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Purification and Subunit Structure of Legumin of *Vicia faba* L. (Broad Bean)

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Zonal isoelectric precipitation was shown to be an effective method for the preparation of legumin which was homogeneous as judged by ultracentrifugation and polyacrylamide-gel electrophoresis. The subunit structure of legumin was investigated by preparative sodium dodecyl sulphate–polyacrylamide-gel electrophoresis and ion-exchange chromatography in urea. Five distinct subunits, of which two were acidic (α) and had a molecular weight of 37000, and three were basic (β) with molecular weights of 20100, 20900 and 23800, were identified. The α and β subunits were present in equimolar amounts in the legumin molecule and, in view of this and molecular-weight considerations, an $\alpha_6\beta_6$ subunit model was proposed for legumin.

In the past, considerable difficulty has been encountered in preparing legumin, the major storage protein of *Vicia faba* seeds, free from contaminating vicilin (Danielsson, 1949; Vaintraub *et al.*, 1962; Kloz *et al.*, 1966; Jackson *et al.*, 1969; Graham & Gunning, 1970; Millerd *et al.*, 1971). Isoelectric precipitation has proved useful, but in order to effect complete separation of legumin from vicilin, this procedure has to be repeated several times (Danielsson, 1949; Bailey & Boulter, 1970). The latter authors reported that legumin consisted of three subunits of molecular weights 56000, 42000 and 23000. By using dye-binding and radiochemical labelling *in vivo*, they suggested that these subunits were present in the legumin molecule in the approximate ratio of 1(56000):3(42000):6(23000).

In the present investigation, zonal isoelectric precipitation (Shutov & Vaintraub, 1965) has been used as an alternative to tedious, repetitious isoelectric precipitations. The legumin so prepared has been shown to be homogeneous and its subunit composition has been investigated by preparative sodium dodecyl sulphate–polyacrylamide-gel electrophoresis and ion-exchange chromatography in urea. The data obtained lead to a reappraisal of the subunit structure of legumin.

Experimental

Materials

Seeds of *Vicia faba* L. (var. 'Triple White') were purchased from the Tyneside Seed Company, Gateshead, Co. Durham, U.K.

Sephadex G-50 (medium grade) was obtained from Pharmacia Ltd., Uppsala, Sweden. Dowex AG1 (X2; 200–400 mesh) was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Bovine serum albumin (fraction V), cytochrome *c* (horse heart), ovalbumin (grade V), creatine kinase (rabbit muscle), β-lactoglobulin, alcohol dehydrogenase (yeast) and lysozyme (egg-white, grade I), were all purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Trypsin and pepsin were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Other chemicals were obtained from British Drug Houses (BDH) Ltd., Poole, Dorset, U.K., or Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K., and were of analytical grade, where necessary.

Methods

Preparation of crude globulin extract. Bean meal (10g) was stirred with 100ml of 1M-NaCl–0.05M-NaH_2PO_4, pH7.0, for 1h, filtered through muslin and then centrifuged at 20000*g* for 30min. The supernatant was adjusted to 90–100% saturation with $(NH_4)_2SO_4$, stirred for 1h and the crude globulin precipitate collected by centrifugation at 20000*g* for 30min.

Zonal isoelectric precipitation. The procedure used for zonal isoelectric precipitation was based on that of Shutov & Vaintraub (1965). The sample was dissolved in 0.2M-NaCl–0.05M-NaH_2PO_4, pH8.0, and applied to a column of Sephadex G-50 equilibrated with 0.2M-NaCl–0.05M-citric acid, pH4.7. Elution of the column was performed with the citrate buffer, and the eluate was monitored continuously at 280nm with an Isco Ultraviolet Analyzer.

Carboxymethylation of legumin. Protein (500mg) was dissolved in 25ml of 6M-guanidine hydrochloride–1M-Tris–HCl, pH8.7, containing 1mM-EDTA, and the solution flushed with N_2. 2-Mercaptoethanol (0.25ml) was added and the solution incubated at 35°C for 4–5h. Iodoacetic acid (1.2g) was added and the reaction allowed to proceed in the dark. After 15min, when a negative nitroprusside reaction was obtained, the solution was dialysed against tap water and then distilled water, and finally freeze-dried.

