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# Immunoaffinity Chromatography as a Means of Purifying Legumin from *Pisum* (Pea) Seeds

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The potential of immunoaffinity chromatography as a means of purifying legumin from a wide range of *Pisum* (pea) types was assessed. The method required small amounts of highly purified legumin from a single *Pisum* type, and this was obtained by salting out with $(NH_4)_2SO_4$ followed by zonal isoelectric precipitation, ion-exchange chromatography on DEAE-cellulose and sucrose-density-gradient centrifugation. Some physicochemical properties of purified legumin were determined, a number of which (Stokes radius, subunit molecular weights, subunit *N*-terminal residues and subunit molar ratios) have not previously been reported for *Pisum* legumin. Examination of *Pisum* legumin by two-dimensional gel isoelectric focusing/electrophoresis indicated the existence of extensive subunit heterogeneity, and polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate showed apparent variation in the nature of this heterogeneity from one *Pisum* variety to another. Despite this variation, immunoaffinity chromatography on immobilized anti-legumin (which was prepared by affinity chromatography on the immobilized purified legumin from the single *Pisum* type) was shown to be a generally applicable method for the purification of undegraded legumin from a range of *Pisum* types, including two primitive lines.

Mature *Pisum* (pea) seeds contain two major storage globulins, vicilin and legumin (Osborne & Campbell, 1898), which may comprise 80% or more of the total seed protein. As part of a programme designed to evaluate the extent of the genetic variation in *Pisum*-seed storage proteins, we wished to purify legumin from a wide range of *Pisum* forms; this required a rapid efficient general method of legumin purification. Several methods have been described for the purification of legumin [see Derbyshire *et al.* (1976) for a review of the relevant literature], and, of these, zonal isoelectric precipitation (Shutov & Vaintraub, 1965; Scholz *et al.*, 1974; Wright & Boulter, 1974) is the most efficient for *Pisum*, producing a legumin preparation that is about 95% pure. To remove the small amount of contaminating proteins, however, requires a second, usually more time-consuming, procedure such as DEAE-cellulose chromatography (Grant & Lawrence, 1964) or sucrose-density-gradient centrifugation (Millerd *et al.*, 1971). The present paper compares the usefulness of a number of different methods of legumin purification, describes some of the properties of legumin from *Pisum sativum* L. and proposes the use of zonal isoelectric precipitation and immunoaffinity chromatography as a means of rapidly purifying legumin from a wide range of *Pisum* forms.

Abbreviations used: Dnp, 2,4-dinitrophenyl; Dns, dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; IgG, immunoglobulin G; SDS, sodium dodecyl sulphate.

## Materials

The *Pisum* seeds were all dry mature specimens from plants grown in the field. Most of the work was carried out on a large yellow smooth-seeded variety (John Innes trial line BS3); the following green wrinkled-seeded cultivars were also used (John Innes accession numbers in parentheses): Dark Skin Perfection, Feltham First (305) and Greenfeast (504); in addition, two primitive small-seeded forms (JI 184 and 186) were used.

Sephadex G-25 (coarse grade), G-50 (medium grade) and G-200, Sepharose 4B and 6B, CNBr-activated Sepharose 4B and Blue Dextran 2000 were purchased from Pharmacia (G.B.) Ltd., London W5 5SS, U.K. Ampholines were from LKB Instruments, South Croydon, Surrey CR2 8YD, U.K. Dry pyridine, dry dimethylformamide, *N*-ethylmorpholine (all Sequanal grade) and dansyl chloride were obtained from Pierce and Warriner (U.K.) Ltd., Chester CH1 4EF, U.K. Urea and guanidinium chloride (both AristaR grade) were obtained from BDH Chemicals, Poole, Dorset BH12 4NN, U.K. Nonidet NP40 was obtained from Fluorochem, Glossop, Derbyshire SK13 9NU, U.K., and diisopropyl phosphorofluoridate from Aldrich Chemical Co., Wembley, Middx. HA0 1PY, U.K. DEAE-cellulose (DE-52) was from Whatman Biochemicals, Maidstone, Kent ME14 2LE, U.K. Polyamide thin layers for the separation of dansyl-amino acids were from the Cheng-Chin Trading Co., Taipei, Taiwan. Collodion membrane sacs for ultrafiltration were

purchased from Sartorius Membrane Filters G.m.b.H., Göttingen, Germany. β-Galactosidase and phosphorylase *a* were from Worthington Chemical Corp., Freehold, NJ, U.S.A. All other marker proteins, agarose (low electro-endosmosis grade), soya-bean trypsin inhibitor, *N*-ε-Dnp-lysine hydrochloride, dansylhydrazine and α-toluenesulphonyl fluoride were obtained from Sigma (London) Chemical Co., Poole, Dorset BH17 7NH, U.K.

All other chemicals were of analytical grade if available. Agarose was treated as described by Lönnerdal & Låås (1976). Acrylamide and *NN'*-methylenebisacrylamide were recrystallized from chloroform and acetone respectively and stored in the dark at 4°C. Water was deionized and glass-distilled.

The molarity of buffers described in the present paper refers to the concentration of the anionic component of the buffer, with the exception of Tris/HCl and glycine/HCl buffers, where the molarities refer to the Tris or glycine concentrations; pH adjustments and measurements were carried out at room temperature (18–22°C).

## Methods

### *Protein extraction*

The seed coats and embryonic axes were removed from the seeds and the cotyledons frozen in liquid $N_2$, finely ground and passed through a 60-mesh sieve. The sieved meal was intimately mixed with an equal weight of polyvinylpyrrolidone and homogenized in a precooled blender (MSE Ato-Mix; four bursts of 15s duration at full speed) with ice-cold 0.5M-NaCl/0.05M-Tris/HCl/0.1mM-dithiothreitol, pH7.0 (10ml of buffer/g of meal). After vigorous stirring on ice for 15min the extract was squeezed through muslin and centrifuged (19000*g* for 10min at 2°C, $r_{av.}$ 8cm) to remove insoluble material. The supernatant solution was filtered through glass-wool to remove traces of low-density material that floated during centrifugation.

To investigate the possibility that proteolysis might occur during extraction, the latter was carried out on ice, at 22°C or at 37°C for periods of up to 4h, in the presence or absence of 1mM-EDTA, 1mM-di-isopropyl phosphorofluoridate, 1mM-α-toluenesulphonyl fluoride or soya-bean trypsin inhibitor (10μg/g of meal). The supernatant solution, obtained as above, was added to 9vol. of boiling SDS/polyacrylamide-gel sample buffer, boiled for 5min and loaded on an SDS/polyacrylamide gel (see below).

### *Legumin purification*

Finely ground solid $(NH_4)_2SO_4$ was added slowly to the stirred supernatant (see above) at room temperature to a final saturation of 40%, the pH being maintained at 7.0 by the addition of NaOH; after 15min the precipitate was removed by centrifugation (19000*g* for 10min, $r_{av.}$ 8cm). The $(NH_4)_2SO_4$ concentration of the supernatant solution was increased to 75% saturation and the precipitate collected after 15min as above. The pellet was dissolved in a minimal volume of 0.2M-NaCl/0.05M-Tris/HCl/0.1mM-dithiothreitol, pH8.0 (buffer A), freed of $(NH_4)_2SO_4$ by passage through a column (2.5cm×100cm) of Sephadex G-25 in buffer A, and either concentrated by ultrafiltration or precipitated by exhaustive dialysis against water at 4°C. The former ('whole protein') was stored in small portions at −20°C; the latter precipitate (globulins) was collected by centrifugation (10000*g* for 10min at 4°C, $r_{av.}$ 8cm), washed three times with cold water, freeze-dried and stored at −20°C.

