Characterization of the Structural Determinants Required for the High Affinity Interaction of Asparagine-linked Oligosaccharides with Immobilized Phaseolus vulgaris Leukoagglutinating and Erythroagglutinating Lectins*

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The carbohydrate binding specificities of the leukoagglutinating phytohemagglutinin (L-PHA) and erythroagglutinating phytohemagglutinin (E-PHA) lectins of the red kidney bean, Phaseolus vulgaris, have been investigated by lectin-agarose affinity chromatography of Asn-linked oligosaccharides. High affinity binding to E-PHA-agarose occurs only with biantennary glycopeptides containing 2 outer galactose residues and a residue of N-acetylglucosamine linked β1,4 to the β-linked mannose residue in the core. This species is not retarded on L-PHA-agarose. In contrast, tri- and tetraantennary glycopeptides containing outer galactose residues and an α-linked mannose residue substituted at positions C-2 and C-4 are specifically retarded on L-PHA-agarose. Triantennary glycopeptides containing outer galactose residues and an α-linked mannose residue substituted at positions C-2 and C-4 are not retarded on L-PHA-agarose. Additionally, the presence of outer sialic acid residues or a core fucose residue does not influence the behavior of complex glycopeptides on either of these lectin-agarose conjugates. This ability of E-PHA and L-PHA to discriminate between Asn-linked oligosaccharides with various branching patterns can be utilized in the fractionation of these glycopeptides (see paper following).

The red kidney bean, Phaseolus vulgaris, contains five tetrameric lectins designated L4, L3E, L2E2, L1E3, and E4, based on the number of L and E polypeptide subunits/molecule (1−3).3 The E subunit accounts for the erythroagglutinating activity of the lectins since the binding affinity of these molecules for human erythrocytes increases with increasing numbers of E subunits/tetramer (3, 4). The L4 lectin does not bind significantly to erythrocytes although it does bind with high affinity to lymphocytes and acts as a mitogen toward these cells (4). These observations demonstrate that the carbohydrate binding specificities of the E4 and L4 lectins are different even though the lectins are similar in molecular weight and amino acid composition (1, 3, 5).

Several studies have been published on the carbohydrate binding specificities of the erythroagglutinating forms of the lectin (6−8). We previously reported that the binding site for these lectins on human erythrocytes was an Asn-linked complex-type oligosaccharide and that the galactose residues of this oligosaccharide were important determinants of binding (6). More recently, Irimura et al. (7), using affinity chromatography on E-PHA-agarose, determined that the biantennary complex-type Asn-linked oligosaccharide of human erythrocyte glycoporphin interacts with high affinity with the lectin. These workers also confirmed that galactose residues are required for this interaction. However, Irimura et al. (7) did not investigate the possibility that immobilized E-PHA might interact with other structurally related glycopeptides.

In contrast, very little is known about the carbohydrate binding specificity of L-PHA. Several investigators have found that high concentrations of N-acetylgalactosamine (>25 mM) can dissociate L-PHA, as well as E-PHA, bound to cells or glycoproteins (6, 9, 10). However, the relationship of this phenomenon to the carbohydrate binding specificity of L-PHA is obscure. For example, L-PHA does not bind significantly to blood group A erythrocytes (1) which contain oligosaccharides with terminal N-acetylgalactosamine residues, but does bind to thyroglobulin (9−11) which lacks N-acetylgalactosamine residues (12).

Since L-PHA is a widely used lectin, we felt that it would be useful to better characterize its carbohydrate binding specificity and to compare this to the binding specificity of E-PHA. To accomplish this, we have immobilized L-PHA and E-PHA on agarose supports and have studied the interaction of a panel of glycopeptides of known structure with these immobilized lectins. Our results indicate that L-PHA and E-PHA interact with different Asn-linked oligosaccharides and that the difference is related to the substitution pattern of the mannose residues of the oligosaccharides.

