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THE LEGUMIN-LIKE STORAGE PROTEIN OF *LUPINUS ALBUS* SEEDS

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Key Word Index—*Lupinus albus*; Leguminosae; white lupin; legumin-like seed globulins; conglutin α; glycopeptides.

Abstract—Legumin-like proteins of *Lupinus albus* seeds, consist of 12*S* and 7*S* species which are in part involved in an association–dissociation equilibrium that is shifted towards association by increasing the ionic strength of the medium. A consistent portion of the 7*S* species does not associate to 12*S*. The 12*S* and the 7*S* associating molecules have the same protomer composition. The 7*S* non-associating molecules differ from them because they have less high M_r acidic protomers. Partial proteolytic breakdown of these polypeptides appears to convert associable to non-associable molecules. The splitting protease is tightly associated to the legumin in the preparation. It is inhibited by sodium azide. The apparent M_r of the heavy and the light species is 315 000 and 185 000 respectively. Also other parameters, such as diffusion coefficients, frictional ratios, Stokes' radii, are in good agreement with those of legumin-like storage proteins from other legume seeds. Binding of the purified legumin to a concanavalin A-Sepharose and incorporation of tritiated *N*-acetylglucosamine into legumin polypeptides during seed development have confirmed that lupin legumin is glycosylated. Only the acidic subunits appear to contain covalently linked carbohydrate.

INTRODUCTION

One of the major components of legume storage proteins is 11–12*S* globulins. They are referred to as legumin-like proteins because of their analogy to legumin of pea seeds. They are of special nutritional interest due to their content in sulphurated aminoacids and in tryptophan that gives them a far more balanced aminoacid composition than the vicilins, the other major group of storage proteins.

As far as the quaternary structure of legumin is concerned, various models of hexameric structure including trigonal antiprism [1] have been proposed, although in a recent contribution it has been claimed that none of these models provides acceptable interpretation of the experimental data [2]. No definite structure of the legumin fraction of lupin seeds has been described so far.

The 11–12*S* forms dissociate to 7–8*S* depending on ionic strength and on pH [3]. In almost all cases dissociation at neutral pH is related to a decrease in ionic strength. Reports on sedimentation behaviour of this group of proteins in lupin on altering the ionic strength vary according to the species: the 12*S* globulin of *Lupinus angustifolius* [4] and *Lupinus luteus* [5] dissociated to 7.8*S* molecules upon decreasing the ionic strength of the medium, whereas in *Lupinus albus* the dissociation of the 12.5*S* protein to 8.7*S* has been reported to occur on raising the ionic strength [6].

The legumin-like fraction of lupin, which has been referred to as conglutin α [7], appears to contain a plurality of components not related to association–dissociation phenomena. In *Lupinus angustifolius*, ammonium sulphate precipitation or chromatography on celite with an ammonium sulphate gradient solubilization yielded at 4° a cold-soluble and a cold-insoluble component [7]. In previous work on *Lupinus albus* two legumin-like fractions were partially resolved by ion exchange chromatography [8]: preliminary evidence indicated that each of these fractions could be resolved further into one heavier and one lighter component [9], but these two forms were not studied in detail.

The legumin-like protein of *Lupinus albus* and *Lupinus angustifolius* were found to contain covalently bound sugar [10, 11]. This appears to be another peculiarity of the lupin since legumins generally are not glycosylated [3, 12, 13]. Bound carbohydrate has been reported in 11*S* seed globulins from faba bean [14], rapeseed and *Brassica campestris* [15].

All these peculiar features of legumin-like proteins in lupin seed prompted a more detailed study. The present work is an approach in this direction. Globulin 8 was considered, that is the most abundant constituent of the legumin-like group in lupin [8]. Some data on globulin 9 are also given.

RESULTS

High and low M_r *components of lupin legumin*

As shown in Fig. 1A two components were resolved from fraction 8 by sedimentation velocity analysis. Sedimentation coefficients did not depend on the ionic strength of the medium in the range investigated (I from 0.06 to 0.8, not shown), while the relative amounts of heavy and light species did. A preparation of fraction 8 in phosphate buffer, pH 7.5 ($I = 0.36$) was desalted, aliquots with increasing ionic strength were prepared and their sedimentation behaviour was assayed: as shown in Fig. 2 a 12*S* and a 7*S* component were present throughout; on raising the ionic strength the 7*S* species decreased and an equivalent amount of 12*S* component was produced. Above $I = 0.1$ further salt addition had no effect.

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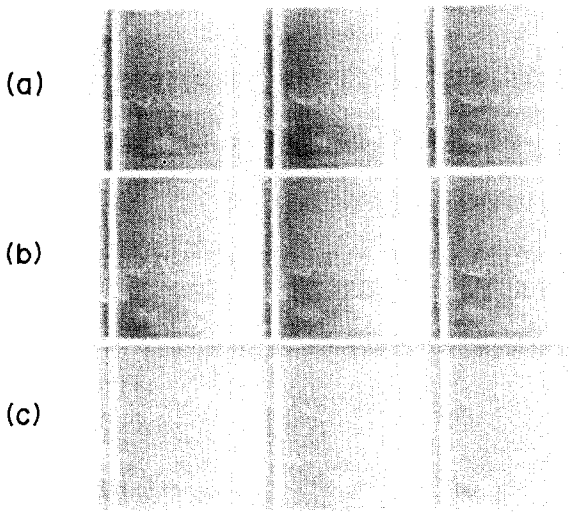

Fig. 1. Sedimentation velocity study of fraction 8 (a) and of its heavy (b) and light (c) component separated by gel permeation chromatography on Sephacryl S-300 at pH 7.5 and I = 0.21. The proteins, 3.85 mg/ml, were spun at 52 000 r.p.m., Schlieren diaphragm 60°. Pictures were taken at 26.5, 34.5 and 50.5 min, left to right (a) and (b) and at 32.4, 39.4 and 50.05 min (c) from start.

