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SPECIFIC SUBUNIT PAIRS OF LEGUMIN FROM *VICIA FABA*

CHRISTIAN HORSTMANN

Zentralinstitut für Genetik und Kulturpflanzenforschung der Akademie der Wissenschaften der DDR, DDR-4325 Gatersleben, German Democratic Republic

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Key Word Index—*Vicia faba*; Leguminosae; field bean; legumin; subunit heterogeneity.

Abstract—Four pairs of disulphide-linked acidic (α) and basic (β) subunits were isolated from legumin of *Vicia faba*. Pairing between α- and β-subunits is nonrandom, supporting the view that each subunit pair arises from a common precursor polypeptide, already containing intramolecular disulphide bonds, when cleavage to the subunit pair takes place. The subunit pairs belong to two structural types: type A contains Met, whereas type B lacks Met. In addition to these four subunit pairs, at least two more pairs are present in legumin in minor amounts.

INTRODUCTION

Legumin, the 11*S* storage protein of *Vicia faba* seeds, is an oligomeric protein of MW *ca* 328 000. The legumin molecule consists of six pairs of subunits, each pair of one α- (MW *ca* 36 000) and one β-subunit (MW *ca* 20 000) linked by disulphide bonds [1, 2]. There exists heterogeneity within each subunit class, i.e. the α-subunits contain two *N*-terminal amino acids, Leu and Thr [1], the *N*-terminal amino acid sequences of the β-subunits exhibit heterogeneity [3], and α- as well as β-subunits show multiple bands in different gel electrophoretic systems [1, 4]. A similar heterogeneity has been described for legumin subunits of *Pisum* seeds [5, 6]. According to Croy *et al.* [7] legumin is synthesized *in vitro* as a single polypeptide chain of MW *ca* 60 000 which, *in vivo*, is cleaved to the corresponding α- and β-subunits. Subunit heterogeneity may be introduced by post-translational modifications [7], or by the existence of different structural genes coding for legumin precursors with different sequences [8]. Three precursors for *Pisum* legumin [9] and two for *Vicia* legumin [10] have been detected. If disulphide links are already formed in the precursors before cleavage to the α- and β-subunits takes place, specific α–β-pairs should occur in native legumin. Evidence is presented here, that legumin of *Vicia faba* contains such specific subunit pairs.

RESULTS AND DISCUSSION

α–β-Subunit pairs were isolated by ion exchange chromatography of legumin under denaturing conditions without reduction of disulphide bonds (Fig. 1). Fractions were pooled as indicated in Fig. 1 and rechromatographed. Fractions 1–4 constitute the major components of legumin, and have been investigated in more detail.

Sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis revealed that these fractions contained subunit pairs consisting of one α- and one β-band each (Fig. 2, upper and lower bands, respectively), with similar positions of the α-bands, but the positions of the β-bands of 1 and 2 being different from those of 3 and 4. Preparative separation of the subunits of pairs 1–4 was

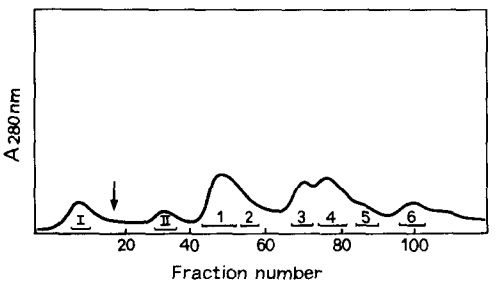

Fig. 1. Ion exchange chromatography of legumin in 6 M urea on DEAE-Sepharose CL-6B. Bars indicate the pooled fractions, and numbers 1–6 over the bars the numbers of the subunit pairs. The arrow denotes the start of gradient elution.

