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Characterization of the Proteins from *Vigna unguiculata* Seeds

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The proteins from *Vigna unguiculata* (L.) Walp. (cowpea) seeds were investigated. Globulins constitute over 51% of the total seed protein, with albumins composing approximately 45%. The globulins may be fractionated by native electrophoresis or anion exchange chromatography into three main components, which were termed (in decreasing order of anodic mobility) α-vignin, β-vignin, and γ-vignin. α-Vignin, with a sedimentation coefficient of 16.5S, is a major, nonglycosylated globulin, composed of a major 80 kDa subunit, which upon reduction, produces two polypeptides (20 and 60 kDa). β-Vignin, with a sedimentation coefficient of 13S, is a major, glycosylated globulin, composed of two main polypeptides (55 and 60 kDa) with no disulfide bonds. Finally, γ-vignin, a minor globulin, is composed by one main type of subunit (22 kDa), which upon reduction, is converted into a single, apparently heavier polypeptide chain (30 kDa) due to the presence of an internal disulfide bond. Immunological analyses revealed structural homology between β-vignin and β-conglutin (the vicilin from *Lupinus* seeds) but not between α- or γ-vignins and their *Lupinus* counterparts. Haemagglutination activity toward trypsinized rabbit erythrocytes was found exclusively in the albumin fraction and was strongly inhibited by *N*-acetylglucosamine or chitin.

KEYWORDS: Cowpea; *Vigna unguiculata*; globulins; proteins; seed; storage

INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp.) is an important crop in many areas around the world, particularly in tropical and subtropical areas (*1*). It is an important legume providing a major source of dietary protein, being used for human consumption and as animal fodder (*1*, *2*). However, few studies have been published on the biochemical composition of cowpea seed protein.

Some studies have focused on the protein composition of cowpea seeds. A study on the conditions for the extraction of cowpea proteins was reported in 1979 (*3*), followed by a preliminary characterization of the water-extractable proteins (*4*). A major globulin was first identified in 1957 (*5*). This 7S or vicilin-like fraction (G-1), termed vignin, was subsequently shown to be a heterogeneous globulin of about 170 kDa (*6*, *7*). Vicilin from cowpea has been shown to strongly associate with chitin, chitosan, and fully acetylated chitin (*8*). For this reason, this protein has been shown to bind to fungal structures, interfering with the germination of spores or conidia from a variety of fungi (*9*) and inhibiting yeast growth (*10*, *11*), and to chitinous structures of the midgut of the cowpea weevil (*Callosobruchus maculatus*), thus interfering with the insect development (*12*−*14*). Antimicrobial peptides (*15*), pathogenesis related (PR) proteins that inhibit the growth of fungi and insects (*16*), and proteins with antiviral and antifungal potency (*17*) have also been detected in cowpea seeds.

In addition, cowpeas, like most other grain legumes, contain anti-nutritional factors, such as trypsin inhibitors and lectins, that decrease protein digestibility and reduce protein quality (*18*, *19*). A lectin that interacts with several saccharides but lacks blood group specificity was isolated from cowpeas (*20*).

Globulins are the major protein component in the storage tissues of legume cotyledons (*21*). In the particular case of *V. unguiculata*, most of the seed protein (72% of the extractable protein from mature seeds, according to Murray et al. (*22*)) is found in the globulin fraction. Therefore, this fraction, as the major seed protein component in cowpea, is also responsible for the nutritional value of the seed (*19*).

In the present work, the protein composition of *Vigna unguiculata* cotyledons was analyzed. Total globulins were fractionated and the major individual globulins, termed α-, β-, and γ-vignins, were purified and thoroughly characterized.

MATERIALS AND METHODS

Plant Material. Dry seeds of cowpea (*Vigna unguiculata* (L.) Walp.) and white lupin (*Lupinus albus* L.) were obtained from a local market.

Total Protein Fractionation Based on Solubility Criteria. Albumins, globulins, prolamins and glutelins from *Vigna unguiculata* cotyledons were sequentially extracted and purified using appropriate extraction solutions (*23*), as described before (*24*).

Isolation of Total Globulins. Total globulins from *V. unguiculata* or *L. albus* seeds were extracted essentially as described before (*25*).

