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Characterization of a Xylose-Specific Antiserum That Reacts with the Complex Asparagine-Linked Glycans of Extracellular and Vacuolar Glycoproteins[1]

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ABSTRACT

Antibodies were raised against carrot (*Daucus carota*) cell wall β-fructosidase that was either in a native configuration (this serum is called anti-βF_1) or chemically deglycosylated (anti-βF_2). The two antisera had completely different specificities when tested by immunoblotting. The anti-βF_1 serum reacted with β-fructosidase and many other carrot cell wall proteins as well as with many proteins in extracts of bean (*Phaseolus vulgaris*) cotyledons and tobacco (*Nicotiana tabacum*) seeds. It did not react with chemically deglycosylated β-fructosidase. The anti-βF_1 serum also reacted with the bean vacuolar protein, phytohemagglutinin, but not with deglycosylated phytohemagglutinin. The anti-βF_2 serum reacted with both normal and deglycosylated β-fructosidase but not with other proteins. These results indicate that the βF_2 antibodies recognize the β-fructosidase polypeptide, while the βF_1 antibodies recognize glycan sidechains common to many glycoproteins. We used immunoadsorption on glycoprotein-Sepharose columns and hapten inhibition of immunoblot reactions to characterize the nature of the antigenic site. Antibody binding activity was found to be associated with $Man_3(Xyl)(GlcNAc)_2Fuc$, $Man_3(Xyl)(GlcNAc)_2$, and $Man(Xyl)(GlcNAc)_2$ glycans, but not with $Man_3(GlcNAc)_2$. Treatment of phytohemagglutinin, a glycoprotein with a $Man_3(Xyl)(GlcNAc)_2Fuc$ glycan, with *Charonia lampas* β-xylosidase (after treatment with jack-bean α-mannosidase) greatly diminished the binding between the antibodies and phytohemagglutinin. We conclude, therefore, that the antibodies bind primarily to the xyloseβ,1→2mannose structure commonly found in the complex glycans of plant glycoproteins.

Antibodies are used extensively as tools to study the biosynthesis and subcellular location of proteins. Interest in the biosynthesis and function of the carbohydrate moiety of glycoproteins has increased the need for antiglycan antisera, while the increased use of glycoproteins as immunogens has heightened the awareness that antisera against glycoproteins often contain complex mixtures of antibodies. Some of these antibodies may be directed at polypeptide domains of the immunogens, while others may bind to the glycans. Plant antiglycan antibodies have been described for asparagine-linked (14), O-linked (22), and arabinogalactan (10) oligosaccharides. Immunizations with pure native or denature plant glycoproteins quite often produce immune sera that react on immunoblots not only with the glycoprotein injected, but also with other, apparently unrelated proteins. Treatment of these blots with periodate usually abolishes the nonspecific interactions with other proteins, leading to the conclusion that the carbohydrate moiety of these glycoproteins is responsible for these unexpected reactions. Because of our interest in glycoprotein biosynthesis and transport (4), we have produced and characterized an anticomplex-glycan serum.

The present report describes the characterization of a polyclonal antiserum raised against carrot cell wall β-fructosidase, a glycoprotein that has high-mannose and complex oligosaccharide sidechains. Analysis of the antibodies, which bind to many plant proteins on immunoblots, shows that they react specifically with the complex Asn-linked oligosaccharides that contain β,1→2 linked xylose residues. Such a carbohydrate structure is widespread among plant glycoproteins (1, 9, 24, 25, 27, and others). Surprisingly, the antiserum contained no antipolypeptide antibodies. Similarities in the antiglycan specificities of antisera raised against other proteins carrying identical or related glycans indicate that xylose-containing glycans are good immunogens.

MATERIALS AND METHODS

Materials

Carrot cells, *Daucus carota* (line WOOIC obtained from R. Sung, University of California, Berkeley) were grown in suspension culture in Murashige-Skoog medium (20) supplemented with 0.1 mg/L 2,4-D at 26°C. Seeds of *Nicotiana tabacum* (var Xanthi) and *Phaseolus vulgaris* (cv Greensleeves) were harvested in our greenhouse. Extracts from cultured mouse macrophages and from yeast (*Saccharomyces cerevisiae*) were gifts from B. Tague (this department). Unless otherwise stated, organic chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). The prestained, mol wt standards were from the Bethesda Research Laboratories (Bethesda,

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MD). α-Mannosidase was obtained from Sigma Chemical Co. and *Charonia lampas* β-xylosidase from Seikagaku Kogyo (Tokyo, Japan). Phaseolin and PHA[6] were extracted from mature cotyledons of *P. vulgaris* and purified as previously described (2, 8). PHA-L was obtained from Vector Laboratories (Burlingame, CA).

