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Specificity of Purified Hemagglutinin (Lectin) from *Lotus tetragonolobus*†

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ABSTRACT: The lectin from *Lotus tetragonolobus* seeds was purified by adsorption on to insoluble polyleucyl hog A + H blood-group substance and subsequent elution with L-fucose. The isolated lectin formed one line in immunodiffusion and immunoelectrophoresis against three rabbit antisera to the crude seed extract. The purified lectin showed three components on acrylamide electrophoresis under alkaline or acid conditions; these were separated by preparative isoelectric focusing having isoelectric points (IEP) of pH 5.4, 6.2, and 7.1 and corresponded to fractions B, C, and A obtained earlier on DEAE-cellulose (Kalb, A. J. (1968), *Biochim. Biophys. Acta 168,* 532). The three fractions and the original lectin are made up of noncovalently linked subunits of molecular weight of about 27,000. The purified lectin precipitated with blood-group H, Le^a and A_2 substances; it did not precipitate with blood group A_1 or B substances, with a precursor blood-group substance having I and i activities, nor with a periodate-oxidized and Smith-degraded H substance. Inhibition of precipitation with various monosaccharides and with milk and blood-group oligosaccharides indicated that the lectin is strongly specific for type 2 chains containing fucose residues on C-2 of the galactose of βDGal(1→4)DGlcNAc, with or without a second fucose on the DGlcNAc, but does not react with similarly substituted type 1 chains of structure βDGal(1→3)DGlcNAc. Inhibition data with the three fractions obtained by isoelectric focusing indicate that they have the same specificity although Kalb found them to have different association constants.

Proteins with the capacity to agglutinate red blood cells are widely dispersed in plants, vertebrates, and invertebrates (for reviews, see Kabat, 1956a, Prokop *et al.,* 1968, and Sharon and Lis, 1972). Some of these proteins, known as lectins, are highly specific in that they agglutinate erythrocytes of the human ABO or MN groups, while others agglutinate independently of these blood groups. Other lectins may precipitate various polysaccharides and glycoproteins specifically (Hammarström and Kabat, 1969; Etzler and Kabat, 1970; Lloyd and Bitoon, 1971), and have been used (Rovis *et al.,* 1973a) in structural studies on oligosaccharides isolated from various blood-group substances and of the other polysaccharides. Lectins also possess a number of other biological properties, such as stimulating resting lymphocytes to divide and form blast-like immature cells, causing agglutination of malignant cells, or of platelets, liberation of thrombin and interference with the fertilization of sea urchin eggs (*cf.* Lis and Sharon, 1973). Although many lectins have been studied as crude extracts (Mäkela, 1957), only a few have been purified, characterized, and studied immunochemically. These include concanavalin A (Agrawal and Goldstein, 1965; Lloyd *et al.,* 1969; Poretz and Goldstein, 1970), the hemagglutinins from *Dolichos biflorus* (Etzler and Kabat, 1970), *Helix pomatia* (Hammerström and Kabat, 1969), lima bean (Galbraith and Goldstein, 1972), soybean (Gordon *et al.,* 1972), wheat germ (Nagata and Burger, 1972; LeVine *et al.* 1972), and others (*cf.* Sharon and Lis, 1972).

The lectin from the seeds of *Lotus tetragonolobus* was shown to agglutinate human O(H) red blood cells (Renkonen, 1948), and the agglutination was specifically inhibited by L-fucose (Morgan and Watkins, 1953). This lectin was precipitated (Yariv *et al.,* 1967) using a trifunctional fucosyl dye, the precipitate dissolved in fucose, and the dye separated from the lectin using an ion-exchange resin. The purified hamagglutinin was shown to be composed of three types of L-fucose-binding molecules (Kalb, 1968), differing in molecular weight and binding constants.

In the present study we report the purification of the lectin from the seeds of *L. tetragonolobus* using, as an immunoadsorbent, an insoluble fucose-containing polyleucyl hog blood-group A + H substance (Kaplan and Kabat, 1966; Moreno and Kabat, 1969; Hammarström and Kabat, 1969; Etzler and Kabat, 1970; Galbraith and Goldstein, 1972), and elution with L-fucose, its characterization, and a study of the nature and specificity of its combining site. The purified lectin precipitated H, A_2, and Le^a substances, but not A_1 substances, proved to have a striking specificity for type 2 chains of blood-group substances containing fucosyl residues on C-2 of the β-

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DGal(1→4)DGlcNAc,[1] and failed to react with the type 1 chain β-DGal(1→3)DGlcNAc similarly substituted. A second fucose on C-3 of the DGlcNAc of the type 2 chain further increased activity. This lectin has proven a highly valuable reagent for elucidating structures of blood-group oligosaccharides (Rovis *et al.*, 1973a).

The purified lectin contains three components which were separated by DEAE-cellulose column chromatography (Kalb, 1968) or by preparative isoelectrofocusing. Each fraction is composed of noncovalently linked subunits of molecular weight of about 27,000. Inhibition data indicate that all three fractions have the same relative specificity in their reactions with L-fucose, methyl α-L-fucopyranoside, and 2′-fucosyllactose and none reacted with the type 1 fucosyl oligosaccharide, lacto-*N*-fucopentaose I. All fractions precipitate equally well with blood-group substance, but differ in their hemagglutination capacity.

Materials

L. tetragonolobus seeds were purchased from Thompson and Morgan, Ltd., Ipswich, England. The blood-group substances used were obtained from human ovarian cysts or salivas, from hog gastric mucin, and from bovine or horse stomach linings, as previously prepared and described in this laboratory (*cf.* Kabat, 1956a; Schiffman *et al.*, 1964; Lloyd *et al.*, 1968; Vicari and Kabat, 1969; Hammarström and Kabat, 1969). Monosaccharides were obtained commercially (Mann Research Laboratories or Nutritional Biochemicals Corp.). The blood-group oligosaccharides used were those described previously (Lloyd *et al.*, 1966). Milk oligosaccharides were gifts from the late Professor Richard Kuhn, Dr. Adeline Gauhe, and Dr. Victor Ginsburg and urine oligosaccharides from Dr. Arne Lundblad.

