GENETIC VARIABILITY IN THE STRUCTURE OF THE α -SUBUNITS OF LEGUMIN FROM *PISUM*—A TWO-DIMENSIONAL GEL ELECTROPHORESIS STUDY

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SUMMARY

Variant forms of legumin (one of the major storage globulins of *Pisum* seeds) were purified by immunoaffinity chromatography from a range of *Pisum* types, including *P. fulvum*, and primitive forms, modern cultivars and advanced breeding selections of *P. sativum*. The purified variants, all of which had sedimentation coefficients of about 12S and leucine, threonine and glycine as N-terminal amino acids, showed subunit heterogeneity on polyacrylamide gels containing sodium dodecyl sulphate and on two-dimensional isofocusing/electrophoresis. Comparison of legumins from different *Pisum* types showed variability, both in the net surface charge of the whole molecule and in the nature of subunit heterogeneity on two-dimensional gels. The usefulness of two-dimensional gels in the genetic analysis of pea seed storage protein structure was stressed and the role of potential artefacts in such analyses assessed.

1. INTRODUCTION

LEGUMIN, one of the major storage proteins of *Pisum* seeds, is an oligomeric protein, of molecular weight about 4×10^5 ; it is composed of two types of subunit with molecular weights of about 40×10^3 and 20×10^3 (α - and β -subunits, respectively, by analogy with *Vicia faba* legumin; Wright and Boulter, 1974). Within each subunit class there exists microheterogeneity (Thomson *et al.*, 1978) and in *Pisum sativum* there are usually three or four major α -subunits with leucine and threonine N-termini (Boulter, 1979; Casey, 1979; Croy *et al.*, 1979).

The α -subunit heterogeneity of legumin has been studied here, using physicochemical and two-dimensional gel analysis of purified protein from a much wider range of *Pisum* types than hitherto examined. The use of two-dimensional gels is shown to be an attractive alternative to the methods of Thomson and Schroeder (1978) for examining the molecular and genetic basis of α -subunit multiplicity and variability in *Pisum* legumin.

2. METHODS

The *Pisum* types examined are described in Table 1. Legumin was purified from bulked mature seed samples of these genotypes and preparations from a given type were compared over a number of seasons and environments. Single F1 and F2 seeds from crosses were examined.

(i) Legumin purification and analysis

Purification of legumin by zonal isoelectric precipitation followed by immunoaffinity chromatography or DEAE-cellulose chromatography,

TABLE 1

Pea types used in the study of legumin variability

Code letter	J. I. accession number	Description
a	227	<i>Pisum abyssinicum</i> ; from Ethiopia
b	184	Single plant selection from the primitive cultivar Khadraa; from Khartoum, Sudan.
c	407	cv. Victory Freezer
d	224 BR	<i>Pisum fulvum</i> (brown seeded) from collection at Hebrew University, Jerusalem, Israel.
e	181	Single plant selection of the Kheeran pea; primitive cultivar from India.
f	224 SP	<i>Pisum fulvum</i> —as 224 BR but speckled seed
g	540	cv. Dark Skin Perfection
h	504	cv. Greenfeast
i	305	cv. Feltham First
j	849	<i>Pisum fulvum</i> (black seeded) from Palestine
k	186	Single plant selection from the primitive cultivar Wiraig; from Khartoum, Sudan.
l	(BS3)	John Innes advanced breeding selection; semileafless type.

electrophoresis on polyacrylamide gels containing sodium dodecyl sulphate (SDS-gels), cellulose acetate electrophoresis, N-terminal group identification by dansylation, analytical ultra-centrifugation and two-dimensional gel electrophoresis were all as previously described (Casey, 1979).

All samples for two-dimensional gel comparisons between *Pisum* genotypes were isofocused at the same time and referred to a pH gradient also determined at that time. Protein samples (5mg/ml) or whole pea meals (25 mg/ml) were incubated for 1 hour at room temperature in lysis buffer [2 per cent (w/v) (pH 3.5-10) Ampholines, 9.5M urea, 5 per cent (v/v) 2-mercaptoethanol, 2 per cent (w/v) Nonidet NP-40; O'Farrell, (1975)] prior to application of 10 µl of sample to the isofocusing gel. The positions of the ends of the first-dimension (isofocusing) gels were marked after second dimension electrophoresis to provide reference points for isoelectric point determination, since the gel expands on destaining. Apparent differences in legumin α -subunit isoelectric points were confirmed either by running mixtures of legumins from different *Pisum* types or from analysis of heterozygote patterns in F1 seeds.

