Phytochemistry, 1973, Vol. 12, pp. 79 to 84. Pergamon Press. Printed in England.

A COMPARISON OF ACID EXTRACTED GLOBULIN FRACTIONS AND VICILIN AND LEGUMIN OF *VICIA FABA*

D. J. WRIGHT and D. BOULTER

Department of Botany, University of Durham, Durham City, England

(*Received* 25 *July* 1972. *Accepted* 15 *August* 1972)

Key Word Index—*Vicia faba*; Leguminosae; broad bean; acidic extraction; globulins.

Abstract—Two globulin fractions, obtained by extraction using acidic conditions, were characterized by their sedimentation properties, subunit compositions and amino acid *N*-termini, and were compared with classical vicilin and legumin preparations. Fraction I had some properties in common with vicilin, but there were differences and it was not possible to equate these two preparations completely. The use of an acidic extractant was discussed.

INTRODUCTION

LEGUMIN and vicilin,[1,2] the two storage proteins of many legume seeds, are normally prepared by extraction into salt solutions buffered to pH 7 or thereabouts. However, the pH of the extractant can have a marked effect on the peptization of the protein.[3] Smith and Circle[4] demonstrated that, in the case of defatted soyabean meal, the amounts of protein extracted varied markedly with the pH. Low yields were obtained in the pH 4–5 region, which corresponded with the isoelectric points and hence minima in solubilities of the proteins, while 85% extraction of proteins was achieved above pH 7 and also in the region of pH 2.

For this reason, therefore, an acidic extraction of globulins from *Vicia faba* was attempted. Ascorbic acid[5] was used since this has been shown to be effective also as an antioxidant for proteins during gel electrophoresis.[6] The extraction was thus carried out under reducing conditions, thereby minimizing the possibility of disulphide formation leading to protein aggregates.[7] The addition of 2-mercaptoethanol or dithiothreitol to the extraction medium has been the usual method for obtaining reducing conditions by other workers.[8,9]

RESULTS

Using the acidic extraction procedure outlined in the Experimental, two globulin fractions were obtained. Fraction I precipitated out when the ionic strength was reduced

[1] T. B. OSBORNE, *The Vegetable Proteins*, 2nd Edn, Longmans-Green, New York (1924).
[2] C. E. DANIELSSON, *Biochem. J.* **44**, 387 (1949).
[3] J. A. SCHELLMAN and C. SCHELLMAN, in *The Proteins* (edited by H. NEURATH), 2nd Edn, Vol. 2, Academic Press, New York (1964).
[4] A. K. SMITH and S. J. CIRCLE, *Ind. Eng. Chem.* **30**, 1414 (1938).
[5] Suggested to us by Dr. T. GRAHAM (Belfast) as essentially the procedure used by T. C. HALL (Wisconsin).
[6] C. POLTER and W. R. MUELLER-STOLL, *Z. Naturforsch. B.* **25**, 695 (1970).
[7] D. R. BRIGGS and W. J. WOLF, *Arch. Biochem. Biophys.* **72**, 127 (1957).
[8] A. O. TUCKER and D. E. FAIRBROTHERS, *Phytochem.* **9**, 1399 (1970).
[9] T. C. HALL, R. C. MCLEESTER and F. A. BLISS, *Phytochem.* **11**, 647 (1972).

by one-fold dilution with deionised water and fraction II was obtained as a precipitate after dialysing the remaining supernatant.

For the purposes of comparison, samples of legumin and vicilin were also subjected to ascorbic acid treatment. When ascorbic acid solutions of legumin and vicilin were diluted one- and even two-fold, no precipitate formed. These solutions were fairly stable, and samples remained clear for many weeks. However, they were susceptible to oxidation since precipitation occurred on passing a stream of air through the solutions, After dialysis of the original solutions for 9 days, a faint precipitate appeared. This was removed by centrifugation, and the solutions, which were very opalescent at this stage, were allowed to stand at 5°. After a further 3 days complete precipitation had occurred, as judged by the lack of absorbance at 280 nm.

