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Vicilin-type globulins follow distinct patterns of degradation in different species of germinating legume seeds

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Abstract

The pattern of degradation of the vicilin-type globulin was followed during germination and seedling growth of a number of legume species, namely *Lupinus albus*, *Lupinus angustifolius*, *Lupinus luteus*, *Lupinus mutabilis*, *Glycine max*, *Pisum sativum*, *Vicia faba*, *Vicia sativa*, *Lathyrus cicera*, *Lathyrus sativus* and *Arachis hypogaea*. Catabolism was fast for some species (ex: *L. albus*, *P. sativum* and *V. sativa*) and particularly slow for others (ex: *L. sativus* and *A. hypogaea*). The pattern of degradation was also species-specific and independent of the rate of proteolysis. In some cases, a transient accumulation of stable intermediates of vicilin catabolism was detected. As a whole, the data indicate that each species evolved a particular mode of vicilin proteolysis that best suited its development under the conditions to which it was most favourably adapted.

Keywords: Globulins; Vicilins; Degradation; Germination; Legumes; Seeds

1. Introduction

Globulins represent the major storage proteins in legume seeds. With very few exceptions, legume seeds contain two major types of globulins, termed vicilin-type and legumin-type globulins, or simply vicilins and legumins, respectively, after the initial isolation by Osborn and Campbell in 1898 of the major proteins from *Pisum sativum* seeds (Ferreira et al., 2003; Osborne and Campbell, 1898). These proteins are also referred to by trivial names; this is the case, for example, of vicilin and legumin from *P. sativum* and *Vicia faba*, of β-conglutin and α-conglutin from *Lupinus* species, of conglycinin and glycinin from *Glycine max*, of β-vignin and α-vignin from *Vigna unguiculata*, of β-lathyrin and α-lathyrin from *Lathyrus sativus*, and of conarachin and arachin from *Arachis hypogaea* (Ferreira et al., 2003; Freitas et al., 2004; Rosa et al., 2000).

Vicilins comprise a well-known class of storage proteins and can constitute as much as 70–80% of the total seed protein (Bewley & Black, 1994). They are glycosylated oligomers of 150–170 kDa formed by three similar subunits of about 40–70 kDa with no disulphide linkages. All vicilins purified from legume seeds are highly heterogeneous and consist of many different types of subunits, thus exhibiting a high degree of polymorphism within each species. This heterogeneity is due to the expression of multigene families whose individual genus are very closely related and also to post-transcriptional processing including glycosylation (Casey et al., 1986; Derbyshire et al., 1976; Ferreira et al., 2003; Nielsen et al., 1989; Wright, 1988).

As storage proteins, vicilins serve primarily as a source of organic nitrogen and carbon skeletons for the biosynthetic reactions of the growing plant. Alternatively, their

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component amino acids may be used as fuel for the energy metabolism. For this reason, vicilins are, as other storage proteins, gradually degraded during germination and seedling growth. Studies performed on *Vicia sativa* showed that during germination, globulin mobilization is initiated by stored cysteine proteinases in protein bodies and that *de-novo*-formed proteolytic enzymes mainly mediate bulk catabolism of stored globulins after germination (Schlereth, Standhardt, Mock, & Muntz, 2001). There, vicilin mobilization precedes legumin mobilization. Apparently, vicilin represents the initial source of amino acids for early growth and differentiation processes in *V. sativa*. Legumin presumably only serves as a bulk amino acid source for subsequent seedling growth during post-germinative globulin proteolysis (Tiedemann, Newbohn, & Muntz, 2000).

In the case of *G. max*, the structures of key intermediary fragments of 30 kDa derived from β-conglycinin degradation were detected and analyzed during germination and seedling growth. β-Conglycinin is first subjected to limited proteolysis at exposed regions on the molecular surface, like domain junctions, generating 30 kDa single-domain fragments before non-specific catabolism (Kawai, Suzuki, Asano, Miwa, & Shibai, 1997). In cotyledons of germinating *Lupinus albus*, the accumulation of a lectin-like breakdown product of β-conglutin catabolism was followed. This intermediate polypeptide of β-conglutin proteolysis abruptly accumulates in *L. albus* cotyledons between 4 and 14 days after the onset of germination, and is rapidly degraded thereafter (Ramos, Ferreira, Franco, & Teixeira, 1997).

