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Effect of α(2-6)-Linked Sialic Acid and α(1-3)-Linked Fucose on the Interaction of *N*-Linked Glycopeptides and Related Oligosaccharides with Immobilized *Phaseolus vulgaris* Leukoagglutinating Lectin (L-PHA)

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The effects of branching and substitution of branches by sialic acid and fucose on the interaction of *N*-linked glycopeptides and related oligosaccharides with immobilized *Phaseolus vulgaris* leukoagglutinating lectin (L-PHA) were examined. Asialo bi-, tri- and tetra-antennary glycans were all retarded but to different extents on a long column of L-PHA-agarose. Asialo tri- and tetra-antennary glycans containing the pentasaccharide unit Galβ1-4GlcNAcβ1-2[Galβ1-4GlcNAcβ1-6]Man were strongly retarded, whereas asialo bi- and tri-antennary glycans lacking the Galβ1-4GlcNAcβ1-6 branch were only weakly retarded. In all instances the interaction with the lectin was completely abolished when either α(2-6)-linked *N*-acetylneuraminic acid or α(1-3)-linked fucose was present at the galactose or *N*-acetylglucosamine residue of the Galβ1-4GlcNAcβ1-2Manα1-6 branch, respectively. The same substitutions on the Galβ1-4GlcNAcβ1-6Manα1-6 branch decreased but did not abolish the affinity of the lectin for the glycans. The presence of NeuAcα2-6 and Fucα1-3 on the other two branches did not interfere with the binding of the glycans to L-PHA. Furthermore, it appeared that the presence of the Manβ1-4GlcNAc unit is required for interaction with the lectin. In order to obtain reliable information on the relative occurrence of tri- and tetra-antennary glycopeptides, this study shows that it is essential to desialylate and to defucosylate the glycans prior to application to L-PHA-agarose.

Abbreviations: L-PHA, leukoagglutinating phytohemagglutinin; CMP-NeuAc, cytidine-5′-monophospho-*N*-acetylneuraminic acid; *GP*, glycopeptide; *OS*, oligosaccharide; suffixes MS, BS and TS indicate mono-, bi- and trisialyl derivatives, respectively; suffix MF indicates monofucosyl derivatives: HPLC, high-performance liquid chromatography; structures of the substrates *OS2, OS3, OS3′, OS4, GP2, GP3, GP4, GP4-MF, OS2(3′)* and *OS2(-)* are presented in Fig. 2; FNR, fraction not retarded; FR, fraction retarded.

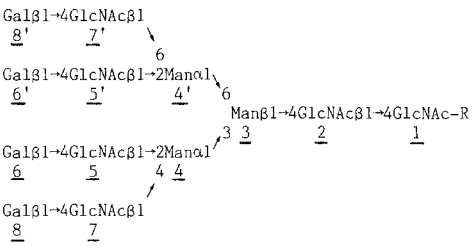


Figure 1. The denoting system used in this study to indicate the constituent monosaccharides of the various oligosaccharides and glycopeptides.

The system is illustrated for glycopeptide *GP4*.

Affinity chromatography with lectins immobilized on solid supports has been demonstrated to be very useful in the fractionation of glycoproteins and glycopeptides. Cummings and Kornfeld [1] have presented a general method for the analysis and isolation of *N*-linked glycopeptides with the aid of a series of immobilized lectins with different carbohydrate-binding specificities. One of the lectins which has been employed frequently for this purpose is the leukoagglutinating lectin (L-PHA) of the red kidney bean (*Phaseolus vulgaris*).

