Seed Proteins of *Lupinus mutabilis*

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Lupinus mutabilis sweet seeds possess the highest levels of oil and protein of all domesticated lupin species. However, no information has been published concerning the structure of its storage proteins. An electrophoretic comparative study of the seed storage protein composition of *L. albus* cv. Multolupa, *L. mutabilis* cv. Potosi, and *L. mutabilis* cv. Inti is reported. Small differences were observed between the two *L. mutabilis* cultivars, but broad differences were detected when the two species were compared. *L. mutabilis* seeds were considerably richer in total nitrogen, total protein, and total globulins but were poorer in albumins than *L. albus*. Besides, *L. mutabilis* total globulins showed less electrophoretic mobility under nondenaturing conditions when compared with *L. albus*. Total albumins and total globulins produced very different electrophoretic patterns in the two *Lupinus* species when analyzed by denaturing, reducing, or nonreducing electrophoresis. When the three individual conglutins were considered, differential results were obtained. γ-Conglutin structure seems to be identical in all cultivars investigated. Subunits and polypeptide chains of α-conglutin showed some degree of heterogeneity among the three cultivars analyzed. β-Conglutin possessed the greatest structural variation, being composed of over 20 polypeptide chains in *L. albus*, covering a broad range of molecular masses (15−65 kDa). Six to seven major polypeptide chains in *L. mutabilis* β-conglutin were observed, covering a limited range of molecular masses (50−67 kDa).

Keywords: *Seed protein; conglutin; L. mutabilis; PAGE*

INTRODUCTION

There is a growing interest in the use of *Lupinus* species as grain crops for animal and human consumption. When compared to soybean, *Lupinus* seeds contain equal or even greater quantities of comparable quality plant protein, together with a high quantity of oil of a composition beneficial for human health (Gladstone, 1970).

Up to now only 4 of the 300 *Lupinus* species have been domesticated. Three of them (*L. albus*, *L. luteus*, and *L. angustifolius*) have their origin in the Mediterranean region, whereas the fourth, *L. mutabilis*—an Andean lupin, is derived from South America. *L. mutabilis* possesses the highest levels in oil and protein of all domesticated lupin species. Indeed, the value of this species is related to the high protein (40−50%) and oil (14−24%) contents of its seeds. Besides, Andean lupin seed protein is relatively rich in cysteine and lysine. Digestibility and nutritive value of the protein are comparable to those of soybean protein (Gross, 1982; Bellido, 1986). Nevertheless, very little is known concerning the protein structure and composition of *L. mutabilis* seeds, when compared with the seeds of *L. albus*, *L. luteus*, and *L. angustifolius*.

Globulins are the major protein component in the storage tissues of legume cotyledons in general and of *Lupinus* cotyledons in particular. Three main storage, glycosylated globulins have been characterized in lupins (*L. albus*, *L. luteus*, and *L. angustifolius*)—γ-conglutin, β-conglutin (vicilin-like), and α-conglutin (legumin-like) (Blagrove and Gillespie, 1975; Mossé and Pernollet, 1983; Esnault et al., 1991; Melo et al., 1994). γ-Conglutin has recently been identified as a lectin-like protein (Duranti et al., 1995). These proteins possess, essentially, identical structure and composition in the three *Lupinus* species. In the case of *L. albus*, γ-conglutin, the minor globulin component, is composed of one main type of subunit (40 kDa) containing two polypeptide chains (18 and 26 kDa) linked by disulfide bonds, and possibly, other minor subunits. β-Conglutin, the major globulin component, is composed of 10−12 major types of subunits (15−72 kDa) and a considerable number of minor subunits, with no disulfide bonds. Finally, α-conglutin contains four main types of subunits (53, 60, 66, and 70 kDa) as well as a number of minor subunits. Upon reduction, each of these four main subunits is split into heavier polypeptide chains (31, 36, 42, and 46 kDa, respectively) and a lighter polypeptide chain (19 kDa), which is common to all four subunits (Melo et al., 1994).