Little is known regarding what roles other than nutrient storage vicilins assume. Some recent reports have raised the possibility that vicilins may, in fact, also play a defensive role in germinating seeds (Chung, Neumann, & Polya, 1997; Yamada, Shimada, Kondo, Nishimura, & Hara-Nishimura, 1999). Indeed, the germination stage is an especially vulnerable one for the developing plant. The seed must survive in a moist and pathogen rich environment during the critical stages of imbibition and germination. Then, the days that coincide and follow the emergence of the plantlet from the soil are probably the most critical and sensitive phase of plant development to predation and often to harsh environmental conditions. Possibly, the intermediary breakdown products of vicilin catabolism referred above may fulfill a role here.

Vicilins from *Vigna unguiculata* and other legume seeds have recently been shown to strongly associate with chitin, chitosan and fully acetylated chitin (Sales, Gomes, Fernandes, & Xavier-Filho, 1996). Recent results have also shown that vicilins interfere with the germination of spores or conidia of phytopathogenic fungi and inhibit yeast growth and glucose stimulated acidification of the medium by yeast cells (Gomes et al., 1997; Gomes, Okorokov, Rose, Fernandes, & Xavier-Filho, 1998). Vicilin-related basic proteins isolated from *Gossypium hirsutum* seeds were also shown to inhibit the growth of various filamentous fungi (Chung et al., 1997). In addition, vicilins of *V. unguiculata* seeds bind to chitinous structures of the midgut of *Callosobruchus maculatus* larvae, suggesting that these proteins may play a role in defending seeds against bruchids (Sales, Gerhardt, Grossi-de-Sa, & Xavier-Filho, 2000; Sales, Pimenta, Paes, Grossi-de-Sa, & Xavier-Filho, 2001).

Vicilins from *Lens culinaris* and *P. sativum* have recently been identified as major allergens (Lopez-Torrejon et al., 2003; Sanchez-Monge et al., 2004). In this respect, vicilins from *Lupinus luteus*, *Vicia sativa*, *Lathyrus cicera*, *G. max*, *P. sativum*, *V. faba*, *L. albus* and *Cicer arietinum* were reported to be immunogenic in weaned piglets (Salgado et al., 2002; Seabra et al., 2001). It is therefore important to follow the rate and pattern of vicilin proteolysis during germination and seedling growth in legume seeds. In this respect, a comparative study was undertaken on the vicilins from *L. albus*, *L angustifolius*, *L. luteus*, *L. mutabilis*, *G. max*, *P. sativum*, *V. faba*, *V. sativa*, *Lath. cicera*, *Lath. sativus* and *A. hypogaea*.

2. Materials and methods

2.1. Plant materials

Dry seeds of white lupin (*L. albus* L.) cultivar Misak, of narrow-leafed lupin (*Lupinus angustifolius* L.), yellow lupin (*Lupinus luteus* L.) and lupin from the Andes (*Lupinus mutabilis* L.) were kindly supplied by Dr. J.N. Martins (Universidade Técnica de Lisboa). Dry seeds of soya bean (*G. max* (L.) Merr.), pea (*P. sativum* L.), broad bean (*V. faba* L.), common vetch (*V. sativa* L.), vetchling (*L. cicera* L.), chickling vetch (*L. sativus* L.) and peanut (*A. hypogaea* L.) were obtained in a local Lisbon market.

Germination was initiated by immersion of the seeds in running tap water for 48 h at room temperature. The germinated seeds were then planted in sand and incubated at 25 °C in a 16 h/8 h light/dark cycle under fluorescent lighting. The plantlets were watered as required with water. Zero (dry seed), 2, 4, 6, 8 and 10 days after the onset of germination, the seed coats were removed and the intact cotyledons dissected from the axes and stored frozen at −80 °C until needed.

2.2. Isolation of total globulins

A previously optimized methodology to isolate the total globulin fraction from dry seeds of lupin, soya bean, pea, broad bean, chickpea, common bean, lentil and peanut was used here (Franco, Ferreira, & Teixeira, 1997; Freitas, Ferreira, & Teixeira, 2000). This methodology is based on the observation that legume globulins self-aggregate into high molecular mass aggregates in the presence of divalent cations in a process mediated by electrostatic interactions (Ferreira et al., 2003). These insoluble large aggregates are readily solubilized under high ionic strength conditions (e.g. 10% (w/v) NaCl) on upon cation removal (e.g. incubation with 10 mM EDTA and 10 mM EGTA) (Ferreira, Franco, & Teixeira, 1999). In this work, the dry cotyledons

