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The (1→3)-linked α-L-fucosyl group of the *N*-glycans of the *Wistaria floribunda* lectins is recognized by a rabbit anti-serum*,†

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ABSTRACT

An increasing number of plant glycoproteins have been shown to possess a characteristic *N*-glycan component containing a β-(1→2)-linked D-xylose unit on the core β-D-mannose unit, and an α-(1→3)-linked L-fucose unit on the asparagine-linked 2-acetamido-2-deoxy-D-glucose unit. *Wistaria floribunda* seeds have two distinct lectins; the erythroagglutinin, WFA, and the lymphocyte mitogen, WFM. Earlier studies indicated that both lectins belong to such a class of glycoproteins. We now report the complete structural analysis of Pronase glycopeptides derived from WFA. On the basis of chemical treatment of the glycopeptides, carbohydrate composition and methylation analysis of fluorescein-labeled glycopeptides, and their susceptibilty to specific exoglycosidases, the structure of the WFA glycan was found to be, α-D-Man*p*-(1→6)-[β-D-Xyl*p*-(1→2)]-[α-D-Man*p*(1→3)]-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*-NAc-(1→*N*).

Quantitative studies on the interaction of the original fluorescein-labeled glycopeptide and its specific degradation products with a rabbit anti-glycan antibody, developed against WFM, showed that the (1→3)-linked α-L-fucose unit is essential for interaction. Loss of the terminal α-D-mannosyl groups resulted in decreased, though detectable binding.

INTRODUCTION

Earlier studies showed that the structure and biosynthesis of asparagine-linked glycans are virtually identical in animals and plants[1]. The report by Ishahara *et al.*[2] of the presence of a (1→2)-linked β-D-xylose unit and a (1→3)-linked core α-L-fucose unit as components of the oligosaccharide of the plant protease, bromelain, demonstrated critical differences in the structures of the *N*-glycans of animal and plant glycoproteins. Shortly afterward, the report by Kaladas *et al.*[3] on the reactivity of an anti-carbohydrate antibody with a number of plant lectins, as well as bromelain, emphasized the probable

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widespread nature of this type of carbohydrate structure. Detailed chemical studies have now demonstrated that *N*-linked components having a $Man_3(GlcNAc)_2$ core unit containing both the characteristic (1→2)-linked β-D-xylose and (1→3)-linked α-L-fucose units is a common feature of plant glycoproteins[4-10].

Expansion of the database for those glycoproteins containing these features requires qualitative reagents specific for characteristic structural elements. In our continuing effort to study the properties of plant lectins, we developed a rabbit antibody toward the mitogenic lectin (WFM) of *Wistaria floribunda* seeds[3]. Approximately 25% of this polyvalent antibody recognizes the glycans of the homologous antigen, the *Sophora japonica* agglutinin (SJA), and the erythroagglutinating lectin (WFA) of *W. floribunda* seeds. Fournet *et al.*[9] demonstrated that the structure of the *N*-linked carbohydrate component of SJA is: α-D-Man*p*-(1→6)-[β-D-Xyl*p*-(1→2)]-[α-D-Man*p*-(1→3)]-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-[α-D-Fuc*p*-(1→3)]-β-D-Glc*p*NAc. We now report herein that WFA has a similar structure and that the anti-carbohydrate antibody raised against WFM recognizes primarily the core linked α-L-fucosyl unit of these lectins.

EXPERIMENTAL

Preparation of electrophoretically-homogeneous fluoresceinated glycopeptides (FGPs). — Glycopeptides were obtained by exhaustive Pronase digestion of purified WFA[11] according to the procedure of Poretz and Pieczenik[12]. The proteolytic digest was applied to a column (1 × 40 cm) of Bio-Gel P6-DG equilibrated with water and the sample was eluted with the same solvent. Fractions containing sugar were pooled, lyophilized, and dissolved in 50mM $Na_2B_4O_7$ (1.0 mL), pH 7.9, containing mM $CaCl_2$. To ensure complete proteolytic digestion, Pronase was added as 0.2% of the substrate weight. After incubation for 24 h at 37°, another aliquot of Pronase was added, and the digestion allowed to proceed for an additional 24 h. The reaction was terminated by boiling the sample for 2 min. After centrifugation and lyophilization of the supernatant, the sample was dissolved in water and subjected to Bio-Gel P6-DG chromatography as described above. Sugar-containing fractions were pooled and lyophilized.

