

# Purification and Characterization of *Griffonia simplicifolia* Leaf Lectins<sup>1</sup>

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## ABSTRACT

Leaves from mature *Griffonia simplicifolia* plants were examined for the presence of leaf lectins possessing sugar binding specificities similar to the four known seed lectins (GS-I, GS-II, GS-III, GS-IV). Three (GS-I, -II, -IV) of the four known *G. simplicifolia* seed lectins were present in the leaves. Leaf *G. simplicifolia* lectins I and IV were similar to the respective seed lectins. Leaf GS-II, however, was composed of two types of subunits ( $M_r = 33,000$  and  $19,000$ ), whereas the seed lectin consists of only one type of subunit ( $M_r 32,500$ ). Seed and leaf GS-II lectins also had different isoelectric points. All leaf and seed lectins were similar with respect to their hemagglutination and glycoconjugate precipitation properties and all subunits contained covalently bound carbohydrate. Leaf GS-IV appeared slightly under-glycosylated compared to seed GS-IV.

The fate of GS-I and GS-II seed lectins in aging cotyledons was investigated. GS-I isolectins usually contain isolectin subtypes associated with each main isolectin. Upon imbibition and germination, these GS-I isolectin subtypes disappeared. Over time, GS-II lectin did not change its disc gel electrophoretic properties.

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*Griffonia simplicifolia* (previously called *Bandeiraea simplicifolia*; 17) is a perennial woody shrub which grows in the tropical rain forests of West Africa to a height of 3 m (13, 14). The seeds of this leguminous plant contain four distinct lectins (GS<sup>5</sup>-I, GS-II, GS-III, GS-IV) each with a different sugar binding specificity (9, 10).

GS-I consists of five tetrameric isolectins (A<sub>4</sub>, A<sub>3</sub>B, A<sub>2</sub>B<sub>2</sub>, AB<sub>3</sub>, B<sub>4</sub>). A<sub>4</sub> and B<sub>4</sub> exhibit a primary sugar specificity for  $\alpha$ -D-GalNAc and  $\alpha$ -D-Gal, respectively, while A<sub>3</sub>B, A<sub>2</sub>B<sub>2</sub>, and AB<sub>3</sub> possess both binding activities. GS-II is a tetrameric protein composed of a single type of subunit (4, 25) and is specific for GlcNAc residues. GS-III and GS-IV, both composed of two types of subunits, have *N*-acetyl-D-galactosamine and Le<sup>b</sup> (Lewis b) sugar binding activ-

ities, respectively.

Our main objective in this study was to isolate *G. simplicifolia* leaf lectins and compare their properties with those of the seed lectins. We felt this was worthwhile inasmuch as so few leaf lectins have been isolated and characterized (*cf.* 28, 30). The leaf and seed lectins were compared on the basis of their sugar binding specificity, subunit mol wt, immunological cross reactivity, agglutination of human erythrocytes, and precipitation of glycoconjugates.

A second objective was to study the fate of two of the seed lectins upon seed imbibition and germination.

## MATERIALS AND METHODS

Fresh seeds were collected by Dr. Len Newton, University of Science and Technology, Kumasi, Ghana.

L-Ascorbic acid-sodium salt, phenylmethyl sulfonyl fluoride, Sepharose 4B, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl were purchased from Sigma Chemical Co. Amino acid standard H and SDS, sequanal grade, were purchased from Pierce Chemical Co. All sugars were purchased from Pfanstiehl Laboratories, Inc. *p*-Nitrophenyl  $\beta$ -D-galactopyranoside was purchased from Vega Biochemicals. All other materials were reagent grade or the best quality available.

**Growing Conditions and Care of *Griffonia simplicifolia* Plants.** *G. simplicifolia* plants were grown at the University of Michigan Matthaei Botanical Gardens. After germination in flats or small pots on a heated bench under 40-w cool-light bulbs, the seedlings were transplanted to clay pots or trays containing Metro mix:leaf mold (3:1, v/v). The plants were grown under a bank of cool-white high-output fluorescent and 36-w incandescent lights. The pH of the water was lowered with phosphoric acid (4.4 ml/gallon) to enhance uptake of iron. Peters 20:20:20 (N:P:K) fertilizer (200 ppm) was applied weekly. 'Sequestered' iron was applied periodically as needed. Various pesticides (Omite, Plictran, Pirimor, Sumethrin, dilute solution of 'Ivory' detergent) were applied as needed, usually at biweekly intervals. To enhance germination, the seeds (obtained directly from Ghana) were soaked in H<sub>2</sub>O for approximately 10 min to loosen the seed coat which was then carefully peeled off. The peeled seeds were planted (half submerged) in soil.

**Purification of Lectins.** The method of Delmotte and Goldstein (3) was employed for the purification of *G. simplicifolia* seed lectins (GS-I, GS-II). A *p*-aminobenzyl-1-thio-*N*-acetyl- $\beta$ -D-glucosaminide-succinylaminohexylaminyl Sepharose 4B (GS-II) affinity column and a *p*-aminophenyl- $\beta$ -D-galactopyranosyl-succinylaminohexylaminyl Sepharose 4B (GS-I) affinity column, for the isolation of leaf lectins, were prepared according to the method of Delmotte and Goldstein (3). Seed and leaf GS-IV were isolated on a Le<sup>b</sup>-glass bead affinity column (Chembiomed, Alberta, Canada). The GS-IV lectin was eluted with 0.1 *N* acetic acid. A GS-III affinity column was prepared by coupling the activated ester of CH-Sepharose 4B with the amino group of galactosamine in a 0.1 M

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<sup>5</sup> Abbreviations: GS, *Griffonia simplicifolia*; GalNAc, 2-acetamido-2-deoxy-galactose; GlcNAc, 2-acetamido-2-deoxy-glucose; PMSF, phenylmethyl sulfonyl fluoride; PBS, 0.1 M sodium phosphate, 0.15 M NaCl, 0.1 mM CaCl<sub>2</sub>, 6 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4; PAGE, polyacrylamide gel electrophoresis; CRM, cross-reacting material.

NaHCO<sub>3</sub> buffer (pH 8.0) for 2 h. Pure seed GS-III was eluted from this affinity matrix with 18 mM D-GalNAc.