(zero days of germination; dry seed) of the species under study were milled (0.2 mm sieve) and the resulting meal was deffated with *n*-hexane (34 mL/g of flour) for 4 h with agitation, decanted and air-dried. The albumin fraction of the proteins was extracted by stirring with water (adjusted to pH 8.0 with highly diluted NaOH) containing 10 mM $CaCl_2$ and 10 mM $MgCl_2$ (34 mL/g of flour) for 4 h. The suspension was centrifuged for 1 h at 30,000*g*. The supernatant was discarded and the total globulin fraction of the proteins extracted by stirring the pellet with 0.1 M Tris–HCl buffer, pH 7.5, containing 10% (w/v) NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM ethyleneglycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (34 mL/g of flour), for 4 h. The globulin-containing solution was centrifuged for 1 h at 30,000*g* and the globulins were precipitated by the addition of ammonium sulphate (561 g/L). The precipitated globulins were centrifuged at 30,000*g* for 20 min, resuspended in 50 mM Tris–HCl buffer, pH 7.5 (5.7 mL/g of flour) and desalted in PD-10 columns (GE Healthcare, Uppsala, Sweden) previously equilibrated in the same buffer. All operations were performed at 4 °C.

For the extraction of total globulins from germinated cotyledons, the same procedure as for the dry cotyledons was used with minor changes. Essentially, 1 mM phenylmethylsulphonyl fluoride (PMSF) was added to both albumin and globulin extraction buffers, 2 mL of extraction buffer/g fresh weight were used instead of 34 mL/g of flour, the incubation times were only of 30 min at 4 °C, and the extract was immediately desalted and not precipitated with ammonium sulphate.

2.3. Purification of vicilins

A one-step methodology previously optimized to fractionate the total globulin fraction and to isolate the major individual globulins (including vicilin) from the dry seeds of lupin, soya bean, pea, broad bean, chickpea, common bean, lentil and peanut was used here (Freitas et al., 2000; Melo, Ferreira, & Teixeira, 1994). In this work, vicilin and legumin were purified from the species under study by FPLC anion exchange chromatography of the total globulin fraction on the Mono Q HR 5/5 column (GE Healthcare, Uppsala, Sweden). The column was equilibrated with 50 mM Tris–HCl buffer, pH 7.5, 2 mL of the solution containing the total globulin fraction were injected into the column, and the bound proteins were eluted with a continuous, linear gradient of NaCl (0–1 M). Vicilins elute from the Mono Q column with ~0.3 M NaCl, whereas legumins are eluted with ~0.4 M NaCl.

2.4. Production of polyclonal antibodies

Polyclonal antibodies were produced in rabbits against the 20 kDa polypeptide that is a breakdown product of a β-conglutin catabolism in the cotyledons of germinating *L. albus* seeds (Ramos et al., 1997). The vicilin containing the 20 kDa polypeptide was purified from the cotyledons of 8-days old *L. albus* plantlets, free of cross-contaminations, as described above. The 20 kDa polypeptide was subsequently isolated by preparative SDS–PAGE. After staining with Coomassie Brilliant Blue, the acrylamide band corresponding to the 20 kDa polypeptide was sliced,

