COTYLEDONARY PROTEINS IN PISUM SATIVUM

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DECLARATION

This thesis contains no material which has been submitted for the award of any other degree or diploma in any University. It contains no material previously published elsewhere except were due reference is made in the text of the thesis.

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Preface

Some decisions had to be made in the presentation of this thesis. In the course of the project, four manuscripts were prepared and submitted for publication in journals. It was decided that these would be presented in the thesis as experimental Chapters 2 to 5, retaining the form in which they were submitted for publication. Of the four manuscripts submitted, that constituting Chapter 2 has already been published, the Chapter 3 one has been accepted in the form presented here. The manuscript of Chapter 4 (co-authored by A.H.D. Brown) has been submitted to Theoretical and Applied Genetics in September 1983, while the Chapter 5 one has been submitted to Journal of Experimental Botany in August 1983.

A consequence of the decision to present the thesis in this form is that each of Chapters 2 to 5 is self-contained, with its own format in accordance with the particular journal's editorial policy, and with its own set of references. The Reference section which appears at the end of the thesis lists those publications cited in the thesis Introduction (Chapter 1) and Conclusion (Chapter 6). Inevitably therefore there will be repetition in the citing of references in these various places. The typeface of Chapter 2, reproduced as published but with new copies of photographs, differs from that of the remainder of the thesis. Page numbers are continuously sequenced throughout the thesis.

The manuscript for Chapter 4 co-authored with A.H.D. Brown presents results of experiments carried out entirely by me as author of the thesis. The contribution by Dr. Brown comprises the statistical treatment of the experimental data provided by me and has been assessed by him as being about one-third of the paper.

SUMMARY

The aim of the present study was to make a detailed quantitative, qualitative and genetic analysis of the cotyledonary proteins of pea seeds. The genetically determined variation in seed protein and protein quality characteristics of 45 lines of peas was examined. The seed protein characters measured were crude protein, extractable protein, globulin, albumin and legumin contents, the protein quality characters measured were total sulfur, protein sulfur, carbon to nitrogen and nitrogen to sulfur ratios. It was shown that quantitatively the albumin fraction is as significant as either of the storage proteins legumin and vicilin. Correlation coefficients were calculated between seed weight and eight of the above characters. A highly significant negative correlation ($r = -0.757$) was found between the two sulfur rich protein fractions legumin and albumin. Without exception lines high in albumin were low in legumin and vice versa. This relationship is an important consideration in breeding to improve protein quality.

The relative abundance and nutritional quality of the albumins, stressed in the first experiment, prompted the characterisation of the albumins at the polypeptide level. The albumins of all 45 lines examined contained two quantitatively major polypeptides of M_r 8000 and 22000. Both of these albumins are cotyledon specific proteins. The M_r 8000 polypeptides were utilised during germination indicating they function as albumin storage proteins. Both polypeptides are relatively rich in sulfur amino acids as was shown by ^{35}S labeling of seed proteins during their synthesis. Consistent with this, the level of these polypeptides was markedly reduced under sulfur deficiency conditions.

The inheritance of albumin and legumin contents was studied in a cross, in which the parents differed in albumin and legumin contents, to investigate whether the negative correlation between these two sulfur amino acid rich fractions could be broken genetically. Four individual F_2 seeds were identified which showed the desired recombination of high albumin with high legumin, indicating that sulfur amino acid content in these seeds should be increased. In this

cross the genetic variations were predominantly additive for legumin but largely dominant for the albumins. These results suggest that breeding for improved protein quality is possible, but must take account of both albumins and legumin as sources of sulfur amino acids.

Quantitative estimates of protein content, and legumin, vicilin and albumin contents were made to measure the effects of exogenously applied growth regulators on seed protein content and composition in peas. Naphthaline-acetic acid (NAA), benzyl adenine (BAP), abscisic acid (ABA) and gibberellic acid (GA_3) were administered for 30 days after full bloom. Specific responses to hormone applications were observed. While GA_3 had no effect on protein composition ABA treatments caused an increase in vicilin content. Legumin increased in response to NAA and BAP treatments and was further increased by application of a mixture of NAA and BAP. This treatment also caused a marked increase in albumin content but a significant decrease in vicilin content. These changes in the level of protein fraction indicate that hormones are involved in regulating seed protein synthesis in peas.

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List of Abbreviations

SDS	=	sodium dodecylsulfate
PAG	=	polyacrylamide gel
PAGE	=	polyacrylamide gel electrophoresis
TCA	=	trichloroacetic acid
M_r	=	molecular weight
PI/G	=	Plant Industry Genetics number

1. INTRODUCTION

1.1. Legume seeds

Because leguminous seeds are of considerable agricultural importance they have understandably been studied in great detail. The high protein content of the seeds and green matter of leguminous plants makes the family to which they belong one of considerable importance, especially in the subtropics and tropics where there is a great need for protein sources for human consumption.

The pea, (Pisum sativum/arvense) as are several other pulses, is a crop with many beneficial qualities. It is comparatively high in protein content, up to 32 percent, with an average of 22 percent. It has wide adaptability, being grown from almost tropical to arctic conditions. The pea has no, or very low, artificial nitrogen requirements, since it fixes atmospheric nitrogen in symbiosis with Rhizobium in root nodules and has a yield potential as high as or higher than that of cereals. The protein of pea seeds is a source of food for man and monogastric animals, but as with other legume seeds these proteins are comparatively low in sulfur amino acids. This is an important factor when designing a mixed diet of cereals and legumes because the total protein will be utilized only to the level of the most limiting essential amino acid. The extent to which peas are grown on a world wide basis as a grain legume crop is shown in Table 1.1.

1.2. Why the Pea?

The pea is one of the most thoroughly investigated organisms genetically. As pointed out by Blixt[2] in a paper "Some genes of importance for the evolution of the pea in cultivation" man has been

Table 1.1. World wide growing of dry peas.

(from: FAO Production Yearbook Vol. 35, 1981

Food and Agricultural Organization of the United Nations[1])

	Area harvested 1000 ha	Yield kg ha	Production megatons
World	7890	1041	8215
Australia	59	1115	66
New Zealand	25	2800	70
Africa	413	746	308
Latin America	168	719	121
Near East	33	1419	47
Far East	624	674	421
North America	114	2106	241
Western Europe	110	2589	285
USSR	4663	858	4000
China	1500	1530	2300
India	600	667	400
France		4040 (highest yield)	
Burrundi		320 (lowest yield)	
Pulses			
World	65693	645	42403
Australia	190	943	179
Soybeans			
World	50219	1751	87941
Australia	46	1522	70
Cereals			
World	740148	2248	1663828
Australia	17267	1346	23242

interested in the pea and has observed spontaneous mutations in Pisum centuries ago. The first such mutant observed was mentioned in 1306 and concerns anthocyanin; recessive a- inhibits anthocyanin production, changing flower colour from red to white and removing the bitter taste of the seed. Other mutants were reported around 1500; R round to r wrinkled seeds, and Le long internode to le short internode zig-zag orientated*. Presently 295 known and symbolised genes are preserved in the pea collection at Weibullsholm Plant Breeding Institute Landskrona, Sweden[2]. The institute is responsible for data storage concerning quantitative and Mendelian characteristics and for linkage analysis of data, collaborating with institutes around the world including Australia[3].

The gene bank in Weibullsholm concentrates on preserving genes rather than genotypes. Until recently only two of the 295 genes were for protein characters. Amp 1- fast and slow moving forms of aminopeptidase and Lap- fast and slow moving forms of leucine aminopeptidase mapped to chromosome 3. Two more structural genes for proteins were mapped by Davies[4], and Matta and Gatehouse[5], Lg-1 a major legumin gene to a locus near the r-tl segment of chromosome 7 and Cvc a convicilin gene to a locus between s and k on chromosome 2. Genes symbols for a number of structural genes for other variant forms of legumin and vicilin are stored in the data bank awaiting mapping. Much needs to be done, particularly with respect to many characters which are polygenic and are important and interesting from an agricultural point of view i.e. polygenic control of yield, protein content and protein composition.

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*Footnote: A list of gene symbols and chromosomal location of genes used here and elsewhere in the thesis is published in Pisum Newsletter Vol. 10, 1977, by S. Blixt.

1.3. Pea Breeding

The practical uses and limitations of peas as a grain legume crop and protein source have been thoroughly examined at the Crop Development Centre, University of Saskatchewan, Canada in research programmes of production, utilisation and marketing of field peas[6]. In most field pea breeding programmes the emphasis is on yield, protein content, adaptation of breeding lines to specific local environments and pest and disease resistance. In Australia the pea is a relatively minor agricultural crop, yet it makes up a third of the pulses grown (Table 1.1). There are only two active pea breeding programmes in Australia. One is at the Victorian Crop Research Institute, Horsham where the grain legume breeder Dr. G.J. Berry has stated: "The objectives for field pea breeding are to produce a cultivar which is structurally adapted to cropping, developmentally attuned to the growing season, with rapid vigorous growth and reliable high yields of good quality seed. It is also important that field peas fix adequate nitrogen for succeeding cereal crops and that optimum sowing time is not the same as for cereals".

The other field pea breeding programme is at the South Australian Department of Agriculture conducted by Dr. S.M. Ali, a Plant Pathologist who breeds for disease resistance to blight, foot rot and charcoal rot, all three of which can severely reduced grain yield[7,8]. In both these programmes no mention is made of breeding for increased protein content and/or protein quality.

1.4. Protein Content of Pea Seeds

Protein content, also often referred to as crude or total protein, of pea seed flour is usually determined by Kjeldahl nitrogen

determinations and multiplying that nitrogen value x 6.25. Considerable variation for protein content exists in the genus Pisum. Slinkard[9] examined 1450 lines, strains and varieties of the USDA world collection for protein content and found a range from 14 to 39 percent protein. In the Weibullsholm collection 836 pea lines of the spontaneous collection and 111 induced mutation lines were examined for variation in protein content by Blixt[10], who found a range in protein content of 17 to 32%. The spontaneous collection, consisting of landraces, wild and primitives, selections, spontaneous mutants, cultivars and cross derivatives averaged 23.8% protein which is lower than that of the 111 induced mutation lines which averaged 25.3%. But the induced mutants, as expected from the narrow origin, had a lower variation in protein content than did the spontaneous material. This contrasts with the finding by Gottschalk[11] who found variation in protein content ranging from 16 to 33 percent in 138 X-ray and neutron induced mutants from one initial line. Of added interest is Gottschalk's claim that nearly all these 138 mutants vary from the initial line in only one gene.

1.5. Genes That Affect Seed Protein Content and Composition

A number of morphological genes have been identified which directly or indirectly affect protein content[10-15]. Lines recessive in a, z, le, r and i were found on average to have higher protein content than A, Z, Le, R and I lines.

[Mutations: A-a lack of anthocyanin production, Z-z no testa decolouration, Le-le reduced internode length, R-r round seed to wrinkled seed, I-i cotyledon colour yellow to green].

Another gene, "mifo" with close set shallow impressions on the testa, the so called golf ball type, is of special interest. Mifo lines show the highest average yield found in the Weibullsholm collection, and out of 10 lines noted for their high protein content no fewer than 9 lines are recessive for mifo combined with either i or r. The effect of seed shape R/r on seed protein content and composition has been studied in considerable detail[4,6,16,17]. Wrinkled seeded lines rr, when compared with genetically similar round seeded lines R-, have a higher protein content by 3 to 4%, but lower seed weight, less starch and lower emergence. rr lines also have lower yields but are equal to R- in protein yield per unit area.

1.6. Protein Content and Yield

Protein content and seed yield are best considered as competitive factors in the energy conversion process of crop productivity, and in most legumes investigated negative correlations between protein content and yield were found. In peas this correlation, protein content to yield, is also significantly negative[6,10], except in le subcollections where the correlation is positive but non-significant. Over a number of years, Polish plant breeders[18] found that in their endeavour to breed cultivars with higher protein content, their progress had been less than expected. A computer analysis of data collected over a number of years from a number of lines and crosses showed that when protein content was not more than 27%, increasing seed yield did not cause a significant reduction in protein content. They concluded that the best possibility of increasing the protein yield would be either by an increase in seed yield when protein content was no more than 27% or by increasing the protein content to

the threshold level of 27% in already high yielding varieties. Another correlation, that between protein weight per seed and seed weight, is also of interest. These two characters are significiantly positively correlated. Narrow sense heritability for seed weight in peas was moderately high, 56 to 79 percent, indicating that this yield component can readily be changed by selection[6]. A breeding strategy to increase protein yield per unit area, that is now generally accepted is to increase seed weight.

The protein content of a seed is determined by a large number of interactions within the genome and between the genome and the environment. Studies of the effects of genotype and environment interaction on percent protein in peas indicated that the environmental effects are as large as or larger than the genetic effects[6,12-14]. For example, it was found that protein content in replications of one variety in one year varied from 19.3 to 22.5%, while the averages over 9 years of the same variety varied from 21.0 to 26.6%. Obviously this variation in protein content by about 5% is an additional obstacle to the breeder.

1.7. Extractable Protein from Pea Cotyledons

In the preceeding paragraph I have referred to protein content measured by Kjeldahl determinations. But the quantitative analyses, biochemical characterisations and genetic studies of seed proteins have been made on the extractable protein from pea cotyledons. It is important that testas be removed before protein extraction from cotyledons since testas often contain substances such as pigments, tannins and phenolics[19] which may interfere with protein extraction procedures and/or give spurious results in colour sensitive protein

determinations by Biuret, Lowry or Hartree methods[20-22]. The cotyledonary protein extractable in buffered saline as a percentage of the crude protein is about 80%. In 45 lines of peas examined by myself[17] the extractable protein ranged from 62.5 to 86.8%. The extractable protein from wild or primitive forms of peas was lowest with a mean of 70.5%, while that of field peas, round and wrinkled garden peas was about 80%. These percentages are in agreement with estimates by Wolff[23] who found that extractable protein ranged from 75 to 85 in 30 Pisum mutant lines examined. Other estimates of extractable protein (globulins plus albumins) by Mueller[24] ranged from 34.5 to 60.0%. These percentages are unrealistically low estimates, and are most likely due to incomplete protein extraction procedures.

1.8. Composition of the Extractable Cotyledonary Protein

The extractable protein consists of two types, the albumins which are metabolic proteins, both enzymatic and structural, concerned with normal cellular activities and the globulins which are primarily storage proteins. In broad terms, storage proteins can be defined as those which are (a) present in the mature seed in quantity, (b) found only in the seed, (c) hydrolysed during germination and early seedling growth, and (d) commonly rich in amides. The storage proteins of peas have been fractionated into two major components, legumin and vicilin on the basis of differential salt solubility, sedimentation velocity, chemical composition, isoelectric point, gel filtration, ion exchange chromatography, electrophoretic behaviour, serological properties and amino acid composition (see reviews by Millerd[25] and Derbyshire[19]).

The synthesis and accumulation of the storage proteins vicilin and legumin in developing cotyledons have been studied from many aspects[26,27]. There are two phases of cotyledonary growth, the initial one of intensive cell division followed by a longer period of growth by cell expansion. The onset of first vicilin and a day later legumin synthesis occurs in the latter part of the cell division phase. However most of the protein is synthesised and accumulated during growth by cell expansion.

During this cell expansion phase legumin and vicilin are accumulated and stored in large amounts in special membrane bound organelles called protein bodies[28-33]. These protein bodies arise by the progressive deformation and gradual breaking up of the large central vacuole. Both vicilin and legumin are found in the same protein bodies. The sequential appearance and accumulation of several distinct proteins, six of which are either vicilin or legumin species, have been analysed by crossed immunoelectrophoresis[34] during cotyledonary development. Both the number of components detected and the order of appearance during development were similar in the genotypes examined, but the quantitative representation of legumin relative to other components was genotype specific.

Thus the extractable protein from mature pea cotyledons is made up of the storage proteins legumin and vicilin[35-39], classical globulins which make up 65 to 80% of the total, while the remaining 20 to 35% are albumins[17]. The legumin, vicilin and albumin contents and their ratios are genotype specific[4,39-43] but are also environment dependent[43-45]. A quantitative study of the cotyledonary proteins of 45 lines of peas divided into four groups, primitive or wild forms, field peas, and round and wrinkled garden peas showed that the

proportions of the seed protein fractions are not only genotype specific but they are also quite group specific (see chapter 2). The proportions of legumin and/or vicilin as percent of either total extractable protein or of total globulins are easily determined by electrophoresis of crude protein extracts or of globulins on cellulose acetate membranes followed by densitometry[4,42,45], or by rocket immunoelectrophoresis[45,67]. Both of these methods are suitable for screening large numbers. For example, quantitation of legumin showed variation in legumin content ranging from 0.5 to 65% in 171 lines of peas examined by Davies[4,42].

Quantitation of the albumin fraction has been more difficult and few reliable estimates are available. Methods which I have developed recently, namely globulin to albumin ratio determinations and rocket immunoelectrophoresis of albumins, make it possible now to measure this protein fraction accurately either as percentage of extractable protein or in absolute amounts as mg/seed. Relative estimates of seed protein fractions expressed as a percentage, are useful indicators of the existing between-line variation but they do no account for differences in absolute amounts of fractions which are determined using a seed weight basis. Seed weight has been shown to be positively correlated with extractable protein and with albumin content[17]. Quantitation of seed protein fractions should therefore be expressed in mg/seed, thus making comparisons between lines more meaningful.

1.9. Protein Quality

Yield and protein content are of prime concern to the pea breeder but protein quality also needs to be assessed in elite lines derived from breeding programmes because it is important in many

diets and animal feeds. A feed based solely on cereals will be deficient in lysine whereas one based entirely on legumes would be deficient in sulfur amino acids. The protein of peas in common with other legume seed proteins is comparatively low in methionine and cysteine. Traditionally protein quality has been assessed in terms of amino acid composition relative to the WHO reference pattern[46], but amino acid analyses are labourious and slow and not suitable for screening the large numbers commonly necessary in breeding programmes.

However, methods other than amino acid analyses have been proposed to study protein quality[47-52]. Total sulfur as percentage of dry weight of flour has been proposed as a coarse indicator of sulfur amino acid levels[47]. It can be measured rapidly and cheaply by X-ray fluorescence spectrometry[51] in the flour of one cotyledon while the other cotyledon containing the embryo can be kept for growing on. The carbon to nitrogen ratio, measured by elemental analysis is another indicator of protein quality. A C:N ratio of 17:1 has been suggested for diets and animal feeds with a higher ratio indicating protein deficiency[49]. Legume seeds including peas, because of their high protein content, have a mean ratio of about 10.5:1. Determination of protein nitrogen and protein sulfur on the other hand are destructive processes and require isolation of the extractable protein. The sulfur deficiency of legume seed proteins is reflected in the protein N:S ratio of about 23.6:1 when compared with the recommended ratio of less than 19:1 for adequate nutritional requirements[49].

Protein quality in peas has also been evaluated by rat feeding experiments measuring parameters such as nitrogen efficiency (NIE),

protein efficiency ratio (PER), true digestibility (TD), biological value (BV) and net protein utilisation (NPU)[40,47,52-55].

In peas most interest in terms of protein quality has centred on the storage proteins legumin and vicilin because these globulins together make up 65-80% of the seed proteins. They are the quantitatively major fraction and biochemically and genetically they have been studied in more detail than have the albumins. It has been suggested that protein quality in peas might be considerably improved by changing the proportions of the two storage proteins, i.e. by increasing legumin content and reducing or even replacing vicilin[42,53-60]. Of the globulins, legumin has a higher sulfur amino acid content while vicilin has the higher lysine content (Table 1.2). However the albumins have a more favourable amino acid composition than either of the globulins, combining a high sulfur amino acid content with a high lysine content. The albumins are a quantitatively significant protein fraction, 20-35% of the seed protein, and it has already been suggested that greater emphasis be put on this fraction when considering protein quality[42,56,59-62].

For comparison I have tabulated amino acid compositions of legumin, vicilin and albumins, total globulins and total protein of different lines of Pisum determined in a number of laboratories (see Table 1.2).

Much of the apparent differences in amino acid composition and individual amino acid contents is probably due to between line variation. Casey[57] found variation in methionine and cysteine content of legumins from 6 lines ranging from 1.09 to 1.75%. In albumins from 5 lines, examined by Hurich[59], methionine and cysteine content varied from 3.75 to 4.59%. As shown in the table, the albumins are

Table 1.2. Amino acid composition of different seed protein fractions g/100 protein

	WHO	Vic	Vic	Vic	Leg	Leg	Leg	Leg	Alb	Alb	Alb	Glob	Glob	To	To
Lysine	5.5	7.9	6.3	8.7	4.4	5.2	4.9	4.5	10.6	7.4	8.9	7.4	7.9	7.3	6.9
Valine	5.0	4.6	4.9	4.2	5.1	4.8	4.4	5.4	5.5	6.7	4.9	4.4	5.3	4.4	4.1
Methionine	3.5	0.2	0.2	0.4	nd	0.5	0.5	0.5	1.1	1.0	1.4	0.6	0.5	0.8	0.7
Cysteine		0.4	0.4	trace	0.6	0.7	0.7	1.1	0.8	1.4	2.5	0.8	0.3	1.0	1.1
Isoleucine	4.0	5.1	4.8	4.6	4.0	4.1	3.6	4.2	6.1	5.0	4.0	4.2	5.9	3.8	3.7
Leucine	7.0	9.2	8.9	9.5	7.6	8.0	8.0	7.5	5.9	6.2	6.1	8.6	8.7	7.4	6.5
Tyrosine	6.0	3.0	2.6	3.4	1.7	2.6	3.5	2.4	5.2	3.2	4.7	3.4	3.1	2.9	-
Phenylalanine		6.2	4.7	6.0	3.6	4.2	4.8	3.6	5.7	3.7	5.0	5.4	3.1	4.4	4.1
Threonine	4.0	3.4	3.5	2.9	3.5	3.2	3.2	3.5	5.2	6.5	5.5	3.5	3.2	3.7	3.6
Tryptophane	1.0	0.1					nd								1.9
Aspartic acid		12.0	11.2	13.2	11.9	12.7	11.6	11.0	11.2	11.1	11.9	12.0	9.9	13.0	
Serine		5.8	6.9	5.7	6.8	5.9	5.0	6.2	3.6	6.6	5.3	5.4	3.6	4.7	
Glutamic acid		19.3	15.8	20.7	20.1	16.5	20.8	19.6	15.9	11.5	15.1	19.5	23.9	18.5	
Proline		3.5	nd	4.2	5.4	nd	5.2	5.5	4.8	5.3	4.6	4.1	4.3	4.2	
Glycine		3.1	5.7	3.0	7.5	6.2	4.2	7.1	5.0	9.4	5.0	3.8	3.6	4.2	
Alanine		3.0	4.5	3.0	6.0	5.8	4.3	5.8	5.2	8.9	6.0	3.9	3.7	4.3	
Histidine		2.1	2.1	2.1	2.6	2.5	3.0	2.6	2.9	2.0	2.9	2.9	2.3	2.1	2.2
Argenine		7.3	6.4	8.6	8.6	7.5	12.5	9.3	5.7	4.4	6.3	9.4	8.8	8.7	9.5
Reference	46	19	63	59	19	63	59	57	55	61	59	55	50	50	

nd - not determined

at least two-fold richer in sulfur amino acids than legumin as well as being richer in lysine. This suggests that increasing the albumin fraction would have a greater effect on protein quality than increasing legumin at the expense of vicilin, especially if one considers the lysine content of vicilin. This view is supported by Bajaj's observation[55] that strains of peas with higher protein efficiency ratios (PER) have a higher albumin content.

Theoretically it can be calculated that high and low legumin lines have a very similar sulfur amino acid content[17]. This apparent similarity is probably largely due to the negative correlation between legumin and albumin content ($r = -0.76$)[17]. For every 2.5-3.0% increase in legumin content, there is a corresponding 1.0% decrease in albumin content and vice versa (see chapter 2). Analyses of methionine content by Slinkard[6] showed that methionine content (as mg methionine per g of protein) was very similar for round and wrinkled seeded lines, high and low legumin content respectively.

To increase protein quality in Pisum the negative correlation between legumin and albumin contents has to be broken. The aim should be to genetically alter the proportions of the three seed protein fractions and to identify and select recombinants that combine high levels of legumin with high albumin content. As reported in chapter 4, this latter proposal has been studied in a cross between two lines of peas, one with high legumin low albumin and the other, low legumin and medium to high albumin.

1.10. The Albumins

The water soluble fraction of the pea seed proteins, the albumins, are thought to consist of mainly metabolic proteins, both

enzymes and structural proteins. At least one of the major albumins however behaves as a seed specific storage protein; it makes up ≅ 4.5 percent of the total extractable cotyledonary protein and is utilised during germination. There are probably many thousands of albumin species, most of which individually contribute little to the total protein content. Collectively, however, the albumins are quantitatively as significant as either of the storage protein fractions legumin and/or vicilin.

1.10.1. Isolation of Seed Albumins

The storage proteins legumin and vicilin have been reviewed by Millerd[25] and Derbyshire et al.[19] with regard to their structure, extraction, purification and characterisation. No similar review of the albumin seed protein fraction is available. Therefore some earlier methods of albumin extraction and purification[35-37,41,42] are described in some detail and these are compared with methods developed by Schroeder.

The extractable protein from cotyledonary flour has been separated into albumin and globulin fractions by methods described by Danielsson[35-37], Davies[42], Przybylska[41]. Proteins were extracted from finely ground flour with phosphate buffer pH 7-7.5 containing 0.2-0.5 M NaCl. The crude extracts are centrifuged and the proteins are precipitated from the supernatant by ammonium sulphate (70-85%). After centrifugation the protein pellet is resuspended in extraction buffer and dialysed against water. This precipitates the globulins, the albumins remain in solution. In another method, developed by Grant[64], an albumin rich protein fraction is prepared by water extraction of flour, removal of the insoluble residue by

centrifugation, after which the protein is precipitated from the supernatant using cold acetone.

The above mentioned methods isolate an albumin fraction which is suitable for qualitative and semi quantitative studies of this fraction, e.g. albumin band patterns on polyacrylamide gels, amino acid analyses and the determination of proportions of albumins and globulins in the protein precipitated with ammonium sulphate. However no attempts were made to investigate what proportion of the total extractable protein, or of individual protein fractions, remained in the supernatant after precipitation. Protein determinations, of the TCA precipitable proteins from the supernatant followed by SDS PAG electrophoresis could have resolved these questions.

Using a method of Schroeder[17] the extractable protein can be separated cleanly and reproducibly into an albumin and a globulin fraction. The purity of the fractionation was monitored by SDS PAG electrophoresis[65] (Fig. 1.1) and immunological studies[66]. An antiserum specific for albumins was raised in sheep and this antiserum was used extensively in the quantitative determinations of albumin contents, (see Chapters 3,4,5 for further details). The albumins prepared by this method retain their enzymatic activities as was shown by isozyme studies (H.E. Schroeder and A.H.D. Brown unpublished data).

1.10.2. Fractionation of Albumins by Polyacrylamide Gel Electrophoresis and uses of Albumin Band Patterns

Electrophoresis of albumins on SDS-PAG under reducing conditions resolves a large number of polypeptides (Figs. 1.1 and 1.2). These range in molecular weight (M_r) from 92000 to 6000.

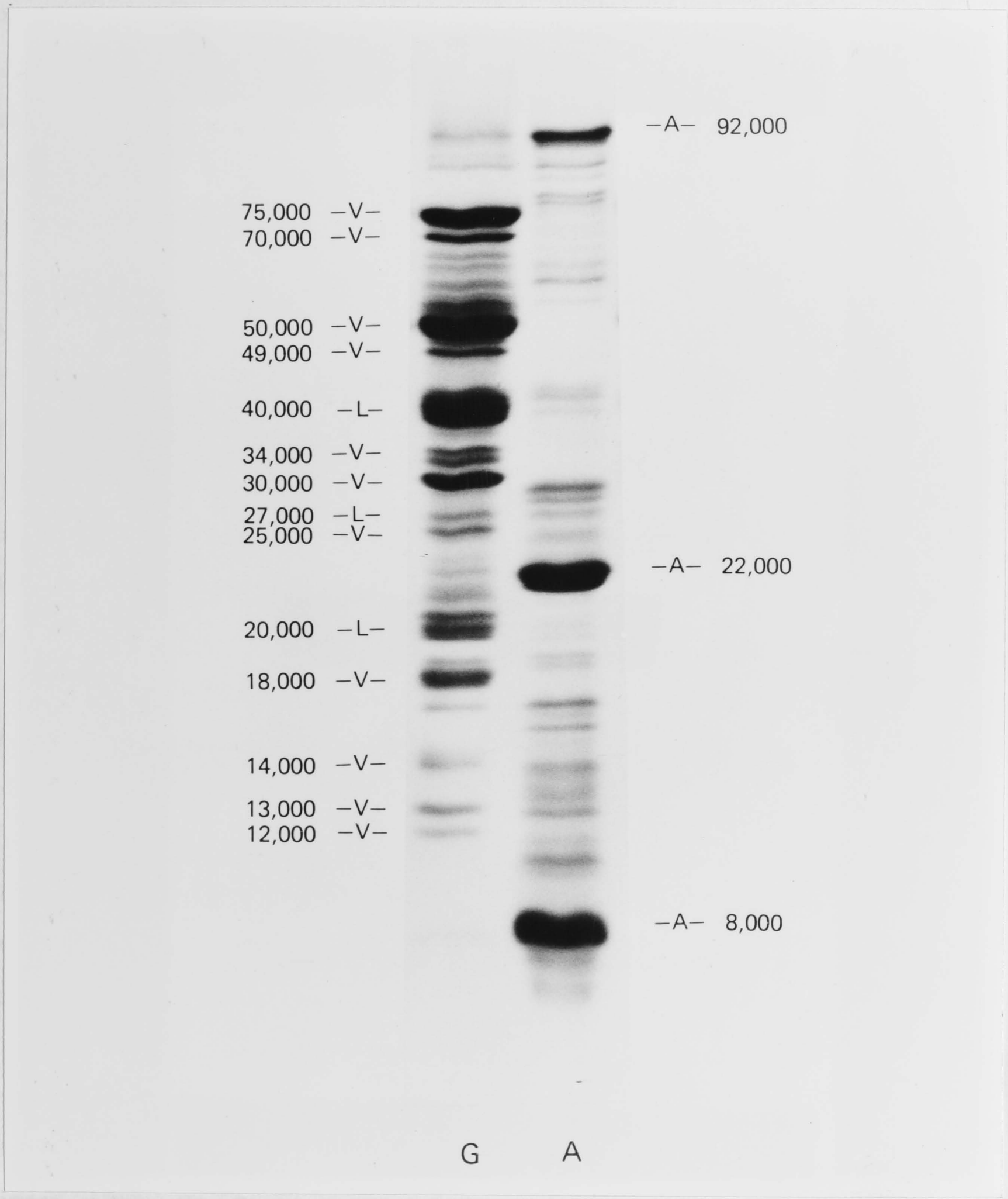

Fig. 1.1. Fractionation of globulins (G) and albumins (A) of cultivar Greenfeast by SDS PAGE under reducing conditions. (Gradient gel 12.5-25 percent). Molecular weights of polypeptides of globulins vicilin (V) and legumin (L) shown on the left, those of the albumin polypeptides (A) on the right vertical axis.

