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# PURIFICATION AND CHARACTERIZATION OF THE MAJOR STORAGE PROTEINS OF *PHASEOLUS VULGARIS* SEEDS, AND THEIR INTRACELLULAR AND COTYLEDONARY DISTRIBUTION

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**Key Word Index**—*Phaseolus vulgaris*; Leguminosae; amino acid composition; protein bodies; storage proteins; *N*-terminus.

**Abstract**—Several extraction and fractionation procedures have been employed to isolate the major storage proteins of mature seeds of *Phaseolus vulgaris* cv. "Seafarer"; three proteins which were soluble at pH 4.7, and one that was insoluble at that pH were identified. The characteristic subunits of the three pH 4.7 soluble proteins had MW's 50000 and 47000, 32000, and 23000 respectively; those of the pH 4.7 insoluble fractions had MW 60000 and 20000. Amino acid compositions, *N*-terminal amino acid residues and the presence of carbohydrate in these proteins have been determined. All these proteins occurred in the protein body fraction and their relative amounts were different in the outer and central parts of the cotyledons.

## INTRODUCTION

Protein laid down during seed development and utilized as a nitrogen and carbon source during germination is termed storage protein; in legumes 80% of the seed protein may be storage protein. Pusztai has purified and characterized several seed proteins from *Phaseolus vulgaris* [1–4] and Racusen and Foote [5] and Hall and his associates [6–8] have also investigated the seed storage proteins of this species; an 11S storage protein from *P. vulgaris* has recently been described [9]. However, when interpreting previously described results, problems arise since the variety of methods of isolation and purification used make comparison difficult. Also, the relationship of a purified fraction to the total protein complement is often unclear, and the analytical methods used, e.g. ultracentrifugation, often do not unequivocally identify the protein.

In this investigation of the storage proteins of *Phaseolus vulgaris*, sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis has been used to identify the component subunits and to assess the purity of the protein fractions obtained. The intracellular localization of the storage proteins and their distribution within the cotyledons has also been determined.

## RESULTS

### *Extraction and fractionation of storage proteins*

*Alkaline salt extracts.* The protein pattern obtained by disc electrophoresis of an alkaline salt extract of cultivar "Seafarer" showed several components which were not always completely resolved. There was a diffuse major band, $R_m$ 0.35–0.4, and in addition there were prominent components, $R_m$ 0.19, 0.1, and several other minor components. A densitometric trace of the protein band pattern obtained by SDS-gel electrophoresis of this extract is shown in Fig. 1. There are two main subunits, MW 50000 and 47000, but four other prominent components, MW 60000, 32000, 23000 and 20000 are also present. SDS extraction and electrophoretic analysis of meal gave a similar electrophoretic profile. SDS extractions of meal of a different cultivar, "Canadian Wonder", gave a similar subunit pattern, except that in this cultivar the equivalent subunits to the 50000 and 47000 MW components were of lower MW, 49000 and 45000 respectively, and an additional major band of MW 53000 was present.

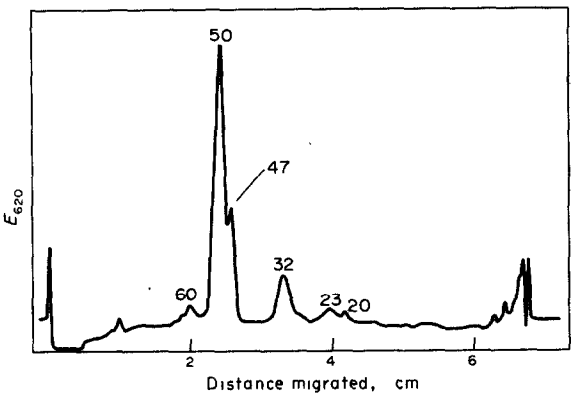


Fig. 1. The SDS electrophoretic pattern of an alkaline salt extract. *Legend.* Numbers above peaks refer to the molecular weights ($\times 10^{-3}$) of the polypeptide chains. A similar band pattern and molecular weights were obtained when a cetyltrimethylammonium-bromide (CTAB)-containing system at pH 5.7 was used [40].

