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Physical–Chemical Characterization and Carbohydrate-Binding Activity of the A and B Subunits of the *Bandeiraea simplicifolia* I Isolectins†

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ABSTRACT: *Bandeiraea simplicifolia* I plant seed isolectins comprise a family of tetrameric α-D-galactopyranosyl-binding glycoproteins composed of various combinations of two different kinds of subunits designated A and B. Subtypes of the A (A^a, A^b, A^c, A^d, and A^e) and B (B^a, B^b, B^c, B^d, and B^e) subunits were demonstrated by isoelectric focusing in 8 M urea. Although the content of subunit subtypes varies from seed to seed (e.g., some seeds contain only B subunits, others only A subunits), subtypes A^c and B^c predominate in a natural mixture of the isolectins. Two-dimensional agar gel diffusion studies indicate that, in addition to common structural features, each subunit contains its own distinct antigenic determinants. Although the A and B subunits have closely similar amino acid compositions, they differ markedly in one respect: the B subunit has one methionine residue whereas the A subunit contains no methionine. The neutral carbohydrate content of both subunits is identical. The ability of biopolymers and synthetic glycoproteins to precipitate A_4 and B_4, as well as the capacity of sugars and oligosaccharides to inhibit precipitate formation, was examined. On the basis of these studies, it is suggested that hydrogen bonding occurs between the *hydrogen* atoms of the C-3 and C-4 hydroxyl groups of α-D-GalNAc*p* and α-D-Gal*p* units and the A and B subunits, respectively.

The seeds of *Bandeiraea simplicifolia* contain at least three distinct species of lectins. These are a family of α-D-galactopyranosyl-binding isolectin (BS I) (Murphy & Goldstein, 1977), an *N*-acetyl-D-glucosamine-binding lectin (BS II) (Shankar Iyer et al., 1976), and a family of *N*-acetyl-D-galactosamine binding isolectins (BS III) (Murphy, 1978). The family of α-D-galactose-binding isolectins contains five principle isolectin forms: BS I A_4, A_3B, A_2B_2, AB_3, and B_4 which are tetrameric structures composed of two major glycoprotein subunit types designated A and B (Murphy & Goldstein, 1977). A detailed description of the binding of the BS I isolectins to human red blood cells has been reported (Judd et al., 1978). An earlier report on the carbohydrate-binding specificity of the two subunits of the BS I isolectins indicated that the A subunit exhibited a primary specificity for α-D-GalNAc but also cross-reacted with α-D-Gal*p* whereas the B subunit showed a sharp specificity for α-D-Gal*p* residues (Murphy & Goldstein, 1977). In this communication we extend the comparative studies on the carbohydrate-binding sites of the two extreme forms of the BS I isolectins (A_4 and B_4) and report on the physiochemical properties of the BS I isolectins.

Experimental Section

Materials

Sugars and Sugar Derivatives. Me-α-D-GalNAc was prepared by the procedure of Neuberger & Wilson (1971). Methyl 4-deoxy-4-fluoro-α-D-galactopyranoside was prepared by the procedure of Marcus & Westwood (1971). Dr. R. U. Lemieux, University of Alberta, donated 3-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-D-galactose, 6-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-D-galactose, and 3-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-D-glucose. *p*-NO_2Ph-α-D-GalNAc*p* was a gift from Dr. B. Weissmann, University of Illinois. Methyl 4-*O*-(α-D-galactopyranosyl)-α-D-galactopyranoside was donated by Dr. E. J. Reist, SRI International, Menlo Park, CA. 1-*O*-(6-*O*-α-D-Galactopyranosyl-1-*O*-β-D-galactopyranosyl)-D-glycerol was a gift from Dr. D. Myhre, Procter and Gamble Co. *p*-NO_2Ph-β-D-GalNAc and *p*-NO_2Ph-α- and -β-D-Gal*p* were obtained from Cyclo Chemical Co., Los Angeles, CA; 2-deoxy-D-galactose was obtained from P-L Biochemicals, Milwaukee, WI. Methyl β-D-thiogalactoside was obtained from Vega-Fox-Biochemicals, Tuscon, AZ. All other sugars were obtained from Pfansteihl Laboratories, Waukegan, IL. D-GalNAc obtained from Pfanstiehl required filtration through a PM-10 membrane (Amicon Corp.) before use.

Glycoproteins and Polysaccharides. Type B ovarian cyst substance was kindly provided by Dr. A. Lundblad of the University of Lund; type A cyst substance and hog A + H mucin were provided by Dr. R. D. Poretz, Rutgers University. The galactomannan (guaran) of *Cyamopsis tetragonolobus* was purified as described by Hayes & Goldstein (1974). The arabinogalactan from *Larix occidentalis* was a gift of Professor B. Lindberg of the University of Stockholm. The carbohydrate–bovine serum albumin conjugate *p*-azophenyl β-D-lactoside–BSA was available from an earlier study in this laboratory (Iyer & Goldstein, 1973). The *p*-azophenyl-*N*-acetyl-α- and -*N*-acetyl-β-D-galactosamine–BSA and the *p*-azophenyl α- and β-D-galactopyranoside–BSA conjugates were prepared similarly. All carbohydrate–protein conjugates

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contained 15–22 sugar units/albumin molecule. Streptococcal group C polysaccharide was provided by Dr. M. McCarty, Rockefeller University. Bovine submaxillary mucin was donated by Dr. G. W. Jourdian of this University. Asialo bovine submaxillary mucin was prepared by treatment with 0.06 N H_2SO_4 at 80 °C for 1.5 h, followed by dialysis against water.