The immunoadsorbent hog A + H blood-group substance (PL-hog A + H) was obtained by copolymerization of the purified hog A + H substance (Kabat, 1956a) with the *N*-carboxyanhydride of L-leucine (Pilot Chemicals) by the technique of Tsuyuki *et al.* (1956) as described by Kaplan and Kabat (1966).

Methods

Analytical Methods. Methylpentose (fucose), hexosamine, *N*-acetylhexosamine, hexose (galactose), and nitrogen were determined by colorimetric methods as described (Kabat, 1961; Lloyd *et al.*, 1966). Periodate uptake has also been described previously (Kabat, 1961).

Physicochemical Methods. Sedimentation velocity was measured in a Spinco Model E ultracentrifuge, equipped with phase-plate schlieren optics and an automatic temperature control.[2] The sedimentation coefficient was calculated at a lectin concentration of 2 mg/ml in phosphate-buffered saline (pH 7.1) from measurements at 50,740 rpm and 8.8°.

Disc electrophoresis was performed in polyacrylamide gels according to the method of Davis (1964) and Reisfeld and Small (1966) as described by Dorner *et al.* (1969). A 10% gel was employed at pH 9.3 and 2.7. Electrophoresis was at an initial constant current of 1 mA/tube, until the buffer line passed from the spacer gel to the small pore gel. The current was then increased to 2.5 mA/tube and the electrophoresis was stopped 30 min after the buffer line reached the end of the small pore gel. The gels were fixed and stained in 0.02% Coomassie Blue in 12.5% trichloroacetic acid and destained in a solution containing methanol (25%) and acetic acid (10%).

The purified *Lotus* lectin and its three fractions were examined by the method of Weber and Osborn (1969) by electrophoresis in 10% polyacrylamide gels prepared in 0.01 M sodium phosphate buffer (pH 7.0), containing 0.1% sodium dodecyl sulfate. The lectin solutions were dialyzed at room temperature against 0.01 M PBS (pH 7.0), containing 0.1% sodium dodecyl sulfate, with or without 0.1% β-mercaptoethanol. Protein markers used were: bovine serum albumin (mol wt 68,000), ovalbumin (mol wt 43,000), pepsin (mol wt 35,000), chymotrypsinogen (mol wt 25,700), trypsin (mol wt 23,300), and lysozyme (mol wt 14,300). Approximately 12 μg of the purified lectin and fractions and of the molecular weight markers were used. Mobility was measured relative to Bromophenol Blue.

Separation Procedures. Preparative isoelectrofocusing was performed according to the directions of the manufacturer (LKB-Producter AB, Sweden) with a 110-ml column at 10°, using 2% carrier ampholytes (Ampholine) (pH 3–10) and a sucrose gradient for 48 hr at 3 W or less.

Gel filtration was carried out on Bio-Gel P-10 at 4°. Protein solutions were concentrated by ultrafiltration using collodion bags (Schleicher Schuell Co., Keene, N. H.).

Immunological Methods. Hemagglutinin titer was determined with the Takatsy microtitrator (Cooke Engineering Co., Alexandria, Va.) using 0.025-ml loops and a 2% suspension of erythrocytes. Equal volumes of the red cell suspension and the lectin dilution were mixed, placed at room temperature for 1 hr, and read.

Quantitative precipitin and inhibition analyses with the purified lectin were carried out by a microprecipitin technique (Kabat, 1961); 5–7 μg of N of the hemagglutinin was mixed with different amounts of blood-group substances and the mixtures were incubated at 37° for 1 hr and then kept at 4° for 1 week. The N in the washed precipitates was determined by the ninhydrin procedure (Schiffman *et al.*, 1964). For inhibition assays, known quantities of sugar were added to amounts of lectin and blood-group substance giving maximum precipitation.

Double diffusion in agar was done using 1.5% agar (Special Noble Agar, Difco Laboratories, Detroit, Mich.) in 0.05 M sodium barbital buffer (pH 8.3) according to the procedure of Ouchterlony (1948). The lectin and the rabbit antisera were allowed to diffuse for 5 days, with daily inspection. Immunoelectrophoresis was performed as reported by Grabar and Williams (1953), using the agar as prepared for immunodiffusion; the lectin was electrophoresed at 150 V for 110 min.

Antisera were produced by immunizing rabbits with crude extracts of *L. tetragonolobus*. The animals were injected into the footpads with a mixture of about 2 mg of lectin extract with Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.) once a week for 3 weeks. The animals were bled by cardiac puncture 1 week after the final injection for 3 consecutive days, and the sera of these three bleedings from each animal were pooled. After 1 month, the immunization and bleeding plan was repeated and a second batch of antisera was obtained.

Experimental Section and Results

Purification of the Lectin. The seeds of the *L. tetragonolobus* were ground in a mortar to a fine powder, which was then extracted with ten volumes 0.001 M phosphate-buffered saline (0.15 M) (pH 7.1) (PBS). The suspension was kept overnight at 4° and centrifuged at 2000 rpm in the cold. The supernatant

[1] Abbreviations used are PBS, phosphate-buffered saline; IEP, isoelectric point; L-fuc, L-fucopyranose; D-Gal, D-galactopyranose; D-Glc, D-glucopyranose; D-GalNAc, 2-acetamido-2-deoxy-D-galactopyranose; DGlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; R, 3-hexenetetrols; Me, methyl.

[2] The authors are indebted to Dr. H. Rosenkranz and to Mr. Samuel Rosenkranz for the ultracentrifugal data.