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The precipitate, obtained from alkaline salt extracts by addition of ammonium sulphate to 70% saturation, was enriched in the 60000, 32000, 23000 and 20000 MW subunits. Two fractions were obtained by zonal isoelectric precipitation of the redissolved precipitate: one eluted immediately after the void volume, in citrate buffer pH 4.7, and was pH 4.7 soluble; the second fraction which accounted for approximately 20% of the protein applied to the column, was insoluble at pH 4.7, and was retarded during chromatography. When these fractions were analysed by disc electrophoresis, the pH 4.7 soluble fraction migrated as a major, poorly defined band, $R_m$ 0.35–0.4, with a subsidiary band $R_m$ 0.19; the pH 4.7 insoluble fraction, which was poorly soluble in the electrophoresis sample buffer, migrated as a single band $R_m$ 0.1. SDS gel electrophoresis showed that the major components of the initial extract, i.e. the MW 50000 and 47000 components, together with the lower MW subunits of 32000 and 23000 MW were confined to the pH 4.7 soluble fraction, and the pH 4.7 insoluble fraction consisted almost entirely of subunits of MW about 80000, 60000 and 20000. This partial purification of components was confirmed when the main band cut from the gel after disc electrophoresis of an alkaline extract was shown, in the SDS gel system, to be mainly composed of 50000 and 47000 MW subunits, with small amounts of the 32000 and 23000 MW subunits. It is of interest to note that when the subsidiary band ($R_m$ 0.19), found on disc gel electrophoresis of the pH 4.7 soluble fraction was similarly cut out and analysed, it also consisted of the 50000, 47000 and 32000 MW subunits. In the absence of 2-mercaptoethanol, the pH 4.7 insoluble fraction gave a single band of MW about 80000 on SDS gel electrophoresis; treatment with 1% (v/v) 2-mercaptoethanol resulted in loss of this component and the concomitant appearance of subunits of MW 60000 and 20000, but a small amount of the 80000 MW band always remained. The pH 4.7 soluble fraction gave the same band pattern in the presence or absence of 2-mercaptoethanol. Carboxymethylation caused no change in the band pattern of any fraction.

When alkaline salt extracts were chromatographed on DEAE-cellulose, the protein was separated into two fractions. Unadsorbed protein was eluted as a small peak with the starting buffer and when analysed by disc electrophoresis it gave a diffuse band $R_m$ 0.27–0.39, together with a band migrating with the bromo-phenol blue marker, $R_m$ 1.0; SDS gel electrophoresis showed this fraction to be mainly composed of the 32000 MW subunit. Protein adsorbed to the column was eluted as a large peak with 0.17 M NaCl; while the leading edge of the elution profile was very sharp, a shoulder occurred on the trailing edge. Disc electrophoresis of the leading edge of this peak resulted in an intense band $R_m$ 0.37, with a weak band $R_m$ 0.21; the protein of the trailing edge contained a slow-moving band $R_m$ 0.1, in addition to these components. The leading edge of the main peak was shown by SDS gel electrophoresis to contain the subunits of 50000 and 47000 MW, together with a trace of the 23000 MW subunit. The middle of the peak contained subunits of MW 80000, 60000, 50000, 47000 and 20000, i.e. the 23000 MW subunit was absent from this region of the peak. The trailing edge contained all the above subunits with enrichment of a 23000 MW subunit. The 32000 MW subunit was not observed in this peak.

Rechromatography of fractions from the leading edge of the main peak failed to remove completely the trace amount of the 23000 MW subunit present. When samples of the trailing edge of the peak were rechromatographed, fractions enriched in the 60000 and 20000 subunits, relative to the 50000 and 47000 MW subunits, were obtained, but the latter subunits were still the major components on SDS gels. Alteration of the pH at which chromatography was carried out, the dimensions of the column, the amount of protein applied, the flow rate, and the steepness of the sodium chloride elution gradient, all failed to give increased resolution of the proteins of the main peak.