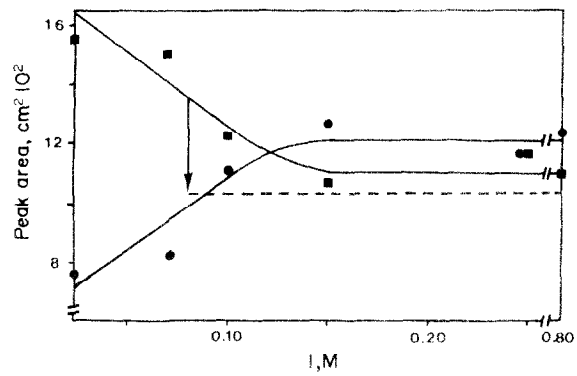

Fig. 2. Distribution of 7*S* and 12*S* components of fraction 8 at varying the ionic strength. Fraction 8, 3.0 mg/ml, was ultracentrifuged at 48 000 r.p.m., Schlieren diaphragm 60°, in 10 mM Na-Pi buffer pH 7.5 and NaCl to give the ionic strength indicated. The areas of Schlieren peaks produced at each ionic strength are given (circle: 12*S*; square: 7*S*). The arrow indicates the calculated amount (see text) of 7*S* species formed by dissociation of the 12*S* globulin at $I = 0.08$. The amount of non-associating 7*S* species (see text) is indicated by the horizontal dashed line.

Further information on transitions between heavy and light species were obtained by gel permeation in Fast Protein Liquid Chromatography (FPLC). This technique resolved fraction 8 into two major components and one excluded fraction. (Fig. 3A). The tubes indicated with bars in the figure were separated and re-chromatographed at various intervals of time. On an immediate run they yielded nearly symmetrical peaks (Fig. 3B and C) indicating only trace contamination by the other component. However upon storage a second species appeared which reproduced in apparent M_r the one removed by the initial chromatography (Table 1) and tended to reach a steady level with time (Fig. 4). In this condition the ratio of light to heavy species was much smaller when the heavy component served as starting material (Fig. 3B') than when the light one was incubated (Fig. 3C'). Sodium azide was present in the medium in order to inhibit endogenous proteolysis of the legumin (see later).

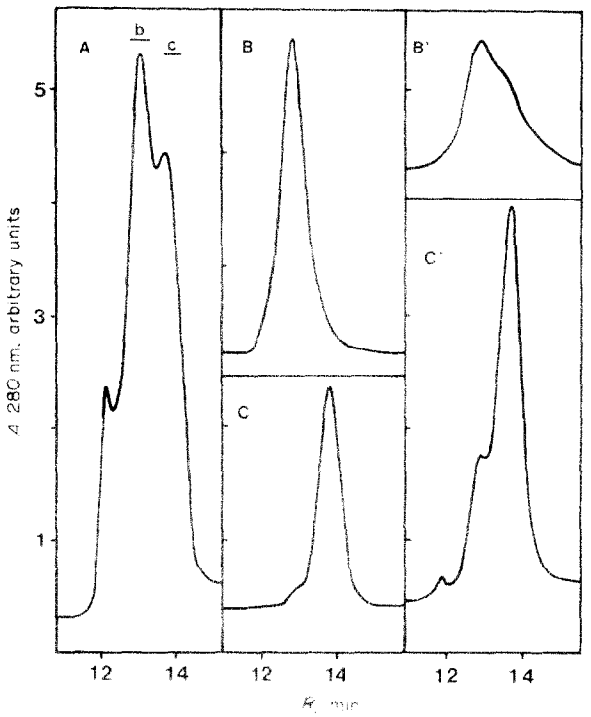

Fig. 3. Fractionation of globulin 8 by FPLC and re-chromatography of the separated heavy and light component. Fraction 8, 6.8 mg/ml, was submitted to gel permeation chromatography on FPLC in 50 mM Na-Pi buffer pH 6.8 ($I = 0.08$), and 3 mM NaN_3 at 20°. The elution profile is shown in A. Tubes indicated as *b* and *c* were pooled and re-chromatographed immediately (B and C respectively) or after 70 hr at 20° (B' and C'). A, B, C and B' were monitored at the same sensitivity; in C' the sensitivity was increased. Void volume in the column 12 min.

The ratio of light to heavy species in conditions approaching equilibrium starting from heavy compound (Fig. 3B', ratio = 0.3, $I = 0.08$) was applied to the data of Fig. 2 and allowed to distinguish within the bulk of 7*S* species the quantity due to dissociation of 12*S* globulin (Fig. 2, arrow). The rest matched the amount of 7*S* component that at I above 0.1 did not associate to 12*S* (Fig. 2, dotted line). This showed that in the experiment of Fig. 2 the association process was complete at $I = 0.1$, and the remaining light species was the light non-associable form in equilibrium with the heavy one: formation of 12*S* molecules speeds up the associating species with respect to non-associable molecules that are finally isolated free of high M_r contaminant as shown by ultracentrifugation and by PAGE.