In the present work we have studied the protein composition of cotyledons from two *L. mutabilis* cultivars. For comparative purposes, the cotyledonary protein of a *L. albus* cultivar was also analyzed. Albumins and γ-, β-, and α-conglutins were extracted and purified and their structure and composition extensively characterized by nondenaturing, by denaturing/nonreducing, and by denaturing/reducing polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Plant Material. Dry seeds of one cultivar of *L. albus* L. (white lupin, cv. Multolupa) and of two cultivars of *L. mutabilis* sweet (Andean or changing lupin, cv. Potosi and Inti) were used.

Isolation of Total Globulins. Total globulins from *Lupinus* seeds were extracted and isolated according to a modification of the procedure described by Melo et al. (1994),

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which is a modified version of the method of Blagrove and Gillespie (1978). The dry cotyledons were milled (0.2 mm sieve), and the resulting meal was defatted with *n*-hexane (34 mL/g of flour) for 4 h with agitation and air-dried after decantation of the hexane. The albumin fraction of the proteins was extracted by stirring with water (pH adjusted to 8.0 with NaOH) containing 10 mM $CaCl_2$, 10 mM $MgCl_2$, and 1 mM phenylmethanesulfonyl fluoride (PMSF) (34 mL/g of flour) for 4 h. The suspension was centrifuged for 1 h at 30000*g* (average), and the supernatant, containing the albumins, was collected. The total globulin fraction of the proteins was subsequently extracted by stirring the pellet with 0.1 M Tris-HCl buffer, pH 8.0, containing 10% (w/v) NaCl, 0.05% (w/v) NaN_3, 1 mM PMSF, 10 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (34 mL/g of flour), for 12 h. The globulin-containing solution was centrifuged for 1 h at 30000*g* (average), and the supernatant was desalted on a PD-10 prepacked Sephadex G-25 column (Pharmacia/LKB), previously equilibrated in 50 mM Tris-HCl buffer, pH 7.5, and used as the source of total globulins. All operations were performed at 4 °C. This procedure completely eliminates the albumin fraction, free of contaminating globulins, allowing an efficient extraction of the globulin fraction. These observations are based on previous experiments that showed the absence of α-, β-, and γ-conglutin polypeptides in the albumin fraction and that albumin and globulin fractions are composed by totally different polypeptide patterns (data not shown).

Total nitrogen was measured according to the method of Kjeldahl, using a Kjeltec system 1030, Tecator, Sweden, according to the instructions of the manufacturer. Protein concentrations were determined according to a modification of the Lowry method (Bensadoun and Weinstein, 1976).

Purification of the Individual Globulins by FPLC Anion Exchange Chromatography. α-Conglutin, β-conglutin, and γ-conglutin were purified by anion exchange chromatography of the total globulin fraction on the Mono Q HR5/5 column of the FPLC (Pharmacia/LKB) as described by Melo et al. (1994). The column was equilibrated with 50 mM Tris-HCl buffer, pH 7.5, and the bound proteins were eluted with a continuous gradient of NaCl (0−1 M). γ-Conglutin, a minor globulin component, does not bind to the anion exchanger at pH 7.5; β-conglutin, the main globulin component, elutes with ca. 0.3 M NaCl; α-conglutin, a major globulin component, elutes with 0.40−0.45 M NaCl (Melo et al., 1994).

Polyacrylamide Gel Electrophoresis (PAGE). A discontinuous buffer system (Laemmli, 1970) was used for PAGE. Electrophoresis was performed in slab gels 16 cm × 18 cm × 1.5 mm. Fifty micrograms of protein was loaded in each lane. Several types of electrophoresis were used, following a methodology similar to that described by Krochko and Bewley (1988).