When samples of alkaline salt extracts were centrifuged in sucrose density gradients a single broad peak was obtained, although some protein remained at the top of the gradient. SDS gel electrophoresis of fractions from the gradient showed that (a) the pH 4.7 insoluble fraction subunits, MW 60000 and 20000, were confined to the lower edge of the peak; (b) as judged by SDS-gel electrophoresis, most of the 50000, 47000 and 32000 MW subunits were found in the broad peak, although some protein composed of these subunits remained at the top of the gradient; (c) while the protein which remained at the top of the gradient was enriched in subunits of MW 23000, traces of subunits of this size were also seen on the SDS gels of fractions from the main peak.

*Water extracts.* When water extracts of meal were cooled a substantial cryoprecipitate formed. When analysed by disc electrophoresis this was found to be composed of a main component which migrated as a wide band, $R_m$ 0.35–0.4, together with a subsidiary band $R_m$ 0.2. The supernatant fluid after removal of the cryoprecipitate contained approximately equal amounts of components with $R_m$s 0.37 and 0.1.

Subunit analysis by SDS gel electrophoresis showed that the cryoprotein consisted of the 50000 and 47000 MW subunits, with traces of the 32000 and 23000 MW subunits; when the supernatant fluid after removal of this protein was analysed, it contained an increased proportion of the 80000, 60000, 32000, 23000 and 20000 MW subunits and about 30% of the total amount of the 50000 and 47000 MW subunits. When the cryoprotein was analysed on disc gels and the main band ($R_m$ 0.35–0.4) cut from the gel and the protein analysed in the SDS system, it was shown to consist of the 50000 and 47000 MW subunits, with a very small amount (*ca* 3% of the total protein) of the 23000 MW subunit; the 32000 MW subunit was not observed.

When the cryoprecipitate from a water extract was subjected to gel isoelectric-focusing in 7 M urea over the pH range 3–6, at least eighteen bands were obtained in the pH region 5.0–5.75. However, similarly complex patterns were also obtained from the pH 4.7 soluble fraction on isoelectric focusing in 7 M urea gels.

*Acidic extracts.* Two acidic extraction methods were used (see Experimental) and both gave the same results when the extracts were examined by gel electrophoresis. When acidic extracts were subjected to disc electrophoresis, the patterns obtained were similar to those obtained with alkaline salt extracts, but owing to the poorer resolution obtained as compared to SDS gels, detailed comparison was not profitable. SDS gel electrophoretic analysis showed that the acidic extracts were also similar in subunit content to alkaline salt extracts.

Table 1. The amino acid compositions and *N*-terminal amino acid residues of protein fractions. All results are expressed as mol%. The amino acid composition of Glycoprotein II [3], recalculated to mol‰ is also included

| Amino acid | pH 4.7 insoluble fraction | Non-adsorbed ion-exchange fraction | Leading edge of main ion exchange peak | Glycoprotein II [3] |
|---|---|---|---|---|
| Asp | 11.9 | 14.8 | 14.9 | 13.4 |
| Thr | 4.1 | 8.2 | 3.8 | 4.2 |
| Ser | 6.6 | 8.3 | 7.1 | 9.6 |
| Glu | 18.3 | 7.3 | 16.2 | 14.6 |
| Pro | 6.5 | 4.6 | 3.6 | 3.7 |
| Gly | 4.7 | 8.0 | 6.1 | 5.8 |
| Ala | 5.5 | 7.2 | 5.2 | 5.2 |
| ½-Cys | N.D.* | 0.8 | 0.4 | 0.2 |
| Val | 6.9 | 7.2 | 5.9 | 6.5 |
| Met | 0.8 | 0.3 | 0.8 | 0.7 |
| Ile | 4.1 | 5.5 | 5.4 | 6.2 |
| Leu | 8.2 | 10.6 | 9.5 | 10.0 |
| Tyr | 2.6 | 2.7 | 2.6 | 2.7 |
| Phe | 2.9 | 5.5 | 5.4 | 5.6 |
| Lys | 8.3 | 5.3 | 6.4 | 5.4 |
| His | 3.7 | 1.4 | 2.5 | 2.4 |
| Arg | 4.9 | 3.0 | 4.8 | 4.0 |
| *N*-terminal amino acids | Gly,Thr. | N.D.* | Thr, Ser, Leu trace Glu | — |

* Not determined.

