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Purification and Characterization of a Lectin (Plant Hemagglutinin) with Blood Group A Specificity from *Dolichos biflorus**

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ABSTRACT: The blood group A specific lectin from *Dolichos biflorus* seeds was purified by adsorption on to insoluble polyleucyl blood group A substance and subsequent elution with 2-deoxy-β-D-galactopyranoside (D-GalNAc). The purified lectin was homogeneous by ultracentrifugation, gave one diffuse band on acrylamide gel electrophoresis under acid and alkaline conditions, formed one line in immunodiffusion and immunoelectrophoresis against rabbit antisera to the crude seed extract, and was totally precipitated by human blood group A substance; it had a tendency to aggregate in solution. Amino acid analyses of the purified lectin showed a large amount of aspartic acid and serine but no cysteine or methionine. The lectin contains about 2% hexose and has a molecular weight of 140,000 and an isoelectric point of pH 4.5. The lectin precipitated with blood group A_1 and A_2 substances as well as with the streptococcal group C polysaccharide. The reactivity of each of these polysaccharides is ascribed to terminal nonreducing α-linked D-GalNAc residues. The lectin did not precipitate with blood group B or H substances, with an ovarian cyst substance lacking A, B, H, Le^a, or Le^b activity, with group A streptococcal polysaccharide, with teichoic acids or with the periodate degradation stages of a blood group H substance. Inhibition of precipitation with various monosaccharides, glycosides, and oligosaccharides indicates that the combining site of the lectin is specific for terminal α-linked D-GalNAc. There is some uncertainty as to the size of the combining site. Although the A-active di- and trisaccharides were equal in inhibiting power to methyl 2-acetamido-2-deoxy-α-D-galactoside, the A-active-reduced pentasaccharide required only about six-tenths the molar concentration for comparable degrees of inhibition. No heterogeneity in the combining site was detected among various lectin fractions differentially eluted from the immunoadsorbent.

Many plants contain agglutinins capable of combining specifically with animal erythrocytes and other cells (for reviews, see Krüpe, 1956, Mäkelä, 1957, and Bird, 1959). These hemagglutinins, called lectins (Boyd and Shapleigh, 1954b), are most frequently found in the seeds of leguminous plants and differ from one another in their specificities, certain of which are directed toward the blood group ABH substances (*cf.* Mäkelä, 1957).

The many similarities in specificity of these plant hemagglutinins to antibodies to blood group substances (Kaplan and Kabat, 1966; Moreno and Kabat, 1969) make it desirable to obtain them in highly purified form, to establish whether they are homogeneous or heterogeneous with respect to their combining sites, to obtain information as to the dimensions of their complementary regions, and ultimately to elucidate the three-dimensional structure of their specific sites.

The seeds of *Dolichos biflorus* contain lectin-agglutinating type A_1 red blood cells (Bird, 1951, 1952a,b) and specifically precipitating with blood group A substance (Boyd and Shapleigh, 1954a; Bird, 1959). Several workers have attempted to purify this lectin by alcohol precipitation (Bird, 1959) and by fractionation procedures based on charge (Kocourek and Jamieson, 1967). The present study describes the purification of the *D. biflorus* lectin by the use of the specific insoluble immunoadsorbent, polyleucyl hog blood group A + H substance (Kaplan and Kabat, 1966; Moreno and Kabat, 1969; Hammarström and Kabat, 1969), its characterization and a study of its combining site.

Materials

D. biflorus seeds were a gift from Col. G. W. G. Bird. The various blood group substances and blood group oligosaccharides used in this study are preparations previously prepared and described in this laboratory (*cf.* Kabat, 1956; Allen and Kabat, 1959; Schiffman *et al.*, 1962, 1964; Lloyd

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et al., 1968; Vicari and Kabat, 1969; Lloyd and Kabat, 1968; Hammarström and Kabat, 1969).

Streptococcal group A and C polysaccharides were a gift from Dr. R. M. Krause of Rockefeller University (Krause and McCarty, 1961, 1962). Monosaccharides were obtained commerically (Nutritional Biochemical Co.). Ethyl 2-acetamido-2-deoxy-β-D-galactopyranoside[1] was prepared by de-O-acetylation with methanolic ammonia of the 3,4,6-tri-*O*-acetyl derivative kindly supplied by Dr. R. W. Jeanloz.

Salmonella typhimurium LT-2 polysaccharide was kindly provided by Dr. Sigfrid Svensson. Teichoic acid from *Staphyloccus aureus* 3528 and Copenhagen (Nathenson *et al.*, 1966) were gifts from Dr. J. L. Strominger. α-D-GNAc-(1→3)-D-GalNAc was generously donated by Dr. W. Willers of Ryksuniversiteit, Utrecht, Holland.

Methods

Analytical Methods. Nitrogen, methylpentose, hexosamine, *N*-acetylhexosamine, and hexose were determined by colorimetric methods previously described (Kabat, 1961; Lloyd *et al.*, 1966). Periodate uptake was measured as previously described (Kabat, 1961).

Amino acid analysis of the purified lectin were kindly performed by Dr. S. Vratsanos and Dr. B. F. Erlanger on salt-free samples hydrolyzed under nitrogen in 6 N HCl at 110° for 24, 48, and 72 hr. Aliquots from these hydrolysates were combined with an internal standard of norleucine and run on a Technicon amino acid analyzer according to the technique of Spackman *et al.* (1958). Response factors were calculated from analyses of a standard mixture of amino acids.

Tryptophan was determined spectrophotometrically by the method of Goodwin and Morton (1946). Cysteine and methionine were determined after performic acid oxidation by the method of Hirs (1956).

Physicochemical Methods. Sedimentation velocity experiments[2] were run in a Spinco Model E analytical ultracentrifuge equipped with schlieren optics. Experiments were done at 10° at 50,740 rpm; four runs were made at concentrations of 175, 391, 782, and 1380 μg of N per ml in 0.001 M phosphate-buffered saline (pH 7.2). The intrinsic viscosity was calculated as described by Schachman (1957) from viscosity measurements of different concentrations of the protein with an Ostwald viscometer. The partial specific volume was determined from the amino acid composition by the method of McMeekin *et al.* (1949).

