Storage Proteins from *Lathyrus sativus* Seeds

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The proteins from *Lathyrus sativus* Linn. (chickling vetch or grass pea) seeds were investigated. Protein constitutes ~20% of the seed dry weight, >60% of which is composed by globulins and 30% by albumins. A single, 24 kDa polypeptide comprises more than half of the protein present in the albumin fraction. The globulins may be fractionated into three main components, which were named α-lathyrin (the major globulin), β-lathyrin, and γ-lathyrin. α-Lathyrin, with a sedimentation coefficient of ~18S, is composed of three main types of unglycosylated subunits (50−66 kDa), each of which produce, upon reduction, a heavy and a light polypeptide chain, by analogy with 11S. β-Lathyrin, with a sedimentation coefficient of 13S, is composed by a relatively large number of subunits (8−66 kDa). Two major polypeptides are glycosylated and exhibit structural similarity with β-conglutin from *Lupinus albus.* One of these possesses an internal disulfide bond. γ-Lathyrin, with a sedimentation coefficient of ~5S, contains two interacting, unglycosylated polypeptides, with no disulfide bonds: the major 24 kDa albumin and the heavier (20 kDa) polypeptide chain of *La. sativus* lectin.

Keywords: *Globulins; Lathyrus sativus; lectins; proteins; seeds; storage*

INTRODUCTION

The search for alternative or new protein sources became an important research trend over the past decades, not only to face an increasing demand of protein but also to look for alternative crops that may be cultivated in marginal soils or to seek plant species capable of supplying a high-quality protein. Among those, legume seeds have certainly played a major role, not only as animal feed but also for human consumption.

Osborne (1924) classified the seed storage proteins into four groups on the basis of their extraction and solubility characteristics: albumins, soluble in water; globulins, the major storage proteins in legume seeds, soluble in dilute salt solutions; prolamins, insoluble in the above solutions but soluble in alcohol/water mixtures; and glutelins, insoluble in the above solutions but soluble in weak acidic or basic solutions (Ashton, 1976; Shewry et al., 1995).

In the particular case of Leguminosae species, Osborne and Campbell (1898) initially separated the globulin fraction from *Pisum sativum* seeds into two major components, which were named vicilin and legumin. The presence of similar fractions was subsequently demonstrated in a variety of legume seeds (Derbyshire et al., 1976). Danielsson (1949) studied the seeds from 34 species of legumes and found, with a few exceptions, that they all contain two globulin components with sedimentation coefficients of approximately 7S and 11 S. These protein components were designated vicilin-like and legumin-like, respectively, on the sole basis of

their sedimentation values. This nomenclature has been adopted and extensively used since the pioneer work of Danielsson (1949), although some uncertainties have recently been raised about its general use (Ferreira et al., 1999).

The use of *Lathyrus* seeds as a source of protein for animals has been hampered by the development of lathyrism—a disease that occurs in humans and other animals as a result of the regular ingestion of seeds from *Lathyrus odoratus* (sweet pea). This is due to the presence of β-aminopropionitrile in the seeds, an irreversible inhibitor of lysyl oxidase. The covalent inactivation of this enzyme results in markedly reduced cross-linking of collagen in lathrytic animals (Voet and Voet, 1990). The outbreak of lathyrism has been considered the result of eating large amounts of *Lathyrus* without proper cooking. New varieties of *Lathyrus* species have been developed. Nevertheless, *Lathyrus sativus* (chickling vetch or grass pea) has been widely cultivated throughout India for pulse and fodder.

Very little is known concerning the proteins present in *Lathyrus* seeds. Almeida (1980) reported that protein constitutes ~25% of the seed dry weight in *La. sativus.* In addition, an electrophoretic analysis of the seed proteins of the genus *Lathyrus* was reported, as well as a study on the changing protein levels in developing and germinating seeds of *La. sativus* (Chandna and Matta, 1994; Sood et al., 1995). However, most of what is known about *Lathyrus* proteins derives from the observation that crude extracts from seeds of a large number of *Lathyrus* species (including *La. odoratus*, *La. sativus*, and *La. ochrus*—ochrus vetch or cyprus vetch) exhibit haemagglutinating activity (Gold and Balding, 1975). The agglutination is inhibited best by D-mannose, D-glucose, or their α-methyl glucosides (Gupta et al., 1980; Kolberg et al., 1980; Kolberg and Sletten, 1982). Several isolectins were subsequently isolated and char-

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acterized from *La. odoratus*, *La. sativus*, and *La. ochrus.* The proteins, with molecular masses of ~50 kDa, were found to consist of two heavy (16−20 kDa) and two light (6 kDa) subunits (Kolberg et al., 1980; Kolberg and Sletten, 1982; Debray and Rougé, 1984; Yarwood et al., 1985; Bourne et al., 1990a,b). Gupta et al. (1980), working with *La. sativus*, described a lectin composed of two identical subunits (21 kDa).

In this work, the protein composition of *La. sativus* seeds was analyzed. The major storage proteins were extracted, fractionated, and isolated following a methodology that was recently developed for other legume species (Franco et al., 1997). Each of the purified proteins was subsequently characterized with respect to sedimentation coefficient, molecular mass, polypeptide and subunit composition, presence of disulfide bonds, presence of carbohydrate residues, and structural similarity with *Lupinus* seed globulins.

MATERIALS AND METHODS

Plant Material. Dry seeds of *La. sativus* Linn. (chickling vetch or grass pea) were harvested in 1998 in an agricultural cooperative at Vendas Novas, Portugal, and stored at 5 °C until required. Dry seeds of *Lu. albus* L. (white lupin), kindly supplied by Dr. J. N. Martins (Instituto Superior de Agronomia, Lisbon, Portugal), were hand-sorted, under ultraviolet (UV) light, to certify their sweet character (Ferreira et al., 1995) and stored at 5 °C until needed.

