Oligosaccharide Specificities of *Phaseolus vulgaris*
Leukoagglutinating and Erythroagglutinating Phytohemagglutinins

INTERACTIONS WITH N-GLYCANASE-RELEASED OLIGOSACCHARIDES*

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The structural determinants required for interaction of oligosaccharides with leukoagglutinating phytohemagglutinin (L-PHA) and erythroagglutinating phytohemagglutinin (E-PHA) from *Phaseolus vulgaris* have been studied by immobilized lectin affinity chromatography. Homogeneous oligosaccharides of known structure, purified following release from Asn with N-glycanase and reduction with NaBH₄, were tested for their ability to interact with columns of L- and E-PHA-agarose. The characteristic elution position obtained for each oligosaccharide was reproducible and correlated with specific structural features. In virtually all cases, L- and E-PHA yielded identical results, indicating that their specificities for reduced oligosaccharides are similar. Both lectins retarded oligosaccharides bearing α2,3- but not α2,6-linked sialic acid. Desialylation of the structural determinants required for interaction of reduced oligosaccharides is similar. Both lectins retarded oligosaccharides bearing α2,3- but not α2,6-linked sialic acid. Desialylated oligosaccharides containing one, two, three, or four peripheral N-acetyllactosamine-type branches were retarded to varying extents by both lectins; however, this interaction was decreased or eliminated by removal of Gal. Desialylated oligosaccharides containing a bisecting GlcNAc residue attached to the β-linked core Man displayed the greatest interaction with both lectins. Structures containing terminal sulfate or GalNAc did not interact with either lectin. In some instances, the specificities of L- and E-PHA lectins for free, reduced oligosaccharides differed from those established using glycopeptides. Therefore, the structural requirements for interaction with lectins such as L- and E-PHA must be fully and systematically defined using the appropriate authentic standards in order to use lectin affinity chromatography for the fractionation and characterization of free oligosaccharides.

The red kidney bean, *Phaseolus vulgaris*, contains five tetrameric isolecits (L₄, Lₑ₁, Lₑ₂, Lₑ₃, and Lₑ₄) with varying proportions of leukocyte reactive and erythrocyte reactive polypeptide subunits (reviewed in Refs. 1–5). The L₄ isolecitin or leukoagglutinating phytohemagglutinin (L-PHA) binds lymphocytes with a high degree of specificity and is mitogenic for these cells. The Lₑ isolectin or erythroagglutinating phytohemagglutinin (E-PHA) agglutinates erythrocytes but not lymphocytes. Despite differences in binding specificities and biological properties, leukocyte reactive and erythrocyte reactive subunits are similar in molecular weight as well as carbohydrate and amino acid compositions.

The specificities of the phytohemagglutinin lectins for carbohydrates have been studied by several different techniques. Hammarstrom et al. (3) examined L-PHA specificity using quantitative precipitation and precipitation-inhibition assays. They found that the most complementary structure for binding to L-PHA is a pentasaccharide containing two N-acetyllactosamine moieties linked to the C-2 and C-6 positions of a Man residue, a structure which is contained within some tri- and tetrabranched Asn-linked oligosaccharides. Using lectin affinity chromatography, Cummings and Kornfeld (4) defined the features of peptide-bound oligosaccharides required for interaction with immobilized L- and E-PHA lectins. Heterogeneous populations of glycopeptides were separated on each lectin, and the structures of the oligosaccharides in each fraction were then determined. Cummings and Kornfeld (4) concluded that glycopeptides containing oligosaccharides with an α-linked core Man bearing an N-acetyllactosamine moiety attached to both the C-2 and C-6 positions are retarded on L-PHA, while those bearing a bisecting GlcNAc attached to the β-linked core Man residue are retarded on E-PHA. In the only study to date separating free oligosaccharides by affinity chromatography with either phytohemagglutinin lectin, Yamashita et al. (5) examined the interaction of hydrazine released, reduced oligosaccharides on immobilized E-PHA. Only structures bearing a bisecting GlcNAc residue were studied.

Asn-linked oligosaccharides can be released from glycoproteins by cleavage of the GlcNAc-Asn bond with N-glycanase (6). Radiolabeling of oligosaccharides is typically performed either by metabolic incorporation of °H- or °C-labeled sugars prior to N-glycanase digestion or by reduction with NaBH₄, following N-glycanase release. A major advantage of characterizing free oligosaccharides, compared to glycopeptides, is the ability to fractionate these oligosaccharides by high-performance liquid chromatography (HPLC) and lectin affinity chromatography in the absence of heterogeneity due to variation in the peptide. Although lectin affinity chromatography is a valuable tool for fractionating and characterizing oligosaccharides, most previous studies describing lectin specificities have utilized glycopeptides rather than free oligosaccharides. The solution conformation of a free oligosaccharide may differ significantly from that of the same structure attached to peptide. Thus, lectin specificity must be evaluated utilizing the identical form of oligosaccharide (free and non-
L-PHA and E-PHA Oligosaccharide Specificities

Oligosaccharide Standards—The oligosaccharide structures used in this study were prepared and characterized in this laboratory. Asn-linked oligosaccharides were derived from human transferrin (Sigma), human chorionic gonadotropin (provided by Drs. Steven Birken and Robert Canfield, Columbia University), human lutropin (luteinizing hormone, provided by the National Hormone and Pituitary Program, National Institutes of Health, Bethesda, MD), and Sialogen (Diplococcus pneumoniae) obtained from E-Y Laboratories. Oligosaccharides were also included in the microfilm edition of the Journal that is available from the Journal of Biological Chemistry. Oligosaccharide Standards are presented in miniprint at the end of this paper.

