Plant Physiol. (1989) 91, 1425–1431
0032-0889/89/91/1425/07/$01.00/0

Received for publication May 23, 1989
and in revised form July 13, 1989

Isolation and Characterization of Protein Bodies in *Lupinus angustifolius*[1]

Kenwyn R. Gayler*, Friederika Wachsmann, Sotirios Kolivas, Roslyn Nott, and Elizabeth D. Johnson
Russell Grimwade School of Biochemistry, University of Melbourne, Parkville, Victoria, 3052, Australia

ABSTRACT

Using Nycodenz, a novel density gradient medium, we isolated intact protein bodies from developing seeds of *Lupinus angustifolius* L. (cultivar Unicrop) and achieved excellent separation from the endoplasmic reticulum, mitochondria, and other organelles. The distribution of the storage protein conglutin-β was taken as evidence that up to 96% of the protein bodies remained intact on the gradients and banded at 1.25 grams per milliliter. The protein bodies also contained the three other abundant proteins present in *L. angustifolius* seeds: conglutins-α, -γ, and -δ. Pulse labeling experiments were carried out to determine the site of proteolytic processing of conglutin-α, a legumin-like 11Svedberg unit storage protein. Cotyledons aged either 33 or 40 days after flowering were pulsed with [^{3}H]leucine. Protein bodies obtained from the cotyledons aged 33 days after flowering contained only the labeled precursors of conglutin-α with molecular weights 85,000, 72,000, and 64,000, even after a 4 hour chase of the radioactivity. Protein bodies obtained from the cotyledons aged 40 days after flowering contained the same radioactive precursors if the tissue had been pulsed for 2 hours, and the processing products of these precursors when the tissue had been chased for 4 hours. These studies confirm that the subcellular location of proteolytic cleavage of this legumin-like protein is the protein body, that this activity is detected only in protein bodies from lupin seeds aged between 33 and 40 days of seed development after flowering and that protein bodies from seeds younger than this contain only unprocessed conglutin-α.

Seeds of the grain legume *Lupinus angustifolius* contain three predominant storage proteins and one relatively minor storage protein. The predominant proteins, conglutins-α, -β, and -δ, are members of the widespread 11S, 7S, and 2S families of storage proteins, respectively. The other protein in seeds of lupins, conglutin-γ, appears to be an example of a fourth type of storage protein (3). Conglutin-γ is a methionine and cysteine-rich protein and has physical and chemical properties quite distinct from the other classes of storage proteins (5, 25). Conglutin-γ in lupins and the homologous relative in soybeans, "basic 7S globulin" (17) are both present only in relatively small amounts in seeds of the respective species.

In common with other well characterized storage proteins from peas and from kidney beans (7, 10), all the conglutins appear to undergo secretion and transport through the endoplasmic reticulum. This was shown specifically for both conglutins-α and -γ (16), and was accompanied in the case of conglutin-α by the removal of signal sequence in the conventional way.

Synthesis of legume storage proteins is further followed by transport to the protein bodies. Initially such proteins accumulate as localized deposits on the edges within the large vacuoles of the cell. However, as seed development progresses these vacuoles develop into smaller protein-filled organelles called protein bodies (11).

Many storage proteins have been shown to undergo a second stage of proteolytic cleavage. In peas, the mature subunits of the 11S protein legumin were cleaved from a family of precursors with M_r approximately 60,000 to families of subunits with M_rs of approximately 40,000 and 20,000, and this occurred within 2 to 3 h after synthesis (9). Vicilin (7S) was likewise cleaved within 6 to 20 h after synthesis in peas and similarly rapid processing was demonstrated for lectins in peas (15), glycinin in soybeans (2) and for other proteins including a soluble 12S globulin from oats (27), and the 1.7S napin from rapeseed (12).

Protein bodies, the generally accepted subcellular location for such processing, have been difficult to isolate and purify and this has hampered further studies of these processes. In this paper we present an improved method for isolation of protein bodies from developing seed material and use it to examine the processing of storage proteins during seed development in lupins.

The capacity for cleavage of the precursors of conglutins-α and -β, the major storage proteins in lupins, is acquired only in the latter stages of cotyledonary development (14). In this paper we show that the protein bodies from lupin seeds differ in their abilities to process the legumin-like conglutin-α at different stages in their development and that the capacity for proteolytic processing of conglutin-α is not acquired by protein bodies until well after the initial accumulation of storage proteins in them. Evidence is also presented which confirms that all four conglutins, α, β, γ, and δ, do accumulate in the protein bodies of the lupin as expected of true storage proteins.

MATERIALS AND METHODS

Plant Material

Lupinus angustifolius L. cultivar Unicrop was grown in 15 cm pots in a mixture of vermiculite and acid-washed sand (3:1) in a controlled environment cabinet (12 h day/night; 24°C day/19°C night) with illumination of 850 μE/m^2/s from

[1] This work was supported by grants from the Australian Research Grants Scheme and from the Rural Development Trust Fund and E. D. J. was supported by a Commonwealth Research Grant Award.

metal arc lamps and fertilized weekly with a complete nutrient solution (4).