**Isolation and Identification of *G. simplicifolia* Leaf Lectins.** *G. simplicifolia* isolectins were extracted from leaves following the method described by Talbot and Etzler (30). Leaves were cut from the plants growing at Matthaei Botanical Gardens and weighed. The leaves were washed with double-distilled H<sub>2</sub>O and cut into small pieces with a pair of scissors. The extraction buffer (0–4°C) containing 0.1 M phosphate (pH 7.2), 0.15 M isoascorbic acid, 1 mM DTT, and 1 mM PMSF was added and the leaves homogenized in small portions in a Waring Blendor (approximately 6 × 30-s intervals). The homogenate was squeezed through three layers of cheesecloth into a cold graduated cylinder. The leaf fiber was reextracted and again squeezed through cheesecloth. Following centrifugation (10,000g, 30 min, 0–4°C, all operations were conducted at 4°C unless otherwise indicated), 40% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to the supernatant and the pH adjusted to 7.2 with NaOH. After stirring for 1 h, the solution was centrifuged (10,000g, 30 min) and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> slowly added to the supernatant to achieve 80% saturation. The pH was adjusted to 7.2 with NaOH. After stirring for approximately 12 h, the solution was centrifuged (10,000g, 30 min) and the pellet was gently resuspended in extraction buffer and dialyzed extensively against PBS.

The solubilized dialyzed pellet (P-80) was applied to a GS-I affinity column. The unbound eluate was applied directly to a GS-II affinity column. This procedure was repeated using a GS-IV and finally a GS-III affinity column. Leaf GS-I isolectins were eluted with 50 mM methyl  $\alpha$ -D-Galp, leaf GS-II was eluted with 2 mM GlcNAc, leaf GS-IV was eluted with 0.1 M acetic acid, and GS-III was eluted with 18 mM D-GalNAc. The leaf lectins were dialyzed against PBS, and characterized by hemagglutination, glycoconjugate precipitation, and, where possible, precipitin analysis with rabbit antisera. SDS-PAGE was used to compare the leaf and seed lectin subunits.

**Extraction of *G. simplicifolia* Lectins from Cotyledon Segments.** The same procedure used by Murphy and Goldstein (20) to extract GS-I isolectins from single seeds was employed with the following modification.

A small piece of cotyledon was removed with a razor blade (without disturbing the embryo) from a single dry *G. simplicifolia* seed and the seed planted. Once germination had begun, additional sections were excised from the cotyledons. The dry-seed segments or the cotyledonary segments were individually extracted by grinding in acetone (–20°C) with a mortar and pestle. The acetone suspension was centrifuged (10,000g, 30 min, 3°C) and the resulting pellet reextracted with methanol (30 min). After a second centrifugation, the pellet was resuspended in PBS and extracted for 3 h. After centrifuging a third time, the supernatant solution was filtered and loaded onto a melibionate Bio-Gel P-300 affinity column (0.7 × 10 cm) (12, 20). The column was washed with PBS until the *A* at 280 nm was <0.01. The five GS-I isolectins were eluted with PBS containing methyl  $\alpha$ -D-Galp (23.5 mM). The unbound eluate from the GS-I affinity column was passed over a GS-II affinity column. Pure GS-II from dry-seed segments or cotyledonary segments was eluted with 2 mM GlcNAc.

**Analysis of Lectins by PAGE.** Nondenaturing pH 8.9 and pH 4.3 PAGE (2) was used to analyze GS-I and GS-II lectins, respectively. The gels (8 × 0.5 cm) were prerun for 30 min at 2.5 mamp/gel. Electrophoresis of the samples was continued at 2.5 mamp/gel for 3 h in Tris/glycine buffer (pH 8.9) or  $\beta$ -alanine/acetate buffer (pH 5.0). SDS-PAGE in the presence and absence of 2-mercaptoethanol was performed on a 9- × 0.5-cm disc and on 15-cm × 9-cm × 1.5-mm slab gels as described by Laemmli (16).

**Production of Antiserum.** Antibodies to GS I-B<sub>4</sub> were raised by injecting each of two New Zealand white rabbits with approxi-

mately 138  $\mu$ g of pure B<sub>4</sub> emulsified (VirTis high-speed homogenizer) in complete Freund's adjuvant (Difco Laboratories). One ml of emulsion, in 0.1-ml aliquots, was injected subcutaneously at multiple sites along the rabbit's back. This protocol was repeated after 3 weeks and again 3 months later. Ten d following the last injection, approximately 50 ml of blood was drawn from the medial auricular artery into a vacutainer tube. Tubes were rimmed with a wooden applicator stick (which was subsequently left in the tube) and the blood was allowed to clot overnight at 3°C. The clot adhering to the stick was removed and the serum was centrifuged at low speed. The clear antiserum was centrifuged at low speed and stored at –20°C, or partially purified by repeated precipitation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (0.25 g/ml) followed by dialysis against PBS. Antisera against crude *G. simplicifolia* seed extract, purified GS I-A<sub>4</sub>, and seed GS-II had been prepared previously using a similar protocol.

**Immunodiffusion and Immunoelectrophoresis.** Immunodiffusion was performed in agarose filled Petri dishes as described by So and Goldstein (26). A protocol from the Gelman Instrument Company manual was employed for immunoelectrophoresis in a Gelman Instrument Company apparatus (Ann Arbor, MI). Samples were placed in the wells and subjected to electrophoresis for 2 h at 3 mamp slide. The agarose from the troughs was removed and the troughs filled with antisera. Immunodiffusion was allowed to proceed for 16 to 24 h (room temperature) in a humid atmosphere.

**Amino Acid Composition Analysis.** Purified lectins were dialyzed against 1 M HCl and lyophilized. Hydrolysis was performed in 6 N HCl *in vacuo*, under N<sub>2</sub> at 110°C. Hydrolyzed samples were analyzed on a Beckman 120°C amino acid analyzer. Cysteine residues were converted to cysteic acid by hydrolysis in the presence of dimethyl sulfoxide (27). Tryptophan was determined according to the method of Edelhoch (5). Amino acid composition was determined using a computer program written by Shaun Black according to the method of Hoy *et al.* (13). Calculations were performed using a Challenger II computer (Ohio Scientific Instruments) and a Tektronics 4051 terminal.

**Hemagglutination Assay.** The hemagglutination assay was performed as described by Hayes (11). A serial dilution of lectin with PBS (0.01 M K-phosphate, 0.15 M NaCl) was made using a 25- $\mu$ l microdiluter and a hemagglutination plate (Cooke Laboratory Products, Alexandria, VA). The respective human erythrocytes were washed several times with PBS (0.01 M K-phosphate, 0.15 M NaCl) and diluted to 2%. A drop of the 2% suspension was added to diluted lectin and the microtiter plate covered with parafilm to prevent desiccation. After 1 h at room temperature, the titer was expressed as the reciprocal of the highest dilution showing detectable agglutination.

