

The major albumin proteins from pea (*Pisum sativum* L.)

Purification and some properties

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A scheme is described for the fractionation of pea (*Pisum sativum*) albumin proteins. By using this scheme, two closely related major albumin proteins have been isolated and purified to homogeneity. The larger protein, designated PMA-L, has $M_r \sim 53\,000$ and consists of two $25\,000$ - M_r subunits, whereas the smaller, PMA-S, has $M_r \sim 48\,000$ and contains two $24\,000$ - M_r subunits. There was no evidence of mixed dimers of the two subunit sizes, despite their close homology as judged by immunological cross-reaction, amino acid composition, *N*-terminal amino acids, tryptic-peptide mapping and CNBr-cleavage products. Both proteins contained significant amounts of sulphur amino acids. The proteins were shown to be located in the soluble cytosol fraction of cotyledon cells and are not significantly degraded on seed germination. Preliminary screening indicates the presence of homologous major albumin proteins in at least three different, though closely related, legume species.

Studies on the seed proteins of pea (*Pisum sativum* L.) have largely concentrated on the predominant proteins, namely the globulin or storage-protein fraction (Gatehouse *et al.*, 1983). This fraction accounts for most (75–80%) of the total seed protein and is therefore, on a quantitative basis, of immediate importance in nutritional aspects of pea breeding. The albumin fraction, variously reported to constitute 14% (Grant *et al.*, 1976), 42% (Murray, 1979) or 15–38% (Davies, 1976) of the total seed protein, depending on the genetic line and method of albumin extraction, has long been regarded as the enzymic or metabolic protein fraction in seeds, containing small amounts of a large number of proteins (Danielson, 1956; Basha & Beevers, 1975; Boulter, 1982; Gatehouse *et al.*, 1983). Recent reports, however, now suggest that the pea albumin fraction contains a few proteins present in amounts large enough to be conveniently isolated and to be considered important nutritionally (Davies, 1976; Grant *et al.*, 1976; Guldager, 1978; Murray, 1979; Tyler, 1981). Furthermore, the levels of the sulphur amino acids

in the pea albumin fraction have been reported to be disproportionately high when compared on an equal weight basis with the globulin fraction (Davies, 1976; Hurich *et al.*, 1977; Jakubek & Przybylska, 1978, 1979), making the albumin proteins even more important nutritionally. The inheritance of different types of pea major albumins and the tentative location of the major albumin gene locus has been reported (Blixt *et al.*, 1980). However, before such proteins can be profitably included in breeding programmes, it is necessary to investigate the properties and location of the individual components, as has been done for the globulin proteins (Gatehouse *et al.*, 1983).

Although it has been suggested (Murray, 1979) that the albumin fraction does contain storage proteins as defined by Derbyshire *et al.* (1976), certain major albumin components do not behave as storage proteins, as judged by their continued presence in germinating seeds long after storage proteins have been degraded (Murray, 1979; Guldager, 1978; Tyler, 1981; Jakubek & Przybylska, 1982). This then raises the question as to what the primary function(s) of these proteins might be. Few reports exist of comprehensive fractionation schemes for the albumins or the isolation and study of individual albumin proteins, except in the case of lectins (Trowbridge, 1974). We describe in the present paper the isolation and properties of two major albumin proteins and

Abbreviations used: SDS, sodium dodecyl sulphate; rel. satn., relative saturation; PMA-L, pea major albumin large; PMA-S, pea major albumin, small; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; h.p.l.c., high-pressure liquid chromatography; IgG, immunoglobulin fraction from serum.

report on some initial work into the elucidation of their function.

Experimental

Materials

Pea seeds (*Pisum sativum* L.), variety Feltham First, were obtained from Suttons Seeds Ltd., Reading, Berks., U.K.; seeds of other legumes (unspecified varieties) were obtained locally. Sephadex G-150, Sephacryl S-200, Blue Dextran and Pharmalyte ampholines were obtained from Pharmacia (G.B.) Ltd., London W.5, U.K. DEAE-cellulose (DE-52) was purchased from Whatman Biochemicals Ltd., Maidstone, Kent, U.K. and hydroxyapatite (Bio-Gel HT grade) was from Bio-Rad Laboratories Ltd., Bromley, Kent, U.K. Standard proteins were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. and BCL Ltd., Bell Lane, Lewes, East Sussex, U.K. Agarose was purchased from BRL, Cambridge, U.K. All other chemicals were obtained from BDH Chemicals, Poole, Dorset, U.K. and were of AnalaR grade wherever possible.