Protein Body Isolation

Protein bodies were isolated from developing cotyledons aged between 30 and 45 DAF by fractionation on continuous density gradients of Nycodenz (Nyegaard and Co, Oslo, Norway). Nycodenz was dissolved in 2 mM $MgCl_2$ by shaking in an orbital shaker for 2 h at 37°C, and diluted to each of 60, 50, and 40% (w/v). The final dilution of 25% (w/v) also included 4% (w/v) sucrose to maintain osmolarity in the range 300 to 390 mOsmol throughout the gradient. Two mL of each dilution of Nycodenz were layered into a Beckman SW40 centrifuge tube and a continuous gradient formed by diffusion overnight with tubes at an angle of 30°. Six whole cotyledons were chopped with a razor blade into a fine suspension in 6 mL of 12% (w/v) sucrose in 2 mM $MgCl_2$ at 4°C. The homogenate was squeezed through two layers of Miracloth (Chickopee Mills), 2 mL loaded directly onto each prepared gradient, and centrifuged at 4°C for 20 min at 2500*g* ($r_{av.}$ 11.3 cm) in a Beckman SW40 rotor unbraked. One mL fractions were collected by aspiration from the gradients. Analysis of marker enzyme activity was carried out directly on these fractions. Protein and Chl contents were assayed after extraction.

Microscopy of Intact Protein Bodies

Protein bodies prepared in this way were viewed directly under a Zeiss photomicroscope using Nomarski interference optics. Protein bodies were also prepared for electron microscopy essentially as described by Peoples *et al.* (22). Isolated protein bodies were washed in 10 volumes of 12% (w/v) sucrose containing 2 mM $MgCl_2$ and pelleted at 1100*g* in a bench centrifuge at room temperature. The protein body pellet was fixed in 3% (w/v) glutaraldehyde in 25 mM Pipes buffer (pH 7.2) for 90 min at room temperature. The fixed pellet was cut into 3 mm cubes, washed in the same buffer, and postfixed in 1% (v/v) aqueous osmium tetroxide for 1 h at room temperature. Tissue was dehydrated through a graded series of ethanol washes, acetone, and finally infiltrated with increasing concentrations of Spurr's resin in acetone over 4 to 5 d. After cutting, blocks were stained with a solution of uranyl acetate in 50% (v/v) methanol followed by lead citrate.

Assays for Marker Enzymes and Chl

Assays for NADH-Cyt *c* reductase were performed directly on the isolated fractions according to Bowles and Kauss (8) except that 1.6 mM NaN_3 replaced NaCN. α-Mannosidase activity was determined by the method of Van der Wilden and Chrispeels (26) using the chromogenic substrate *p*-nitrophenyl-α-D-mannoside (Sigma) in the presence of 4 mM $ZnSO_4$, 0.1% Triton-X-100, and 100 mM sodium acetate buffer (pH 4.5). One unit of α-mannosidase activity hydrolyzed 1×10^{-6} μmol substrate/min. Cyt *c* oxidase was assayed according to Bollini and Chrispeels (6) to identify the position of mitochondrial membranes. Chl was estimated either by absorption spectroscopy in ethanolic extracts or by fluorescence in acetone extracts excited at 420 nm and measured at 667 nm on a Perkin-Elmer MPF-3 dual beam fluorescence spectrometer.

Labeling of Proteins in Detached Cotyledons

Incorporation of radioactive amino acid into proteins in detached cotyledons was performed as previously described (16). Cotyledons were placed on 20 μL drops of L-[4,5-^{3}H] leucine (10 μCi) for 2 h and then rinsed in sterile distilled water and incubated in 1 mM leucine (unlabeled) for the duration of the 'chase.' After incubation a 1 mm slice was taken from the face of each cotyledon exposed to the radioactivity and used for isolation of protein bodies.

Protein Analysis

Proteins were extracted by homogenization in 60 mM Tris-HCl buffer (pH 7.5), 10% (w/v) NaCl, incubation at room temperature for 2 h, centrifugation to remove insoluble material, and dialysis into 60 mM Tris-HCl buffer (pH 7.5).

For analysis of conglutin-δ, proteins were extracted into 60 mM Tris-HCl buffer (pH 7.5) containing 10% (w/v) NaCl and 5 mM *N*-ethyl maleimide and incubated for 2 h at room temperature to block free sulphydryl groups. After centrifugation the supernatant was loaded onto a reversed-phase Sep-Pak cartridge (Waters-Millipore, Bedford, MA) and the loosely bound material removed by washing first with distilled water and then with 8% (v/v) acetonitrile in 0.1% TFA. Conglutin-δ was quantitatively recovered from the column by elution with 42% (v/v) acetonitrile in 0.1% TFA followed by freeze-drying.

Quantitation of Storage Proteins

Total protein was determined by the method of Lowry *et al.* (19) after precipitation in 9% (w/v) TCA. Levels of conglutins-β and -γ were measured in competition assays by ELISA. Anti-rabbit IgG[2] or anti-mouse IgG conjugated to horseradish peroxidase (Amersham International) was used to oxidize 2.2[1]-azino-di-[3-athyl-benzthiazolinosulfonat(6)] (Boehringer Mannheim, West Germany) and the absorbance at 414 nm was recorded in a Bio-Rad plate reader.

Antibodies

Antisera to conglutins-α, -β and -γ were raised in rabbits. For conglutin-γ, cell lines of specific monoclonal antibodies were injected into the peritoneal cavity of mice, and ascites fluid containing the monoclonal antibodies was collected. Both the rabbit antisera and mouse ascites fluid were purified by affinity chromatography on columns prepared by the coupling of the respective purified proteins to CNBr-activated Sepharose 4B.