Crude legumin was purified from whole protein (or occasionally from globulins) by zonal isoelectric precipitation as described by Wright & Boulter (1974), except that all buffers contained 0.1mM-dithiothreitol and the elution buffer was buffer A. Up to 1g of protein was applied to a column (4.4cm×55cm) of Sephadex G-50 at pH4.8 and the column eluted with buffer A, at room temperature, at a flow rate of 60ml/h (4ml/h per $cm^2$); 10ml fractions were collected. Those fractions corresponding to the tailing half of the second major peak (crude legumin) were pooled, adjusted to pH7.0 with HCl, concentrated by ultrafiltration and stored at −20°C where necessary.

Crude legumin was further purified by DEAE-cellulose chromatography as described by Grant & Lawrence (1964), with slight modification. Crude legumin (20–100mg) was dialysed into 0.1M-NaCl/0.015M-sodium phosphate buffer/0.1mM-dithiothreitol, pH8.0. After removal of a small amount of precipitate by centrifugation (19000*g* for 10min, $r_{av.}$ 8cm), the supernatant solution (2–10ml) was applied to a column (1.6cm×27cm) of DEAE-cellulose equilibrated with the same buffer at room temperature. A trace of material absorbing at 280nm was removed by washing the column with the same buffer, before development with a linear gradient (300ml of 0.1M-NaCl and 300ml of 0.4M-NaCl, both in 0.015M-sodium phosphate/0.1mM-dithiothreitol, pH8.0) at room temperature. The flow rate was 60ml/h (30ml/h per $cm^2$), and 5ml fractions were collected. Fractions containing legumin were dialysed into buffer A, concentrated to about 10mg/ml by ultrafiltration and stored at −20°C.

Portions (200μl, 2mg) of this legumin were applied to linear 5–20% (w/v) sucrose gradients in buffer A and centrifuged at 4°C for 18h at 36000rev./min by using a Beckman SW41 rotor and a Beckman Spinco L3.50 ultracentrifuge. The bottoms of the tubes were punctured and 550μl fractions were collected, 20μl

portions of which were assayed for protein as described below. The fractions corresponding to 12S legumin were pooled, freed of sucrose by passage through a column (2.5cm × 85cm) of Sephadex G-25 in buffer A and the protein (pure legumin) was either concentrated by ultrafiltration or dialysed against water, freeze-dried and stored at −20°C. Legumin for sedimentation or diffusion studies was subjected to a second sucrose-density-gradient centrifugation to remove all traces of 16–17S contaminants (see below).

### *$(NH_4)_2SO_4$-gradient solubilization*

As an alternative to DEAE-cellulose chromatography, crude legumin was fractionated by interfacial 'salting-out' with $(NH_4)_2SO_4$ on unsubstituted agarose gel (von der Haar, 1976; Mevarech *et al.*, 1976). Crude legumin (about 1mg/ml in buffer A) was made 55% saturated with $(NH_4)_2SO_4$, and after 15min at room temperature the precipitate was removed by centrifugation (19000*g* for 10min, $r_{av.}$ 8cm). The supernatant solution was applied to a column (2.5cm × 32cm) of Sepharose 4B equilibrated with 55%-satd. $(NH_4)_2SO_4$ in 0.015M-sodium phosphate buffer/0.1mM-dithiothreitol, pH7.0, and the column was washed with the same buffer to remove unbound material. The column was developed with a linear gradient [500ml of 55%-satd. $(NH_4)_2SO_4$ and 500ml of 10%-satd. $(NH_4)_2SO_4$, both in 0.015M-sodium phosphate buffer/0.1mM-dithiothreitol, pH7.0] at a flow rate of 75ml/h (15ml/h per $cm^2$); 10ml fractions were collected, and those containing legumin were dialysed against water and freeze-dried.

### *Gel filtration*

Pure legumin [5–10mg in 0.5–1ml of 0.5M-NaCl/0.05M-Tris/HCl/0.1mM-dithiothreitol, pH8.0 (buffer B)] was applied to a column (2.5cm × 89cm) of Sepharose 6B in buffer B and the column eluted at room temperature at a flow rate of 30ml/h (6ml/h per $cm^2$); 2.8ml fractions were collected, and those containing legumin were pooled, dialysed against water and freeze-dried.

The column was calibrated by determining the elution volumes ($V_e$) of the following proteins of known Stokes radius and molecular weight (Andrews, 1970; Koshiyama, 1972): bovine thyroglobulin, rabbit muscle aldolase, human haemoglobin and horse heart cytochrome *c*. The void volume ($V_0$, 151ml) and the total column volume ($V_t$, 427ml) were determined by using Blue Dextran 2000 and *N*-ε-Dnp-lysine respectively. Values of $K_{av.}$ $[(V_e-V_0)/(V_t-V_0)]$ were determined (Laurent & Killander, 1964), between six and nine individual determinations being made for each protein. The data were used to obtain a Stokes radius for legumin [from the linear plot of $(-\log K_{av.})^{\frac{1}{2}}$ against Stokes radius] and an estimate of molecular weight [from a plot of $K_{av.}$ against log (molecular weight)]; in each case the appropriate straight line was obtained by linear regression analysis.

### *Analytical ultracentrifugation*

Pure legumin (0.7–10.7mg/ml in 0.5M-NaCl/0.05M-sodium phosphate buffer/0.1mM-dithiothreitol, pH8.0) was centrifuged at 52000rev./min in the An-D rotor of a Beckman Spinco model E ultracentrifuge equipped with a rotor-temperature-control unit and diagonal Schlieren optics.

Photographs were taken at 16min intervals. Sedimentation coefficients were measured as described by Markham (1960) and corrected to $s_{20,w}$ values, by using density and viscosity values given by Svedberg & Pederson (1940), and a value for $\bar{v}$ of 0.715$cm^3/g$, calculated as described by Schachman (1957) from the amino acid composition of legumin (see below). Several replicate values of $s_{20,w}$ were obtained for a number of legumin concentrations and $s^0_{20,w}$ was obtained by linear regression analysis of the data.

### *Measurement of diffusion coefficient*

The diffusion coefficient of pure legumin (2.5mg/ml in 0.5M-NaCl/0.05M-sodium phosphate buffer, pH 8.0) was measured by using the model E ultracentrifuge equipped with a monochromatic u.v.-light source, u.v. optics, a rotor-temperature-control unit and a cup-and-valve-type artificial-boundary cell (Beckman part no. 305088), as described by Markham (1962). After boundary formation, the An-D rotor was operated at 3219rev./min and photographs were taken at 64min intervals. *D* was calculated from the densitometer scans of the photographs and corrected to $D_{20,w}$ as described by Markham (1962).

### *Amino acid analysis*

Pure legumin was hydrolysed for 24h in redistilled constant-boiling HCl and the excess of HCl removed by using a rotary evaporator at 40°C. The amino acid composition of the dried hydrolysate was determined as described by Spackman *et al.* (1958) by using a Beckman 120 amino acid analyser. Methionine and cysteine were determined as methionine sulphone and cysteic acid respectively, after performic acid oxidation (Moore, 1963). Threonine and serine values were corrected by 5% and 10% respectively for hydrolytic losses (Rees, 1946). Tryptophan was not measured.

