Phytochemistry, Vol. 20, No. 9, pp. 2077–2083, 1981.
Printed in Great Britain.

0031–9422/81/092077–07 $02.00/0

SUBUNIT COMPOSITION OF THE SEED GLOBULINS OF *LUPINUS ALBUS*

PATRIZIA RESTANI, MARCELLO DURANTI, PAOLO CERLETTI and PIERA SIMONETTI

Department of General Biochemistry, University of Milan, Milano, Italy

(*Revised received* 8 *January* 1981)

Key Word Index—*Lupinus albus*; Leguminosae; white lupin; seed globulins; globulin subunits; glycopeptides.

Abstract—The seed globulins separated from *Lupinus albus* are all oligomeric proteins. Vicilins, i.e. globulins 4, 5, 6 and 7, and to a minor extent legumins, i.e. globulins 8 and 9a, consist of a large number of protomers which differ in MW and/or isoelectric point and in carbohydrate content. Vicilins are rather similar in protomer composition. They are heterogeneous, i.e. each of them contains molecules which differ slightly for some protomers. Globulins 1 and 9b have a simpler structure and the ratio of protomers within their molecule was established. In legumins, and in proteins 1 and 9b, some of the protomers are linked by disulphide bridges. Carbohydrate is distributed specifically on some protomers; the others contain only minute amounts or none.

INTRODUCTION

In a preceding paper we reported the fractionation and properties of the seed globulins of *Lupinus albus* which include 87.2% of total seed proteins [1]. Twelve components were found, most of them with high MW, all containing carbohydrates. Globulins 4, 5, 6 and 7 represent in lupine the group of vicilins, 8 and 9a that of legumins. They are quantitatively the most relevant protein constituents together with globulins 1 and 9b. The latter has relatively low MW, and displays a peculiar amino acid composition. Within each group, proteins differ in MW and the ratio of component monosaccharides, but are similar in other properties, e.g. electrophoretic behaviour, amino acid composition, type of neutral sugar and total carbohydrate content per mg protein, so that to previous investigators they appeared as the same species [2, 3]. Vicilins have quite poor nutritional value but legumins are nutritionally better than the total protein extract and globulin 1 is very good [1, 4].

Our earlier study [1] also indicated that some of the globulins undergo association–dissociation phenomena and that they might be multi-subunit structures. This fitted with what is known about the sub-molecular arrangement of legume seed globulins [5–8]. In particular, in *L. angustifolius* all three conglutins have been found to contain more than one subunit [9] and the subunit distribution appeared to vary in different genotypes [10]. Evidence is available that *L. albus* seed proteins in the presence of a reducing agent resolve on sodium dodecyl sulphate (SDS) electrophoresis into a number of smaller fragments [11], but the material treated contained a mixture of proteins and the information is therefore poor.

In view of the several components evidenced by our previous studies in the group of legumins and in that of vicilins and of the several other globulins found [1], it appeared worthwhile revising the problem of subunit composition of lupine proteins. Our investigations were therefore directed to establish the distribution of peptide and sugar, and the association characteristics of the subunits in the various molecules separated. The results are reported in the present paper.

RESULTS

Protomer composition

The purified globulins were resolved into their constituent subunits by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The distribution of interpeptide disulphide bonds was investigated by the two-dimensional technique: the protein was first run in the absence of 2-mercaptoethanol and in the second dimension the separated subunits were submitted to a reducing medium.

Vicilins gave the same protomer distribution under either condition. Each released many protomers covering a wide range of MWs. Some, however, were very close in size (Table 1). Bands demonstrated with the carbohydrate stain coincided with peptide bands on the electrophoretic pattern (Fig. 1B). Carbohydrate concentrated on some protomers and a few did not contain any. A group of subunits were similar in size and molar percentage in different proteins, and also the presence of carbohydrate was quite similar, suggesting some relationship in structure. Each protein, however, had some protomers of its own. Globulins 4 and 5 were most similar to each other, then came 6. There is a greater difference in protomer composition between globulin 7, a minor component, and the other vicilins than amongst themselves.