Electrophoresis and Western Blotting

Electrophoresis on SDS-polyacrylamide gels, fluorography and Western blot transfers were carried out as previously

[2] Abbreviations: IgG, immunoglobulin G.

described (16). Antibodies on such blots were detected using alkaline phosphatase conjugated to anti-rabbit or anti-mouse IgG and Promega Protoblot color reagents.

RESULTS

Isolation of Intact Protein Bodies

Non-aqueous procedures which have been used previously to isolate protein bodies from dry lupin seeds (23, 24) are not readily applicable to hydrated developing cotyledonary tissue. Likewise most of the aqueous procedures previously used in lupins (3) and in other legumes (10, 28) produced low yields of intact protein bodies due to the osmotic instability of the protein bodies. When preliminary attempts were made to isolate protein bodies from developing lupin cotyledons in aqueous sucrose by the method of Chrispeels *et al.* (9, 10), protein bodies rapidly lysed both at high (>30%) and at low (<12%) sucrose concentrations. Improved fractionation required a medium which had a relatively low osmolarity (300–400 mOsmol) at the high densities (1.3 g/mL) needed to separate the protein bodies from cellular debris. Mixed gradients of Nycodenz and sucrose were used to produce a continuous density gradient which increased from 1.05 g/mL to 1.31 g/mL whilst retaining an osmolarity in the range 300 to 390 mOsmol.

Tissue chopped into a fine suspension and centrifuged through such gradients separated into several distinct fractions. Total protein assays showed a small peak at a density of 1.10 g/mL at the interface between the 12% sucrose sample layer and the top of the Nycodenz gradient and a large peak of protein further down the gradient at a density of 1.25 g/mL (Fig. 1). Under a light microscope large numbers of protein bodies were clearly visible in this fraction as spherical membrane-bound organelles density 1.25 g/mL (Fig. 2A).

Electron micrographs also showed intact protein bodies in this fraction at density 1.25 g/mL organelles filled with electron dense material and apparently surrounded by a single membrane (Fig. 2B). This is equivalent to the structure seen for protein bodies in sections of intact cells of *L. angustifolius* (21). Le Gal and Rey (18) have observed an additional type of protein body in mature cotyledons of *Lupinus albus* which contains small lightly staining inclusions. These appear to be absent from *L. angustifolius* at the more juvenile stages of cotyledonary development studied in this paper.

The major contaminants of the protein bodies were Chl-containing membrane fragments, presumably the remnants of chloroplasts. Measurements of the distribution of Chl throughout the gradient (Fig. 1) and of fluorescence under the light microscope both showed the presence of Chl in the only significant membrane fragments contaminating the protein bodies. Other membranes, particularly those detected by the marker enzymes for endoplasmic reticulum, NADH-Cyt *c* reductase, and for mitochondria, Cyt oxidase, were only minor contaminants. The bulk of the activities of these enzymes was located in the fraction density 1.10 g/mL at the interface at the top of the gradients (Fig. 1). Inosine diphosphatase activity was found at densities equal to or less than the NADH-Cyt *c* reductase activity when tissue was fractionated on similar (10–40%) Nycodenz gradients. Both the Golgi membranes and the endoplasmic reticulum membranes therefore were concentrated in these fractions with density approximately 1.10 g/mL, well away from the protein bodies.

The majority of protein bodies appeared to be recovered intact. Not only was a high proportion of the total protein recovered in the protein body fraction (Figs. 1 and 6), but the distribution of particular proteins on the gradients also suggested that there was little or no release of the contents of the protein bodies during the isolation procedure. This was shown both by electrophoresis of the total proteins in each fraction and by immunoassays for particular proteins.

As shown in Figure 3 the proteins present in the protein body fractions on gradients of this kind were predominantly of mol wt 64,000 to 85,000, that is the high mol wt precursors of conglutins-α and -β previously shown to be the major forms of storage proteins in cotyledons of lupins of this age (28–33 DAF) (14). A small pool of similar proteins was also present in the fraction at density 1.10 g/mL, presumably associated with the endoplasmic reticulum. By contrast the top three fractions, where the contents of burst protein bodies

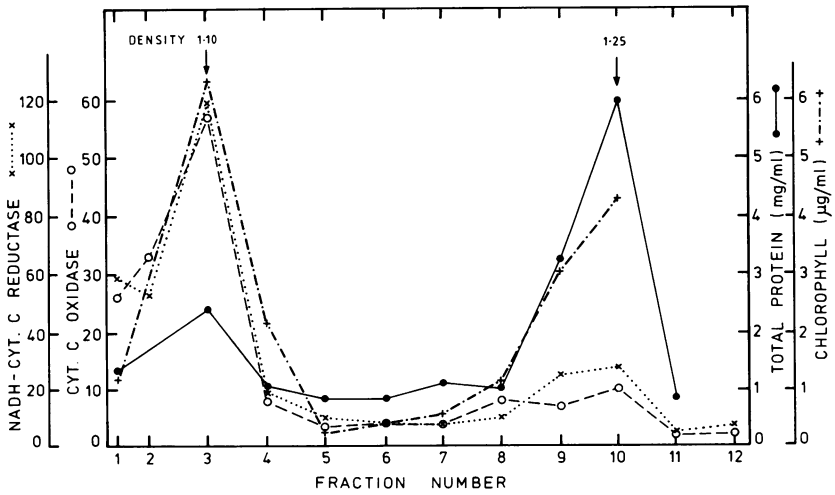

Figure 1. Distribution of protein, NADH-Cyt *c* reductase, Cyt oxidase, and Chl on a Nycodenz-sucrose gradient. Subcellular extracts were prepared from six cotyledons aged 28 to 33 DAF and fractionated on a 25% to 60% (w/w) Nycodenz gradient containing sucrose to maintain osmolarity between 300 and 390 mOsmol/mL. All media also contained 2 mM $MgCl_2$. Gradients were centrifuged at 2500*g* for 20 min and fractions (1 mL) collected at 4°C. NADH-Cyt *c* reductase (x·····x) and Cyt *c* oxidase (O-----O) activities are expressed as units/mL. One unit NADH-Cyt *c* reductase = 1 µmol Cyt *c* reduced/min; 1 unit Cyt *c* oxidase = 1 µmol Cyt *c* oxidized / min.