Materials and Methods

RESULTS

Interaction of Dibranched Oligosaccharides with L-PHA—Purified N-glycansase released, reduced oligosaccharides with the structures shown in Table I and products derived from these oligosaccharides were chromatographed on columns of L- and E-PHA-agarose. Results of these analyses are shown in Figs. 1-9, along with schematically illustrated structures of the oligosaccharides examined. For ease of discussion, four regions (I-IV) have been defined to indicate differing degrees of interaction with the immobilized lectins. Although reproducibility is such that precise elution positions could be determined so as to define more subtle differences in lectin interaction, this was not done for the analyses shown here.

Dibranched oligosaccharides bearing α2,3- versus α2,6-linked sialic acid moieties differ in their interaction with L-PHA. Dibranched structures bearing two terminal α2,6-linked sialic acid residues (Di-1) elute virtually unretracted in region I (Fig. 1A), whereas similar oligosaccharides bearing two terminal α2,3-linked sialic acid residues (Di-2) elute in region III (Fig. 1C). Removal of sialic acid from Di-1 (Fig. 1B) and Di-2 (Fig. 1D) results in elution of both oligosaccharides in region II. Thus, the presence of α2,6-linked sialic acid prevents significant interaction of Di-1 with L-PHA, since the desialylated (asialo) form of this oligosaccharide is retarded by the lectin (Fig. 1B). In contrast, the presence of α2,3-linked sialic acid markedly enhances the interaction of Di-2 oligosaccharides with L-PHA, since the sialylated form of this oligosaccharide is retarded to a greater extent than the asialo form. The affinity of L-PHA for desialylated dibranched oligosaccharides is dependent on the presence of Gal, since removal of Gal from either asialo Di-1 (data not shown) or asialo Di-2 (Fig. 1E) by digestion with β-galactosidase (Diplococcus) results in elution of the agalacto products within region I. The core region, consisting of Man₃GlcNAc₂, also elutes in region I.

A slight difference in elution position is evident between Di-1 and Di-2 with two α2,6-linked sialic acid moieties, and the agalacto derivatives of Di-1 (Fig. 2) and Di-2. Although each of these oligosaccharides elutes within region I, the sialylated form of Di-1 consistently elutes 1-2 fractions later than the agalacto structures. Polyasaccharides structurally unrelated to Asn-linked oligosaccharides but of similar size, for example (GlcNAc)₄hexa, coelute with agalacto Di-1 and agalacto Di-2 (at a position corresponding to the column void), rather than with Di-1. Thus, α2,6-linked sialic acid does not completely prevent interaction of Di-1 with L-PHA, whereas removal of Gal abolishes all interaction with the lectin.

Di-1 and Di-2 oligosaccharides differ in both the linkage of sialic acid residues (α2,3 versus α2,6) and in the variable...
<table>
<thead>
<tr>
<th>Oligosaccharide Structure</th>
<th>Designation</th>
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<tbody>
<tr>
<td>Sia\textsubscript{a}2,6Gal\textsubscript{b}1,4GlcNAc\textsubscript{b}1,2Manal</td>
<td>Di-1</td>
</tr>
<tr>
<td>\textit{Man}\textsubscript{b}1,4GlcNAc\textsubscript{b}1,4GlcNAc</td>
<td></td>
</tr>
<tr>
<td>Sia\textsubscript{a}2,6Gal\textsubscript{b}1,4GlcNAc\textsubscript{b}1,2Manal</td>
<td>Di-2</td>
</tr>
<tr>
<td>\textit{Man}\textsubscript{b}1,4GlcNAc\textsubscript{b}1,4GlcNAc</td>
<td></td>
</tr>
<tr>
<td>Sia\textsubscript{a}2,3Gal\textsubscript{b}1,4GlcNAc\textsubscript{b}1,2Manal</td>
<td>Di-3</td>
</tr>
<tr>
<td>\textit{Man}\textsubscript{b}1,4GlcNAc\textsubscript{b}1,4GlcNAc</td>
<td></td>
</tr>
<tr>
<td>Sia\textsubscript{a}2,3Gal\textsubscript{b}1,4GlcNAc\textsubscript{b}1,2Manal</td>
<td>Di-4</td>
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<tr>
<td>\textit{Man}\textsubscript{b}1,4GlcNAc\textsubscript{b}1,4GlcNAc</td>
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<td>Sia\textsubscript{a}2,6Gal\textsubscript{b}1,4GlcNAc\textsubscript{b}1,2Manal</td>
<td>Di-5</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>Sia\textsubscript{a}2,6Gal\textsubscript{b}1,4GlcNAc\textsubscript{b}1,2Manal</td>
<td>Di-6</td>
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<tr>
<td>\textit{Man}\textsubscript{b}1,4GlcNAc\textsubscript{b}1,4GlcNAc</td>
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We next verified that the difference in the interaction of Di-1 and Di-2 with L-PHA is due exclusively to the linkage of sialic acid and not to the presence of Fuc and/or some other previously undetected modification of the sialic acid moieties. The desialylated forms of Di-1 and Di-2 coelute from the L- or E-PHA columns, yet only asialo Di-2 contains Fuc; and 3) affinity chromatography of Di-2 and Di-2 derived oligosaccharides on L- and E-PHA is not altered by removal of Fuc with α-fucosidase.