**Capillary Ring Test.** Lectins were tested for their ability to precipitate glycoconjugates (29) by adding, by capillary action, a solution of glycoconjugate followed by a lectin solution, to a microhematocrit tube (Dade, Miami, FL). The microhematocrit tube was sealed with critoseal (Lancer, St. Louis, MO) and left for 1 h or more before observing under high intensity light for the presence of a precipitate at the interface between the two solutions. The specificity of the reaction was tested by adding a few crystals of the appropriate sugar to the microhematocrit tube and observing the dissipation of the precipitate.

**Protein and Neutral Sugar Assay.** An extinction coefficient of 1.41 cm<sup>-1</sup> · (mg/ml)<sup>-1</sup> was used to convert the GS-I *A* at 280 nm to protein concentration (12). Protein was also determined by the method described by Lowry *et al.* (18) using BSA as standard. Covalently bound carbohydrate was detected in SDS-polyacrylamide gels by the method of Racusen (23).

## RESULTS

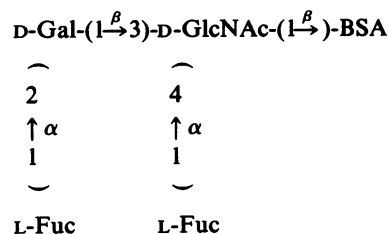
**Isolation of *G. simplicifolia* Leaf Lectins.** Three lectins were isolated from leaves of this plant by extraction with buffer con-

taining a protease inhibitor (PMSF) and a sulfhydryl protecting agent (DTT) (30). Triton X-100 was initially used in a Hepes buffer in the belief that the lectins might be membrane bound (1). However, the detergent-containing buffer failed to extract any lectins.

Leaf GS-I isolectins were purified on a *p*-aminophenyl  $\beta$ -D-galactoside affinity column and eluted specifically with 50 mM methyl  $\alpha$ -D-Galp. Leaf GS-II lectin was eluted from a *p*-aminobenzyl-1-thio-*N*-acetyl- $\beta$ -D-glucosaminide-Sepharose column with 2 mM D-GlcNAc. Unbound crude seed extract from the two aforementioned columns was passed over a Le<sup>b</sup>-glass bead affinity column and eluted with 0.1 N acetic acid. After passage over a GS-I, GS-II, and GS-IV affinity column, the crude leaf extract was passed over a GS-III galactosamine-Sepharose 4B affinity column in order to isolate GS-III. A small amount of material was eluted from this affinity column with 0.1 M D-Galp. This material, unlike authentic seed GS-III, did not react with a galactosamine-BSA glycoconjugate and did not exhibit the same electrophoretic mobility on SDS-polyacrylamide gels as authentic seed GS-III. No further characterization of this material was undertaken. With the exception of GS-III, the same affinity chromatography conditions used to isolate seed lectins were therefore successful in isolating three leaf lectins possessing the same sugar binding specificity as the seed lectins.

**Characterization of the *G. simplicifolia* Leaf Lectins.** The sugar binding specificity of the GS leaf lectins was inferred from their

ability to bind to, and be eluted from specific affinity matrices. Further evidence of the binding specificity was adduced from capillary precipitation tests and hemagglutination studies. Leaf GS-I isolectins formed a precipitate with the galactomannan, guaran (inhibited by D-Gal). Leaf lectin GS-II precipitated an *N,N'*-diacetylchitobioside-BSA conjugate. Leaf GS-IV precipitated only with Le<sup>b</sup>-BSA (structure shown below):



The purified leaf lectins (GS-I, -II, -IV) were examined for their ability to agglutinate human erythrocytes. Equal concentrations of seed and leaf lectins were tested in parallel. Seed lectin thus functioned as a positive control. Leaf and seed GS-I lectins agglutinated both type A and type B erythrocytes with virtually the same titer ( $\pm 1$  well). Leaf and seed GS-II agglutinated only type O T/Tk human erythrocytes, again with virtually the same titer. Similarly both seed and leaf GS-IV lectins agglutinated type O Le<sup>a-b+</sup> but not Le<sup>a-b-</sup> human erythrocytes. Thus, all three leaf and seed lectins exhibited identical hemagglutinating properties.

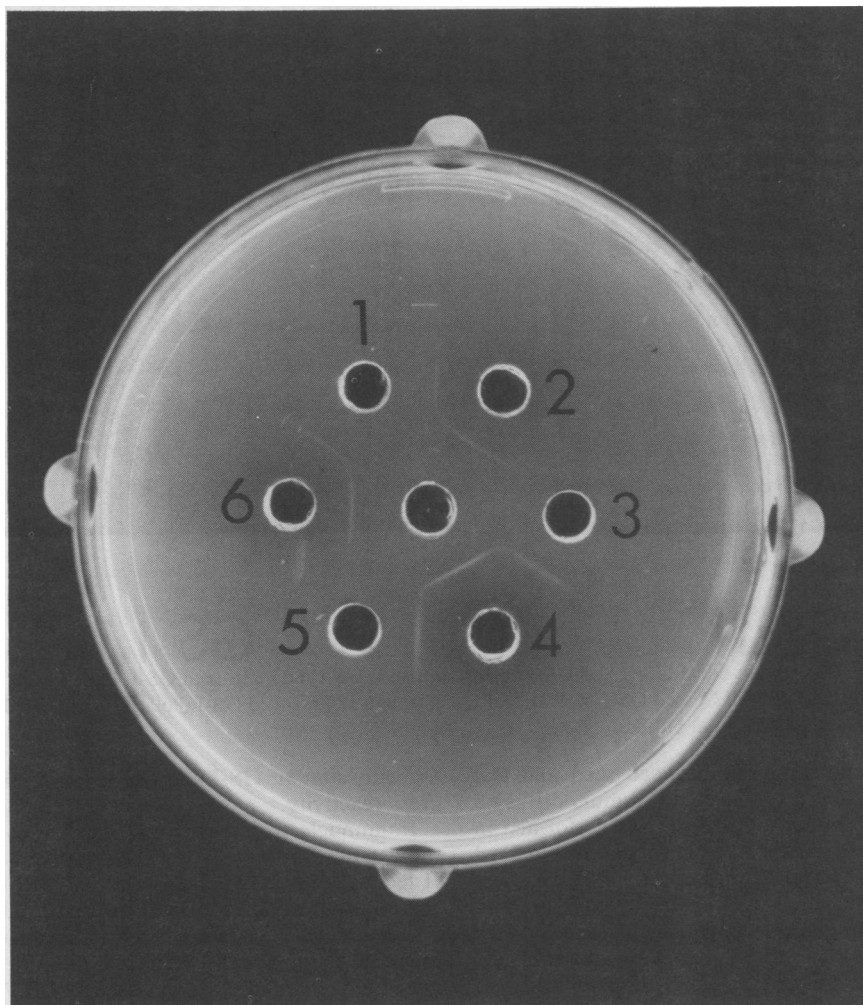


FIG. 1. Immunodiffusion in agarose of leaf and seed GS-I and GS-II against anti-seed GS-I and anti-seed GS-II. Well 1, leaf GS-I; well 2, anti-seed GS-I; well 3, leaf GS-II; well 4, anti-seed GS-II; well 5, leaf GS-II; well 6, anti-seed GS-I; central well, mixture of seed GS-II and GS-I.