Nondenaturing PAGE (ND-PAGE). The final concentrations in the separation gel were as follows: 5% (w/v) acrylamide, 0.26% (w/v) *N*,*N*′-methylenebis(acrylamide) (bisacrylamide), 0.375 M Tris-HCl buffer, pH 8.8, 0.03% (w/v) ammonium persulfate (AMPS). The gel was polymerized by the addition of 0.03% (v/v) *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED). The final concentrations in the stacking gel were as follows: 4% (w/v) acrylamide; 0.13% (w/v) bisacrylamide; 0.125 M Tris-HCl buffer, pH 6.8; and 0.1% (w/v) AMPS. The stacking gel was polymerized by the addition of 0.05% (v/v) TEMED. The electrode buffer (pH 8.3) contained 0.025 M Trizma base and 0.192 M glycine. The protein samples were diluted in sample buffer to obtain the following final concentrations: 0.08 M Tris-HCl buffer, pH 6.8; 15% (v/v) glycerol; and 0.006% (w/v) *m*-cresol purple. Electrophoresis was carried out under constant current (20 mA per gel) at 5 °C.

Nonreducing Sodium Dodecyl Sulfate PAGE (NR-SDS-PAGE). The separation gel mixture contained 12.5% (w/v) acrylamide, 0.1% (w/v) bisacrylamide, 0.375 M Tris-HCl buffer, pH 8.8, and 0.03% (w/v) AMPS. Polymerization was initiated by the addition of 0.03% (v/v) TEMED. The stacking gel mixture consisted of 5% (w/v) acrylamide, 0.13% (w/v) bisacrylamide, 0.125 M Tris-HCl buffer, pH 6.8, and 0.1% (w/v) AMPS. The stacking gel was polymerized by the addition of 0.05% (v/v) TEMED. The electrode buffer (pH 8.3) contained 0.025 M Trizma base, 0.192 M glycine, and 0.1% (w/v) SDS. Sodium acetate (0.1 M) was also included in the anode buffer. The protein samples were prepared for electrophoresis by boiling for 3 min in the presence of 0.08 M Tris-HCl buffer, pH 6.8, containing 2% (w/v) SDS, 15% (v/v) glycerol, and 0.006% (w/v) *m*-cresol purple. Electrophoresis was carried out under constant current (40 mA per gel) at room temperature.

Reducing SDS-PAGE (R-SDS-PAGE). The procedure followed for R-SDS-PAGE was identical to that described for NR-SDS-PAGE except that a reducing agent (2-mercaptoethanol, 0.1 M) was included in the buffer during the preparation of samples for electrophoresis.

The use of *m*-cresol purple as the tracking dye and, in the case of NR-SDS-PAGE and R-SDS-PAGE, the inclusion of 0.1 M sodium acetate in the anode buffer allows the resolution of polypeptides with molecular masses ranging from 2.5 to >200 kDa (Christy et al., 1989).

Staining. The proteins in gels were stained with Coomassie Brilliant Blue R-250. In brief, the proteins were fixed by incubating the gels in 12% (w/v) trichloroacetic acid for 20 min. The gels were subsequently washed with water, stained in 0.25% (w/v) Coomassie Brilliant Blue R-250, 25% (v/v) 2-propanol, and 10% (v/v) acetic acid, destained in 25% (v/v) 2-propanol and 10% (v/v) acetic acid, and stored in 10% (v/v) acetic acid.

Table 1. Protein Content of *L. albus* and *L. mutabilis* Seeds

	L. albus cv. Multolupa	*L. mutabilis* cv. Potosi	*L. mutabilis* cv. Inti
total N			
mg of N/g of fr wt	51.10 ± 0.03	77.00 ± 0.02	85.0 ± 0.8
albumins			
mg of protein/g of fr wt	50 ± 3	41 ± 4	46 ± 2
N albumins/total N (%)	16 ± 1	8.5 ± 0.8	8.7 ± 0.5
globulins			
mg of protein/g of fr wt	101 ± 2	209 ± 19	238 ± 19
N globulins/total N (%)	31.7 ± 0.6	43 ± 4	45 ± 4