On fractionation of the acidic ascorbate extract, subunits of 80000, 60000 and 20000 MW were confined to the F-I fraction (see Experimental for terminology of fractions) but those of MW 50000 and 47000 were found in all three fractions. The 23000 MW subunit was enriched in the F-I fraction, while the 32000 MW subunit was confined to the F-II fractions.

When acidic extracts were prepared in the absence of ascorbate, the F-I fraction contained the same subunits as the F-I fraction from the ascorbate-containing extraction, but there was markedly less of the 50000 and 47000 MW subunits, i.e. the 80000, 60000 and 20000 subunits were enriched. Whilst the presence or absence of ascorbate had less effect on the composition of the F-II fraction, nevertheless, in the absence of ascorbate the F-IIa fraction consisted almost entirely of the 50000 and 47000 MW subunits, and the F-IIb fraction of the 32000 MW subunit. Using either extractant, the F-II fractions agglutinated human group O erythrocytes.

In contrast to the situation with acidic extracts, protein was not precipitated by two-fold dilution of alkaline salt extracts. When the latter were dialysed overnight against running tap water, a fraction with subunits similar to those of the F-I fraction from the ascorbate-containing extract was precipitated.

*Characterisation of protein fractions*

*Amino acid analysis.* The amino acid compositions of the pH 4.7 insoluble fraction (60000 and 20000 MW subunits), the non-absorbed fraction from DEAE-cellulose ion-exchange chromatography (32000 MW subunit), and the leading edge of the main peak eluted during ion-exchange chromatography (50000 and 47000 MW subunits), are presented in Table 1, together with the *N*-terminal amino acid residues found for each fraction. The amino acid composition of Glycoprotein II (3), recalculated to mole‰, is included in Table 1 for comparison.

*Carbohydrate content.* When SDS gels of the pH 4.7 soluble and insoluble fractions were stained for the presence of carbohydrate, only the 50000, 36000 and 32000 MW subunits took up stain. The 32000 MW subunit stained relatively strongly; the 36000 MW subunit, which was an insignificant component on the gels when stained for protein, showed up as a prominent component on periodic acid-Schiff (PAS) staining. When gels of SDS extracts of *P. vulgaris* cv. "Canadian Wonder", were similarly stained, the 53000, 49000 and 32000 MW subunits took up stain.

*Protein composition of protein bodies*

SDS gel electrophoresis showed that all the main storage protein subunits (see Fig. 1) were found in the protein body fraction. Protein bodies sedimented during sucrose density gradient centrifugation as an opaque band centred at approximately 80% (w/v) sucrose. When fractionation of the gradient was monitored at 280 nm, the protein body peak was usually symmetrical with respect to 280 nm absorption. Occasionally shoulders of apparently more or less dense particles were observed, but no differences in subunit pattern were detected when these regions were analysed. Only about 10% of the protein applied to the gradients was recovered in the protein body fraction. All the subunits present in the protein body fraction were also found at the top of the gradients.

Isolation of protein bodies with glycerol at pH 5.0 resulted in a protein body fraction which accounted for approximately 40% of the total protein extracted from meal. The protein subunit composition of these protein body preparations resembled that of those isolated by sucrose density gradient centrifugation but in contrast to the sucrose method none of the pH 4.7 insoluble com-


Fig. 2. Electron micrographs of protein body preparations. *Legend*: (a) prepared by sucrose density gradient centrifugation; the arrow indicates one of the protuberances noted in the text. The bar in the lower right-hand corner represents 5 μm. (b) prepared by the glycerol method; the bar represents 2 μm.

ponents were found in the supernatant. When the glycerol procedure was carried out at pH 7.5, some of the pH 4.7 insoluble fraction protein subunits were found in the supernatant, but at this pH the yield of protein bodies was lower (about 25% of the protein present in the pH 5.0 protein body fraction) and the protein body fraction contained an even higher proportion of the pH 4.7 insoluble fraction subunits relative to the total.

Figure 2a is an electron micrograph of protein bodies prepared by sucrose gradient centrifugation. The protein bodies are membrane-bound, although the membranes of several have been ruptured and leakage of their contents is apparent. In some protein bodies, distinct and often sharp protuberances (arrowed) are found.

