Purification and Properties of the Hemagglutinin from *Sophora japonica* Seeds†

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ABSTRACT: Purification of the erythroagglutinating protein from the seeds of the Japanese pagoda tree (*Sophora japonica*) was performed in an overall yield of 72%. The lectin, which represents 8.5% of the saline extractable protein, was adsorbed onto insolubilized hog gastric mucin and eluted by a batch procedure with 0.2 M D-galactose. Homogeneity of the purified protein was demonstrated by immunoelectrophoresis, isoelectric focusing, polyacrylamide gel disc electrophoresis, gel filtration, and ultracentrifugation. Calibrated gel filtration indicated a molecular weight of 132,800 daltons. Amino acid analysis demonstrated that the lectin contained relatively large amounts of hydroxy and acidic amino acids, no methionine, and approximately 5 residues of half-cystine/mol. The presence of covalently bound carbohydrate was determined as 14–15 residues of 2-amino-2-deoxyglucose and 5.9% neutral saccharide composed of mannose and xylose. An eightfold greater concentration of lectin was needed to agglutinate human type A erythrocytes than required for the agglutination of type B cells. The lectin was unable to agglutinate type O cells at a concentration 500 times greater than needed for the agglutination of type B erythrocytes. The optimum pH for agglutination of B red blood cells was 8.3–9.0 with the activity at pH 7.0 only one-eighth as great as at the optimum pH. Essentially 100% functional homogeneity of the purified agglutinin is suggested by the complete precipitation of the protein by B-active human blood-group substance. The lectin was shown to be inhibited by *N*-acetyl-D-galactosaminides more easily than by D-galactosides and displays a preference for binding β anomers of these saccharides.

In light of the numerous ways lectins have been employed as serological (see Boyd, 1970) and analytical reagents (Poretz and Goldstein, 1967), specific precipitants (Goldstein *et al.*, 1965a; Kass *et al.*, 1969), and relatively simple models for the study of protein–carbohydrate interactions (Goldstein *et al.*, 1965b; Poretz and Goldstein, 1970; Poretz and Goldstein, 1971), the availability of purified and well-characterized lectins is of increasing interest.

Since the first report (Krüpe and Braun, 1952) of the hemagglutinin in *Sophora japonica* seeds a number of attemps have been made to purify the active component and to determine the specificity of the binding site. Shortly afterward, Morgan and Watkins (1953) demonstrated by adsorption and elution techniques that a single component of the seed extracts was responsible for the agglutination of both type A and B erythrocytes. Employing classical fractionation techniques, Osawa and Akiya (1961a) obtained partially purified fractions that exhibited anti-A, anti-B, and anti-O erythrocyte activity. Inhibition of agglutination experiments (Morgan and Watkins, 1953; Mäkelä, 1957; Osawa and Akiya, 1961b; Yosizawa and Miki, 1963) demonstrated that the protein reacts with D-galactose and *N*-acetyl-D-galactosamine. In order to define the binding properties of the protein responsible for the agglutination of A and B red blood cells, we have prepared a chemically homogeneous protein employing a specific insoluble adsorbant. The purification and partial characterization of this lectin are reported subsequently.

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Materials and Methods

Immunochemical Procedures. Hemagglutination activity was assayed with the microtiter apparatus (Cooke Laboratory Products, Alexandria, Va.) employing 0.01 M sodium phosphate buffer (pH 7.8) containing 0.14 M sodium chloride (PBS) as diluent (unless noted otherwise) and a 2% suspension of A, B, or O erythrocytes. The degree of agglutination was observed microscopically after 30-min incubation at room temperature.

Quantitative precipitation analysis was performed by the method of So and Goldstein (1967) in 3-ml conical centrifuge tubes using 80 μg of lectin and 0–100 μg of appropriate blood-group active mucin in a total volume of 0.7 ml. Following incubation at room temperature for 1 hr and 4° for 48 hr, the solutions were centrifuged at 1000*g* for 15 min at 4°, the supernatants were decanted, and the precipitates were resuspended in 0.5 ml of cold PBS. The precipitates were washed two more times and then collected by centrifugation. The protein content of each precipitate, as well as the original lectin solution, was determined by the micro-Lowry method (Mage and Dray, 1965) employing bovine serum albumin as a standard.

The quantitative precipitation reaction of concanavalin A and the *S. japonica* lectin was conducted with 65 μg of concanavalin A and 0–76 μg of purified lectin in a total volume of 0.3 ml. After incubation at 4° for 48 hr the tubes were centrifuged at 1000*g* for 15 min at 4° and the precipitates were washed and analyzed as described above. The supernatants were retained and examined for the presence of *S. japonica* lectin by immunodiffusion against rabbit anti-lectin serum.

