvest	Vicilin (area under the peak)	Legumin (area under the peak)	Lg-1 (acidic subunits)	Lg-2 (basic subunits)	Lg-1 / Lg-2	Legumin, vicilin ratio
English Wonder	year I	45.42	18.11	12.4	5.71	2.17:1	0.4:1
	year II	28.52	19.03	12.74	6.29	2.03:1	0.7:1
Kelvedon Wonder	year I	49.57	36.38	25.88	10.5	2.4:1	0.7:1
	year II	17.69	9.81	6.6	3.2	2.06:1	0.6:1
Feltham First	year I	69.72	117.47	84.4	33.07	2.5:1	1:0.6
	year II	19.1	21.68	14.79	6.89	2.15:1	1:0.9

(ii) Rocket immunoelectrophoresis : Legumin and vicilin were determined by using appropriate antisera in the immunoelectrophoretic method given by Laurell (1966). Legumin and vicilin contents of each line were estimated from the calibration curves prepared for the 'rocket' heights of standard legumin and vicilin samples respectively. Results in Table 1.3 show the variation of legumin and vicilin in these Pisum lines. Lines 1238 and JI 181 had a very high content of legumin and the lines 1263 and 1238 were rich in vicilin. On the other hand, lines Mangtout and 1263 were low in legumin and lines JI 181 and Meteor low in vicilin content.

Table 1.3 Quantitative analysis of legumin and vicilin in total protein extract of pea seeds by immunoelectrophoresis

S.No	Pisum line	% age of legumin	% age of vicilin	Legumin: vicilin ratio
1	Feltham First	24.8	8.48	2.92:1
2	Meteor	21.6	5.9	3.66:1
3	Mangtout	11.6	6.3	1.84:1
4	JI 181	34.4	5.3	6.50:1
5	807	22.0	8.0	2.75:1
6	851	20.8	9.8	2.12:1
7	1238	50.8	22.0	2.31:1
8	1263	12.4	10.2	1.20:1
9	110	31.6	8.0	3.95:1

(iii) Cellulose acetate membrane electrophoresis : Legumin and vicilin in globulins and total protein extract were also quantified by cellulose acetate membrane electrophoresis. Vicilin and legumin protein bands were identified by running these purified proteins along with the samples. Mobility of vicilin was lower than that of legumin but in low legumin lines the legumin band was not clearly resolved from the vicilin band. These results were thus inferior to those obtained on gels and were not in quantitative agreement.

b) Quantitation by column chromatography

Chromatographic separation by stepwise elution on hydroxylapatite column was used to measure the legumin and vicilin in Vicia globulins. Optical densities of the fractions under the second and third peak were measured and fractions pooled to get the relative amounts of proteins eluted in these fractions. The second peak contained about 5 times more protein than that in the third peak. However, this method of quantitation of legumin was also found not to give reproducible results and also the proteins eluted under the peaks were not pure legumin and vicilin.

E. Conclusions

Single pore gels were found to be the best system for qualitative analysis. These gels were very easy to make and gave a wide and good separation of legumin subunits. On the basis of differing patterns for

various subunits, a number of lines were selected for further studies on the structure and genetics of legumin. These lines were 'Felix' of Vicia faba and 110, 807, 1238, 1263, 'Feltham First' and 'Meteor' of Pisum sativum. SDS-polyacrylamide gel electrophoresis suitably modified was found to be most suitable for studies on structure and genetics of legumin. Variety 'Felix' of Vicia faba was selected for studies on the structure of legumin only. Genetical studies on Vicia legumin could not be carried out as the pattern for Lg-1 subunits was not constant from generation to generation and differences in the subunit were more quantitative than qualitative.

Part II. STRUCTURE OF LEGUMIN OF VICIA FABA L.

A. SDS-Polyacrylamide gel electrophoresis of Vicia faba globulins

Globulins of Vicia faba variety 'Felix' isolated as in the Methods section were analysed on 12.5% SDS-polyacrylamide gels. Under reducing conditions (Fig.2.1d) legumin subunits were identified by reference to the Wright and Boulter (1974) model of legumin. Acidic subunits were represented by two bands of mol. wt. 37,500 and 35,000 and basic subunits by bands of mol. wt. 23,000, 21,600, 21,200 and 21,000. Bands representing polypeptides previously identified as convicilin (CV), mol. wt.64,000 (Croy et al., 1980b) and as vicilin (V), mol. wt. 48,000 and 32,000 (Wright, 1973) as well as a number of other bands, were also present. When examined under non-reducing conditions (Fig.2.1a), the protein banding pattern was found to be different with new bands of mol. wt. 79,000, 73,000, 67,000, 53,000 and 40,000-37,000. One of these, the band H/I representing the subunit pair of conventional legumin model (Wright and Boulter, 1974) had a mol. wt. 53,000. The bands (A-J) under non-reducing conditions were found to be due to components undergoing reductive cleavage i.e. a series of disulphide bonded subunit pairs, by two dimensional SDS-polyacrylamide gel electrophoresis, employing non-reducing conditions in the first dimension and reducing conditions in the second dimension (Fig.2.2). The mol. wts. (in brackets) of two components of each of the bands 'A-J'

were as follows:

A(79,000) = (58,000) + (21,000)

B(73,000) = (54,000) + (21,200)

C(70,000) = (52,000) + (21,200)

D(67,000) = (50,000) + (21,200)

E(61,500) = (46,500) + (21,000)

F(60,000) = (43,500) + (21,000)

G(56,500) = (37,500) + (23,000)

H/I(53,000) = (35,000 + 21,600), (35,500 + 23,000) and (35,500 + 21,600)

J(40,000 - 37,000; three bands)

= (22,700 - 23,100) + (21,200 - 21,000)

Thus, whereas the mol.wts. of the larger subunits, depending on the size of the subunit pair, varied from 23,100 to 58,000, those of the smaller subunits of all the pairs lay approximately in the same range (mol.wt. 21,000-23,000). Also, whereas mol.wts. of the larger subunits decreased linearly from band 'A' to 'H', band 'I' was peculiar in having a mol.wt. (35,500) greater than that of 'H' (35,000). Another characteristic of the subunit pairs 'I' was that in comparison to one smaller subunit contained in all other pairs, there were two smaller subunits of mol.wt. 23,000 and 21,600 respectively associated with subunit pair of the type 'I'.

Fig. 2.1 : SDS-polyacrylamide gel electrophoresis of globulins and legumin of Vicia faba variety 'Felix'. Track 'a' : globulins under non-reducing conditions; track 'b' : immunoprecipitated legumin obtained by double diffusion of Vicia faba globulins and affinity purified anti-Pisum sativum legumin; track 'c' : legumin purified by hydroxylapatite column chromatography; track 'd' : globulins under reducing conditions. A-J represent legumin subunit pairs as defined in the text. CV, V and IgG represent convicilin, vicilin and immunoglobulins respectively.

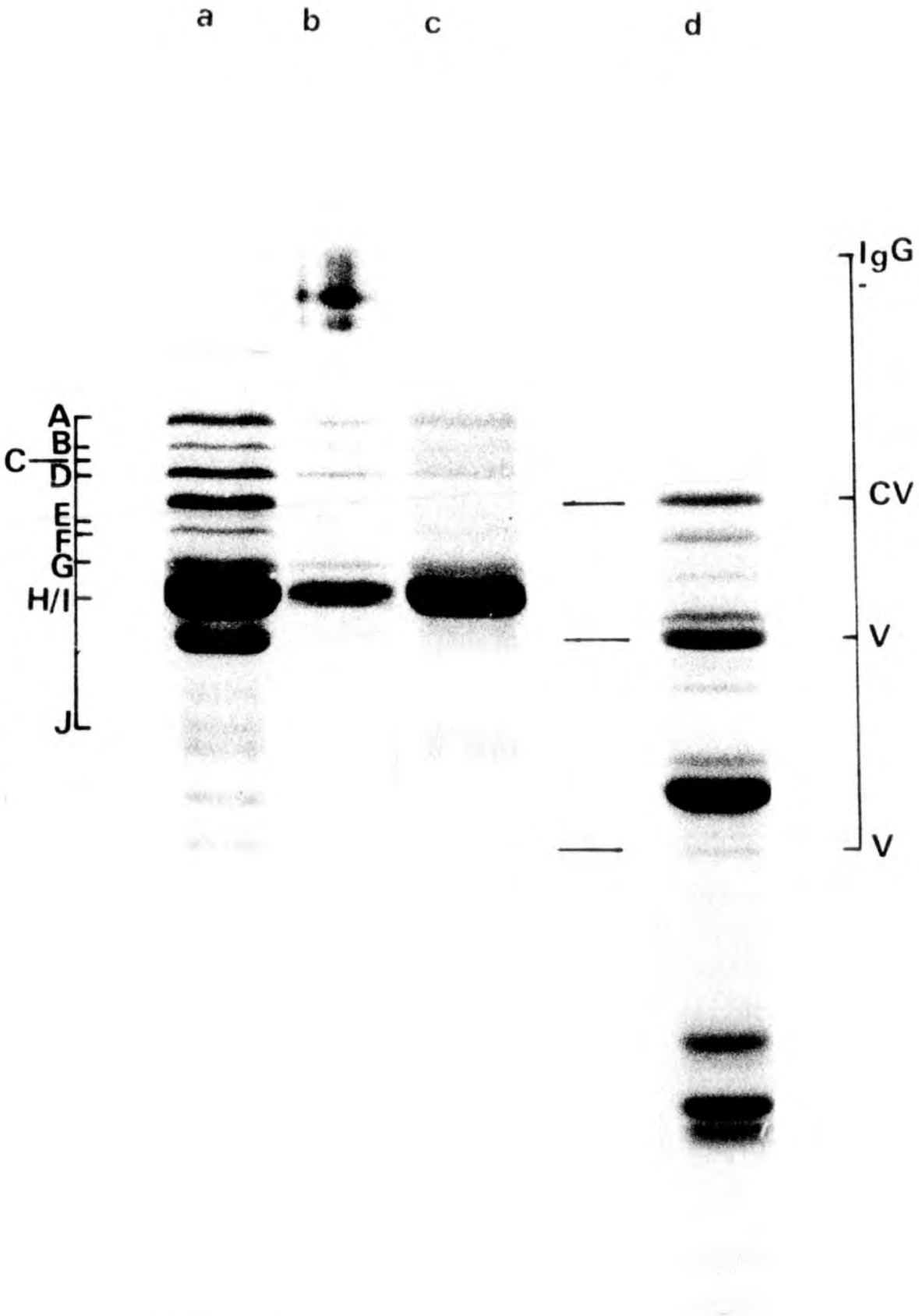


Fig. 2.1

B. Legumin purification by hydroxylapatite column chromatography

Legumin of Vicia faba variety 'Felix' was fractionated on hydroxylapatite columns and examined under non-reducing conditions on 12.5% gels (Fig.2.1c). It contained all the bands of fig.2.1a except those representing convicilin and vicilin; all the bands present corresponded to ones containing disulphide bonded subunits i.e. bands 'A-J'. Densitometric scanning of the stained gels showed that the subunit pair H/I constituted approx. 80% of the total legumin whereas subunit pairs 'A-G' and 'J' were each present as minor components.

C. Immunodiffusion studies

Immunoprecipitates were formed by double diffusion of Vicia faba globulins against the affinity purified antibodies directed against legumin of Pisum sativum, which is known to cross-react with Vicia faba legumin (Croy et al.,1979). The sharp precipitin arcs were excised, washed and when run on SDS-gels (Fig.2.1b), contained all the bands 'A-J' except 'F' seen in the purified legumin in Fig.2.1c. This showed that all the subunit pairs except 'F' had determinants in common with the pea legumin.

Fig. 2.2 : Two dimensional SDS-polyacrylamide gel electrophoresis of Vicia faba globulins. 1D : analysis under non-reducing conditions; band pattern given as track (i) in the first dimension. 2D : analysis under reducing conditions in the second dimension; band pattern given as track (ii). A-J represent legumin subunit pairs and their components as defined in the text CV and V represent convicilin and vicilin respectively.

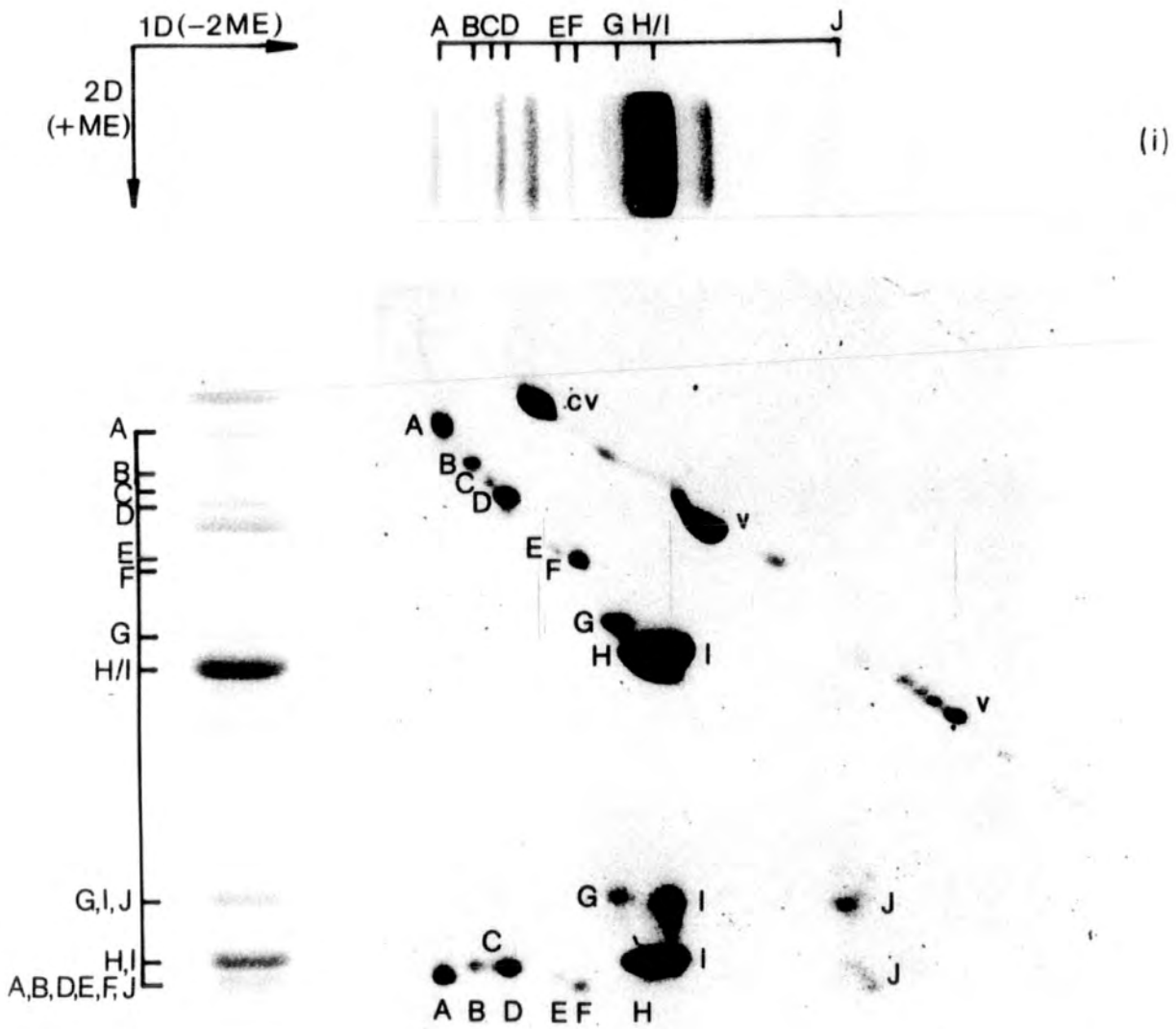

1D(-2ME)
2D
(+ME)
A BCD EF G H/I J
(i)
A
B
C
D
E
F
G
H/I
G,I,J
H,I
A,B,D,E,F,J
cv
v
v
G
I
J
C
I
J
A B D E F H
(i i)

Fig. 2.2

D. Two dimensional electrophoreses

(i) Polyacrylamide gel electrophoresis/SDS-polyacrylamide gel electrophoresis

Globulins were run in the first dimension under non-dissociating conditions on 10% polyacrylamide gels (Fig.2.3). Three bands 'DLg', 'F' and 'Lg' of relative mobilities 0.09, 0.11 and 0.19 respectively were observed, the last being the major band. A smear was also observed along the sides of the gel track beyond band 'Lg'. Gel slices from the first dimension were run under reducing and non-reducing conditions in the second dimension on 12.5% SDS-polyacrylamide gels (Fig.2.3). Bands 'DLg' and 'Lg' were found to contain all the bands except 'F' of Fig.2.1d and Fig.2.1a under reducing and non-reducing conditions respectively. Band 'DLg' was considered to be due to dimers of legumin molecules. Band 'F' corresponded to band 'F' of Fig.2.1a under non-reducing conditions and to the subunit products of band F of Fig.2.2 under reducing conditions. Components of smears corresponded to the bands of vicilin and convicilin.

(ii) Polyacrylamide gradient gel electrophoresis/SDS-polyacrylamide gel electrophoresis

The first dimension electrophoresis of globulins was carried out on 5-15% polyacrylamide concentration gradient gels. Three bands of relative mobilities 0.275, 0.337 and 0.520 were seen (Fig.2.4). From the mol.wt. calibration curve of these gels, the mol.wt.

Fig. 2.3 : Two dimensional analysis of Vicia faba globulins. 1D : analysis by gel electrophoresis (non-dissociating conditions) in the first dimension. Bands DLg, F and Lg of increasing mobility shown in tracks (i) are defined in the text. 2D : analysis by SDS-polyacrylamide gel electrophoresis in the second dimension under non-reducing (b) and reducing (a) conditions. Band patterns shown in tracks (ii)b (non-reducing) and (ii)a (reducing). A-J represent legumin subunit pairs and their components as defined in the text.

