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COMPARISON OF GLOBULIN PROTEINS FROM *PHASEOLUS VULGARIS* WITH THOSE FROM *VICIA FABA*

R. C. McLeester, T. C. Hall, S. M. Sun and F. A. Bliss
Department of Horticulture, University of Wisconsin, Madison, WI 53706, U.S.A.

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Key Word Index—*Phaseolus*; *Vicia*; Leguminosae; globulin; seed protein; electrophoresis; legumin; vicilin.

Abstract—Extraction of maturing *Phaseolus vulgaris* seeds with an ascorbic acid—NaCl medium facilitated the preparation of two globulin fractions which were shown by acrylamide gel electrophoresis and by analytical ultracentrifugation to be completely separated from each other. These two fractions, which are equivalent to legumin and vicilin, were shown to differ from similar fractions from *Vicia faba*. Dissociation of the proteins of each globulin fraction by sodium dodecyl sulphate resulted in differing peptide band patterns for these two plants. Conditions necessary for fractionating the individual globulin components from bean seeds are discussed, and the usefulness of the specific names legumin and vicilin is questioned.

INTRODUCTION

An electrophoretic study of globulins of several members of the Leguminosae revealed both similarities and differences between the seed proteins of the species studied.[1] Sufficient variability of the total protein complement in cultivars of one species has been shown to exist[2] to suggest that there is potential for increasing the nutritional value of individual species by selective breeding;[3] and, despite the preponderance of globulin proteins in the seed, the albumin fraction has also been shown to be important in this respect.[4]

Characterization of the individual seed proteins is a fundamental requirement for both basic and applied studies on the legume proteins. However, considerable difficulty has been encountered in obtaining pure samples of even the major globulin components of legume seed proteins.[5–10] This article details the electrophoretic separation of proteins extracted from different tissues of the seed of *Phaseolus vulgaris* and presents a rapid method for the isolation of two globulin fractions from the cotyledons which are electrophoretically distinct. These globulins appear to be markedly different from the fractions obtained by similar procedures from the seed of *Vicia faba*, and the peptide subunits of these globulins also indicate major differences between the storage proteins of these plants.

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RESULTS

The profiles of proteins separated from different tissues of *P. vulgaris* by acrylamide gel electrophoresis of extracts made in an alkaline-salt buffer are shown in Fig. 1a–d. The proteins of the cotyledons are of nutritional importance, and intensive synthesis of globulins in the cotyledon tissues results in marked changes in the total seed proteins during development.[11] The proteins of the cotyledons may be fractionated into globulins, acid-soluble albumins and alkaline-soluble albumins (Fig. 1e–g).

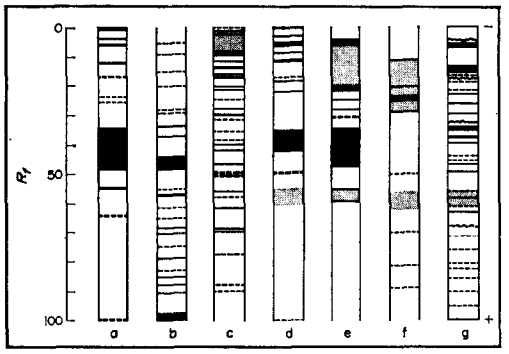

Fig. 1.

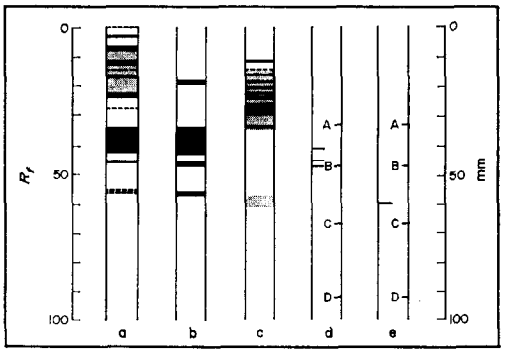

Fig. 2.