Protein Fractionation Based on Solubility Criteria. Albumins, globulins, prolamins, and glutelins from *La. sativus* cotyledons were sequentially extracted and purified using appropriate extraction solutions. The cotyledons were separated from teguments and embryos and ground to a fine powder using an electric mill. The albumins were extracted by stirring the flour for 4 h at 4 °C in water (pH adjusted to 8.0) containing 10 mM $CaCl_2$ and 10 mM $MgCl_2$ (34 mL per g dry weight) (Franco et al., 1997). The insoluble proteins were removed by centrifugation at 30000 g and 4 °C for 1 h. For globulin extraction, the pellet was resuspended in Tris-HCl buffer, pH 7.5, 100 mM, containing 10% (w/v) NaCl, 10 mM ethylenediamine-tetraacetic acid (EDTA), and 10 mM ethylene glycol bis (β-aminoethyl ether*)-N,N,N',N'*-tetraacetic acid (EGTA) (34 mL/g of dry weight) and stirred for 4 h at 4 °C. The solubilized globulins were obtained by centrifugation at 30000*g* and 4 °C for 1 h. The presence of $CaCl_2$ and $MgCl_2$ during the extraction of albumins, and of EDTA and EGTA during the extraction of globulins, increases the extraction efficiency of these proteins and avoids cross-contamination, as shown by Franco et al. (1997) for several legume seeds. The procedure was sequentially repeated to obtain prolamin and glutelin fractions from the pellet containing the insoluble material. The pellet was resuspended in 75% (v/v) ethanol (5 mL/g of dry weight), and the prolamins were extracted overnight at 4 °C with agitation. After centrifugation at 30000*g* and 4 °C for 1 h, the glutelin fraction was obtained by treating the precipitate with sodium borate buffer, pH 10, 50 mM, containing 1% (v/v) β-mercaptoethanol and 1% (w/v) SDS (5 mL/g of dry weight). The suspension was stirred at room temperature (to keep SDS in soluble form) for 4 h and centrifuged at 30000*g* and 20 °C for 1 h. All samples were kept at −70° until used.

Isolation of Total Globulins. Total globulins from *Lu. albus* and *La. sativus* seeds were extracted essentially as described by Ferreira et al. (1999). The dry cotyledons were milled (0.2 mm sieve), and the resulting meal was defatted with *n*-hexane (34 mL/g of dry weight) 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 (adjusted to pH 8.0) containing 10 mM $CaCl_2$ and 10 mM $MgCl_2$ (34 mL/g of dry weight) for 4 h. The suspension was centrifuged for 1 h at 30000*g*. The supernatant was discarded and the total globulin fraction of the proteins extracted by stirring the pellet with Tris-HCl buffer, pH 7.5, 100 mM, containing 10% (w/v) NaCl, 10 mM EDTA, and 10 mM EGTA (34 mL/g of dry weight), for 4 h. The globulin-containing solution was centrifuged for 1 h at 30000*g*, and the globulins were precipitated by the addition of ammonium sulfate (561 g/L). The precipitated globulins were centrifuged at 30000*g* for 20 min, resuspended in Tris-HCl buffer, pH 7.5, 50 mM (5.7 mL/g of dry weight), and desalted on PD-10 columns previously equilibrated in the same buffer. All operations were performed at 4 °C.

Purification of Individual Globulins. α-Conglutin, β-conglutin, and γ-conglutin were purified from *Lu. albus* seeds by FPLC anion-exchange chromatography of the total globulin fraction on Mono Q (1 mL bed volume) or Q-Sepharose (8 mL bed volume) columns (Pharmacia) as described by Ferreira et al. (1999). The individual globulins from *La. sativus* seeds were fractionated and purified by an identical methodology.

Purification of the Lectin from *La. sativus*. The lectin was purified from *La. sativus* essentially as described by Richardson et al. (1984) for the lectin from *La. ochrus*. Cotyledons were milled, and the lectin was extracted from the crude meal with Tris-HCl buffer, pH 7.6, 50 mM, containing 150 mM NaCl (2.5 mL/g of dry weight). The homogenate was filtered through four layers of cheesecloth, centrifuged at 30000*g* and 4 °C for 1 h, dialyzed against the same buffer, and subjected to affinity chromatography on a Sephadex G-200 column equilibrated with the same buffer. The lectin, retained by the column, was eluted by adding 100 mM glucose to the buffer. The protein was subsequently concentrated and transferred to Tris-HCl buffer, pH 7.5, 50 mM, by ultrafiltration/ diafiltration and subjected to FPLC anion-exchange chromatography on a Mono Q column equilibrated in the same buffer. The bound proteins were eluted with a step gradient of NaCl (0−1 M).

Production of Polyclonal Antibodies. Polyclonal antibodies were produced in rabbits against α-conglutin and β-conglutin from *Lu. albus* as described before (Seabra et al., 1999). Samples containing the purified antigens (200 μg) were mixed with an equal volume of Freund's complete adjuvant (l mL final volume) and injected subcutaneously into female New Zealand rabbits. To obtain a high titer, three booster injections were given every 2 weeks in complete Freund's adjuvant diluted 1:10 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.

Isopycnic Glycerol or Sucrose Density Gradient Ultracentrifugation and Determination of Sedimentation Coefficients. The glycerol gradients were prepared in ultraclear 38 mL centrifuge tubes and were composed of 45% (4 mL), 40% (4 mL), 35% (4 mL), 30% (4 mL), 25% (4 mL), 20% (4 mL), 15% (4 mL), 10% (4 mL), and 5% (4 mL) glycerol (v/v) made up in Tris-HCl buffer, pH 7.5, 50 mM (Ferreira et al., 1999). The sucrose gradients were also prepared in ultraclear 38 mL centrifuge tubes and were composed of 30% (7.2 mL), 25% (7.2 mL), 20% (7.2 mL), 15% (7.2 mL), and 10% (7.2 mL) sucrose (w/v) made up in phosphate buffer, pH 7.6, 35 mM, containing 400 mM NaCl and 10 mM β-mercaptoethanol (Hill and Breidenbach, 1974). The gradients were prepared, loaded, and ultracentrifuged as described before (Ferreira et al., 1999). Sedimentation coefficients of protein fractions were also estimated as reported by Ferreira et al. (1999).

Electrophoresis, Western Blotting, Affinoblotting, 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. Several types of electrophoresis were used, namely, nondenaturing PAGE (ND-PAGE), nonreducing sodium dodecyl sulfate PAGE (NR-SDS−PAGE), and reducing SDS−PAGE (R-SDS−PAGE), following the methodology described by Santos et al. (1997). Before electrophoresis, all protein samples were either added to nondenaturing sample buffer (PAGE) or boiled for 3 min in the presence of SDS (2% w/v) (NR-SDS− PAGE) or SDS and β-mercaptoethanol (0.1 M) (R-SDS− PAGE).

Proteins separated by R-SDS–PAGE were blotted onto a poly(vinylidene difluoride) (PVDF) membrane (previously soaked in methanol and 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 semidry 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 sulfosalicylic acid, and washed for 5 min with water.