Fractions I and II, as well as vicilin and legumin preparations, were examined in the ultracentrifuge. Fraction I in 0·5 M NaCl gave a single sedimenting species with a $S_{20,w}$ value of 7·3*S*. Using Halsall's equation,[10] this gives a MW of 167 000 ± 25 000 for fraction I. However, when dissolved in 0·5 M NaCl containing 0·25 M ascorbic acid, no single peak was observed. Instead a sedimenting 'plateau' was observed with its origin at the meniscus. Even after 2 hr in the centrifuge there was still no evidence of peak formation. Similar results were obtained with fraction II dissolved in either 0·5 M NaCl or 0·5 M NaCl, 0·25 M ascorbic acid. The sedimentation coefficient, determined for the leading edge of the 'plateau', was 3·1*S* which corresponds to a MW of 46 000 ± 7000. Legumin dissolved in 0·5 M NaCl produced one major peak with $S_{20,w} = 11{\cdot}8S$ and two minor components with $S_{20,w}$ values of 8·6*S* and 17*S*. MW calculations give values of 340 000 ± 50 000, 211 000 ± 30 000 and 585 000 ± 90 000 respectively for these three components. If it is assumed that the 11·8*S* component is similar to the 12*S* protein isolated from *Pisum sativum*,[2] then another estimate of its MW can be obtained from the Svedberg equation,[11] using $D^\circ = 3{\cdot}49$ as the value[2] for the diffusion constant. This gives a value of 314 000 for legumin. Vicilin, in 0·5 M NaCl, produced one component in the ultracentrifuge with $S_{20,w} = 7{\cdot}1S$, giving a MW of 150 000 ± 25 000.

Electrophoretic patterns obtained from the various samples are shown in Fig. 1. Fraction I dissolved in 0·5 M NaCl gave one main band with R_m 0·18 and several other minor bands, notably one at 0·22 (Fig. 1a). When dialysed overnight against 0·5 M NaCl before electrophoresis these minor bands disappeared leaving only the band at 0·18. In the presence of ascorbic acid, one major component of fraction I appeared, R_m 0·22, with four other components of greater mobilities (Fig. 1b).

Several well-defined components separated out on gels when fraction II, dissolved in 0·5 M NaCl, was electrophoresed (Fig. 1c). R_m's of major bands are 0·28, 0·62, 0·75, 0·91 and 1·0. In the presence of ascorbic acid, a similar pattern was produced although the slower moving bands were much less in evidence.

When samples from the ascorbic acid solutions of legumin and vicilin were electrophoresed, they produced numerous bands, indicating quite extensive dissociation. However, analysis of samples after extended dialysis gave much simplified patterns (Fig. 1d and e). Prominent bands in the legumin sample are at 0·14 and 0·22 while those in the vicilin sample are at 0·14 and 0·28. Figures 1f and 1g show typical band patterns obtained when legumin and vicilin in dil. NaCl are electrophoresed.

[10] H. B. HALSALL, *Nature, Lond.* **215**, 880 (1967).
[11] T. SVEDBERG and K. O. PEDERSON, *The Ultracentrifuge*, Oxford University Press, London (1940).

The subunit compositions of fractions I and II were examined by SDS (sodium dodecyl sulphate) gel electrophoresis. The results are presented in Fig. 2. Similar electrophoretic patterns were produced by fraction I, (a) after carboxymethylation, (b) after incubation in 0·2% (w/v) SDS for 3 hr, and (c) after incubation in 0·2% (w/v) SDS containing 2% (v/v) 2-mercaptoethanol for 3 hr (Fig. 2a). Fraction II gave very diffuse bands when electrophoresed after reduction in 0·2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol; only after carboxymethylation were the separate bands identifiable (Fig. 2b). The pattern produced by fraction II after incubation in SDS alone is shown in Fig. 2c. The low MW bands seen in Fig. 2b are still in evidence, but the presence of higher MW species probably indicates incomplete dissociation.

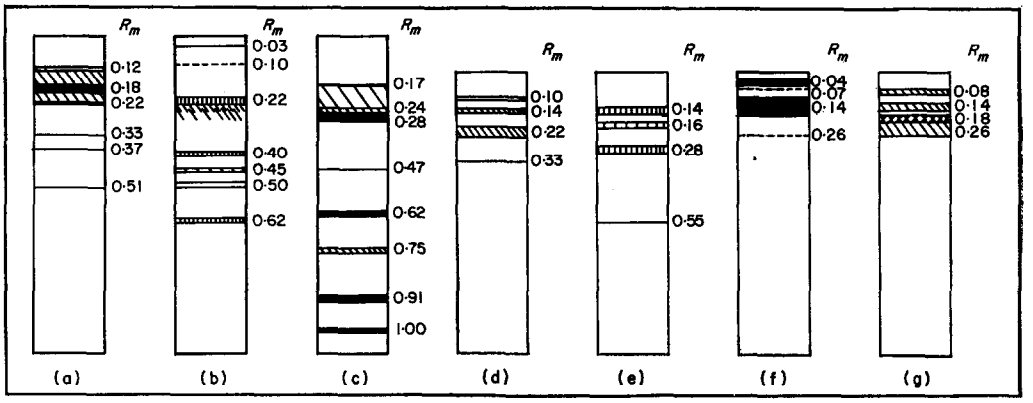

FIG. 1. ACRYLAMIDE GEL ELECTROPHORESIS ON 7·5% GELS OF (a) FRACTION I IN 0·5 M NaCl, (b) FRACTION I IN 0·5 M NaCl, 0·25 M ASCORBIC ACID, (c) FRACTION II IN 0·5 M NaCl, (d) ASCORBIC ACID—TREATED LEGUMIN, (e) ASCORBIC ACID—TREATED VICILIN, (f) LEGUMIN, AND (g) VICILIN.