Fig. 1. Discontinuous gel electrophoresis of various tissues and protein fractions from *Phaseolus vulgaris* seeds.
Alkaline-salt extracts of proteins from, (a) the whole seed, (b) the testa, (c) the embryo, (d) the cotyledons. The cotyledon extract was separated into, (e) the globulin fraction, (f) the acid-soluble albumins, and (g) the alkaline-soluble albumins. Further details are given in the text and in the Experimental.

Fig. 2. Acrylamide gel electrophoresis of globulins extracted from *Phaseolus vulgaris* cotyledons.
Discontinuous gel patterns are shown for (a) the total globulin extract prepared by extensive dialysis of the acid-extracted cotyledon proteins against distilled water, (b) the Gl globulin fraction, (c) the G2 globulin fraction. The globulin fractions were dissociated with SDS-urea-mercaptoethanol and run in split gels together with an equal volume of marker proteins (right-hand side of the gels) using a continuous electrophoretic system, (d) G1 fraction peptides, (e) G2 fraction peptide. The proteins used as markers were: A, bovine serum albumin (MW 68 000), B, ovalbumin (43 000); C, chymotrypsinogen A (25 700); D, lysozyme (14 300); insulin (2900) is not shown in the figure, but ran at 119 mm from the top of the gel. The scale at the left denotes the movement of proteins in the discontinuous gels relative to bromophenol blue tracking dye, that at the right indicates the distance traveled by the polypeptides in the continuous gels from the top of the gel. All samples were run from the top of the figure towards the anode at the bottom.

The globulin fraction of *P. vulgaris* is usually regarded as consisting of two major components termed vicilin and legumin.[6] Despite the high proportion of the total seed protein which such globulins represent in edible legumes (90% in the case of *V. faba*)[12] complete resolution of these fractions from *P. vulgaris* has not been demonstrated previously. Ammonium sulphate precipitation has been frequently employed to isolate legume globulins.[5,6,10,13] The ammonium sulphate procedure yields fractions rich in individual globulin components, but is less effective in separating them than is water precipitation.[6] As an

[11] T. C. Hall, R. C. McLeester and F. A. Bliss, *Phytochem.* **11**, 647 (1971).
[12] C. J. Bailey, A. Cobb and D. Boulter, *Planta* **95**, 103 (1970).
[13] N. Catsimpoolas, D. A. Rogers, S. J. Circle and E. W. Meyer, *Cereal Chem.* **44**, 631 (1967).

alternative to the usual procedure of initial extraction in alkaline-salt buffer, we tested the use of acidic extraction conditions. This seemed to be a useful approach because the globulins of *P. vulgaris* are soluble at acid pH values (e.g. in 1 M acetic acid, pH 2·4) while the bulk of proteins of the albumin fraction are precipitated under such conditions; hence they are not extracted by the ascorbate system (see Experimental) and will not contaminate the globulin fraction. The globulins then can be readily separated from the relatively low amount of acid-soluble albumins by decreasing the salt concentration of the extract. The appearance of the total globulin extract obtained by acid extraction and dialyses against distilled water is shown in Fig. 2a, and is similar to that obtained from alkaline extracts (Fig. 1e).

Dilution of the acid-extracted globulins with an equal volume of distilled water (resistivity greater than 10^6 ohms/cm^2) resulted in the precipitation of a fraction (G1) which is equivalent to that designated as legumin by Danielsson.[6] This fraction was pelleted by centrifugation, and the salt concentration of the supernatant further reduced by dialysis against distilled water when a second globulin fraction (G2) precipitated. The G2 fraction corresponds to the vicilin fraction obtained by Danielsson.[6] The fractions G1 and G2 were distinct from each other when subjected to disc electrophoresis (Fig. 2b,c). It is not immediately apparent that the banding pattern obtained on gel electrophoresis of the total globulin fraction (Fig. 2a) is equivalent to a summation of the G1 and G2 fractions (Fig. 2b,c). However, since the G1 fraction preponders, application of a large amount of protein to the gel is necessary to show the presence of bands in addition to those of the G1 fraction.