Oligosaccharides bearing a single sialylated branch display marked variation in their interaction with L-PHA, depending on the composition and attachment site of the peripheral branch. For example, Di-3, which contains a single α2,3-sialylated branch attached to the α1,3-linked core Man, elutes between regions II and III (Fig. 3A), while asialo Di-3 elutes between regions I and II (Fig. 3B). In contrast, an oligosaccharide with a single α2,6-sialylated branch attached to the...
FIG. 1. L-PHA-agarose affinity chromatography of dibranched oligosaccharides. N-Glycanase-released, reduced Di-1 (panel A), desialylated Di-1 (panel B), Di-2 (panel C), desialylated Di-2 (panel D), and β-galactosidase (Diplococcus) digested desialylated Di-2 (panel E) oligosaccharides (5000 cpm each) were chromatographed on L-PHA-agarose. Elution regions I-IV are indicated by brackets. Structures of the oligosaccharides are shown in detail in Table I and schematically illustrated in each panel. The symbols used for structures analyzed in Figs. 1-10 are: Sia (▲); Gal (●); GlcNAc (□); Man (○); Fuc (●); and GalNAc (●).

α1,6-linked core Man (derived by addition of an α2,3-linked sialic acid residue to Di-4) elutes in region II (Fig. 3C), while asialo Di-4 elutes in region III (Fig. 3D). Thus, the affinity of L-PHA for oligosaccharides bearing a single α2,3-linked sialic acid is more pronounced when this moiety is present on the branch attached to the α1,6-linked core Man. Di-2 and α2,3-sialylated Di-4 coelute during affinity chromatography on L-PHA (Figs. 1C and 3C), yet these structures contain 2 and 1 α2,3-linked sialic acid residues, respectively. Thus, the number of α2,3-linked sialic acid moieties does not affect the interaction of dibranched oligosaccharides with L-PHA as much as the attachment site of the α2,3-sialylated branch.

Oligosaccharide structures which are similar to Di-3 and α2,3-sialylated Di-4, but bear an α2,6- rather than α2,3-linked sialic acid, elute in region I (data not shown). Thus, neither the presence of 2 α2,6-linked sialic acid residues nor 1 α2,6-linked sialic acid residue (on either peripheral branch) promotes interaction of dibranched oligosaccharides with L-PHA. A minor subpopulation of Di-1 elutes as a small peak in region III (Fig. 1A). This minor Di-1 fraction was isolated by preparative L-PHA-agarose affinity chromatography. Digestion with Newcastle disease virus neuraminidase, which cleaves α2,3- but not α2,6-linked sialic acid (25), released only 1 sialic acid residue from this oligosaccharide, as assessed by anion-exchange HPLC. In contrast, digestion with C. perfringens neuraminidase, which cleaves both α2,3- and α2,6-linked sialic acid (11), released 2 sialic acid residues. Thus, this minor Di-1 structure contained one α2,3- and one α2,6-linked sialic acid moiety. Since this minor oligosaccharide species elutes in region III (rather than between regions II and III), these structures likely bear the α2,3-sialylated branch attached to the α1,6-linked core Man. Hence, an α2,6-sialylated branch attached to the α1,3-linked core Man probably does not compromise L-PHA interaction with the α2,3-sialylated branch attached to the α1,6-linked core Man. Di-5 oligosaccharides contain one branch with the sequence SO₃GalNAcβ1,4GlcNAcβ1,2 and one branch with the sequence SiaγGalβ1,4GlcNAcβ1,2 attached to the α1,3- and α1,6-linked core Man residues, respectively. Separation of Di-5 structures by L-PHA affinity chromatography yields two peaks, eluting in regions I and III, respectively (Fig. 3E). Oligosaccharides in each peak were isolated by preparative L-PHA affinity chromatography, digested with either Newcastle disease virus neuraminidase or C. perfringens neuraminidase, and analyzed by anion-exchange HPLC. For the earlier eluting Di-5 oligosaccharides (region I), C. perfringens but not Newcastle disease virus neuraminidase released a single sialic acid residue, indicating the presence of an α2,6-linked sialic acid moiety. For the later eluting Di-5 oligosaccharides (region
L-PHA and E-PHA Oligosaccharide Specificities

region II (Fig. 3F), while agalacto Di-5 elutes in region I (data not shown). These latter findings with Di-5 further illustrate the importance of the branch attached to the α1,6-linked core Man for L-PHA interaction and the apparent lack of influence (either positive or negative) of the branch attached to the α1,3-linked core Man.