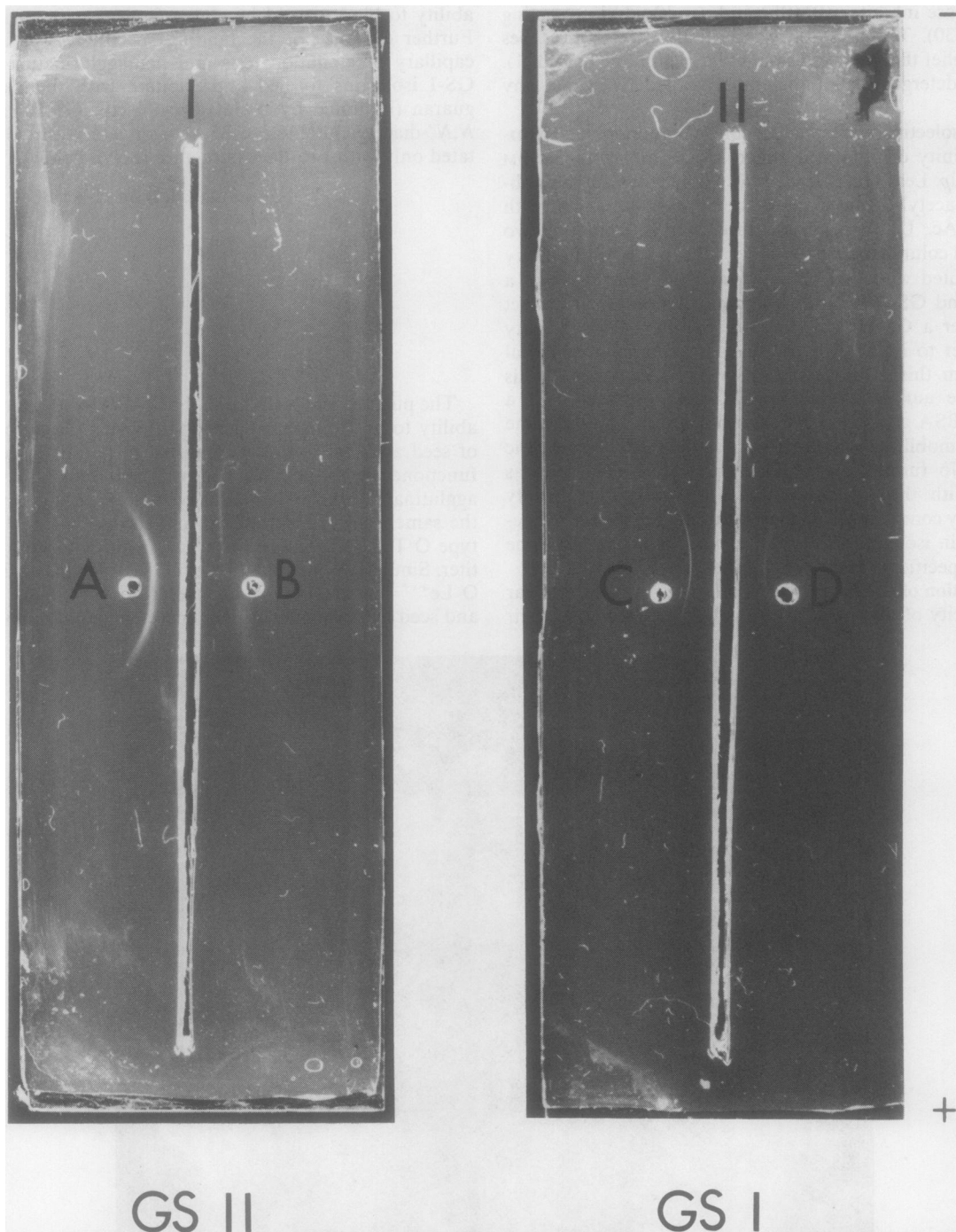


FIG. 2. Immunoelectrophoresis of seed and leaf GS-II and GS-I against anti-seed GS-II and anti-seed GS-I. Plate I: well A, seed GS-II; well B, leaf GS-II; trough I, anti-seed GS-II. Plate II: well C, seed GS-I; well D, leaf GS-I; trough II, anti-seed GS-I. Polarity is indicated at right. Glass slides were covered with agarose dissolved in pH 8.4 barbital buffer.

To determine the antigenic similarity between seed and leaf lectins, all three leaf lectins were subjected to immunodiffusion and immunoelectrophoresis. Immunodiffusion was performed in agarose using antiserum raised against a crude *G. simplicifolia* seed extract, anti-seed-GS-I mixture and anti-seed GS-II lectin. Leaf GS-IV reacted only with antisera raised against a crude extract from *G. simplicifolia* seeds and formed no lines of identity with the other leaf lectins. Leaf GS-I lectin reacted only with anti-seed GS-I and formed a line of identity with seed GS-I. Similarly, leaf and seed GS-II formed a line of identity against anti-seed GS-II. The results of the immunodiffusion analysis are shown in Figure 1. Seed and leaf GS-I and GS-II lectins were therefore immuno-

logically similar; GS-I and GS-II leaf lectins failed to exhibit any cross-reactivity.

