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# The Lectins of *Sophora japonica*

## I. PURIFICATION, PROPERTIES, AND N-TERMINAL AMINO ACID SEQUENCES OF TWO LECTINS FROM LEAVES

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CHARLES N. HANKINS*, JUANITA KINDINGER, AND LELAND M. SHANNON
*Department of Biochemistry, University of California, Riverside, California 92521*

### ABSTRACT

**Two lectins, Leaf Lectin I and Leaf Lectin II (LLI and LLII) were purified from the leaves of *Sophora japonica*. Like the *Sophora* seed lectin, LLI and LLII are tetrameric glycoproteins containing a single subunit with respect to size. The subunits of LLI (32 kilodaltons) and LLII (34 kilodaltons) are slightly larger than those of the seed lectin (29.5 kilodaltons). The three *Sophora* lectins display indistinguishable specificities, amino acid compositions, specific hemagglutinin activities, and extinction coefficients. Although very closely related to the seed lectin, the leaf and seed lectins are not immunologically identical and they differ in subunit molecular weights, carbohydrate content, and in the pH sensitivity of their hemagglutinin activities. N-terminal amino acid sequence analysis shows that although they are homologous proteins, the three *Sophora* lectins are products of distinct genes.**

Legume seeds have proven to be a rich source of lectins and many of these proteins (classic seed lectins) have been purified and extensively characterized (2, 6). The classic seed lectins have not been unequivocally demonstrated in nonseed tissues of their respective plants, but proteins very similar to them have been observed in the roots, stems, and/or leaves of many legumes (3).

The best characterized of the nonseed legume lectins are those found in the leaves of *Dolichos biflorus* and *Griffonia simplicifolia* (10, 16). In both these cases, the leaf lectins and the respective seed lectins are structurally very similar with each lectin containing two closely related, but not identical subunits. Also, in both cases, the leaf and seed lectins apparently share a common subunit (7, 9). As a result of amino acid sequence and peptide map comparison, it was suggested that the *Dolichos* leaf and seed lectins might differ only as the result of differential posttranslational modifications of one subunit (9). More recent studies (4), however, suggest that these proteins are distinct products from a family of lectin genes. If different posttranslational modifications account for the structurally distinct lectins present in different tissues, then lectins might provide model systems for studying developmentally regulated (tissue specific) differential posttranslational modifications of proteins. If, on the other hand, structurally different lectins from a single legume species are products of distinct genes, then lectins provide a system for studying the developmentally regulated expression of different genes within a closely related gene family. It is, of course, quite possible that both of the above cases may be operative in legumes.

*Sophora japonica* is a leguminous tree from which a seed lectin (SJA) has been purified and well characterized (11). SJA is a tetrameric glycoprotein containing subunits of a single size (29.5 kD). This lectin is homologous to the other well characterized legume lectins by virtue of both primary amino acid sequence (15) and immunological homologies (8). With respect to its specificity and structure, SJA is typical of the 'galactose' specific legume lectins. The only unusual property distinguishing SJA from most other galactose lectins is the pronounced sensitivity of its carbohydrate binding activity to pH (11).

This property (pH-sensitivity) has been observed in several other cases, however, even in a legume lectin of very different structure (12).

In previous studies, we noted that crude extracts of *Sophora* leaves contained hemagglutinin activity and also displayed strong immunological cross-reactions with antisera raised against the *Sophora* seed lectin. In this report, we describe the purification and some of the properties of two lectins from *Sophora* leaves.

### MATERIALS AND METHODS

The tissues used in these studies were harvested from a single specimen of *Sophora japonica*, growing on the campus of the University of California, Riverside. Seeds were harvested when fully desiccated and stored at room temperature after removal from their pods. Fully expanded, mature leaves were harvested, washed with distilled $H_2O$, and if not extracted immediately, were frozen with liquid $N_2$ and stored at −20°C for later use.