Polypeptides of M_r 22000 and 8000 are quantitatively significant and of widespread occurrence in the genue Pisum[67]. Together they make up about 35% of the albumin proteins and account for about 50% of the sulfur amino acid content of this fraction. Electrophoretic fractionation of albumins on PAG under non-dissociating conditions resolve a number of major and minor bands (Fig. 1.2). These banding patterns have been used in studies of the albumin fraction[68-72]. They are genetically determined and are not influenced by environmental factors[69]. Band patterns of the predominant albumins have been useful in taxonomic studies, Przybylska[41] examined the relationship of 50 widely divergent lines of peas by their albumin pattern, she distinguished five characteristic patterns I-V. Two of these band patterns, I and III, when analysed genetically by Blixt et al.[69] were found to be determined by two alleles of one locus. This is a rather surprising result unless they were looking at a regulatory gene, because each of the major bands making up band pattern I and III when electrophoresed in the second dimension on SDS-PAG under reducing conditions, is made up of a number of polypeptides of different molecular weight (personal observation).

The albumin band patterns have also been used by Wolff[71] in phylogenetic and taxonomic studies. She compared mutants of one initial line, subspecies of the genus Pisum and species of different genera within the Papilionaceae on the basis of their albumin band patterns.

In genetic, biochemical and physiological studies of X-ray and neutron induced mutants[12-14,72,73], which according to Gottschalk[11] differ from the initial line in nearly all cases in one single gene, the

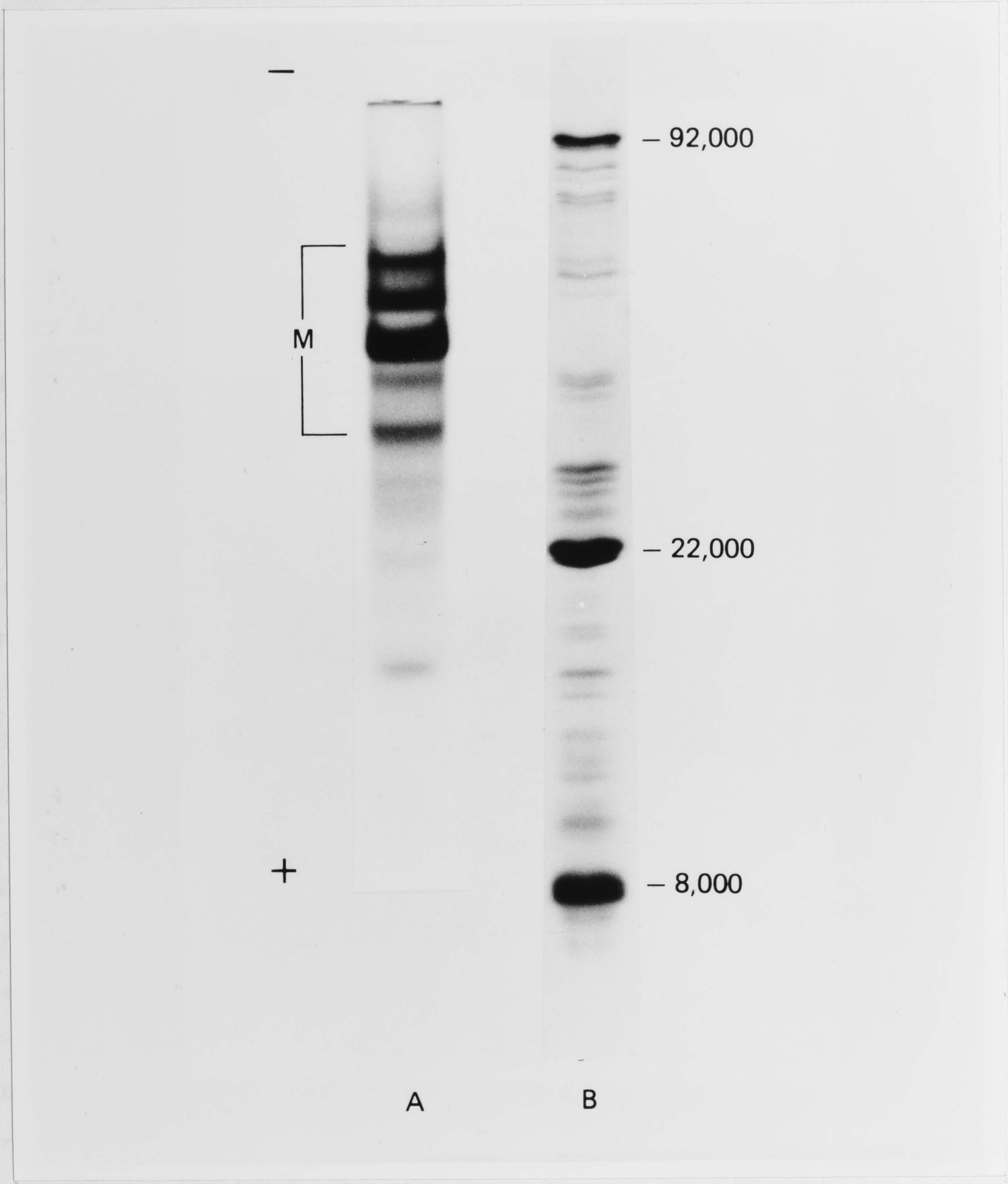

Fig. 1.2. Fractionation of pea seed albumins of cultivar Greenfeast by PAGE, (A) under non dissociating conditions (B) under dissociating and reducing conditions. In (A) M indicates major band region. In (B) molecular weights of quantitatively major polypeptides are shown on the vertical axis.

albumin band patterns were used to investigate whether the mutated gene directly or indirectly affected the seed protein composition of these mutants and whether changes were quantitative or qualitative. "If they show constant deviation from the initial line with regard to the amount or the composition of the seed proteins, they can only be due to the influence of the respective mutated gene", a quote by Gottschalk[11]. A number of mutants in which the albumin patterns differ from the initial line were actually identified. However, except for studies with recombinants no attempts have been made to determine genetically whether these changes in the protein profiles of mutants were due to concurrent mutations of structural genes for specific proteins or whether the one mutated gene identified, indirectly affected seed protein composition.

1.10.3. Immunological Characterisation and Isozyme Studies of Albumins

Compared with the number of albumin proteins thought to be present in the seed protein, a relatively small number of different albumins is resolved by PAG electrophoresis (Fig. 1.2A). More sensitive methods such as immunological techniques and specific enzyme assays have been used to resolve either additional or different albumin proteins. Analysing pea seed proteins by crossed immunoelectrophoresis, Guldager[74] identified 26 different albumins, three of these A_1-A_3 being quantitatively major. Twenty two of these albumins were found to be synthesised prior to the synthesis of the storage proteins vicilin and legumin but the major albumins, A_1-A_3 were synthesised after the onset of legumin synthesis. Labelling of the seed proteins with ^{35}S showed these major albumins to be rich in sulfur amino acids.

Isozyme studies, for the purpose of detecting genetic variability and biochemical and genetic markers in Pisum have been undertaken by Almgard[75], Scandalios and Espirita[76], Przybylska et al.[77], Gottschalk et al.[72,73], and Brown and Schroeder (unpublished).

The use of the albumins in the study of gene regulation and gene expression was advocated by Mueller[78]. His hypothesis was, that in trisomics and aneuploidy an additional copy of a chromosome or lack of a chromosome should result in quantitative changes in proteins from that chromosome. However, those studies were inconclusive.

1.10.4. Biochemical Studies of Pea Seed Albumins

Seed albumins from 5 lines of peas, showing the characteristic albumin band patterns I-V (Przybylska[41]), have been fractionated by Sephadex G-100 column chromatography by Jacubek et al.[70]. Four chromatographic fractions S_1-S_4 were obtained, the percentages of each fraction being 16, 26, 48 and 11 respectively. Similar fractionation of albumins by Grant[64] using Sephadex G-150 resulted in only three fractions and these are thought to correspond to Jacubek's S_1-S_3 fractions. The proteins of the S_1-S_4 fractions were examined by PAG electrophoresis under both non-dissociating and dissociating conditions. This fractionation resolved specific banding patterns and polypeptide compositions of each fraction. Amino acid analysis of individual fractions, S_1-S_4, showed that corresponding albumin fractions from different lines of peas had rather uniform amino acid composition. However, considerable variation in amino acid composition between fractions was observed. In this regard the S_3 fraction is of particular interest. It is the quantitatively major

fraction (48%) and in terms of protein quality it is much richer in sulfur amino acids, especially cysteine, than the other fractions.

The various studies of the albumin seed proteins and their quantitative and qualitative analyses have shown that at least the predominant albumins are amenable to biochemical characterisation and genetic analysis and that selection of desirable components may be possible.

1.11. Seed Storage Proteins Vicilin and Legumin

Seed storage proteins have previously been defined as proteins which accumulate in the seed in significant quantities. The main storage proteins of peas are vicilin and legumin, classical globulins salt soluble water insoluble, which together make up 65 to 80% of the extractable cotyledonary protein. It was also pointed out earlier that vicilin and legumin contents, as percent of total protein or globulins, can vary greatly from line to line but that the proportions are genotype specific.

1.11.1. Characterisation of Storage Proteins

In mature seeds legumin is a 12S protein (M_r ~ 360000) that consists of six acidic subunits (M_r ~ 40000) and six basic subunits (M_r ~ 20000) linked together in pairs by disulfide bridges[19]. A number of quantitatively minor polypeptides (M_r ~ 37000, 27000 and 18000) nearly always copurify with the major legumin and are recognized as belonging to the legumin fraction[78]. Heterogeneity and hereditary variations in the major and minor polypeptides of legumin are resolved by polyacrylamide gel electrophoresis[79-81]. Vicilin in mature seeds is a 7-9 S protein (M_r ~ 150000-200000) and it has a more complex subunit structure than legumin. Vicilin can be

fractionated into distinct oligomers on the basis of electrophoretic mobility, differential solubility and isoelectric precipitation[19,25,39,79,82]. These vicilin oligomers are made up of at least 13 different polypeptides in various proportions. The abundant polypeptides of the vicilin fractions have molecular weights of 75000, 50000 (several), 30000 and 18000. The less abundant polypeptides have molecular weights of 70000, 49000, 34000, 25000, 14000 (doublet), 13000 and 12000. Polypeptide patterns of legumin and vicilin fractionated by SDS-PAGE under reducing conditions are shown in Fig. 1.3.

1.11.2. Synthesis of Legumin and Vicilin

The time course of synthesis of the polypeptides of vicilin and legumin and the site of their assembly into protein oligomers was studied by Chrispeels et al.[83,84]. It was shown that legumin and vicilin are synthesised and initially sequestered into oligomers of 7-9 S vicilin and 8S legumin in the endoplasmic reticulum. Legumin is present in the ER as polypeptides of M_r 60000-65000, whereas vicilin is represented by polypeptides of M_r 75000, 70000, 50000 and 49000. Further it was found that legumin is transported to the protein bodies as an 8S oligomer.

Proteolytic processing of these precursors (M_r 60000-65000) and assembly of 12S legumin occurs in the protein bodies. Vicilin is transported to the protein bodies already assembled into 7-9S proteins, but containing only polypeptides with $M_r > 49000$. The smaller vicilin polypeptides are the result of post-translational processing of some of the $M_r \sim 50000$ precursor polypeptides in protein bodies.

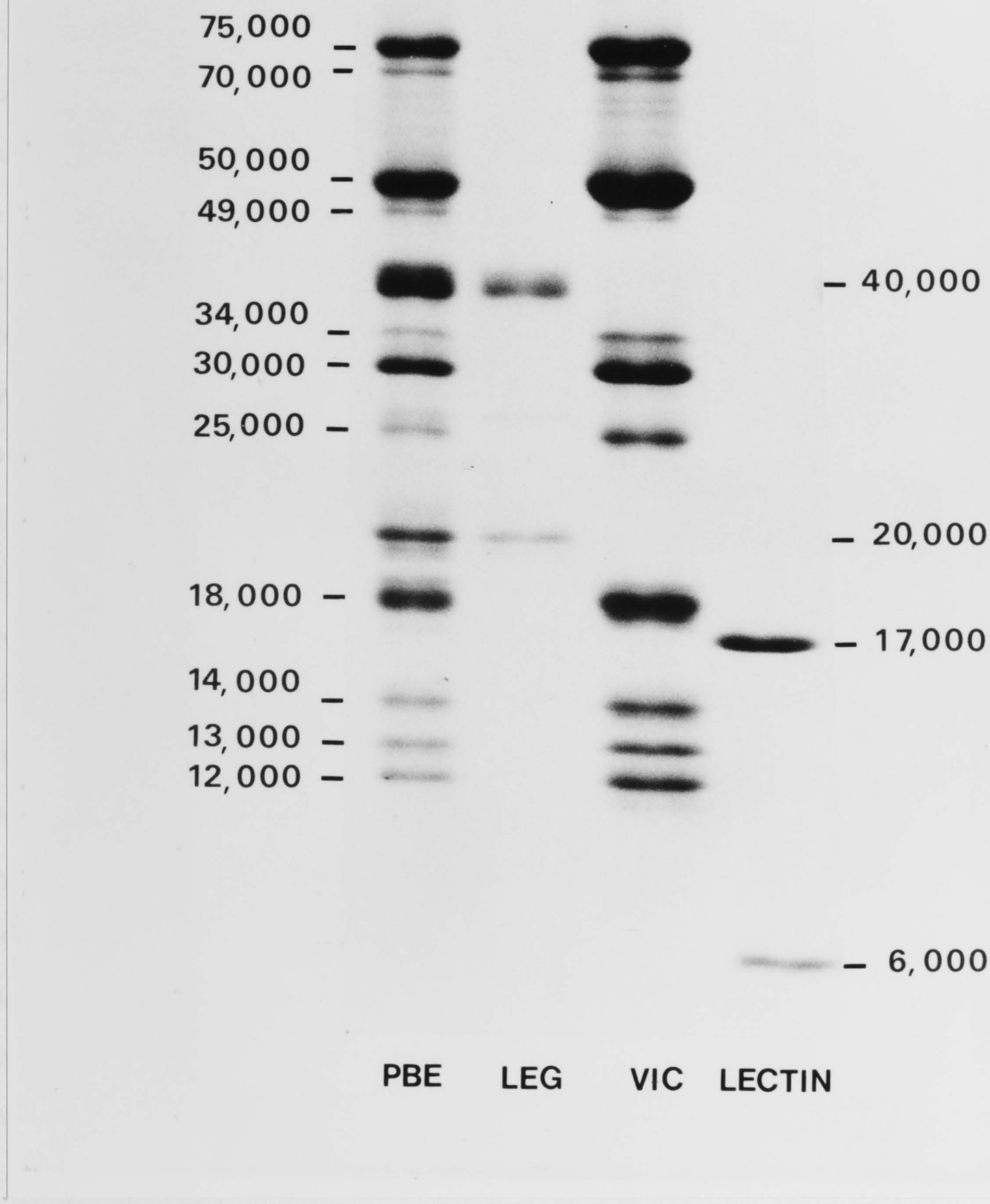

Fig. 1.3. Fractionation of protein body extract (PBE), legumin (Leg), vicilin (Vic) and lectin of cultivar Greenfeast by SDS PAGE under reducing conditions. Molecular weights of polypeptides of the different seed protein fractions are shown on the vertical axes. Polypeptides of vicilin on the left, polypeptides of legumin and lectin on the right.

1.11.3. The Precursor Product Relationships within the Legumin and Vicilin Families

One of the first indications that storage proteins might be synthesised as precursors was provided by the finding that when RNA from membrane bound polysomes of pea was translated in an initiating cell-free system, the products, although they were not of the same size as authentic polypeptides, did contain storage protein[85-89]. This indicated at least two processing steps; one, removal of signal peptides, the other, post-translational cleavage. The precursors of legumin, M_r 60000-65000 subunits are proteolytically cleaved in protein bodies to yield M_r 40000 and 19000 polypeptides[90,91]. These polypeptides are assembled to make up the 12S legumin oligomers (M_r 360000) which consist of 6 x 60000 molecular weight subunits, each consisting of an acidic (M_r 40000) and basic (M_r 20000) polypeptide linked by disulfide bonds. Legumin extracted from protein bodies and fractionated by SDS-PAGE under reducing conditions resolves families of both acidic and basic polypeptides. Within each family there exists microheterogeneity, the molecular and genetic basis of subunit multiplicity and variability in legumin has been studied in detail[39,80,81,92]. But it is still not clear whether the observed heterogeneity in the legumin subunit classes arises from initial heterogeneity in legumin genes or whether it arises by post-translational modification of initially homogeneous gene products.

In vicilin the primary translation products are M_r 75000, 70000 and 50000 (3-6) subunits[83,84,93,94]. The group of M_r 50000 polypeptides has been resolved into 3-6 components by SDS-PAGE, but an even more complex pattern is revealed by isoelectric focusing.

In protein bodies of mature seeds vicilin exists as 7-9 S oligomers, fractionation of vicilin by SDS-PAGE resolves more than 10 subunits (Fig. 1.3). There is now good evidence that all subunits of less than M_r 50000 arise by processing[94-97]. The interrelationship of the group of M_r 50000 subunits and the relationship of smaller than Mr 50000 subunits with the putative M_r 50000 parent molecules has been studied by serological methods, by pulse-chase labelling in vivo, amino acid analyses and sequences, and by nucleotide sequencing of cDNA clones complementary to mRNA for members of the M_r 50000 group[94-97]. These studies have led to the identification of processing sites in the M_r 50000 parent molecules which allowed ordering of the positions of the smaller subunits relative to the precursor. Serological techniques (Western-blot) and sequence analysis of the M_r 50000 and lower molecular weight polypeptides have shown that the small polypeptides are homologous to different regions of the high molecular weight chains.

Evidence of molecular heterogeneity in the vicilin fraction has accumulated from many sources[39,82,93,97,98]. What has been difficult to reconcile was the M_r of different vicilin oligomers and vicilin fractions with respect to the number and M_r of polypeptides resolved by SDS-PAGE. The post-translational processing of only a small number of the larger vicilin polypeptides could account for these anomalies and the precursor studies have given new insight into how these different 7-9 S vicilin molecules may be assembled.

Another quantitatively minor seed storage protein in protein bodies is pea lectin. It should be mentioned, because lectin also exhibits the precursor-product relationship. Pea lectin is also synthesised with a leader sequence and is initially detected as

precursor of M_r 23000 which is cleaved post translationally to yield M_r 17000 and 6000 polypeptides[99,100].

1.11.4. Analysis of Pea Seed Proteins using recombinant DNA Technology

In the preceeding section it was pointed out that most of the precursor product relationships were elucidated by pulse-chase labelling experiments, amino terminus analysis and the use of cDNA clones. In Canberra a representative set of cDNA clones for primary translation products of the major pea seed proteins have been constructed[101,104]. These clones are for vicilin M_r 75000, and three M_r 50000, an unidentified M_r 70000, legumin M_r 60000-65000, lectin M_r 25000, and two major seed albumins M_r ~ 22000 and 13000. These nine clones correspond to nine of the most abundant seed polypeptides. They were used to measure by hybridization (Northern-blot) the relative levels of specific mRNAs throughout seed development. In Northerns total RNA is electrophoresed on agarose gels, blotted onto nitrocellulose paper and then hybridized to radioactively labelled cDNA clones which correspond to mRNA for particular polypeptides. In all cases there was a close correlation between the time at which maximal mRNA levels occurred for a particular polypeptide and when the rate of synthesis of that polypeptide was highest.

Most of the clones are close to the full length of the specific mRNA and therefore contain almost the entire amino acid coding sequence. This has facilitated the comparison of amino acid sequencing of specific polypeptides with sequences of cDNA complementary to those polypeptides[101]. Legumin and vicilin are

products of multigenic families, in the case of vicilin at least three families of genes coding for the M_r 50000 subunits have been identified. Within each family up to six genes are resolved by Southern-blot, indicating perhaps as many as 18 genes for the Mr 50000 vicilins. In legumin 4-6 genes coding for M_r 60000-65000 have been identified. In Southerns, total DNA is digested with restriction endonucleases, electrophoresed on agarose gels, blotted onto nitro-cellulose paper and hybridized with labeled cDNA clones for respective legumin and vicilin subunits.

1.11.5. Studies of Seed Proteins under Conditions of Nutrient Deficiencies

The quantitative and qualitative effects of deficiency of S,P,K, and Mg on the cotyledonary proteins, particularly storage proteins, have been studied[45]. While Mg deficiency had little effect on the proportions of the storage proteins, deficiency of S,P, and K caused characteristic and consistent changes in the proportion of certain proteins. An increase in a quantitatively minor vicilin and a marked increase in legumin was caused by P and K deficiency. S deficiency resulted in relative decreases in legumin, a minor vicilin and two albumins[45,67,102]. Under severe S deficiency legumin and one albumin are virtually undetectable[45,67]. This has led to a study of the cellular mechanisms involved in the regulation of legumin synthesis under S deficiency[103]. Three main questions were asked: i) is there a normal rate of legumin synthesis but an increased rate of degradation, ii) is there a normal level of legumin mRNA but reduced translation and iii) is there a reduced level of legumin mRNA. The results showed that a reduced level of legumin mRNA is

the main factor responsible for the reduced accumulation of legumin. Further studies are now in progress to determine whether the regulatory effect of the sulfur status in the developing seed on the legumin mRNA occurs at the transcriptional or post-transcriptional level. Studies of seed proteins under less severe sulfur deficiency levels[104] have shown that there is little or no depression of total protein synthesis. Under these conditions the decreased accumulation of legumin and sulfur-rich albumins is accompanied by a compensating increased accumulation of the other storage protein, vicilin. This is achieved in two ways, there is an increase in the rate of vicilin synthesis in S-deficient compared with healthy cotyledons, and this is reflected in increased levels of mRNA for the major vicilin polypeptides of Mr 50000. In addition the period of vicilin synthesis is greatly extended in S-deficient seeds.

Another approach to study the effect of S-deficiency on seed proteins was to determine the levels of the sulfur amino acids methionine and cysteine in the free amino acid- and the aminoacyl-tRNA pools[105]. It was concluded that sulfur deficiency during seed development resulted in an 80 percent reduction in the relative level of cysteine in the free amino acid pool of the cotyledons but had no effect on the level of methionine. However, in the aminoacyl-tRNA pool there was no effect on either cysteine or methionine levels.

1.12. Genetics of Pea Seed Proteins

Implementation of breeding strategies aimed at improved seed protein content, protein yield and quality depends on adequate knowledge of the genetic basis and regulation of the functional units

involved. Co-ordinated analyses at both the holoprotein and subunit levels are required if genetic and biochemical investigations of the seed proteins are to be useful to the breeder. The gene action systems responsible for the protein phenotypes of mature seeds not only involve structural loci, specifying primary sequences of the polypeptides comprising these proteins, but also regulatory loci affecting total protein and the relative proportions of different seed protein fractions. Other loci controlling post-translational modifications, transport, assembly and packaging of these various protein components would be of interest to the molecular geneticist rather than the plant breeder.

1.12.1. Quantitative Genetics of Protein Content

Seed protein content (percent protein) in peas has been studied over many years by many authors[6,9,10,15,18,72,73,106-110], and protein contents ranging from 14 to 39% were reported. However, much of the usable genetic variability of this quantitative character is masked by environmental factors[6,12-14]. It is also important to note that in self fertilizing species such as the pea, only the additive variance component (narrow sense heritability) can be used to make progress by selection. The dominance variance (broad sense heritability) even if in the desired direction, cannot be utilised in breeding for higher protein content.

Studies on the inheritance and heritability of protein content in peas indicated that narrow sense heritability for protein content is moderately low. Genetic analysis of protein content in nearly homozygous F_6 and F_7 generations of crosses by Weber[110] showed that the additive variance between progenies which can be used for

selection was only about 15-20 percent of the non-genetic (environmental) variance. Swiecicki et al.[109] studied the inheritance and heritability of protein content in two crosses, high x intermediate and intermediate x low protein content. The authors found that the inheritance of protein content had an additive - dominance character. In the first cross, dominance in the direction of the lower protein content was evident, and the additive variance was moderately low. In the second cross the dominance effect was practically zero and the whole genetic variance was of an additive nature (heritability coefficient 70.4 percent) suggesting good progress by selection can be made. However, these results need to be viewed in terms of firstly the threshold level of 27% protein mentioned previously, and secondly the relationship of protein content as a percentage with other quantitative characters important in protein production such as protein weight per seed, protein yield, seed weight and seed yield. Some of these relationships are listed below. There is no correlation between percent protein and seed weight but a significant negative correlation between percent protein and seed yield. On the other hand protein yield is highly positively correlated with seed yield but only slightly correlated with percent protein, this suggests that greater progress can be made in breeding for increased protein yield by increasing seed yield than by increasing percent protein. In addition there is a high positive correlation between protein weight per seed and seed weight indicating successful selection for protein weight per seed would result in much larger seeds with little or no effect on % protein. Narrow sense heritability for seed weight is moderately high, 56-79%, therefore the yield component can be readily changed by selection. Thus an increase in protein production could be achieved by selecting for larger seeds holding % protein constant.

Another consideration in breeding to increase protein is the individual gene effects on % protein[10-15]. i.e. Wrinkled seeded lines averaged about 3 percent higher in percent protein than genetically similar round seeded lines. Yet this higher protein content cannot be utilised because in hybrid populations where most of the genetic variation in % protein was directly attributable to differences between seed types narrow sense heritability was essentially zero[6].

1.12.2. Genetics of Seed Protein Fractions

As has already been pointed out the cotyledonary protein is made up of the globulins vicilin and legumin (storage proteins) and the albumins. The vicilin, legumin and albumin contents and their proportionate abundance are genotype, line and group specific. Genetic variability in the amounts and proportions of seed proteins has been reported by a number of authors who have used different methods of quantitation[4,17,42,43,45,78,111] e.g. reported variation, globulin to albumin ratio 1.88 to 4.51, vicilin to legumin ratio 0.50 to 3.13, % legumin of globulins 0.5 to 68, % albumins of total extractable protein 18 to 35.

While biochemical studies and genetic analyses of structural genes or families of genes coding for subunits of vicilin and legumin have been steadily progressing no regulatory loci affecting the relative proportions of seed protein fraction have as yet been identified. The genetic analysis and quantitative inheritance of protein fractions until recently were lacking altogether. Quantitative analysis of the cotyledonary proteins revealed a highly significant negative correlation between legumin and albumin contents[17]. Since these two fractions are relatively rich in sulfur amino acids their

relative proportions largely determine protein quality. The joint inheritance of legumin and albumin contents was studied in a cross between two contrasting lines of peas, one with high legumin and low albumin and the other with low legumin and medium to high albumin[112]. Legumin content showed predominant additive genetic variation whereas the dominant variance was the largest component for albumin content. Four F_2 seeds were identified which apparently broke the negative correlation and combined relatively high levels of legumin with high albumin content. The results indicated that when both legumin and albumin contents are subject to positive selection improvement in sulfur amino acid content should be possible. But further studies are needed to determine whether these high levels of legumin and albumin contents can be fixed genetically.

1.12.3. Genetics of Legumin and Vicilin

Gel electrophoresis has been used to investigate genetically controlled variation in storage protein constituents from legumin and vicilin. Using starch gel electrophoresis Hynes[113] identified specific genetically determined variants involving a set of vicilin subunits. These variants were shown to be inherited additively in hybrids between contrasting phenotypes, the patterns were controlled by alternative alleles at a single locus. SDS- and urea-PAGE were used by Thomson and Schroeder[79] to investigate genetically controlled variation of five subunits of legumin and three subunits of vicilin. In each variant system the phenotypes of legumin and vicilin polypeptides from F_1 seeds were additive with respect to the subunit pattern of the parental lines and in reciprocal crosses, no dominance was observed. Variation involving major legumin polypeptides M_r

40000 and vicilin polypeptides M_r 75000, 30000, 12000 and 14000 components were found to be based on allelic alternatives at single loci. Inheritance of minor legumin polypeptides M_r 37000 and 20000 was found to be multigenic. Results of two-dimensional gel electrophoretic studies of variant forms of M_r 40000 legumin polypeptides by Casey[80], who used SDS-PAGE and isoelectric focusing in the second dimension, showed generally good agreement with those by Thomson and Schroeder[79]. Both studies showed that there is both micro-heterogeneity of legumin at the subunit level and genetic variation in the nature of the heterogeneity (see also Thomson et al.[39]).

1.12.4. Mapping of Storage Protein Genes

A survey of the proportion of legumin in a wide range of genotypes suggested that the r_a locus had an influence on the composition of the storage proteins in peas[4]. Alleles at this locus determine seed type, R_a- round seeds, $r_a r_a$-wrinkled seeds. All wrinkled seeds had markedly lower proportions of legumin. F_2 seeds from a cross between wrinkled and round lines were analysed for seed type, legumin content and legumin subunits M_r 40000, linkage studies established the location of the structural genes for the M_r 40000 subunits on chromosome 7 close to the r_a locus[4]. These structural genes for legumin would be equivalent to the locus Lgc described by Thomson and Schroeder[79]. One and two-dimensional SDS PAGE techniques were used by Matta and Gatehouse[5] to study the inheritance of legumin and convicilin subunits in crosses of various peas. It was shown that the major acidic subunit M_r 40000 of legumin behaved as products of a single Mendelian gene with at least five

different alleles. Genetic analysis of F_2 seeds was used to map the major legumin genes, here designated Lg-1, to a locus near the r_a-tl segment of chromosome 7, which is in agreement with Davies[4]. From the same analyses[5] the convicilin gene Cvc was mapped to a locus between s and k on chromosome 2. The Cvc locus appears to be identical to the vicilin locus VcA previously found by Thomson and Schroeder[79], but it is now claimed that convicilin is a storage protein distinct from the rest of the vicilin fraction[5,114]. The mapping of legumin and convicilin genes in peas resolved that storage protein genes are not clustered on the pea genome. A simple polytene chromosome system in peas and its use for in situ hybridization[115] may facilitate mapping of other seed protein genes by-passing lengthy genetic analyses.

1.13. RATIONALE FOR EXPERIMENTAL WORK

A study of the literature of the quantitative and qualitative analyses of pea seed proteins revealed some shortcomings in those analyses. These deficiencies have been mentioned in the appropriate sections of the introduction. The experimental work in Chapters 2 to 5 was undertaken to rectify some of the deficiencies. Although the aims of the present investigations are stated in the introduction of each manuscript, brief reasons why this work should have been undertaken are given below.

The need to study the pea seed proteins quantitatively in their entirety.