*Determination of N-terminal amino acids*

Pure legumin and its isolated subunits (see below) were treated with dansyl chloride in SDS/*N*-ethylmorpholine as described by Gray (1972). Dns-proteins were hydrolysed in a sealed tube at 108°C with constant-boiling HCl for 4, 18 and 48h. Excess of HCl was removed *in vacuo* over NaOH pellets. The dried hydrolysates were dissolved in 1–2$\mu$l of aq. 50% (v/v) pyridine and applied to polyamide thin-layer plates (5cm×5cm) (Woods & Wang, 1967). Identification of the *N*-terminal Dns-amino acid was made by two-dimensional chromatography against marker Dns-amino acids by using the solvent systems described by Hartley (1970).

*Phenol partitioning and neutral-sugar analysis*

Pure legumin (5mg/ml in 0.5M-NaCl/0.05M-sodium phosphate buffer, pH7.0) was partitioned into phenol as described by Pusztai (1966), precipitated and washed with diethyl ether, and dried *in vacuo* over $P_2O_5$. Neutral-sugar analysis of legumin was carried out by the phenol/$H_2SO_4$ method of Dubois *et al.* (1956), with D-glucose as a standard, before and after partitioning into phenol.

*Carboxymethylation*

Crude legumin (60mg) was dissolved in 6ml of 6M-guandinium chloride / 0.1M-Tris/HCl / 10mM-dithiothreitol, pH8.6, and reduced under $N_2$ for 4h at 45°C. Iodoacetic acid (0.133g) was added, and the mixture incubated at 19°C in the dark under $N_2$ until a negative nitroprusside reaction was obtained (30min). 2-Mercaptoethanol (0.303ml, equivalent to a 6-fold molar excess over iodoacetate) was added; the pH of the solution was lowered to about 4 with HCl and the carboxymethylated protein dialysed exhaustively against 0.2M-acetic acid and freeze-dried.

*Polyacrylamide-gel electrophoresis*

Polyacrylamide-gel electrophoresis in the presence of 1% (w/v) SDS was carried out in a slab gel (15cm× 14cm×0.2cm) with an 18% running gel (14cm long) and a 5% stacking gel (1cm long), in the system described by Laemmli (1970), except that the contents of cross-linker in the running and stacking gels were 0.5% (w/w) and 5% (w/w) respectively of the total monomers. Samples were prepared by boiling for 5min at 1mg/ml in Laemmli's (1970) sample buffer. Then 3–50$\mu$g of protein was loaded and electrophoresis carried out at 42V (room temperature) until the Bromophenol Blue marker had reached the bottom of the gel (22–24h). Gels were stained and destained as described by O'Farrell (1975). The following proteins (molecular weights in parentheses) were used to calibrate the gels: lysozyme (14300); myoglobin (17200); $\beta$-lactoglobulin (18500); concanavalin A (26000); carbonic anhydrase (29000); aldolase (40000); catalase (60000); bovine serum albumin (68000); phosphorylase *a* (94000); $\beta$-galactosidase (130000). Mobilities were measured relative to Bromophenol Blue and used to obtain approximate molecular weights for legumin subunits as described by Weber & Osborn (1970). Periodate/Schiff and Dns-hydrazine staining were carried out as described by Segrest & Jackson (1972) and Eckhardt *et al.* (1976) respectively.

*Two-dimensional isofocusing/electrophoresis*

Two-dimensional gels were prepared as described by O'Farrell (1975) except that (*a*) the Ampholine content of the 4% (w/v) polyacrylamide-isofocusing-gel mixture was 0.4% (v/v) pH3.5–10, 0.8% (v/v) pH4–6 and 0.8% (v/v) pH6–8; (*b*) the final voltage (400V) was applied for 18h at room temperature; (*c*) the first-dimension gels were equilibrated for 20min at room temperature before electrophoresis in the second dimension; (*d*) the second dimension was a uniform 15% gel, rather than a gradient gel. The apparent pH gradient, measured after elution of 0.5cm portions of the first-dimension gel into 1ml of 8M-urea prepared in degassed water, was linear over the range pH5.4–7.9. Samples (5mg/ml) were reduced under $N_2$ for 2h at 37°C in 5% (v/v) 2-mercaptoethanol/2% (w/v) Nonidet NP40/9.5M-urea/2% (v/v) Ampholines (pH range 3.5–10) and a 10$\mu$l sample (50$\mu$g of protein) was loaded.

*Cellulose acetate electrophoresis*

Electrophoresis on cellulose acetate membranes was carried out by using a Beckman micro-zone electrophoresis system; 2–3$\mu$g of protein in 0.25$\mu$l of buffer was applied, and electrophoresis carried out at 18V/cm for 20min at room temperature. The buffers used were 0.05M-sodium phosphate, pH7.0, 0.2M-Tris/10mM-EDTA/borate, pH9.2, and Tris/glycine/barbital, pH8.8 (*I* 0.08) (Weeke, 1973). After electrophoresis the membranes were stained for 2min at room temperature in methanol/water/acetic acid (9:9:2, by vol.) containing 0.2% (w/v) Coomassie Brilliant Blue G250 and destained in the same solvent without dye.

*Agarose-gel electrophoresis*

Protein (3–20$\mu$g in 3–20$\mu$l of buffer) was applied to a 1% (w/v) agarose gel (0.15cm×8.4cm×9.4cm for analytical separations or 0.6cm×16cm×14cm for preparative separations) and subjected to electrophoresis at 10V/cm in either Tris/EDTA/borate

buffer, pH9.2, or Tris/glycine/barbital buffer, pH8.8 (see above). Analytical separations were carried out on a water-cooled plate and preparative separations in a cold-room at 4°C. Analytical gels, or guide-strips from the long edges of preparative gels, were stained for 15min with Coomassie Brilliant Blue in methanol/water/acetic acid and destained, as described above. Appropriate regions of preparative gels were excised, cut into small pieces with a razor blade and protein was eluted from them by centrifugation as described by Thomson *et al.* (1978).

*Immunochemical techniques*

Antiserum to crude legumin or to globulins was raised in rabbits. For this, 1mg of protein in 1ml of 0.3M-NaCl/0.02M-sodium phosphate buffer, pH7.0, +1ml of Freund's complete adjuvant was injected subcutaneously and an identical booster injection given 8 days later. Thereafter the animals were bled at biweekly intervals and received monthly subcutaneous injections of 0.5mg of protein in 0.5ml of 0.3M-NaCl/0.02M-sodium phosphate buffer, pH7.0, +0.5ml of Freund's incomplete adjuvant. Sera exhibiting high titre were pooled and IgG was prepared by $(NH_4)_2SO_4$ precipitation and DEAE-cellulose chromatography (Livingston, 1974). Before the injection series, the rabbits were bled to obtain pre-immune (control) sera.

Double immunodiffusion was carried out at room temperature for 24–48h in 1% (w/v) agar gel in 0.5M-NaCl, or in 1% (w/v) agarose gel in Tris/glycine/barbital buffer ($I$ 0.08), pH8.8 (see above), containing 0.2M-NaCl.