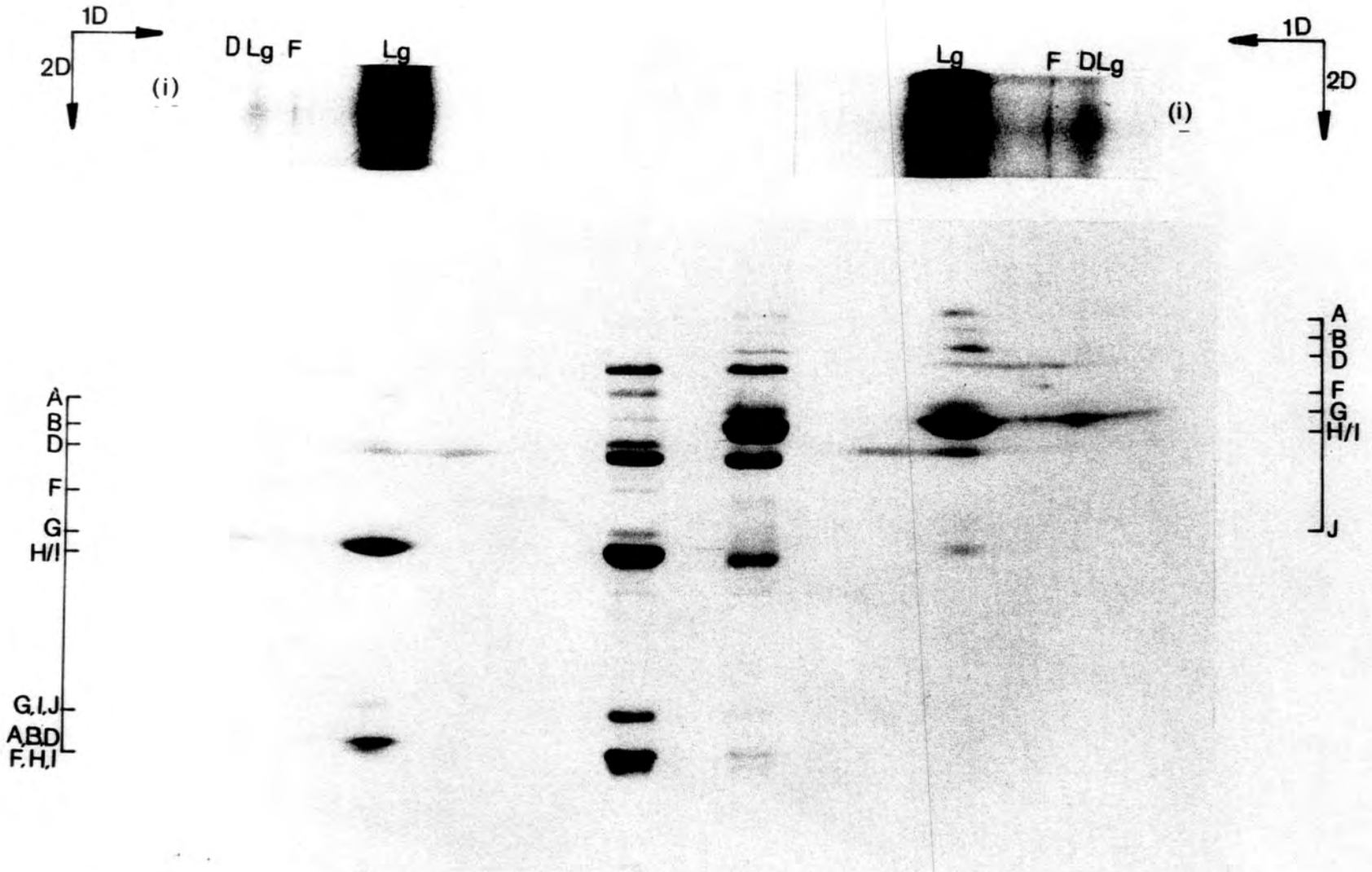

1D
2D
(i)
D Lg F
Lg
Lg
F DLg
1D
2D
(i)
A
B
D
F
G
H/I
G,I,J
A,B,D
F,H,I
A
B
D
F
G
H/I
J
a
(ii)a
(ii)b
b

Fig.23

Fig. 2.4 : Two dimensional gel analysis of Vicia faba globulins. 1D : analysis by gradient pore gel electrophoresis under non-reducing conditions in the first dimension. 2D : analysis by SDS-polyacrylamide gel electrophoresis in the second dimension. A-J represent large acidic subunits as defined in the text.

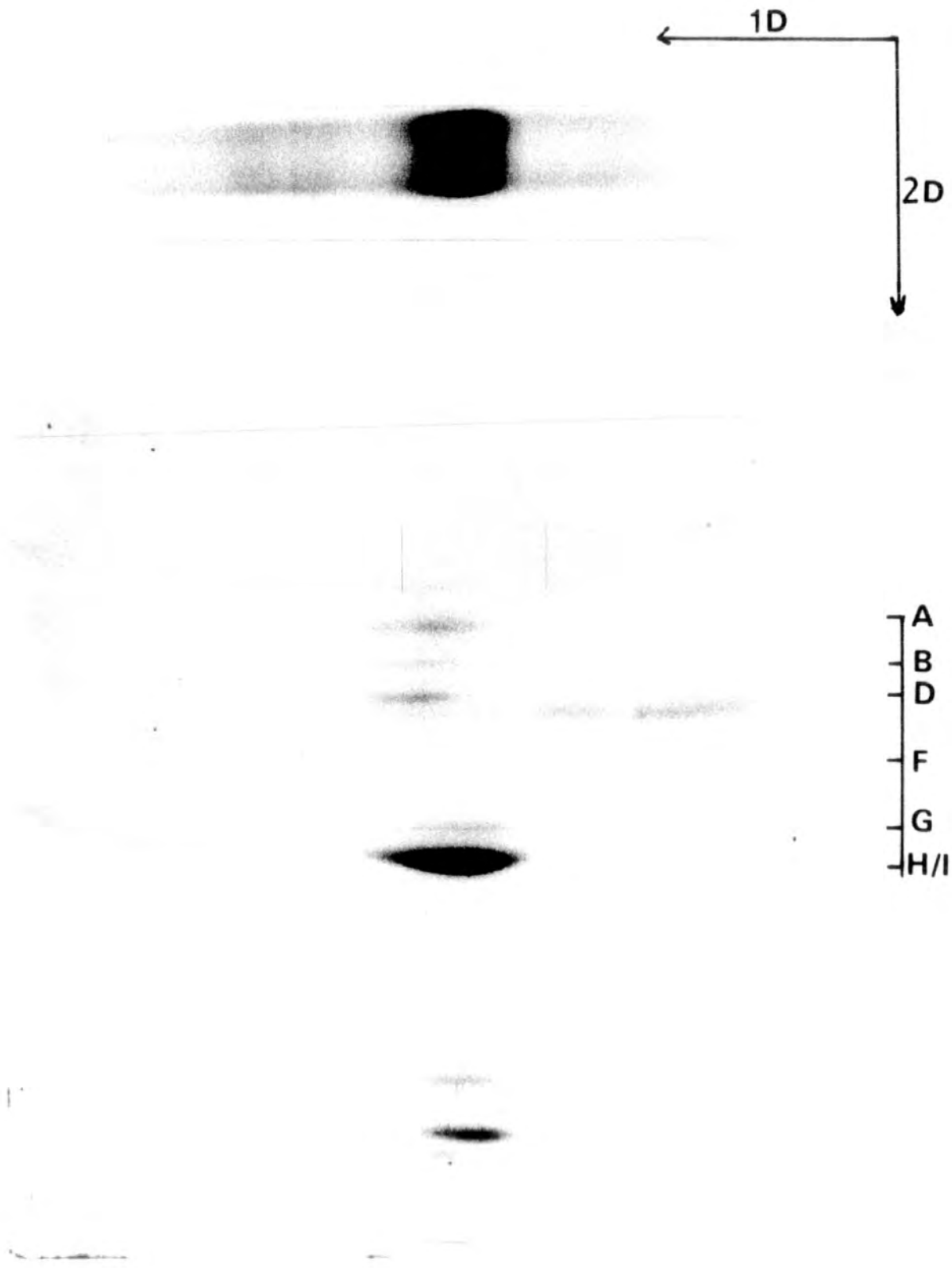


Fig. 2.4

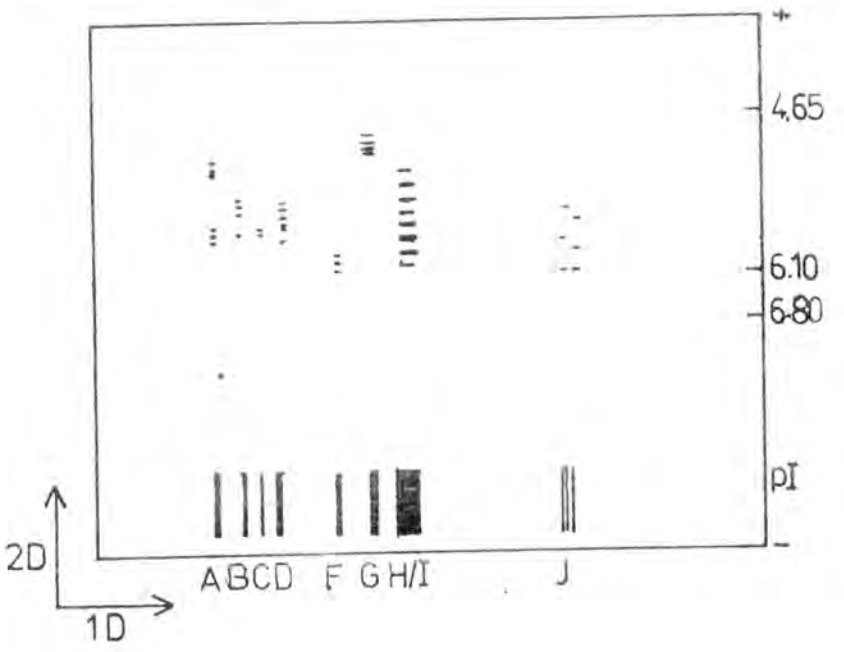
values of these bands were found to be 630 - 650,000, 560,000 and 330,000 respectively. Mol. wt. 330,000 of the third band represented the mean value of a large number of closely placed dark bands (as many as 10 in number) in the mol. wt. range of 280,000 to 380,000. The subunit pairs present in these bands (mol.wt. 280 - 380,000) were revealed by analysis in the second dimension by using SDS-polyacrylamide gel electrophoresis under reducing conditions (Fig.2.4). Although the resolution in the first dimension did not allow complete analysis of species present in the major band, subunit pair A, B, C, D were partially separated and had a lower mobility in the first dimension whereas 'F' was partially separated to a higher mobility. The legumin subunit pairs 'H/I' and the subunit pair 'G' were found in all the legumin species analysed. Subunit pair 'J' was also found to be associated with the subunit pairs 'H/I'. The minor bands at lower mobility in the first dimension also contained legumin subunits with a distribution similar to the major legumin bands.

(iii) SDS-polyacrylamide gel electrophoresis/Isoelectric focusing

This technique involved the analysis of Vicia globulins on SDS-polyacrylamide gels in the first dimension followed by isoelectric focusing in the pH range 4-6.5 (for acidic subunits) and pH 6-10 (for basic subunits). Results are shown in Fig.2.5a and 2.5b for acidic and basic subunits respectively. All the bands 'A-J' of Fig.2.1a separated in the first dimension under non-reducing

Fig. 2.5 : Two dimensional analysis of Vicia faba legumin. 1D : analysis by SDS-polyacrylamide gel electrophoresis under non-reducing conditions in the first dimension. 2D : analysis by isoelectric focusing in the second dimension in the acidic range (a) and in the basic range (b). A-J represent legumin subunit pairs as defined in the text.

(a)



(b)

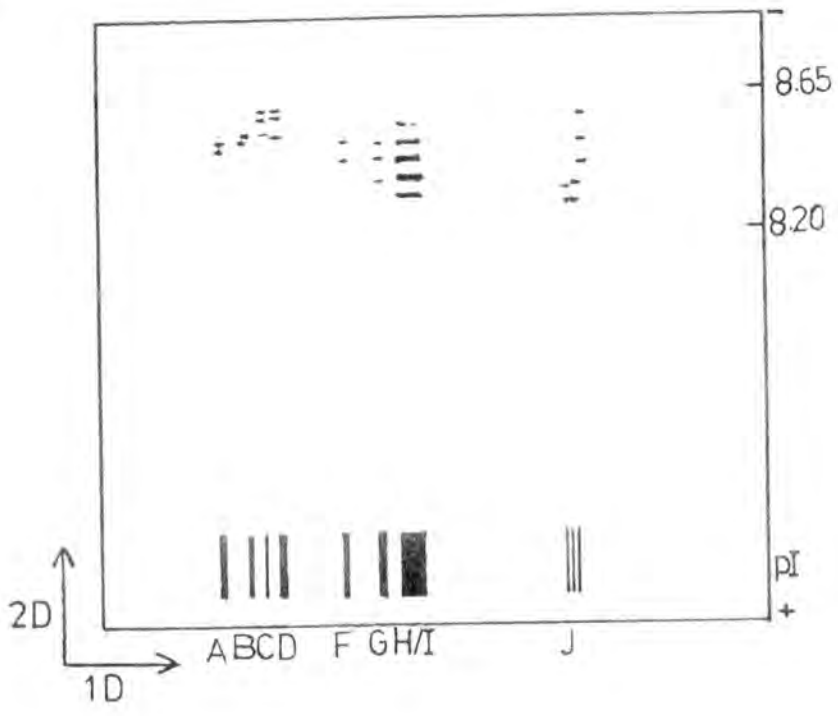


Fig. 2.5

conditions were shown to consist of acidic and basic subunits in the second dimension. The pI range of acidic subunits was 4.6-6.1 and of basic subunits 8.2-8.5. When subunit-pair-products separated in the first dimension under reducing conditions were subjected to isoelectric focusing in the second dimension (results not shown) it was observed that in each case the large subunit of a pair was acidic and the smaller subunit basic. All the subunits showed charge heterogeneity and the pI range of the subunit pairs 'A-G' and 'J' was similar to those of the subunits of conventional legumin H/I.

E. Ultrogel column chromatography

Fig. 2.6 shows the elution profile of Vicia faba total proteins on a column of ultrogel ACA 22. Proteins in fractions (No.31-47) representing globulins in the total protein extract were precipitated individually by adding trichloroacetic acid to a final concentration of 12%. The precipitates were analysed on 12.5% SDS-gels under reducing conditions. Products of subunit pairs 'A-J' of Fig.2.1d, representing legumin subunits, dominated the fractions 31-44 whereas vicilin and convicilin subunits were present in fractions 40-47. Stained gel strips of individual fractions were scanned densitometrically and the peak areas of acidic subunits of pairs A, B, D, F and G and the basic subunits of all the subunit pairs taken collectively were plotted to

Fig. 2.6 : Elution profile from ultrogel column chromatography of Vicia faba total proteins. Lg represents the fractions in which legumin is eluted; Vo stands for void volume.

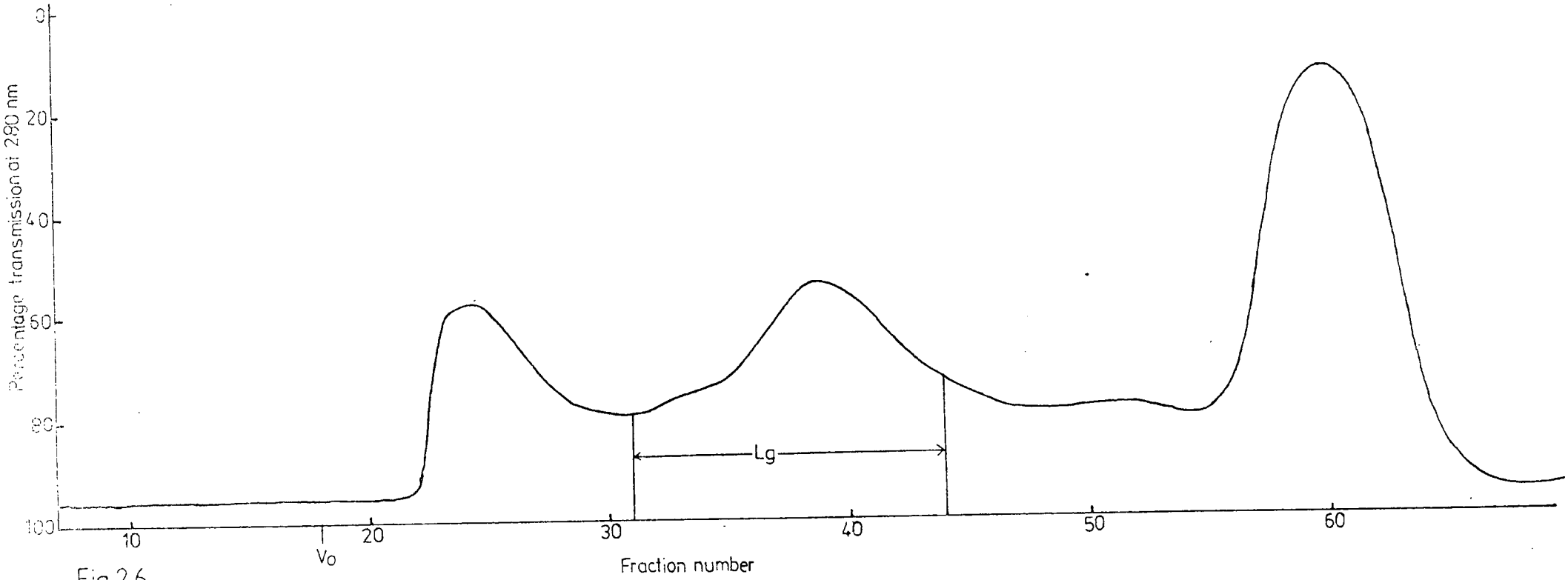

Percentage transmission at 280 nm
0
20
40
60
80
100
10
20
30
40
50
60
Vo
Lg
Fraction number

Fig.2.6

get the elution pattern relative to that of the acidic subunits of pair H/I (Fig.2.7). Fractions 32-33, representing higher mol.wt. contained significant amounts of all the subunit pairs and were due to dimerised legumin. Acidic subunit 'G' followed the distribution of conventional legumin subunits H/I in being present in higher amounts in the fractions 37 and 38. Acidic subunits A, B and D though eluting throughout the legumin fractions were concentrated relative to H/I in the faster-eluting fractions 35 and 36. Acidic subunit 'F' showed the anomalous distribution by eluting predominantly only in the fraction No. 33, between monomer and dimer peaks of H/I.

Thus all the bands 'A-J' in the globulin fraction of Vicia faba which represented disulphide bonded subunits were considered as representing legumin as they were eluted by hydroxylapatite column chromatography along with H/I, were antigenically similar and ran together under non-dissociating conditions on polyacrylamide gels and on ultrogel column.

Fig. 2.7 : Distribution of legumin subunits after gel filtration chromatography determined by densitometric scanning of stained SDS-polyacrylamide gel electrophoretic analysis of individual fractions. Elution volume increases with fraction number. Curve H/I : distribution of large acidic subunits of subunit pairs H/I. Curves A, B, D, F, G : distribution of large acidic subunits of subunit pairs A, B, D, F, G respectively relative to H/I.

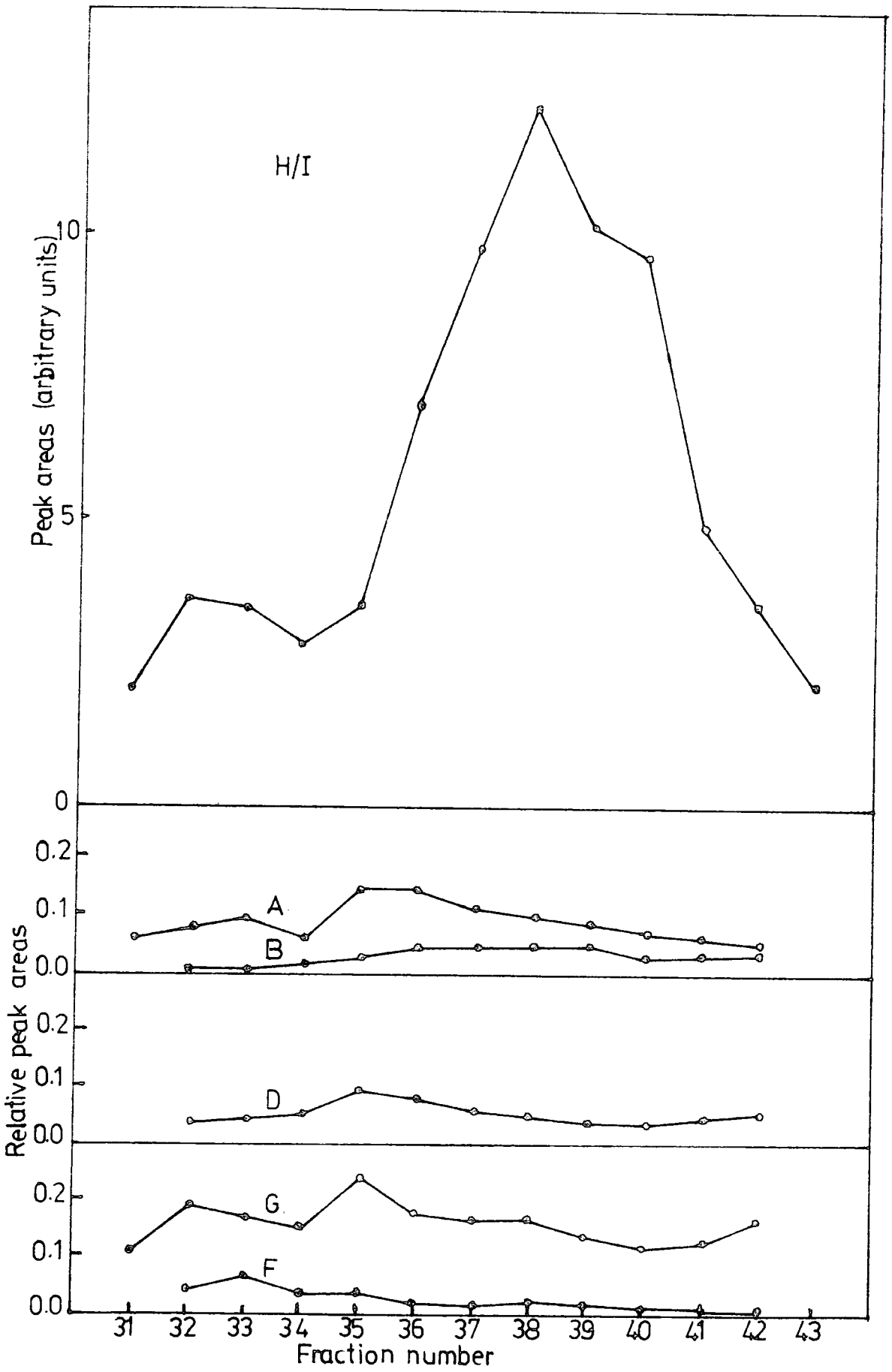

H/I
Peak areas (arbitrary units)
10
5
0
0.2
0.1
0.0
A
B
D
G
F
Relative peak areas
31
32
33
34
35
36
37
38
39
40
41
42
43
Fraction number

Fig.2.7

Part III STRUCTURE OF LEGUMIN OF PISUM SATIVUM L.