In the past major interest has centred on the seed storage proteins legumin and vicilin, the albumin fraction has largely been ignored. Legumin, the more sulfur amino acid rich globulin fraction, has been studied more extensively both quantitatively and biochemically than vicilin. While legumin content has been determined by many authors in a large number of lines, widely divergent in origin and often from spatially different sources and grown under field and/or glasshouse conditions, relatively few reliable estimates of vicilin and albumin contents are available.

Many suggestions on how to improve protein content and quality have also been made, namely, altering the proportions of the storage proteins, or identifying sulfur rich components within protein fractions and determining the genetic basis of their inheritance for possible genetic manipulations. However, these suggestions were mostly based on experimental results involving only a few lines of peas (1-6) or they came from purely hypothetical considerations. The aim of the present study in Chapter 2 was to measure nine seed

protein characters in a large number of lines grown under environmentally uniform conditions.

The seed albumins of peas

As mentioned already, relatively few reliable quantitative estimates of the albumin content have been available. Determinations of the albumin content, by a method described in chapter 2, showed that this seed protein fraction is quantitatively as significant as either of the storage proteins. Fractionation of the albumins of 45 lines by SDS-PAGE resolved a large number of polypeptides. M_r 22000 and 8000 polypeptides were found to be quantitatively major components in all lines examined. The aim of the study in Chapter 3 was to characterise these major albumin polypeptides.

Inheritance of legumin and albumin contents

In the introduction it was pointed out that quantitative genetics of pea seed protein fractions was totally lacking. The important finding that legumin and albumin contents are significantly negatively correlated ($r = -0.75$, chapter 2) showed that protein quality in peas could not be improved by simply altering the proportions of the storage proteins. This led to the study of the inheritance and heritability of legumin and albumin content in Chapter 4. The aim was to identify recombinants with high legumin and high albumin contents and to assess these in terms of their potential use in breeding programmes to increase protein quality.

Hormonal regulation of seed protein composition

Studies in Chapter 2 and 4 showed that legumin, vicilin and albumin contents are genetically determined. However, an intriguing question remains; why a high legumin line makes 2 to 3 times as much legumin as a low legumin line? Assuming that the same number of

structural genes for legumin are operative in high and low legumin lines, an assumption which is currently under investigation, are there factors, other than the environment, which could be involved in the regulation of the synthesis and accumulation of legumin, vicilin and the albumins during cotyledonary development. Hormonal control is one of the possibilities. There is evidence to suggest that hormones play a role in storage protein synthesis from in vitro experiments with Phaseolus vulgaris[116,117] and Brassica napus[118]. Further, seeds of Pisum are a relatively rich source of hormones[119-123] particularly at the time of maximum liquid endosperm content. In our standard line, cv Greenfeast, the onset of vicilin and legumin synthesis coincides with maximum liquid endosperm content of the developing seeds. Preliminary in vivo experiments with cv Greenfeast in which hormones were applied by various means indicated that hormones could affect seed protein composition at maturity. However, these results were inconclusive because differences within treatments were often as large as differences between treatments. These findings suggest that the technique of uniform hormone application was the key factor. In this experiment, in Chapter 5, the effects of four growth regulators on pod growth and seed protein composition in peas were investigated.

CHAPTER 2:

A Manuscript entitled:

Quantitative Studies on the Cotyledonary Proteins in the Genus Pisum published in the Journal of Science Food and Agriculture, August 1982.

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Quantitative Studies on the Cotyledonary Proteins in the Genus *Pisum*

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Forty-five lines of peas including primitive or wild forms, field peas, and round and wrinkled garden peas, were grown under uniform conditions and the seeds examined for variation in protein characteristics likely to influence nutritional value. The characters measured were crude protein, extractable protein, globulins and albumins, the percentages of legumin, total sulphur and protein sulphur, carbon:nitrogen and nitrogen:sulphur ratios. The extractable protein was separated quantitatively into an albumin fraction (20–35%) and a globulin fraction (legumin and vicilin). Without exception lines high in albumin content were low in legumin content (correlation coefficient $r = -0.757$). As both the albumin fraction and legumin are rich in sulphur amino acids, this negative correlation has important implications for attempts through plant breeding to improve the nutritional quality of legume seed proteins, by increasing the sulphur amino acid content. Total sulphur was not correlated with any other protein character.

1. Introduction

The proteins of pea (*Pisum*) seeds are a source of food for man and monogastric animals, but as with other legume seeds these proteins are comparatively low in sulphur amino acids. Major interest has centred on the storage proteins legumin and vicilin,[1-7] classical globulins which together make up 65–80% of the buffer extractable protein of *Pisum* cotyledons. The albumin, legumin and vicilin contents, and their ratios, are genotype specific,[7-11] but also environment dependent.[11-14] It has been suggested[15-21] that protein quality in legumes, particularly in *Pisum* and *Vicia*, might be considerably improved if legumin, the more sulphur amino acid rich component of the globulin fraction could be increased, reducing or even replacing much of vicilin, the other major globulin component. That the albumins should be considered in breeding programmes, in particular to increase protein quality, has also been proposed.[7,13,15,21-23] The albumins have a more favourable amino acid profile[8,21,22] than the globulins and are quantitatively significant, contributing 20–35% of the buffer extractable cotyledonary protein. It has been reported that strains of peas with higher protein efficiency ratios (PER) have a higher albumin content.[8]

This paper describes the variation in protein characteristics likely to be relevant in determining nutritional value, and in particular seeks correlations between protein fractions. The characters measured were crude protein, extractable protein, globulins and albumins, the percentages of legumin, total sulphur and protein sulphur, carbon:nitrogen and nitrogen:sulphur ratios. The last four characteristics have been proposed as indicators of protein quality.[24-28] Forty-five lines of peas were examined including, primitive or wild forms of field peas, round seeded garden peas and wrinkled seeded garden peas. The results are presented as a correlation matrix of the nine seed characters examined and are discussed in relation to assessment of protein quality.

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Table 1. A description of 45 lines of *Pisum*

PI/G line no.	Name of line and other information		Seed weight (mg per seed)	Study code no.
	Group I: Primitive or wild forms			
293	*P. cinereum*	WL 1490	82.3	1
294	*P. formosum*	WL 1389	33.3	2
295	*P. humili*	WL 936	90.2	3
296	*P. fulcum*	WL 1256	59.4	4
297	*P. elatius*	WL 805	159.5	5
298	*P. zeylanicum*	WL 1201	89.4	6
299	*P. elatius*	WL 226	260.9	7
300	*P. tibetanicum*	WL 611	90.8	8
301	*P. abyssinicum*	WL 808	147.7	9
303	*P. tibetanicum*	WL 1477	206.7	10
304	*P. asiaticum*	WL 809	99.0	11
308	*P. elatius*	WL 1293	118.0	12
277	*P. fulcum* var. *amphicarpum*		72.4	13
	Group II: Field peas			
012	From cv. *Collegian* (ex Dr Y. Aitken)		232.4	14
013	Austrian Winter (CPI 1260)		152.3	15
037	From cv. Dunn (ex Dr R. Oram, CSIRO)		195.2	16
038	From cv. Derrimut (ex Dr R. Oram CSIRO)		206.7	17
077	Collegian (PV 156, CSIRO)		177.6	18
083	*P. arvense*		156.1	19
132	Graue niedrige		216.7	20
160	*P. arvense* (Sparta, CPI 30919)		214.1	21
162	*P. arvense* (Afganistan, ex USDA)		212.0	22
163	*P. arvense* (Russia, CPI 31444)		219.1	23
	Group III: Garden peas, round seeded			
001	*P. sativum* (ex USA)		207.5	24
007	*Pisum*	WL 1072	378.4	25
009	Var. Roi de Carouby Switzerland		353.9	26
011	From cv. Alaska		229.0	27
034	Origin Chile (ex Dr Y. Aitken)		273.4	28
040	Dippes Gelbe Victoria (ex Professor W. Gottschalk)		378.4	29
076	From cv. White Brunswick		284.7	30
142	CPI 65349 (ex Dr Marx)		290.9	31
151	Trapper Canada (ex. G. Child)		162.6	32
272	Express (ex I. Murfet)		262.6	33
307	*Pisum Kungsärt*	WL 110	384.3	34
	Group IV: Garden peas, wrinkled seeded			
002	*P. sativum* (ex USA)		271.4	35
006	*Pisum*	WL 851	336.5	36
008	*Pisum*	WL 1238	217.8	37
086	From cv. Greenfeast, control line Canberra		291.1	38
117	Witham Wonder		305.0	39
122	Acacia (ex Lamprecht)	WL 102	295.7	40
148	Laxtons Progress (ex G. Child, Canada)		328.0	41
291	*P. sativum* (CPI 76951)		308.8	42
292	*P. sativum* (CPI 76952)		274.0	43
305	From cv. Minnesota Early Sweet	WL 1685	260.5	44
306	*Pisum*	WL 1688	258.2	45

PI/G, Plant Industry Genetics Number.
WL, Pisum Genebank, Weibullsholm, Plant Breeding Institute, Landskrona, Sweden.
CPI, Commonwealth Plant Introduction Number.

2. Experimental

2.1. Materials

Lines of peas used in the analyses are listed in Table 1. Plants were grown in a loam/sand/peat moss mixture in 23-cm pots. A complete fertiliser was added to the mix before potting. Pots were placed in a glasshouse under natural light with increasing day length from August to December. The temperature range was from 18°C (night) to a maximum of 26°C (day). Seeds were harvested from completely desiccated plants, air dried to a moisture content of 6.5–7.5% and then weighed before dehulling, milling to a fine flour and passing through a 0.2 mm screen. The dehulled, milled cotyledons will be referred to as 'flour'. Between 30–50 seeds per line were taken to make up a bulk sample of each line for protein analyses. An exception was *P. formosum*, a perennial form, in which 50 seeds used were grown by Dr S. Blixt, Weibullsholm, Sweden.

2.2. Buffer extractable protein

Flour (1 g) was extracted twice with 0.1M phosphate buffer (pH 7.2) containing 0.5M NaCl for 1.5 h at 20°C on an end-over-end shaker. The final weight:volume ratio was 1:25. Extracts were centrifuged at 12 000*g* for 20 min at 15°C. The combined supernatants of the two extracts were filtered through two layers of Miracloth pre-wetted with extraction buffer. Protein was determined by a micro-biuret method[29] and expressed relative to crude protein.

2.3. Globulin and albumin contents

The filtered extract was dialysed at 4°C against distilled water, using eight changes of pre-cooled water during 84 h. It proved important to resuspend the contents of the dialysis bags with each change. After centrifuging at 12 000*g* for 20 min at 4°C the supernatant albumin solution was decanted into pre-weighed flasks. The globulin pellet was washed once by suspension in cold distilled water and collected by centrifuging as described. The final globulin fraction was then suspended in distilled water for transfer to a pre-weighed flask. Both globulins and albumins were lyophilised, their weights were determined and their ratios calculated.

2.4. Legumin contents of flour and globulins

Legumin was determined as a percentage of total extractable protein from flour and of globulins by Laurell's rocket immunoassay,[30] using a specific antilegumin serum raised in sheep. Samples (10 μl amounts of extracts) were applied to wells cut in an agarose gel into which antilegumin had been mixed before cooling. Standards of pure legumin (5–20 μg) were included on each gel. The distance from the origin to the tip of the precipitate was proportional to the amount of legumin in the sample. By reference to a standard curve plotted for each gel, the amount of legumin in the extracts was then estimated and expressed as a percentage of the total protein content.

2.5. Total sulphur and total nitrogen of flour

The total sulphur content of flour was determined by X-ray fluorescence spectrometry according to Norrish and Hutton.[31] Total nitrogen was determined by a micro-Kjeldahl method.

2.6. Crude protein

This was calculated as total N × 6.25.

2.7. Protein sulphur and protein nitrogen

Extraction of protein from flour was performed as described in section 2.2. To the pre-cooled supernatant, 50% trichloroacetic acid (TCA) was added to give a final concentration of 10% and the mixture was left on ice for 2 h before centrifuging at 12 000*g* for 20 min at 4°C. The supernatant was discarded and the precipitate was washed twice with cold acetone to remove any TCA and to facilitate air drying. The precipitated protein was left to dry overnight before grinding into a fine powder. Determinations of sulphur and nitrogen were made as described in section 2.5.

2.8. C:N ratio of flour

This was determined using an elemental analyser, Carlo Erba Model 1106.

2.9. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were prepared for electrophoresis by either dissolving the freeze-dried powder in digestion buffer (see later) or by precipitation of dilute solutions with 4 vols of cold (−20°C) acetone and

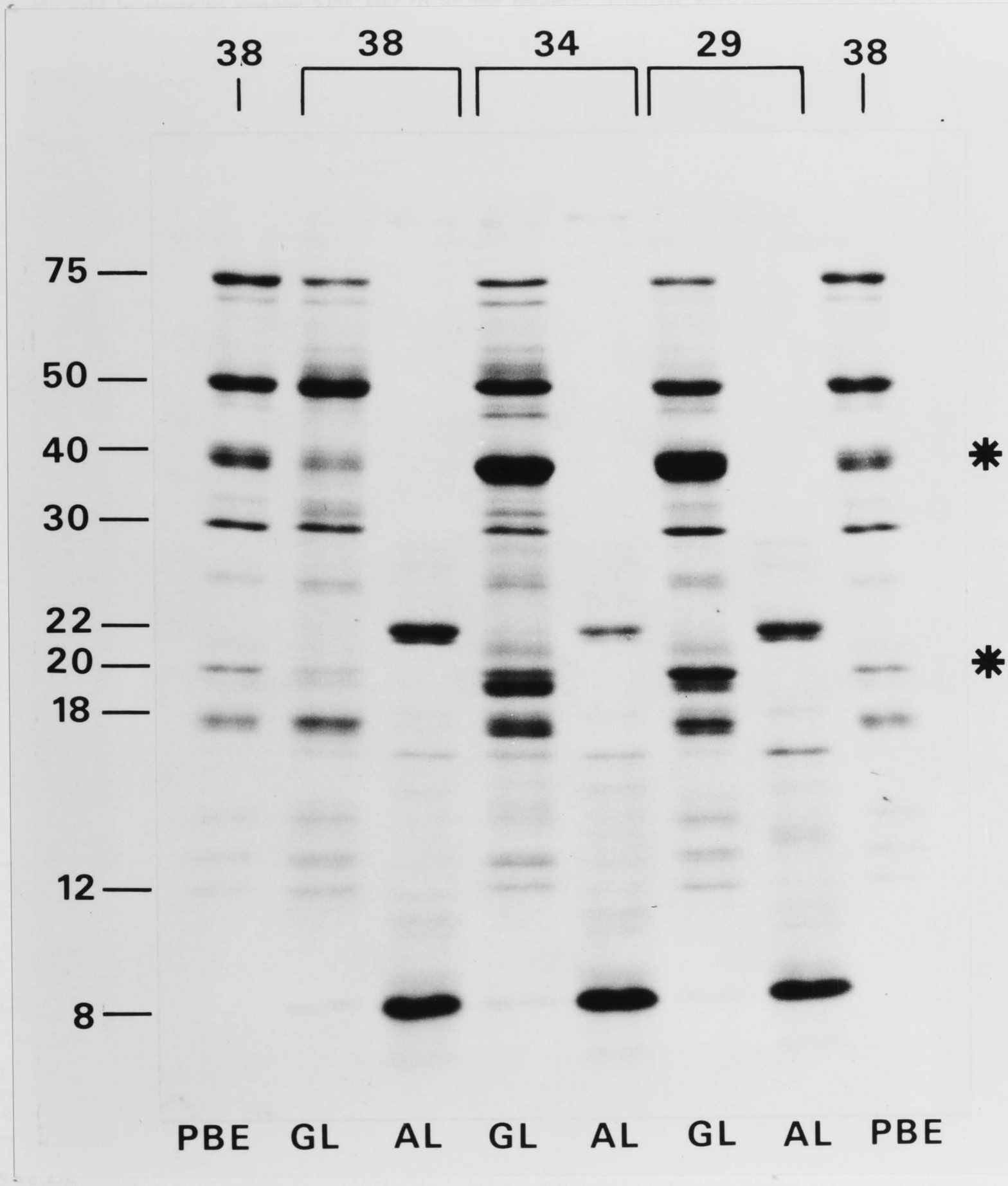

Figure 1. SDS-PAGE of reduced polypeptides of protein body extract (PBE), globulins (GL) and albumins (AL) of three representative lines: no. 38 a low legumin line and 34 and 29, two high legumin lines. Apparent molecular weights ($\times 10^{-3}$) are indicated on the vertical axis; *, acidic (40) and basic (20) polypeptides of legumin. Protein bodies were prepared according to Thomson *et al.*[6]

collecting the precipitate by centrifuging at 10 000*g* for 10 min. The pellets were dissolved in digestion buffer [0.125M Tris–HCl (pH 6.7), 2% SDS, 2% β-mercaptoethanol, 5% glycerol and 0.01% bromophenol blue] and heated at 90°C for 2 min. Samples (50–100 μl) containing 50–100 μg of protein were loaded in slots of slab gradient (12.5–25%) polyacrylamide gels (150 × 140 × 1.5 mm) prepared as described elsewhere.[32] Electrophoresis was carried out (100 V, 14 mA) at room temperature for 16–20 h. The gels were washed twice for 30 min with a solution of 25% ethanol–7% acetic acid in water to remove SDS and to fix the proteins. The gels were stained with 0.5% Coomassie Brilliant Blue R in 50% methanol–40% acetic acid for 15 min and destained in 25% ethanol–7% acetic acid.

3. Results

3.1. Characterisation of albumin and globulin fractions by SDS-PAGE

The extractable protein from pea cotyledons was cleanly and reproducibly separated into albumin and globulin components (see section 2.3). SDS-PAGE of the two fractions is illustrated in Figure 1 for three representative lines: a low legumin line (no. 38) and two high legumin lines (no. 34 and 29). For comparison, the polypeptide composition of protein bodies (the organelles in which storage proteins are accumulated) is also shown for line no. 38.

The electrophoretic pattern of storage protein polypeptides from isolated protein bodies and globulins of line no. 38 are qualitatively very similar; minor quantitative differences can be discerned, which could be due to differential losses during the protein body isolation procedure. The quantitative difference in legumin content, between the high and low legumin lines is clearly evident in the $M_r \sim 40\,000$ and $M_r \sim 20\,000$ regions. With the exception of one polypeptide ($M_r \sim 17\,000$) the patterns of the globulin and albumin fractions are very different. In addition, no cross-contamination was detected by immunodiffusion using either anti-protein body serum or anti-albumin serum (not shown). The albumin polypeptide pattern of the three lines is very similar, although minor qualitative differences were observable. Two components ($M_r \sim 22\,000$ and $M_r \sim 8\,000$) are quantitatively very conspicuous. The polypeptide of $M_r \sim 22\,000$ may be of particular interest since there is some evidence that it may be relatively rich in sulphur.[14]

3.2. Quantitative protein characters

Between the members of four groups of *Pisum* lines a comparison was made of seed weight and eight protein characters. The protein characters assayed were: crude protein, percentage extractable protein, legumin as a percentage of extractable protein and of globulins, albumin as a percentage of extractable protein, globulin:albumin ratio, sulphur as a percentage of flour and C:N ratio of flour.

Table 2. Group means for quantitative protein characters and seed weight

Group	Number of lines per group	Mean seed weight (mg)	Crude protein (%)	Extractable protein* (%)	Legumin: Percentage of extractable protein (flour)	Legumin: Percentage of globulins	Globulin: albumin ratio	Albumins as percentage of extractable protein	Sulphur as percentage dry weight of flour	C:N ratio of flour
I	13	116.1 c†	26.8 ab	70.5 b	34.2 a	54.6 a	3.89 a	20.8 c	0.259 a	10.2:1 bc
II	10	198.2 b	23.2 c	80.5 a	23.4 b	42.3 b	3.15 b	24.6 b	0.215 c	11.8:1 a
III	11	291.5 a	24.4 bc	80.2 a	28.9 ab	47.6 ab	2.96 b	25.6 b	0.222 c	11.3:1 ab
IV	11	286.1 a	28.2 a	81.0 a	16.3 c	32.9 c	2.39 c	29.7 a	0.243 ab	9.7:1 c

* Protein extractable in buffered saline measured by a biuret method as percentage of the crude protein (total N × 6.25).

† Means followed by the same letter are not significantly different (5% level).

Percentage extractable protein was lowest in the primitive forms (group I). Closest resemblance of the protein characters analysed was found between groups II (field peas) and III (garden peas), both round seeded phenotypes. The round (group III) and wrinkled garden peas (group IV) are similar only in seed weight, extractable protein and percentage total sulphur of flour, but differ significantly in the other six protein characters.

3.3. Matrix of correlation coefficients

Correlation coefficients were calculated between all combinations of the parameters listed in Table 2.

Table 3. Correlation matrix of seed weight and the protein characters observed

	1	2	3	4	5	6	7	8	9
1 Seed weight	1.0000								
2 Crude protein (%)	−0.0586	1.0000							
3 Extractable protein (%)	0.6012	−0.4109	1.0000						
4 Legumin of flour (%)	−0.3770	0.2376	−0.6454	1.0000					
5 Legumin of globulins (%)	−0.3326	0.1944	−0.5740	0.9346	1.0000				
6 Globulin:albumin ratio	−0.5565	0.1844	−0.6944	0.7446	0.7678	1.0000			
7 Albumins (%)	0.5328	−0.1609	0.6759	−0.7571	−0.7600	−0.9759*	1.0000		
8 Sulphur as percentage of dry weight of flour	−0.3034	0.2335	−0.1388	0.0736	0.0465	0.0782	−0.0670	1.0000	
9 C:N ratio of flour	0.0318	−0.9229	0.4255	−0.2425	−0.2204	−0.1609	0.1434	−0.1297	1.0000

* This correlation coefficient is of mathematical value only.
On 43 degrees of freedom significant at: 5%, 0.295; 1%, 0.381; and 0.1%, 0.475.

Seed weight is positively correlated (0.1% level) with percentage extractable protein and albumin content. These latter two highly desirable characters are also positively correlated (0.1% level). Consequently, larger seeds are negatively correlated with percentage legumin and with the globulin: albumin ratio. The negative correlation of seed weight with total sulphur, barely significant at the 5% level, is entirely due to the small-seeded primitive forms having the highest total sulphur content (see Table 2). Total sulphur is not correlated with any other protein character examined. The highly significant correlation of percent legumin of flour and percent legumin of globulins shows that levels of legumin can be accurately determined using flour. The negative correlation of extractable protein with crude protein is a consequence of the primitive forms having a relatively high crude protein but a low extractable protein content. The C:N ratio is negatively correlated with crude protein content because groups I and IV have the highest crude protein contents but the lowest C:N ratios (see Table 2). Albumin content is negatively correlated at the 0.1% level with legumin content.

3.4. Correlation of albumin with legumin content

The highly negative correlation of percentage albumin with percentage legumin (Table 3) could be important if protein quality is dependent on sulphur amino acid content of the cotyledonary proteins. The individual analyses for all lines are shown in Figure 2.

No line was found which was either high or low in *both* legumin and albumin, among the 45 lines examined. The albumin and legumin content appear to be line and group specific. Primitive

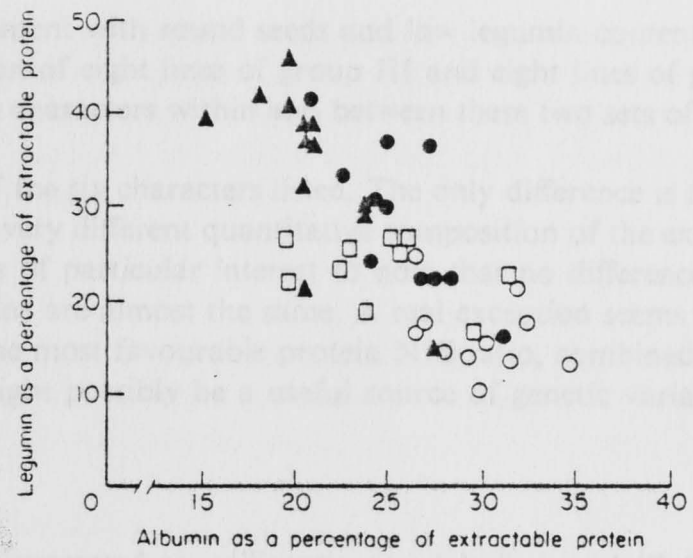

Figure 2. Relationship of albumin and legumin contents of 45 lines of peas. Results are expressed as the percentages of each fraction in the protein extracted from the flour; ▲, primitive or wild forms, □, field peas; ●, garden peas (round seeded); ○, garden peas (wrinkled seeded)—correlation coefficient $r = -0.757$.

or wild forms (group I) generally have the highest legumin and lowest albumin contents, with the exception of no. 13—an unusual subterranean form of *P. fulvum*. Field peas (group II) show the least variation for legumin content but the widest range of albumin contents. Round seeded garden peas (group III) exhibit the greatest variation for legumin content while wrinkled seeded garden peas (group IV) show the least variation for both characters.

3.5. Protein analyses of round and wrinkled seeds

Because of the significant negative correlation between albumin and legumin contents (Figure 2)

Table 4. Comparative analyses of seed weight and protein characters of round and wrinkled seeded lines

Study code no.	Dehulled seed weight[a] (mg)	Crude protein (%)	Extractable protein (%)	Globulin: albumin ratio	Sulphur as percentage dry weight of flour	Protein N:S ratio
			Round seeded lines			
24	188.1	24.81	77.9	2.69	0.256	23.8
27	209.7	21.56	79.4	2.54	0.227	22.6
28	241.0	18.63	84.2	3.00	0.206	22.6
29	345.9	23.63	80.4	3.45	0.216	26.8
30	259.8	27.63	75.3	3.12	0.214	22.5
31	241.6	31.50	76.3	3.02	0.229	25.1
33	241.6	20.69	84.6	2.21	0.240	19.5
34	349.5	28.19	80.8	3.81	0.206	24.6
Mean	*259.7*	*24.58*	*79.9*	*2.98*	*0.224*	*23.4*
			Wrinkled seeded lines			
36	297.8	26.38	80.1	1.88	0.231	23.6
37	182.6	31.69	78.2	2.80	0.195	28.7
38	258.0	27.13	82.9	2.61	0.220	22.7
39	269.7	29.50	75.6	2.10	0.196	27.9
40	262.2	26.70	84.0	2.42	0.269	21.2
41	297.3	27.19	77.2	2.75	0.249	23.8
42	274.3	26.94	83.3	2.13	0.260	21.2
44	238.6	28.00	86.8	2.33	0.295	22.8
Mean	*260.1*	*27.94*	*81.0*	*2.38*	*0.239*	*23.9*
S.e. of means	17.0	1.17	1.3	0.15	0.010	0.9
Significance of difference	NS	NS	NS	*	NS	NS

[a] Seed weight, mean of 100 seeds; other data, mean of two observations with < 5% deviation; average bulk sample analysed, 30–50 seeds per line.

* Significant at the 5% level; NS = Not significant.

and the close correlation of high legumin content with round seeds and low legumin content with wrinkled seeds,[10] a more detailed examination of eight lines of group III and eight lines of group IV was made. Individual variation of protein characters within and between these two sets of eight lines are presented in Table 4.

The two sets are not different in five out of the six characters listed. The only difference is found in the globulin:albumin ratio. In view of the very different quantitative composition of the extractable protein of these lines (see Table 5), it is of particular interest to note that no difference was found in protein N:S ratio. The two set means are almost the same. A real exception seems to be the round seeded line no. 33. This line has the most favourable protein N:S ratio, combined with a low globulin:albumin ratio; such a line might possibly be a useful source of genetic variability for a protein quality breeding programme.

3.6. Protein fractions per pair of cotyledons

In Table 5, analyses of protein fractions are expressed as milligrams per dehulled seed (Table 5) rather than in percentages, so that the weight of cotyledons is taken into account.

The albumin component of the extractable protein is consistently higher in the wrinkled seeds while the globulin component is very similar for both sets. Even though percentage legumin differs

Table 5. Protein fractions of round and wrinkled seeded lines (mg per pair of cotyledons) and vicilin:legumin ratio

Study code no.	Albumin (mg)	Globulin (mg)	Legumin (mg)	Vicilin: legumin ratio[a]
		Round seeded lines		
24	9.84	25.50	12.44	1.05
27	10.14	25.74	9.29	1.77
28	9.46	28.36	15.68	0.81
29	14.27	50.95	28.17	0.81
30	13.12	40.94	19.73	1.08
31	14.44	43.62	23.99	0.82
33	13.18	29.12	8.59	2.39
34	16.54	63.04	41.98	0.50
Mean	*12.62*	*38.41*	*19.98*	*1.15*
		Wrinkled seeded lines		
36	21.84	41.05	14.90	1.76
37	11.91	33.36	10.14	2.29
38	16.08	41.97	15.40	1.73
39	19.30	40.82	13.96	1.85
40	17.19	41.60	12.73	2.27
41	16.55	45.88	11.10	3.13
42	19.66	41.89	15.09	1.71
44	17.42	40.58	15.26	1.66
Mean	*17.49*	*40.89*	*13.57*	*2.05*
S.e. of means	1.00	4.80[b]	4.00[b]	0.20
		1.20[c]	0.70[c]	
Significance of difference	**	NS	NS	**

[a] Vicilin content was estimated by subtracting the amount of legumin (mg) from the amount of globulin (mg).

[b, c] S.e. of round (*b*) and wrinkled (*c*) lines derived separately.

** Significant at the 1% level; data, mean of two observations with < 5% deviation, average bulk sample analysed 30–50 seeds per line.

significantly (Table 2) between groups III and IV (round and wrinkled seeded garden peas), no difference in milligrams of legumin per seed could be established. Legumin content (mg per seed) is quite uniform within the wrinkled seeded set, but shows wide variation (see also Figure 2) in the round seeded set and this accounts for the large, separately calculated standard error. However, vicilin:legumin ratios of the two sets differ significantly.

4. Discussion

All lines, except *P. formosum*, were grown under similar environmental conditions because temperature[11] and nutrient status[14] have previously been shown to affect the relative proportions of pea seed proteins. Thus, the variation in seed and seed protein characteristics, observed in the present study, must be genetically determined.

The close correlation between round and wrinkled seeds and high and low levels of legumin established by Davies [10] was also found in this study. But correlations between quantitative protein characters, namely, of globulin:albumin ratio with legumin content and of albumin content with legumin content have not been reported previously. A high legumin content was always reflected in a lower albumin content and vice versa. None of the 45 lines examined broke these correlations.

Total sulphur as a percentage of dry weight of flour was not correlated with any of the protein characters measured (Table 3). Comparing sets of high and low legumin lines (round and wrinkled seeds, Table 4) some variation in total sulphur was observed within sets but no significant difference between set means could be established. The N:S ratio in extractable protein also varies within sets and again set means are almost identical.