Protein bodies prepared by the glycerol method at pH 5.0 were more intact (Fig. 2b), and their membranes were more clearly defined. However, small fragments of cell wall were present and protein bodies were frequently present in clusters, surrounded by cytoplasmic material.

*Protein distribution within the cotyledon*

Salt extracts from abaxial sections of cotyledons were richer in protein than those from the centre and trypsin inhibitor activity was also greater in the abaxial sections. Not only is there relatively more protein in the abaxial portion of the cotyledon, but the proportion of the subunits there differs from that of the central region. Thus, the abaxial region is enriched relative to the central portions in 60000 and 20000 MW subunits and impoverished in 32000 and 23000 MW subunits. By comparison with the whole seed the abaxial region has less 47000 MW subunit relative to the 50000 MW subunit. In contrast, the adaxial surface contained relatively large amounts of 32000 and 23000 MW subunits when compared with other parts of the seed.

The distribution of protein subunits established by the direct analysis of SDS extracts of different regions of the cotyledon was confirmed when protein bodies were isolated, using the glycerol procedure at pH 5.0, and their constituent protein was analysed by SDS gel electrophoresis.

## DISCUSSION

The major storage protein of *P. vulgaris* cv. "Seafarer" accounts for about 60% of the total seed protein and is composed of two subunits with MW 50000 and 47000, in the approximate ratio 2–3:1. This protein is pH 4.7 soluble and, as described by Derbyshire and Boulter [9], sediments in the ultracentrifuge at pH 7.0 as a 7S molecular species which partially associates to an 18S species at pH 6.2. Pusztai and Watt (3) have isolated a glycoprotein, Glycoprotein II, from *P. vulgaris* and Racusen and Foote [5] have equated this protein with the major salt soluble protein of *P. vulgaris* on the evidence of disc electrophoresis and amino acid composition. Although the subunit composition of Glycoprotein II has not been reported, the similarity between the major purified protein of this investigation and Glycoprotein II in sedimentation coefficient, association behaviour and amino acid compositions clearly indicate that they are the same protein. Furthermore, although the mobility in 5% acrylamide gels obtained by us (not given here) was slightly greater than that reported by Racusen and Foote [5], there was a general similarity in the appearance of the band on disc electrophoresis in the two investigations.

This major protein behaved as a cryoprotein and precipitated when a water extract was cooled to 0–4°. A vicilin (i.e. a 7S, pH 4.7 soluble protein) from *Pisum sativum*, which was cryoprecipitated from a water extract of the meal, has also been described [10]. In contrast, a cryoprotein obtained from water extracts of *Glycine max* [11] has been shown to be composed mainly of an 11S protein (legumin-like) [12]. Cryoprecipitation has been used as an initial step in the purification of this protein [13] but the effectiveness of water as an extractant for seed proteins varies from legume to legume [14].

A second pH 4.7 soluble fraction, corresponding to the 6S fraction observed in the ultracentrifuge by Derbyshire and Boulter [9], was composed of glycoprotein subunits with MW approximately 32000. The amino acid composition of this fraction is in reasonable agreement with that of the lectin (subunit MW 30000–32000) isolated by Andrews [15] from navy bean; a notable common feature is a low glutamic acid content as compared to the other storage proteins of this species.

Recently, Pusztai and Watt [4] have separated and partially characterized a number of agglutinins from *P. vulgaris*, which differ in their isoelectric points but are also characterized by low glutamic acid contents. It would appear, therefore, that the protein fraction described in this investigation is related to the preparations

of Pusztai and Watt, but their precise relationship is not clear.

The pH 4.7 insoluble fraction obtained here by zonal isoelectric precipitation is similar to that described in greater detail by Derbyshire and Boulter [9], i.e. it is a legumin-like protein. It is the only one of the storage proteins described here that appears to contain inter-subunit disulphide bonds.