The legumin fraction of seed proteins of Pisum sativum was purified by hydroxylapatite chromatography and analysed by SDS-polyacrylamide gel electrophoresis under reducing and non-reducing conditions (Fig. 3.1a and 3.1b respectively). Under reducing conditions, mol. wts. of the components varied from 20,700 to 43,000. Bands of mol. wt. 35,000 to 43,000 represented acidic and those of mol. wt. 20,700 to 22,700 represented basic subunits of legumin (Gatehouse et al., 1980). A number of bands in mol. wt. range of 35,000 to 58,000 were observed under non-reducing conditions. Faint bands at mol. wt. 26,500 and 26,000 were also observed in the absence as well as presence of 2-mercaptoethanol and thus these bands represented the subunits that were not disulphide bonded. These subunits (mol. wt. 26,000 and 26,500) and subunit pair of mol. wt. 35,000 have previously been reported as impurities (Gatehouse et al., 1980).

A. Immunodiffusion studies

The total protein extract of pea seeds was immunoprecipitated against purified anti-legumin antibodies of Pisum sativum by double diffusion (Fig. 3.2). The sharp precipitin arc when excised and examined on SDS-gel under non-reducing conditions (Fig. 3.1c) showed subunit components similar to those of legumin purified by hydroxylapatite column chromatography. Hence all

Fig. 3.1 : Polyacrylamide gel electrophoresis of pea seed proteins under dissociating conditions (tracks 'a-d') and under non-dissociating conditions (track 'e'). Tracks 'a' and 'b' : purified legumin under reducing and non-reducing conditions respectively; track 'c' : immunoprecipitated legumin under non-reducing conditions; track 'd' : total protein extract under non-reducing conditions; track 'e' : purified legumin. A, B, C and D represent legumin molecular forms and impurities as defined in the text; IgG stands for immuno-globulins.

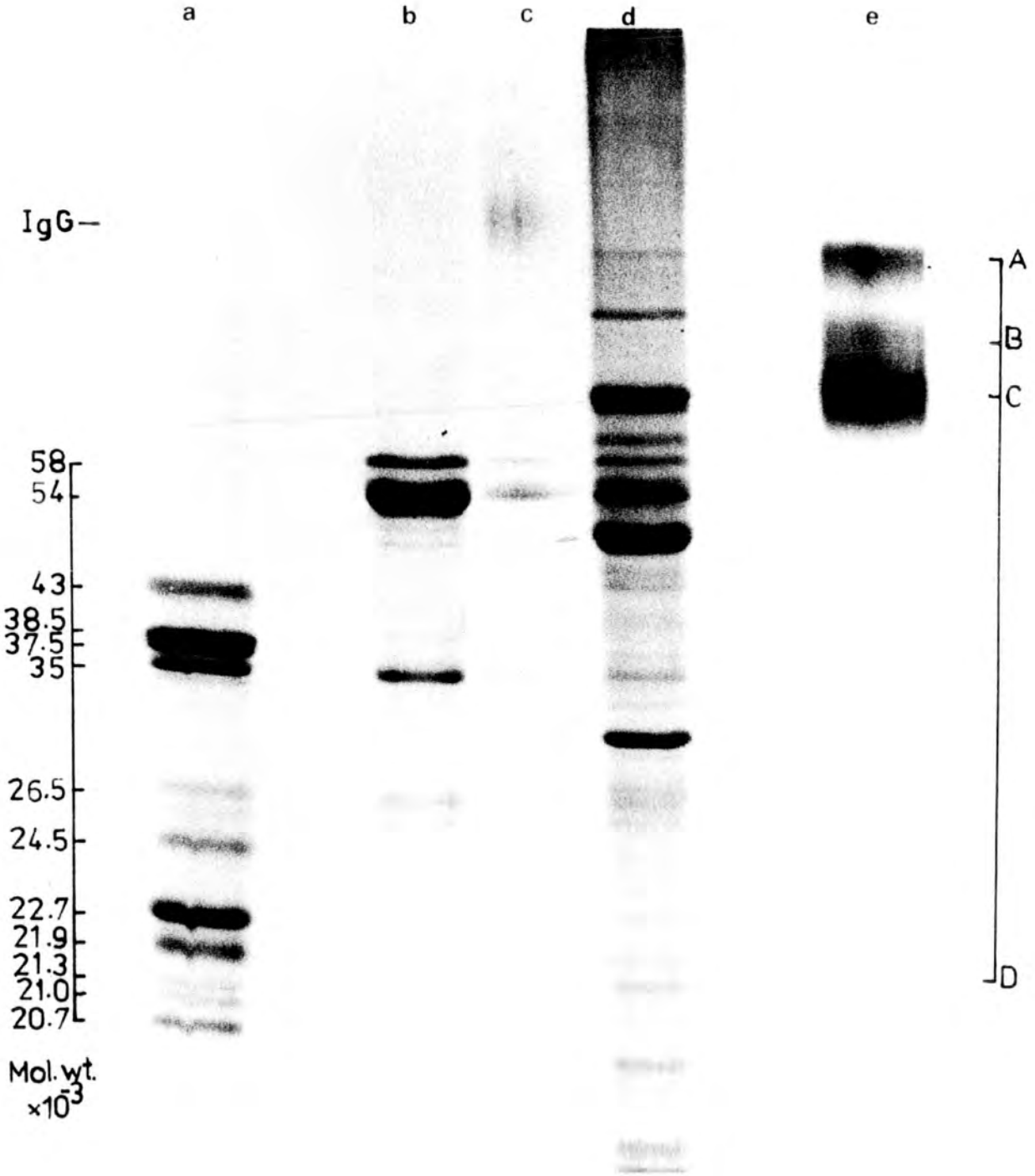

a
b
c
d
e
IgG—
A
B
C
D
58
54
43
38.5
37.5
35
26.5
24.5
22.7
21.9
21.3
21.0
20.7
Mol. wt.
×10⁻³

Fig. 3.1

Fig. 3.2 : Ouchterlony double-diffusion experiment. (a) - Outer wells : 1, 4 Pisum sativum total protein extract; 2, 5 Pisum sativum legumin; 3, 6 Pisum sativum vicilin (b) - Outer wells : 1 - 6 Pisum sativum total protein extract. (a) and (b) - inner wells : affinity purified anti-Pisum legumin IgG.

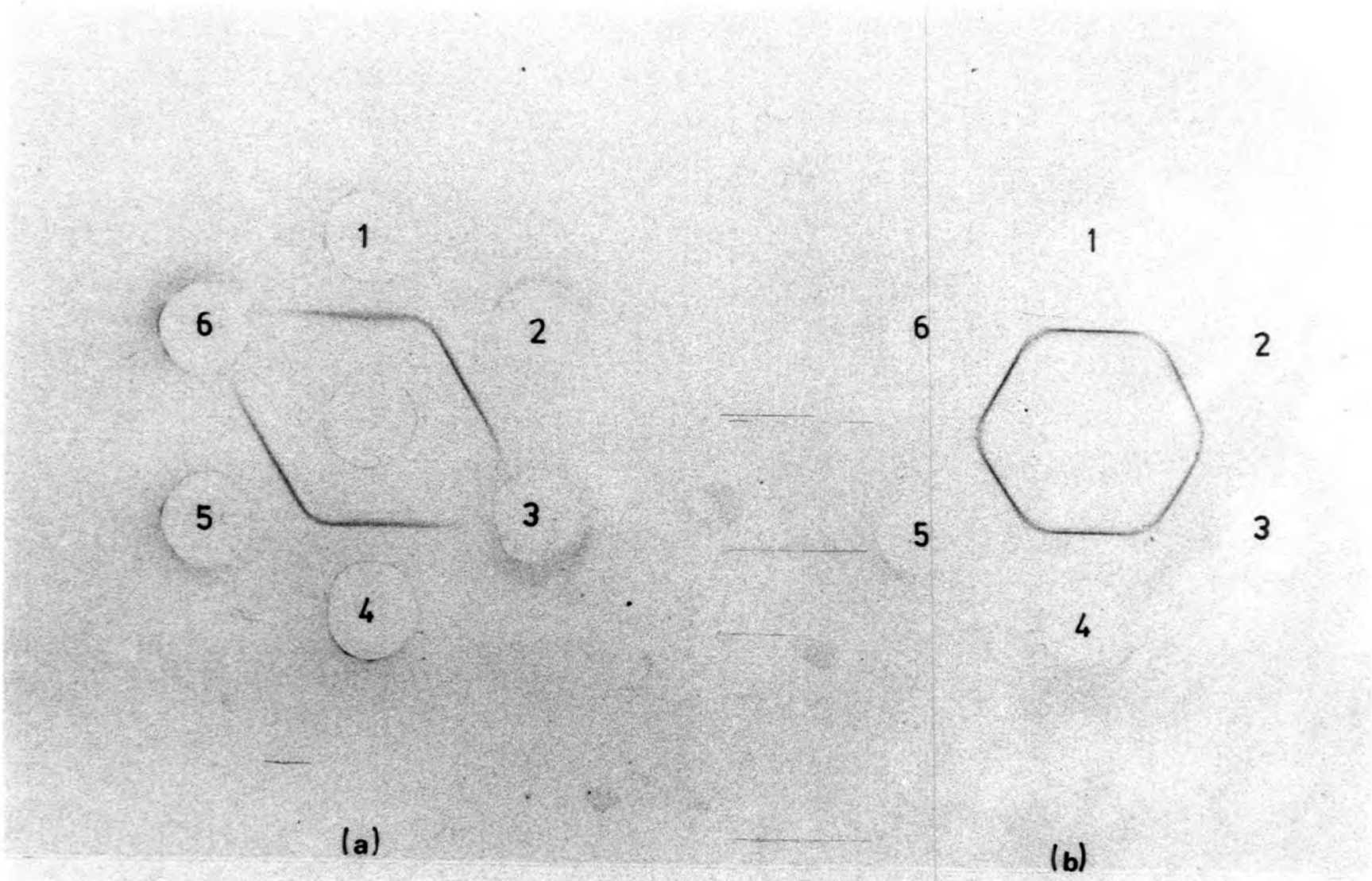


Fig. 3.2

these subunit pairs and their products were considered as representing different constituents of legumin.

B . Composition of subunit pairs

Two dimensional SDS-polyacrylamide gel electrophoresis of legumin separating subunit pairs under non-reducing conditions in the first dimension followed by separation of respective subunits of each pair after reduction by 2-mercaptoethanol in the second dimension was employed to understand the relationship of subunit pairs to their respective subunits (Fig. 3.3). The following subunit pairs (mol. wt. in brackets) were deduced :

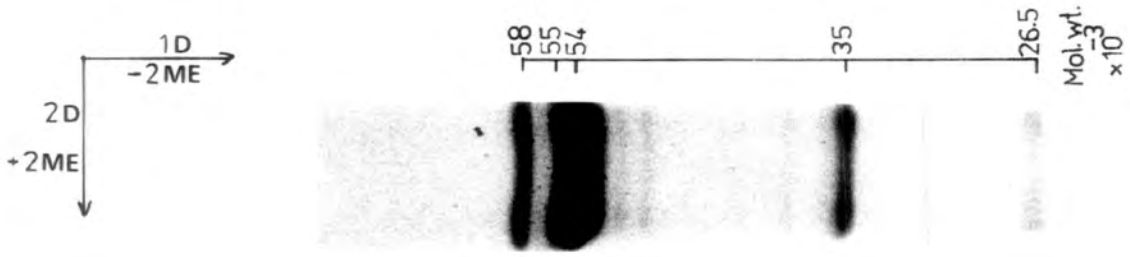
1. (58,000) = (43,000) + (21,300)
2. (58,000) = (43,000) + (21,900)
3. (55,000) = (35,000) + (21,900)
4. (54,000) = (38,500, 37,500 and 35,000) + (22,700)
5. (35,000) = (24,500) + (21,000 and 20,700)

The subunits lacking disulphide bonds ran to the same mol. wt. in both the dimensions.

C . Composition of legumin molecules

Legumin when analysed under non-dissociating conditions on 7.5% polyacrylamide gels, showed separation into a number of components as shown in Fig. 3.1e. A number of faint bands of very high mobility (0.8-0.86) and three incompletely separated bands 'A-C' of fairly low mobility (0.27-0.37) were obtained (Table 3.1).

Fig. 3.3 : Two dimensional SDS-polyacrylamide gel electrophoresis of Pisum legumin.
1D : analysis under non-reducing conditions in the first dimension; band pattern given as track (i).
2D : analysis under reducing conditions in the second dimension; band pattern given as track (ii).
1, 2, 3, 4 and 5 represent components of subunit pairs as defined in the text.


1D
−2ME
2D
+2ME
58
55
54
35
26.5
Mol. wt.
−3
×10

(i)

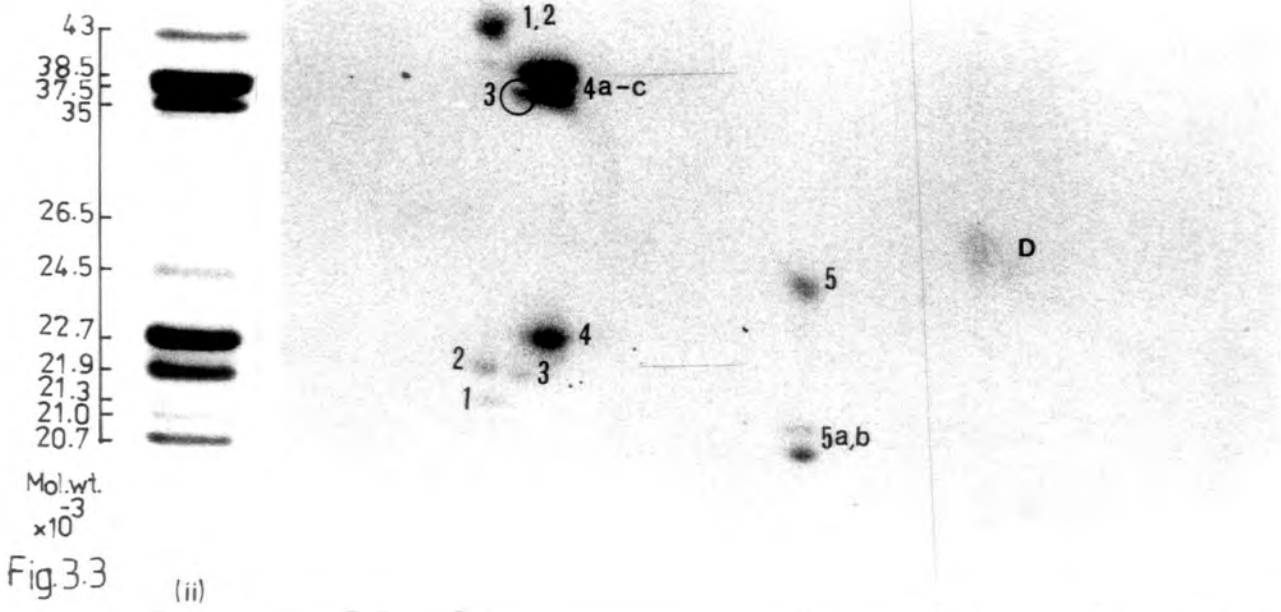

43
38.5
37.5
35
26.5
24.5
22.7
21.9
21.3
21.0
20.7
Mol.wt.
×10-3
1,2
3
4a-c
D
5
4
2
3
1
5a,b

Fig. 3.3 (ii)

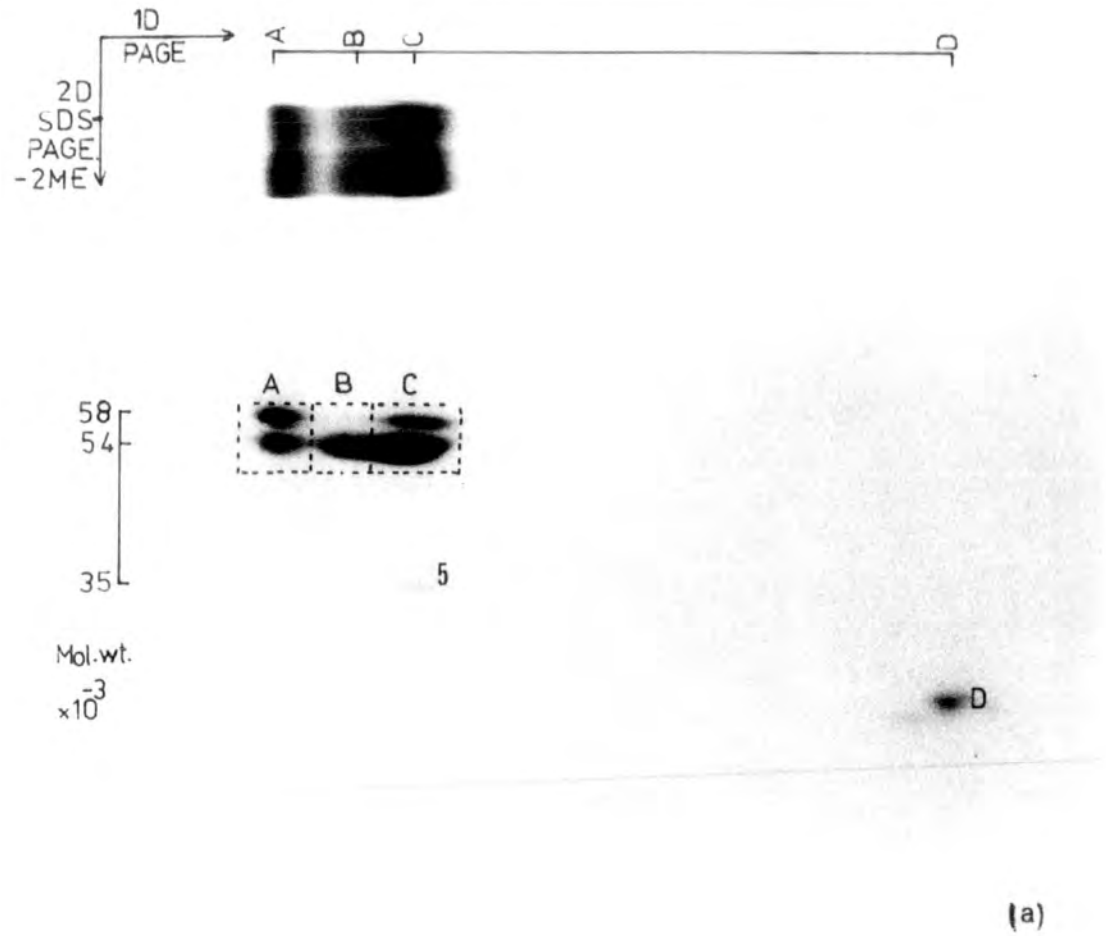
Table 3.1 Composition of molecular species