Inhibition of precipitation studies were conducted using 20 μg of B-active blood-group substance and 80 μg of purified lectin with varying concentrations of inhibitor in a total volume of 0.7 ml. The reaction was incubated and treated in

manner identical with the quantitative precipitation reaction.

Antisera were produced by footpad (1.0 ml) and intraperitoneal (1.0 ml) immunization of New Zealand white rabbits with either the crude extract (1.3 mg/ml) or purified lectin of *S. japonica* seeds (2.9 mg/ml) emulsified with an equal volume of Freund's complete adjuvant. Booster injections without adjuvant were given intramuscularly at week 2 and intravenously at week 7. The animals were bled by cardiac puncture after the eighth week.

Immunodiffusion was conducted essentially as described by Ouchterlony (1948) using 1% agarose in pH 7.8 PBS. The procedure of Grabar and Williams (1953) was followed for immunoelectrophoresis using commercial precoated 1% agarose plates containing 0.05 M sodium barbital buffer (pH 8.6) (Analytical Chemists, Palo Alto, Calif.). Electrophoresis was conducted at 100 V for 35 min and developed with the appropriate antiserum in a humidity chamber at 20°.

Electrophoresis Methods. Disc electrophoresis in 7.5% polyacrylamide gels at pH 8.9 was conducted as described by Davis (1964) except that samples containing 20% sucrose were layered on top of the separation gels and at pH 4.3 in a manner similar to that of Reisfeld *et al.* (1962).

Liquid isoelectric focusing employing a sucrose density gradient, 2% concentration of ampholytes, and 15 mg of purified lectin was accomplished over a period of 48 hr in a 110-ml column (LKB Instruments, Rockville, Md.) at 4° as described by Vesterberg and Svensson (1966).

Analytical Gel Filtration. Molecular weight determination was performed by calibrated gel filtration employing Sephadex G-200 equilibrated in PBS and packed in a glass column (2.5 × 80 cm). Samples of purified lectin (2.5 mg/ml), proteins of known molecular weight (2.0 mg/ml), and Blue Dextran (1 mg/ml) were successively applied and eluted from the column essentially as described by Andrews (1965). The protein content of the eluate was followed by a flow-through absorptometer at 280 nm as well as by optical density measurements at 280 nm of each 3-ml fraction.

Ultracentrifugation. Sedimentation velocity ultracentrifugation was conducted with a Spinco Model E ultracentrifuge equipped with schlieren optics and using the AN-D rotor with standard 12-mm aluminum cells. Photographs were taken before and after the rotor attained a running speed of 59,780 rpm at 4°. Boundary positions were measured with a Nikon shadowgraph microcomparator.

Amino Acid Analysis. Protein solutions containing 0.01 M NaCl were hydrolyzed with constant-boiling HCl at 100° for 24, 48, and 72 hr in evacuated thick-walled Pyrex tubes. Aliquots were removed and analyzed according to the method of Spackman *et al.* (1958) employing a Beckman Model 120C automatic amino acid analyzer. Lectin samples which had been oxidized by performic acid (Hirs, 1967) were hydrolyzed and analyzed as above. The spectrophotometric method of Goodwin and Morton (1946) was used for the estimation of tryptophan.

Carbohydrate Analysis. Analysis of the total carbohydrate of the unhydrolyzed protein was accomplished by the anthrone procedure (Roe, 1955) and the phenol–sulfuric acid method (Dubois *et al.*, 1956) using D-galactose as a standard. Fractionation of the neutral and amino sugars of the lectin after hydrolysis with 3 N HCl for 12 hr at 100° was performed as described by Spiro (1966). Quantitation of these fractions was accomplished using the anthrone and Elson–Morgan (Elson and Morgan, 1933) procedures, respectively.

Neutral sugars were qualitatively identified by paper chromatography on Whatman No. 4 paper and thin-layer chromatography on cellulose plates irrigated with ethyl acetate–pyridine–water (10:4:3, v/v) (Whistler and Hickson, 1955) or phenol–water–ammonia (160:40:1, w/w) (Smith, 1960) and visualized with alkaline silver nitrate (Trevelyan *et al.*, 1950) and aniline–phosphoric acid (Smith, 1960) spray reagents.

Glycoside Hydrolase Activity. The presence of α- and β-D-galactosidase, β-D-glucosidase, *N*-acetyl-β-D-glucosaminidase, and *N*-acetyl-β-D-galactosaminidase activities were detected at pH 4.1 (0.01 M sodium acetate in 0.13 M NaCl) using the appropriate *p*-nitrophenyl derivative as substrate. The assays were conducted essentially as described by Lederberg (1950) and the liberated *p*-nitrophenol was measured by absorption of light at 420 nm using *p*-nitrophenol as a standard.

Preparation of Specific Adsorbant. Insolubilized polyleucyl A- and H-active hog gastric mucin was prepared according to the procedure of Kaplan and Kabat (1966). Phenol–sulfuric acid analysis of the hydrolyzed (2 N HCl, 100°, 16 hr) insolubilized mucin indicated approximately 50% mucin, the remainder presumably being leucyl residues.

