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Characterization of Globulins from Common Vetch (*Vicia sativa* L.)

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The proteins from *Vicia sativa* L. (common vetch) seeds were investigated. Protein comprises ∼11.4% of the seed fresh weight, >50.8% of which is composed by globulins and 43.6% by albumins. The globulins may be fractionated into two main components, which were named α-vicinin (comprising 73% of the total globulin fraction, and hence >37% of the total seed protein) and β-vicinin. Two minor globulin components are also present, γ-vicinin and δ-vicinin. α-Vicinin, the legumin-like globulin, with a sedimentation coefficient of 10.6 S, is a nonglycosylated, disulfide-bond-containing globulin, composed of a group of subunits with molecular masses ranging from 50 to 78 kDa. Upon reduction, each of these subunits releases a heavy polypeptide chain (34−66 kDa) and a light polypeptide chain (21−23 kDa). β-Vicinin, the vicilin-like globulin, with a sedimentation coefficient of 7.7 S, is a nonglycosylated globulin that contains no disulfide bonds and consists of two major polypeptides with molecular masses of 58 and 66 kDa. γ-Vicinin is a minor, glycosylated, disulfide-bond-containing globulin. In the reduced form, it comprises six polypeptide chains with molecular masses of 12, 19, 21, 22, 23, and 31 kDa. Finally, δ-vicinin is a minor, highly glycosylated globulin that exhibits hemagglutinating activity. It is composed of a major 47 kDa polypeptide and two minor (33 and 38 kDa) polypeptides. N-terminal sequencing of the δ-vicinin 47 kDa polypeptide revealed no homology to any other known storage protein.

KEYWORDS: Proteins; seeds; storage proteins; vetch; *Vicia sativa*

INTRODUCTION

Vicia sativa L. (common vetch) is widely cultivated as food for humans and livestock and has been used extensively for soil improvement (*1*, *2*). It is certainly an attractive alternate grain legume for many areas of the world, particularly for dryland farming. However, relatively few studies have been performed and published on the biochemistry and physiology of its seeds. This assumes great importance because legume grains constitute a major source of protein, oil, and other nutrients in the diets of almost all countries around the world (*3*). As legume seeds in general, vetch seeds are deficient in the sulfur-containing amino acids (*4*).

The observation that common vetch is toxic to livestock and many other animals (*5*, *6*) prompted researchers to perform a number of studies on the neurotoxic amino acid β-cyano-L-alanine and its γ-glutamyl derivative, known to be present in vetch seeds and seedlings (*7*). Other studies have been reported on a mitogenic lectin from vetch seeds. This is a tetrameric lectin, a glycoprotein $\alpha_2\beta_2$ complex with a molecular mass of 40 kDa, which agglutinates red blood cells and stimulates mitosis in lymphocytes. It consists of two large β-subunits (14 kDa) and two small α-subunits (6 kDa) (*8*, *9*). The complete amino acid sequence (52 residues) of the α chain has been determined and was found to be homologous to those of the α subunits of the lectins from *Pisum sativum*, *Lens culinaris*, and *Vicia faba*, except that the *V. sativa* α subunit has an additional serine at the N terminus (*10*). *V. sativa* seeds were shown to contain a protein (lectin binder) which associates with their lectin (*11*).

A number of studies have been performed on cysteine proteinases from *V. sativa*, including proteinases A and B, that are apparently involved in seed storage protein mobilization. A simplistic picture was initially formulated for the degradation of storage globulins in vetch seeds. According to this model, proteinase A, synthesized de novo in an axis-dependent manner after the onset of germination, modifies 7S and 11S globulins by limited proteolysis, thus initiating the degradative process (*12*). As a consequence, the globulins become partially soluble and susceptible to the action of other endo- and exopeptidases, including proteinase B (*13*). However, is is becoming increasingly evident that storage protein breakdown may be rather more

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complicated. Indeed, mobilization of storage globulins is probably brought about by a complex of different cysteine proteinases (CPRs). Proteinase A, a papain-like cysteine endopeptidase, and proteinase B, an asparagine-specific endopeptidase, have been extensively characterized in vetch seeds (*14*, *15*). Both proteinases are synthesized de novo during seed germination. Although proteinase A is capable of hydrolyzing storage globulins in vitro, recent evidence suggests that it is not involved in the early steps of storage protein mobilization (*15*). In addition, at least eight developmentally regulated proteolytic activities have been detected in germinating *V. sativa* cotyledons, including proteinase A (*16*), CPR1 and CPR2 (*17*), and proteinase B (*12*). CPR1, CPR2, and CPR4 are papain-like CPRs, whereas VsPB2 and proteinase B are legumain-like CPRs with specificity for Asn in the P1 position at the peptide bond to be cleaved. CPR1, CPR2, and proteinase B were shown to degrade storage globulins in vitro (*18*). CPR2, CPR4, and VsPB2, together with their mRNAs, are already present in the axes and cotyledons of dry seeds, indicating that they were formed during seed maturation. Additional papain-like CPRs are formed later during germination and seedling growth (*19*). These observations suggest that storage globulin mobilization in germinating vetch seeds is initiated by stored CPRs, whereas the mobilization of the bulk of globulin is predominantly mediated by de novo synthesized CPRs.