Crossed immunoelectrophoresis was carried out in 1% (w/v) agarose gel on glass plates (8.4cm×9.4cm) in Tris/glycine/barbital buffer ($I$ 0.08) at pH8.8 as described by Weeke (1973). Electrophoresis in the first dimension (10V/cm for 6h at room temperature) was followed by immunoelectrophoresis (6V/cm for 20h at room temperature) in 1% agarose supplemented with 10% (v/v) rabbit antiserum to globulins. Plates were washed in 0.2M-NaCl, dried and stained for 15min as above. Tandem crossed electrophoresis was carried out similarly, the samples being applied 0.8cm apart, as described by Krøll (1973).

*Immunoaffinity chromatography*

The methods used for preparation of monospecific antibody and for immunoaffinity chromatography on IgG–Sepharose columns were based on those of Anderson *et al.* (1975) and Kerckaert *et al.* (1977).

Pure legumin (49.6mg in 16.5ml of 0.5M-NaCl/0.1M-$NaHCO_3$, pH8.3) was coupled to CNBr-activated Sepharose 4B (4.1g) for 18h at 4°C. The extent of coupling was estimated, from $A_{278}$ (see below), to be 98%. After incubation in 0.5M-ethanolamine/HCl, pH8.5 (2h at room temperature), the legumin–Sepharose was washed five times successively with 100ml of 0.5M-NaCl/0.1M-sodium acetate buffer, pH4.0, and 100ml of 0.5M-NaCl/0.1M-$NaHCO_3$, pH8.0, then with 500ml of 0.5M-NaCl/0.2M-glycine/HCl, pH2.8, and equilibrated with 0.5M-NaCl/0.1M-Tris/HCl, pH8.0 (buffer C). Between 6.6 and 20mg of IgG, prepared from antisera to crude legumin, was applied in buffer C to a column (1cm×15cm) of legumin–Sepharose and the column washed with buffer C until the $A_{280}$ of the effluent was zero. Bound anti-legumin IgG was eluted with 0.5M-NaCl/0.2M-glycine/HCl, pH2.8, immediately neutralized with $Na_2HPO_4$, precipitated with 50%-satd. $(NH_4)_2SO_4$ and collected by centrifugation (10000g for 10min, $r_{av.}$ 8cm). IgG from pre-immune (control) sera did not bind to the legumin–Sepharose.

The IgG pellet was dissolved in and dialysed against 0.5M-NaCl/0.1M-$NaHCO_3$, pH8.3, the protein concentration adjusted to 3mg/ml and the IgG bound to CNBr-activated Sepharose 4B as above; 96% of the protein was bound on the basis of $A_{280}$. The IgG–Sepharose was treated and washed as above, then washed with 100ml of each of 3M-KSCN/0.1M-Tris/HCl, pH8.0, and 8M-urea/0.1M-Tris/HCl, pH8.0, before equilibration with buffer C.

Crude legumin (3mg/ml in buffer C) was applied to a column (1cm×3cm) of IgG–Sepharose and the column washed with buffer C to elute a trace of material absorbing at 278nm. Bound legumin was eluted with 3M-KSCN/0.1M-Tris/HCl, pH8.0, rapidly diluted with 2vol. of buffer A, dialysed into buffer A and concentrated to 4mg/ml by ultrafiltration.

*Estimation of legumin subunit ratios*

Legumin is composed of two classes of subunit with mol.wts. of approx. 40000 and 20000 (Higgins & Spencer, 1977). Attempts were made to determine the relative molar ratios of one subunit class to the other, by using two methods based on different principles. In method A, the subunits were separated by SDS/polyacrylamide-gel electrophoresis, as above. Photographic negatives of the stained gels were scanned by using a Joyce–Loebl microdensitometer mark IIIB, and the peak areas obtained from the scans were used to estimate the relative proportions of the two subunits.

In method B, carboxymethylated legumin was separated into the two classes of subunits by gel filtration. First 15mg of CM-legumin was dissolved in 1ml of 8M-urea/0.2M-acetic acid and applied to a column (2.5cm×92cm) of Sephadex G-200 equilibrated with 0.2M-acetic acid at 4°C. The column was eluted at 9ml/h (1.9ml/h per $cm^2$), 1.8ml fractions were collected and the $A_{278}$ of each fraction was determined. Fractions corresponding to the subunits

of about 40000 and 20000 mol.wt. were each pooled, freeze-dried and subjected to a second identical gel-filtration step, freeze-dried again and the purity of each peak was ascertained by SDS/polyacrylamide-gel electrophoresis. The estimated peak areas from the Sephadex G-200 column (see Fig. 1) were corrected for absorption coefficients (see below) and for subunit molecular weights (assuming average values of 39000 and 22000; see Table 1) to give a final molar ratio of large to small subunit.

*Protein determination and specific absorption coefficient*

Protein concentrations were measured by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard; bovine serum albumin concentrations were estimated from $A^{1\%}_{1cm,280}=6.6$. The u.v. spectrum of pure legumin in 0.5M-NaCl/0.05M-Tris/HCl, pH8.0, obtained with a Pye-Unicam SP.1700 spectrophotometer, showed a maximum at 277–278nm. The readings at 278nm were corrected for non-specific absorption (by extrapolating the absorption in the 350nm region of the spectrum) and used for the calculation of an approximate specific absorption coefficient, $A^{1\%}_{1cm}$. This value (8.05) was used routinely for measuring the concentration of pure legumin preparations. Approximate specific absorption coefficients were determined for legumin subunits (in 0.2M-acetic acid) in identical fashion, except that readings were made at 275nm; $A_{350}$ was negligible. The values obtained were 8.2 (large subunits) and 7.8 (small subunits).

IgG concentrations were measured from $A_{280}$ at pH7.0 by assuming $A^{1\%}_{1cm}$ 14.6 (Livingston, 1974).

Protein concentrations in the presence of thiol were measured by dye-binding (Bradford, 1976), by using bovine serum albumin or pure legumin as a standard, as appropriate.

Protein contents of ground dry *Pisum* meals were measured by ninhydrin assay (Jacobs, 1959), after hydrolysis of 20mg of dry meal in 1ml of constant-boiling redistilled HCl for 24h at 108°C. The meal was extracted successively with 10% (w/v) trichloroacetic acid, acetone and diethyl ether and dried *in vacuo* before hydrolysis. Globulins that had been hydrolysed under identical conditions were used as a standard.

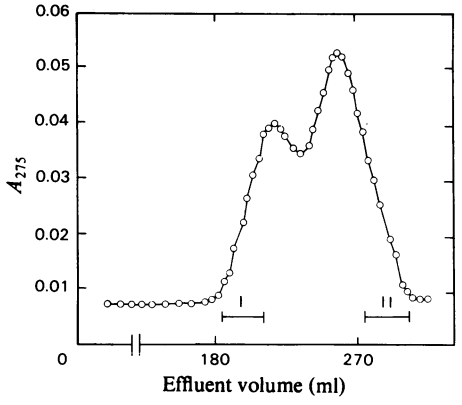


Fig. 1. *Separation of the large and small subunits of CM-legumin by gel filtration on Sephadex G-200*

Conditions of chromatography are described in the Methods section. ○, $A_{275}$. Fractions were pooled as indicated by the bars and each pool subjected to a second identical cycle of gel filtration. SDS/polyacrylamide-gel electrophoresis of each peak after re-chromatography showed peak I to contain only the large (40000-mol.wt.) subunits and peak II to contain only the small (20000-mol.wt.) subunits, with no cross-contamination.