**Extraction.** All procedures were carried out at 0 to 5°C unless otherwise specified. Leaves (200 g) were blended 2 min in a 4 L Waring Blendor with 1 L of distilled $H_2O$ containing 10 mM 3-mercaptoethanol and then squeezed through four layers of cheesecloth. The extract was then centrifuged (10,000*g*) for 20 min to yield a clarified crude extract. The pH of the crude extract was lowered to 4.0 by the dropwise addition of glacial acetic acid while stirring and after 30 min the resulting precipitate removed by centrifugation and discarded as before, yielded the pH supernatant. An equal volume of ice-cold acetone was added dropwise to the pH supernatant over a period of 30 min while stirring on ice, then allowed to stand on ice for 30 min. The material precipitated by acetone was collected by centrifugation (5000*g* × 10 min) and suspended in 100 ml of distilled $H_2O$. A substantial amount of insoluble material was removed by centrifugation (5000*g*, 10 min) and discarded. The solubilized acetone precipitate was then dialyzed 15 h against excess distilled $H_2O$ using large pore-size (Spectrapor 50 kD cutoff) dialysis tubing.

*Sophora* leaves contain tannins and other colored materials which interfere with the purification of the lectin. The inclusion of reducing agent in crude extracts and the acetone precipitation step aid in inhibiting and removing most of these materials. Because the high pH required for carbohydrate binding activity accelerates the reaction of colored materials (tannins) with the lectin, the pH of the lectin preparation was not adjusted until immediately prior to loading onto the affinity column.

**Affinity Chromatography.** A 1.5 × 15 cm column of *N*-acetyl-

D-galactosamine-Sepharose-4B was equilibrated with 0.5 M Tris-HCl (pH 8.5) containing 0.1 mM $CaCl_2$, 0.1 mM $MnCl_2$, and 0.4 M NaCl. The dialyzed lectin extract was fortified just prior to loading, with concentrated Tris-metal buffer to give a final composition equivalent to the equilibration buffer. All column procedures were performed by pumping at a constant flow rate of 1 ml/min. After sample application, the column was washed with equilibration buffer until the $OD_{280}$ was ≤0.05. Bound lectins were eluted by 50 mM citrate buffer at pH 5 or 0.1 M galactose-containing buffer. Purified lectins were stored at −20°C for 6 months with little or no loss in hemagglutinin activity. Also, the lectins may be lyophilized following dialysis against distilled water and are readily resolubilized in distilled water.

Standard hemagglutinin assays using washed rabbit erythrocytes were performed as previously described (13) except the assay buffer was 0.05 M Tris/HCl, 0.15 M NaCl (pH 8.5). For assays at other pH values, lectin dilutions and erythrocyte suspensions were prepared in Tris-NaCl buffers at the indicated pH. Ouchterlony double diffusion in agar was performed as previously described (8, 13).

Amino acid analysis and N-terminal sequences were performed by the Biotechnology Instrumentation Facility at UCR. Gel filtration was performed on a 1.5 × 100 cm column of either Sephacyl S-200 or S-300 (Pharmacia). Elution was downward at a constant flow rate of 30 ml/h. *N*-Acetyl-D-galactosamine-Sepharose-4B was prepared by the method of Porath (5). Carbohydrate was estimated by the microquantitative phenol-$H_2SO_4$ assay (1) using glucose as the standard.

## RESULTS AND DISCUSSION

Crude extracts of the leaves of *Sophora japonica* contain both hemagglutinin activity and material (CRM) which cross-reacts with antisera to the SJA. The hemagglutinin activity of SJA is known to be very pH-sensitive, with maximal activity displayed only at pH values above 8.0. In fact, we have observed that SJA can be eluted from affinity columns as effectively by low pH (pH < 5.0) buffers as by elution with hapten sugars. Since the hemagglutinin activity in *Sophora* leaves was inhibited by *N*-acteyl-D-galactosamine (as is SJA) and also appeared to be somewhat pH-sensitive, we decided to utilize an affinity purification scheme similar to that which we have used for SJA. The results of these studies are depicted in Figure 1. Note that a single peak of protein (lower elution profile) containing two very similar sized polypeptides (right inset) is obtained if the affinity column is eluted with a galactose-containing buffer. However, if the column is first eluted with pH 5.0 citrate buffer and subsequently with a galactose-containing buffer, two distinct protein peaks (upper elution profile) are obtained, each containing a single-sized polypeptide (left and middle insets). We refer to these proteins as leaf lectin I (LLI) and leaf lectin II (LLII), respectively.

