Isolation, Characterization, and Subunit Structures of Multiple Forms of *Dolichos biflorus* Lectin*

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The *Dolichos biflorus* lectin was isolated from seed homogenates by adsorption onto insoluble poly-leucyl hog blood group A + H substance and subsequent elution with *N*-acetyl-*d*-galactosamine. Although the lectin was homogeneous as determined by discontinuous polyacrylamide gel electrophoresis, isoelectric focusing, sedimentation equilibrium, and immunodiffusion against rabbit antisera prepared against the crude seed extract, the lectin was fractionated into at least two electrophoretically distinguishable forms (A and B) by chromatography on concanavalin A-Sepharose. Approximately 12% of the original lectin sample did not bind to the concanavalin A and contains the B form. The bound lectin was eluted specifically and quantitatively as a biphasic peak from the concanavalin A-Sepharose with a gradient of methyl *α*-glucopyranoside. Carbohydrate analyses of lectin fractions obtained from different portions of the elution profile showed variation in the amount of mannose and *N*-acytetylgalactosamine, thus confirming the heterogeneity of the electrophoretic A form. Both the A and B forms of the lectin are active and are apparently present in the dry seeds. Once separated, the two electrophoretic forms of the *D. biflorus* lectin are distinguishable by electrophoresis. The separated A and B forms show a high degree of similarity in molecular weights (113,000 and 109,000, respectively), antigenic character, and amino acid compositions. Both forms have alanine as *NH₂*-terminal residues and either leucine or valine as the only detectable *COOH*-terminal residues.

The A and B forms specifically agglutinate and have similar titers for type A human red blood cells. They gave similar precipitin curves with hog blood group A + H substance and show similar inhibition curves with methyl *α*-N-acytetylgalactosamine and *N*-acytetylgalactosamine.

Discontinuous polyacrylamide gel electrophoresis of the unfraccionated *D. biflorus* lectin in 0.1% sodium dodecyl sulfate-8.0 M urea produced two major bands, corresponding to subunits IA and IIA of the A form of the lectin and two minor bands corresponding to subunits IB and IIB of the B form.

Subunit molecular weight determinations by electrophoresis in 0.1% sodium dodecyl sulfate gels showed molecular weights of 26,500 for subunits IA and IIA and 26,000 for subunits IB and IIB, thus indicating that each form of the lectin is composed of four subunits.

The seeds of the *Dolichos biflorus* plant contain a lectin that specifically agglutinates type A erythrocytes and precipitates blood group A substance (1-5). This lectin has been isolated by adsorption onto insoluble poly-leucyl hog blood group A + H substance (6) and subsequent elution by *N*-acytetylgalactosamine (4, 5), the immunodominant sugar of the blood group A substance (7). Inhibition studies of the isolated lectin have shown that its blood group A specificity is due to its ability to recognize terminal nonreducing *α*-N-acytetylgalactosamine residues (4, 5).

The isolated lectin was found to be a glycoprotein, homogeneous by a number of criteria, including disc gel electrophoresis under acid and basic conditions, sedimentation velocity, isoelectric focusing, and immunodiffusion against rabbit antisera to the crude seed extract. The lectin was precipitated totally by human blood group A substance (4).

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† To whom reprint requests should be addressed.

The present investigation describes the fractionation of this lectin into two electrophoretically distinguishable forms by passage through concanavalin A-Sepharose. The binding specificities, physical characteristics, and subunit structures of these two forms are described along with the carbohydrate variations observed during subfractionation of the predominant electrophoretic form.