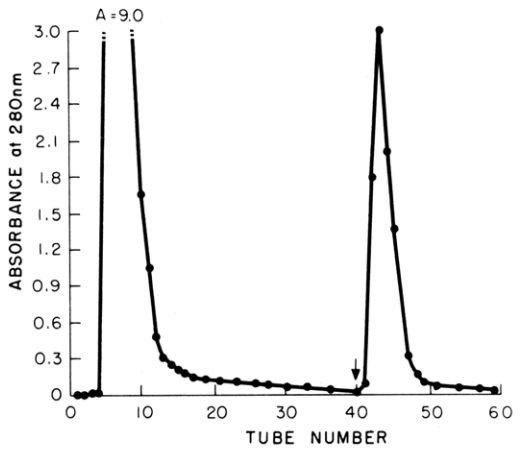


FIGURE 1: Isolation of the *L. tetragonolobus* lectin from a PL-hog A + H column (3.5 × 0.9 cm). The seed extract was applied to the column and, after the immunoadsorbent was saturated with the lectin, it was washed with 0.001 M PBS (pH 7.1) and then eluted (arrow) with 0.01 M L-fucose, collecting 5.0 ml/tube; flow rate 1.0 ml/min at 4°.

was filtered through a Millipore filter (0.45 μ). It agglutinated human O erythrocytes to a titer of 32 and also agglutinated A_2 and B cells weakly (titer 4); no agglutination was observed with A_1 cells.

PL-hog A + H was ground in a mortar with washed Celite, in a proportion of 1:5, and the mixture was poured into a small column and washed extensively with 0.001 M PBS (pH 7.1). The crude *Lotus* extract was then applied continuously to the column, and 5.0-ml fractions were collected until the titer against O(H) erythrocytes of the effluent equaled that of the initial extract. Crude extract (20 ml) was necessary to saturate a column of 100 mg of PL-hog A + H. The column was then washed with buffer until the absorbance at 280 nm was less than 0.030.

Specific elution of the hemagglutinin was effected by 0.01 M L-fucose in 0.001 M PBS (pH 7.1) (Figure 1). The peak obtained was pooled and concentrated by ultrafiltration, and the fucose was separated from the lectin on a Bio-Gel P-10 column (63 × 1.8 cm) (Hammarström and Kabat, 1969; Etzler and Kabat, 1970). The lectin appeared as a single symmetrical peak in the excluded volume and the fucose was retarded giving a separate peak as detected by periodate uptake. The protein peak was pooled, concentrated by ultrafiltration, and rechromatographed on the same Bio-Gel P-10 column; periodate-positive material was not found. The isolated lectin agglutinated a 2% suspension of O(H) erythrocytes at a minimum protein concentration of 33 μg/ml.

The purity of the lectin was assayed by double diffusion and immunoelectrophoresis using rabbit antisera against the crude extract. As can be seen in Figure 2A,B, the isolated hemagglutinin gave only one band in gel diffusion, at concentrations ranging from 26 to 250 μg of N per ml, while the crude extract revealed at least five bands. The lines of the isolated lectin showed complete fusion and fused with but one of the lines of the crude extract.

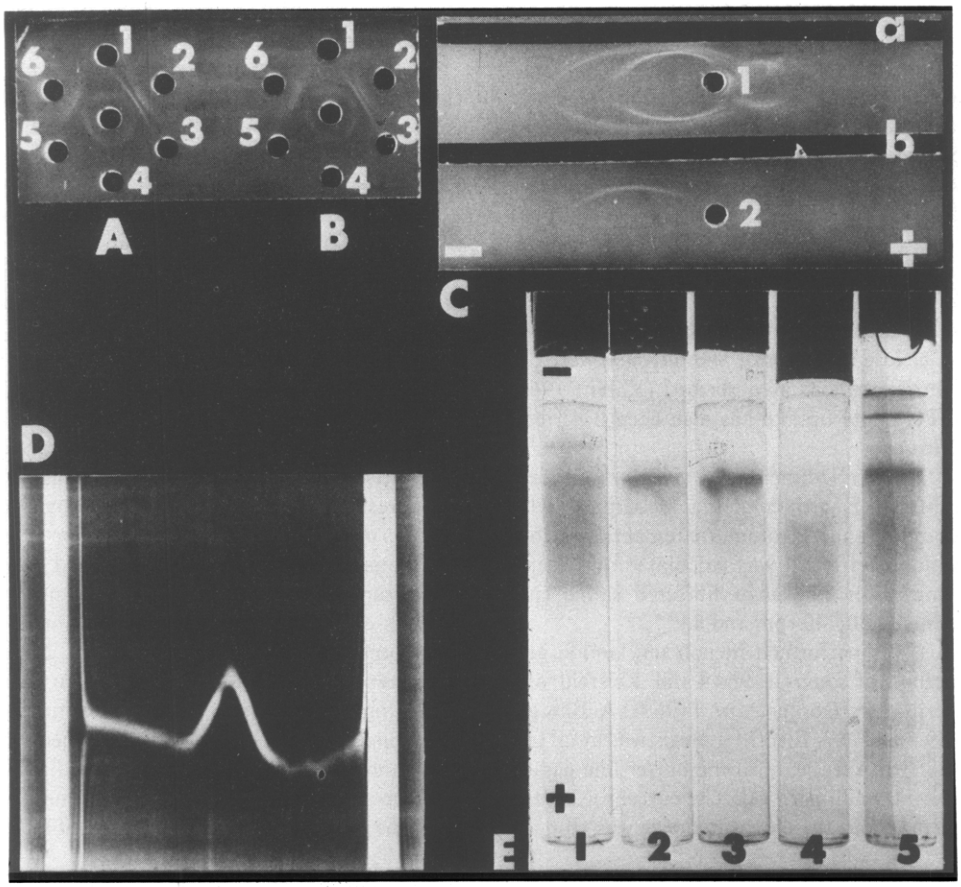


FIGURE 2: Immunodiffusion, immunoelectrophoretic, and ultracentrifugal patterns of the Lotus lectin. (A and B) Immunodiffusion of crude extract and purified lectin preparations. Center wells: two different rabbit antisera to crude *L. tetragonolobus* extract. Peripheral wells: (1) purified lectin, 81.7 μg of N/ml; (2) crude seed extract; (3) purified lectin, 150 μg of N/ml; (4) purified lectin, 26 μg of N/ml; (5) purified lectin, 250 μg of N/ml; (6) crude seed extract. (C) Immunoelectrophoresis of crude extract (well 1) and of purified lectin (250 μg of N/ml, well 2). Troughs a and b contain rabbit antiserum to the crude seed extract. (D) Schlieren pattern of purified lectin (2.0 mg/ml in 0.001 M PBS, pH 7.1). Photograph after 124 min at 50,740 rpm. (E) Disc electrophoresis at pH 9.2: (1) purified lectin, 15 μg of N; (2) fraction I, 10.7 μg of N; (3) fraction II, 17.0 μg of N; (4) fraction III, 19 μg of N; (5) seed extract, about 22 μg of N.