Immunoelectrophoresis was employed to characterize further the extent of homology between the seed and leaf GS-I and GS-II lectins (Fig. 2). The seed and leaf GS-II lectins (plate I) both reacted with anti-seed GS-II antisera; but, as shown in plate I/well B, the leaf GS-II appeared to have a different mobility from the seed lectin. At pH 8.4, the leaf GS-II lectin was, therefore, less negatively charged than was seed GS-II. These data support the notion that leaf GS-II has a higher isoelectric point than seed GS-II. Inspection of plate I (Fig. 2) reveals a faint diffuse, and unidentified, precipitin arc surrounding well B. In contrast, seed

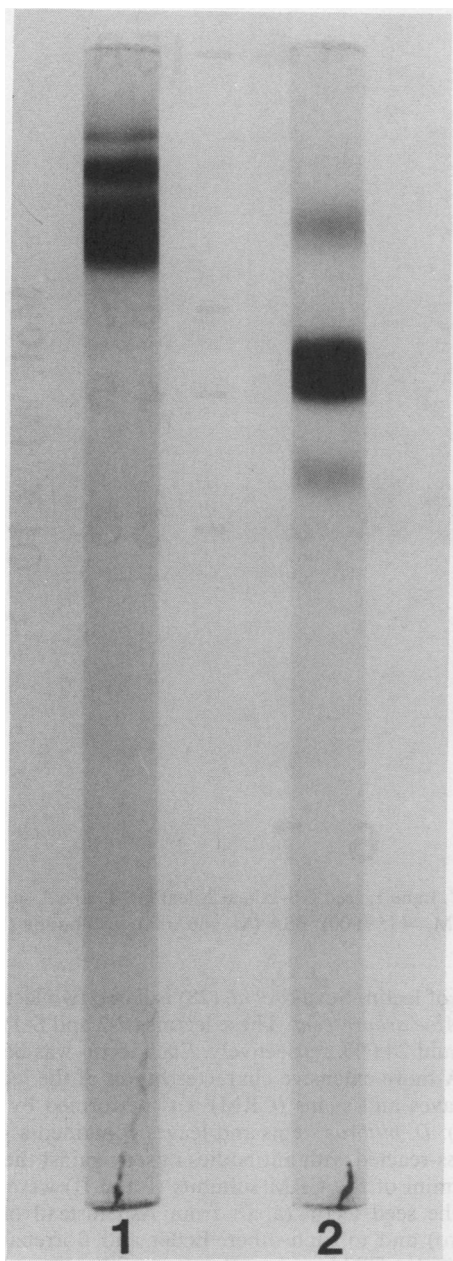


FIG. 3. pH 4.3 nondenaturing PAGE in 7.5% polyacrylamide gels of seed and leaf GS-II. Gel 1, seed GS-II; gel 2, leaf GS-II. Direction of migration is from top (+) to bottom (-).

and leaf GS-I lectins appeared to have the same mobility at pH 8.4, which suggests they have the same isoelectric point (see Fig. 2, wells C and D).

Acidic (pH 4.3) nondenaturing PAGE was performed to compare the mobility of leaf and seed GS-II (Fig. 3). The most striking observation was that the leaf GS-II (gel 2) has a higher mobility than seed GS-II (gel 1). Characteristic protein aggregates of seed GS-II were observed at the top of gel 1 but were not present in leaf GS-II (gel 2). Leaf GS-II migrated as a major band with two very faint minor bands above and below it (gel 2). This electrophoretic analysis was consistent with the results obtained by immunoelectrophoresis of seed and leaf GS-II—the leaf lectin possesses a higher positive charge than seed GS-II and thus a greater cathodic mobility. These data support the notion that leaf GS-II has a higher isoelectric point than seed GS-II.

Seed and leaf GS-I lectins were examined by pH 8.9 nondenaturing

PAGE. Although seed GS-I isolectins migrated as five distinct bands (20), leaf GS-I lectin did not form discrete bands but instead formed a smear, indicative of a very large number of satellite bands. Repeated attempts to improve the resolution of leaf GS-I lectin on pH 8.9 polyacrylamide gels were unsuccessful.

Seed and leaf GS-I, -II, -IV lectins were examined by SDS-PAGE in the presence of 2-mercaptoethanol. For ease of comparison, seed and leaf lectins were placed in adjacent lanes (Fig. 4). No difference in subunit composition was noted between GS-I and GS-IV lectins from seeds (lanes 1 and 5) and leaves (lanes 2 and 6). Seed and leaf GS-II lectins, however, were composed of subunits of a different size. Leaf GS-II lectin contained a large subunit ( $M_r$ , 33,000) and a small subunit ( $M_r$ , 19,000), whereas seed GS-II was composed of only one type of subunit ( $M_r$ , 32,500).

The presence of covalently bound carbohydrate in leaf and seed lectin subunits was determined by staining a SDS-polyacrylamide gel (similar to the 12.5% polyacrylamide gel shown in Fig. 4) with a thymol sulfuric acid reagent (23) and visualizing the glycoproteins under UV light. All bands visible in Figure 4, except for the mol wt markers aldolase (158,000 D) and BSA (67,000 D) stained for carbohydrate. The leaf GS-IV lectin did not react as intensely as the seed GS-IV lectin, which may indicate that it was underglycosylated. Both seed and leaf GS-IV lectins reacted with concanavalin A in a ring capillary test, although the leaf GS-IV lectin again reacted less vigorously than the seed lectin—indicative of a smaller amount of covalently bound carbohydrate.

Seed and leaf GS-II lectins were analyzed by SDS-PAGE in the presence and absence of 2-mercaptoethanol (Fig. 5). Leaf GS-II lectin (gel 2), in the absence of 2-mercaptoethanol, migrated as two bands ( $M_r$  = ~325,000 and 155,500, respectively); seed GS-II lectin (gel 4) under similar conditions also migrated as two bands but with higher mol wt ( $M_r$ , ~410,000 and 220,000, respectively). In the absence of 2-mercaptoethanol, therefore, both seed and leaf GS-II lectins formed multimers of their respective subunits. These data indicated that the subunits were held together by disulfide bonds.

A comparison between the amino acid composition of seed and leaf GS-II lectins is presented in Table I. Although the lectins were similar with respect to most amino acid residues, there were some notable exceptions. The number of lysine residues was significantly higher in leaf GS-II than in seed GS-II lectin. Other amino acids which were somewhat higher in the leaf GS-II lectin were Asp, Ser, Glu, Ala, Gly, and Leu. Cys and Trp were present in higher amounts in the seed GS-II. The increase in lysine content undoubtedly contributed significantly to the observed increase in the isoelectric point of leaf GS-II lectin. Leaf GS-II lectin subunit had a higher mol wt (~28,000) than seed GS-II lectin (24,000). These mol wt do not include any contribution from covalently bound carbohydrate, however. A more accurate comparison between the respective amino acid compositions will be possible when the small and large leaf GS-II lectin subunits are analyzed separately.