S.No	Band (Fig 3.1e)	Relative Mobility	Percentage	Subunit pairs and percentage ( )
1	A	0.27	23	1 : 4 : 5 (45) (52) (3)
2	B	0.32	20	2 : 3 : 4 : 5 (4) (4) (90) (2)
3	C	0.37	47	2 : 3 : 4 : 5 (15) (32) (47) (6)
4	D	0.8-0.86	10	Not legumin subunits (impurity)

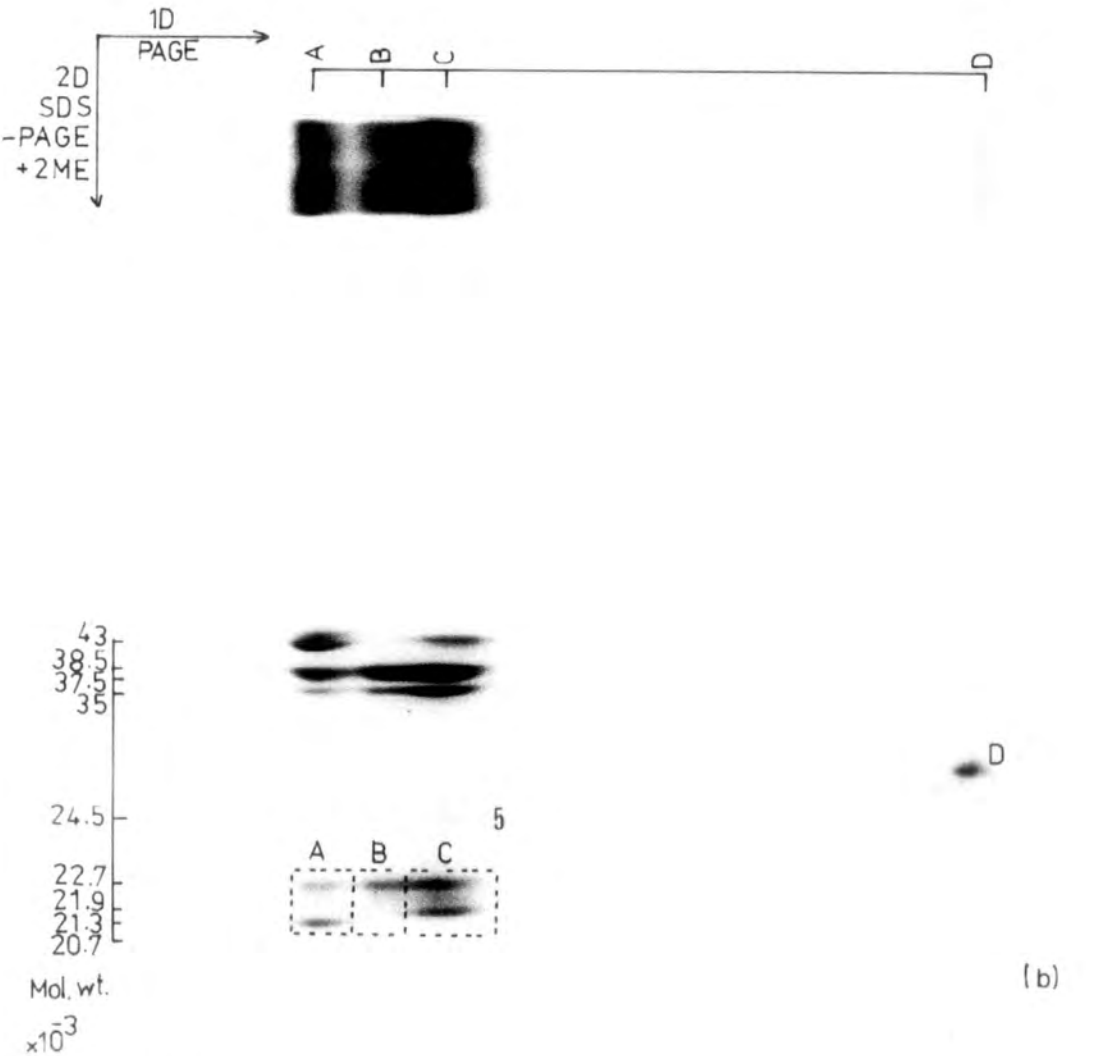
Densitometric measurements of these stained bands showed that legumin form represented by the band 'C' constituted about half of the total legumin.

The subunit composition of these bands 'A-D' was established by running the separated bands in second dimension in the absence and presence of 2-mercaptoethanol (Fig. 3.4a and 3.4b respectively). This analysis clearly showed that bands of high mobility (D) contained only the polypeptides of mol. wt. 26,500 and 26,000 demonstrating thereby that these polypeptides belong to a protein impurity in the legumin fraction. The remaining bands of lower mobility (A-C) contained subunit pairs and their respective subunits in different combinations as detailed in Table 3.1. Whereas, the subunit pair of mol. wt. 54,000 was common to all the bands, the subunit pair of mol. wt. 58,000 was present

Fig. 3.4 : Two dimensional gel electrophoresis of purified Pisum legumin. 1D : (a) and (b) - polyacrylamide gel electrophoresis under non-dissociating conditions in the first dimension, see text for A, B, C and D.
2D : SDS-polyacrylamide gel electrophoresis in the second dimension.
(a) : under non-reducing conditions. Boxed areas excised for 'third dimension' analysis shown in Fig. 3.5.(b) : under reducing conditions. Boxed areas excised for 'third dimension' analysis shown in Fig. 3.6.


1D
PAGE
A
B
C
D
2D
SDS
PAGE
-2ME
A
B
C
58
54
35
5
Mol.wt.
×10⁻³
D

(a)


1D
PAGE
A
B
C
D
2D
SDS
-PAGE
+2ME
43
38.5
37.5
35
24.5
22.7
21.9
21.3
20.7
5
A
B
C
D
Mol. wt.
×10⁻³

(b)

Fig. 3.4

predominantly in bands A and C and those of mol. wt. 55,000 and 35,000 in band C only. Traces of polypeptides of mol. wt. 26,500 and 26,000 were also found in band C. The constitution of the three bands became clearer when run under reducing conditions. The subunit pair of mol. wt. 58,000 which appeared to be common to A and C, was actually represented by two different types which differed in their subunit composition. Thus, the 58,000 pair had subunits of mol. wt. 43,000 + 21,300 and 43,000 + 21,900 in band A and C respectively.

Assignment of the subunit pairs to molecular forms of legumin A, B and C obtained on non-dissociating gels was confirmed by taking the subunit pairs obtained after second dimension under non-reducing conditions and running these into a 'third dimension' SDS-gel under reducing conditions. Thus, this analysis made apparent the presence of an additional subunit pair (mol. wt. 55,000) in form C. It consisted of a large subunit of mol. wt. 35,000 and a small subunit of mol. wt. 21,900 (Fig. 3.5).

D. Isoelectric points of the legumin subunits

Isoelectric focusing (IEF) of the acidic and basic subunits of legumin subunit pairs was carried out by second dimension IEF after separation of the subunits by SDS-polyacrylamide gel electrophoresis under reducing conditions in the first dimension. The observed band patterns were then compared with band patterns obtained

Fig. 3.5 : 'Third dimension' SDS-polyacrylamide gel electrophoresis (under reducing conditions) of legumin molecular forms A, B and C separated by two dimensional gel analysis shown in Fig. 3.4a. 1, 2, 3, 4 represent components of subunit pairs as defined in the text.

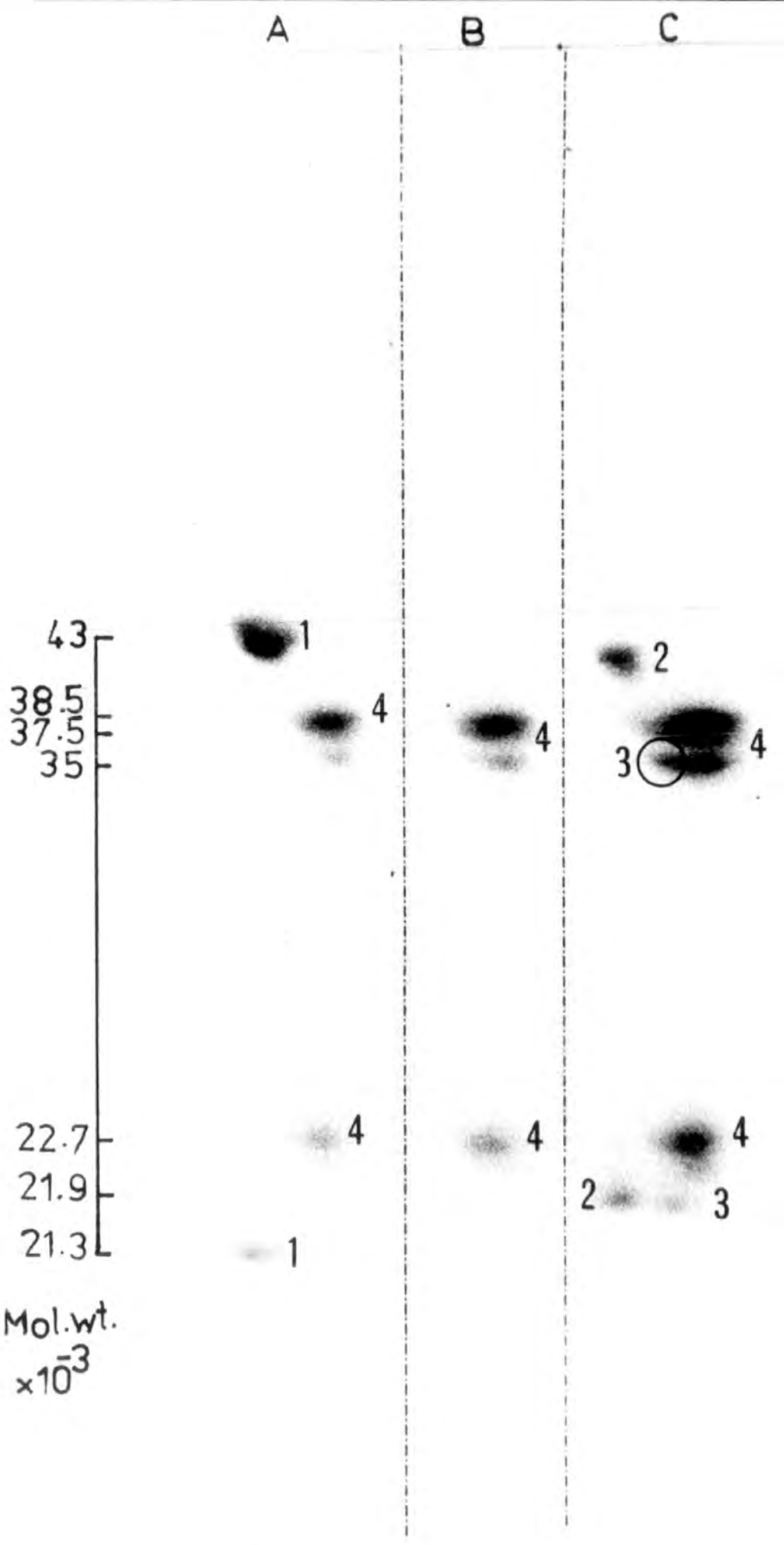


Fig.3.5

by isoelectric focusing in the third dimension of component subunits of molecular forms separated in two dimensions by electrophoreses under non-dissociating and dissociating conditions in the presence of 2-mercaptoethanol (Fig. 3.6). Table 3.2 lists the isoelectric points of the subunits. In all subunit pairs, the large subunits had lower pIs i.e. were more acidic. Most acidic subunits fall in the pI range 5.0-5.8 (excepting those belonging to subunit pair 2 and subunit pair 5) and most basic subunits fall in the pI range 7.4-8.0. The subunit pair 1 had pIs of its basic subunits below 7 (6.2-6.4). Twenty two separate acidic subunits and 11 separate basic subunits were identified.

Fig. 3.6 : 'Second' and 'third dimension' gel analysis by isoelectric focusing of legumin basic subunits separated by SDS-PAGE under reducing conditions (for 'second' dimension) and by two dimensional gel analysis as shown in Fig. 3.4b (for 'third' dimension). 1, 2, 3, 4, 5 represent basic subunits as defined in Table 3.2. 2 and 3 in form B are due to its incomplete resolution from C.

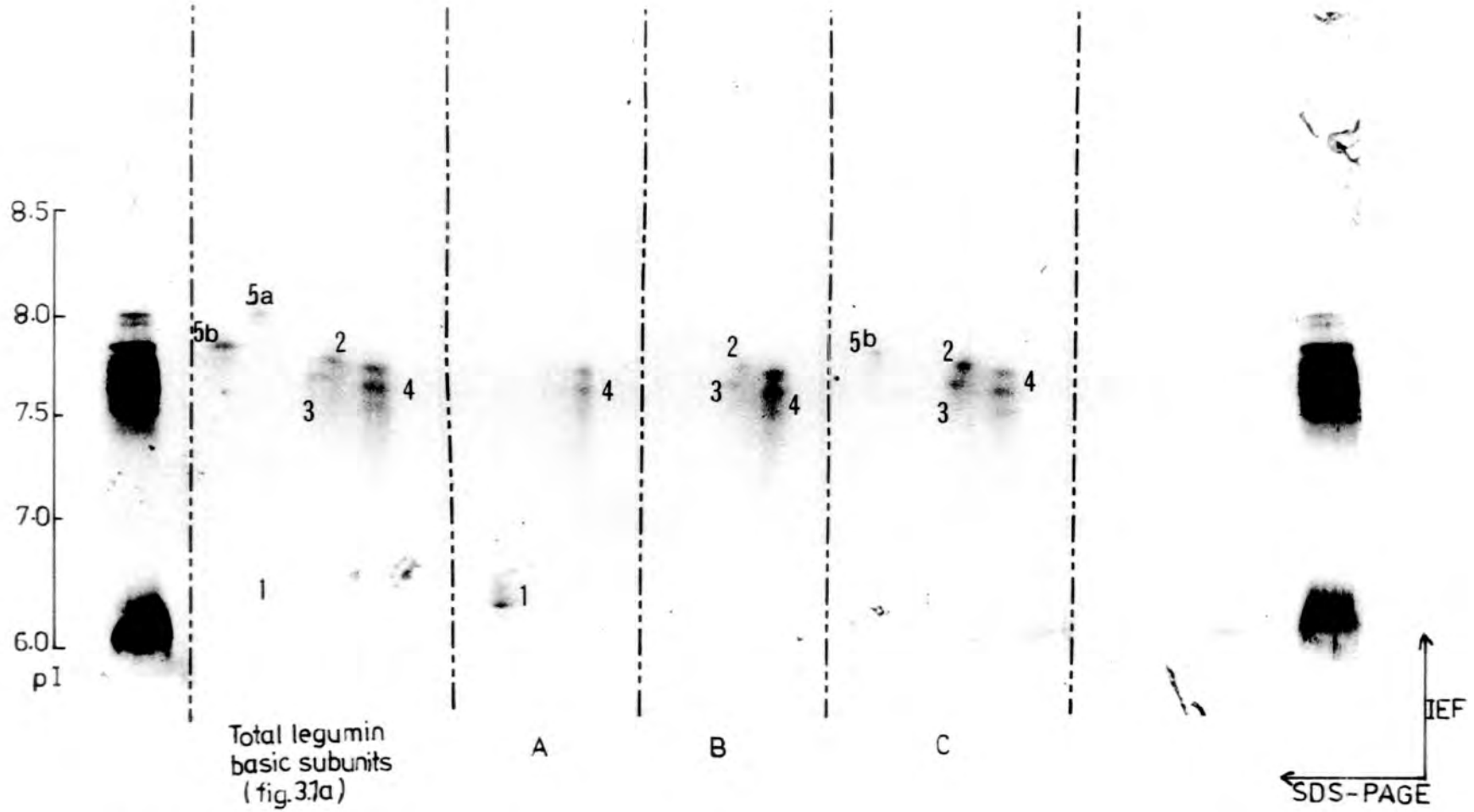

8.5
8.0
7.5
7.0
6.0
pI
5a
5b
2
3
4
1
Total legumin
basic subunits
(fig. 3.1a)
A
B
C
IEF
SDS-PAGE

Fig. 3.6

Table 3.2 : Properties of subunit pairs of legumin

Subunit pair	Mol. wt.	Acidic subunits			Basic subunits		
			Mol. wt.	pI range ( )*		Mol. wt.	pI range ( )*
1.	58,000		43,000	5.48-5.76 (6)		21,300	6.22-6.35 (2)
2.	58,000		43,000	4.85-4.95 (2)		21,900	7.69 (1)
3.	55,000		35,000	5.1 -5.22 (2)		21,900	7.45-7.55 (2)
4.	54,000	a	38,500	5.25-5.52 (4)		22,700	7.4 -7.62 (3)
		b	37,500	5.05-5.18 (2)			
		c	35,000	5.28-5.55 (4)			
5.	35,000		24,500	5.95-6.15 (2)	a	21,000	7.92-8.0 (2)
					b	20,700	7.8 (1)

* Values within brackets denote the number of subunits

E. Molecular weight of the molecular forms

a) Polyacrylamide gradient gel electrophoresis

Legumin was run in the first dimension on 5-15% polyacrylamide gradient gel for 70h at a constant voltage of 100V. A band of relative mobility 0.425 (mol. wt. 420,000) and a group of bands of relative mobilities 0.529-0.571 (mol. wt. 310-270,000) were observed. An analysis of these bands in the second dimension on SDS-polyacrylamide gels showed that the bands of mol. wt. 420,000 corresponded to form A in having subunit pairs 1 and 4 and the lower group of bands contained the remaining subunit pairs 2, 3, 4 and 5 (Form C). Impurities of mol. wt. 26,500 and 26,000 were present in forms having mol. wt. in this lower group of bands.

b) Ultrogel column chromatography

Analysis of the mol. wt. distribution of legumin molecules carried out by gel filtration showed that most of the legumin eluted with a mean mol. wt. of 400,000 ($\pm$40,000) i.e. similar to that previously reported (Croy et al., 1979). However, subunits characteristic of legumin molecular form A were distributed about a significantly higher mol. wt. (450,000 $\pm$ 40,000). No other separation of legumin molecular forms was observed.

F. Fractionation of legumin molecular forms

The occurrence of legumin in three forms with the subunit composition already observed was also confirmed by ion-exchange chromatography of legumin on DEAE-cellulose column. Legumin was eluted in a broad peak and the fractions pooled in four parts were analysed by PAGE and SDS-PAGE. Starting fractions of the peak (elution molarity 0.16-0.18) contained forms A and B (Fig. 3.7); the middle fractions (elution molarity 0.19-0.23) contained A, B and C forms, form A decreasing and C increasing in concentration; and the later fractions (elution molarity 0.23-0.26) contained no subunits characteristic of form A but contained forms B and C. The impurity proteins in legumin (mol. wt. 26,500 and 26,000) also eluted with the later legumin fractions.

