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LECTINS AS CARBOHYDRATE-BINDING PROTEINS

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Abstract - Lectins are cell-binding and agglutinating (glyco)proteins found in a variety of living forms. They will be defined here as homogeneous carbohydrate-binding (glyco)proteins principally of plant (seed) origin but also occurring in invertebrate (and vertebrate?) forms.

In recent years, lectins have been employed in studying a wide range of biological phenomena including cell differentiation and maturation (*e.g.*, mitogenesis and spermatogenesis); cell agglutination; tumor growth and inhibition; and the nature and distribution of carbohydrates on cell surfaces.

Lectins have recently been found to be valuable tools for investigating the structure of complex carbohydrates (polysaccharides, glycoproteins, and glycolipids), for studying the molecular basis of carbohydrate-protein interactions, and for isolating and purifying polysaccharides and glycoproteins. However, in order to be truly useful as structural probes, the physical-chemical properties of lectins must be characterized and, most importantly, their carbohydrate-binding specificity must be defined.

Lectins with specificity for a rather large number of sugars have been isolated and characterized. The use of carbohydrate-protein conjugates for the detection of lectins, and natural and synthetic affinity columns for the isolation of lectins will be described.

The isolation and characterization of concanavalin A, the jack bean lectin, is described along with an enumeration of its carbohydrate binding specificity and the localization of its binding site by x-ray crystallography.

A comparison of the carbohydrate-binding specificity of four N-acetyl-D-galactosamine binding lectins from *Helix pomatia*, *Dolichos biflorus*, *Phaseolus lunatus*, and *Glycine max* is discussed.

As an example of how lectins may be used in structural studies, an investigation of the interaction of several lectins with *pneumococcus* S XIV capsular polysaccharide is described.

The α-D-galactopyranosyl-binding lectin from *Bandeiraea simplicifolia* seeds is shown to comprise a family of five isolectins. Each isolectin is a tetramer composed of two different subunits, A which binds N-acetyl-D-galactosamine and B which is specific for α-D-galactosyl units. The separation, characterization and some properties of these isolectins are presented.

For almost a century we have known that many plant seed extracts clump or agglutinate animal erythrocytes. Specific proteins or glycoproteins isolated from these extracts cause the agglutination. These proteins were called plant agglutinins or phytohemagglutinins until about 25 years ago when Boyd coined the term lectin from the Latin *legere* - to pick out or choose (1). Boyd chose this term to call attention to their serological specificity since he and Renkonen had discovered that some of these seed extracts could distinguish among human blood groups (2,3). Testing lima bean (*Phaseolus lunatus*) extract against a panel of blood samples, Boyd discovered that the phytohemagglutinin of lima beans was quite specific for type A erythrocytes (2). Several years ago, the lima bean lectin was purified and its specificity for type A cells was confirmed (4,5).

Building upon these observations, Morgan and Watkins (6,7) followed by numerous other investigators (8,9), employed lectins as probes for studying the immunochemical, determinant sugars of blood group antigens of substances. All blood group substances contain the

same four sugars - D-galactose, L-fucose, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine. The specific way these sugars are linked together determines blood group specificity. This is illustrated in Fig. 1 which shows fragments of the ABO blood group substances. The terminal nonreducing sugar of each of these structures plays a dominant

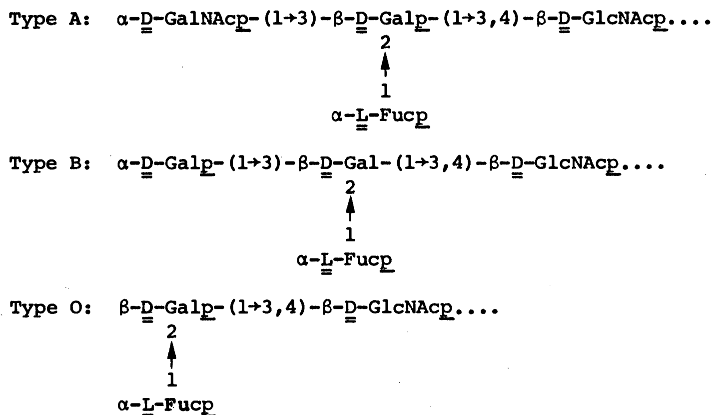

Fig. 1 Sequence of sugars in oligosaccharide chains of blood group substances.