The presence of at least a fourth protein is suggested by the occurrence of subunits of MW 23000 which cannot be assigned to any of the three previously described proteins. Subunits of this size were found in two different fractions during ion-exchange chromatography and sucrose density gradient centrifugation. One fraction eluted and sedimented with Glycoprotein II, while the other sedimented more slowly and was eluted at a higher salt concentration during ion-exchange chromatography.

The use of an acidic extractant either with or without ascorbate to prevent oxidation, has been advocated by Hall and his associates [6–8]. These same procedures were used here but our interpretation of the results is different to that of Hall and his colleagues. We consider Hall's G-I fraction to be equivalent to our FI and FIIa fractions (prepared in the absence of ascorbate) combined, i.e. to an impure preparation of Glycoprotein II. Hall's G-II fraction, containing a single major subunit of MW 30000 appears similar to our F-IIb which we equate with the agglutinins described from *Phaseolus* by Pusztai and Watt [4]. The use of an acid extraction method with *Vicia faba* led to the formation of lower MW polypeptides [16] and the method cannot be recommended for general use with legumes.

Isoelectric focusing in urea gels of several globulin preparations has led to the demonstration of more protein components than expected from the results obtained with other methods [17–19] and this is also true for the data on Glycoprotein II presented here. It is not possible to say whether some components are artefacts caused by carbamylation [20] and/or deamidation [21] or if, in fact, all the components are native subunits, the complexity of the pattern resulting from microheterogeneity, possibly in the carbohydrate moiety [22].

The major storage proteins of *P. vulgaris* described above have been shown to be located in the protein body preparations by the presence of their subunits and the protein bodies were morphologically similar to those previously described *in situ* by Opik [23]. The glycerol method for protein body preparation is rapid and gives a higher yield than from sucrose density gradient centrifugation, but glycerol preparations appeared to be less pure.

Differences in the amount of protein in different parts of legume and cereal seeds have been reported [24, 25]. Vogel and Wood [26] demonstrated that the outer regions of the cotyledons of *Phaseolus vulgaris* were richer in protein-bound sulphydryl groups than the inner regions; Wood and Cole [27] subsequently showed that individual disc electrophoretic components were found in different ratios in the outer and central regions of the cotyledons. We have confirmed the gradient of protein and trypsin inhibitor activity described by Zimmermann *et al.* [25] and have shown differences in the distribution of storage protein subunits in different parts of the cotyledon. Although the sulphur content of different regions has not been analysed, the presence of greater amounts of legumin subunits in the abaxial region of the cotyledon is compatible with a higher protein bound sulphydryl concentration there.

A striking result was the difference in the ratio of the 50000 and 47000 MW subunits observed between the abaxial surface and the central regions of the cotyledon. The fact that this difference was also seen when the protein subunits of protein bodies derived from these regions were analysed by SDS gel electrophoresis suggests that the difference is not simply an extraction artefact. Further work will be necessary to determine whether this difference is upheld in purified preparations of this protein isolated from different regions of the cotyledons.

## EXPERIMENTAL

*Preparation of seed meals.* Seeds or cotyledons of *Phaseolus vulgaris* cvs. "Seafarer" and "Canadian Wonder" were finely ground in a Janke and Kunkel water-cooled mill.

*Extraction of proteins.* (a) *Alkaline salt extracts.* 6 g of meal was stirred with 60 ml 0.5 M NaCl, 0.05 M NaPi buffer pH 7.5 for 30 min at 4°. Slurry was clarified by centrifugation at 38000 *g* for 90 min at 4°.

(b) *Water extracts.* 6 g meal was stirred with 60 ml distilled water for 30 min at room temp (18–20°). After clarification at 38000 *g* for 90 min (at 20°) the supernatant fluid was cooled in an ice bath for 90 min. The ppt. which formed during cooling was collected by centrifugation at 23000 *g* for 30 min at 4°.