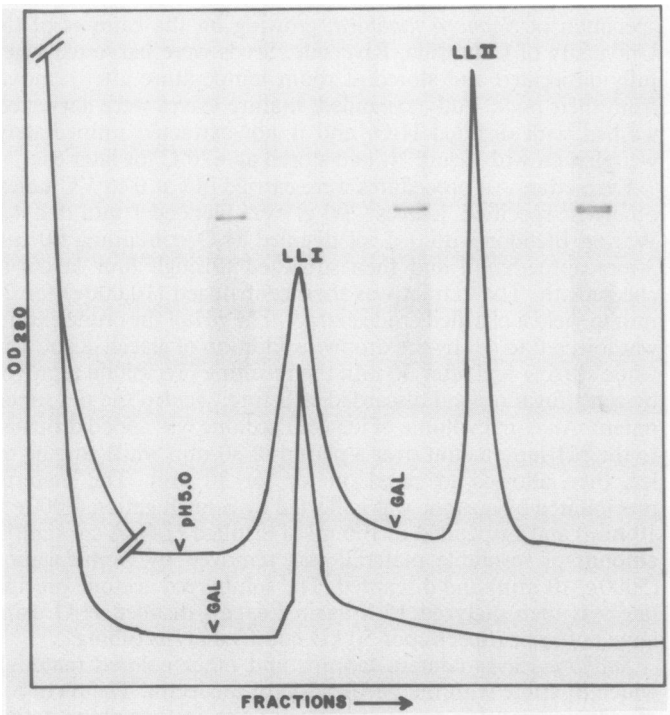


FIG. 1. Affinity purification of the *Sophora* leaf lectins on *N*-acetyl-D-galactosamine-Sepharose. Lower trace, coelution of LLI and LLII with 0.1 M galactose; upper trace, elution of LLI by pH 5 buffer and subsequent elution of LLII with 0.1 M galactose. Insets, SDS gel patterns of peaks; left, pH eluted LLI; middle, galactose eluted LLII after pH elution; right, LLI and LLII coeluted (lower trace) by galactose.

Table I. *Purification Summary of Sophora Leaf Lectins*

| Step | Volume | Protein | HA Titer | CRM Titer[a] |
|---|---|---|---|---|
| 1. Crude extract | 1000 | 2.6 | 32–64 | 1/4 |
| 2. pH 5 supernatant | 980 | 0.9 | 32–64 | 1/4 |
| 3. Acetone precipitate | 100 | 4.4 | 128–256 | 1/8–1/16 |
| 4. *GalNAc Sepharose* | | | | |
| 4a. pH 5 eluate (LLI) | 8 | 0.83 | 2000 | ≥1/32 |
| 4b. Galactose eluate (LLII) | 8 | 1.02 | 2000 | ≥1/32 |
| 4c. Void | 100 | 4.3 | 0 | 1/2 |

[a] Maximum dilution of extract that gives a precipitin band by Ouchterlony double diffusion following 24 h incubation against antisera to SJA.

Table I gives a summary of a typical purification of lectin from 200 g (fresh weight) of mature (mid-summer, 1985) *Sophora* leaves. From these results, one can see that the *Sophora* leaf lectins, although much less abundant than SJA in seeds, are significant (total from 0.5% to 1% of extractable protein) components of leaves. It is important to note, however, that leaf extracts totally depleted of hemagglutinin activity by passage over the affinity column still contain material(s) immunologically related (CRM) to SJA (to be discussed below).