role in determining immunochemical specificity. It is also chiefly these sugar residues with which lectins interact. Thus, α-linked N-acetyl-D-galactosamine is the immunodominant sugar of type A substance. The lima bean lectin interacts with this sugar. Similarly, D-galactose-linked α- is the immunodominant sugar of blood group B substance and α-L-fucosyl groups of O-substance. The lectins from Lotus tetragonolobus seeds and eel serum agglutinate type O erythrocytes. Morgan and Watkins discovered that this agglutination reaction between type O red blood cells and Lotus extracts was best inhibited by L-fucose (6,7). This is the way they showed L-fucose to be the immunodominant sugar of O-cells. The same investigators showed N-acetyl-D-galactosamine to be the best inhibitor of the agglutination reaction between type A erythrocytes and lima bean extracts (7).

The finding that simple sugars inhibited lectin-induced hemagglutination reactions indicated that these sugars were interacting with sites on the lectin molecules. These observations indicate that lectins are a class of carbohydrate-binding proteins.

Since these milestones in lectin research, substances capable of binding carbohydrates and interacting with and agglutinating animal cells have been found in many diverse sources: bacteria and molds, lichens, fish sera and roe, numerous invertebrates and even vertebrate forms (10).

Approximately 25 lectins have been purified and their physical-chemical properties and carbohydrate-binding specificity studied. A group of representative lectins is presented in Table 1 along with their blood group specificity, sugar specificity and some of their molecular properties.

TABLE 1. Some properties of purified lectins[a]

Source	Sugar Specificity	Blood Group Specificity	Mol. Wt. Subunits
Canavalia ensiformis (jack bean)	α-D-Man α-D-Glc (GlcNAc)	-	104,000 (4)
Lens culinaris (lentil)	α-D-Man α-D-Glc (GlcNAc)	-	49,000 (2)
Pisum sativum (pea)	α-D-Man α-D-Glc (GlcNAc)	-	55,000 (4)
Glycine max (soy bean)	D-GalNAc	A	120,000 (4)
Dolichos biflorus (horse gram)	α-D-GalNAc	A	113,000 (4)

TABLE 1. (Continued)

Source	Sugar Specificity	Blood Group Specificity	Mol. Wt. Subunits
Phaseolus lunatus (lima bean)	α-D-GalNAc	A	125,000 (4) 250,000 (8)
Helix pomatia (edible snail)	α-D-GalNAc	A	100,000 (6)
Bandeiraea simplicifolia I	α-D-Gal	B	114,000 (4)
Sophora japonica (Japanese pagoda tree)	α-D-GalNAc α-D-Gal	B,A	132,800 (?)
Ricinus communis (castor bean RCA_I)	β-D-Gal	-	120,000 (4)
Lotus tetragonolobus (asparagus pea)	α-L-Fuc	O	120,000 (4)
Anguilla anguilla (eel)	α-L-Fuc	O	123,000 (12)
Triticum vulgaris (wheat)	GlcNAc(1$\overset{\beta}{\rightarrow}$4)GlcNAc	-	36,000 (2)
Solanum tuberosum (potato)	[GlcNAc(1$\overset{\beta}{\rightarrow}$4)GlcNAc]$_2$	-	100,000 (2)
Bandeiraea simplicifolia II	β-D-GlcNAc α-D-GlcNAc	-	113,000 (4)
Phaseolus vulgaris	(SA)→β-D-Gal... (SA)→β-D-Gal...		136,000 (4)
Streptomyces 27 S 5	L-Rha	B	11,000
Limulus polyphemus (horse shoe crab)	Sialic acid	-	400,000 (20)

a. Sugar abbreviations are standard. SA, sialic acid.

The physiological function of lectins is still completely unknown although there have been many speculations (Table 2). However, none of these possibilities has been convincingly demonstrated.

TABLE 2. Possible functions of plant lectins

Sugar transport, storage and immobilization
Carbohydrases
Plant antibodies
Binding of nitrogen fixing bacteria
Involvement in germination, differentiation, maturation, cell division

Despite our ignorance of the biological role of lectins, these carbohydrate-binding proteins have proved to be exceptionally valuable and versatile substances for examining a variety of cellular activities. Some biological properties of lectins are enumerated in Table 3.