Di-6 oligosaccharides, which bear two branches containing sulfate and GalNAc, but no sialic acid or Gal, do not significantly interact with L-PHA (Fig. 3G). Similarly, desulfated Di-6, which is identical to desialylated Di-2 except bearing terminal β1,4-linked GalNAc; instead of β1,4-linked Gal, also does not interact with the lectin (Fig. 3H). The latter result indicates that the presence of an N-acetyl- rather than an HO- moiety at the C-2 position of terminal Gal results in a discernible decrease in L-PHA interaction. Thus, L-PHA does not interact with oligosaccharides bearing branches with terminal sulfate or GalNAc, regardless of the attachment sites (α1,3- versus α1,6-linked core Man) of the peripheral branches.

Interaction of Dibranched Oligosaccharides Containing a Bisecting GlcNAc Residue with L-PHA—Oligosaccharides containing a bisecting GlcNAc residue attached to the β-linked core Man, such as Di-7, have been shown to strongly interact with E-PHA (4, 5, 29). The interaction of such structures with L-PHA has to date been studied using peptide-bound oligosaccharides but not with free oligosaccharides. Di-7, containing two α2,6-linked sialic acid moieties, elutes in region I during L-PHA affinity chromatography (Fig. 4A), whereas asialo Di-7 elutes in region IV (Fig. 4B). The latter interaction is greatly decreased, but not completely eliminated, by removal of Gal (Fig. 4C), resulting in elution of the oligosaccharide between regions I and II. The presence of a bisecting GlcNAc residue, even in the absence of Gal, increases the interaction of an oligosaccharide with L-PHA, since agalacto Di-7 (Fig. 4C) elutes later than agalacto Di-1 (Fig. 4E). Thus, a bisecting GlcNAc residue markedly en-

![Fig. 3. L-PHA-agarose affinity chromatography of sialylated and sulfated dibranched oligosaccharides.](image)

![Fig. 4. L-PHA-agarose affinity chromatography of dibranched oligosaccharides containing a bisecting GlcNAc residue.](image)
hances the interaction of a dibranched oligosaccharide with L-PHA, but this enhancement is abolished by the presence of α2,6-linked sialic acid.

**Interaction of Tri-1 and Tetra-branched Oligosaccharides with L-PHA**—The interaction of tri- and tetrabranched oligosaccharides with L-PHA reflects both the linkages of sialic acid moieties and the attachment sites of peripheral branches to core Man residues. Tri-1 oligosaccharides consist of a heterogeneous array of species, each containing 3 sialic acid residues and the identical underlying carbohydrate structure (Table I). These oligosaccharides differ in the linkage (α2,3 versus α2,6) of sialic acid residues and in the distribution of α2,3- and α2,6-linked sialic acid moieties among the peripheral branches. Tri-1 oligosaccharides elute as three major peaks within regions I, II-III, and III-IV, respectively, during L-PHA affinity chromatography (Fig. 5A). Removal of sialic acid (Fig. 5B) or both sialic acid and Gal (Fig. 5C) from Tri-1 structures results in elution of the oligosaccharides in a single, uniform peak. Asialo Tri-1 coelutes with asialo forms of dibranched oligosaccharides (Di-1 and Di-2), whereas agalacto Tri-1 elutes slightly later than the agalacto forms of Di-1 and Di-2. Fractionation of the sialylated forms of Tri-1 oligosaccharides into three peaks during L-PHA affinity chromatography (Fig. 5A) likely reflects the linkage and/or distribution of the three sialic acid moieties present on each oligosaccharide. The proportion of α2,3- and α2,6-linked sialic acid residues on the oligosaccharides in the “early,” “middle,” and “late” peaks was assessed, as summarized in Table II. Structures eluting within region I (early peak) bear predominantly α2,6-linked sialic acid, those eluting within regions II-III (middle peak) contain relatively less α2,6- but more α2,3-linked sialic acid, and those eluting within regions III-IV (late peak) bear mostly α2,3-linked sialic acid. Thus, the linkage of sialic acid moieties on Tri-1 oligosaccharides markedly influences interaction of these structures with L-PHA.

Previous studies indicated that glycopeptides (4) and synthetic carbohydrates (3) bearing Man residues substituted at the C-2 and C-6 positions by N-acetyllactosamine strongly interact with L-PHA. This specificity is also displayed for N-glycanase released, reduced oligosaccharides. Like Tri-1, Tri-2 contains three peripheral branches; however, Tri-2 bears a core Man with N-acetyllactosamine moieties attached at the C-2 and C-6 rather than C-2 and C-4 positions. Tri-2, containing no sialic acid, elutes between regions II and III (Fig. 5D), whereas asialo Tri-1 elutes between regions I and II (Fig. 5B). Agalacto Tri-2 elutes between regions I and II (Fig. 5E) at virtually the same position as agalacto Tri-1 (Fig. 5C).

