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 polyleucyl 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-D-glucopyranoside. Carbohydrate analyses of lectin fractions obtained from different portions of the elution profile showed variation in the amount of mannose and N-acetylglucosamine, 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-acetyl-d-galactosamine and N-acetyl-d-galactosamine.

Discontinuous polyacrylamide gel electrophoresis of the unfractionated *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 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 polyleucyl hog blood group A + H substance (6) and specific elution by N-acetyl-d-galactosamine (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-acetyl-d-galactosamine 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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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 orcinol procedure (8) was used to determine the concentration of methyl α-n-glucosyranoside (Pfanstiehl Laboratories).

Protein samples for amino acid analysis were washed with nitrogen and hydrolyzed in vacuo in 6 N constant boiling HCl (Sequanaid Grade, Pierce Chemical Co., Rockford, Ill.) at 110°C for 24, 48, and 72 hours. After removal of HCl by vacuum, amino acid analyses were performed by Eldex Laboratories, Inc. (Menlo Park, Calif.) using a Durrum model D-500 amino acid analyzer. Tryptophan 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 amino 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 (Quantum 1 gram Q1, Quantum Industries, Fairfield, N.J.) in Solvent 1, toluene:pyridine-acetic acid (150:50:5.5); Solvent 2, N-Heptane-1-butanol-acetic acid (9:9:2); Solvent 3, M-Cresol-acetic acid-cobalt-28% amino acids (9:8:4); or on MN-polynylgumolyamid 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 (Sequanaid Grade, Pierce Chemical Co., Rockford, Ill.: 21 mg CaP/ml). Lectin samples were denatured at 100°C in freshly distilled 0.5 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 dried in vacuo over P₂O₅. The dry residue was dissolved in 50 µl of H₂O and spotted on either (a) Quartz 1 gram Q1 thin layer chromatography plates and run in 1-propanol-H₂O (70-30) or 1-butanol-acetic acid-H₂O (4:1:1) or (b) Whatman No. 1 chromatography paper and developed in 1-butanol-acetone-dichloromethane-H₂O (10:10:2.5). Thin layer chromatography plates were sprayed with ninhydrin-collidine-CuNO₃ (18). Quantitation was performed by visual inspection and quantification was done by high voltage paper electrophoresis. All paper chromatograms were developed by dipping in ninhydrin-cadmium indicator (19). Identification and quantitation of COOH-terminal amino acids residues released by carboxypeptidase were also checked by amino acid analyses (AAA Laboratories, Seattle, Wash.).

Hemagglutination was performed with a Takatsy microtitrator using 0.025 ml loops and 0.2 ml human erythrocyte suspensions. Hemagglutination was recorded after 1 hour incubation at room temperature. Quantitative precipitin analyses were done as previously described in a final volume of 250 µl (4) using hog blood group A + H substance as antigen. The tubes were incubated at 37°C for 1 hour and kept at 4°C for 4 days. Nitrogen in the washed precipitates was determined by the ninhydrin procedure (10).

Immunodiffusion was performed by the Ouchterlony (20) method in their technical assistance in performing these molecular weight determinations.

**Preparation of Concanavalin A-Sepharose**—The N-hydroxysuccinimide ester of succinyl-aminoethylpropionylamino-Sepharose was prepared as described by Custodio and Parikh (29, 30). This ester was added to a 1.5-mg/ml sol. of concanavalin A (three times crystallized, Miles-Yeda Ltd., Kankakee, Ill.) in 0.1 M sodium phosphate buffer, pH 7.2, and 0.3% sodium chloride and stirred for 4 hours at 4°C. Under these conditions about 45% of the concanavalin A was bound to the Sepharose. The Sepharose was then washed extensively with 5 M urea and 0.1% sodium dodecyl-sulfate (SDS). Lectins were included in the above electrophoresis system as described by Wu and Bruening (25) except that 12.5% w/v sucrose was used in the sample buffer. In the case of the urea-sodium dodecyl sulfate gels, the sample buffer also contained 2.0 mg/ml of sodium dodecyl sulfate and the sample was heated at 65°C for 15 min prior to application to the gels.