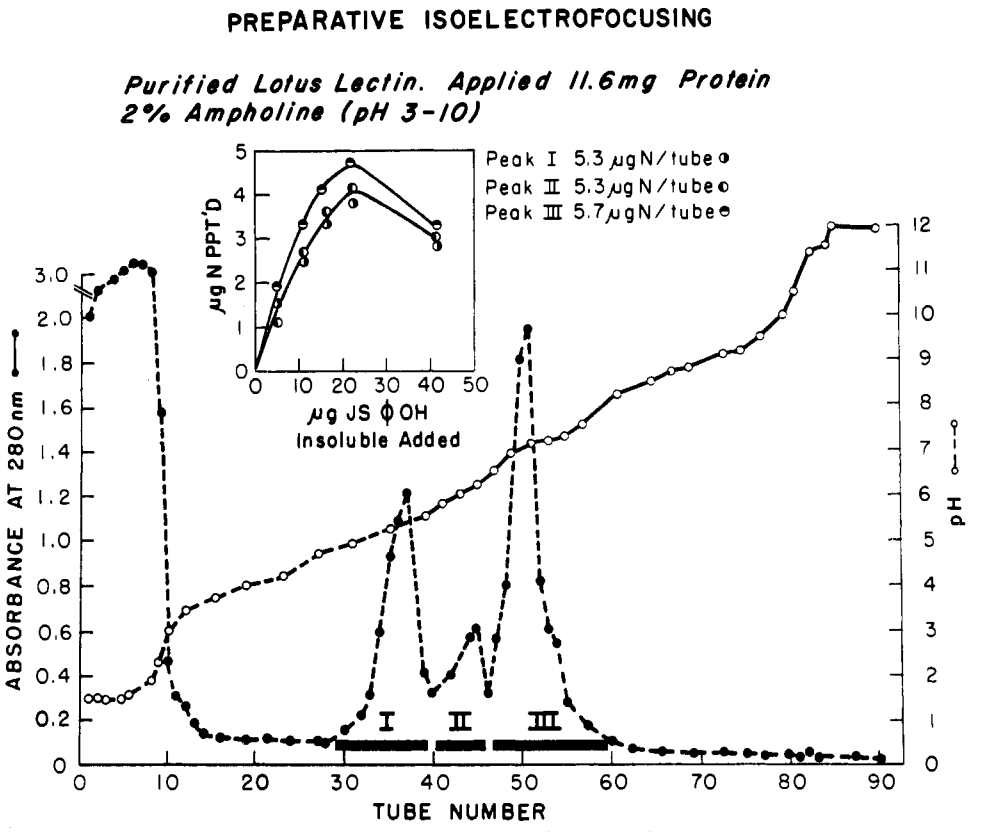


FIGURE 3: Isoelectric focusing of purified *Lotus* lectin. Inset shows the precipitin curves of the isolated fractions with human blood-group H substance JS.

Further evidence for the purity of the isolated lectin was disclosed by immunoelectrophoresis which showed one arc for the isolated lectin with three rabbit antisera to the crude extract (Figure 2C). These antisera reacted with crude extracts of the lectin to give at least ten arcs; 7.5 mg of purified lectin was recovered from the 20 ml of crude extract which saturated 100 mg of PL-hog A + H.

Physical and Chemical Analyses. In the analytical ultracentrifuge using schlieren optics the purified *Lotus* lectin, at the concentration of 2 mg/ml in 0.001 M PBS (pH 7.1), sedimented as a single peak with a s_{20w} of 7.2 S plus a considerable amount of diffuse material behind the peak (Figure 2D) which amounted to 55% of the total refractive index. Disc electrophoresis in polyacrylamide gels at pH 9.3 and 2.7 showed that the purified lectin consisted of three fractions; the fastest moving fraction was very broad while the others were relatively sharp (Figure 2E); these were separated by preparative isoelectric focusing (Figure 3) using the 110-ml LKB column. Components I, II, and III appeared as single peaks, with isoelectric points of 5.4, 6.2, and 7.1 and with relative proportions of 33, 14, and 53%, respectively. When tested in polyacrylamide gel electrophoresis at concentrations of 10–19 μg of nitrogen, under both alkaline and acid conditions components I and II gave a single sharp band, while component III was quite diffuse (Figure 2E). Component I required 410–450 μg of protein/ml to agglutinate a 2% suspension of O(H) human erythrocytes, while the mini-

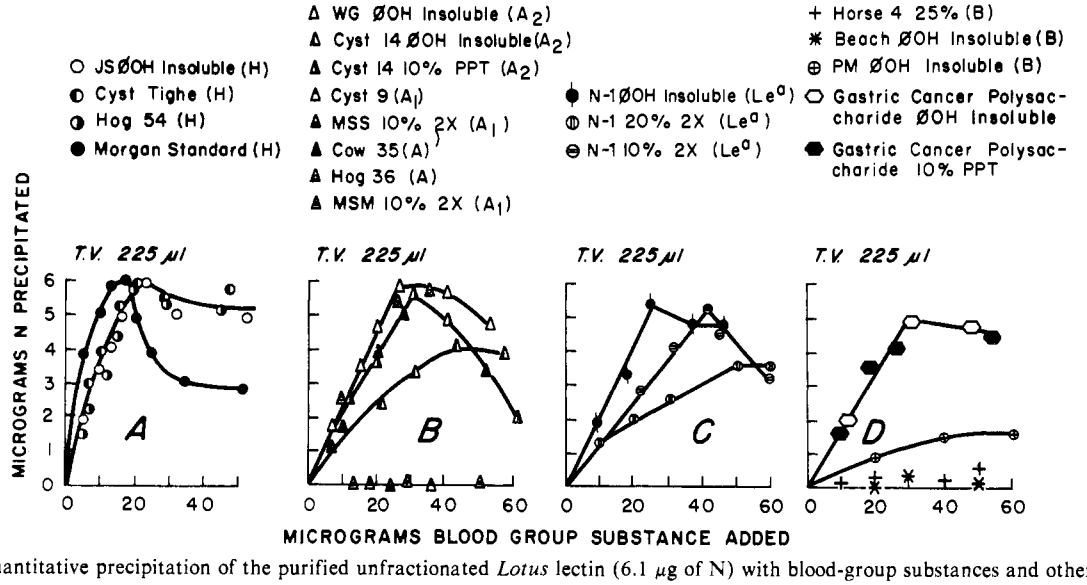


FIGURE 4: Quantitative precipitation of the purified unfractionated *Lotus* lectin (6.1 μg of N) with blood-group substances and other polysaccharides.

