Isoelectric-Focusing Properties and Carbohydrate Content of Pea (*Pisum sativum*) Legumin

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Legumin from pea (*Pisum sativum*) is a molecule made up of six pairs of subunits, each pair consisting of an 'acidic' subunit (mol.wt. about 40000) and a 'basic' subunit (mol.wt. about 20000) linked by one or more disulphide bonds. The heterogeneity of legumin has been investigated by isoelectric focusing; undissociated legumin could not be focused satisfactorily, but legumin subunits could be analysed under dissociating conditions. 8M-Urea was not found to be a satisfactory medium for isoelectric focusing of legumin, as the 'basic' subunits showed a shift in pI with time of incubation in urea. A new dissociating medium for isoelectric focusing, namely 50% (v/v) formamide, was used for analysis of legumin, which gave pI values of 5.0–5.3 for the 'acidic' subunits, and 8.3–8.7 for the 'basic' subunits. Both types of subunits were shown to be heterogeneous in charge and molecular weight by two-dimensional analysis employing isoelectric focusing in the first dimension and sodium dodecyl sulphate/polyacrylamide gel electrophoresis in the second. The 'basic' and 'acidic' subunits of legumin were separated on the preparative scale by ion-exchange chromatography in 50% formamide. Carbohydrate attached to the protein was investigated as a possible cause of the heterogeneity of legumin subunits. However, both a fluorescent-labelling technique and a sensitive radioactive-labelling technique failed to show any carbohydrate bound to legumin subunits, and it was concluded that legumin is not a glycoprotein.

Legumin is the major storage protein of pea (*Pisum sativum*). As isolated, the legumin molecule is thought to contain six pairs of subunits held together in a structure of mol.wt. 390000 by non-covalent bonding, each pair of subunits consisting of an 'acidic' subunit (mol.wt. about 40000) and a 'basic' subunit (mol.wt. about 20000) joined by one or more disulphide bonds (Wright & Boulter, 1974; Croy *et al.*, 1979). Heterogeneity is present in legumin isolated from pea seeds in that both 'acidic' and 'basic' subunits can be shown to be heterogeneous with respect to molecular weight and isoelectric point (Casey, 1979; Krishna *et al.*, 1979), although heterogeneity in molecular weight in legumin molecules has not been demonstrated (Croy *et al.*, 1979) and the insolubility of legumin at or near its isoelectric point (Derbyshire *et al.*, 1976) has prevented successful analytical isoelectric focusing.

The nature of the heterogeneity observed in pea legumin is not well established. Some confusion

exists in literature reports of the behaviour of legumin on isoelectric focusing under dissociating conditions. Casey (1979) reported pI values of 6.3–6.6 for the 'acidic' subunits, and 6.8–7.1 for the 'basic' subunits, whereas Krishna *et al.* (1979) claimed pI values of 4.6–4.9 for the 'acidic' subunits and 8.5–8.8 for the 'basic' subunits. These very considerable discrepancies cannot be accounted for by varietal differences. There are also conflicting reports concerning the carbohydrate content of legumin; Basha & Beevers (1976) reported the presence of 1.0% neutral sugars and 0.1% amino sugar, whereas other authors (Bailey & Boulter, 1970; Casey, 1979) claim that the amount of carbohydrate in the protein is very low (less than 0.1%). It is important to clarify this issue, since variability in the carbohydrate portions of glycoproteins is known to be a cause of protein microheterogeneity (Spiro, 1970).

The observed heterogeneity in the two legumin subunit types may arise from an initial heterogeneity in legumin cistrons (genes), or from post-translational modification of initially homogeneous, single-gene products. An understanding of the

Abbreviations used: QAE-, quaternary aminoethyl-; SDS, sodium dodecyl sulphate; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

molecular basis of the heterogeneity is a prerequisite to undertaking a study of the genetic control of legumin synthesis. The present paper is intended to clarify the previous data on the heterogeneity of legumin and to examine whether carbohydrate attached to the protein is a source of this heterogeneity. The work has involved the use of a new dissociating medium, 50% (v/v) formamide, for isoelectric focusing and ion-exchange chromatography of proteins, and a highly sensitive radioactive-labelling method for detecting protein-bound carbohydrate.