The hog gastric mucin used for the preparation of insolubilized adsorbant was a 10–15% ethanol precipitate of granular mucin type 1701-W (Wilson Laboratories, Chicago, Ill.) as described by Morgan and King (1943).

All human blood-group substances used in this work were the generous gifts of Professors Winifred Watkins and W. T. J. Morgan.

Purification of the Lectin. Freshly ground *S. japonica* seeds (100 g, F. W. Schumacher, Sandwich, Mass.) were suspended by stirring in 1 l. of PBS at 4°. After 15 hr the suspension was filtered through a double layer of cheesecloth, the residue was washed with 100 ml of PBS, and the combined filtrates were centrifuged at 15,000*g* and 4° for 45 min. The supernatant was decanted and retained (fraction F-1).

To each stirred volume of fraction F-1 was slowly added at −20°, 1.1 volumes of 95% ethanol so that the final concentration of ethanol was 50%. After complete addition of ethanol the suspension was stirred for 1 hr and centrifuged at 15,000*g* and −10° for 45 min. The supernatant was decanted (fraction F-2) and the precipitate was suspended in 200 ml of PBS and dialyzed against 3 × 4 l. of PBS. The dialyzed suspension was centrifuged at 25,000*g* for 1 hr and the supernatant was filtered through a glass wool plug yielding fraction F-3.

To 1.0 g of insolubilized hog gastric mucin or insolubilized B-active human blood-group substance was added the total of fraction F-3 (220 ml; 1.15 g of protein) and this suspension was stirred at 4° for 3–15 hr. After centrifugation at 25,000*g* for 1 hr the supernatant was decanted (fraction F-4), the precipitate was suspended in 100 ml of cold PBS for 15 min and centrifuged, and the supernatant was removed. This wash was repeated until the optical density at 280 nm of the supernatant was below 0.05 ODU. The sediment was suspended in the cold with 100 ml of PBS containing 2% D-galactose. After 1 hr the suspension was centrifuged at 25,000*g* for 1 hr and the supernatant was decanted through a glass wool plug and exhaustively dialyzed against PBS yielding fraction F-5, the purified lectin.

Results

The characteristics of the fractions obtained at various stages of purification of the crude extract of ground *S. ja-*

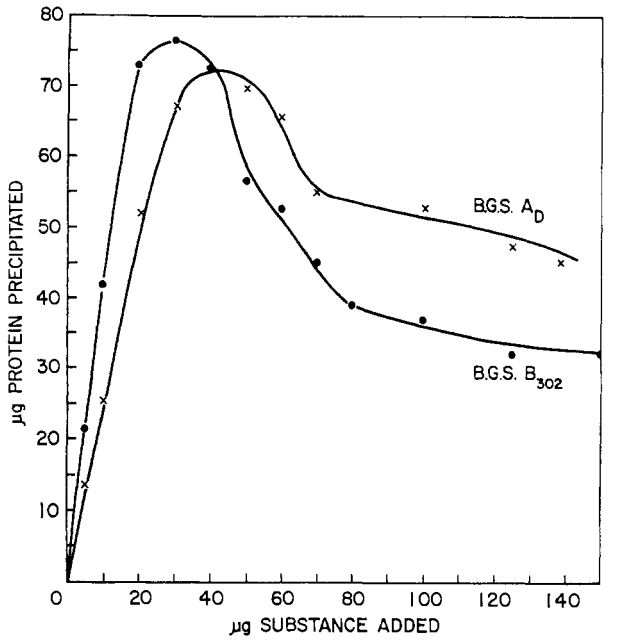

FIGURE 1: Quantitative precipitation curves of the *S. japonica* lectin with human blood-group susbtances: A-active substance, ×; B-active substance, ●.

ponica seeds are presented in Table I. From each 100 g of ground seeds, 2.4 g of protein is extracted containing 200 mg of lectin as determined by precipitation analysis with human type B blood-group substance.

Ethanol precipitation (fraction F-3) resulted in a two-fold purification of the lectin with essentially no loss of activity and a 65% decline in contaminating α-D-galactosidase activity (see Table II). Fraction F-2, the dialyzed supernatant of the ethanol fractionation, contained about one-third of the protein found in the crude extract and no hemagglutinating activity even when concentrated tenfold as compared to the original volume of fraction F-1. The protein which is not resolubilized after ethanol precipitation probably accounts for the discrepancy of the quantities of protein found in fractions F-1, F-2, and F-3. Specific adsorption of the lectin with insolubilized (A + H)-active hog gastric mucin resulted in a supernatant (fraction F-4) containing less than 3% of the original lectin activity. However, after washing of the adsorbant followed by elution with the monosaccharide inhibitor of the lectin, D-galactose, 149 mg of protein was recovered with a functional purity of 97%. The overall purification resulted in a 72% recovery of activity calculated from the functional activities of the lectin in fractions F-1 and F-5. Additional elution of the adsorbant after D-galactose treatment with 5% *N*-acetyl-D-galactosamine or 0.05 N NaOH removed protein equivalent to only 10% that eluted by D-galactose.