TABLE 3. Some biological properties of lectins

Agglutination of cells: Erythrocytes, lymphocytes, spermatozoa, tumor cells, microorganisms, viruses
Induction of mitogenesis in lymphocytes
Inhibition of enzymatic activity: 5'-nucleosidase, ß-glucosidase
Insulin-like action of fat cells
Cellular poisons: Ricin, abrin
Immunological activity: Immunosuppression and enhancement; histamine release

The use and application of lectins especially as probes for structural studies on complex carbohydrates is shown in Table 4.

TABLE 4. Some uses of lectins

Blood typing; structural studies on blood group substances
Detection, preliminary characterization, and structural studies on polysaccharides, glycoproteins and glycolipids
Isolation and purification of carbohydrate-containing polymers
Studies of carbohydrate-containing structures on cell surfaces
Models for carbohydrate-protein interaction and for antibody

In order for a lectin to be a useful probe in molecular biology and a structural probe in carbohydrate chemistry it is necessary to isolate it in pure form and to characterize its physical and chemical properties as completely as possible. Above all, its carbohydrate binding specificity must be studied in detail. In only one case has such a complete characterization been accomplished - that of concanavalin A.

First isolated by Sumner and Howell, in 1936 (11), concanavalin A (con A, from Canavalia ensiformis beans) was obtained in pure form by affinity chromatography on cross-linked dextran gel (Sephadex) (12,13). The carbohydrate-binding specificity of this lectin has been studied in great detail (14). As illustrated in Fig. 2, the con A combining sites are most complementary to terminal, nonreducing α-D-mannopyranosyl units, although 2-O-substituted α-D-mannopyranosyl residues will also interact with the lectin (15). We have used a series of deoxy, O-methyl, and fluoro derivatives of D-glucose and D-mannose to identify the precise atoms of each hydroxyl group that may be involved in binding to the protein (14). Our findings are summarized in Fig. 2.

Con A is a metalloprotein composed of subunits, molecular weight 26,000. At pH 5-6 the lectin occurs as a dimer; at pH 7 as a tetramer. The protein has been sequenced (16) and its x-ray crystallographic structure solved at a resolution of 2 Å (17) and 2.4 Å (18). Con A has one Mn^{2+} and one Ca^{2+} per protomer; both ions are required for carbohydrate binding activity (19). The con A carbohydrate binding site has been located in crystals of con A (20,21) after considerable controversy (22-25).

Although lectins are most commonly detected by screening extracts from plant seed and animal tissue for their ability to agglutinate animal cells, more sophisticated procedures are now available. We use precipitation reactions between lectins and polysaccharides (26), and naturally occurring and synthetic carbohydrate-protein conjugates (27,28). Recently we have developed a simple procedure for linking carbohydrates to proteins (29). The method is illustrated in Fig. 3. A peptide linkage is established between sugar acid and protein amino groups. This linkage leads to far less nonspecific interactions compared to the azophenyl linkage we have used previously.

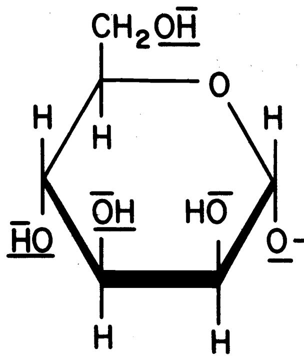

Fig. 2 Sugar binding specificity of concanavalin A. Essential hydroxyl groups (C-3, 4 and 6) are underlined. The hydrogen and oxygen atoms thought to participate in hydrogen bonding to the protein are overscored.

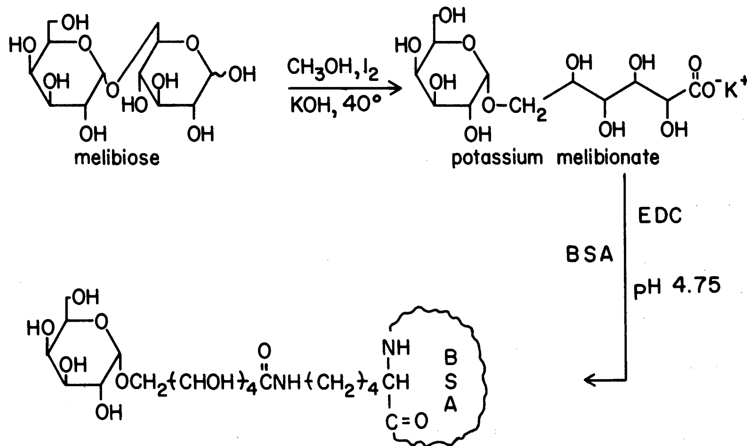

Fig. 3 Aldonate coupling reaction. BSA, bovine serum albumin; EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide.