Acrylamide gel disc electrophoresis was performed at pH 9.3 and 2.9 according to the Davis (1964) and Reisfeld and Small (1966) procedures as described by Dorner *et al.* (1969). Electrophoresis was run in 10% gels, at a constant initial current of 1 mA/tube, which was increased to 2.5 mA/tube after the buffer line entered the small pore gel. Electrophoresis was stopped 1 hr after the buffer line passed through the gel, and the gels were stained for 1 hr in a 1% Amido Black solution in 15% acetic acid. The gels were destained by electrophoresis in 15% acetic acid.

Isoelectric focusing was performed in a 110-ml LKB column at 10° using a 1% concentration of LKB ampholine carrier ampholytes and a sucrose gradient. Preliminary experiments to establish the pH range were done by incorporating a 1% concentration of the LKB ampholytes in acrylamide gels and performing the gel isoelectrofocusing according to the technique of Wrigley (1968) and Awdeh *et al.* (1968). The gels were stained with coomassie blue according to the procedure of Chrambach *et al.* (1967).

Separation Procedures. Gel filtration was performed at 4° on Bio-Gel P-10 and Bio-Gel P-300 according to the directions of the Bio-Rad laboratories, Richmond, Calif.

Immunochemical Methods. Hemagglutination was performed at room temperature with a Takatsy microtitrator (Cooke Engineering Co., Alexandria, Va.) using 0.025-ml loops and a 2% suspension of erythrocytes.

Quantitative precipitin analyses were done by a microprecipitin technique (Kabat, 1961) employing a final volume of 240 μl unless otherwise stated. The tubes were incubated for 1 hr at 37° and kept for 1 week at 4°. Nitrogen in the washed precipitates was determined by the ninhydrin procedure (Schiffman *et al.*, 1964).

Immunodiffusion was by the Ouchterlony (1948) method in 1% ionagar (Consolidated Laboratories, Inc.) in 0.01 M phosphate-buffered saline (pH 7.2). Immunoelectrophoresis was performed according to the technique of Grabar and Williams (1953) in 1.5% agar containing 0.05 M sodium barbital buffer (pH 8.3) at 150 V for 100 min.

Antisera were produced in rabbits against a crude extract of *D. biflorus* seeds. After obtaining preimmunization sera, the footpads of the rabbits were injected once a week for 3 weeks with an emulsion of about 1 mg of seed extract and Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.). The animals were bled on 3 successive days beginning 1 week after the final injection and the three bleedings were pooled; after 3 weeks the immunization and bleeding schedule was repeated and a second batch of antiserum was obtained.

Preparation of Immunoadsorbent. Hog A + H blood group substance was purified by ethanol precipitation from hog gastric mucin (Wilson) according to procedures previously described (Kabat, 1956). The blood group substance was copolymerized with the *N*-carboxyanhydride of L-leucine (Pilot Chemicals) by the technique of Tsuyuki *et al.* (1956) to form the insoluble polyleucyl derivative as described by Kaplan and Kabat (1966). This derivative will be referred to as polyleucyl hog A + H blood group substance (PL hog A + H).

Experimental Section and Results

Purification of Lectin. D. biflorus seeds were ground in a mortar and suspended to a concentration of 10% (w/v) in 0.001 M phosphate-buffered saline (pH 7.2). The suspension was stored at 4° overnight and then centrifuged at 2000 rpm. The clear supernatant agglutinated type A_1 erythrocytes with a titer of 32 and did not agglutinate type B or O cells. Quantitative precipitin analysis of the extract showed 8–9% of the total nitrogen to be precipitable by human blood group A substance (MSM).

Two separate batches of lectin were prepared at 4° by

[1] Abbreviations used are: Et-β-D-GalNAc, ethyl 2-acetamido-2-deoxy-β-D-galactopyranoside; GalNAc, 2-acetamido-2-deoxy-D-galactopyranose; GNAc, 2-acetamido-2-deoxy-D-glucopyranose; Gal, D-galactopyranose; Fuc, L-fucopyranose; R, 3-hexenetetrols.

[2] We are indebted to Mr. S. and Dr. H. Rosencranz for performing these experiments.

TABLE I: Fractions of Lectin Eluted from Immunoadsorbent.

Fraction	Eluting Agent (M)	Total μg of N Eluted	Hemagglutination Assay with A_1 Erythrocytes: Initial Concn (μg of N/ml)	Titer[a]
	Experiment I			
I_1	GalNAc (0.005)	480	80	256
I_2	GalNAc (0.005)	521	82	512
I_3	GalNAc (0.005)	337	63	256
I_4	GalNAc (0.01)	452	84	256
I_5	GalNAc (0.01)	490	88	512
I_6	GalNAc (0.01)	773	91	256
I_7	GalNAc (0.01)	245	65	256
I_8	GalNAc (0.02)	248	69	512
I_9	GalNAc (0.1)	119	99	1024
I_{10}	KSCN (2)	536	179	8
I_{11}	KSCN (2)	1945	572	8
	Experiment II			
II	GalNAc (0.01)	18,880	78	512
	GalNAc (0.01)	With A_2 erythrocytes	78	4

[a] Titer represents final dilution of solution capable of agglutinating a 2% erythrocyte suspension.

TABLE II: Amino Acid Composition of Purified Lectin (Fraction II).