achieved by ion exchange chromatography on DEAE-Sepharose CL-6B after reduction of the disulphide links and *S*-carboxymethylation. The isolated α-subunits have different *N*-terminal amino acids: α1 and α2 (numbers of the subunits pairs and their subunits refer to the fraction numbers in Fig. 1) have *N*-terminal Leu; α3 and α4, *N*-terminal Thr; whereas all the β-subunits have *N*-terminal Gly [1]. These results suggest that at least two types of subunit pairs exist in *Vicia faba* legumin: type A comprising subunit pairs 1 and 2, each pair consisting of one α-subunit with *N*-terminal Leu and one β-subunit of higher apparent MW than β3 and β4 in SDS polyacrylamide gels (Fig. 2); and type B comprising subunit pairs 3- and 4-: αs with *N*-terminal Thr and βs of lower apparent MW. The data of the amino acid analyses also support this classification. The amino acid compositions of the corresponding subunits of each type are similar, but differences exist between the subunits of types A and B (Table 1). Tryptic peptide maps of the α- (Fig. 3) and β-subunits (Fig. 4) show different patterns for types A and B subunits, but similarities of the α- and β-subunits within each type. Differences between the two types were also obvious after reaction with cyanogen bromide, which cleaves peptide bonds on the carboxyl side of Met residues [11]: only α- and β-subunits of type A were cleaved showing, in SDS

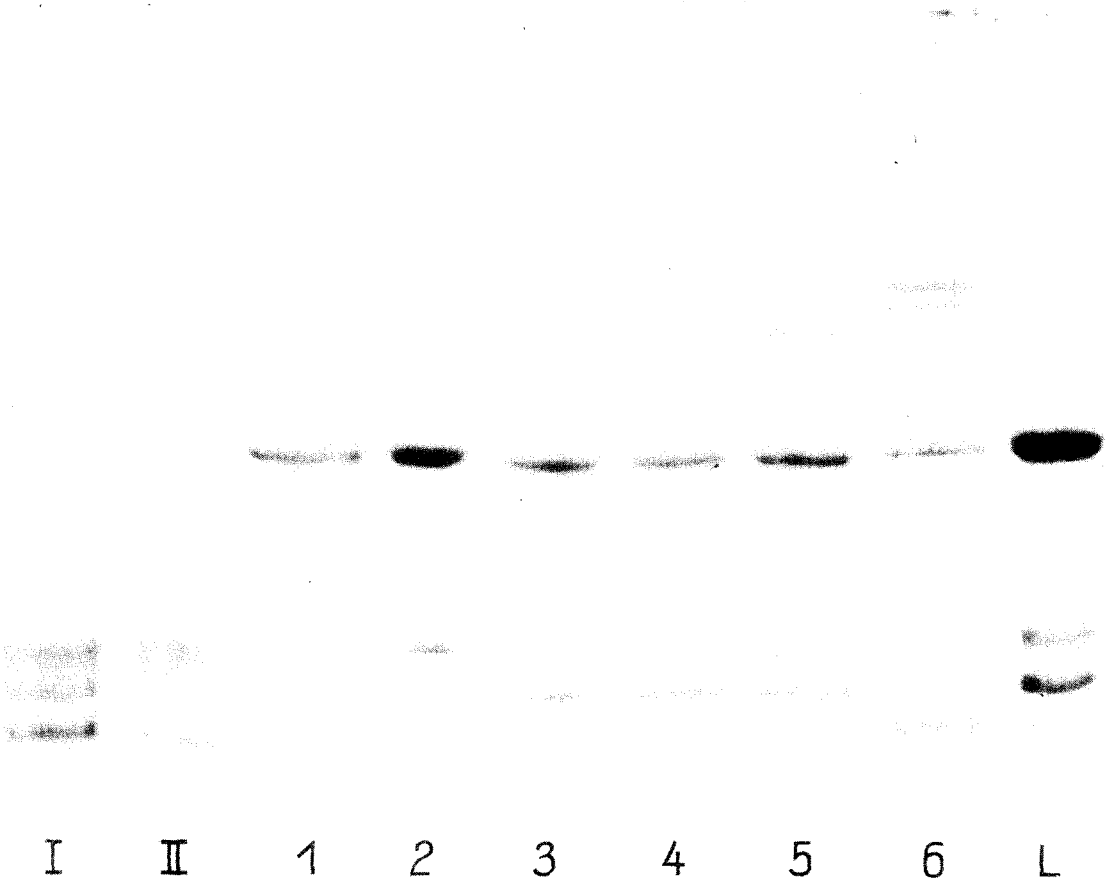

Fig. 2. SDS polyacrylamide gel electrophoretic analysis of the fractions of Fig. 1. Numbers correspond to the numbers over the bars in Fig. 1. L = legumin.