Detection and quantification of a non associable species allowed us to interpret as an

Table 1. Molecular parameters of the heavy and light components of globulin 8

	Isolated 12S	Isolated 7S	Globulin 8 Heavy component	Globulin 8 Light component
S^0_{20w}	12.3±0.4 ($n=3$)	7.4±0.7 ($n=3$)	12.0±0.7 ($n=5$)	7.1±0.8 ($n=5$)
D^0_{20w}				
Calculated	3.80	3.89	n.d.	n.d.
Determined	n.d.	3.10	n.d.	n.d.
Stokes' radius (nm)	5.61	5.48	n.d.	n.d.
Frictional ratio, (f/f_0)	1.24	1.45	n.d.	n.d.
M_r				
sedimentation equilibrium	315 700±5300 ($n=3$)	185 400±3200 ($n=3$)	n.d.	n.d.
sedimentation velocity	n.d.	190 000	n.d.	n.d.
Gel permeation on:				
TSK G3000 SW	*	180 000±2000 ($n=6$)	*	188 000
Sephacryl S 300	n.d.	n.d.	315 000±1700 ($n=3$)	188 000±500 ($n=3$)

n.d.: Not determined.
*: Out of the fractionation range.

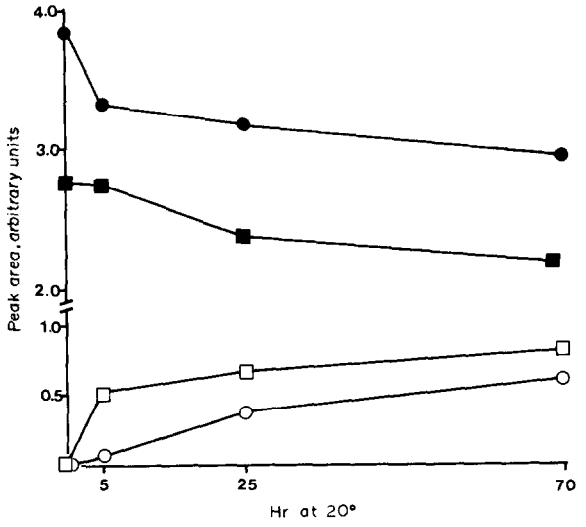

Fig. 4. Time dissociation–association behaviour of isolated heavy and light component of fraction 8. The heavy and the light component purified by FPLC as detailed in Fig. 3A, were re-chromatographed after various intervals at 20° (abscissa). Media as in Fig. 3. In composite elution peaks components were made evident by assuming for overlapped parts a behaviour symmetrical to the uncontaminated slope. Planimetric measurement of areas allowed to establish the amount of heavy and light species present at each time in the analyzed sample. Full symbols, starting material; empty symbols, newly formed species. Heavy component of fraction 8, circles; light component, squares.

association–dissociation process the results of ultracentrifugations done at increasing ionic strength. The ascertained association of 7S protein to yield 12S globulin as I is increased, modifies the data reported by Joubert [6] and sets the legumin-like proteins of *Lupinus albus* within the general scheme of behaviour as those of *Lupinus angustifolius* and of *Lupinus luteus* [4, 5] and of other legume seeds.

Loss of association capacity in the light species appears to be related to proteolytic splitting of the acidic polypeptides into its constituent subunits: this event occurs in the legumin preparation itself and its agent is tightly associated with it. A lupin albumin has been partially purified elsewhere, that hydrolyses the acidic protomers [16, 17] of pea and lupin legumins only.

Different ratios of heavy to light component in separate preparations of fraction 8 show that non-associable molecules were in different amounts; probably because proteolysis had involved a different number of molecules. Proteolysis however did not play a role in determining the ratio of heavy to light species during ultracentrifugation or gel permeation studies because of the presence of the inhibitor, sodium azide or the temperature and duration of the the run. This non-associable molecule appears also in FPLC if one considers the higher ratio of light to heavy species in Fig. 3C′ as compared to 3B′.

Gel permeation chromatography of globulin 8 on Sephacryl S 300 at pH 7.5 and $I=0.21$ gave one excluded fraction followed by a broad peak (Fig. 5A) that, depending on preparation and ionic strength, partially separated into two. On polyacrylamide gel electrophoresis (PAGE) the excluded fraction did not penetrate the gel. Early tubes of the peak displayed two close spots with low anodic mobility; the slower one soon disappeared. They were accompanied by a faster species that increased as elution progressed and separated alone at the end of the peak. Intermediate tubes in the elution contained the species in varying ratios (Fig. 5B). Overall data (see Discussion) relate slow movement to high M_r and fast movement to lower M_r. Two components were separated by direct electrophoresis of fraction 8 (Fig. 5B).