Experimental

Materials

All chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., unless stated otherwise, and were of analytical grade or of the highest grade obtainable. Formamide and solutions of urea were deionized by stirring for 1 h with Amberlite MB-1 mixed-bed resin (1 g/100 ml) and were stored at 4°C in the dark for up to 1 week; within this time the conductivity of the liquids remained almost constant, indicating that negligible quantities of ionic species (e.g. cyanate) were present. Ampholines for isoelectric focusing were obtained from LKB-Produkter A.B., Bromma, Sweden, and Pharmacia Fine Chemicals, Uppsala, Sweden. Agarose for isoelectric focusing was from LKB. Sodium boro[³H]hydride (555 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. QAE-Sephadex A50 was a product of Pharmacia Fine Chemicals. Hydroxyapatite was obtained from Bio-Rad Laboratories Ltd., Watford, Herts., U.K.

Methods

Legumin was prepared from pea (*Pisum sativum*, var. Feltham First; Suttons Seeds Ltd., Reading, Berks., U.K.) by hydroxyapatite chromatography of an extract of seed meal. The extract was prepared by grinding 10 g of dry seeds for 30 s in a water-cooled mill (Janke and Kunkel, Staufen in Breisgau, Germany) and stirring the resulting fine meal with 100 ml of 50 mM-potassium phosphate buffer, pH 8.0, for 16 h at 4°C. The mixture was centrifuged at 29 000 g for 20 min and the clear supernatant taken and applied to a column (3.2 cm diam., 120 ml volume) of hydroxyapatite that had been equilibrated with 50 mM-potassium phosphate buffer, pH 8.0, as described above. The column was washed with equilibrating buffer to elute non-bound material and then eluted with a linear concentration gradient of potassium phosphate buffer, pH 8.0 (300 ml of 50 mM-buffer + 300 ml of 1 M-buffer). Under these conditions legumin elutes as a single peak of protein. All buffers used in column chromatography were sterilized by filtration through a 0.45 µm pore-

size cellulose acetate filter. Legumin-containing fractions were pooled, dialysed against water and freeze-dried.

Isoelectric focusing was carried out in polyacrylamide slab gels containing 7.5% monomer, of which 2.5% was *NN'*-methylenebisacrylamide, as described by Vesterberg (1975). Deionized formamide was used to replace part of the water in the gel mix so that the final formamide concentration was 50% (v/v). Alternatively, deionized urea solutions were used to give a final urea concentration of 8 M. Slabs were prefocused for 30 min at 5 W, and then samples (25 µl; 2 mg of protein/ml) were applied to the gel on small rectangles (10 mm × 15 mm) of glass-fibre filter 'paper'. Focusing was carried out for 3 h at 10–12 W. Slices were removed from the focused gels for determination of the pH gradient; the slices were sectioned, and the gel pieces (5 mm × 10 mm) were each soaked overnight in 1 ml of water. The pH values of the solutions were then measured. After focusing, gels were washed overnight in 15% (w/v) trichloroacetic acid (at least 400 ml). They were then stained with 0.025% Coomassie Blue R-250 in methanol/acetic acid/water (5:1:4, by vol.) and destained in methanol/acetic acid/water (3:1:6, by vol.). The gels frequently distorted on drying, so the staining patterns were copied from the wet gels. Isoelectric focusing was also carried out in agarose slabs, prepared, run and stained as described in the appropriate LKB product-information sheet. Ion-exchange chromatography of legumin subunits was carried out on a column (2.5 cm diam., 50 ml volume) of QAE-Sephadex A50 initially equilibrated with 50 mM-Tris/HCl buffer, pH 7.5, containing 50% (v/v) formamide. Legumin samples (10 mg in 2 ml of column buffer), unreduced, or reduced by the addition of 2% (v/v) 2-mercaptoethanol, were applied and the column was washed with buffer as described above until all non-bound material had been removed, and eluted with a linear gradient (0–0.5 M) of NaCl in column buffer (400 ml total volume). Protein-containing fractions were pooled, dialysed against water, and freeze-dried.