We have already seen that lectins bind a rather broad range of different sugars. However, no lectin is specific for a single sugar. Concanavalin A binds α-D-mannopyranosyl units most avidly; but the lectin also interacts with α-linked D-glucosyl and N-acetyl-D-glucosaminyl residues although with only about 20% and 10%, respectively, of the affinity the lectin exhibits for D-mannose (30).

Even within a group of lectins that have a primary specificity for the same sugar there are important differences. Let us consider, for example, four N-acetyl-D-galactosamine-binding lectins from the soy bean, the lima bean, Dolichos biflorus and the snail Helix pomatia.

My colleagues (S. Hammarström, M. Etzler and L. Murphy) and I have studied the ability of these four lectins to precipitate with a series of biopolymers (31). All four lectins precipitate with hog A + H substance which contains terminal α-linked N-acetyl-D-galactosaminyl units. Only the snail and soy bean lectins precipitated with the galactomannan, guaran, which contains α-D-galactopyranosyl stubs. Similarly, only the snail and soy bean lectins will precipitate a p-azophenyl β-D-galactosaminyl-BSA conjugate. Soy bean agglutinin is the only lectin that precipitates with the p-azophenyl α-D- and β-D-galactopyranoside conjugates; and none of the lectins precipitate with larch arabinogalactan containing terminal, nonreducing β-D-galactopyranosyl residues.

These results demonstrate that important specificity differences exist and must be considered when drawing conclusions about the reactions of lectins with carbohydrate-containing polymers of unknown constitution. The fact that the snail lectin has 6 combining sites, whereas the other three *N*-acetyl-*D*-galactosamine-binding proteins possess only two sites, is an important consideration.

As an example of how lectins may be used in structural studies, I will describe our investigation of *Pneumococcus* S XIV with wheat germ agglutinin and the hemagglutinin from the castor bean. The lectin from wheat (WGA) has a combining site that interacts with β-linked *N*-acetyl-*D*-glucosaminyl residues that occur at polysaccharide or glycoprotein chain ends, or with internal units linked at the O-4 position (32-34). In fact, the combining site of this lectin can accommodate three β-(1→4)-linked units as occur in *N*,*N*',*N*"-triacetyl chitotriose. The castor bean hemagglutinin interacts with both α- and β-*D*-galactopyranosyl end groups, with a slight preference for the β-anomer. Thus, the castor bean agglutinin will precipitate with both guaran and larch arabinogalactan (35-37).

The structure of S XIV, as elegantly worked out by Lindberg *et al.* (38), is presented in Fig. 4. The site of reaction with wheat germ agglutinin and the castor bean agglutinin are also indicated. Indeed, both of these lectins readily precipitate with S XIV. It is most interesting to observe that wheat germ agglutinin is capable of interacting with a β-*D*-GlcNAc unit substituted at both the O-4 and O-6 positions. On the other hand, Allen and his coworkers showed that O-3 substitution is not tolerated by wheat germ agglutinin (33).

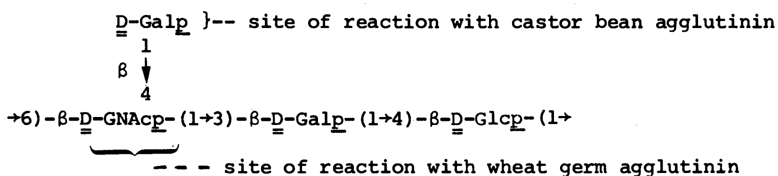

Fig. 4 Structure of *Pneumococcus* S XIV capsular polysaccharide illustrating the site of interaction of wheat germ agglutinin and the castor bean agglutinin.

We also conducted periodate oxidation studies on S XIV with some interesting results (Fig. 5). One mole of oxidant is consumed in less than a day followed by a slower oxidation which required 15 days for the theoretical 3 moles to be consumed. At the end of this time the polyaldehyde was reduced to the corresponding polyalcohol. The S XIV polyalcohol failed to precipitate with either *R. communis* hemagglutinin or wheat germ agglutinin.