Ion-exchange chromatography of legumin. This was performed on Dowex AG1(X2) resin, prepared as outlined by Weber & Kuter (1971). The resin was successively washed with 2M-NaOH, distilled water, 4M-acetic acid, distilled water, and finally 50mM-Tris–acetate, pH8.0. Just before use, the resin was equilibrated on a glass sinter with 50mM-Tris–acetate, pH8.0, containing 6M-urea. The same buffer was used for sample application and also for initial elution. Subsequent elution was performed with 50mM-acetic acid–6M-urea, adjusted to pH4.5 with dilute NaOH. The column eluate was monitored at 280nm with an Isco Ultraviolet Analyzer.

Polyacrylamide-gel electrophoresis. (*a*) Analytical. Gel electrophoresis was performed in both denaturing and non-denaturing systems. Native proteins were analysed in the pH8.3 system of Ornstein (1964) and Davis (1964), with 5% gels. Subunit structures were investigated by using the sodium dodecyl sulphate-containing buffer systems described by Schapiro *et al.* (1967) and Laemmli (1970) and a modification of the method of Reisfeld *et al.* (1962) in which 6M-urea was included in all the gel solutions. In most systems, after electrophoresis, gels were stained immediately in 1% (w/v) Amido Black in 7% (v/v) acetic acid for 1h and then diffusion-destained with several changes of 7% (v/v) acetic acid. With the Laemmli (1970) buffer system, gels were first fixed overnight in 50% (w/v) trichloroacetic acid and then rinsed for 3h in 7% (v/v) acetic acid before staining. When the gels had completely destained, densitometric traces were obtained by scanning the gels in transmission at 620nm with a Joyce–Loebl Chromoscan apparatus.

(*b*) Preparative. Preparative-scale gel electrophoresis was performed in a Quickfit 'Prep-P.A.G.E.' apparatus. The assembly and operation were carried out as described in the manufacturer's manual, obtainable from Quickfit Instrumentation, Stone, Staffs., U.K. The gel system used was a modification of that of Davis (1964), in that the reservoir buffer and gel were made 0.1% (w/v) in sodium dodecyl sulphate. The elution buffer (pH8.6) comprised 52g of Tris, 14ml of acetic acid, 1g of sodium dodecyl sulphate and distilled water to 1 litre. Gels were 6cm in height and contained 10% (w/v) acrylamide. Pre-electrophoresis was carried out at 100V until the u.v.-absorbing materials had been eluted. The sample (approx. 25mg) was dissolved in about 2ml of tenfold-diluted reservoir buffer re-adjusted to 0.1% (w/v) in sodium dodecyl sulphate, density-stabilized with glycerol and then layered on to the surface of the gel by using fine-bore Teflon tubing attached to a peristaltic pump. Electrophoresis was performed at 100V (100mA) at room temperature. Pooled fractions were dialysed against distilled water and then freeze-dried.

Determination of subunit ratios. The ratio of subunits present in the legumin molecule was determined by a dye-binding method, in which the ratio of dye bound to each subunit on the gels was used as a measure of the amount of protein. The relative amounts of dye bound to each subunit were obtained by integrating the densitometric traces of the gels with a Technicon integrator/calculator.

Another estimate of the subunit ratio was obtained from amino acid-composition data (Burgess, 1969). By using the latter, determined for the isolated subunits and the intact protein, it was possible to calculate weighting coefficients which, in effect, represented the relative proportions of each subunit required to produce a protein of the desired amino acid composition. The 'best fits' for these coefficients were obtained by regression analysis.

Determination of subunit molecular weights. Molecular weights of polypeptide chains were determined on 10% polyacrylamide gels containing sodium dodecyl sulphate by the methods of Weber & Osborn (1969) and Laemmli (1970). Gels were calibrated by using the following protein standards (molecular weights are given in parentheses): lysozyme (14300), β-lactoglobulin (18400), trypsin (23300), pepsin (35000), alcohol dehydrogenase (37000), creatine kinase (40000), ovalbumin (43000), γ-globulin (25000 and 55000) and serum albumin (64000). In the system of Weber & Osborn (1969), relative mobilities were calculated relative to cytochrome *c*. When the discontinuous system of Laemmli (1970) was used, the Bromophenol Blue marker band remained very sharp throughout the experiment, and so mobilities could be measured relative to this.

Analytical ultracentrifugation. This was performed in a Christ Omega II 70000 ultracentrifuge. Runs were carried out at 40000rev./min and 20°C. Samples were dissolved in and dialysed against 0.5M-NaCl–0.05M-NaH_2PO_4, pH8.0. Sedimentation coefficients and $s_{20,w}$ values were determined as described by Svedberg & Pedersen (1940).