The *Sophora* lectins were compared by Ouchterlony double diffusion using SJA-antisera and LLI-antisera. Figure 2 shows the results of these studies and reveals the extremely close relationships between these proteins. Leaf lectins I and II could not be distinguished by this method using either seed lectin antisera or antisera against LLI (Fig. 2A). Antisera against SJA or LLI were, on the other hand, able to discriminate between SJA and leaf lectin II (as evidenced by the classic 'spur' between wells 2 and 3 of Fig. 2B), but as can be seen, the immunological difference is slight and these sera did not clearly distinguish SJA from LLI. The results shown in Figure 2C reveal that the CRM present in extracts after removal of the leaf lectins by affinity chromatography is related to but not identical (note substantial spur formation between wells 1, 2, and 5) to either leaf lectin. The CRM is also related to, but not identical to, SJA (not shown).

Although some of the properties of SJA described herein were previously reported (11), we felt that the three *Sophora* lectins must be examined under identical conditions to permit a direct comparison of properties. Additionally, we purified and utilized SJA from the seeds of the same tree used to isolate the leaf lectins, thus eliminating potential confusion due to possible varietal differences.

The apparent 'native' mol wt of the *Sophora* lectins were determined by gel filtration on Sephacryl S-200 and S-300 columns (data not shown). The average mol wt extrapolated from