Globulins are the major storage proteins of legume seeds (*20*). With a few exceptions, legume seeds contain two major globulins with sedimentation coefficients of approximately 7 and 11 S. On the sole basis of their sedimentation values, these proteins were termed vicilin-like and legumin-like globulins, respectively. These globulins are also referred to by trivial names; this is the case, for example, of vicilin and legumin from *P. sativum* and *V. faba*, of β-conglutin and α-conglutin from *Lupinus albus*, of conglycinin and glycinin from *Glycine max*, and of conarachin and arachin from *Arachis hypogaea*. In the particular case of mature dry vetch seeds, the vicilin-type and legumin-type globulins are found in protein bodies, which are present not only in the cotyledons, but also in the radicle, axis, and shoot (*19*). Recent immunochemical studies revealed that the vicilin-type mobilization precedes the legumin-type mobilization, with the vicilin type representing the initial source of amino acids for early growth and differentiation processes. Presumably, the legumin type serves as a bulk amino acid source for subsequent seedling growth during postgermination globulin degradation (*19*, *21*). These studies employed anti-legumin and anti-vicilin antibodies raised against proteins from *V. faba* seeds or, in the case of vicilin, also against recombinant legumin B1 from *V. sativa*.

Nevertheless, both the vicilin type and legumin type have already been purified from *V. sativa* seeds to be used as substrates for the vetch seed proteinases (*15*, *18*, *22*, *23*). However, to our knowledge, these proteins have not yet been characterized. A detailed characterization of these vicilin-type and legumin-type globulins is therefore lacking, as well as of the other protein components of *V. sativa* seeds.

In this work, a study was conducted on the analysis and properties of the protein fraction from mature vetch seeds. The major globulins (termed α- and β-vicinins) as well as two minor globulins were extensively characterized.

MATERIALS AND METHODS

Plant Material. Dry, mature seeds of common vetch (*V. sativa* L.), var. *caia*, were obtained from a local market. The integuments and embryos were removed. and the dry cotyledons were ground to a fine powder using an electric mill (0.2 mm sieve). The resulting meal was used as the source of proteins in all the experiments.

Protein Sequential Fractionation Based on Solubility Criteria. The meal was defatted with *n*-hexane (34 mL/g of flour) for 4 h with agitation and air-dried after decantation of the hexane. Albumins, globulins, prolamins, and glutelins from *V. sativa* were subsequently sequentially extracted and purified using appropriate extraction solutions. The albumins were extracted by stirring the powder 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/g of dry mass) (*24*). 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 ethylenediaminetetraacetic acid (EDTA), and 10 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) (34 mL/g of dry mass), and the suspension was 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-contaminations, as shown for a considerable number of legume seeds (*24*, *25*). 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 mass), and the prolamins were extracted for 2 h at 4 °C with agitation. After centrifugation at 30000*g* and 4 °C for 15 min, 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 mass). The suspension was stirred at room temperature (to keep SDS in soluble form) for 2 h and centrifuged at 30000*g* and 20 °C for 15 min. All samples were kept at −70 °C until use.

Isolation of Total Globulins. Total globulins from *V. sativa* seeds were extracted essentially as described before for *L. albus* (*26*). The flour obtained after the dry cotyledons were milled was defatted with *n*-hexane (34 mL/g of dry mass) 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 mass) 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 mass) 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 mass), and desalted on PD-10 columns previously equilibrated in the same buffer. All operations were performed at 4 °C.

Purification of Individual Globulins. Total globulins were fractionated by FPLC anion-exchange chromatography on a Q-Sepharose (8 mL bed volume) column (Pharmacia/Amersham) as described before for *L. albus* conglutins (*26*). The column was equilibrated in Tris−HCl buffer, pH 7.5, 50 mM. Three 2 mL aliquots were loaded into the column. The flow rate was 1.5 mL/min, and the bound proteins were eluted with a linear gradient (0−1 M) of NaCl. Fractions 1 mL in volume were collected.