Acid hydrolysis and amino acid analysis. Duplicate samples in 6M-HCl (approx. 2mg/ml) were hydrolysed *in vacuo* at 105°C in sealed Pyrex tubes. Hydrolysates were dried *in vacuo* over solid NaOH and then analysed on a Locarte automatic-loading amino acid analyser.

N-Terminal amino acid analysis. The *N*-terminal amino acids of legumin were determined qualitatively by the dansylation procedure of Gros & Labouesse (1969).

Results

The elution profile obtained when a crude globulin extract was subjected to zonal isoelectric precipitation is illustrated in Fig. 1. The fractions separated correspond to the pH4.7-soluble (I) and pH4.7-insoluble

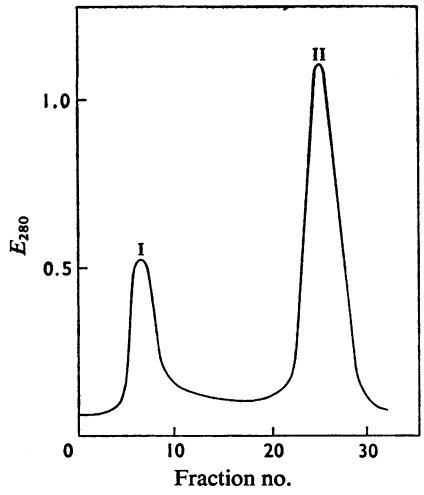

Fig. 1. *Zonal isoelectric precipitation of Vicia faba globulins*

Details of the procedure are given under 'Methods'. Approx. 500mg of crude globulin was applied to a column (2.2cm×53cm) of Sephadex G-50. Elution was performed at a rate of 36ml/h and 4.8ml fractions were collected.

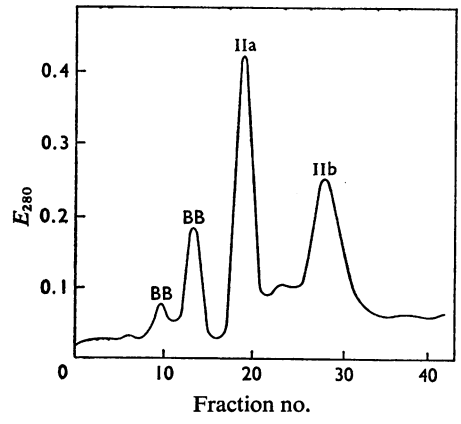

Fig. 2. *Preparative gel-electrophoresis elution profile of Cm-legumin*

The procedure is described in 'Methods'. Cm-legumin (25mg), dissociated in 0.1% (w/v) sodium dodecyl sulphate was applied to a 6cm 10% polyacrylamide-gel column. Electrophoresis was performed at a constant 100V (100mA). Elution buffer flow rate was 25ml/h; 5ml fractions were collected. Abbreviation: BB, Bromophenol Blue.

II) globulins, i.e. vicilin and legumin (Danielsson, 1949), respectively. The latter was shown to be homogeneous by ultracentrifugation, showing a sedimentation coefficient of approx. 11S, and by polyacrylamide-gel electrophoresis at pH8.3. Three *N*-terminal amino acids were identified for legumin, namely glycine, leucine and threonine.

When examined in the ultracentrifuge, fraction I gave two components, corresponding to approx. 7S and 11S species. The contaminating legumin (11S) could be removed by isoelectric precipitation at pH4.7 at lower ionic strength, i.e. 0.1M-NaCl.

When analysed in the pH7.0 sodium dodecyl sulphate–polyacrylamide-gel system of Schapiro *et al.* (1967), legumin (fraction II) gave two major components (α and β) with estimated mol.wts. of 36200 and 22000 respectively, and a lesser component with mol.wt. 48500. Molecular weights were obtained from a calibration graph constructed from proteins of known molecular weight.

The ratio of α to β subunits of Cm-legumin* was determined by a dye-binding method and found to be 1:1.26 or approx. 4:5. When samples of Cm-legumin were electrophoresed in polyacrylamide gels in the preparative apparatus, the elution profile illustrated in Fig. 2 was obtained. There were only two principal protein-containing peaks (IIa and IIb), and a comparison of the electrophoretic patterns of the proteins recovered from these fractions with the subunit pattern of Cm-legumin showed that this method gave a good separation of the α and β subunits, i.e. there was no cross-contamination.