**Experimental Procedures**

Materials—Concanavalin A-Sepharose was obtained from Pharmacia Fine Chemicals and PHA-P was purchased from P-L Biochemicals. Affi Gel 10 was obtained from Bio-Rad. ['H]Acetate (100 mCi/mmol) was purchased from New England Nuclear. Sulfopropyl-Sephadex, Sephadex G-150, Sephadex G-25, bovine epididymal α1-fucosidase, and bovine thyroglobulin were obtained from Sigma. Vibrio cholera neuraminidase and pronase (B grade) were obtained from Calbiochem-Behring. β-Galactosidase, β-N-acetylgalactosaminidase, and α-mannosidase were prepared from jack bean meal by the method of Li and Li (13).

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**Sources of Test Glycopeptides**—Desialylated fibrinogen glycopeptide was obtained from a preparation of the glycoprotein supplied by Drs. J. Miletich and G. Broze of the Washington University School of Medicine. The structure of this glycopeptide is shown in Table I as L-IgA-II-C glycopeptide (Table I, II) containing 2 terminal NeuAc residues which was prepared and labeled with \(^{13}C\)acetic anhydride (15). IgA-A glycopeptide was also labeled with \(^{13}C\)acetic anhydride (14) and labeled with \(^{1}C\)acetic anhydride (15). This glycopeptide has the structure of glycopeptide III in Table I. Mouse IgM glycopeptide I-A (labeled with \(^{12}C\)acetic anhydride) and fetuin glycopeptide were generously provided by Dr. Rosalind Kornfeld and Dr. Jacques Baenziger, respectively, of the Washington University School of Medicine. These glycopeptides have related structures (16-18), but differ in the number and linkage of sialic acid residues. The structure of the fetuin glycopeptide is shown in Table I as V. A glycopeptide with the structure of VI (Table I) was prepared from the mouse lymphoma cell line (BW5147), labeled in \(^{12}C\)mannose, as described previously (19).

**Glycopeptides with the structures of II, IV, VII, and VIII in Table I** were prepared from bovine thyroglobulin by the following technique. Crude bovine thyroglobulin (1 g) was dissolved in 5 ml of 0.1 M Tris-HCl, 2 mM CaCl\(_2\), pH 8.0, and digested with pronase (40 mg) for 24 h in a solution atmosphere at 60 °C. The additional pronase (5 mg) was then added and the reaction continued for 5 more days in a solution of 5% 1-propanol in H\(_2\)O. The void fractions were pooled and the solvent evaporated. The residue was dissolved in 0.1 M Tris-HCl, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), 0.02% NaN\(_3\), pH 8.0, and one-tenth of the sample was applied to a column of ConA-Sepharose G-25 (1 x 10 cm) in 7% 1-propanol in H\(_2\)O. The void fractions were pooled and the solvent evaporated. The residue was dissolved in a final volume of 30 mM NaHCO\(_3\), 0.2 M N-acetylgalactosamine, pH 8.0. A slurry of Affi Gel 10 (10 ml) was washed as described by the manufacturer, and the moist cake was added quickly to the solution of L-PHA. The solution was mixed gently for 18 h at 4 °C and the gel was then allowed to settle. Uncoupled L-PHA was recovered in the supernatant, and the gel was resuspended in 0.1 M NaHCO\(_3\), pH 8.0, containing 0.1 M ethanolamine to block any remaining coupling sites. After 2 h at 4 °C, the L-PHA-agarse was placed in a column (0.5 x 30 cm) at room temperature and washed with PBS/Na\(_3\). Coupling efficiency was estimated by determining the amount of protein remaining in the supernatant after the coupling reaction. L-PHA was coupled to Affi Gel 10 with approximately 60% efficiency, and the amount of coupled protein was estimated to be 1.8 mg/ml of gel.

**Table I** lists the various Asn-linked oligosaccharides which were tested for their ability to interact with L-PHA-agarse. An example of the behavior of three of these glycopeptides on the L-PHA-agarse column is shown in Fig. 1. Glycopeptide IV is not retarded on the column (A) and is therefore scored as noninteracting (Table I). In contrast, the elution of glycopeptides VI and VII is retarded significantly (B and C), indicating a higher affinity interaction between the lectin and these glycopeptides. This behavior is scored as a positive interaction in Table I. Of the glycopeptides listed in Table I, only glycopeptides VI, VII, and VIII are retarded on the L-PHA-agarse. These data indicate that L-PHA interacts with the highest affinity with tri- and tetraantennary glycopeptides having a branched residue substituted at positions C-2 and C-4. The interaction of L-PHA with biantennary glycopeptides (I, II, and III) and the trimannosyl glycopeptides having a branched mannose residue substituted at positions C-2 and C-4 (IV and V) is not sufficiently strong to result in retardation of the glycopeptides under the conditions of the experiment.