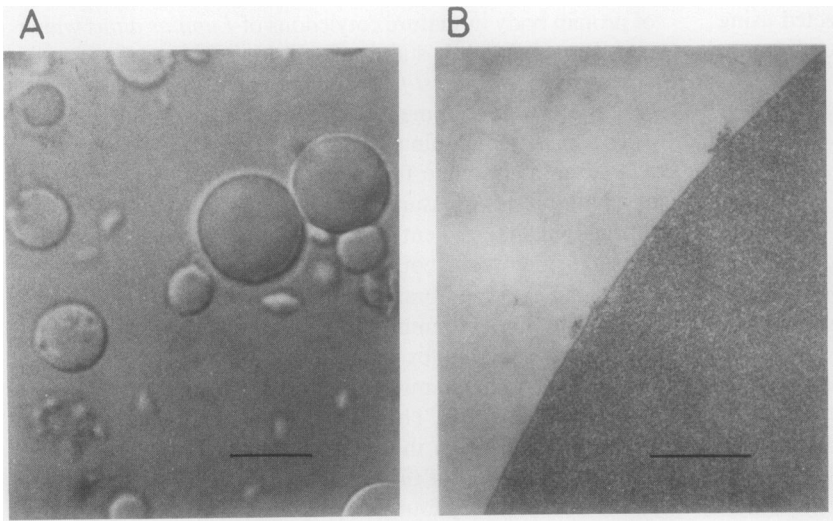

Figure 2. Protein bodies isolated fraction density 1.25 g/mL on a standard Nycodenz gradient viewed (A) by Nomarski interference light microscopy and (B) by electron microscopy. The single membrane surrounds electron dense material. Bars: A, 10 μm; B, 0.5 μm.

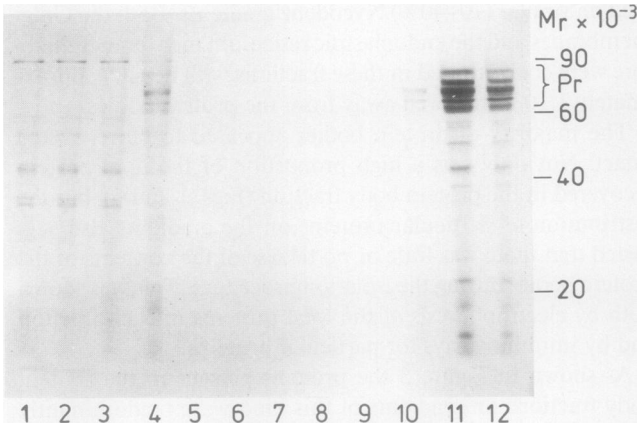

Figure 3. Fractionation by SDS-PAGE of the polypeptides present in each fraction (1–12) of the Nycodenz gradient shown in Figure 6. Lanes were loaded with a standard aliquot (2 μL) of the collected fraction. Proteins were detected with Coomassie blue staining. Pr = high mol wt precursors of conglutins-α and -β.

were expected, contained a diverse group of proteins with little resemblance to the major storage proteins.

Further characterization of the proteins associated with organelles separated on Nycodenz gradients was obtained using ELISA immunoassays to measure the level of individual conglutins. As shown in Figure 4A, conglutin-β, the authentic 7S storage protein from *L. angustifolius*, was located almost entirely in the protein body fraction. On this particular gradient only 4% of conglutin-β was detected outside of the protein bodies; in the fraction at density 1.10 g/mL. This not only confirmed the identity of the protein bodies but also suggested that at least 96% of the protein bodies retained their contents during the extraction and isolation.

Subcellular Location of Other Stored Proteins

The Nycodenz procedure for the isolation of protein bodies was used to determine the location of the other putative storage proteins, conglutins-γ and -δ.

Detection of conglutin-δ, the 2S sulfur-rich storage protein in lupins, was not possible immunologically as this protein failed to produce antibodies in either rabbits or mice. Conglutin-δ was measured instead by selectively binding the protein to a C_{18}-reversed phase column and then the protein was detected by electrophoresis and densitometric scanning. It was estimated in this way that at least 98% of conglutin-δ was located in the protein bodies (Fig. 4C).

The distribution of conglutin-γ, the other sulfur-rich putative storage protein in lupins was determined using an ELISA assay with monoclonal antibodies which reacted specifically with the small subunit of conglutin-γ in Western blot analyses. As shown in Figure 4B, the majority of the conglutin-γ detected by the monoclonal antibodies on gradients was located in the protein body fraction and a small proportion was found in the fractions at the top which include the endoplasmic reticulum and soluble proteins. Independent Western Blot analysis using polyclonal antibodies (Fig. 5) showed that much of the conglutin-γ found at the top of the gradient was present as a polypeptide of M_r 51,000; that is, it was the precursor of conglutin-γ, M_r 51,000, which is in transit through the endoplasmic reticulum. This precursor was not expected and was not detected in the protein bodies (Fig. 5 track 10). The remainder of the conglutin-γ at the top of these gradients was shown by these semiquantitative Western blots to be conglutin-γ in its mature form. This pool may be from the larger and more fragile central vacuoles.