Isopycnic Sucrose Density Gradient Ultracentrifugation and Determination of Sedimentation Coefficients. The sucrose gradients were prepared in ultraclear 38 mL centrifuge tubes and were composed of 20% (4.85 mL), 17.5% (4.85 mL), 15% (4.85 mL), 12.5% (4.85 mL), 10% (4.85 mL), 7.5% (4.85 mL), and 5% (4.85 mL) sucrose (w/v) made up in phosphate buffer, pH 7.6, 35 mM, containing 400 mM NaCl and 10 mM β-mercaptoethanol (*28*). The gradients were prepared, loaded, ultracentrifuged, and fractionated as described before (*26*). Sedimentation coefficients of protein fractions were also estimated as reported previously (*26*).

Electrophoresis, Western Blotting, and Affinoblotting. A discontinuous buffer system (*29*) 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 previously described (*30*). 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.

When appropriate, protein samples were analyzed by two-dimensional electrophoresis. γ-Vicinin (100 μg) was subjected to ND-PAGE (first dimension) and subsequently transferred onto a membrane and visualized by staining with Ponceau S as described above. The membrane band containing γ-vicinin was sliced, incubated for 15 min at 60 °C, with agitation, in Tris−HCl buffer, pH 6.8, 0.2 M, containing 1% (w/v) agarose, 2% (w/v) SDS, and 65 mM dithiothreitol and placed on top of an SDS−PAGE gel (second dimension). The well containing the membrane was filled with Tris−HCl buffer, pH 6.8, 20 mM, containing 1% (w/v) agarose. After electrophoresis, the total polypeptides were stained with Coomassie Brillant Blue R-250.

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 (*31*). 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 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 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_2O_2 (30% (v/v)). After specific detection of the glycosylated polypeptides present, the membrane was washed with water and immediately photographed.

N-Terminal Polypeptide Sequencing. N-Terminal sequencing was performed as described previously (*27*).

Hemagglutination Assays. Blood (5 mL) was collected from New Zealand female rabbits, and the erythrocytes were subsequently sedimented by centrifugation at 3000*g* for 10 min at room temperature. The erythrocytes were washed (40 mL per 5 mL of initial blood) three times with 0.9% (v/v) saline (water containing 150 mM NaCl, pH adjusted to 6.0), resuspended in the same solution (120 mL), and trypsinized by treatment with trypsin (1 mg/mL) for 1 h at 37 °C with agitation. The trypsinized erythrocytes were then pelleted, washed three times, and resuspended as described above, to give a 4% (v/v) suspension of trypsinized rabbit erythrocytes.

Agglutination tests were performed in microtiter U-plates with serial dilutions (1:3) of the vetch protein solution with 0.9% saline. Hemagglutination was observed visually, after incubation at 37 °C for 1 h of 70 μL of 0.9% saline, plus 70 mL of the 4% suspension of trypsinized erythrocytes and 70 mL of the protein solution. A negative control (140 mL of 0.9% saline plus 70 mL of the 4% suspension of trypsinized erythrocytes) and a positive control (70 mL of 0.9% saline, plus 70 mL of the 4% suspension of trypsinized erythrocytes and 70 mL of a concanavalin A solution (500 mg/mL in 0.9% saline)) were always included in each microtiter plate.

Sugar specificity was determined by incubation of 0.9% saline and the vetch protein sample with various sugar concentrations at 37 °C for 1 h, followed by incubation of the sugar−protein mixture with the erythrocytes at 37 °C for 1 h. One hemagglutination unit (HU) is defined as the lowest concentration (μg/mL of the final mixture) of the lectin that originates total agglutination.

General Assays. Protein was determined according to a modification of the Lowry method (*32*), using bovine serum albumin as the standard. Total seed nitrogen was measured by the method of Kjeldahl, using a Kjeltec system 1030 (Tecator, Sweden), following the instructions of the manufacturer.

Table 1. Protein Content of *V. sativa* Seeds

total seed protein	
mg of protein/g of fr mass	114.1
protein N/total N (%)	41.6
albumins	
mg of protein/g of fr mass	49.7
albumin N/total N (%)	18.1
globulins	
mg of protein/g of fr mass	58.0
globulin N/total N (%)	21.2
prolamins	
mg of protein/g of fr mass	0.5
prolamin N/total N (%)	0.2
glutelins	
mg of protein/g of fr mass	5.9
globulin N/total N (%)	2.2

RESULTS AND DISCUSSION

The first approach to study the proteins present in *V. sativa* seeds was to extract sequentially these polymers with appropriate solutions, following the classification proposed by Osborne (*33*) 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 procedure to initiate the characterization of the seed storage proteins from a species that has not been studied in detail (*34*), not only for comparative purposes but also as an initial purification step.