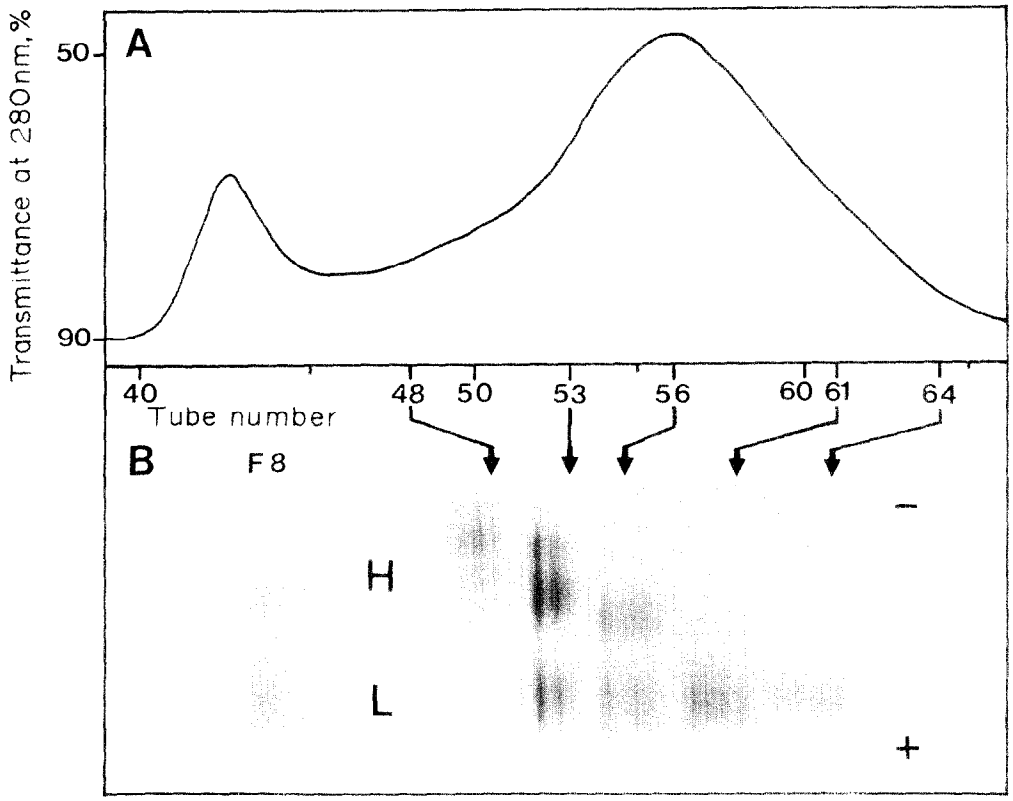

Fig. 5. Gel permeation chromatography of fraction 8 (A) and PAGE of the separated fractions (B). Fraction 8, 35 mg, was chromatographed on Sephacryl S-300 superfine at pH 7.5, $I = 0.21$ and 4°. Aliquots of 3 ml per tube were collected. A: elution curve. B: PAGE of tubes indicated, 20–28 μg protein depending on concentration in tube. H = heavy, L = light protein. F8: untreated fraction 8.

The low M_r species isolated on Sephacryl at $I = 0.21$ displayed in the ultracentrifuge a single symmetrical boundary with little broadening during the run (Fig. 1B) whereas the high M_r one contained a small amount of lighter component (Fig. 1C). This species was not removed by repeated gel permeation before ultracentrifugation.

As shown in Table 1 different separation procedures and starting material (i.e. unresolved fraction 8 or the components of it) were in very good agreement as far as determined M_r of the heavy and the light species and similarly for values of $S_{20,w}$ in the ultracentrifuge.

Fraction 9 displayed in the ultracentrifuge and on gel permeation chromatography the same type of separations as obtained with fraction 8 (not shown).

Protomer composition

The proteins separated by PAGE following gel permeation were submitted to sodium dodecyl sulphate (SDS)-PAGE under non-reducing and reducing conditions. The two slow moving species were analysed together: their protomer pattern was in both media practically the same as in the low M_r species of early tubes from Sephacryl (Fig. 6). In non reducing medium they displayed major polypeptides of apparent M_r 81 000 and 69 000 and two minor protomers of 75 000 and 72 000 that were evident as thin bands in gels at constant acrylamide concentration (e.g. Fig. 7) and in laser scannings were recognized as separate peaks or humps in the main peaks (Fig. 6A). (Calculated average M_r, $n = 7$, were 81.2 ± 2.4, 69.2 ± 2.6, 75.0 ± 2.4, 72.0 ± 0.6). Under reducing conditions (Fig. 6B) a 21 000 polypeptide was present that corresponded to the basic polypeptide of the legumin separated according to ref. [18] (not shown). Acidic polypeptides had apparent M_r 52 000, 50 000, 47 000 and 43 000 (calculated averages, $n = 3$, 52.3 ± 0.5, 49.9 ± 0.5, 47.4 ± 0.4, 42.8 ± 0.8, 20.7 ± 0.7). The light protein eluted alone in the last tubes from Sephacryl was deficient in high M_r protomers (Fig. 6, A and B) while a protomer of 63 000 (unreduced, calculated average M_r, $n = 7$, 63.0 ± 1.9) increased (Fig. 6A). The area of acidic polypeptides was 81% of total protomer area in the heavy protein while it was 77% in the light protein eluted last (Fig. 6B).

Fraction 8 and 9 displayed qualitatively and quantitatively the same protomer composition both on direct SDS-PAGE (Fig. 6C) and in second dimension after PAGE (not shown).

Autolysis

Protomer analysis by SDS-PAGE showed that high M_r polypeptides in the globulins were progressively degraded when the protein was incubated at 37° for more than 36 hr (Fig. 7). The basic polypeptide did not change in amount with time (not shown). The same degradation was found