Other Materials. Human erythrocytes were supplied by John Judd, University of Michigan Blood Bank. *B. simplicifolia* seeds were routinely obtained from Calbiochem, La Jolla, CA; fresh seeds were provided by Dr. L. E. Newton, Kumasai University, Ghana. Rabbit antisera to the natural mixture of *B. simplicifolia* I isolectins, the crude seed extract, and BS II isolectin were available from previous studies (Hayes & Goldstein, 1974; Shankar Iyer et al., 1976). All other chemicals used in this investigation were of reagent grade or the best quality available.

Methods

Purification of B. simplicifolia I Isolectins. B. simplicifolia I isolectins A_4 and A_3B were purified on a Bio-Gel melibionate column, and A_2B_2, AB_3, and B_4 were separated on a column of insolubilized blood group A substance according to the procedure of Murphy & Goldstein (1977). Free amino groups remaining after preparation of the Bio-Gel melibionate column by the method of Hayes & Goldstein (1974) were blocked by acetylation with acetic anhydride as outlined by Frankel-Conrat (1955).

A modification of the standard protocol was developed in order to purify *B. simplicifolia* I isolectins from individual seeds. Each seed was soaked in absolute ethanol for 25 min. The loosened seed coat was removed with a razor blade and the seed quartered. Seed sections were pulverized in a porcelain mortar in the presence of acetone (10 mL at −10 °C), the seed meal was extracted with acetone (35 min), and the slurry was centrifuged at 12000*g* for 10 min. The supernatant was discarded, and the resulting seed meal was extracted with methanol (9 mL; 40 °C) for 40 min and centrifuged at 12000*g* for 10 min. The supernatant was discarded, and protein was extracted by stirring for 1 h at 0 °C with 10 mL of PBS–Ca (0.01 M phosphate, 0.15 M NaCl, and 0.1 mM $CaCl_2$, pH 7.4) containing 1% poly(vinylpyrrolidone). The mixture was centrifuged at 12000*g* for 15 min. The supernatant was removed and filtered through a 0.45 μM Milex disposable filter (Millipore Corp., Bedford, MA). The clarified extract was applied to a column of Bio-Gel melibionate (0.7 × 10 cm), the column was washed with PBS–Ca until the absorbance was at 280 ≤ 0.02 nm, and the bound isolectins were eluted with 10 mL of PBS–Ca containing Me-α-D-Gal*p* (5 mg/mL), 2-mL fractions being collected. Fractions with an absorbance at 280 ≥ 0.1 nm were pooled. Approximately 1–2 mg of isolectins was obtained per seed.

Assay Procedures. Protein concentration was determined by absorbance at 280 nm, using $E = 1.41$ cm^{-1} mg^{-1} mL as the extinction coefficient determined for pure lectin solutions (Hayes & Goldstein, 1974). Alternatively, the microbiuret protein assay of Janatova et al. (1968) with bovine serum albumin as the standard was employed. Neutral sugar was determined by the phenol–sulfuric acid colorimetric assay of Dubois et al. (1956).

Electrophoresis. Electrophoresis on polyacrylamide gels was performed at pH 9.5 in Tris–glycine buffer according to Brewer & Ashworth (1969). The gels were prerun for 4 h at 2 mA/gel before use. Discontinuous polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to Laemmli (1970). Discontinuous polyacrylamide gel electrophoresis was carried out on an anionic glycine system (pH 9.7) in the presence of 8 M urea and 0.1% sodium dodecyl sulfate according to the method of Wu & Bruening (1971) as modified by Carter & Etzler (1975). Isoelectric focusing in polyacrylamide gels in the presence of 8 M urea and 0.8% NP-40 was done according to the procedure of Ames & Nikaido (1976) with the following modification: the acrylamide concentration was increased to 5.1%, the final concentration of NP-40 was reduced to 0.8%, and only narrow-range physiolytes of pH range 4–6 were used.

Antibody Preparation. Purified *B. simplicifolia* I A_4 was emulsified in Freund's complete adjuvant (Difco Laboratories) giving a product containing 390 μg of lectin per mL. Fifteen aliquots (0.1 mL) of the emulsion were injected intradermally into the lower back region of each of two New Zealand White rabbits. Three weeks later the rabbits were bled from the marginal ear vein. The antisera were stored at −20 °C until further use.

Amino Acid Analysis. Amino acid analyses were carried out by using the single column, three buffer system (Durham Chemical Corp.) and a Beckman 120B amino acid analyzer equipped with long path length cells. Samples (0.3 mg) were hydrolyzed in 1 mL of 6 M HCl for 22, 48, and 72 h, and the values for serine and threonine were obtained by extrapolation to zero time of hydrolysis. A separate sample was hydrolyzed in the presence of 20 μL of Me_2SO according to the procedure of Spencer & Wold (1969) for the determination of cysteic acid. Tryptophan and tyrosine were determined as described by Edelhoch (1967).

Analysis of the Carbohydrate Moiety. Neutral sugars were determined by the phenol–sulfuric acid method of Dubois et al. (1956) using methyl α-D-mannopyranoside as the standard and quantified by gas–liquid chromatography according to the procedure of Lehnhardt & Winzler (1968).

Results and Discussion

Electrophoresis. Discontinuous polyacrylamide gel electrophoresis at pH 9.7 in the presence of 8 M urea and sodium dodecyl sulfate gives considerable separation of the A and B subunits (Figure 1). Isoelectric focusing of the subunits of *B. simplicifolia* I isolectin mixture, A_4, and B_4 in the presence of 8 M urea, 0.8% NP-40, and narrow-range physiolytes (pH 4–6) is shown in Figure 2 with a graphic analysis derived from these data presented in Figure 3. The *B. simplicifolia* I isolectin mixture gave three major bands (B^c, B^e, and A^c) and seven minor bands (B^a, B^b, B^d, A^a, A^b, A^d, and A^e); B_4 gave two major bands (B^c and B^e) and three minor bands (B^a, B^b, and B^d); A_4 gave one principle band (A^c) and four minor bands (A^a, A^b, A^d, and A^e). Of the two major B_4 bands (B^c and B^e), B^c was present in greater amount.