The carbohydrate content of undissociated proteins was analysed in solution by the phenol/H₂SO₄ method (Dubois *et al.*, 1956). Carbohydrate was detected in protein subunits after SDS/polyacrylamide-gel electrophoresis on 10% (w/v) acrylamide disc gels (Gabriel, 1971) by the periodic acid/Schiff-reagent method (Zacharias *et al.*, 1969) or by using dansylhydrazine (Eckhardt *et al.*, 1976). Alternatively glycoproteins were ³H-labelled in their carbohydrate side chains by a modification of the method of Lotan *et al.* (1975). Proteins (1–2 mg) were dissolved in 1 ml of 1% (w/v) periodic acid/1% (w/v) glycine/3% (v/v) acetic acid in water. Oxidation was allowed to proceed for 1 h at room temperature; the samples were then dialysed extensively

against 50mM-sodium borate buffer, pH 8.0, containing 1% (w/v) glycine. NaB^3H_4 (25 mCi; 1.8 mg) was dissolved in 0.2 ml of 50mM-NaOH and 40 μl of this solution was added to each dialysed sample. Reduction was allowed to proceed for 5 h at room temperature, and the samples were subsequently dialysed against water and freeze-dried. The labelled proteins were analysed by SDS/polyacrylamide-gel electrophoresis and fluorography (Bonner & Laskey, 1974).

SDS/polyacrylamide-gel electrophoresis was carried out in 17% acrylamide slab gels by the method of Laemmli (1970) unless specified otherwise. Two-dimensional-gel-electrophoretic analysis of proteins was carried out by a modification of the systems of O'Farrell (1975) and Ames & Nikaido (1976), which employ isoelectric focusing on polyacrylamide-gel slabs (0.6 mm thick) containing formamide (as described above) in the first dimension, and SDS/polyacrylamide-gel electrophoresis on 17% polyacrylamide-gel slabs (1.2 mm thick) in the second dimension. Proteins were also analysed by SDS/polyacrylamide-gel electrophoresis in 12% polyacrylamide-gel slabs (0.6 mm thick) in the first dimension, and in 17% polyacrylamide gel slabs (1.2 mm thick) in the second dimension. The unreduced protein samples electrophoresed in the first dimension were reduced before electrophoresis into the second dimension by placing a strip of the first-dimension gel containing the protein on top of the second-dimension gel and covering with a solution of 1% agarose in electrophoresis sample buffer [0.2 M-Tris/HCl, pH 6.8, containing 2% (w/v) SDS] containing 5% (v/v) 2-mercaptoethanol at 60°C. Electrophoresis into the second dimension was carried out when the agarose solution had solidified and cooled to room temperature.

Results

Identification of impurities in the legumin preparation used

Legumin, unreduced or reduced by the addition of 2-mercaptoethanol, was analysed by SDS/polyacrylamide-gel electrophoresis. The results of a combined two-dimensional analysis, legumin being unreduced in the first dimension and reduced in the second dimension, are shown in Fig. 1. Unreduced legumin gave species at indicated approx. mol.wts. of about 60 000 (at least two bands), 38 000, 31 000 and 29 000. Reduced legumin gave species at indicated approx. mol.wts. of about 40 000 (several bands), 31 000, 29 000, 25 000, about 20 000 (several bands) and about 18 000 (two bands). As Fig. 1 shows, the bands corresponding to about 60 000 mol.wt. in unreduced legumin gave bands at about 40 000 and about 20 000 mol.wt. on reduction; the bands corresponding to 31 000 and 29 000 mol.wt. are unchanged on reduction, where-