Cotyledons from *V. sativa* seeds were subjected to a sequential extraction procedure, to analyze by R-SDS−PAGE the polypeptide composition of the albumin, globulin, prolamin, and glutelin fractions. In addition, the total seed nitrogen was determined by the Kjeldahl method, and the total albumins, globulins, prolamins and glutelins were quantified by a modification of the Lowry technique, as described under Materials and Methods. The 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 nonprotein nitrogenous compounds. Because a portion of the total nitrogen measured originates from alkaloids (*35*), Gross and Baer (*36*) proposed a factor of 5.7 for legume proteins and Pompei and Lucisano (*37*) 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 seed fresh mass. The results of these experiments are presented in **Table 1** and in **Figure 1**. Several features deserve a brief comment. (i) After the total seed nitrogen is estimated as 43.85 mg/g of fresh mass, the total seed protein (114.1 mg/g of fresh mass) comprises 41.6% of the total seed nitrogen (**Table 1**). (ii) The total protein constitutes ∼11.4% of the seed fresh mass. (iii) The fractions containing total albumins (**Figure 1**, lane 1), total globulins (**Figure 1**, lane 2), and total prolamins (**Figure 1**, lane 4) are composed of distinct sets of polypeptides. The albumins are composed of

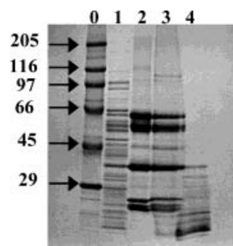

Figure 1. Polypeptide patterns of albumin (lane 1), globulin (lane 2), glutelin (lane 3), and prolamin (lane 4) fractions from *V. sativa* cotyledons. The protein fractions were sequentially extracted as described in the Materials and Methods and the resulting samples analyzed by R-SDS–PAGE. Lane 0: molecular mass markers (kDa). A 50 μg sample of protein was loaded in each lane.

Table 2. Hemagglutination Activity in Extracts from *V. sativa* Seeds

fraction	hemagglutination activity (HU),[a] μg/mL
total seed extract	1.2
albumin fraction	2.22
globulin fraction	17.6
δ-vicinin	0.137

[a] One HU is defined as the lowest concentration (μg/mL of the final mixture) of the lectin that originates total agglutination of rabbit erytrocytes (see Materials and Methods).

many different polypeptides covering a wide range of molecular masses (14−116 kDa), whereas the globulins are essentially represented by five major polypeptides (21, 23, 36, 55, and 66 kDa). Prolamins exhibit low molecular masses, ranging from 14 to 36 kDa. However, the polypeptide composition of the glutelin fraction (**Figure 1**, lane 3) is almost identical to that of globulins, suggesting that a small proportion of these proteins may have been coextracted with the glutelin fraction. (iv) Globulins compose the major protein component in *V. sativa* seeds (50.8%; this value is most certainly underestimated; see above), whereas the albumins are also abundant, reaching 43.6% of the total seed proteins. Prolamins (0.4% of the seed protein) and glutelins (5.2% of the seed protein; this value is most certainly largely overestimated; see above) represent minor protein fractions in vetch seeds.

Hemagglutination activity was detected in several *V. sativa* seed extracts (**Table 2**). Thus, the total seed extract and the

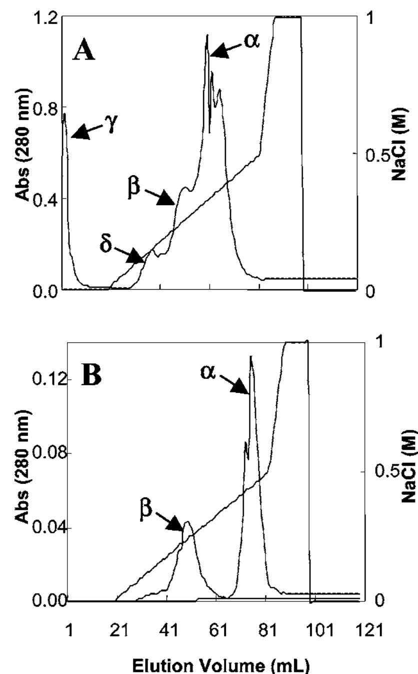

Figure 2. Fractionation of the total globulin fraction from *V. sativa* seeds by FPLC anion-exchange chromatography. Total globulins were fractionated by anion-exchange chromatography at pH 7.5 into four major components, termed α-, β-, γ-, and δ-vicinins (**A**). Due to cross-contamination between the main components, the fractions enriched in α- and β-vicinins were pooled and further subjected to two consecutive anion-exchange chromatographies performed at pH 8.0 and 8.5 (**B**). In each case, the bound proteins were eluted with a linear (0–1 M) gradient of NaCl.

albumin fraction exhibit considerable hemagglutination activities, whereas the globulin fraction possesses a lower activity. The fraction with the highest hemagglutination activity corresponds to a minor, specific globulin component (δ-vicinin; see below). The major globulin components (see below) either do not possess hemagglutination activity (α-vicinin) or exhibit reduced but varying levels of activity (β-vicinin), most probably reflecting contamination with δ-vicinin (**Figure 2**).