N-Terminal analyses were performed on fractions I and II and the following *N*-termini were obtained. Fraction I: leucine, glycine and alanine with strong traces of aspartic acid, glutamic acid, serine, threonine and phenylalanine; fraction II: leucine, valine, alanine, glycine and a strong trace of threonine.

DISCUSSION

Danielsson observed[2] that when globulin solutions were diluted at 0°, legumin precipitated out, while vicilin remained in solution. Also earlier experiments of Osborne[1] had shown that vicilin was soluble in more dilute salt solutions than legumin. On this basis, fraction I, which precipitated out on dilution, could be legumin, and fraction II, which remained in solution, vicilin.

However, analysis of these two fractions in the ultracentrifuge indicates that this is not so. Analysis of fraction I taken up in 0·5 NaCl, in the ultracentrifuge demonstrated that it consisted of one component, with a $S_{20,w}$ value of 7·3*S*. Analysis of fraction II under the same conditions gave a sedimenting plateau, the leading edge of which had a sedimentation coefficient of 3·1*S*. Ultracentrifugation studies of vicilin and legumin by us gave results which are in agreement with those of other workers,[2,12,13] namely a $S_{20,w}$ value of 7·1*S* for vicilin and 11·8*S* for legumin. Thus, if fractions I and II are related to legumin and

[12] B. P. BRAND and P. JOHNSON, *Trans. Faraday Soc.* **54**, 1911 (1958).
[13] A. D. SHUTOV and I. A. VAINTRAUB, *Biokhimiya* **31**, 726 (1966).

vicilin, they represent approximately half- and third-molecules respectively, on the basis of their determined MWs (158 000 for vicilin, and 340 000 for legumin). When fraction I and fraction II were taken up in ascorbic acid solution, they both gave a sedimenting plateau with a $S_{20,w}$ value for the leading edge of about 3*S*.

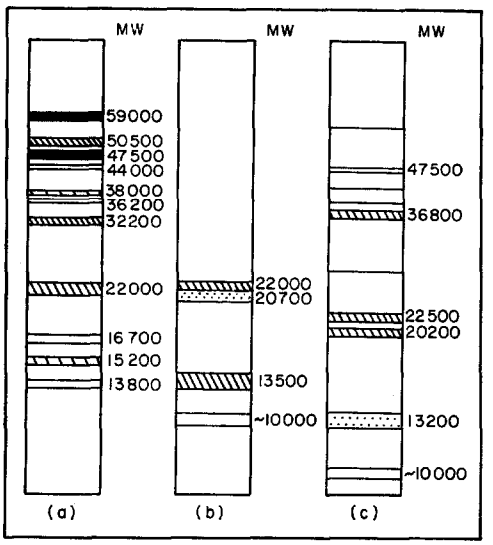

FIG. 2. SDS ELECTROPHORETIC PATTERNS OF (a) CARBOXYMETHYLATED FRACTION I, (b) CARBOXYMETHYLATED FRACTION II AND (c) FRACTION II IN SDS WITHOUT 2-MERCAPTOETHANOL.

The sedimenting plateau probably consists of a variety of low MW species which are either not resolved in the ultracentrifuge, or undergoing dissociation–association reactions; the value of 46 000 $\pm$ 7000 is the upper limit of a range of MW components.

These analyses indicate the dissociation of the globulins under acidic conditions. Other workers have reported similar results. Reversible or irreversible dissociation of the 11–12*S* seed protein of various legumes into the 8*S* half-molecules has been demonstrated.[14–16] Further dissociation was shown[17–20] to occur under acid conditions, thus Shutov and Vaintraub[13] found that legumin was completely dissociated into a 2*S* form in pH 2·8 buffer, and a value of 1·9*S* has been reported by Danielsson[2] when legumin was dissociated at pH 3. Vicilin was also shown[13] to undergo partial dissociation into a 3·4*S* form at both pH 2·5 and 2·1.

The minor components in the legumin, seen in the ultracentrifuge, are presumably vicilin contaminants indicating the difficulty of preparing pure legumin, free of traces of vicilin.