Table 1. The amino acid compositions (mol %) of subunits of legumin from *Vicia faba*

Amino acid	α1	α2	α3	α4	β1	β2	β3	β4
Asp	14.37	14.22	10.41	10.24	12.92	12.84	11.00	10.91
Thr	2.68	2.84	4.55	4.58	5.73	5.56	3.67	3.56
Ser	5.25	5.66	8.05	8.23	7.05	7.43	6.51	6.63
Glu	21.99	21.27	22.16	22.29	7.85	7.47	12.14	13.01
Pro	5.08	5.17	7.17	7.22	4.98	5.08	4.57	4.50
Gly	8.72	8.45	8.07	7.95	5.75	5.82	6.62	6.72
Ala	4.22	4.22	3.15	3.06	11.40	11.11	9.68	9.54
Val	3.63	3.59	3.65	3.55	8.32	8.87	9.66	9.83
Ile	4.66	4.75	5.52	5.61	2.91	2.96	4.55	4.60
Leu	5.82	5.90	7.69	7.74	12.03	11.65	10.81	10.85
Tyr	2.74	2.77	2.12	2.14	2.73	2.56	3.73	3.77
Phe	3.90	3.86	2.67	2.61	3.91	3.89	2.94	2.88
Lys	3.88	3.97	3.04	3.08	5.81	5.79	3.16	2.94
His	2.88	2.74	2.80	2.91	1.21	1.16	2.15	1.80
Arg	10.19	10.58	8.95	8.79	7.39	7.83	8.81	8.48

Values of Thr and Ser extrapolated from 24 and 48 hr hydrolysates.