The diffuse "satellite" bands observed in polyacrylamide gel electrophoresis at pH 9.5 (Murphy & Goldstein, 1977) and the multiple bands exhibited by the A and B subunits on isoelectric focusing in 8 M urea may be due to genetic variations in the charged residues of the A and B subunits. The *B. simplicifolia* I isolectins were purified from a pool of seeds collected in the forests of Ghana, Africa. No genetically defined strains were available for this work. The results of polyacrylamide gel electrophoresis of a number of preparations of *B. simplicifolia* I isolectin mixture obtained from individual seeds are shown in Figure 4. The *B. simplicifolia* I isolectin mixture obtained from individual seeds varied considerably in the ratio of A to B subunits and in the subtype of A and B subunits present. Several seeds contained subunits of only one type, A or B. Isoelectric focusing in 8 M urea showed multiple bands for A_4 and B_4 purified from pooled seeds, but

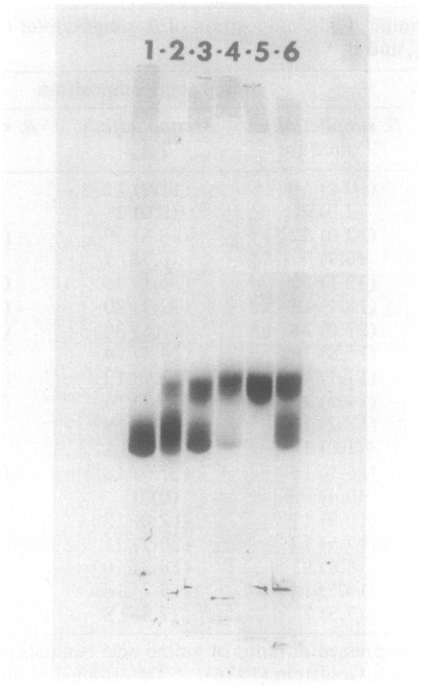

FIGURE 1: Polyacrylamide disc gel analysis of *B. simplicifolia* I isolectins in the presence of sodium dodecyl sulfate and urea, pH 9.7. (1) A_4; (2) A_3B; (3) A_2B_2; (4) AB_3; (5) B_4; (6) A_4, A_3B, A_2B_2, AB_3, and B_4. Twenty-five micrograms of protein was applied to each gel.

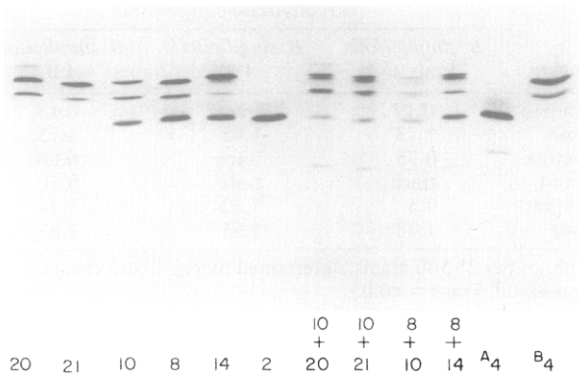

FIGURE 2: Isoelectric focusing in 8 M urea and 0.8% NP-40 of the subunits of *B. simplicifolia* I. Numbers refer to Bio-Gel melibionate purified isolectins purified from single seeds or mixtures of two seeds. A_4 and B_4 are from pooled seeds.

one principle band accounted for the major proportion of the protein present in each subunit. These principle bands corresponded to subtype A^c and B^c. This explains why only five *major* bands are observed when the *B. simplicifolia* I isolectin mixture prepared from pooled seeds is electrophoresed on polyacrylamide gels at pH 9.5. The lectin is a tetramer and, if it is composed solely of two different subunits, five bands should theoretically be observed. The diffuse satellite bands are also observed because other subtypes of the A and B subunits with different electrophoretic mobility are also present.

Theoretically, a tetramer with 3 different subunits would give rise to 15 isolectins. This number of bands is approached in gels 9 and 11 (Figure 4A); overlap in the electrophoretic mobility of some of the isolectins probably occurs.

The seed to seed variation in the ratio of A to B subunits, the absence of the A or B subunits in some seeds, and the variation in the subtypes of the A (A^a, A^b, A^c, A^d, and A^e) and B (B^a, B^b, B^c, B^d, and B^e) subunits present in individual

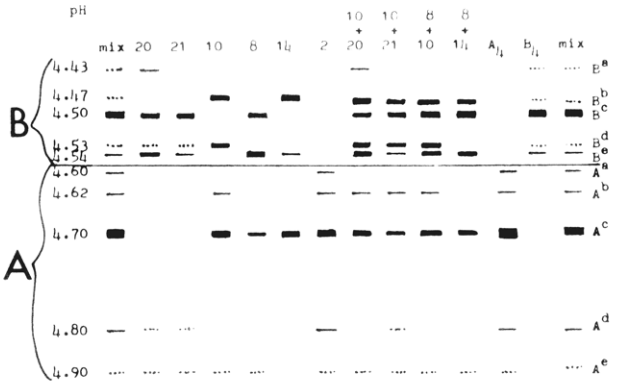

FIGURE 3: Graphic analysis of the isoelectric focusing in 8 M urea of the subunits of the *B. simplicifolia* I isolectin mixture isolated from single seeds. Data were obtained from the composite photograph shown in Figure 2. Mix refers to the mixture of isolectin subunits from a pooled seed preparation.