Other Functions Associated with the Protein Bodies

Mannosidases

By contrast with the storage proteins, α-mannosidase, one of the hydrolases found in plant protein bodies, was spread through several subcellular fractions from the lupin cotyledons. Protein bodies from developing lupin cotyledons did contain a significant pool of α-mannosidase as shown in Figure 6, and there was in addition a large amount of α-mannosidase activity associated with the endoplasmic reticulum and soluble fractions at the top of the gradients. In *Phaseolus vulgaris*, α-mannosidase activity has been demonstrated in three compartments, the cell wall, the endoplasmic

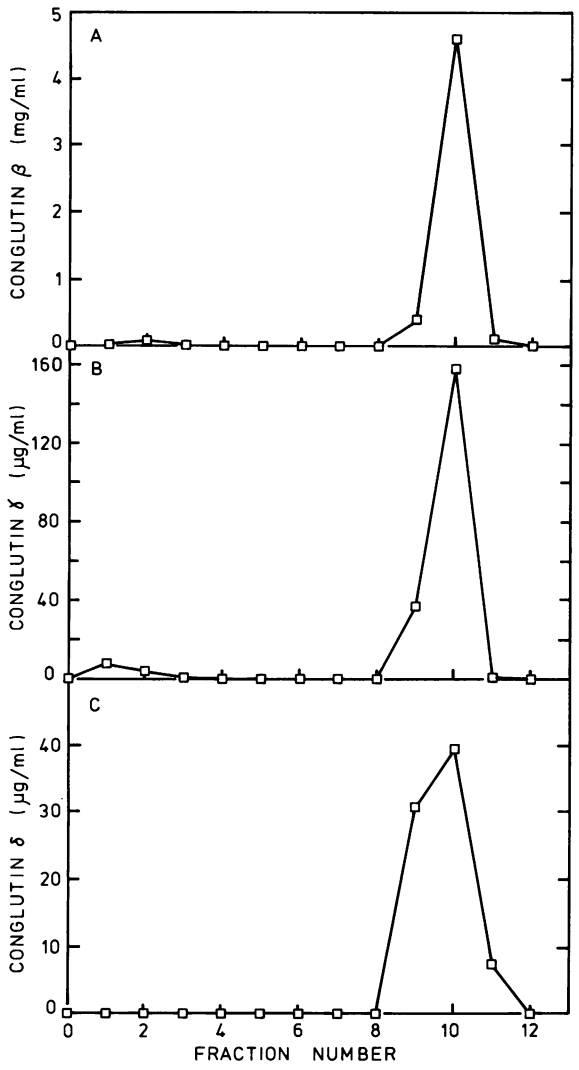

Figure 4. Distribution of the conglutins on Nycodenz gradients. Fraction numbers correspond to 1 mL fractions taken sequentially from the top of a standard gradient. Protein was extracted (A and B) in 60 mM Tris-HCl buffer (pH 7.5), 10% (w/v) NaCl and (C) in the same buffer containing 5 mM *N*-ethyl maleimide. Individual conglutins were detected by either ELISA (A and B) or chemically (C). Conglutin levels are expressed in units/ml of the original fractions. (A) Conglutin-β detected with affinity purified rabbit anti-conglutin-β antibodies. Four per cent of conglutin-β was present in fractions 2, 3, and 4. (B) Conglutin-γ detected with affinity purified mouse ascites fluid containing monoclonal anti-conglutin-γ antibodies. (C) Conglutin-δ detected after reverse phase chromatography on C_{18} Sep-pak.

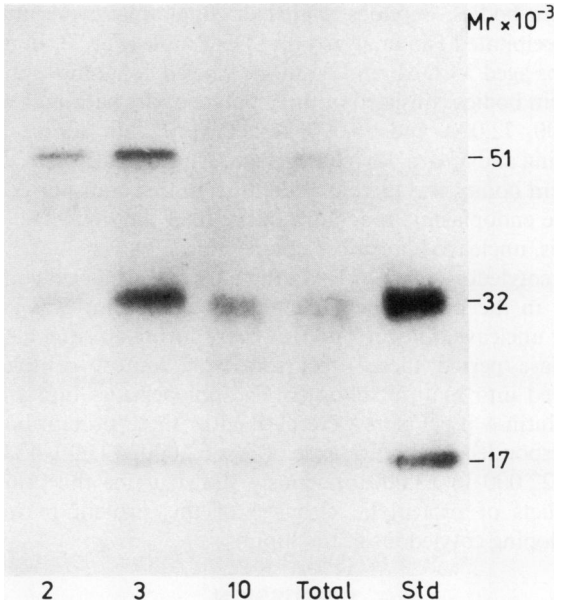

Figure 5. Western blot analysis showing the distribution of conglutin-γ in subcellular organelles fractionated on a standard Nycodenz gradient. Lanes marked 2, 3, and 10 contain 50 μg total protein from fractions equivalent to 2, 3, and 10 in Figure 1; lanes marked 'Total' and 'STD' contained, respectively, 50 μg of the unfractionated protein prior to loading onto the gradient and 1 μg of purified conglutin-γ. Proteins were separated by SDS-PAGE, transferred to cellulose nitrate and detected with affinity purified rabbit anti-conglutin-γ antibodies using alkaline phosphatase conjugated to anti-rabbit IgG.