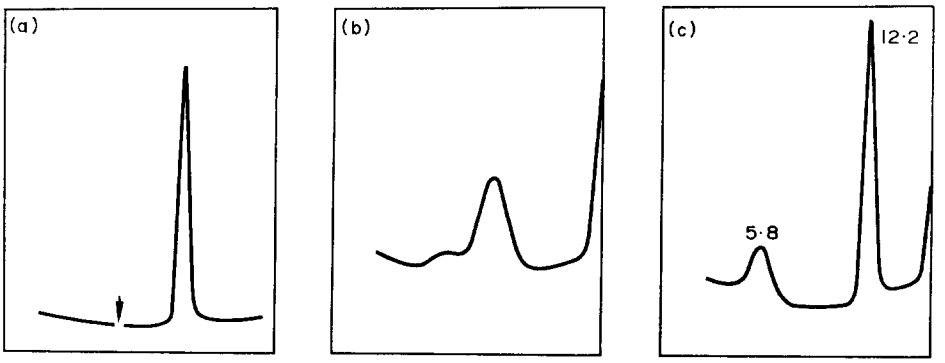

FIG. 3. ULTRACENTRIFUGATION OF THE G1 AND G2 FRACTIONS FROM *Phaseolus vulgaris*. The G1 fraction had a sedimentation coefficient of 12·2 S and is shown in (a); the photograph was taken 24 min after reaching a speed of 56 000 rpm at a phase plate angle of 63° and temperature of 20°. Protein concentration was 4 mg/ml, the high concentration shown being chosen to illustrate the very small amount of other components present in this fraction (arrow). The G2 fraction had a sedimentation coefficient of 5·8 S and is shown in (b); the photograph was taken 72 min after reaching a speed of 56 000 rpm at a phase plate angle of 43° and a temperature of 20°. Protein concentration was 4 mg/ml. A mixture of G1 (2 mg protein) and G2 (2 mg protein) fractions is shown in (c); the photograph being taken 32 min after reaching a speed of 56 000 rpm at a phase plate angle of 53° and a temperature of 20°. In all photographs sedimentation proceeds from left to right (*Note.* The above figures were redrawn from a photograph).

Despite the heavy loading, the bands corresponding to the proteins of the G2 fraction are not clear because of their low concentration relative to that of the G1 fraction. Further, the high amount of protein present in the gel slows the migration of the individual proteins, hence a lack of correlation in R_p values with those obtained for levels of the separated G2 fraction used (Fig. 2c) to show clearly the multiple protein bands present. A high amount of

G1 fraction protein was chosen for Fig. 2b to demonstrate the absence of G2 fraction proteins after separation of the two fractions, and also to show the presence of the bands at R_p 18, 46 and 58 which are often not apparent when low levels of G1 protein are electrophoresed.

The G1 and G2 fractions were clearly different when characterized by analytical ultracentrifugation (Fig. 3), and dissociation of the proteins of these fractions with SDS yielded entirely different peptide components (Fig. 2d,e), having the apparent MWs presented in Table 1.

Table 1. Apparent MWs of polypeptides of the G1 and G2 fractions from *Phaseolus vulgaris* and *Vicia faba* cotyledons

Phaseolus fraction		*Vicia* fraction	
G1	G2	G1	G2
53 000	32 000	68 000	23 000
47 000		55 000	13 000
43 000		47 000	10 500
		36 000	
		21 250	

These values were obtained from the positions of the polypeptides on discontinuous gels (see Figs. 2 and 4) in relation to the marker proteins. A logarithmic plot of the MW of the marker proteins against the distance traveled from the top of the gel yielded a straight line.