Subunit molecular weight determinations on sodium dodecyl sulfate gels were conducted as described by Weber and Osborn (27) with the exception that the protein reduction was carried out in 5 mM dithiothreitol as well as 1% mercaptoethanol.

Molecular weights and charge to friction ratios of the lectins and subunits were determined by measurements of mobility retardation with increasing gel concentration as described by Hedrick and Smith (28).

**Carbohydrate Analyses**—The carbohydrate content of each lectin fraction was determined by gas-liquid chromatography of the aldol acetate derivatives (39) of sugars released after acid hydrolysis. Preliminary hydrolysis experiments on unfractuated lectin were run for varying time intervals in 1, 2, and 3 N HCl at 100°C. Hydrolysis of 0.1 N HCl for 3.0 hours was found to give maximal sugar release and minimal sugar destruction and was adopted as a standard condition for comparison of the various forms of the lectin. Lectin samples (1 to 2 mg) were hydrolyzed in sealed tubes under nitrogen, cooled, neutralized with Na₂CO₃, and the aldol acetate derivatives of the released sugars were prepared as described by Grigs et al. (31). Inositol was included as an internal standard throughout the analysis; deoxyribose and erythritol internal standards were added as further checks of quantitation at various stages in the procedure.

For gas-liquid chromatography, the aldol acetate derivatives were dissolved in acetone and injected on a 6-foot column of 3% OV-225 (Applied Science Laboratories, Inc., P. O. Box 440, State College, Pa.) on Chromosorb Q (Applied Science Laboratories, Inc.) using helium as carrier gas at a flow rate of 20 ml/min. The temperature was maintained at 160°C for 7 min and then programmed to 205°C at 1°C/min. Temperature was maintained at 205°C for 30 min. The temperature of the injection port was 215°C and the flame ionization detector tempera-

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1 We would like to thank Edwina Beckman and David Bylund for their technical assistance in performing these molecular weight determinations.
ture was 250°. Flow rates of H₂ and air through the detector were 30 and 300 ml/min, respectively.

RESULTS

Physical Characteristics and Criteria of Homogeneity—The isolated Dolichos biflorus lectin formed a single peak with a pI of 5.05 upon isoelectric focusing in pH 3 to 10 and pH 4 to 6 gradients in glycerol. Immunodiffusion of the lectin with rabbit antisera prepared against the crude seed extract produced a single precipitin band which formed a line of identity with the lectin preparation described in previous work (4).

The lectin formed a single, diffuse band in discontinuous polyacrylamide gel electrophoresis on pH 9.7 glycine gels. The diffuseness of the band was independent of the gel concentration and quantity of lectin applied to the gel. An apparent molecular weight range of 107,000 to 112,000 was estimated from measurements of retardation of lectin mobility in increasing concentrations of pH 9.7 glycine gels.

Sedimentation equilibrium of the lectin at a concentration of 0.638 A₂₈₀ unit/ml in 0.1 M Tris- HCl, 0.2 M NaCl was performed at 18,000 rpm at 25°. The plot of fringe displacement versus centrifugal radius was linear. The weight average molecular weight of the lectin was determined to be 111,000 using a partial specific volume of 0.728 ml/g calculated from the amino acid composition.

Fractionation of Lectin on Concanavalin A-Sepharose—Preliminary experiments showed the D. biflorus lectin was only partially precipitated by concanavalin A and partially bound to concanavalin A-Sepharose. A similar observation was made by Bessler and Goldstein (34). Of 158 mg of lectin chromatographed on concanavalin A-Sepharose, 12.1% of the lectin did not bind to the column and 87.6% of the lectin was bound and specifically eluted with a concave gradient of 0 to 0.3 M methyl α-D-glucopyranoside (35, 36) in PBS (Fig. 1). The biphasic character of the elution profile of the bound lectin indicates a variation in number of concanavalin A receptor sites on the lectin as described in previous work (4).

Characterization of Multiple Forms of Lectin—The eluates of D. biflorus lectin from the concanavalin A-Sepharose column were divided into seven fractions, I – VII (Fig. 1), and analyzed for carbohydrate content by gas-liquid chromatography of alditol acetate derivatives of the sugars obtained after acid hydrolysis. As shown in Table I, each of the fractions contain mannose and N-acetylglucosamine but differ from one another in relative quantities of these sugars.