MATERIALS AND METHODS

Purification of Lectin—The *Dolichos biflorus* lectin was isolated as previously described (4, 5) from seed (S. B. Penick and Co., Church Street, N. Y., N.Y.) extract by adsorption onto insoluble poly-leucyl hog blood group A + H substance (6) and specific elution from this immunoadsorbent with 0.01 M *N*-acytetylgalactosamine (Pfanstiehl Laboratories, Inc., Waukegan, III.). The hapten was removed by chromatography of the lectin on Bio-Gel P-10 (Bio-Rad Laboratories, Richmond, Calif.) and detected as a separate peak by periodate uptake (8). The purification procedure was carried out at 4-5°C in a buffer of 0.01 M sodium phosphate, pH 7.2, containing 0.9% sodium chloride and 0.02% sodium azide. The lectin isolated by this procedure was concentrated by ultrafiltration in a Diaflo ultrafiltration device using...
an XM-50 or PM-10 filter and stored at 4 °C in concentrations of about 1 mg/ml.

**Analytical Methods**—Protein concentration was determined by a modification of the Lowry procedure (9) or by nitrogen determination using the ninhydrin method (10). The ornithine procedure (8) was used to determine the concentration of methyl α-N-glucopyranoside (Pfanstiehl Laboratories).

Protein samples for amino acid analysis were flushed with nitrogen and hydrolyzed in vacuo in 6 N constant boiling HCl (Sequana Grade, Pierce Chemical Co., Rockford, Ill.) at 110 °C for 24, 48, and 72 hours. After hydrolysis of the HCl by vacuum, amino acid analyses were performed by Eldex Laboratories, Inc. (Menlo Park, Calif.) using a Dionex model D-50 amino acid analyzer. Trypsophan was analyzed spectrophotometrically by the method of Goodwin and Morton (11) in the presence of 0.1 N NaOH and 8.0 M urea (12). Cysteine, cystine, and methionine were analyzed by performic acid oxidation (13). Sample protein concentrations were calculated from the amino acid analyses.

NH₂-terminal amino acids were determined by labeling the protein with 5-dimethylaminonaphthalene-1-sulfonic chloride (Eastman Kodak Co.) (14) and subsequent acid hydrolysis as previously described. The dansyl derivatives of the amine acids were identified by co-chromatography on thin layer plates with standard dansyl-amino acids. Thin layer chromatography was done on Silica Gel G plates (Quanta 1 gram Q1, Quantum Industries, Fairfield, N. J.) in Solvent 1, toluene-pyridine-acetic acid (100:50:5.5), Solvent 2, N-Heptane-1-butanol-acetic acid (85:15:2.5), Solvent 3, N-Butanol-28% ammonia (9:8:4) or on MN-polygram polyamide-6-plates (Brinkmann Industries) in Solvent 4, benzene-acetic acid (9:1); and Solvent 5, formic acid-H₂O (1.5:100). The developed plates were dried, equilibrated in an atmosphere of NH₃·H₂O (15), and read under an ultraviolet light at 365 nm.

COOH-terminal amino acids were determined by hydrolysis with 6 N constant boiling HCl (Carbco, Miss., A DFP, 47 units/21 mg CPA/ml). Lecithin samples were denatured at 100 °C in freshly distilled 0.2 M N-ethylmorpholine-acetate, pH 8.5 (10), 0.1% sodium dodecyl-sulfate for 5 min. Carboxypeptidase A was solubilized in 1.0 M NH₄HCO₃ and then added to the denatured lectin samples and incubated at 37°C for various time intervals. Enzymatic cleavage was stopped by immersion of the samples in a boiling water bath for 5 min. The samples were then frozen and lyophilized. The lyophilized protein was extracted with 67% ethanol (17) and the pooled extracts derivatized with 5-dimethylamino naphthalene-1-sulfonic chloride (Eastman Chemical Co., Rochester, N. Y.). 5-Dimethylaminonaphthalene-1-sulfonfyl; PBS, 0.01 M sodium phosphate buffer, pH 7.2, 0.9% sodium chloride, and 0.02% sodium azide.

1 The abbreviations used are: dansyl, 5-dimethylaminonaphthale-1-sulfonic; PBS, 0.01 M sodium phosphate buffer, pH 7.2, 0.9% sodium chloride, and 0.02% sodium azide.