Amino Acid	Residues per 140,000 g: 24 hr	48 hr	72 hr	Av[a]
Asp	170.9	173.5	175.0	173.1
Thr	99.5	100.3	96.7	99.9
Ser	176.8	159.8	143.6	194.1
Glu	97.7	96.4	101.1	98.4
Pro	50.1	48.0	46.6	48.2
Gly	92.2	90.8	90.6	91.2
Ala	132.3	131.7	135.2	133.1
Val	104.2	107.1	105.9	105.7
Cys				0
Met				0
Ile	91.0	91.4	91.4	91.3
Leu	100.0	106.0	105.3	103.8
Tyr	33.4	38.3	36.1	35.9
Phe	58.8	75.3	73.8	74.6
Lys	57.2	58.7	58.0	58.0
His	19.5	17.8	15.9	17.7
Arg	43.1	44.9	43.3	43.8
Trp[b]				22.9

[a] The serine value was obtained by a linear extrapolation to zero hydrolysis time; the threonine value is the average of the 24- and 48-hr hydrolysates. The phenylalanine value is the average of the 48- and 72-hr hydrolysates; an unknown peak probably a dipeptide was seen in the 24-hr sample. [b] Tryptophan was determined spectrophotometrically by the method of Goodwin and Morton (1946).

applying the supernatant of the seed extract to columns containing a 1:5 mixture of PL hog A + H and Celite. The extract was continuously applied until the titer of the column eluate against type A erythrocytes approached that of the initial extract. At this point, the columns were washed extensively with 0.001 M phosphate-buffered saline (pH 7.2) until the optical density at 280 mμ of the eluate was less than 0.050.

The lectin was specifically eluted from the immunoadsorbent by *N*-acetyl-D-galactosamine which is the immunodominant sugar of blood group A specificity (*cf.* Kabat, 1956). In the first experiment, the column was eluted with different concentrations of *N*-acetyl-D-galactosamine in order to establish the optimal conditions for elution and to check for heterogeneity of the lectin. After collecting three fractions from the column with 0.005 M GalNAc, four fractions were eluted with 0.01 M GalNAc and two small fractions with 0.02 and 0.1 M GalNAc. These fractions (and their titers) are listed in Table I and account for about 50% of the lectin applied to the column. The column was finally eluted with 2 M KSCN to remove nonspecifically any remaining material (Avrameas and Ternynck, 1967; Dandliker *et al.*, 1967).

The second experiment was done on a larger scale on a column not completely saturated with seed extract; 118 mg of lectin was eluted with 0.01 M GalNAc. This preparation was compared with the fractions from the first experiment and is the material used for the physicochemical analyses.

In both experiments, hapten was removed by dialysis of the fraction against phosphate-buffered saline and by several passages through Bio-Gel P-10 in which the protein is recovered in the exclusion volume and the hapten detected as a separate peak by periodate uptake.

The lectin preparations were stored at concentrations of 1–5 mg/ml at 4° in phosphate-buffered saline in the presence of toluene. Under these conditions, the activity remained stable for many months. When stored at concentrations above 5 mg/ml however, a small amount of precipitate formed after several weeks. Passage of such solutions (after removal of the precipitate) through Bio-Gel P-300 columns results in the separation of a small amount of high molecular weight material in the exclusion volume. If the main peak is concentrated and rerun immediately, no high molecular weight material is detected; however, if chromatographed after standing for several days at concentrations greater than 5 mg/ml, heavy material is again seen. Since this material forms with time and has activity against blood group A substance, it may represent an aggregated form of the lectin.

The lectin preparations were tested for purity by immunodiffusion and immunoelectrophoresis against antisera prepared against the seed extract. Fractions 1–8 from expt 1 and the material obtained in expt II each formed a single precipitin band when tested at 1 mg of protein/ml (Figure 1a). These preparations also gave a single band in immunodiffusion against hog blood group A substance. Fraction I_9 was not