The summed MWs of the protomers largely exceeded the MW of the protein itself: 187 000 for globulin 4, 225 000 for globulin 5, 260 000 for globulin 6 and 143 000 for globulin 7 [1]. This indicates that the molecules contained some of the alternative protomers, namely they were hetergeneous. This may be one reason for the defective stoichiometry between protomers. Other contributing factors are the following: SDS and Coomassie Blue do not bind to the carbohydrate, therefore where the sugar component significantly contributed to the MW of the subunit, (a) mobility in SDS-PAGE was reduced and consequently the apparent MW artefactually increased and (b) only peptide

Table 1. Subunit composition of vicilins

4			5			6			7	
MW ($\times 10^{-3}$)	Peptide	Sugar	MW ($\times 10^{-3}$)	Peptide	Sugar	MW ($\times 10^{-3}$)	Peptide	Sugar	MW ($\times 10^{-3}$)	Peptide
60.5	8.8	—	59.6	3.9	—	61.4	2.8	6.7		
56.6	4.5 } 5.2					56.1	16.0	—		
54.9	7.3 }		54.9	9.1 } 9.7						
			52.5	13.9 }						
47.5	5.1	62.2	47.8	5.9	61.9	47.0	9.8	—		
42.6	8.4	10.4				43.3	7.2	47.1	43.2	1.1
			40.7	4.0	8.8					
38.3	7.9	—				38.0	1.9	16.0	38.6	2.2
			37.1	10.1	—					
35.2	9.1	1.1								
			33.9	8.3	4.7	33.7	11.8	1.5		
30.7	9.2	3.8	30.2	5.8	1.7	30.1	6.7	—		
26.9	8.2	3.9	27.6	9.0	—					
			25.1	5.5	6.3	25.4	5.6	2.8		
22.5	8.7	2.1				21.7	14.9	3.1	20.5	10.6
						18.2	10.3	3.1	17.8	11.9
17.3	13.4	4.3	17.0	9.3	—				17.2	6.1
						16.2	5.8	7.7		
15.0	19.4	7.0	15.5	15.2	6.9				14.6	30.2
									13.4	9.6
						12.9	7.2	12.0	12.6	11.1
									10.6	17.2

The central tubes of the DE 52 eluate of each separated globulin were analysed by SDS-PAGE as described in Experimental. Carbohydrates were not determined in protein 7. Peptide (in mol) and sugar (by wt) are given as per cent of total peptide and sugar determined.

developed colour and the per cent in weight appeared less than it is in reality. All these facts together prevent the establishment of the ratio of subunits within the molecule based on SDS-PAGE data.

Legumins differed in subunit pattern from vicilins. Their heavier subunits contained disulphide-linked peptides and were dissimilar in the two proteins for number and MW and for the size of their constituent protomers (Table 2). The MW of the subunits summed up to smaller values than that measured on the intact proteins: 330000 for globulin 8 and 430000 for globulin 9a [1]. The data, however, were insufficient to establish a stoichiometry for the oligomeric molecule.

Globulin 9b separated in non-reducing media into two subunits, the heavier one containing two disulphide-linked protomers of equal MW (Table 2). Since the MW of the protein is 44000 [1], the heavier and lighter subunits appear in a 1:2 stoichiometry.

In globulin 1, two different protomers linked by a disulphide bond were shown (Table 3). Their MWs and molar fractions were determined on the protein itself and after separating the subunits by gel filtration as detailed in a further section where the results are jointly examined.

Globulin 2 showed the same pattern as globulin 1 under non-reducing and reducing conditions. Only qualitative data were obtained since it is present in very small amounts. Globulin 3, another very small component, has a unique SDS-electrophoresis behaviour.

Isoelectric focusing

The role of ionic interactions in the association between subunits and the ionic characteristics of the subunits were investigated by isoelectric focusing (IEF). Low ionic strength media dissociated the vicilins into several bands which focused in slightly differing acidic pH range (Fig. 2A). Protein 1 focused as a single component at pH 7.9 under non-reducing conditions. Legumins precipitated at pH values close to the isoelectric point and addition of 0.5 M glycine or 8 M urea was necessary in order to resolve sharp bands.* However, glycine allowed band separation only in a limited range around its isoelectric pH (pH 5.5–6.5) (Fig. 2B) and components with different pI values were therefore lost. In all proteins, the number of bands seen progressively increased with increasing ionic strength or denaturing capacity of the medium.

