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Purification and partial characterization of storage proteins in *Lupinus angustifolius* seeds

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RESUMEN

Purificación y caracterización parcial de proteínas de almacenamiento de semillas de *Lupinus angustifolius*.

Se ha realizado una purificación por diálisis secuencial y cromatografía de intercambio iónico de las proteínas de la semilla de *L. angustifolius*, y su composición en aminoácidos ha sido estudiada para determinar su valor nutricional como fuentes de aminoácidos esenciales. Las albúminas están compuestas por una compleja mezcla de proteínas. Las globulinas se resolvieron en conglutinas α, β y δ. La conglutina α es la proteína más abundante en las semillas de *L. angustifolius* representando el 76.6% del total. Las proteínas de la semilla de *L. angustifolius* son limitantes en lisina. Las albúminas son limitantes en tirosina y las globulinas en metionina y lisina. La α conglutina es limitante en lisina, metionina e histidina.

PALABRAS-CLAVE: Albúminas - Conglutinas α β y δ. - Globulinas - Lupinus angustifolius - Proteínas de semilla.

SUMMARY

Purification and partial characterization of storage proteins in *Lupinus angustifolius* seeds.

Lupinus angustifolius seed proteins have been purified by sequential dialysis and ion exchange chromatography, and their amino acid composition has been studied in order to determine their nutritional value as sources of essential amino acids. Albumins include a great variety of proteins. Globulins were resolved in α, β and δ conglutins. Conglutin α is the main protein in the seeds of *L. angustifolius*, representing 76.6% of the total. While lysine was found to be the limiting amino acid in *L. angustifolius* seed proteins as a whole, tyrosine was the limiting amino acid in albumins, and methione and lysine were limiting in globulins. Lysine, methionine and histidine were limiting amino acids in α conglutin.

KEY-WORDS: Albumins - α, β and δ conglutins - Globulins - Lupinus angustifolius - Seed proteins.

1. INTRODUCTION

Legume seeds are one of the most promising alternative sources of protein for human and animal nutrition (Cerletti et al., 1978). Soybean is by far the most important among them, and many countries import soybean from producing countries such as USA, Brazil and Argentina. Other legumes are being studied as potential alternative sources of protein in order to reduce the need to import soybean. The seeds of the legume lupine have a high protein content, ranging from 33.8% in *Lupinus angustifolius* (Lqari et al., 2002) to 44% in *L. luteus*, quite similar to soybean which has 35% protein (Duranti et al., 1981). One interesting aspect of lupine as a new source of protein is that it can be grown in soils and in climates where soybean can not grow. Considering the chemical, functional and nutritional properties of its proteins, lupine seeds can be considered as a source of high quality protein (Lampart-Szczapa et al., 1997; Lqari et al., 2002).

Globulins are the main protein component in lupine seeds and legumes in general. Three main globulins have been described in *L. albus*, *L. luteus*, and *L. angustifolius*: γ-conglutin, β-conglutin (vicilin-like), and α-conglutin (legumin-like) (Blagrove and Gillespie, 1975; Melo et al., 1994, Santos et al., 1997). Although γ-conglutin has been recognized as a lectin-like protein (Duranti et al., 1995) the physiological role of conglutins in general has not clearly been established (Duranti et al., 2000). These proteins possess, essentially, identical structures in the three lupine species considered. In *L. albus*, γ-conglutin, the minor globulin component, is composed of one main subunit (40 kDa) containing two polypeptide chains (18 and 26 kDa) linked by disulfide bonds. Conglutin β, the major globulin, is made of up to 10 to 12 different types of major subunits (15-72 kDa) and a considerable number of minor globulin subunits, with no disulfide bonds. Finally, α-conglutin contains four main types of subunits (53, 60, 66 and 70 kDa). Upon reduction, each of these four main subunits is split into heavy polypeptide chains (31, 36, 42, and 46 kDa) and a light polypeptide chain (19 kDa) (Melo et al., 1994).