Proteins separated by R-SDS–PAGE and blotted onto a PVDF membrane were also utilized in the detection of glycopolypeptides essentially by the concanavalin A/peroxidase method developed by Faye and Chrispeels (1985). The membrane, containing the fixed polypeptides, was washed (5 min) with water and then (1 min) with 20 mM Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl (TBS), immersed for 1 h in TBS containing 0.1% (v/v) Tween-20 (TBST), and incubated in TBST containing 25 μg mL^{-1} concanavalin A, 1 mM CaCl$_2$, and 1 mM MgCl$_2$ (TBSTS). After 1 h, the membrane was washed (4 × 10 min) with TBSTS, incubated for 1 h in TBSTS containing 50 μg.mL^{-1} peroxidase and washed (4 × 10 min) with TBSTS and once (5 min) with TBS containing 1 mM CaCl$_2$ and 1 mM MgCl$_2$ (TBSS). The membrane was finally immersed for 5 min in 50 mL of a freshly prepared solution of TBSS containing 30 mg of 4-chloro-1-naphthol (previously dissolved in 10 mL of methanol) and 60 μL of H$_2$O$_2$ (30% v/v). After specific detection of the glycosylated polypeptides present, the membrane was washed with water and immediately photographed.

When appropriate, the proteins separated by R-SDS–PAGE and blotted onto a PVDF membrane were subjected to immunoblotting. The blots were probed with anti-α-conglutin (5000-fold diluted) or anti-β-conglutin (5000-fold diluted) antibodies and processed as described by Ramos et al. (1997).

N-Terminal Polypeptide Sequencing. The protein to be sequenced was purified, denatured in the presence of SDS and β-mercaptoethanol, and subjected to R-SDS–PAGE. The polypeptide was transferred onto a PVDF membrane, and the area corresponding to the polypeptide was sliced, washed with water, and subjected to N-terminal sequencing by the Edman reaction in an Applied Biosystems sequencer, following the instructions recommended by the manufacturer.

In addition to the methodologies described above for electrophoresis and Western blotting, electrotransfer of polypeptides for microsequencing requires a number of additional steps. The SDS utilized in the preparation of the SDS–PAGE gels was recrystallized twice in ethanol and water, as described by Hunkapiller et al. (1983). To reduce the risks of N-terminal blockage of polypeptides (which impedes N-terminal sequencing), gels were pre-electrophoresed (30 min at 200 V) in the presence of 50 μM glutathione in the cathodic solution (to avoid tryptophan and methionine degradation). Protein samples were electrophoresed in the presence of 100 μM glutathione in the cathodic solution (Yuen et al., 1986). These alterations remove charged impurities, reduce the amount of peroxides and free radicals, and take away uncharged reactive species, such as acrylamide monomers and other reactive compounds (Cholin and Wittman-Liebold, 1990). In the electrotransfer of polypeptides, a specialized membrane was used: Immobilon-P^{SQ}, 0.1 μm pore size (Millipore). The transfer buffer utilized was 10 mM CAPS [β-(cycloheximide)-l-propanesulfonic acid) containing 10% (v/v) methanol] (Yuen et al., 1990), not only to reduce the concentrations of Tris and glycine (which interfere with the sequencing process) in the polyacrylamide gel but also to increase the efficiency of the transfer process (LeGendre and Matsudaira, 1989). After transfer, the membranes were treated with the irreversible stain Ponceau S (0.1% w/v in an aqueous solution containing 1% v/v acetic acid), and the polypeptide bands were sliced and stored at −20 °C until used.

After sequencing, the N-terminal sequence obtained was compared with that of other proteins using the protein

Table 1. Protein Content of *La. sativus* Seeds in Albumins, Globulins, Prolamins, and Glutelins[a]

|	protein concn	
protein fraction	mg g^{-1} of dry wt	% of total
albumins	54.4 ± 4.3	31
globulins	112.2 ± 11.7	64
prolamins	1.7 ± 0.7	1.1
glutelins	6.8 ± 5.3	3.9

[a] Protein content in fractions was determined in triplicate by a modification of the Lowry method (Bensadoun and Weinstein 1976).

sequence database SWISS–PROT and the automatic server BLITZ (BLITZ@ebi.ac.uk). The online database Expasy Proteomic Tools (Expasy.hcuge.ch/www/tools.html) was also utilized.

General Assays. Protein was determined according to a modification of the Lowry method (Bensadoun and Weinstein, 1976) using bovine serum albumin as the standard. Several methods were utilized, when required, to concentrate dilute protein solutions, namely, precipitation with acetone, precipitation with ammonium sulfate, lyophilization, FPLC anion-exchange chromatography on the Mono Q column (elution of the bound proteins with a 1 M step NaCl gradient), and ultrafiltration with Centricon 10 microconcentrators (Amicon). In a similar way, desalting/buffer exchange was achieved whenever needed by dialysis, diafiltration (using Centricon 10), or gel filtration chromatography on the prepacked Sephadex G-25M PD-10 columns (Pharmacia).

RESULTS AND DISCUSSION

The first approach to study the proteins present in *La. sativus* seeds was to extract sequentially these polymers with appropriate solutions, following the classification proposed by Osborne (1924) that divides proteins according to their solubility characteristics into albumins, globulins, prolamins, and glutelins. This classification of proteins is an ambiguous system, resulting in many inaccuracies that have been widely discussed in the literature. Nevertheless, fractionation based on solubility criteria is a convenient system to initiate the characterization of the seed storage proteins from a species that has not been studied in detail (Sood et al., 1995), not only for comparative purposes but also as an initial purification step. We have therefore subjected the cotyledons of *La. sativus* seeds to a sequential extraction procedure, to analyze by R-SDS–PAGE the polypeptide composition of the albumin, globulin, prolamin, and glutelin fractions. The results of this experiment are presented in Table 1 and Figure 1. Several features deserve a brief comment: (i) Total protein constitutes ~20% of the seed dry weight, a value that is in reasonably good agreement with the value of 25% reported by Almeida (1980). (ii) Globulins comprise the vast majority of the seed proteins. This observation was expected because *La. sativus* belongs to the Leguminosae family, and globulins are known to be the major protein component in the storage tissues of legume cotyledons. (iii) The globulins, comprising >60% of the seed protein, are composed by many different polypeptides covering a wide range of molecular masses (Figure 1, lane 2). On the contrary, the albumins, comprising ~30% of the seed protein, contain a 24 kDa polypeptide that corresponds to more than half (as determined by densitometric analysis) of the protein present in this fraction (Figure 1, lane 1). (iv) Prolamins and glutelins represent minor fractions in *La. sativus* seeds. Moreover, the glutelin polypeptide pattern illustrated in

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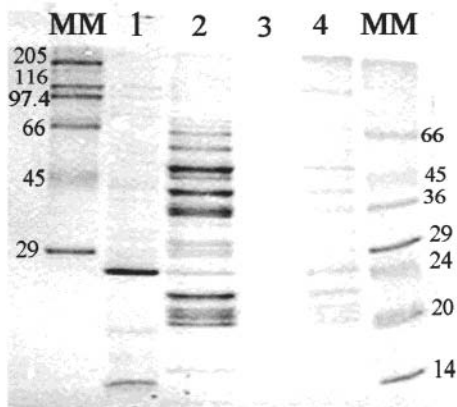
Figure 1. Polypeptide patterns of albumin (lane 1), globulin (lane 2), prolamin (lane 3), and glutelin (lane 4) fractions from *La. sativus* cotyledons. The protein fractions were sequentially extracted as described under Materials and Methods and the resulting samples (corresponding to 500 μg of dry weight of plant material) analyzed by R-SDS−PAGE. Lanes MM: molecular mass markers (kDa).