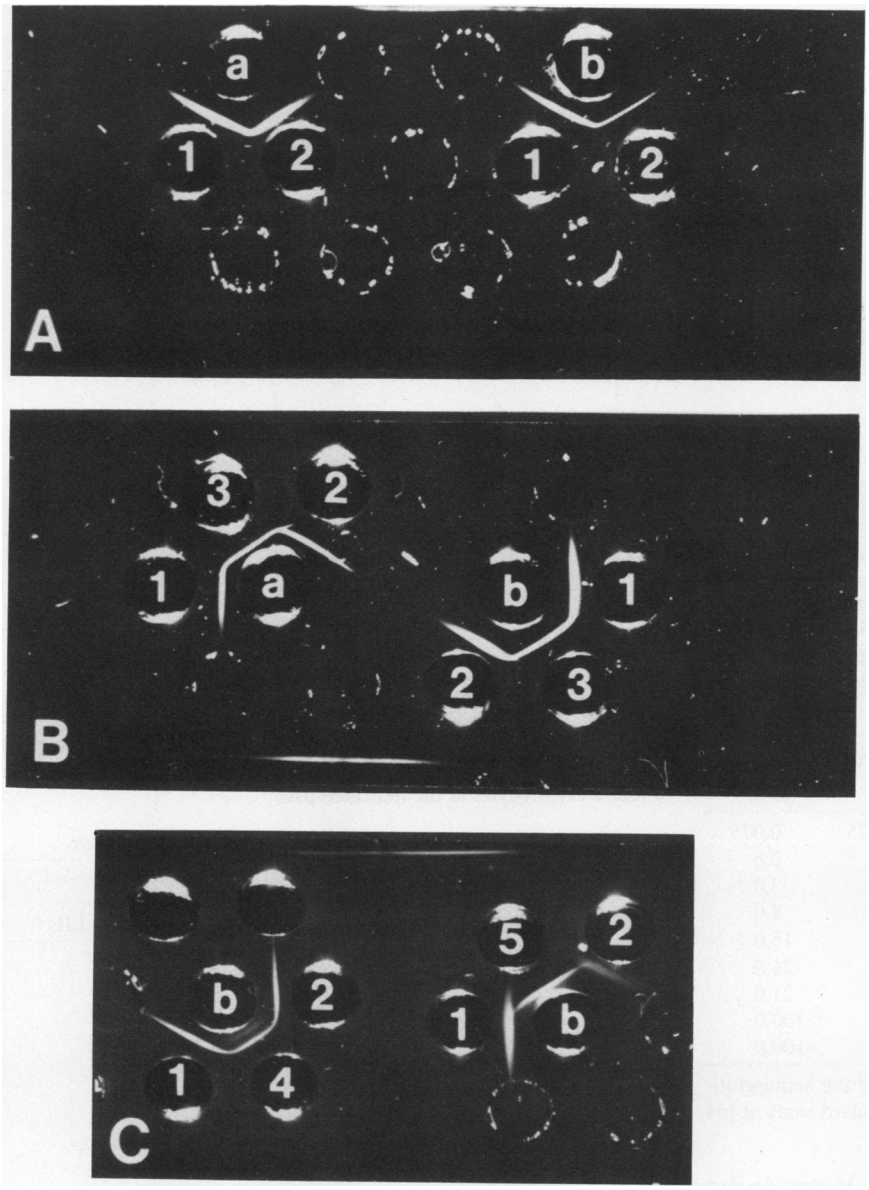


FIG. 2. Ouchterlony double diffusion patterns of *Sophora* lectins. 1: LLI, 10 μg; 2: LLII, 10 μg; 3: SJA, 10 μg; 4: crude leaf extract, 10 μl; 5: affinity column void, 10 μl; a: anti-SJA sera, 10 μl; b: anti-LLI-sera, 10 μl.

a number of trials was about 120,000 for SJA and about 130,000 for both leaf lectins. These studies were carried out at both pH 8.5 and pH 5 with the same results, indicating that at pH 5 the lectins did not show any tendency to disassociate into smaller mol wt forms.

The subunit structures of the *Sophora* lectins were examined by SDS-PAGE, in the presence of reducing agent. Electrophoresis of the reduced proteins reveals (Fig. 3) that each of the *Sophora* lectins contains a single type of subunit with respect to size (SDS-migration) and that each lectin subunit is slightly different. Therefore, each native lectin would appear to be a tetramer of identical or nearly identically sized subunits. The extrapolated mol wt (averaged from numerous trials) for the subunits of the *Sophora* lectins are 29,500 for SJA, 32,000 for LLI and 34,000 for LLII. These size differences should not, however, be taken as evidence for absolute differences in the sizes of the lectin polypeptide chains since a major portion of the size variations seen could be the result of differences in the quantity of attached carbohydrate and its influence on SDS gel migration.

The hemagglutinin activities of the *Sophora* lectins were studied using freshly washed rabbit erythrocytes. At pH 8.5, the lectins possessed equal specific activities, each requiring a minimum concentration of about 0.05 μg to give detectable hemagglutination under our standard assay. Also, as summarized in Table II, the three lectins possessed (as judged by inhibition of hemagglutination) virtually indistinguishable carbohydrate specificities at pH 8.5. The hydrophobic effect of the nitrophenol derivatives observed by Poretz *et al.* (11) with SJA is also readily apparent with the leaf lectin. Although there appears to be no major qualitative or quantitative differences in the carbohydrate binding properties of these lectins at pH 8.5, a major difference is seen at lower pH value. The data shown in Figure 4 reveals that the hemagglutinin activities of SJA and LLI are very sensitive (and equally sensitive) to pH whereas LLII retains maximal activity over a significantly broader pH range and shows a less rapid decline in activity as the pH is lowered. The lack of activity of SJA and LLI at low pH is not due to inactivation since activity can be restored by raising the pH. In fact, one can cause the dissolution of red blood cells already agglutinated by SJA or LLI by lowering the pH of the mixture and subsequently observe reaggregation by raising the pH. The use of pH in resolving LLI and LLII in the affinity purification scheme show that the pH effect is not an effect on red blood cells or the hemagglutination process *per se*. The results of the gel filtration studies (at both