A number of sugars were tested for their inhibitory effect upon hemagglutination. With the exception of *N*-acetylglucosamine for the total extract and of sucrose for the total extract

Table 3. Inhibition of Hemagglutination by Sugars[a]

	albumin fraction		total extract	
sugar	lowest inhibitory sugar concn	highest concn of protein inhibited, μg/mL	lowest inhibitory sugar concn	highest concn of protein inhibited, μg/mL
glucose			0.1 M	32
glucosamine			0.1 M	32
N-acetylglucosamine			33 mM	32
galactose				
galactosamine				
N-acetylgalactosamine				
lactose				
mannose	0.1 M	59	33 mM	32
raffinose				
fucose				
melezitose	3.7 mM	59	11 mM	32
methyl α-glucopyranoside	0.1 M	59	0.1 M	32
methyl α-mannoside	33 mM	59	33 mM	32
sucrose				
maltose	0.1 M	59		

[a] One HU is defined as the lowest concentration (μg/mL of the final mixture) of the lectin that originates total agglutination of rabbit erytrocytes (see the Materials and Methods).

and the albumin fraction, the sugars that do not inhibit hemagglutination activity (**Table 3**) are in good agreement with those reported in the literature (*9*). With respect to those sugars that inhibit hemagglutination, a higher number of sugars were found for the total extract than for the albumins (**Table 3**), suggesting the presence of lectins with distinct sugar specificities in the albumin and globulin fractions. Considering those sugars that inhibit hemagglutination in both the total extract and the albumin fraction (i.e., mannose, melezitose, methyl α-glucopyranoside, and methyl α-mannoside), melezitose is the most potent (**Table 3**).

Four main protein peaks are obtained when the total globulin fraction from *V. sativa* seeds is fractionated at pH 7.5 by FPLC anion-exchange chromatography (**Figure 2A**). Following a terminology similar to that used to designate the major globulins from *L. albus* (α-, β-, and γ-conglutins) and *Lathyrus sativus* (α-, β-, and γ-lathyrins) (*27*), we propose the terms α-vicinin (peak α in **Figure 2A**), β-vicinin (peak β in **Figure 2A**), γ-vicinin (peak γ in **Figure 2A**), and δ-vicinin (peak δ in **Figure 2A**). However, the electrophoretic analysis of all fractions eluting from the anion-exchange column (**Figure 2A**) reveals that α- and β-vicinins are eluted from the column in an overlapping manner, resulting in cross-contamination in the fractions collected, whereas the γ-vicinin fraction contains several polypeptide bands, suggesting the presence of more than one protein. For these reasons, the fractions enriched in α- or β- vicinin in **Figure 2A** were pooled and further subjected to two consecutive FPLC anion-exchange chromatographies performed under the same conditions described for **Figure 2A**, except that the column was equilibrated with buffer of increasing pH. In other words, the column was equilibrated with buffer at pH 7.5 during the first run (**Figure 2A**), at pH 8.0 during the second run (chromatogram not show), and at pH 8.5 during the third run (**Figure 2B**). The outcome of this experiment, illustrated in **Figure 2B**, indicates that α- and β-vicinins are now purified, free of cross-contaminations.

The peak corresponding to γ-vicinin was collected from the anion-exchange column (pH 7.5; **Figure 2A**), cleaned by passage through a diethylaminoethylcellulose (DE-52) column (equilibrated in Tris−HCl buffer, pH 7.5, 50 mM), desalted, lyophylized, resuspended in Tris−HCl buffer, pH 9.0, 50 mM, and resubjected to FPLC anion-exchange chromatography performed at pH 9.0. The unbound protein fraction was finally subjected to affinity chromatography in a concanavalin A−Sepharose column previously equilibrated in Tris−HCl buffer, pH 7.4, 20 mM, containing 0.5 M NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$. γ-Vicinin was eluted with 0.3 M methyl α-D-glucopyranoside.