Fractons II to VII had the same electrophoretic mobility as the unfractoned 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 unfractoned lectin on pH 9.7 glycine gels regardless of the amount of protein applied (Fig. 2), its presence in the unfractoned 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

Carbohydrate analyses of lectin fractions obtained by chromatography of the D. biflorus lectin on concanavalin A-Sepharose. Alditol acetate derivatives of sugars released by acid hydrolysis of the seven lectin fractions (I to VII) were compared with the alditol acetate derivatives of standard sugars (erythrose, 2-deoxyribose, fucose, xylose, mannose, galactose, inositol, N-acetylglucosamine, and N-acetylglactosamine) by gas-liquid chromatography. All carbohydrate content values were calculated assuming molecular weights of 109,000 for Fraction I and 113,000 for Fractions II to VII.

| Fraction | Mannose/lectin | N-Acetylglucosamine/lectin |
|----------|----------------|---------------------------|
|          | Per cent carbohydrate | Mannose | N-Acetylglucosamine |
|          | nmol/nmol | mg/liter | mg/liter |
| I        | 4.3       | 3.2 | 0.7 | 0.6 |
| II       | 12.4      | 10.2 | 1.9 | 1.9 |
| III      | 13.3      | 9.4 | 2.1 | 1.9 |
| IV       | 11.6      | 5.8 | 1.9 | 1.1 |
| V        | 21.1      | 6.6 | 3.3 | 1.3 |
| VI       | 20.5      | 7.7 | 3.2 | 1.5 |
| VII      | 18.0      | 7.3 | 9.6 | 1.4 |

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 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). Eluates were pooled as indicated into Fractions I to VII.
FIG. 2. 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.

TABLE II

| Amino acid | Fraction VII* | Fraction I* |
|------------|--------------|-------------|
|            | Residues/113,000 MW | Residues/109,000 MW |
| Asp        | 11.2 | 114 | 11.2 | 114 |
| Thr*       | 7.1  | 72  | 6.5  | 66  |
| Ser*       | 15.6 | 159 | 16.3 | 165 |
| Glu        | 6.4  | 65  | 6.3  | 64  |
| Pro        | 5.0  | 51  | 4.8  | 48  |
| Gly        | 6.4  | 65  | 6.4  | 64  |
| Ala        | 8.8  | 80  | 8.9  | 80  |
| Val        | 7.4  | 56  | 7.5  | 56  |
| Met        | 0.5  | 5   | 0.5  | 5   |
| Ile        | 3.1  | 26  | 3.4  | 34  |
| Leu        | 8.0  | 81  | 8.1  | 81  |
| Tyr        | 3.3  | 33  | 3.4  | 34  |
| Phe        | 4.8  | 49  | 4.8  | 49  |
| His        | 1.2  | 12  | 1.2  | 12  |
| Lys        | 3.9  | 39  | 3.9  | 39  |
| Arg        | 2.4  | 25  | 2.4  | 25  |
| Trp*       | 1.9  | 22  | 1.5  | 16  |
| Cys*       | 0    | 0   | 0    | 0   |

*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 113,000 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 two fractions were identical when tested in immunodiffusion against antiserum prepared against the seed extract and had very similar amino acid compositions (Table II).

Fraction 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 3. 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-α-galactosamine and N-acetyl-D-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 IIA in Form A and of subunits designated IB and IIB in Form B (Fig. 3). Analysis of the two predominant subunits of unfractionated lectin on pH 9.7 glycine gels in urea by the technique 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.

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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-d-galactosamine; ●, Form B inhibited by N-acetyl-d-galactosamine. □, unfractionated *D. biflorus* lectin inhibited by N-acetyl-d-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.

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 L-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-acetylgalactosamine 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 unfractonated 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 NH-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 horse blood group A + H substance and show similar inhibition curves with methyl a-N-acetyl-d-galactosamine and N-acetyl-β-galactosamine.

The unfractonated 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 make its presence undetectable by most techniques, or that is present in the native lectin mixture in such small quantities as to obscure the observation of the B form. The first possibility is not readily acceptable due to the identification of significant amounts of the B form to be detectable on the sulfate-urea-disruptive gels, indicating that the unfractionated 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 (47-56). 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).

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