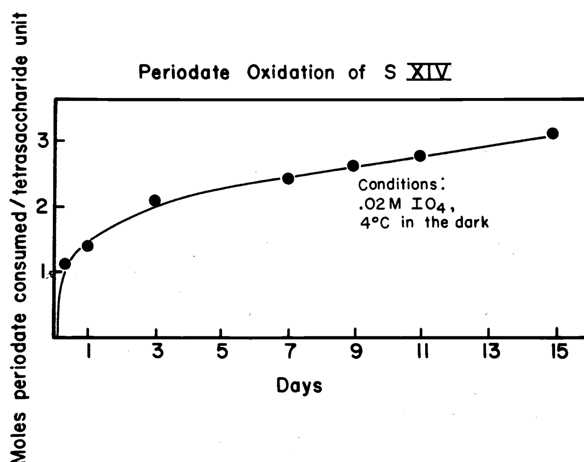

Fig. 5 Periodate oxidation of S XIV polysaccharide.

We did not expect the polyalcohol to precipitate with ricin, since the galactosyl stubs were fragmented. But we were surprised when S XIV polyalcohol failed to react with wheat germ agglutinin. It is possible that steric hindrance caused by the polyalcoholic groups at the O-4 position of GlcNAc units prevented reaction with wheat germ agglutinin.

If the oxidation was allowed to proceed for just one day before the reduction step, the β-D-galactosyl stub was preferentially cleaved. Controlled hydrolysis (Smith degradation) at pH 1.0, 25° for 24 hours removed the (1→4)-β-D-galactosyl unit. As expected the derived linear polymer no longer precipitated with ricin; but it did react vigorously with wheat germ agglutinin. This is the first instance of a β-D-GlcNAc unit, substituted solely at the O-6 position, reacting with wheat germ agglutinin.

These studies, along with hapten inhibition data on several-score simple sugars and oligosaccharides, now make wheat germ agglutinin a valuable reagent for studying GlcNAc residues in biopolymers.

Mäkelä and Mäkelä first reported a lectin in B. simplicifolia seeds which preferentially agglutinated type B erythrocytes (39). They indicated that melibiose was the best inhibitor of the agglutination reaction between the B. simplicifolia lectin and type B erythrocytes. Dr. Colleen Hayes in our laboratory isolated the lectin by affinity chromatography on a melibionate-Bio-Gel P-300 column (40). The lectin so isolated behaved as a single component on polyacrylamide gel electrophoresis at pH 4.3 and in the analytical ultracentrifuge (pH 5.0). Results of serological studies indicated the lectin to be specific for B and AB erythrocytes and to cross-react with A_1 but not A_2 cells. A summary of the lectin's properties is presented in Table 5.

TABLE 5. Some physical, chemical and biological characteristics of Bandeiraea simplicifolia lectin (BS I)

Molecular weight:	114,000
Subunit structure:	4 subunits, 28,500 mol. wt.
Percentage carbohydrate:	6.7%
Metal requirement:	Ca^{2+}
Sulfhydryl content:	one thiol
Isoelectric point:	5.0 ± 0.2
Carbohydrate binding specificity:	α-D-galactopyranosides, one binding site/subunit, $K_a = 3.3 \times 10^4$ M^{-1} at 20° C
Human Blood group specificity:	type B with weak A_1 cross reactivity; no reaction with types A_2 or O

The B. simplicifolia lectin gives a typical antibody-antigen type precipitin curve with guaran, precipitates readily with type B substance and cross-reacts to a limited extent with type A_1 substance. Carbohydrate-binding specificity studies indicate the lectin to be most complementary to α-D-galactopyranosyl end units, but to cross-react to a limited extent with N-acetyl-D-galactosaminyl units (40).

Recently we found that N-acetyl-D-galactosamine completely abolished the agglutination of A_1 erythrocytes by the lectin, but did not reduce its titer against B cells. This led us to reexamine our data and question the homogeneity of the B. simplicifolia (BS I) lectin. The amino acid analysis indicated approximately 0.4 mole methionine/subunit. We also noted that the hapten inhibition curve for N-acetyl-D-galactosamine was atypical in that it was not sigmoidal as for other carbohydrate inhibitors. Furthermore, the titer for A erythrocytes appeared to vary considerably with different lectin preparations. Finally, we observed that polyacrylamide gel electrophoresis at pH 8.9 gave 5 major bands, each of which stained with our new fluorescent glycoprotein stain (41).

These data led us to postulate that the B. simplicifolia (BS I) lectin comprises a family of five isolectins. We had already established that the lectin contained four subunits. Our hypothesis required two different kinds of subunits: B subunit displaying a primary specificity for α-D-galp units and A subunit specific for α-D-GalNAcp units. These isolectins have been designated as indicated in Figure 6.

