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Purification and Properties of the Hemagglutinin from *Wistaria floribunda* Seeds†

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ABSTRACT: Extracts of *Wistaria floribunda* seeds contain separable erythroagglutinating and lymphocyte mitogenic activities. We wish to report the purification and characterization of the erythroagglutinating lectin of these seeds. A phosphate-buffered saline (PBS) extract of the ground seeds was made to 50% ethanol and the precipitate, which contained both the agglutinin and mitogen, was dissolved in PBS. The erythroagglutinating activity was adsorbed onto insoluble polyleucyl derivatized A+H active hog gastric mucin. After desorption with 0.2 M D-galactose and removal of the sugar by dialysis, the eluate displayed three protein bands on polyacrylamide gel electrophoresis. The major component represented 85% of the mixture. Immunoelectrophoresis of the mixture demonstrated immunochemical identity among the proteins. Gel filtration through Sephadex G-200 resulted in purification of the major component. Based upon the composition and subunit molecular weight, it was concluded that the three components represent a dimer, tetramer, and octamer of a single glycopolypeptide chain of 28 000. The erythroagglutinin has a p*I* at pH 5.4 and one cystine per dimeric unit.

In order to develop sensitive carbohydrate specific reagents for the detection of saccharides of complex glycoconjugates, we have been purifying lectins which exhibit reactivity with D-galactosyl and *N*-acetyl-D-galactosaminyl residues. This report describes the purification, using affinity adsorption, and partial characterization of the hemagglutinin from *Wistaria floribunda*.

Extracts of *W. floribunda* seeds had been shown to possess both erythroagglutinating (Boyd et al., 1958) and lymphocyte mitogenic activities (Barker & Farnes, 1967). Though Boyd et al. (1958) were the first to report the hemagglutinating properties of *W. floribunda* seed extracts, Boyd & Reguera (1949) had noted, 9 years earlier, this activity in a related species, *W. chinensis*. Using aqueous extracts of *W. chinensis*, Mäkelä (1957) demonstrated that the hemagglutinating activity was inhibited best by sugars containing nonreducing terminal D-galactosyl residues. Toyoshima et al. (1971) have since shown that the protein of *W. floribunda* seeds responsible for mitogenic activity is distinct from that which causes hemagglutination. Recently, Kurokawa et al. (1976) have reported on the isolation, by classical techniques, and properties of a hemagglutinin from *W. floribunda* seeds.

Experimental Procedure

Purification of the W. floribunda Hemagglutinin. Ground *W. floribunda* seeds (100 g, F. W. Schumacher, Sandwich, MA) were extracted with 1 L of PBS for 18 h. The particulate material was removed by filtration through cheesecloth and centrifugation at 10000*g* at 4 °C for 45 min. To the resulting supernatant (fraction I) cold ethanol was slowly added at 0 °C to yield a final concentration of 50%, after which the suspension was centrifuged at 10000*g* at 4 °C for 45 min. The precipitate was resuspended in 150 mL of PBS and dialyzed against PBS (2 × 4 L). Following centrifugation of the suspension at 25000*g* for 1 h at 4 °C, the resulting supernatant (fraction II) was mixed with 1 g of polyleucyl hog gastric mucin (PLHGM)[1] (Kaplan & Kabat, 1966; Poretz, 1973) for 1 h at 4 °C. This suspension was centrifuged at 1000*g* and 4 °C for 20 min yielding a supernatant (fraction III) and the PLHGM–lectin complex. The lectin-bound adsorbant was repeatedly washed with cold PBS until the supernatant displayed an optical density at 280 nm of less than 0.05 unit. It was suspended in 100 mL of PBS containing 0.2 M D-galactose, a known inhibitor of the hemagglutinin. Following solubilization of the lectin, a supernatant was obtained by centrifugation at 1000*g* and 4 °C for 20 min and exhaustively

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[1] Abbreviations used: PLHGM, polyleucyl hog gastric mucin; PBS, phosphate-buffered saline.

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dialyzed against PBS (4 × 4 L). The final supernatant was designated fraction IV.