Protein Purification

Carrot cell wall β-fructosidase (EC 3.2.1.26) was purified from carrot cell suspension cultures. Cell walls were prepared by homogenization of carrot cells in water and by repeated (5 times) resuspension and sedimentation at 1,000*g* for 10 min. The cell walls were extracted with 5 volumes of 1 M NaCl for 18 h at 0°C. The salt extract was recovered by centrifugation at 13,000*g* for 15 min and dialyzed overnight at 4°C against 100 mM Na-acetate (pH 5.6), containing 100 mM NaCl. The dialyzed solution was centrifuged again at 13,000*g* for 15 min and chromatographed on carboxymethyl Sephadex (CM Sephadex C 50, Pharmacia) equilibrated in the same buffer. The bound proteins were eluted with a linear gradient from 0.1 to 0.6 M NaCl in 100 mM Na acetate (pH 5.6). The fractions with β-fructosidase activity were successively chromatographed on a Sephacryl S200 (Pharmacia) column (1.5 cm × 2 m) in 100 mM Na-acetate (pH 5.6), containing 1 M NaCl, and passed over a 2 mL column of DEAE cellulose (DE 52 Whatman) in 10 mM Na phosphate (pH 7.0). The active fractions were dialyzed and bound to an octyl agarose column (0.7 × 18 cm) in 50 mM Na-acetate (pH 5.0) with $(NH_4)_2SO_4$ at 40% saturation. Pure β-fructosidase was eluted in the same buffer with a decreasing linear gradient of $(NH_4)_2SO_4$ from 40 to 10% of saturation. The β-fructosidase activity was monitored at each step and the purity of each fraction was checked by SDS-PAGE. The fractions with the highest activity were pooled, then concentrated and dialyzed by ultrafiltration through a PM 10 membrane (Amicon Corp., Lexington, MA).

Isolation of Glycopeptides

Pineapple stem bromelain (3.3 g, Behring Diagnostics, La Jolla, CA) was dissolved in 50 mL H_2O and cleared by centrifugation. The clear brown supernatant was diluted with 50 mL 10% TCA and put on ice for 30 min. The protein was collected by centrifugation and washed twice with 5% TCA, and twice with 80% acetone and was dried under vacuum. The greyish powder was suspended in 20 mL 100 mM Tris/HCl (pH 8.0), containing 5 mM $CaCl_2$ and heated in a boiling waterbath for 5 min. After cooling, the protein suspension was digested with Pronase (Behring Diagnostics, La Jolla, CA) (3 × 40 mg, 24 h per aliquot, 37°C, toluene atmosphere). The glycopeptide was purified by Biogel P4 (minus 400 mesh, Bio-Rad Laboratories, Richmond, CA) chromatography as described by Sturm *et al.* (24). The fractions were assayed for neutral sugar according to Hodge and Hofreiter (12). Some of the purified bromelain glycopeptide was digested with jackbean α-mannosidase and the resulting glycopeptide was isolated by Biogel P4 chromatography. $Man_3(GlcNAc)_2Asn$ was isolated from human transferrin (Sigma) as described by Gleeson and Schachter (11).

Analytical Methods

For immunoaffinity chromatography, phaseolin and phytohemagglutinin were coupled separately to cyanogen bromide-activated Sepharose CL-4B (Pharmacia) according to the specifications of the manufacturer. The antiserum was tumbled with the Sepharose beads overnight at 4°C and bound IgG was eluted with 0.2 M glycine-HCl (pH 2.3), containing 0.5 M NaCl. Enzymic removal of the high-mannose glycans with endo-β-*N*-acetylglucosaminidase H (EC 3.2.1.96) was performed according to Trimble and Maley (26). Deglycosylation with TFMS was carried out according to Edge *et al.* (6). Polypeptides were separated by SDS-PAGE as described by King and Laemmli (15) and transferred to Trans-Blot (Bio-Rad) nitrocellulose paper. The free polypeptide binding sites on the nitrocellulose sheet were blocked with 3% gelatin in Tris-buffered saline (TBS; 20 mM Tris/HCl [pH 7.5], 500 mM NaCl) by gently agitating the solution on a shaker platform for 1 h at room temperature. The sheet was transferred to the first antibody solution (anti-βF_1 or anti-βF_2) containing the serum (diluted 1:2500 or 1:1000, respectively) with 1% gelatin in TBS, and gently agitated for an additional 2 h. For the hapten competition experiments, the first antibody solutions in 1% gelatin in TBS (same dilutions as described above) were incubated for 3 h with or without 5 mM purified glycopeptide prior to the incubation of the nitrocellulose sheets (22). The free and nonspecifically bound antibodies were removed by three 10-min washes in 0.05% Tween 20 in TBS followed by 10-min wash in TBS. The nitrocellulose sheet was then incbuated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) diluted 1:1000 with 1% gelatin TBS, followed by four washes as described above. The horseradish peroxidase color reaction was carried out in a fresh solution of 15 mg 4-chloro-1-napthol (Bio-Rad) in 5 mL methanol mixed with a solution of 15 μl H_2O_2 in 25 ml TBS. The color reaction was stopped by rinsing the nitrocellulose sheet with water.