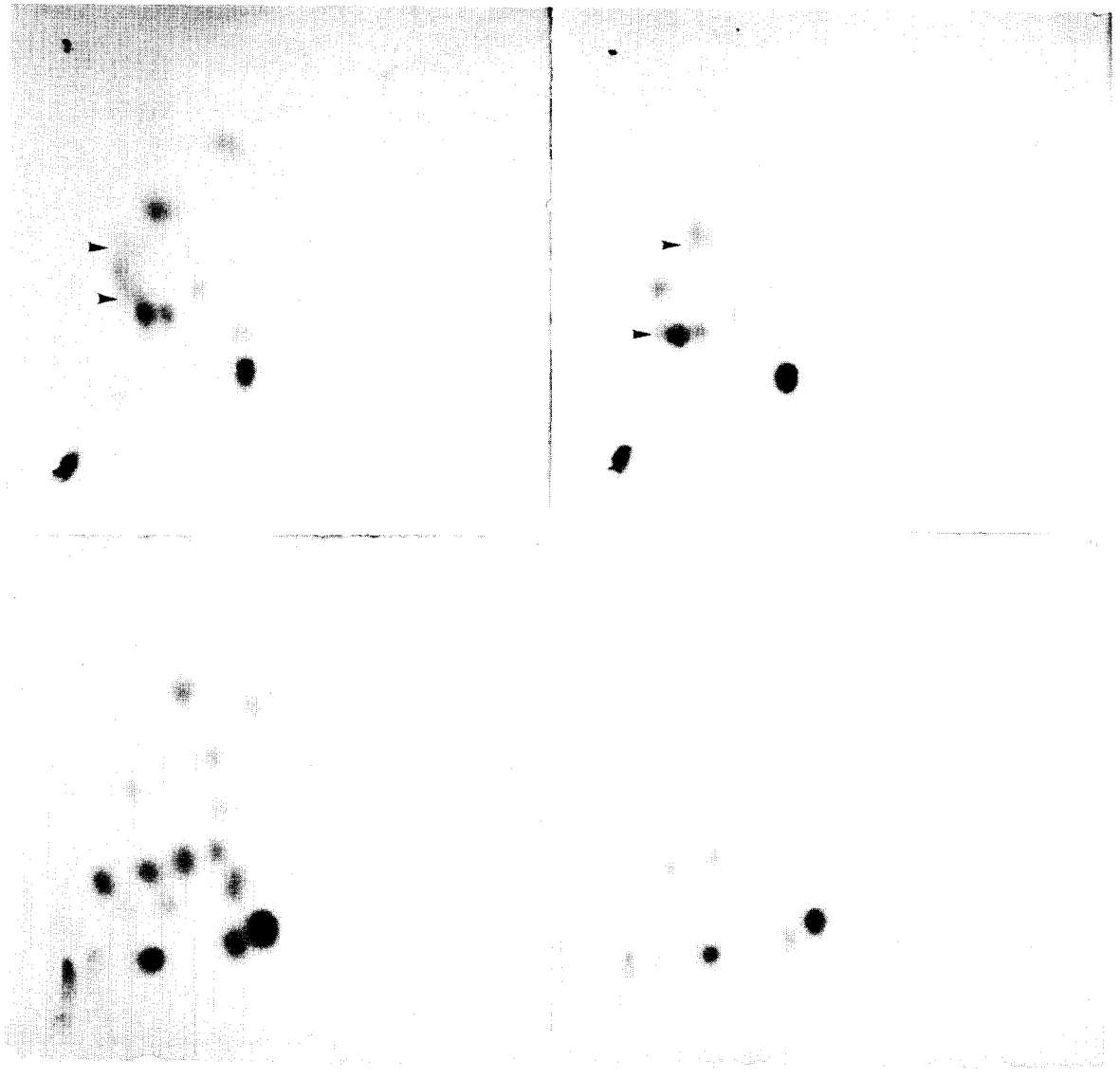

Fig. 3. TLC of tryptic digests from legumin CM-α-subunits. First dimension from bottom to top; second dimension from left to right. Upper row: left, α1; right, α2. Lower row: left, α3; right, α4. Arrows indicate spots different for α1 and α2.

gels, three cleavage products of the α- and four of the β-subunits, with no differences between α1/α2 and β1/β2. On the contrary, α- and β-subunits of type B remained unchanged after reaction with cyanogen bromide, indicating that these subunits contain no Met. Although Met of the different subunits has not been quantified, after automatic amino acid analysis only type A subunits show a Met peak, no corresponding peak is visible at the Met position in hydrolysates of type B subunits.

The results of amino acid analyses, peptide mapping, and cyanogen bromide cleavage suggest that the differences between types A and B subunits are due to different amino acid sequences of their respective precursors, and not to post-translational modifications. The appearance of specific disulphide linked subunit pairs supports the idea that the disulphide links are formed before the precursors are cleaved to α- and β-subunits which, therefore, are specifically paired by S–S-bonds. This is also in agreement with the observation of R. Bassüner (personal communication) that only one of the two legumin precursors [10] is labelled by ^{35}[S]Met during *in vitro* biosynthesis and, hence, should be a precursor of the type A subunit pairs, while the other one is devoid of Met and should give rise to the type B subunit pairs.

Similar relationships may exist for the subunit pairs within each type. Peptide maps of subunits α1 and α2 (Fig. 3), as well as of subunits β1 and β2 (Fig. 4), exhibit minor differences for each class of subunits. Subunits α3 and α4 are indistinguishable by peptide mapping (Fig. 3), whereas the maps of β3 and β4 (Fig. 4) are rather different. These differences might reflect slightly different amino acid sequences of the comparable subunits, post-translational modifications, or artifacts arising during