Methods

Fractionation of the pea albumins and isolation of the major protein components. Pea seeds were dehulled and the cotyledons milled to pass through a 365 μm -mesh sieve. The meal (50g) was extracted twice with 20mM-sodium acetate buffer, pH 5.0, at a ratio of 1g of meal to 5ml of buffer, for 1–2h at 4°C. Extracts were clarified by centrifugation at 10000g for 30min and then fractionated by $(\text{NH}_4)_2\text{SO}_4$ precipitation at 0°C. Precipitates in the ranges 0–50% and 50–90% rel. satn. were prepared, redissolved in 10ml of 50mM-Tris/HCl buffer, pH 7.5, and either dialysed against water and freeze-dried or used immediately for further fractionation. $(\text{NH}_4)_2\text{SO}_4$ -precipitated fractions were chromatographed on a column of Sephadex G-150 (3.2cm diam. \times 56cm, flow rate 20ml/h) in 50mM-Tris/HCl buffer, pH 7.5 (Fig. 1a). Appropriate fractions from the G-150 chromatography were pooled and applied directly to a column of DE-52 cellulose (2.5cm diam. \times 26cm, flow rate 40ml/h) equilibrated with 50mM Tris-HCl, pH 7.5, and then eluted with a linear NaCl gradient (0–0.5M, 500ml total volume) in the same buffer (Fig. 1b below). Fractions eluted from the columns were analysed by SDS/polyacrylamide-gel electrophoresis and pooled accordingly. Globulin and albumin fractions were prepared from mature, developing or germinating seeds and from protein bodies as follows: total protein extracts were made from defatted seed meal or protein bodies, in 50mM-sodium borate buffer, pH 8.0, and clarified by centrifugation. The extracts were then dialysed

overnight against a large excess of 20mM-acetate buffer, pH 5.0, at 4°C (Pusztai & Watt, 1970) and the globulin proteins, which were quantitatively precipitated, recovered by centrifugation at 40000g for 30min, leaving the albumin proteins in the supernatant. All separated fractions were dialysed against distilled water and freeze-dried. Albumin protein extracts were prepared from small samples of meal (100–200mg) from seeds of different species, by extraction with 20mM-ammonium acetate buffer, pH 5.0 (1ml) for 1h at 4°C. After centrifugation at 13000g for 10min the supernatants were used directly for immunodiffusion or were freeze-dried before SDS/polyacrylamide-gel electrophoresis.

M_r determinations. A column of Sephacryl S-200 (1.6cm diam. \times 40cm, flow rate 7ml/h), equilibrated with 0.1M-Tris/HCl buffer, pH 8.0, containing 0.25M-NaCl and 0.1% NaN_3 , was calibrated with standard proteins [$(M_r$ in parentheses) catalase (240000); pea vicilin (150000); bovine serum albumin (68000); ovalbumin (43000); and myoglobin (16200)], to obtain a standard graph of V_e/V_0 against $\log M_r$. Albumin protein samples were subsequently chromatographed on the column and their molecular weights calculated from the corresponding V_e/V_0 values.

Gel electrophoresis. The subunit compositions of protein preparations were analysed by SDS/polyacrylamide-gel electrophoresis in slab gels by the methods of Laemmli (1970) and as modified by Matta *et al.* (1981) for various one-dimensional and two-dimensional techniques. M_r values for albumin protein subunits were obtained from a standard graph prepared for the following standard subunits analysed by SDS/polyacrylamide-gel electrophoresis (subunit M_r in parentheses): transferrin (76600); bovine serum albumin (68000); catalase (60000); ovalbumin (43000); lactic dehydrogenase (36000); soya-bean trypsin inhibitor (20100); ferritin (18500); β -lactoglobulin (17500); and pea seed lectin (18000). Non-dissociating electrophoresis was carried out in 8.5% (w/v)-acrylamide gels as described by Laemmli (1970), but omitting SDS and mercaptoethanol from all buffers. One-dimensional isoelectric focusing was performed in 7.5% (w/v)-acrylamide slab gels as described by Gatehouse *et al.* (1980). Urea was omitted from gels used for analysis of non-dissociated protein samples. Gels were stained for proteins with Coomassie Brilliant Blue R and for glycoproteins with the dansylhydrazine fluorescent stain as described by Eckhardt *et al.* (1976).

Amino acid analyses. Protein samples were reduced and carboxymethylated with iodoacetamide (Glazer *et al.*, 1975) before acid hydrolyses, as described by Croy *et al.* (1980), and analysed on a Variant 5060 h.p.l.c. system, with a

Micropak A.A. column (Varian; 15 cm × 4 mm diam.) using the Varian PCR-1 post-column derivatization and Fluorichrom detection system.

N-Terminal amino acid determination. *N*-Terminal amino acids were determined by the methods of Gray (1972) and Woods & Wang (1967).