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The dry cotyledons were milled (0.2 mm sieve) and the resulting meal was deffated 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 30 000*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 30 000*g*, and the globulins were precipitated by the addition of ammonium sulfate (561 g/L). The precipitated globulins were centrifuged at 30 000*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-Vignin, β-vignin, and γ-vignin were fractionated and purified from the *V. unguiculata* total globulin fraction by FPLC anion-exchange chromatography on Q-Sepharose columns, as described before (*25*).

α-Conglutin, β-conglutin, and γ-conglutin were fractionated and purified from the *L. albus* total globulin fraction by an identical procedure. The light (γ_L) and heavy (γ_H) polypeptide chains of γ-conglutin and the nonglycosylated 19 kDa polypeptide of α-conglutin were subsequently isolated following preparative SDS-PAGE of the corresponding native proteins.

Production of Polyclonal Antibodies. Polyclonal antibodies were produced in rabbits as described before (*26*) against α-vignin and β-vignin from *V. unguiculata*, against α-conglutin and β-conglutin from *L. albus*, and against the light (γ_L) and heavy (γ_H) polypeptide chains of γ-conglutin, and the nonglycosylated 19 kDa polypeptide of α-conglutin from *L. albus*. The native proteins were used in soluble form, whereas the polypeptides were used in polyacrylamide bands sliced from the SDS-PAGE gels. Samples containing the purified antigens (200 μg) were mixed with an equal volume of Freund's complete adjuvant (1 mL final volume) and injected subcutaneously into female New Zealand rabbits. To obtain a higher 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 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, 17.5, 15, 12.5, 10, 7.5, and 5% sucrose (w/v) (4.85 mL each) made up in phosphate buffer, pH 7.6, 35 mM, containing 400 mM NaCl and 10 mM β-mercaptoethanol (*27*). The gradients were prepared, loaded, and ultracentrifuged as described before (*25*). Sedimentation coefficients of protein fractions were also estimated as reported previously (*25*).

Electrophoresis, Western Blotting, Affinoblotting, and Immunoblotting. A discontinuous buffer system (*28*) 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. (*29*). 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). The presence of disulfide bonds in the storage proteins was determined by two-dimensional electrophoresis using a method based on the lateral diffusion of 2-mercaptoethanol during electrophoresis (*30*).

Proteins separated by R-SDS-PAGE were blotted onto a nitrocellulose (NC) membrane (previously soaked for 15 min in transfer buffer: 39 mM Tris, 48 mM glycine, 0.1% (w/v) SDS, 20% (v/v) methanol, pH 8.3) at 15 V for 1 h 15 min using a semidry transfer unit (BIO-RAD). After protein transfer, the polypeptides in the membrane were fixed for 5 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 15 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 NC 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 $\mu g \cdot mL^{-1}$ 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 $\mu g \cdot mL^{-1}$ 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_2O_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 an NC membrane were subjected to immunoblotting. The blots were probed with anti-α-conglutin (5000-fold diluted), anti-β-conglutin (5000-fold diluted), anti-γ_L (1500-fold diluted), anti-γ_H (1500-fold diluted), anti-nonglycosylated, 19 kDa polypeptide of α-conglutin (1500-fold diluted), anti-α-vignin (1500-fold diluted), or anti-β-vignin (1500-fold diluted) antibodies and processed as described by Ramos et al. (*32*).

General Assays. Protein was determined according to a modification of the Lowry method (*33*, *34*) using bovine serum albumin as the standard.

Haemagglutination assays were performed in U-shaped bottom microtiter plates, with trypsinized rabbit erythrocytes, essentially as described before (*35*).

RESULTS AND DISCUSSION

When characterizing the total seed protein from a poorly studied species, the first logical experiment to do is to perform a sequential extraction of proteins according to their solubility characteristics, as defined by Shewry and collaborators (*23*) (i.e., albumins, globulins, prolamins and glutelins). When such an experiment was performed for cowpea (*Vigna unguiculata* seeds), the results illustrated in **Figure 1A** were obtained, indicating that globulins (composing 51% of the total seed protein) and albumins (composing 45% of the total seed protein) constitute the vast majority of the seed protein. The gel presented in **Figure 1B** shows the polypeptide pattern of each of the four solubility classes of proteins. The polypeptide pattern obtained for the glutelin fraction (lane 4) parallels that achieved for the globulin fraction (lane 2), suggesting some degree of inefficiency during the extraction of globulins. This observation implies that the proportion of glutelins given in **Figure 1A** is overestimated, whereas the proportion of globulins is underestimated. The large proportion of albumins (45%) found in this work for cowpea seeds contrasts with a report found in the literature claiming that globulins compose 72% of the extractable protein from mature seeds (*22*). In addition, the albumin fraction usually composes 12−17% of the total proteins in a number of legume seeds (*36*, *37*), although higher values (30−40%) have also been reported for plants from the genera *Pisum* and *Acacia* (*38*). Nevertheless, it is important to note that an improved methodology that was developed in our laboratory was employed in this work to extract the albumins and globulins from legume seeds (*37*). This method is based on the observation that globulins are typically insoluble because calcium and/or magnesium ions