Two fractions were also obtained when Cm-legumin, dissolved in 6M-urea, was chromatographed on the anion-exchange resin AG1-X2. The first (basic fraction) passed unhindered through the column, and the other (acidic fraction) was bound to the resin, and was subsequently removed by elution with a pH4.5 buffer. When these two chromatographic fractions were analysed in the pH7.0 sodium dodecyl sulphate–polyacrylamide-gel system, only one major component was evident in either case, and these corresponded to the α (acidic fraction) and β (basic fraction) subunits of legumin. In the sodium dodecyl sulphate gel system of Laemmli (1970), the acidic fraction, as in the pH7.0 system, gave only one component, with mol.wt. 37000; on the other hand, the basic fraction separated into three species, with mol.wts. of 23800 (β_1), 20900 (β_2) and 20100 (β_3). When electrophoresed in an urea-containing gel system at pH4.3, however, the basic fraction migrated as a single component with a relative mobility of 0.32. In this same system, the acidic fraction separated into two closely-spaced components, α_1 and α_2, with relative mobilities of 0.16 and 0.21 respectively.

* Abbreviation: Cm-legumin, carboxymethylated legumin.

Table 1. *Amino acid analyses of hydrolysates of the α (acidic) and β (basic) subunits of Cm-legumin*

Values are means±errors from duplicate analyses.

Amino acid	Subunit ...	Recovery (g of amino acid residue/100g of protein)			
		α		β	
	Hydrolysis time ...	20h	72h	20h	72h
Asp		10.85±0.13	10.97±0.21	10.72±0.03	10.55±0.39
Thr		2.21±0.04	2.28±0.11	3.31±0.05	2.98±0.0
Ser		3.85±0.02	3.21±0.0	4.57±0.07	4.07±0.2
Glu		20.92±0.84	19.59±0.56	10.57±0.12	10.54±0.23
Pro		3.89±0.04	3.57±0.51	3.63±0.04	3.51±0.17
Gly		3.24±0.0	3.03±0.02	2.76±0.04	2.68±0.03
Ala		1.97±0.03	1.88±0.06	5.58±0.12	5.51±0.06
Val		2.37±0.02	2.48±0.09	6.33±0.16	7.61±0.25
Met		0.58±0.02	0.71±0.09	0.46±0.0	0.48±0.04
Ile		3.51±0.12	3.72±0.11	3.10±0.03	3.21±0.07
Leu		5.36±0.15	5.41±0.34	9.94±0.08	9.92±0.06
Tyr		2.82±0.02	2.72±0.16	3.98±0.04	3.68±0.13
Phe		3.17±0.07	3.09±0.08	3.69±0.06	3.88±0.04
His		2.64±0.10	2.48±0.09	1.37±0.01	1.49±0.06
Lys		4.60±0.10	4.30±0.02	6.02±0.10	6.12±0.2
Arg		10.25±0.20	11.83±0.71	9.65±0.21	10.47±0.33
		82.23	81.27	85.68	86.7

Table 2. *Amino acid composition of Cm-legumin and its α (acidic) and β (basic) subunits*

For details see the text.

Amino acid	Cm-legumin (mole %)	Content (amino acid residues)					
		α subunits			β subunits		
		(g/100g of protein)	(mol/10^5g of protein)	(mole %)	(g/100g of protein)	(mol/10^5g of protein)	(mole %)
Asp	12.86	10.97	95.3	13.00	10.72	93.1	11.60
Thr*	3.25	2.28	22.5	3.07	3.45	34.1	4.25
Ser*	6.60	4.13	47.4	6.47	4.79	55.0	6.86
Glu	18.03	20.92	162.0	22.11	10.57	81.9	10.21
Pro	6.16	3.89	40.0	5.46	3.63	37.4	4.66
Gly	6.91	3.24	56.8	7.75	2.76	48.4	6.03
Ala	5.91	1.97	27.7	3.78	5.58	78.5	9.78
Val	5.21	2.48	25.0	3.41	7.61	76.8	9.57
Met	0.76	0.71	5.41	0.74	0.48	3.66	0.46
Ile	4.13	3.72	32.9	4.49	3.21	28.4	3.54
Leu	8.08	5.41	47.8	6.52	9.94	87.8	10.94
Tyr*	2.67	2.85	17.5	2.39	4.11	25.2	3.14
Phe	3.40	3.17	21.5	2.93	3.88	26.4	3.29
His	1.96	2.64	19.2	2.62	1.49	10.9	1.36
Lys	5.03	4.60	35.9	4.90	6.12	47.7	5.95
Arg	9.05	11.83	75.7	10.33	10.47	67.0	8.35
		84.81	732.6		88.81	802.3	

* Extrapolated to zero time.