Tetra-1 oligosaccharides consist of a heterogeneous population of species, each containing 4 sialic acid residues and the same underlying carbohydrate structure (Table I). Like Tri-1, these oligosaccharides differ in the linkage (α2,3 versus α2,6) of sialic acid residues and in the distribution of α2,3- and α2,6-linked sialic acid moieties among the peripheral branches. During L-PHA affinity chromatography, Tetra-1 structures separate into two peaks: a sharp peak eluting within region I (early peak) and a broad peak eluting within regions II-IV (late peak) (Fig. 6A). As with Tri-1, the earlier eluting Tetra-1 oligosaccharides contain mostly α2,6-linked sialic acid, whereas the later eluting structures bear larger proportions of α2,3-linked sialic acid (Table II). Following removal of sialic acid, Tetra-1 elutes as a single, uniform peak between regions II and III (Fig. 6C), indicating that the heterogeneity displayed by sialylated Tetra-1 is a function of the sialic acid moieties themselves. Asialo Tetra-1 and Tri-2 coelute (Figs. 5D and 6C), consistent with the presence of a Man residue substituted at positions C-2 and C-6 by N-acetyllactosamine on both Tetra-1 and Tri-2. Following removal of Gal, Tetra-1 elutes between regions I and II (Fig. 6D), at the same position as the corresponding forms of both Tri-1 (Fig. 5C) and Tri-2 (Fig. 5E).

Unfractionated preparations of desialylated Tetra-1 were found to contain a significant fraction of oligosaccharides...
bearing one or more peripheral polylactosamine (Galβ1,4GlcNAc)n units. Such preparations separate into two peaks during L-PHA affinity chromatography (Fig. 6B). Oligosaccharides eluting between regions II and III were identical to asialo Tetra-1, i.e. containing no peripheral polylactosamine. In contrast, those structures eluting in region I contained polylactosamine, as indicated by their sensitivity to E. freundii endo-β-galactosidase. Thus, the presence of peripheral polylactosamine sequence(s) precludes the interaction of desialylated tetrambranched oligosaccharides with L-PHA.

Interaction of Oligosaccharides with E-PHA—The specificity of E-PHA for N-glycanase-released, reduced oligosaccharides is virtually identical to that of L-PHA. Each of the oligosaccharides analyzed by affinity chromatography on L-PHA-agarose (Figs. 1–6) was found to behave in an analogous manner on E-PHA. Representative profiles are shown in Figs. 7–9. The same four regions (I–IV) can be defined for affinity chromatography on E-PHA as was done for L-PHA, although the precise location of each region differs for the two lectin-agarose conjugates. Dibranched oligosaccharides bearing α2,6-linked sialic acid elute in region I (Fig. 7A), while those bearing α2,3-linked sialic acid elute in region III (Fig. 7B). Asialo Di-1 and asialo Di-2 (Fig. 7C) elute in region II. In contrast to L-PHA, removal of Gal from di- (Fig. 7D), tri-, and tetrabranch (data not shown) oligosaccharides does not completely eliminate interaction with E-PHA, since the resulting agalacto structures elute between regions I and II. Subsequent removal of GlcNAc from the agalacto structures, however, abolishes E-PHA interaction (Fig. 7E). Analysis of dibranched oligosaccharides bearing a bisecting GlcNAc, such as Di-7 (Fig. 8A), asialo Di-7 (Fig. 8B), and agalacto Di-7 (Fig. 8C), yields the identical results as obtained with L-PHA (Fig. 4). Desialylated oligosaccharides containing a peripheral branch attached to the C-6 position of the α1,6-linked core Man also preferentially interact with E-PHA. Thus, as was found for L-PHA (Figs. 5 and 6), the asialo forms of Tri-2 (Fig. 9B) and Tetra-1 (Fig. 9C) are slightly more retarded than asialo Tri-1 (Fig. 9A) during affinity chromatography on E-PHA. Analysis of the sialylated forms of Tri-1 and Tetra-1 by E-PHA affinity chromatography yielded the same results as were obtained with L-PHA.

The nearly identical chromatographic behavior of N-glycanase released, reduced oligosaccharides on L- and E-PHA-agarose columns raised the question of potential cross-contamination of these lectins. Three independent criteria indicated that the preparations of L- and E-PHA-agarose contain pure L4 and E4 isolectins, respectively. First, sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis performed by E-Y Laboratories (the commercial source of the lectin-agarose conjugates) demonstrated that the L- and E-PHA

| TABLE II |
| Relative distributions of α2,3- and α2,6-linked sialic acid residues on Tri-1 and Tetra-1 oligosaccharide fractions separated by L-PHA-agarose affinity chromatography |

<table>
<thead>
<tr>
<th>Tri-1 oligosaccharides</th>
<th>α2,3-Linked sialic acid (No.)</th>
<th>%</th>
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<tr>
<td>Early peak</td>
<td>0 0 20 80 0</td>
<td></td>
</tr>
<tr>
<td>Middle peak</td>
<td>0 0 20 80 0</td>
<td></td>
</tr>
<tr>
<td>Late peak</td>
<td>8 44 41 7 0</td>
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</table>

<table>
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<tr>
<th>Tetra-1 oligosaccharides</th>
<th>α2,3-Linked sialic acid (No.)</th>
<th>%</th>
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<td>0 0 20 80 0</td>
<td></td>
</tr>
<tr>
<td>Late peak</td>
<td>8 44 41 7 0</td>
<td></td>
</tr>
</tbody>
</table>

*Tri-1 oligosaccharides were fractionated by preparative L-PHA-agarose affinity chromatography into three peaks (Early (region I), Middle (regions II–III), and Late (regions III–IV)), as shown in Fig. 5A. Analysis of each fraction by anion-exchange HPLC confirmed the uniform presence of 3 sialic acid residues. Following digestion with Clostridium perfringens neuraminidase, which cleaves both α2,3- and α2,6-linked sialic acid moieties (11), oligosaccharides in each fraction eluted as neutral (desialylated) structures. Each fraction was also digested with Newcastle disease virus neuraminidase, which cleaves α2,3- but not α2,6-linked sialic acid moieties (11, 25), and analyzed by anion-exchange HPLC (11). The resulting loss of anionic charge indicated the number of α2,3-linked sialic acid residues (11). The % of oligosaccharides within each fraction containing the indicated number of α2,3- and α2,6-linked sialic acid residues is indicated.