We would like to thank Edwina Beckman and David Bylund for their technical assistance in performing these molecular weight determinations.

**Preparation of Concanavalin A-Sepharose**—The N-hydroxysuccinimide ester of succinyl-aminohippurinol-Sepharose was prepared as described by Cuesta and Parikh (29, 30). This ester was added to a 1.5 mg/ml solution of concanavalin A (three times crystallized, Miles-Yeda Ltd., Kankakee, Ill.) in 0.1 M sodium phosphate buffer, pH 7.2, and 0.9% sodium chloride and stirred for 4 hours at 4°C. Under these conditions about 45% of the concanavalin A was bound to the Sepharose, and the Sepharose was measured by gel filtration chromatography. This sample was then activated by high voltage paper electrophoresis. The activated Sepharose was then washed extensively with 0.5 M NaCl in 0.1 M sodium phosphate buffer, pH 7.2, containing 0.02% sodium azide to reduce the constant leakage of the ligand (31).
phy of alditol acetate derivatives of the sugars obtained after acid hydrolysis. As shown in Table I, each of the fractions contain mannose and N-acetylgalactosamine but differ from one another in relative quantities of these sugars.

**Fractions I to VII** had the same electrophoretic mobility as the unfractonated lectin on pH 9.7 glycine gels whereas Fraction I had a different electrophoretic mobility (Fig. 2). These two electrophoretic forms of the lectin, designated as Forms A and B, have apparent molecular weights of 116,000 and 103,000, respectively, as estimated from measurements of mobility retardation with increasing gel concentration. A similarity in retardation plot intercepts indicated very similar charge to friction ratios for the two forms.

Although Form B of the lectin could not be detected in electrophoresis of the unfractonated lectin on pH 9.7 glycine gels regardless of the amount of protein applied (Fig. 2), its presence in the unfractonated lectin could be verified by discontinuous electrophoresis on sodium dodecyl sulfate-urea gels in which subunits of Form B have different mobilities than subunits of Form A (Fig. 3). To test the possibility that Form B of the lectin originated as a degradation product of Form A during the 8- to 10-day purification procedure, small quantities of the lectin were isolated batchwise from a common seed

**Table I**

**Carbohydrate content of Dolichos biflorus lectin**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Mannose/lectin (nmol/nmol)</th>
<th>N-Acetylglucosamine/lectin (nmol/nmol)</th>
<th>Per cent carbohydrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>4.3</td>
<td>3.2</td>
<td>0.7</td>
</tr>
<tr>
<td>II</td>
<td>12.4</td>
<td>10.9</td>
<td>1.9</td>
</tr>
<tr>
<td>III</td>
<td>13.3</td>
<td>9.4</td>
<td>2.1</td>
</tr>
<tr>
<td>IV</td>
<td>11.6</td>
<td>5.8</td>
<td>1.9</td>
</tr>
<tr>
<td>V</td>
<td>21.1</td>
<td>6.6</td>
<td>3.3</td>
</tr>
<tr>
<td>VI</td>
<td>20.5</td>
<td>7.7</td>
<td>3.2</td>
</tr>
<tr>
<td>VII</td>
<td>18.0</td>
<td>7.3</td>
<td>3.0</td>
</tr>
</tbody>
</table>

**Fig. 1. Chromatography of the Dolichos biflorus lectin on concanavalin A-Sepharose.** Lectin (158 mg) at a concentration of 3.7 mg/ml of PBS was applied to a column containing 300 ml of concanavalin A-Sepharose. 12.1% of the applied Dolichos lectin was not retained (tubes 1 to 100). 87.6% of the bound lectin was specifically and quantitatively eluted with a 3.0-liter concave gradient of 0 to 0.3 M methyl α-D-glucopyranoside (gradient began at tube 101). Further application of a 0.3 M wash of methyl α-D-glucopyranoside failed to release any more Dolichos lectin. ▲, concentration of methyl α-D-glucopyranoside (mol/liter); ●, retention times of the fractions were indicated by numbers.
FIG. 2. (left). Discontinuous polyacrylamide gel electrophoresis of the A and B forms of the Dolichos biflorus lectin on pH 9.7 glycine gels. 1, original unfractionated lectin; 2, Form A (Fractions II to VII); 3, Form B (Fraction I); 4, recombined Form A and Form B. Form B was not detected in electrophoresis of unfractionated lectin even when gels were overloaded.