Consistent with this, using sulphur amino acid analyses from the literature[8,17,21,22] and either percentages of protein fractions from Table 2, or albumin, globulin, legumin and vicilin:legumin ratio data from Table 5 it can be calculated that high and low legumin lines have a very similar total sulphur amino acid content. Amino acid analysis [Poulsen, R. (Plant Breeding Institute, Cambridge, England), private communication] of two high and low legumin lines from the Canberra collection, supported this conclusion. An analysis of methionine content by Slinkard's group[33] revealed that the methionine content (expressed as mg methionine g^{-1} protein) was very similar for round and wrinkled seeded lines. They concluded that most of the observed variation in methionine content was due to variation in protein content and was largely environment dependent. It appears then, that sulphur amino acid content relative to protein content, is a fairly constant factor, varying little if any from line to line.

When assessing both protein quality and quantity the vicilin fraction must be considered. The lysine content of vicilin[17,21,22] is significantly higher than that of legumin. This is an important factor when designing a mixed diet of cereals and legumes, because the total protein will be utilised only to the level of the most limiting essential amino acid. The albumins combine high levels of sulphur amino acids with a high lysine content and as a protein fraction fully satisfy the WHO[34] requirement pattern in terms of g of amino acid 100^{-1} g protein.

The present results suggest that when considering protein quality more emphasis should be given to the nutritionally superior[8,17,21–23] albumin fraction. In the present study 20–35% of the total extractable protein as albumins, but variation in albumin content ranging from 14 to 40% have been observed[23] and Bajaj's study[8] correlating PER with albumin content indicates at least a two-fold difference in albumin content. There are probably many thousands of albumin species, most of which individually contribute little to the total protein content. On this basis it would be difficult or impossible to alter the composition of this fraction significantly by selecting a particular desirable albumin component in a quality breeding programme. But the present study shows that the predominant albumins are amenable to biochemical characterisation and possibly genetic analysis and some polypeptides are individually of quantitative significance. A relatively small number of native albumin proteins displayed by PAGE are of major quantitative significance[9,35,36] and these have been used to distinguish between *Pisum* forms taxonomically, resulting in five albumin band patterns. Two of these band patterns when analysed genetically[37] were found to be determined by two alleles of one locus. Elution profiles of albumins[38] fractionated on Sephadex

G-100 yielded four fractions, fraction 3 being six times richer in cystine content. Electrophoretic analysis of albumins on SDS-PAG under reducing conditions revealed two band regions of major quantitative significance (Figure 1). One of the predominant polypeptides ($M_r \sim 22\,000$) is markedly reduced in staining density in the polypeptide spectrum of extracts from sulphur-deficient peas.[14] Thus, even without full analysis of the albumins it may be possible to use available data to identify and select desirable albumin components, and to establish the genetic basis of particularly sulphur-rich albumins with nutritional potential.

Round and wrinkled lines of peas have been compared in detail genetically by Blixt[39,40] and for agronomically important characters by Slinkard's group.[33] Both studies showed wrinkled seeds to have a higher protein content but round seeded lines to have higher yields and better field establishment. Protein yield per unit area was not significantly different in the two groups of peas. Genetic variation in protein content, partly due to the pleiotropic effect of the gene for wrinkled,[41] is limited, making any improvement in the percentage protein of peas by breeding slow and difficult.

Two highly positive correlations, namely, protein yield with seed yield and protein weight per seed with seed weight,[33] coupled with the highly positive correlation of seed weight and albumin content found in this study (Table 3), appear to offer hope for useful advances in breeding for protein quantity and quality. But a breeding strategy[42] for the improvement of protein in pulses, based on the coordinated analysis at both the subunit and holoprotein levels, will be required[15,43] if genetical and biochemical investigations of the proteins are to be useful to breeders trying to improve seed protein yield and quality.

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References

1. Danielsson, C.-E. Investigation of vicilin and legumin. *Acta Chem. Scand.*, 1949b, **3**, 41–49.
2. Danielsson, C.-E. A contribution to the study of the synthesis of the reserve proteins in ripening pea seeds. *Acta. Chem. Scand.*, 1952, **6**, 149–159.
3. Danielsson, C.-E. The breakdown of the high molecular reserve proteins of peas during germination. *Acta Chem. Scand.*, 1951, **5**, 541.
4. Raacke, I. D. Protein synthesis in ripening peas seeds. *Biochem. J.* 1957a, **66**, 101–110.
5. Derbyshire, E.; Wright, D. J.; Boulter, D. Legumin and vicilin, storage proteins of legume seeds. *Phytochemistry* 1976, **15**, 3–24.
6. Thomson, J. A.; Schroeder, H. E.; Dudman, W. F. Cotyledonary storage proteins in *Pisum sativum*. I. Molecular heterogeneity. *Aust. J. Plant Physiol.* 1978, **5**, 263–279.
7. Davies, D. R. Variation in the storage proteins of peas in relation to sulphur amino acid content. *Euphytica* 1976, **25**, 717–724.
8. Bajaj, S.; Mickelsen, O.; Lillevik, H. A.; Baker, L. R.; Bergen, W. G.; Gill, J. L. Prediction of protein efficiency ratio of peas from their albumin content. *Crop Sci.* 1971, **11**, 813–815.
9. Przybylska, J.; Blixt, S.; Hurich, J.; Przybylska, Z. Z. Comparative study of seed proteins in the genus *Pisum*, I. Electrophoretic patterns of different protein fractions. *Genetica Polonica* 1977, **18**, 27–38.
10. Davies, D. R. The r_a locus and legumin synthesis in *Pisum sativum*. *Biochemical Genetics* 1980, **18**, 1207–1219.
11. Thomson, J. A.; Millerd, A.; Schroeder, H. E. Genotype dependent patterns of accumulation of seed storage proteins in *Pisum*. In *Seed Protein Improvement in Cereals and Grain Legumes* Volume II, IAEA-SM-230/61, Vienna, 1979, pp. 231–240.
12. Füredi, J. Importance of breeding peas with increased protein content. In *Protein Growth by Plant Breeding* (Balint, A., Ed). Akademiai Kiado, Budapest, 1970, pp. 99–128.
13. Gottschalk, W.; Müller, H. P.; Wolff, G. Relations between protein production. Protein quality and environmental factors in *Pisum* mutants. IAEA-PL-570/14, Vienna, 1975, pp. 105–123.

14. Randall, P. J.; Thomson, J. A.; Schroeder, H. E. Cotyledonary storage proteins in *Pisum sativum*. IV. Effects of sulphur, phosphorous, potassium and magnesium deficiencies. *Aust. J. Plant Physiol.* 1979, **6**, 11–24.
15. Nelson, O. E. Genetic modifications of protein quality in plants. *Adv. Agron.* 1969, **21**, 171–194.
16. Boulter, D.; Evans, I. M.; Derbyshire, E. Proteins of some legumes with reference to environmental factors and nutritional value. *Qual. Plant. Plant Foods Hum. Nutr.* 1973, **23**, 239–250.
17. Boulter, D. Quality problems in protein plants with special attention paid to protein of legumes. In *Protein Quality from Leguminous Crops* EEC Seminar, Dijon, 1977, pp. 11–47.
18. Boulter, D. Ontogeny and development of biochemical and nutritional attributes in legume seeds. *Advances in Legume Science* (Summerfield, R.; Bunting, A., Eds), 1980, pp. 127–134.
19. Casey, R.; Short, M. N. Variation in amino acid composition of legumin from *Pisum*. *Phytochemistry* 1981, **20**, 21–23.
20. Boulter, D. Improving protein content and quality in legumes. In *Genetic Diversity in Plants* (Muhammed, A.; Aksel, R.; von Borstel, R. C., Eds), Plenum Publishing Corp. 1977, pp. 387–397.
21. Hurich, J.; Parzysz, H.; Przybylska, J. Comparative study of seed proteins in the genus *Pisum* II. Amino acid composition of different protein fractions. *Genetica Polonica* 1977, **18**, 241–252.
22. Boulter, D.; Derbyshire, E. In *Chemotaxonomy of the Leguminosae* (Harborne, J. B.; Boulter, D.; Turner, B. L. Eds), Academic Press, London & New York, 1971, pp. 285–308.
23. Murray, D. R. A storage role for albumins in pea cotyledons. *Plant, Cell and Environment* 1979, **2**, 221–226.
24. Boulter, D.; Evans, I. M.; Thompson, A. Screening for protein quality in legumes. *Qual. Plant. Plant Foods Hum Nutr.* 1976, **26**, 107–119.
25. Evans, I. M.; Boulter, D. Crude protein and sulphur amino acid content of some commercial varieties of peas and beans. *J. Sci. Food Agric.* 1980, **31**, 238–242.
26. Russel-Hunter, W. D. In *Aquatic Productivity* Macmillan & Co., 1979, 174–182.
27. Blagrove, R. J.; Gillespie, J. M.; Randall, P. J. Effect of sulphur supply on the seed globulin composition of *Lupinus angustifolius*. *Aust. J. Plant Physiol.* 1976, **3**, 173–184.
28. Eppendorfer, W. H. Effects of S, N and P on amino acid composition of field beans (*Vicia faba*) and responses of the biological value of the seed protein to S-amino acid content. *J. Sci. Food Agric.* 1971 **22**, 501–505.
29. Goa, J. A micro-biuret method for protein determination. *Scand. J. Clin. Lab. Invest.* 1953, **5**, 218–222.
30. Weeke, B. Rocket immunoelectrophoresis. In *A Manual of Quantitative Immunoelectrophoresis* (Axelson, N. H., Knoll, J.; Weeke, B., Eds), Universitetsforlaget, Oslo, pp. 47–56.
31. Norrisk, K.; Hutton, J. T. Plant analyses by X-ray spectrometry. I. Low atomic number elements, sodium to calcium. *X-ray Spectrometry* 1977, **1**, 6–11.
32. Spencer, D.; Higgins, T. J.; Button, S. C.; Davey, R. A. Pulse-labelling studies on protein synthesis in developing pea seeds and evidence of a precursor form of legumin small subunit. *Plant Physiol.* 1980, **66**, 510–515.
33. Slinkard, A. E. In *Production, Utilization and Marketing of Field Peas* Volume 1, Crop Development Centre, University of Saskatchewan, Canada, Subprojects I–VI, 1980.
34. World Health Organization Energy and protein requirements. *WHO Tech Report Series* 1973, p. 522.
35. Fox, D. J.; Thurman, D. A.; Boulter, D. Studies on the proteins of seeds of the Leguminoseae. I. Albumins. *Phytochemistry* 1964, **3**, 417–419.
36. Guldager, P. Immunoelectrophoretic analysis of seed proteins from *Pisum sativum* L. *Theor. Appl. Genet.* 1978, **53**, 241–250.
37. Blixt, S.; Przybylska, J.; Przybylska, Z. Z. Comparative study of seed proteins in the genus *Pisum*. V. Genetics of the electrophoretic patterns I and III. *Genetica Polonica* 1980, **21**, 153–160.
38. Jakubek, M.; Przybylska, J. Comparative study of seed proteins in the genus *Pisum* III. Electrophoretic patterns and amino acid composition of albumin fractions separated by gel filtration. *Genetica Polonica* 1979, **20**, 369–380.
39. Blixt, S. Problems relating to pea breeding. *Agric. Hortique Genetica* 1978, **36**, 56–87.
40. Blixt, S. Natural and induced variability for seed protein in temperate legumes. In *Seed Protein Improvement In Cereals and Grain Legumes*, Volume II, IAEA-SM-230/85, Vienna, 1979, pp. 3–21.
41. Shia, G.; Slinkard, A. E. Relationship of seed shape and cotyledon color to percent protein of peas. *Crop Science* 1977, **17**, 183–184.
42. Smartt, J.; Winfield, P. J.; Williams, D. A strategy for the improvement of protein quality in pulses by breeding. *Euphytica* 1975, **24**, 447–451.
43. Thomson, J. A.; Doll, H. Genetics and evolution of seed storage proteins. In *Seed Protein Improvement in Cereals and Grain Legumes* Volume I, IAEA-SM-230/60, Vienna, 1979, pp. 109–124.

CHAPTER 3:

A Manuscript entitled:

The Major Albumins of Pisum Cotyledons

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The major albumins of Pisum cotyledons

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Summary

The albumin fraction of the cotyledons of Pisum contains two major polypeptides which together make up 34 percent (17% each) of the total albumin fraction. Both of these albumins (M_r ~ 8000 and ~ 22000) are cotyledon specific proteins. In many Pisum lines the M_r ~ 22000 fraction resolves into two components on Na-dodecylsulfate-polyacrylamide gels. The M_r ~ 8000 polypeptide was broken down during germination and early seedling growth, indicating that it functions as a storage protein, while the M_r ~ 22000 polypeptides were degraded relatively slowly. The level of both of these polypeptides was markedly reduced under sulfur deficiency conditions, the M_r ~ 22000 components being affected to a lesser extent than the M_r ~ 8000 component. Consistent with this, when [^{35}S]-sodium sulfate was injected into the pedicel of control plants during seed development and albumins were isolated at seed maturity, polypeptides of M_r ~ 8000 and ~ 22000 together accounted for a major proportion of the radioactivity in the total albumin fraction. The abundance and relatively high sulfur content of these two albumins could be significant factors in determining the nutritional value of pea seed proteins.

Abbreviations SDS = sodium dodecylsulfate

PAGE = polyacrylamide gel electrophoresis

PI/G = Plant Industry Genetics number

1. Introduction

The seed proteins from peas have been studied from many aspects in recent years; most attention has been given to the composition of the globulin fraction because it contains the major seed storage proteins vicilin and legumin. However, the albumin fraction contributes a quantitatively important proportion of the seed proteins in Pisum. It accounts for 20-35 percent of the total extractable cotyledonary proteins[1] and includes most of the enzymatic and metabolic proteins. Analysis of this complex fraction by SDS-PAGE revealed the presence of two major polypeptide components, of M_r ~8000 and ~22000[1].

The aim of this study was to further investigate some of the characteristics of these major components of the seed albumin fraction. These two albumin polypeptides were present in all of the 45 lines of Pisum examined. Polypeptides of Mr ~ 8000 and Mr ~ 22000 contributed 34 percent of the protein of the albumin fraction and accounted for about 50 percent of the ^{35}S incorporated into this fraction throughout seed development, indicating they are rich in sulfur-containing amino acids. These polypeptides are cotyledon-specific; their behavior during germination and early seedling growth suggests that at least the Mr ~ 8000 polypeptides function as storage proteins.

2. Experimental

2.1. Materials and Methods

Plant material, protein isolation, in vivo incorporation of [^{35}S]sodium sulfate, SDS-PAGE, fluorography and densitometry. Our standard line Pisum sativum L. PI/G 086, a selection from cv Greenfeast stock

collection CSIRO Canberra, was grown under conditions described by Schroeder[1]. Albumins from pea seeds of 45 lines were extracted and protein sulfur was determined according to Schroeder[1]. Albumins from peas grown under sulfur deficiency conditions were obtained from previously described experimental material[2]. Seed proteins of our standard line were labelled with ^{35}S by injecting [^{35}S]sodium sulfate (50 µl of carrier free, 185×10^3 MBq/ml, New England Nuclear, Boston,. Mass, U.S.A.) into the pedicel on days 15, 20 and 25 after flower opening and albumins were isolated at maturity. The labelled albumins were fractionated by SDS-PAGE and fluorographed according to Spencer et al.[3] and Gill et al.[4] respectively. Protein samples were loaded to give optimum resolution for major and minor polypeptide bands and to bring their staining by Coomassie blue within the linear response range and their fluorography to below-saturating intensities when measured by densitometry using a Joyce Loebl Denisitometer. I have assumed that there is no differential dye-binding between the albumin polypeptides. Contribution of the selected components, $M_r \sim 22000$ and ~ 8000 polypeptides was expressed as the areas under the peaks, relative to the total area.

2.2. Extraction of leaf, stem, pod and root proteins of pea plants.

Fresh leaf, stem, pod and root tissue (1 g) was ground in liquid nitrogen and extracted with phosphate buffer pH 7.2 (0.1 M sodium phosphate, 0.5 M NaCl, 1 mM $MgCl_2$, 5 mM ethylene diaminetetra-acetic acid (EDTA) and 0.14 M 2-mercaptoethanol) for 30 min with gentle stirring on ice. The homogenate was centrifuged at 20000 g for 30 min at 5°C. Aliquots of the supernatants were either tested directly against seed albumin antiserum in Ouchterlony gel plates according to Dudman and Millerd[5], or were precipitated with 10% trichloroacetic acid for SDS-PAGE and protein determinations[6].

2.3. Extraction of proteins during germination. Pea seeds of equal weight of line PI/G 086 were imbibed overnight and grown in vermiculite in the dark at a constant temperature of 22°C using only distilled water. Proteins were extracted from cotyledons on days 0,1,3,6,9. Cotyledons were ground in a mortar in protein extraction buffer (5 ml per seed, 0.5 M NaCl, 0.02 M Tes pH 7.8, 1 mM phenylmethylsulfonylfluoride) and vortexed intermittently for 30 min. The homogenate was centrifuged at 10000g for 20 min at 5°C and 10 µl of the supernatant was used directly for SDS-PAGE.

3. Results

3.1 Albumin-polypeptides - The albumin fraction of of 45 lines of Pisum was fractionated on SDS-PAGE and the results for 10 representative lines are shown in Fig. 1. In all lines examined two major polypeptide classes, one of $M_r \sim 22000$ and the other of $M_r \sim 8000$, were observed together with a number of minor components.

Insert Fig. 1

Quantitative and qualitative heterogeneity was observed between lines. The $M_r \sim 22000$ fraction consisted of either two polypeptides or one or other member of the pair, while only small differences were apparent in the $M_r \sim 8000$ polypeptide region. Similar variations were seen among all 45 lines examined (data not shown).

3.2. Cotyledon specificity - The albumin polypeptides of $M_r \sim 22000$ and ~ 8000 are restricted to the cotyledons. When total protein extracts of leaves, stems, pods and roots of actively-growing pea plants of our standard line were fractionated on SDS-PAGE, no polypeptides corresponding to the two major cotyledonary albumins were detected (Fig. 2).

Insert Fig. 2

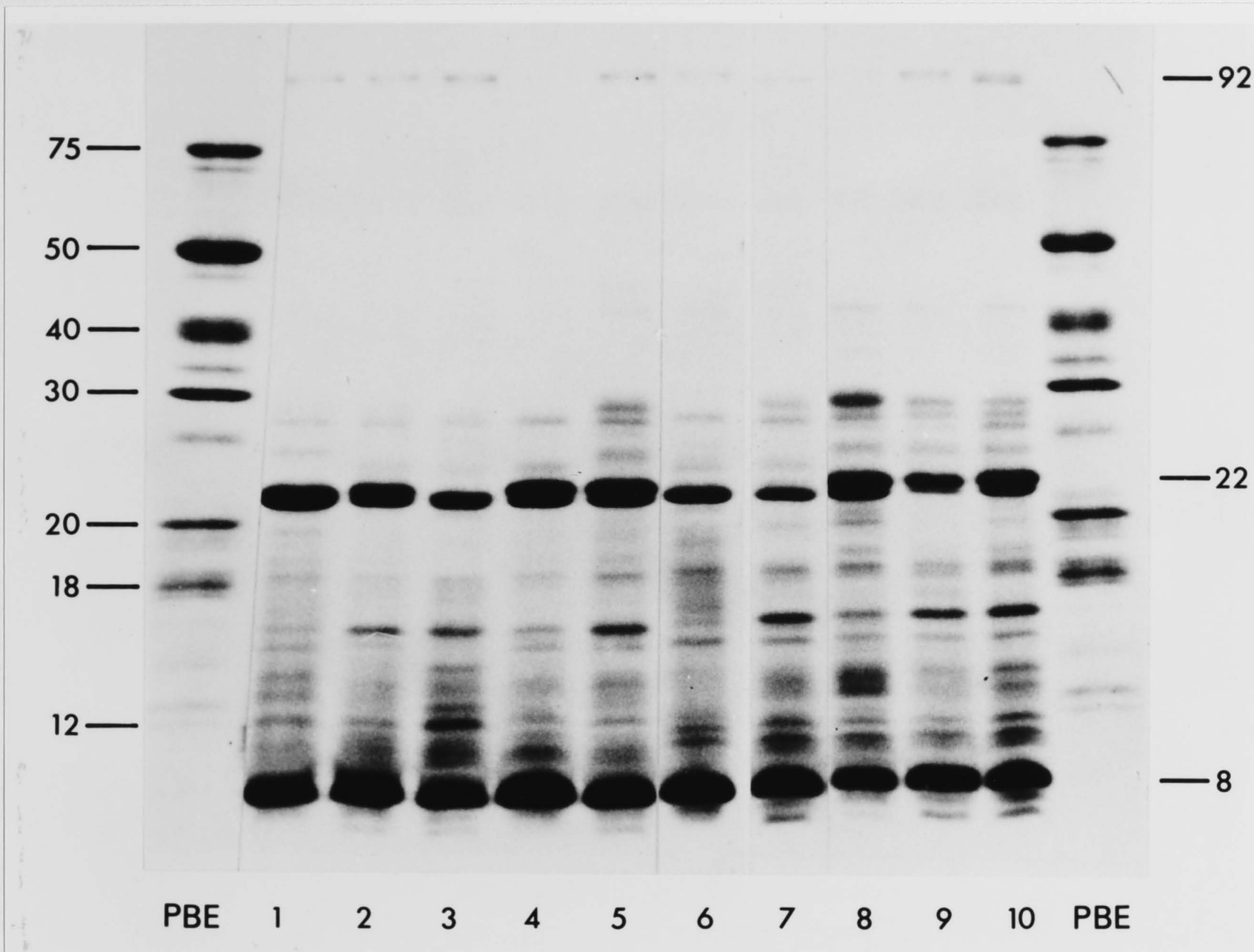


Fig. 1. Fractionation of albumins of 10 lines of Pisum by SDS-PAGE under reducing conditions. (1) P. cinereum, (2) P. humile, (3) P. fulvum, (4) P. elatius, (5) P. tibetanicum, (6) P. abyssinicum, (7) P. asiaticum, (8,9 and 10) P. sativum. A protein body extract (PBE) from P. sativum was used to provide marker polypeptides in the outer two tracks. The $M_r \times 10^{-3}$ for globulins and albumins are shown on the left and right vertical axis, respectively. Each track was loaded with equal amounts of protein.

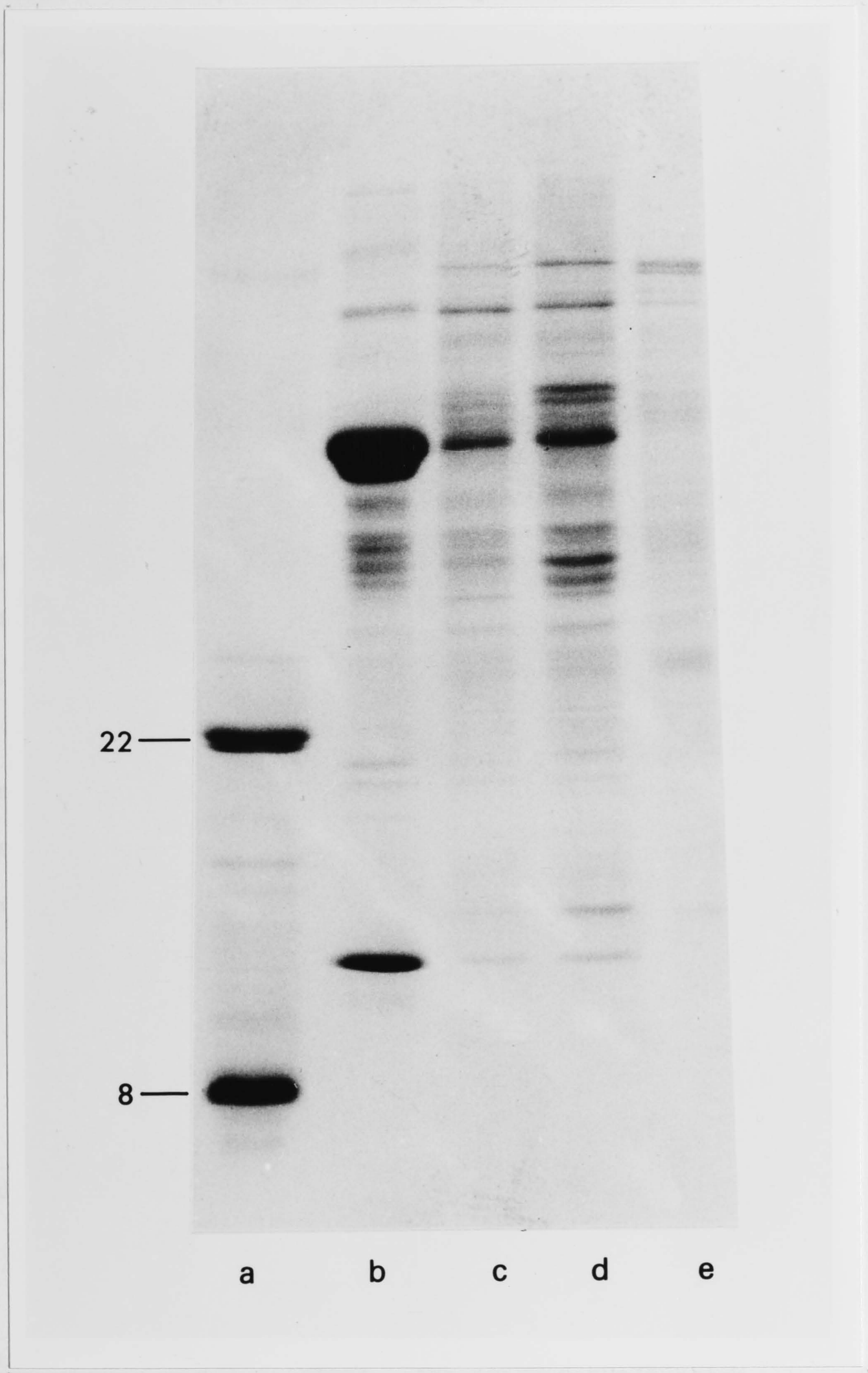


Fig. 2. Polypeptides from various tissues of our standard Pisum line PI/G 086 compared with seed albumins fractionated by SDS-PAGE under reducing conditions. (a) seed albumins; total extractable protein from (b) leaf, (c) stem, (d) pod and (e) root. Each track was loaded with equal amounts of protein determined by Biuret.

Consistent with this, when compared on an equal protein concentration basis, these plant protein extracts failed to react with antiserum against seed albumins when tested by immunodiffusion in Ouchterlony gel plates within 10 days (data not shown).

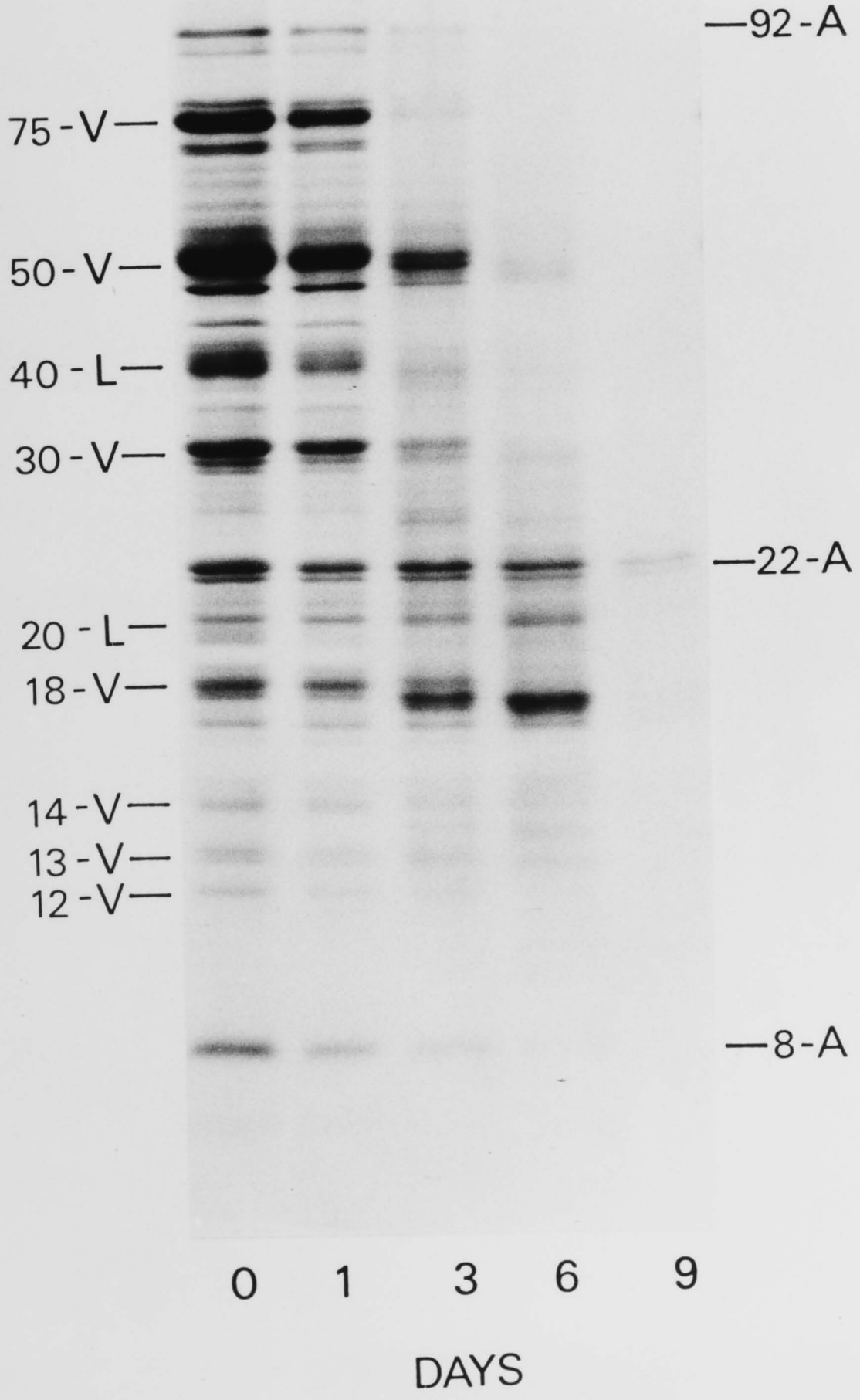
3.3. Protein utilization during germination - The fact that these two albumins were restricted to the cotyledons suggested that they may function as storage proteins. Their rate of degradation during germination was therefore compared with that of the globulin storage proteins legumin and vicilin. Total cotyledonary protein was extracted from germinating pea seeds, grown in complete darkness and their polypeptide patterns were examined by SDS-PAGE, from days 0 to 9 after imbibition (Fig. 3).

Insert Fig. 3

Utilization of Mr ∿ 75000 (vicilin) and Mr ∿ 40000 (legumin) polypeptides was observed first. Vicilin polypeptides of Mr ∿ 50000 and ∿ 30000 and albumin polypeptides of Mr ∿ 8000 were broken down more slowly. In contrast Mr ∿ 22000 albumin polypeptides, although markedly reduced quantitatively, were still resolved on day 9. The loading of equal volume of seed extract (see methods 2.3) reflected the absolute decrease in protein content of cotyledons from day 0 to 9. By day 9 most of the degradation products were sufficiently small to be no longer retained on the gel.