FGPs were prepared according to the procedure described for fetuin[12]. The glycopeptides were dissolved in a minimum volume of 50mM $Na_2B_4O_7$, pH 9.0. Fluorescein isothiocyanate (FITC) was added in an amount greater than the molar quantity required to derivatize all potential α-amino groups generated by the complete digestion of the protein. After incubation with gentle shaking at room temperature for 20 h, the samples were centrifuged (2000*g* for 15 min). Filtration through a column (0.9 × 40 cm) of Sephadex G-10 and elution with water yielded the FGPs at the exclusion volume.

Poly(acrylamide) gel electrophoresis of FGPs. — Electrophoretically-homogeneous FGPs were obtained according to the procedure developed by Poretz and Pieczenik[12]. Discontinuous poly(acrylamide) gel electrophoresis (PAGE) was performed in slab gels using the Tris·glycine–Tris·HCl buffer system described by Davies[13]. The gel consisted of a 25% poly(acrylamide) separating gel [containing 1.25% *N,N'*-methylene

bis(acrylamide)] and a 7% poly(acrylamide) stacking gel. To the samples was added glycerol to a concentration of 10% and electrophoresis was conducted at 4° with a constant current of 20 mA. Fluorescent components were detected and recorded as described previously[12]. Glycopeptides were obtained after preparative PAGE by excising the fluorescent bands and extracting with water. After concentration *in vacuo*, the eluates were desalted in a column (1 × 40 cm) of Bio-Gel P-2 with water as the solvent.

Enzyme digestion of FGPs. — The appropriate enzyme (2.5 mU/μL of reaction volume) was mixed with an aliquot of fluorescent material in sterile 50mM sodium acetate buffer, pH 4.5. The mixture was covered with toluene and incubated for 20 min or 18 h at 37°, and the reaction was terminated by boiling for 2 min.

Preparation of L-*fucose-free glycopeptides.* — Partial acid hydrolysis of glycopeptides was performed by a modification of the method described for bromelain glycopeptides[14]. A mixture of WFA glycopeptides (40 μL; 0.25 mg total sugar content), which was eluted in the exclusion volume of the Bio-Gel P6-DG column, and 13M trifluoroacetic acid (400 μL) was incubated at room temperature for 24 h. The sample was dried under a stream of N_2 and excess acid was removed by repeated addition and evaporation of methanol. The glycopeptides were then labeled with FITC, and FGPs were purified by PAGE as described above.

Borate-buffered poly(acrylamide) gel electrophoresis. — The homogeneity of FGPs were ascertained by PAGE using a borate-buffered system[15]. Samples were applied to 7.5% poly(acrylamide) gel slabs [containing 0.38% of *N,N'*-methylene bis(acrylamide)] prepared with 0.1M $Na_2B_4O_7$, pH 9.1. The same buffer was employed as electrode solutions and the electrophoresis was performed at 200 V.

Carbohydrate analysis. — The carbohydrate composition of FGPs was determined by g.l.c.–m.s. of per-*O*-trimethylsilyl derivatives after methanolysis[16]. The derivatized methyl glycosides were analyzed with a Hewlett–Packard model 5890 chromatograph and a model 5970 mass detector. Separation was accomplished with a Hewlett–Packard ultra 1 capillary column (30 m × 0.2 mm, 0.11-μm film) and a linear temperature gradient of 2°/min from 90 to 240°. Methylation analysis was performed by the production of partially methylated alditol acetates of FGPs and their subsequent identification by g.l.c.–m.s. as reported earlier[17].

Amino acid analysis. — The amino acid composition of WFA FGPs was determined essentially according to the procedure of Jones and Gilligan[18].

Immunochemical methods. — Immunoglobulin G (IgG) of the anti-WFM serum was obtained by use of the procedure of Miller and Stone[19]. The binding capacity of the FGPs (before and after the removal of specific sugar residues) to the IgG was determined by a microfluorometric assay[3].