Figure 1, lane 4, appears to be similar to the albumin plus globulin patterns (Figure 1, lane 1 and 2), suggesting that a small proportion of these proteins may have been coextracted with the glutelin fraction. In this case, the glutelin fraction may represent far less than 3.9% of the seed protein.

The total globulin fraction from *La. sativus* cotyledons was subjected to FPLC anion-exchange chromatography on a Q-Sepharose column (Figure 2A), as described by Melo et al. (1994), to purify α-conglutin, β-conglutin, and γ-conglutin from *Lu. albus.* Each of the three major protein peaks obtained was subsequently analyzed by ND-PAGE (Figure 2B), NR-SDS−PAGE (Figure 2C), and R-SDS−PAGE (Figure 2D).

The terminology utilized to designate the three main globulins of *Lu. albus* cotyledons as γ-conglutin, β-conglutin, and α-conglutin was introduced in 1961 by Morawieska and subsequently used by Blagrove and Gillespie (1978) to indicate the increase in mobility toward the anode observed when these globulins are subjected to paper electrophoresis on cellulose acetate. Cerletti et al. (1978) observed that these proteins were eluted in the same sequence, that is, γ, β, and α, from an anion-exchange column. In a similar way, the three major *Lathyrus* globulins that elute sequentially from the anion-exchange columns (peaks 2−4 in Figure 2A) exhibit increased mobility toward the anode when analyzed by PAGE (Figure 2B). For this reason, the three main *Lathyrus* globulins may be designated γ-lathyrin (peak 2 in Figure 2A and lane 2 in Figure 2B−D), β-lathyrin (peak 3 in Figure 2A and lane 3 in Figure 2B−D), and α-lathyrin (peak 4 in Figure 2A and lane 4 in Figure 2B−D).

The data illustrated in Figure 2 allow a preliminary characterization of α-, β-, and γ- lathyrins. α-Lathyrin is by far the most abundant storage protein, comprising ∼80% of the total globulin fraction. β- and γ-lathyrins are minor storage proteins, constituting ca. 14 and 6% of the total globulin fraction, respectively. This differs from *Lupinus* storage proteins, for which α- and β-conglutins are the major globulins (Melo et al., 1994), but is in agreement with the observation that the legumin-like globulin is generally considered to be the most important storage protein of legume seeds (Shewry et al., 1995).
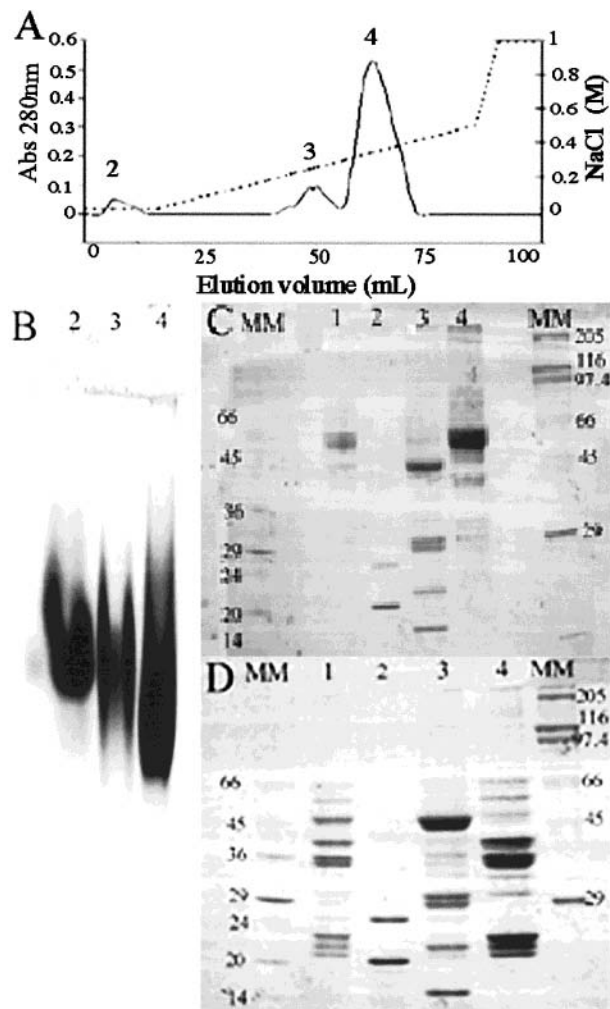
Figure 2. Fractionation of the total globulin fraction from *La. sativus* seeds by FPLC anion-exchange chromatography (A) and subsequent analysis of each of the three major globulins by ND-PAGE (B), NR-SDS−PAGE (C), and R-SDS−PAGE (D). (Lanes) 1, total globulin fraction; 2, peak 2, γ-lathyrin; 3, peak 3, β-lathyrin; 4, peak 4, α-lathyrin; MM, molecular mass markers (kDa). Fifty micrograms (C, D) or 100 μg (B) of protein was loaded in each lane.