For analysis of crude protein extracts, the biological material was extracted directly at 100°C in the denaturing sample buffer (4% SDS, 4% β-mercaptoethanol, 15% glycerol, and 10 mM Tris [pH 6.8]) and centrifuged at 10,000*g* for 5 min. Protein was quantitated by a modification of the Coomassie binding assay (21).

Antisera

Antibodies to cell wall β-fructosidase, PHA, and phaseolin were raised in rabbits by injecting the purified proteins in their native or chemically deglycosylated (TFMS) form. Deglycosylated proteins were solubilized in 0.1% SDS in TBS prior to immunization. For the first injection, 300 μg of protein in complete Freund adjuvant were injected in the lymph nodes followed by subsequent subcutaneous injections (every 2 weeks) of the same amount of protein in incomplete

[6] Abbreviations: PHA, phytohemagglutinin; anti-βF_1, anti-native β-fructosidase antiserum; anti-βF_2, anti-deglycosylated β-fructosidase antiserum; endoH, endo-β-*N*-acetylglucosaminidase H; PHA-L, PHA-leucoagglutinating; TFMS, trifluoromethanesulfonic acid.

adjuvant. Blood samples were taken at 2 week intervals. The third bleedings were used for all experiments.

Digestion with β-Xylosidase and α-Mannosidase

PHA (1 μg in 5 μL) was denatured by heating for 3 min at 100°C in the presence of 0.5% SDS. This solution was diluted to 50 μL containing 0.3% Triton X-100, 10 mM sodium acetate (pH 5.8), 5 mM $ZnSO_4$, and 0.25 unit of jack-bean α-mannosidase. The mixture was incubated for 24 h at 37°C under a toluene atmosphere. The pH was then changed by adding 6 μL of 1 M citrate-phosphate (pH 4.0), and two 5 μL aliquots of 0.4 m unit of β-xylosidase were added at 24 h intervals as the mixture incubated at 37°C under a toluene atmosphere. SDS-PAGE and immunoblots were run using 0.1 volume of the reaction mixture (100 ng of PHA). Controls lacking α-mannosidase and/or β-xylosidase were included in the experiment.

RESULTS

Characteristics of Two Different Antisera

The cell wall β-fructosidase from suspension-cultured carrot (*Daucus carota*) cells was purified to homogeneity, and the purity of the preparation was checked by SDS-PAGE. The preparation used for the immunizations contained a single polypeptide band with M_r 63,000 (18). Two kinds of immune sera were prepared in rabbits with this pure enzyme as antigen: anti-βF_1 was obtained by injecting the native enzyme and anti-βF_2 by injecting the denatured enzyme after deglycosylation with TFMS. After three injections of the antigen, anti-βF_2 contained precipitating antibodies. We obtained a single precipitin band by double immunodiffusion with 2 to 5 μg of β-fructosidase and 20 μl of anti-βF_2 antiserum, but only after the enzyme was denatured by boiling in 0.1% (w/v) SDS and 1% (w/v) Triton X-100. Under the same conditions, no precipitin band was observed between anti-βF_1 and either the native or the denatured β-fructosidase. The two immune sera were tested by immunoblotting after SDS-PAGE against protein extracts of bean (*Phaseolus vulgaris*) cotyledons, tobacco (*Nicotiana tabacum*) seeds and carrot cell walls. A single polypeptide band with an M_r expected for β-fructosidase (18) was detected in the carrot cell wall extract with anti-βF_2, but there was no cross-reactivity with tobacco seed or bean cotyledon proteins (Fig. 1, right panel). Anti-βF_1 reacted with numerous proteins in all three extracts (Fig. 1, left panel), as well as with extracts from other plants (data not shown). However, no reaction was observed with crude protein extracts from yeast (*Saccharomyces cerevisiae*) or mouse macrophages (data not shown). The preimmune sera of the rabbits used for immunization reacted with none of the protein extracts (data not shown).