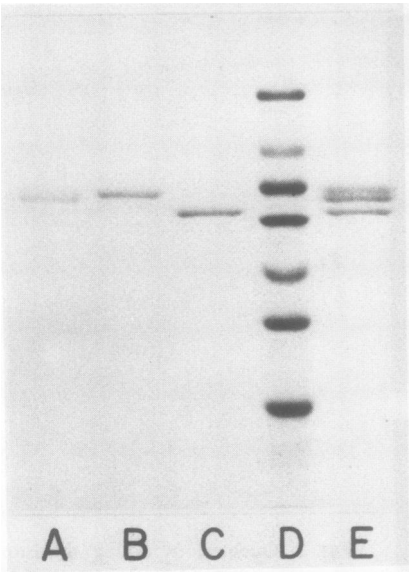


FIG. 3. SDS-gel pattern of *Sophora* leaf and seed lectins. A, LLI; B, LLII; C, SJA; D, mol wt standards: from top BSA, 66 kD; egg albumin, 45 kD; G-3-P, 36 kD; carbonic anhydrase, 29 kD; trypsinogen, 24 kD; trypsin inhibitor, 20.1 kD; α-lactalbumin, 14.2 kD; E: mix of LLI, LLLII and SJA.

Table II. *Carbohydrate Specificities of Sophora Leaf and Seed Lectins*

| Inhibitor | LLI | LLII | Seed |
|---|---|---|---|
| ρ-NØβGalNAc | 0.0375 | 0.0375 | 0.075 |
| ρ-NØαGalactose | 0.6 | 0.6 | 0.6 |
| *N*-acetyl-D-Galactosamine | 1.5 | 1.5 | 3.0 |
| Galactosamine | 8.0 | 8.0 | 8.0 |
| Galactose | 15.0 | 15.0 | 15.0 |
| Raffinose | 21.0 | 21.0 | 21.0 |
| Stachyose | 21.0 | 21.0 | 21.0 |
| Mannose | >100.0 | >100.0 | >100.0 |
| Glucose | >100.0 | >100.0 | >100.0 |

[a] Minimum concentrations required to totally inhibit the hemagglutination of rabbit erythrocytes by 1 μg of lectin in a standard assay at pH 8.5.

low and high pH) indicate that these tetrameric lectins do not undergo pH induced subunit disassociations; thus, pH must directly or indirectly influence the carbohydrate binding affinity of the *Sophora* lectins. Although pH could differentially affect the conformations or the metal ion binding properties of these lectins, it is also possible that they possess a difference in charged residues within their active sites.

The amino acid compositions (Table III) of the *Sophora* lectins were found to be insignificantly different from each other and typical of legume lectins (low cysteine, methionine, and high serine and threonine). Consistent with their amino acid compositions, the lectins possess nearly identical UV-extinction coefficients.

All three *Sophora* lectins are adsorbed by immobilized ConA and stain positive for carbohydrate by the periodate-Schiff reaction indicating that they are glycoproteins possibly containing carbohydrate side chains of the high mannose type due to the strong interaction with ConA. It should be noted, however, that the carbohydrate content of the three lectins differ somewhat (Table III).

All of our observations thus far reveal an extremely close relationship between SJA and the leaf lectins, but do not reveal their genetic relationship. This relationship is, however, clarified by an examination of their *N*-terminal amino acid sequences as