Comparison of the electrophoretic patterns of the acidic and basic fractions with that of unfractionated legumin showed that all the components present in legumin are accounted for in these fractions; their chromatographic separation was complete, since they had no common electrophoretic components. When unreduced legumin (i.e. no 2-mercaptoethanol present) was electrophoresed in the Laemmli (1970) gel system, only two major bands, with mol.wts. of 49500 and 50500, were present, although two much

fainter bands with mol.wts. of 60500 and 63800 were also apparent.

The results of duplicate analyses of 20-h and 72-h hydrolysates of the acidic and basic subunits (fractions) are presented in Table 1, and the amino acid compositions derived from them in Table 2.

An estimate for the subunit ratio in legumin was obtained by using the amino acid-composition data for legumin and its acidic and basic subunits. Thus, for any particular amino acid, if X, A and B represent the mole percentage of that amino acid in legumin, the acidic subunits and the basic subunits respectively, then $X = aA + bB$, where a and b are weighting coefficients. By using values for A and B given in Table 2 and values for X previously determined (Wright, 1973), and applying regression analysis, the values obtained for a and b were 0.66 ± 0.02 and 0.34 ± 0.04 respectively, with a multiple correlation of 0.996. Values for methionine and proline were not included in the computation because of the greater analytical error involved in their determination. When these values are expressed on a molar basis, the subunit ratio in the legumin molecule is given by $(0.66 \times 0.31):(0.34 \times 0.52) = 1.1:1.0$, or approx. 1:1.

The *N*-terminal amino acids of the subunits of legumin, separated by both electrophoresis and chromatography, were qualitatively identified by the dansylation procedure. Glycine was the only *N*-terminus found for the basic subunits and fraction IIa, and leucine and threonine for the acidic subunits and fraction IIb.

Discussion

The method of zonal isoelectric precipitation gave a preparation of legumin which was homogeneous by various criteria and corresponded in its characteristics to those of the preparations of other workers: insolubility at pH4.7 and sedimentation coefficient (Danielsson, 1949), amino acid and *N*-terminal analyses and numbers of subunits (Bailey & Boulter, 1970). However, the molecular weights calculated in the present report differ by as much as 10% from those of Bailey & Boulter (1970), and there are some differences in the amino acid compositions reported. The component of highest molecular weight (48500) found in the Schapiro *et al.* (1967) method of analysis was not present on Laemmli (1970)-type gels, which used a higher sodium dodecyl sulphate concentration for sample dissociation, suggesting that it is an association product. The two systems gave subunits with almost identical molecular weights.

The two subunits found in the Schapiro *et al.* (1967) system had molecular weights of 36200 and 22000; these same fractions were separated by ion-exchange chromatography and hence are described as acidic and basic, in view of their charge. The basic subunits, which were not separated one from another by the Schapiro *et al.* (1967) system, were separated into three components in the Laemmli (1970) system, as a result of the increased resolution afforded by the discontinuous gel system in the 15000–30000 molecular-weight range (Weber *et al.*, 1972). The possibility that these β_1, β_2 and β_3 subunits resulted from the action of proteolytic enzymes during extraction was excluded, since the same three β subunits were shown to be present in sodium dodecyl sulphate extracts of meal (Wright, 1973), and it is known that proteolytic degradation is inhibited by the presence of sodium dodecyl sulphate. The acidic subunits α_1 and α_2 were resolved into two components when electrophoresed in urea gels.

The molar ratio of the α and β subunits present in the legumin molecule was found to be 4:5 by dye-binding, and 1:1 by the procedure of Burgess (1969). This latter value is considered the more reliable because, first, it was determined by regression analysis, in which a multiple correlation of 0.996 was obtained, and secondly, there were fewer assumptions involved in its derivation.