The goal of the present work was to determine whether the proteins in *L. angustifolius* seeds can be purified by using a method previously described for purification of proteins in chickpea seeds, and to study the amino acid composition of the purified proteins in order to determine their content in essential amino acids.

2. MATERIALS AND METHODS

Materials

The Lupine seeds were a gift from Koipesol Semillas, S.A. (Sevilla, Spain). Blue dextran 2000, thyroglobulin, -amilase, bovine serum albumin and ribonuclease were obtained from Amersham Pharmacia Biotechnology (Uppsala, Sweden). Diethyl ethoxymethylenemalonate was purchased from Fluka (Buchs, Switzerland).

Purification of *L. angustifolius* seed proteins

The Lupine seeds (100 g) were ground in a blender (particle size 0.2-0.5 mm) and extracted by stirring in a 1000 mL 0.1 M sodium borate buffer (pH 8.3) at room temperature for 1 h, according to the method by Singh et al. (1988). The extract was centrifuged at 8000 g for 15 min. The supernatant was dialyzed against a 25 mM sodium citrate buffer (pH 4.6) at 4°C for 15 h and centrifuged as before. The supernatant and the pellet correspond to the albumin and globulin fractions, respectively. The globulin precipitate was dissolved in a 0.1 M sodium borate 0.2 M sodium chloride buffer (pH 8.3), and dialyzed against a 25 mM sodium citrate buffer (pH 4.6) at 4°C for 15 h, and centrifuged. The supernatant correspond to β-conglutin, and the pellet formed by the legumin fraction was freeze-dried.

The pellet was redissolved by shaking in a 0.1 M sodium borate and a 0.2 M sodium chloride buffer (pH 8.3) (1:20 w/v), and insoluble material was eliminated by centrifugation at 8000 g for 15 min. In order to remove non-protein compounds the supernatant was applied to a PD-10 Sephadex G-25M column and eluted with 0.19 M sodium phosphate and a 19 mM sodium citrate buffer (pH 7.0). The resulting solution was then loaded onto a protein Pak DEAE 8 H 1000 A 8 μm ion exchange column (Waters, Milford, MA) for purification of conglutin γ and α using a binary gradient system made up of buffers A (0.19 M sodium phosphate, 19 mM sodium citrate buffer (pH 7.0)) and B (0.19 M sodium phosphate, 19 mM sodium citrate, 0.5 M sodium chloride buffer (pH 7.0)) delivered to the column as follows: from 0 to 10 minutes elution with 100% buffer A; from 10 to 25 minutes linear gradient from 0 to 28% buffer B; from 25 to 35 minutes elution with 28% buffer B; from 35 to 45 minutes linear gradient from 28% to 100% buffer B; from 45 to 55 minutes elution with 100% buffer B; and from 55 to 60 minutes linear gradient to 100% buffer A. The flow rate was 1 mL/min, and volume injection and sample concentration were 10 mL and 18 mg of protein/mL, respectively. Legumin enriched fractions were pooled for further purification by gel filtration chromatography.

Gel filtration chromatography

Lyophilized samples (1 g) were dissolved in 10 mL 0.1 M sodium borate, 0.2 M sodium chloride, pH 8.3 buffer. Gel filtration was carried out in an FPLC system equipped with a Superose 12 HR 10/30 column from Pharmacia LKB Biotechnology. Volume injection and protein concentration of the samples were 200 μl and 1.6 mg/mL, respectively. The eluent borate buffer was as above at a flow rate of 0.4 mL/min. Elution was monitored at 280 nm. The approximate molecular masses were determined using blue dextran 2000 (2000 kDa), β-amylase (200 kDa), bovine serum albumin (67 kDa) and ribonuclease A (13.7 kDa) as molecular weight standards.