## Results and Discussion

*Protein extraction*

*Pisum* seed meal (line BS3) that had been extracted with 10% (w/v) trichloroacetic acid contained 24.8% [±0.8S.D. (4)] protein on a dry-weight basis; the moisture content of unextracted meal was 9% (w/w) and thus the meal protein content was 22.6% (w/w). Although up to 85% of this protein could be recovered by repeated re-extraction of the meal over several hours, extraction was usually limited to 70–75% of the protein over 15min at 0°C.

The inclusion of proteinase inhibitors (see the Methods section) did not significantly alter the SDS/polyacrylamide-gel electrophoresis pattern of the extracts; neither did increasing the extraction temperature to 25 or 37°C or prolonging extraction at these temperatures to 4h. It was concluded that the inclusion during extraction of any of these proteinase inhibitors was unnecessary. The electrophoresis pattern in each case was virtually identical with that of the control extract (made with boiling SDS sample buffer), which suggests that, if any proteolysis of storage protein has occurred, it has taken place before extraction.

The presence of either, or both, of 0.1mM-dithiothreitol and 1mM-EDTA had no effect on the degree of extraction, the analytical-ultracentrifuge pattern of the globulins or the SDS/polyacrylamide-gel electrophoresis pattern of the whole protein. However, 0.1mM-dithiothreitol was included in all solutions as a precautionary measure.

Mature dry seed was used as a source of legumin to ensure that a molecule was isolated that had completed any possible post-translation processing (cf. Higgins & Spencer, 1977).

*Legumin purification*

*General strategy.* It is known that variation exists in the net charge and the subunit composition of legumin within the genus *Pisum* (R. Casey, unpublished work; D. R. Davies, personal communication; Thomson & Schroeder, 1978). A number of methods for the purification of legumin were therefore assessed and, as discussed below, none was considered satisfactory for the rapid production of pure legumin from a wide range of *Pisum* varieties. Immunodiffusion against antiserum to legumin (results not shown) established that legumins isolated from mature seeds of a number of cultivars show a reaction of identity, even though they appear to differ in subunit composition (see below); immunoaffinity chromatography was therefore considered as a potentially rapid and efficient purification method. The antiserum used as a source of IgG was raised in response to crude legumin, and monospecific antibody was purified from this by affinity chromatography on pure legumin–Sepharose. This approach obviates the necessity for a constant supply of pure legumin for injections, but does require a small amount of pure legumin to prepare the legumin–Sepharose.

*Preparation of crude legumin.* Zonal isoelectric precipitation was an extremely effective method of obtaining crude legumin (about 95% pure) from whole protein extracts (15–50% legumin) in a single step (Fig. 2). The NaCl concentration in the pH 4.8 buffer was important; if it was increased from 0.2 to 0.5 M, legumin was not precipitated, indicating that the solubility of legumin at its isoelectric point is a function of ionic strength. Crude legumin contained traces of proteins with a lower electrophoretic mobility than legumin on agarose gel at pH 8.8 (Fig. 2*a*) and contained small amounts of 7–9 S and 16–17 S material (pure legumin sediments at around 12 S; see below). SDS/polyacrylamide-gel electrophoresis of crude legumin showed the presence of traces of subunits of mol.wts. about 90000, 70000, 45000 and 30000 in addition to the major subunits of mol.wt. about 40000 and 20000 (see below). The first half of the crude legumin peak obtained by zonal isoelectric precipitation contained relatively more of the subunits of 90000, 70000, 45000 and 30000 mol.wt. than did the second half of the peak, and for this reason the latter was used as crude legumin.

Crude legumin could be concentrated, at pH 7, to at least 20 mg/ml and stored in buffer A at −20°C without loss of solubility (although it was precipitated reversibly on standing at 4°C). In this respect, it differs markedly from legumin that had been precipitated by titration or dialysis to pH 4.8 (Danielsson, 1949; Joubert, 1955) and that is only poorly soluble in buffer A.

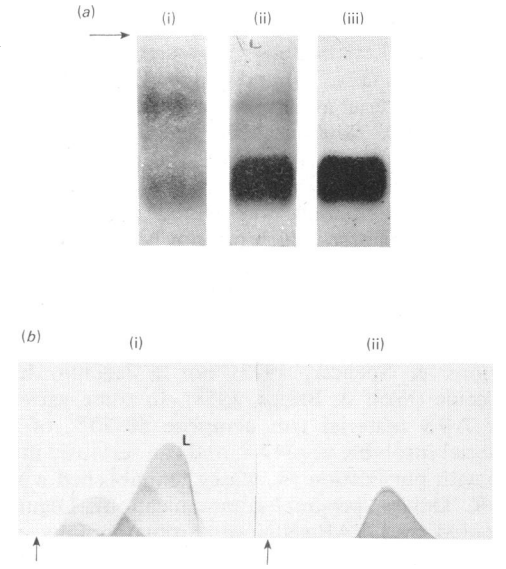


Fig. 2. *Electrophoresis and two-dimensional crossed immunoelectrophoresis in agarose gel of Pisum seed proteins (line BS3)*

(*a*) Agarose-gel electrophoresis of (i) whole protein, (ii) crude legumin and (iii) pure legumin. Migration is from top to bottom, the anode being at the bottom. In each case approx. 20 μg of protein was analysed. (*b*) Two-dimensional crossed immunoelectrophoresis of (i) whole protein (L=legumin) and (ii) legumin prepared by immunoaffinity chromatography, in agarose gel with rabbit antiserum to *Pisum* seed globulins (line BS3). Approx. 50 and 20 μg respectively of protein was analysed. For further details see the Methods section. The arrows denote the point of sample application.

Precipitation of crude legumin with $(NH_4)_2SO_4$ resulted in an increase in the amount of 16–17 S material, even in the presence of thiol; this material is an aggregated form of 12 S legumin, since it contains the same subunits as legumin after purification by sucrose-density-gradient centrifugation (see below).

*Preparation of pure legumin and comparison of purification methods.* The contaminants of slow electrophoretic mobility (Fig. 2*a*) could be removed from legumin in a number of ways. Gel filtration on Sepharose 6B (or 4B) only partly resolved the contaminants from legumin, and was not a useful purification procedure (although it specifically removed the subunit of about 90000 mol.wt.). Fractional interfacial 'salting-out' by using a gradient of decreasing $(NH_4)_2SO_4$ concentration and a Sepharose 4B column also effected only a partial separation of legumin from the slow-moving contaminants, the latter being eluted very slightly later than legumin.

DEAE-cellulose chromatography of crude legumin from line BS3 completely removed the slow-moving contaminants (Fig. 2*a*) and concomitantly removed the 7–9 S material and the contaminating subunits of about 90000, 70000, 45000 and 30000 mol.wt. Legumin (line BS3) was eluted at 0.17–0.18 M-NaCl under the conditions used, whereas the 7–9 S (slow-moving) contaminant was eluted slightly earlier (Fig. 3). The latter, which has not been prepared in sufficient quantity for detailed characterization, appeared to give rise to a major subunit of mol.wt. about 70000 and possibly a trace subunit of about 45000 mol.wt. It is therefore neither vicilin (see Higgins & Spencer, 1977) nor a legumin half-molecule (Wolf & Briggs, 1958). In some varieties this 7–9 S material can comprise 60–70% of the material insoluble at pH 4.8, and can seriously interfere with purification (R. Casey, unpublished work; D. R. Davies, personal communication). Legumin prepared by DEAE-cellulose chromatography usually contained some 16–17 S material (aggregated legumin; see above) that could be removed by sucrose-density-gradient centrifugation.