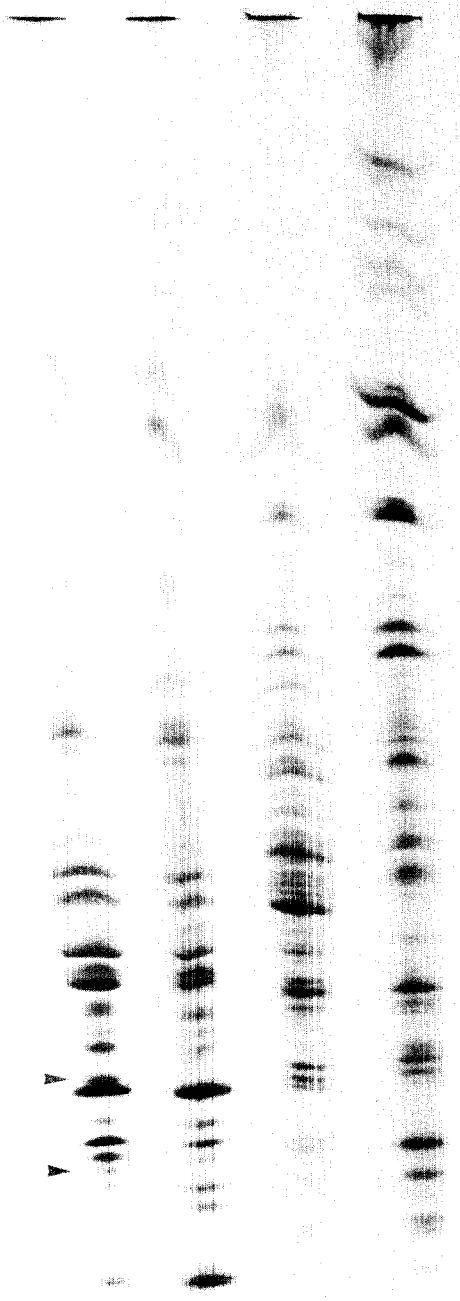

Fig. 4. Isoelectric focusing of tryptic digests from legumin CM-β-subunits. From left to right: β1, β2, β3, β4. Arrows indicate bands different for β1 and β2.

extraction or chromatography. The formation of artifacts can be excluded because legumins purified by different methods, i.e. ion exchange or hydroxylapatite chromatography, always showed the same pattern of subunit pairs as depicted in Fig. 1, and the subunit pairs eluted at the same positions when rechromatographed under identical conditions.

The occurrence of specific pairs of acidic and basic subunits was first proposed by Kitamura *et al.* [12] for glycinin, the 11*S* storage protein of the soybean. Staswick *et al.* [13] isolated five subunit pairs from glycinin and showed, by means of *N*-terminal sequence analyses of the constituent acidic and basic subunits, that pairing between these subunits is nonrandom. A similar conclusion was drawn for glycinin by Iyengar and Ravestein [14], using sequence analyses and gel electrophoretic studies of the subunits. The detection of specific subunit pairs in *Vicia faba* legumin fits well into those results and it is most likely that other comparable storage proteins of legume seeds contain similar subunit pairs, e.g. legumin from *Pisum sativum*, which also exhibits subunit heterogeneity [5, 6].

It is interesting to note that only type A subunits contain Met. Since Met is a nutritionally limiting factor in *Vicia* seed proteins, it would be worthwhile to look for *Vicia* varieties containing an increased ratio of type A/B subunits as material for the improvement of protein quality through plant breeding.

The other fractions, I, II, 5 and 6 (Fig. 1), obtained by ion exchange chromatography of legumin represent only minor constituents of the protein. In SDS polyacrylamide gels I and II exhibit multiple bands at the position of the β-subunits, a weak band with MW *ca* 30 000, but only traces at the α-position (Fig. 2). The main *N*-terminal amino acid of this mixture is Gly, besides small amounts of Leu and His. This fraction seems to correspond to subunits J according to Matta *et al.* [4].

SDS gel electrophoresis of fractions 5 and 6 (Fig. 2) shows, besides α- and β-subunits as in 3 and 4, additional α- ('α5' and 'α6') and β-bands ('β5' and 'β6'). It has not been possible to separate α5, with apparent MW *ca* 55 000, from the accompanying α, the *N*-terminal end-groups of the α-mixture are Thr and His. Since Thr is the *N*-terminus of α3/4, His most probably constitutes the *N*-terminus of α5. Subunit α6, showing a double band of MW *ca* 60 000 in SDS gels (Fig. 2), could be isolated, but a separation of its two components was not achieved and some minor contaminants of higher MW are still visible in SDS gels. The *N*-terminal end-group of α6 is His, other end-groups detected in small amounts can be attributed to the contaminants. That α5 and α6 are linked by S–S-bonds to β5 and β6, respectively, could be demonstrated by 2D SDS gel electrophoresis, running the first dimension without and the second dimension with reduction of the disulphide bands. Subunits α5 and β5 arise from a single band with apparent MW *ca* 75 000, α6 and β6 from a double band with MW *ca* 80 000, and the accompanying α- and β-subunits from a band with MW *ca* 60 000.