G. Legumin and storage effect

a) Molecular weights

In the recently harvested seeds of a number of pea varieties, legumin basic subunits on SDS-polyacrylamide gels were seen to have a pattern different to that in the old seeds. An additional band of mol. wt. 22,200 was observed in the new seeds. To study this observation in detail, a line 'Meteor" was selected. The normal 'Meteor' legumin had a pattern of acidic and basic subunits similar to that described for 'Feltham First' except that it had an extra acidic subunit of

Fig. 3.7 : Two dimensional gel analysis of Pisum legumin fractions from ion-exchange chromatography. 1D : polyacrylamide gel electrophoresis of the initial fractions (1), middle fractions (2, 3) and last fractions (4) of the legumin peak. 2D : SDS-polyacrylamide gel electrophoresis of A, B, C bands from 1D of 1, 2, 3 and 4. A, B, C represent legumin forms separated in 1D and various components of these forms as defined in the text. Form C is absent in the initial fractions (1) and form A is absent in the last fractions (4).

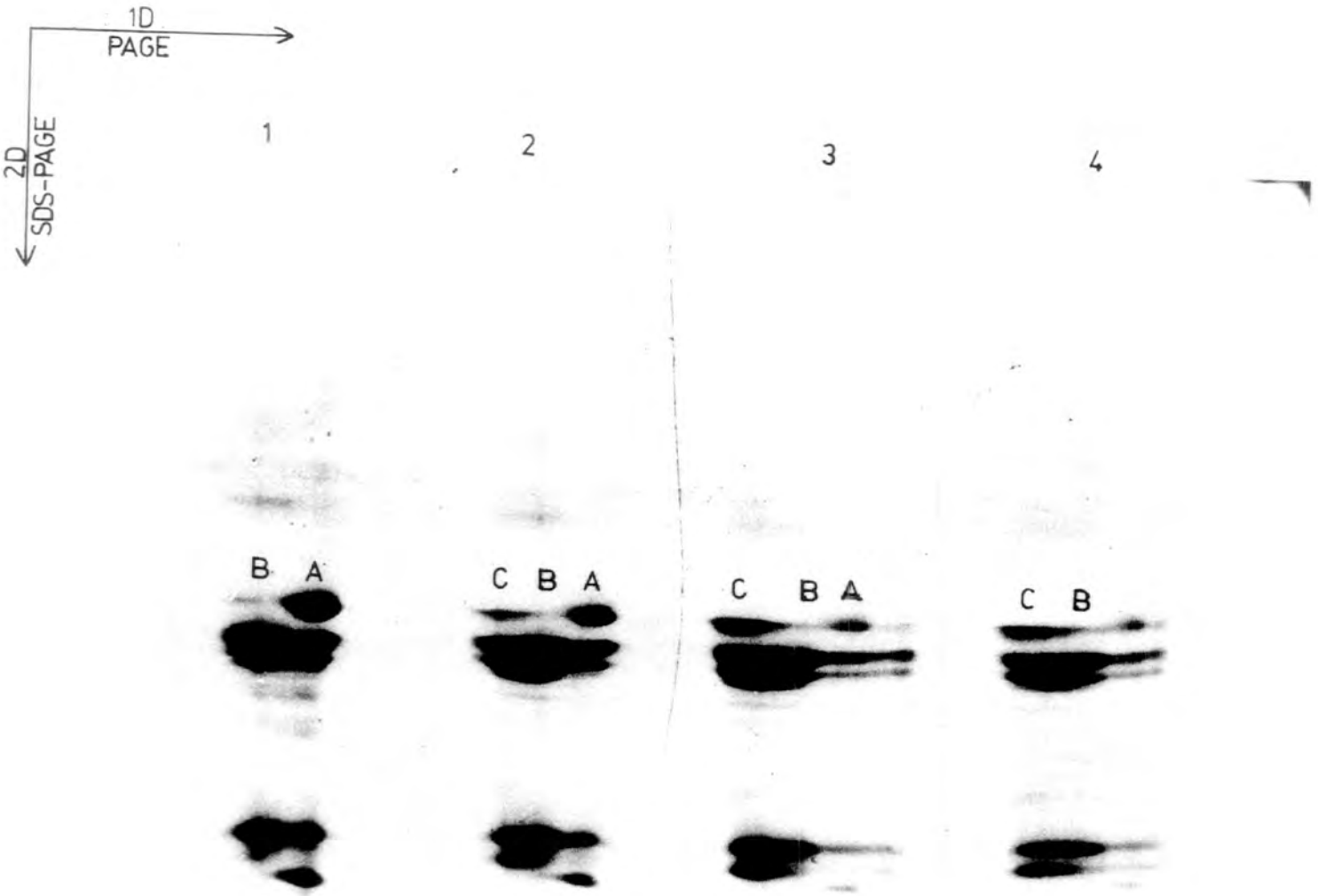


Fig.3.7

mol. wt. 40,000. A pod with dry mature seeds was harvested. Two seeds were frozen in liquid nitrogen immediately after harvest and one of these finely pulverized. Total protein extracts from these single seeds were prepared on the day of harvest, after a period of 2 weeks, 4 weeks and 6 weeks. An analysis of these extracts on SDS-polyacrylamide gel (Fig. 3.8) showed that the major basic subunit was represented by a dark band of mol. wt. 22,200 (white arrow) on the day of harvest (zero day of storage). A new band of mol. wt. 22,700 (black arrow) appeared in the 2 week old seeds. The concentration of this band increased and that of mol. wt. 22,200 decreased relatively with increase in storage period (4 and 6 week old seeds). This effect was more pronounced in eight months old seeds. In two year old seeds, basic subunit was represented by the band of mol. wt. 22,700 only and that of 22,200 had completely disappeared. Thus, mol. wt. 22,200 of the basic subunit appeared to be shifting to 22,700.

The basic subunit of mol. wt. 22,200 behaved in a similar way in the legumin purified by hydroxylapatite column chromatography of 6 week , 8 month and 2 year old seeds (Fig. 3.8). When run on non-dissociating polyacrylamide gel, legumin of the two year old seeds showed slight decrease in mobility as compared to 6 weeks old seeds. Also an alteration in the elution behaviour of legumin was observed on hydroxylapatite

Fig. 3.8 : SDS-polyacrylamide gel electrophoresis of total protein extract and purified legumin from seeds of pea variety 'Meteor' stored for various periods. White arrows indicate legumin basic subunit band (mol. wt. 22,200) decreasing in amount and black arrows indicate the band (mol. wt. 22,700) increasing in amount with storage.

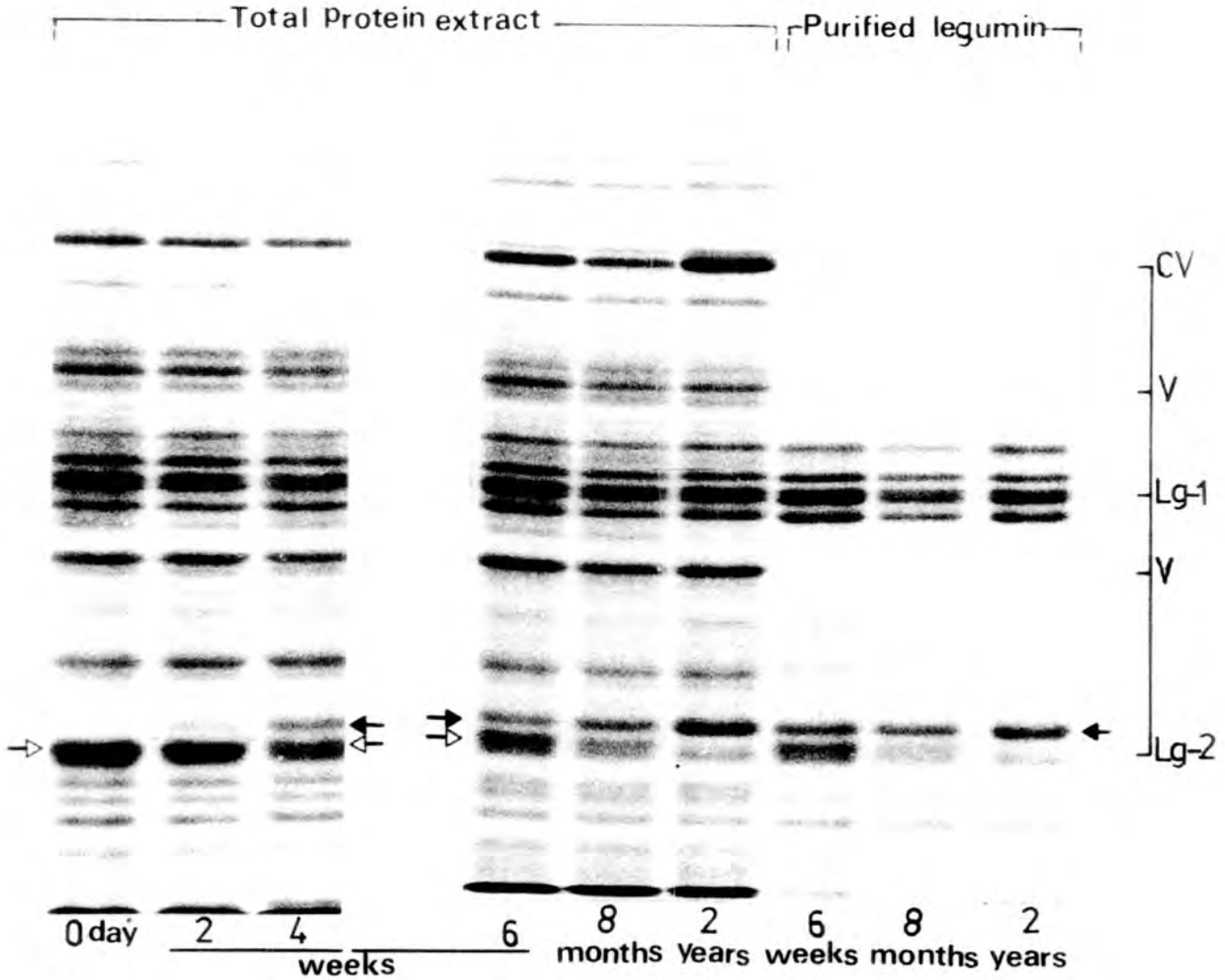

Total Protein extract
Purified legumin
CV
V
Lg-1
V
Lg-2
0 day
2
4
weeks
6
8
months
2
years
6
weeks
8
months
2
years

Fig. 3.8

column chromatography whereby legumin of the older seeds was eluted comparatively at higher molar concentration.

b) Isoelectric points

A two dimensional gel electrophoresis of the basic subunits separated on an SDS-gel in the first dimension and the isoelectric focusing in the second dimension showed that isoelectric points of all the basic subunit bands except those of mol. wt. 22,200 remained the same (Fig. 3.9). The 22,200 mol. wt. band consisted of three subunits and with the shift of this band to 22,700, isoelectric points of all the three subunits fell by 0.05 units each.

There is a possibility that this change in pIs is causing a shift in the basic subunit band on SDS-gel as these gels have been seen to give separation on the basis of charge as well.

Fig. 3.9 : Two dimensional electrophoretic analysis and isoelectric focusing of legumin basic subunits of 6 week (a), 8 month (b) and 2 year (c) old seeds of pea variety 'Meteor'. 1D, IEF : isoelectric focusing in the first dimension; a′ is the subunit pattern redrawn from a. 1D, SDS-PAGE (SDS-polyacrylamide gel electrophoresis in the first dimension) of a, b and c was followed by 2D, IEF (isoelectric focusing in the second dimension). White arrows indicate subunits decreasing in amount with storage; black arrows indicate subunits increasing in amount with storage.

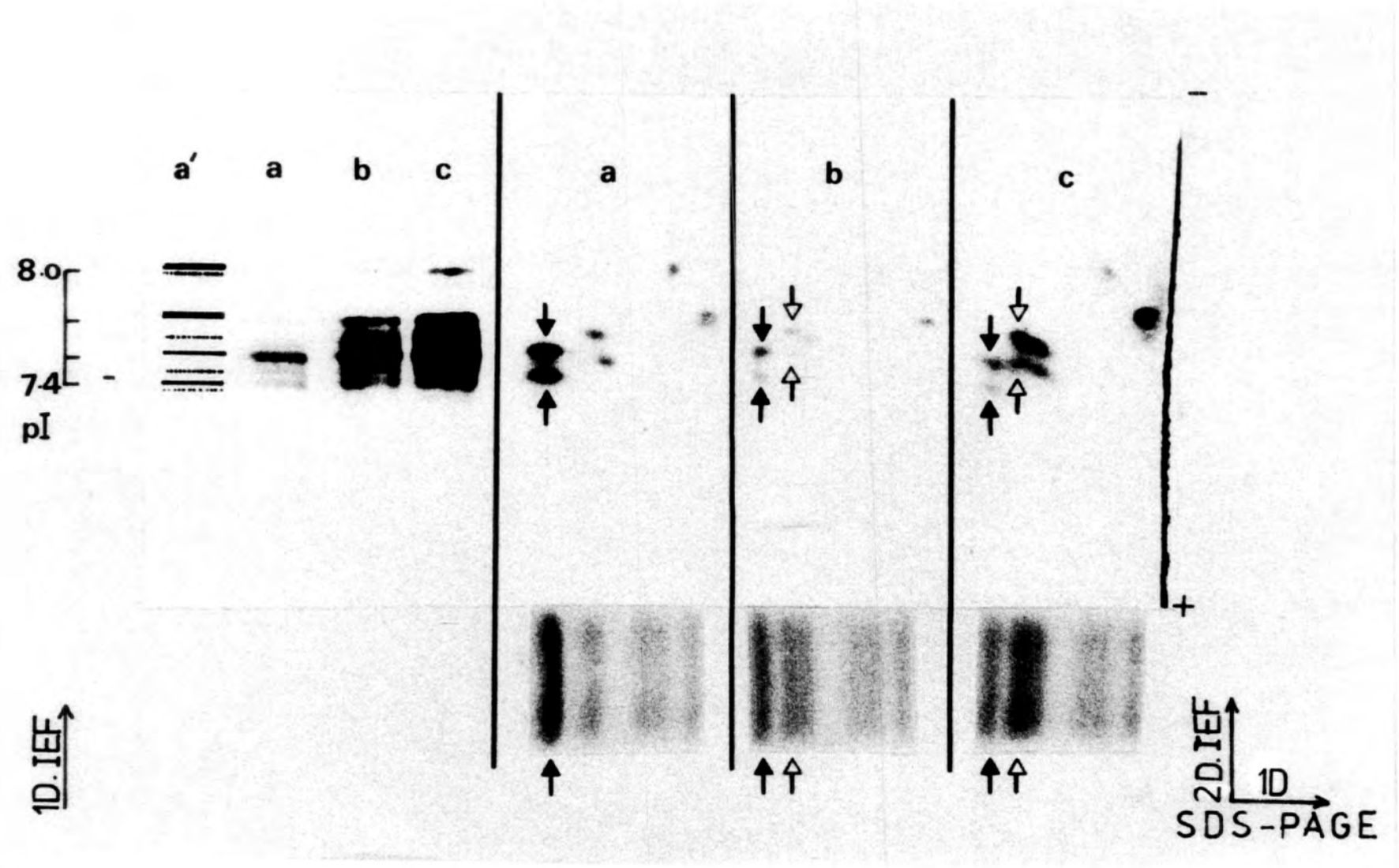


Fig. 3.9

Part IV. GENETICS OF LEGUMIN SUBUNITS IN PISUM SATIVUM L.

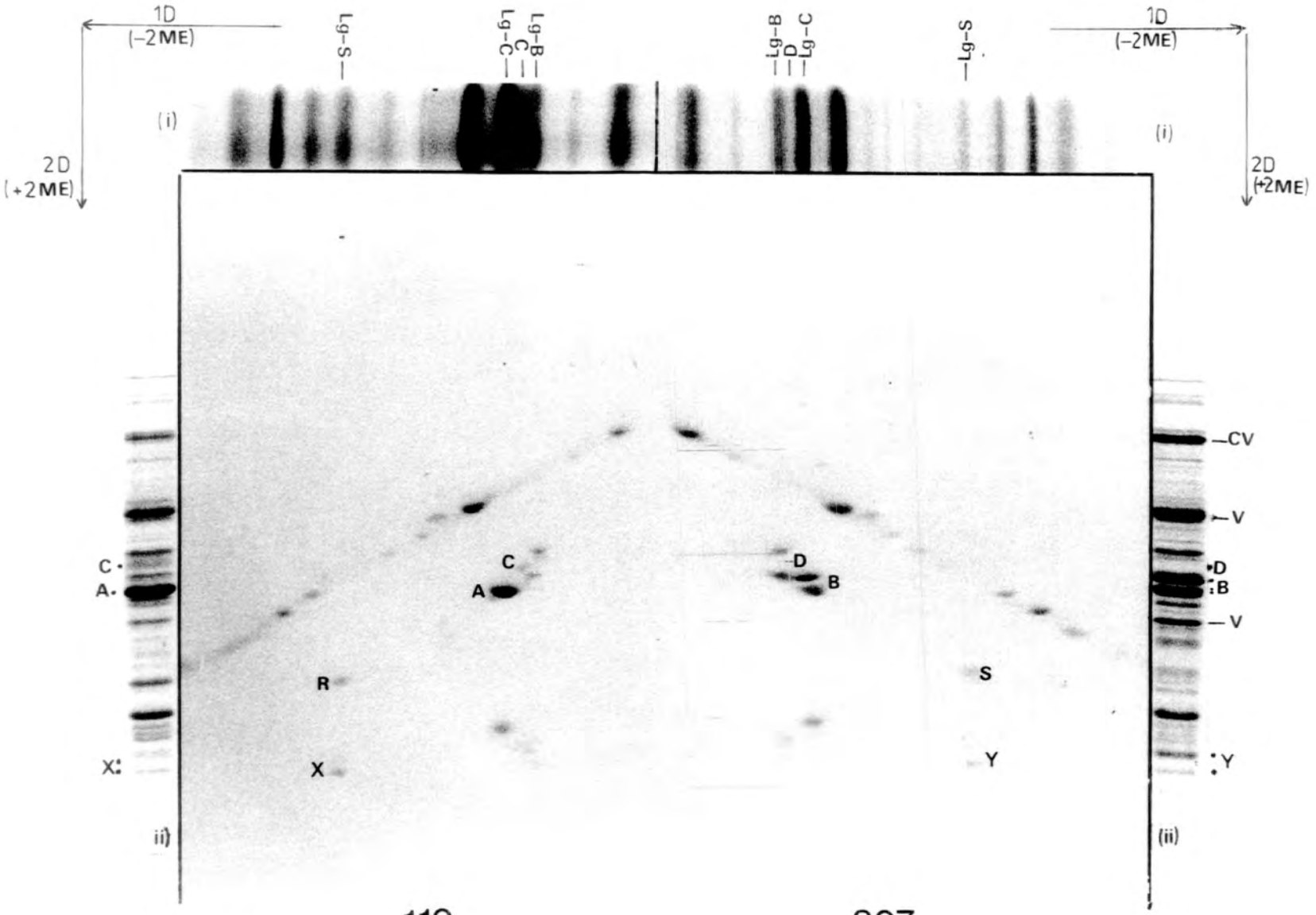
A number of Pisum lines were examined for legumin subunit variation and four lines namely 110, 807, 1238 and 1263 were selected. Appropriate crosses were made to study the inheritance of legumin subunits of various types classified (see Materials and Methods section) as conventional, big and small legumin. F_2 plants of the cross 110 x 807 were analysed on 12.5% SDS-polyacrylamide gels in order to study the inheritance of these subunits and the linkage relationships of the genes controlling them with respect to each other. The cross between lines 1238 and 1263 was made to study the location of the gene controlling acidic subunits of conventional legumin (Lg-1C) in relation to convicilin gene and the morphological markers selected for different linkage groups.