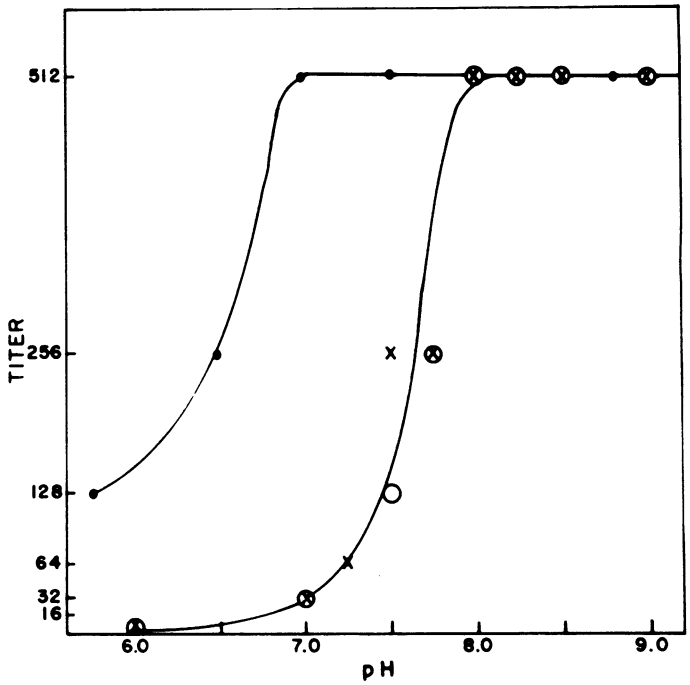


FIG. 4. pH optima curves for the hemagglutinin activities of *Sophora* lectins. SJA, (○); (×); LLII, ⊗. Titer is the reciprocal of the highest dilution of 250 μg/ml lectin samples which gave positive agglutination of rabbit erythrocytes at the indicated pH.

Table III. *Amino Acid Composition and Other Properties*

| Amino Acid | Residues/mol | | |
|---|---|---|---|
| | SJA | LLI | LLII |
| Asp (Asn) | 26 | 27 | 28 |
| Thr | 27 | 24 | 27 |
| Ser | 28 | 39 | 31 |
| Glu (Gln) | 23 | 23 | 25 |
| Pro | 12 | 12 | 15 |
| Gly | 21 | 23 | 21 |
| Ala | 24 | 23 | 24 |
| Cys (1/2) | 1–2 | 1–2 | NA[a] |
| Val | 26 | 23 | 25 |
| Met | 0 | 0 | 0 |
| Ile | 12 | 11 | 13 |
| Leu | 22 | 25 | 24 |
| Tyr | 9 | 10 | 11 |
| Phe | 14 | 14 | 14 |
| Trp | NA | NA | NA |
| Lys | 9 | 9 | 8 |
| His | 4 | 4 | 4 |
| Arg | 6 | NA | 6 |
| Other properties | | | |
| $E^{1\%}_{283}$ | 16.7 | 16.2 | 16.5 |
| Carbohydrate content[b] | 6.2% | 7.9% | 11.3% |

[a] Not done/available. [b] Amino sugars not determined.

given in Table IV. These results show that within the first *N*-terminal 23 residues, SJA differs from LLI by two residues (positions 12 and 19), and from LLII by three residues (positions 3, 12, and 19). Also, LLI and LLII differ by one residue (position 3). Since the residues which differ among these lectins are not amino acids which are easily confused or known to be modified posttranslationally, we conclude that SJA, LLI, and LLII are each products of distinct genes.

We are aware that the sequence data, reported herein, for SJA

Table IV. *N-Terminal Amino Acid Sequences of Sophora Lectins*

| | 1 | | | | 5 | | | | 10 | | | | 15 | | | | 20 | | | | | 25 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SJA | A | E | I | L | S | F | S | F | P | K | F | A | S | N | Q | E | D | L | L | L | Q | G | D | A | L |
| LLI | A | E | I | L | S | F | S | F | P | K | F | V | S | N | Q | E | D | L | V | L | Q | G | D | — | — |
| LLII | A | E | V | L | S | F | S | F | P | K | F | V | S | N | Q | E | D | L | V | L | Q | G | — | A | — |

differs at residues 15 and 16 from that previously reported (15). We do not know the reason for this discrepancy, but the results shown were obtained from two analyses, one using commercially available SJA (Vector Labs) and one with SJA purified from the seeds of 'our' tree. The two SJA samples gave identical results and, in fact, all three *Sophora* lectins analyzed were homologous at positions 15 and 16.

We have made preliminary observations with immature *Sophora* seeds which show that SJA is synthesized as a larger mol wt precursor and, thus, undergoes posttranslational modifications as do several other legume seed lectins (14). Whether or not the leaf lectins undergo any posttranslational modifications and whether any of the other leaf CRMs are the results of such processes remains to be discovered. With the use of Western blot techniques, we have been unable to detect any polypeptide in leaves which corresponds to the SJA subunit, nor have we observed any LLI or LLII subunits in seed extracts. Independent of any lectin processing which may occur in *Sophora* leaves, the three lectins described herein arise from distinct genes and the expression of the leaf and seed genes would appear to be under distinct developmental regulation.

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