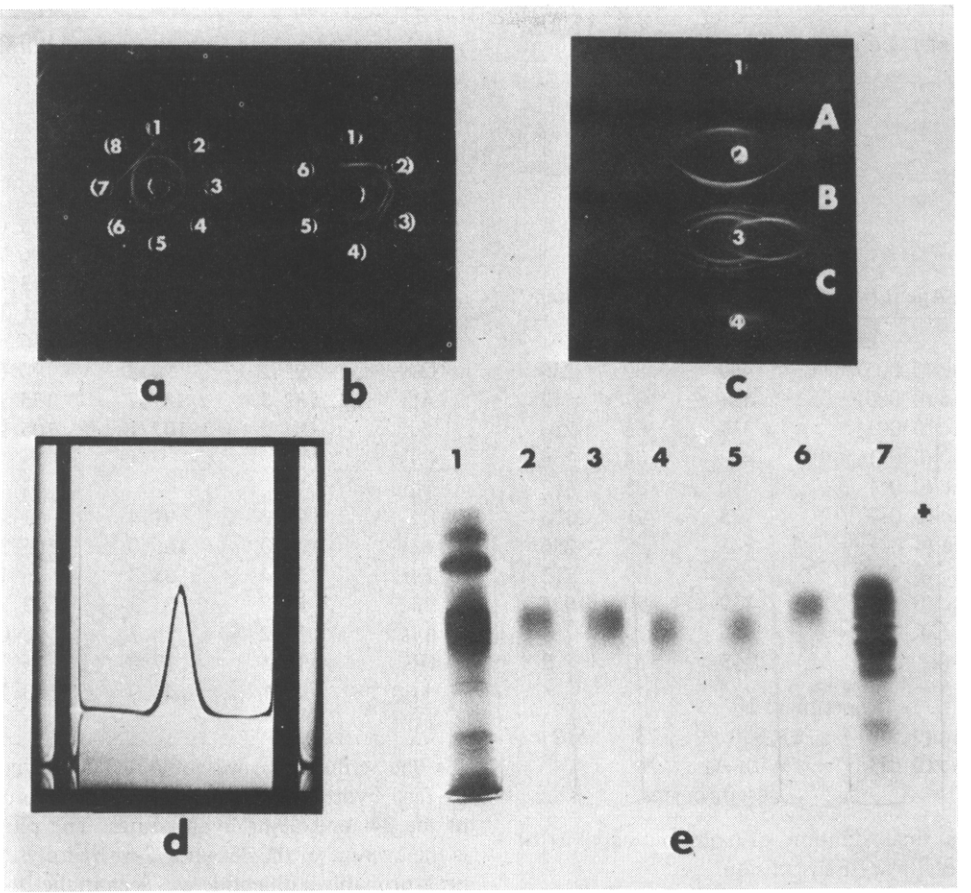

FIGURE 1: Studies of seed extract and lectin. (a) Immunodiffusion of seed extract and purified lectin preparations. Center well: rabbit antiserum against crude *D. biflorus* seed extract. Peripheral wells: (1) fraction I_1, 159 μg of N/ml; (2) fraction I_2, 163 μg of N/ml; (3) fraction I_4, 167 μg N/ml; (4) fraction I_6, 151 μg of N/ml; (5) fraction I_7, 129 μg of N/ml; (6) fraction I_8, 69 μg of N/ml; (7) fraction II, 176 μg of N/ml; and (8) crude seed extract. (b) Immunodiffusion of seed extract and lectin preparations. Center well: rabbit antiserum against crude *D. biflorus* seed extract. Peripheral wells: (1) crude seed extract; (2) fraction I_{10}, KSCN eluate, 178 μg of N/ml; (3) fraction I_{11}, KSCN eluate; 572 μg of N/ml; (4) fraction II, 782 μg of N/ml; and (5 and 6) high molecular weight material from P-300 column, 61 μg of N/ml. (c) Immunoelectrophoresis of seed extract (wells 1 and 3). Fraction II, 782 μg of N/ml (well 2) and high molecular weight material from P-300 column (well 4). Troughs B and C contain rabbit antiserum against seed extract. Trough A contains hog A + H substance. (d) Schlieren pattern of purified lectin (fraction II, 1.38 mg of N/ml in 0.001 M phosphate-buffered saline, pH 7.2). Photograph was taken after 128 min at 50,740 rpm. (e) Disc electrophoresis at pH 9.2. (1) Seed extract; (2) fraction I_1, 8.0 μg of N; (3) fraction I_4, 8.4 μg of N; and (4) fraction I_7, 7.7 μg of N; (5) fraction I_8, 7.0 μg of N; (6) fraction II, 7.9 μg of N; and (7) fraction I_{11}, KSCN eluate, 43 μg of N.

available in sufficient quantity for testing. The precipitin band obtained fused with one of five precipitin bands found in immunodiffusion of the seed extract against the antisera. The material eluted with KSCN also contained a precipitin band identical with that of the lectin; however, three other components were present.

When immunodiffusion was conducted on preparations at concentrations of 5 mg/ml, a faint second band was seen which appeared to fuse partially with a precipitin band found in immunodiffusion of the heavy material obtained from P-300 columns (Figure 1b). The isolated heavy material also has a different electrophoretic mobility as seen in Figure 1c.

Fractions I_1, I_2, I_4, I_6, I_7, I_8, and II each formed a single band in acrylamide gel electrophoresis when tested at concentrations of 6–8 μg of nitrogen under alkaline and acid conditions. Fractions I_3, I_5, and I_9 were not tested. The crude extract formed 10 bands and at least 9 bands were present in the KSCN fraction (Figure 1d).

Physicochemical Analyses. Sedimentation velocity experiments on fraction II, run at concentrations from 175 μg to 1.38 mg of N/ml of phosphate-buffered saline (pH 7.2) showed a single peak with $s^0_{20,w}$ values of 6.42, 6.09, 6.30, and 6.55 S, respectively (Figure 1d). This fraction had an intrinsic viscosity of 0.064 dl/g and its partial specific volume as calculated from the amino acid composition (Table II) was 0.73. Using the average $s^0_{20,w}$ value of 6.34×10^6 and 2.16×10^6 S for the β function (Schachman, 1957), the molecular weight was calculated to be 141,000.

Isoelectric focussing (Figure 2) showed a very large main peak of isoelectric point pH 4.5. Amino acid analyses showed a high concentration of aspartic acid, and serine; no cysteine or methionine residues were detected (Table II).

Colorimetric analyses showed a sugar content of 2.4% hexose, 1.6% hexosamine, and 1.5% *N*-acetylhexosamine. No methylpentose (6-deoxyhexose) was present.

Immunochemical Specificity. Fractions I_1–I_7 and fraction II give similar precipitin curves when reacted with human ovarian cyst blood group A substance (MSM) (Figure 3a).

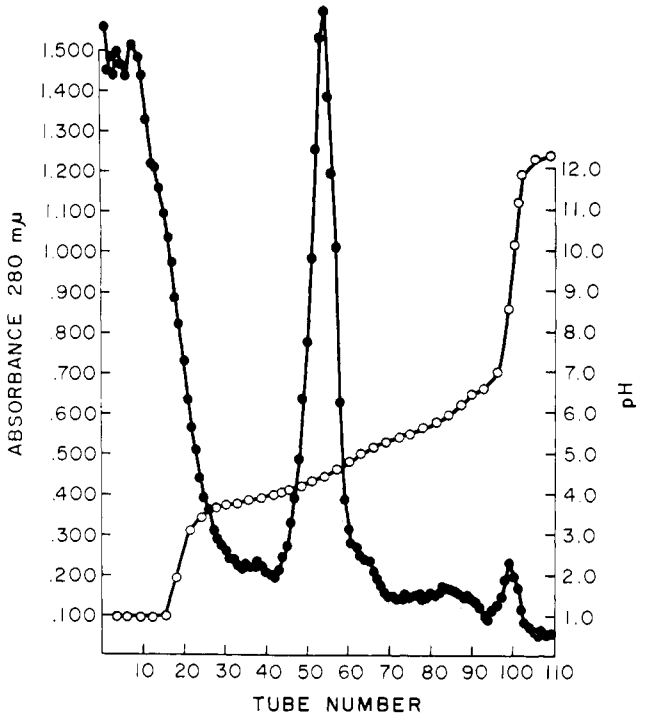

FIGURE 2: Isoelectrofocusing of purified lectin (fraction II) at pH 3–6. (●—●) Absorbance at 280 mμ; (O–O) pH.

Subtraction of the antigen nitrogen added from the total nitrogen precipitated at the equivalence zone shows that 87–99% of the nitrogen in the lectin preparation is precipitated by the human blood group A substance. The fractions also give identical precipitin curves with hog blood group A substance (hog 76) (Figure 3b); however with this antigen only 60–70% of the lectin nitrogen is precipitated. Two other hog blood group A preparations (hog 39 C and hog 60) gave similar results (*cf.* Figure 6). The lectin reacted equally well with the fraction of hog 76 precipitated by concanavalin A as well as with the nonprecipitable fraction (Lloyd *et al.*, 1969), as would be expected since both had comparable blood group A activity.