3.4. Sulfur content of the major albumins - The albumin polypeptide pattern of seeds from plants grown under sulfur deficiency conditions (-S) (Fig. 4) showed almost complete suppression of polypeptides in the M_r ∿ 8000 region while the M_r ∿ 22000 polypeptides appeared not to be as markedly affected. However, since the total albumin fraction from

Fig. 3. The remobilization of seed proteins during germination. Total cotyledonary proteins were extracted from germinating seeds, between 0 to 9 days after imbibition and fractionated by SDS-PAGE under reducing conditions. $M_r \times 10^{-3}$ of major globulins vicilin (V) and legumin (L) are shown on the vertical axis, left, those of major albumins (A) on the right. The PAG was stained with Coomassie blue. By day 9 most of the protein has been degraded to small polypeptides which apparently have run off the gel, except the M_r 22000 albumin polypeptides which could still be discerned.


—92-A
75-V—
50-V—
40-L—
30-V—
—22-A
20-L—
18-V—
14-V—
13-V—
12-V—
—8-A
0
1
3
6
9
DAYS

S-deficient peas is reduced by 30 percent, (from 27 percent in control seeds to 19 percent of total seed protein in S-deficient seeds), there is also a reduction in the absolute amount of the $M_r \sim 22000$ polypeptides. Furthermore, the equal loading of protein in both tracks of Fig. 4, and the almost total absence of $M_r \sim 8000$ protein tends to overrepresent the contribution of the $M_r \sim 22000$ protein in this comparison.

Insert Fig. 4

3.5. Quantitative analyses - The contributions of the Mr ∿ 22000 and Mr ∿ 8000 polypeptides to the total albumin fraction were determined and their relative sulfur content was estimated. In line 086, cv Greenfeast, the albumins made up 27 percent of the extractable cotyledonary protein but contain 68 percent of the total protein sulfur. Since it was not feasible to prepare sufficient Mr ∿ 22000 and Mr ∿ 8000 polypeptide protein for sulfur determinations by X-ray fluorescence spectometry, an indication of their contribution to the total albumin sulfur was sought by labelling developing seeds with [^{35}S] sodium sulfate throughout the major phase of protein accumulation. At seed maturity the albumin fraction was isolated and then fractionated by SDS-PAGE. The gel was first stained with Coomassie Blue and then subjected to fluorography. Densitometry of the stained gel showed that Mr ∿ 22000 and Mr ∿ 8000 bands together accounted for 34 percent (17 percent each) of the albumin fraction. (Fig. 5a), or 9 percent of the total cotyledonary protein. Densitometry of the fluorograph indicated that the Mr ∿ 22000 and Mr ∿ 8000 polypeptides together accounted for about 50 percent of the total label in the albumin fraction (Fig. 5b). Taking ^{35}S incorporation as a measure of the sulfur-amino acid content, this indicates that the $M_r \sim 8000$ polypeptides are particularly rich in these amino acids.

Insert Fig. 5a and b

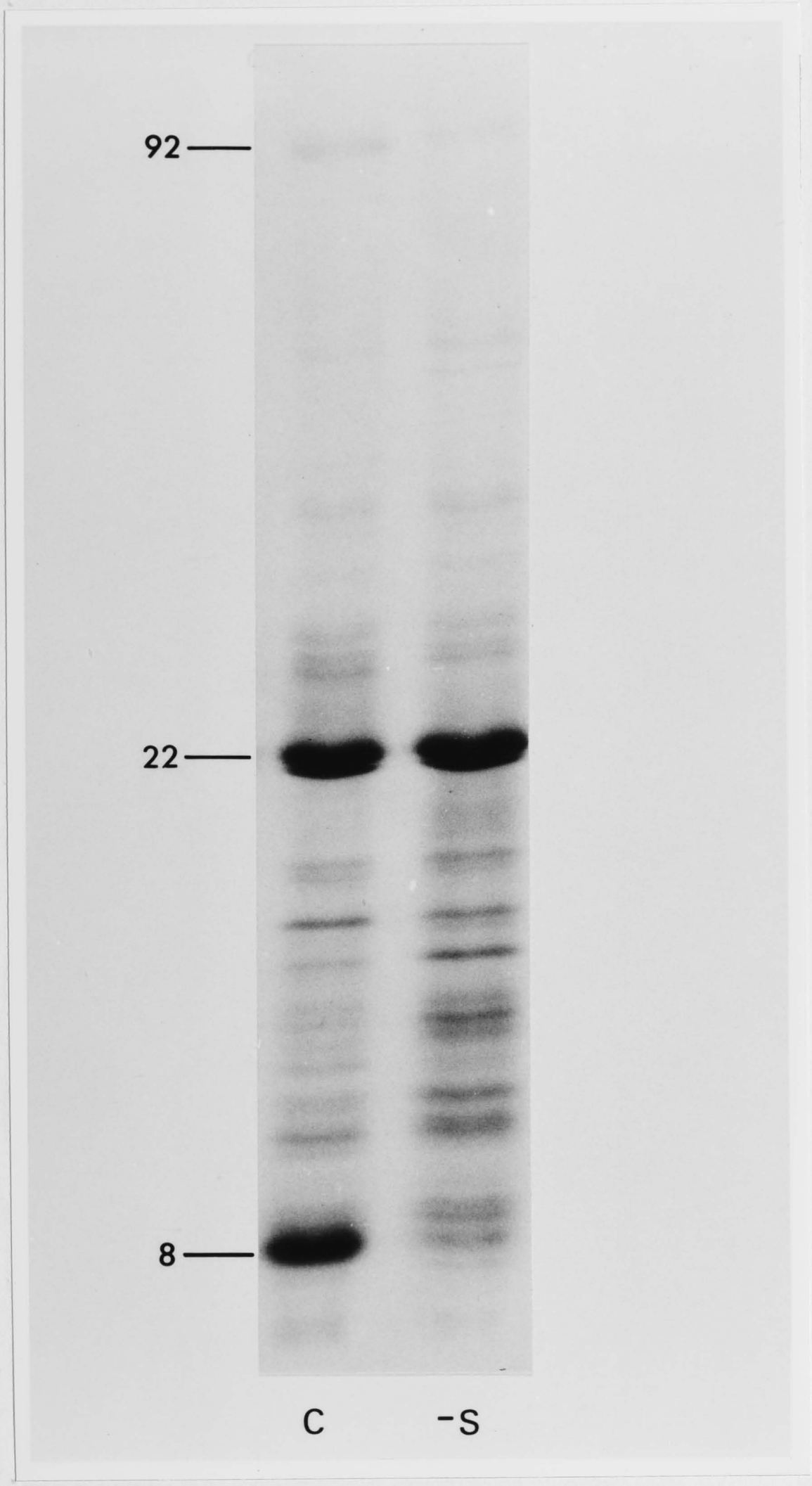
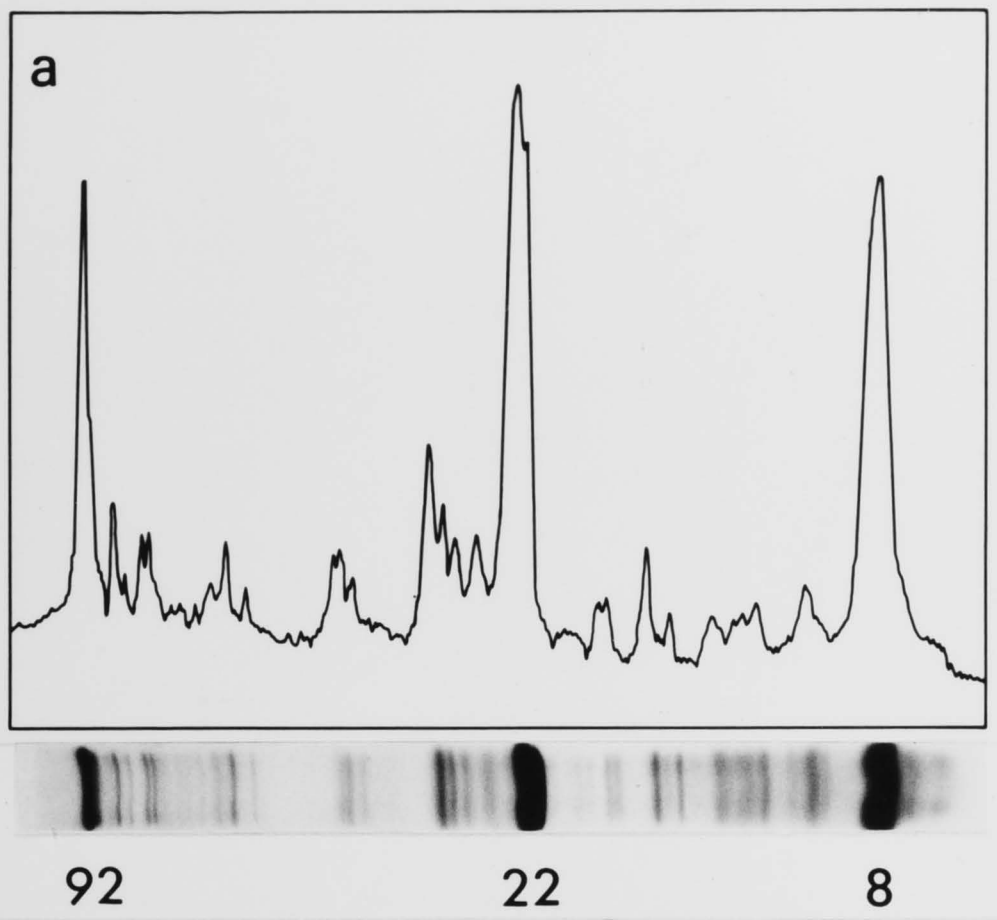


Fig. 4. The effects of sulfur deficiency on the albumin fraction of cotyledons of our standard line P. sativum PI/G 086. (C) control and (-S) sulfur deficient. $M_r \times 10^{-3}$ are shown on the vertical axis. There was a strong reduction of M_r 8000 polypeptides due to S deficiency. The M_r 22000 polypeptides are also reduced but this is largely masked by the equal protein loading in both tracks. Sulfur deficiency results in a 30 percent reduction in the albumin content of the cotyledons.

Fig. 5a and b. The contribution of the major albumin polypeptides to the total albumin polypeptide fraction of pea seeds and the distribution of sulfur-containing amino acids in these polypeptides. Developing seeds were labelled in situ with (^{35}S) sodium sulfate and at seed maturity the albumins were isolated and fractionated by SDS-PAGE under reducing conditions. The gel was first stained with Coomassie blue and then subjected to fluorography. (a) Coomassie-blue-stained gel and the densitometric scan of the stained gel, (b) Fluorograph of the same gel and the densitometric scan of the fluorograph.


a
92
22
8
b
92
22
8

4. Discussion

Studies on the albumins of pea seeds have shown that this protein fraction is amenable to biochemical characterisation and genetic analysis[7-10].

The albumins have a well-balanced amino acid profile and are relatively rich in S-amino acids[9-12]. Since the albumins in Pisum make up 20 to 35 percent of the total extractable cotyledonary proteins[1] it is likely that they are an important factor in determining the nutritional quality of legume seed protein. Here, I have shown that 34 percent of the albumin proteins of line 086 can be accounted for by equal contributions of two polypeptide classes ($M_r \sim 22000$ and $M_r \sim 8000$) Fig 5a. Both these polypeptides are quantitatively significant and of widespread occurrence throughout the genus Pisum (Fig. 1) and are restricted to the seed. The $M_r \sim 8000$ polypeptides appear to function as storage proteins since they were degraded during germination and early seedling growth; the $M_r \sim 22000$ polypeptides were not broken down to the same extent. When peas were grown under sulfur deficiency conditions there was a substantial reduction in the level of the $M_r \sim 22000$ components and an almost complete reduction of the $M_r \sim 8000$ polypeptides (Fig. 4). Consistent with this was the finding that the $M_r \sim 22000$ and $M_r \sim 8000$ polypeptides accounted for a major proportion of the [^{35}S] labelled albumins in the mature seed (Fig. 5b).

The occurrence of a S-amino-acid-rich, low-molecular-weight albumin fraction in Pisum has already been reported by Jakubek and Przybylska[10]. They fractionated pea seed albumins by G-100 column chromatography and obtained four fractions, S_1 to S_4. The S_3 fraction was shown to be sixfold richer in cystine than the other fractions but yielded no visible polypeptides on 10% SDS-PAG. The $M_r \sim 8000$ polypeptides described here, are also rich in sulfur-containing amino acids and are not retained on 10%

SDS-PAG (data not shown), but are clearly resolved on the gradient gels (12.5 to 25% polyacrylamide) used in this study. Given the similarities in their properties it is likely that polypeptides of the S_3 fraction correspond to the $M_r \sim 8000$ polypeptides reported in this paper. A comprehensive study of oil seeds by Youle and Huang[13] also reported the widespread occurrence of low molecular weight and high-cysteine-containing albumin proteins in a number of species.

Globulins have long been considered as storage proteins while albumins generally have been thought of as enzymatic and metabolic proteins[11,14]. Yet, at least some of the albumins appear to function as storage proteins in germination[15,16]. Murray[17] also proposed a storage role for albumins in pea cotyledons and results reported in this paper agree in principle with his conclusion since at least polypeptides in the M_r 8000 regions are shown to behave as storage proteins during germination. However, Murray based his interpretation on an albumin fraction which was largely contaminated with globulin polypeptides. It can be shown that the dialysis of a pea protein solution against running tap water and distilled water for only 18 h, as used by Murray[17], does not result in a clean separation of globulins from albumins (data not shown). The presence of vicilin and legumin polypeptides in Murray's albumin fraction of cv. Greenfeast makes the $M_r \sim 8000$ polypeptides a relatively minor albumin component when in fact in our standard line, also cv. Greenfeast, these polypeptides account for 17 percent of the albumin proteins, or even more significantly, 4.5 percent of the total extractable protein of the cotyledons. Of added significance is the sulfur richness of the $M_r \sim 8000$ polypeptides, which is important if one equates protein quality of legume seed protein with S amino acid content. These particular albumins represent a significant sulfur storage form in seeds and one would predict that nutritionally they are more valuable than other storage proteins (globulins) in the same seed[12].

References

1. Schroeder, H.E., Quantitative studies on the cotyledonary proteins in the genus Pisum. J. Sci. Food Agri. 1982, 33, 623-633.

2. Randall, P.J.; Thomson, J.A.; Schroeder, H.E., Cotyledonary storage proteins in Pisum sativum IV. Effects of sulphur, phosphorous, potassium and magnesium deficiencies. Aust. J. Plant Physiol. 1979, 6, 11-24 (1979).

3. Spencer, D.; Higgins, T.J., Button; S.C., Davey, R.A., Pulse-labelling studies on protein synthesis in developing pea seeds and evidence of a precursor form of legumin small subunit. Plant Physiol. 1980, 66, 510-515.

4. Gill, D.S., Kumarasamy, R., Symons, R.H., Cucumber mosaic virus induced RNA replicase: Solubilization and partial purification of the particulate enzyme. Virology 1981, 113, 1-8.

5. Dudman, W.F.; Millerd, A., Immunochemical behaviour of legumin and vicilin from Vicia faba: a survey of related proteins in the Leguminosae subfamily Faboideae. Biochemical Systematics and Ecology 1975, Vol. 3, 25-33.

6. Goa, J., A micro biuret method for protein determinations. Scand. J. Clin. Lab. Invest. 1953, 5, 218-222.

7. Przybylska, J.,; Blixt, S.; Hurich, J.; Przybylska, Z.Z., Comparative study of seed proteins in the genus Pisum I. Electrophoretic patterns of different protein fractions. Genetica Polouica 177, 18, 27-36.

8. Blixt, S.; Przybylska, J.; Przybylska, Z.Z., Comparative study of seed proteins in the genus Pisum V. Genetics of the electrophoretic Patterns I and III. Genetica Polonica 1980, 21, 153-160.

9. Hurich, J.; Parzysz, H.; Przybylska, J., Comparative study of seed proteins in the genus Pisum II. Amino acid composition of different protein fractions. Genetica Polonica 1977, 18, 241-252.

10. Jakubek, M.; Przybylska, J., Comparative study of seed proteins in the genus Pisum III. Electrophoretic patterns and amino acid composition of albumin fractions separated by gel filtration. Genetica Polonica 1979, 20, 369-380.

11. Boulter, D.; Derbyshire, E., In Chemotaxonomy of the Leguminosae (Harborne, J.B., Boulter, D., Turner, B.L. Eds.). Academic Press, London and New York 1971, pp. 285-308.

12. Bajaj, S.; Mickelsen, O.; Lillevik, H.A.; Baker, L.R.; Bergen, W.G.; Gill, J.L., Prediction of protein efficiency ratio of peas from their albumin content. Crop Science 1971, 11, 813-815.

13. Youle, R.J.; Huang, A.H.C., Occurrence of low molecular weight and high cysteine containing albumin storage proteins in oilseeds of diverse species. Amer. J. Bot. 1981, 68(1) 44-48.

14. Millerd, A., Biochemistry of legume seed proteins. Ann. Rev. Plant Physiol. 1975, 26, 53-72.

15. Youle, R.J.; Huang, A.H.C., Albumin storage proteins in the protein bodies of castor bean. Plant Physiol. 1978, 61, 13-16.

16. Manickam, A.; Carlier, A.R., Isolation and function of a low molecular weight protein of mung bean embryonic axis. Planta 1980, 149, 234-240.

17. Murray, D.R.; A storage role for albumins in pea cotyledons. Plant, Cell and Environment 1979, 2, 221-226.

CHAPTER 4:

A Manuscript entitled:

Inheritance of Legumin and Albumin Contents in a Cross Between Round and Wrinkled Peas

Submitted for publication to the Journal Theoretical and Applied Genetics, September 1983.

Inheritance of Legumin and Albumin Contents in a Cross Between Round and Wrinkled Peas

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Summary. Legumin and albumin are the fractions of pea seed proteins preferred to vicilin because of their high sulfur amino acid contents. The joint inheritance of legumin and albumin contents was studied in a cross between two contrasting lines of peas - one with high legumin and low albumin, and the other with low legumin and medium to high albumin. Single seed determinations were made in the parental, F_1, F_2 and backcross generations using rocket immunoelectrophoresis. In the non-segregating generations (P_1, P_2 and F_1), legumin and albumin contents were negatively correlated ($r = -Z\ 0.50$). The estimates of correlation coefficients in the segregating generations (F_2, BC_1 and BC_2) were also about -0.5. However, the two estimates based on the round and on the wrinkled seeds separately in the F_2 generation were not significantly different from zero. At least four individual round F_2 seeds showed the desired recombination of high legumin with high albumin indicating that the unfavorable correlation can be broken. In this cross legumin content showed predominantly additive genetic variation whereas the dominance variance was the largest component for albumin content. A combined "relative sulfur index", proposed as a convenient measure for selection, showed a narrow sense heritability of 47 per cent. In general these results support the view that sulfur amino acid content of peas can be improved by breeding, but that the required selection regime must take both legumin and albumin content into account.

Key words: Seed proteins - legumin - albumin - heritability

Introduction

The seed proteins in pea (Pisum sativum L.) seeds are made up of the storage globulins legumin and vicilin together with the albumins. Globulins account for 65-80 percent and albumins for 20-35 percent of the extractable protein of pea cotyledons (Schroeder, 1982). The legumin, vicilin and albumin contents are genotype-specific but also environment-dependent (Füredi, 1970; Bajaj et al. 1971; Gottschalk et al. 1975; Davies, 1976, 1980; Przybylska et al. 1977; Thomson et al. 1979; Randall et al. 1979). Of the storage proteins, legumin has a higher sulfur amino acid content than vicilin and is therefore a more desirable protein fraction in terms of animal nutrition. It appears then, that sulfur amino acid content could be increased by genetically altering the proportions of storage proteins, i.e., raising legumin content at the expense of vicilin content. However, the albumins, as a protein fraction, have a considerably higher sulfur amino acid content than legumin with an overall more favourable amino acid profile than the globulins (Bajaj et al. 1971; Hurich et al. 1977; Boulter and Derbyshire, 1971) and they are a quantitatively significant protein fraction. Furthermore it was previously found (Schroeder, 1982) that legumin and albumin contents of a diverse set of lines are negatively correlated ($r = -0.76$) indicating that protein quality, if assessed in terms of sulfur amino acid content, cannot be improved by raising only the legumin content. The aim then should be to break the negative correlation and to combine high levels of legumin with high levels of albumins. Therefore a realistic assessment of the prospects of increasing protein quality in peas depends on an understanding of the joint genetic control of legumin and albumin contents.

The purpose of this experiment was to study the joint inheritance and heritability of legumin and albumin contents in P. sativum. The results of the genetic analysis show that to be successful, breeding for increased protein quality must take account of both these sources of sulfur amino acids.

Materials and Methods

Plant Material

Crosses were made between a low legumin, medium to high albumin, wrinkled seeded line (Parent 1), and a high legumin, low albumin, round seeded line (Parent 2). Parent 1 was cv. Greenfeast, the Canberra control line PI/G 086 (Plant Industry/Genetics). Parent 2 was PI/G 307 (Pisum Genebank, Weibullsholm number WL110, Kungsärt). Seeds from parent lines were harvested only from those plants used in reciprocal crosses for the production of F_1, BC_1 and BC_2 generation seed. Cross combinations were made in both directions. F_2 generation seed was represented by seeds borne on F_1 plants. The characters measured were seed weight, percent extractable protein and legumin and albumin contents as percentages of extractable protein.

All plants were grown in a sand, loam, peat moss mixture (1:1:1) in 23 cm pots, with complete fertilizer added, in a glasshouse under natural light. The temperature range was 18°C (night) to a maximum of 24°C (day). Seed was harvested from completely desiccated plants, air-dried to a moisture content of 6.5-7.5 percent and then weighed.

Buffer Extractable Protein

Single seeds were dehulled and the cotyledons milled to a fine flour passing through a 0.2 mm screen. Duplicate samples of 50 mg flour per seed were extracted twice with 0.5 ml TBE extraction buffer (0.5 M tris (hydroxymethyl) aminomethane, 0.01 M ethylene diaminotetraacetic acid,

titrated with boric acid to pH 9.2 containing 1 mM phenylmethyl-sulfonylfluoride). After vortexing, the samples were put on an end-over-end shaker for 30 min at room temperature. The homogenate from each flour sample was centrifuged at 20000 g for 20 min at 5°C. Aliquots of the supernatant were used directly for protein determinations by biuret (Goa, 1953) and to measure legumin and albumin contents.

Determination of Legumin and Albumin Contents

Legumin and albumin contents as percentages of total extractable cotyledonary protein from flour were determined by Laurell's rocket immunoassay (Weeke 1973) using specific antisera raised in sheep, against purified legumin and albumin protein fractions. Samples (10 µl of single seed extracts were applied to wells cut into agarose gels (0.9%) into which either antilegumin or antialbumin serum had been mixed before cooling. Standards of pure legumin (9-19 µg) and pure albumin (7-19 µg) were included in the respective gels. The distance from the origin to the tip of the precipitin line was proportional to the amount of legumin in the samples. Rocket immunoelectrophoresis of the albumin fraction resulted in a rocket with 5-7 precipitin lines. However, the height of the major line of the rocket was directly proportional to the albumin concentration of albumin standards and albumin in the samples tested. By reference to a standard curve plotted for each gel, the amount of either legumin or albumin in single seed extracts was determined and expressed as percent of extractable protein. The duplicate extracts from each seed provided replicates of each variable legumin and albumin. Subsequent computations used the means of these two replicates.

Relative Sulfur Index

By using sulfur amino acid data from the literature (Casey and Short, 1981; Hurich et al. 1977; Boulter and Derbyshire, 1971) and percentages of legumin, vicilin and albumin, it can be calculated that most lines of peas differ little if at all in sulfur amino acid content (Schroeder 1982). This finding led us to calculate a relative sulfur index value for each of the seeds analysed in this cross. The sulfur index is proportional to the sum of 1.40 x legumin %, 0.38 x vicilin %, and 3.53 x albumin %. Since the three fractions account for all the extractable cotyledonary protein this relationship can conveniently be expressed as -

relative sulfur index = legumin % + 3.1 x albumin %

Genetic Analysis

Genetic analysis was performed on the following characters; seed weight, protein content, the concentrations of the protein fractions legumin and albumin and the calculated relative sulfur index value. The additive (D) and dominance (H) genetic components of variance, and the environmental (E) component, as well as the narrow and broad sense heritabilities for each character were calculated by standard procedure (Mather and Jinks, 1971). These used the generation variances for the parental lines, the two reciprocal F_1's, the four backcrosses and the two F_2's. The four backcrosses arose because each of the two reciprocal F_1's were used as either male or female parents in backcrosses. In generations segregating for round versus wrinkled seed (backcrosses and F_2's) each seed was classified for seed type and the means and variances for all characters computed first within seed types. In backcrosses every seed harvested was analysed. In the F_2's all seeds harvested (total of 759) were grouped into round and wrinkled phenotypes to determine the phenotypic ratio. However, equal numbers of round and wrinkled seeds, taken at random

from the two groups, were analysed. The means for each of the segregating generations were derived by averaging these groups weighted by the expected ratio. The variances of these whole populations included both the variation within seed phenotypes and the variation among seed phenotypes.

Results

Legumin and Albumin Contents

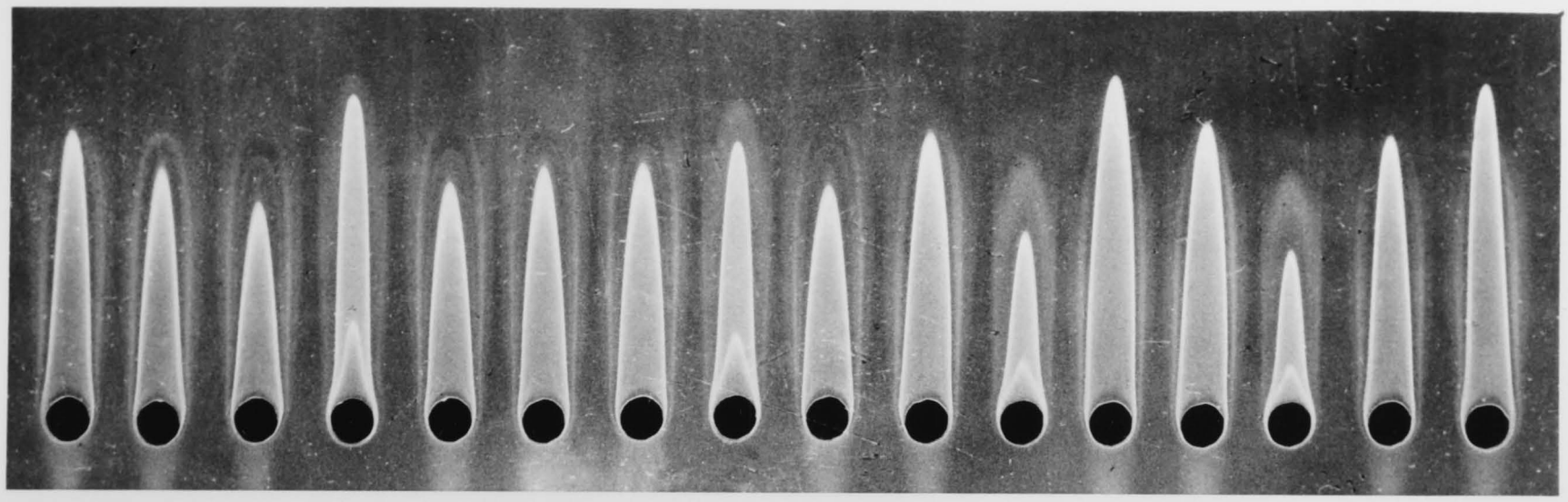
Determination of legumin and albumin content by rocket immunoelectrophoresis of a number of single seed extracts of P_1, P_2, F_2's and pure legumin and albumin standards are shown in Fig. 1a and b.

Insert Fig 1a and b.

In rockets of legumin standards, a second (minor) rocket is observed which is not seen in seed extracts. Presumably the concentration of this legumin component is too low in seed extracts to give a reaction. Heterogeneity in the albumin fraction is indicated by the number of minor rockets within the major rocket. The antiserum against albumins was made against an equal mixture of pure albumins from 20 lines of peas; this explains differences observed in the intensity of the minor rockets of standards and of seed extracts of parents and crosses. But chemical analyses of albumin contents and globulin to albumin ratio determinations (Schroeder, 1982) of all the lines tested so far (data not shown) indicated that the height of the major rocket is proportional to the albumin concentration in protein extracts. A calibration curve showing the relationship between amounts of legumin and albumin standards and the length of the immunoprecipitates (rockets) formed are shown in Fig. 2.

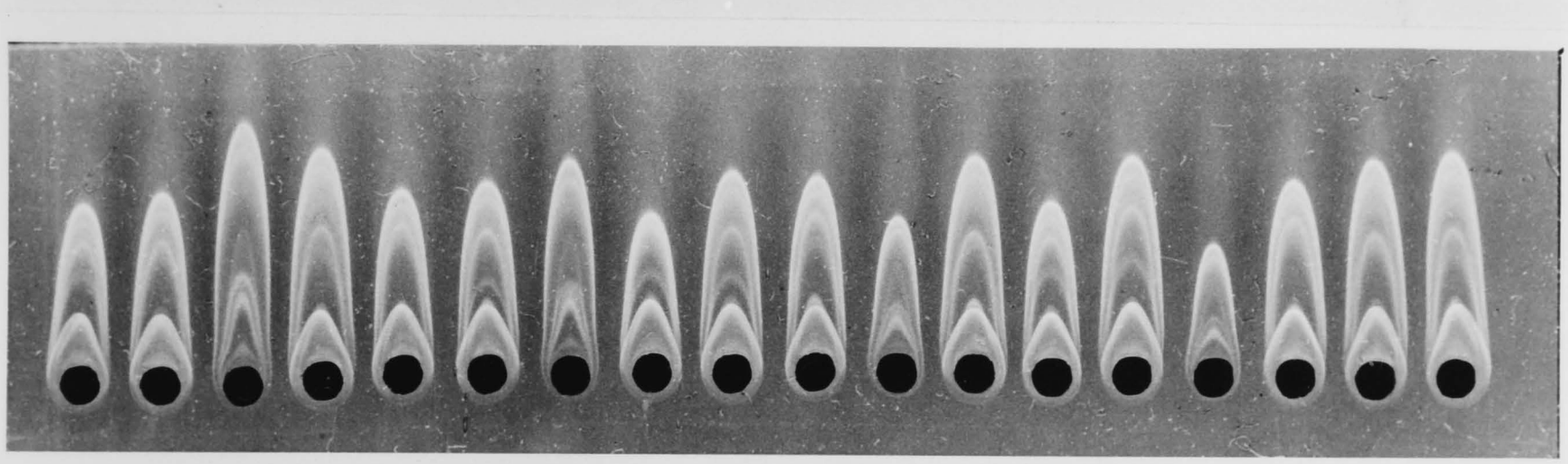
Insert Fig. 2

LEGUMIN



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

ALBUMIN



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Fig. 1a and b. Determination of legumin and albumin contents by rocket immunoelectrophoresis. (a) rockets; 4,8,11 and 14 are legumin standards, 3 and 5 are $parent_1$, 13 and 15 are $parent_2$, others are F_2's. (b) rockets; 3,7,11 and 15 are albumin standards, 4 is $parent_1$, 8 is $parent_2$, others are F_2's.