(ii) *Analysis of protein subunit patterns of F2 seeds*

The micro-scale two-dimensional gel system of Lin *et al.* (1976) was used to rapidly examine small portions of large numbers of F2 seeds. A small piece (1-2 mg) of the seed was removed from opposite the embryonic axis, the testa discarded and the portion of cotyledon finely ground in a pestle and mortar. The meal was suspended in lysis buffer in a sealed conical centrifuge tube, incubated for 1 hour at room temperature, centrifuged at 3000 g for 10 min and a 5 µl portion of the supernatant liquid (equivalent to 25-35 µg of protein, depending on meal protein content) was loaded onto a 5 cm × 0.15 cm prefocused isofocusing gel of composition identical to that of large isofocusing gels (Casey, 1979). Isofocusing was at 400 V for 15 hours, equilibration with second dimension sample buffer (O'Farrell, 1975) was for 20 min. at room temperature and SDS-gel electrophoresis (Laemmli, 1970)

in the second dimension was on an 18 per cent (w/v) polyacrylamide slab (6.5 cm wide $\times$ 4.5 cm long $\times$ 0.15 cm thick) for 16 hours at 3 V/cm. Gels were stained for 30 min. at room temperature in 0.1 per cent (w/v) Brilliant Blue G in 50 per cent (w/v) trichloroacetic acid and destained in 7 per cent (v/v) acetic acid.

Legumin α -subunits were identified on micro-scale two-dimensional gels of whole meal extracts by co-electrophoresis of about 30 μ g purified legumin (from an appropriate genotype) in the presence of 10-15 μ g of total protein from an extract.

Ten samples were examined simultaneously and 1 mg of meal afforded enough material for several analyses if necessary; the remainder of each seed was sown to provide a further generation.

3. RESULTS

(i) *Variation in legumin*

The relative electrophoretic mobilities at pH 7.0 of the purified variant legumins are shown in Fig. 1. Although the genotypes were selected for variability in legumin α -subunit composition on SDS-gels there is also clearly variation in the net surface charge of the native legumin molecule. The legumins shown in fig. 1 were purified by immunoaffinity chromatography on a column of IgG which was monospecific for legumin from genotype BS3 (Casey, 1979). The same electrophoretic mobilities were observed if legumins were purified by DEAE-cellulose, instead of immunoaffinity, chromatography; some legumin variants (notably those of lower electrophoretic mobility—see fig. 1, d and f) were, however, difficult to separate from vicilin by DEAE-cellulose chromatography.

All of the purified legumins had a sedimentation coefficient of about 12 ($S_{20,w} = 11.4$ - 11.9 , depending on protein concentration) and hence a molecular weight of about $3.5-4 \times 10^5$. Despite the considerable variation in subunit patterns exhibited by the legumin variants (see below) they all possessed only the three N-terminal groups (leucine, threonine and glycine) previously observed for *Pisum sativum* (Jackson *et al.*, 1969) and *Vicia faba* (Wright and Boulter, 1974) legumin. In every case the relative intensities of dansyl-end-groups were leucine > glycine > threonine and in one case (JI 849) dansyl-threonine was barely discernible.

(ii) *Variation in the subunit composition of legumin*

(a) *One-dimensional (SDS)-gels*

Fig. 2 shows the broad range of variation in the mobilities of the α - and β -subunits of the purified legumins on SDS-gels; fig. 3 shows the result of decreasing the gel concentration and lowering the amount of sample to accentuate the multiplicity seen in the α -subunit region. Repeat extractions on three to ten different samples from a given genotype showed that such patterns are genotype-specific (and as will be seen below, are accurately repeated in the second dimension of a two-dimensional gel); the subunit pattern was consistent, regardless of the environment in which the plants were grown.

(b) *Two-dimensional gels*

Two-dimensional gels are subject to a number of potential artefacts (O'Farrell, 1975), the most important of which, in the context of this work, is spot multiplicity in the isofocusing dimension. This can result from partial deamidation of subunits, or from carbamylation of proteins by traces of cyanate (formed in the concentrated urea solutions used for sample preparation and isoelectric focusing).