Some differences in banding pattern have been observed to result from changes in the extraction and final solution media. When Dow Corning FG-10 antifoam emulsion was used a single band at R_p 18 was observed in the G1 fraction (Fig. 2b), while polyvinylpyrrolidone-extracted material had two sharply resolved bands at R_p 17, 19. If the G1 fraction was dissolved in 0·5 M NaCl then an additional band at R_p 10 can be detected which is absent when the G1 fraction is dissolved in 0·5 M NaCl containing 0·25 M ascorbate (Fig. 2b). This band at R_p 10 was also much more intense when G1 from alkaline-salt extracted cotyledons was dissolved in 0·5 M NaCl buffered at pH 7·6 with 0·1 M Tris than from the same extract dissolved in 0·5 NaCl alone.

The seed globulins of *V. faba* are also separable into fractions designated legumin and vicilin,[8] and protein was also extracted from the cotyledons of *V. faba* with acid. While we obtained two protein fractions from the seed which were procedurally analagous to the G1 and G2 fractions from *P. vulgaris*, it was evident that considerable differences existed in the properties of these globulin fractions obtained from the two genera. Electrophoretically the G1 fraction from *P. vulgaris* gave protein bands with R_ps differing from those obtained from the *V. faba* G1 fraction (Fig. 4a,c). The G2 fractions also showed entirely different band patterns. When the G1 fraction obtained from *V. faba* was dissolved in 0·5 M NaCl containing 0·25 M ascorbic acid it dissociated into material giving many bands on disc gel electrophoresis (Fig. 4b). This dissociation was reversible, and removal of ascorbate by dialysis against distilled water followed by solution of the precipitated globulin in 0·5 M NaCl resulted in an electrophoretic pattern (Fig. 4c) identical to that obtained for *V. faba* G1 protein not treated with ascorbate (Fig. 4a).

Studies on the structure of *V. faba* legumin[14] and vicilin[15] have shown that different peptide components are resolved for each fraction by treatment with SDS. *Vicia* globulin fractions G1 and G2 were dissociated with SDS and run under the same conditions used for similarly treated *Phaseolus* globulins (Fig. 2d,e). The banding pattern for *V. faba* fractions, shown in Fig. 4e,f, was different from that for *P. vulgaris* peptides. One polypeptide from the G1 fractions prepared from the two plants gave an apparent MW of 47 000, while the remaining polypeptides were distinct from each other (Table 1).

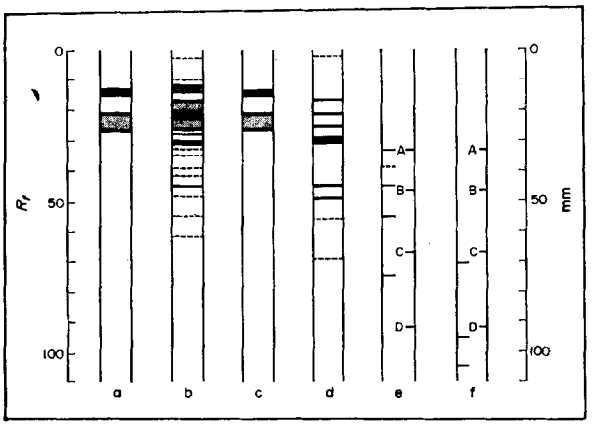

FIG. 4. ACRYLAMIDE GEL ELECTROPHORESIS OF GLOBULINS EXTRACTED FROM *Vicia faba* COTYLEDONS. Discontinuous gel patterns are shown for, (a) the G1 fraction dissolved in 0·5 M NaCl, (b) the G1 fraction dissolved in 0·5 M NaCl containing 0·25 M ascorbate, (c) reconstituted G1 fraction by dialysis of the ascorbate-treated extract, (d) the G2 fraction. The globulin fractions were dissociated and electrophoresed on split gels in a similar manner to those from *Phaseolus* (see Fig. 2), (e) the G1 fraction peptides, (f) the G2 fraction peptides. The marker proteins, scales and direction of electrophoresis were the same as in Fig. 2.