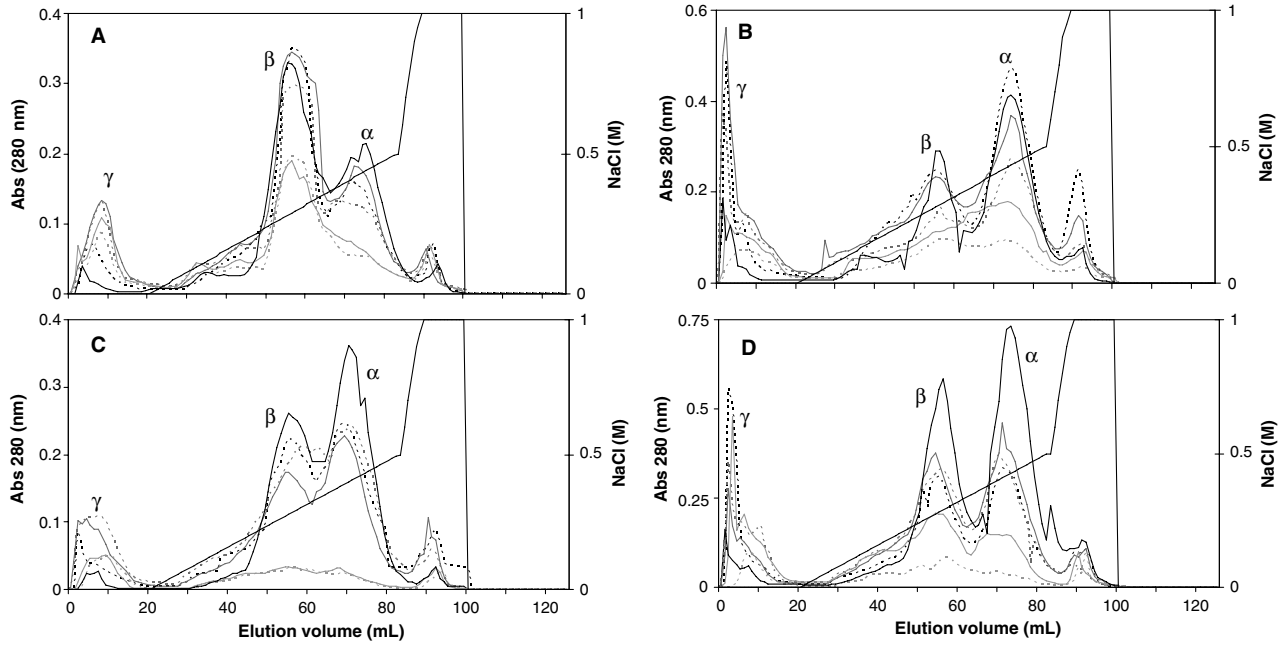

Fig. 1. Changes in the major globulins during germination and seedling growth of *Lupinus albus* (A), *Lupinus angustifolius* (B), *Lupinus luteus* (C), and *Lupinus mutabilis* (D). *Lupinus* seeds were germinated for 0 (–), 2 (--), 4 (–), 6 (--), 8 (–) and 10 (--) days. After each time period, the cotiledonary total globulin fraction was isolated and fractionated by FPLC anion exchange chromatography into α-, β- and γ-conglutins.

macerated and utilized for immunization. Samples containing the purified antigen were mixed with an equal volume of Freud's complete adjuvant and injected subcutaneously into New Zealand female rabbits. To obtain a higher titre, three booster injections were given every 2 weeks in complete Freund's adjuvant diluted 1:10 (by volume) with incomplete adjuvant. Total blood was taken from the heart 9 days after the third booster injection. Blood samples were allowed to clot, and the serum was collected and stored frozen at −70 °C.

2.5. Electrophoresis and immunoblotting

A discontinuous buffer system (Laemmli, 1970) was used for polyacrylamide gel electrophoresis (PAGE). Electrophoresis was performed in slab gels, 16 cm × 18 cm × 1.5 mm. Before electrophoresis, all protein samples were boiled for 3 min in the presence of SDS (2% w/v) and β-mercaptoethanol (0.1 M) (R–SDS–PAGE). Total protein in gels was stained with Coomassie Brilliant Blue R.

Proteins separated by R–SDS–PAGE were blotted onto a nitrocellulose membrane (previously soaked for 15 min in transfer buffer: 39 mM Tris, 48 mM glycine, 0.1% (w/v) SDS, 20% (v/v) methanol, pH 8.3) at 15 V for 1.5 h using a semi-dry transfer unit (Bio-Rad). After protein transfer, the polypeptides in the membrane were fixed for 15 min in a solution containing 10% (v/v) acetic acid and 25% (v/v) 2-propanol. Total polypeptides in the membrane were visualized with Ponceau S. In brief, the membrane was washed for 1 min with water, incubated for 5 min with 0.026 M Ponceau S, 1.8 M trichloroacetic acid, and 1.2 M sulphosalicylic acid, and washed for 5 min with water. After protein transfer, the membrane was subjected to immunoblotting. The blots were probed with anti-20 kDa polypeptide antibodies (500-fold diluted) and processed essentially as described before (Ramos et al., 1997).

2.6. Protein determination

Protein was measured by a modification of the Lowry method (Bensadoun & Weinstein, 1976).