The carbohydrate-binding specificity of L-PHA was studied by Hammarström *et al.* [2] by assaying the inhibition of precipitation of carcinoembryonic antigen by glycopeptides and oligosaccharides. These authors concluded that the minimal structural requirement for L-PHA-binding was contained in the disaccharide GlcNAcβ1-2Man. The most effective structure appeared to be the pentasaccharide Galβ1-4GlcNAcβ1-2[Galβ1-4GlcNAcβ1-6]Man. A different approach was used by Cummings and Kornfeld [3], who examined the extent to which *N*-linked glycopeptides differing in carbohydrate structure were retarded on columns of immobilized L-PHA. These authors established that tri- and tetra-antennary glycopeptides containing the aforementioned pentasaccharide structure were specifically retarded; bi- and tri-antennary glycopeptides lacking the Galβ1-4GlcNAcβ1-6 branch showed no interaction with the lectin. These results were consistent with those of Hammarström *et al.* [2] indicating the importance of the pentasaccharide unit for interaction with L-PHA. Very recently Green and Baenziger [4] showed with free and reduced oligosaccharides that interaction with L-PHA of bi- and tri-antennary glycans can also occur, but is dependent on the substitution of galactose by e.g. sialic acid. In a preliminary report [5] we had reached the same conclusion for glycopeptides. However, Green and Baenziger noted that the elution patterns obtained with certain glycopeptides were in marked contrast to those obtained using analogous reduced oligosaccharides. In most studies using L-PHA-agarose affinity chromatography, glycopeptide mixtures are employed [6-8]. Therefore, it is essential to know whether the high level of specificty of L-PHA for free and reduced oligosaccharides can also be obtained for glycopeptides.

In this study we have re-examined the carbohydrate-binding specificity of L-PHA for *N*-linked glycopeptides and extended the existing knowledge concerning the influence of substitution of tri- and tetra-antennary glycopeptides by α(2-6)-linked sialic acid and α(1-3)-linked fucose on the retardation of the glycopeptides. For these studies a series of partially sialylated and fucosylated glycopeptides were used which are currently available in our laboratory. A preliminary report has appeared [5].