Purified α-, β-, γ-, and δ-vicinins were subsequently analyzed by ND-PAGE (**Figures 3A** and **4A**), by NR-SDS−PAGE (**Figures 3B** and **4B**), and by R-SDS−PAGE (**Figures 3C**, **4B** and **5A**). The proteins were also subjected to isopycnic sucrose density gradient ultracentrifugation (to determine their sedimentation coefficients) and to affinoblotting (to detect their glycosidic character) (**Figures 4C** and **5B**). It should be noted that the methodology employed to detect glycopolypeptides (the concanavalin A/peroxidase method proposed by Faye and Chrispeels (*31*); see the Materials and Methods) recognizes only high-mannose-type glycoproteins, since concanavalin A specifically binds to molecules which contain α-D-mannopyranosyl, α-D-glucopyranosyl, and sterically related residues. In other words, glycoproteins exist that are not detected by this method. Concanavalin A has, nevertheless, a rather broad binding specificity, being able to bind to most glycoproteins. On the other hand, previous studies have shown that mannose residues appear to be present in all glycosylated globulin subunits.

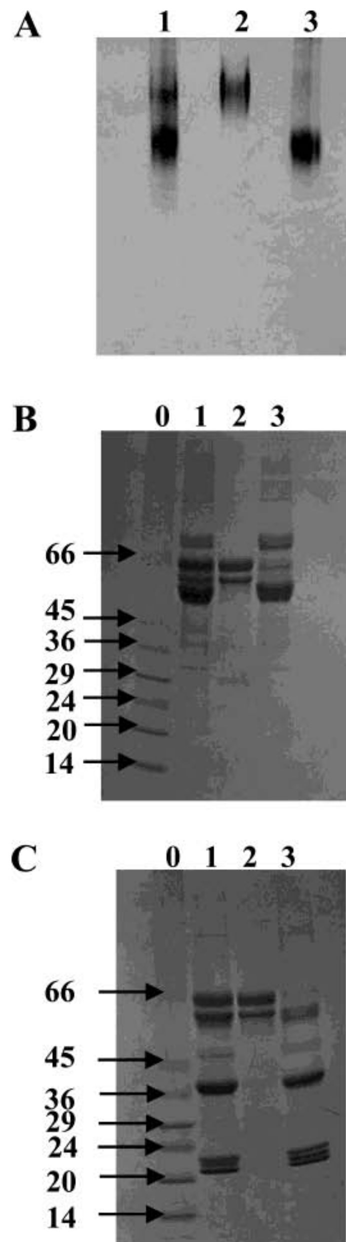

Figure 3. Analysis of the total globulin fraction (lane 1), β-vicinin (lane 2), and α-vicinin (lane 3) by ND-PAGE (**A**), NR-SDS–PAGE (**B**), and R-SDS–PAGE (**C**). Lane 0: molecular mass markers (kDa). The protein mass loaded in each lane was 150 μg (**A**, lane 1), 100 mg (**A**, lanes 2 and 3), or 50 mg (**B** and **C**).

To double check the degree of purity of α-, β-, and γ-vicinins, and to detect the presence of possible cross-contaminations, the purified proteins were analyzed by two-dimensional electrophoresis; i.e., they were subjected to native electrophoresis (ND-PAGE), transferred onto a membrane, stained with Ponceau S, sliced, and fractionated by R-SDS−PAGE as illustrated in **Figure 6**. In all cases, the polypeptide patterns obtained after 2-D electrophoresis are identical to those obtained by simple R-SDS−PAGE (**Figures 3C** and **4B**).

α-Vicinin, a legumin-like globulin, comprising 73% of the total globulin fraction (and hence >37% of the total seed protein), is by far the most abundant protein component in vetch seeds. This is in agreement with the data reported for *P. sativum* (pea), *V. faba* (broad bean), and *Vicia cracca* (tufted vetch), in