3. Results and discussion

After isolation of the total globulin fraction, the three major globulins from *L. albus* seeds (i.e., γ-conglutin, β-conglutin or the vicilin-type globulin, and α-conglutin or the legumin-type globulin) were promptly isolated, using FPLC-anion exchange chromatography on the Mono Q column (Melo et al., 1994). Indeed, using a suitable, continuous salt gradient, β-conglutin was eluted from the Mono Q column, at pH 7.5, with ca. 0.3 M NaCl, whereas α-conglutin was eluted with 0.40–0.45 M NaCl. Subsequently, this same procedure was extended to fractionate and isolate the vicilin-type and legumin-type globulins from a variety of legume species, namely *L. mutabilis*, *P. sativum*, *G. max*, *V. faba*, *Cicer arietinum*, *Phaseolus vulgaris*, *Lens culinaris*, *A. hypogaea*, *L. sativus*, *Lupinus luteus*, *V. sativa*, *L. cicera*, and *Vigna unguiculata* seeds (Ferreira et al., 1999; Freitas et al., 2000; Freitas et al., 2004; Ribeiro, Teixeira, & Ferreira, 2004; Rosa, Ferreira, & Teixeira, 2000; Sanchez-Monge et al., 2004; Santos, Ferreira, & Teixeira, 1997). It was subsequently shown that the major globulins from germinating *L. albus* cotyledons were also eluted from the Mono Q column at salt concentrations that were identical to those used for the proteins from dry seeds, despite changes in protein concentration and/or composition in subunits. Therefore, an identical methodology was

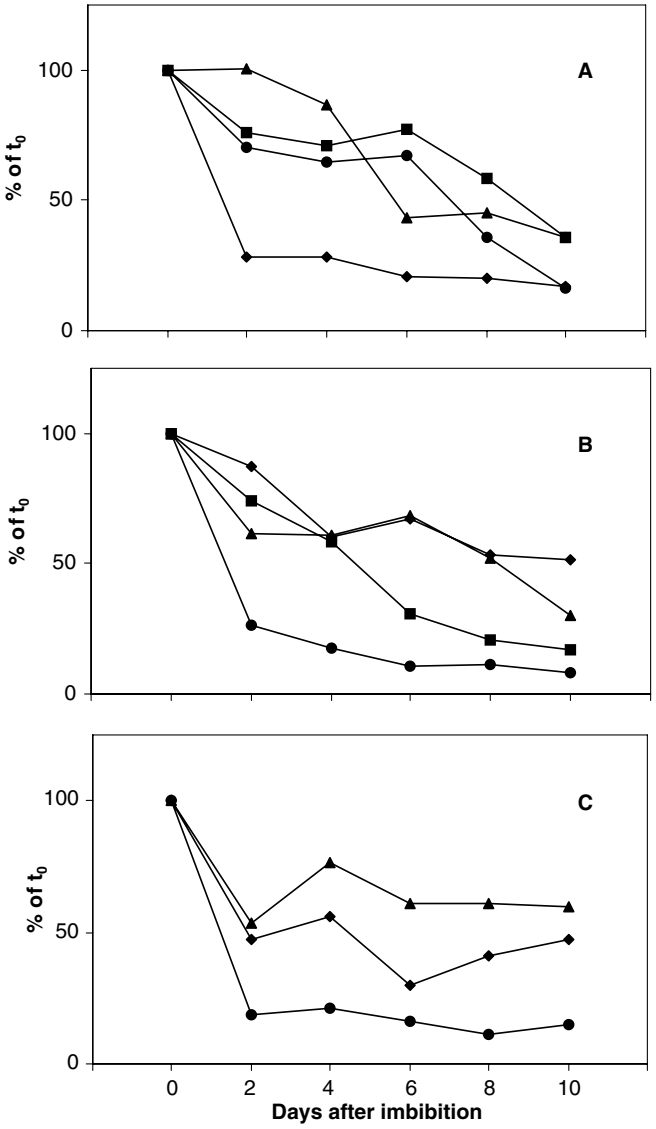

Fig. 2. Rate of vicilin proteolysis during germination and seedling growth. (A) *Lupinus albus* (◆), *Lupinus angustifolius* (▲), *Lupinus luteus* (●), *Lupinus mutabilis* (■); (B) *Lathyrus sativus* (◆), *Lathyrus cicera* (▲), *Vicia sativa* (●), *Vicia faba* (■); (C) *Glycine max* (◆), *Arachys hypogaea* (▲), *Pisum sativum* (●). Total globulins were extracted at various times from the cotyledons of legume seedlings and subsequently fractionated by FPLC anion exchange chromatography as described in the legend to Fig. 1. For each species analyzed, the relative amount of vicilin was estimated from the size of its A_{280} peak in the chromatogram and expressed as a percentage of the value obtained for the dry seed.

also utilized to follow the pattern of degradation of each of the three major globulins during germination and seedling growth of *L. albus* (Ferreira, Melo, & Teixeira, 1995). In the present work, we have extended the same process to perform a comparative study on the rate and pattern of proteolysis of vicilins during the germination and seedling growth of a variety of legume seeds, videlicet *L. albus*, *L. angustifolius*, *L. mutabilis*, *G. max*, *P. sativum*, *V. faba*, *V. sativa*, *L. cicera*, *L. sativus*, and *A. hypogaea*.