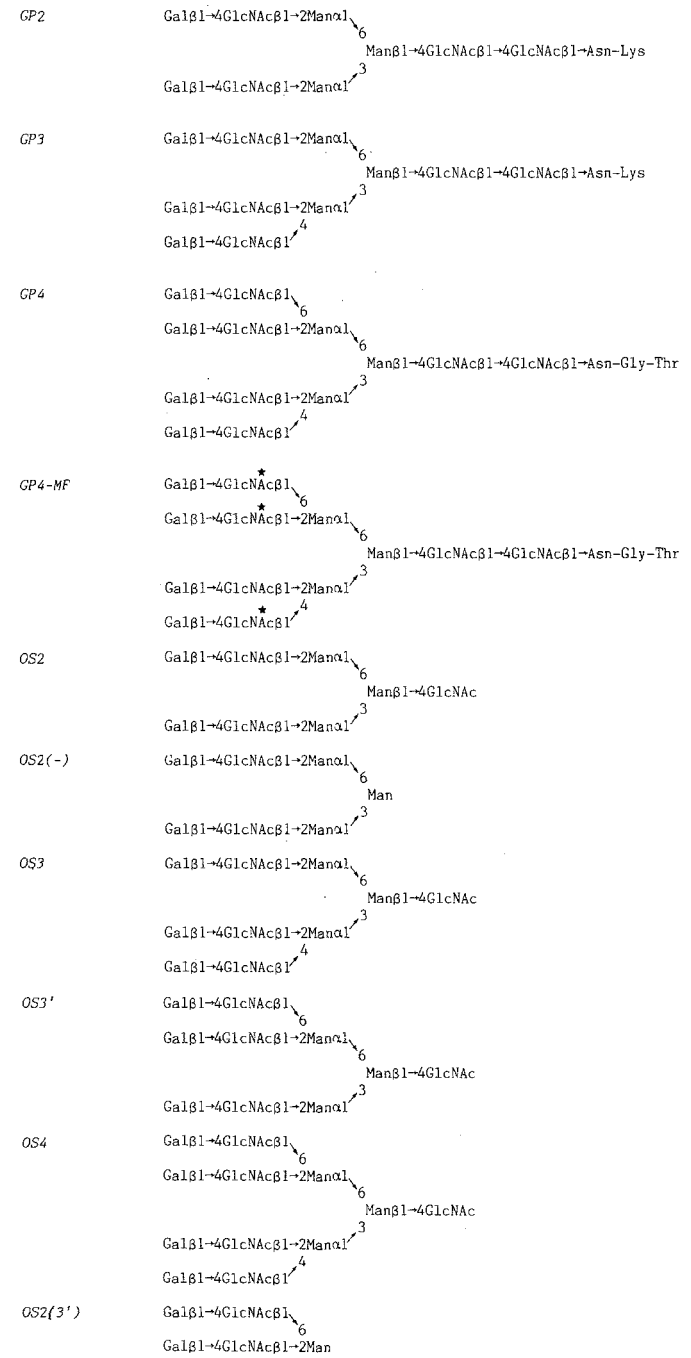


Figure 2. Structures of the various glycopeptides and oligosaccharides used in the present study.

Structures of the branched acceptor substrates referred to in this study are presented in the figure. Glycopeptide *GP4-MF* is a mixture of monofuco-glycopeptides, in which fucose is present on either of the branches marked with an asterisk.

Experimental Procedures

Materials

The complete structures of the various oligosaccharides and glycopeptides referred to in this study and the system which is used to denote their constituent monosaccharides are presented in Figs. 1 and 2. Glycopeptides *GP2, GP3, GP4* and *GP4-MF* were donated by Dr. K. Schmid (Boston University, Boston, MA, USA) and correspond to glycopeptides GPII-6, GPII-5, GPV-4 and GPV-3 obtained from desialylated human α_1-acid glycoprotein, respectively [9]. Glycopeptide *GP4-MF* is a mixture of monofucoglycopeptides with L-fucose α(1-3)-linked to one of the *N*-acetylglucosamine residues **7'**, **5'** or **7** (numbering refers to Fig. 1). GlcNAc-**7'** contains 25% of the fucose, GlcNAc-**5'** 15% and GlcNAc-**7'** 60% [10]. The oligosaccharides *OS2, OS3, OS3'* and *OS4* (isolated from the urine of patients with GM1 gangliosidosis) were a gift from Dr. G. Strecker (Université de Lille, Villeneuve d'Ascq, France). The synthetic pentasaccharide *OS2(3')*, representing a partial structure of *OS3'*, and the heptasaccharide *OS2(-)* were kindly donated by Dr. J. Lönngren (University of Stockholm, Stockholm, Sweden). α(2-6)-Sialylated oligosaccharides and glycopeptides were prepared by incubation of the various acceptors with bovine colostrum CMP-sialic acid:Galβ1-4GlcNAc-R α(2-6)-sialyltransferase and CMP-[^{3}H]NeuAc as described previously [10-11]. All the above mentioned structures had been identified by ^{1}H-NMR spectroscopy [10-14], except for *OS3'-TS* which could not be obtained in sufficient quantity. For the preparation of [^{3}H]Gal-labeled *OS3'* and *OS2(3')*, the original oligosaccharides were digested with jack bean β-galactosidase and subsequently re-galactosylated using UDP-[^{3}H]Gal and calf thymus UDP-Gal:*N*-acetylglucosaminide β(1-4)-galactosyltransferase according to [15]. The radioactive forms of *OS3'* and *OS2(3')* were isolated from the product mixture by HPLC using the system described previously [15].

L-PHA-agarose (lot No. 031202 and 040822, 3-5 mg of protein/ml of gel) was purchased from E-Y Laboratories, Inc., San Mateo, CA, USA. Bio-Gel P-4 (200-400 mesh) was obtained from Bio-Rad Laboratories, Richmond, CA, USA. [1-^{14}C]Acetic anhydride (25 mCi/mmol) was purchased from Amersham International, Amersham, UK.