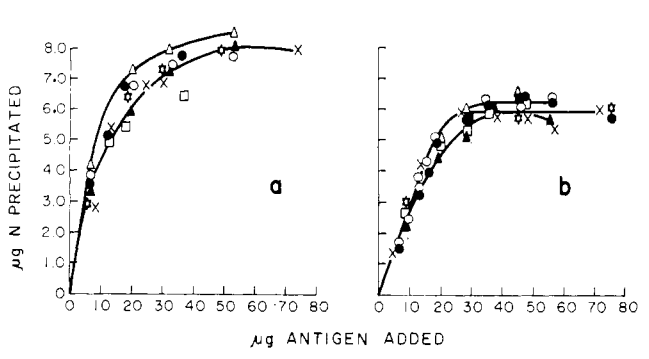

FIGURE 3: Precipitation of purified lectin fractions by (a) purified human blood group A substance (MSM) and (b) purified hog A blood group substance (hog 7.6). (O–O) Fraction I_1, 6.39 μg of N/tube; (●–●) fraction I_2, 6.53 μg of N/tube; (□–□) fraction I_3, 6.40 μg of N/tube; (■–■) fraction I_4, 6.32 μg of N/tube; (△–△) fraction I_5, 6.29 μg of N/tube; (▲–▲) fraction I_6, 6.53 μg of N/tube; (☆–☆) fraction I_7, 6.52 μg of N/tube; and (X–X) fraction II, 6.33 μg of N/tube.

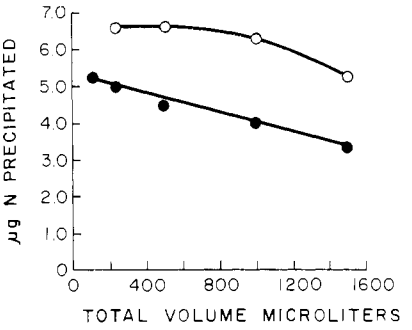

FIGURE 4: Effect of volume on precipitation of human and hog blood group A substances by purified lectin. (O–O) Human blood group A substance, MSM, 48.9 μg/tube; (●–●) hog blood group A substance (hog 76) 55.4 μg/tube. *Dolichos* lectin (55.4 μg of N) fraction I_3 was added to each tube.

Since the lectin was prepared by adsorption and elution from an immunoadsorbent of hog blood group substance and since fractions I_1–I_7 and fraction II react essentially identically with hog blood group A substance, it may be inferred that all of the purified lectin can combine with hog blood group substance. The failure of the hog material to precipitate all of the purified lectin may therefore be due to the formation of soluble complexes between lectin and hog blood group substance. A study of the effect of volume on the ability of the lectin to precipitate human and hog blood group A substances shows that the reaction with the hog material is more sensitive to volume than is the reaction with human material (Figure 4).

Another indication that soluble complexes are formed between hog 76 and lectin is obtained from a comparison of the percentage of added antigen remaining in the supernatants after precipitation of fraction II with MSM and with hog 76. Figure 5 shows that essentially all of the methylpentose of human A (MSM) is precipitated throughout the range studied

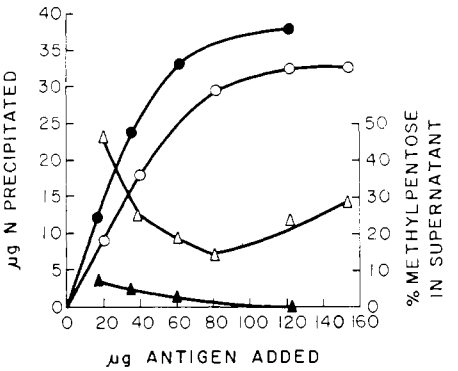

FIGURE 5: Analysis of supernatants after precipitation of human and hog blood group A substances by purified lectin (fraction II, 31.3 μg of N/tube). (●–●) μg of N precipitated by human blood group A substance (MSM), (O–O) μg of N precipitated by hog blood group A substance (hog 76); (▲–▲) per cent methylpentose remaining in supernatant after precipitation of human blood group A substance, (△–△) per cent methylpentose remaining in supernatant after precipitation of hog blood group A substance.