Although legumin preparations containing some 16–17 S material, but free of the slow-moving 7–9 S contaminant, could be prepared on a small scale by agarose-gel electrophoresis, pure legumin was generally prepared by zonal isoelectric precipitation, DEAE-cellulose chromatography and sucrose-density-gradient centrifugation. To assess its suitability for affinity chromatography of IgG, the homogeneity and some properties of pure legumin were determined.

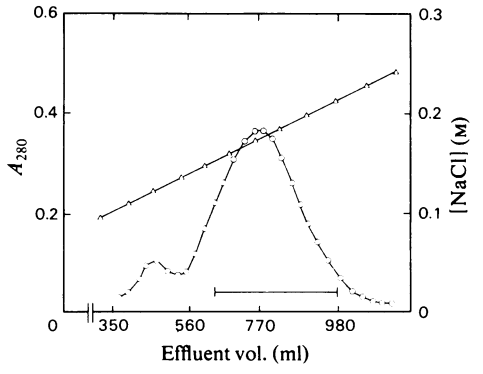


Fig. 3. *Purification of legumin by DEAE-cellulose chromatography*

Conditions of chromatography are described in the Methods section. Fractions corresponding to legumin were pooled as indicated by the bar. ○, $A_{280}$; △, [NaCl] (M). For the sake of clarity some experimental points have been omitted.

*Purity and properties of legumin*

Examination of pure legumin by electrophoresis in agarose gels (or on cellulose acetate membranes) at a range of pH values and ionic strengths showed a single broad diffuse band (Fig. 2*a*).

Pure legumin was homogeneous in the analytical ultracentrifuge over the concentration range 0.7–10.7 mg/ml in 0.5 M-NaCl/0.05 M-sodium phosphate buffer, pH 8.0. An $s^0_{20,w}$ value of 12.23 S (Table 1) was obtained from a linear regression analysis, which showed the slight concentration-dependence of $s_{20,w}$ to obey the relationship $s_{20,w}=12.23-0.152C$, where $C$ is the protein concentration in mg/ml.

Gel filtration of pure legumin on Sepharose 6B showed legumin to be eluted as a single symmetrical peak ($K_{av.}=0.399$). This value of $K_{av.}$ was used to obtain a Stokes radius of 7.1 nm and a mol.wt. of 395200. The diffusion coefficient ($D_{20,w}$) was calculated to be $2.66\times10^{-7}$ cm$^2$/s, a value that is considerably lower than that reported by Danielsson (1949) but close to that determined by Johnson & Richards (1962). Calculation of legumin molecular weight from $D_{20,w}$ and $s_{20,w}$ (obtained on the samples used for the diffusion studies), by using the Svedberg equation (Svedberg & Pederson, 1940), gave a value of 420000, whereas that calculated from $s_{20,w}$ and Stokes radius, by using the equation:

$$\text{molecular weight} = \frac{6\pi\eta r N s^0_{20,w}}{(1-\bar{v}\rho)}$$

where $\eta$=the viscosity of the medium (1.0854 mPa·s), $r$=Stokes radius (7.1 nm), $N$=Avagadro's number and $\rho$=the density of the medium (1.0256 g/ml), was 415000. Thus the molecular weight of legumin appears to be about 400000, which agrees well with those reported by Brand *et al.* (1955), Brand & Johnson (1958) and Johnson & Richards (1962), but less well with that of Danielsson (1949).

Dansylation of legumin showed the presence of *N*-terminal leucine, threonine and glycine. No trace of any other Dns-amino acid (except *O*-Dns-tyrosine and ε-Dns-lysine) was detected, which is an indication of the purity of the preparation. Vaintraub & Gofman (1961), Grant & Lawrence (1964) and Jackson *et al.* (1969) have noted the same *N*-terminal amino acids, although two of these groups also noted traces of other *N*-terminal residues, and Vaintraub & Gofman (1961) did not detect threonine; legumin from *Vicia faba* (broad bean) also has *N*-terminal leucine, threonine and glycine (Wright & Boulter, 1974).

The pattern obtained on SDS/polyacrylamide-gel electrophoresis of reduced legumin from line BS3 [Fig. 4, gel (*a*)] showed the subunits of mol.wt. about 40000 and 20000, which are characteristic of legumin from *Pisum* (Higgins & Spencer, 1977). There were apparently two major and one minor subunits of mol.wt. about 40000, and one major, plus three, or

Table 1. *Some physicochemical properties of legumin from Pisum sativum (line BS3)*

| Property | Value | Literature value | Reference |
|---|---|---|---|
| Molecular weight | 395200 [±13800S.D. (3)]* | 330000 | Danielsson (1949) |
| | 415000 [±11700S.D. (3)]† | 388000 | Brand *et al.* (1955) |
| | 420000 [±24700S.D. (2)]‡ | 398000 | Johnson & Richards (1962) |
| | | 410000 | Brand & Johnson (1958) |
| $s^0_{20,w}$ | 12.23 | 12.14 | Danielsson (1949) |
| | | 13.7 | Brand & Johnson (1958) |
| $D_{20,w}$ | 2.66 [±0.16S.D. (2)] | 3.49 | Danielsson (1949) |
| | | 2.99 | Johnson & Richards (1962) |
| $\bar{v}$ | 0.715 | 0.735 | Danielsson (1949) |
| | | 0.733 | Johnson & Richards (1962) |
| Stokes radius | 7.1nm [±0.2S.D. (3)] | — | |
| Subunit molecular weights§ | 40370 [±880S.D. (18)] | ~40000 | Higgins & Spencer (1977) |
| | 38800 [±700S.D. (18)] | and | |
| | 37600 [±400S.D. (18)] | ~20000 | |
| | 22280 [±780S.D. (18)] | | |
| Subunit molar ratios (large/small)‖ | | | |
| A | 1.24 [±0.19S.D. (11)] | — | |
| B | 0.94 [±0.007S.D. (2)] | | |
| Subunit *N*-terminal residues | Leu, Thr (40000-mol.wt. subunits) Gly (20000-mol.wt. subunits) | Leu, Thr, Gly | Jackson *et al.* (1969) |

* Calculated from gel-filtration data [$K_{av.}$ versus log(molecular weight)].
† Calculated from $s^0_{20,w}$ and Stokes radius.
‡ Calculated from $s_{20,w}$ and $D_{20,w}$.
§ Determined as described by Weber & Osborn (1970).
‖ A and B refer to the Methods section.