**Effect of Outer Chain Sugar Residues on Glycopeptide Binding to L-PHA-agarse**—The role of the outer sugar
\section*{Table I}

Structures of glycopeptides tested for their ability to interact with either L-PHA-agarose or E-PHA-agarose

| Glycopeptide Designation | Glycopeptide Structure | Elution Retarded by L-PHA | Elution Retarded by E-PHA |
|--------------------------|------------------------|---------------------------|---------------------------|
| I                        |                        |                           |                           |
| II                       |                        |                           |                           |
| III                      |                        |                           |                           |
| IV                       |                        |                           |                           |
| V                        |                        |                           |                           |
| VI                       |                        | +                         |                           |
| VII                      |                        | +                         |                           |
| VIII                     |                        | +                         |                           |
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residues of the Asn-linked oligosaccharides in the interaction with L-PHA-agarose was determined by treating the glycopeptides with various glycosidases and reapplying the partially digested samples to the lectin-agarose column. Treatment of glycopeptide VI with neuraminidase has no effect on the interaction of the compound with L-PHA-agarose. However, the subsequent removal of the galactose residues with \( \beta \)-galactosidase abolishes the high affinity interaction of glycopeptide VI with L-PHA-agarose (data not shown). These results indicate that a second determinant necessary for the interaction of Asn-linked oligosaccharides with L-PHA is the presence of outer galactose residues. We have been unable to obtain homogeneous preparations of glycopeptide VI containing only 1 or 2 galactose residues, and, therefore, the positional effects, if any, and the minimal number of galactose residues required for the interaction of glycopeptides with L-PHA are unknown.

Removal of the internal fucose residue of glycopeptide VI by treatment with \( \alpha \)-L-fucosidase does not affect the behavior of the glycopeptide on the L-PHA column. However, this treatment did abolish binding of glycopeptide VI to pea lectin-Sepharose, confirming studies by Kornfeld et al. (19) which demonstrated that core fucose is a requirement for binding of glycopeptides to this lectin.

Interaction of Glycopeptides with E-PHA-agarose—Fig. 2 shows an example of the chromatography of two glycopeptides on E-PHA-agarose. As shown in A, glycopeptide II is not retarded, whereas glycopeptide III (B) is significantly retarded on E-PHA-agarose. The results of the chromatography of the other Asn-linked oligosaccharides are indicated in Table I. The only glycopeptide that is retarded on the column is glycopeptide III. Since this is the only glycopeptide with a residue of N-acetylglucosamine linked \( \beta 1,4 \) to the \( \beta \)-linked mannose, we conclude that this residue is an important determinant for high affinity binding to E-PHA-agarose. This is best illustrated by comparing the structures of glycopeptide I (which fails to interact with E-PHA) and glycopeptide III. The only difference between these two glycopeptides is the presence of the “bisecting” N-acetylglucosamine residue on glycopeptide III, as well as the presence of a residue of N-acetylneuraminic acid on the glycopeptide. Since removal of the N-acetylneuraminic acid from glycopeptide III has no effect on the interaction with E-PHA-agarose (see below), the difference in the behavior of the two glycopeptides must be due to the bisecting N-acetylglucosamine residue.

Effect of Outer Chain Sugar Residues on Glycopeptide Binding to E-PHA-agarose—Removal of the N-acetylneuraminic acid from glycopeptide III with neuraminidase has no effect on the interaction of the glycopeptide with E-PHA-agarose. However, the removal of 1 galactose residue from the glycopeptide by treatment with \( \beta \)-galactosidase abolishes the high affinity interaction with E-PHA-agarose (data not shown). These results indicate that the 2 outer galactose residues of the glycopeptide constitute a second determinant in the lectin-oligosaccharide interaction.