mum agglutinating activity of fractions II and III was about 13–20 and 12–24 μg per ml, respectively, as compared with 33–50 μg/ml for the original mixture of the three components. When applied to a DEAE-cellulose column, component III was eluted with the starting 0.01 M sodium phosphate buffer (pH 7.3), while fractions I and II appeared in the effluent using a linear gradient of NaCl in the same buffer, as described by Kalb (1968). Fraction B by isoelectric focusing required about 700 μg/ml while fraction A and C required 72–110 and 175 μg per ml, respectively. Fractions B, A, and C correspond to I, III, and II by isoelectric focusing, respectively.

On sodium dodecyl sulfate polyacrylamide gels, the unfractionated purified *Lotus* lectin migrated as a homogeneous molecular species with a molecular weight of about 27,000. Fractions I, II, and III obtained by isoelectric focusing also formed single sharp bands, with maximum molecular weights (*cf.* Segrest and Jackson, 1972) of 27,800, 27,000, and 27,800, respectively. The same values were obtained in sodium dodecyl sulfate, with or without β-mercaptoethanol; the lectin contains no cysteine (Kalb, 1968). Colorimetric analyses gave values of 2% hexosamine, 2.2% *N*-acetylhexosamine, and 1.2% galactose; no fucose was detected.

Immunochemical Specificity. Figure 4A shows the precipitation of the purified unfractionated *L. tetragonolobus* lectin with blood-group H substances. It is seen that two human ovarian cyst H substances (JS C_6H_5OH insoluble and Tighe) and a hog H substance (hog 54) precipitate equally well with the purified lectin, while another human H substance (Morgan standard H) is more active on a weight basis; for 50% precipitation of the lectin, 4 μg of the Morgan standard H was required as compared with 9 μg for the other three H substances. Another difference was noted in the region of excess H substance in that precipitation of the purified lectin was much more strongly inhibited by the Morgan standard H than by the other three substances. Subtraction of H substance nitrogen added from the total nitrogen precipitated at the equivalence zone shows that 83–88% of the purified *Lotus* lectin is precipitable by the H substances used.

The reaction of blood-group A substances with the purified unfractionated *Lotus* lectin is shown in Figure 4B; the hemagglutinin can distinguish human A_1 (MSS 10% 2×, MSM 10% 2×, and cyst 9) from human A_2 blood-group substance (WG C_6H_5OH insoluble, cyst 14 C_6H_5OH insoluble, and cyst 14 10% precipitate). This is quite striking as the lectin does not precipitate A_1 substances, while the precipitin curves with WG C_6H_5OH insoluble (A_2), cyst 14 10% precipitate and cyst 14 C_6H_5OH insoluble show them to be about 70, 58, and 33% as active as blood-group H substance JS C_6H_5OH insoluble. Hog A substance (hog 36) was as active as WG C_6H_5OH insoluble (human A_2), while cow 35 (A) gave a precipitin curve identical with that of cyst 14 10% precipitate (A_2).

The purified *Lotus* lectin was also precipitated by Le^a substances (N-1 fractions, Figure 4C); N-1 C_6H_5OH insoluble has almost twice the activity of N-1 20% 2×, while N-1 10% 2× was poorer than the other two N-1 fractions.

The B substances precipitated relatively poorly with the purified *L. tetragonolobus* lectin (Figure 4D); thus, Beach C_6H_5OH insoluble and horse 4 25% precipitated negligible amounts of lectin, and PM C_6H_5OH insoluble from human saliva had slight activity when used in high amounts.

The lectin did not react with the precursor substance with I and i activity, OG 20% 2×, nor did it react with a periodate-oxidized Smith-degraded H substance JS 1st IO_4^-, nor with cow 45 P1 (Allen and Kabat, 1959) nor Beach P1 (Allen and Kabat, 1959) obtained by mild acid hydrolysis. It reacted quite well with two gastric cancer polysaccharide fractions (Iseki *et al.*, 1962) which were shown to have weak Le^a and H activity (Vicari and Kabat, 1969).

The three fractions of the purified *Lotus* lectin obtained by isoelectric focusing precipitate equally well with human H substance JS C_6H_5OH insoluble (Figure 3, inset). It should be noted that although component I was less active in agglutinating human red blood cells, it gave a typical precipitin curve with human H substance JS.