RESULTS AND DISCUSSION

Isolation of glycopeptides. — Pronase digestion of the lectin, followed by gel filtration through Bio-Gel P6-DG, and redigestion of the carbohydrate-containing material in the column flow-through yielded a glycopeptide fraction upon rechromato-

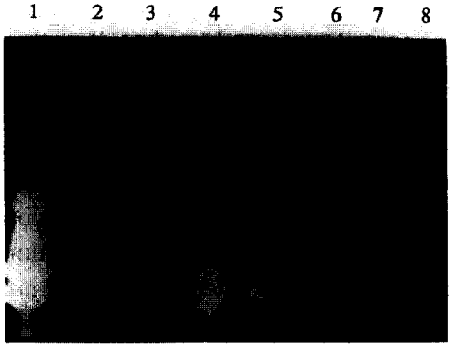

Fig. 1. Composite PAGE electrophoretogram of the fluoresceinated WFA glycopeptide fraction after gel filtration through Bio-Gel P6-DG. Lane 1, total fluoresceination mixture; lanes 2–6, elution pools 1–5, respectively, resulting from Sephadex G-10 gel filtration of the total fluoresceination mixture; lane 7, purified FGP 2; and lane 8, purified FGP 1.

graphy through the same column (data not shown). A greater than 80% yield of glycopeptide was obtained, based upon a value of 3.5% of neutral sugars for the original glycoprotein, as determined by the phenol–sulfuric acid method[20]. The pooled glycopeptide fractions were labeled with FITC. Fig. 1 shows the PAGE electrophoretogram of the fluoresceination mixture before and after filtration through Sephadex G-10. The chromatography procedure resolved the high-molecular-weight FGPs (lanes 2 and 3) from the other products of the fluoresceination reaction (lanes 4–6). Preparative PAGE was performed to isolate the two major FGPs (FGP 1 and FGP 2) shown in lanes 7 and 8, respectively. The homogeneity of these FGPs was verified by borate PAGE. Rago and Poretz[15] demonstrated that borate-buffered gels are able to resolve ovalbumin FGPs which have coincident mobility on 25% poly(acrylamide) sizing gels lacking borate. FGP 1 and FGP 2 each migrated as a single band on borate PAGE (data not shown).

Structural analysis of the glycopeptides. — The carbohydrate composition of FGP 1 and FGP 2 (Table I) confirmed that both samples are glycopeptides. The ratio of fucose:xylose:mannose (1:1:3) is the same for both FGPs and other plant glycoprotein lectins[6,9,10].

TABLE I

Neutral carbohydrate composition of FGPs from WFA

Fluoresceinated glycopeptide	*Molar ratio[a] of methyl per-O-trimethylsilyl-*		
	Fucoside	*Xyloside*	*Mannoside*
FGP 1	0.81	0.91	3.00
FGP 2	0.74	1.00	3.00
FGP 1-M	0.78	0.89	2.00
FGP 1-2M	0.83	0.95	1.00
FGP 1-F	<0.05	0.89	3.00

[a] Expressed as values relative to methyl 2,3,4,6-tetra-*O*-(trimethylsilyl)-α-D-mannopyranoside.

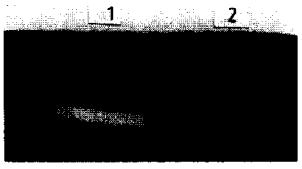

Fig. 2. Composite PAGE electrophoretogram of jack bean α-D-mannosidase-treated FGP 1 (lane 1), and untreated FGP 1 (lane 2). The incubation was performed for 20 min at 37°.

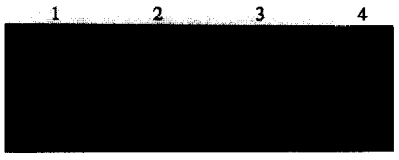

Fig. 3. Composite PAGE electrophoretogram of FGPs: Lane 1, FGP 1 after digestion with jack bean α-D-mannosidase for 18 h at 37° (FGP 1-2M); lane 2, purified FGP 1-M; lane 3, purified FGP 1; and lane 4, purified FGP 1-F.

Sequence and anomeric information was obtained by sequential exoglycosidase digestion of the FGPs. Exoglycosidase treatment of FGPs, followed by PAGE in sizing gels allowed for the determination of the number of neutral sugar residues removed by the enzyme. Poretz and Pieczenik[12] demonstrated that the logarithm of the change in electrophoretic mobility of an FGP is proportional to the number of sugars removed. The fluorescent bands arising from α-D-mannosidase degradation (20 min) of FGP 1, as shown in Fig. 2, are: Undigested FGP 1 (same mobility as the original band); partially digested FGP 1 (change in mobility that corresponds to the loss of one sugar residue, FGP 1-M); and the limit digest of FGP 1 (change in mobility indicated the removal of two sugar units, FGP 1-2M). Complete α-D-mannosidase digestion (18 h) of FGP 1 produced only a single fluorescent band with a mobility change corresponding to the loss of two mannose units (Fig. 3, lane 1). Also shown in Fig. 3 are the fluorescent bands corresponding to purified FGP 1 and FGP 1-M. These results indicated the presence of two nonreducing terminal α-D-mannosyl groups, or an α-linked α-D-mannobiosyl group. FGP 2 exhibited the same susceptibility to jack bean α-D-mannosidase (data not shown).