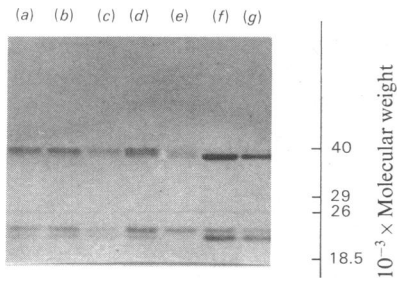


Fig. 4. *Electrophoresis of legumin from a number of Pisum varieties in polyacrylamide-gel slabs containing sodium dodecyl sulphate*

Migration was from top to bottom, the anode being at the bottom. (*a*) Pure legumin from line BS3, prepared by zonal isoelectric precipitation, DEAE-cellulose chromatography and sucrose-density-gradient centrifugation; (*b*)–(*g*), legumin from lines BS3, JI 504, JI 305, Dark Skin Perfection, JI 184 and JI 186 respectively prepared by zonal isoelectric precipitation and immunoaffinity chromatography. For experimental details see the Methods section. Approx. 20$\mu$g of legumin was loaded per slot. The numbers at the side indicate the positions adopted by marker proteins of these particular molecular weights.

possibly four, subunits of mol.wt. about 20000. These groups of subunits are henceforth referred to as the 40000-mol.wt. and 20000-mol.wt. subunits on the understanding that these values are used for descriptive purposes only. It should be stressed that there exists considerable variation in the numbers, relative staining intensities and apparent molecular weights of the 40000-mol.wt. subunits [see Fig. 4, gels (*b–g*), and Thomson & Schroeder (1978)] and thus there is no such thing as a 'typical' SDS/polyacrylamide-gel-electrophoresis pattern for legumin. Carboxymethylation of reduced legumin did not alter the SDS/polyacrylamide-gel-electrophoresis pattern. Estimated values for the subunit molecular weights are shown in Table 1.

Separation and subsequent dansylation of the two classes of subunit showed the 20000-mol.wt. subunits to have *N*-terminal glycine and the 40000-mol.wt. subunits to have *N*-terminal leucine and threonine, as was also found for *Vicia faba* legumin (Wright & Boulter, 1974). The subunit molar ratios (40000-mol.wt. subunits/20000-mol.wt. subunits) were calculated as 1.24 (method A, average of 11 determinations) and 0.94 (method B, average of two closely concordant determinations). A subunit ratio of unity was therefore assumed; accepting a mol.wt. of about 400000 for legumin, a model similar to that proposed by Wright & Boulter (1974) in which legumin contains six large (mol.wt. about 37000–41000) and six small (mol.wt. about 22000–23000) subunits, would be consistent with these data. Since unreduced legumin gives rise to subunits of mol.wt. about 58000 on SDS/polyacrylamide-gel electro-

phoresis, with a concomitant reduction in the amounts of 40000- and 20000-mol.wt. subunits (results not shown), some of the 40000- and 20000-mol.wt. subunits may be linked by disulphide bonding in the intact oligomer (Wright & Boulter, 1974; Kitamura *et al.*, 1976).

The amino acid composition of legumin from line BS3, which was used to calculate a value of 0.715 for $\bar{v}$, is shown in Table 2. The analysis was similar to those reported by Goa & Strid (1959), Grant & Lawrence (1964), Jackson *et al.* (1969) and Hurich *et al.* (1977), except that the histidine content was lower and the half-cystine and methionine contents were higher.

The neutral-sugar content of legumin was negligible (less than 0.1%, w/w), both before and after phenol partition, a finding that is at variance with the results of Basha & Beevers (1976); it is possible that there is genetic variation for legumin carbohydrate content. Periodate/Schiff and Dns-hydrazine staining of SDS/polyacrylamide gels after electrophoresis of 100$\mu$g and 20$\mu$g respectively of legumin were both negative; the sensitive Dns-hydrazine stain resulted in some u.v.-fluorescent bands, but these also appeared on control gels that had not been oxidized with periodic acid. Thus it appeared that, within the limits of the detection methods, none of the subunits of legumin from line BS3 contains covalently linked carbohydrate.

Table 1 summarizes the physical and chemical data obtained for *Pisum* legumin and compares these with previously published results where appropriate.

Table 2. *Amino acid composition of legumin from Pisum sativum (line BS3)*

For details see the Methods section. Values are the means ± S.E.M. from duplicate analyses.

| Amino acid | Content (mol %) |
|---|---|
| Lys | 4.24 ± 0.25 |
| His | 1.75 ± 0.08 |
| Arg | 9.99 ± 0.12 |
| Asp | 13.07 ± 0.29 |
| Thr | 3.08 ± 0.29 |
| Ser | 5.77 ± 0.21 |
| Glu | 19.74 ± 0.28 |
| Pro | 5.50 ± 0.36 |
| Gly | 6.89 ± 0.52 |
| Ala | 6.01 ± 0.28 |
| ½CyS | 1.21 ± 0.15 |
| Val | 4.97 ± 0.09 |
| Met | 0.66 ± 0.02 |
| Ile | 3.95 ± 0.18 |
| Leu | 7.60 ± 0.03 |
| Phe | 2.30 ± 0.11 |
| Tyr | 3.64 ± 0.03 |

*Subunit heterogeneity of legumin*

Two-dimensional gel isoelectric focusing/electrophoresis of crude legumin (Fig. 5) showed the apparent presence of six or seven subunits of mol.wt. about 40000, but of different isoelectric point, and three or four 20000-mol.wt. subunits, also differing in isoelectric point. It is unlikely that this multiplicity of subunits is a result of carbamoylation during sample preparation in 9.5M-urea, since the pattern is the same if the sample is incubated at room temperature for 30min or at 45°C for 24h; moreover, one legumin form has been isolated that shows only one 40000-mol.wt. subunit after two-dimensional gel isoelectric focusing/electrophoresis (results not shown). It is difficult to rule out the possibility that subunit multiplicity is due to partial deamidation of labile sequences containing asparagine residues (see, e.g., McKerrow & Robinson, 1971; Shotton & Hartley, 1973); however, the genetic evidence of Thomson & Schroeder (1978) is consistent with heterogeneity of the 40000-mol.wt. subunits comparable with that indicated by two-dimensional gels. It also seems possible that there are three (or four) types of 20000-mol.wt. subunit, differing in amino acid sequence and hence in isoelectric point, but having the same *N*-terminal residue (glycine).

Subunit heterogeneity has been noted by Catsimpoolas & Wang (1971) for glycinin (the counterpart of legumin in soya-bean seeds), and is also evident from the data of Beachy *et al.* (1978); similarly, Derbyshire *et al.* (1976) suggest that subunit multiplicity exists in *Vicia faba* legumin subunits. Although it is possible that such multiplicity reflects true sequence microheterogeneity, until more is known

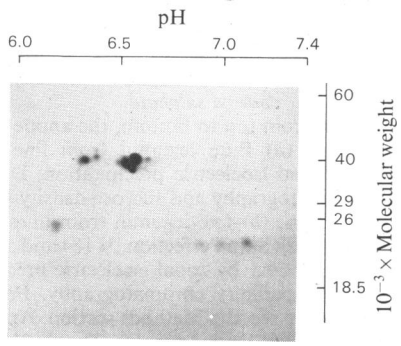


Fig. 5. *Two-dimensional isoelectric focusing/electrophoresis of legumin (line BS3) in polyacrylamide gel under denaturing conditions*

For details see the Methods section. Approx. 50$\mu$g of legumin was analysed. The apparent pH values were measured in 8M-urea; positions adopted by marker proteins in the second dimension are indicated as in Fig. 4.

about the primary structure of legumin subunits it is premature to speculate on the molecular basis of the observed subunit heterogeneity.