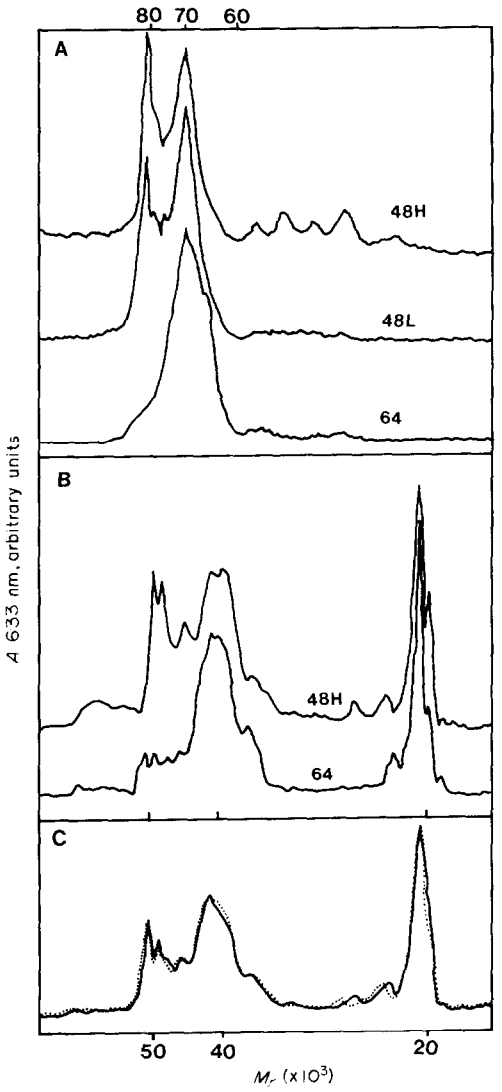

Fig. 6. Protomer composition of the various legumin-like proteins of *L. albus* seed. Proteins separated by PAGE in the experiment displayed in Fig. 5B were analysed by SDS-PAGE. Indications for tubes and bands are the same as in Fig. 5B. Laser scannings of proteins run under non-reducing, A, or reducing, B, conditions are shown. C, superimposed scannings of untreated fraction 8 (full line) and fraction 9 (dots) analysed under reducing conditions. M_r (abscissa) were determined in comparison with known standards.

in assays run under sterile conditions. At 72 hr autolysis was more pronounced in the light globulin (Fig. 7, L vs H). The total area of protomers decreased after incubation, 9% in the heavy globulin and 72% in the separated light one: this indicates that small fragments not detected by SDS-PAGE were formed. Sodium azide added in the reaction medium inhibited autolysis (Fig. 7).

The contaminating proteinase(s) were removed only in part by repeatedly passing of fraction 8 on diethylaminoethyl (DEAE) cellulose or by chromatography on Concanavalin A Sepharose as detailed under methods.

Modifications in the assembled oligomeric protein were investigated by sedimentation analysis: a sample that had the 12*S* and 7*S* components in a 3.5 to 1 ratio (52 000 rpm, sodium phosphate buffer pH 8.0, $I=0.29$) was again ultracentrifuged in the same conditions after 48 hr incubation at 37°. At this ionic strength the 12*S* globulin should not dissociate: nevertheless the ratio was found to have decreased to 1.8:1.

Protein glycosylation

Fraction 8 was fully retained on a column of concanavalin A-Sepharose in the binding buffer: it was eluted on adding 0.1 M α-methylmannoside to the buffer (Fig. 8). Samples taken throughout the elution peak had the same polypeptide composition on SDS-PAGE as untreated fraction 8 (Fig. 8).

Duplicate runs of these samples and of the separated basic and acidic subunits of globulin 8 were stained either with Coomassie Blue or with PAS stain for carbohydrate detection. The densitometric scannings of the differently stained gels run under reducing conditions are superimposed in Fig. 9. They show that no sugar was bound to the basic component, while all the acidic polypeptides were glycosylated.

Incorporation of radioactivity in the proteins of total extracts of cotyledons of developing lupin and pea seeds incubated with tritiated *N*-acetylglucosamine was measured after SDS-PAGE under non-reducing and reducing conditions. The fluorography and the densitometric scanning of a Coomassie Blue stained electropherogram in non-reducing medium are compared in Fig. 10. Bands due to protomers of legumin-like proteins were recognized because they disappeared under reducing conditions. In lupin radioactivity was incorporated in protomers of legumin-like proteins, of conglutin γ and in some belonging to vicilins (Fig. 10A). Small sized fragments appeared not to incorporate radioactivity. In the pea only the protomers of vicilin were labelled (Fig. 10B). When the total extract was resolved under reducing conditions (not shown) results were consistent with those mentioned but identification of protomers of legumin-like and vicilin-like proteins in lupin was less clearcut.

DISCUSSION

The legumin-like component of *Lupinus albus* seeds appears heterogeneous, although its constituent proteins are in many respects alike. These globulins had been partially resolved in previous work [8] by ion exchange chromatography into fractions 8 and 9 (this latter being named 9a in earlier work [8–10]. They displayed the same bound hexoses, though in slightly different amounts [8]; the aminoacid composition was very alike but amidation of dicarboxylic aminoacids was less in fraction 9, which thereby increased its acid character [8, 9]: indeed fraction 9 had a slightly more acidic isoelectric point than fraction 8 [8]. This diversity may be the reason for their different behaviour on DEAE cellulose. In the present research these proteins displayed the same M_r on gel permeation and in the ultracentrifuge, and contained the same type of protomers. Heterogeneity in isoelectric point in legumin-like proteins has been documented in other legume seeds [3, 19].

Each legumin contains one 12*S* and one 7*S* component that can be separated in the ultracentrifuge and by gel permeation chromatography but not by ion exchange.