**G. simplicifolia Lectins (GS-I, -II) from Germinating Seeds.** The second part of this study involved a small scale developmental analysis of seed GS-I and GS-II lectins. We undertook to monitor the fate of these lectins in senescent cotyledons. One small segment (~0.7 g) was excised from each dry seed and the seeds planted. Of the 35 seeds planted, only five germinated. Additional cotyledon segments (0.1–0.4 g) were removed from each seed at approximately monthly intervals and stored at  $-80^\circ\text{C}$  for analysis. GS-I and GS-II lectins were extracted from the small segments and purified using a melibionate Bio-Gel P-300 affinity column and a D-GlcNAc affinity column, respectively. Following elution of GS-I (23.5 mM methyl  $\alpha$ -D-Galp) and GS-II (2 mM D-GlcNAc), the lectins were dialyzed against double-distilled  $\text{H}_2\text{O}$  and freeze dried. Analysis of GS-I isolectins by pH 8.9 nondenaturing PAGE (Fig. 6), revealed that the satellite bands, generally associated with



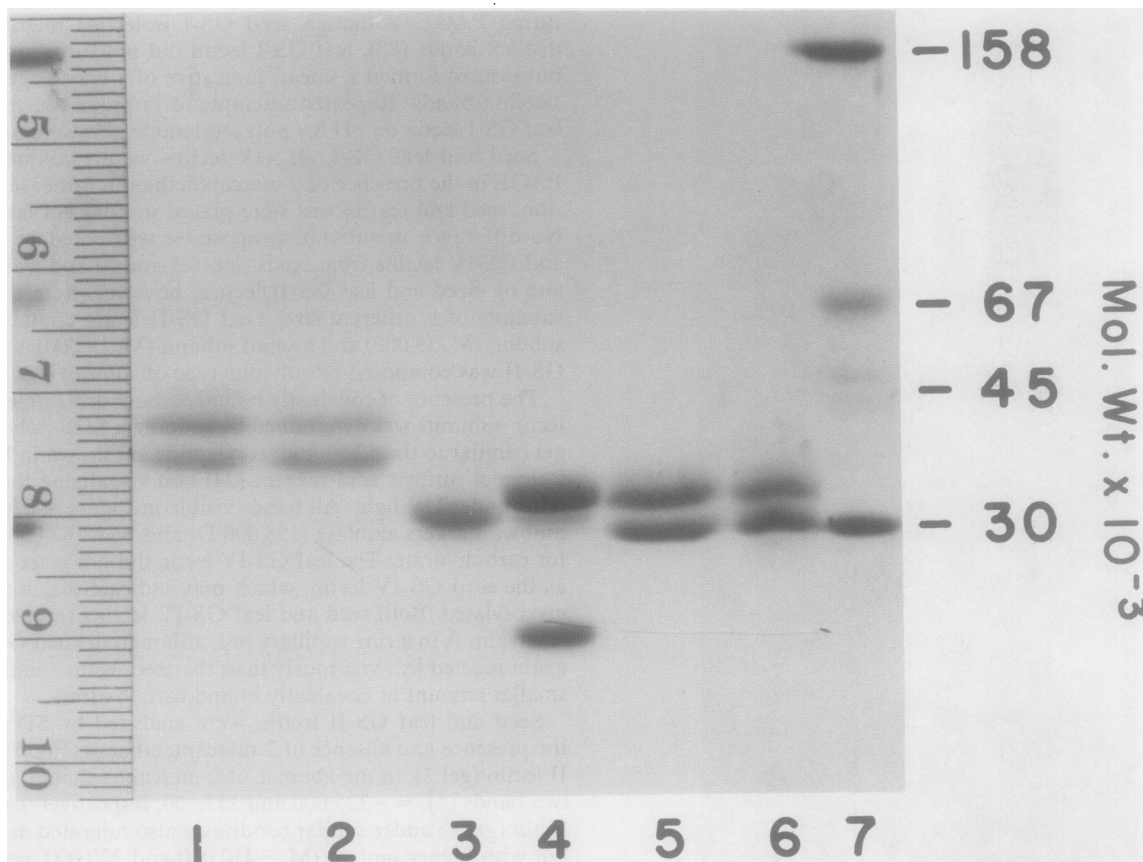


FIG. 4. SDS-PAGE in a 12.5% polyacrylamide slab gel of seed and leaf GS-I, -II, -IV. Lane 1, seed GS-I; lane 2, leaf GS-I; lane 3, seed GS-II; lane 4, leaf GS-II; lane 5, seed GS-IV; lane 6, leaf GS-IV; lane 7, mol wt markers: aldolase ( $M_r = 158,000$ ), BSA ( $M_r = 67,000$ ), ovalbumin ( $M_r = 45,000$ ), carbonic anhydrase ( $M_r = 30,000$ ).

each major isolectin band, disappeared when the seeds germinated. After 1 month (Fig. 6, gel 2), there were five distinct bands ( $A_4$ ,  $A_3B$ ,  $A_2B_2$ ,  $AB_3$ ,  $B_4$ ) and only very faint satellite bands between each major band. A comparison between gels 1 and 2 (Fig. 6) reveals the extent of the decrease in intensity and number of satellite bands. No change in this pattern was apparent 1 month later (*cf.* gels 2 and 3).

GS-II was also isolated from the cotyledon segments by affinity chromatography and analyzed by pH 4.3 PAGE. Examination of the acidic, nondenaturing polyacrylamide gels of the seed GS-II lectins (data not shown) indicated that no changes occurred in the mobility of the GS-II lectins although there was a decrease in the amount of the lower mobility aggregates which are characteristically associated with the seed GS-II lectin. Thus, any temporal changes which occurred did not alter the mobility of the GS-II seed lectin.

## DISCUSSION

A major objective of this study was to determine whether *G. simplicifolia* leaves contain lectins and, if so, whether the leaf lectins resemble the seed lectins. This endeavor was made possible when we fortuitously discovered that removing the seed coat enhanced seed germination. Having successfully grown a number of *G. simplicifolia* plants, we were able to provide answers to these questions.