DISCUSSION

Isolation of Seed Proteins

Legume seeds are very suitable materials for the study of plant proteins because of the high proportion of proteins they contain, and also because there appear to be a very limited number of molecular species present in the storage proteins in the developing cotyledons. The storage globulin accumulating in maturing *P. vulgaris* cotyledons effectively dilutes out the other proteins, resulting in a differing electrophoretic profile during development.[11] In Fig. 1a the profile of proteins from a 15-mm long seed is dominated by the cotyledon proteins (Fig. 1d), and the globulin fraction (Fig. 1e) is predominant in the cotyledon extract. However, many other proteins are present throughout the development of the seed, as demonstrated by electrophoresis of extracts from the testa and embryo (Fig. 1b,c). That the cotyledons have proteins in addition to the globulins is evidenced by the albumin fractions shown in Fig. 1f,g. Enzymes are present in protein extracts of the seeds, and their activity can be detected in the presence of the globulin fraction.[16,17] In attempting to purify

[14] C. J. BAILEY and D. BOULTER, *Eur. J. Biochem.* **17**, 460 (1970).
[15] C. J. BAILEY and D. BOULTER, *Phytochem.* **11**, 59 (1972).
[16] D. A. THURMAN, D. BOULTER, E. DERBYSHIRE and B. L. TURNER, *New Phytol.* **66**, 37 (1967).
[17] T. C. HALL, *J. Am. Soc. Hort, Sci.* **95**, 355 (1970).

the globulin fractions the numerous proteins present in the seed should, therefore, be taken into consideration and suitable precautions followed to prevent them contaminating the globulin preparation.

Separation of the cotyledons from the testa and embryo is a simple procedure for removing proteins which would otherwise derive from these seed tissues. Extraction in alkaline-salt buffer results in extraction of most of the seed albumins in addition to the globulins, and their subsequent complete removal may prove difficult. The use of an acid extraction medium is useful in that only a small part of the albumin fraction is solubilized. The globulins of *P. vulgaris*, while soluble in ascorbate–NaCl solution are then less soluble than in alkaline conditions, and may readily be precipitated isoelectrically. This avoids the necessity for dialysis steps requiring several days, and the opportunity for microbial degradation of the sample is greatly reduced. Since there is a high level of glutamic and aspartic acids present in legume globulins[8] these proteins readily form esters, and the solubility of the globulin will in part depend upon the salt form it is in.[18] Separation of globulin fractions by ammonium sulphate precipitation[5,10,13] is a useful procedure but does not completely remove one fraction of globulin from others, as was clearly shown for pea vicilin and legumin by Danielsson.[6] Extraction of globulins in water alone[19] is not particularly effective, since the globulins are, by definition, salt soluble. Osborne[20] noted that *Phaseolus* proteins were poorly extracted with water compared with many other legume seed proteins. It would seem that water extraction will favour solubilization of globulins requiring relatively low salt concentrations (the G2 fraction) over the fractions requiring higher salt concentrations. Indeed, it is possible to extract the *P. vulgaris* G2 globulin fraction alone (little or no G1) by extracting the cotyledons with 0·25 M ascorbate containing 0·2 M NaCl. This procedure reduces preparation time to less than 5 hr in place of the several days required by other methods.[15] Reduction of the temperature of salt extracts decreases the conductivity of the solution, which appears to result in precipitation of protein as a 'cold-insoluble fraction'. A combination of water extraction and decreasing temperature has been used to give relatively homogeneous preparations of soya bean protein.[13,21]

The necessity for protection of thiol groups during the extraction of legume globulins appears debatable. Koshiyama reported[22] that disulphide bonding had nothing to do with binding between subunits of 7*S* soya bean globulin, while precipitation of the 11*S* component as a result of disulphide bonding was described by Hashizume, Kakiuchi, Koyama and Watanabe.[23] In general, the maintenance of reducing conditions during protein extraction appears desirable. Ascorbate provides an inexpensive means to this end, but it is probably not suitable for proteins which dissociate in acid conditions,[24] such as was observed for the G1 fraction from *V. faba* (Fig. 4b). Mercaptoethanol[13] and dithiothreitol[11] have been used as thiol protectants in legume seed extractions.