Figure 5 summarizes the ability of monosaccharides, milk oligosaccharides, and blood-group oligosaccharides to inhibit precipitation of the purified unfractionated lectin by human H substance JS C_6H_5OH insoluble. Table I shows the structures and activities of the oligosaccharides involved. It is evident that the two H-active difucosyl oligosaccharides JS R_{IM5} 2.5 (Lloyd *et al.*, 1966) and lacto-difucotetraose were the best inhibitors, 40 and 80 nmol giving 50% inhibition. Two monofucosyl oligosaccharides JS R_L 0.75 (H active) (Lloyd *et al.*, 1966) and 2′-fucosyllactose were also good inhibitors, although less active than the corresponding difucosyl oligosaccharides, 105 and 150 nmol giving 50% inhibition, respectively. All of these have a structure involving a βDGal(1→4) linkage to DGlcNAc or DGlc; they represent the type 2 chain of blood substances (Watkins, 1972; Kabat, 1973) with L-fucosyl residues attached (Table I). However if the fucosyl residue is linked to C-2 of the terminal nonreducing β-linked galactose of the type 1 chain (*e.g.*, lacto-*N*-fucopentaose I), the resulting oligosaccharide is inactive, no inhibition being obtained with up to 580 nmol. Even the presence of a second fucose in the molecule, linked to DGlcNAc (lacto-*N*-difucohexaose I), does not give significant inhibitory activity in this range. The non-fucose-containing oligosaccharides lactose and lacto-*N*-tetraose are also inactive. One may thus infer that an L-fucosyl residue on C-2 of galactose of these H-active type 2 chains (JS R_L 0.75 and 2′-fucosyllactose) is necessary for inhibition, and that addition of a second fucose to the DGlcNAc or DGlc (JS R_{IM5} 2.5 and lacto-difucotetraose) increases inhibitory power. The mono- and difucosyl compounds containing DGlcNAc were more active than those containing DGlc. Similarly substituted oligosaccharides of the type 1 chain fail to inhibit the precipitation of the purified *Lotus* lectin by human H substance JS.

The immunodominant group of A(αDGalNAc) and B(αDGal) substances linked 1→3 to the DGal completely blocked the activity of the H-active oligosaccharides, since the A-active (A R_L 0.52 and urine-A) oligosaccharides and the B-active (Beach R_{IM5} 1.2 and urine-B) oligosaccharides were completely inactive at concentrations up to 500, 450, 625, and 650 nmol, respectively.

Oligosaccharides containing a single fucosyl residue linked only to the DGlcNAc of the type 1 and type 2 chains (lacto-*N*-fucopentaose II and lacto-*N*-fucopentaose III, respectively) or to DGlc of the type 2 chain (3-fucosyllactose) show some activity, but less than the galactose-substituted monofucosyl type 2 or difucosyl type 2 compounds, 700, 450, and 575 nmol, respectively, giving 50% inhibition.

Inhibition of precipitation by L-fucose and methyl α-L-fucopyranoside is also shown in Figure 5. L-Fucose is slightly poorer than 2′-fucosyllactose and methyl α-L-fucopyranoside is almost as good an inhibitor as the difucosyl H JS R_{IM5} 2.5 and somewhat better than lactodifucotetraose. The hydroxyl at C-3 of L-fucose is apparently important for the binding of this sugar to the *Lotus* lectin, since 3-*O*-methyl-L-fucose is inactive. The reduced L-fucose (fucitol) is also inactive. No significant inhibition was observed with *N*-acetyl-D-glucosamine (3720 nmol), D-mannose (1520 nmol), *N*-acetyl-D-mannosamine

TABLE I: Structures and Activities of Milk and Blood-Group Oligosaccharides Reacting with the Lectin of *Lotus tetragonolobus*.

	nmol for 50% Inhibn		nmol for 50% Inhibn
αLFuc1 ↓ 2 βDGal(1→4)DGlc 2′-Fucosyllactose	150	αLFuc1 αLFuc1 ↓ ↓ 2 3 βDGal(1→4)DGlc Lacto-difucotetraose	80
αLFuc1 ↓ 2 βDGal(1→4)βDGlcNAc(1→6)R H JS R_L 0.75	105	αLFuc1 αLFuc1 ↓ ↓ 2 3 βDGal(1→4)βDGlcNAc(1→6)R H JS R_{IM5} 2.5	40
αLFuc1 ↓ 2 αDGalNAc(1→3)βDGal(1→4)βDGlcNAc(1→6)R A R_L 0.52	Inactive	αLFuc1 αLFuc1 ↓ ↓ 2 3 αDGal(1→3)βDGal(1→4)βDGlcNAc(1→6)R Beach R_{IM5} 1.2	Inactive
αLFuc1 ↓ 3 βDGal(1→4)βDGlcNAc(1→3)βDGal(1→4)DGlc Lacto-*N*-fucopentaose III	450	αLFuc1 αLFuc1 ↓ ↓ 2 3 αDGal(1→3)βDGal(1→4)DGlc Urine-B oligosaccharide	Inactive
αLFuc1 ↓ 4 βDGal(1→3)βDGlcNAc(1→3)βDGal(1→4)DGlc Lacto-*N*-fucopentaose II	700	αLFuc1 αLFuc1 ↓ ↓ 2 3 αDGalNAc(1→3)βDGal(1→4)DGlc Urine-A oligosaccharide	Inactive
αLFuc1 ↓ 3 βDGal(1→4)DGlc 3-Fucosyllactose	575	αLFuc1 αLFuc1 ↓ ↓ 2 4 βDGal(1→3)βDGlcNAc(1→3)βDGal(1→4)DGlc Lacto-*N*-difucohexaose I	Inactive
αLFuc1 ↓ 2 βDGal(1→3)βDGlcNAc(1→3)βDGal(1→4)DGlc Lacto-*N*-fucopentaose I	Inactive	βDGal(1→3)βDGlcNAc(1→3)βDGal(1→4)DGlc Lacto-*N*-tetraose	Inactive

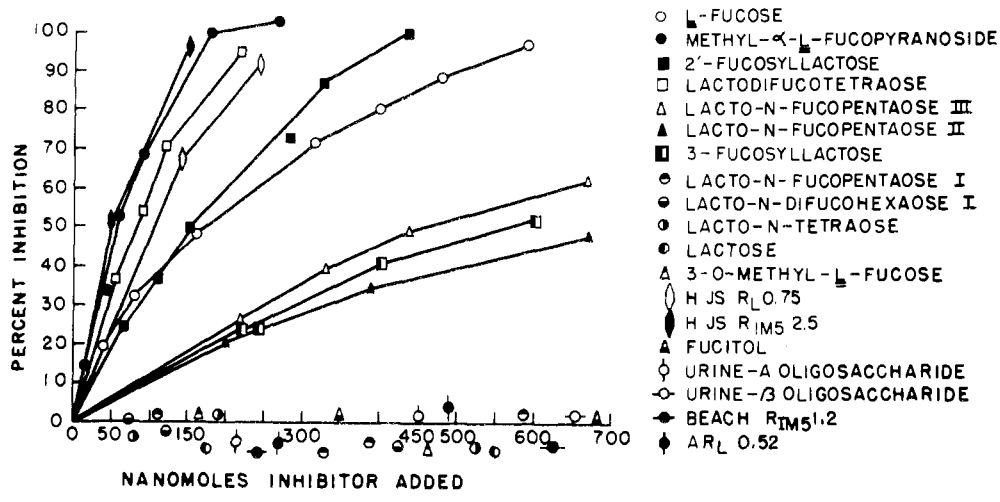


FIGURE 5: Inhibition by monosaccharides, milk, blood-group, and urine oligosaccharides of precipitation of human blood-group H substance JS with purified unfractionated *Lotus* lectin.