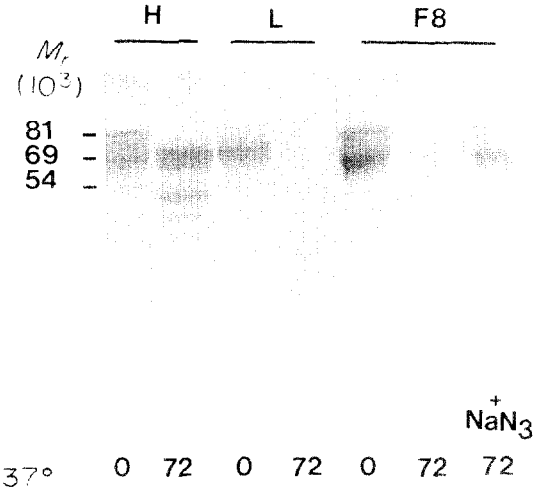

Fig. 7. Autolysis of globulin 8. Fraction 8 (F8) and its heavy (H) and light (L) component separated on Sephacryl S-300, were incubated at 37° in 0.05 M Na-Pi buffer pH 8.0 and 0.15 M NaCl. Sodium azide, where present, was 3 mM. Equal aliquots were removed at intervals and were assayed by SDS-PAGE under non reducing conditions. Results at $t=0$ and after 72 hr are shown. The M_rs were established with reference to standard proteins.

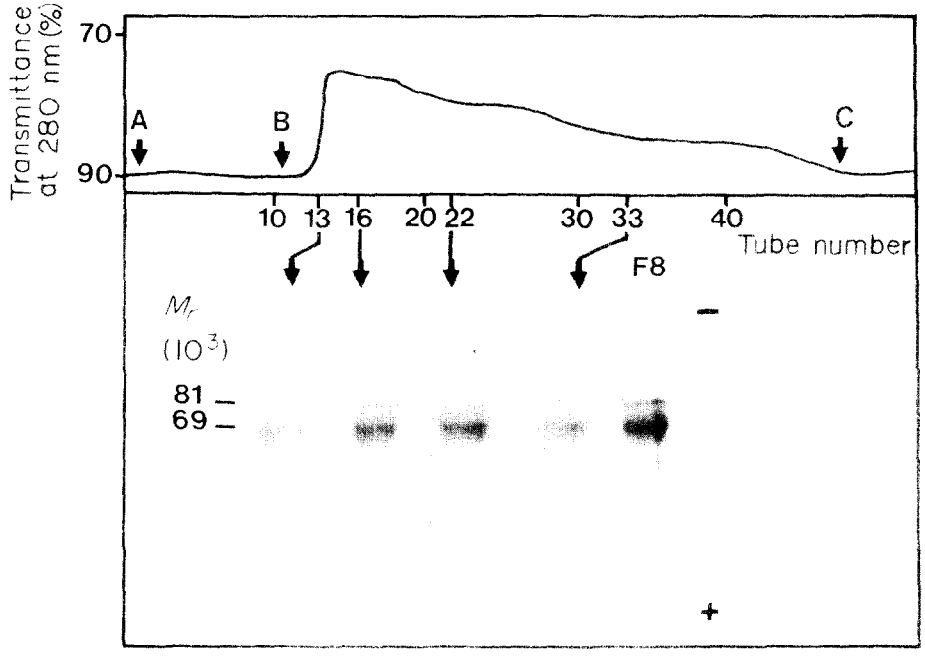

Fig. 8. Chromatography on Concanavalin A-Sepharose of fraction 8. Fraction 8 (26 mg) was dissolved and applied in 0.1 M Na-Pi buffer pH 7.2 containing 0.2 M NaCl, 1 μM $MnCl_2$ and 1 μM $CaCl_2$. Elution was first with buffer as above (A) then with buffer containing 0.1 M α-methylmannoside (B), finally with 0.2 M borate buffer pH 8.5 (C). Aliquots of 1.75 ml per tube were collected. SDS-PAGE analysis of tubes indicated with arrows and of the starting protein (F8) are shown in the lower part of the figure.

This behaviour indicates that different mobilities observed in PAGE were not due to deamidation of asparagine or glutamine residues in the same compound but to differences in M_r. The legumin appears to form also high M_r aggregates that were excluded in separations based on molecular size; their amount depended on the course of experimental manipulations of the protein. The very slow species in PAGE may represent a dimeric form of the 12*S* component; a similar situation occurs in the broad bean [20].

Gel permeation chromatography of the 12*S* and the 7*S* globulin made it possible to follow quantitatively how the light species formed from the heavy one and vice versa, in a reversible association–dissociation process that tended

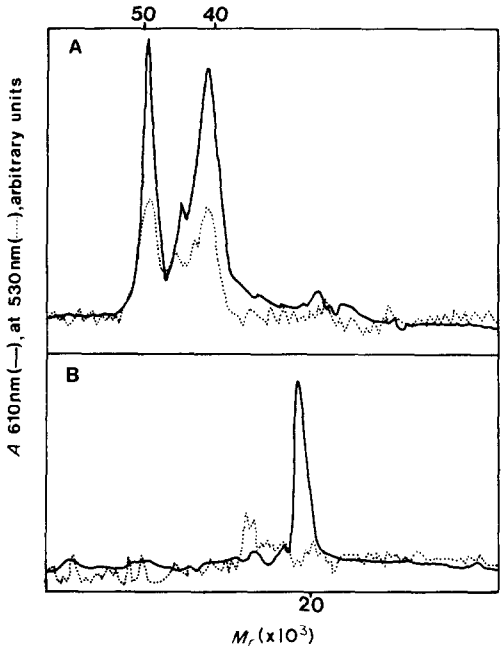

Fig. 9. Polypeptide and sugar distribution in legumin-like protein of lupin. The separated acidic (A) and basic (B) polypeptides of the legumin (fraction 8) were analysed by SDS-PAGE in the presence of 2% 2-mercaptoethanol. Each sample was run twice, in parallel lanes. At the end of the run one lane was stained for polypeptide with Coomassie Blue and scanned at 610 nm (full line), the other one for sugar with a Schiff reagent, and was scanned at 530 nm (dots). Scannings are superimposed. The M_rs were determined in comparison to known standards.