A. Cross 110 x 807

a) Legumin banding patterns

Total protein extracts of single seeds of the two Pisum lines 110 and 807 when examined on 12.5% SDS-polyacrylamide gels under reducing conditions differed in their legumin banding patterns. The bands in the total protein extract were identified by running the extracts under non-reducing conditions in the first dimension followed by reducing conditions in the second (Fig.4.1). Subunits obtained in the second dimension

Fig. 4.1 : Two dimensional SDS-polyacrylamide gel electrophoresis of total protein extracts of Pisum lines 110 and 807. 1D : electrophoresis under non-reducing conditions in 10% gels in the first dimension; band patterns shown in tracks (i). 2D : electrophoresis under reducing conditions in the second dimension; band patterns shown in tracks (ii). A,B, C,D, R,S, X,Y, Lg-B, Lg-C and Lg-S represent various legumin species as defined in the text. CV and V stand for convicilin and vicilin respectively.



110 807

Fig. 41

and their mol. wt. were found as follows:

Subunit type	Pisum line 110		Pisum line 807	
	coded as	mol.wt.	coded as	mol. wt.
Lg-1B	1	43,000	1	43,000
Lg-1B	C	40,000	D	(absent)
Lg-1B	2	38,000	2	38,000
		37,500		
Lg-1C	A	35,500	B	37,500
				35,500
				35,000
Lg-1S	R	24,500	S	25,000
				24,500
Lg-2C	3	22,700	3	22,700
Lg-2B	4	22,300-21,300	4	22,300-21,900
Lg-2S	X	21,000	Y	21,300
		20,700		20,700

b) Inheritance of legumin subunits

Legumin subunits 1-1, C-D, 2-2, A-B, R-S, 3-3, 4-4 amd X-Y in the lines 110 and 807 respectively, were assumed to be synthesized by alleles of the same gene since these subunits had similar mol. wts. in the two lines and were reduction products of similar subunit pairs. As the two lines differed in the pattern of a number of subunits only, inheritance of A and B, C and D, R and S and X and Y only was studied.

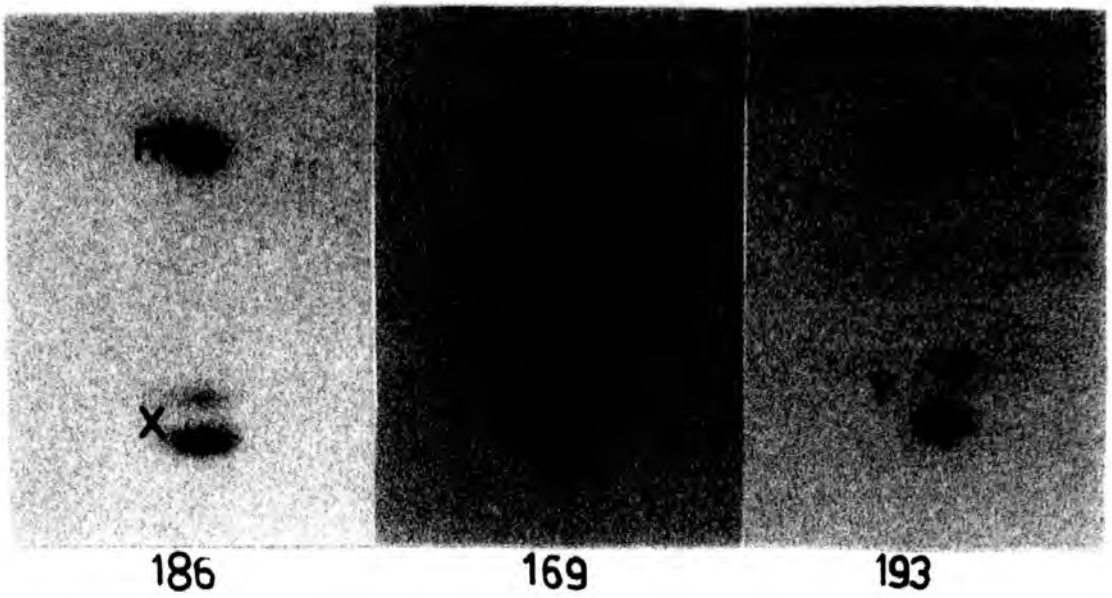
(i) Products of subunit pair Lg-C

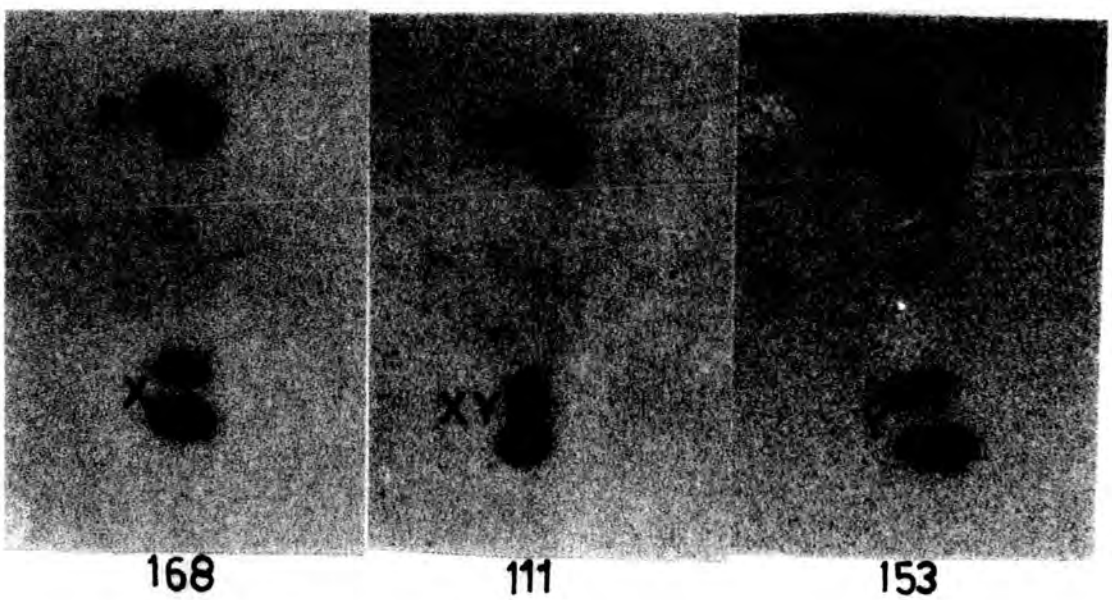
This pair represents the conventional legumin subunit pair (mol. wt. 53,000) which on reduction gives rise to two groups of subunits. The acidic subunits in line 110 consisted of only one dark band of mol. wt. 35,500 (Pattern A) but those of line 807 gave three bands of mol. wt. 37,500, 35,500 and 35,000 (Pattern B). This is illustrated in Fig. 4.1 from two-dimensional SDS-polyacrylamide gel electrophoresis. Electrofocusing studies followed by SDS-polyacrylamide gel electrophoresis showed that the band in line 110 actually consisted of two subunits and 3 bands of line 807 consisted of one subunit each. One hundred and seventy plants were analysed (Fig. 4.3) to calculate the chi-square values (Table 4.1). Chi-square value for Lg-1C subunit ($\chi^2_{(2)} = 1.2$) was found to be statistically insignificant and was in good agreement with the hypothesis that these subunits are controlled by a single pair of co-dominant genes.

Table 4.1 : Analysis of F_{2_n} segregation of various legumin subunit patterns and their chi-square values

Subunit	Frequency of the segregating patterns in F_2 generation			χ^2 (n=2)
Lg-1C	A	AB	B	
Observed	48	84	38	1.2
Expected	42.5	85	42.5	
Lg-2S	X	XY	Y	
Observed	43	92	35	1.9
Expected	42.5	85	42.5	
Lg-1B	C	CD	D	
Observed	15	20	17	2.92
Expected	13	26	13	
Lg-1S	R	RS	S	
Observed	10	26	16	1.38
Expected	13	26	13	

Fig. 4.2 : Cut-out sectors from SDS-polyacrylamide gel electrophoresis in the second dimension under reducing conditions (first dimension being under non-reducing conditions) showing different combinations of independently inherited subunit patterns R,S and X,Y (Lg-1S and Lg-2S respectively) in the F_2 plants (No. 186, 169,----177) of the cross between Pisum lines 110 and 807.





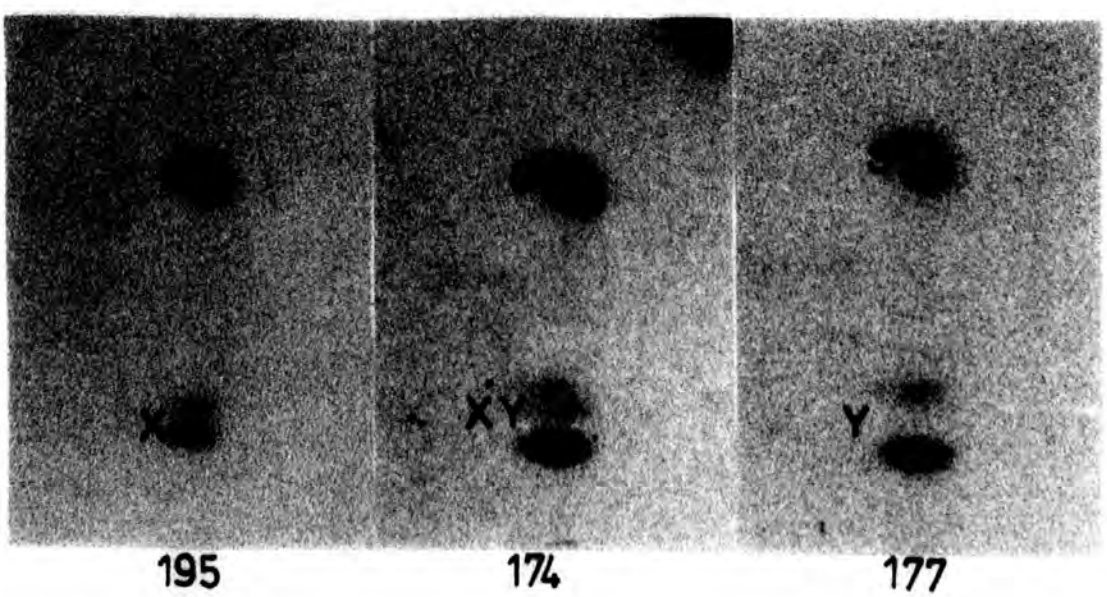


Fig.4.2

(ii) Products of subunit pair Lg-B

This subunit pair represents the legumin of mol. wt. higher than that of conventional legumin - hence the name Lg-B (Big legumin). A band of mol. wt. 55,000 was present in the line 110 (Pattern C) and absent in the line 807 (Pattern D) under non-reducing conditions. Two dimensional electrophoresis showed that this subunit pair on reduction gave rise to an Lg-1B subunit of mol. wt. 40,000 and Lg-2B subunit of mol. wt. 22,000 of which only the acidic subunit was clearly analysed and studied. The observed variation in the amount of protein present was ascribed to a dosage effect,i.e. depended on the number of genes. Thus, the pattern 'C', a dark spot, was assumed to be due to a pair of homozygous alleles of the same gene of the line 110, 'CD' - a fainter spot of half the intensity of 'C' due to a single allele contributed by line 110 in the heterozygote and 'D' - absence of this spot in the absence of any effective allele was taken as contributed by the line 807. Chi-square value, with the probability of chance variation being 20-30%, was found to be insignificant. Thus the subunit of big legumin was also thoughtto be controlled by a single pair of co-dominant alleles of the same gene.

(iii) Products of subunit pair Lg-S

This pair is called 'Lg-S' because it has a smaller mol. wt. than that of conventional legumin. The acidic and basic subunits differed in their pattern in the two

lines. Fifty two plants of the F_2 generation were analysed by two dimensional SDS-polyacrylamide gel electrophoresis for Lg-1S subunits represented by R and S in lines 110 and 807 respectively (Fig. 4.2) and 170 plants for Lg-2S represented by X and Y (Fig. 4.3). Chi-square values calculated (Table 4.1) were within the statistical bounds for monogenic segregation and co-dominance of the alleles responsible for these subunits.

c) Linkage relationships of the subunits

Chi-square values were calculated to determine the status of the genes for various legumin subunits as to whether linkage occurred between the genes for various legumin subunits or if they were located on different chromosomes (Table 4.2). The assumption was made that they followed the mendelian law of independent assortment with respect to each other in the F_2 generation. The subunit combinations given below were studied for these linkage relationships:

(i) Lg-1C (AB) . Lg-2S (XY)

(ii) Lg-1C (AB) . Lg-1S (RS)

(iii) Lg-1C (AB) . Lg-1B (CD)

(iv) Lg-1S (RS) . Lg-2S (XY)

(v) Lg-1B (CD) . Lg-1S (RS)

(vi) Lg-1B (CD) . Lg-2S (XY)

Some of these combinations and their behaviour in F_2 generation are illustrated in Fig.4.3 and 4.2.

Fig. 4.3 : SDS-polyacrylamide gel electrophoresis, under reducing conditions, of total protein extracts showing different combinations of A,B and X,Y in F_2 plants of the cross between Pisum lines 110 x 807. A, B, C, D, X and Y represent the legumin species as defined in the text. CV and V stand for convicilin and vicilin respectively.

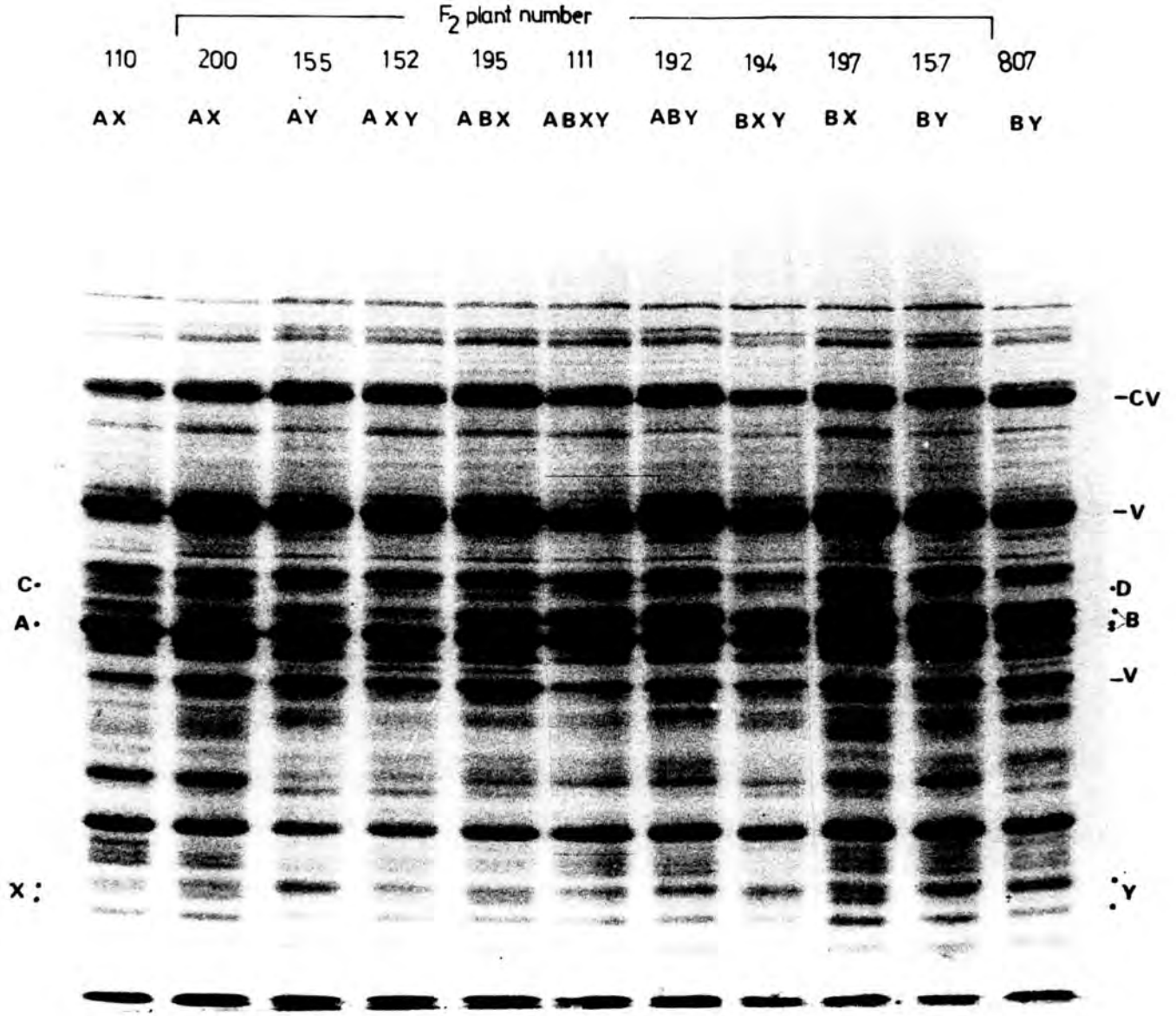

F2 plant number
110 200 155 152 195 111 192 194 197 157 807
AX AX AY AXY ABX ABXY ABY BXY BX BY BY
-CV
-V
C·
A·
·D
·B
-V
X :
·Y

Fig.4.3

Table 4.2 : Subunit patterns of the dihybrid cross, their frequency and chi-square values in F_2 generation for various legumin subunit combinations

Subunit Combination	Pattern of subunit Combination	Expected ratio and frequency of subunit patterns in F_2 generation									χ^2 (n = 8)
		1	1	2	2	4	2	2	1	1	
Lg-1C.Lg-2S	A X / B Y	AX	AY	AXY	ABX	ABXY	ABY	BXY	BX	BY	4.51
	Observed	11	12	25	23	47	14	20	9	9	
Lg-1C.Lg-1S	A R / B S	AR	AS	ARS	ABR	ABRS	ABS	BRS	BR	BS	8.30
	Observed	4	4	6	3	18	9	2	3	3	
Lg-1C.Lg-1B	A C / B D	CA	CB	CAB	CDA	CDAB	CDB	DAB	DA	DB	7.13
	Observed	4	2	9	4	13	3	8	6	3	
Lg-1S.Lg-2S	R X / S Y	RX	RY	RXY	RSX	RSXY	RSY	SXY	SX	SY	12.71
	Observed	5	4	1	3	16	7	6	3	7	
Lg-1B.Lg-1S	C R / D S	CR	CS	CRS	CDR	CDRS	CDS	DRS	DR	DS	11.46
	Observed	6	5	4	2	13	5	9	2	6	
Lg-1B.Lg-2S	C X / D Y	CX	CY	CXY	CDX	CDXY	CDY	DXY	DX	DY	53.72
	Observed	8	2	5	3	15	2	3	0	14	

For all the aforementioned subunit combinations except the last one, chi-square values were insignificant and low enough not to indicate any linkage between various subunits. However, acidic subunits of big legumin and basic subunits of small legumin for which chi-square values were highly significant, seemed to show a considerable degree of linkage.