(2500 nmol), D-galactose (3220 nmol), *N*-acetyl-D-galactosamine (650 nmol), D-glucose (3700 nmol), or D-fucose (2600 nmol).

The ability of L-fucose, methyl α-L-fucopyranoside, 2′-fucosyllactose, and lacto-*N*-fucopentaose I to inhibit precipitation of human H substance (JS C_6H_5OH insoluble) with comparable amounts of the three fractions of the *L. tetragonolobus* lectin obtained by isoelectric focusing is shown in Figure 6, and Table II. With all three fractions, methyl α-L-fucopyranoside (Me-α-L-Fuc) is the most effective inhibitor on a molar basis followed by 2′-fucosyllactose and L-fucose. The inhibitory power of any individual sugar such as Me-α-L-Fuc, for each fraction, is not the same. Thus, with fractions I, II, and III 50% inhibition was obtained with 13, 90, and 30 nmol of methyl α-L-fucopyranoside, respectively. However, a comparison of the ratios of the inhibiting powers of these sugars to one another shows them to be the same within experimental error for each fraction and for the purified unfractionated lectin (Table II). Figure 6 also shows that lacto-*N*-fucopentaose I did not inhibit the precipitation of any of these fractions when tested at concentrations of up to 330 nmol.

Discussion

The results presented show that the lectin from *L. tetragonolobus* seeds may be isolated in highly pure form by adsorption to insoluble PL-hog A + H and specifically eluted with L-fucose in the same way as had been done in earlier studies with other lectins. In the analytical ultracentrifuge, the isolated lectin sedimented as a single peak and showed some diffusely slow sedimenting material. It formed one line in double diffusion and immunoelectrophoresis when tested against three rabbit antisera to the crude *Lotus* extract, although the original extract showed at least ten components. On isoelectric focusing three fractions were obtained which could be matched with the three fractions obtained by Kalb (1968) on DEAE-cellulose and had similar biological activities. Our fractions I, II, and III of IEP 5.4, 6.2, and 7.1 corresponded to Kalb fractions B, C, and A, respectively. Fraction B (IEP 5.4) was less potent in agglutinating erythrocytes (*cf.* Vlodavsky *et al.*, 1972) but did precipitate with H substance JS C_6H_5OH insoluble. Of the three fractions it was most readily inhibitable by the fucosyl compounds and Kalb (1968) had reported it to have the lowest association constant by equilibrium dialysis. The relative inhib-

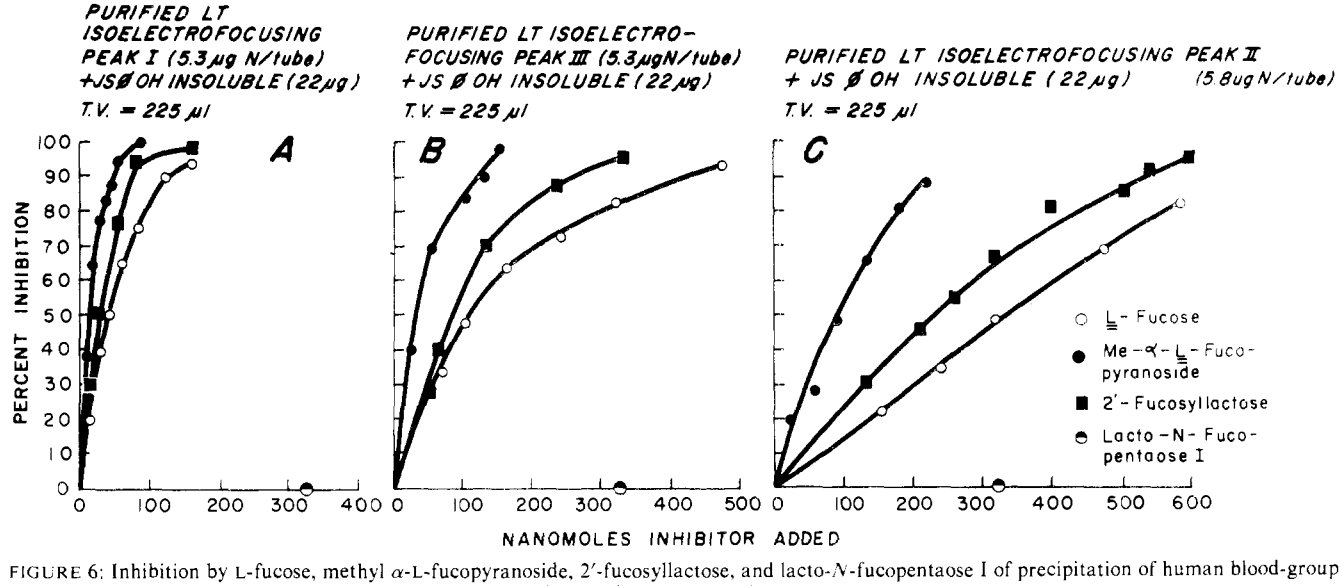


FIGURE 6: Inhibition by L-fucose, methyl α-L-fucopyranoside, 2′-fucosyllactose, and lacto-*N*-fucopentaose I of precipitation of human blood-group H substance JS with the three fractions of *Lotus* lectin obtained by isoelectrofocusing.

TABLE II: Comparison of Fractions Obtained by Isoelectric Focusing and of the Original Lectin in Inhibitory Power and in Association Constant.