Preparation of N-[^{14}C]Acetylated Glycopeptides

Glycopeptides were *N*-[^{14}C]acetylated by conditions modified slightly from those of Koide and Muramatsu [16]. About 50 nmol of the glycopeptides *GP2, GP3, GP4* and *GP4-MF* were incubated with 10 μmol [1-^{14}C]acetic anhydride (25 mCi/mmol) in 1 ml of 60 mM $NaHCO_3$ at room temperature for 2 h. The incubation mixtures were subsequently subjected to gel filtration on a column (1.5 × 45 cm) of Bio-Gel P-4 eluted with 0.1 M ammonium acetate, pH 5.0. Fractions containing the ^{14}C-labeled glycopeptides were pooled and lyophilized, and stored at -20°C until used.

L-PHA-Agarose Affinity Chromatography

Glycopeptides or oligosaccharides (0.2-3.0 nmol; 0.5-2.5 nCi ^{3}H or ^{14}C) were dissolved in 1 ml of phosphate-buffered saline/NaN_3 (6.7 mM KH_2PO_4/K_2HPO_4, 0.15 M NaCl, 0.02% NaN_3, pH 7.4) and analyzed on a column (0.6 × 50 cm) of L-PHA-agarose. The capacity of the L-PHA-agarose column was found to be at least 10 nmol of *N*-[^{14}C]acetylated asialo

tri- or tetra-antennary glycopeptide corresponding to 68 nCi ^{14}C. Elution of the glycopeptides or oligosaccharides was performed by the same buffer at a flow of 5 ml/h and 20°C. Fractions (1 ml) were collected and the radioactivity in each fraction was determined by liquid scintillation. The radioactivity of each of the eluted materials was expressed as a percentage of that totally eluted. Recovery of glycopeptides and oligosaccharides was routinely 90-100%. L-PHA-Agarose was used repeatedly for over a year without apparent loss of activity. We observed, however, a large variation in activity of different batches of L-PHA-agarose. We therefore tested the different batches with a number of *N*-linked oligosaccharides of known structure before using them in this study. Two out of four batches (lot No. 040822 and 031202) showed identical properties and yielded sufficient resolution to perform these studies.

Based on their elution behaviour on the L-PHA-agarose columns the materials were indicated as not retarded (FNR, elution between 12 and 18 ml), weakly retarded (FR1, elution between 19 and 27 ml) and strongly retarded (FR2, elution between 29 and 41 ml).

^{1}H-NMR Spectroscopy

Prior to ^{1}H-NMR spectroscopic analysis, 25-120 nmol of the glycopeptides were repeatedly treated with ^2H_2O (99.75 atom % ^{2}H) at pH 7 and room temperature. After each exchange treatment the materials were lyophilized. Finally each sample was dissolved in 40 μl ^2H_2O (99.95 atom % ^{2}H). ^{1}H-NMR spectroscopy was performed at 400-MHz on a Bruker MSL-400 spectrometer (Department of Physical and Theoretical Chemistry, Vrije Universiteit, Amsterdam) operating in the Fourier-transform mode. Resolution enhancement of the spectra was achieved by Lorenzian-to-Gaussian transformation. The probe temperature was kept at 300°K. Chemical shifts are expressed downfield from internal sodium 4,4-dimethylsilapentanesulfonate, but were actually measured by reference to internal acetone δ = 2.225 ppm) with an accuracy of 0.002 ppm.

Results and Discussion

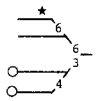
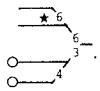
Various *N*-linked glycopeptides and related oligosaccharides were subjected to L-PHA-agarose affinity chromatography. The structures of the tetra-, tri- and bi-antennary glycopeptides and oligosaccharides used, together with their relative distribution of radioactivity in the eluate, are listed in Tables 1, 2 and 3, respectively. Three examples of the elution profiles obtained are shown in Fig. 3.