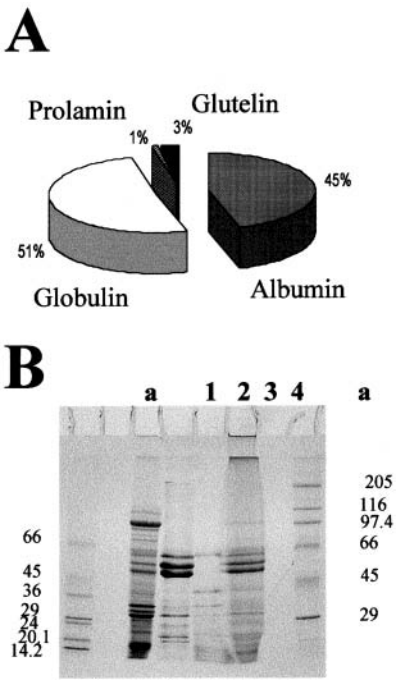

Figure 1. (**A**) Total protein composition of *Vigna unguiculata* cotyledons. (**B**) Polypeptide patterns of each of the solubility classes of proteins present in *V. unguiculata* seeds. The protein fractions were sequentially extracted as described under Materials and Methods and the resulting samples analyzed by R-SDS-PAGE (50 μg of protein were loaded in each lane). Lane a: molecular mass markers (kDa). Lanes 1, 2, 3, 4: albumin, globulin, prolamin, and glutelin fractions, respectively.

(positively charged) form electrostatic bridges between negatively charged globulin molecules, leading to a self-aggregation of the protein molecules, which results in the formation of large and insoluble macromolecular aggregates (*25*, *39*). Therefore, the improved procedure involves extraction of the albumins in water containing Ca^{2+} + Mg^{2+} (to insolubilize the globulins) and the subsequent extraction of the globulins in high ionic strength buffer containing EDTA + EGTA. This methodology, developed for *Lupinus* seeds but tested for a number of other legume seeds, allows the complete solubilization of albumins, free of contaminating globulins (*25*). For example, these authors found that incubation of the hexane-treated flour in water (the traditional way to extract albumins) solubilized 76% of *Lupinus albus* seed protein, but this value decreased to 13% when calcium and magnesium were included.

Fractionation of the total globulin fraction from *V. unguiculata* cotyledons by FPLC anion exchange chromatography at pH 7.5 resulted in three major A_{280} peaks, as illustrated in the chromatogram presented in **Figure 2**. Each of these protein peaks was subsequently analyzed by ND-PAGE (**Figure 3A**), NR-SDS-PAGE (**Figure 3B**) and R-SDS-PAGE (**Figure 3C**).

The term vignin has been used by several authors to designate the major, 7S globulin from *V. unguiculata* seeds (*7*, *22*, *40*). The terminology utilized to designate the three main globulins from some legume seeds (such as γ-, β-, and α-conglutins in the case of *Lupinus albus* (*41*−*43*) or γ-, β-, and α-lathyrins in the case of *Lathyrus sativus* (*24*)) is based on their increased mobility toward the anode observed when these globulins are subjected to paper electrophoresis on cellulose acetate or nondenaturing PAGE, or on their elution sequence from an anion-exchange column. In a similar way, the three major *Vigna* globulins that elute sequentially from the anion-exchange column (peaks γ, β, and α in **Figure 2**) exhibit increased