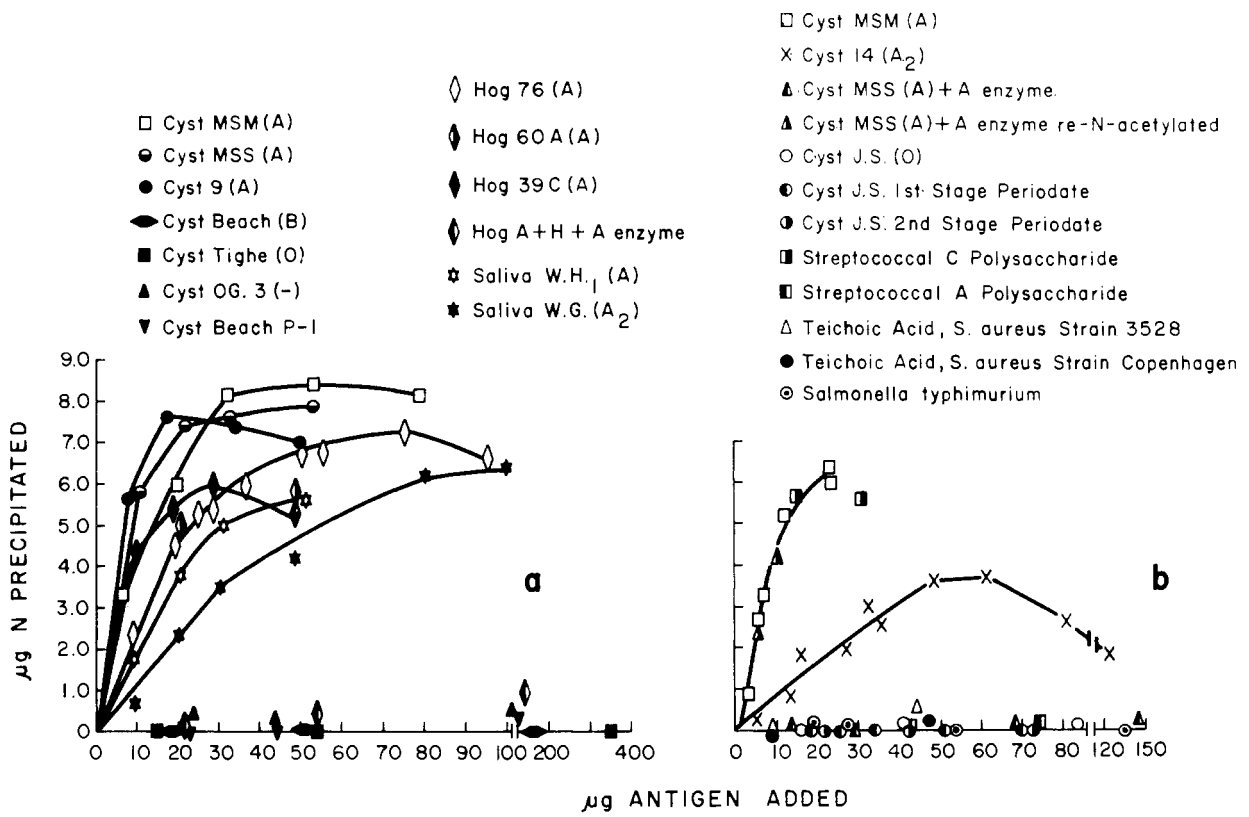

FIGURE 6: Precipitation of purified blood group substances and other polysaccharides by purified lectin. (a) Fraction I_6, 6.53 μg of lectin N/tube; (b) fraction II, 5.09 μg of lectin N/tube.

while with hog 76 a substantial proportion of the added methylpentose remains in the supernatant. Moreover with hog 76, the amount of methylpentose precipitated varied with the proportion of antigen to lectin throughout the precipitin curve. Moreover lectin was completely adsorbed on the polyleucyl A + H substance and could not be detected in the effluent until the column was saturated.

Addition of MSM to supernatants obtained after precipitation with hog 76 precipitates residual lectin.

A comparison of the ability of fraction I_6 to precipitate various blood group substances is shown in Figure 6. The lectin reacts strongly with the three human ovarian cyst blood group A preparations but gives no reaction with type B (Beach) or O (Tighe, J. S.) cyst blood group substances nor does it react with an A-, B-, H-, Le^a-negative cyst substance (OG), with the nondialyzable portion of a B cyst subjected to mild acid hydrolysis (Beach P-1), or with O cyst substance (J. S.) after one and two stepwise Smith degradations with periodate (Lloyd and Kabat, 1968). The lectin also precipitates human saliva blood group A substance showing a stronger reaction with A_1 saliva (W. H.) than with A_2 saliva (W. G.) and with A_1 cyst substance than with A_2 cyst substance. Similar precipitin curves are obtained with three different hog blood group A preparations (hog 76, 60, and 39C). The lectin did not react with hog A + H substance or with human A substance (MSM) after they had been treated with the de-N-acetylating enzyme from *Clostridium tertium* (Marcus *et al.*, 1964) which destroys type A specificity by removing the *N*-acetyl group on the terminal nonreducing *N*-acetyl-D-galactosamine; re-N-acetylation of MSM restored reactivity. The lectin is also precipitated by group C streptococcal polysaccharide (Krause and McCarty, 1962) which has terminal nonreducing GalNAc residues but does not precipitate with the group A streptococcal polysaccharide with terminal nonreducing GNAc residues (Krause and McCarty, 1961), or with teichoic acid preparations containing α- or β-D-GNAc residues. It failed to precipitate with a polysaccharide from *Salmonella typhimurium* containing nonreducing terminal residues of 2-*O*-acetyl-D-abequose.

Various monosaccharides and oligosaccharides were tested for their ability to inhibit the precipitation of human A substance (MSM) with lectin (Figure 7). Of the monosaccharides tested, only *N*-acetyl-D-galactosamine was able to inhibit precipitation. No significant inhibition was observed with *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, D-galactosamine hydrochloride, D-glucosamine hydrochloride, *N*-acetyl-D-galactosaminitol, D-galactose, L-fucose, D-mannose, or D-glucose when tested at concentrations higher than necessary to obtain complete inhibition with D-GalNAc. The glycoside, Me-α-D-GalNAc, was 2.5-fold better than D-GalNAc whereas Et-β-D-GalNAc was poorer than D-GalNAc. No inhibition was obtained with methyl α-D-galactoside, Me-α-D-GNAc, or E-β-D-GNAc. The A-active disaccharide, α-D-GalNAc-(1→3)-D-Gal, and trisaccharide, α-D-GalNAc-(1→3)-β-D-Gal-(1→3)-D-GNAc, were equal in inhibiting power on a molar basis to Me-α-D-GalNAc. The A-active reduced pentasaccharide required only 0.32 μmole for 50% inhibition as compared with 0.55 μmole for Me-α-D-GalNAc