TABLE I: Characteristics of Fractions from the Purification of the *Sophora japonica* Hemagglutinin.[a]

Fraction	Total Protein[b]	Hemagglutinin Act.[c]	Functional Act.[d]	% Functional Purity[e]
F-1	2400	12	200	8.4
F-2	735	>1200	0	0
F-3	1145	7.5	195	17
F-4	795	670	<6	<1
F-5	149	1.1	144	97

[a] From 100 g of seeds. [b] As milligrams of bovine serum albumin. [c] Hemagglutinin activity as minimum concentration (μg/ml) of protein required to completely agglutinate type B erythrocytes. [d] Maximum protein in milligrams precipitated with the optimal quantity of B-active blood-group substance. [e] Maximum protein precipitated with B-active blood-group substance/total protein × 100.

TABLE II: Glycoside Hydrolase Activity of Fractions from the Purification of the *S. japonica* Hemagglutinin.

Enzyme	Act. of Fraction[a] F-1	F-3	F-5
α-D-Galactosidase	19.0	6.5	0
β-D-Galactosidase	57.1	52.0	0
α-D-Glucosidase	7.2	8.6	0
N-Acetyl-β-D-glucosaminidase	15.3	5.4	0
N-Acetyl-β-D-galactosaminidase	NT[b]	NT[b]	0

[a] Expressed as nmoles of substrate hydrolyzed per min per mg of protein. [b] NT, not tested.

The minimum concentrations of purified lectin needed to agglutinate type A and B erythrocytes were 8.8 and 1.1 μg per ml, respectively. The precipitability of the purified lectin with both A- and B-active blood-group substances is shown in Figure 1. Approximately 97% of the total protein added to the reaction is specifically precipitated at the point of maximum precipitation (*i.e.*, at 30 μg of B-active blood-group substance). Consistent with the weaker reactivity of the lectin with A erythrocytes than with B cells, more A-active than B-active blood-group substance is required to maximally precipitate the lectin. Similarly, the lectin does not precipitate with H-active human blood-group substance and does not agglutinate type O erythrocytes at concentrations less than 500 μg/ml. As shown in Figure 2A immunoelectrophoresis of the purified lectin produces only one precipitin band when developed with antisera raised against the crude extract or purified protein. In contrast, the crude extract yielded at least seven components when developed with the anti-crude extract serum.

Though the crude extract (fraction F-1) displayed α- and β-D-galactosidase, α-D-glucosidase, and *N*-acetyl-β-D-glucosaminidase activities (Table II), ethanol fractionation (fraction F-3) resulted in a decrease of these enzymes in the hemagglutinin fraction and the purified lectin (fraction F-5) lacked the activity exhibited by these enzymes as well as *N*-acetyl-β-D-galactosaminidase.

The physiochemical purity of the lectin preparation was examined by isoelectric focusing. Figure 3 displays the isoelectric focusing profiles of fraction F-5 in gradients of pH 3–10 and 5–7. Essentially, a single symetrical peak was obtained in each gradient with an observed isoelectric pH of 5.45. Polyacrylamide gel disc electrophoresis at pH 4.3 produced a single Amido Black staining component. However, electrophoresis at pH 8.9 resulted in three to four diffuse bands. An investigation of the nature of the multiple banding of the lectin during electrophoresis at pH 8.9 is in progress.

Sephadex G-200 chromatography in PBS resulted in the elution of a single symetrical protein peak. The elution volume of the lectin on a calibrated Sephadex G-200 column

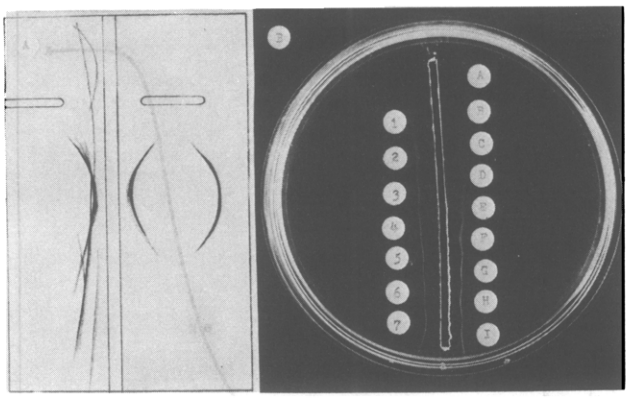

FIGURE 2: Immunodiffusion patterns. (A) Immunoelectrophoretogram of fraction F-1 (left-hand well) and fraction F-5 (right-hand well) developed with anti-F-1 (center trough) and anti-F-5 (right-hand trough) sera. The anode is at the bottom. (B) Immunodiffusion pattern of the supernatants of the *S. japonica* lectin–concanavalin A reaction (see Figure 6) with rabbit anti-F-5-serum (trough). Well A, concanavalin A only, 213 μg/ml; wells B–I, supernatants of the concanavalin A precipitation of the *S. japonica* lectin (first eight tubes); wells 1–7, original concentration of *S. japonica* lectin used for the precipitation reaction in tubes 2–8 respectively.