Lupinus seeds were germinated for up to 10 days. The results presented in Fig. 1 show the chromatograms obtained when the total globulin fraction from *L. albus* (Fig. 1A), *L. angustifolius* (Fig. 1B), *L. luteus* (Fig. 1C) and *L. mutabilis* (Fig. 1D) is fractionated by FPLC anion exchange chromatography, at pH 7.5, in the Mono Q column. As expected, the total globulin fraction extracted from the dry seeds (0 days germination) is fractionated into three main peaks, previously identified as α-conglutin (the legumin-type globulin), β-conglutin (the vicilin-type globulin) and γ-conglutin. As germination and seedling growth proceed, there is a gradual but steady reduction in the amounts of α- and β-conglutins, but an apparent increase in the concentration of γ-conglutin. This effect may be attributed to the fact that a variable fraction of γ-conglutin has been reported to be lost in the albumin fraction, even when divalent cations are present in the extraction procedure (Franco et al., 1997).

The data illustrated in Fig. 2A show the rate of β-conglutin proteolysis during germination and seedling growth of the four *Lupinus* species analyzed in Fig. 1A–D, as well as the rates of vicilin proteolysis during germination and seedling growth of *G. max*, *P. sativum*, *V. faba*, *V. sativa*, *L. cicera*, *L. sativus*, and *A. hypogaea* (Fig. 2B and C) analyzed in a similar way to that depicted in Fig. 1 (results not shown). The results exhibited in Fig. 2 indicate that the rate of vicilin catabolism during germination and seedling growth varies widely, regardless of the genus or species of legume considered. Three species in particular, *L. albus* (Fig. 2A), *V. sativa* (Fig. 2B), and *P. sativum* (Fig. 2C), exhibit an almost identical and very fast rate of vicilin proteolysis. On the contrary, the vicilins of some legumes, such as *L. sativus* (Fig. 2B) and *A. hypogaea* (Fig. 2C), undergo a particularly slow rate of degradation.

The observation that the rate of vicilin degradation in legume seedlings is species dependent, even within the same genus, focused our attention on the pattern of vicilin proteolysis. The SDS–PAGE gels displayed in Fig. 3A–D illustrate the changes in the polypeptide profile of β-conglutin during the germination and seedling growth of four *Lupinus* species. In all cases, the pattern of change is remarkably similar – mature β-conglutin, present in the dry seed and composed of a considerable number of distinct polypeptides, undergoes a dramatic change in its structure and concentration, involving the appearance of a new set of polypeptides including a higher molecular mass group, whose concentration steadily declines until complete disappearance after 10 days, and a lighter molecular mass group, whose concentration surprisingly increases from 5 to 10 days. Among the lighter polypeptides is a 20 kDa polypeptide that exhibits lectin-like activity (Ramos et al., 1997).

When β-conglutin extracted from growing seedlings of the four *Lupinus* species was blotted onto a membrane and probed with antibodies specific to the 20 kDa polypeptide, the four immunoblots presented in Fig. 3E–H were obtained. These blots highlight the β-conglutin polypep-