*α2,6-Linked sialic acid (No.)

| Early peak | 0 0 78 22 |
| Late peak  | 59 27 14 |

In contrast to L-PHA, removal of Gal from di- (Fig. 7B), tri-, and tetrabranch (data not shown) oligosaccharides does not completely eliminate interaction with E-PHA, since the resulting agalacto structures elute between regions I and II. Subsequent removal of GlcNAc from the agalacto structures, however, abolishes E-PHA interaction (Fig. 7E).
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Fig. 7. E-PHA-agarose affinity chromatography of di-branched oligosaccharides. N-Glycanase-released, reduced Di-1 (panel A), Di-2 (panel B), desialylated Di-2 (panel C), β-galactosidase- (Diplococcus) digested desialylated Di-2 (panel D), and β-N-acetylhexosaminidase- (Diplococcus) and β-galactosidase- (Diplococcus) digested desialylated Di-2 (panel E) oligosaccharides (5000 cpm each) were chromatographed on E-PHA-agarose. Elution regions I-IV are indicated by brackets. Symbols for the schematically illustrated oligosaccharides are the same as in Fig. 1, with detailed structures shown in Table I.

Fig. 8. E-PHA-agarose affinity chromatography of di-branched oligosaccharides containing a bisecting GlcNAc residue. N-Glycanase-released, reduced Di-7 (panel A), desialylated Di-7 (panel B), and β-galactosidase- (Diplococcus) digested desialylated Di-7 (panel C) oligosaccharides (3000 cpm each) were chromatographed on E-PHA-agarose. Elution regions I-IV are indicated by brackets. Symbols for the schematically illustrated oligosaccharides are the same as in Fig. 1, with detailed structures shown in Table I.

preparations utilized in this study each consist of a single, well-resolved protein band which was free of contamination by the other. Second, each islectin preparation was found by E-Y Laboratories to be free of cross-contamination based on differential immunoprecipitation studies. Third, certain glycopeptide preparations were found to behave differently during affinity chromatography on L- versus E-PHA-agarose. For example, glycopeptides prepared by affinity chromatography on L-PHA and known to contain oligosaccharides bearing an α-linked core Man residue substituted at the C-2 and C-6 positions by N-acetyllactosamine (4) were analyzed with each lectin column. Desialylated forms of these glycopeptides eluted from E-PHA in region I, yet eluted from L-PHA in region III. Glycopeptides prepared by affinity chromatography on E-PHA and known to contain oligosaccharides bearing a bisecting GlcNAc residue (4) were also analyzed with each lectin column. Desialylated forms of these glycopeptides eluted from L-PHA in region I and from E-PHA in region IV. The elution patterns obtained with these glycopeptides are in marked contrast to those obtained using analogous reduced oligosaccharides (discussed above). Thus, by physical-chemical, immunological, and functional properties, the L- and E-PHA-agarose preparations used are free of cross-contamination. These results indicate that L- and E-PHA discriminate among certain oligosaccharides when examined in their glycopeptide form but not as reduced oligosaccharides.

DISCUSSION

Lectin affinity chromatography has become a popular and useful adjunct for the characterization of oligosaccharide structures (2, 30-32). With few exceptions, the specificities of immobilized lectins have been established utilizing glycopeptides rather than free oligosaccharides. Specificities for free oligosaccharides and glycopeptides bearing the identical structures may, however, differ significantly. The availability of enzymatic (6, 12, 33) and chemical (34) methods for release of intact oligosaccharides, in conjunction with the establishment of HPLC techniques for their fractionation and analysis (9-11), have allowed development of new approaches for the structural characterization of free oligosaccharides. These approaches, including lectin affinity chromatography, are particularly suited for the analysis of free oligosaccharides which can only be obtained in trace quantities and radiolabeled metabolically or by reduction with NaB[3H]4.

Prior to use of lectin affinity chromatography for characterization of oligosaccharide structures, it is essential that the specificities of immobilized lectins for free oligosaccharides be established. Comparisons of lectin specificities for peptide-bound versus free oligosaccharides have not previously been carried out in a systematic fashion with large numbers of
known oligosaccharide structures. Our present characterization of the interactions of L- and E-PHA has been performed with a broad spectrum of homogeneous oligosaccharides, produced by release from Asn with N-glycanase followed by reduction. In addition to N-glycanase-released oligosaccharide structures which had previously been characterized in the form of glycopeptides (1, 2, 4, 29), we have examined the sulfated oligosaccharides obtained from pituitary glycoprotein hormones (8, 21) as well as structures with sialic acid in α2,3 and/or α2,6 linkage, structures which have not previously been examined. Our analyses have provided new insights into the specificities of L- and E-PHA. Using this approach, we have also examined the specificity of other lectins, including concanavalin A (ConA), *Datura stramonium* agglutinin (DSA), *Vicia villosa* agglutinin, *Ricinus communis* agglutinin I, and *Ricinus communis* agglutinin II (15, 35). The remarkable specificity of these lectins allows their use in characterizing the structures of free oligosaccharides by lectin affinity chromatography on columns previously calibrated with appropriate oligosaccharide standards; such an approach has also been used with glycopeptides (1, 2, 30–32).