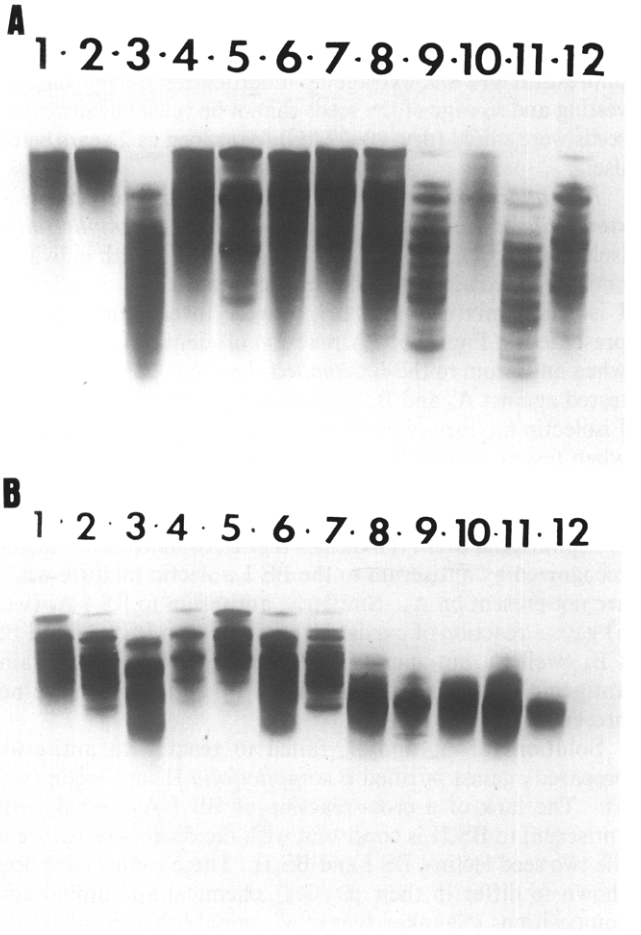

FIGURE 4: Alkaline polyacrylamide disc gel analysis of *B. simplicifolia* I isolectins purified from individual seeds. (A) (1) A_4 from pooled seeds; (2) *B. simplicifolia* I isolectin mixture from single seed (SS) 1; (3) BS I from SS 2; (4) BS I from SS 3; (5) BS I from SS 4; (6) BS I from SS 5; (7) BS I from SS 6; (8) BS I from SS 7; (9) BS I from SS 8; (10) BS I from SS 9; (11) BS I from SS 10; (12) BS I from SS 11. (B) (1) BS I from SS 12; (2) BS I from SS 13; (3) BS I from SS 14; (4) BS I from SS 15; (5) BS I from SS 16; (6) BS I from SS 17; (7) BS I from SS 8; (8) BS I from SS 18; (9) BS I from SS 19; (10) BS I from SS 20; (11) BS I from SS 21; (12) B_4 from pooled seeds.

seeds suggest genetic variation. Although isolation of the isolectins from half of the meal of an individual seed immediately after grinding and processing the second half of the seed meal 2 months later did not result in a change in the isolectin pattern as revealed by polyacrylamide gel electro-

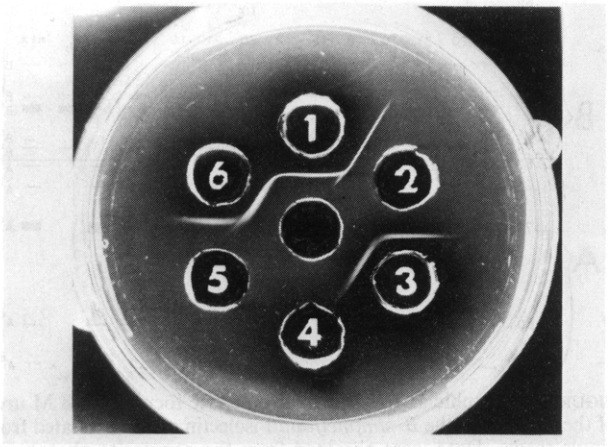

FIGURE 5: Ouchterlony double diffusion in agar patterns. Central well, BS I A_4; well 1, antiserum to BS I natural mixture; well 2, BS I B_4; well 3, antiserum to crude protein extracts of *B. simplicifolia* seeds; well 4, antiserum to BS II; well 5, BS I B_4; well 6, antiserum to BS I A_4. All lectins were 0.30 mg/mL in PBS–Ca.

phoresis at pH 8.9, extragenic modification during the harvesting and storage of the seeds cannot be ruled out since most seeds were stored (dry; 20–27 °C) for as long as 2 years before use.

Immunochemical Studies. The reaction of crude *B. simplicifolia* seed extract, affinity-purified *B. simplicifolia* I isolectin mixture, BS I A_4, BS I B_4, and BS II with individual antisera raised against crude BS I seed extract, *B. simplicifolia* I isolectin mixture, and BS II were investigated and are presented in Figure 5. A reaction of identity was observed when antiserum to the *B. simplicifolia* crude seed extract was tested against A_4 and B_4. Antiserum against the natural BS I isolectin mixture (well I) gave a reaction of partial identity when tested against BS I A_4 (central well) and B_4 (well 2). This pattern suggests that BS I A_4 and B_4 share many common antigenic determinants; however, the spurring of the B_4 precipitin band over A_4 indicates that B_4 contains determinants recognized by antiserum to the BS I isolectin mixture which are not present on A_4. Similarly, antiserum to BS I A_4 (well 6) gave a reaction of partial identity between BS I A_4 and BS I B_4 (well 5), but spur formation denotes that A_4 contains antigenic determinants recognized by anti-A_4 that are not present on B_4.

Solutions of A_4 and B_4 failed to react with antiserum prepared against purified *B. simplicifolia* II seed lectin (well 4). The lack of a cross-reaction of BS I A_4 and B_4 with antiserum to BS II is consistent with the distinctive nature of the two seed lectins, BS I and BS II. These lectins have been shown to differ in their physical–chemical and amino acid compositions (Shankar Iyer et al., unpublished results), and in their carbohydrate binding properties (Shankar Iyer et al., 1976; Ebisu et al., 1978; Wood et al., 1978).