Immunochemical Procedures. Antisera were produced by footpad (1.0 mL) immunization of New Zealand White rabbits using either fraction I (5.0 mg/mL) or fraction IV (1.5 mg/mL) emulsified with an equal volume of Freund's complete adjuvant. Booster injections of emulsions of the appropriate fraction with Freund's incomplete adjuvant were administered intramuscularly at 2-week intervals. The animals were bled by cardiac puncture after the eighth week. Agglutination titrations of various solutions with human type A, B, and O erythrocytes were performed in a manner described earlier (Poretz, 1973).

Immunoelectrophoresis was conducted using polyacrylamide gel cylinders prepared by the method of Davis (1964) excluding the stacking gel. Following electrophoresis the gel cylinders were placed into Petri dishes containing liquified 1% agarose in 0.01 M sodium phosphate containing 0.12 M sodium chloride buffer (PBS, pH 6.8). Troughs, to which antiserum was added, were cut into the agarose parallel to the polyacrylamide gel cylinders. Diffusion and immunoprecipitation were allowed to occur at 4 °C.

Mitogen-stimulating activity was measured with human type O peripheral lymphocytes as previously described (Poretz & Barth, 1976).

Hemagglutination and saccharide inhibition of hemagglutination were performed as detailed by Bausch & Poretz (1977).

Protein Analysis. The protein content of the various solutions was routinely measured by the colorimetric procedure of Mage & Dray (1965) using bovine serum albumin as a reference or by absorption of light at 280 nm. The extinction coefficient [$E_{1\%}^{1cm}$ (280 nm)] of the purified lectin determined from the dry weight was 9.9. One milligram of bovine serum albumin produced an optical density in the colorimetric protein analysis equivalent to 0.75 mg of purified *W. floribunda* hemagglutinin.

Carbohydrate Analysis. The total neutral carbohydrate content of fraction IV was determined by the anthrone procedure (Spiro, 1966). Hexosamines were separated and quantified during amino acid analysis. A linear extrapolation to zero time from values for timed hydrolyses was employed as the final result. Hydrolysis and preparation of the lectin for the gas chromatographic analyses of the alditol acetates of neutral sugars were performed as described by Metz et al. (1971).

Amino Acid Analysis. Separation and quantification of amino acids were performed by the method of Spackman et al. (1958). Hydrolysis of untreated and performic acid oxidized (Hirs, 1967) lectin samples was accomplished in evacuated tubes with 6 N HCl at 110 °C for 24, 48, and 72 h.

Glycoside Hydrolase Activity. The method of Lederberg (1950) was employed to detect and quantify the presence of α- and β-D-galactosidase, α- and β-D-glucosidase, α-D-mannosidase, *N*-acetyl-β-D-glucosaminidase, and *N*-acetyl-β-D-galactosaminidase activities at pH 4.1 and 6.8 in various fractions obtained during the purification of the lectin. Appropriate commercially available *p*-nitrophenyl glycosides were employed and the liberated *p*-nitrophenol was determined spectrophotometrically at 420 nm using *p*-nitrophenol as a reference.

Gel Permeation Chromatography. Preparative recycle gel filtration was conducted at 4 °C using a glass column (2.5 × 100 cm) containing Sephadex G-200 equilibrated with PBS.

Table I: Characteristics of Fractions Obtained during Purification of *Wistaria floribunda* Hemagglutinin[a]

fraction[b]	protein (mg/mL)	total protein (g)	sp hemagglutination titer[c]	sp mitogenic act.[d]
I	18.5	18.5	0.9	19.2
II	14.0	2.01	4.6	2.9
III	6.1	1.71	0.3	3.7
IV	7.0	0.154	36.6	>400

[a] From 100 g of seeds. [b] I, crude extract; II, ethanol precipitate; III, nonadsorbable protein; IV, affinity purified agglutinin. [c] Titer with O erythrocytes/protein concentration. [d] Micrograms of protein needed to induce mitogenesis of human lymphocytes.