The specificities of L- and E-PHA for oligosaccharides and glycopeptides have been examined by a number of laboratories (3–5, 29). Hammarstrom et al. (3) utilized quantitative precipitation and precipitation-inhibition to study the specificity of L-PHA. The minimal structural unit required for binding was found to be GlcNAcβ1,2Man, and the most complementary structure was the pentasaccharide Galβ1,4GlcNAcβ1,2-(Galβ1,4GlcNAcβ1,6)Man. The latter structure is found in tetrambranched and some tribranched oligosaccharides (Tetra-1 and Tri-1), did not significantly interact with L-PHA. These observations were largely in agreement with those of Cummings and Kornfeld (4), who found that glycopeptides retarded on L-PHA affinity columns contained tri- and tetrambranched oligosaccharides such as Tetra-2 and Tri-1, respectively. They also noted that Gal, but not sialic acid or Fuc, was required for interaction with L-PHA.

Cummings and Kornfeld (4) and Narasimhan et al. (29) reported that only glycopeptides bearing oligosaccharides with a bisecting GlcNAc were significantly retarded on E-PHA affinity columns. Furthermore, binding by E-PHA requires the presence of Gal but not sialic acid or Fuc. Yamashita et al. (5) further defined the specificity of E-PHA using hydrazine-released oligosaccharides containing a bisecting GlcNAc residue and found that interaction of these structures with E-PHA required 1) a Gal residue on the branch arising from the α1,6-linked core Man but not on other branches, and 2) a peripheral β1,2-linked GlcNAc attached to each α-linked core Man residue.

Conclusions have differed about the extent to which L- and E-PHA have similar specificities. Cummings and Kornfeld (4) reported distinct specificities of the two lectins for glycopeptides. Glycopeptides bearing oligosaccharides with a bisecting GlcNAc (such as Di-7) were found to interact with E-PHA but not L-PHA, while glycopeptides bearing oligosaccharide structures such as Tri-2 and Tetra-1 were found to interact with L-PHA but not E-PHA. In contrast, based on the finding that Galβ1,4GlcNAcβ1,2Man inhibited binding of both L- and E-PHA, Hammarstrom et al. (3) suggested that the specificities of L- and E-PHA must be grossly similar.

The present findings are in general agreement with the studies summarized above; however, there are a number of important departures. First, using free, reduced oligosaccharides rather than glycopeptides, we find no discernible difference in the specificities of L- and E-PHA. Second, the specificities of L- and E-PHA for free oligosaccharides are not identical to those reported for glycopeptides. This indicates that the use of lectin affinity chromatography for structural characterization of free oligosaccharides requires prior detailed examination of lectin specificity with appropriate oligosaccharide standards rather than glycopeptides. Third, L- and E-PHA display a far greater degree of specificity for oligosaccharide structural features than has previously been appreciated. This level of specificity can only be taken advantage of by carefully monitoring the highly reproducible, characteristic elution positions of oligosaccharides.

Hammarstrom et al. (3) reported that structures with the sequence Siaα2,3Galβ1,4GlcNAc display enhanced binding to L-PHA, whereas those with α2,6-linked sialic acid do not bind to the lectin. Although this study suggested that sialic acid linkage might be of considerable importance for the interaction of oligosaccharides with L-PHA, the effect of α2,3- versus α2,6-linked sialic acid on oligosaccharide interaction with L- and E-PHA had not been systematically studied. The presence of α2,6-linked sialic acid has an inhibitory effect on interaction of oligosaccharides with both L- and E-PHA. This is most dramatically illustrated with structures such as Di-7, which bears a bisecting GlcNAc. In the absence of sialic acid, Di-7 is retarded on L- and E-PHA columns to a greater extent than any other oligosaccharide (Figs. 4B and 8B), yet the presence of terminal α2,6-linked sialic acid completely abolishes this interaction (Figs. 4A and 8A). This may reflect the folding of α2,6-linked sialic acid back over the Galβ1,4GlcNAcβ1,2Man sequence (36), thereby blocking access to the region recognized by the lectin. In contrast, the presence of α2,3-linked sialic acid significantly enhances in-
interaction of oligosaccharides with L- and E-PHA. Since structures bearing a single branch with the sequence Siaα2,3Galβ1,4GlcNAcα1,2 attached to the α1,6-linked core Man (α2,3-sialylated Di-4) interact with L- and E-PHA to a greater extent than oligosaccharides with the same sequence attached to the α1,3-linked core Man (α2,3-sialylated Di-3), the branch arising from the α1,6-linked core Man likely dominates in the interaction with both L- and E-PHA. The presence of an additional branch on α2,3-sialylated Di-4, terminating with either α2,3-sialic acid (Di-2) or SO₄GalNAc (α2,3-sialylated Di-5), does not alter interaction with L- and E-PHA. This suggests that the branch arising from the α1,3-linked core Man does not strongly influence oligosaccharide interaction with either lectin. A similar difference in interaction with glycopeptides bearing oligosaccharides with a single peripheral branch attached to the α1,3- versus α1,6-linked core Man has been observed by Narasimhan et al. (29) for L-PHA.