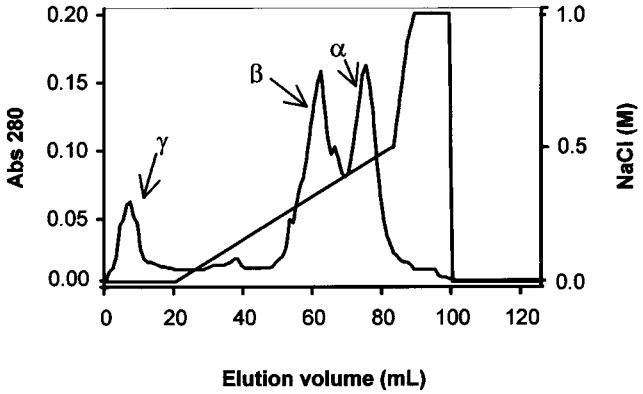

Figure 2. Fractionation of the total globulin fraction from *Vigna unguiculata* cotyledons by FPLC anion exchange chromatography. Total globulins were extracted, isolated as described in Materials and Methods, and loaded on a Q-Sepharose column, previously equilibrated in 20 mM Tris-HCl buffer, pH 7.5. The bound proteins were eluted with a gradient (0–1 M) of NaCl. Peaks γ, β, and α correspond to γ-vignin, β-vignin, and α-vignin, respectively.

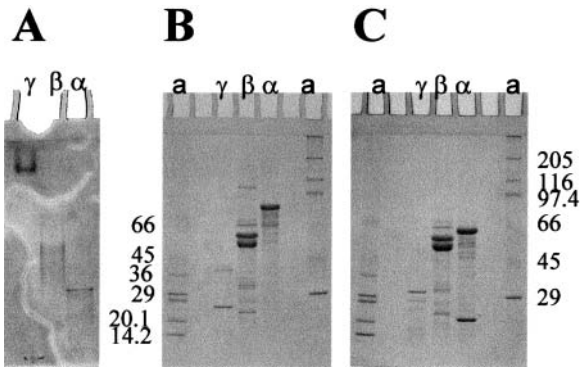

Figure 3. Electrophoretic analysis of γ-vignin (lane γ), β-vignin (lane β), and α-vignin (lane α). γ-, β-, and α-vignin were purified as shown in **Figure 2** and subsequently subjected to ND-PAGE (**A**), NR-SDS-PAGE (**B**) and R-SDS-PAGE (**C**). Protein (50 μg) was loaded in each lane. Lane a: molecular mass markers (kDa).

mobility toward the anode when analyzed by ND-PAGE (**Figure 3A**). For these reasons, we propose the designations of γ-vignin (peak γ in **Figure 2** and lane γ in **Figure 3A**), β-vignin (peak β in **Figure 2** and lane β in **Figure 3A**), and α-vignin (peak α in **Figure 2** and lane α in **Figure 3A**) for the three main *Vigna* globulins.

α-Vignin, a major *Vigna* globulin, binds in a strong manner to the anion exchanger at pH 7.5, being eluted with approximately 0.4 M NaCl (**Figure 2**). For this reason, under native conditions, the protein exhibits a high mobility toward the anode (**Figure 3A**). When analyzed by nonreducing but denaturing electrophoresis (**Figure 3B**), α-vignin is essentially composed of a major 80 kDa subunit. In addition, a number of minor and smaller subunits were also detected (44−58 kDa). Denaturing electrophoresis performed under reducing conditions (R-SDS-PAGE) revealed that the major 80 kDa subunit (and each of the minor, smaller subunits) is composed of a 60 kDa polypeptide (or a minor and smaller polypeptide) and a 20 kDa polypeptide, linked by disulfide bonds. The presence of disulfide bonds was further assessed according to a method developed by Melo et al. (*30*), which is based on the lateral diffusion of β-mercaptoethanol during electrophoresis. The result of this experiment is illustrated in **Figure 4C** and confirms the conclusions drawn from **Figure 3**. α-Vignin subunits appear to

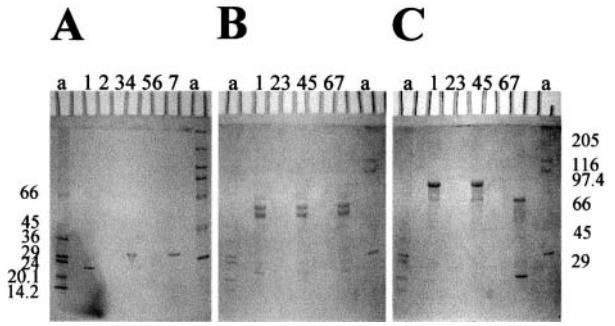

Figure 4. Detection of disulfide bonds in the subunits of γ-vignin (**A**), β-vignin (**B**), and α-vignin (**C**). Lanes 1 and 4, pure protein in sample buffer without β-mercaptoethanol (SB); lane 7, pure protein in sample buffer containing β-mercaptoethanol (SBME); lane 2, SB; lanes 3, 5, and 6, SBME; lane a, molecular mass markers (kDa). Protein (50 μg) was loaded in each lane.