Comparison of the sodium dodecyl sulphate–polyacrylamide-gel-electrophoretic patterns of reduced and unreduced legumin indicates that the two major components of mol.wts. 49500 and 50500 found only in the absence of reducing agents represent products of disulphide-bond formation between the α and β subunits. It is likely that the disulphide bonds exist in the legumin molecule itself, since random disulphide-bond formation as a result of disruption of secondary and tertiary structure by sodium dodecyl sulphate would have produced a whole range of molecular-weight species. The molecular weights of these 'intermediary subunits' can only be explained by postulating a structure for them in which one α and one β subunit are combined. The discrepancy in the apparent molecular weights of these proposed $\alpha\beta$ intermediary subunits (49500 and 50500) and the combined molecular weight of an α and a β subunit (57200–60800, according to which β subunit is considered) probably reflects the increased secondary and tertiary structure of the former, produced as a result of the disulphide bonding. The presence of inter- and intra-molecular disulphide bonds in proteins has been shown to affect their electrophoretic mobilities in sodium dodecyl sulphate gels (Griffith, 1972), and thus can lead to erroneous estimates of molecular weights (Dunker & Rueckert, 1969; Trayer *et al.*, 1971).

Clearly, in the light of all these new data, a reappraisal of the subunit structure of legumin, as described by Bailey & Boulter (1970), is required. It is proposed that legumin consists of equimolar proportions of α- and β-type subunits, which differ in molecular weight and charge, and that it has the subunit structure $\alpha_6\beta_6$. A similar subunit structure has been proposed for the 11S protein of soya beans

(Catsimpoolas, 1969). By using the molecular weights determined for reduced (i.e. not carboxymethylated) legumin subunits (35800 for α and an average of 21300 for β subunits) this structure is equivalent to mol.wt. of 342600, a value in good agreement with the mol.wt. (328000) obtained from sedimentation studies (Wright, 1973). In addition, the following dissociation scheme is tentatively proposed:

$$\alpha_6\beta_6 \xrightarrow{\text{Sodium dodecyl sulphate}}$$

$$6\alpha\beta \xrightarrow[\text{Sodium dodecyl sulphate}]{\text{2-Mercaptoethanol}} 6\alpha + 6\beta$$

The α and β subunits of legumin of *V. faba* correspond, on a molecular-weight basis, to the B and A subunits of legumin extracted from *Vicia sativa* (Vaintraub & Nguyen Thanh Thien, 1971), although a subunit, corresponding to the 32600-molecular-weight subunit (C) reported by these authors in *V. sativa*, has not been observed in legumin of *V. faba*.

It is also apparent that the β-subunits contain more hydrophobic amino acids, such as leucine, valine and alanine, a fact that agrees well with experimental observations that they are much less soluble in aqueous media than the α subunits. The amino acid compositions of the isolated A, B and C subunits of legumin of *V. sativa*, determined by Vaintraub & Nguyen Thanh Thien (1971), are presented in Table 3. For the purposes of comparison, the results are recalculated and expressed in mole percentages. The amino acid composition of the two subunits of low molecular weight (β and A) from both species of *Vicia* agree exceptionally well; in most cases the variation is considerably less than 10%. On the other hand, the amino acid composition of the α subunits lies somewhere between those of the B and C subunits. When this is considered, together with the fact that the *N*-terminal amino acids of the B (leucine) and C (threonine) subunits are the same as those of the α subunits and that there are two α subunits, it is likely that the α_1 and α_2 subunits correspond to the B and C subunits of *V. sativa*, although their molecular weights differ.

Table 3. *Amino acid composition of the subunits (A, B and C) of legumin from Vicia sativa*

Data are recalculated from Vaintraub & Nguyen Thanh Thien (1971).

Amino acid	Content (mole%) A	B	C
Asp	12.57	13.38	9.69
Thr	4.39	2.56	3.25
Ser	6.45	5.86	7.83
Glu	9.51	19.24	24.27
Pro	5.12	4.97	6.15
Gly	6.17	7.93	7.04
Ala	9.92	5.52	3.65
½-Cys	0.91	1.05	1.04
Val	8.73	3.55	3.54
Met	0.46	0.96	0.11
Ile	3.98	5.06	4.90
Leu	10.15	6.04	6.94
Tyr	3.06	3.05	1.79
Phe	3.52	3.82	3.00
His	1.60	2.47	3.18
Lys	4.16	3.76	4.18
Arg	8.28	9.81	8.01
Trp	1.01	0.99	1.43

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