A number of control experiments were carried out to determine the extent of carbamylation and deamidation and their possible effect on α -subunit multiplicity on two-dimensional gels. Attempts were made to increase carbamylation by incubating samples in lysis buffer for 5 hours at 45°C. Attempts to decrease possible carbamylation included (a) routine prefocusing of *pH* gradients to remove cyanate (*pK_a* 3.75); (b) sample application immediately after solubilisation; (c) sample preparation in [8M urea, 0.1M acetic acid, 5 per cent (v/v) 2-mercaptoethanol, *pH* 3.95] (cyanate is unstable below *pH* 5); (d) application of samples, prepared as in (c), to the low *pH* end of preformed gradients, which ensured that samples never encountered a *pH* greater than 5. Ampholines (which contain reactive amines and protect proteins from carbamylation by "scavenging" cyanate) were omitted from some control samples. Attempts were made to induce deamidation by incubating samples for 5 hours at 45°C in a phosphate-containing buffer of ionic strength 1.0, *pH* 10 (McKerrow and Robinson, 1971). When the two-dimensional gel patterns of these controls were compared with the "normal" pattern of a sample which had been incubated for 1 hour at room temperature in lysis buffer, no differences were seen, which suggested that little, if any, of the observed α -subunit charge heterogeneity was due to chemical artefact.

Continuation of isofocusing for 42 hours had no effect on the two-dimensional gel pattern and thus the observed subunit multiplicity is not a result of under-focusing.

Aggregation through incomplete reduction probably did not occur, since none of the legumin samples (see fig. 2) show any evidence of the 60×10^3 mol. wt. band observed with unreduced legumin (Thomson *et al.*, 1978). Multiplicity of α -subunits as a result of random dimerisation of β -subunits is unlikely since the two-dimensional gel patterns closely reflect the SDS-gel patterns (see, for instance, figs. 3 and 5, c and g); one would not expect the same aggregation patterns in urea/Nonidet/mercaptoethanol as in SDS/mercaptoethanol.

We therefore conclude that the observed two-dimensional gel patterns probably reflect genuine α -subunit microheterogeneity.

Two-dimensional gel electrophoresis (fig. 4) of the purified legumin from genotype BS3 (which was used as antigen in the preparation of the immuno-affinity matrix) shows even further α -subunit multiplicity when compared to SDS gels (fig. 2, channel 1); the α -subunits fall into a major (α^M) and minor (α^m) group. The β -subunits of *Pisum* legumin do not appear on the two-dimensional gels because their isoelectric point (>9 in 6M urea; Croy *et al.*, 1979) falls outside the range of the *pH* gradient (5.5-8.0, in 8M urea) used in this study. Although minor subunits with a molecular weight of about 20×10^3 are present (see fig. 4) their identity is uncertain; their isoelectric points are too low for normal β -subunits. The subunits of about 25×10^3 mol. wt. (which have also been reported by Thomson *et al.*, 1978),

have been tentatively designated γ until their chemical identity becomes more certain. These γ -subunits are very minor components (scarcely visible on SDS-gels) which consistently co-purify with legumin during a number of non-denaturing purification procedures, including density-gradient centrifugation and hydroxyapatite chromatography.

Although the relative staining intensities in fig. 2 suggest a preponderance of α - over β -subunits, chemical evidence (Casey, 1979) indicates that α - and β -subunits are in fact present in equimolar proportions, as in *Vicia faba* legumin (Wright and Boulter, 1974).

Major α -subunits

These can vary in number from one (fig. 5, j and possibly also d) to four (fig. 5, c and g). Their apparent isoelectric points in concentrated urea span the range about p*H* 6.0-6.5 and their apparent molecular weights range from about 39×10^3 to about 42×10^3 .

Minor α -subunits

It should be emphasised that these are very minor in amount when compared with the α^M -subunits and are scarcely visible on a two-dimensional gel which has been slightly underloaded to optimise the separation of α^M -subunits. The apparent numbers of subunits range from one (fig. 6, d) to seven or eight (fig. 6, c and g), but all patterns are entirely reproducible for a given genotype. There is no obvious correlation of α^m patterns with α^M patterns. The apparent p*H* range covered by the α^m -subunits is narrow (p*H* 5.6-5.8), while the apparent molecular weights vary from 40×10^3 to about 43×10^3 .

γ -subunits

No variability was observed amongst the γ -subunits of the legumins from different genotypes.

There was no obvious correlation between the apparent isoelectric points of the α -subunits and the relative electrophoretic mobility (fig. 1) of a particular variant legumin.