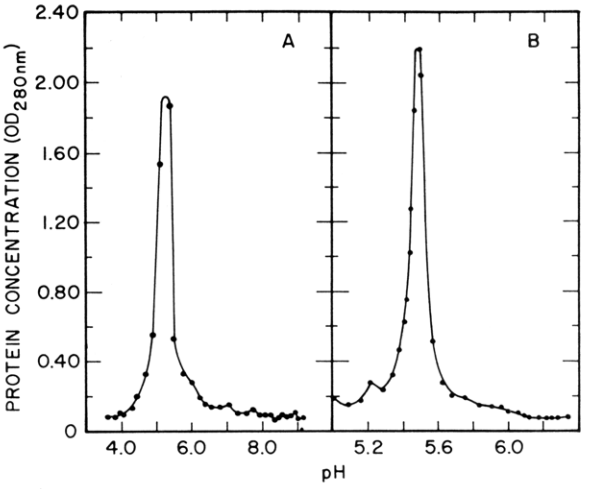

FIGURE 3: Liquid isoelectric focusing profile of the purified *S. japonica* lectin. (A) Gradient range pH 3–10; (B) gradient range pH 5–7.

corresponded to a molecular weight of 132,800 daltons (Figure 4).

Additonal evidence of the homogeneity of the purified lectin is shown by the sedimentation velocity pattern of the protein in the ultracentrifuge (Figure 5). The single symetrical peaks obtained at concentrations from 0.4 to 2.5% protein indicate a single molecular weight species with a sedimentation coefficient ($s^0_{20,w}$) of 6.8 S.

Amino acid analysis of the purified lectin is shown in Table III. The protein is relatively rich in hydroxy and acidic amino acids and contains small amounts of basic amino acids, consistent with the observed acidic isoelectric point. No methionine was detected in the hydrolysate of the protein or methionine sulfone in the performic acid oxidized material. However, approximately 5 residues of half-cystine/132,800 g of lectin were obtained with the oxidized hemagglutinin. The total recovery of nitrogen, excluding amide nitrogen, represents 90% of the applied sample. The nitrogen content of the lectin was found to be 14.0% employing the Kjeldahl (Kabat and Mayer, 1964a) and Kjeldahl–ninhydrin (Schiffman *et al.*, 1964) procedures.

Glucosamine was established as the only amino sugar by use of the automated amino acid analyzer and was quantified as 14 residues/132,800 g by this procedure and 15 residues/132,800 g by the Elson–Morgan reaction. Carbohydrate analysis with the phenol–sulfuric acid and anthrone reagents directly on the protein as well as on the neutral sugar fraction of the acid hydrolysate indicated the neutral sugar content to be 5.9% (as D-galactose) by weight. Qualitative paper chromatography and thin-layer chromatography of the neutral sugar fraction using a number of solvent systems yielded only mannose and xylose; the pentose was confirmed and distinguished from fucose by the red color formed with the aniline–phosphoric acid spray reagent (Smith, 1960) as well as its paper chromatographic mobility. No other pentose

TABLE III: Composition and Physiochemical Characteristics of the *Sophora japonica* Lectin.

Residue	Residues/ 132,800 g		
Lys	35.7	Glucosamine	14.3
His	23.4	Neutral carbohydrate[d]	43.1
Arg	17.1	Total N	14.0%
Asp	98.3	$E^{1\,cm}_{1\%}$ (283 nm)	15.7
Thr[a]	88.8	p*I*	5.45
Ser[a]	115.8	$s^0_{20,w}$ (S)	6.8
Glu	79.7	Molecular weight	132,800
Pro	42.0		
Gly	79.9		
Ala	94.7		
$^1/_2$-Cys[b]	5.2		
Val	86.4		
Met	0		
Ile	59.5		
Leu	88.0		
Tyr	33.3		
Phe	51.1		
Trp[c]	33.3		

[a] Obtained by linear extrapolation to zero time of the results of the 24-, 48-, and 72-hr hydrolysates. [b] As cysteic acid from the performic acid oxidized protein. [c] Determined spectrophotometrically. [d] By the anthrone procedure; as D-galactose.