The focusing patterns in urea are shown in Fig. 2C. Within the group of vicilins and that of legumins, separate proteins had similar band distribution but bands differed in intensity and specific components were also present. Globulin 9b (not shown) displayed the most acidic character: in a non-reducing medium it gave a rather diffuse band around pH 4.3. For this protein, for globulin 1 and for the legumins the number of bands increased and focusing values changed dramatically when a reducing agent was added (Fig. 2C,b). This confirmed the presence of disulphide-linked protomers. Vicilins were not modified by adding 2-mercaptoethanol.

Sequential analysis by IEF and SDS-PAGE of the proteins denatured in 8 M urea indicated that focusing

*The use of glycine to maintain proteins close to their isoelectric point in solution was suggested by Dr. E. Gianazza, to whom we are grateful.

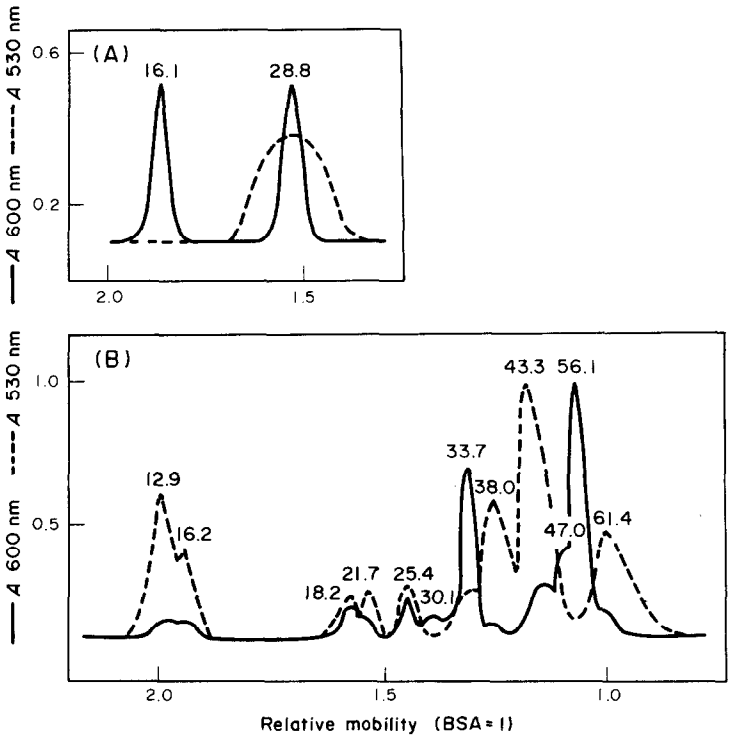

Fig. 1. Peptide and sugar distribution in the subunits of isolated globulins. Proteins shown are (A) globulin 1, MW see text, sugar content 4.3 mg/100 mg protein and (B) globulin 6, MW 260 000, sugar 2.1 mg/100 mg protein. They were treated with 0.4 % SDS and 0.5 % 2-mercaptoethanol and then submitted to SDS-PAGE. Strips run in parallel were stained with Coomassie Blue or with the Schiff reagent as detailed in Experimental. The bands developed were scanned respectively at 600 and 530 nm. MWs multiplied by 10^{-3} are given.

shows a larger number of bands because of the different isoelectric point in protomers with the same behaviour on SDS-PAGE. It also allowed the establishment of the apparent MW of the bands separated on IEF. Results for globulin 1 are shown in Fig. 3. The four bands focusing at more acidic pH exhibit on SDS-PAGE the same apparent MW of 16 000, whereas the four less acidic bands all have a MW of 28 800.

Also in the other globulins, groups of bands separated on IEF displayed the same behaviour on SDS-PAGE; in general more acidic components appeared to be heavier (not shown).

Subunit assay by gel filtration

In a different approach to their molecular composition, the isolated globulins were dissociated in 8 M urea under non-reducing or reducing conditions and were then submitted on a preparative scale to gel filtration chromatography. The peptide composition of the separated fractions was established by SDS-PAGE.