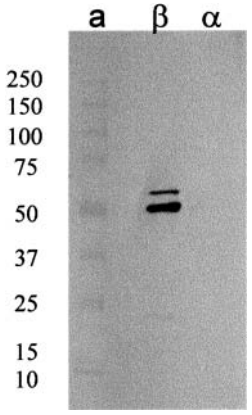

Figure 5. Detection of glycosylated polypeptides on β- and α-vignin. Samples of pure proteins were subjected to R-SDS-PAGE and the polypeptides transferred to an NC membrane and probed with concanavalin A-peroxidase for glycoprotein detection. Lane a, prestained molecular mass markers (kDa). Protein (50 μg) was loaded in each lane.

be nonglycosylated (**Figure 5**). Sucrose density gradient ultracentrifugation allowed the estimation of a sedimentation coefficient of 16.5S for α-vignin. Khan et al. (*6*) reported the presence in cowpea seeds of a globulin of 300−400 kDa, containing disulfide-linked pairs of subunits of 18.5 and 62 kDa, which most probably corresponds to α-vignin.

β-Vignin, another major *Vigna* globulin, elutes from the anion exchanger at pH 7.5 with 0.25−0.30 M NaCl (**Figure 2**) and exhibits an intermediate mobility (when compared to α- and γ-vignins) when subjected to ND-PAGE (**Figure 3A**). It is composed of two major polypeptides (55 and 60 kDa) and a number of minor polypeptides (22, 24, 30, and 66 kDa) (**Figure 3C**). Identical protein patterns were obtained by SDS-PAGE performed under reducing (**Figure 3C**) and nonreducing (**Figure 3B**) conditions, indicating the absence of disulfide bonds in β-vignin (**Figure 4B**). Two β-vignin polypeptides are glycosylated (**Figure 5**). Sucrose density gradient ultracentrifugation originated a value of 13S for the sedimentation coefficient of β-vignin. Cowpea seeds have been previously shown to contain a major heterogeneous globulin of 170 kDa (*6*); two components were separated by gel electrophoresis at pH 4.0, one predominating, containing subunits of 52 and 58 kDa, the other containing subunits of 52, 58, and 63 kDa in roughly equal amounts. This globulin corresponds most certainly to β-vignin. Contrasting with our data is the observation that the 58 and 63 kDa subunits contained covalently bound carbohydrate.