RESULTS AND DISCUSSION

Among the domesticated species of the *Lupinus* genus, *L. mutabilis* seeds possess the highest level of protein (Bellido, 1986). Davin and Brillouet (1986) reported that *L. albus* seeds exhibited protein levels equivalent to those of soybean seeds, whereas *L. mutabilis* seeds contained higher values. Other authors have confirmed this observation (Pompei and Lucisano, 1976; Sathe et al., 1982; Brillouet and Riochet, 1983). The values reported in the literature are in good agreement with the data presented in Table 1 obtained for the cotyledons of *L. albus* cv. Multolupa, *L. mutabilis* cv. Potosi, and *L. mutabilis* cv. Inti. We have analyzed total nitrogen according to the Kjeldahl method and total albumins and total globulins by a modification of the Lowry technique, as described under Materials and Methods. Total protein content is often estimated by multiplying the total nitrogen value by the factor 6.25. This procedure overestimates the protein values because living tissues, and legume seeds in particular, contain considerable amounts of non-protein nitrogenous compounds. Because a portion of the total nitrogen measured originates from alkaloids (Hatzold et al., 1983), Gross and Baer (1977) proposed a factor of 5.7 for legume proteins and Pompei and Lucisano (1976) a factor of 5.4 for lupin seeds. To overcome these uncertainties, we have preferred to express the data obtained by the Kjeldahl method in terms of milligrams of nitrogen per gram of fresh weight. The data included in Table 1 show that *L. mutabilis* seeds are considerably richer in total nitrogen than *L. albus* seeds. With respect to total cotyledonary albumins, *L. albus* contains

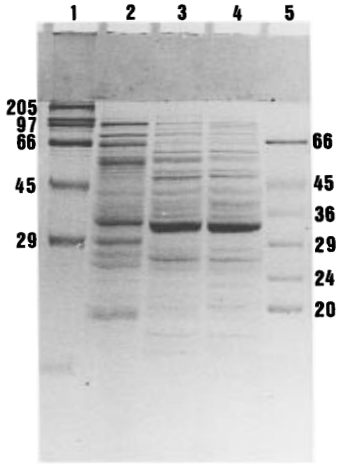

Figure 1. Polypeptide patterns of the total albumin fraction from *L. albus* cv. Multolupa (lane 2), *L. mutabilis* cv. Potosi (lane 3), and *L. mutabilis* cv. Inti (lane 4) analyzed by R-SDS-PAGE. Fifty micrograms of protein was loaded in each lane. Lanes 1 and 5: molecular mass markers (kDa).

approximately 50 mg of protein/g of fresh weight, accounting for 16% of the seed nitrogen, whereas the two cultivars of *L. mutabilis* analyzed contain 40−45 mg of albumins/g of fresh weight, comprising 8−9% of the seed nitrogen. Thus, the cotyledons of *L. albus* are richer in albumins than those of *L. mutabilis*. However, the reverse is true when the globulins are concerned. *L. mutabilis* seeds contain 210−240 mg of globulins/g of fresh weight, corresponding to 43−45% of the seed nitrogen, whereas *L. albus* possesses 100 mg of globulins/g of fresh weight, accounting for 32% of the seed nitrogen (Table 1).

Nothing is known, as far as we are aware, about the structure and composition of *L. mutabilis* albumins and globulins when compared with those of *L. albus*, *L. luteus*, and *L. angustifolius*. The total albumin fractions from the three *Lupinus* cultivars studied were analyzed by NR-SDS-PAGE and R-SDS-PAGE. The gel presented in Figure 1 shows the polypeptide patterns of the albumin fractions obtained after R-SDS-PAGE. Electrophoresis performed under nonreducing conditions (NR-SDS-PAGE) produced identical polypeptide patterns (data not shown), indicating that the presence of disulfide bonds is not apparent in *L. albus* and *L. mutabilis* albumins. The polypeptide patterns of the two *L. mutabilis* cultivars are virtually identical (Figure 1, lanes 3 and 4) but considerably different from that of the *L. albus* cultivar (Figure 1, lane 2). Particularly evident is the presence of an abundant 34 kDa albumin in *L. mutabilis* cotyledons.