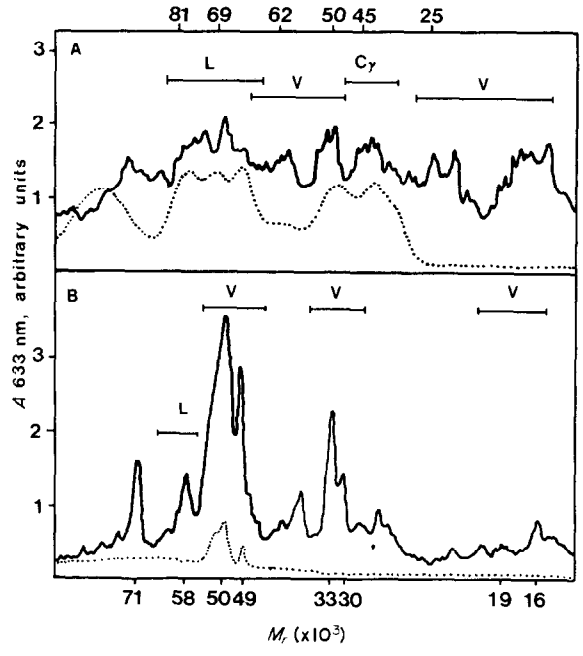

Fig. 10. Incorporation of tritiated *N*-acetylglucosamine in developing cotyledons of lupin (A) and of pea (B) seeds. The total globulin extract of cotyledons incubated with tritiated *N*-acetylglucosamine was analysed by SDS-PAGE under non-reducing conditions. Laser scannings of electropherograms stained with Coomassie Blue (full line) are compared with labelled polypeptides evidenced by fluorography (dots). Lupin peaks belonging to legumin-like (L) or vicilin-like (V) proteins and to conglutin γ (Cγ) are indicated (horizontal full lines). Pea polypeptides are attributed according to literature data [37]. Apparent M_rs were determined with reference to standard proteins.

to equilibrium. These results agree with data obtained in the ultracentrifuge and indicate that 12*S* molecules are formed by associating 7*S* units. An equilibrium between a monomer of M_r 180 000 and a dimer of 350 000 has been reported for arachin [21].

Dissociation of the heavy species explains why it was not possible to obtain it free of a light component in the ultracentrifuge and by PAGE. Proteolysis measured on the non associable light species indicates that modifications due to proteolysis are likely to make the molecule more susceptible to further action of proteolytic enzymes. The isolated legumin-like protein of sunflower seeds, helianthinin [22], and that of *Nigella damascena* [23], undergo degradations quite similar to those shown in our experiments when kept at 4°.

The legumin contains several polypeptides that differ slightly in M_r. A similar situation occurs in other legume seeds [24–27]. The pattern of proteolysis observed in the present experiments, as well as results on the albumin endopeptidase of lupin seed [17] indicate that larger protomers (81 000, 75 000 and 72 000) may be the precursors of those of 69 000 and 63 000 upon proteolytic splitting. What physiological role these modifications play in the seed is not known. They might prepare stored protein for the action of other endopeptidases and of exopeptidases on germination.

The determined M_r of lupin legumin compare well with those of most legumin-like proteins. Values for 7*S* and 12*S* components close to 1:2 indicate that 12*S* is made up of two 7*S* units as in other legumin-like proteins [3]. The constituent polypeptides found by protomer analysis hardly fit a trimer:hexamer scheme, because of their high M_r. Nevertheless they may display on SDS-PAGE an apparent M_r higher than that assumed from the assembled oligomer because of either conformational constraints in the oligomer or by virtue of the bound sugar and high content of dicarboxylic aminoacids: these last factors may interfere with correct binding of SDS, and affect the mobility in the gel. Also the sum of M_rs of acidic and basic subunits is less than the M_rs of the unreduced unsplit protomers: in this case binding of acidic and basic peptides, before reduction, is an additional hindering factor to mobility.

Precursors of conglutin α, the legumin-like globulin of *Lupinus angustifolius* seeds, form in protein bodies a family of polypeptides of M_r 64 000–85 000 [28]. These values are in good agreement with those found by us for the mature polypeptides: in phaseolin precursor and mature polypeptide differ by 1000–2000 [29].

Presence of bound sugar in legumin-like proteins has long been debated: for some seeds it has been attributed to co-purification of a contaminant glycosylated polypeptide [12]. The present data confirm our previous evidence [8] indicating that legumin-like seed globulin in *Lupinus albus* is glycosylated. No carbohydrate was

bound to the basic polypeptide, whereas all acidic polypeptides were glycosylated. This result and the higher content of hydrophobic aminoacid residues in the basic subunit (Duranti, M. and Cerletti, P., unpublished results) agree well with the view that makes the acidic subunits exposed to solvent and the basic one hidden inside the native molecule of legumin.

EXPERIMENTAL

All chemicals were of the highest purity commercially available. *Lupinus albus* seeds were of the sweet Multolupa variety (alkaloid content 0.05%).

Purification of the legumin-like proteins and of their subunits. The procedure detailed below is a modification of the method previously described in ref. [8]. All proteins quoted were identified by SDS-PAGE.