Globulin 1 gave on gel filtration under non-reducing conditions one peak, and when mercaptoethanol was present two peaks, each including one different protomer. All of the carbohydrate was with the heavier protomer both after gel filtration and after resolution by SDS-

Table 2. MWs ($\times 10^{-3}$) of subunits in native and reduced legumins and in globulin 9b

8			9a				9b		
No addition	+2-Mercapto-ethanol		No addition	+2-mercaptoethanol			No addition	+2-mercapto-ethanol	
68.9	47.0	17.3	67.8	32.7	32.7				
			60.0	31.0	18.4	16.6			
			56.9	27.8	27.8				
45.0	24.5	17.3	46.0	23.0	16.6				
			41.3	21.4	21.4				
31.0	35.0								
21.6	24.5		25.3	25.3					
20.9	20.9		21.5	21.5			21.6	10.5	10.5
18.8	17.3		19.0	19.0			13.7	12.8	

The central tubes of the DE 52 eluate of each globulin were analysed by two-dimensional SDS-PAGE as described in Experimental. Peptides liberated under reducing conditions from a given subunit are given on the same line.

Table 3. Protomers separated from globulin 1 under reducing conditions

Gel filtration		SDS-PAGE		
MW ($\times 10^{-3}$)	Mol %	of gel filtration peaks MW ($\times 10^{-3}$)	MW ($\times 10^{-3}$)	of the entire protein Mol %
29.5	44.3	28.8	28.8	40.6
20.9	55.7	16.8	16.1	59.4

Globulin 1 was gel-filtered on a column of Sepharose CL 6B in the presence of 8 M urea and 0.1 % 2-mercaptoethanol. The central tubes of the separated peaks were analysed by SDS-PAGE. The proteins were also separated by SDS electrophoresis in reducing medium before gel filtration.

PAGE of the entire globulin (Fig. 1A). MWs calculated on gel filtration data were somewhat higher than those obtained from SDS-PAGE (Table 3). Overestimation of peptide size due to incomplete unfolding is a known possibility in gel filtration in urea. The discrepancy was more conspicuous for the smaller protomer since the large one contains carbohydrate; its weight was also overestimated in SDS-PAGE as detailed before.

The mentioned effects of the carbohydrate on colour development and on apparent MW alter the molar ratio between the two protomers calculated from SDS-PAGE data in favour of the lighter protomer. The ratio is probably unity. Gel filtration results shift slightly from this figure, but this may depend on the values for MW being incorrect, as discussed above; the quantitative estimation of the protomers may also be a cause if they