Discussion

The data presented in this paper demonstrate that E-PHA and L-PHA have similar but distinct carbohydrate binding specificities. Both lectins are capable of high affinity interactions with Asn-linked complex-type oligosaccharides and, in both instances, the galactose residues of these oligosaccharides are important in this interaction. On the other hand, there are significant differences in the binding specificities of these lectins. E-PHA interacts with highest affinity only with galactosylated glycopeptides containing an N-acetylglucosamine residue linked to the \( \beta \)-linked mannose residue of the core.

Only biantennary glycopeptides with this type of substitution were available in this study. It is possible that tri- or tetrataennary glycopeptides with this substitution pattern can interact strongly with E-PHA-agarose. Irimura et al. (7) and Yoshima et al. (24) have shown that the Asn-linked oligosaccharide on glycoporphin is a biantennary oligosaccharide with a bisecting N-acetylglucosamine residue. Thus, the data available indicate that E-PHA binds to glycoporphin molecules on human erythrocytes (6), and that this binding results from the high affinity interaction of E-PHA with the bisected biantennary oligosaccharide of glycoporphin.

L-PHA, which does not bind significantly to human erythrocytes, fails to interact with the bisected biantennary glycopeptide with high affinity. L-PHA interacts best with galactosylated tetrantennary and tetroantennary glycopeptides that have at least 1 of the \( \alpha \)-linked mannose residues substituted at positions C-2 and C-6 with \( \beta \)-linked N-acetylgalactosamine residues. The other \( \alpha \)-linked mannose may be singly substituted at C-2 (as in the tetroantennary species) or doubly substituted at C-2 and C-4 (as in the tetraantennary species). Neither of these tri- or tetroantennary glycopeptides interacts with high affinity with E-PHA.

The carbohydrate binding specificity of L-PHA also has some similarities to that reported for the pea and lentil lectins (19). These latter lectins bind tetroantennary glycopeptides that have an \( \alpha \)-linked mannose residue substituted at C-2 and C-6, but fail to bind tetroantennary species that have \( \alpha \)-linked mannose residues substituted at C-2 and C-4. However, they also require the presence of an \( \alpha \)-linked fucose residue in the core, whereas L-PHA binding is not influenced by this fucose.
Asn-linked oligosaccharides and their interaction with L-PHA.

In this study, we have not tested glycopeptides with O-linked oligosaccharides for their ability to interact with L-PHA or E-PHA-agarose. It is unlikely, however, that either of these lectins interact strongly with O-linked oligosaccharides. Previous studies have shown that only Asn-linked oligosaccharides interact strongly with E-PHA (6, 23). The finding that L-PHA fails to bind to human erythrocytes, even after neuraminidase treatment, suggests that O-linked oligosaccharides do not serve as binding sites for this lectin since human erythrocytes contain high amounts of O-linked oligosaccharides.

It is important to note that our study has utilized E- or L-PHA coupled to agarose, and we have not investigated the specificity of the tetrameric lectins L_{E^+}, L_{E^-}, L_{E^+}, and L_{E^-} coupled to agarose. It is possible that these latter lectins may have unusual differences from either L-PHA- or E-PHA-agarose in their binding requirements or affinities for glycopeptides.

Previous reports have demonstrated the value of lectin affinities of cell membrane Asn-linked oligosaccharides. A comparison of our findings with L-PHA- and E-PHA-agarose with those previously reported with Concana-valin A-, pea lectin-, and lentil lectin-Sepharose indicate that the use of all these lectins in the correct sequence can provide an extremely simple, rapid, and sensitive technique for the fractionation of Asn-linked oligosaccharides. In the following paper, we present a scheme for serial lectin affinity chromatography and illustrate the usefulness of this approach for the separation of Asn-linked oligosaccharides of cultured cell lines (30).

Note Added in Proof—A recent report (Hammarström, S., Hammarström, M. L., Sundblad, G., Arnarp, J., and Löngren, J. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 1611-1615) described a study of the specificity of L-PHA using the techniques of quantitative precipitation and precipitation-inhibition by a number of different glycopeptides and synthetic oligosaccharides. They concluded that the most complementary structure to the binding site of L-PHA was a pentasaccharide composed of two N-acetyllactosamine disaccharides linked through the GlcNAc residues to a mannose residue at both positions C-2 and C-6. This finding is consistent with our data indicating that glycopeptides with this structural feature are selectively retarded in their elution from a column of L-PHA-agarose.

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