Samples were applied and circulated using a peristaltic pump and monitored at 280 nm with a UV monitor (ISCO Model UA-2, Lincoln, NB). The recycle valve (Laboratory Data Control, Riviera Beach, FL) was positioned such that all protein, except the component with the largest K_{av}, would pass through the column for a second time. Collection of four 1-mL fractions was initiated at the time of recycle valve switching.

Analytical gel filtration was performed as described by Andrews (1965) using a glass column (0.9 × 108 cm) packed with Sephadex G-200 equilibrated with PBS. One-milliliter fractions were collected.

Polyacrylamide Gel Electrophoresis. Discontinuous electrophoresis in polyacrylamide gels was conducted in a manner as described by Davis (1964) except that samples, containing 20% sucrose, were layered directly on top of the separation gels. Protein bands were visualized with either Amido black or Coomassie brilliant blue. Electrophoresis of sodium lauryl sulfate denatured protein was performed by the method of Weber & Osborn (1969).

Isoelectric Focusing. The purity of fraction IV was examined by liquid isoelectric focusing, as described in the LKB electrofocusing manual (LKB Instruments, Rockville, MD), using the LKB Model 8121 column and 2% Ampholine in the presence and absence of 6 M urea. One-milliliter fractions were collected and the protein content and pH determined.

Determination of the Free Thiol Groups. Reactivity of component B of fraction IV with *N*-ethylmaleimide was examined using both the spectrophotometric method and amino acid analysis procedure for *S*-succinylcysteine (a hydrolysis product of the reaction condensate *S*-ethylsuccinylcysteine) as described by Riordan & Vallee (1972). The reaction of 1.2 mg of protein with *N*-ethylmaleimide was monitored at 305 nm for up to 220 min prior to termination of the reaction.

Results

The *W. floribunda* hemagglutinin can be purified by specific adsorption onto PLHGM. The results shown in Table I demonstrate that 154 mg of purified lectin may be isolated from 100 g of seeds with a recovery of at least 34% of the hemagglutination activity. It is apparent that the crude extract of the ground seeds contains both hemagglutinating and mitogenic activities as reported previously (Toyoshima et al., 1971). Ethanol precipitation results in a recovery of 55% of the hemagglutinating and 70% of the mitogenic activities, with a five- to sixfold purification in both activities. Adsorption onto PLHGM yields a supernatant with approximately 50% of the original mitogenic and only 4% hemagglutinating activities. However, desorption of bound protein from the washed adsorbent with 0.2 M D-galactose produced a preparation with no detectable mitogenic activity and a 40-fold

Table II: Glycoside Hydrolase Activity of Fractions from Purification of *Wistaria floribunda* Hemagglutinin

	act. of fractions[a,b]			
	I	II	III	IV[c]
α-D-galactosidase	1.83	5.50	2.78	0.0
β-D-galactosidase	2.17	14.0	11.1	0.0
α-D-glucosidase	0.82	2.60	1.44	0.0
β-D-glucosidase	0.86	5.05	5.88	0.0
α-D-mannosidase	1.20	5.83	1.55	0.0
N-acetyl-β-D-galactosaminidase	3.13	12.5	12.7	0.0
N-acetyl-β-D-glucosaminidase	3.26	18.5	22.2	0.0

[a] I, crude extract; II, ethanol precipitate; III, nonadsorbable protein; IV, affinity purified agglutinin. [b] Expressed as nmol of substrate hydrolyzed per min per mg of protein at pH 4.1. [c] Identical results were obtained at pH 6.8.