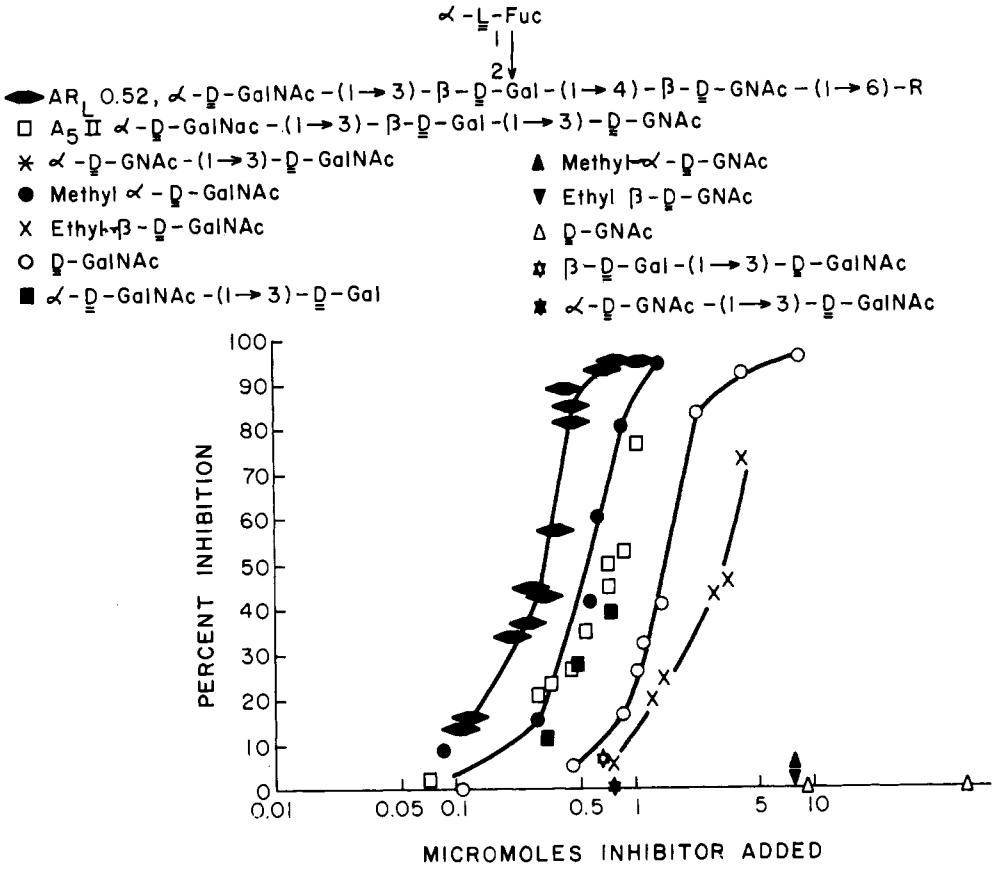

FIGURE 7: Inhibition by monosaccharides, methylglycosides and oligosaccharides of precipitation of human blood group A substance with purified lectin. Fraction II (5.09 μg of N) plus A substance (15.84 μg) (MSM). The following compounds were also tested and gave no significant inhibition, up to the amount indicated: D-ManNAc, 18.8 μmoles; D-GalNH$_2$, 11.7 μmoles; D-GlcNH$_2$, 27.5 μmoles; D-GalNAcol, 2,3 μmoles; D Gal 25.5 μmoles; L-Fuc, 26.2 μmoles; D-Glc, 27.4 μmoles; D-Man, 26.9 μmoles; Me-α-D-Gal, 8.0 μmoles. All glycosidically linked residues are in the pyranose form.

The disaccharides, β-D-Gal-(1→3)-D-GalNAc and α-D-GNAc-(1→3)-D-GalNAc did not give any inhibition at the concentrations tested.

α-L-Fuc
1
2
α-D-GalNAc-(1→3)-β-D-Gal-(1→4)-β-D-GNAc-(1→6)-R

A comparison of inhibition of precipitation of MSM with fractions I_1–I_8 and fraction II with D-GalNAc and Me-α-D-GalNAc is shown in Figure 8. No difference in inhibition was obtained with any of the fractions.

Discussion

The results outlined above describe the purification of the *D. biflorus* lectin by adsorption onto polyleucyl hog blood group A + H and specific elution with *N*-acetyl-D-galactosamine. The eluted lectin sediments as a single peak in the analytical ultracentrifuge, forms a single broad band in acrylamide electrophoresis, and gives one band in immunoelectrophoresis and immunodiffusion against antisera prepared against the crude seed extract, although the original extract contained at least ten components in acrylamide and five distinct antigens. The purified lectin tends to aggregate to a small extent on standing in concentrated solutions.

The homogeneity of the lectin preparation by the above criteria (Figure 1) and the finding that 87–99% of the nitrogen in the preparation is precipitated by human blood group A substance attest to the utility of the immunoadsorbent method for preparing the lectin in a highly purified state. Similar studies with insoluble blood group immunoadsorbents have recently been carried out on a hemagglutinin of the snail *Helix pomatia* (Hammarström and Kabat, 1969), on the blood group specific lectin *Lotus tetragonolobus* with a synthetic fucosyl immunoadsorbent (Yariv *et al.*, 1967; Kalb, 1968) and on non blood group specific lectins such as concanavalin A (Agrawal and Goldstein, 1965) and the lectin from *Lens culinaris* (McGregor and Sage, 1968; Howard and Sage, 1969), using Sephadex as an insoluble adsorbent as had been described for fractionation and purification of human antidextran (Schlossman and Kabat, 1962; Gelzer and Kabat, 1964).

The lectin is a glycoprotein containing about 2% hexose. In this respect it resembles the soybean lectin (Lis *et al.*, 1966a,b) and the lectins from *Lotus tetragonolobus* (Kalb, 1968), *Lens culinaris* (Howard and Sage, 1969) and *Phaseolus vulgaris* (Takahashi and Liener, 1968) which are reported to have carbohydrate contents of 2–10%. It is also similar to other

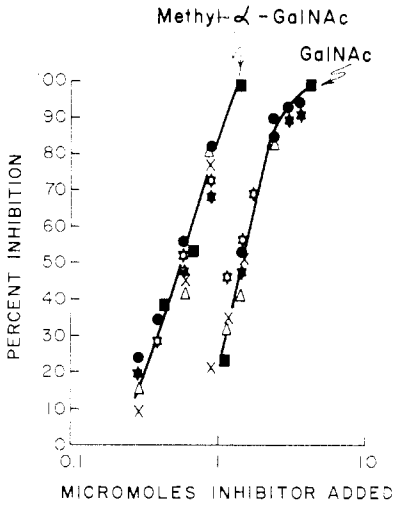

FIGURE 8: Comparison of inhibition of precipitation of six different purified lectin fractions with Me-α-D-GalNAc and D-GalNAc. (●–●) Fraction I_2, 4.78 μg of N/tube; (■–■) fraction I_4, 4.90 μg of N/tube; (△–△) fraction I_5, 5.13 μg of N/tube; (☆–☆) fraction I_7, 4.98 μg of N/tube (★–★) fraction I_8, 5.21 μg of N/tube; (X–X) fraction II, 5.09 μg of N/tube. Human A substance (15.84 μg) (MSM) was added to each tube. The same ratio of effectiveness of the two inhibitors at 50% inhibition was also obtained in inhibition of precipitation of hog A substance (hog 76) with fraction I although in this case the curves were shifted to the left.