In order to confirm the identity of the sugar units removed by α-D-mannosidase, carbohydrate compositional analysis (Table I) was performed on the purified digestion products. A decrease in the amount of mannose correlated with the number of monosaccharide units removed by α-D-mannosidase as determined by PAGE. Fucose and xylose were detected in amounts similar to those in the untreated FGPs. The FGPs derived from WFA were not susceptible to the following glycosidases: *Aspergillus niger* β-D-xylosidase, bovine kidney α-L-fucosidase, and jack bean *N*-acetyl-β-D-glucosaminidase. Considering the substrate specificity of these enzymes and assuming that xylose and fucose were present as non-reducing, terminal sugar units, the results suggested

TABLE II

Methylation analysis of FGPs from WFA

O-Acetyl-O-methylalditol	*Molar ratio*[a]		
	FGP 1	*FGP 2*	*FGP 1-F*
1,5-Di-*O*-acetyl-2,3,4-tri-*O*-methylxylitol	0.77	0.50	0.69
1,5-Di-*O*-acetyl-2,3,4-tri-*O*-methylfucitol	0.91	0.89	<0.05
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol	2.00	2.00	2.00
1,2,3,5,6-Penta-*O*-acetyl-4-*O*-methylmannitol	1.12	1.20	1.07
1,4,5-Tri-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-2-(methyl-acetamido)glucitol	0.88	0.89	1.81
1,3,4,5-Tetra-*O*-acetyl-2-deoxy-6-*O*-methyl-2-(methyl-acetamido)glucitol	0.46	0.51	<0.05

[a] Expressed as values relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol.

that: (a) The D-xylose residue is not β-(1→4)-linked[21]; (b) the L-fucosyl group is not α-(1→6)-linked[22]; and (c) 2-acetamido-2-deoxy-D-glucose is not present as a non-reducing, terminal group[23].

Methylation analyses of FGPs 1 and 2 (Table II) showed that the FGPs contain two nonreducing, terminal mannosyl groups (di-*O*-acetyl-tetra-*O*-methylmannitol) and confirmed the results of α-D-mannosidase digestion. The other terminal sugar groups are one each of xylose and fucose (both di-*O*-acetyltri-*O*-methylalditol). Molar ratios of less than 1 were obtained for these two sugars, as reported by others. Fournet *et al.*[9] explained the lower values for the pentose and the deoxyhexose by the high volatility of their methylated derivatives. According to Ashford *et al.*[10], the loss was due to destruction during hydrolysis. A combination of both factors may account for our low values. The occurence of 1,2,3,5,6-penta-*O*-acetyl-4-*O*-methylmannitol indicated the presence of a single, internal D-mannopyranosyl residue substituted at O-2, -3, and -6. This substitution pattern suggested that the monomethylmannitol was derived from the β-D-mannosyl residue of the trimannosyl core unit of an *N*-linked glycan. Numerous structural studies of the glycan component of plant glycoproteins have shown that the β-D-mannopyranosyl residue is often substituted at O-3 and -6 by two α-D-mannopyranosyl groups[1], the remaining substituent being the (1→2)-linked D-xylopyranosyl group. The identification of 1,3,4,5-tetra-*O*-acetyl-2-deoxy-6-*O*-methyl-2-(*N*-methyl-acetamido)glucitol indicated that one 2-acetamido-2-deoxy-D-glucose unit is substituted at both O-3 and -4. 1,4,5-Tri-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)glucitol arose from the single internal 2-acetamido-2-deoxy-D-glucose unit of the pentasaccharide core di-*N*-acetylchitobiose unit. Though the molar ratios for both *O*-acetyl-*O*-methyl-2-(*N*-methylacetamido)glucitols were lower than expected, no other methylated 2-amino-2-deoxyhexose derivatives were detected. The high stability of the 2-acetamido-2-deoxy-D-glucose–asparagine linkage towards hydrolysis may be responsible for the low yields[9]. In addition, the fluoresceination of the peptide may decrease the susceptibility of the oligosaccharide to hydrolysis.