Tryptic-peptide analyses. Tryptic peptides from carboxymethylated protein samples were prepared and analysed by h.p.l.c. using a Micropak MCH-10 (C₁₈, 10 μm) column as described by Gatehouse *et al.* (1982). The peak elution profiles were compared as peptide maps.

CNBr cleavage. Albumin proteins were digested for different times (0, 3, 6, 24 and 48 h) with CNBr as described by Croy *et al.* (1980) and the fragments were analysed on urea/SDS-containing gels as described by Hashimoto *et al.* (1983). Standard proteins used for estimating the *M_r* values for the fragments were: soya-bean trypsin inhibitor (*M_r* 20100); β-lactoglobulin (17500); myoglobin (16200); lysozyme (13200); cytochrome *c* (12700); and insulin (5600).

Carbohydrate analysis. Albumin proteins were precipitated and washed extensively with 10% (w/v) trichloroacetic acid and their sugar contents measured by the phenol/H₂SO₄ acid method of Dubois *et al.* (1956).

Antibody production, immunodiffusion and immunoelectrophoresis. Antibodies against purified albumin proteins were raised in New Zealand White rabbits and IgG fractions isolated from the antisera as described previously (Evans *et al.*, 1979). Immunodiffusions were performed in 1% (w/v) agarose gels as described by Croy *et al.* (1979). The identity of precipitin lines was confirmed by excision from the gel and analysis by SDS/polyacrylamide-gel electrophoresis as described by Croy *et al.* (1980). Rocket immunoelectrophoresis was carried out by the method of Weeke (1973), with antibodies (IgG) raised against purified major albumins (anti-PMA). Protein subunits were transferred from SDS/polyacrylamide gels on to nitrocellulose filters (Schleicher and Schüll) by electroblotting (Bio-Rad Trans Blot Cell). The filters were allowed to react with anti-PMA and then with peroxidase-coupled goat anti-rabbit IgG, before staining with 4-chloro-1-naphthol according to the standard 'Western blotting' protocols (Towbin *et al.*, 1979).

Haemagglutination assays. Assays were carried out by a serial-dilution method on microtitration plates with a 2% (v/v) suspension of untreated rabbit erythrocytes in phosphate-buffered saline [50 mM-sodium phosphate buffer (pH 7.5)/0.15 M-NaCl]. Pea lectins and concanavalin A were used as standard haemagglutinins.

Protein-body isolation. All operations were carried out at 0°–4°C. Cotyledons from 20-day-old

developing seeds were chilled, grated into homogenization buffer [0.5 M-sucrose/50 mM-sodium phosphate buffer, pH 7.5 (buffer A)], and stirred gently for 5–10 min to release the organelles. The homogenate was strained through muslin, centrifuged for 5 min at 300 *g* to remove starch grains and protein bodies pelleted by centrifugation for 10 min at 10000 *g* and gently resuspended in buffer A. Samples of the homogenate, or of protein bodies, were layered on to 20 ml linear (30–90% (w/v)-sucrose gradients and centrifuged in an MSE Prepspin 65 ultracentrifuge for 15 h at 60000 *g* (MSE 3 × 25 ml swing out rotor). Gradients were unloaded through an ISCO-UV monitor and fractions collected according to the *A*₂₈₀ profile. Only the two major protein fractions from the gradients, corresponding to the total soluble (cytosol) proteins (top of the gradient) and the protein bodies [density (*ρ*) = 1.3 g/ml] were analysed for protein composition. Total soluble proteins and protein-body proteins were fractionated into globulins and albumins as described previously, and freeze-dried (Pusztai & Watt, 1970).

Enzyme activities. Albumin proteins were tested for potential enzyme and inhibitor activities as follows: α- and β-D-glucosidases, α- and β-D-galactosidases and α-D-mannosidase were assayed using the *p*-nitrophenyl derivatives of the corresponding sugars according to the methods of Anstee & Charnley (1977). Amylase and amylase inhibitors were estimated by the method of Mestechy *et al.* (1969). Trypsin inhibitors were estimated by the method of Erlanger *et al.* (1961).

Concanavalin A. Precipitation reactions of the major albumins with concanavalin A were performed in 1% (w/v) agarose gels as described by Croy *et al.* (1980).

Results and discussion

Purification of major albumin proteins

The scheme described in the Experimental section has been used to purify two major pea albumin proteins to homogeneity, as judged by SDS/polyacrylamide-gel electrophoresis. Each consists of single-*M_r* subunits.