Fig. 1. Two-dimensional SDS/polyacrylamide-gel analysis of legumin

Unreduced legumin was subjected to SDS/polyacrylamide-gel electrophoresis in a 12% (w/v) polyacrylamide-gel slab. A strip from the first-dimension gel was applied to the top of an SDS/17% polyacrylamide-gel slab, reduced by incubation with 5% 2-mercaptoethanol (2-ME) in 1% agarose solution, and the whole was then electrophoresed. Track A (horizontal), unreduced legumin, stained strip from first-dimension gel; track B (vertical), reduced-legumin standard.

as the band corresponding to 38 000 mol.wt. gave bands corresponding to mol.wts. of 25 000 and about 18 000 on reduction. On the basis of these results and the accepted model for the structure of legumin (Wright & Boulter, 1974), the bands at about 60 000 mol.wt. (unreduced) or about 40 000 and about 20 000 mol.wt. (reduced) were considered to represent legumin. These bands were stained much more intensely than the others. The resolution of the legumin bands on SDS/polyacrylamide-gel electrophoresis was found to depend on sample loading, so that, on some gels, closely spaced bands were resolved, whereas on others they were not. Other bands were considered to represent impurities, in agreement with analyses of legumin as further purified by ion-exchange chromatography on DEAE-cellulose (Croy *et al.*, 1979). Densitometric scans of SDS/polyacrylamide gels stained with Coomassie Blue R-250 showed that the legumin as defined was about 95% pure.

Isoelectric focusing of undissociated legumin

Legumin, if undissociated, could not be focused to its isoelectric point in polyacrylamide gels. The protein precipitated in the gel before it had reached its

pI, resulting in an ill-defined smear of material in the pH 5–6 region. Addition of 2% (v/v) Nonidet P-40 and/or 1% (w/v) glycine to the gel and/or sample did not prevent this precipitation and allow isoelectric focusing to take place. However, in an agarose gel, isoelectric focusing appeared to take place, since legumin focused to essentially a single band at a pI value of about 4.7, although this band was broader than those of standard proteins, indicating heterogeneity. To investigate the nature of this heterogeneity, it was necessary to use dissociating media for isoelectric focusing.

Isoelectric focusing of legumin in gels containing 8M-urea

The behaviour of legumin on isoelectric focusing in slab gels containing 8M-urea was found to vary considerably with prior treatment of the sample. Incubation of legumin samples in urea, even without 2-mercaptoethanol, caused extensive dissociation of the 'acidic' from the 'basic' subunits; this was shown both by SDS/polyacrylamide-gel electrophoresis and by isoelectric focusing. The dissociation was favoured at acidic pH values. Significantly, when legumin samples incubated a short time in urea were focused, 'basic'-subunit bands at pI 7 were seen, whereas legumin samples incubated in urea for 3

days or more at 4°C showed much more strongly stained 'basic'-subunit bands at pI values of about 9 (Fig. 2). Including 2-mercaptoethanol in the dissociating media had no effect on the resulting gel patterns. As previously reported, considerable heterogeneity was observed in the legumin. The band pattern was often very complex, owing to the presence of material focusing in the pI 6–7 region as well as the legumin 'acidic'- (pI around 5) and 'basic'-subunit bands; results were often not reproducible.

Isoelectric focusing of legumin in gels containing 50% formamide

Reproducible results were obtained when legumin was focused in gel slabs containing 50% formamide. Dissociation of 'acidic' from 'basic' subunits in the absence of 2-mercaptoethanol was not observed in samples dissolved in 50% formamide, and the legumin under these conditions focused to a poorly defined series of bands at pI 6–6.5. If the legumin in 50% formamide was reduced by addition of 2% 2-mercaptoethanol, and then subjected to isoelectric focusing, two series of sharp bands, one at pI 5.0–5.3, representing 'acidic' subunits (two major, three minor bands) and one at pI 8.3–8.7, representing 'basic' subunits (three major bands, several minor

Fig. 2. *Isoelectric focusing of legumin in 8M-urea* Legumin, incubated in 8M-urea at 4°C for the stated times, was subjected to isoelectric focusing on a polyacrylamide-gel slab containing 8M-urea.