The defatted lupin flour was extracted with H_2O (1:20 w:v) at pH 5, for 12 hr at 4° and then centrifuged 30 min at 16000 *g*; the pellet was extracted for 3 hr at 4° with 1 M NaCl in 50 mM Na-Pi buffer pH 7 (1:10 w:v) and then centrifuged as above. The supernatant was desalted by gel filtration on Sephadex G 50 in the same buffer as above, at pH 7.5. This buffer was present also in all subsequent purification steps. The desalted soln was adjusted to 0.18 M NaCl and was applied on a column of DEAE 52 cellulose (34 × 4 cm) equilibrated with 0.18 M NaCl in the buffer at pH 7.5. Elution was carried out with a discontinuous NaCl gradient: 0.18 M NaCl eluted conglutin γ and the vicilin-like proteins (conglutin β). Some legumin associated with the vicilins was thereby lost in a separate peak but no contaminating vicilins were left in the column. The salt concn was raised to 0.25 M: a broad peak was eluted which, depending on the experiment, partially separated into two. The initial part of the peak was taken as fraction 8, while the rest was resolved on a column of Ultrogel AcA 54 (55 × 2 cm) into fraction 9 and a non-legumin low M_r component corresponding to conglutin δ [8] (In previous work [8–10] fraction 9 was named 9a and conglutin δ 9b.) Both fraction 8 and 9 displayed on SDS-PAGE a pattern typical for the legumins. Their relative amount was 7:3 (w:w). Basic and acidic subunits of globulin 8 were separated as described in ref. [18] with minor modifications.

The heavy and the light components of globulin 8 were prepared from fraction 8 by gel permeation chromatography on a column (94 × 1.9 cm) of Sephacryl S-300 superfine (Pharmacia) at 4° in 50 mM Na-Pi buffer, pH 7.5 containing 0.1 M NaCl at a flow rate of 30 ml/hr.

Analytical ultracentrifugation. A Beckman Spinco model E analytical ultracentrifuge with Schlieren optics was used for sedimentation velocity studies. Runs were done at 20°. Procedures and calculations were those described in ref. [30] and [31]. The value $v = 0.75$ ml/g was assumed as partial specific vol. as suggested in ref. [30]. Sedimentation equilibrium studies were done with a MSE Centriscan 75 analytical ultracentrifuge equipped with a data processor computer. The meniscus depletion method of ref. [32] was used: a linear plot of ln C vs $r^2/2$ (correlation coefficient = 0.997) was obtained with the proteins tested.

Diffusion coefficients at 20° ($D^0_{20,w}$) were either determined by sedimentation velocity with the height-area method or calculated from determined M_rs and sedimentation coefficients. For association–dissociation equilibria measurements, the areas of the peaks measured by planimetry after magnification of the photographs were corrected for radial dilution.

Protomer composition. Protomer composition of the proteins was determined by SDS-PAGE on a 15% acrylamide bis-acrylamide gel according to ref. [33]. Apparent M_r determinations were done on an 11–19% acrylamide–bis-acrylamide gradient in the presence or not of 2% 2-mercaptoethanol. Quantitative estimation of the separated bands was done by densitometric scanning of the gels using either a LKB 2202 Ultroscan laser densitometer interfaced with an Apple II C computer or a Varian 634 spectrophotometer equipped for variable wavelength densitometer scannings.

Assays for protein glycosylation. Chromatography on concanavalin A Sepharose 4B Sigma was done on a column of 110 × 2 cm in 0.1 M Na-Pi buffer pH 7.2, containing 0.1 M NaCl, 1 μM $MnCl_2$ and 1 μM $CaCl_2$ (binding buffer). Elution was first with the binding buffer then with buffer containing 0.1 M α-methylmannoside and finally with 0.2 M Na borate buffer, pH 8.5. Glycosylated polypeptides after SDS-PAGE were shown with the *p*-aminosalicylate (PAS) stain according to ref. [34].

Tritiated *N*-acetyl-D-glucosamine was incorporated in developing seeds of *Lupinus albus* and of *Pisum sativum* 49 days after flowering in lupin and 14 days after flowering in pea as described in ref. [35]. Proteins were extracted from separated cotyledons with sample denaturing buffer containing 2% SDS and the extract was analysed by SDS-PAGE. The polypeptide bands were fixed with a soln of MeOH–HOAc–H_2O (4:1:5 v:v) then the gel was soaked for 2 hr with Autofluor, National Diagnostics, U.K. Fluorography was done with Fuji X ray films at −80° for 5 weeks. The developed and fixed films were compared with Coomassie Blue stained gels.

Other methods. Gel permeation in FPLC was run on an LKB equipment having a TSK-G 3000 SW column with fractionation range 10,000–300,000. Na-Pi buffer, 50 mM, pH 6.8, containing 3 mM NaN_3 was the eluting agent. PAGE under non denaturing conditions was on a 7.5% acrylamide bis-acrylamide gel in 0.06 M Tris–glycine buffer, pH 9.0 ($I = 0.05$). Apparent M_r were determined according to ref. [36]. Protein markers were glucose oxidase, bovine serum albumin (BSA), transferrin, egg albumin, β-lactoglobulin, myoglobin, ribonuclease A, cytochrome *c*, for gel permeation studies; phosphorylase B, BSA, egg albumin, carbonic anhydrase and lysozyme, for PAGE and SDS-PAGE, all from SIGMA. Unless otherwise specified, other methods were as previously detailed [8, 10].

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