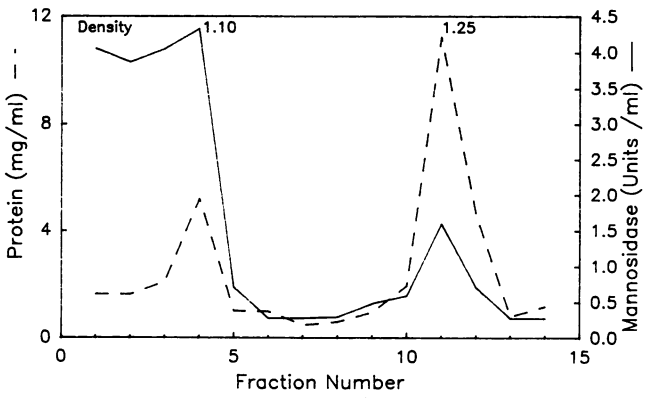

Figure 6. Distribution of α-mannosidase activities in subcellular fractions of developing lupin cotyledons. Fraction numbers correspond to 1 mL fractions taken sequentially from the top of a standard gradient. Distribution of α-mannosidase is shown relative to the distribution of total protein on the gradient. One unit of α-mannosidase catalyzed hydrolysis of 1×10^{-6} μmol substrate/min.

reticulum, particularly in developing cotyledons where synthesis and secretion are active, and in the protein bodies (26). It appears that in developing lupin seeds α-mannosidase may similarly reside outside of the protein bodies in the endoplasmic reticulum or cell walls. We cannot however rule out the possibility that some of the soluble α-mannosidase activity may have been released through lysis of large central vacuoles.

Proteolysis by Protein Bodies

The Nycodenz procedure for preparation of intact protein bodies from developing cotyledons was also used to identify the intracellular site of the delayed proteolytic processing of the precursors of conglutins in lupins, particularly conglutin-α the 11S protein.

Protein bodies were prepared from cotyledons either 33 DAF, or 40 DAF, *i.e.* either before or after cotyledons acquire the capacity for proteolysis of the precursors of conglutins-α and -β (16). The cotyledons were labeled by incubation with [^{3}H]leucine and chased for up to 4 h before preparation of

protein bodies. Peptides related to conglutin-α were immunoprecipitated and analyzed on SDS-PAGE (Fig. 7). In cotyledons aged 33 DAF and younger, labeled conglutin-α in the protein bodies consisted of three polypeptides with mol wt of 85,000, 72,000, and 64,000, respectively, both after 2 h of labeling and after a 4 h 'chase' (Fig. 7A). Conglutin-α in these protein bodies was therefore identical to that transported out of the endoplasmic reticulum in tissue of the same age (16), that is, uncleaved precursor polypeptides.

In cotyledons aged 40 DAF, the bulk of the labeled conglutin-α in the protein bodies after 2 h of labeling was again these uncleaved precursors. However, during an equivalent 4 h chase period these precursors were almost completely cleaved into mature subunits. The polypeptides into which conglutin-α precursors were cleaved in these protein bodies corresponded exactly to those of M_rs 62,000, 49,000, 42,000, and 22,000 to 23,000 previously shown to be the primary products of proteolytic cleavage of this protein in intact developing cotyledons of the lupin.

DISCUSSION

The method for isolation of protein bodies developed above appears to have several advantages over previous methods. We have shown that Nycodenz gradients with narrow osmotic range can be used to isolate protein bodies from developing cotyledons of the lupin in high yield and to separate them from the other possible pools of storage proteins: the endoplasmic reticulum, golgi apparatus and cytoplasm. As previously shown by Ford and Rickwood (13) Nycodenz is relatively inert. It did not interfere in the current work with either the integrity of the protein bodies, the binding of antibodies or the enzyme assays used in these subcellular localization studies. Nycodenz should therefore have wider application in organelle studies in plants.

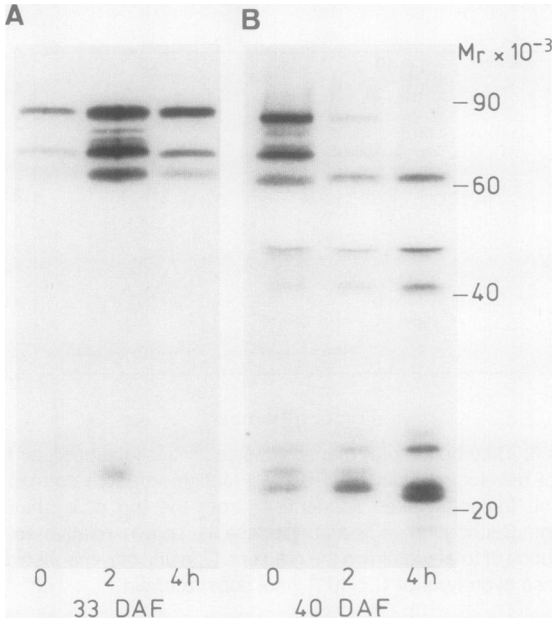

Figure 7. Fluorograph of conglutin-α polypeptides recovered from the protein body fraction of developing cotyledons after labeling in a pulse-chase incubation. Cotyledons were labeled with 10 μCi of [^{3}H] leucine for 2 h, then protein bodies isolated as described in Figure 1 after a further 0, 2, or 4 h 'chase' with unlabeled leucine. Polypeptides related to conglutin-α were recovered by immunoprecipitation with anti-conglutin-α antibodies and separated by SDS-PAGE. (A) Cotyledons aged 33 DAF and (B) cotyledons aged 40 DAF. Minor contaminant bands of conglutin-β polypeptides are visible in some tracks.