B. Cross 1238 x 1263

Reciprocal crosses were made between lines 1238 and 1263 in order to study the relative assignment of the legumin gene to a particular linkage group. In contrast to line 1263, line 1238 was double recessive for the characters dwarfness, procumbent branches, absence of maculum ring, absence of tendrils, pink flowers, keel like wings, yellow pods, sticky seeds due to tragacanth excretion on the outside, absence of air pockets below the leaf epidermis, green cotyledons and wrinkled seeds, thus of the genotype le, pro, d, tl, b, k, gp, s, fl, i and r respectively. On the other hand, line 1263 was characterised by the absence of anthocyanin pigment, thus of genotype recessive a. The two lines differed in the pattern of Lg-1C subunits and convicilin subunits. Lg-1C subunits were represented by three bands of mol. wt. 38,000, 37,000 and 35,000 in the line 1238 (pattern E) and by two bands of mol. wt. 35,500 and 35,000 in the line 1263 (pattern F). Convicilin was represented by bands of mol. wt. 70,000 and 68,000 (pattern U) in the

Fig. 4.4 : Two dimensional SDS-polyacrylamide gel electrophoresis of total protein extracts of pea lines 1263 and 1238. 1D : electrophoresis under non-reducing conditions in 10% gel in the first dimension; band patterns shown in tracks (i). 2D : electrophoresis under reducing conditions in the second dimension; band patterns shown in tracks (ii). E, F, Lg-1, Lg-2, Lg-1S, Lg-B, Lg-C and Lg-S are the legumin species and, U and V the convicilin patterns as defined in the text. CV and V stand for convicilin and vicilin respectively.

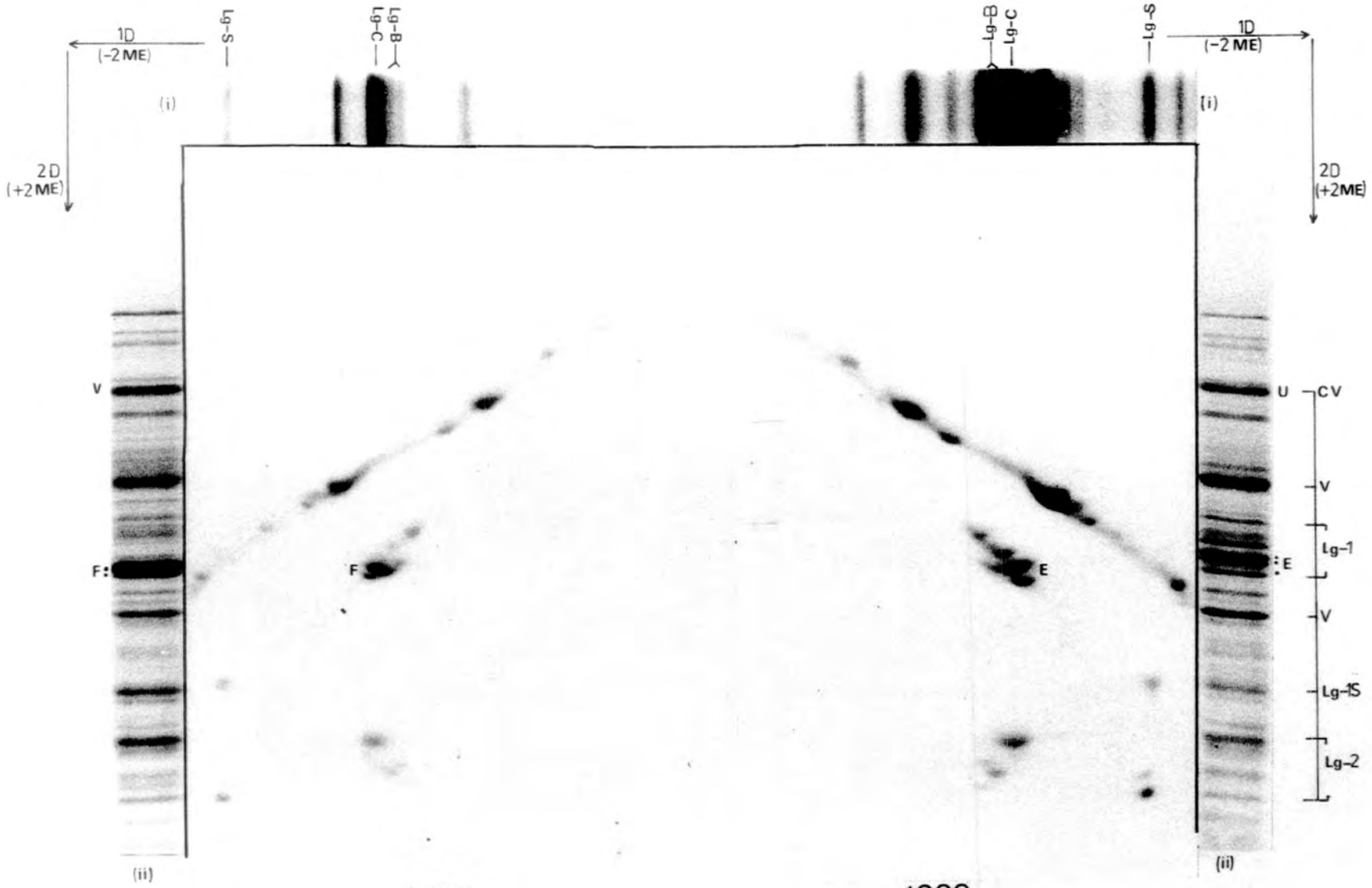


Fig. 4.4

line 1238 and bands of mol. wt. 70,000 and 67,000 (pattern V) in the line 1263 (Fig. 4.4).

a) F_1 hybrids

F_1 plants of reciprocal crosses between the two parental lines showed the expected dominant expression for the genes Le, Pro, D, Tl, B, K, Gp, S, I, R and A. The pattern of legumin acidic subunits and convicilin in these F_1 plants was that of a hybrid combination of the two parental lines (Fig. 4.5).

b) Segregation of legumin and convicilin subunit patterns

The observed frequency of F_2 plants with the parental and hybrid patterns for acidic subunits of legumin and convicilin subunits is given in Table 4.3. For both of these polypeptides, chi-square values provided support for the hypothesis that genes controlling these subunits occur as a single pair of co-dominant alleles.

Table 4.3 : Analysis of F_2 segregation and chi-square values for legumin subunits Lg-1C and convicilin

Subunit	F_2 segregants and their frequency			χ^2 (n = 2)
Lg-1C	E	EF	F	
Observed	35	67	26	1.55
Expected	32	64	32	
Convicilin	U	UV	V	
Observed	34	58	36	1.19
Expected	32	64	32	

Fig. 4.5 : SDS-polyacrylamide gel electrophoresis (reducing conditions) of the total protein extracts of Pisum parental lines 1238 and 1263, and the F_{1s} of the reciprocal cross between them. Lg-1, Lg-2, CV and V represent acidic subunits of legumin, basic subunits of legumin, convicilin and vicilin respectively.

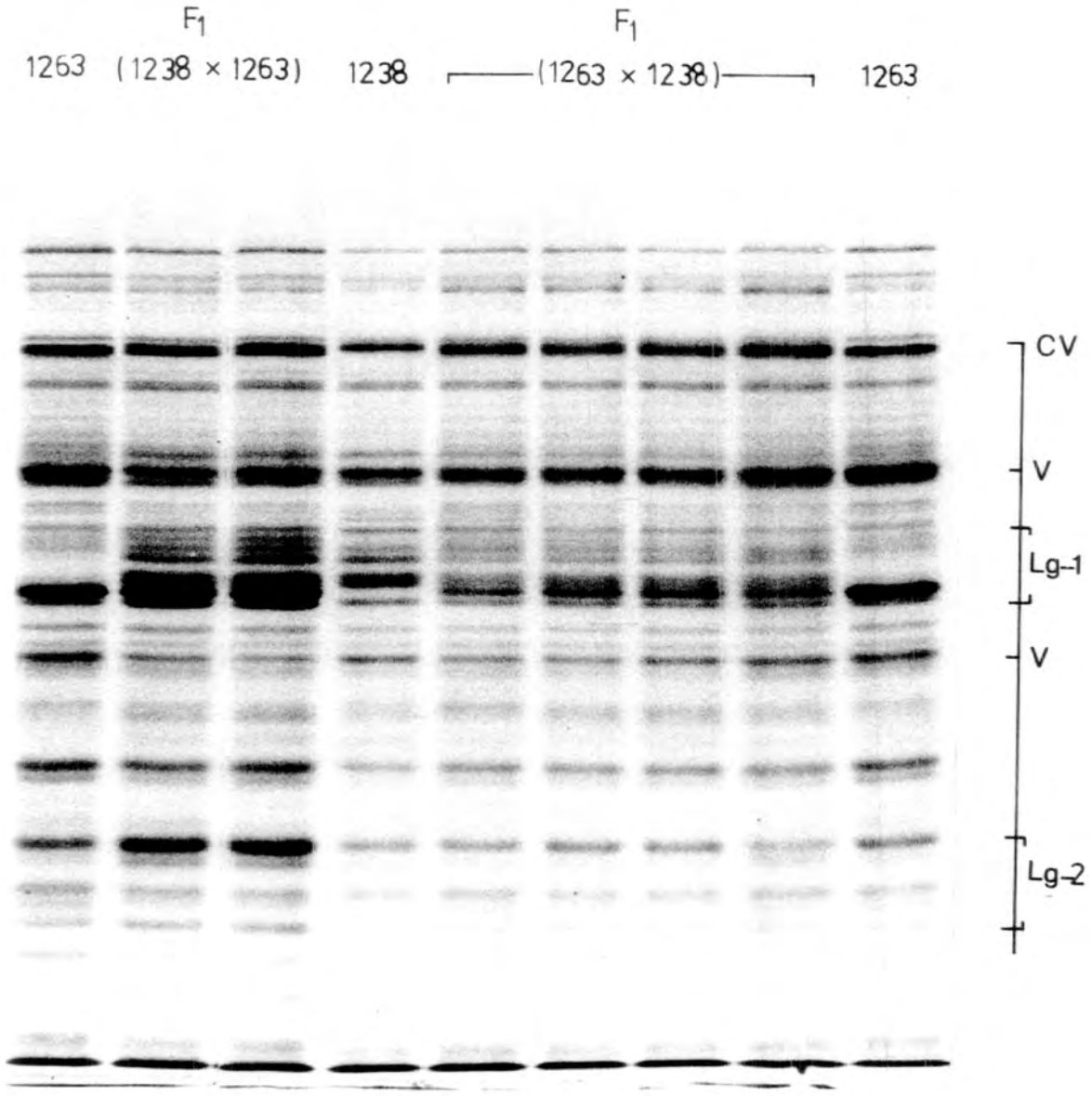


Fig. 4.5

c) Testcross for linkage of legumin subunit genes

To get an idea of linkage between the genes for the acidic subunits of legumin and the genes for the selected morphological markers, F_1 plants were backcrossed with the double recessive parent line 1238. The genotype of 19 plants as determined by this testcross made with respect to various characters is given in Table 4.4. As can be seen from the results of the table, genes for the acidic subunits of conventional legumin appeared to be linked with tl-r segment of the chromosome No.7. Plant No.2 and 8 show recombination between legumin and the tendril gene and plant No. 2, 8, 15 and 19 represent recombinants for legumin and seed surface gene r. A higher number of recombinants between Lg-1C and r genes points towards the location of Lg-1C gene being on the side of tl locus away from r locus.

Table 4.4 : Lg-1C pattern and genotype (tl and r markers of linkage group 7) of testcross progeny from a cross between F_1 of the cross 1238 x 1263 and double recessive parent line 1238

Plant No.	Lg-1C pattern	Genotype of the testcross plants	
	E/F	Tl/tl	R/r
1	F	Tl	R
2	F	tl	r
3	E	tl	r
4	F	Tl	R
5	F	Tl	R
6	F	Tl	R
7	F	Tl	R
8	E	Tl	R
9	E	tl	r
10	F	Tl	R
11	E	tl	r
12	E	tl	r
13	F	Tl	R
14	F	Tl	R
15	F	Tl	r
16	F	Tl	R
17	F	Tl	R
18	E	tl	r
19	F	Tl	r

d) Linkage relationship of legumin subunit gene

Patterns for acidic subunits of conventional legumin in the F_2 generation assorted independently of the pattern for the storage protein convicilin. The segregation of patterns for these two storage proteins in the F_2 generation is shown in fig. 4.6. Chi-square value (5.06 at 8 degree of freedom) calculated from the observed number of different F_2 classes showed that the genes for these polypeptides were present on different chromosomes, or if present on the same chromosome, were distantly located from each other. However, when these legumin subunit patterns were studied in relation to the F_2 segregation of seed surface type (round 'RR' 'Rr' or wrinkled 'rr') and tendril character (present 'TlTl', 'Tltl' or absent 'tl tl'), the gene for acidic subunits of conventional legumin was found to be linked with tl-r segment. The crossover values calculated by the method of Immer (1930) were $18.3 \pm 2.6\%$ between the legumin gene and the r locus and $15.1 \pm 2.3\%$ between the legumin gene and tl locus (Table 4.5). This confirms the location of legumin gene at 15 map units from the tl locus on the side opposite to r locus since r and tl loci are separated by 5 recombination units according to Blixt (1972).

Fig. 4.6 : SDS-polyacrylamide gel electrophoresis, under reducing conditions, of total protein extracts showing different combinations of U,V and E,F in F_2 plants of the cross between Pisum lines 1238 and 1263. E, F and U,V represent Lg-1C and convicilin subunit patterns respectively as defined in the text; Lg-1, Lg-1S and Lg-2 represent different legumin species as defined in the text.

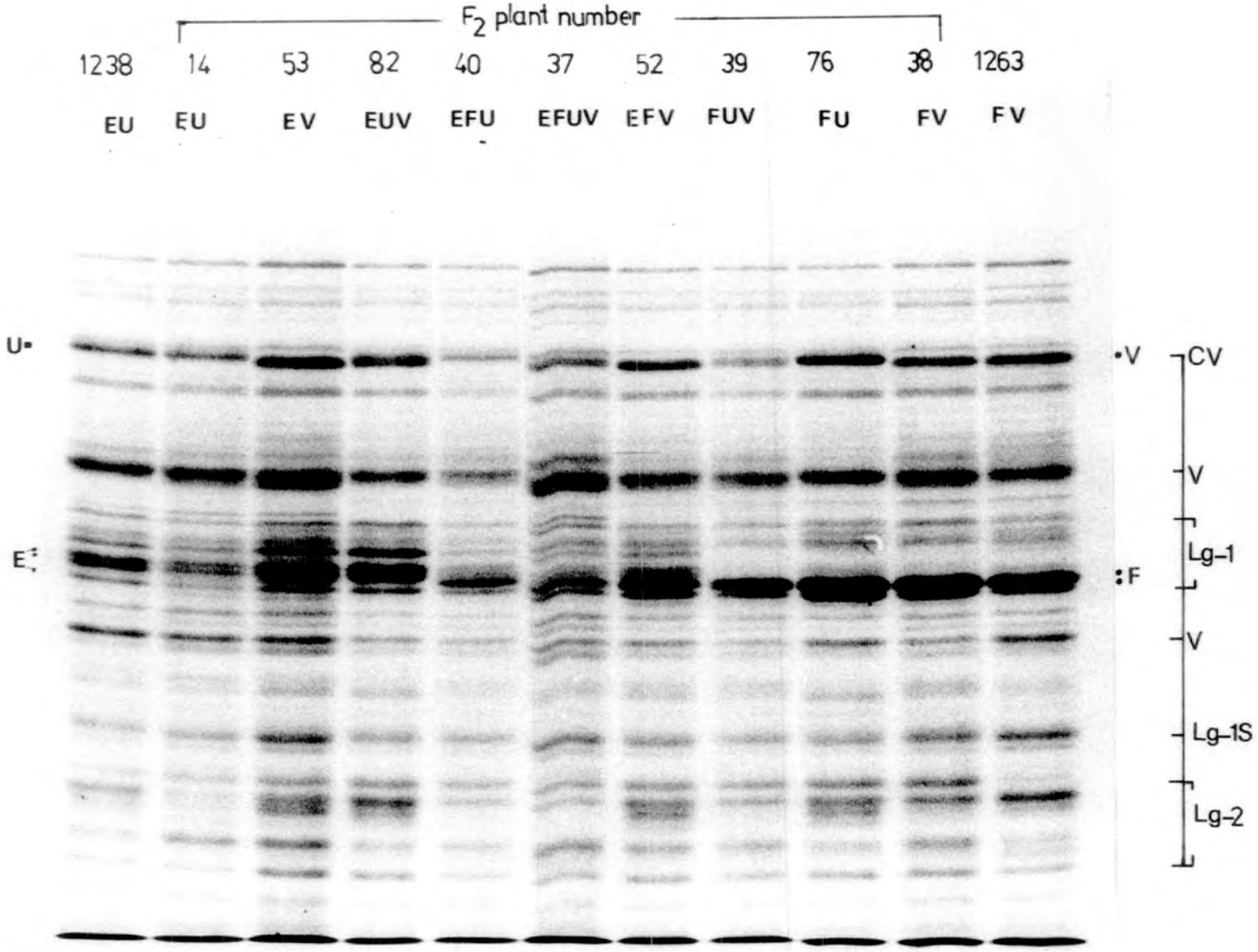

F2 plant number
1238
14
53
82
40
37
52
39
76
38
1263
EU
EU
EV
EUV
EFU
EFUV
EFV
FUV
FU
FV
FV
U
E
V
F
CV
V
Lg-1
V
Lg-1S
Lg-2

Fig. 4.6

Table 4.5 : Segregation of acidic subunits of legumin and convicilin subunits in F_2 generation with respect to the selected markers and their crossover values.
($Lg\text{-}1C^E$ and $Lg\text{-}1C^F$ stand for the patterns E and F respectively of legumin subunits; 'R' for round and 'r' for wrinkled seeds, 'Tl' for presence and 'tl' for absence of tendrils. Cvc^U and Cvc^V stand for U and V, patterns of convicilin respectively, 'K' for normal wings and 'k' for keel like wings).

Character pair	F_2 classes and observed frequency				$p = \frac{bc}{ad}$	Crossover value $\pm$ probable error
	a	b	c	d		
Lg-1C. seed surface	$Lg\text{-}1C^E$.r 27	$Lg\text{-}1C^E$.R 7	$Lg\text{-}1C^F$.r 18	$Lg\text{-}1C^F$.R 76	0.0614	$18.3 \pm 2.6\%$
Lg-1C. Tendrils	$Lg\text{-}1C^E$.tl	$Lg\text{-}1C^E$.Tl	$Lg\text{-}1C^F$.tl	$Lg\text{-}1C^F$.Tl	0.0397	$15.1 \pm 2.3\%$
Cvc. Wingtype	Cvc^U.k 30	Cvc^U.K 2	Cvc^V.k 2	Cvc^V.K 90	0.0015	$3.2 \pm 1.1\%$

e) Linkage relationships of convicilin gene

As stated earlier, the convicilin subunit patterns 'U' and 'V' were inherited independently of the Lg-1C subunit patterns, genes for which are located on chromosome 7. In the F_2 generation, the Cvc patterns and the wing type in the flowers mostly showed the parental combinations and thus the gene Cvc for convicilin subunits showed a strong linkage with the locus k on chromosome 2. The crossover value between Cvc and k loci was found to be $3.2 \pm 1.1\%$ (Table 4.5). The data for segregation of k and Cvc loci with respect to s was not sufficient enough to give the exact location of Cvc locus in the linkage group. However, the detection of linkage between Cvc and s loci supports the location of Cvc in this segment of linkage group 2.