Analysis of globulin fractions by gel electrophoresis confirmed the results obtained in the ultracentrifuge. Thus, fraction I dissolved in 0·5 M NaCl gave a single band R_m 0·18,

[14] F. J. JOUBERT, *J. South Afric. Chem. Inst.* **10**, 21 (1957).
[15] P. JOHNSON, *Trans. Faraday Soc.* **42**, 28 (1956).
[16] W. J. WOLF and D. R. BRIGGS, *Arch. Biochem. Biophys.* **76**, 377 (1958).
[17] P. JOHNSON and E. G. RICHARDS, *Arch. Biochem. Biophys.* **97**, 260 (1962).
[18] E. M. PETRI, A. J. STAVERMAN and D. T. F. PALS, *Biochim. Biophys. Acta* **17**, 446 (1955).
[19] W. J. WOLF, J. J. RACKIS, A. K. SMITH, H. A. SASAME and G. E. BABCOCK, *J. Am. Chem. Soc.* **80**, 5730 (1958).
[20] V. L. KRETOVICH, T. I. SMIRNOVA and S. YA. FRENKEL', *Biokhimiya* **23**, 547 (1958).

which had a diffuse leading edge and appeared very similar to the 0·18 band obtained with vicilin on gel electrophoresis. Fraction I, when dissolved in ascorbic acid and electrophoresed gave a band of R_m value 0·22. Fraction II yielded a variety of low molecular weight species on gels, irrespective of whether it was dissolved in NaCl or in ascorbic acid. Thus, it is apparent from the electrophoretic analyses that fraction I and fraction II under the ascorbic acid treatment, undergo dissociation, which, in the case of fraction II, is reversible only to a minor extent.

The subunit pattern of fraction I is very complex, and remains unaltered by carboxymethylation and different incubation conditions. The pattern is very similar to that produced by vicilin samples on SDS gels, although this similarity does not mean that they necessarily have similar primary structures of their subunits or similar subunit ratios. It does indicate, however, that similar association reactions are occurring since the same MW species occur in both samples. The diffuse nature of the electrophoretic bands produced by fraction II may be due to association–dissociation reactions taking place during electrophoresis; the fact that carboxymethylation results in an improved separation seems to confirm this view. The MWs determined for the subunits of fraction II, viz. 22 000, 20 700, 13 500 and approximately 10 000 are very low compared to those previously determined for either legumin[12] or vicilin.[22] The appearance of some higher MW species on gels of SDS-treated fraction II, probably indicates association via disulphide bridges, since in the presence of 2-mercaptoethanol these bands virtually disappear. At the same time the higher MW components observed on gels of fraction I may also be association products of more elementary units. However, in this case addition of 2-mercaptoethanol does not produce further dissociation, but this may be due to the presence of some residual ascorbic acid, which has been shown[23] to oxidize the thiol groups of proteins at pH 7. Most of the ascorbic acid will have been removed from fraction II by the 4 hr dialysis, but some may have precipitated out with fraction I.

The fact that sodium dodecyl sulphate is known to produce both association[24] as well as incomplete dissociation[25] and that 2-mercaptoethanol has limitations as a reducing agent,[26] lends support to the suggestion that the higher MW species of fraction I are due to association of smaller components. If it is accepted that the majority of bands are products of association, it is possible, by eliminating all components which can be formed from multiples of lower MW species, to arrive at the following 'subunit' compositions: fraction I: 22 000, 16 700, 15 200 and 13 800; fraction II: 22 000, 20 700, 13 500 and approximately 10 000. As the MW calibration curve is only linear as far down as 15 000,[27] the approximate nature of those values below 15 000 is emphasized. It has also been reported that substances of MW less than 15 000 are not sieved by 10% gels.[28] Although referring to a different buffer system, it may still be applicable to the system used here.

From the *N*-terminal analysis there is no immediate correlation between the acid extracted globulins and legumin or vicilin. Increased quantities of some amino acids, notably alanine and valine, in fractions I and II may be a result of cleavage of particularly

[21] C. J. Bailey and D. Boulter, *Europ. J. Biochem.* **17**, 460 (1970).
[22] C. J. Bailey and D. Boulter, *Phytochem.* **11**, 59 (1972).
[23] J. Meacham, *Experientia* **24**, 125 (1968).
[24] A. Ray, *Chem. Soc. London Spec. Publ.* No. 23, 49 (1968).
[25] R. L. Katzman, *Biochim. Biophys. Acta* **266**, 269 (1972).
[26] A. S. L. Hu, R. G. Wolfe and F. J. Reithel, *Arch. Biochem. Biophys.* **81**, 500 (1959).
[27] A. L. Schapiro, E. Vinuela and J. V. Maizel, *Biochem. Biophys. Res. Commun.* **28**, 815 (1967).
[28] U. K. Laemmli, *Nature, Lond.* **227**, 680 (1970).

labile peptide bonds. However this would seem unlikely under the extraction conditions employed.