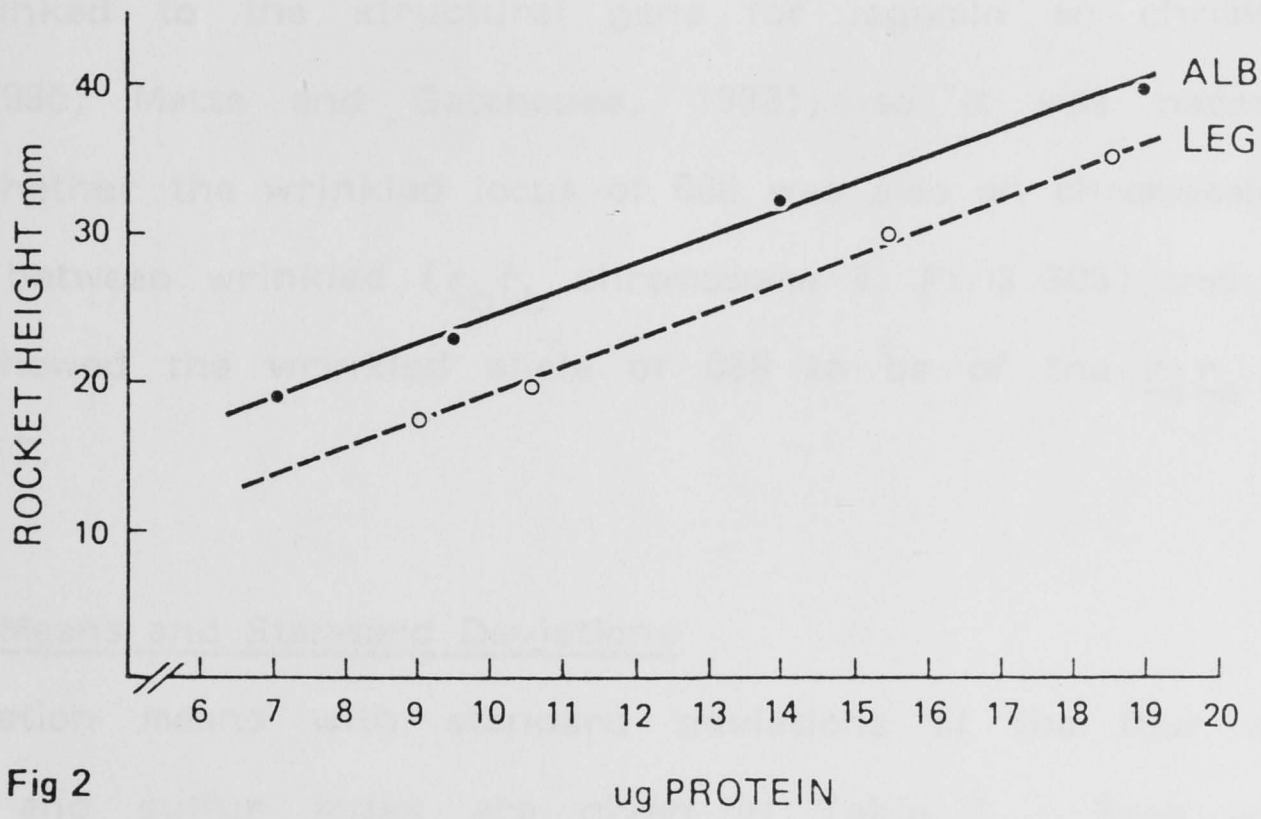


Fig 2

Standard curves of legumin and albumin. Rocket height is proportional to the concentration of legumin and albumin in the total protein extracted from flour.

Segregation of Round Versus Wrinkled

The present cross segregated for the classic character round versus wrinkled seeds in the expected 3:1 ratio in the F_2's (572:187, x^2 = 0.0295). From the standpoint of seed protein fractions this major gene difference complicated the analysis. The choice of parents was largely determined by results from a previous survey of over 100 P. sativum lines which found that high legumin content was associated with round seeds while high albumin content was associated with wrinkled seeds. Furthermore, the wrinkled phenotype can be due to recessivity at either the r_a locus on chromosome 7 or at the r_b locus on chromosome 3. The r_a locus is linked to the structural gene for legumin on chromosome 7 (Davies, 1980; Matta and Gatehouse, 1982), so it was necessary to establish whether the wrinkled locus of 086 was also on chromosome 7. A test cross between wrinkled ($r_b r_b$ chromosome 3, PI/G 305) and wrinkled (rr 086) showed the wrinkled allele of 086 to be of the $r_a r_a$ type on chromosome 7.

Generation Means and Standard Deviations

The generation means with standard deviations of the four measured characters and sulfur index are given in Table 1. Seed weight is dominated by the R/r gene. Legumin and albumin percentage means in reciprocal F_1's were close to the midparent value although the direction of the cross had some bearing; legumin was higher and albumin lower when P_2 was the female parent. In the segregating populations the genes for round versus wrinkled markedly affected the levels of legumin and albumin. As shown in Table 1 and seen in more detail in Fig. 3 higher legumin was associated with the round phenotype (R-) while higher albumin is associated with wrinkled (rr) in F_2 populations. This result differs from BC_1 populations (Table 1, Fig. 4), where in both Rr and rr

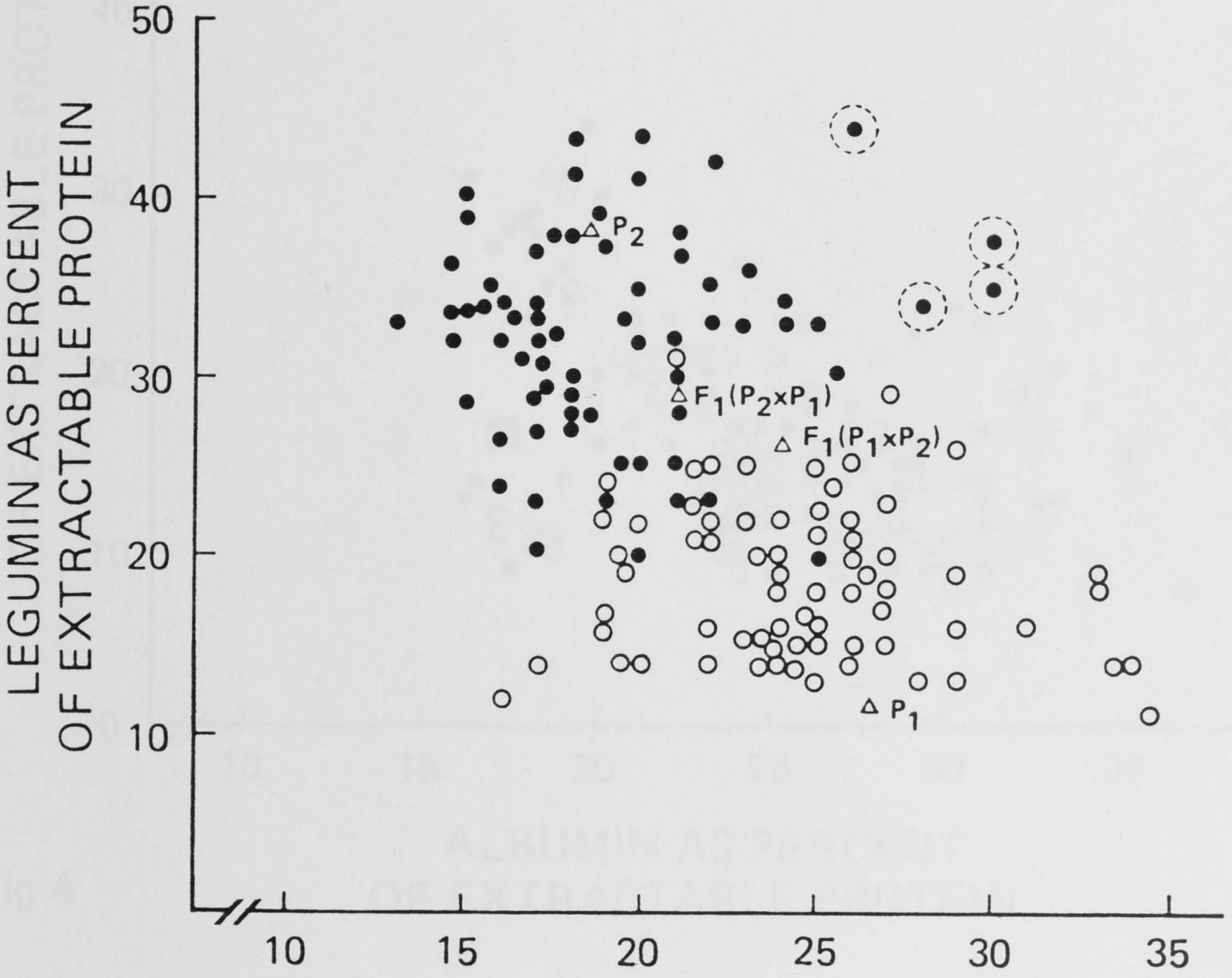


Fig 3

Relationship of legumin and albumin contents of individual F_2 seeds. Results are expressed as the percentages of each fraction in the protein extracted from flour; ●, round (R-) phenotypes, 0, wrinkled (rr) phenotypes. Correlation coefficients: r= -0.513 $p < 0.001$, R-phenotypes only r= -0.040, rr phenotypes only r= -0.154.

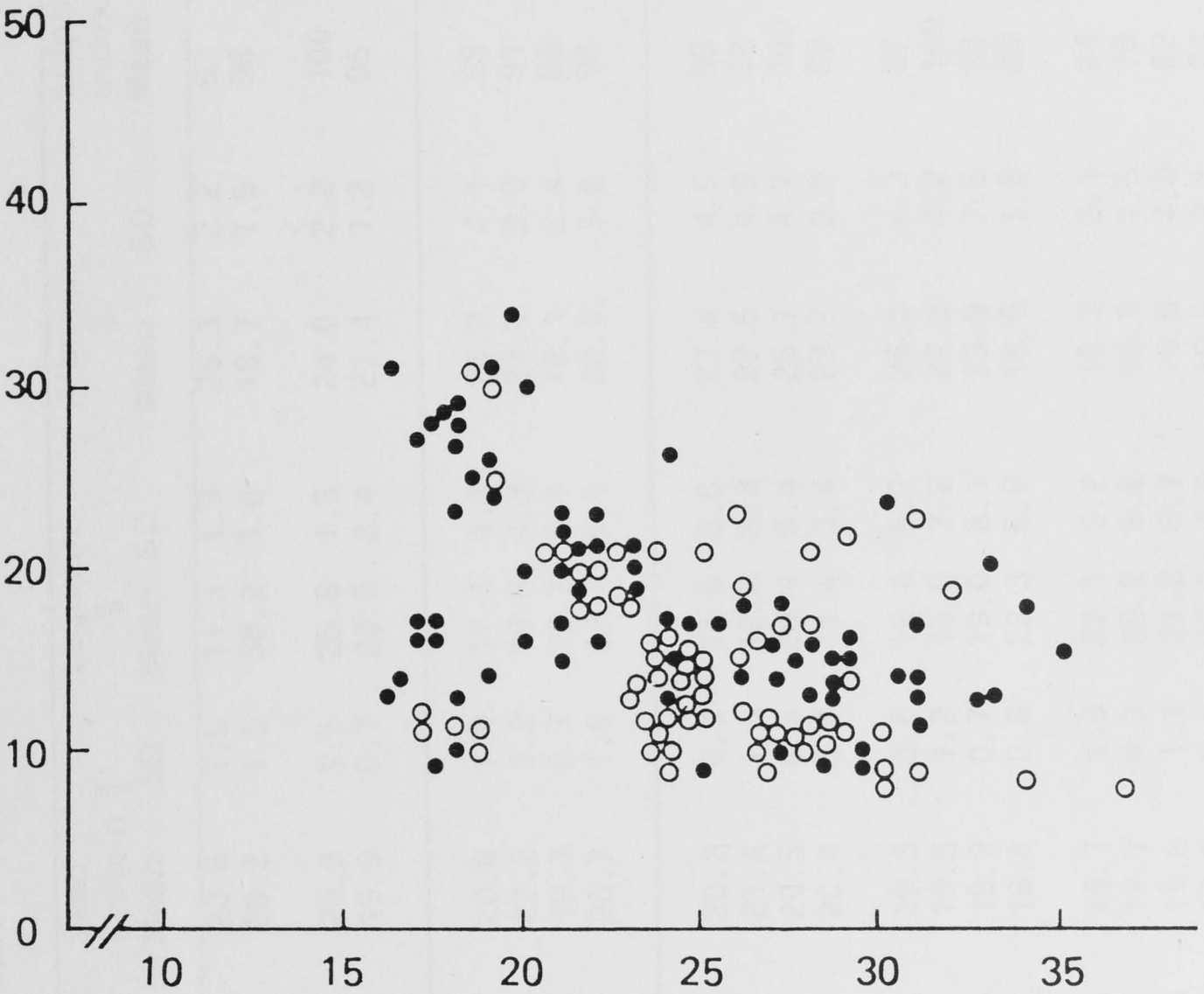


Fig 4

Relationship of legumin and albumin contents of individual BC_1 seeds. Results are expressed as the percentages of each fraction in the protein extracted from flour; ● round (R-) phenotypes, 0 wrinkled (rr) phenotypes. Correlation coefficient $r = -0.419$ $p < 0.01$.

Table 1. Means and standard deviations (SD) of seed and protein characters in various generations of the cross between Pisum sativum cv. Greenfeast PI/G086 and PI/G307

	Seed Phenotype	Seed Weight		Seed Protein %		Legumin %		Albumin %		Sulphur Index		Sample Size
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
P_1 = 086	rr	295	19	20.6	1.5	11.3	1.3	26.3	2.2	93	6	28
P_2 = 307	RR	381	21	20.2	1.5	38.2	3.6	18.7	1.9	96	5	28
F_1												
086 x 307	Rr	389	16	20.9	1.1	25.8	1.8	24.0	2.2	100	6	14
307 x 086	Rr	357	10	19.9	0.7	29.0	2.4	21.4	1.2	95	4	14
F_2												
086 x 307	rr	342	23	20.6	1.0	17.4	4.3	26.2	4.1	99	12	34
307 x 086	rr	349	28	22.2	1.2	19.6	4.3	23.1	3.0	91	11	36
086 x 307	R-	382	19	19.7	0.7	32.2	6.1	18.1	3.4	88	12	35
307 x 086	R-	398	26	20.7	1.0	32.7	5.1	20.5	3.6	96	13	36
BC_1												
086 x (086 x 307)	rr	292	16	20.2	0.9	13.6	6.0	27.4	4.5	99	14	20
086 x (307 x 086)	rr	349	23	21.4	1.3	12.6	5.4	27.5	4.9	98	13	13
(086 x 307) x 086	rr	400	39	20.5	0.6	20.4	6.4	25.7	4.7	100	10	8
(307 x 086) x 086	rr	370	25	20.4	0.9	14.4	3.4	23.7	3.0	89	9	21
086 x (086 x 307)	Rr	294	19	19.3	0.6	16.4	5.7	26.0	4.3	97	12	25
086 x (307 x 086)	Rr	373	28	19.3	1.2	15.9	3.2	27.2	4.2	100	11	17
(086 x 307) x 086	Rr	415	39	19.2	0.7	19.0	8.1	23.9	7.5	93	24	7
(307 x 086) x 086	Rr	396	27	19.8	0.8	22.3	6.9	18.6	1.6	80	9	24
BC_2												
307 x (086 x 307)	R-	363	35	19.7	1.0	27.1	5.2	18.3	3.1	84	8	20
307 x (307 x 086)	R-	359	26	19.7	0.7	28.2	3.6	16.3	1.5	79	6	26
(086 x 307) x 307	R-	378	25	19.6	1.1	21.2	6.7	19.6	4.0	82	7	36
(307 x 086) x 307	R-	378	21	19.8	0.6	23.5	4.6	19.6	2.1	84	8	32

Table 2. Generation variances, variance components and heritability estimates for seed protein characters.

Generation	Legumin content		Albumin content		Sulphur Index	
P_1	1.6		5.1		42	
P_2	13.3		3.7		27	
F_1	7.0		4.8		29	
F_2*	64.7	32.0	19.1	13.5	170	178
BC_1*	40.6	41.7	25.5	28.7	192	222
BC_2	35.4		10.3		55	
Variance Components						
D-Additive	106.8	0	4.8	0	186	159
H-Dominance	16.4	113.4	48.4	60.8	180	284
E-Environmental	7.2	10.2	4.6	4.3	32	28
Heritability						
h^2 narrow	82	0	8	0	47	34
h^2 broad	94	92	92	93	93	94

where $E = (P_1 + P_2 + 2F_1)/4$

$D = 4F_2 - 2(BC_1 + BC_2)$

$H = 2(BC_1 + BC_2) - D - 4E$

*. In the two generations segregating for round (R-) versus wrinkled (rr), two variance estimates are given. The first is a combined estimate over both phenotypes, the second is for variances within the round phenotype. This leads to two sets of genetic statistics.

populations two doses of 086 (P_1) caused lower legumin but higher albumin levels.

The Effect of the Major Gene Round Versus Wrinkled

The variation in legumin and albumin content of protein extracts from 141 individual F_2 seeds are shown in Fig. 3. For comparison, the mean values of these two characters for P_1, P_2 and reciprocal F_1's are also shown. Round and wrinkled F_2's separate into two almost distinct groups. The previously established negative correlation between legumin and albumin content among diverse genotypes (Schroeder 1982) was again apparent in this segregating population, r = -0.513, p F 0.001. On the other hand the joint distribution of legumin and albumin within the genetically uniform populations (P_1, P_2, F_1) resulted in correlation coefficients of r = -0.53, r = -0.50 and r = -0.56 respectively; all these estimates were statistically significant at the p F 0.01 level. Thus, environmental variation can cause a negative correlation between legumin and albumin. However, this is not the sole cause of the correlation in the F_2 generation because the phenotypic variances of legumin and albumin are much greater in the F_2 generation than in P_1, P_2 and F_1 generations (see Table 2).

When the correlation coefficients were calculated separately for the two F_2 subpopulations, the round phenotypes had r = - 0.040 and the wrinkled r = -0.154, i.e., they were close to zero and within these subpopulations legumin and albumin behaved like independent characters. This contrasted with the behavior of legumin and albumin in the BC_1 populations (Fig. 4), which showed no difference in segregation patterns between round and wrinkled subpopulations. Two doses of P_1 (086) caused lower legumin but high albumin population means (Table 1) in this generation. In BC_2 populations with all round seeds, two doses of P_2 (307) caused low albumin and lower than expected legumin levels. A significant negative correlation (r = -0.657 p F 0.001) was observed.

Genetic Variances and Heritability

In this cross legumin content is controlled largely by genes with additive effects whereas albumin content is much more influenced by dominant genes (Table 2). For both legumin and albumin contents, the additive component is zero when the estimates are based on only the subpopulation of round seeds from the segregating generations. This arises because the variances of legumin and albumin contents in the F_2 generation are markedly less within the round subpopulation than for the whole generation whereas the BC_1 variances are not so affected. Compared with the F_2 generation, two doses of P_1 (086) reduced the variance of legumin content but increased the variance of albumin content, possibly indicating a pleiotropic effect of wrinkled (rr). The character sulfur index was different to both legumin and albumin content. Its inheritance in this cross showed both additive and dominance effects. Further, when the data from the round seeds are considered alone, the estimates of additive variance and heritability are not different from those based on all seeds. If legumin and albumin were independent characters, the variance for sulfur index in any generation should be predictable as the sum of variance of legumin content plus $(3.1)^2$ times the variance of albumin content. The theoretical estimates are 248 for the whole F_2 population and 162 for the round F_2 subpopulation. The difference (248-170) arises from the negative correlation between legumin and albumin (Fig. 3). For the round subpopulations however, there was no difference between the theoretical variances. These results clearly support selection based on sulfur index.

Discussion

In Pisum, the two seed protein fractions rich in sulfur amino acids legumin and albumin, were previously found to be negatively correlated (Schroeder 1982). Hences seed protein quality may not be improved simply by altering the proportions of the major storage proteins, i.e., by

increasing legumin at the expense of vicilin. This raises the important question, can the correlation be broken genetically and recombinant genotypes be identified and selected that combine high legumin with high albumin contents.

The inheritance of legumin and albumin content in peas was studied in a cross in which the parents had similar protein content, but differed in seed weight, seed type, and legumin and albumin content (Table 1 and 2). Seed weight and protein content were of lesser interest in this study. Seed weight is important in determining yield and protein yield, but this cross was not set up to study this variable. Inheritance of protein content has already been studied in more appropriate crosses (for example see: Slinkard, 1980; Weber, 1981; Swiecicki, 1981). Little variation for this variable existed in this cross.

In this cross it was found that the genes for seed type (R/r) had a major effect on seed protein composition, namely legumin and albumin contents (Fig. 3 and 4). Variation at this locus also had a marked effect on estimates of the components of variances for legumin content, albumin content and sulfur index (Table 2). Out of a total of 141 F_2 generation seeds four seeds with the round phenotype (circled, Fig. 3) were identified which apparently combine relatively high levels of legumin and albumin. No comparable phenotypes were found in the wrinkled F_2 subpopulation indicating the confounding effect of the rr genotype on seed protein composition.

The combined high legumin and high albumin contents of the four F_2 progeny indicate that total sulfur amino acid content should be considerably increased in the protein of these seeds. Total sulfur estimates have been proposed as coarse indicators of S-amino acid levels (Boulter et al 1976). In this case, measurements of total sulfur, 0.233% for the four F_2's, 0.206% for P_1 and 0.220% for P_2, indicate an increase in

S-amino acid content in the four F_2 seeds. The mean value of the relative sulfur indices for the four F_2's was 126.6. This represents a substantial increase over the values of 93 for P_1, 96 for P_2, 93.5 for the F_2 populations and 93.5 ± 7.8 for the 45 lines of peas previously examined. The sulfur index is a function of the individual legumin and albumin determinations. While it affords a ready way of combining the major components of pea seed proteins into one comparative value an important reservation concerning the sulfur index and it's use in plant breeding needs to be stated. This is the assumption that there will be no change, quantitatively or qualitatively, in the protein composition of legumin and/or albumin when their proportions are altered genetically.

High legumin high albumin recombinants were only found in the round $\underline{R}$-F_2 subpopulation, but because sampling procedures were destructive, progeny testing was not possible. This leaves open the question whether these four F_2 seeds were in fact correlation breakers and whether they would breed true for high legumin and high albumin contents, and consequently higher S-amino acid contents. Further studies are needed in this cross and other crosses to confirm the existence of high legumin high albumin recombinants by non-destructive sampling of F_2 seeds and progeny testing, or by assaying F_3 families.

Literature

Bajaj, S.; Mickelsen, O.; Lillevik, H.A.; Baker, L.R.; Bergen, W.G.; Gill, J.C. (1971): Prediction of protein efficiency ratio of peas from their albumin content. Crop Sci. 11, 813-815

Boulter, D.; Derbyshire, E. (1971): In Chemotaxonomy of the Leguminosae (Harborne, J.B.; Boulter, D,; Turner, B.L. Eds) Academic Press, London & New York, 285-308

Boulter, D.; Evans, I.M.; Thompson, A. (1976): Screening for protein quality in legumes. Plant. Plant Foods Hum. Nuts. 26,, 107-119

Casey, R.; Short, M.N. (1981): Variation in amino acid composition of legumin from Pisum. Phytochem. 20, 21-23

Davies, D.R. (1976): Variation in the storage proteins of peas in relation to sulphur amino acid content. Euphytica 25, 717-724

Davies, D.R. (1980): The r_a locus and legumin synthesis in Pisum sativum. Biochem. Gen. 18, 1207-1219

Füredi, J: (1970): Importance of breeding peas with increased protein content. In Protein Growth by Plant Breeding (Balint, A., Ed) Akademiai Kiado, Budapest pp 99-128

Goa, J.A. (1953): A micro-biuret method for protein determination. Scan. J. Clin. Lab. Invest. 5, 218-222

Gottschalk, W.; Mueller, H.P.; Wolff, G. (1975): Relations between protein production. Protein quality and environmental factors in Pisum mutants. IAEA-PI-570/14 Vienna pp 105-123

Hurich, J.; Parzysz, H.; Przybylska, J. (1977): Comparative study of seed proteins in the genus Pisum II. Amino acid composition of different protein fractions. Genetica Polanica 18, 241-252

Mather, K.; Jinks, J.L. (1971): Biometrical Genetics, London

Matta, N.K.; Gatehouse, J.A. (1982): Inheritance and mapping of storage protein genes in Pisum sativum L. Heredity 48(3), 383-392

Przybylska, J.; Blixt, S.; Hurich, J.; Przybylska, Z.Z. (1977): Comparative study of seed proteins in the genus Pisum I. Electrophoretic patterns of different protein fractions. Genetica Polonica 18, 27-38

Randall, P.J.; Thomson, J.A.; Schroeder, H.E. (1979): Cotyledonary storage proteins in Pisum sativum IV. Effects of sulfur, phosphorus, potassium and magnesium deficiencies. Aust. J. Plant Physiol. 6, 11-24

Schroeder, H.E. (1982): Quantitative studies on the cotyledonary proteins in the genus Pisum. J. Sci. Food Agric. 33, 623-633

Thomson, J.A.; Millerd, A.; Schroeder, H.E. (1979): Genotype dependent patterns of accumulation of seed storage proteins in Pisum. In Seed Protein Improvement in Cereals and Legumes. Vol.II, IAEA-SM-230/61 Vienna pp 231-240

Slinkard, A.E. (1980): In Production, Utilization and Marketing of Field Peas Volume I, Crop Deveopment Centre, University of Saskatchewan, Canada, Subprojects I-IV

Swiecicki, W.K.; Kaczmarek, Z.; Surma, M. (1981): Inheritance and heritability of protein content in seeds of selected crosses of pea (Pisum sativum L.). Genetica Polonica 22, 189-194

Weber, W.E. (1981): Untersuchungen zur Vererbung des Proteingehaltes in Erbsen (Pisum sativum L.). Z. Pflanzenzüchtg. 86, 201-209

Weeke, B. (1973): Rocket immunoelectrophoresis. In A Manual of Quantitative Immunoelectrophoresis. (Axelson, N.H.; Knoll, J.; Weeke, B.; Eds) Universitetsforlaget, Oslo, pp 47-56

CHAPTER 5:

A Manuscript entitled:

Effects of Applied Growth Regulators on Pod Growth and Seed Protein Composition in Pisum sativum L.

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Effects of Applied Growth Regulators on Pod Growth and Seed Protein Composition in Pisum sativum L.

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Abstract

Plants of Pisum sativum L. raised from seed of an isogenic line selected from cv. Greenfeast were grown under glasshouse conditions. The plant growth regulators naphthalene-acetic acid, benzyl adenine, abscisic acid and gibberellic acid (GA_3) were administered to developing fruits by daily injections into the pedicels of pods between 2 and 32 days after full bloom, thereby spanning the time of maximum protein synthesis. Changes were observed in growth rates and dry weights of pods at maturity. Total protein content per cotyledon increased in naphthalene-acetic acid, benzyl adenine and abscisic acid treatments. Legumin increased in response to naphthalene-acetic acid and benzyl adenine while vicilin increased in response to abscisic acid. The albumin content was not affected. Gibberellic acid (GA_3) caused no changes in total protein content or in levels of individual protein fractions. The level of legumin was further increased by application of an equal mixture of naphthalene-acetic acid and benzyl adenine; this treatment also resulted in a marked increase in the albumin fraction but a considerable decrease in vicilin content. The results imply that hormones play a role in regulating protein synthesis and accumulation in Pisum.

Keywords - Hormones - Seed - Protein - Composition

Introduction

The occurrence of hormones in developing and mature seeds of many species has been firmly established (Bewley and Black, 1978; Letham, Goodwin and Higgins 1978). Little is known about the role of hormones in seed development and seed protein synthesis. This contrasts with the extensive knowledge on the hormonal regulation of enzyme synthesis during seed germination, particularly in the cereal, barley (see for example, Higgins, Jacobsen and Zwar, 1982). Seeds of Pisum are a relatively rich source of auxins, cytokinins, gibberellins and abscisic acid (Eeuwens and Schwabe, 1975; Burrows and Carr, 1970; Davies, Emshwiller, Gianfagne, Probsting, Noma and Pharis, 1982; Engvild, 1975; Engvild, Egsgaard and Larsen, 1978). Work on P. sativum and P. arvense has demonstrated fluctuating levels of these four hormones during the course of seed development and some correlations have been made between high levels of one or other of these hormones and particular phases of seed growth and development (Eeuwens and Schwabe, 1975; Burrows and Carr, 1970). No attempt has been made to relate endogenous levels of hormones to the accumulation of pea seed proteins, although Davies and Bedford (1982) showed that exogenous abscisic acid did not affect storage protein accumulation in isolated pea embryos. Similarly, Dure and Galau (1981) found that abscisic acid did not affect the expression of storage protein genes in developing cotton seeds. However, hormones have been implicated in the regulation of seed protein synthesis and accumulation in other species. For example, in cultured embryos of Phaseolus vulgaris, phaseolin increased in response to abscisic acid while showing no response to indole-acetic acid, kinetin and gibberellic acid (Sussex and Dale, 1979; Sussex, Dale and Crouch, 1980). Similarly, the accumulation of the 12S protein in Brassica napus embyros in vitro was enhanced by abscisic acid (Crouch and Sussex, 1981).

This paper reports a detailed study of the possible role of hormones in regulating the accumulation of seed proteins in Pisum. Exogenous hormones were applied daily to developing fruits until the end of the phase of protein accumulation and at full seed maturity the quantitative and qualitative effects of these treatments on defined seed protein fractions were determined. Naphthalene-acetic acid, benzyl adenine and abscisic acid each had specific effects on legumin and vicilin levels whereas gibberellic acid treatment was without effect on these proteins.

Materials and Methods

Plant Materials

Pea plants (P. sativum L. PI/G 086) were raised from seeds harvested from a population of our standard line cv. Greenfeast which, over a number of generations, had been selected for uniformity in terms of seedling growth, plant height, number of nodes to first flower, seed weight and protein content. Plants were grown in a loam-sand-peat moss mixture in 23 cm pots. A complete fertiliser was added to the mix before potting. From 5 weeks after sowing a complete nutrient solution was added to each pot once a week. Plants were grown in a glasshouse under natural light, the temperature range was 18°C (night) to a maximum of 24°C (day). Experiments I and II (twelve months later) were timed so that seed maturation occurred in long days (Nov. in Australia).