γ-Lathyrin, a minor globulin component, does not bind to the anion-exchange column at pH 7.5 (Figure 2A) and exhibits the lowest mobility when analyzed by ND-PAGE (Figure 2B). It contains two polypeptide chains (20 and 24 kDa) with no disulfide bonds (Figure 2C,D). β-Lathyrin, another minor globulin component, elutes from the anion-exchange column with 0.2−0.3 M NaCl (Figure 2A) and shows intermediate electrophoretic mobility (Figure 2B). It is composed by several polypeptides covering a wide range of molecular masses (14−66 kDa) and apparently no disulfide bonds (Figure 2C,D). Finally, α-lathyrin, the major globulin component, elutes from the anion-exchange column with 0.3−0.4 M NaCl (Figure 2A) and presents the highest mobility when resolved in its native form by electrophoresis (Figure 2B). It consists of three main types of subunits, in the molecular mass range of 50−66 kDa, which, upon reduction, produce two major sets of polypeptide chains: a heavy group and a light group, with molecular masses of 35−40 and 21−22 kDa, respectively. This result clearly indicates the presence of disulfide bonds in α-lathyrin. The apparent great similarity of lathyrins with *Lu. albus* conglutins (Melo

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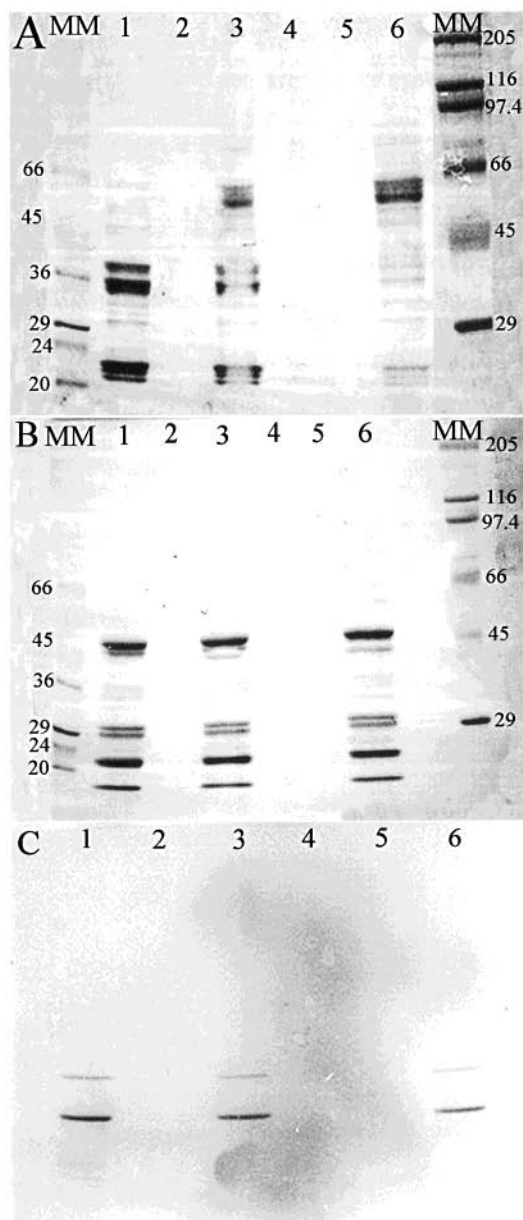
Figure 3. Detection of disulfide bonds in the subunits of α-lathyrin (A), β-lathyrin (B), and γ-lathyrin (C). (Lanes) 1, pure protein in sample buffer containing β-mercaptoethanol (SBME); 3 and 6, pure protein in sample buffer without β-mercaptoethanol (SB); 2 and 4, SBME; 5, SB; MM, molecular mass markers (kDa). Fifty micrograms of protein was loaded in each lane.

et al., 1994), and possibly with the globulins of other legume seeds, is surprising.

The presence of disulfide bonds was further assessed according to a method developed by Melo et al. (1994), which is based on the lateral diffusion of β-mercaptoethanol during electrophoresis. The results of this experiment, illustrated in Figure 3A,B,C for α-, β-, and γ-lathyrins, respectively, clearly show the following: (i) γ-Lathyrin possesses no disulfide bond. (ii) β-Lathyrin contains an internal polypeptide chain disulfide bond in a 45 kDa subunit (compare lanes 1, 3, and 6 in Figure 3B); this conclusion is based on the observation that the 45 kDa subunit originates, upon reduction, a single polypeptide chain with an apparent higher molecular mass. (iii) Each of the α-lathyrin three main types of subunits (50–66 kDa) is split, upon reduction, into a heavy polypeptide chain (35–40 kDa) and a light
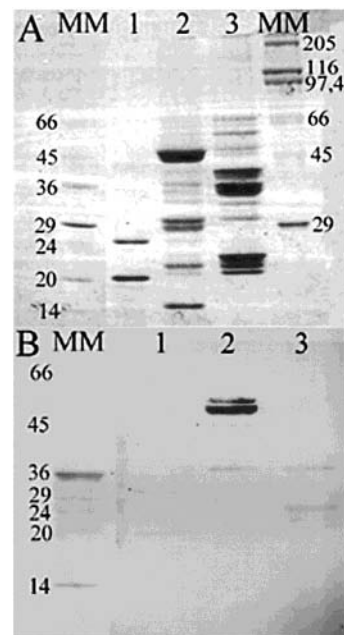
Figure 4. Detection of glycosylated polypeptides. Samples of pure γ-lathyrin (lane 1), β-lathyrin (lane 2), and α-lathyrin (lane 3) were analyzed by R-SDS−PAGE and the polypeptides stained with Coomassie Brilliant Blue (A) or transferred to a PVDF membrane and probed with concanavalin A−peroxidase for glycoprotein detection (B). Lane MM: molecular mass markers (kDa). Fifty micrograms (A) or 15 μg (B) of protein was loaded in each lane.

polypeptide chain (21−22 kDa) in a way similar to that reported for α-conglutin from *Lu. albus* (Blagrove and Gillespie, 1975; Melo et al., 1994). Many seed storage proteins from different plants are glycosylated. This is certainly true for legume seeds (Derbyshire et al., 1976) and, in particular, for *Lu. albus* seeds. Indeed, all three conglutins are heavily glycosylated (Ferreira et al., 1995). In an attempt to determine the extent of glycosylation of *La. sativus* seed proteins, the polypeptide pattern of each of the three lathyrins was probed with concanavalin A−peroxidase, as described under Materials and Methods. The results shown in Figure 4 indicate that α- and γ-lathyrins are not glycosylated and that β-lathyrin contains two glycosylated subunits (with molecular masses of ~45 kDa; Figure 4B, lane 2). A more detailed investigation revealed that one of the glycosylated subunits is the polypeptide containing an internal disulfide bond (Figure 5).