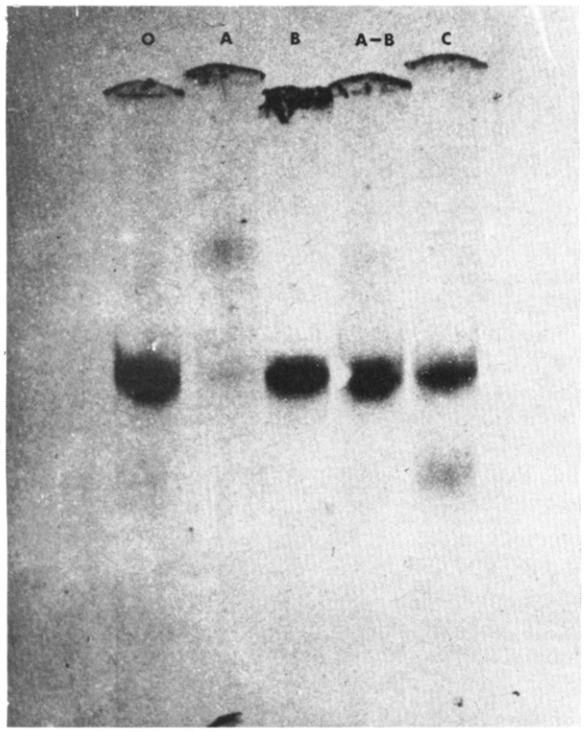

FIGURE 1: Polyacrylamide gel electrophoresis of (O) original fraction IV; (A) pool A; (B) pool B; (A–B) pool between pools A and B; (C) pool C from gel permeation chromatography of fraction IV (see Figure 4). Approximately 60 μg of protein was applied to each gel.

purification of the hemagglutinin. Similarly, as shown in Table II, fractions I, II, and III demonstrated considerable α- and β-D-galactosidase, α- and β-D-glucosidase, α-D-mannosidase, and *N*-acetyl-β-D-glucosaminidase and -galactosaminidase activities, but the purified lectin preparation was completely devoid of these glycosidase activities measured at both pH 4.1 and 6.8.

Electrophoretic analysis of the affinity purified lectin indicates a major band with two minor staining components (Figure 1). Immunoelectrophoresis (Figure 2) demonstrates that the two minor components are immunochemically identical with the major electrophoretic component. The gel filtration profile of fraction IV (Figure 3) exhibits a minor component (A) of high molecular weight, the main peak B with a slight shoulder C, and the very low molecular weight component D. As shown in Figure 4, recycle gel filtration results in the partial resolution of peak A and an improved resolution of components B and C. The first peak collected in the recycle experiment is peak D emerging from the column at the end of the first cycle.

Figure 1 shows polyacrylamide gel electrophoresis cylinders

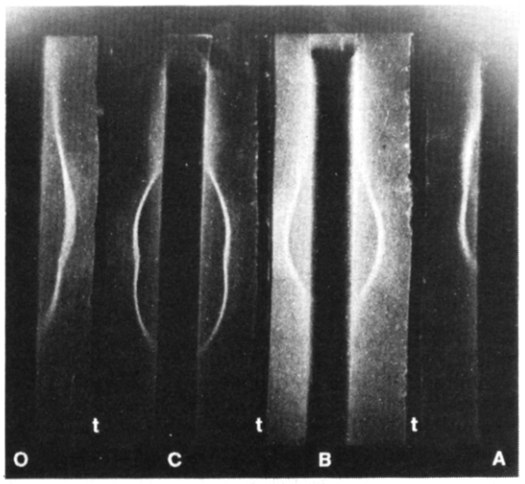

FIGURE 2: Polyacrylamide gel disc immunoelectrophoresis of (O) original fraction IV; (C) pool C; (B) pool B; (A) pool A from gel permeation chromatography of fraction IV (see Figure 4). Antiserum to fraction IV was added to troughs, t.

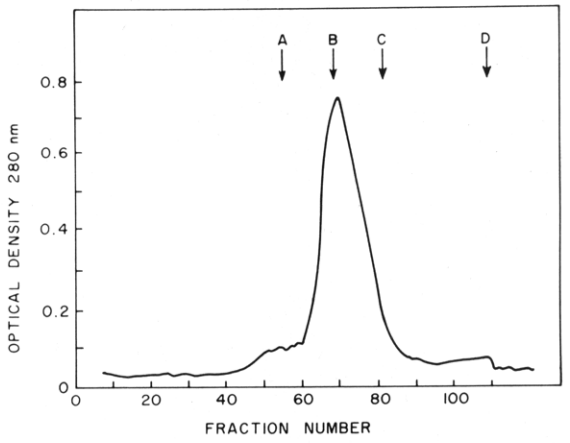

FIGURE 3: Gel permeation chromatogram of fraction IV indicating the location of components A, B, C, and D.