Oligosaccharide Added as Inhibitor	*Lotus* lectin	Oligosaccharide for 50% Inhibn (nmol)	Rel Inhibiting Power Compared to Methyl α-L-fucopyranoside (%)	Ass. Constant × 10^{-4} (Kalb, 1968)	Ass. Constant Relative to Fraction I	Inhibitory Power Rel to Fraction I
Methyl α-L-fucopyranoside	Fraction I	13	(100)			1.0
2′-Fucosyllactose		31	39	0.6	1.0	1.0
L-Fucose		41	29			1.0
Methyl α-L-fucopyranoside	Fraction II	90	(100)			6.9
2′-Fucosyllactose		240	38	3.7	6.2	7.7
L-Fucose		330	27			8.0
Methyl α-L-fucopyranoside	Fraction III	30	(100)			2.3
2′-Fucosyllactose		90	33	1.2	2.0	2.9
L-Fucose		110	27			2.7
Methyl α-L-fucopyranoside	Unfractionated	50	(100)			
2′-Fucosyllactose		150	33			
L-Fucose		180	28			

iting capacity of the three fractions paralled the association constants within experimental error (Table II).

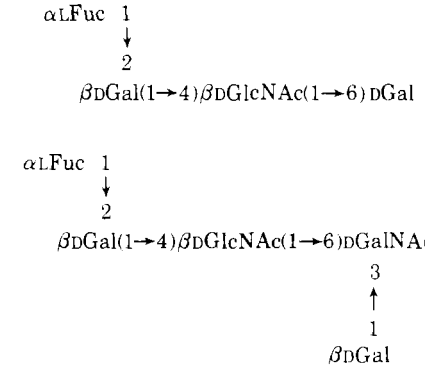
The inhibition studies tend to indicate that the saccharide-binding sites of the three *Lotus* fractions are homogeneous in the extent of their complementary areas. This is a consequence of the findings that the ratios of the inhibiting powers of the haptens relative to one another is constant for each fraction and for the original *Lotus* lectin when the same amounts of lectin and blood-group substance are used (*cf.* Hammarström and Kabat, 1969; Etzler and Kabat, 1970). If the combining sites of these three fractions had different complementary areas, the ratios of the inhibiting powers of the various sugars to one another would differ, as shown (Kabat, 1956b) in studies of the combining regions of human anti-dextran (*cf.* Cisar *et al.*, 1974).

The molecular weight of fractions I, II, and III in sodium dodecyl sulfate polyacrylamide gels is about 27,000. The finding that the subunits are dissociated by sodium dodecyl sulfate independently of reducing agent and absence of cysteine in the lectin (Kalb, 1968) indicate that the subunits are held together by noncovalent bonds.

The *Lotus* lectin provides a unique reagent of considerable value in identifying fucosyl oligosaccharides because of its specificity for the type 2 chain and its failure to react with the type 1 chain. Indeed it has already contributed (Rovis *et al.*, 1973a) to the elucidation of the structure of several blood-group oligosaccharides.

The studies with *Lotus* lectin and blood-group A and B substances reopen the entire question of the structural basis of A_1 and A_2 specificity. Figure 4B shows that the lectin is precipitated by the A_2 but not by the A_1 substances tested, with the most active A_2 substance, WG C_6H_5OH insoluble, being about 70% as active as H substance JS. This implies that A_2 but not A_1 substance must have substantial numbers of H determinants accessible to the *Lotus* combining site. The inhibition data (Figure 5) indicate that the only fucosyl oligosaccharides which bind to the lectin are those with type 2 chains and that addition of the A immunodominant group, αDGalNAc(1→3) to these H-active oligosaccharides completely blocks the binding activities. It then follows that A_2 substances must possess some type 2 determinants lacking the αDGalNAc(1→3) residue for precipitation to occur, while in A_1 substances all type 2 chains are substituted by αDGalNAc(1→3) residues. An indication that A_2 specificity involves DGalNAc on the type 1 chain comes from enzyme-splitting studies of A_2 substances (Stealey and Watkins, private communication) in which removal of DGalNAc from A_2 substances increased Le^b activity. On the other hand, immunochemical studies with A_1 and A_2 glycoproteins using human anti-A antibodies (Lloyd and Kabat, 1968; Moreno *et al.*, 1971) strongly favor a qualitative difference between A_1 and A_2 substances, with the type 2 A immunodominant group associated with A_2 glycoproteins. This evidence came from absorption experiments, in which human anti-A antisera absorbed with A_2 substance removed antibodies reacting with A_2 substance leaving anti-A_1 activity; furthermore, inhibition studies with mono- and difucosyl type 2 A-active oligosaccharides showed that type 2 receptors are present in A_2 substance.

It has recently been shown that there are two types of type 2 determinants in H substance (Rovis *et al.*, 1973b).



Thus there can be two types of type 2 A determinants by the substitution of αDGalNAc(1→3) on the DGal of the above two structures. Since A_1 substances do not react with *Lotus* lectin, all such type 2 structures would be blocked by αDGalNAc(1→3) residues. It is thus possible that in A_2 substances only one of the two kinds of type 2A determinants is present. This would explain the simultaneous reactivity of the *Lotus* lectin to A_2 substances as well as the presence of type 2 A determinants.

Schachter *et al.* (1973) showed that the *N*-acetyl-D-galactosaminyltransferases obtained from A_1 and A_2 donors are qualitatively different, although the A_1 and A_2 transferases act on both type 1, lacto-*N*-fucopentaose I, and type 2, 2′-fucosidolactose, oligosaccharide acceptors. These A_1 and A_2 enzymes differed in their cation requirements, pH optima and K_m values; they could still show differences not only in their reactions with the macromolecular H substances as substrate but even toward the two kinds of type 2 H determinants. Thus a qualitative structural difference between A_1 and A_2 substances based on the two kinds of type 2 A determinants is still reasonable.

Added in Proof

Moreover, Schenkel-Brunner and Tuppy (1973) have just demonstrated that the A_1 and A_2 enzymes have different specificities toward macromolecular H substance.

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