The total globulin fractions from the three *Lupinus* cultivars studied were also analyzed by ND-PAGE (Figure 2A), NR-SDS-PAGE (Figure 2B), and R-SDS-PAGE (Figure 2C). The gels presented in Figure 2 reveal that the electrophoretic patterns of globulins obtained for the two *L. mutabilis* cultivars are identical (lanes 3 and 4), but considerably different from those corresponding to the *L. albus* cultivar (lane 2)—a situation similar to that observed for the albumins. A closer analysis indicates that *L. mutabilis* total globulins exhibit lower electrophoretic mobility than their *L. albus* counterparts when studied by ND-PAGE (Figure 2A). When compared with *L. albus*, denaturing PAGE analysis shows that the total globulins of *L. mutabilis* are composed of a group of polypeptides with a higher range of molecular masses. Some of these polypeptides are linked by disulfide bonds (Figure 2B,C).

The total globulin fraction from each of the three *Lupinus* cultivars under study was subjected to anion exchange chromatography on the Mono Q column of the FPLC, as described by Melo et al. (1994) for *L. albus*, to fractionate the individual conglutins. The chromatograms obtained (data not shown) are identical for the three cultivars. Three main protein fractions are clearly fractionated: γ-conglutin, not binding to the anion exchanger, and β-conglutin and α-conglutin, eluting with 150−250 and 300−400 mM NaCl, respectively.

The individual conglutins from *L. albus* cv. Multolupa (Figure 3A−C), *L. mutabilis* cv. Potosi (Figure 3D−F), and *L. mutabilis* cv. Inti (Figure 3G−I) were subsequently analyzed by ND-PAGE (Figure 3A,D,G), NR-SDS-PAGE (Figure 3B,E,H), and R-SDS-PAGE (Figure 3C,F,I). The main features are as follows: (i) ND-PAGE produces a similar result for the three cultivars examined. The presence of a high mobility protein band in β-conglutin from *L. mutabilis* cv. Inti (Figure 3G, lane 4) may be tentatively explained by a small amount of α-conglutin contamination or by a different protein composition of this globulin. The absence of cross-contamination observed in the gels presented in parts