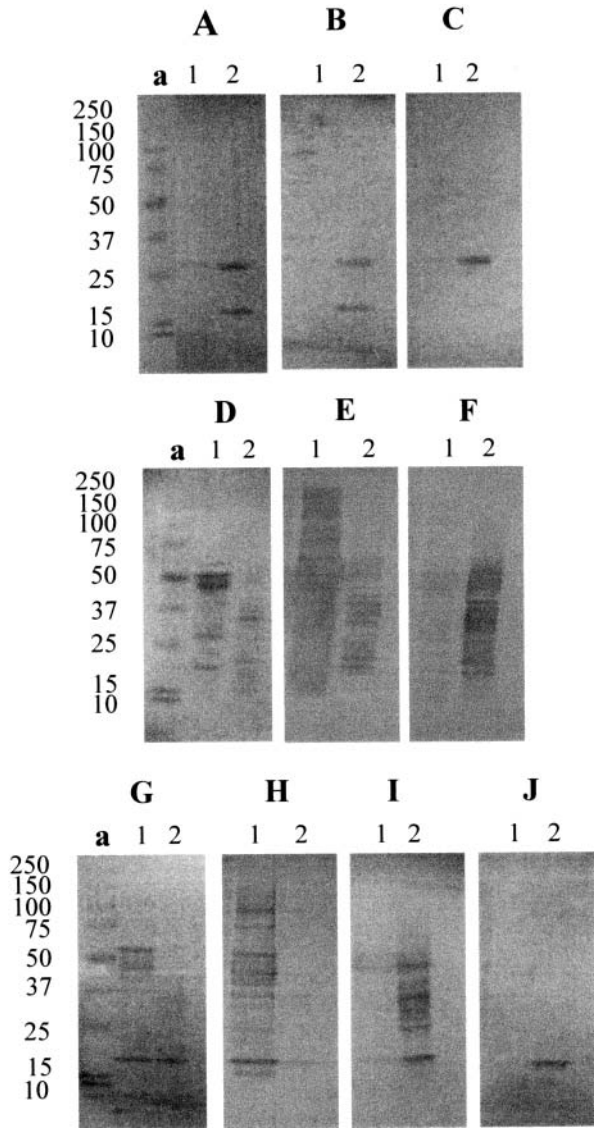

Figure 6. Structural similarity between *Vigna* α-, β-, and γ-vignins and *Lupinus* α-, β-, and γ-conglutins. γ-Vignin (lane 1 in **A**, **B**, and **C**), γ-conglutin (lane 2 in **A**, **B**, and **C**), β-vignin (lane 1 in **D**, **E**, and **F**), β-conglutin (lane 2 in **D**, **E**, and **F**), α-vignin (lane 1 in **G**, **H**, **I**, and **J**), and α-conglutin (lane 2 in **G**, **H**, **I**, and **J**) were subjected to R-SDS-PAGE, blotted onto a membrane, and either stained for total protein with Ponceau S (**A**, **D**, and **G**) or probed with anti-light polypeptide chain of γ-conglutin (**B**), anti-heavy polypeptide chain of γ-conglutin (**C**), anti-β-vignin (**E**), anti-β-conglutin (**F**), anti-α-vignin (**H**), anti-α-conglutin (**I**) or anti-nonglycosylated, 19 kDa polypeptide of α-conglutin antibodies. Lane a, prestained molecular mass markers (kDa). Protein (50 μg) was loaded in each lane.

γ-Vignin, a minor *Vigna* globulin, does not bind to the anion exchanger at pH 7.5, because it does not possess a net negative charge at this pH (**Figure 2**). For this reason, it exhibits a very low mobility when subjected to electrophoresis performed under native conditions (**Figure 3A**). It is composed by a major type of subunit (22 kDa, **Figure 3B**), which upon reduction, originates a single and apparently heavier polypeptide (**Figures 3C** and **4A**). This apparent inconsistency can be explained by the presence in the 22 kDa subunit of an internal (or intrapolypeptide) disulfide bond.

The considerable structural similarity detected between *Lupinus* conglutins (*30*) and *Vigna* vignins in subunit and polypeptide composition, and the 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 vignins. To this end, polyclonal antibodies were produced specifically to α- and β-conglutins, to α- and β-vignins, to the heavy and light polypeptide chains of γ-conglutin, and to the nonglycosylated, 19 kDa polypeptide of α-conglutin. These antibodies were utilized to probe α-, β-, and γ-vignins and α- and β-conglutins, previously blotted onto an appropriate membrane. The results obtained, presented in **Figure 6**, show that the vicilins (i.e., β-vignin and β-conglutin) are the only proteins to show some degree of structural homology. In fact, anti-β-vignin antibodies do recognize β-conglutin (lane 2 in **Figure 6E**), whereas anti-β-conglutin antibodies originate a weak signal when utilized to probe β-vignin (lane 1 in **Figure 6F**). No cross-reactivity was observed for γ-vignin/γ-conglutin (**Figure 6**, parts **B** and **C**) and only a very weak signal was obtained when anti-α-conglutin antibodies were used to probe α-vignin (lane 1 in **Figure 6I**).

Like most other legume seeds, a *V. unguiculata* total seed extract exhibits a strong haemagglutination activity toward trypsinized rabbit erythrocytes (results not shown). This activity was found exclusively in the albumin fraction, with the purified total globulin fraction, α-, β-, or γ-vignins were incapable of agglutinating erythrocytes. *N*-acetylglucosamine and chitin strongly inhibit the haemagglutination of trypsinized rabbit erythrocytes by the albumin fraction (data not shown). A 55 kDa lectin without blood group specificity was isolated from cowpea (*20*). This lectin was reported to interact with several saccharides, including galactose, glucosamine, methyl-galactopyranoside, methyl-glucopyranoside, and methyl-mannopyranoside.

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