FIG. 3 (right). Discontinuous polyacrylamide gel electrophoresis of the A and B forms of the Dolichos biflorus lectin on pH 9.7 glycine gels in the presence of 8.0 M urea and 0.1% sodium dodecyl sulfate. 1, original unfractionated lectin; 2, Form A (Fractions II to VII had the same electrophoretic patterns.); 3, Form B; 4, recombined form A and form B. The four bands from top to bottom have been designated IA, IB, IIA, and IIB. A very faint band that is electrophoretically identical with concanavalin A can be seen below IIB.

extract at various times after the initial seed disruption. No variation in relative proportions of Forms A and B was detected in sodium dodecyl sulfate-urea gel electrophoresis of lectins prepared over times ranging from 4 hours to 21 days, thus indicating that both electrophoretic forms are present in the seed as native proteins and do not arise from modification or degradation during the purification procedure. Fractions VII and I were selected for further characterization of the A and B electrophoretic forms, respectively, since they represent the two extreme forms of the lectin.

Sedimentation equilibrium studies on Fractions I and VII at respective concentrations of 0.643 and 0.726 A280 unit per ml of 0.1 M Tris-HCl, pH 7.3, 0.2 M NaCl gave weight average molecular weights of 109,000 and 113,000 for electrophoretic Forms B and A, respectively. The partial specific volumes used in this estimation were 0.732 ml/g for Form A and 0.731 ml/g for Form B as determined from the amino acid compositions.

<table>
<thead>
<tr>
<th>Table II</th>
<th>Amino acid composition of Fraction I and Fraction VII of Dolichos biflorus lectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid</td>
<td>Fraction VII*</td>
</tr>
<tr>
<td></td>
<td>Mol %</td>
</tr>
<tr>
<td>Asp</td>
<td>11.2</td>
</tr>
<tr>
<td>Thr*</td>
<td>7.1</td>
</tr>
<tr>
<td>Ser*</td>
<td>15.6</td>
</tr>
<tr>
<td>Glu</td>
<td>6.4</td>
</tr>
<tr>
<td>Pro</td>
<td>5.0</td>
</tr>
<tr>
<td>Gly</td>
<td>6.4</td>
</tr>
<tr>
<td>Ala</td>
<td>8.8</td>
</tr>
<tr>
<td>Val</td>
<td>7.4</td>
</tr>
<tr>
<td>Met</td>
<td>0.5</td>
</tr>
<tr>
<td>Ile</td>
<td>6.1</td>
</tr>
<tr>
<td>Leu</td>
<td>8.0</td>
</tr>
<tr>
<td>Tyr</td>
<td>3.3</td>
</tr>
<tr>
<td>Phe</td>
<td>4.8</td>
</tr>
<tr>
<td>His</td>
<td>1.2</td>
</tr>
<tr>
<td>Lys</td>
<td>3.9</td>
</tr>
<tr>
<td>Arg</td>
<td>2.4</td>
</tr>
<tr>
<td>Trp*</td>
<td>1.9</td>
</tr>
<tr>
<td>Cys*</td>
<td>0</td>
</tr>
</tbody>
</table>

*The mole per cent and number of residues was calculated by averaging the values obtained from acid hydrolysis in 6 N constant boiling HCl for 24, 48, and 72 hours.

*The number of amino acid residues was calculated assuming a molecular weight of 133,000 minus a 4.3% carbohydrate content for Fraction VII and a molecular weight of 109,000 minus a 1.5% carbohydrate content for Fraction I.