Fig. 3. *Isoelectric focusing of legumin in 50% formamide*

Legumin was dissolved in 50% (v/v) formamide and subjected to isoelectric focusing on a polyacrylamide-gel slab containing 50% formamide. (a) Unreduced legumin; (b) legumin reduced by the addition of 2% (v/v) 2-mercaptoethanol; (c), (d) and (e) fractions I, II and III respectively from ion-exchange chromatography of legumin (unreduced and reduced) in 50% formamide (see Fig. 5).

bands) were seen. These results are summarized in Fig. 3. No changes in band patterns were seen between samples focused immediately after dissolution in 50% formamide and samples focused after incubation for up to 3 days at 4°C in this medium.

Two-dimensional analysis of legumin

The molecular weights of the 'acidic' and 'basic' subunits of legumin, as separated by isoelectric focusing in gels containing 50% formamide, were determined by a two-dimensional analysis, which employed SDS/polyacrylamide-gel electrophoresis under reducing conditions as the second dimension to separate the focused subunits according to size and to compare their molecular weights with legumin subunits subjected to SDS/polyacrylamide-gel electrophoresis. The results of such an analysis are shown in Fig. 4. The 'acidic' subunits correspond to the 40000-mol.wt. subunits of legumin, and the 'basic' subunits correspond to the 20000-mol.wt. subunits. The heterogeneity shown by both groups of subunits in charge and molecular weight is clearly shown by this analysis.

Ion-exchange chromatography of legumin in 50% formamide

Legumin subunits could be isolated on the preparative scale by ion-exchange chromatography on a column of QAE-Sephadex A50 equilibrated at pH 7.5 with buffer containing 50% formamide; protein fractions were eluted by a salt gradient. If unreduced legumin was applied to the column, it was eluted at a salt concentration of 0.1–0.15 M essentially as a single peak (Fig. 5). No other material was eluted up to salt concentrations of 1 M. The

eluted material contained 60000-mol.wt. subunits, as shown by SDS/polyacrylamide gel electrophoresis, which gave 40000- and 20000-mol.wt. subunits after reduction with 2-mercaptoethanol; this material also had a pI corresponding to unreduced legumin (Figs. 3 and 6). If legumin was reduced with 2-mercaptoethanol before chromatography, two protein fractions were obtained from the column: one was not bound, indicating a pI greater than 7.5; the other was

Fig. 5. Ion-exchange chromatography on legumin in 50% formamide

Elution profiles for ion-exchange chromatography of legumin in 50 mM-Tris/HCl buffer, pH 7.5, containing 50% (v/v) formamide on a column of QAE-Sephadex A50. —, Unreduced legumin; ----, legumin reduced by the addition of 2% 2-mercaptoethanol; ····, [NaCl] gradient.

Fig. 4. Two-dimensional analysis of legumin
Reduced legumin was subjected to isoelectric focusing in 50% formamide. A strip from the focusing slab gel was applied to the top of an SDS/polyacrylamide slab gel, and the whole was electrophoresed. Track A, reduced-legumin standard. The pH scale is taken from the focusing slab.