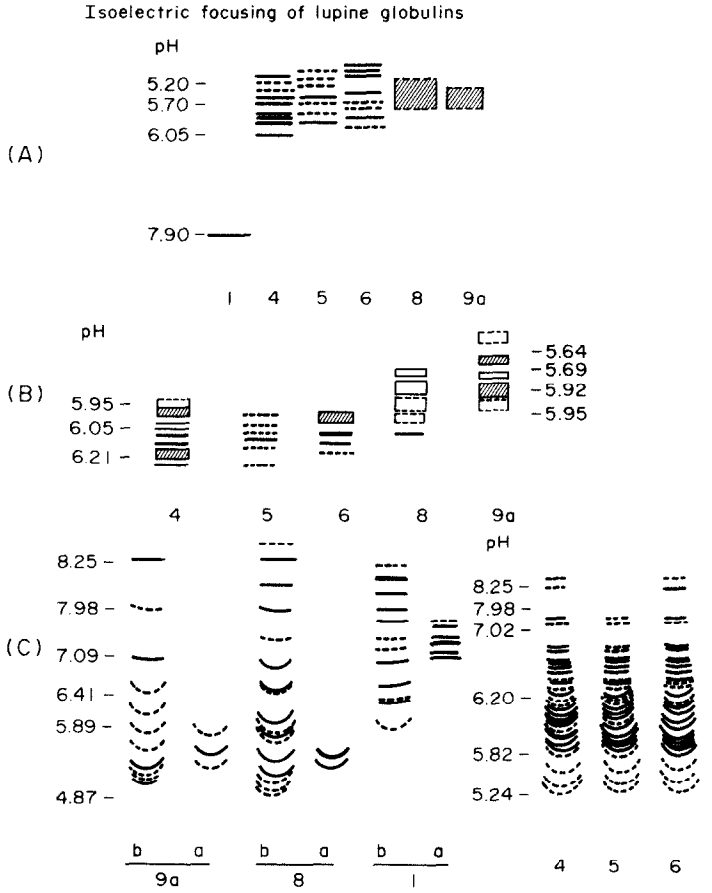

Fig. 2. Isoelectric focusing of lupine globulins (A) and after adding 0.5 M glycine (B) or 8 M urea (C). In C, 0.5 % 2-mercaptoethanol was added to the sample (b). The gels contained Ampholine pH 5–9 in A, pH 5–7 in B and pH 3.5–10 in C.

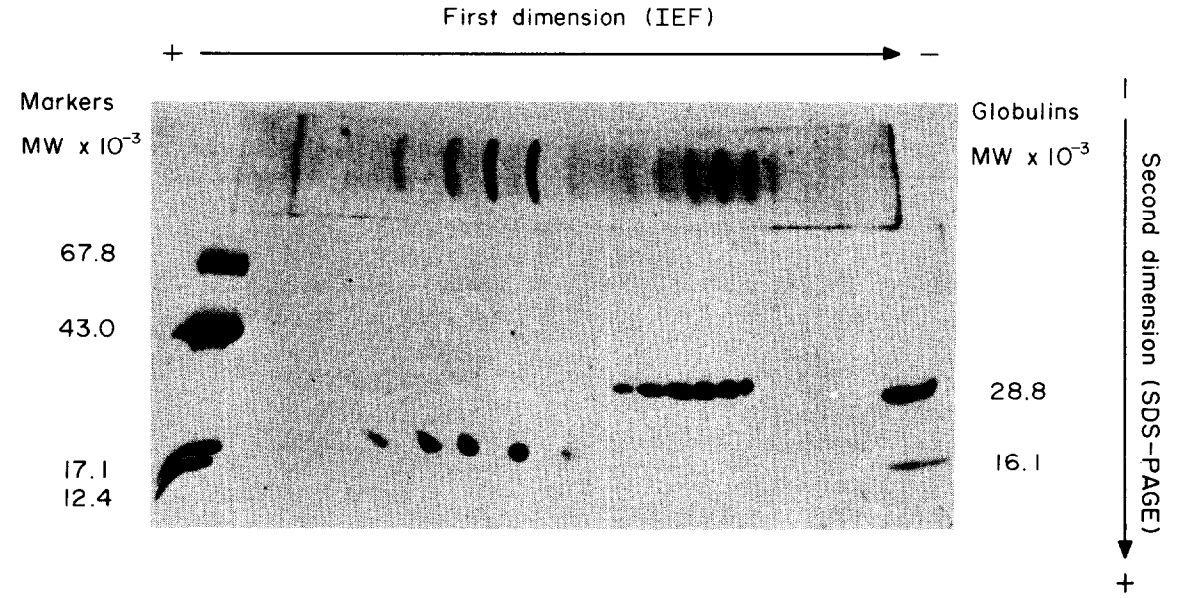

Fig. 3. Two-dimensional sequential IEF and SDS-PAGE of globulin 1. The strip resolved by IEF was placed in contact with a polyacrylamide gradient gel slab where the second dimension was developed by SDS-PAGE. Both runs were done under reducing conditions. Proteins of known MW and untreated globulin 1 were run in parallel on SDS-PAGE as markers.

differ significantly in amino acid composition, especially aromatic amino acids, since concentrations were determined by absorbancy measurements. The mentioned composition, one subunit of apparent MW 45–50 × 10^3 composed of two disulphide-linked peptides of MW 16–20 × 10^3 and 29 × 10^3 (this latter one containing carbohydrate), fits well with data indicating that the protein contains species of apparent MW 92000, 150000 and 300000 in association–dissociation equilibrium [1]. It may be concluded that globulin 1 exists as oligomers containing either two or three or six subunits.

Vicilins resolved on gel filtration into several peaks with the same pattern under non-reducing and reducing conditions. Also legumins produced different peaks; their number increased when the reducing agent was present and elution volumes were displaced towards smaller MWs.