The protein fraction precipitating from total seed albumins between 50 and 90%-rel.-satn. (NH₄)₂SO₄ contained all or most of the major albumin proteins. This fraction was subsequently chromatographed on a Sephadex gel-filtration column. SDS/polyacrylamide-gel electrophoresis of the chromatographed fractions showed that the main peak (Fig. 1*a*) consisted largely of the major albumin proteins (subunit *M_r* about 25000). Pea seed lectins, also precipitating in this (NH₄)₂SO₄ fraction, were bound to the Sephadex, and the purified major albumins were completely free from

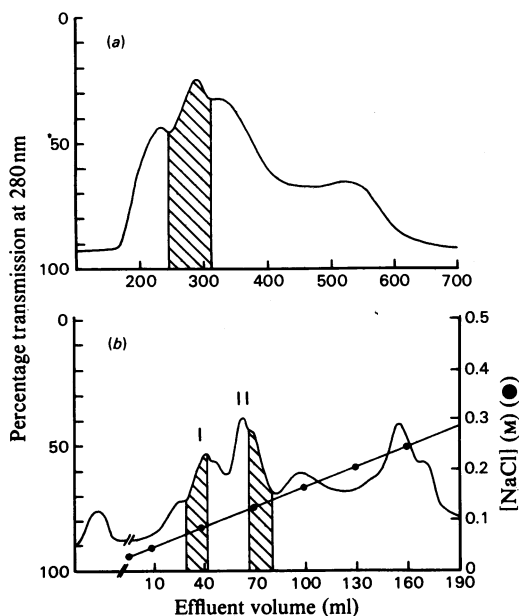


Fig. 1. U.v.-absorbance (280 nm) elution profiles of albumin proteins from (a) Sephadex G-150 and (b) DEAE-cellulose columns

Cross-hatched areas indicate fractions pooled for further purification and analysis. Peaks I and II in (b) contained the small and large major albumin proteins respectively.

lectin as judged by the absence of haemagglutination activity or reaction with anti-(pea lectin) antibodies (Fig. 5a below).

Separation and purification of the major albumins was achieved by chromatography on DEAE-cellulose. SDS/polyacrylamide-gel analysis (Fig. 2a) of fractions in the leading edge of the first large peak (I) eluted from the column (Fig. 1b) contained major albumin protein consisting only of the small subunits (subunit M_r 24000) (Fig. 2a, track 5), whereas the trailing edge of the second large peak (II) (Fig. 1b) containing major albumin consisting only of the larger subunits (subunit M_r 25000) (Fig. 2a, track 6). For simplicity the large and small *Pisum* major albumins were termed PMA-L and PMA-S respectively. Other fractions from the two peaks contained variable amounts of large and small subunits. PMA-L, PMA-S and the total major albumin proteins (total pooled fractions from Peaks I and II, Fig 1b) were used in the subsequent analyses (Fig. 2a). Total major albumin preparations showed traces of contaminating proteins absent from PMA-L or PMA-S (Fig. 2a, track 4). The success of the present method is due largely to the very efficient extraction of the albumin proteins with low-ionic-strength acidic buffer, which fails to extract any detectable amounts of globulin proteins, in agreement with the reports of Guldager (1978). Quantitative data obtained by

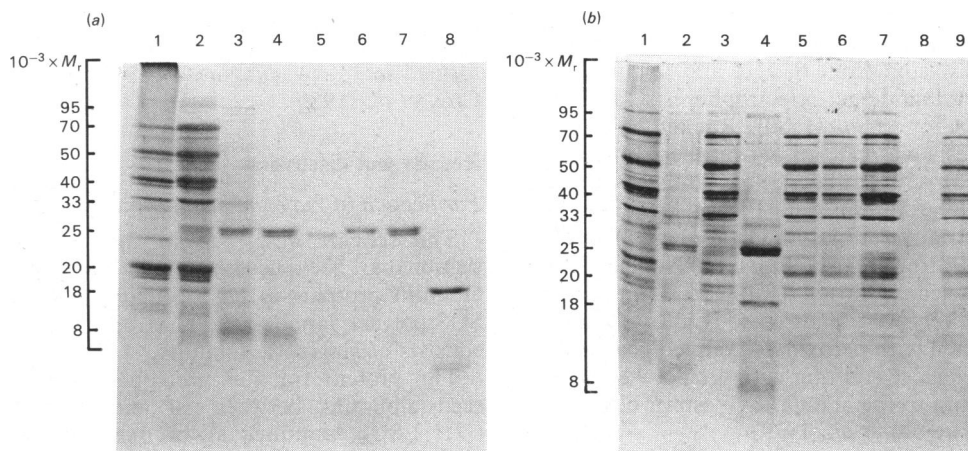


Fig. 2. SDS/polyacrylamide-gel analyses of isolated fractions as described in the Experimental section (a) Purification of the major albumin proteins: track 1, total seed globulin proteins; track 2, total seed proteins; track 3, total seed albumin proteins; track 4, total major albumin proteins; track 5, small major albumin protein; track 6, large major albumin protein; track 7, equal amounts of purified large and small major albumin proteins, mixed and co-electrophoresed; track 8, purified pea lectin. (b) Subcellular fractions from developing pea cotyledons purified on sucrose density gradients. Track 1, total seed proteins; track 2, total seed albumin proteins; track 3, total soluble proteins from the top of the gradient; track 4, albumin proteins from the top of the gradient; track 5, globulin proteins from the top of the gradient; tracks 6 and 9, globulin proteins from protein bodies; track 7, total protein-body proteins; track 8, albumin proteins from protein bodies.