*Immunoaffinity chromatography*

Immunoaffinity chromatography of crude legumin, from line BS3, resulted in the specific binding of legumin, which could be eluted with 3M-KSCN, pH8.0. Although chaotropic ions such as thiocyanate have a tendency to dissociate multimeric proteins, the legumin thus produced had an $s_{20,w}$ of 11.2S (at 4.3mg/ml), with no trace of low-molecular-weight proteins. It had the same subunit pattern on SDS/polyacrylamide-gel electrophoresis as did pure legumin and had *N*-terminal glycine, leucine and threonine. Crossed immunoelectrophoresis (Fig. 2*b*) indicated the presence of a single component. Tandem immunoelectrophoresis with pure legumin gave smoothly confluent peaks, consistent with it being identical with legumin (results not shown); thus zonal isoelectric precipitation followed by immunoaffinity chromatography affords a rapid method of preparing pure legumin. Crude legumin has been used for the affinity chromatography because experiments with crude whole protein extracts resulted in the non-specific binding of traces of proteins other than legumin to the (anti-legumin)–Sepharose.

The applicability of the method to other *Pisum* forms is demonstrated in Fig. 4 (gels *b*–*g*); it should be noted that two of these lines (JI 184 and 186) are primitive types, so the method appears to have general application. Decreasing the size of the zonal precipitation column to 1cm×25cm has permitted the isolation of 4mg of crude legumin in less than 1h; hence it should be feasible to prepare small amounts of pure legumin from a large number of varieties in a short time. The range of legumins thus prepared can be used to assess the extent of variation in the amino acid composition and subunit structure of legumin from the pea.

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## References

Anderson, N. G., Willis, D. D., Holladay, D. W., Caton, J. E., Holleman, J. W., Eveleigh, J. W., Attrill, J. E., Ball, F. L. & Anderson, N. L. (1975) *Anal. Biochem.* **68**, 371–393

Andrews, P. (1970) *Methods Biochem. Analysis* **18**, 1–53

Basha, S. M. M. & Beevers, L. (1976) *Plant Physiol.* **57**, 93–97

Beachy, R. N., Thompson, J. F. & Madison, J. T. (1978) *Plant Physiol.* **61**, 139–144

Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254

Brand, B. P. & Johnson, P. (1958) *Trans. Faraday Soc.* **54**, 1911–1921

Brand, B. P., Goring, D. A. I. & Johnson, P. (1955) *Trans. Faraday Soc.* **51**, 872–876

Catsimpoolas, N. & Wang, J. (1971) *Anal. Biochem.* **44**, 436–444

Danielsson, C. E. (1949) *Biochem. J.* **44**, 387–400

Derbyshire, E., Wright, D. J. & Boulter, D. (1976) *Phytochemistry* **15**, 3–24

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956) *Anal. Chem.* **28**, 350–356

Eckhardt, A. E., Hayes, C. E. & Goldstein, I. J. (1976) *Anal. Biochem.* **73**, 192–197

Goa, J. & Strid, L. (1959) *Arch. Mikrobiol.* **33**, 253–259

Grant, D. R. & Lawrence, J. M. (1964) *Arch. Biochem. Biophys.* **108**, 552–561

Gray, W. R. (1972) *Methods Enzymol.* **25**, 121–138

Hartley, B. S. (1970) *Biochem. J.* **119**, 805–822

Higgins, T. J. V. & Spencer, D. (1977) *Plant Physiol.* **60**, 655–661

Hurich, J., Parzysz, H. & Przybylska, J. (1977) *Genet. Pol.* **18**, 241–251

Jackson, P., Boulter, D. & Thurman, D. A. (1969) *New Phytol.* **68**, 25–33

Jacobs, S. (1959) *Nature (London)* **183**, 262

Johnson, P. & Richards, E. G. (1962) *Arch. Biochem. Biophys.* **97**, 260–276

Joubert, F. J. (1955) *J. S. Afr. Chem. Inst.* **8**, 75–79

Kerckaert, J.-P., Bayard, B., Debray, N., Sautière, P. & Biserte, G. (1977) *Biochim. Biophys. Acta* **493**, 293–303

Kitamura, K., Takagi, T. & Shibasaki, K. (1976) *Agric. Biol. Chem.* **40**, 1837–1844

Koshiyama, I. (1972) *Int. J. Peptide Protein Res.* **4**, 167–176

Krøll, J. (1973) *Scand. J. Immunol.* **2**, *Suppl.* **1**, 57–59

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

Laurent, T. C. & Killander, J. (1964) *J. Chromatogr.* **14**, 317–330

Livingston, D. M. (1974) *Methods Enzymol.* **34**, 723–731

Lönnerdal, B. & Låås, T. (1976) *Anal. Biochem.* **72**, 527–532

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Markham, R. (1960) *Biochem. J.* **77**, 516–519

Markham, R. (1962) *Adv. Virus Res.* **9**, 241–270

McKerrow, J. H. & Robinson, A. B. (1971) *Anal. Biochem.* **42**, 565–568

Mevarech, M., Leicht, W. & Werber, M. M. (1976) *Biochemistry* **15**, 2383–2387

Millerd, A., Simon, M. & Stern, H. (1971) *Plant Physiol.* **48**, 419–425

Moore, S. (1963) *J. Biol. Chem.* **248**, 235–237

O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021

Osborne, T. B. & Campbell, G. F. (1898) *J. Am. Chem. Soc.* **20**, 348–362

Pusztai, A. (1966) *Biochem. J.* **99**, 93–101

Rees, M. W. (1946) *Biochem. J.* **40**, 632–640

Schachman, H. K. (1957) *Methods Enzymol.* **4**, 70–71

Scholz, G., Richter, J. & Manteuffel, R. (1974) *Biochem. Physiol. Pflanzen.* **166**, 163–172

Segrest, J. P. & Jackson, R. L. (1972) *Methods Enzymol.* **28**, 54–63

Shotton, D. M. & Hartley, B. S. (1973) *Biochem. J.* **131**, 643–675

Shutov, A. D. & Vaintraub, I. A. (1965) *Ukr. Biokhim. Zh.* **37**, 177–181
Spackman, D. H., Stein, W. H. & Moore, S. (1958) *Anal. Chem.* **30**, 1190–1206
Svedberg, T. & Pederson, K. O. (1940) *The Ultracentrifuge*, Clarendon Press, Oxford
Thomson, J. A. & Schroeder, H. E. (1978) *Aust. J. Plant Physiol.* **5**, 281–294
Thomson, J. A., Schroeder, H. E. & Dudman, W. F. (1978) *Aust. J. Plant Physiol.* **5**, 263–280
Vaintraub, I. A. & Gofman, Y. Y. (1961) *Biokhimiya* **26**, 13–17
von der Haar, F. (1976) *Biochem. Biophys. Res. Commun.* **70**, 1009–1013
Weber, K. & Osborn, M. (1970) *J. Biol. Chem.* **244**, 4406–4412
Weeke, B. (1973) *Scand. J. Immunol.* **2**, *Suppl.* **1**, 47–56
Wolf, W. J. & Briggs, D. R. (1958) *Arch. Biochem. Biophys.* **76**, 377–393
Woods, K. R. & Wang, K.-T. (1967) *Biochim. Biophys. Acta* **133**, 369–370
Wright, D. J. & Boulter, D. (1974) *Biochem. J.* **141**, 413–418