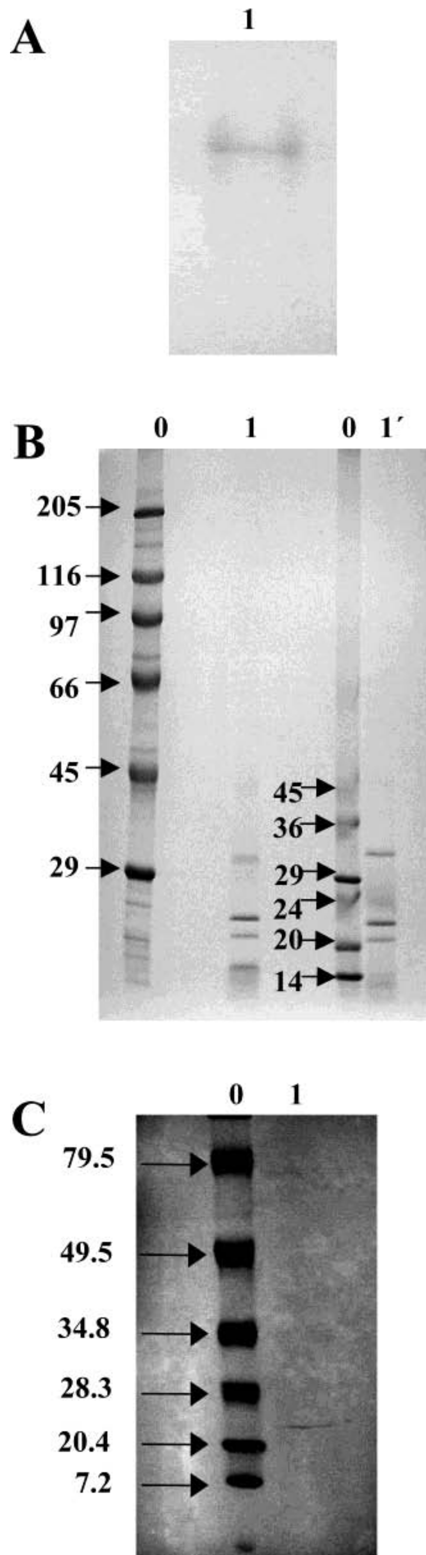

Figure 4. Electrophoretic analysis of γ-vicinin (lanes 1 and 1′) performed under native conditions (ND-PAGE, **A**) nonreducing, denaturing conditions (NR-SDS–PAGE, **B**, lane 1), and reducing, denaturing conditions (R-SDS–PAGE, **B**, lane 1′). (**C**) Affinoblotting of γ-vicinin for detection of glycosylated polypeptides. Lane 0: molecular mass markers (kDa). A 50 mg (**A**, **B**) or 30 mg (**C**) sample of protein was loaded in each lane.

which legumin is the predominant storage globulin, but contrasts with those for *G. max* (soybean) and *L. albus* (white lupin), in which vicilin is more abundant than legumin (*38*, *39*). At pH 7.5 the protein displays a strong negative charge, being eluted from the anion-exchange column between 0.28 and 0.42 M NaCl (**Figure 2A**). For this reason, it exhibits the highest mobility when analyzed by native PAGE (**Figure 3A**, lane 3). With a sedimentation coefficient of 10.6 S, it is composed of a group of subunits ranging in molecular mass from 50 to 78 kDa (**Figure 3B**, lane 3). Upon reduction, each of these subunits is split into a heavier polypeptide chain (34−66 kDa) and a lighter polypeptide chain (21−23 kDa) (**Figure 3C**, lane 3), indicating the presence of disulfide bonds. The polypeptide pattern obtained under reducing conditions (**Figure 3C**, lane 3) is essentially the same as that observed for the pure protein used as a protein substrate for vetch seed proteinases (*15*, *18*). α-Vicinin is not glycosylated (result not shown). The structure of α-vicinin resembles that reported for the legumin-like protein of many other legume seeds, including *Lupinus mutabilis* (*30*) and *L. sativus* (*27*).

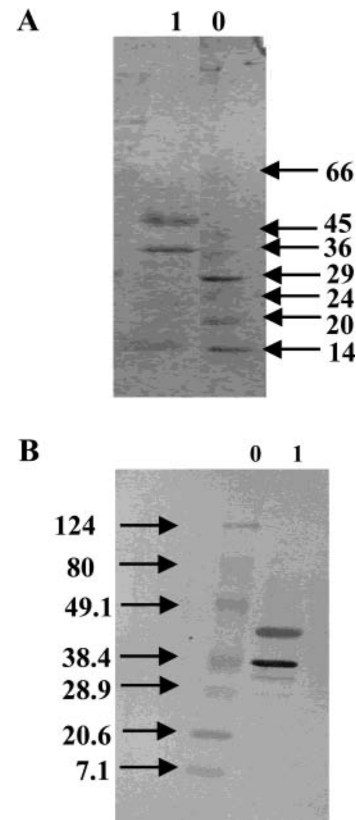

Figure 5. Electrophoretic analysis of δ-vicinin (lane 1) performed under reducing, denaturing conditions (R-SDS–PAGE, **A**). (**B**) Affinoblotting of δ-vicinin (lane 1) for detection of glycosylated polypeptides. Lane 0: molecular mass markers (kDa). A 30 mg sample of protein was loaded in each lane.

β-Vicinin, a vicilin-like globulin, exhibits an intermediate negative charge at pH 7.5 and is eluted from the anion-exchange column between 0.17 and 0.27 M NaCl (**Figure 2A**). When it is analyzed by native PAGE, its mobility is intermediate between those of α- and γ-vicinins (**Figure 3A**, lane 2). With a sedimentation coefficient of 7.7 S, the protein is composed of two major polypeptides, with molecular masses of 58 and 66 kDa, without disulfide bonds (**Figure 3B,C**, lane 2). However, unlike most other vicilins, β-vicinin is not glycosylated (result not shown). The polypeptide pattern obtained for this protein (**Figure 3B,C**, lane 2) is essentially the same as that observed for the pure protein used as a protein substrate for vetch seed proteinases (*15*, *18*, *23*).