lectins in that the amino acid analyses show a high content of serine and aspartic acid as also noted by Kocourek and Jamieson (1967) and no detectable methionine or cysteine (cystine). No sulfur-containing amino acids are reported in the *Lens culinaris* lectin (McGregor and Sage, 1968) while concanavalin A (Olson and Liener, 1967), and the lectins from *Phaseolus vulgaris* (Takahashi *et al.*, 1967), *Lotus tetragonolobus* (Kalb, 1968), and soybean (Lis *et al.*, 1966a,b) contain small amounts of methionine and no cysteine. The *Dolichos* lectin has an approximate molecular weight of 140,000 as determined by sedimentation and viscosity experiments.

In addition to confirming the specificity of the lectin for blood group A substance, the quantitative precipitin studies with the various A, B, and H substances showed two points of interest. (1) The lectin was able to react with both A_1 and A_2 blood group substances thereby confirming the report of Bird (1959) and contradicting previous studies in which the lectin was said to be A_1 specific (*cf.* Boyd and Shapleigh, 1954a,b). The early studies had been done with seed extracts which may not have contained lectin in amounts sufficient enough to detect the weak A_2 hemagglutination (Table I). (2) The lectin appears to form specific precipitates which are somewhat more soluble with hog A substance than with human A substance. This may be due to differences in the number of antigenic determinants per molecule since a similar solubility phenomenon has been reported in the precipitation of dextran with concanavalin A in which more soluble complexes occur with those dextrans that are not highly branched (Goldstein *et al.*, 1968).

Inhibition of precipitation of the lectin and human blood group A substance was used in an effort to determine the specificity and size of the combining site. *N*-Acetyl-D-galactosamine, which is the immunodominant sugar of blood group A specificity (*cf.* Kabat, 1956; Morgan, 1960), was the only monosaccharide tested that was able to inhibit precipitation. The failure of galactosamine hydrochloride and *N*-acetyl-D-mannosamine to inhibit precipitation show the importance of the *N*-acetyl group and its orientation on C-2 to the specificity of the lectin. The importance of the *N*-acetyl group is also indicated by the failure of the lectin to precipitate with blood group A substance treated by the de-N-acetylating enzyme from *Clostridium tertium* and the restoration of activity by re-N-acetylation.

The specificity of the lectin is also critically determined by the proper stereochemical arrangement of the hydroxyl on C-4 as shown by the failure of *N*-acetyl-D-glucosamine to inhibit precipitation. At present no information is available on the stereochemical requirements of C-3 and C-6 although failure to precipitate the *S. typhimurium* (LT-2) polysaccharide which contains terminal nonreducing α-linked 2-*O*-acetyl-D-abequose residues (2-*O*-acetyl-3,6-dideoxy-D-xylo-hexopyranosyl residues.) (Hellerqvist *et al.*, 1969) suggests that one or both of these positions are important. The finding that the methyl α-glycoside of GalNAc was a more effective inhibitor than GalNAc and the large amounts of the ethyl β-glycoside required show a strong specificity of the lectin for the α linkage.

The A-active di- and trisaccharides were equal to Me-α-D-GalNAc as inhibitors; however the A-active reduced pentasaccharide was definitely better than Me-α-D-GalNAc. The A-active tri- and pentasaccharides differ in three respects: (1) a fucose is present on carbon 2 of the galactose; (2) the linkage of the Gal to the GNAc is 1→3 as compared with 1→4; and (3) in the pentasaccharide the GNAc is linked to a hexenetetrol.

Since the disaccharide, α-D-GalNAc-(1→3)-D-Gal, is as active as the trisaccharide, α-D-GalNAc-(1→3)-β-D-Gal-(1→3)-D-GNAc, it is inferred that the hexenetetrol and the linkage of the Gal to the GNAc are probably not involved in the specificity of the lectin site. One would then be inclined to infer that the increased potency of the pentasaccharide is attributable to the α-L-Fuc-(1→2) linkage. This might be ascribed either to the fucose stabilizing a favorable conformation of the α-D-GalNAc linkage to the rest of the molecule thus facilitating entry into the lectin site or to the fucose creating a more hydrophobic environment on the determinant and thus facilitating contact with the site or by some type of hydrophobic interaction between the fucose and the lectin. L-Fucose itself does not react with the lectin. These findings result in some ambiguity in the estimation of the size of the combining site. While it may be no larger than an α-linked D-GalNAc as has been reported for the snail hemagglutinin (Hammarström and Kabat, 1969) the contribution attributed to the fucose whether specific or nonspecific provides a high degree of blood group A specificity which might not otherwise be seen. More precise evaluation of the size of the combining site will require further study.

The inhibition studies show that the specificity of the lectin for blood group A substance is due predominately to its ability specifically to recognize terminal nonreducing *N*-acetyl-D-galactosamine residues bound with an α linkage to other sugars. The strong precipitation with streptococcal group C polysaccharide which contains terminal D-GalNAc residues (Krause and McCarty, 1962) indicates that they are linked α to the rhamnose backbone. The lectin should thus

prove to be a valuable reagent for detecting terminal α-D-GalNAc linkages in oligo- and polysaccharides.

The comparison of the properties and inhibition of the various fractions eluted from the immunoadsorbent has resulted in the detection of no heterogeneity in the materials eluted from the immunoadsorbent in the 50% of the original material recovered.

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