To extract the leaf lectins from *G. simplicifolia*, we used a modification of the extraction buffer employed by Talbot and Etzler (30) to extract the CRM from *Dolichos biflorus* leaves and stems. Until recently, scant attention was given to the purification of leaf lectins, presumably because these plant tissues contain low

quantities of lectin. Suzuki *et al.* (28) isolated two lectins from the leaves of *Aloe arborescens*. These lectins (P-2 and S-1) had mol wt of 18,000 and 24,000, respectively. Each lectin was believed to be a dimer. A more extensive characterization of the lectin from *D. biflorus* leaves and stems (CRM) was performed by Talbot and Etzler (30). *D. biflorus* stems and leaves contained a glycoprotein which cross-reacted with antibodies raised against the seed lectin. The *N*-termini of the CRM subunits (I and II) were identical to those of the seed lectin (apart from Asn instead of an Asp at residue two) and to each other. Etzler and Borrebaeck (6) suggested that the CRM and seed lectin subunits might represent different degrees of completion or modification of a common polypeptide chain. Noteworthy, however, was the difference in carbohydrate binding specificity between CRM and the seed lectin. The binding of CRM to blood group A + H substance was inhibited by D-GalNAc and D-GlcNAc, whereas the seed lectin was inhibited solely by D-GalNAc. The CRM, however, failed to agglutinate red blood cells or bind to carbohydrates in the presence of physiological saline; one of these properties is usually deemed necessary to describe a protein as a lectin. Although lectin activity has been detected in wheat leaves (19), soybean leaves (22), phloem exudate (24), peanut leaves (1), flowers of *Datura stramonium* (15), and in sieve-tube sap of various trees (8), few if any of these lectins have been thoroughly characterized.

We found low concentrations of three lectins in *G. simplicifolia* leaves which had the same sugar binding activity as the seed lectins. The CRM from *Dolichos biflorus* leaves appears to differ somewhat in its sugar binding properties from the seed lectin (6). Two of the leaf lectins, GS-I and GS-IV, appeared to be very similar if not identical to the seed lectins. The seed and leaf GS-II lectins differed, however, with respect to subunit mol wt and

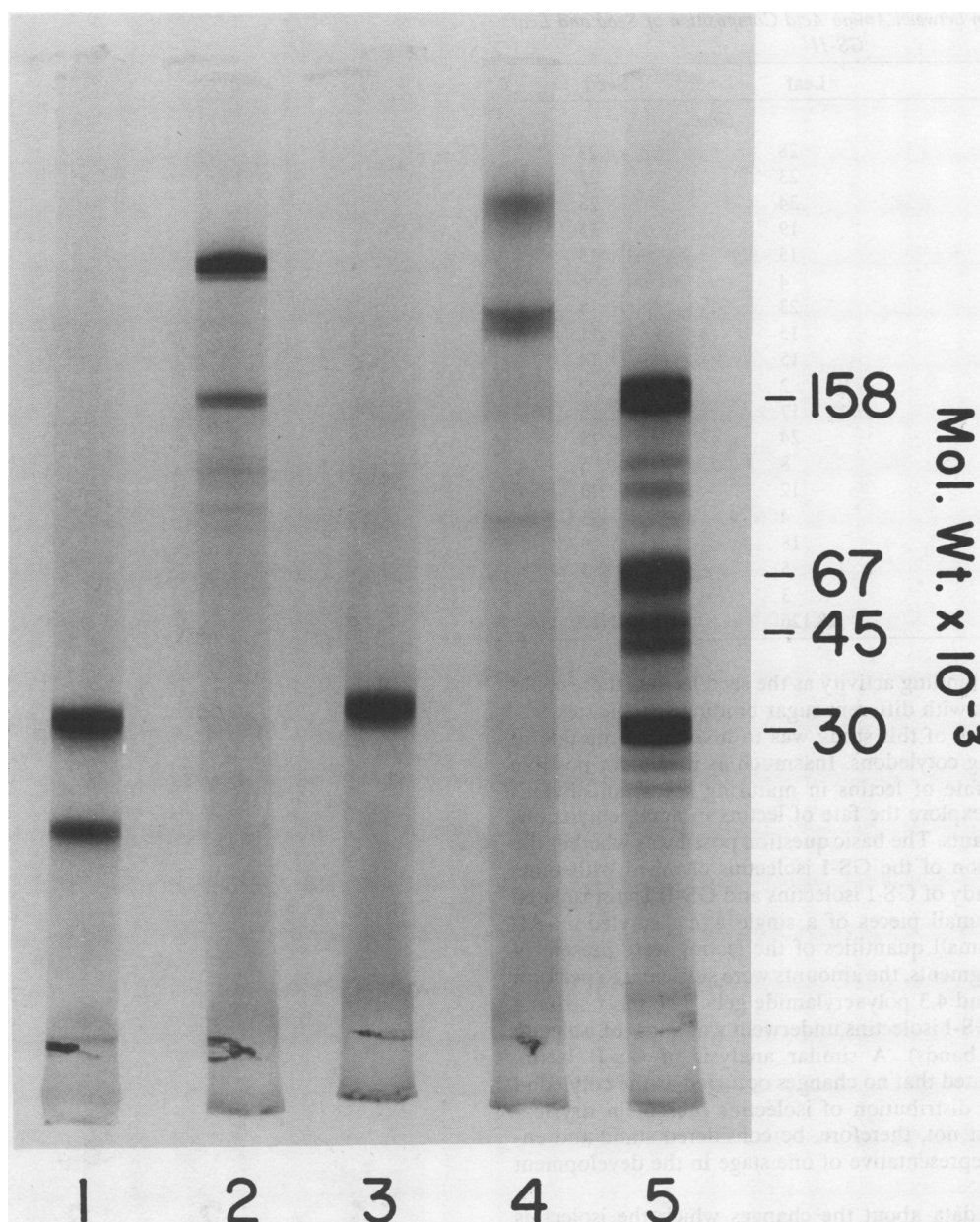


FIG. 5. SDS-PAGE in 12.5% polyacrylamide disc gels of leaf and seed GS-II in the presence and absence of 2-mercaptoethanol. Gels 1 and 2, leaf GS-II; gels 3 and 4, seed GS-II. Gels 1 and 3 contain 2-mercaptoethanol; gels 2 and 4 contain only SDS. Gel 5, mol wt markers: aldolase ( $M_r = 158,000$ ), BSA ( $M_r = 67,000$ ), ovalbumin ( $M_r = 45,000$ ), carbonic anhydrase ( $M_r = 30,000$ ). Direction of migration is from top (-) to bottom (+).

isoelectric point. Leaf GS-II was composed of one large subunit ( $M_r = 33,000$ ) and one small subunit ( $M_r = 19,000$ ), whereas seed GS-II was composed of one type of subunit ( $M_r = 32,500$ ). Both leaf and seed GS-II subunits were glycosylated. In addition, the pI of the GS-II leaf lectin was higher than that of the seed lectin. A possible reason for this observation was revealed when the amino acid compositions of the seed and leaf GS-II lectins were compared. Leaf GS-II lectin had a significantly greater number of lysine residues than the seed GS-II lectin. There were also differences in other amino acid residues which could contribute to the increase in pI of the leaf lectin.