stained for the proteins of pools A, B, and C (Figure 4) as well as the material between pools A and B. It is apparent that the major component may be obtained in a homogeneous state and represents approximately 85% of the original mixture. Pools A and C, respectively, represent enriched preparations of the slowest and fastest migrating components on polyacrylamide gel electrophoresis. Immunoelectrophoresis of pools A, B, and C (Figure 2) clearly demonstrates that the protein of pool B produces a single sharp precipitation arc and pools A and C each show two arcs of complete fusion (one arc of each pool produced by component B). However, sodium lauryl sulfate–polyacrylamide gel electrophoresis illustrates that each of pools A, B, and C have one protein staining band with a mobility reflecting a molecular weight of 28 000 when compared to the mobility of standard proteins (bovine serum albumin, ovalbumin, chymotrypsinogen, cytochrome *c*). Consistent with the proposal that components A, B, and C represent various stages of aggregation of the same sodium lauryl sulfate dissociable subunit, analytical gel filtration through Sephadex G-200 results in a K_{av} corresponding to a molecular weight for component C of 57 000, component B of 116 000, and component A, 235 000.

In contrast to the distribution seen in the analysis of the molecular weight variants by gel filtration, electrofocusing of fraction IV yields a single zone of precipitation at pH 5.4 (Figure 5) with no evidence of protein focusing outside of this band. Electrofocusing of the protein in the presence of 6 M

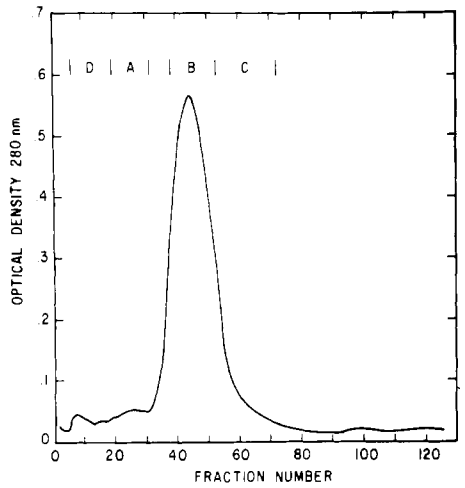

FIGURE 4: Recycle gel permeation chromatogram of fraction IV. Fractions were pooled as indicated.

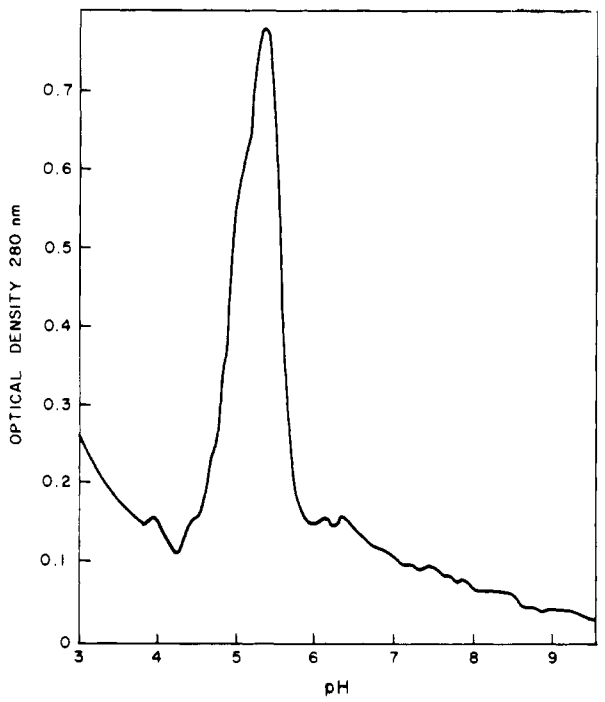

FIGURE 5: Column isoelectric focusing elution pattern of fraction IV.

urea reduces, but does not eliminate, the degree of precipitation produced at pH 5.4.