Binding of Asialo Bi-, Tri- and Tetra-antennary Glycans to L-PHA-Agarose

We observed a clear interaction of asialo bi- and tri-antennary glycopeptides lacking the Galβ1-4GlcNAcβ1-6 branch (Table 2, *GP3* and Table 3, *GP2*) with the lectin. In accordance with the specificity of L-PHA described previously [2-4] asialo tri- and tetra-antennary oligosaccharides and glycopeptides containing the pentasaccharide Galβ1-4GlcNAcβ1-2[Galβ1-4GlcNAcβ1-6]Man interacted with high affinity with immobilized L-PHA (Table 1, *GP4* and Table 2, *OS3'*). Obviously, the Galβ1-4GlcNAcβ1-4 branch did not contribute to the interaction.

Table 1. Behaviour of tetra-antennary oligosaccharides (*OS*) and glycopeptides (*GP*) on L-PHA-agarose.
The structures are depicted in a schematic form, with numbers indicating the positions of substitution at the branching points (compare Fig. 1).

Glycopeptide or oligosaccharide	Schematic structure[a]	Isomers in structure[b] (%)	Relative distribution of radioactivity in eluate (%)		
			FNR	FR1	FR2
GP4			3	3	94
GP4-MF1		60			
GP4-MF2		25	15[c]	25[c]	60[c]
GP4-MF3		15			
GP4-MS			10	n.d.[d]	90
OS4-MS			15	n.d.	85
GP4-MF1/MS					
GP4-MF2/MS			21	19	60
GP4-MF3/MS					
GP4-BS1		65			
GP4-BS2		35	28[c]	n.d.	72[c]

GP4-MF1/BS1				
GP4-MF1/BS2				
GP4-MF2/BS1				
GP4-MF2/BS2		65	35	n.d.
GP4-MF3/BS1				
GP4-MF3/BS2				
GP4-TS1		80		
GP4-TS2		20	76	n.d. 24

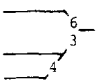
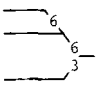
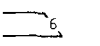
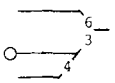
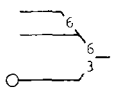
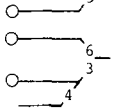
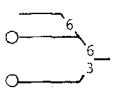
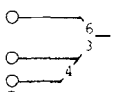
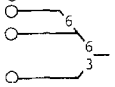
[a] ○ = *N*-Acetylneuraminic acid linked α2-6 to galactose; ★ = Fucose linked α1-3 to *N*-acetylglucosamine.

[b] *GP4-MF* is a mixture of monofuco-glycopeptide isomers. *GP4-BS* and *GP4-TS* are mixtures of two isomers differing in the branch to which the secondly or thirdly introduced *N*-acetylneuraminic acid residue, respectively, is attached. *GP4-MF/BS* is a mixture of six isomers differing in the branch to which the secondly introduced *N*-acetylneuraminic acid residue or the fucose residue is attached. Relative occurrence of the isomers is derived from [10, 12].

[c] When mixtures were analyzed, we attempted to characterize the peak materials that were resolved upon chromatography on the lectin column by 400-MHz ^{1}H-NMR spectroscopy. The two fractions (FNR and FR2) that were obtained from glycopeptide *GP4-BS* were each found to consist of a single isomer which could be identified as *GP4-BS2* (bearing α(2-6)-linked *N*-acetylneuraminic acid residues at Gal-**6** and Gal-**6'**) and *GP4-BS1* (bearing *N*-acetylneuraminic acid residues at Gal-**6** and Gal-**8**), respectively. This identification was based on the comparison of the chemical shift values of the *N*-acetyl resonances of the *N*-acetylglucosamine residues as well as the H-1 of Man-**4'** (Table 4) with those established for these isomers [10]. Similarly, the three materials (FNR, FR1 and FR2) which were resolved upon chromatography of *GP4-MF* appeared to be single isomers. They could be identified as tetra-antennary glycopeptides containing one fucose residue attached to GlcNAc-**5',7'** and **7**, respectively (Table 4) [10, 13]. Other glycopeptides and oligosaccharides which also were resolved, however, were available in too small amounts to allow analysis of the fractions by ^{1}H-NMR spectroscopy.

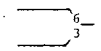
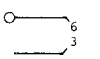
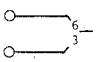
[d] n.d. = not detectable.