The occurrence of α6 subunits with MW *ca* 60 000 leads to speculation that they could be non-processed precursors of legumin subunits which exhibit a similar MW [7, 10]. In contradiction to this assumption are the following observations: (a) α6-subunits are linked via S–S-bonds to corresponding β-subunits; and (b) isoelectric focusing gels of the tryptic peptides from α6 exhibit a single band (α1–α4 show 2–4 bands under these conditions) and not the typical peptide pattern of β-subunits as shown in Fig. 4, i.e. the α6 subunits do not contain amino acid sequences of β-subunits. These results support the view that α6 belongs to the group of α-

subunits, and it seems to be reasonable to infer that α5 also is a member of this group.

Thus, α5–β5 and α6–β6 are two more pairs of specifically linked subunits of legumin, though constituting only minor components of the protein. The observation that none of them is cleaved by cyanogen bromide suggests a relationship to the type B subunits.

The results presented indicate, for the *Vicia* variety investigated, that at least two different gene families code for the two main classes of legumin precursor polypeptides and, hence, the corresponding subunit pairs of types A and B. The heterogeneity within each of these types may be a result of mutational alterations of a common ancestral gene. It cannot be excluded that the two gene families also arose from a single gene. The high degree of homology encountered in the *N*-terminal amino acid sequences of the mixture of β-subunits of *Vicia faba* [3] supports this idea. More information on sequences of the different α- and β-subunits is necessary to answer this question.

The final assembly of the different subunit pairs to form the hexameric legumin holoprotein still remains an unresolved question. It has been demonstrated [4, 15, 16] that various molecular species of legumin exist, which differ in their subunit compositions, but it is unknown whether all conceivable combinations of subunit pairs are possible, or whether certain restrictions exist at the molecular level favouring distinct combinations.

EXPERIMENTAL

Isolation of legumin. Seeds of *Vicia faba* L. var. minor cv 'Fribo' were finely ground. The meal was extracted with 0.05 M Tris–HCl–0.5 M NaCl, pH 7.5 (5 ml buffer/g meal). After centrifugation, solid $(NH_4)_2SO_4$ was added to the supernatant at room temp. to 45% satn. The resulting ppt was discarded and the $(NH_4)_2SO_4$ concn of the supernatant was increased to 65% satn. The ppt was exhaustively dialysed against H_2O at 4° and, subsequently, freeze-dried. 1 g of this 'crude legumin' in 25 ml 0.15 M NaPi buffer, pH 6.5, was applied to a 2 × 23 cm column of DEAE-Sepharose CL-6B (Pharmacia), equilibrated with the same buffer. After elution with *ca* 60 ml buffer, a linear gradient to 0.5 M NaCl in this buffer was applied (gradient vol.: 500 ml). Fractions containing legumin were dialysed against H_2O at 4° and freeze-dried. The legumin contained only traces of vicilin, as judged by SDS gel electrphoresis. Legumin of similar purity could also be obtained by hydroxyapatite chromatography [17] of 'crude legumin'.

Separation of subunit pairs. pH values of all buffers containing urea were adjusted in the presence of urea. Buffer A: 50 mM Tris–HCl–6 M urea, pH 7.5; buffer B: buffer A + 0.3 M NaCl.

Legumin (1 g) in 25 ml buffer A was applied to a 2 × 23 cm column of DEAE-Sepharose CL-6B equilibrated with buffer A. After washing the column with *ca* 100 ml buffer A, further elution was performed with a linear gradient of 500 ml buffer A and 500 ml buffer B. Fractions of 125 drops (*ca* 6.5 ml) were collected. Fractions were pooled as indicated in Fig. 1, dialysed against H_2O at 4° and freeze-dried.