Chemical Characterization. (1) Amino Acid Composition. The amino acid compositions of *B. simplicifolia* I isolectins A_4 and B_4 are presented in Table I. The analysis of the *B. simplicifolia* I isolectin mixture (Hayes & Goldstein, 1974) is included for comparison. The most striking difference between B_4 and A_4 resides in the content of methionine: B_4 contains one residue per subunit and A_4 has none; the isolectin mixture contains 0.4 residue/subunit. Comparison of the content of charged amino acids reveals some differences in the ratio of acidic to basic amino acids; such differences would account for the more anodic migration of the B subunit at alkaline pH values. Minor differences in the content of neutral amino acids are also apparent. Although both the A and B subunits contain a single cysteine residue, the reaction of these residues with 5,5′-dithiobis(2-nitrobenzoic acid) (Habeeb, 1972) differs in that the A_4 isolectin can be titrated only in the presence of 6 M guanidine hydrochloride (Murphy & Goldstein, 1977).

Table I: Amino Acid Composition of *B. simplicifolia* I Isolectin Mixture, A_4, and B_4[a]

amino acid	amino acid composition		
	B. simplicifolia I[b] mixture	*B. simplicifolia* I A_4	*B. simplicifolia* I B_4
Lys	(10.2) 10	(11.9) 12	(8.7) 9
His	(2.0) 2	(2.0) 2	(2.0) 2
ammonia	(32.0) 32	(28.8) 29	(35.0) 35
Arg	(6.9) 7	(6.78) 7	(7.36) 7
Asp	(32.1) 32	(36.1) 36	(32.9) 33
Thr	(18.1) 18	(19.7) 20	(20.9) 21
Ser	(27.6) 28	(29.6) 30	(31.4) 31
Glu	(15.8) 16	(14.5) 14	(19.0) 19
Pro	(11.1) 11	(11.7) 12	(12.6) 13
Gly	(14.9) 15	(16.1) 16	(15.5) 15
Ala	(18.2) 18	(18.9) 19	(19.5) 19
1/2-Cys[c]	(1.0) 1	(0.85) 1	(1.1) 1
Val	(16.1) 16	(17.9) 18	(18.9) 19
Met	(0.4) 0.4	(0) 0	(1.02) 1
Ile	(10.9) 11	(12.8) 13	(11.4) 11
Leu	(17.5) 17	(20.7) 21	(18.9) 19
Tyr	(8.8) 9	(10.3) 10	(9.17) 9
Phe	(14.5) 14	(15.7) 16	(16.5) 16
Trp[d]	(7.3) 7	(7.86) 8	(6.8) 7

[a] Data are expressed in terms of amino acid residues per subunit. [b] From Hayes & Goldstein (1974). [c] Determined by hydrolysis in the presence of dimethyl sulfoxide. [d] Determined spectrophotometrically.

Table II: Carbohydrate Composition[a] of *B. simplicifolia* I Isolectin Mixture, A_4, and B_4

sugar	carbohydrate composition		
	B. simplicifolia I mixture	*B. simplicifolia* I A_4	*B. simplicifolia* I B_4
arabinose	0.19	trace[b]	0.13
fucose	1.73	1.65	1.73
galactose	0.75	trace	0.60
glucose	trace	trace	0.50
mannose	7.5	7.72	7.70
xylose	1.78	1.65	1.85

[a] Moles per 28 500 grams, determined by gas–liquid chromatography. [b] Trace = <0.05.

The minimum molecular weights for A_4 and B_4, calculated on the basis of amino acid composition and neutral sugar and glucosamine content, are 30 800 and 30 400, respectively. These values contrast with the more marked difference in apparent molecular weights determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis: 32 000 for A_4 and 33 000 for B_4 (Murphy & Goldstein, 1977).

(2) Carbohydrate Composition. Hayes & Goldstein (1974) have already established that the natural mixture of *B. simplicifolia* I isolectins is a glycoprotein (6.7% neutral sugars) consisting of mannose, fucose, xylose, and glucosamine in the approximate molar ratio of 10:2:2:4. Carbohydrate analysis of the *B. simplicifolia* I isolectins A_4 and B_4 gave 6.9 and 7.2% neutral sugar, respectively, by the phenol–sulfuric acid assay. The composition of the neutral carbohydrate moieties of *B. simplicifolia* I isolectin mixture, A_4, and B_4, determined by gas–liquid chromatography of neutral sugar alditol acetates, is shown in Table II. Determined to the nearest integer, the molar ratio of mannose, fucose, and xylose is 8:2:2 per subunit (M_r 28 500) for the isolectin mixture, for A_4, and for B_4. The

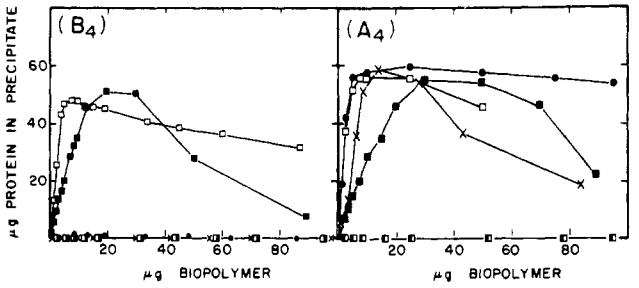

FIGURE 6: Precipitation of biopolymers by *B. simplicifolia* I isolectins B_4 and A_4. Fifty-three micrograms of B_4 and sixty micrograms of A_4 were added per tube; total volume was 500 μL. (●) Streptococcal group C cell wall polysaccharide; (×) type A cyst substance; (■) type B cyst substance; (□) guaran; (◨) larch *arabino*-galactan.