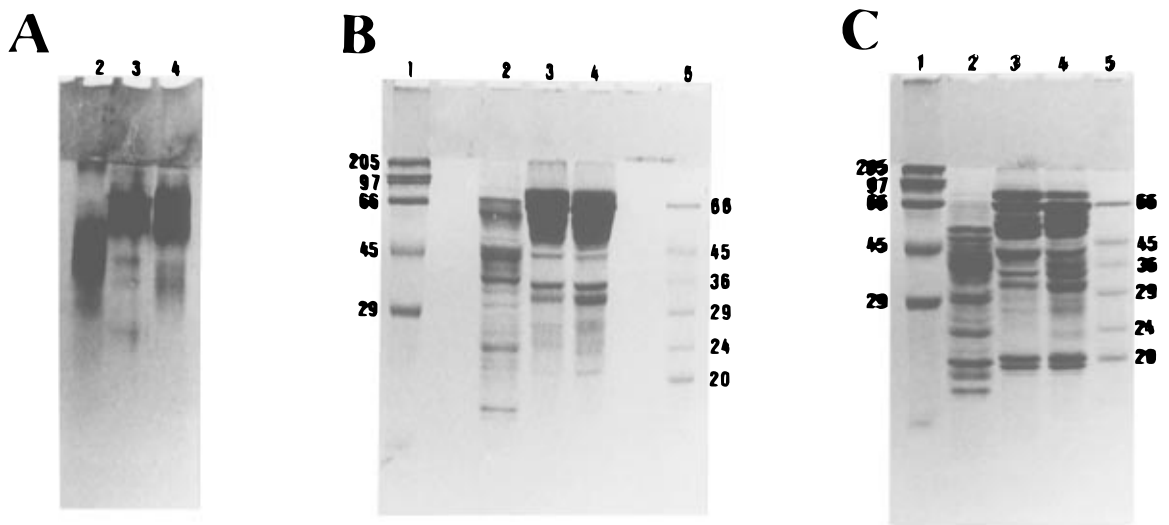

Figure 2. Electrophoretic patterns of the total globulin fractions from *L. albus* cv. Multolupa (lane 2), *L. mutabilis* cv. Potosi (lane 3), and *L. mutabilis* cv. Inti (lane 4) obtained by ND-PAGE (A), NR-SDS-PAGE (B), and R-SDS-PAGE (C). Fifty micrograms of protein was loaded in each lane. Lanes 1 and 5: molecular mass markers (kDa).