Table 2. Behaviour of tetra-antennary oligosaccharides (*OS*) and glycopeptides (*GP*) on L-PHA-agarose.
The structures are depicted in a schematic form, with numbers indicating the positions of substitution at the branching points (compare Fig. 1).

Glycopeptide or oligosaccharide	Schematic structure[a]	Isomers in structure[b] (%)	Relative distribution of radioactivity in eluate (%)		
			FNR	FR1	FR2
GP3			2	94	4
OS3'			9	8	83
OS2(3')[c]			100	n.d.[d]	n.d.
GP3-MS			19	81	n.d.
OS3-MS			16	84	n.d.
OS3'-MS			10	7	83
GP3-BS1		90			
GP3-BS2		10	87	13	n.d.
OS3'-BS1		90			
OS3'-BS2		10	83	17	n.d.
GP3-TS			100	n.d.	n.d.
OS3'-TS			100	n.d.	n.d.

[a] O = NeuAc linked α2-6 to galactose.
[b] *GP3-BS* and *OS3'-BS* are mixtures of two isomers differing in the branch to which the secondly introduced NeuAc residue is attached. Relative occurrence of the isomers is derived from [10].
[c] The pentasaccharide *OS2(3')* represents a partial structure of *OS3'* (compare Fig. 2).
[d] n.d. = not detectable.

Table 3. Behaviour of bi-antennary oligosaccharides (*OS*) and glycopeptides (*GP*) on L-PHA-agarose.
The structures are depicted in a schematic form, with numbers indicating the positions of substitution at the branching points (compare Fig. 1).

Glycopeptide or oligosaccharide	Schematic structure[a]	Isomers in structure[b] (%)	Relative distribution of radioactivity in eluate (%)		
			FNR	FR1	FR2
GP2			2	98	n.d.[c]
GP2-MS			15	85	n.d.
OS2-MS			6	94	n.d.
OS2(—)-MS1		40			
OS2(—)-MS2		60	100	n.d.	n.d.
GP2-BS			100	n.d.	n.d.

[a] O = NeuAc linked α2-6 to galactose.
[b] *OS2(—)-MS* is a mixture of two isomers differing in the branch to which the NeuAc residue is attached. Both isomers lack the GlcNAc-**2** residue, Man-**3** is the reducing end of the oligosaccharides (compare Figs. 1 and 2). Relative occurrence of the isomers is derived from [11].
[c] n.d. = not detectable.

Effect of Acetylneuraminic Acid Linked α2-6 to Galactose

With sialylated tri- and tetra-antennary glycans, a pronounced effect on the interaction with L-PHA was observed depending on the branch at which *N*-acetylneuraminic acid was present. Our data show that the presence of *N*-acetylneuraminic acid linked α2-6 to Gal-**6'** completely abolished the affinity of the lectin for the glycans (Table 1, *GP5-BS* and *GP4-TS*; Table 2, *GP3-BS, OS3'BS* and *GP3-TS*; Table 3, *GP2-BS*). The influence of *N*-acetylneuraminic acid linked α2-6 to Gal-**8'** on binding to L-PHA was not always the same. In one case a decrease in interaction from FR2 to FR1 between the oligosaccharide and the immobilized lectin was observed (Table 2, *OS3'BS*). With another glycan there was no detectable change in the high affinity interaction due to the addition of *N*-acetylneuraminic acid to this galactose residue (Table 1, *GP4-TS*). The presence of *N*-acetylneuraminic acid residues linked α2-6 to Gal-**6** and Gal-**8** did not interfere with the interaction between the glycopeptides or oligosaccharides and the immobilized L-PHA (Table 1, *GP4-MS, OS4-MS, GP4-BS*, and *GP4-TS*; Table 2, *GP3-MS, OS3-MS, OS3'MS*, and *PG3-BS*; Table 3, *GP2-MS* and *OS2-MS*).