In this paper it has been used to study transport and post-translational modification of storage proteins in developing lupin seeds. As already indicated, the outstanding feature of both conglutins-α and -β is the persistence of the precursor forms in juvenile seeds of the lupin and the sudden onset of specific proteolysis 30 to 35 days after flowering. Such proteolysis has been shown in other species to occur in the protein bodies (9, 15). Initially it was thought that in lupins the precursors might simply be physically separated from the protein bodies in younger seeds and hence from the proteolytic enzymes. However, examination of subcellular transport showed that newly synthesized polypeptides did arrive intact in the protein bodies. Although these proteins remained unchanged in such young tissue, the same precursors of conglutin-α were rapidly cleaved in protein bodies in older cotyledons (aged 40 DAF) (Fig. 7). It must be concluded therefore that at a stage of development between 33 and 40 DAF that protein bodies acquire the capacity to cleave selected proteins accumulated within them, presumably through the synthesis or activation of one or more specific enzymes. The enzymes presumably include one equivalent to the thiol-dependent protease isolated from pumpkin cotyledons which catalyzes cleavage of the precursor form of the 11S globulin in pumpkin and has been shown to be similarly developmentally regulated (20). The biosynthesis of this processing enzyme in pumpkin cotyledons is delayed for several days after the onset of synthesis of the 11S globulin molecules (20), so that processing of 11S proglobulin molecules in pumpkins is delayed for several days after synthesis in a manner equivalent to that previously shown for the precursors of the conglutins in lupins (14, 16).

Whereas the major role served by the complement of hydrolytic enzymes, both glycosidases and proteases in seeds is to interact during seed development, maturation and germination to prepare the storage proteins for their ultimate degradation, it is possible that such hydrolysis also plays an important role in the secretion of such proteins. Legume storage proteins must contain inherent or potential signals to direct the protein to the protein bodies and these signals are required for successful function of the storage protein. Post-translational proteolytic cleavage to the mature subunit polypeptides however does not appear to be a signal for transport of the major storage proteins in developing lupin seeds. That such cleavage is not essential for transport of conglutin-α, is shown clearly by the fact that its precursors are able to make their way to the protein body uncleaved (Fig. 7A).

A more likely functional role for proteolysis may in fact be to facilitate packing in the developing storage organelle. The change observed in the proteolytic capacity of these protein bodies may be related indirectly to an alteration in protein body structure which occurs in such legumes during seed development. During the early stages of development of the cotyledon there are substantial changes in protein body struc-

ture. Protein is deposited against the internal side of the central vacuole in both peas and lupins (11, 21) although in soybeans subdivision of large vacuoles is reported to precede protein accumulation (1). During the period of rapid protein synthesis in peas and lupins, protein bodies become larger and more numerous (21, 29) and are thought to be derived from fragmentation of the central vacuole. In these smaller vacuoles the concentration of deposited protein must increase. If post-translational proteolysis is required for efficient packing of storage proteins, it is possible that cleavage begins when the protein concentration within the protein body or the ratio between individual proteins reaches a critical level. This may be the signal for production of the relevant proteolytic enzyme. Delayed post-translational proteolysis has also been observed for glycinin in the very early stages of development in soybean (2). However, in this case the capacity to cleave the glycinin precursor is acquired much earlier than in the lupin; before the seed is capable of maximum synthesis and possibly before the protein bodies reach mature size.

Finally in this paper we have considered the status of conglutins-γ and -δ as storage proteins. From the labeling studies of conglutin-γ (16) it is clear that conglutin-γ is synthesized as a precursor of M_r 51,000 and that this precursor can be isolated from the endoplasmic reticulum fraction. Qualitative studies of Blagrove and Gillespie (3) and Plant and Moore (24) both indicated that conglutin-γ was present in protein body fractions isolated from mature dried seeds. Using the improved gradients which avoid osmotic extremes, we have now shown that most of this protein does accumulate in the protein body in developing tissue along with conglutins-α and -δ. On this basis we conclude that not only conglutins-α, -β, and -δ but also conglutin-γ must indeed be regarded as authentic storage proteins in the lupin.

ACKNOWLEDGMENTS

The authors thank Mrs. V. Beilharz for preparation of the electron micrographs and Drs. G. G. Lilley and D. Hewish, CSIRO Division of Biotechnology, for assistance with monoclonal antibody preparation and reversed phase chromatography of conglutin-γ.