Plant growth regulators and mode of application

Plant growth regulators were obtained from the following sources: naphthalene-acetic acid (NAA) and N6-benzyladenine (BAP) from ICN Pharmaceuticals Inc., Cleveland, Ohio; gibberellic acid (GA_3) from Sigma Chemical Company; abscisic acid (ABA) cis-trans from Hoffman La-Roche,

Switzerland. All hormones were made up as a 10^{-3} M stock solution. NAA, GA and ABA were dissolved in 1.25 ml ethanol (AR grade) then made up to 25 ml with distilled water. BAP was first dissolved in 2 drops of 0.2 M HCl before ethanol and water were added. The control treatment was 5% ethanol in water. An untreated control was also included in the experiment. Because the analysis of variance showed no differences between this control and the treated control (injected with 5% ethanol) in any of the parameters measured it will not be referred to again.

Each treatment, including a 5% ethanol control, was applied to 3 pods on each of 4 plants (12 pods total). Only pods on the main stem were used and stem branches and laterals were cut off throughout the experiment. The first two flowers were also removed and the next three flowers, referred to as pod position 1,2 and 3, were treated. At blooming of flower 6, both it and the apex of the main stem were cut off. Pruning of plants is desirable because it was found previously that number of stem branches and laterals, and consequently the number of pods, can vary from plant to plant. Hormone and control solutions were injected daily into the pedicel of pods from 2 to 32 days after full bloom using a 50 µl (Hamilton 705) syringe. For the first 10 days 30 µl, and for the following 20 days 45 µl, were injected. It proved important to puncture both ends of the pedicels to ensure retention of the injected solution. If done carefully the same two holes could be used for the duration of the treatment. Flowers emerged at two day intervals.

A second experiment examined the effect of a mixture of NAA and BAP (10^{-3}M solutions of each, mixed 1:1) on protein composition. Control and NAA and BAP treatments were repeated, experiment II treatments involved 3 pods on each of 2 plants.

Seed harvest and protein analyses

Individual pods were harvested at full seed maturity. After seed removal pods were oven dried (90°C, 48 h) to determine pod dry weight. Seeds were air dried to a moisture content of 6.5 to 7.5%. The seeds were then de-hulled and cotyledon weight sum of two cotyledons per seed, was recorded before milling them to a fine flour. Except for cotyledon weight, duplicate determinations of all protein parameters were made on each of 12 and 6 samples per treatment in experiment I and II respectively. Methods for the determination of extractable protein and legumin content are described in detail by Schroeder (1982). Albumin content, as a percentage of total extractable protein, was measured by Laurell's rocket immunoassay (Weeke 1973) using a specific antiserum against albumins raised in sheep. The antiserum was raised against an equal mixture of purified albumins from mature seeds of 20 lines of Pisum. Rocket immunoelectrophoresis of the albumin fraction results in a rocket with 6 to 7 precipitin lines. However, preliminary experiments showed that the height of the major line of the rocket is directly proportional to the albumin concentration in standards of pure albumins and in the samples tested (data not shown). Vicilin content was determined by calculating the difference between total protein per cotyledon and the sum of the legumin and albumin contents.

Results

Physiological observations, pod elongation and pod dry weight

Observations on pod development indicated that the hormone treatments were eliciting a morphological response. Pod growth, as determined by daily measurements of pod length, followed an approximately sigmoid curve (Fig. 1).

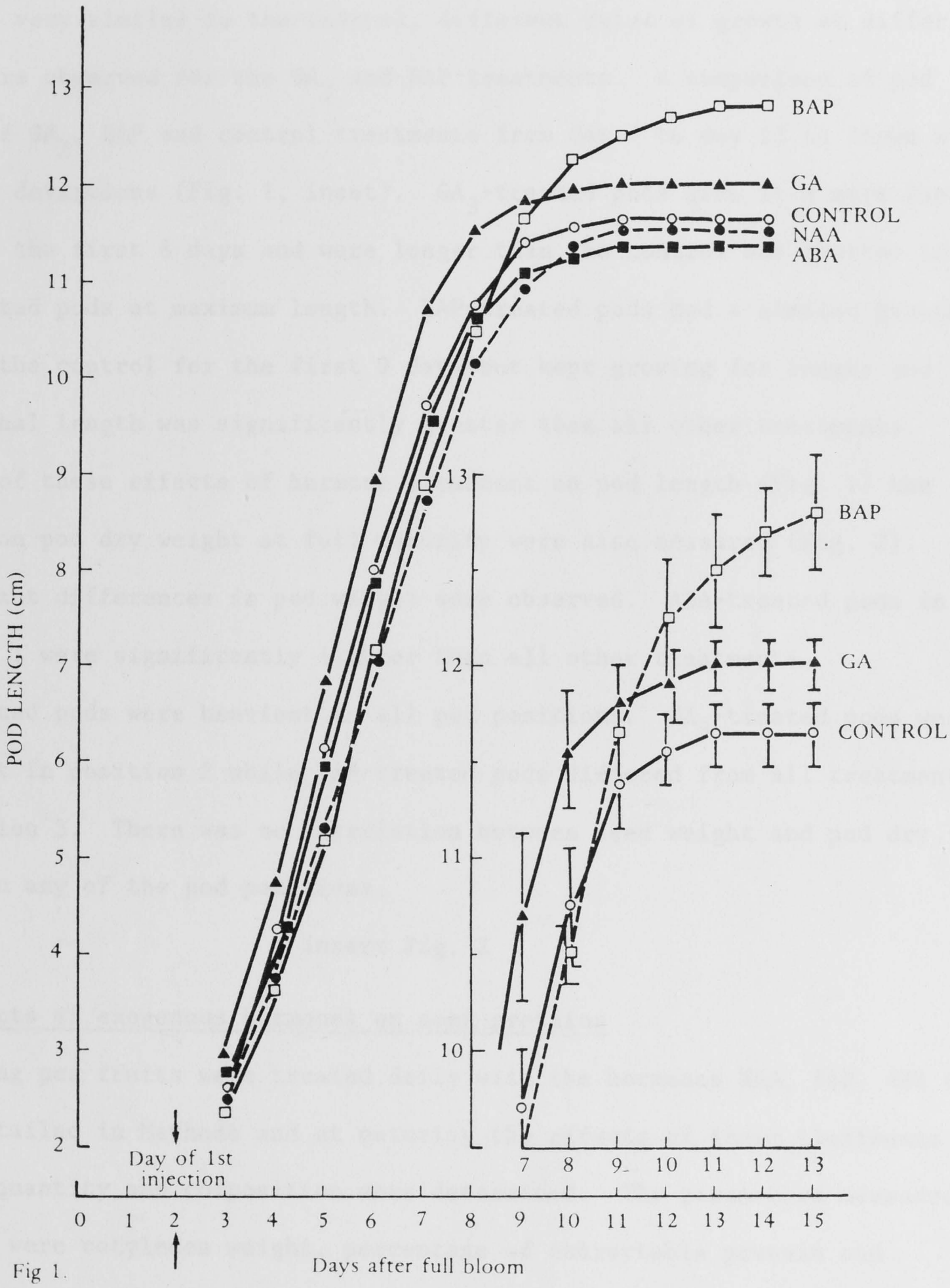

Fig 1.

The effect of exogenous hormones on pod growth. A comparison of pod growth of BAP, GA_3 and control treatments from day 7 to 13 with standard deviations is shown in the inset.

Insert Fig. 1

Hormone injection into the pedicel had differential effects on the rate of growth and maximum length of pods. While growth of NAA- and ABA-treated pods was very similar to the control, different rates of growth at different times were observed for the GA_3 and BAP treatments. A comparison of pod growth of GA_3, BAP and control treatments from day 7 to day 13 is shown with standard deviations (Fig. 1, inset). GA_3-treated pods grew at a more rapid rate for the first 8 days and were longer than the control but shorter than BAP-treated pods at maximum length. BAP-treated pods had a similar growth rate to the control for the first 9 days but kept growing for longer and their final length was significantly greater than all other treatments. Because of these effects of hormone treatment on pod length (Fig. 1) the effects on pod dry weight at full maturity were also measured (Fig. 2). Significant differences in pod weight were observed. ABA-treated pods in position 1 were significantly lighter than all other treatments. BAP-treated pods were heaviest in all pod positions. GA_3-treated pods were different in position 2 while NAA-treated pods differed from all treatments in position 3. There was no correlation between seed weight and pod dry weight in any of the pod positions.

Insert Fig. 2

The effects of exogenous hormones on seed proteins

Developing pea fruits were treated daily with the hormones NAA, BAP, ABA or GA as detailed in Methods and at maturity the effects of these treatments on protein quantity and composition were determined. The parameters measured directly were cotyledon weight, percentage of extractable protein and legumin and albumin contents as a proportion of extractable protein. From these data the absolute amounts (mg per cotyledon) of total protein, legumin, vicilin and albumin were calculated. Except for cotyledon weight

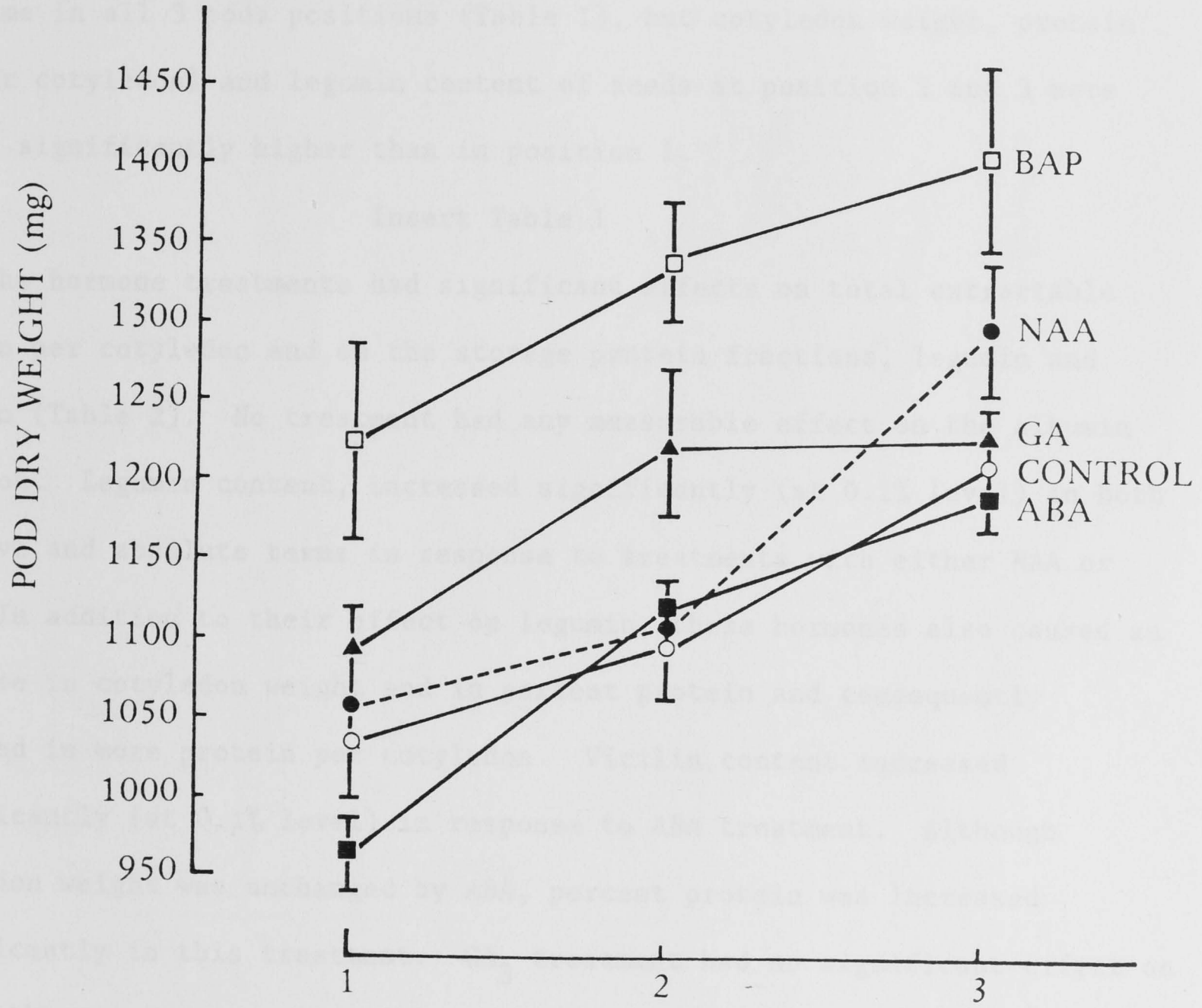

Fig 2.

Effects of hormone treatments on pod dry weight in relation to pod positions.

each entry in Table 1 and 2 is the mean of duplicate determinations (coefficient of variation < 3%) on each of 12 samples per treatment. The statistical analysis showed significant treatment and pod position effects but no interactions between either treatments and plants (replications) or treatments and pod position.

Pod position affected protein quantity and composition in seeds of pod position 1, 2 and 3 but response to hormone treatments was consistent at all 3 positions. Percent protein, vicilin and albumin contents were essentially the same in all 3 pods positions (Table 1), but cotyledon weight, protein (mg per cotyledon) and legumin content of seeds at position 2 and 3 were always significantly higher than in position 1.

Insert Table 1

The hormone treatments had significant effects on total extractable protein per cotyledon and on the storage protein fractions, legumin and vicilin (Table 2). No treatment had any measurable effect on the albumin fraction. Legumin content, increased significantly (at 0.1% level) in both relative and absolute terms in response to treatments with either NAA or BAP. In addition to their effect on legumin, these hormones also caused an increase in cotyledon weight and in percent protein and consequently resulted in more protein per cotyledon. Vicilin content increased significantly (at 0.1% level) in response to ABA treatment. Although cotyledon weight was unchanged by ABA, percent protein was increased significantly in this treatment. GA_3 treatment had no significant effect on any of the parameters measured.

Insert Table 2

The extent to which legumin, vicilin and albumin each contributed to the changes in total protein in hormone-treated seeds is shown in Fig. 3. The changes are shown as differences from the corresponding values in control seeds. The net increases in total protein content, as a result of

Table 1. The relationship between pod position and seed protein quantity and composition.

	Pod position 1	2	3	LSD 0.1%
Cotyledon weight mg	400.5	412.9	418.0	6.80
Percent extractable protein	25.07	25.27	25.35	NS
Protein mg per cotyledon	100.4	104.3	106.0	1.90
Vicilin mg per cotyledon	53.20	54.71	54.98	NS
Percent legumin	22.78	23.69	24.24	0.64
Legumin mg per cotyledon	22.90	24.73	25.73	0.71
Percent albumin	24.22	23.88	23.86	NS
Albumin mg per cotyledon	24.31	24.90	25.27	NS

Table 2. The effect of exogenous hormone treatments on seed protein quantity and composition.

	Control	NAA	BAP	ABA	GA	LSD 0.1%
Cotyledon weight mg.	412.9	422.3	417.3	413.9	405.5	NS
Percent extractable protein	24.63	25.74	25.50	25.94	24.60	0.53
Protein mg per cotyledon	101.8	108.7	106.4	107.4	99.8	4.40
Vicilin mg per cotyledon	53.87	55.21	55.12	58.14	51.68	2.29
Percent legumin	22.45	25.21	25.15	21.98	23.79	1.38
Legumin mg per cotyledon	22.85	27.43	26.75	23.66	23.78	2.04
Percent albumin	24.61	23.99	23.05	23.83	24.38	NS
Albumin mg per cotyledon	25.05	26.07	24.50	25.59	24.31	NS

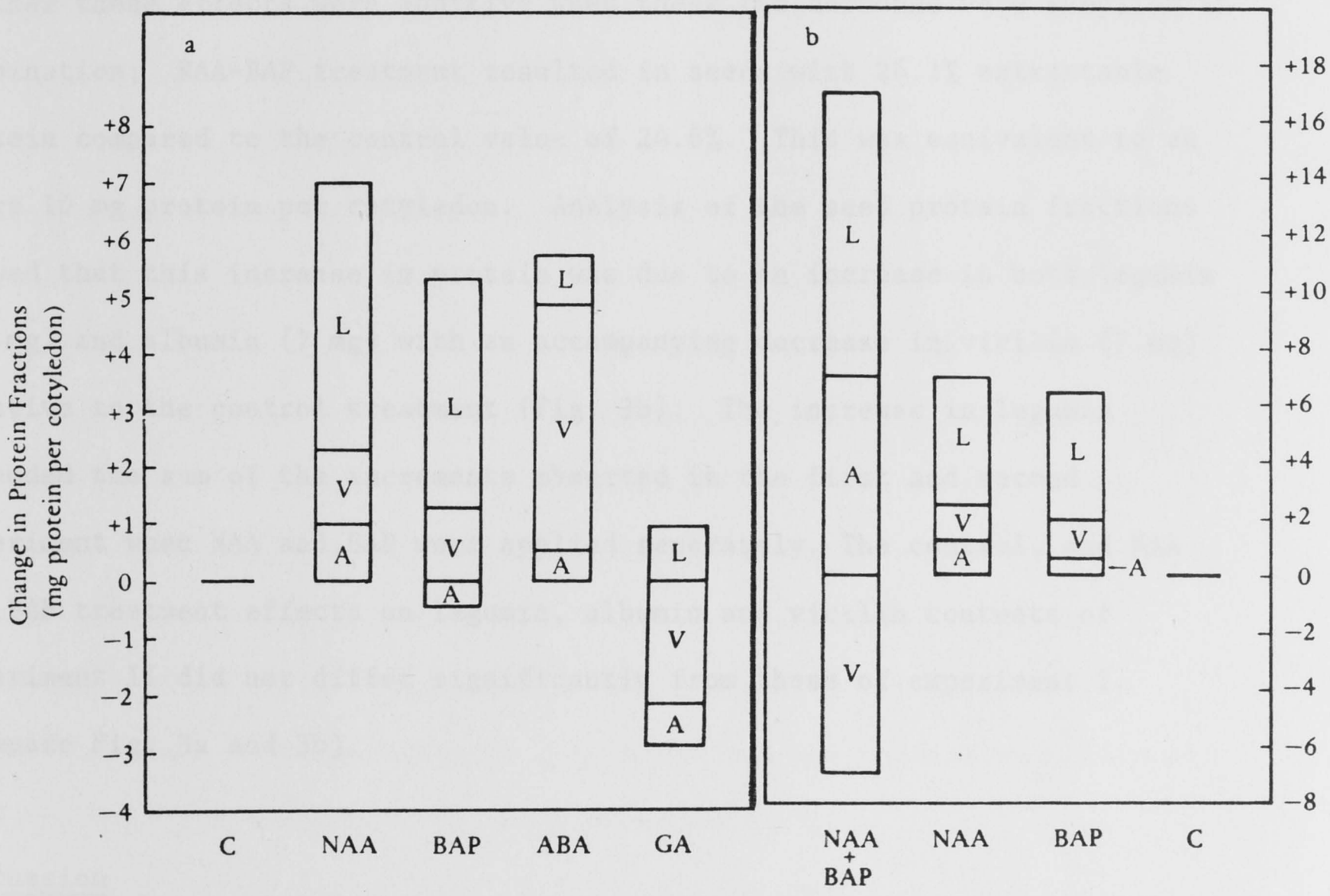

Fig. 3a and b. Changes in albumin (A) vicilin (V) and legumin (L) contents (mg per cotyledon) in response to treatments with NAA, BAP, ABA and GA_3 experiment 1(a), and NAA, BAP and a combination of NAA and BAP experiment 2(b). Changes are shown as differences from the corresponding values in control seeds.

NAA and BAP treatments, were largely due to increases in legumin, while the increased protein in the ABA treatment was largely due to changes in vicilin content. Treatment with GA_3 resulted in a small, non-significant, overall decrease in total protein, without a significant change in any particular protein fraction.

Insert Fig. 3a and b.

Since treatment with either NAA or BAP resulted in an increase in legumin (Table 2, Fig. 3a) a second experiment was carried out to test whether these effects were additive when these two hormones were supplied in combination; NAA-BAP treatment resulted in seeds with 26.1% extractable protein compared to the control value of 24.6%. This was equivalent to an extra 10 mg protein per cotyledon. Analysis of the seed protein fractions showed that this increase in protein was due to an increase in both legumin (10 mg) and albumin (7 mg) with an accompanying decrease in vicilin (7 mg) relative to the control treatment (Fig. 3b). The increase in legumin exceeded the sum of the increments observed in the first and second experiment when NAA and BAP were applied separately. The control, and NAA and BAP treatment effects on legumin, albumin and vicilin contents of experiment II did not differ significantly from those of experiment I. (Compare Fig. 3a and 3b).

Discussion

In the present study I have established that exogenously applied growth regulators caused changes in pod growth and weight and in seed protein levels and composition in Pisum. The initial indication of hormone elicited responses was the observation that hormones, specifically GA_3 and BAP, affected rate of growth, maximum length and dry weight of pods. Subsequent analyses showed that injections of NAA, BAP and ABA into the pedicel resulted in changes in seed weight, seed protein and the proportions of the

storage proteins legumin and vicilin; no significant changes in the albumin fraction were observed. Injection of GA_3 had no effect on any of the seed protein parameters analysed (Table 2, Fig. 3a). Some relatively specific effects were noted. Vicilin increased in response to ABA while NAA and BAP caused an increase in legumin. Legumin content was increased even further (Fig. 3b) by the application of a mixture of NAA and BAP. These two hormones apparently acted in a synergistic manner, since in combination they increased legumin content to a level greater than the sum of the increases observed when they were applied separately. This treatment also resulted in a marked increase in albumins but a considerable decrease in vicilin content. Analysis of the polypeptide composition of total protein and protein fractions of NAA-BAP treatment by SDS-polyacrylamide gel electrophoresis showed changes which clearly reflected the observed quantitative changes in the individual protein fractions (data not shown). The change in albumin content is of particular interest, firstly because application of any of the hormones separately had no effect on albumin levels and secondly, because the concurrent increase of albumin and legumin breaks the very strong negative correlation between these two protein fractions seen in earlier experiments. This negative correlation ($r = -0.76$) was shown to be genetically determined and was consistent within the 45 lines of Pisum examined previously (Schroeder 1982).

Hormones were injected into the pedicels because this was considered to be the most uniform mode of application in closest proximity to the developing seeds. Previously it was found that when ^{35}S[methionine] was injected into the pedicel the label was readily taken up and incorporated into the seed protein (Millerd, Thomson and Randall, 1979; Schroeder 1983 in preparation). The choice of daily injections was based on the finding that ABA had to be present continually to effect changes in the protein synthesis in Phaseolus vulgaris embryos in vitro (Sussex and Dale, 1979). The daily

injections, from 2 to 32 days after full bloom, in this experiment, should have ensured the continuous presence of hormones throughout the time of synthesis of all protein fractions. Repeated foliar applications, addition of hormones to the liquid endosperm by injection *in vivo* and hormones applied directly to the developing pod in lanolin had no effect on seed protein levels (data not shown).

Developmental patterns relating to seed and pod formation and protein accumulation have been studied in detail (Beevers and Poulson, 1972; Millerd and Spencer, 1974; Pate, 1975; Millerd, Thomson and Schroeder, 1979). Endogenous hormones have been measured in immature and mature peas and fluctuating levels of these plant growth regulators have been related to phases of pod, seed and embryo development and volume of liquid endosperm (Eeuwens and Schwabe, 1975; Burrows and Carr, 1970). No studies have been made to investigate the possible relationship between endogenous hormone levels and protein synthesis and accumulation.

Exogenous hormones have been shown to cause changes in the rate of storage protein accumulation in *P. vulgaris* and *B. napus in vitro* (Sussex and Dale, 1979; Sussex et al., 1980; Crouch and Sussex, 1981). This raised the question whether exogenous hormones would also affect pea seed protein levels and composition. Davies and Bedford (1982) studied the effect of ABA on protein synthesis in cultured embryos of *P. sativum* and found that ABA had no effect on the accumulation of the storage protein legumin. In the present *in vivo* study, ABA also had no effect on legumin levels, but a significant increase in vicilin in response to treatment with ABA was found.

In *Pisum*, levels of legumin, vicilin and albumins are genotype-dependent (Thomson, Millerd and Schroeder, 1979; Schroeder, 1982), but these levels can be markedly affected by environmental factors such as temperature and nutrient status (Millerd et al., 1978; Randall, Thomson and

Schroeder, 1979). Exogenously applied hormones are another such non-genetic factor and although changes in levels of protein fractions reported in this *in vivo* study are not of the same magnitude as those obtained in some *in vitro* studies, the results nevertheless suggest that hormones can play a role in the regulation of storage protein fraction accumulation in *Pisum*.

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Literature cited

Beevers, L., and Poulson, R; 1972. Protein synthesis in cotyledons of Pisum sativum L. Plant Physiology, 49, 476-481.

Bewley, J.D., and Black, M.; 1978. Physiology and biochemistry of seeds Vol. 1, 88-101. Springer Verlag Berlin, Heidelberg. New York.

Burrows, W.J., and Carr, D.J.; 1970. Cytokinin content of pea seeds during growth and development. Physiologia Plantarum, 23, 1064-1070.

Crouch, M.L. and Sussex, I.M.; 1981. Development and storage protein synthesis in Brassica napus L. embryos in vitro and in vivo. Planta, 153, 64-74.

Davies, D.R. and Bedford, I.D.; 1982. Abscisic acid and storage protein accumulation in P. sativum embryos grown in vitro. Plant Science Letters, 27, 337-343.

Davies, P.J., Emshwiller, E., Gianfagna, T.J., Probsting, W.M., Noma, M., and Pharis, R.P.; 1982. The endogenous gibberellins of vegetative and reproductive tissues of G2 peas. Planta, 154, 266-272.

Dure III. L., and Galau, A; 1981. Developmental biochemistry of cotton seed embryo genesis and germination. Plant Physiology, 68, 187-194.

Eeuwens, C.J., and Schwabe, W.W.; 1975. Seed and pod wall development in P. sativum L. in relation to extracted and applied hormones. Journal of Experimental Botany, 26, 1-14.

Engvild, K.C.; 1975. Natural chlorinated auxins labelled with radioactive chloride in immature seeds. Plant Physiology, 34, 286-287.

Engvild, K.C., Egsgaard, H., and Larsen, E.; 1978. Gas chromatographic-mass spectrometric identification of 4-chloroindolyl-3-acetic acid methyl ester in immature green peas. Plant Physiology, 42, 365-368.

Higgins, T.J.V., Jacobsen, J.V., and Zwar, J.A.; 1982. Gibberellic acid and abscisic acid modulate protein synthesis and mRNA levels in barley aleurone layers. Plant Molecular Biology, 1, 191-215.

Letham, D.S., Goodwin, P.B., and Higgins, T.J.V.: (Eds) 1978. Phytohormones and Related Compounds : A comprehensive Treatise Vol. 1, Elsevier North Holland, Amsterdam, Oxford, New York.

Millerd, A., and Spencer, D.; 1974. Changes in RNA synthesizing activity and template activity in nuclei from cotyledons in developing pea seeds. Australian Journal of Plant Physiology. 1, 331-341.

Millerd, A., Thomson, J.A., and Schroeder, H.E. 1978. Cotyledonary storage protein in Pisum sativum III. Pattern of accumulation during development. Australian Journal of Plant Physiology 5, 519-534.

Millerd, A., Thomson, J.A., and Randall, P.J. 1979. Heterogeneity of sulfur content in the storage proteins of pea cotyledons. Planta, 146, 463-466.

Pate, J.S.: 1975. Pea. In: Crop Physiology. Evans, L.T.; (ed) Cambridge : Uni. Press, 191-224.

Randall, P.J.; Thomson, J.A., and Schroeder, H.E.; 1979. Cotyledonary storage proteins in Pisum sativum IV. Effects of sulfur, phosphorus, potassium and magnesium deficiencies. Australian Journal of Plant Physiology 6, 11-24.

Schroeder, H.E.; 1982. Quantitative studies on the cotyledonary proteins in the genus Pisum. Journal of Science Food and Agriculture 33, 623-633.

Schroeder, H.E.; 1983. The major albumins of Pisum cotyledons. (in preparation).

Sussex, I.M., and Dale, R.M.K.; 1979. Hormonal control of storage protein synthesis in Phaseolus vulgaris. in: I. Rubenstein et al (eds). The plant seed development preservation and germination. Academic Press 129-141.

Sussex, I.M., Dale, R.M.K., and Crouch, M.L.; 1980. Developmental regulation of storage protein synthesis in seeds. In: C.J. Leaver (ed). Genome organisation and expression in plants. Plenum Press, 283.

Thomson, J.A., Millerd, A., and Schroeder, H.E.; 1979. Genotype dependent patterns of accumulation of seed storage proteins in Pisum. In: Seed protein improvement in cereals and grain legumes. Vol. II. IAEA-SM-230/61 Vienna, 231-240.

Weeke, B. 1973. Rocket immunoelectrophoresis. In: A. manual of quantitative immunoelectrophoresis. (Axelson, N.H., Knole, J., Weeke, B. (eds.). Universitetsforlaget, Oslo, 37-56.

6. CONCLUSION

Experimental results of the quantitative and qualitative analyses of pea seed proteins and the genetic analysis of protein fractions presented in this thesis, I believe, will contribute towards a more thorough understanding of seed proteins of the genus Pisum.

The development of improved and new techniques to quantitatively determine the albumin content namely, the clean and reproducible fractionation of the extractable cotyledonary protein into a globulin and an albumin fraction (Chapter 2) and determination of albumin content by rocket immunoelectrophoresis (Chapters 4 and 5), combined with already existing techniques of measuring legumin and vicilin contents made it possible to study the pea seed proteins quantitatively in their entirety.

The quantitative study of 10 seed protein and protein quality characteristics of 45 lines in Chapter 2 was set up so as to include a representative number of lines from each of four groups of peas, primitive or wild forms, field peas, and round and wrinkled garden peas. The seed protein characters measured were; crude protein, extractable protein, globulins, albumins, and legumin content of flour and globulins. The protein quality characters measured were; total sulfur, protein sulfur, carbon to nitrogen and nitrogen to sulfur ratios. Variation in seed protein and protein quality characteristics was examined and correlation coefficients were calculated between seed weight and 8 of the above characters.

Results presented in the correlation matrix (Chapter 2) highlighted the importance of the nutritionally desirable albumin fraction. However, the most significant finding was the significantly

negative correlation between albumin and legumin content (r = -0.76), in none of the lines examined was that correlation broken. This result showed clearly that in plant breeding attempts to improve the nutritional quality of pea seed proteins, legumin content can no longer be considered in isolation, instead the major emphasis should be on the albumin fraction.