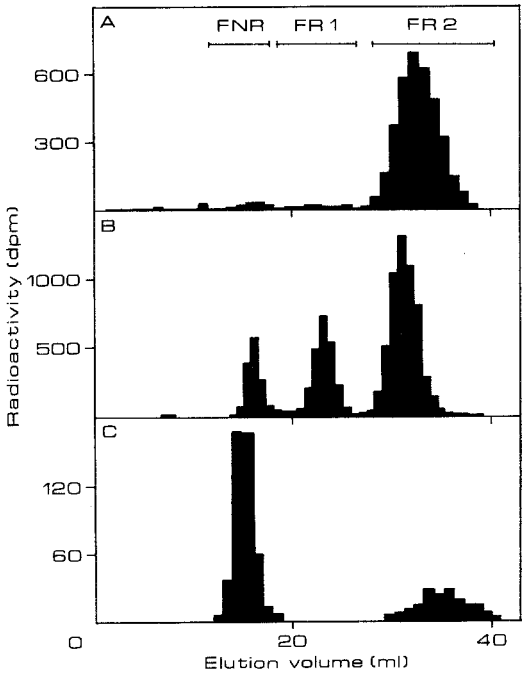


Figure 3. Affinity chromatography of oligosaccharides and glycopeptides on L-PHA-agarose.

Radioactively-labeled glycopeptides *GP4* (A), *GP4-MF* (B) and *GP4-TS* (C) were chromatographed on L-PHA-agarose as described under "Experimental Procedures". Fractions of 1 ml were collected and monitored for radioactivity. Recovery of radioactivity was greater than 90%.

The latter result might explain why α(2-6)-linked *N*-acetylneuraminic acid has been suggested to have no effect on L-PHA-binding [3]. Probably in the glycopeptides used in those studies the location of NeuAcα2-6 has been confined to Gal-**6** and Gal-**8**. Indeed, it is known that in tetra-antennary glycans these galactose residues are preferentially α(2-6)-sialylated, whereas Gal-**6'** and Gal-**8'** are very resistant to α(2-6)-sialylation [10].

Recently, a similar effect of *N*-acetylneuraminic acid linked α2-6 to galactose on the binding to a column of immobilized *Datura stramonium* agglutinin has been reported [17].

Effect of Fucose Linked α1-3 to N-Acetylglucosamine

The presence of fucose linked α1-3 to GlcNAc-**5'** completely abolished the affinity of L-PHA for asialo tetra-antennary glycopeptides, whereas the presence of such a fucose residue on GlcNAc-**7'** only diminished the interaction. The presence of fucose linked α1-3 to GlcNAc-**7** did not influence the behaviour of the asialo tetra-antennary glycopeptides on the column (Table 1, cf. *GP4-MF* with *GP4*). The interference of fucose linked α1-3 to peripheral *N*-acetylglucosamine residues of *N*-linked glycopeptides with the

Table 4. 400-MHz ^{1}H-NMR spectral data of subfractions of glycopeptides GP4-BS and GP4-MF.

Reporter group	Residue	Chemical shift values of glycopeptide fraction (ppm)				
		GP4-BS		GP4-MF		
		FNR	FR2	FNR	FR1	FR2
H-1	Man-**4'**	4.886	4.875	N.D.[a]	N.D.	N.D.
NAc	GlcNAc-**1**	2.006	2.006	2.006	2.007	2.007
	GlcNAc-**2**	2.078	2.077	2.077	2.077	2076
	GlcNAc-**5**	2.071	2.071	2.052	2.053	2.053
	GlcNAc-**5'**	2.065	2.042	2.033	2.041	2.040
	GlcNAc-**7**	2.078	2.102	2.077	2.077	2.068
	GlcNAc-**7'**	2.039	2.042	2.038	2.030	2.040

[a] N.D. = not determined

binding to an L-PHA-agarose column has been noticed before by Santer *et al.* [18], but the structural background for this observation was not established. A similar influence of fucose linked α1-3 to *N*-acetylglucosamine on the binding to columns of immobilized *Datura stramonium* agglutinin, *Phaseolus vulgaris* erythroagglutinating lectin, Concanavalin A and four different *Erythrina* lectins has been reported [17-20].