The most interesting leaf lectin, and fortunately the one present in highest concentrations, is the GS-II lectin. Conceivably, the small seed lectin subunit is related to the larger leaf lectin subunit via some form of proteolytic modification. This suggestion has been proposed for the CRM and seed lectin of *Dolichos biflorus* (6). Perhaps the small subunit of the leaf GS-II lectin is a protein

which binds to the GS-II large subunit. Gansera *et al.* (7) reported that lectins from the seeds of pea, jack bean, *Vicia faba*, *Vicia sativa*, and castor bean contained 'protein binders'; all these proteins, however, exhibited mol wt considerably higher than the small subunit of GS-II leaf lectin.

The leaf and seed GS-II lectins were shown in this study to have similar carbohydrate binding affinities, hemagglutination properties, and cross-reactivity with seed GS-II antibodies. They appear, therefore, to be very closely related.

In conclusion, *G. simplicifolia* leaves contain three agglutinins which closely resemble three of the seed lectins. One of these leaf lectins (GS-II) appears to have the same binding specificity as the seed lectin but is composed of two types of subunits unlike seed GS-II which is composed of one type of subunit. The GS-II leaf lectin, in addition, has a higher isoelectric point than the GS-II seed lectin. A fourth lectin, present in the seeds (GS-III), was not detected in the leaves. Although we looked only for leaf aggluti-

Table I. Comparison between Amino Acid Composition of Seed and Leaf GS-II

Amino Acid	Leaf	Seed
	<i>no.</i>	
Asp	28	23
Thr	23	22
Ser	34	28
Glu	19	13
Pro	15	15
Cys	4	6
Gly	23	18
Ala	15	11
Val	15	14
Met	2	2
Ile	17	15
Leu	24	19
Tyr	8	5
Phe	12	13
His	4	3
Lys	18	9
Arg	5	3
Trp	3	6
Mol Wt	28,126	24,417

nins with the same binding activity as the seed lectins, there could be other leaf lectins with different sugar binding specificities.

A second objective of this study was to investigate the fate of seed lectins in aging cotyledons. Inasmuch as it was not possible to investigate the fate of lectins in maturing seeds, an obvious alternative was to explore the fate of lectins in aging cotyledons from developing plants. The basic question posed was whether the relative concentration of the GS-I isolectins changed with time. We undertook a study of GS-I isolectins and GS-II lectin isolated periodically from small pieces of a single aging cotyledon. Although only very small quantities of the lectins were present in the cotyledonary segments, the amounts were sufficient to perform PAGE in pH 8.9 and 4.3 polyacrylamide gels. The most striking change which the GS-I isolectins underwent was a loss of isolectin subtypes (satellite bands). A similar analysis of GS-II lectin, however, demonstrated that no changes occurred as the cotyledon aged. The familiar distribution of isolectins present in dry leguminous seeds must not, therefore, be considered static and enduring but rather representative of one stage in the development of the plant.

Some intriguing data about the changes which the isolectins undergo after the seeds imbibe water and germinate was obtained by Pueppke (22). He noted that peanut isolectins did not all decline at the same rates in aging cotyledons of growing plants. In both peanut varieties he studied (Jumbo Virginia and Spanish), the more basic isolectins decreased more rapidly relative to acidic isolectins with the concomitant appearance of new acidic bands. He suggested that the newly appearing isolectin bands were fragments of the more basic isolectins which retained binding activity, but exhibited more acidic pI values (21).

There are several plausible explanations for the loss of satellite bands. Perhaps the GS-I isolectins undergo selective degradation—the proteins representing the satellite bands being preferentially degraded first; or the loss of satellite bands represents a change in the form of isolectins synthesized from preexisting or newly synthesized mRNA.

These findings underscore the fact that lectins may change during plant development and that the changes may vary in different plant tissues. These conclusions must be considered when postulating role(s) for plant lectins.

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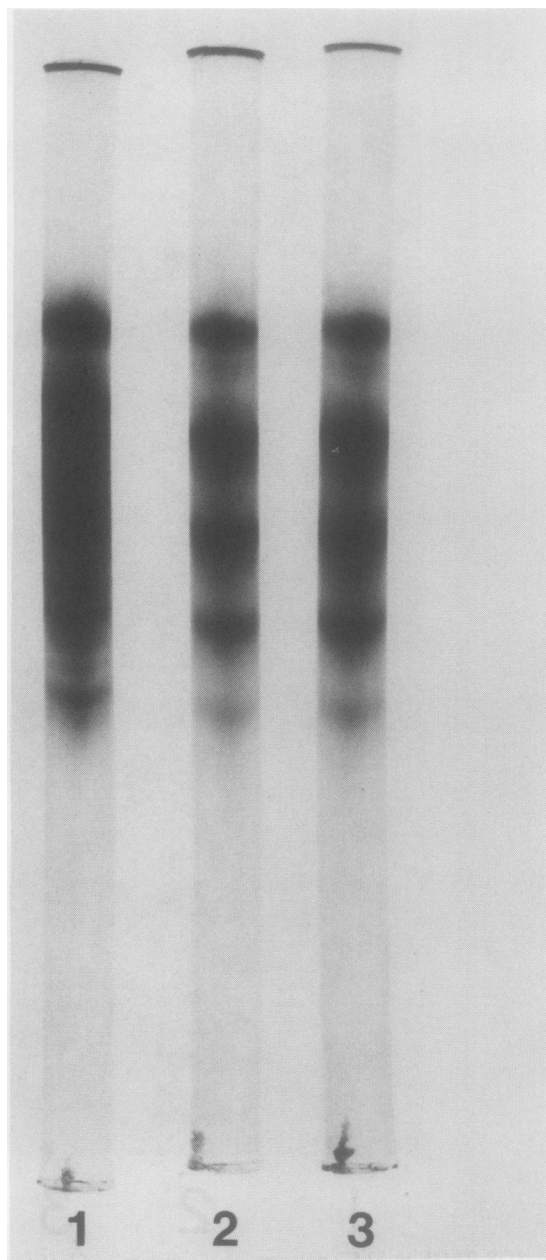


FIG. 6. pH 8.9 nondenaturing PAGE of GS-I isolectins isolated from cotyledon segments of one seed prior to and following imbibition. Gel 1, GS-I isolectins extracted from one segment of a dry seed planted on 8/14/80; gel 2, GS-I isolectins isolated from a different segment of the same seed on 9/30/80; gel 3, GS-I isolectins extracted from another segment of the same seed on 10/17/80.

glutination assays. The assistance of Shaun Black in the amino acid analyses is gratefully acknowledged.

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