Electrophoretic pattern of Phaseolus *Seed Proteins*

Boulter *et al.* commented[1] that the number of bands observed in a particular region of a gel may vary according to the amount of protein electrophoresed. The bands observed will

[18] L. J. Henderson, in *The Vegetable Proteins*, Longmans-Green, New York (1924).
[19] R. C. Roberts and D. R. Briggs, *Cereal Chem.* **42**, 71 (1965).
[20] T. B. Osborne, in *The Vegetable Proteins*, Longmans-Green, New York (1909).
[21] A. C. Eldridge and W. J. Wolf, *Cereal Chem.* **44**, 645 (1967).
[22] I. Koshiyama, *Agric. Biol. Chem.* **35**, 449 (1971).
[23] K. Hashizume, K. Kakiuchi, E. Kotama and T. Watanabe, *Agric. Biol. Chem.* **35**, 449, (1971).
[24] A. M. Altschul, L. Y. Yatsu, R. L. Ory and E. M. Engleman, *Ann. Rev. Plant Physiol.* **17**, 113 (1966).

also depend upon the relative amounts of tissues taken (e.g. proportion of cotyledon to testa), and whether any particular fraction (such as the globulin) has been removed, as discussed above. Some bands are quite characteristic: in the albumin fraction (Fig. 1g) there are four bands (R_p, 3, 18, 32, 68) which always appear squiggly, even when the other bands, including those next to them, are sharp and straight. The globulin fraction G1 invariably gives a rather diffuse banding pattern having the appearance shown in Fig. 2b. The multiple bands are not artifacts resulting from overloading since they may be seen when less than 100 μg protein are applied to the gel and visualized with Coomassie Brilliant Blue. Evidence is accumulating to suggest that the G1 fraction exists in an equilibrium state of the four bands. It is possible that the native globulin dissociates during electrophoresis since a single, sharp, peak can be seen when the G1 fraction is subjected to sedimentation velocity centrifugation (Fig. 3a). The powerful resolution of electrophoresis may permit separation of the equilibrium mixture of the G1 fraction after it has stacked on the gel. However, as the protein runs down the separating gel each band tends to dissociate so as to reestablish the equilibrium, resulting in hazy edges to the bands. If the lack of sharpness was caused by overloading or retention in the gel any streaking should trail rather than precede the individual bands, while observation of the globulin bands shows that there are diffuse edges at both the leading and trailing ends, hence dissociated molecules of a relatively high charge-density are electrophoresing ahead of the intact protein.

Differences between Phaseolus *and* Vicia *Globulins: Validity of the Terms Legumin and Vicilin*

The electrophoretic behaviour of the G1 and G2 fractions obtained for proteins extracted from these two species on both discontinuous and continuous gel systems (Figs. 2 and 4) strongly suggests that they are very different proteins. The banding pattern for the *V. faba* globulin G1 appears identical to the published pictures of *V. faba* legumin prepared by other investigators.[1,8] The reversible dissociation of the G1 fraction from *V. faba* (Fig. 4b,c) by ascorbate was striking and requires further study. No such dissociation has been observed for the G1 fraction from *Phaseolus*. The G1 fraction from *P. vulgaris* is soluble in 1 M HOAc while that from *V. faba* is only partially soluble. To solubilize 2·5 mg protein of the *V. faba* G1 fraction 1 ml of 2 M NaCl was required, while a similar amount of *P. vulgaris* G1 will completely dissolve in 1 ml of 0·5 M NaCl.