Fig. 6. SDS/polyacrylamide-gel analysis of fractions from ion-exchange chromatography of legumin in 50% formamide

(a) Unreduced legumin; (b) and (c) fraction I from ion-exchange chromatography of unreduced legumin in 50% formamide (see Fig. 4), unreduced or reduced by addition of 2% 2-mercaptoethanol, respectively; (d) and (e) fractions II and III respectively from ion-exchange chromatography of reduced legumin in 50% formamide (see Fig. 4); (f) legumin reduced by addition of 2% 2-mercaptoethanol. ●, Bands due to impurities in legumin [note that fraction I has been purified from impurities corresponding to 31000 and 29000 mol.wt., and that fractions II and III have been purified additionally from the (reduced) impurity corresponding to 25000 mol.wt.].

eluted at salt concentrations of 0.4–0.5 M (Fig. 5). SDS/polyacrylamide-gel electrophoresis showed the first fraction to consist of 20000-mol.wt. subunits, and the second fraction to consist of 40000-mol.wt. subunits; these were confirmed to be 'basic' and 'acidic' subunits of legumin respectively by isoelectric focusing (Figs. 3 and 6). The analyses of fractions from ion-exchange chromatography of legumin in 50% formamide showed that certain bands assigned to impurities were removed by this technique.

Carbohydrate analyses on legumin

No reaction for carbohydrate was given by 2 mg of legumin under conditions where carbohydrate could be clearly detected in pea vicilin, which contains 0.5% carbohydrate (Basha & Beevers, 1976) [shown by using the phenol/H₂SO₄ method of Dubois *et al.* (1956) (lower limit of detection 2 μ g of carbohydrate, i.e. 0.1% in the legumin)]. Further, when carbohydrate was shown to be present in vicilin subunits after SDS/polyacrylamide-gel electrophoresis by staining the gels with periodic acid/Schiff reagent (Zacharias *et al.*, 1969) or with the more sensitive fluorescent reagent dansylhydrazine (Eckhardt *et al.*, 1976), gels containing similar amounts of legumin run in parallel gave no bands that stained for carbohydrate. As a further method of analysis, various proteins and glycoproteins were oxidized with periodic acid and

reduced with NaB³H₄, thus labelling any carbohydrate present. The protein preparations used were ovomucoid, ovalbumin, bovine serum albumin, pea vicilin and pea legumin, with carbohydrate contents (respectively) of 24.7%, 3.2%, very low or none, 0.5%, and an unknown amount (Eylar, 1965; Basha & Beevers, 1976). The specific radioactivities of the protein preparations after labelling were: ovomucoid, 4×10^7 c.p.m./mg; ovalbumin, 0.9×10^7 c.p.m./mg; bovine serum albumin, 0.4×10^7 c.p.m./mg; vicilin 0.3×10^7 c.p.m./mg; legumin, 0.1×10^7 c.p.m./mg. When the preparations were analysed by SDS/polyacrylamide-gel electrophoresis and fluorography, radioactivity was shown to be incorporated into ovalbumin, ovomucoid and some vicilin subunits, but comparison of the fluorograph with a gel stained for protein showed that the radioactivity incorporated into bovine serum albumin was not in the albumin (mol.wt. 67000) but in impurities (mol.wt. about 60000). In agreement with the low incorporation of radioactivity into the legumin preparation as a whole, only a band at about 30000 mol.wt. was ³H-labelled, and this remained unchanged whether legumin was reduced or unreduced, showing that it represented an impurity in the preparation and was not a part of the legumin molecule. The labelling procedure did not affect the running position of the proteins on SDS/polyacrylamide-gel electrophoresis. These results are summarized in Fig. 7.

Discussion

The use of 50% (v/v) formamide in isoelectric focusing demonstrates the value of this highly efficient dissociating medium in investigating the nature of multisubunit proteins, or proteins not readily soluble in non-dissociating media. In addition to the described methods, isoelectric focusing of proteins dissolved in SDS solutions can be carried out in gels containing 50% formamide if non-ionic detergent [2% (v/v) Nonidet P40] is added to the samples before focusing to dissociate the SDS–protein complexes (Tuszynski *et al.*, 1979); we have successfully focused extracts of whole pea seeds in SDS solutions by this method (J. Gatehouse, R. R. D. Croy & D. Boulter, unpublished work). However, pI values for protein subunits measured by this technique may differ from pI values measured by other methods, owing to the suppression of protein ionization caused by the formamide; the use of urea as a denaturant is thought to alter the pI values of some proteins for this reason (Josephson *et al.*, 1971), and formamide would be expected to cause similar alterations. Consequently, it is probable that the pI values for legumin 'acidic' subunits measured in 50% formamide as 5.1–5.3 are higher than the 'true' pI values, which would be expected to be lower than the pI value of whole legumin molecules (about 4.7).