rocket immunoelectrophoresis indicates that the major albumins may represent as much as 2% of the dry weight of the seed, i.e. 8–10% of the total cotyledonary protein (results not shown).

M_r and subunit compositions

Chromatography of the major albumin proteins on Sephacryl S-200 and comparison of V_e/V_0 values with those for standard proteins gave M_r values of $53000 \pm 10\%$ for PMA-L and $48000 \pm 10\%$ for PMA-S. Subunit M_r values for these proteins, estimated from SDS/polyacrylamide-gel electrophoresis, were 25000 for PMA-L and 24000 for PMA-S. Such estimates are consistent with dimeric molecules for the major albumin proteins.

The major albumin proteins isolated contained only large (M_r 25000) or small (M_r 24000) subunits (Fig. 2a, tracks 5 and 6) in the dimeric molecules, suggesting that molecules containing one of each type of subunit are not present. In support of this suggestion, analysis of fresh albumin extracts or total protein extracts by non-dissociating polyacrylamide-gel electrophoresis (Fig. 3, track 6), and on two-dimensional gels combining this technique with SDS/polyacrylamide-gel electrophoresis (results not shown), indicated two major albumin components, one of low electrophoretic mobility, containing only small subunits (Fig. 3, track 3), and the other of higher mobility, containing only large subunits (Fig. 3, track 5). However, purified preparations of PMA-L, PMA-S, or total major albumins, examined by polyacrylamide-gel electrophoresis, showed variable amounts of additional bands of higher mobility, corresponding to an increase in negative charge in the proteins. This modification occurs in a discontinuous manner, i.e. there is a shift from one form to another with no intermediate forms being observed (cf. Fig. 3, tracks 2 and 3, 4 and 5). It is apparent that this change occurs during prolonged protein purification or on storage, since freshly prepared PMA-L and PMA-S show predominantly those bands present in fresh total albumin extracts and very little of the modified forms (cf. Fig. 3, tracks 3, 5 and 6). The modification may be analogous to that observed by Matta & Gatehouse (1981) in pea legumin β -subunits on storage, and is possibly due to chemical or enzymic deamidation of amino acid amide residues. These results are thus not inconsistent with a homodimer structure for PMA-L and PMA-S.

Two-dimensional electrophoresis, combining isoelectric focusing under dissociating conditions and SDS/polyacrylamide-gel electrophoresis, showed that each subunit size class consists of several (six to eight) charge forms (Fig. 4), as has been shown for several other pea proteins (Krishna *et al.*, 1979; Gatehouse *et al.*, 1980, 1981). Since the

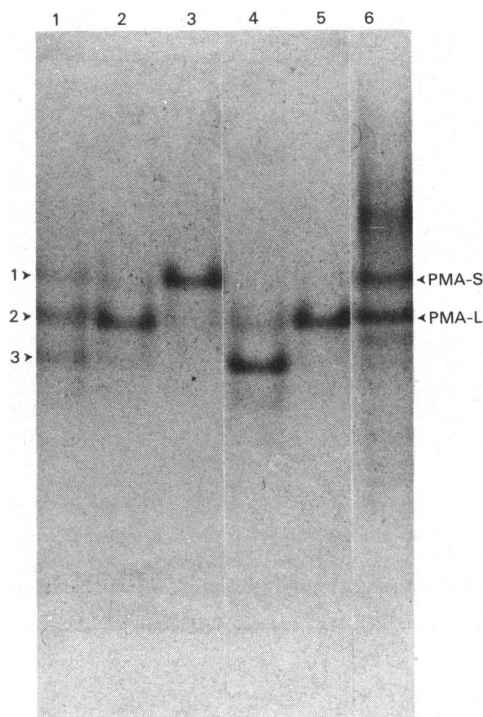


Fig. 3. Polyacrylamide-gel electrophoresis of pea albumin proteins under non-dissociating conditions, pH 8.3

The direction of electrophoresis is from the top to the bottom of the Figure. Track 1: total major albumin protein (PMA-L + PMA-S); stored sample; arrowheads denote the three components (1, 2, 3). Tracks 2 and 3: PMA-S, stored sample (track 2) and fresh sample (track 3). Tracks 4 and 5: PMA-L, stored sample (track 4) and fresh sample (track 5). Track 6: total albumin proteins extracted from pea cotyledons (fresh sample); arrowheads denote bands shown to be due to PMA-S and PMA-L on analysis by SDS/polyacrylamide-gel electrophoresis in the second dimension (results not shown).

subunit arrays of PMA-L (Fig. 4c) and PMA-S (Fig. 4b) closely resemble those in the total seed albumins (Fig. 4a), it seems likely that they are genuine charge isomers and not artefacts due to the charge modification discussed above.