(iii) *Genetics of α^M -subunit composition*

The α^m -subunit patterns in single F2 seeds were complex and could not be adequately analysed.

The inheritance of variant α^M -subunit patterns was studied in crosses of JI 224 BR and JI 224 SP. Two-dimensional gels show that the single α^M -subunit in JI 224 BR legumin (fig. 5, d) can readily be differentiated from the three α^M -subunits of JI 224 SP (fig. 5, f). These subunit patterns can be unambiguously detected in two-dimensional gels of seed meals (fig. 7, a and b) and analysis of five F1 seeds from each of the reciprocal crosses showed additive patterns for the α^M -subunits. The F1 patterns were the same, irrespective of which parent was the paternal one, confirming the findings of Thomson and Schroeder (1978) that there is no maternal effect in the inheritance of legumin α^M -subunit patterns.

Examination of 25 single F2 seeds by micro-scale two-dimensional gels showed the segregation of α^M -subunit types to be 1 JI 224 BR : 1 JI 224 SP :

2 F1 ($\chi^2_{(2)} = 0.33, p > 0.75$). Fig. 7 shows the three types of pattern obtained from individual F2 seeds and indicates the value of two-dimensional gels in such an analysis; it would be very difficult to differentiate between the JI 224 SP and F1 phenotypes by SDS gel analysis alone.

4. DISCUSSION

On the basis of analytical ultracentrifugation, cellulose acetate electrophoresis, N-terminal group analysis and immunological behaviour we concluded that the legumin preparations were pure. Despite variability in the nature of the α - and β -subunits and the variation in net surface charge (figs. 1 and 2), all legumin variants have retained the characteristic features of insolubility at low, but solubility at high, ionic strength at pH 4.8, sedimentation coefficient of about 12S (and hence a molecular weight of about $3.5\text{--}4.0 \times 10^5$), the same N-terminal amino acids and the ability to react with monospecific anti-legumin IgG.

It is clear from this work and from the studies of Thomson *et al.* (1978) and of Thomson and Schroeder (1978) that there is both micro-heterogeneity of legumin at the subunit level and genetic variation in the nature of this heterogeneity.

It is difficult at present to ascribe a molecular basis to α -subunit heterogeneity. The α -subunits have two different N-terminal groups (Leu and Thr) and the relative intensities of the dansyl derivatives suggest that Leu might be derived from the α^M -, and Thr from the α^m -, subunits; there are, however, inherent difficulties in such an evaluation stemming from variation in stability and quantum fluorescence yield of dansyl amino acids (Hartley, 1970). There is some evidence, discussed below, which indicates gene duplication of α^M loci and suggests that α^M -subunit genes may be closely linked.

Little information exists on the genetics of the seed storage protein subunits from any legume, other than the work of Romero *et al.* (1975) and Hall *et al.* (1977) on heritable variation in a subunit of a major storage protein of *Phaseolus vulgaris* and the study of *Pisum sativum* by Thomson and Schroeder (1978). These latter authors have described five genetic systems (LA-LE) determining the structure of *Pisum* legumin. Genetic system LA refers to the least acidic of the bands which migrate to the anode in urea at pH 8.3, which are relatively major and which have subunit molecular weights of about 40×10^3 ; these correspond to our α^M -subunits. System LB refers to subunits which are minor, more acidic than the LA subunits and which have subunit mol. wts. of 37×10^3 . These would appear to correspond to our α^m -subunits in all but molecular weight, since we consistently observe α^m -subunit mol. wts. of $40\text{--}43 \times 10^3$; this difference may represent genetic variation. Alternatively, although the relative mobilities of the LB subunits in urea gels at alkaline pH appear to be consistent with our definition of them as the α^m -subunits, it is possible that there is some aggregation of legumin subunits in 8M urea at alkaline pH (cf. Grant and Lawrence, 1964) and that the inclusion of Nonidet and higher concentrations (9.5M) of urea reduces or eliminates aggregation; hence LB subunits and α^m -subunits may not be strictly comparable. The LC system refers to slight differences in the subunit molecular weight of the major 40×10^3 mol. wt. subunits

FIG. 1.—Cellulose acetate electrophoresis at pH 7 of purified legumins from different genotypes of *Pisum* (table 1). The arrow denotes the point of sample application; migration towards the anode. Approx. $2.5 \mu g$ of protein was loaded per lane except for lanes (f)-(h), where approx. $1.5 \mu g$ was loaded.