Our procedure for the isolation of the G1 and G2 fractions is a modification of the method described by Danielsson[6] for the isolation of legumin and vicilin, and analytical ultracentrifugation (Fig. 3) confirmed our G1 fraction to be equivalent to legumin and the G2 fraction to be equivalent to vicilin. While we believe that the *Phaseolus* G1 fraction represents homogeneous protein, we believe the G2 fraction to contain several proteins. The multiple banding pattern observed on disc gels (Fig. 2c) appears too complex for a molecule having a particle weight of less than 200 000 daltons. While one polypeptide can be clearly seen on SDS gels (Fig. 2e) several other bands, which are poorly defined, may be seen. While the profile of the G2 fraction from *V. faba* (Fig. 4d) appeared rather clearer than that recently published by Bailey and Boulter,[15] we concur with the opinion of these authors that the band pattern is not typical of a pure protein. The banding pattern obtained for SDS-dissociated G1 protein (Fig. 4e) is different to that obtained by Bailey and Boulter[14] for legumin, although when a lower amount of material is added to the gel the band at 39 mm is difficult to distinguish. Our estimates (Table 1) of MW values for the G1 polypeptides appear rather lower than those published by the above authors for carboxymethyl

derivatives of the *Vicia* legumin polypeptides. The dissociated G2 fraction from *V. faba* shows four major bands on SDS gels, as was observed for the carboxymethyl derivative of vicilin.[15] In our experience the additional bands appear more distinct than in Bailey and Boulter's material, but this could easily be due to differences in sample preparation and amount of protein applied to the gel. As with the material described by the above authors, it is most unlikely that any of the bands observed in the G2 fraction derive from the G1 fraction (i.e. that the vicilin is contaminated with legumin). This is especially the case for our *P. vulgaris* preparations with which we have had considerable experience, and which show widely differing band patterns for the G1 and G2 fractions both by discontinuous and by continuous gel electrophoresis as well as being completely distinct by analytical ultracentrifugation (Fig. 3a–c). The above evidence leads us to conclude that the G1 and G2 fraction globulins from *P. vulgaris* are different from each other, and also from *V. faba* globulins. This is not to deny that there are many similarities between these proteins, such as their generally similar solubility properties and amino acid composition in addition to their similar function as storage proteins of related plants.

The terms legumin and vicilin have been applied to globulin fractions obtained from the seeds of many different legume plants, hence it may well be thought that the isoelectrically-prepared fraction called legumin obtained from *Phaseolus* is identical to that of *Vicia*. This is not the case, nor is vicilin from *Phaseolus* identical to vicilin from *Vicia*. Previous studies have also shown considerable differences between the globulins of several genera of the Leguminosae.[1,8] Osborne and Campbell[25] detailed several tests for legumin, and noted that the name legumin was first proposed by Braconnot in 1827. It is doubtful if all the legume globulins currently termed legumin in the literature would conform to these tests, and certainly the more rigorous tests for homogeneity of proteins now available distinguish between these proteins obtained from differing species. A similar argument can be advanced against the use of vicilin as denoting a uniform globulin fraction from seeds of different species. We feel that the terms legumin and vicilin are no longer meaningful, and indeed are confusing since they indicate similarities which do not exist. Recent articles on soya bean proteins refer to the protein preparations by centrifugal characteristics, such as the 7S component[19,23] and the 11S protein.[26] In this paper we have referred to the globulins as the preparative fractions G1 and G2, and we suggest that specific names should not be used until the individual proteins have been characterized by modern procedures and shown to be homogeneous.

EXPERIMENTAL

Plant culture. Bean plants (*Phaseolus vulgaris cv.* 'Tendergreen') were germinated in moist vermiculite and then transferred to tanks where they were grown in aerated Hoagland's nutrient solution at the University of Wisconsin Biotron. The plants were illuminated for 16 hr each day, with day and night temp. and humidities of 29° and 74% and 18° and 77% respectively. Light and temperature were gradually increased over 1 hr 'dawn' and 'dusk' periods. Under these conditions the seeds attained a length of 15 mm in *ca.* 52 days from sowing the seed. Seeds of 15–18 mm were used for the studies reported here. Extracts of the broad or horse bean (*Vicia faba*) were made from mature seed obtained commercially.