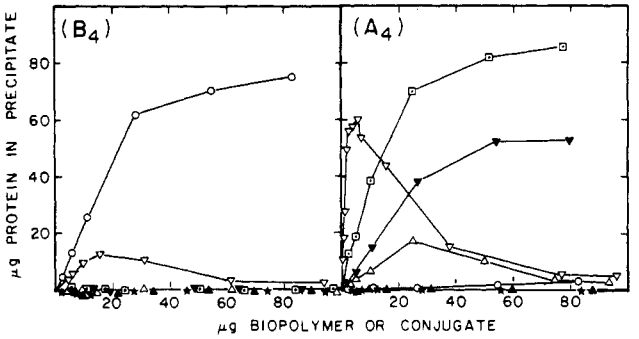

FIGURE 7: Precipitation of biopolymers and bovine serum albumin (BSA) glycoconjugates by *B. simplicifolia* I isolectins B_4 and A_4. Fifty-three micrograms of B_4 and 60 sixty micrograms of A_4 were added per tube; total volume was 500 μL. (△) Bovine submaxillary mucin; (▽) asialo bovine submaxillary mucin; (★) *p*-azophenyl α-D-galactopyranoside–BSA; (○) *p*-azophenyl β-D-galactopyranoside–BSA; (⊡) *p*-azophenyl-*N*-acetyl-α-D-galactosamine–BSA; (▼) *p*-azophenyl-*N*-acetyl-β-D-galactosamine–BSA; (▲) *p*-azophenyl β-D-lactoside–BSA.

carbohydrate compositions of A_4 and B_4 appear to be very similar if not identical.

Precipitin Reactions with Biopolymers and Glycoconjugates. The results of a large number of precipitin reactions of *B. simplicifolia* I isolectins A_4 and B_4 with naturally occurring biopolymers and carbohydrate–bovine serum albumin (BSA) conjugates are summarized in Figures 6 and 7. Both A_4 and B_4 formed precipitates with biopolymers containing multiple, nonreducing α-D-Gal*p* end groups; these include guaran (a galactomannan) and type B blood group substance. Biopolymers containing multiple, nonreducing β-D-Gal*p* end groups such as larch arabinogalactan did not form precipitates with either isolectin.

Interestingly, the *p*-azophenyl α-D-galactopyranoside–BSA conjugate did not precipitate with either A_4 or B_4 whereas *p*-azophenyl β-D-galactopyranoside–BSA reacted very weakly with A_4 and strongly with B_4. The *p*-azophenyl β-lactoside–BSA conjugate did not form a precipitate with either A_4 or B_4. A number of biopolymers containing multiple, nonreducing α-D-GalNAc*p* end groups were also tested for their ability to form precipitates with A_4 and B_4. Type A blood group substance, a streptococcus type C cell wall polysaccharide, and asialo bovine submaxillary mucin precipitated strongly with A_4, whereas only asialo bovine submaxillary mucin precipitated a small amount of B_4. Native bovine submaxillary mucin which probably contains a small number of incomplete carbohydrate chains with exposed terminal, nonreducing α-D-GalNAc*p* residues reacted weakly with A_4 but not with B_4. A_4 precipitated twice as much *p*-azophenyl-*N*-acetyl-α-D-galactosamine–BSA as the β anomer (*p*-azophenyl-*N*-acetyl-β-D-galactosamine–BSA) whereas B_4 did not precipitate either of these conjugates.

Table III: Inhibition of *B. simplicifolia* I Isolectins A_4- and B_4-Guaran Precipitation by Low Molecular Weight Sugars

sugar	[I] (mM) required for 50% inhibn of A_4	[I] (mM) required for 50% inhibn of B_4
D-Gal	1.6	0.86
Me-α-D-Gal*p*	0.45	0.31
Me-β-D-Gal*p*	2.7	1.7
p-NO_2Ph-α-D-Gal*p*	0.22	0.32
p-NO_2Ph-β-D-Gal*p*	1.55	0.27
Me-1-thio-β-D-Gal*p*	ND[a]	6.0
D-GalNAc	0.12	48
Me-α-D-GalNAc*p*	0.031	29
p-NO_2Ph-α-D-GalNAc*p*	0.0084	4.2 (4%)[b]
p-NO_2Ph-β-D-GalNAc*p*	0.135	4.2 (0%)
methyl 2-deoxy-2-amino-α-D-galactopyranoside	1.7	0.6
2-deoxy-2-trifluoroacetamido-D-galactose	0.137	4.0
2-deoxy-D-galactose	8.6	35
D-Fuc	ND	23
Me-α-D-Fuc	ND	5.6
Me-β-L-Ara*p*	ND	35
D-GlcNAc	84 (13.5%)	83 (3%)

[a] ND = not determined. [b] Numbers in parentheses give percentage inhibition at the concentration tested.

Table IV: Inhibition of *B. simplicifolia* I Isolectins A_4- and B_4-Guaran Precipitation by Oligosaccharides

oligosaccharide	[I] (mM) required for 50% inhibn of A_4	[I] (mM) required for 50% inhibn of B_4
6-*O*-α-D-Gal*p*-D-Glc	0.49	0.43
1-*O*-(6-*O*-α-D-Gal*p*-1-β-D-Gal*p*)-D-glycerol	ND[a]	0.56
Me-4-*O*-α-D-Gal*p*-α-D-Gal*p*	ND	0.90
4-*O*-β-D-Gal*p*-D-Glc	47 (29%)[b]	32
3-*O*-β-D-Gal*p*-D-Ara	ND	13
6-*O*-(α-D-GalNAc*p*)-D-Gal	0.026	5.6 (8%)
3-*O*-(α-D-GalNAc*p*)-D-Gal	0.023	5.6 (7.8%)
3-*O*-(α-D-GalNAc*p*)-D-Glc	0.029	5.6 (26%)

[a] ND = not determined. [b] Numbers in parentheses give percentage inhibition at the concentration tested.