Consistent with the proposal that component B represents a tetramer of a single sodium lauryl sulfate dissociable subunit, amino acid analysis (Table III) of the performic acid oxidized protein indicates 1 residue of cysteic acid per 28 000 g. The native protein contains approximately 2 residues of D-glucosamine per monomeric unit, a value suggestive of a serum-type glycoprotein structure. In agreement with this type of structure, 8 units of mannose per 28 000 g was obtained by gas phase chromatographic analysis (Table III) of the alditol acetates. Furthermore, fucose and arabinose were detected in significant quantities as probable constitutents of the lectin. The overall amino acid analysis of the protein is relatively unremarkable for a lectin; that is, it contains no methionine, high levels of hydroxy and acidic aminoacyl residues, and relatively low levels of basic aminoacyl moieties consistent with the acidic isoelectric point of the protein.

The complete lack of reactivity of component B with *N*-ethylmaleimide as indicated by the absence of a change in optical density at 305 nm during the analysis, as well as by the lack of *S*-succinylcysteine as determined by amino acid analysis of the hydrolysis products of the condensation reaction, demonstrates that the thiol function of the single cysteine residue per monomer is not available for reaction with this reagent.

Table III: Composition and Physicochemical Characteristics of *Wistaria floribunda* Hemagglutinin

residue	residues/ 28 000 g	residue	residues/ 28 000 g
Lys	11.1	Ile	10.0
His	7.6	Leu	21.0
Arg	4.5	Tyr	5.3
Asp	34.4	Phe	12.5
Thr	19.2	Trp	2.4
Ser	23.9	Cys-SO_3H	1.1
Glu	18.6	GlcNAc	1.5
Pro	13.6	Man	8.1
Gly	12.4	Ara	3.8
Ala	16.9	Xyl	2.0
Val	21.7	Fuc	2.0
Met	0		

$E_{1\%}^{1\,cm}$ (280 nm): 9.9
p*I*: 5.4
mol wt: 57 000, 116 000, 235 000

The hemagglutinating activity of the *W. floribunda* agglutinin is inhibited by simple sugars. Saccharides related to D-galactose and *N*-acetyl-D-galactosamine are capable of causing complete inhibition of hemagglutination of human erythrocytes at millimolar concentrations. *N*-Acetyl-D-galactosamine, which requires a concentration of 0.064 mM to cause complete inhibition of lectin induced agglutination of human type O erythrocytes, is 130 times more potent as an inhibitor than D-galactose. Methyl α-D-galactopyranoside which causes inhibition of the lectin at 2.1 mM is four times more effective as an inhibitor than is the corresponding β-anomeric derivative. Surprisingly, lactose (a β-linked D-galactopyranosyl-D-glucose) is four times more active than melibiose (an α-linked D-galactopyranosyl-D-glucose) which caused complete inhibition of the lectin at a concentration of 1.0 mM.

Discussion

Polyleucyl hog gastric mucin (PLHGM) has been successfully employed as an affinity adsorbant for the plant lectins from the seeds of *Dolichos bifloris* (Etzler & Kabat, 1970), *Sophora japonica* (Poretz et al., 1974), and *Maclura pomifera* (Bausch & Poretz, 1977). This paper reports the utilization of this adsorbant for the specific isolation of the hemagglutinin from *W. floribunda* seeds. The use of such an adsorbant allows for the separation of the hemagglutinin from the mitogenic lectin also extracted from these seeds. The solution of protein not adsorbed onto PLHGM may then be used for the rapid isolation of the mitogen (Kaladas & Poretz, 1978).

The hemagglutinin as isolated from the affinity adsorbent appears to exist as a mixture of dimeric, tetrameric, and octameric forms constituted from a single glycopolypeptide chain of 28 000 g/M. Recycle gel filtration through Sephadex G-200 as well as a single pass through Sephacryl S-200 (column size 2.5 × 80 cm) (S. Morss and R. D. Poretz, unpublished results) allows for isolation of the tetramer free from other protomers. Traces of lymphocyte mitogenic activity (equivalent to 1–2% mitogen contamination) are removed during the gel filtration process and are localized in the protein peak corresponding to the dimer. It is now well documented that plant lectins may be isolated as mixtures of protomers of varying degrees of aggregation. This is seen with conca-

navalin A (McCubbin & Kay, 1971) and the agglutinins from lima bean (Gould & Scheinberg, 1970; Galbraith & Goldstein, 1972) and soy bean (Lotan et al., 1974) which have been detected as a mixture of aggregated protomers.