Separation of α- and β-subunits. (a) Subunit pair (100–200 mg) was dissolved in 15 ml buffer A + 20 mg dithioerythritol (DTE) and chromatographed on DEAE-Sepharose CL-6B with buffer A containing 1 mM DTE. After elution of the β-subunit, a linear gradient of 250 ml buffer A and 250 ml buffer B, each containing 1 mM DTE, was applied to elute the corresponding α-subunit. The subunits were dialysed against H_2O and freeze-dried.

(b) To obtain S-CM subunits, 100–200 mg of a subunit pair was dissolved in 10 ml 0.1 M Tris–HCl–6 M urea, pH 8.6, and after adding 15.4 mg DTE reduced at room temp. under N_2 for 2 hr. ICH_2COOH (46.5 mg) was added and the mixture incubated in the dark for 20 min. 2-Mercaptoethanol (0.16 ml) and 10 ml 6 M urea in H_2O were added, the pH was adjusted to 7.5, and the soln chromatographed on DEAE-Sepharose CL-6B as under (a) without addition of DTE to the buffers.

SDS polyacrylamide gel electrophoresis. SDS polyacrylamide gel electrophoresis was carried out in 1 mm thick slab gels with a 15% running gel and a 5% stacking gel according to ref. [3] with the modification that stacking gel buffer and reservoir buffer were those of the borate–sulfate system described in ref. [18].

Determination of N-terminal amino acids. Subunit (0.5–1 nmol) in 0.1 ml 0.5% SDS–0.1 M Na_2HPO_4 was dansylated with 10 μl dansyl chloride (0.2 M in MeCN) for 1 hr at room temp. in the dark, ppted with 10% TCA, the ppt washed with 1 M HCl and 80% Me_2CO, and hydrolysed with 0.5 ml 6 M HCl for 18 hr at 110°. After drying in *vacuo* the residue was dissolved in 10 μl 50% C_6H_5N and chromatographed on polyamide sheets with solvent systems as described in refs. [19, 20].

Peptide mapping of legumin subunits. Trypsin soln (5 μl) (2 mg trypsin, Boehringer, in 1 ml 1 mM HCl) was added to 1 mg subunit in 0.1 ml 0.1 M NH_4HCO_3 and the mixture was incubated at 37° for 2 hr. After adding an additional 5 μl trypsin soln, the incubation was continued for 2 hr and, thereafter, the digestion was stopped by freeze-drying. Separation of the peptides was performed by two different methods: (a) *TLC (for peptides from α-subunits).* The freeze-dried peptides were dissolved in 20 μl 0.2 M NH_3 and 5 μl was spotted on a Si gel TLC plate. The first dimension was run with $CHCl_3$–MeOH–17% NH_3 (2:2:1) and, after drying at room temp. for 1 hr, the second dimension was run with *n*-BuOH–MeOH–HOAc-H_2O–C_5H_5N (45:30:6:24:20). Peptides were visualized with 0.5% ninhydrin in Me_2CO. (b) *Isoelectric focusing in polyacrylamide gels (for peptides from β-subunits).* Isoelectric focusing was performed in 7% polyacrylamide gel (acrylamide–*N*,*N*′-methylene-bisacrylamide, 25:1) similar to the method in ref. [21]. The slab gels contained 2% Ampholine, pH 3.5–10 (LKB Produkter AB), and 7 M urea. The gel slots were filled with 0.5% sucrose–2% Ampholine, pH 3.5–10. Freeze-dried peptides were dissolved in 0.2 ml 8 M urea–0.04% Ampholine and 20 μl of the soln was layered onto the gel. H_3PO_4 (25 mM) and NaOH (0.1 M) were used as anolyte (top) and catholyte (bottom), respectively. Electrofocusing was run with 2000 V·hr for a gel length of 18 cm. During the run the voltage was raised from 200 to 900 V. Gels were stained with Coomassie Brilliant Blue G-250 according to ref. [22].

Cleavage of subunits with CNBr. A soln of 1 mg CNBr in 0.3 ml 70% HCO_2H was added to 1 mg subunit, the mixture incubated at room temp. in the dark for 24 hr, diluted with 1.5 ml H_2O and freeze-dried. No cleavage of the subunits due to the acidic conditions occurred when CNBr was omitted.

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