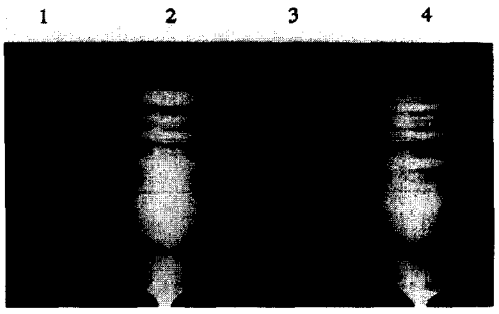

Fig. 4. PAGE electrophoretogram of the fluoresceination mixture of unmodified and chemically treated WFA glycopeptides: Lane 1, purified FGP 1; lane 2, unmodified glycopeptides (arrow denotes FGP 1); lane 3, purified FGP 2; and lane 4, trifluoroacetic acid-treated glycopeptides (arrow denotes new band with a change in mobility corresponding to one hexose unit compared to FGP 1).

The location of the L-fucosyl group was determined by removing it by mild acid hydrolysis. To overcome the apparent lability of FGPs to mild acid, and to allow the use of this condition for removal of fucose, mild acid hydrolysis was performed on the total glycopeptide fraction of the Pronase-digested lectin. After fluoresceination, the treated FGPs were resolved by PAGE (Fig. 4). It is apparent that the acid-treated sample lacked the fluorescent band with a mobility corresponding to the original FGP 1, but possessed a new band with a mobility difference corresponding to the loss of one hexose residue. The modified FGP was purified by preparative PAGE. Fig. 3 (lane 4) shows the electrophoretically purified FGP obtained after mild acid hydrolysis.

Evidence supporting the selective removal of fucose from the glycopeptide was obtained by carbohydrate (Table I) and methylation (Table II) analysis. The carbohydrate composition of this glycopeptide was identical to that of FGP 1, except for the loss of fucose. The methylation data also showed the absence of a fucose derivative and 1,3,4,5-tetra-*O*-acetyl-2-deoxy-6-*O*-methyl-2-(*N*-methylacetamido)glucitol, both of which present in the intact glycopeptides. The concomitant appearance of a residue of 1,4,5-tri-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)glucitol established that the fucosyl group is linked to O-3 of a 2-acetamido-2-deoxyglucose unit.

The results of parallel studies indicated that the glycans of FGP 1 and 2 are identical. The amino acid composition of the FGPs (Table III) showed that the components of the sequence, Asn-X-Ser/Thr, required for peptide *N*-glycosylation[24], are present in both FGPs. FGP 1 contains two additional amino acyl residues (alanine and serine), which probably account for its slower mobility than FGP 2 in PAGE. Since the results of quantitative and compositional studies indicated that each 28-Kd subunit of WFA contains one glycosylation site, the isolation of two major Pronase glycopeptides is related to the incomplete action of the protease. Exhaustive digestion of glycoproteins by Pronase may yield heterogeneous digestion products[25,26]. Accordingly, the amino acid composition of FGP 2 suggested that the single glycosylation site is Asn-X-Ser, where X is either Glx or Gly.

TABLE III

Amino acid composition of FGPs from WFA

Amino acid	*Molar ratio*[a]	
	FGP 1	*FGP 2*
Asx	1.00	1.00
Glx	0.92	1.04
Ser	1.78	0.81
Gly	1.89	1.88
Ala	1.06	0.05

[a] Expressed as values relative to Asx. Amino acids with quantities equivalant to <0.05 residue are taken as absent.

Pronase digestion, followed by PAGE purification of FGPs[12], may be the technique to be preferred over alternative methods to produce pure samples for complex carbohydrate analysis. The widely-used hydrazinolysis procedure has been reported to give low yields owing to the formation of unusual degradation products. For example, this method resulted in the loss of the α-(1→3)-linked L-fucosyl group and in degradation of the reducing, terminal 2-acetamido-2-deoxy-D-glucose unit from bromelain[27] and *Caesalpinia pulcherrima* protease-inhibitor oligosaccharides[7].