It has been considered well established that many legume seeds contain two major globulin components with sedimentation coefficients of approximately 7S and 11S (Derbyshire et al., 1976). Although the sedimentation coefficients of α- and β-conglutins had never been clearly established, α-conglutin was generally considered to be the 11S globulin, whereas β-conglutin was regarded as the 7S globulin. However, using isopycnic glycerol or sucrose density gradient centrifugation performed under low or high ionic strength and gel filtration techniques, Franco et al. (1997) found that β-conglutin (with a sedimentation coefficient of approximately 11S) is considerably heavier than α-conglutin (with a sedimentation coefficient of approximately 7S). In a subsequent study, Ferreira et al. (1999) suggested that the traditional 7S and 11S protein components of legume seed proteins may simply reflect a preferential degree of globulin association under the conditions existing in vitro. In favor of this suggestion

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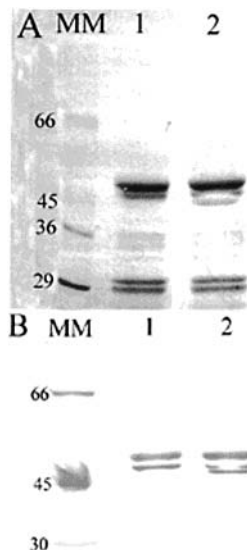
Figure 5. Detection of glycosylated polypeptides on β-lathyrin. Samples of pure β- lathyrin were subjected to SDS−PAGE under reducing conditions (lane 1) and under nonreducing conditions (but surround by lanes containing β-mercaptoethanol; lane 2) and the polypeptides stained with Coomassie Brilliant Blue (A) or transferred to a PVDF membrane and probed with concanavalin A−peroxidase for glycoprotein detection (B). Lanes MM: molecular mass markers (kDa). Fifty micrograms (A) or 15 μg (B) of protein was loaded in each lane. To achieve a better resolution between the two glycosylated polypeptides, the electrophoretic run was prolonged until the small polypeptides (<30 kDa) left the gel.

Table 2. Sedimentation Coefficients of *La. sativus* Lathyrins[a]

| | sedimentation coefficient, S | | |
type of gradient	α-lathyrin	β-lathyrin	γ-lathyrin
glycerol	19.1 (a)	13.0 (a)	5.0 (a)
	19.0 (b)	12.8 (b)	4.6 (b)
sucrose	16.8 (a)	12.9 (a)	4.4 (a)
	16.5 (b)	12.9 (b)	4.6 (b)

[a] *La. sativus* total globulins (a) or each of the individual lathyrins (b) (1.5 mg) were subjected to isopycnic glycerol or sucrose density gradient ultracentrifugation. The gradients were collected, the protein bands identified by R-SDS−PAGE, and the sedimentation coefficients determined as described under Materials and Methods.

are results presented in Table 2, which show the sedimentation coefficients of α-, β-, and γ-lathyrins estimated by isopycnic glycerol or sucrose density gradient ultracentrifugation. Under the conditions utilized in vitro, α-, β-, and γ-lathyrins exhibit sedimentation coefficients rather different from the generally assumed values of 11S, 7S, and 2S, respectively.

The similarity detected between *Lupinus* conglutins and *Lathyrus* lathyrins in subunit and polypeptide composition (Figures 2 and 3 and Melo et al., 1994) and a number of studies published on structural similarity among vicilin-like and legumin-like globulins from different legume species prompted us to investigate the occurrence of structural analogies between conglutins and lathyrins. To this end, polyclonal antibodies were produced specifically to α- and β-conglutins and utilized to probe α- and β-lathyrins, previously blotted onto an appropriate seed membrane. The results obtained, presented in Figure 6A−D, show that only the anti-β-conglutin antibodies were able to recognize three β-lathyrin polypeptides specifically, the two glycosylated subunits
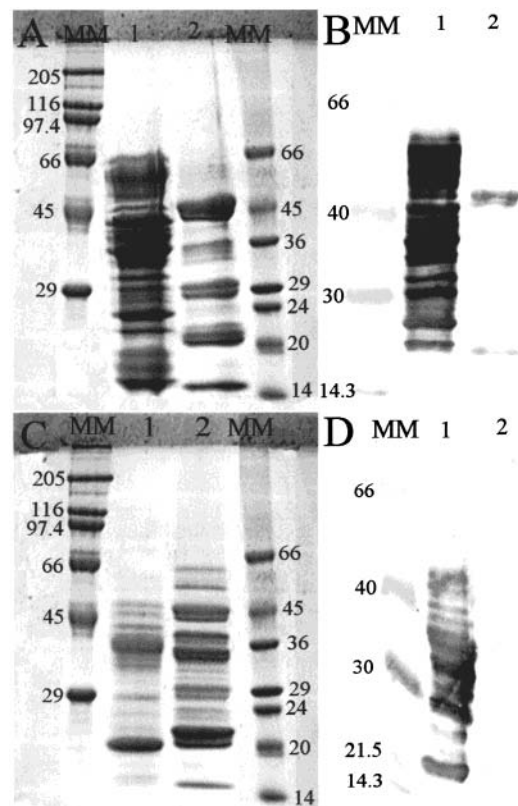
Figure 6. Structural similarity between *Lathyrus* α- and β-lathyrins and *Lupinus* α- and β-conglutins. β-Conglutin (lane l, A and B), β-lathyrin (lane 2, A and B), α-conglutin (lane 1, C and D), and α-lathyrin (lane 2, C and D) were analyzed by R-SDS−PAGE and either stained for total protein (A, C) or blotted onto a membrane and probed with anti-β-conglutin antibodies (B) or anti-α conglutin antibodies (D). MM: molecular mass markers (kDa). Fifty micrograms (A, C) or 15 μg (B,D) of protein was loaded in each lane.

(see Figures 4 and 5) and a 20 kDa subunit. Thus, although there seems to be some structural similarity between the vicilin-like globulins from *Lathyrus* and *Lupinus*, the opposite occurs for α-lathyrin and α-conglutin.

A major difference became evident between γ-conglutin and γ-lathyrin. Although they both possess no net negative charge at pH 7.5, γ-conglutin is composed by one main type of subunit (40 kDa) containing two polypeptide chains (18 and 26 kDa) linked by disulfide bonds (Melo et al., 1994), whereas the γ-lathyrin fraction contains two polypeptides (20 and 24 kDa) with no disulfide bonds. Two extreme hypotheses were formulated to try to explain the difference: the 20 and 24 kDa polypeptides from *Lathyrus* constitute two subunits of the same protein or they are independent proteins that coelute from the anion-exchange column just because they do not exhibit net negative charge at pH 7.5. To try to elucidate this issue, the 20 kDa polypeptide was subjected to N-terminal sequencing and the resulting sequence compared with the published sequences of other proteins. The results, expressed in Table 3, indicate that the sequence of the 22 N-terminal amino acid resides of the 20 kDa polypeptide shows a very high homology with a family of legume lectins, exhibiting 95% homology with the heavier polypeptide from *La. ochrus* lectin and 90% with the lectin from *Lens culinaris. La. ochrus* lectin has been reasonably studied and is known to be a 50 kDa protein composed of two heavy (16−20 kDa) and two light (6 kDa) subunits (Debray