Preparation of alkaline extracts. The seeds were removed from the pods, and the testa, embryo and cotyledons separated. The protein was obtained by grinding the tissue in 0·1 M Hepes [1-(*N*-2-hydroxyethylpiperazin-*N'*-yl) ethanesulphonic acid] buffer brought to pH 7·4 with KOH, and containing 5 mM dithiothreitol, 0·5 M NaCl and 15% (w/v) insoluble PVP. The extracts were filtered, dialysed and concentrated as described previously.[11]

Preparation of acidic extracts. Cotyledons (5 gm fr. wt) were excised and ground at 4° under N_2 using a VirTis '45' homogenizer into a medium (50 ml) of 0·5 M NaCl, 0·25 M ascorbic acid and 0·5% (v/v) Dow

[25] T. B. Osborne and G. F. Campbell, *J. Am. Chem. Soc.* **18**, 583 (1896).
[26] W. J. Wolf and D. A. Sly, *Cereal Chem*, **44**, 653 (1967).

Corning FG-10 antifoam emulsion. Six 30-sec bursts at full speed usually completely macerated the tissue, which was then filtered through acetate taffeta cloth (previously washed and rinsed in distilled H_2O). The filtrate was centrifuged (4°) at 30 000 *g* for 30 min. The supernatant was decanted and further clarified by centrifugation at 30 000 *g* for 30 min (2×). The pellet from each of these three centrifugations was discarded. To the final clear opalescent supernatant an equal volume (40 ml) of distilled H_2O having a resistivity greater than 10^6 ohms/cm^2 was added. A white precipitate formed immediately, and was pelleted by centrifugation at 30 000 *g* for 30 min. The pellet was designated globulin fraction G1, and was dissolved in either 0·5 M NaCl or in 0·5 M NaCl containing 0·25 M ascorbic acid. A small portion of the supernatant was diluted with twice its volume of distilled water to check that no further precipitation occurred. The remainder of the supernatant was dialysed at 4° against distilled water (4 l.) for a total of 4 hr., the water being changed at least 3×. A white precipitate formed which was pelleted by centrifugation at 30 000 *g* for 30 min. This pellet was designated globulin fraction G2, and was dissolved in either 0·5 M NaCl or in 0·5 M NaCl containing 0·25 M ascorbate. The supernatant gave no further precipitate on prolonged dialysis, and was designated the acid albumin fraction.

Dissociation of protein fractions. The proteins were dissociated into their polypeptide components either by dialysis against a solution containing 8 M urea, 1·5% (w/v) SDS and 1·5% (v/v) mercaptoethanol or by heating in the same solution to 100° for 2 min in a water bath. No differences in the final banding patterns were noted between the two procedures, although incomplete stages of dissociation were observed if the sample was not dialyzed long enough: further dialysis resulted in complete dissociation.

Electrophoresis. The discontinuous system for electrophoresis was that of Davis,[27] using 7% (w/v of monomer) acrylamide gels at pH 8·9 (Tris-glycine, 4°). SDS gels were run in the continuous buffer system of Maizel,[28] as modified by Linda Hall (cited by Medappa, McLean and Rueckert[29]) to contain 0·01 M mercaptoproprionic acid to prevent protein association during electrophoresis. The SDS gels contained 10% (w/v) of monomer. The amount of protein applied to the gel was varied to yield clear banding patterns, and depended upon the stain used for visualization. For extracts containing few bands, such as the dissociated G1 globulin fraction, as little as 25 μg protein were applied, while extracts having many bands required as much as 300 μg protein, even when Coomassie Brilliant Blue was used as the stain.[30] R_ps were assigned relative to the distance traveled by the bromophenol blue marker dye from the top of the separating gel.

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