Inhibition by Low Molecular Weight Sugars. Inhibition by low molecular weight sugars of the precipitation reaction between *B. simplicifolia* I isolectins A_4 and B_4 and guaran is shown in Tables III and IV. The concentrations of sugar required to inhibit the A_4- or B_4-guaran interaction by 50% are taken from *complete* inhibition curves.

The best inhibitors of the *B. simplicifolia* I isolectin B_4–guaran system were Me-α-D-Gal*p*, *p*-NO_2Ph-α-D-Gal*p*, and *p*-NO_2Ph-β-D-Gal*p*. Comparison of Me-α-D-Gal*p* with Me-β-D-Gal*p* and a series of oligosaccharides containing nonreducing α- and β-D-galactopyranosyl end units reveals that the α anomer is bound more strongly than the corresponding β anomer. Comparison of Me-α-D-Gal*p*, Me-β-D-Gal*p*, and D-galactose supports the conclusion that a methoxyl group in the α position contributes positively to stabilization of the B_4–sugar complex, whereas a β-methoxyl group represents a destabilizing factor. Substitution of the oxygen atom at the C-1 position of methyl β-D-galactoside by a sulfur atom (methyl β-D-thiogalactoside) produced a 3.5-fold decrease in

the inhibiting potency of this glycoside.

Replacement of the methyl aglycon of methyl α- and β-D-galactopyranoside by sugars produces disaccharides which generally interact more weakly with the *B. simplicifolia* isolectins. Thus, as is evident in Table III, oligosaccharides with terminal nonreducing α-D-galactopyranosyl groups are somewhat poorer inhibitors than methyl α-D-galactopyranosides. β-Linked disaccharides, e.g., 3-*O*-β-D-galactopyranosyl-D-arabinose and lactose, are poor inhibitors, being 7–18 times less reactive than methyl β-D-galactopyranoside. On the other hand, it is evident from Table III that oligosaccharides containing nonreducing terminal 2-acetamido-2-deoxy-α-D-galactopyranosyl groups interact just as strongly with the A_4 isolectin as methyl 2-acetamido-2-deoxy-α-D-galactopyranoside.

The interaction of the *p*-nitrophenyl α- and β-D-galactosides with B_4 is remarkable. In sharp contrast to the results obtained with the α- and β-galactosides with aliphatic aglycons, *p*-nitrophenyl α- and β-D-galactoside were equally inhibitory. Inasmuch as methyl and *p*-nitrophenyl α-D-galactoside were equally potent as inhibitors, it is apparent that the aromatic aglycon does not enhance the capacity of the galactoside to bind to the lectin. However, *p*-NO_2Ph-β-D-Gal*p* was 6 times more potent an inhibitor than Me-β-D-Gal*p*, indicating a strong enhancement of binding by the aromatic aglycon of this β-D-galactoside.

Substitution at the O-2 position of D-galactose has a substantial effect on its binding to B_4. D-Galactose is 41 times more inhibitory than 2-deoxy-D-galactose (2-deoxy-D-*lyxo*-hexose) and 56 times more potent an inhibitor than D-GalNAc. Me-α-D-Gal*p* is only 2 times more potent an inhibitor than methyl 2-amino-2-deoxy-α-D-galactopyranoside and 94 times more inhibitory than Me-α-D-GalNAc*p*. *p*-NO_2Ph-α-D-GalNAc*p* and *p*-NO_2Ph-β-D-GalNAc*p* were both poor inhibitors (<4%) at the highest concentration tested (4.2 mM). Limited solubility prevented the *p*-nitrophenyl glycosides from being studied at higher concentrations. 6-*O*-(2-Acetamido-2-deoxy-α-D-galactopyranosyl)-D-galactose, 3-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-D-galactose, and 3-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-D-glucose were poor inhibitors of B_4 at the highest concentration tested (5.6 mM). Insufficient material prevented these disaccharides from being assayed at higher concentrations.

The C-6 hydroxymethyl group of D-galactose is an important binding locus for interaction with B_4. This statement is supported by the observations that D-galactose is 27 times more potent an inhibitor than 6-deoxy-D-galactose (D-fucose) and Me-α-D-Gal*p* is 18 times more inhibitory than methyl 6-deoxy-α-D-galactopyranoside and 113 times better than methyl β-L-arabinopyranoside which lacks a C-6 hydroxymethyl group.

The hydrogen atom of the C-3 and C-4 hydroxyl groups of D-galactose is probably involved in the binding of D-galactose to B_4. Methyl 4-deoxy-4-fluoro-α-D-galactopyranoside (0% at 20 mM) and *p*-nitrophenyl 3-deoxy-3-fluoro-β-D-galactopyranoside (0% at 10 mM) were both noninhibitors of the *B. simplicifolia* I isolectin mixture–guaran interaction. Since no inhibition of the precipitation occurred at these relatively high concentrations of inhibitors, these sugar derivatives must not interact with either A_4 or B_4. The fluorine atom is isosteric with a hydroxyl group and is known to participate in hydrogen-bond formation (Pauling, 1960). The absence of inhibition by these two derivatives suggests that the *hydrogen* atoms and not the *oxygen* atoms of the C-3 and C-4 hydroxyl groups are involved in hydrogen bonding of D-galactose to B_4. The fact that D-GlcNAc is a noninhibitor in the B_4–guaran system at 83 mM, whereas D-GalNAc gives 50% inhibition at 48 mM, suggests that an axial C-4 hydroxyl group is an important sugar binding locus for B_4–D-galactose interaction.

Examination of the data in Tables III and IV indicates that the *B. simplicifolia* I A_4 isolectin also has a preference for the α-anomeric configuration: α-GalNAc*p* groups are bound more strongly than the anomeric β-glycosides. It was also observed that the *p*-nitrophenyl α- and β-D-glycosides of D-GalNAc are 2–4 times better inhibitors than the corresponding methyl glycosides of the same anomeric configuration. These results indicate a role for aromatic aglycons in the binding mechanism of glycosides to the A_4 isolectin [cf. Poretz & Goldstein (1971)].