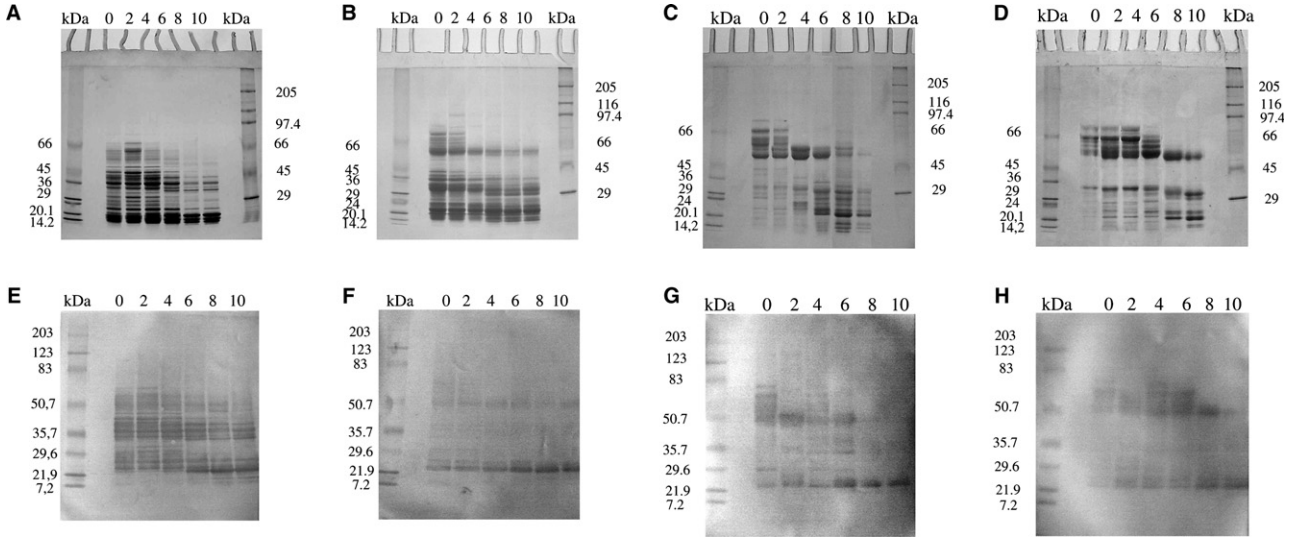

Fig. 3. Pattern of β-conglutin proteolysis during germination and seedling growth. (A, E) *Lupinus albus*; (B, F) *Lupinus angustifolius*; (C, G) *Lupinus luteus*; (D, H) *Lupinus mutabilis*. Total globulins were extracted at various times (days indicated at the top of gels and blots) from the cotyledons of legume seedlings and β-conglutin purified by FPLC anion exchange chromatography and subsequently analyzed by SDS–PAGE (A–D, 50 μg protein loaded in each lane) or blotted onto a membrane and probed with anti-20 kDa polypeptide antibodies (E–H, 15 μg protein loaded in each lane). Molecular masses of standards are indicated in kDa.