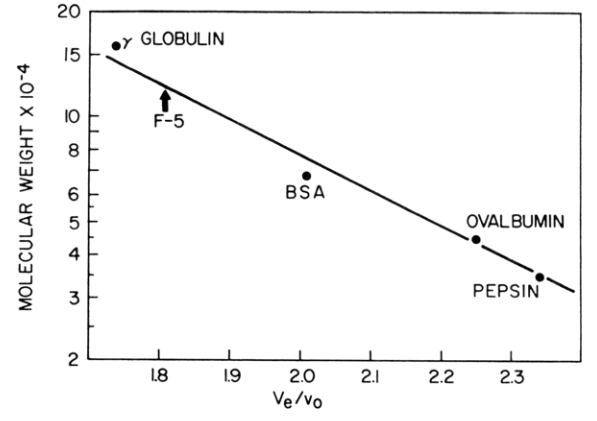

FIGURE 4: Calibration graph for the determination of the molecular weight of purified *S. japonica* lectin.

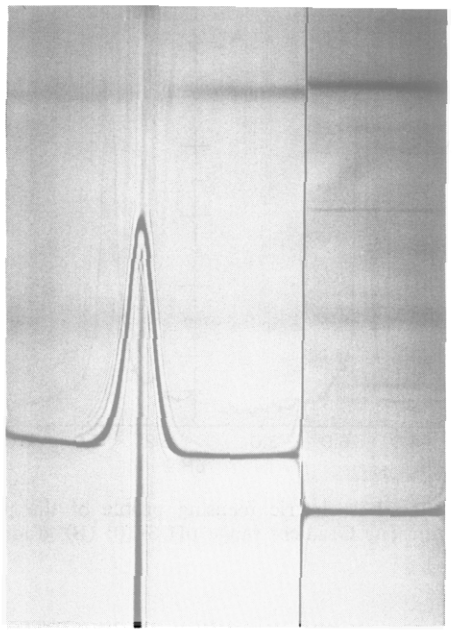

FIGURE 5: Sedimentation pattern of the purified *S. japonica* lectin (14.9 mg/ml) in PBS at 4°. The photograph was taken 32 min after attaining full speed of 59,780 rpm. Sedimentation is from right to left.

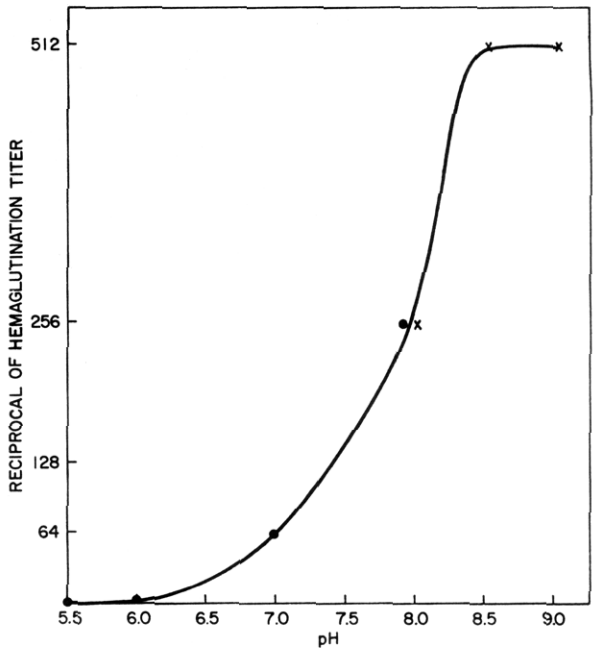

FIGURE 7: Effect of hydrogen ion concentration on the agglutination of type B human erythrocytes by the *S. japonica* lectin. Sodium phosphate buffer, ●; tris(hydroxymethyl)aminomethane buffer, ×.

or neutral sugars were detected in the acid hydrolysate of the purified protein.

The existence of α-linked D-mannopyranosyl residues as nonreducing terminal units or as internal structures substituted only at the 2 position is suggested by the strong reaction exhibited between the *S. japonica* lectin and concanavalin A (So and Goldstein, 1968; Poretz and Goldstein, 1970). Evidence for the very tight association or covalent attachment of the carbohydrate to the protein is shown in Figure 6. Here it may be seen that the precipitation reaction involving the two lectins yields a curve quite similar to that reported for the interactions of concanavalin A with glycoproteins (Goldstein *et al.*, 1969). An indication that the carbohydrate-specific reagent, concanavalin A, precipitates the protein component of the lectin along with the saccharides is shown in Figure 2B. The immunodiffusion pattern demonstrates that the component responsible for the interaction of the *S. japonica* lectin with rabbit antiserum (presumably the protein) decreases in the supernatant of the precipitin reaction of concanavalin A and the *S. japonica* lectin even though the complete precipitation of both lectins was never reached.