In most peaks separated from the vicilins SDS-PAGE showed several peptides slightly differing in MW. Table 4 gives the results obtained with globulin 5 which are typical of the situation. Weighted averages were calculated for each gel filtration peak from the MW of the peptides indicated by SDS-PAGE and their molar distribution: a reasonable agreement with the MW displayed in gel filtration was found for the heavier components but for peaks containing small protomers, with little or no sugar, calculated values were lower than those found. As discussed for Table 3, this discrepancy may depend on the absence of carbohydrate.

In legumins, fewer fractions displayed more than one peptide and these peaks contained a smaller number of different peptides than in vicilins.

DISCUSSION

As detailed in a previous paper, globulins in the group of vicilins and those of the legumin type are, within each group, similar in MW, amino acid composition, neutral sugar content and electrophoretic behaviour [1]. The results reported here indicate that their molecular structures also have common features. With either group of proteins it was not possible to establish the subunit structure due to the complicated protomeric pattern. In *L. angustifolius* conglutin α, which represents the legumins, has been found to release three or four types of subunits with an apparent MW in the range 55000–80000, each of which may contain a disulphide-bonded moiety with a MW near 20000 and conglutin β, which corresponds to the vicilins, yielded four subunits in the range 20000–80000 with no covalent links [12].

Globulins 1 and 9b differ in molecular properties from the vicilins and legumins and also have a much simpler composition: their structure is given under Results. For globulin 1, data agree well with those found in *Lupinus angustifolius* for conglutin γ, the corresponding protein, namely that it contains one subunit of apparent MW 40000 composed of two disulphide-linked peptides of MW 17000 and 30000 [9, 12].

All globulins are glycosylated but only some of the protomers contained carbohydrate in sizeable amounts whereas the others had little or none. Similarly, only two of the four subunits of *Phaseolus aureus* vicilin are PAS-positive [13] and data are available suggesting preferential glycosylation of particular subunits in *Pisum sativum* [14]. In *L. angustifolius* the presence of carbohydrate on all subunits separated from conglutin α, β and γ has been reported [9]. This finding, however, may depend on insufficient resolution since lupine seeds contain more than three individual molecular species [1] and also the number of subunits described for conglutins α and β [9, 10] is smaller than found in this study for legumins and vicilins.

The presence of sugar on some peptides affects the behaviour of the protomers in the techniques used in

Table 4. Protomer composition of the peaks separated from globulin 5 by gel filtration in 8 M urea

Gel filtration		SDS electrophoresis		
MW ($\times 10^{-3}$)	Mol %	MW ($\times 10^{-3}$)	Mol %	weighted average MW $\times 10^{-3}$
54.9	13.8	59.5	15.1	53.1
		54.6	32.4	
		52.7	35.3	
		45.9	17.1	
38.9	18.3	40.2	18.6	35.3
		37.9	19.0	
		34.5	40.2	
		30.5	22.1	
33.9	18.3	27.0	24.4	24.6
		23.8	75.6	
20.9	41.8	17.2	76.7	16.9
		15.7	23.3	
13.7	7.8	nothing visible		

The central tubes of gel filtration were analysed by SDS-PAGE. For other experimental details see text. Weighted averages were calculated on MW and molar per cent of components evidenced by SDS-PAGE in each gel filtration peak.

different ways. Determined values of MW are therefore relative to the procedure applied as shown by comparing data from gel filtration and SDS electrophoresis. With these limitations, they will be used for our discussion. Equal MW by itself does not speak for similarity of peptides in separate proteins. Nonetheless, in view of the great similarity in amino acid composition in the vicilins [1, 4] and also because of the similar subunit pattern, we assume that peptides having the same MW in globulins 4, 5 and 6 are similar in amino acid composition.

The ionization behaviour of protomers on isoelectric focusing was consistent with the neutral character of globulin 1 on electrophoresis on cellulose acetate and with the acidic properties of the other proteins, increasing from vicilins to legumins and globulin 9b. The marked acidity of globulin 9b fits well with its content in glutamate, that represents 41 % by weight of the total amino acids [1, 4].