Amino acid analysis

Samples containing 2 mg of protein were hydrolyzed in 6 N HCl at 110°C for 24 h and derivatized by reaction with diethyl ethoxymethylenemalonate. The derivatives were analyzed by reversed-phase high-performance liquid chromatography (HPLC) using D, L-α-amino butyric acid as internal standard (Alaiz et al., 1992). The HPLC apparatus (Waters) consisted of a Model 600E multi-solvent delivery system, a Wisp Model 712 automatic injector and a 484 UV-Vis detector. Data acquisition and processing were carried out using a Maxima 820 3.3 version software (Waters). A 300 x 3.9 mm i.d. reversed phase column (Nova Pack C_{18}, 4 μm, Waters) and a binary gradient system with 25 mM sodium acetate pH 6.0 and acetonitrile was used. The column was maintained at 18°C. Amino acid composition was expressed as molar percentage.

Protein determination

Protein was determined according to the method of Bradford (1976) using bovine serum albumin as standard.

3. RESULTS AND DISCUSSION

Fractionation of *L. angustifolius* seed proteins

Fig. 1 shows the procedure followed for fractionation of *L. angustifolius* seed proteins. *L. angustifolius* flour was extracted in a 0.1 M pH 8.3 borate buffer for 60 minutes at room temperature, which allowed the extraction of 96% of the protein present in the flour (Fig. 1). Figure 2 shows the gel filtration chromatographic profile of the extracted proteins. The main peak, labeled A in figure 2, corresponds to a molecular weight of 180 kDa, and includes the main seed storage protein, α conglutin, forming a trimer of 60 kDa subunits. Fractions B and C are

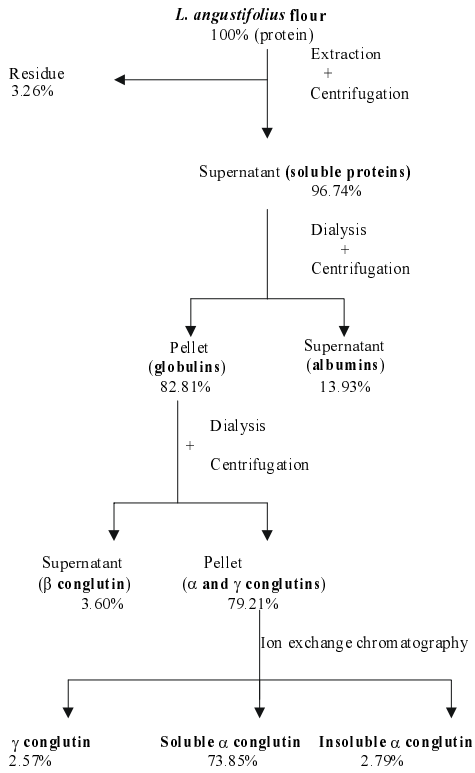

Figure 1
Procedure followed for fractionation of *L. angustifolius* defatted flour to obtain albumins, globulins and a, ß, and γ conglutins.

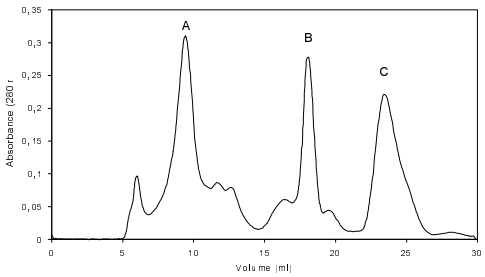

Figure 2
Gel filtration chromatography of protein extracted from *L. angustifolius* defatted flour.

probably non-protein compounds such as phenols or nucleic acids that also adsorb at 280 nm.

Protein extracts were dialyzed against a pH 4.6 25 mM sodium citrate buffer according to the method described by Singh et al. (1988) (see Materials and Methods). This dialysis causes precipitation of the globulins that represent the main fraction of *L. angustifolius* seed storage proteins. Proteins that remain soluble constitute the albumin fraction. Globulins and albumins represent 82% and 14% of *L. angustifolius* seed proteins, respectively. This distribution of globulins and albumins in *L.*