Characterization of PMA-L and PMA-S

Although subunits of PMA-L and PMA-S do not associate together *in vivo*, the two proteins are structurally very similar. PMA-L and PMA-S gave a reaction of complete identity in immunodiffusion against anti-(total major albumin) antibodies (Fig. 5a) and gave no cross-reactions with other seed proteins or with antibodies raised against other albumin proteins (e.g. anti-lectin; Fig. 5a) or globulin proteins.

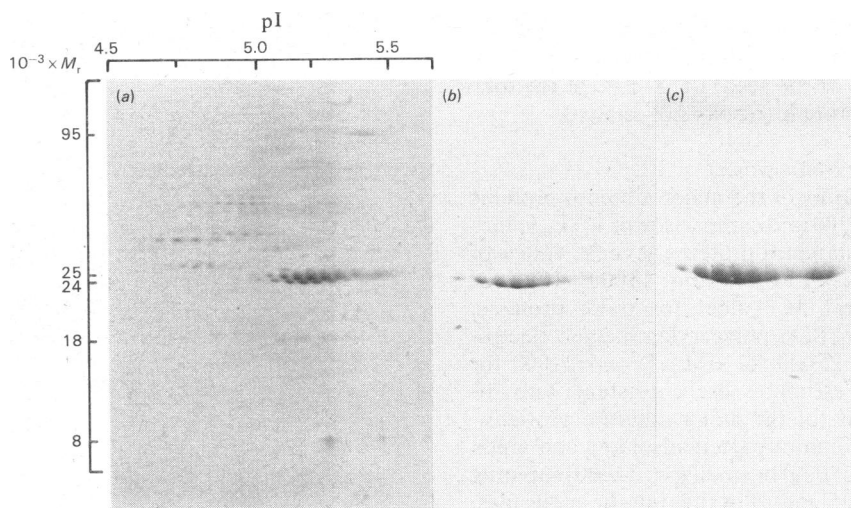


Fig. 4. Two-dimensional gel analyses of albumin proteins combining isoelectric focusing in urea gels in the first dimension and SDS/polyacrylamide-gel electrophoresis in the second dimension
(a) Total seed albumin proteins; (b) small major albumin protein; (c) large major albumin protein.

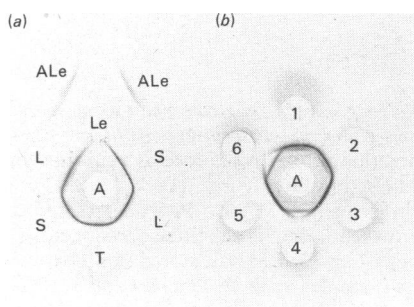


Fig. 5. Immunodiffusion of (a) purified pea albumin proteins and (b) total albumin extracts

(a) Abbreviations used: A, anti-(major albumin) antibodies; ALe, anti-lectin antibodies; L, large major albumin protein; S, small major albumin protein; T, total major albumin proteins; Le, lectin.
(b) A, anti-(major albumin) antibodies; other wells contained total albumin extracts from: (1, 3, 5) *Pisum sativum* (pea), (2) *Lens culinaris* (lentil), (4) *Lathyrus odoratus* (sweet pea), and (6) *Cicer arietinum* (chickpea).

The amino acid compositions of both proteins (Table 1) were very similar, with higher levels of the sulphur amino acids (cysteine and methionine) than the average for globulin proteins. These results support the reports of Grant *et al.* (1976), Hurich *et al.* (1977), Jakubek & Przybylska (1979) and Murray (1979) of the sulphur-rich nature of the albumin protein fraction and suggest the major albumin proteins as further candidates for investigations into improving the nutritional quality of peas (Davies, 1976; Boulter, 1982).

Table 1. Amino acid and carbohydrate compositions of large and small major albumin proteins compared with that of total seed albumins

Amino acid data is expressed as mol/100 mol of the total analysed and (in parentheses) as residues per subunit molecule of PMA-L and PMA-S. Residues per molecule were calculated from the average M_r for amino acids of 112 and subunit M_r values of 25000 for PMA-L and 24000 for PMA-S. Results are means of duplicate or triplicate determinations. Variation between determinations was less than 10%. Abbreviations used: CM-Cys, carboxymethyl-cysteine; ND, not determined.