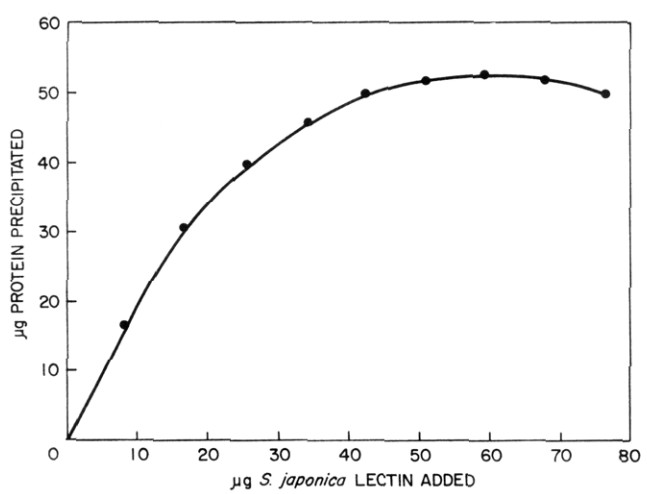

FIGURE 6: Precipitation curve of purified *S. japonica* lectin with concanavalin A (64.1 μg/0.3 ml).

Examination of the pH profile of the interaction of the *S. japonica* lectin with type B human erythrocytes (Figure 7) indicates no detectable agglutinating activity at pH 6, though at higher pH values a rapid rise in activity is observed reaching a maximum plateau at pH 8.5. It is interesting to note that at pH 7 (that pH at which erythroagglutination is often conducted) the lectin demonstrates only 12% the activity displayed at pH 8.5. A similar pH dependency of agglutination was observed employing type A erythrocytes.

The ability of the purified lectin to react with a number of saccharides is shown in Table IV. Reported is the quantity of each saccharide required to produce 50% inhibition of the precipitate resulting from the reaction of the *S. japonica* lectin with B-active blood group substance. Each value was obtained from a curve generated by plotting the per cent inhibition caused by at least five different concentrations (each

TABLE IV: Inhibition of the *Sophora japonica* Lectin Blood-Group Substance Reaction by Various Saccharides.

Inhibitor	μmol for 50% Inhibn
D-Galactose	25
Methyl α-D-galactopyranoside	18
Methyl β-D-galactopyranoside	11
p-Nitrophenyl β-D-galactopyranoside	1.2
2-Acetamido-2-deoxy-D-galactose	4.6
Methyl 2-acetamido-2-deoxy-α-D-galactopyranoside	3.5
p-Nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside	0.21
Methyl β-D-glucopyranoside	>200

in duplicate) of inhibitor with respect to the logarithm of the concentration of inhibitor. The curves so produced are similar to those obtained in hapten inhibition of antibody–antigen reactions (Kabat and Mayer, 1964b) as well as in other lectin–saccharide systems (Poretz and Goldstein, 1970). It is interesting to note that *N*-acetyl-D-galactosamine is a more potent inhibitor of the lectin than D-galactose. As with concanavalin A (Poretz and Goldstein, 1971), though not the lima bean lectin (Galbraith and Goldstein, 1972) aromatic glycosides are active inhibitors of the *S. japonica* lectin. Thus, either as D-galactosides or 2-acetamido-2-deoxy-D-galactosides the *p*-nitrophenyl derivative is approximately 21 times more effective an inhibitor than is the corresponding free sugar.

Discussion

Earlier reports of studies utilizing crude extracts (Yosizawa and Miki, 1963) or partially purified (Osawa and Akiya, 1961a) preparations of agglutinin of *S. japonica* seeds noted the presence of anti-A and -B erythroagglutinin activity as well as anti-O erythrocyte activity. In order to relate these properties to a particular protein as well as to obtain a reagent capable of interacting with D-galactopyranosyl residues, we have purified the protein in *S. japonica* seeds responsible for the agglutination of type A and B human red blood cells.

After preliminary purification with ethanol, a batch method of specific adsorption with insolubilized hog gastric mucin (A + H active) followed by a one-step desorption employing a competitive inhibitor D-galactose produced a functionally homogeneous lectin in 70% yield based on the activity of the original crude dialyzed extract. The lectin preparation did not show α- and β-D-galactosidase, α-D-glucosidase, *N*-acetyl-β-D-galactosaminidase, or *N*-acetyl-β-D-glucosaminidase activities, all of which were detected in the crude extract.

Physiochemical homogeneity of the lectin was indicated by gel filtration, sedimentation velocity ultracentrifugation, polyacrylamide disc gel electrophoresis as pH 4.3, and isoelectric focusing. Preliminary data (Poretz and Timberlake, 1972) suggest that the multiple banding detected in polyacrylamide gel electrophoresis at pH 8.9 is not due to impurities in the lectin preparation. Similarly, Boos and Gordon (1971) reported that the purified galactose-binding protein of *Escherichia coli* produces two bands on polyacrylamide gel electrophoresis at pH 8.4, both of which appear to be interconvertible forms of the same protein. It is interesting to note that Hammarström and Kabat (1969) reported that the anti-A agglutinin purified from snail, *Helix pomatia*, shown homogeneous by a number of criteria, exhibited more than one band on polyacrylamide gel disc electrophoresis at pH 8.9.