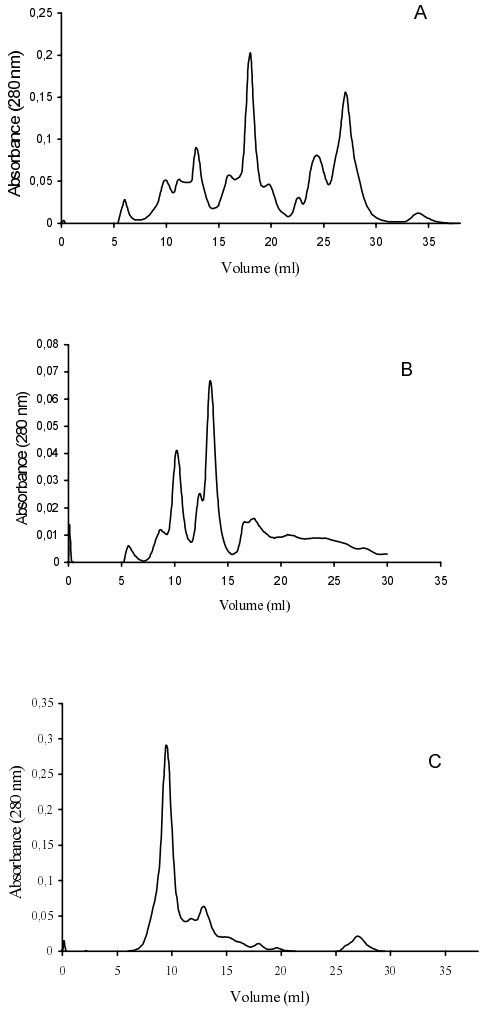

Figure 3
Gel filtration chromatography of *L. angustifolius* albumins (A), β conglutin (B) and globulins (C).

angustifolius is typical of other legumes such as chickpea (Sánchez-Vioque, 1998). Figure 3 shows the gel filtration chromatographic profile of globulins and albumins. Apparently, globulins are almost completely constituted by the trimeric form of conglutin. On the other hand, albumins include a number of proteins with different molecular weights. Accordingly, most enzymes as well as other soluble proteins such as lectins and protease inhibitors are included in this fraction.

Dialysis of the resuspended globulins yielded soluble β conglutin, while α and γ conglutin precipitated. Figure 3 shows the gel filtration chromatographic profile of β conglutin, indicating that this fraction contains a number of peptides and proteins of different molecular weights, including 125, 56 and 20 kDa as observed in other legumes such as chickpea (Sánchez-Vioque, 1998). Also, the proportion

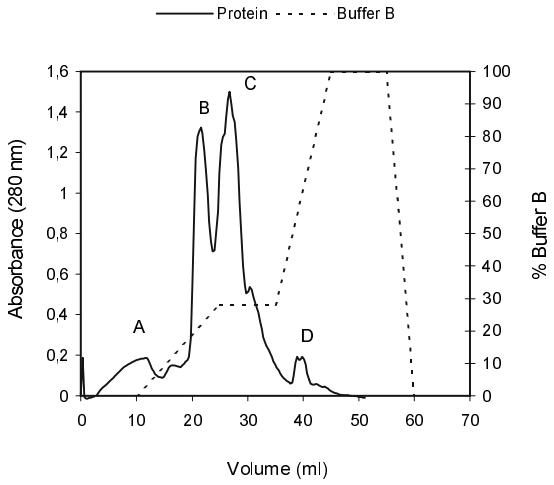

Figure 4
Ion exchange chromatography of *L. angustifolius* and conglutins. A = γ conglutin; B and C = soluble α conglutin; D = insoluble α conglutin. The composition of the elution buffer (% buffer B as described in materials and methods) is shown by a broken line.