FIG. 2.—SDS-gel electrophoresis of purified legumin variants from different genotypes (table 1). The patterns for (c) and (g) are the same, as are (b) and (k). Approx. $20 \mu g$ of legumin was loaded per slot; 18 per cent gel.

FIG. 3.—SDS-gel electrophoresis of purified legumin variants from different genotypes (table 1) showing the 40×10^3 mol. wt. region in more detail. Approx. $12 \mu\text{g}$ of legumin was loaded per slot; 15 per cent gel.

FIG. 4.—Two-dimensional gel of purified legumin from genotype BS3. The apparent pH values were measured in 8M urea; positions adopted by marker proteins in the second dimension are indicated at the side.

FIG. 6.—The α^M -regions from two-dimensional gels of purified legumins from different genotypes (table 1). The arrows indicate the position of a marker protein of mol. wt. 40×10^3 in the SDS dimension.

FIG. 6.—The α^M -regions from two-dimensional gels of purified legumins from different genotypes (table 1). The arrows indicate the position of a marker protein of mol. wt. 40×10^3 in the SDS dimension.

FIG. 7.—Two-dimensional gels of meal from F₂ seeds from the cross JI 224 BR × JI 224 SP showing (a) the JI 224 BR parental type; (b) the JI 224 SP parental type and (c) the F₁ phenotype (with respect to legumin α^M -subunits). The arrows show the relevant legumin α^M -subunits.

within SDS gels of reduced whole legumin, *i.e.*, size differences between α^M -subunits on SDS-gels. Genetic system LD refers to the set of bands which migrate cathodically in urea at *pH* 7.5 (*i.e.*, which are basic) and which have subunit mol. wts. of about 20×10^3 . These correspond to the β -subunits, which do not appear on the two-dimensional gels. The LE system refers to a group of acidic, very minor polypeptides with subunit molecular weights of $25\text{--}27 \times 10^3$ and corresponds to the γ -subunits. Thomson and Schroeder (1978) noted genetic variability in the LE system but ascribed no genetic basis to it; we observed no variability.

On the whole the agreement between our results, using two-dimensional gels, and those of Thomson and Schroeder (1978), using urea gels and SDS gels, is good, despite the use of different conditions for the separation of subunits on the basis of charge. Two-dimensional gels offer some advantages: in particular, it is possible to unambiguously assign an apparent isoelectric point and subunit molecular weight to a given α -polypeptide without the uncertainty resulting from cutting, eluting and re-electrophoresing bands; hidden complexity is quickly and clearly revealed; the use of micro-scale two-dimensional gels in the analysis of F2 seeds enables us to rapidly obtain information on the segregation patterns of seed protein subunits from very small portions of large numbers of seeds, whilst the remainder of each seed can be grown for subsequent analysis of plant phenotype and for further generations.

The two-dimensional gel patterns of α^m -subunits in F2 seeds were complex, difficult to score and included combinations of subunits which were not seen in either parental type. We therefore conclude, as did Thomson and Schroeder (1978) (for their LB variant system), that the basis of α^m -subunit variability is complex. Thomson and Schroeder (1978) also noted that the variability in β -subunits has a multigenic basis; we have not examined β -subunit variability.

The F2 patterns of α^M -subunits from the cross 224 BR $\times$ 224 SP indicated that variation in α^M -subunit number, charge and molecular weight has a simple genetic basis and that the one-polypeptide α^M -pattern of 224 BR and the three-polypeptide pattern of 224 SP are allelic alternatives. Within the limited number of F2 seeds examined, no recombination between α^M -subunit patterns was observed, which would imply that the structural loci for the three α -polypeptides of 224 SP are present as a linked group (which would be consistent with α^M gene duplication).

There seems to be a discrepancy between the results of Thomson and Schroeder (1978) and the present data on the inheritance of α^M -subunits. Thomson and Schroeder (1978) state that phenotypes of the LA and LC systems segregated independently in F2 seeds; that is, they observed independent segregation of size and charge variants of the major 40×10^3 mol. wt. subunits. Our results, however, show that the charge and size of phenotypes of a given subunit do not segregate independently.

This report indicates that two-dimensional gels, especially in miniaturised form, are useful techniques in the study of inheritance patterns of *Pisum* legumin α -subunits.

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