The present results do not allow one to conclude that fraction I and fraction II correspond to either vicilin or legumin or vice versa. At first sight it might appear that fraction I corresponds to vicilin since both have $S_{20,w}$ values of about 7 and their subunit patterns on SDS gels are similar. However, legumin can dissociate to a 7–8*S* component, and it occurs in greater amounts than vicilin as does fraction I relative to fraction II. As legumin and vicilin are known to dissociate under acid conditions the validity of an acidic extraction procedure is questionable.

EXPERIMENTAL

Seeds of *Vicia faba* L. (var. Triple White) were purchased from the Tyneside Seed Stores and grown in the University Botanic Gardens during the summer of 1971. After harvesting, seeds were stored at −20°.

Preparation of legumin and vicilin. The extraction and purification of legumin and vicilin were carried out as previously described.[21,22]

Acidic extraction of globulins. All operations in the extraction were performed at 5°. Approx. 5 g of cotyledons were homogenized with 50 ml of a 0·5 M NaCl, 0·25 M ascorbic acid solution. The homogenate was filtered through muslin and then centrifuged at 25000 *g* for 30 min. The supernatant was decanted, clarified again at 25000 *g* for 30 min and then stirred during the addition of an equal volume of deionised water. The resultant precipitate (fraction I) was centrifuged down at 25000 *g* for 30 min. The supernatant was dialysed for 4 hr firstly against tap H_2O, then glass distilled H_2O and finally deionized H_2O. The precipitate (fraction II) was collected by centrifugation at 25 000 *g* for 30 min.

Ascorbic acid treatment of legumin and vicilin. 250 mg legumin and 200 mg vicilin were dissolved in separate 40 ml portions of a 0·5 M NaCl, 0·25 M ascorbic acid solution, and the solutions clarified twice by centrifugation at 25 000 *g* for 30 min. The method used for acidic extraction of globulins was then applied to these solutions.

Ultracentrifugation analysis. These studies were performed on a Christ Omega II 70 000 Ultracentrifuge. Samples for analysis were dissolved in either 0·5 M NaCl or 0·5 M NaCl, 0·25 M ascorbic acid and then dialysed against the same solution overnight. Sedimentation studies were carried out at 40 000 rpm and 20°. Serial dilutions of the samples were also run to examine the effect of concentration on sedimenting species. Sedimentation velocities, $S_{20,w}$ values and MWs were then calculated as described elsewhere.[10,11] Values for the partial molar volumes of legumin and vicilin were calculated[29] from the amino acid compositions,[21,22] and were found to be 0·722 (legumin) and 0·733 (vicilin).

N-*terminal amino acids* of protein samples were investigated by the method of Gros and Labouesse.[30]

Polyacrylamide gel electrophoresis. Analysis of globulin fractions was conducted on gels containing 7·5% acrylamide.[31,32] The sample gel was omitted and samples, density stabilized in 25% (v/v) glycerol, were applied directly to the stacking or spacer gels. Protein samples were dissolved in either 0·5 M NaCl or 0·5 M NaCl containing 0·25 M ascorbic acid. Bromophenol blue was used as the marker band, and relative mobilities (R_m) were calculated relative to this band.

Sodium dodecyl sulphate gels. The method used was that of Schapiro *et al.*[27] Samples to be electrophoresed were (a) carboxymethylated, (b) incubated in 0·2% (w/v) SDS for 3 hr, or (c) incubated in 0·2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol for 3 hr. Bromophenol blue was used as an indicator of the progress of the run, and horse heart cytochrome-*c* as the reference protein. MWs of subunits were determined from a calibration plot of standard proteins.[33]

Acknowledgement—One of us (D.J.W.) is in receipt of an S.R.C. studentship.

[29] H. K. SCHACHMAN, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 4, p. 70, Academic Press, New York (1957).
[30] C. GROS and B. LABOUESSE, *Europ. J. Biochem.* **7**, 463 (1969).
[31] L. ORNSTEIN, *Ann. N.Y. Acad. Sci.* **121**, 321 (1964).
[32] B. J. DAVIS, *Ann. N.Y. Acad. Sci.* **121**, 404 (1964).
[33] K. WEBER and M. OSBORN, *J. Biol. Chem.* **244**, 4406 (1969).