*The serine and threonine values were obtained by linear extrapolation to zero hydrolysis time.

*Tryptophan was determined spectrophotometrically by the method of Goodwin and Morton (11). Limited quantities of Form B restricted the number of tryptophan determinations to 1.

* Determined after performic acid oxidation.

The two fractions were identical when tested in immunodiffusion against antisera prepared against the seed extract and had very similar amino acid compositions (Table II).

Fractions I and VII and unfractionated lectin were labeled with dansyl-chloride and hydrolyzed in acid. Dansyl-alanine co-chromatographed with the dansylated NH₂-terminal residue from each of the samples in Solvents 1 to 5. COOH-terminal amino acids were determined by hydrolysis with carboxypeptidase A. The kinetics of released COOH-terminal amino acids indicated an essentially simultaneous release of both leucine and valine residues for both the A and B forms.

Fractions I and VII had similar titers for type A human erythrocytes and showed similar precipitin curves with hog blood group A + H substance (Fig. 4). Similar inhibition curves were obtained for both fractions with methyl α-N-acetyl-β-galactosamine and N-acetyl-α-galactosamine (Fig. 5).

Subunit Characterization—Sodium dodecyl sulfate-urea gel electrophoresis of the isolated A and B forms of the lectin showed an increased proportion of subunits designated IA and IIB in Form A and of subunits designated IB and IIB in Form B (Fig. 3). Analysis of the two predominant subunits of unfracionated lectin on pH 9.7 glycine gels in urea by the technique

The methyl α-N-acetyl-β-galactosamine was a gift from Dr. I. J. Goldstein, Department of Biological Chemistry, University of Michigan.
Fig. 4. Precipitation of purified Forms A and B of Dolichos biflorus lectin and unfractionated D. biflorus lectin by hog blood group A+H substance. ○, Form B (Fraction I) 5.37 µg of nitrogen; △, Form A (Fraction VII), 5.19 µg of nitrogen. ■, unfractionated D. biflorus lectin, 6.10 µg of nitrogen.

Fig. 5. Inhibition by monosaccharides and methylglycosides of precipitation of hog blood group A+H substance with Form A and Form B of Dolichos biflorus lectin and unfractionated D. biflorus lectin. △, Form A inhibited by methyl α-D-N-acetylgalactosamine; ○, Form B inhibited by methyl α-D-N-acetylgalactosamine; ■, unfractionated D. biflorus lectin inhibited by methyl α-D-N-acetylgalactosamine. ▲, Form A inhibited by N-acetyl-β-p-galactosamine; ☐, Form B inhibited by N-acetyl-β-p-galactosamine. □, unfractionated D. biflorus lectin inhibited by N-acetyl-β-p-galactosamine. Form A (5.64 µg of nitrogen) and Form B (5.31 µg nitrogen) were combined with 2.27 µg of nitrogen of hog blood group A+H substance. Unfractionated D. biflorus lectin (4.88 µg of nitrogen) was combined with 1.63 µg of nitrogen hog blood group A+H substance.

Fig. 6. Continuous pH acrylamide gel electrophoresis of the A and B forms of the Dolichos biflorus lectin in the presence of 0.1% sodium dodecyl sulfate (27). Electrophoresis of the unfractionated lectin (1) produces two bands. These two bands correspond to Form A (2) and Form B (3). The recombined purified A and B forms ran as single resolvable bands. Gels in this figure are oriented showing direction of electrophoresis from top to bottom.

Forms A and B, respectively. This result indicates that the disruption and separation of the A and B forms into two subunit types each on discontinuous sodium dodecyl sulfate-urea at pH 9.7 may not be based totally on differences in subunit molecular weight.