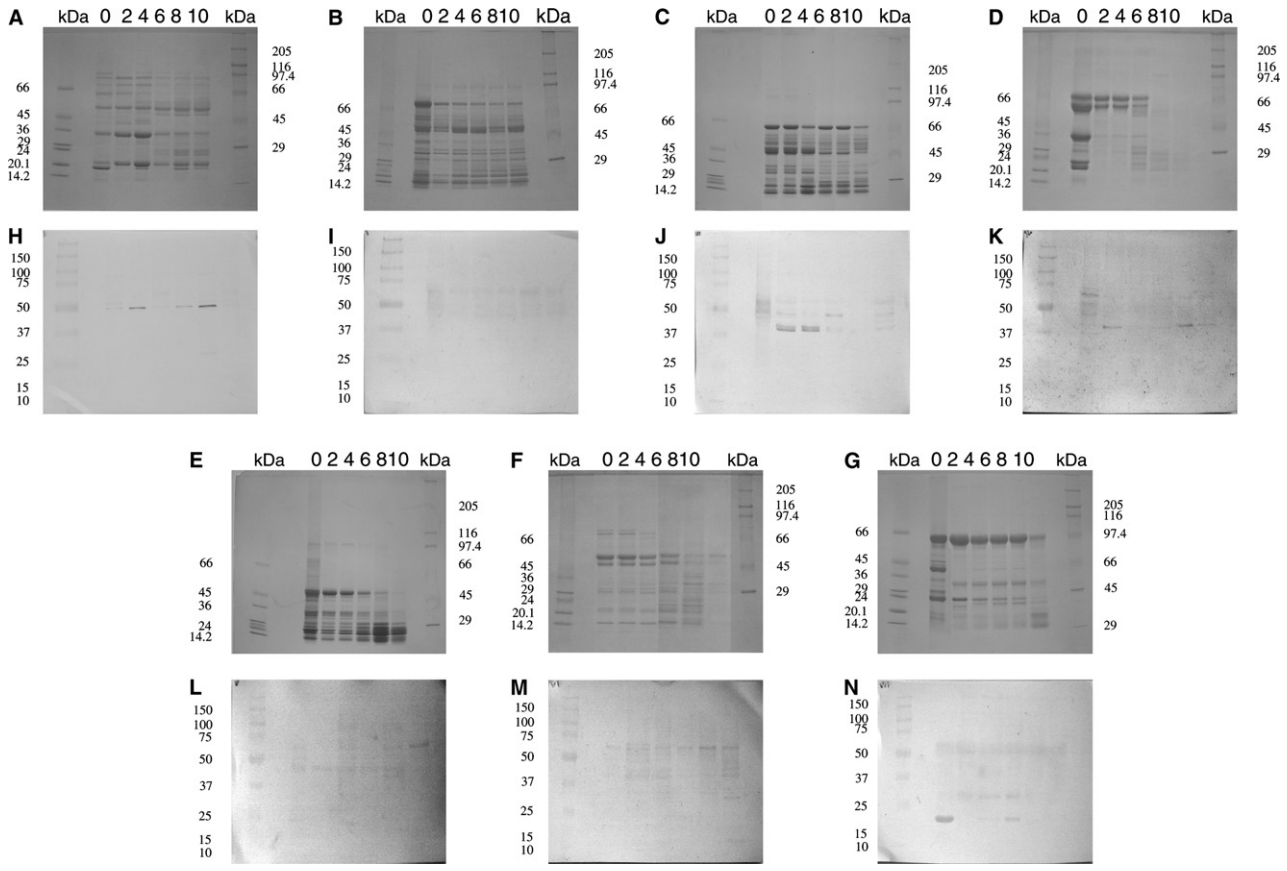

Fig. 4. Pattern of vicilin proteolysis during germination and seedling growth. (A, H) *Glycine max*; (B, I) *Pisum sativum*; (C, J) *Vicia faba*; (D, K) *Vicia sativa*; (E, L) *Lathyrus cicera*; (F, M) *Lathyrus sativus*; (G, N) *Arachis hypogaea*. Total globulins were extracted at various times (days indicated at the top of gels and blots) from the cotyledons of legume seedlings and vicilin purified by FPLC anion exchange chromatography and subsequently analyzed by SDS–PAGE (A–G, 50 μg protein per lane) or blotted onto a membrane and probed with anti-20 kDa polypeptide antibodies (H–N, 7.5 μg protein per lane). Molecular masses of standards are indicated in kDa.

tides which are precursors of the 20 kDa polypeptide and demonstrate that this polypeptide is a stable intermediate breakdown product of β-conglutin catabolism in all four *Lupinus* species examined.

The experiment outlined in Fig. 3 for the four *Lupinus* species was extended to seven other legume species belonging to five different genus. The results are displayed in Fig. 4 for *G. max* (Fig. 4A, H), *P. sativum* (Fig. 4B, I), *V. faba* (Fig. 4C, J), *V. sativa* (Fig. 4D, K), *L. cicera* (Fig. 4E, L), *L. sativus* (Fig. 4F, M), and *A. hypogaea* (Fig. 4G, N). Unlike the four *Lupinus* species, the patterns of vicilin proteolysis depicted in Fig. 4 vary from species to species, even within the same genus. For example, whilst *L. cicera* (Fig. 4E) exhibits a pattern of vicilin degradation similar to that of *Lupinus* (Fig. 3A–D), *L. sativus* (Fig. 4F) exhibits a gradual decrease in the major vicilin subunits concomitant with the appearance of a wide range of low molecular mass intermediate breakdown products. In the case of *P. sativum* (Fig. 4B) and *G. max* (Fig. 4A) the pattern of vicilin polypeptides along germination and seedling growth remained essentially unaltered. *V. faba* (Fig. 4C), *V. sativa* (Fig. 4D) and *A. hypogaea* (Fig. 4G) revealed intermediate patterns of vicilin catabolism.

A number of vicilin polypeptides were recognized by the anti-20 kDa polypeptide antibodies (Fig. 4H–N), indicating some degree of homology to the stable intermediate breakdown product of β-conglutin catabolism. However, none of these polypeptides corresponded in molecular mass to the *Lupinus* 20 kDa polypeptide.

As a whole, the data indicate that the rate and pattern of vicilin catabolism along germination and seedling growth is species specific. Possibly, each legume species evolved a particular mode of vicilin proteolysis that best suited its development under the environmental conditions to which it is most favourably adapted.

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