We propose that the oligomeric forms of the *W. floribunda* agglutinin are composed of a dimer consisting of two identical monomers covalently associated through a disulfide linkage. Each monomer has a molecular weight of 28 000 as determined by polyacrylamide gel electrophoresis in sodium lauryl sulfate and a single $^1/_2$-cystine residue. The tetramer represents the noncovalent association of two dimers, and the octamer, the noncovalent association of four dimeric units. A similar interaction of polypeptide chains has been reported for the hemagglutinins from *S. japonica* (Timberlake, 1974) and *Phaseolus lunatus* (Galbraith & Goldstein, 1972). The finding of approximately 2 residues of glucosamine and 8 residues of mannose per 28 000 g of hemagglutinin is consistent with a monomeric glycopolypeptide structure containing a single serum-type glycopeptide linkage. Such a linkage, which is prevalent in glycoproteins of animal and plant origin (Kornfeld & Kornfeld, 1976), has been reported to be an element of other lectins (Carter & Etzler, 1975; Miller et al., 1975; Misaki & Goldstein, 1977; Lis & Sharon, 1978).

However, the *W. floribunda* agglutinin also contains xylose, arabinose, and fucose and may well possess a second glycopeptide structure containing xylose and arabinose. Pentoses have previously been reported as constituents of lectins. This is the case with the lectins from *S. japonica* (Poretz et al., 1974) which contains xylose and from *Solanum tuberosum* (Allen & Neuberger, 1973) possessing arabinose. *Ulex europeus* II (Matsumoto & Osawa, 1970) and *P. lunatus* (Galbraith & Goldstein, 1972) have been shown to contain both arabinose and xylose.

The fucose we report to be a component of the *W. floribunda* agglutinin may be localized as a branch substituent on *N*-acetyl-D-glucosamine (a structure found in a number of glycoproteins (Kornfeld & Kornfeld, 1976)) and as a nonreducing terminal residue of the mannose or pentose chains. The former structure has been suggested for the carbohydrate component of the lima bean lectin (Misaki & Goldstein, 1977).

Kurokawa et al. (1976) have reported on the isolation, by classical biochemical techniques, of an agglutinin from *W. floribunda* seeds. This lectin which possesses an amino acid composition similar to the protein reported in this communication was obtained only as a dimer of a single subunit of 32 000 g/M. This is in contrast to our work which resulted in the isolation of a mixture of polymeric forms with the tetramer of 116 000 g/M predominating. Furthermore, it is noteworthy that the lectin isolated by Kurokawa et al. (1976) was reported to contain only 3.2% carbohydrate consisting of galactose in addition to mannose and glucosamine and apparently lacked the pentoses reported in this paper. Though the differences may be due to differences in the methods of isolation and characterization of the lectins, the proteins may display some fundamental structural differences due to the source of the seeds from which they were extracted.

Like the agglutinin obtained by Kurokawa et al. (1976), we have found that *N*-acetyl-D-galactosamine is a potent inhibitor of the hemagglutinin. However, lactose is a more efficient inhibitor of the protein than methyl β-D-galactopyranoside, methyl α-D-galactopyranoside, or melibiose. These results may well suggest that the carbohydrate binding site of the lectin is most complementary to a β-linked structure larger than a monosaccharide and that the D-glucose moiety of lactose may contribute a positive force for the interaction of the saccharide with the protein.

Now with the convenient purification of the major protomeric form of the *W. floribunda* hemagglutinin which will allow further characterization of the protein with respect to its structure and carbohydrate binding properties, it is anticipated that this lectin will become a useful reagent to study the structure and function of cell surface carbohydrates.

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