Binding of Tetra-antennary Sialo/fuco-glycopeptides to L-PHA-Agarose

The elution profiles obtained with the sialylated products of the fucose-containing glycopeptide *GP4-MF* (Table 1, *GP3-MF/MS* and *GP4-MF/BS*) are consistent with the aforementioned results and the relative occurrence of isomers as reported previously [10]. No FR2 fraction was observed upon chromatography of *GP4-MF/BS* on L-PHA-agarose (Table 1).

It is known that a Galβ1-4GlcNAc branch that carries a fucose residue linked α1-3 to *N*-acetylglucosamine cannot be α(2-6)-sialylated at the galactose [21]. So in all isomers of *GP4-MF/BS* three out of the four branches were carrying an *N*-acetylneuraminic acid or fucose residue. Thus the presence of *N*-acetylneuraminic acid linked α2-6 to Gal-**8'** as well as of fucose linked α1-3 to GlcNAc-**7'** obviously diminished the high affinity interaction of these glycans with the lectin.

Effect of the Core on L-PHA-Binding

The results obtained with glycopeptides and the corresponding oligosaccharides having a reducing *N*-acetylglucosamine residue were essentially the same (compare e.g. *GP4-MS* and *OS4-MS*, Table 1). This implies that in the glycans studied the presence of the GlcNAcβ1-Asn does not appear to be required for binding to L-PHA. However, structures having Man-**3** at the reducing end appeared to have lost the capability to interact with L-PHA (compare *OS2(-)-MS* and *OS2-MS*, Table 3). It is of interest to note that the presence of GlcNAc-**2** in the core structure is essential for bovine colostrum α(2-6)-sialyltransferase to retain its branch specificity towards bi-antennary glycans [11]. It has

been suggested that this residue acts as a recognition site mediating the correct positioning of the substrate on the enzyme. Likewise L-PHA might specifically interact with this *N*-acetylglucosamine residue. Alternatively the presence of GlcNAc-**2** might give steric constraints for the glycan branches yielding conformational features that are essential for binding to the lectin [22, 23].

Green and Baenzinger [4] noted that differences in elution pattern could be obtained with certain reduced oligosaccharides in comparison to the corresponding glycopeptides. This discrepancy in results might be explained by a difference in solution conformation of free oligosaccharides as a result of reduction of GlcNAc-**1** [4]. So our studies once more emphasize the necessity to calibrate the L-PHA column with the appropriate standards, prepared in a similar way as the structures to be analysed.

Conclusions

L-PHA-Agarose can be used to separate bi-, tri- and tetra-antennary glycans, of which tri- and tetra-antennary structures containing the pentasaccharide Galβ1-4GlcNAcβ1-2 [Galβ1-4GlcNAcβ1-6]Man interact with high affinity. The most important part of this pentasaccharide unit recognized by the lectin appears to be the Galβ1-4GlcNAcβ1-2 branch; α(2-6)-sialylation of the galactose or α(1-3)-fucosylation of the *N*-acetylglucosamine residue of this branch completely abolishes the interaction. It is not unlikely that this effect is caused by shielding of an essential binding site on GlcNAc-**5'**, particularly since it has been proposed that the *N*-acetylneuraminic acid residue in the NeuAcα2-6Galβ1-4GlcNAc sequence folds back towards the *N*-acetylglucosamine residue [24]. Substitution at C-6 of Gal-**8'** by *N*-acetylneuraminic acid or at C-3 of GlcNAc-**7'** by fucose generally diminishes the interaction with L-PHA, whereas other branch locations of *N*-acetylneuraminic acid and fucose do not interfere with the glycan-lectin interaction. Based on its specificity, L-PHA-agarose chromatography may also be very useful in the fractionation of α(1-3)-fucosylated glycans.

In general it is essential to desialylate and to defucosylate the glycans prior to application to the column, in order to obtain correct information on the relative occurrence of tri- and tetra-antennary glycopeptides.

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