(c) *Acidic extraction and fractionation.* All procedures were at 4°. Extraction and fractionation were carried out as described by McLeester *et al.* [6] using 0.5 M NaCl, 0.25 M ascorbic acid (measured pH 2.4); a similar procedure was followed in the absence of ascorbate, using 0.5 M NaCl, adjusted to pH 2.0 with dil HCl, as the extractant. The solvent to meal ratio was 10:1 (v/w). The fraction precipitated by addition of 2 vols of distilled water to the initial supernatant was collected by centrifugation, and is referred to as the F-I fraction. Overnight dialysis of the supernatant, after removal of the F-I fraction, against running $H_2O$ resulted in the formation of a ppt; this was collected by centrifugation and is referred to as the F-IIa fraction. A further ppt. was obtained by dialysis of the remaining supernatant fluid against several changes of dist. $H_2O$; this was also collected and is referred to as the F-IIb fraction.

(d) *SDS extraction.* 20 mg samples of meal were stirred with 4 ml of 0.01 M NaPi buffer pH 7.0, 1.0% (w/v) SDS and 1.0% (v/v) 2-mercaptoethanol for 60 min at 37°; they were then heated to 100°C for 3 min and allowed to cool at room temp; extracts were clarified by centrifugation and analysed by SDS gel electrophoresis.

*Preparation of protein bodies.* (a) *By sucrose density gradient centrifugation.* All operations were carried out at 0–4°. Seeds were soaked overnight in running $H_2O$ and their testas and embryo axes removed. 3 g cotyledons were finely sliced and extracted with 10 ml of 35% (w/v) sucrose in 0.025 M NaPi, 0.025 M citric acid, pH 7.5, by gentle grinding for 1 min in a chilled mortar. Extracts were stirred for 10 min and then centrifuged at 165 *g* for 5 min. Approximately 1.5–2.0 ml of the supernatant fluid was carefully layered onto linear 50–90% (w/v) sucrose gradients in 0.025 M NaPi, 0.025 M citric acid, pH 7.5, prepared in 23 ml polycarbonate tubes. Gradients were centrifuged for 3 hr in a 3 × 23 aluminium swing-out rotor operating at 4° and 30000 rpm (R av. 94000 *g*). Gradients were fractionated by inserting a thin capillary tube through the gradient to the bottom of the tube and pumping the contents out. The gradient was monitored at 280 nm. Sucrose concentration was measured using a Bellingham and Stanley sugar refractometer. Samples were taken for electron microscopy, SDS gel electrophoresis and protein estimation.

(b) *By centrifugation in glycerol solutions.* The method used was communicated by Chrispeels [28]. 3 g meal was gently blended with 30 ml 80% glycerol in 0.05 M NaPi, 0.05 M citric acid, pH 5.0, containing 0.1% 2-mercaptoethanol at room

temp. The suspension was centrifuged for 10 min at 2000 *g*. When the supernatant was layered onto a cushion of 8 ml 90% (v/v) glycerol in water in a 50 ml centrifuge tube and centrifuged at 38000 *g* for 60 min at 15°, protein bodies were obtained as a pellet. Samples of some pellets were taken for electron microscopy and for SDS-gel electrophoresis; pellets were resuspended in 0.5 M NaCl, 0.05 M NaPi buffer, pH 7.5, for protein estimation. The procedure was also carried out in the glycerol Pi–citrate buffer adjusted to pH 7.5 with NaOH.

*Column chromatography.* (a) *Zonal isoelectric precipitation.* Supernatant fluid from an alkaline salt extract was adjusted to 70% saturation with respect to ammonium sulphate; precipitated protein was collected by centrifugation, dispersed in 0.2 M NaCl, 0.05 M NaPi, pH 8.0, made 0.1% in 2-mercaptoethanol, and dialysed against this buffer. It was then subjected to zonal isoelectric precipitation (29,30) using buffer of the same composition for elution.

(b) *DEAE-cellulose ion-exchange chromatography.* Ion-exchange chromatography was carried out on columns of Whatman DE 52 cellulose equilibrated in 0.025 M NaPi buffer, pH 7.2. Samples for chromatography were dialysed against this buffer prior to application. Elution was commenced with buffer of the same composition, subsequently with a linear NaCl gradient (routinely 0–0.5 M) in the same buffer.