Based upon the chemical and immunochemical data presented herein (see below), and in analogy to the structures of fucose- and xylose-containing plant glycoproteins reported earlier[4–10], it is clear that each 28 000-mol. wt. polypetide of WFA[11] possesses a single glycan having structure **1**.

α-D-Man*p*-(1→6)-[β-D-Xyl*p*-(1→2)]-[α-D-Man*p*-(1→3)]-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-[(α-D-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-(1→*N*)

1

Binding specificity of the anti-WFM IgG. — To define the carbohydrate epitope for antibody recognition, the binding capacity of modified and intact FGPs to anti-WFM IgG was examined. Binding profiles (Fig. 5) were obtained by measuring the percentage of added FGP fluorescence remaining in solution after the addition of fixed amounts of specific IgG and *S. aureus*, as compared to controls[3] with normal rabbit IgG. Unmodified FGP 1 and FGP 2, which contain identical glycans, exhibited the same binding ability. Removal of one or two α-D-mannopyranosyl groups from FGP 1 resulted in a loss of ~45% of the affinity of the glycopeptide or binding capacity of the antibody (or both), when compared to the intact FGP. In contrast, FGP 1 lacking fucose (FGP 1-F) was unable to bind to the antibody. These data indicated that the presence of the (1→3)-linked α-L-fucopyranosyl group in the glycopeptide is crucial for binding to the total antibody population, and that a terminal α-D-mannopyranosyl

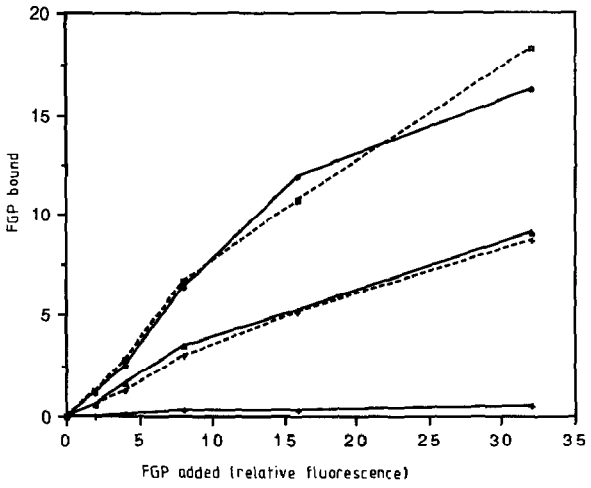

Fig. 5. Binding profiles of FGPs to the IgG fraction of anti-WFM sera: (--□--□--) FGP 1, (—●—●—) FGP 2, (—▲—▲—) FGP 1-M, (--+--+--) FGP 1-2M, and (—+—+—) FGP 1-F.

group contributes to the binding to at least a subpopulation of antibody. Earlier work[3] regarding the specificity of the anti-WFM carbohydrate antibody showed that (a) the D-xylosyl group of glycopeptides, similar in structure to the glycan of WFA, does not contribute to the binding of FGPs to the antibody and (b) the nonreducing, terminal (1→3)-linked α-D-mannopyranosyl group is not part of the epitope. These earlier results and those in the present study demonstrated that (a) the anti-carbohydrate antibody is directed principally toward the (1→3)-linked α-L-fucopyranosyl group and (b) though a part of the epitope, the (1→6)-linked α-D-mannopyranosyl group is, in itself, not sufficient for interaction with the antibody.

The production of rat monoclonal and other rabbit polyclonal anti-glycan antibodies against plant glycoproteins containing complex glycans having xylose/fucose components has been reported[28,29]. Although the epitopes of the corresponding antigens have not yet been determined, crossreactivities with oligosaccharides and glycoproteins of known glycan structures suggested that they require the (1→3)-linked α-L-fucopyranosyl or (1→2)-linked β-D-xylopyranosyl group (or both). Recently, Laurière *et al.*[30] reported that a polyclonal antibody raised against carrot cell-wall β-D-fructosidase is directed towards the (1→2)-linked β-D-xylopyranosyl group of *N*-glycans. The development of an antibody that requires the (1→3)-linked α-L-fucopyranosyl and not the β-D-xylopyranosyl group in the epitope, as reported herein, and those immunochemical reagents that interact with the D-xylosyl groups, should prove useful analytical tools for the identification and characterization of specific plant glycoproteins, as well as probes to monitor biosynthesis, processing, and subcellular localization of oligosaccharides containing these epitopes.

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