Fig. 7. SDS/polyacrylamide-gel analysis of protein preparations showing labelling for carbohydrate

(a) Unreduced legumin; (b) legumin reduced by addition of 2% 2-mercaptoethanol; (c) vicilin; (d) ovalbumin; (e) ovomucoid; (f) bovine serum albumin. ▷, Bands labelled with dansylhydrazine after periodate oxidation; ◄, bands ³H-labelled after periodate oxidation and reduction with NaB³H₄; ●, bands containing impurities in the proteins.

The discrepancies between the present pI values for legumin 'acidic' and 'basic' subunits and those previously reported by Krishna *et al.* (1979) are not large, and may be a result of similar causes; however, the values reported by Casey (1979) differ considerably, and cannot be due to suppression of protein ionization. The present results of isoelectric focusing of legumin in 8 M-urea show that the 'basic' subunits, incubated in that medium, undergo a slow alteration that raises their pI values from about 7 to nearly 9. The pI values found by Casey (1979) appear to represent the beginning of this process, and those of Krishna *et al.* (1979) its completion. Since the effect of carbamylation by cyanate present in impure urea solutions would be to decrease protein pI values, this effect may be ruled out. The marked increase in staining intensity with Coomassie Blue R-250 during this process indicates a conformational change, or a denaturation. Other proteins are known to be resistant to denaturation in urea solutions [e.g. papain (Nair *et al.*, 1976)]. Contrary to the case of the basic subunits, we cannot account for the pI values reported by Casey (1979) for the 'acidic' legumin subunits.

The preparative separation of legumin 'acidic' and 'basic' subunits achieved by ion-exchange chromatography in 50% formamide is a superior method to gel filtration in urea solutions (Casey, 1979), since the separation is clear-cut, and to the use of strongly basic ion-exchange materials in 8 M-urea (Wright & Boulter, 1974), since the conditions used are relatively mild, and are unlikely to cause chemical alterations to the proteins. Further, the technique has potential for separating individual subunits within the 'acidic' and 'basic' classes. It may be of general usefulness in preparative protein subunit purification, since it is simple and quick (high flow rates were possible, and decomposition of the Sephadex was not observed).

The heterogeneity in pI and molecular weight observed in legumin subunits in the present study and others does not appear to be due to the presence of carbohydrate in the molecule, since none could be detected, in agreement with the results of Casey (1979). This contrasts with the results of Basha & Beevers (1976), which suggested the presence of about 0.1% amino sugar and 1.0% neutral sugar. We have also found small amounts of sugars (mainly glucose) by g.l.c. analysis of phenol-partitioned legumin (J. Gatehouse, R. R. D. Croy & D. Boulter, unpublished work). These results may be due to contamination of the legumin with a glycoprotein or with carbohydrate. The conclusion that legumin is not a glycoprotein is in contrast with the glycoprotein nature of many other legume-seed storage proteins of the vicilin type (Derbyshire *et al.*,

1976). The observed heterogeneity in legumin is thus a reflection of differences in the primary structures of the subunits, and thus, provided the differences do not arise as a result of post-translational modifications, should be able to be used as a genetic characteristic. Differences in band patterns among pea lines on isoelectric focusing have been observed in the course of the present study, and other authors have observed differences among lines in SDS/polyacrylamide-gel-electrophoretic studies (Casey, 1979; Croy *et al.*, 1979).

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