In view of the high content of polar amino acids in all globulins [1, 4], it is not surprising that ionic interactions play an important role in holding together these oligomeric associations as shown by partial resolution of the vicilins at pH values close to the isoelectric point. The increasingly complicated focusing pattern upon addition of glycine and of urea suggests stepwise dissociation of preferential and more solid associations between the numerous peptides of these molecules; the more extensive resolutions induced by urea indicate that hydrogen bonding and molecular conformation are also important for the associations to occur. Interpeptide disulphides are also involved in building the molecular structure in legumins and in globulins 1 and 9b. Similar behaviour has been described for other legumes and has been clearly documented for the soy bean [7, 8].

Heterogeneity in protomer composition is another facet in the structure of the seed globulins. It has long been described for the vicilins of soy bean and of other legumes [6, 7] and has recently been documentated for the legumin of *Pisum sativum* [15]. Our data extend this condition to lupine. Differences are evidently small enough to allow the same behaviour of the overall molecules in the separation techniques used in our earlier studies [1], as well as in ultracentrifugation and on cellulose acetate electrophoresis [2, 3]. For vicilins heterogeneity was evident already from the MWs derived from SDS-PAGE data. Results from gel filtration of the urea-denatured proteins confirmed the observation. Indeed the several similar protomers shown in the peaks from gel filtration having MWs close to that of the peak itself were not derived from breakdown of a larger unit but very likely were alternatively present in the molecules. A similar situation for legumins proposes heterogeneity also for this group of proteins. However, here the sum of MWs of separated protomers does not provide straightforward support of the contention.

Electrophoretic mobilities in SDS-PAGE depend on the amount of SDS bound, i.e. on the size and composition of peptides, and on the quality and quantity of sugar in carbohydrate-containing protomers. Heterogeneity therefore refers to these variables. In addition, sequential IEF and SDS-PAGE demonstrated different ionic properties in protomers with same behaviour in SDS-PAGE: they can be referred to differences in type, number and interaction with the solvent of polar amino acid residues in the peptide.

Nevertheless, if heterogeneity is taken into account, the molecules appeared to have a specific composition; indeed proteins isolated from different seed samples yielded the same protomers. Since each analysed sample consisted of a large number of seeds it is possible that although the sample was homogeneous upon breeder's

criteria, vicilins, and to a minor extent legumins, differ slightly in seeds from different plants. However, the alternate hypothesis, namely heterogeneity in the same seed, cannot be excluded and data concerning *Pisum sativum* support this view [15].

It is interesting that the proteins with poorest nutritional quality, the vicilins, are also those with highest heterogeneity. This confirms that they serve as unspecific storage material and suggests that room for genetic improvement is available.

EXPERIMENTAL

Unless otherwise specified, materials, methods and the preparation of protein were as described previously [1].

Isoelectric focusing was done according to ref. [16]; the gel slabs were stained in Fast Stain. The pH gradient on the slab was measured at 0.5 cm intervals. Glycine and urea, when used, were added during the prepn of the gel. For expts with urea, proteins previous to application were denatured overnight in 8 M urea.

SDS-polyacrylamide gel electrophoresis. SDS-PAGE was performed according to ref. [17]; the weighed freeze-dried samples were suspended in 0.1 ml of H_2O and 0.1 ml 0.25 M Tris–HCl, pH 6.8, containing 15% glycerol and 2% SDS, was added; for separations under reducing conditions the buffer also contained 5% 2-mercaptoethanol (2-ME). For peptide assay the amount of sample loaded corresponded to *ca* 10 μg protein for each separated band and Coomassie Blue [18] was the dye used. The carbohydrate component of glycopeptides was shown with a Schiff's reagent according to ref. [19]: for this assay the sample applied contained *ca* 50 μg protein per band: it was concd by reverse osmosis using CX immersibles from Millipore with a cut-off limit of MW *ca* 12000.

Two-dimensional SDS-PAGE. The procedure of ref. [20] was used with some modifications. Samples were run in both dimensions on gel slabs prepared in a gradient of polyacrylamide as described in ref. [17]. Strips developed in the first run were cut and soaked for 30 min in 1.5 M Tris–HCl buffer, pH 8.8, containing 0.4% SDS and 1% 2-ME; each was then laid on a new slab and the second dimension was run. Slabs were stained and destained as described in ref. [17].