*Sucrose density gradient centrifugation of proteins.* Samples of clarified alkaline salt extracts were dialysed against excess 0.5 M NaCl, 0.05 M NaPi pH 7.6. Approximately 1.5 ml was then carefully layered on to linear 5–20% (w/v) sucrose gradients in the same buffer and the gradients were centrifuged for 18 hr at 4° and 30000 rpm (R av. 94000 *g*) in a 3 × 23 ml swing-out rotor. Gradients were fractionated as described for the preparation of protein bodies by sucrose density gradient centrifugation. Fractions of 1.0 ml were collected.

*Estimation of protein.* Protein concentration was determined by the method of Lowry *et al.* [31] using bovine serum albumin as standard. Crude globulin preparations, prepared by lyophilisation of the precipitate formed by overnight dialysis against running tap water of an alkaline salt extract of *Phaseolus vulgaris*, gave a similar calibration curve up to a protein concentration of 200 μg/ml.

*Polyacrylamide gel electrophoresis.* Undissociated proteins were examined in 7.0% (w/v) acrylamide gels by the disc electrophoretic procedure of Ornstein and Davis [32] except that the spacer gel was omitted. The method of Weber and Osborn [33] was used to determine the apparent MWs of the subunits of protein dissociated with SDS (1%, w/v) and 2-mercaptoethanol (0.2%, v/v). MWs were routinely determined on 7.0% and 10.0% (w/v) acrylamide gels. To obtain maximum dissociation of subunits (i.e. the lowest MW's) it was found necessary to incubate samples in a boiling water bath for 3 min. Gels were stained for protein in 0.2% (w/v) Amido Black in 7.0% (v/v) HOAc and destained by diffusion in 7.0% (v/v) HOAc; densitometric traces were obtained by scanning the gels in transmission at 620 nm using a Joyce–Loebl Chromoscan. Gels were stained for glycoprotein by the PAS method of Zacharius *et al.* [34]. The location of bands after disc electrophoresis is described by their mobility relative to that of the bromophenol blue marker used (relative mobility, $R_m$). Molecular weights were determined in the SDS system by comparison of the mobility of subunits with that of standard proteins of known MW's.

*S-Carboxymethylation.* This was carried out by the method of Crestfield *et al.* [35].

*Direct analysis of bands in disc gels.* After electrophoresis of undissociated proteins in disc gels, the region corresponding to a particular band was cut from several gels in which the same sample had been electrophoresed; the pooled gel sections were ground and extracted with 0.025 M NaPi buffer pH 7.0 containing 2% (w/v) SDS and 2% (v/v) 2-mercaptoethanol.

*Gel isoelectric focusing in 7 M urea.* The method used was that of Wrigley [36].

*Amino acid analysis.* Duplicate samples of proteins in 6 N-HCl were hydrolysed *in vacuo* at 105° for 24 and 72 hr; their amino acid compositions were determined on a Locarte automatic-loading amino acid analyser. The cysteine-cystine content was determined as cysteic acid following performic acid oxidation [37].

*N-terminal amino acid analysis.* *N*-terminal amino acids were determined by the dansyl-method of Gros and Labouesse [38].

*Electron microscopy.* Samples of protein bodies were fixed in 2.5% (v/v) glutaraldehyde, 1% (w/v) osmic acid, 0.05 M sodium cacodylate buffer pH 7.0 in 80% sucrose (for samples from sucrose gradients) or in 80% glycerol (for samples from the glycerol procedure) for 3 hr at 4°. They were dehydrated in an alcohol series and embedded in Spurr's resin. Thin sections were post-stained with uranyl acetate and alkaline lead citrate and examined in an A.E.I. EM 66 electron microscope.

*Agglutination test.* Protein samples were prepared in 0.9% (w/v) sodium chloride pH 7.0, and mixed with an equal vol of 2% (v/v) suspension of Human group O erythrocytes in the same solution, at room temp.

*Trypsin inhibitor activity.* This was determined by the effect of alkaline salt extracts on the trypsin-catalysed hydrolysis of benzoyl-arginine-*p*-nitroanilide (BAPA); the second method described by Erlanger *et al.* [39] was used except that the assay was carried out in 0.05 M NaPi, 0.05 M citric acid buffer pH 7.5, at 35°. Assays were carried out such that the total inhibition did not exceed 60% of the control trypsin activity.

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