Section IV DISCUSSION

Previous work on the structure of legumin has led to the general acceptance of the hexameric model of Wright and Boulter (1974) in which each legumin molecule is postulated to consist of six pairs of disulphide bonded acidic (approx. 40,000 mol. wt.) and basic (approx. 20,000 mol. wt.) subunits. However, there were already observations which indicated that the situation was more complicated than this. Bailey and Boulter (1970) had clearly demonstrated the presence of a 56,000 mol. wt. subunit in addition to the 40,000 and 20,000 mol. wt. subunits of the hexameric model in the legumin of Vicia faba and that the subunits (mol. wt. 56,000, 42,000 and 23,000) studied by them were present in the ratio of 1:3:6 respectively. The larger subunits were also subsequently noted by Utsumi and Mori (1980). This additional complexity of the legumin molecules has been thoroughly supported by the results presented in this thesis showing the presence of subunit pairs 'A-J' in Vicia legumin (Fig. 2.2) and '1-5' in Pisum legumin (Fig. 3.3). However, all these subunit pairs and the resultant legumin molecules can still be fitted easily into the operational definition of legumin given above. Subunit pairs H/I in Vicia and subunit pair 4 in Pisum constitute about 80% of the total legumin subunit pairs, i.e. represent the predominant subunit species. These are the subunit pairs previously considered in the hexameric

legumin model (Wright and Boulter, 1974; Croy et al., 1979). We have, therefore, used the following nomenclature : subunit pairs H/I and No. 4 in Vicia and Pisum respectively are called Lg-C (conventional); subunit pairs 'A-G' in Vicia legumin and '1-3' in Pisum legumin which have a mol. wt. greater than H/I and 4 are called big legumin subunits i.e. Lg-B. Subunit pairs of mol. wt. 37-40,000 (J) in Vicia and mol. wt. 35,000 (5) in Pisum which have a lower mol. are called Lg-S (small) legumin subunits. Pisum is different to Vicia in that it does not have the subunit pairs (Lg-B) with mol. wt. greater than that of the convicilin subunits and with acidic subunits (Lg-1B) of mol. wt. greater than 50,000. Polypeptides of mol. wt. 26,500 and 26,000 which always co-purify with Pisum legumin but which are not disulphide bonded i.e. are impurities, seem to be entirely absent in the case of Vicia legumin.

Two dimensional electrophoresis employing electrophoretic techniques in different combinations is a powerful analytical method which has been used to study the characteristics of many proteins by various workers (Raymond, 1964; Margolis and Kenrick, 1969; Kaltschmidt and Wittman, 1970; O'Farrell, 1975). In this study, two dimensional SDS-polyacrylamide gel electrophoresis with non-reducing conditions in the first dimension and reducing conditions in the second dimension has been shown to be of great value in studies

of the storage protein legumin. This is because legumin is a protein containing disulphide-bonded subunits which run as subunit pairs in the first dimension but are separated by 2-mercaptoethanol used in the second dimension, causing the two types of subunits to leave the diagonal path whereas other proteins remain along the diagonal. In this way, bands representing legumin subunits in a complex mixture of seed proteins can be recognised very easily; this is not the case with other 2-D systems. However, this system, like others, does not give a complete analysis of the heterogeneity present and 2-D systems should be used complementary to one another.

The mol. wts. of unreduced legumin subunit pairs were lower than the sum of the mol. wts. of the component reduced subunits. This discrepancy can be accounted for by the presence of inter- and intrachain disulphide bonds in the subunit pairs which can prevent complete unwinding of the unreduced polypeptides resulting in an apparent mol. wt. lower than the true one. This has been very clearly seen in bovine serum albumin which has an apparent mol. wt. of approx. 55,000 when unreduced as compared to 67,000 when reduced (data not presented). Consequently the mol. wt. values for reduced subunits are the more correct ones.

Molecular weights of the large subunits of all the subunit pairs of Vicia faba legumin decreased linearly according to mol. wt. of subunit pair, except

that of subunit pair 'I' which had a mol. wt. higher than that of 'H' (Fig. 2.2). Thus, sum of the mol. wt. of components of 'I' was higher whereas that of the unreduced 'I' was lower when compared to 'H'. This was also observed by Utsumi and Mori (1980) for the subunit pair of composition 36,000 + 23,000 which had a lower mol. wt. as compared to the one with subunit composition 36,000 + 20,500. Utsumi and Mori (1980) also reported the presence of a subunit pair ('IV') equivalent to subunit pair 'I' here,having one acidic subunit but two basic subunits, thus confirming the present results. These anomalies in the mol. wt. of subunit pairs relative to their components can be attributed to the presence of inter- and intrachain disulphide bonds.

Further studies of Vicia legumin showed greater heterogeneity in the number of subunits when additional separatory techniques were used. The 10 subunit pairs seen under non-reducing conditions were shown to consist of at least 21 components in a two-dimensional analysis using both reducing and non-reducing conditions. Utsumi and Mori (1980) detected 3 acidic and 3 basic subunits and on the basis of their results with gradient pore gel electrophoresis they proposed the existence of 7 molecular forms of the complete legumin molecule with four different mol. wts. Studies carried out in this investigation using ultrogel column chromatography and gel electrophoresis also point to the existence of several legumin molecular forms. However, as the 13

subunits (Fig. 2.1d) observed in first dimension under reducing conditions exist in a number of charge variants (Fig. 2.5a and 2.5b), the number of potential legumin molecules seems to be very large. The results of gel filtration and gel electrophoresis of Vicia legumin where subunit pair 'F' runs independently of the other subunit pairs can be explained by assuming that in this case subunit pairs 'F' assemble individually into hexameric molecules. This is also supported by the fact that the subunit pair 'F' was the only subunit pair not to be immunoprecipitated with the other subunit pairs by anti-legumin antibodies of Pisum sativum. It was not possible, however, to separate under non-dissociating conditions other legumin molecules consisting of only one type of subunit pair. Apart from the legumin with 'F' subunits, several other legumins were partly separated but most subunit pairs were represented in most molecular forms suggesting the possibility of random assembly of subunit pairs to form hexameric molecules. As the subunit pair H/I constitutes about 80% of the total legumin, it would be feasible to predict that the most common legumin species was 6 x (H/I) (although never isolated) followed by various 5 x (H/I) + 1 x (X) species of slightly different mol. wt. and charge. The partial separation of A, B, D from H/I on gel filtration and gradient pore gel electrophoresis suggests that certain combinations of subunit pairs in hexameric molecule are more likely

than others. These results thus do not entirely support or disprove the conclusions of Utsumi and Mori (1980).

Legumin of Pisum sativum also shows considerable heterogeneity in the range of mol. wt. and isoelectric points of the subunits and has been partially separated into different molecular forms. As many as 13 subunits have been observed by two-dimensional analysis in contrast to 21 subunits seen in Vicia. The subunits of all the subunit pairs include charge heterogeneity, i.e. each subunit contains a number of charge isomers as specified in table 3.2. Subunit pair types are assigned firstly on the basis of their mol. wt. and the mol. wt. values of their component acidic and basic subunits and secondly, on the basis of the common subunits and their distribution in molecular species. For example, in subunit pair 4, the three acidic subunits a, b and c all have a common basic subunit (with three charge isomers) and occur in approximately equal proportions to each other in all molecular forms whereas subunit pairs 1 and 2 have no subunit in common, pair 1 occurs predominantly in legumin molecular form A and pair 2 predominantly in form C. Form B legumin containing subunit pair 4 seems to be the common form, its charge decreasing or increasing by its association with pair 1 or 2 respectively. Form A, of higher mol. wt. than the other legumin molecular types, contains subunit pairs 1 and 4 in approximately equal proportions, i.e. each hexameric molecule contains 3 subunit pairs each

of type 1 and 4 i.e. [3 x (1) + 3 x (4)] . From the gel electrophoresis and gel filtration studies it appears that form 'C' actually consists of different forms which have varying proportions of subunit pairs 2, 3 and 5; possibly these forms are [2 x (2) + 4 x (4)] , [2 x (3) + 4 x (4)] and [2 x (5) + 4 x (4)] . However, since a complete separation of the different molecular forms cannot be achieved, these conclusions may be considered as tentative.

Thus from these studies of Vicia and Pisum legumin a modified model for the legumin molecule which contains a number of subunit pairs other than that of conventional legumin (Wright and Boulter, 1974) may be put forward. In this case, each hexameric molecule is formed by the association of individual subunit pairs of one or more types, association being regulated by the relative abundance of subunit pairs and by structural constraints to give the observed selection of molecular species. Legumin is thus not a single protein species of defined amino acid sequence but is a generic name for a series of closely related proteins. This situation is analogous to that found in other proteins e.g. the isoenzymes of lactate dehydrogenase or immunoglobulins. We have adopted the operational definition of legumin outlined above.

Certain anomalies are raised by the results presented herein. For example, it has been demonstrated that separation on gradient pore gel is not solely

according to mol. wt. but a charge factor is also operative. Molecular weight values measured by this pore limit electrophoresis in the present work are lower for Pisum legumin and higher for Vicia legumin. But gel filtration (Croy et al., 1979) and ultra-centrifugation studies (Derbyshire et al., 1976) have shown that Pisum legumin has a higher mol. wt. than the Vicia legumin. The higher charge at pH 8.0 of Pisum legumin in comparison to Vicia legumin (Croy et al., 1979) may explain the higher mobility of Pisum legumin in gradient pore gels. It has also been pointed out by Rodbard et al. (1971) that the concept of a 'pore limit' in gradient gels is theoretically invalid.

The terms acidic and basic for legumin large and small subunits respectively must be considered as relative since both the types of subunits of subunit pair 1 in Pisum legumin have their pIs below 7, although the small (basic) subunits have higher pIs than the large (acidic) subunits. All other legumin basic subunits have pIs above 7. Small subunits of legumin (mol. wt. 21,300) with pIs below 7, i.e. in the acidic range, were reported by Casey (1979b) but were not assigned as components of any particular subunit pair.

Acidic subunits of subunit pairs 1, 2, 3 and 4 of Pisum legumin can be compared with the 'LA-LC' groups of legumin as given by Thomson and Schroeder

(1978) and with minor and major α subunits given by Casey (1979b). On the other hand basic subunits of all the subunits represent 'LD' group of Thomson and Schroeder (1978) and β subunits of Casey (1979b). 'LE' group of Thomson and Schroeder (1978) and γ subunits of Casey (1979b) are represented by subunits of mol. wt. 26,500, 26,000 and 24,500. Of these, the subunits of mol. wt. 24,500 is actually the only legumin acidic subunit i.e. of subunit pair 5 in Pisum legumin. The polypeptides of mol. wt. 26,500 and 26,000 (pI 4.5 - 4.7) designated as 'D' bands on the non-dissociating gel (Fig. 3.1e) are clearly not legumin species since these are not disulphide bonded and can be completely removed from legumin subunit pairs under non-dissociating conditions. These polypeptides are, therefore, associated with legumin molecules by weak non-covalent bonds which are possibly dependent on ionic strength. No further characterization of this protein was attempted and its functional role is therefore unknown.

The high degree of heterogeneity observed in the legumin of Vicia faba and Pisum sativum may be expressed in terms of a hierarchial model as follows, where the levels are given in ascending order.

(i) Single substitutions in amino acid sequences of polypeptides; these may be responsible for the charge heterogeneity of individual subunits, although many single substitutions may be unrecognised by the

procedure (isoelectric focusing) used.

(ii) More extensive differences in sequence among polypeptides including differences in the total number of amino acids; these may be considered responsible for the size heterogeneity among subunits.

(iii) The formation of legumin molecules containing subunit pairs in different combinations; this may be considered responsible for the charge and size heterogeneity of legumin under non-dissociating conditions.

It is possible that heterogeneity of types (i) and (ii) is a direct expression of genetic heterogeneity evident in the translation products synthesized *in vitro* on the polysomes purified from developing seeds of *Vicia faba* and *Pisum sativum*. Translation products were specifically immunoprecipitated against anti-legumin antibodies and were shown to be heterogeneous in mol. wt. when analysed by SDS-polyacrylamide gel electrophoresis and fluorography (Gatehouse, unpublished). The evidence that heterogeneity has a genetic basis also comes from the studies made by Thomson *et al.* (1978) who found the F_1 progeny to show a pattern additive of the two parental patterns. Muller and Gottschalk (1973) found some induced mutant lines with patterns of globulins different from those of the parent lines; this also suggests some mechanism at the primary level responsible for the heterogeneity in these seed proteins.

Heterogeneity may also arise as a result of some post-translational modification of the legumin

precursor which is synthesized as a 60,000 mol. wt. polypeptide and is nicked in some way to give rise to two types of disulphide bonded subunits after its synthesis (Croy et al., (1980). Higgins and Spencer (1980) attributed the increase in mol. wt. of legumin basic subunit from 19,000 to 20,000 to the post-translational modification of the long lived precursor for legumin. A similar observation made by us appears to be more of a storage effect rather than post-translational modification. In the pea line 'Meteor', during storage of the dry seeds, a basic subunit band seemed to show an apparent increase in mol. wt. from 22,200 to 22,700. This apparent shift in mol. wt. is accompanied by a decrease in the pIs of the three subunits constituting the 22,200 mol. wt. band. It seems likely that this change, as it occurs on storage in the freeze dried and pulverized seeds, is due to a non-enzymic chemical alteration of legumin rather than a directed enzyme catalysed process. It may be suggested that the most likely alteration in the legumin basic subunits to account for these observations is deamidation of asparagine or glutamine residues - this will lower the pI of the subunit and decrease its positive charge. This decrease in positive charge might cause an upward shift of the subunit on SDS-gel as SDS-gels have been shown to give separation on the basis of mol. wt. as well as charge. Thus heterogeneity may sometimes be artificial and generated during storage.

Heterogeneity in mol. wt. and isoelectric points is greater for acidic subunits than that for basic subunits. This effect is more evident in Vicia legumin. Also, the treatment of the acidic and basic subunits belonging to various subunit pairs of Vicia legumin with cyanogen bromide showed the cleavage products of basic subunits to be more similar. On the other hand, the number and mol. wt. of the cleavage polypeptides for individual acidic subunits were much different from those of each other (data not presented). All these observations indicate that the structure of basic subunits is more constrained and that the genes responsible for the basic subunits have been more stable whereas those for acidic subunits seem to have been more prone to mutations and other changes during the course of evolution. From this assumption, a possible arrangement of the two types of subunits can also be deduced which is that basic subunits are situated towards the interior and acidic subunits towards the exterior of the hexameric molecule. This model is supported by the observation that the acidic subunits carry the antigenic determinants for the legumin molecule, and that the acidic subunits are proteolysed first on germination (Croy, unpublished results).

Recently a great deal of work has been undertaken regarding the inheritance of seed proteins of Pisum sativum. A number of proteins like 'vicilin' (Hynes, 1969), aminopeptidase (Scandalios and Espiritu, 1969),

acidic subunits of legumin (Thomson and Schroeder, 1978; Casey, 1979b), vicilin subunits (Thomson and Schroeder, 1978) and 'albumin a' (Blixt et al., 1980) have been found to be under the control of a single pair of co-dominant genes. Legumin basic subunits, however, have been reported to be under multigenic control (Thomson and Schroeder, 1978). These reports invalidate the earlier reports of maternal effects on the inheritance of storage proteins in pea (Davies, 1973). Results presented in table 4.1 and 4.3 of this thesis also support the monogenic control by co-dominant alleles for Lg-1B, Lg-1C, Lg-1S, Lg-2S and convicilin subunits. The subunit pattern for various legumin subunit types may consist of two or more than two subunit bands e.g. 3 bands in line 807 and 1238 and two bands in line 1263 for Lg-1C subunits, 2 bands for Lg-1S in line 807 and 2 bands for Lg-2S in lines 110 and 807. It may be assumed that each of these bands is controlled by one gene. Occurrence of either parental or the combination of two parental patterns in F_2 suggests that genes for these subunits are strongly linked. As the 2-D electrophoretic studies (SDS-PAGE $\longrightarrow$ IEF) have shown that each subunit band separated on the SDS-gel further consists of a number of charge variants, it is very likely that there exist a number of gene copies (more or less identical) for each subunit so closely located that there is no recombination at all among them and the genes for various subunits of a kind act as a single gene.

Four types of legumin subunits (Table 4.2) and, Lg-1C and convicilin ($\chi^2_{(n=8)} = 5.06$) seem to be under independent control by the genes which are located either on different chromosomes or are syntenic,i.e. if located on the same chromosome are distantly located from each other so that they do not show any linkage. The only exception appears to be in the case of Lg-1B and Lg-2S which show a considerable degree of linkage with each other. The situation for most of the legumin subunit genes, thus, seems to be similar to zein polypeptide genes, which though being identical in effect are located on different chromosomes (Valentini et al., 1979).

Considerable evidence exists to support the hypothesis that legumin subunits are synthesized as precursor polypeptides of 60,000 mol. wt. (Croy et al., 1980; and unpublished results) containing both parts of the subunit pair. This implies an adjacent location of the genes for acidic and basic subunits on the same chromosome. (More correctly, each subunit pair is the product of a single gene). No evidence exists to show that this is not the case with the conventional legumin subunit pair i.e. 60,000 mol. wt. However, the results of independent inheritance of the genes for acidic and basic subunits of small legumin would suggest that a different pathway of synthesis takes place in this case. The absence of a 35,000 mol. wt. precursor in the translation products supports this suggestion.

Since the earliest days of classical genetics, Mendel's study material, Pisum sativum, has been one of the most extensively used plants for genetical studies. The first reports on linkage by Vilmorin and Bateson (1912) were followed by the extensive and outstanding work of H. Lamprecht (Lamprecht, 1948; Blixt, 1969). Nearly 200 genes, mostly for morphological traits, (Blixt, 1977; Marx and Provvidenti, 1979; Marx, 1980; Murfet, 1980; Gritton and Hagedorn, 1980 and Blixt et al., 1980) have been assigned to the seven linkage groups of pea. The tl and r genes linked to each other (Lamprecht, 1948) are located on the short arm of chromosome 7 (Blixt, 1972). The gene for the acidic subunit (Lg-1C) in the present studies has been found to show linkage with these loci and is located 15 map units from the tl locus on the side away from r locus. Recently there has been a controversy regarding the position of tl-r segment. Lamm (1978) on the basis of his studies on translocation lines has suggested the position of tl-r segment to be on the long arm of chromosome 5 rather than the short arm of chromosome 7. However, we have followed the gene map given by Blixt (1972) in considering the Lg-1C gene linked to tl-r segment as being on chromosome 7. As suggested under the rules for gene symbols (Annonymous, 1977) this Lg-1C gene maps as given below

Lg-1C --- 15 --- tl --- 5 --- r

The gene for convicilin subunits which shows independent assortment with respect to Lg-1C gene ($\chi^2_{(n=8)} = 5.06$) shows a strong linkage with the gene k for keel like wings. It is about 3 recombination units apart from k locus, k in turn being located on chromosome 2. However, the exact location of the convicilin gene (Cvc) could not be ascertained because there was no other suitable marker for this linkage group in the cross 1238 x 1263.

Lastly, it is appropriate to discuss the implications of the results presented in this thesis on the structure and genetics of legumin to the importance of legumes as high protein crops.

Existing work on seed protein improvement in cereals (Nelson and Burr, 1973) has indicated that changing the ratio of major seed storage proteins may well be the most easy route to protein improvement. There are two requirements : (i) the existence of seed proteins with improved amino acid profile, (ii) the possibility of largely increasing the content of those proteins relative to others. As pointed out in the introduction, legumin potentially satisfies these criteria. Its sulphur amino acid content is superior to the other major storage protein vicilin and different lines show varying proportions of legumin in the total seed protein.

The complexity of the legumin molecule demonstrated in these results would suggest that the sulphur amino

acid content of individual subunit pairs needs to be determined in order to identify where improvement should be made. Furthermore, although the genetic position was not fully elucidated, it would appear complex and, therefore, considerable work would be needed to accomplish the desired results. On the positive side, however, since no linkage occurs between the genes responsible for different storage proteins, improving the protein profile nutritionally would appear feasible in principle. A discussion of the precise dietary situation in which such improvements are desirable lies out of the scope of this thesis.

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