DISCUSSION

Heterogeneity in lectin forms has been detected in hemagglutinins from many plant sources (37-45). Allen et al. (37) have described the fractionation of commercial wheat germ agglutinin into three active proteins. Likewise, Kalb (40) has found three different 1-fucose-binding proteins in preparations of Lotus tetragonolobus. Two electrophoretically distinguishable hemagglutinins have been obtained from Lens culinaris (39) and as many as four hemagglutinins have been found chromatographically separable in soybean oil meal (41). The above data now show that the D. biflorus lectin which previously had been considered homogeneous can be fractionated into two electrophoretically distinguishable forms (A and B) by chromatography on concanavalin A-Sepharose. The predominant form (Form A) has been further subfractionated on the basis of its affinity for concanavalin A. All the forms are active.

Concanavalin A has a specificity for the α-methylglycosides of D-mannose, D-glucose, and N-acetyl-D-glucosamine (35, 36). The differential abilities of the various forms of the D. biflorus lectin to bind to concanavalin A suggests that these forms may differ from one another in their carbohydrate content. This possibility of carbohydrate heterogeneity has been evaluated by gas-liquid chromatography of alditol acetate derivatives of monosaccharides released by acid hydrolysis. Each fraction was found to contain different amounts of both mannose and N-acetylglucosamine residues which may account for the
differences in affinities of the various fractions to concanavalin A.

Mannose and N-acetylglucosamine have been detected as the primary carbohydrate constituents of several lectins, including, the soybean agglutinins (Glycine max) (41), the lima bean lectin (Phaseolus lunatus) (46), the red kidney bean agglutinin (Phaseolus vulgaris) (38), L. tetragonolobus lectins (40) and Ulex europaeus phytohemagglutinin (42).

Several physical characteristics for the unfraccionated D. biflorus lectin showed minor variations from previously published results by Etzler and Kabat (4). Since the seeds were obtained from a different supplier, the discrepancies detected in molecular weight, isoelectric pH, and methionine content may represent variation in lectin character resulting from differences in the seed source. Despite these minor differences the lectins from both seed suppliers had similar amino acid compositions and specificities and were found to be antigenically identical when tested with antisera prepared against extracts from each of the two seed sources.

Physical and chemical evaluation of the A and B forms of the D. biflorus lectin indicates very similar molecular weights of 113,000 and 109,000, respectively, and similar amino acid compositions. Both forms contain alanine as the NH₂-terminal residue and leucine or valine as detectable COOH-terminal residues. The A and B forms of the lectin also show strong similarities in their activities and specificities. Both forms specifically agglutinate and have similar titers for type A human red blood cells. They show similar precipitin curves with hog blood group A + H substance and show similar inhibition curves with methyl α-N-acetyl-d-galactosamine and N-acetyl-α-galactosamine.

The unfraccionated D. biflorus lectin fails to indicate the presence of any material that is electrophoretically similar to the B form when run on the nondisruptive anionic pH 9.7 glycine gel system, no matter how much protein is applied to the gels. The above observation indicates that the B form is not readily acceptable due to the identification of significant and detectable quantities of both the A and B forms in unfraccionated lectin samples when run on sodium dodecyl sulfate-urea-disruptive gels, indicating that the unfraccionated lectin does contain enough of the B form to be detectable on the glycine gels. The possibility of some interaction between the A and B forms is under investigation at present.

The subunit structures of a number of lectins have been recently described (47-55). In the present study, the predominant A form of the D. biflorus lectin (113,000 g/mol) has been disrupted into two major classes of subunits (IA and IIA) by electrophoresis on sodium dodecyl sulfate-urea-gels with a corresponding molecular weight of 26,500 each thus suggesting a total of four subunits for each native A form molecule. The minor B form of the lectin (109,000 g/mol) has also been disrupted into two predominant types of subunits (IB and IIB) which differ slightly from subunits IA and IIA in electrophoretic mobility and in molecular weight (26,000).

Acknowledgment—We greatly appreciate the technical assistance of Naomi Miyao.
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W G Carter and M E Etzler


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