The disaccharides 3-*O*-(α-D-GalNAc*p*)-D-Gal, 6-*O*-(α-D-GalNAc*p*)-D-Gal, and 3-*O*-(α-D-GalNAc*p*)-D-Glc were all excellent inhibitors in the A_4–guaran system, being 7–25% better inhibitors than methyl α-D-GalNAc*p*.

The 2-acetamido-2-deoxy group appears to represent an important binding locus for the A_4–D-GalNAc*p* interaction. The observation that D-GalNAc is 13 times more inhibitory than D-Gal and 72 times better than 2-deoxy-D-galactose supports this view. Furthermore, Me-α-D-GalNAc*p* is 55 times more potent an inhibitor than methyl 2-amino-2-deoxy-α-D-galactopyranoside.

As is the case for the B_4–D-galactose interaction, the fact that methyl 4-deoxy-4-fluoro-α-D-galactopyranoside and *p*-nitrophenyl 3-deoxy-3-fluoro-β-D-galactopyranoside are noninhibitors of the *B. simplicifolia* I isolectin mixture–guaran system supports the hypothesis that the *hydrogen* atoms of the C-3 and C-4 hydroxyl groups are important binding loci for A_4–D-GalNAc interaction. The importance of the C-4 hydroxyl as a binding locus is supported by the observation that D-GalNAc is approximately 1000 times more inhibitory than D-GlcNAc in the A_4–guaran system.

The carbohydrate binding specificity of *B. simplicifolia* I isolectin A_4 may be summarized as follows: α-D-GalNAc*p* > β-D-GalNAc*p* ≃ α-D-Gal*p*. This specificity is apparent when substances containing a relatively small number of determinant sugars (low valency) such as carbohydrate–*p*-azophenyl–BSA conjugates and A and B blood group active cyst substances are compared. The specificity is also observed when the agglutination of types A and B erythrocytes is considered. However, in its interaction with substances containing a high density of cross-reactive α-D-Gal*p* units such as guaran and the Bio-Gel melibionate affinity column, the preferred specificity of A_4 for α-D-GalNAc*p* groups is no longer apparent. Biopolymers containing a high local concentration of α-D-Gal*p* residues react with A_4 as well as most of the α-D-GalNAc*p*-containing substances tested.

From the results of the above inhibition studies, several points of hydrogen-bond interaction between A_4 and α-D-GalNAc*p* may be inferred. These include the hydrogen atoms of the C-3 and C-4 hydroxyl groups.

The carbohydrate binding specificity of *B. simplicifolia* I isolectin B_4 may be summarized as follows: α-D-Gal*p* ≫ β-D-Gal*p* ≃ α-D-GalNAc*p*. For methyl glycosides, the specificity is Me-α-D-Gal*p* > Me-β-D-Gal*p* ≫ Me-α-D-GalNAc*p*, and, for the special case of *p*-nitrophenyl glycosides, the reactivity of B_4 is *p*-NO_2Ph-α-D-Gal*p* ≃ *p*-NO_2Ph-β-D-Gal*p*. This specificity is confirmed by the results of precipitation and agglutination studies with but few exceptions: e.g., asialo bovine submaxillary mucin is weakly precipitated by B_4. This may be due to (1) the very high content of α-D-GalNAc*p* terminal residues (one for each six amino acid

residues) or (2) the interaction of B_4 with a small number of D-galactose-terminated oligosaccharide chains which have not yet been characterized.

The precipitation or lack thereof of certain *p*-azophenyl α- and β-galactopyranoside–BSA conjugates with B_4 was unexpected. *p*-Azophenyl β-D-galactopyranoside–BSA is an excellent precipitant of B_4, whereas *p*-azophenyl α-D-galactopyranoside–BSA is nonreactive. This is just the reverse of what one would have predicted for an α-D-Gal*p*-binding lectin. Interestingly, a similar situation was observed when the interaction of *p*-azophenyl α- and β-galactopyranoside–BSA conjugates was studied with concanavalin A (Iyer & Goldstein, 1973), a lectin with specificity for α anomers. *p*-Azophenyl β-D-galactopyranoside–BSA was precipitated by concanavalin A more readily than *p*-azophenyl α-D-galactopyranoside–BSA. Enhanced binding of β-linked aromatic aglycons occurs with both *B. simplicifolia* I B_4 and concanavalin A. *p*-NO_2Ph-β-D-Glc*p* is 5 times more potent than Me-β-D-Glc*p*, and *p*-NO_2Ph-β-D-Gal is 6 times more potent than Me-β-D-Gal*p* as inhibitors of concanavalin A and *B. simplicifolia* I isolectin B_4, respectively. *p*-NO_2Ph-α-D-Glc*p* and Me-α-D-Glc*p* are equally potent as inhibitors of concanavalin A. In a parallel fashion, *p*-NO_2Ph-α-D-Gal*p* and Me-α-D-Gal*p* are equally potent inhibitors of B_4.

Several points of hydrogen bonding between B_4 and α-D-Gal*p* may be inferred from the results of inhibition studies carried out on *B. simplicifolia* B_4. These include the hydrogen atoms of the C-3 and C-4 hydroxyl groups and either the oxygen or hydrogen atoms of the C-2 and C-6 hydroxyl groups. The fact that methyl α-D-Fuc*p* (methyl 6-deoxy-α-D-galactopyranoside) is 6 times more potent an inhibitor than Me-β-L-Ara*p* suggests a nonpolar interaction between the C-6 methylene group and the lectin. An aromatic binding site for the β-linked aglycon of *p*-NO_2Ph-β-D-Gal*p* is also proposed.

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