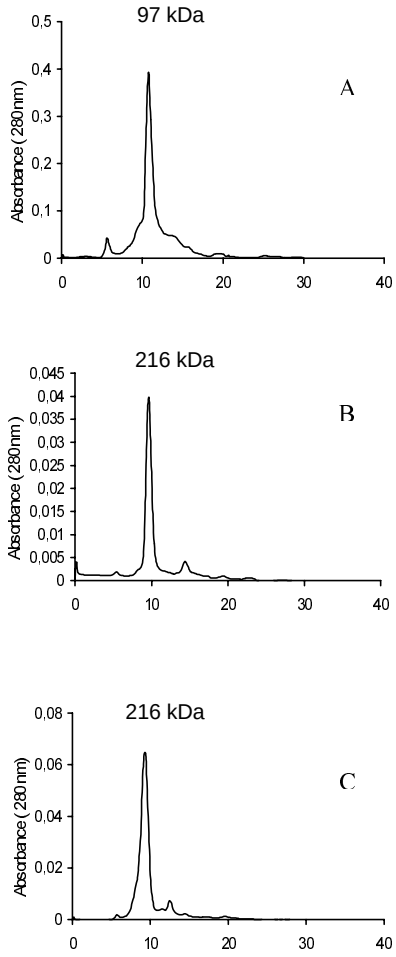

Figure 5
Gel filtration chromatography of fractions A to D obtained by ion exchange chromatography. (A) γ conglutin, (B) soluble α conglutin, (C) insoluble α conglutin.

between conglutin β (vicilin like) and conglutins α and δ (legumin like) is similar to that observed ion other legumes such as chickpea (Sánchez-Vioque, 1998). The precipitate including conglutins α and γ was resolved by ion exchange chromatography (Figure 4). Fractions A, B, C and D were collected and further resolved by gel filtration chromatography (Figure 5). Fraction A includes a single peak of 97 kDa that corresponds to conglutin γ, while fractions B and C were made up of a single peak of 216 kDa that corresponds to α conglutin. Probably different degrees of aggregation of α conglutin are responsible for the fractionation of this protein in ion exchange chromatography. According to Blagrove and Gillespie (1975), peaks B and C in figure 4 would correspond to soluble α conglutin, and peak D would correspond to insoluble α conglutin. After ion exchange chromatography, α conglutin represents the most abundant protein of *L. angustifolius* seeds (76.6% of the total, soluble plus insoluble).

Amino acid composition of *L angustifolius*

Amino acid analysis showed that lysine is a limiting amino acid in *L. angustifolius* seed proteins according to F.A.O. standards, while tyrosine is limiting in albumins, and methionine and lysine are at limiting amounts in globulins (Table I). According to this data, the albumin fraction has a better amino acid composition than the globulin fraction from the nutritional point of view. Unfortunately, albumins represent only a small portion of the total seed proteins (14%) and include antinutritional components such as lectins and protease inhibitors, and therefore offers little possibility for improvement of the nutritional value by breeding (Oomah and Bushuk, 1983).

Lysine was at limiting amounts in conglutins α, β and δ (Table II). In addition, methionine and histidine were limiting in conglutin, while histidine, valine and methionine were limiting in β conglutin. Thus, the most abundant seed proteins in *L. angustifolius*, α conglutin, is limiting in four essential amino acids. Conglutin δ, in which only lysine is limiting, has the best nutritional value considering the content in essential amino acids. Unfortunately, this protein only represents 2.6% of total seed proteins in *L. angustifolius*.

In conclusion, we have found that the procedure described by Singh et al. (1988) for the purification of chickpea seed proteins is also useful for the purification of seed proteins in *L. angustifolius*. The major protein component, α conglutin, has been purified by ion exchange chromatography in a single chromatographic step. Thus, this procedure allows for a rapid and efficient purification of the major proteins in *L. angustifolius* seeds providing pure protein fractions for further studies.