The apparent significance of the albumin fraction, the lack of albumin characterisation at the polypeptide level, and the availability of pure albumin proteins from 45 lines of peas studied in Chapter 2, led to the investigations of the major albumins of pea cotyledons in Chapter 3.

Albumins fractionated by SDS-PAGE resolved a large number of polypeptides ranging in molecular weight from 6000 to 92000. Densitometric scans of Coomassie blue stained gels showed that polypeptides of M_r 22000 and 8000 were quantitatively major components in all lines examined. In our standard line, cv Greenfeast, these major polypeptides together account for more than 30% of the total albumin fraction. Both these albumins were found to be cotyledon specific proteins and polypeptides in the M_r 8000 region were shown to behave as storage proteins during germination. Further, it was shown that the M_r 8000 polypeptides are particularly rich in sulfur amino acids. Consistant with this, under sulfur deficiency conditions these polypeptides were markedly reduced. In cv Greenfeast, the albumin fraction made up 27% of the total extractable cotyledonary protein but accounted for 68% of the total protein sulfur. The M_r 8000 polypeptides accounted for 17.5% of the albumin fracton or about 5% of the total seed protein. However, they made up 35% of the protein sulfur of the albumins or more significantly 24% of the total seed protein sulfur.

The data presented above indicate that, quantitatively and nutritionally, the M_r 8000 albumin polypeptides are the most important protein components of the pea seed proteins. For these reasons further examination of this particular albumin species would be desirable.

Investigations of the M_r 8000 polypeptides are now progressing to establish; firstly, whether these polypeptides are the same as the low molecular weight, cysteine rich albumin polypeptides reported by Jacubek and Przybylska[70]. Secondly, variant forms of M_r 8000 polypeptides have been identified in 2 lines of peas and these could facilitate the genetic analysis and possibly mapping of the structural genes for these polypeptides. Thirdly, cDNA clones for these polypeptides are in hand and these clones may be used to examine mRNA levels of this protein during cotyledonary development and to establish the number of genes coding for these polypeptides.

In the discussion of Chapter 2 it was concluded that low and high legumin lines have a very similar sulfur amino acid content. Largely responsible for this similarity is the negative correlation between the two sulfur rich fractions legumin and albumins. This indicated that pea seed protein quality improvements by breeding would not be achieved by selecting independently for either high legumin or high albumin content. To be successful, the negative correlation has to be broken. The prospects of doing this, depends on an understanding of the joint genetic control of legumin and albumin contents. Therefore, the inheritance and heritability of legumin and albumin contents was studied (Chapter 4) in a cross in which the parents had similar protein content but differed in seed type, legumin and albumin contents. It was found that the genes for

seed type had a major effect on legumin and albumin contents, and this complicated the genetic analysis and the interpretation of results. However, at least four individual F_2 seeds were identified which showed the desired recombination of high legumin with high albumin, showing that the negative correlation can be broken. This combination indicated that total sulfur amino acid content in the four F_2 seeds should be increased. This was supported by measurements of total sulfur and sulfur indices values which showed higher values for the four F_2's than for either parent. The question concerning the breeder is whether these F_2's would breed true for higher legumin and albumin contents and consequently higher sulfur amino acid content. Variance estimates from the genetic analysis suggest that protein quality can be improved by breeding, but the required selection regime must take account of the genetic variations which are predominantly additive for legumin but largely dominant for albumins.

Only further studies of the inheritance of legumin and albumin contents in other crosses, sampling of larger F_2 populations and following generations, and finally amino acid analyses of the protein of selected seeds, can answer the question of increased sulfur amino acid content.

The question, addressed in Chapter 5, is whether so called none genetic factors, such as growth regulators, can play a role in the regulation of protein synthesis and accumulation in peas. One needs to keep in mind that these factors are likely to be also under line specific genetic control.

The quantitative analyses of seed proteins at maturity was the selected method of assessing the effects of four exogenously applied hormones on seed protein content and composition. The hormones

used were NAA, BAP, ABA and Ga_3. Pod growth was measured primarily because of the known correlation between pod and seed development[119], and observed responses in pod development to Ga_3 and BAP treatments indicated that hormones were eliciting a response. Ga_3 treatments caused no changes in seed protein content or composition but specific responses to ABA, NAA and BAP treatments were observed. While vicilin content increased in response to ABA treatment, legumin content increased in response to NAA and NAP treatments. Changes in seed protein content and composition in response to hormone applications in this in vivo experiment were relatively smaller than those observed in in vitro experiments with P. vulgaris and B. napus[116-118]. However, the results suggest some interaction between non genetic and genetic factors which control the seed protein synthesis and/or accumulation.

Questions that could be answered by further investigations are, is the increase in protein fractions, of legumin in response to NAA and BAP and of vicilin in response to ABA, due to an increase in the rate of synthesis or prolonged synthesis of these protein components. Further, are daily applications of hormones for 30 days necessary or could application of hormones at specific times of cotyledonary development and for only short periods have similar effects.

REFERENCES

1. FAO Production Year-Book Vol. 35, 1981. Food and Agricultural Organization of the United Nations

2. Blixt, S. 1979. Some genes of importance for the evolution of the pea in cultivation. Proc. Conf. Broadening Genet. Basis Crops, Wageningen 1978. Pudoc, Wageningen

3. Blixt, S. and Williams D. eds. 1982. Documentation of Genetic Resources : A Model. Nordic Gene Bank. International Board for Plant Genetic Resources IBPGR Secretariat Rome AGPG : IBPGR/83/21 Dec. 1982

4. Davies, D.R. 1980. The r_a locus and legumin synthesis in Pisum sativum. Biochemical Genetics, Vol. 18, 1207-1219

5. Matta, N.K. and Gatehouse, J.A. 1982. Inheritance and mapping of storage protein genes in Pisum sativum L. Heredity, 48, 383-392

6. Slinkard, A.E. 1980. In : Production, Utilization and Marketing of Field Peas Vol. 1. Crop Development Centre University of Saskatchewan, Canada. Subproject I-VI

7. Ali, S.M., Nitschke, L.F., Dube, A.J., Krause, M.R. and Cameron, B. 1978. Selection of pea lines for resistance to pathotypes of Ascochyta pinodes, A. pisi and Phoma medicaginis var. pinodella. Aust. J. Agric. Research 29, 841-849

8. Ali, S.M., Paterson, J., and Crosby, J. 1982. A standard technique for detecting seed borne pathogens in peas, chemical control and testing commercial seed in South Australia. Aust. J. of Exp. Agric. and Animal Husbandry 22, 348-352

9. Slinkard, A.E. 1972. Breeding and protein studies. Crop Development Centre, University of Saskatchewan S7N OWO Canada

10. Blixt, S. 1979. Natural and induced variability for seed protein in temperate legumes. In : Seed protein improvement in cereals and grain legumes. Vol. II, IAEA-SM-230/85 Vienna 1979

11. Gottschalk, W. 1975. The influence of mutated genes on quantity and quality of seed proteins. Indian Agric. Vol. 19, 205-223

12. Gottschalk, W. 1978. Gene - ecological studies in Pisum mutants and recombinants. Genetica, Vol. 10, 43-61 Acta Biologia Jugoslavica Beograd 1978

13. Gottschalk, W., Mueller, H.P. and Wolff, G. 1976. Further investigations on the genetic control of seed protein production in Pisum mutants. Evaluation of seed protein alterations by mutation breeding. (Proc. Meeting Hahnenklee 1975), IAEA Vienna 151

14. Gottschalk, W., Mueller, H.P. and Wolff, G. 1975. Relations between protein production, protein quality and environmental factors in Pisum mutants. Breeding for seed protein improvements using nuclear techniques. ISEA-PI-570/14 Vienna, 105-123

15. Blixt, S. 1978. Problems relating to pea-breeding. Agric. Hortique Genetica XXXVI, 56-87

16. Shia, G., Slinkard, A.E. 1977. Relationship of seed shape and cotyledon colour to percent protein in peas. Crop Science Vol. 17 Jan-Feb

17. Schroeder, H.E. 1982. Quantitative studies on the cotyledonary proteins in the genus Pisum. Journal of the Science of Food and Agriculture 33, 623-633

18. Swiecicki, W.K., Blazcale, P., Hauke, J. and Mezza, S. 1981. Inheritance of protein content in Pea III. Correlation between protein content and seed yield. Pisum News Letter Vol. 13, 52-53

19. Derbyshire, E., Wright, D.J., and Boulter, D. 1976. Legumin and vicilin storage proteins of legume seeds. Phytochemistry, 15, 3-24

20. Goa, J.A. 1953. A micro biuret method for protein determinations. Scand. J. Clin. Lab. Invert. 5, 218-222

21. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275

22. Hartree, E.F. 1972. Microbiuret protein assay. Anal. Biochem., 48, 422-427

23. Wolff, G. 1975. Quantitative Untersuchungen ueber den Proteingehalt in Samen von Pisum sativum. Zeitschrift fuer Pflanzenzuechtung 75, 43-54

24. Mueller, H.P. 1973. Genetic control of the quantitative composition of seed proteins in mutants and recombinants of Pisum sativum. Genetica, Vol. 5, 51-58. Acta Biologica Jugoslavica Beograd

25. Millerd, A. 1975. Biochemistry of legume seed proteins. Ann. Review of Plant Physiol. 26, 53-72

26. Millerd, A., Spencer, D. 1974. Changes in RNA-synthesizing activity and template activity in nuclei from cotyledons of developing pea seeds. Aust. J. Plant Physiol. 1, 311-341

27. Millerd, A., Spencer, D., Dudman, W.F. 1974. Studies on the regulation of storage protein synthesis in developing pea seeds. In : Mechanisms of Regulation of Plant Growth (Bieleski, R.L., Ferguson, A.R., Cresswell, M.M. eds). Bulletin 12, Royal Society of New Zealand, Wellington, pages 799-803

28. Varner, J.E. and Schidlovky, G. 1963. Intracellular distribution of proteins in pea cotyledons. Plant Physiol. 38, 139-144

29. Craig, S., Goodchild, D.J. and Hardham, A.R. 1979a. Structural aspects of protein accumulation in developing pea cotyledons. I. Qualitative and quantitative changes in parenchyma cell vacuoles. Aust. J. Plant Physiol. 6, 81-98

30. Craig, S., Goodchild, D.J. and Millerd, A. 1979b. Immunofluorescent localisation of pea storage proteins in glycol methacrylate embedded tissue. J. Histochem. Cytochem 27, 1312-1316

31. Craig, S., Goodchild, D.J. and Miller, C. 1980. Structural aspects of protein accumulation in developing pea cotyledons. II. Three-dimensional reconstructions of vacuoles and protein bodies from serial sections. Aust. J. Plant Physiol. 7, 329-337 Part I

32. Craig, S., Millerd, A. and Goodchild, D.J. 1980. Structural aspects of protein accumulation in developing pea cotyledons. III. Immunocytochemical localisation of legumin and vicilin using antibodies shown to be specific by the enzyme-linked immunosorbent assay (ELISA). Aust. J. Plant. Physiol. 7, 339-351, Part II

33. Craig, S. and Millerd, A. 1981. Pea seed storage proteins, immunocytochemical localisation with protein A-Gold by electron microscopy. Protoplasm 105, 333-339

34. Millerd, A., Thomson, J.A., Schroeder, H.E. 1978. Cotyledonary storage proteins in Pisum sativum. III. Patterns of accumulation during development. Aust. J. Plant Physiol. 5, 519-534

35. Danielsson, C.E. 1949b. Investigation of vicilin and legumin. Acta Chem. Scand. 3, 41-49

36. Danielsson, C.E. 1952. A contribution to the study of the synthesis of the reserve proteins in ripening pea seeds. Acta Chem. Scand. 6, 149-159

37. Danielsson, C.E. 1951. The breakdown of high molecular reserve proteins of peas during germination. Acta Chem. Scand. 5, 541

38. Raacke, I.D. 1957a. Protein synthesis in ripening pea seeds. Biochem. J. 66, 101-110

39. Thomson, J.A., Schroeder, H.E., Dudman, W.F. 1978. Cotyledonary storage proteins in Pisum sativum. I. Molecular heterogeneity. Aust. J. Plant Physiol. 5, 263-279

40. Bajaj, S., Michelsen, O., Lillevik, H.A., Baker, L.R., Bergen, W.G., Gill, J.L. 1971. Prediction of protein efficiency ratio of peas from their albumin content. Crop Science 11, 813-815

41. Przybylska, J., Blixt, S., Hurich, J., Przybylska, Z.Z. 1977. Comparative study of seed proteins in the genus Pisum I. Electrophoretic patterns of different protein fractions. Genetica Polonica 18, 27-38

42. Davies, D.R. 1976. Variation in the storage proteins of peas in relation to sulphur amino acid content. Euphytica, 25, 717-724

43. Thomson, J.A., Millerd, A., Schroeder, H.E. 1979. Genotype dependent patterns of accumulation of seed storage proteins in Pisum. In : Seed protein improvement in cereals and grain legumes. Vol. II, IAEA-SM-230/61 Vienna, 231-240

44. Fueredi, J. 1970. Importance of breeding peas with increased protein content. In : Protein Growth by Plant Breeding (Balint, A., Ed.) Akademiai Kiado, Budapest. 99-128

45. Randall, P.J., Thomson, J.A., Schroeder, H.E. 1979. Cotyledonary storage proteins in Pisum sativum. IV. Effects of sulphur, phosphorous, potassium and magnesium deficiencies. Aust. J. Plant Physiol. 6, 11-24

46. World Health Organization, Energy and Protein Requirements WHO Tech. Report Series 1973, 522

47. Boulter, D., Evans, I.M., Thompson, A. 1976. Screening for protein quality in legumes. Qual. Plant. Plant Foods Hum. Nutr. 26, 107-119

48. Evans, I.M., Boulter, D. 1980. Crude protein and sulphur amino acid content of some commercial varieties of peas and beans. J. Sci. Food Agric. 31, 238-242

49. Russel-Hunter, W.D. 1970. In Aquatic Productivity. Macmillan & Co., 174-182

50. Evans, R.J., Bandemer, L. 1967. Nutritive value of legume seed proteins. J. Agr. Food Chem. Vol. 15, 3, 439-443

51. Norrish, K., Hutton, J.T. 1977. Plant analysis by X-ray spectrometry. I. Low atomic number elements, sodium to calcium. X-ray Spectrometry I, 6-11

52. Eppendorfer, W.H. 1971. Effects of S. N and P on amino acid composition of field beans (Vicia faba) and responses of the biological value of the seed protein to S-amino acid content. J. Sci. Food Agric. 22, 501-505

53. Boulter, D., Evans, I.M., Derbyshire, E. 1973. Proteins of some legumes with reference to environmental factors and nutritional value. Qual. Plant. Plant Foods Hum. Nutr. 23, 239-250

54. Boulter, D. 1977. Quality problems in protein plants with special attention paid to protein of legumes. In : Protein Quality from Leguminous Crops, EEC Seminar, Dijon, 11-47

55. Bajaj, S., Michelsen, O., Baker, L.R., Markarian, D. 1971. The Quality of protein in various lines of peas. British Journal of Nutrition 25, 207-212

56. Boulter, D. 1980. Ontogeny and development of biochemical and nutritional attributes in legume seeds. Advances in Legume Science (Summerfield, R., Bunting, A. Eds.). 127-134

57. Casey, R., Short, M.N. 1981. Variation in amino acid composition of legumin from Pisum. Phytochemistry 20, 21-23

58. Boulter, D. 1977. Improving protein content and quality in legumes. In : Genetic Diversity in Plants (Muhammed, A., Aksel, R., von Borstel, R.C., Eds.) Plenum Publishing Corp. 387-397

59. Hurich, J., Parzysz, H., Przybylska, J. 1977. Comparative study of seed proteins in the genus Pisum. II. Amino acid composition of different protein fractions. Genetica Polonica 18, 241-252

60. Boulter, D., Derbyshire, E. 1971. In : Chemotaxonomy of the Leguminoseae (Harborne, J.B., Boulter, D., Turner, B.L. Eds.) Academic Press London and New York, 285-308

61. Murray, D.R. 1979. A storage role for albumins in pea cotyledons. Plant, Cell and Environment 2, 221-226

62. Nelson, O.E. 1969. Genetic modification of protein quality in plants. Adv. Agron. 21, 171-194

63. Jackson, P., Boulter, D., Thurman, D.A. 1969. A comparison of some properties of vicilin and legumin isolated from seeds of Pisum sativum, Vicia faba and Cicer arietinum. New Phytologist 68, 25-33

64. Grant, D.R., Summer, A.K., Johnson, J. 1976. An investigation of pea seed albumins. Can. Inst. Food Sci. Technol. 9, 84-91

65. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature, 227, 680-685

66. Weeke, B. 1973. Rocket immunoelectrophoresis. In : A Manual of Quantitative Immunoelectrophoresis (Axelson, N.H., Weeke, B. Eds.) Universitetsforlaget Islo. 47-56

67. Schroeder, H.E. 1983. The major albumins of Pisum cotyledons. J. Sci. Food Agric. (in press)

68. Fox, D.J., Thurman, D.A., Boulter, D. 1964. Studies on the proteins of seeds of the Leguminoseae. I. Albumins. Phytochemistry 3, 417-419

69. Blixt, S., Przybylska, J., Przybylska, Z.Z. 1980. Comparative study of the seed proteins in the genus Pisum. V. Genetics of the electrophoretic patterns I and III. Genetica Polonica 21, 153-160

70. Jakubek, M., Przybylska, J. 1979. Comparative study of the seed protein in the genus Pisum. III. Electrophoretic patterns and amino acid composition of albumin fractions separated by gel filtration. Genetica Polonica 20, 369-380

71. Wolff, G. 1980. Investigations on the relations within the family Papilionaceae on the basis of electrophoretic banding patterns. Theor. Appl. Genetics, 57, 225-232

72. Gottschalk, W., Mueller, H.P., Wolff, G. 1976. Genetic of seed protein production in Pisum mutants. In : Evaluation of Seed Protein Alteration by Mutation Breeding. IAEA Vienna 157-177

73. Gottschalk, W., Mueller, H.P., Wolff, G. 1975. The genetic control of seed protein production and composition. Egyptian J. of Genetics and Cytology. Vol. 4(2) 453-468

74. Guldager, P. 1978. Immunoelectrophoretic analysis of seed proteins from Pisum sativum L. Theor. Appl. Genetics 53, 241-250

75. Almgard, G. 1970. Cultivar distinction by means of isozyme techniques in Pisum. Pisum News Letter 2, 9

76. Scandalios, J.G., Espiritu, L.G. 1969. Mutant aminopeptidases of Pisum sativum L. Developmental genetics and chemical characteristics. Molec. Genetics. 105, 101-112

77. Przybylska, J., Blixt, S., Parzysz, H., Przybylska, Z.Z. 1982. Isozyme variation in the genus Pisum. I. Electrophoretic patterns of several enzyme systems. Genetica Polonica, 23, 103-121

78. Mueller, H.P. 1978. Gene mapping on chromosomes and some aspects of gene regulation in Eukaryotic cells. The Nucleus Vol. 21(2) 135-142

79. Thomson, J.A., Schroeder, H.E. 1978. Cotyledonary storage proteins in Pisum sativum. II. Hereditary variation in components of the legumin and vicilin fractions. Aust. J. Plant Physiol. 5, 281-294

80. Casey, R. 1979. Genetic variability in the structure of the a subunits of legumin from Pisum - A two-dimensional gel electrophoresis study. Heredity, 43(2), 265-272

81. Matta, N.K., Gatehouse, J.A., Boulter, D. 1981. Molecular and subunit heterogeneity of legumin of Pisum sativum L. (Garden Pea) - A multi-dimensional gel electrophoretic study. J. of Exp. Botany 32(131), 1295-1307

82. Thomson, JA., Schroeder, H.E., Tassie, A.M. 1980. Cotyledonary proteins in Pisum sativum. V. Further studies on molecular heterogeneity in the vicilin series of holoproteins. Aust. J. Plant Physiol. 7, 271-282

83. Chrispeels, M.J., Higgins, T.J.V., Craig, S., Spencer, D. 1982. The role of the endoplasmic reticulum in the synthesis of reserve proteins and the kinetics of their transport to the protein bodies in developing cotyledons. J. Cell Biol. 93, 5-14

84. Chrispeels, M.J., Higgins, T.J.V., Spencer, D. 1982. Assembly of storage protein oligomers in the endoplasmic reticulum and processing of the polypeptides in the protein bodies of developing cotyledons. J. Cell Biol. 93, 306-313

85. Higgins, T.J.V., Spencer, D. 1977. Cell-free synthesis of pea seed proteins. Plant Physiol. 60, 655-661

86. Croy, R.R.D., Gatehouse, J.A., Evans, I.M., Boulter, D. 1980. Characterisation of the storage protein subunits synthesised in vitro by polyribosomes and RNA from developing pea (Pisum sativum L.) Planta 148, 49-56

87. Evans, I.M., Croy, R.R.D., Hutchinson, P., Boulter, D. 1979. Cell-free synthesis of some storage protein subunits by polyribosomes and RNA isolated from developing seeds of pea (Pisum sativum L.) Planta 144, 455-462

88. Higgins, T.J.V., Spencer, D. 1980. Biosynthesis of pea seed proteins : evidence for precursor forms from in vivo and in vitro studies. In : Genome Organisation and Expression in Plants (Ed. C.J. Leaver) pp. 245-258. New York : Plenum 607 pp

89. Higgins, T.J.V., Spencer, D. 1981. Precursor forms of pea vicilin subunits : modification by microsomal membranes during cell free translation. Plant Physiol. 67, 205-211

90. Spencer, D., Higgins, T.J.V., Button, S.C., Davey, R.A. 1980. Pulse-labelling studies on protein synthesis in developing pea seeds and evidence of a precursor form of legumin small subunit. Plant Physiol. 66, 510-515

91. Spencer, D., Higgins, T.J.V. 1980. The biosynthesis of legumin in maturing pea seeds. Biochem. International. Vol. 1, No. 5, 502-509

92. Gatehouse, J.A., Croy, R.R.D., Boulter, D. 1980. Isoelectric-focusing properties and carbohydrate content of pea (Pisum sativum) legumin. Biochem. J. 185, 497-503

93. Gatehouse, J.A., Croy, R.R.D., Morton, H., Tyler, H., Boulter, D. 1981. Characterization and subunit structure of the vicilin storage proteins of pea (Pisum sativum L.). Eur. J. Biochem. 118, 627-633

94. Spencer, D., Chandler, P.M., Higgins, T.J.V., Inglis, A.S., Rubira, M. 1983. Sequence interrelationship of the subunits of vicilin from pea seeds. Plant Mol. Biol. in press

95. Gatehouse, J.A., Lycett, G.W., Delauney, A.J., Croy, R.R.D., Boulter, D. 1983. Sequence specificity of the post-translational proteolytic cleavage of vicilin, a seed storage protein of pea (Pisum sativum L.). Biochem. J. 212, 427-432

96. Gatehouse, J.A., Lycett, G.W., Croy, R.R.D., Boulter, D. 1982. The postranslational proteolysis of the subunits of vicilin from pea (Pisum sativum L.). Biochem. J. 207, 629-632

97. Gatehouse, J.A., Evans, I.M., Bown, D. Croy, R.R.D., Boulter, D. 1982. Control of storage protein synthesis during seed development in pea (Pisum sativum L.). Biochem. J. 208, 119-127

98. Grant, D.R., Lawrence, J.M. 1964. Effects of sodium dodecyl sulfate and other dissociating reagents on the globulins of peas. Arch. Biochem. Biophys. 108, 552-561

99. Higgins, T.J.V., Chandler, P.M., Zurawski, G., Button, S.C., Spencer, D. 1983a. The biosynthesis and primary structure of pea seed lectin. J. Biol. Chem. 258, 9544-9549

100. Higgins, T.J.V., Chrispeels, M.J., Chandler, P.M., Spencer, D. 1983b. Intracellular sites of synthesis and processing of lectin in developing pea cotyledons. J. Biol. Chem. 258, 9550-9552

101. Chandler, P.M., Arrifin, Z., Blagrove, R.J. 1984a. Sequence relationships between pea vicilin cDNA clones : implications for mRNA structure and protein processing. Submitted

102. Millerd, A., Thomson, J.A., Randall, P.J. 1979. Heterogeneity of sulfur content in the storage proteins of pea cotyledons. Planta, 146, 463-466

103. Chandler, P.M., Higgins, T.J.V., Randall, P.J., Spencer, D. 1983. Regulation of legumin levels in developing seeds under conditions of sulfur deficiency. Rates of legumin synthesis and levels of legumin mRNA. Plant Physiol. 71, 47-54

104. Chandler, P.M., Spencer, D., Randall, P.J., Higgins, T.J.V. 1984b. The influence of sulfur nutrition on developmental patterns of the major pea seed proteins and their mRNAs. Submitted

105. Macnicol, P.K. 1983. Differential effects of sulfur deficiency on the composition of the aminoacyl-tRNA and free amino acid pools of the developing pea. FEBS Letters 156, 55-57

106. Quednau, H.D., Wolff, G. 1978. Investigations of the seed protein content of several pea genotypes grown in two different years. Theor. Apply. Genet. 53, 181-190

107. Pandley, S., Gritton, E.T. 1975. Inheritance of protein and other agronomic traits in a diallel cross of pea. J. Amer. Soc. Hort. Sci. 100(1) 87-90

108. Swiecicki, W.K., Kaczmarek, Z., Surma, M. 1981. Diallel analysis of protein content in selected lines of peas. Genetica Polonica 22, 79-84

109. Swiecicki, W.K., Kaczmarek, Z., Surma, M. 1981. Inheritance and heritability of protein content in seeds of selected crosses of pea (Pisum sativum L.) Genetica Polonica 22, 189-194

110. Weber, W.E. 1981. Untersuchungen zur Vererbung des Proteingehaltes bei Erbsen (Pisum sativum L.). Z. Pflanzenzuechtung 86, 201-209

111. Casey, R., Sharman, J.E., Wright, D.J., Bacon, J.R., Guldager, P. 1982. Quantitative variability in Pisum seed globulins, its assessment and significance. Qual. Plant, Plant Foods Hum. Nutr. 31, 333-346

112. Schroeder, H.E., Brown, A.H.D. 1983. Inheritance of legumin and albumin contents in a cross between round and wrinkled peas. Theoretical and Applied Genetics. In preparation

113. Hynes, M.J. 1968. Genetically controlled electrophoretic variants of a storage protein in Pisum sativum. Aust. J. Biol. Sci. 21, 827-829

114. Croy, R.R.D., Gatehouse, J.A., Evans, I.M., Boulter, D. 1980. Characterisation of the storage protein subunits synthesised in vitro by polyribosomes and RNA from developing pea (Pisum sativum L.) II. Vicilin Planta 148, 57-63

115. Davies, D.R., Cullis, C.A. 1982. A simple plant polytene chromosome system and its use for in situ hybridization. Plant Molecular Biology 1, 301-304

116. Sussex, I.M., and Dale, R.M.K. 1979. Hormonal control of storage protein synthesis in Phaseolus vulgaris. In : I. Rubenstein et al (Eds.). The plant seed development preservation and germination. Academic Press 129-141

117. Sussex, I.M., Dale, R.M.K., and Crouch, M.L. 1980. Developmental regulation of storage protein synthesis in seeds. In : C.J. Leaver (Ed.). Genome organisation and expression in plants. Plenum Press

118. Crouch, M.L. and Sussex, I.M. 1981. Development and storage protein synthesis in Brassica napus L. embryos in vitro and in vivo. Planta 153, 64-74

119. Eeuwens, C.J., and Schwabe, W.W. 1975. Seed and pod wall development in P. sativum L. in relation to extracted and applied hormones. Journal of Experimental Botany, 26, 1-14

120. Engvild, K.C. 1975. Natural chlorinated auxins labelled with radioactive chloride in immature seeds. Plant Physiology, 34, 286-287

121. Davies, P.J., Emshwiller, E., Gianfagna, T.J., Probsting, W.M., Noma, M., and Pharis, R.P. 1982. The endogenous gibberellins of vegetative and reproductive tissues of G2 peas. Planta 154, 266-272

122. Burrows, W.J., and Carr, D.J. 1970. Cytokinin content of pea seeds during growth and development. Physiologia Plan tarum 23, 1064-1070

123. Letham, D.S., Goodwin, P.B., and Higgins, T.J.V. (Eds.) 1978. Phytohormones and Related Compounds : A comprehensive Treatise Vol. 1, Elsevier North Holland, Amsterdam, Oxford, New York

ADDENDUM TO MSc. THESIS:

Cotyledonary Proteins in Pisum sativum

by H.E. SCHROEDER

The following information, additional to the text of the thesis, is offered in response to questions raised in one of the examiners' reports.

1) Gels presented in figures 1.1 and 1.2 (pp. 14,16) were done by myself in the course of the experiments. The gel presented in figure 1.3 (p.19) was kindly supplied by Dr. T.J. Higgins CSIRO Division of Plant Industry Canberra. Methods of SDS-PAGE of globulins, albumins, protein body extract, legumin, vicilin and lectin under dissociating and reducing conditions in figures 1.1, 1.2 and 1.3 are described in detail in Chapter 2 (pp. 38,39), and reference No. 32 of that chapter (see p.45). PAGE of albumins under non-dissociating conditions (Fig. 1.2A) was performed according to methods described by Davis, B.J. (1964). Ann. New York. Academ. Sci. 121:404-427.

2) The origin of lines of Pisum, sources and methods of protein amino acid analyses for each set of vicilin, legumin, albumin, globulins and total protein are listed at the bottom of each column by a reference number in Table 1.2 (between pp. 11 and 12).

3) In the final chapter (Conclusion) the following statement appears on page. 88. "However, they [$M_r \sim 800$ polypeptides] made up 35% of the protein sulfur of the albumins or more significantly 24% of the total seed protein sulfur". These derived estimates refer to the quantitative work reported in Chapter 3, and were obtained as follows.

Total sulfur measurements, by X-ray fluorescence spectrometry of globulins and albumins showed 0.422% for globulins and 0.896% for albumins, a total of 1.318%. The albumin proportion was thus 68% of total protein sulfur (quoted in section 3.5, p.53). The albumin $M_r \sim 8000$ polypeptides accounted for about 35% of the ^{35}S label incorporated into the albumin fraction (quantitation of the gel scan presented in figure 5b following p.53), representing 24% of the total protein sulfur (35% of 68%).

It should be pointed out that the gels and scans presented in figures 5a,b were expected to give only an indication of the contribution to the total albumin sulfur of the various polypeptides. This was clearly stated in section 3.5, p.53. The results were taken to have demonstrated the relatively high nutritional importance of the $M_r \sim 8000$ polypeptides.

4) The data presented graphically in Fig. 3a (p. 80) from experiment I are also given in Table 2. (p.80). LSD 0.1% values for each protein are listed. Results from experiment II are presented only graphically in Fig. 3b and data from experiment II were not analysed statistically. Fig. 3a, b, is a pictorial comparison of results of experiments I and II.