	Composition (mol/100mol)		Total Albumin Proteins
	PMA-S	PMA-L	
Asp	15.4 (33)	15.0 (34)	12.0
Thr*	7.5 (16)	7.2 (16)	4.5
Ser*	6.3 (14)	6.1 (14)	6.5
Glu	6.7 (14)	7.0 (16)	11.6
Gly	8.1 (17)	7.3 (16)	8.4
Ala	7.8 (17)	7.7 (17)	9.7
Val	5.5 (12)	5.6 (13)	7.5
Met	1.1 (2)	1.2 (3)	1.2
Ile	6.6 (14)	6.6 (15)	5.5
Leu	5.4 (12)	5.5 (12)	7.1
Tyr	6.6 (14)	7.2 (16)	3.9
Phe	7.8 (17)	8.4 (19)	5.1
Lys	7.5 (16)	7.9 (18)	7.9
His	1.3 (3)	1.1 (3)	2.1
Arg	4.9 (11)	5.3 (12)	4.6
CM-Cys	1.2 (3)	1.2 (3)	2.3
Pro	ND -	ND -	ND
Trp	ND -	ND -	ND
Carbohydrate	0 -	0 -	ND

* Corrected for decomposition.

CNBr cleaves both large and small subunits into three distinct fragments showing the presence of at least two methionine residues per subunit (Fig. 6, tracks 4, 6 and 7). Since two to three methionine residues per subunit are predicted by the amino acid composition (Table 1), these results are in reasonably good agreement, but they do not eliminate the possibility that an additional, presumably small, CNBr fragment may also be produced. The three polypeptide fragments obtained after 3h incubation were unchanged on prolonged reaction times (6, 24 and 48 h). By using highly resolving urea/SDS gels, comparison of these polypeptide fragments with those obtained from the total major albumin confirmed that one fragment (M_r 11 500) was coincident in both large and small major albumin subunits, whereas the other two fragments were of slightly higher M_r in the large major albumin subunit (M_r 8400 and 7200 in PMA/L and M_r 7600 and 6600 in PMA-S; Fig. 6, tracks 4 and 6). Summation of the M_r values for the CNBr fragments agrees with the calculated subunit M_r values. Such results, although confirming the similarity of the subunit polypeptides, indicated that the primary sequences of the two subunits vary slightly in at least two regions but may have at least one large highly conserved region.

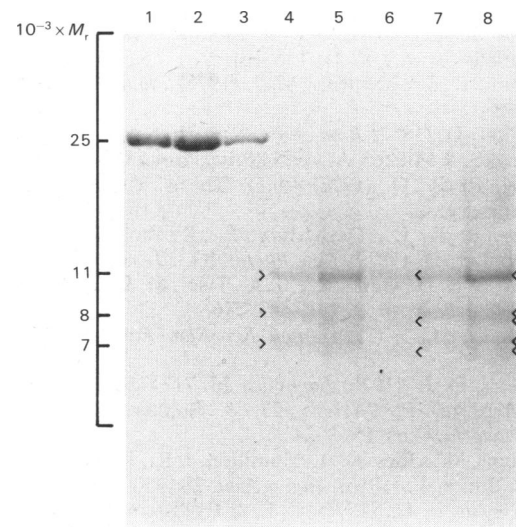


Fig. 6. SDS/urea/polyacrylamide gel analysis of CNBr cleavage fragments from large, small and total major albumin proteins

Tracks 1, 2 and 3, untreated large, total and small major albumin proteins; tracks 4 and 7, CNBr-cleaved large major albumin (6 and 24h treatment); tracks 5 and 8, CNBr-cleaved total major albumins (6 and 24h treatments); track 6, CNBr-cleaved small major albumin (6h treatment).

Tryptic peptide maps in the form of h.p.l.c. elution profiles were similar for PMA-L and PMA-S, reinforcing the above conclusions of structural relatedness. Both PMA-L and PMA-S were found to have threonine as the *N*-terminal amino acid.

Neither of the major albumin proteins is a glycoprotein as judged by: chemical analysis (Table 1); running known glycoproteins (and non-glycoproteins for comparison) on SDS/polyacrylamide gels followed by glycoprotein-specific staining (Croy *et al.*, 1980); and the absence of any reaction with concanavalin A (Gleeson & Jermym, 1977).