Besides α-vicinin (the 11S or legumin-like protein) and β-vicinin (the 7S or vicilin-like protein), two other storage

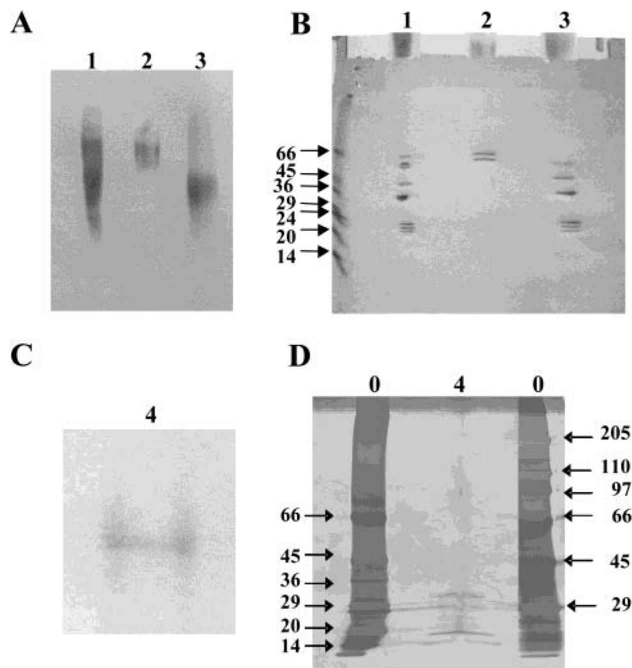

Figure 6. Two-dimensional electrophoresis of total globulins (lane 1, **A** and **B**), β-vicinin (lane 2, **A** and **B**), α-vicinin (lane 3, **A** and **B**), and γ-vicinin (lane 4, **C** and **D**) to confirm their polypeptide composition. The proteins were subjected to ND-PAGE, transferred onto a membrane, and stained with Ponceau S (**A** and **C**). The protein bands were sliced, subjected to R-SDS–PAGE, and stained with Coomassie Brilliant Blue (**B**) or subjected to silver staining (**D**). Lane 0: molecular mass markers (kDa). A 150 mg sample of protein was loaded in lane 1, and 50 mg in lanes 2–4 (**A** and **C**).

globulins were detected and characterized in vetch seeds: γ- and δ-vicinins. γ-Vicinin does not bind to the anion exchanger at pH 7.5, thus exhibiting the lowest mobility when subjected to native PAGE (**Figure 4A**). It is composed of six major subunits, with molecular masses of 14, 21, 22, 26, 31, and 43 kDa (**Figure 4B**, lane 1). Upon reduction, a slightly different pattern of polypeptides is obtained (12, 19, 21, 22, 23, and 31 kDa) (**Figure 4B**, lane 1′), revealing the existence of disulfide bonds. γ-Vicinin is glycosylated (**Figure 4C**), an observation that allowed the use of concanavalin A−Sepharose affinity chromatography in its purification.

δ-Vicinin displays a weak negative charge at pH 7.5, eluting from the anion exchanger between 0.08 and 0.16 M NaCl (**Figure 2A**). The electrophoretic analysis of this protein revealed the presence of a major 47 kDa polypeptide and two minor polypeptides (33 and 38 kDa) (**Figure 5A**). The protein exhibits hemagglutination activity (see above), suggesting that it may be a lectin. Because a protein corresponding to δ-vicinin has not been identified in most legume seeds, the sequence of amino acid residues was determined for its N-terminal. The sequence obtained for the 13 N-terminal residues was

Lys-Ser-Gln-Ser-His-Pro-Pro-Lys-Pro-Asn-Leu-Leu-Val

A search through the database Swissprot (www.ebi.ac.uk/servicestmp/.5321.html) revealed relatively low homologies with other known proteins. The highest homologies were found for a heat shock transcription factor from *Xenopus laevis* (African clawed frog) (match 61.5%; eight matches, two conservative, three mismatches), the B4 protein (a histone H1-like protein) from *X. laevis* (match 53.8%; seven matches, four conservative, two mismatches), and the hypothetical protein MG369 homologue from *Mycoplasma pneumoniae* (match 53.8%; seven matches, three conservative, three mismatches).

ABBREVIATIONS USED

FPLC, fast protein, peptide, and polynucleotide liquid chromatography; fr mass, fresh mass; HU, hemagglutination unit; SDS, sodium dodecyl sulfate.

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