LITERATURE CITED

1. **Adams CA, Norby SW, Rinne RW** (1985) Production of multiple vacuoles as an early event in the ontogeny of protein bodies in developing soybean seeds. Crop Sci **25:** 255–262
2. **Barton KA, Thompson JF, Madison JT, Rosenthal R, Jarvis NP, Beachy RN** (1982) The biosynthesis and processing of high molecular weight precursors of soybean glycinin subunits. J Biol Chem **257:** 6089–6095
3. **Blagrove RJ, Gillespie JM** (1975) Isolation, purification and characterization of the seed globulins of *Lupinus angustifolius*. Aust J Plant Physiol **2:** 13–27
4. **Blagrove RJ, Gillespie JM, Randall PJ** (1976) Effect of sulphur supply on the seed globulin composition of *Lupinus angustifolius*. Aust J Plant Physiol **3:** 173–184
5. **Blagrove RJ, Gillespie JM, Lilley GG, Woods EF** (1980) Physicochemical studies of conglutin-γ, a storage globulin from seeds of *Lupinus angustifolius*. Aust J Plant Physiol **7:** 1–13
6. **Bollini R, Chrispeels MJ** (1979) The rough endoplasmic reticulum is the site of reserve protein synthesis in developing *Phaseolus vulgaris* cotyledons. Planta **146:** 487–501
7. **Bollini R, Van der Wilden W, Chrispeels MJ** (1982) A precursor of the reserve-protein, phaseolin, is transiently associated with the endoplasmic reticulum of developing *Phaseolus vulgaris* cotyledons. Physiol Plant **55:** 82–92
8. **Bowles DJ, Kauss H** (1976) Characterization, enzymic and lectin properties of isolated membranes from *Phaseolus aureus*. Biochim Biophys Acta **443:** 360–374
9. **Chrispeels MJ, Higgins TJV, Spencer D** (1982) Assembly of storage protein oligomers in the endoplasmic reticulum and processing of the polypeptides in the protein bodies of developing pea cotyledons. J Cell Biol **93:** 306–313
10. **Chrispeels MJ, Higgins TJV, Craig S, Spencer D** (1982) Role of the endoplasmic reticulum in the synthesis of reserve proteins and the kinetics of their transport to protein bodies in developing pea cotyledons. J Cell Biol **93:** 5–14
11. **Craig S, Goodchild DJ, Hardham AR** (1979) Structural aspects of protein accumulation in developing pea cotyledons. 1. Qualitative and quantitative changes in parenchyma cell vacuoles. Aust J Plant Physiol **6:** 81–98
12. **Ericson ML, Rodin J, Lenman M, Glimelius K, Josefsson L, Rask L** (1986) Structure of the rapeseed 1.7S storage protein, napin, and its precursor. J Biol Chem **261:** 14576–14581
13. **Ford TC, Rickwood D** (1982) Formation of isotonic Nycodenz gradients for cell separations. Anal Biochem **124:** 293–298
14. **Gayler KR, Boadle BG, Snook M, Johnson ED** (1984) Precursors of storage proteins in *Lupinus angustifolius*. Biochem J **221:** 333–341
15. **Higgins TJV, Chrispeels MJ, Chandler PM, Spencer D** (1983) Intracellular sites of synthesis and processing of lectin in developing pea cotyledons. J Biol Chem **258:** 9550–9552
16. **Johnson ED, Knight J, Gayler KR** (1985) Biosynthesis and processing of legumin-like storage proteins in *Lupinus angustifolius* (lupin). Biochem J **232:** 673–679
17. **Kagawa H, Yamauchi F, Hirano H** (1987) Soybean basic 7S globulin represents a protein widely distributed in legume species. FEBS Lett **226:** 145–149
18. **Le Gal MF, Rey L** (1986) The reverse proteins in the cells of mature cotyledons of *Lupinus albus* var Lucky. 1. Quantitative ultrastructural study of the protein bodies. Protoplasma **130:** 120–127
19. **Lowry OH, Rosebrough NJ, Farr AL, Randall RJ** (1951) Protein measurement with the folin phenol reagent. J Biol Chem **193:** 265–275
20. **Nishimura IH, Nishimura M** (1987) Proglobulin processing enzyme in vacuoles isolated from developing pumpkin cotyledons. Plant Physiol **85:** 440–445
21. **Parker ML** (1984) Cell wall storage polysaccharides in cotyledons of *Lupinus angustifolius* L. I. Deposition during seed development. Protoplasma **120:** 224–232
22. **Peoples MB, Beilharz VC, Waters SP, Simpson RJ, Dalling MJ** (1980) Nitrogen redistribution during grain growth in wheat (*Triticum asetivum* L.). II. Chloroplast senescence and the degradation of ribulose-1-5-bisphosphatase carboxylase. Planta **149:** 241–251
23. **Plant AR, Moore KG** (1982) α-D-Mannosidase and α-D-galactosidase from protein bodies of *Lupinus angustifolius* cotyledons. Phytochemistry **21:** 985–989
24. **Plant AR, Moore KG** (1983) The protein, lipid and carbohydrate composition of protein bodies from *Lupinus angustifolius* seeds. Phytochemistry **22:** 2359–2363
25. **Tulloch PA, Blagrove RJ** (1985) Electron microscopy of seed-storage globulins. Arch Biochem Biophys **241:** 521–532
26. **Van der Wilden W, Chrispeels MJ** (1983) Characterization of the isozymes of α-mannosidase located in the cell wall, protein bodies, and endoplasmic reticulum of *Phaseolus vulgaris* cotyledons. Plant Physiol **71:** 82–87
27. **Walburg G, Larkins BA** (1983) Oat seed globulin: subunit characterization and demonstration of its synthesis as a precursor. Plant Physiol **72:** 161–165
28. **Weber E, Manteuffel R, Neumann D** (1978) Isolation and characterization of protein bodies of *Vicia faba* seeds. Biochem Physiol Pflanzen **172:** 597–614
29. **Wozny A, Mlodzianowski F, Stefaniak B** (1984) Protein bodies formation in yellow lupin seed. Acta Soc Bot Pol **53:** 3–9