What is the evidence in support of this hypothesis? First we have been partially successful in resolving this mixture. The form which we call A_4 is readily displaced from a melibionate-Bio-Gel column by low concentrations of D-GalNAc. This is followed by A_3B. Elution with methyl α-D-galactopyranoside gave the remaining three forms.

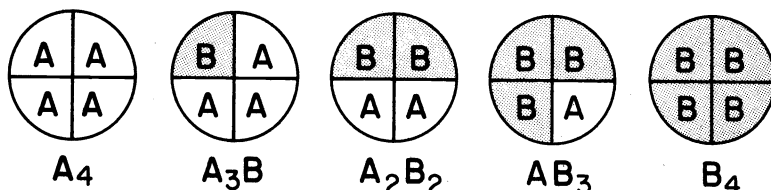

Fig. 6 Isolectins of B. simplicifolia (BS I) lectin. Each form contains four subunits. Subunit B binds α-D-Galp >> α-D-GalNAcp; subunit A has the specificity α-D-GalNAc > α-D-Galp.

On the other hand, when the isolectin mixture is added to an insolubilized blood group A substance column, B_4 and B_3A pass through the column whereas the other forms are bound. The bound isolectins (A_4, A_3B, and A_2B_2) can be eluted subsequently with methyl α-D-galactopyranoside.

Preliminary serological experiments indicate that the mixture of B_4 and B_3A are absolutely specific for type B erythrocytes and A_4 for A erythrocytes with some of the intermediate forms falling between these two extremes.

Amino acid analyses of A_4, the native mixture of isolectins and B_4, B_3A revealed some important differences between the two kinds of subunits: the B subunit contains approximately one mole methionine whereas the A subunit has none. There are also some expected differences in the ratio of acidic to basic amino acids, with the B subunit moving more rapidly toward the anode at alkaline pH values. The sulfhydryl groups on the A and B subunits also titrate differently with 5,5'-dithiobis(2-nitrobenzoic) acid. Rabbit antisera against A_4 or the mixture of five isolectins do not distinguish between the subunits. These reactions of apparent immunochemical identity taken together with the amino acid analyses indicate considerable homology between the two types of subunits.

We have also carried out a preliminary investigation of the carbohydrate binding specificity for A_4 and the mixture of B_4, B_3A. A_4 precipitates both blood groups A and B substances as well as guaran, but not larch arabinogalactan. B_4, B_3A precipitates guaran and type B substance but not type A substance.

Hapten inhibition studies demonstrate the 100-fold preference of B_4, B_3A for α-D-galactosyl units over α-D-GalNAc units, whereas A_4 exhibits only a 20-fold preference for α-D-GalNAc over α-D-Gal. These studies indicate the B subunit to be highly specific for α-D-Gal units. We believe this series of isolectins will prove to be valuable structural probes for the carbohydrate chemist, once the individual species are fully characterized.

We have already employed the immobilized mixture of isolectins to isolate a new galactomannan from the seeds of Cassia alata (42).

Finally, we have isolated a sixth lectin from B. simplicifolia seeds, one that has a primary specificity for N-acetyl-D-glucosaminyl units (43). We discovered this new lectin (BS II) when we screened B. simplicifolia extracts with a series of synthetic carbohydrate-protein conjugates and discovered a reaction with a p-azophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside-bovine serum albumin conjugate. The lectin was isolated by affinity chromatography on chitin (43). It gave a single protein band by polyacrylamide gel electrophoresis at pH 4.3 with exactly the same mobility as B. simplicifolia I.

Immunochemically it is distinct from B. simplicifolia I, a reaction of complete nonidentity being exhibited by the antisera of each purified component against the second lectin.

Amino acid analyses of the two lectins (BS I and BS II) also indicate gross differences. We are now studying this interesting new lectin with N-acetyl-D-glucosamine-containing biopolymers.

In summary, lectins are substances of great versatility. They have the advantage of being rather cheap and simple to prepare in relatively large quantities. We believe that in the years to come purified lectins of known specificity will be the subject of intensive investigation both as valuable tools in studies of carbohydrate structure and in molecular biology, and also as subjects of study in their own right: proteins and glycoproteins of great inherent interest to immunologists, biochemists, and plant physiologists.

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