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Table 3. Comparison between the N-Terminal Amino Acid Sequence of the 20 kDa Polypeptide (γ20) and Those of the Heavier Polypeptide from *La. ochrus* Lectin (L*Lo*) and *Lens culinaris* Lectin (L*Lc*)[a]

	1									10									20		22
L*Lo*	T	E	T	T	S	F	S	I	T	K	F	G	P	D	Q	Q	N	L	I	F	Q G
						*															
γ20	T	E	T	T	S	F	L	I	T	K	F	G	P	D	Q	Q	N	L	I	F	Q G
						*			*												
L*Lc*	T	E	T	T	S	F	S	I	T	K	F	S	P	D	Q	Q	N	L	I	F	Q G
	1									10									20		22

[a] Asterisks indicate nonhomologous residues between γ20 and those of the other polypeptides.
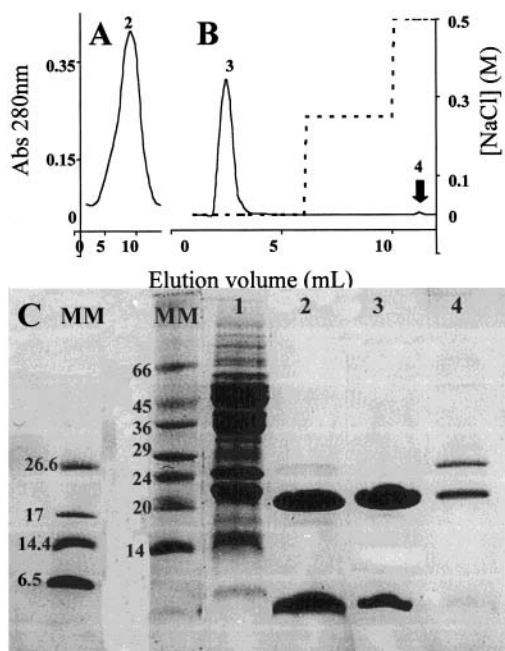
Figure 7. Purification of *La. sativus* lectin. The lectin was extracted and the dialyzed crude extract subjected to affinity chromagraphy on a Sephadex G-200 column (A). The protein eluted from the column with glucose (peak 2) was fractionated by FPLC anion-exchange chromatography (B) in two protein fractions (peaks 3 and 4). The polypeptide composition of the crude extract (lane 1; 100 μg) and of peaks 2 (50 μg), 3 (50 μg), and 4 (5 μg)—lanes 2, 3, and 4, respectively—was subsequently analyzed by R-SDS−PAGE (C). MM: molecular mass markers (kDa).

and Rougé, 1984; Yarwood et al., 1985; Bourne et al., 1990 a, 1990b). Considering this information, how can the presence of only two polypeptides (20 and 24 kDa) in the γ-lathyrin fraction be explained? To try to resolve this uncertainty, *La. sativus* lectin was purified following the methodology described by Richardson et al. (1984) to isolate *La. ochrus* lectin. This methodology was not enough because a 24 kDa contaminant is still present after chromatography on Sephadex G-200 (Figure 7C, lane 2). For this reason, another purification step was introduced (anion-exchange chromatography, Figure 7B) to obtain the lectin in pure form (Figure 7C, lane 3). The overall purification process is illustrated in Figure 7. Confirming the results reported by Richardson and co-workers, the lectin from *La. sativus* is composed by two types of polypeptide chains, with

molecular masses of 6 and 20 kDa. Two important conclusions may therefore be drawn from Figure 7: (i) The 20 kDa polypeptide present in the γ-lathyrin fraction seems to be a component or subunit of the *La. sativus* lectin. (ii) The protein fraction eluting with 0.5 M NaCl from the anion-exchange column (Figure 7B, peak 4) contains both polypeptides (20 and 24 kDa) present in the γ-lathyrin fraction, suggesting that they interact with each other in some way, in the absence of the light subunit of the lectin (6 kDa) (Figure 7C, lane 4). Moreover, it was found that the 24 kDa polypeptide that copurifies with the heavy lectin subunit during the purification of this protein (Figure 7C, lane 4) is the same as the heavy polypeptide present in γ-lathyrin obtained after globulin isolation (Figure 2D, lane 2) and the major 24 kDa polypeptide that occurs in the total albumin fraction from *La. sativus* (Figure 1, lane 1). The failure to detect carbohydrate residues in both the 20 and 24 kDa polypeptides (Figure 4) ruled out a possible interaction of the type lectin−glycoprotein between the two polypeptides.

In conclusion, the results presented suggest that γ-lathyrin may be a naturally occurring globulin or a globulin formed in vitro by the interaction between a major 24 kDa albumin and the heavy (20 kDa) polypeptide chain of *La. sativus* lectin. The nature of this interaction is currently under investigation.

ABBREVIATIONS USED

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

LITERATURE CITED

Almeida, J. M. A cultura de leguminosas para grão, fava, tremoço, chicharos e grão de gramicha. In *Documentação sobre Forragens e Pastagens*; INIA: Lisbon, Portugal, 1980.

Ashton, F. M. Mobilisation of storage proteins of seeds. *Annu. Rev. Plant Physiol.* **1976**, *27*, 95−117.

Bensadoun, A.; Weinstein, D. Assay of proteins in the presence of interfering materials. *Anal. Biochem.* **1976**, *70*, 241−250.

Blagrove, R.; Gillespie, J. M. Isolation, purification and characterisation of the seeds globulins *of Lupinus angustifolius. Aust. J. Plant Physiol.* **1975**, *2*, 13−27.

Blagrove, R.; Gillespie, J. M. Comparative studies on the proteins from seeds of *Lupinus angustifolius. Aust. J. Plant Physiol.* **1978**, *5*, 561−663.

Bourne, Y.; Albergel, C.; Cambillau, C.; Frey, M.; Rouge, P.; Fontecilla-Camps, J. C. X-ray crystal structure determination and refinement at 1.9A resolution of isolectin I from seeds *of Lathyrus ochrus. J. Mol. Biol.* **1990a**, *214*, 571−584.

Bourne, Y.; Anguille, C.; Fontecilla-Camps, J. C.; Rouge, P.; Cambillau, C. Co-crystallisation and preliminary X-ray diffraction studies of *Lathyrus ochrus* isolectin I with di- and trisaccharides, and biantennary octosaccharide. *J. Mol. Biol.* **1990b**, *213*, 211−213.