The presence of terminal Gal and/or bisecting GlcNAc increases interaction of dibranched oligosaccharides with L- and E-PHA. Dibranched oligosaccharides with terminal GlcNAc do not significantly interact with L- and E-PHA (Figs. 1E and 1D), whereas the additional presence of a bisecting GlcNAc residue on these structures enhances interaction with both lectins (Figs. 4C and 8C). Dibranched oligosaccharides with terminal Gal are retarded by L- and E-PHA (Figs. 1B, 1D, and 7C), and the additional presence of a bisecting GlcNAc residue markedly increases the extent of this retardation (Figs. 4B and 8B).

The interactions of free tri- and tetrabranched oligosaccharides with both L- and E-PHA are similar to those described for glycopeptides with L-PHA (4). Both asialo Tri-1 and Tri-2 interact with L- and E-PHA. However, as seen with peptide-bound oligosaccharides and L-PHA, asialo Tri-2, with a branch attached to the C-6 position of the α1,6-linked core Man, is more retarded by these lectins than asialo Tri-1, with a branch attached to the C-4 position of the α1,3-linked core Man (Figs. 5B versus 5D, and 9A versus 9B). Asialo Tetra-1 elutes at the same position as asialo Tri-2 (Figs. 6C and 9C), indicating that interaction with the branch containing a β1,6-linked GlcNAc predominates. Notably, the presence of either peripheral polylactosamine sequences or large amounts of α2,6-sialic acid abolishes interaction of tetra- and tribranched structures with L- and E-PHA, whereas the presence of a greater proportion of α2,3-sialic acid enhances interaction. The identical specificity of L- and E-PHA for tri- and tetrabranched oligosaccharides represents a significant difference from the distinct specificities of these lectins for glycopeptides (1, 2, 4).

Specific interaction with L- and E-PHA involves at a minimum the presence of the sequence GlcNAcα1,2Man, as suggested by Hammarstrom et al. (3). The enhanced interaction seen in the presence of various substituents suggests that these structural alterations 1) change the conformation of the region recognized by the lectin(s) sufficiently to permit better binding, 2) improve access to this recognition region, and/or 3) provide an additional structural domain for the lectin(s) to bind. In some instances, structural differences which appear at first to be subtle, such as the presence of GalNAc rather than Gal, markedly alter oligosaccharide-lectin interaction. Information about the three-dimensional structures of a limited number of oligosaccharides is now available (37, 38). More extensive knowledge of the solution conformation of oligosaccharides may ultimately permit a better understanding of the influence of various structural features on lectin interaction. Regardless of the precise recognition phenomenon involved, it is clear that affinity chromatography on L- and E-PHA can be utilized to distinguish among closely related oligosaccharide structures. It is, however, essential to establish the specificities of these lectins for each newly encountered structure, since it is not possible to predict with confidence the elution position of an oligosaccharide not previously examined.

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REFERENCES
L-PHA and E-PHA Oligosaccharide Specificities


Analysis of Tri-2 oligosaccharides by ConA and DSA affinity HPLC (13) also supports the structural assignment shown in Table 1. Tri-2 structures do not bind to ConA (Fig. 1A), consistent with the presence of a tri- or tetra-branching oligosaccharide (mentioned in Ref. 13). Tri-2 structures do, however, bind tightly to DSA (Fig. 1B). Based on the established specificity of DSA (13), including a recent examination of the behavior of free oligosaccharides, it is clear that Tri-2 structures bind to DSA at a site located on the non-reducing end of the oligosaccharide. Moreover, the presence of a β-1,3-linked GlcNAc attached to the α-L-rhamnose core (Tri-1 or Tri-2 type structures; Ref. 11) may be lost upon conjugation (Ref. 12). Therefore, the addition of three rather than two DSAlactosyl residues to the structure during biosynthesis with galactosyltransferase, and the pattern of sensitivity to sequential endoglycosidase digests (Fig. 1D), further support the proposed structure for Tri-2 shown in Table 1.

![Graph showing binding of ConA and DSA to Tri-2 oligosaccharides.](image_url)

**Fig. 1.** Lectin Affinity HPLC of Tri-2 oligosaccharides. Tri-2 oligosaccharides (100 ng) were analyzed by ConA-affinity (Panel A) and DSA-affinity (Panel B) affinity HPLC, as described (12). In Panel A, bound oligosaccharides were eluted with 0.5 M methyl-α-D-mannoside (MAN) (100% of peak) as shown. In Panel B, bound oligosaccharides were eluted with 1.0 M glucosamine, 1.0 M M6P, 1.0 M aminophosphonic acid, and 1.0 M ascorbic acid in PBS at pH 6.0 to 7.0 (trace).