Table I
Amino acid composition of *L angustifolius* flour, albumin fraction and globulin fraction. Data is expressed as % molar and are the means ± SD of two analyses

	FAO[a]	Flour	Albumins	Globulins
Aspartic acid [b]		11.6 ± 0.55	11.9 ± 1.8	12.1 ± 1.4
Glutamic acid [c]		25.6 ± 1.25	22.1 ± 1.5	28.1 ± 2.7
Serine		7.1 ± 1.3	5.5 ± 0.1	6.2 ± 0.8
Histidine	1.9	2.7 ± 0.05	2.4 ± 0.8	2.3 ± 0.4
Glycine		4.6 ± 0.1	6.7 ± 0.2	4.0 ± 0.1
Threonine	3.4	4.9 ± 0.2	6.6 ± 0.7	3.5 ± 0.5
Arginine		11.5 ± 1.55	7.9 ± 0.5	12.3 ± 1.0
Alanine		3.8 ± 0.25	7.5 ± 1.2	3.1 ±0.2
Proline		4.6 ± 0.95	7.7 ± 2.6	5.5 ± 1.3
Tyrosine	6.3 [d]	5.9 ± 0.2	3.2 ± 0.3	4.3 ± 0.5
Valine	3.5	3.9 ± 0.2	4.7 ± 0.5	3.5 ± 0.1
Methionine	2.5 [e]	1.3 ± 0.4	1.0 ± 0.2	0.3 ± 0.1
Cysteine		3.5 ± 0.2	2.6 ± 0.5	1.4 ± 0.1
Isoleucine	2.8	5.5 ± 0.75	3.6 ± 0.1	4.3 ± 0.1
Leucine	6.6	8.7 ± 0.35	6.9 ± 0.8	8.2 ± 0.4
Phenylalanine		5.2 ± 0.6	2.8 ± 0.8	4.3 ± 0.2
Lysine	5.8	5.4 ± 0.0	8.2 ± 0.2	4.2 ± 0.2

[a]FAO (1991).

[b]Aspartic acid + asparagine.

[c]Glutamic acid + glutamine

[d]Tyrosine + phenylalanine

[e]Methionine + cysteine

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Table II

Amino acid composition of *L. angustifolius* α; β and γ conglutins. Data are given as % molar and represent means ± SD of two analyses

	FAO[a]	α-conglutin	γ-conglutin	β-conglutin
Aspartic acid[b]		8.4 ± 0.9	8.7 ± 1.3	13.5 ± 1.1
Glutamic acid[c]		20.2 ± 1.4	9.0 ± 1.2	27.1 ± 3.2
Serine		7.8 ± 0.0	9.1 ± 1.3	6.6 ± 0.5
Histidine	1.9	1.6 ± 0.2	3.2 ± 0.7	1.6 ± 0.5
Glycine		7.2 ± 0.1	9.8 ± 0.9	5.9 ± 0.3
Threonine	3.4	4.6 ± 0.2	7.9 ± 0.4	3.4 ± 0.1
Arginine		8.9 ± 1.0	3.6 ± 0.1	9.2 ± 1.2
Alanine		4.4 ± 0.6	6.1 ± 0.3	3.9 ± 0.1
Proline		3.3 ± 0.1	1.3 ± 1.2	4.2 ± 0.7
Tyrosine	6.3[d]	4.3 ± 0.2	3.5 ± 0.1	3.8 ± 0.2
Valine	3.5	5.1 ± 1.9	12.1 ± 6.1	3.3 ± 0.6
Methionine	2.5[e]	1.3 ± 0.0	3.0 ± 0.0	0.2 ± 0.0
Cysteine		0.9 ± 0.3	1.5 ± 0.0	0.5 ± 0.2
Isoleucine	2.8	4.4 ± 0.4	3.9 ± 0.2	3.5 ± 1.5
Leucine	6.6	10.8 ± 1.6	9.0 ± 0.1	8.3 ± 0.5
Phenylalanine		3.9 ± 0.4	4.1 ± 0.1	2.9 ± 1.3
Lysine	5.8	3.4 ± 0.0	5.6 ± 0.1	3.5 ± 0.4
Tryptophan		0.5 ± 0.0	0.5 ± 0.0	0.1 ± 0.0

[a] FAO Energy and protein requirement, (1991).
[b] Aspartic acid + asparagine.
[c] Glutamic acid + glutamine.
[d] Tyrosine + phenylalanine.
[e] Methionine + cysteine

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