Cerletti, P.; Fumagalli, A.; Venturin, D. Protein composition of seeds of *Lupinus albus. J. Food Sci.* **1978**, *43*, 1409−1411.

Chandna, M.; Matta, N. K. Studies on changing protein levels in developing and germinating seeds *of Lathyrus sativus L. J. Plant Biochem. Biotechnol.* **1994**, *3*, 59−61.

Cholin, T.; Wittman-Liebold, B. Protein blotting followed by microsequencing. *Electrophoresis* **1990**, *11*, 562−568.

Danielson, C. E. Seed globulins of the *Gramineae* and *Leguminosae. Biochem. J.* **1949**, *44*, 387−400.

Debray, H.; Rouge, P. The fine sugar specificity of the *Lathyrus ochrus* seed lectin and isolectins. *FEBS Lett.* **1984**, *176*, 120−124.

Derbyshire, E.; Wright, D. J.; Boulter, D. Legumin and vicilin, storage proteins of legume seeds. *Phytochemistry* **1976**, *15*, 3−24.

Faye, L.; Chrispeels, M. J. Characterisation of N-linked oligosacharides by affinoblotting with concanavalin A-Peroxidase and treatment of the blots with glycosidases. *Anal. Biochem.* **1985**, *149*, 218−224.

Ferreira, R. B.; Melo, T. S.; Teixeira, A. N. Catabolism of seed storage proteins from *Lupinus albus:* Fate of globulins during germination and seedling growth. *Aust. J. Plant Physiol.* **1995**, *22*, 373−381.

Ferreira, R. B.; Franco, E.; Teixeira, A. R. Calcium- and magnesium-dependent aggregation of legume seed storage proteins. *J. Agric. Food Chem.* **1999**, *47*, 3009−3015.

Franco, E.; Ferreira, R. B.; Teixeira, A. R. Utilisation of an improved methodology to isolate *Lupinus albus* conglutins in the study of their sedimentation coefficients. *J. Agric. Food Chem.* **1997**, *45*, 3908−3913.

Gold, E. R.; Balding, P. *Receptor-Specific Proteins, Plant and Animal Lectins*; Excerta Medica: Amsterdam, The Netherlands, 1975.

Gupta, B. K. D.; Chatterjee-Ghose, R.; Sen, A. Purification and properties of mitogenic lectins from seeds of *Lathyrus sativus* Linn. (chickling vetch). *Arch. Biochem. Biophys.* **1980**, *201*, 137−146.

Hill, J. E.; Breidenbach, R. W. Proteins of soybean seeds I. Isolation and characterisation of the major components. *Plant Physiol.* **1974**, *53*, 742−746.

Hunkapiller, M. W.; Lujan, E.; Ostander, F.; Hood L. E. In *Methods in Enzymology*; Hirs, C. H. W., Timasheff, S. N., Eds.; Academic Press: New York, 1983; Vol. 91, p 227.

Kolberg, J.; Sletten, K. Purification and properties of a mitogenic lectin from *Lathyrus sativus* seeds. *Biochim. Biophys. Acta* **1982**, *704*, 26−30.

Kolberg, J.; Michaelson, T. E.; Sletten, K. Subunit structure and N-terminal sequences of the *Lathyrus odoratus* lectin. *FEBS Lett.* **1980**, *117*, 281−283.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

LeGendre, N.; Matsudaira, P. T. Purification of proteins and peptides by SDS-PAGE. In *A Practical Guide to Protein and Peptide Purification for Microsequencing*; Matsudaira, P. T., Ed.; Academic Press: San Diego, CA, 1989; pp 52−66.

Melo, T. S.; Ferreira, R. B.; Teixeira, A. N. The seed storage proteins from *Lupinus albus. Phytochemistry* **1994**, *37*, 641−648.

Morawieska, B. Changes in proteins of yellow lupin (*Lupinus luteus* L.) during germination. *Acta Biochem. Pol.* **1961**, *8*, 313−319.

Osborne, T. B., Ed. *The Vegetable Proteins*; Longmans-Green: London, U.K., 1924.

Osborne, T. B.; Campbell, G. F. Proteins of the pea. *J. Am. Chem. Soc.* **1989**, *20*, 348−362.

Ramos, P. C. R.; Ferreira, R. M. S. B.; Franco, E.; Teixeira, A. R. N. Accumulation of a lectin-like breakdown product of β-conglutin catabolism in cotyledons of germinating *Lupinus albus* L. seeds. *Planta* **1997**, *203*, 26−34.

Richardson, M.; Rougé, P.; Sousa-Cavada, B.; Yarwood, A. The amino acid sequences of the *al* and *a2* subunits of the isolectins from seeds of *Lathyrus ochrus (L)* DC. *FEBS Lett.* **1984**, *175*, 76−81.

Santos, C. N.; Ferreira, R. B.; Teixeira, A. R. Seed proteins *of Lupinus mutabilis. J. Agric. Food Chem.* **1997**, *45*, 3821−3825.

Seabra, M. A.; Freire, J. P. B.; Ferreira, R. B.; Cunha, L.; Teixeira, A. R. The use of lupinseed (*Lupinus albus*), faba bean (*Viciafaba*) and cowpea (*Vigna unguiculara*) on the piglet weaning diet: antigenicity and effect on growth peformances. *Rev. Portuguesa Zootecnia* **1999**, *6*, 133−148.

Shewry, P. R.; Napier, J. A.; Tatham, A. S. Seed storage proteins: structures and biosynthesis. *Plant Cell* **1995**, *7*, 945−56.

Sood, A.; Chandna, M.; Matta, N. K. Electrophoretic studies on seed proteins of the genus *Lathyrus. J. Plant Biochem. Biotechnol.* **1995**, *4*, 121−124.

Voet, D., Voet, J. G., Eds. *Biochemistry*; Wiley: New York, 1990.

Yarwood, A.; Richardson, M.; Sousa-Cavada, B.; Rougé, P. The complete amino acid sequences of the β_1- and β_2-subunits of the isolectins LoL I and LoL II from seeds *of Lathyrus ochrus* (L.) DC. *FEBS Lett.* **1985**, *184*, 104−109.

Yuen, S.; Hunkapiller, N. W.; Wilson, K. J.; Yuan, R. M. *SDS−PAGE Electroblotting*; Applied Biosystems User Bulletin 25; CA, 1986.

Yuen, S.; Sheer, D.; His, R. L.; Mattaliano, R. *Problott, an Improved PVDF-type Media for Protein Sequencing*; Applied Biosystems, Researcher News: CA, 1990.