Degradation and subcellular location of the major albumins

The breakdown of the major albumins during germination is quite different from that of the globulin storage proteins. The pea globulins, legumin, vicilin and convicilin, were all rapidly degraded during the first few days of germination, whereas PMA-L, PMA-S and the lectins were all maintained longer than seven days after germination (results not shown; Tyler, 1981). This result agrees with the observations of Murray (1979) and Jakubek & Przybylska (1982), who described the continued presence of a major albumin polypeptide of M_r ~22 000–24 000 in pea cotyledons up to 20 days after germination, and of Guldager (1978), who described significant amounts of albumins and lectin remaining after the disappearance of vicilin and legumin.

Clearly the absence of significant degradation of such a protein, coupled with its high sulphur content, indicates that its role is different from that of the storage proteins. This has similarly been suggested by Murray (1979) and Jakubek & Przybylska (1982), although the suggestion of a structural function made by the latter authors seems unlikely in view of the protein's high aqueous solubility. In an effort to elucidate its function we have carried out preliminary localization studies by subcellular fractionation. The results strongly support a cytoplasmic (the soluble fraction of the cytosol) location for the major albumins, since SDS/polyacrylamide-gel analysis of the total albumin proteins isolated from the soluble cytoplasmic fraction at the top of the density gradients showed the albumin polypeptides (PMA-L, PMA-S, lectin and others) present as the main components (Fig. 3b, track 4). By contrast, no such polypeptides were observed in the albumin fraction from protein bodies, even at high sample loadings (Fig. 2b, track 8). Despite the large yield of protein from the protein bodies, the amount of albumin fraction recovered from them was very low, as reported by other groups (Konopska, 1978; Weber & Newmann, 1980; Varner & Schidlovsky, 1965) and differ from the

results obtained from subcellular fractionation of other legume seeds (e.g. those of the French bean, *Phaseolus vulgaris*), where the predominant albumin proteins are located within the protein bodies (Croy, 1977; Pusztai *et al.*, 1977; Youle & Huang, 1978; Bollini & Chrispeels, 1978). The present results support the suggestions of Murray (1979) of a cytoplasmic site for the pea albumins, but are at variance with the reports of Weber *et al.* (1978), who reported that the homologous albumin lectin in broad bean (*Vicia faba*) was protein-body-associated. In the present study the major storage proteins were all mainly present in the protein-body fraction (Fig. 2b, tracks 6 and 9), with lesser amounts of released globulins present in fractions from the top of the gradient (Fig. 2b, track 5). Thus it is likely that most of the isolated protein bodies are intact. Croy (1977) and Pusztai *et al.* (1977), who used a similar system, were able to show that a significant redistribution of *Phaseolus vulgaris* protein-body albumin proteins did not take place. This makes the present results unlikely to be an artefact, since it is improbable that all the major albumin proteins and lectins would have been quantitatively released from the protein bodies. However, unequivocal localization of these proteins awaits quantitative subcellular-fractionation studies and immuno-histochemical studies at the electron-microscopic level as have been performed for the pea globulins (Craig & Millerd, 1981).

Assays for functional properties

The cytoplasmic location of PMA-L and PMA-S suggests some kind of metabolic or anti-metabolic role for these proteins. However, neither PMA-L nor PMA-S showed activity in any of the enzyme assays tested (α - and β -D-glucosidases, α - and β -D-galactosidases, α -D-mannosidase, amylase). Furthermore, neither protein exhibited inhibition in amylase-inhibition assays with porcine pancreatic amylase or trypsin-inhibition assays with bovine trypsin, and no haemagglutinating activity was associated with the proteins. The role of the major albumin proteins therefore remains unknown.

Species distribution of pea major albumin type proteins

Total albumin extracts from a variety of different legume species were screened for PMA-type proteins by immunodiffusion against anti-PMA IgG. *Lathyrus oderatus* (sweet pea), *Lens culinaris* (lentil) and *Cicer arietinum* (chick-pea), were the only three species to show specific immunoprecipitations as judged by reaction of identity (lentil) or partial identity (chick-pea and sweet pea) with PMA (Fig. 5b).

No reactions were obtained with extracts from *Vicia faba*, *Glycine max* (soya-bean), *Dolichos lablab* (horse gram), *Cajanus cajan* (pigeon pea), *Phaseolus vulgaris*, *Phaseolus coccineus* (runner bean), *Vigna unguiculata* (cowpea) and *Vigna mungo* (mung bean). Analysis of the three immunoprecipitating albumin extracts on SDS/polyacrylamide gels showed major polypeptides of $M_r \sim 22000$ – 24000 , which when examined by 'Western blotting' showed specific anti-PMA binding, confirming their homology with the pea major albumin (results not shown). This type of protein thus seems to be characteristic of *Pisum* and closely related species, and suggests that the lentil is the most closely related species to *Pisum sativum*, as has been shown with other seed proteins (e.g. legumin; R. Sammour, unpublished work).

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