Two-dimensional sequential IEF and SDS-PAGE. The procedure of ref. [21] was used with some modifications. Samples were dissolved (5 mg/ml) in 8 M urea containing 4% 2-ME and allowed to stay at room temp. overnight. For each sample 40–50 μl was applied in appropriate pockets in the polyacrylamide gel slab and IEF was run as described, for 6 hr. The developed strips were cut and laid in contact with a polyacrylamide gradient gel slab for SDS-PAGE and the second dimension was run.

Gel filtration in urea. Freeze-dried proteins (20 mg) were dissolved in 2 ml 1% NaOAc–1% acid ammonium phosphate buffer brought to pH 5 with HOAc containing 8 M urea; in expts under reducing conditions the buffer also contained 1% 2-ME and the pH was adjusted to 8.6 with methylamine. The sample was allowed to stay at room temp. overnight under N_2. The soln was then chromatographed in a column (114 × 2.1 cm) packed with Sepharose CL 6B. The mentioned buffer at pH 5, degassed and continuously flushed with N_2, was percolated from the bottom of the column. In expts under reducing conditions the eluant contained also 0.1% 2-ME. The urea soln used in the eluant was deionized on AG 501-X8 (Bio-Rad) before the addition to the buffer. To prevent crystallization of urea in the column the buffer flowed without interruption at a rate of 3 ml/hr/cm^2. The outflow was monitored at 279 nm and recorded in a Uvicord II (LKB).

REFERENCES

1. Duranti, M., Restani, P., Poniatowska, M. and Cerletti, P. (1981) *Phytochemistry* **20,** 2071.
2. Gillespie, J. M. and Blagrove, R. J. (1975) *Aust. J. Plant Physiol.* **2,** 29.
3. Joubert, F. J. (1956) *Biochim. Biophys. Acta* **19,** 172.
4. Duranti, M. and Cerletti, P. (1979) *J. Agric. Food Chem.* **27,** 977.
5. Derbyshire, E., Wright, D. J. and Boulter, D. (1976) *Phytochemistry* **15,** 3.
6. Thanh, V. H. and Saibasaki, K. (1978) *J. Agric. Food Chem.* **26,** 692.
7. Badley, R. A., Atkinson, D., Hauser, H., Oldani, D., Green, J. P. and Stubls, J. M. (1975) *Biochim. Biophys. Acta* **412,** 214.
8. Kitamura, K., Takagi, T. and Shibasaki, K. (1976) *Agric. Biol. Chem.* **40,** 1837.
9. Eaton-Mordas, C. and Moore, K. G. (1979) *Phytochemistry* **18,** 1775.
10. Blagrove, R. J. and Gillespie, J. M. (1978) *Aust. J. Plant Physiol.* **5,** 651.
11. Sgarbieri, V. C. and Galeazzi, M. A. (1978) *J. Agric. Food Chem.* **26,** 1438.
12. Blagrove, R. J. and Gillespie, J. M. (1975) *Aust. J. Plant Physiol.* **2,** 13.
13. Ericson, M. C. and Chrispeels, M. J. (1976) *Aust. J. Plant Physiol.* **3,** 763.
14. Basha, S. M. M. and Beevers, L. (1976) *Plant Physiol.* **57,** 93.
15. Krishna, T. G., Croy, R. R. D. and Boulter, D. (1979) *Phytochemistry* **18,** 1879.
16. Righetti, P. G. and Drysdale, J. W. (1976) *Isoelectric Focusing*, p. 419. North-Holland, Elsevier.
17. Arosio, P., Adelman, T. G. and Drysdale, J. W. (1978) *J. Biol. Chem.* **253,** 4451.
18. Powell, L. W., Alpert, K., Isselbacher, K. J. and Drysdale, J. W. (1975) *Br. J. Haematol.* **30,** 47.
19. Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) *Biochemistry* **10,** 2606.
20. Wang, K. and Richards, F. M. (1974) *J. Biol. Chem.* **249,** 8005.
21. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250,** 4007.