The purified lectin from *S. japonica* seeds displayed a molecular weight of 132,800 daltons by gel filtration and contains little half-cystine, no methionine, and a large percentage of hydroxyamino acids, similar to a number of other purified phytohemagglutinins (see Sharon and Lis, 1972).

Carbohydrate analysis of the purified lectin showed the presence of mannose, xylose, and glucosamine. The absence of fucose was verified by chromatographic procedures as well as the reactivity of chromatographically fractionated sugars with specific color spray reagents. It is interesting to note that the saccharide employed for elution of the protein from the adsorbant, D-galactose, is not detected in the final product. The implication of α-D-mannopyranosyl residues as nonreducing terminal structures or as 2-O-substituted internal residues of the associated carbohydrate can be made based upon the strong reaction of concanavalin A with the purified lectin. In the examination of the reactions of concanavalin A with glycoproteins, Goldstein *et al.* (1969) noted a strong reaction of the lectin with human IgM, which has been reported to have nonreducing terminal α-D-mannopyranosyl residues (Kornfeld *et al.*, 1971). However, the finding by Leon (1967) that human IgG, which appears to possess nonreducing β-D-galactopyranosyl units and internal α-D-mannopyranosyl residues (Kornfeld *et al.*, 1971), is also capable of reacting with concanavalin A, emphasizes the fact that reactivity with concanavalin A may be indicative of a substance containing either nonreducing terminal or internal 2-O-substituted α-D-mannopyranosyl moieties (So and Goldstein, 1968; Poretz and Goldstein, 1970). Goldstein (personnal communication) has also noted the reactivity of concanavalin A with internal units of D-mannose-containing oligosaccharides. In comparison to the reactivity of concanavalin A with various dextrans (Goldstein *et al.*, 1968), the incomplete precipitation of concanavalin A by the *S. japonica* lectin may suggest that the carbohydrate is attached to the protein at only a few loci and/or the carbohydrate portion of the lectin is only slightly branched. The covalent attachment or very tight complexation of the carbohydrate to the protein of the lectin is indicated by the parallel removal of carbohydrate by concanavalin A and the loss of reactivity of the resulting solution with anti-lectin serum. Though one of the first lectins to be purified, concanavalin A, has been shown to lack carbohydrate (Agrawal and Goldtein, 1967), a number of other purified phytohemagglutinins are reported to contain covalently linked carbohydrate and polypeptide (Lis *et al.*, 1966; Johnson and Rigas, 1972; Galbraith and Goldstein, 1972).

An eightfold greater concentration of purified *S. japonica* lectin was required to agglutinate type A erythrocytes than needed for the agglutination of type B cells; no agglutination of type O red blood cells was detected even at concentrations 500 times greater than needed to agglutinate B active erythrocytes. However, no anti-O activity was detected in either the crude extract or fraction F-3 even when examined with O cells from five individuals. These findings are in disagreement with those of Osawa and Akiya (1961a) who reported appreciable anti-O activity in crude extracts of *S. japonica* seeds. Though these differences in ability to agglutinate type O erythrocytes may be due to the source of seeds used in the experiments, a possibility may exist that the lectin recognizes antigenic structures other than the A, B, and O (H) determinants, which may vary with geographically distinct populations (Poretz and Chien, 1972).

Preliminary examination of the saccharide binding specificity of the purified agglutinin indicated a preferential binding of *N*-acetyl-β-D-galactosaminyl residues. This property is in contrast to that expected for a lectin which displays greater reactivity with B-active than A-active determinants. However, D-galactopyranosides are capable of reacting with the lectin, being approximately 20% as potent inhibitors as the analogous *N*-acetyl-D-galactosaminides. The absolute requirement of the protein for binding of an axial C-4 hydroxyl group of the ligand is inferred by the noninhibitory property of methyl β-D-glucopyranoside.

It appears that consistent with the concept that both the A and B determinants of erythrocytes of blood-group substance bind to the same site on the *S. japonica* lectin, the ratio of inhibiting power of either the *p*-nitrophenyl or methyl D-galactoside to D-galactose is identical with the ratio of in-

hibiting power of the analogous *N*-acetyl-D-galactosaminides to *N*-acetyl-D-galactosamine. That is, the effect of either a *p*-nitrophenyl or methyl aglycone alters the potency of the saccharide quantitatively the same, regardless of whether the glycose moiety is D-galactose or *N*-acetyl-D-galactosamine. However, a complete understanding of the binding specificity of the *S. japonica* lectin for simple sugars and cellular antigens requires further investigation.

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