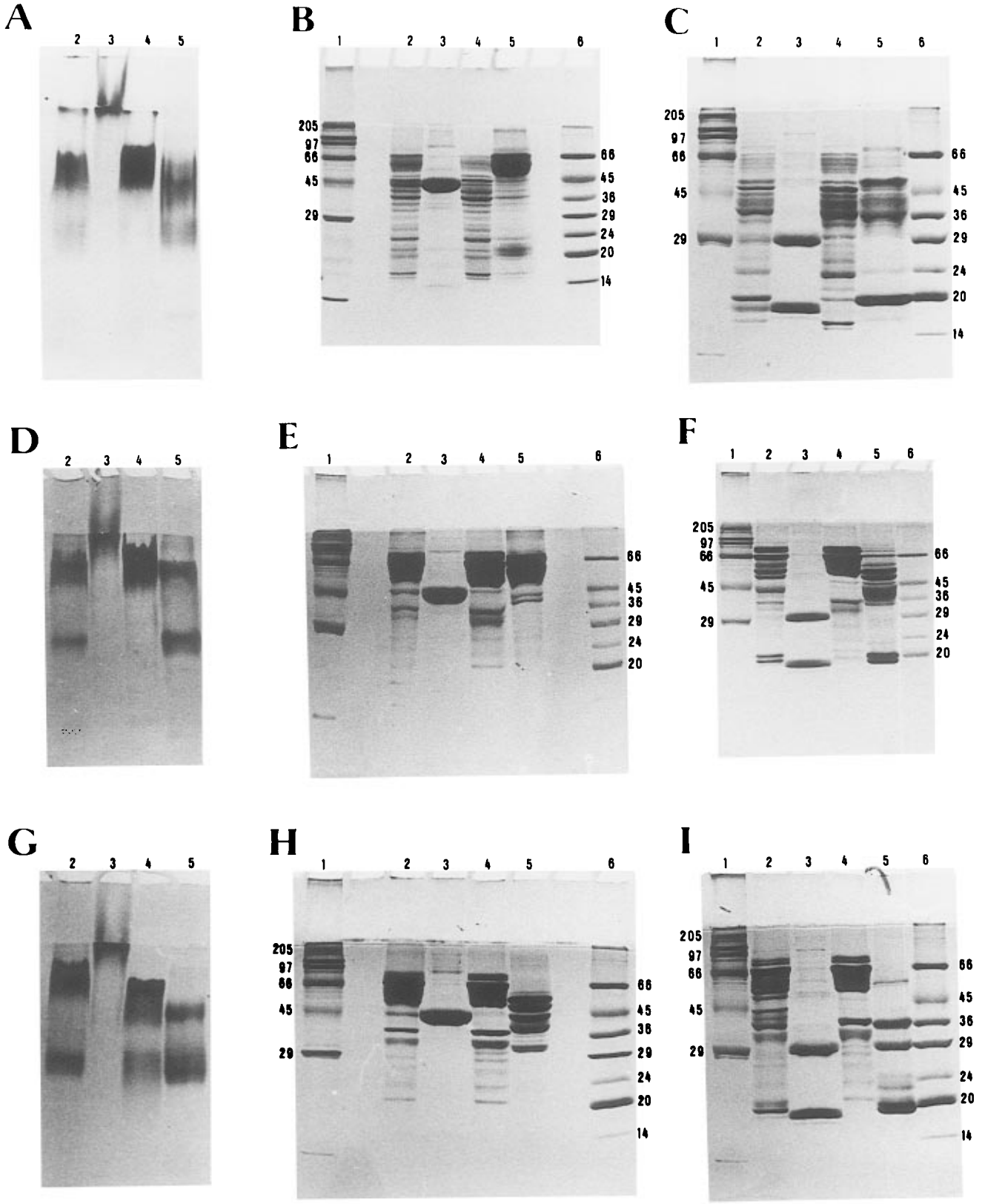

Figure 3. Electrophoretic analysis of the purified conglutins from *L. albus* cv. Multolupa (A−C), *L. mutabilis* cv. Potosi (D−F), and *L. mutabilis* cv. Inti (G−I) obtained by ND-PAGE (A, D, G), NR-SDS-PAGE (B, E, H), and R-SDS-PAGE (C, F, I). Fifty micrograms of protein was loaded in each lane. Lanes 1 and 6: molecular mass markers (kDa). Lane 2: G_T, total globulin fraction. Lanes 3−5: γ-, β-, and α-conglutins, respectively.

H and I of Figure 3, lanes 4 and 5, favors the second hypothesis. (ii) γ-Conglutin possesses an identical structure in the three cultivars studied—it consists of a single type of subunit, with a molecular mass of 42−43 kDa, composed of two polypeptide chains linked by disulfide bonds (18 and 30 kDa). (iii) β-Conglutin from *L. albus* is composed of over 20 polypeptide chains with no disulfide bonds, with molecular masses ranging from 15 to 65 kDa. However, β-conglutin from the two *L. mutabilis* cultivars under study shows a completely different structure, being composed of seven major polypeptide chains (with molecular masses ranging from

50 to 67 kDa), two polypeptide chains with molecular masses in the range 33−38 kDa, and a number of minor, lower molecular mass polypeptides. The presence of disulfide bonds was not detected. (iv) α-Conglutin from *L. albus* is composed of four main types of subunits, with molecular masses between 50 and 60 kDa, and a considerable number of minor subunits, including a 19 kDa polypeptide. Upon reduction, each of the main subunits is split into a heavier polypeptide chain (molecular masses between 38 and 50 kDa, depending on the subunit considered) and a lighter polypeptide chain (19 kDa), which is common to all four main subunits. A small amount of the 19 kDa polypeptide chain is also detected under nonreducing conditions (Figure 3B, lane 5). α-Conglutin from *L. mutabilis* seems to be slightly different from the *L. albus* globulin. α-Conglutin from cv. Potosi is composed of four main types of subunits (molecular masses of 50−65 kDa) and two minor types of subunits (40−42 kDa), which upon reduction produce a number of heavier polypeptide chains and two lighter polypeptide chains (18 and 19 kDa). α-Conglutin from cv. Inti differs from that of cv. Potosi as it consists of five main types of subunits (32, 40, 45, 49, and 53 kDa), which upon reduction produce four main types of polypeptide chains (18, 19, 31, and 37 kDa).

In summary, it is possible to conclude that the two major storage proteins from *L. mutabilis*, i.e. α- and β-conglutins, show considerable differences in structure and composition when compared with *L. albus* conglutins. The significance of these observations is unknown, although they may simply reflect a divergent evolutionary course of the two species. However, minor variations are found when different *L. mutabilis* cultivars are compared. γ-Conglutin, a minor globulin component, possesses identical polypeptide and subunit compositions in all *Lupinus* cultivars studied.

ABBREVIATIONS USED

EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FPLC, fast protein liquid chromatography; ND-PAGE, nondenaturing PAGE; NR-SDS-PAGE, nonreducing SDS-PAGE; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; R-SDS-PAGE, reducing SDS-PAGE; SDS, sodium dodecyl sulfate.

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