Anti-βF_1 has Antibodies Against the Complex Glycan of β-Fructosidase

To find out if the extensive cross-reactivity of anti-βF_1 was due to antiglycan antibodies, we determined its reactivity on immunoblots with purified β-fructosidase and with PHA, a vacuolar bean protein (4), after treatment of these glycoproteins with endoglycosidase H (endo H; to remove high-mannose glycans) or TFMS (to remove all glycans). The results (Fig. 2) show that anti-βF_1 reacts with the endoH-treated proteins, but not with the deglycosylated proteins. Treatment of parallel blots with anti-βF_2 and with antiserum to deglycosylated PHA showed that chemical deglycosylation of the proteins did not result in the degradation of the polypeptides. Together, these experiments allow us to conclude that the anti-βF_2 serum recognizes the β-fructosidase polypeptide, while the anti-βF_1 serum recognizes its complex glycan. Although the structures of the complex glycans of β-fructosidase and PHA have not been determined, the composition of the glycan of PHA (30) and a partial structural determination (31) indicate that PHA is likely to have a typical $Man_3(Xyl)(GlcNAc)_2Fuc$ structure. Such complex asparagine-linked glycans have been found in many glycoproteins. They differ from the asparagine-linked glycans of mammalian glycoproteins by the presence of a β,1→2 xylose residue and fucose-linked α,1→3 instead of α,1→6 (17). The antibodies in anti-βF_1 also bind to pineapple stem bromelain (data not shown), a glycoprotein with a $Man_2(Xyl)(GlcNAc)_2Fuc$ side-chain (27). The cross-reactivity of anti-βF_1 with many other proteins is probably caused by the presence of such complex glycans on these proteins in the extracts that we tested.

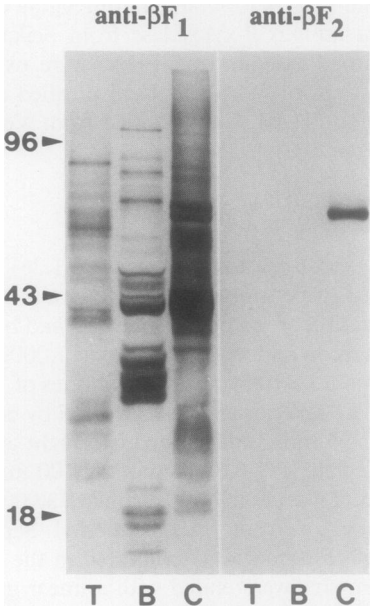

Figure 1. Analysis of the specificities of two immune sera against native β-fructosidase (anti-βF_1) and against deglycosylated β-fructosidase (anti-βF_2) by immunoblotting after SDS-PAGE. Twenty μg protein from tobacco seeds (T), bean cotyledons (B), and carrot cell walls (C) were loaded per lane. Relative mol wt in kilodaltons are indicated by arrows.

The conclusion concerning the reason for the cross-reactivity of antiβF_1 is confirmed and extended by the analysis of the immunoreaction between phaseolin and anti-βF_1. Phaseolin is also a vacuolar bean glycoprotein. It is composed of four polypeptides, A, B, C, and D (3, 24; Fig. 2, where A and B are not separated). A and C each have two high-mannose

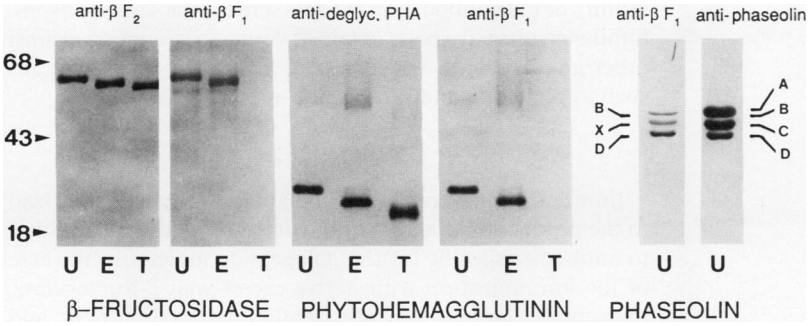

Figure 2. Correlation between the state of glycosylation of β-fructosidase, phytohemagglutinin, and phaseolin and the reactivity of these proteins with various antisera (indicated above panels). Partially purified β-fructosidase from carrot cell walls and purified phytohemagglutinin-L and phaseolin from cotyledons of *P. vulgaris* were subjected to SDS-PAGE as untreated (U), EndoH-treated (E), or TFMS-deglycosylated (T) samples. (Untreated phaseolin was included in the analysis because its complex glycans have xylose but, in contrast to phytohemagglutinin, no fucose [see refs. 24 and 31].) After electrotransfer, the blots were immunostained with anti-βF_1 or with the indicated anti-polypeptide immune sera. A, B, C, D on the far right panel identify the four polypeptides of phaselin (see ref. 24); X refers to an unknown polypeptide with at least one complex glycan. Relative mol wt in kilodaltons are indicated by arrows.

glycans, while B and D have one complex glycan (24). Anti-βF_1 reacts strongly with B and D but not with A and C (Fig. 2). In addition, anti-βF_1 reacts with a polypeptide (labeled X) which could be a hitherto undetected glycoform of phaseolin with one high-mannose and one complex glycan. The complex glycans of PHA and phaseolin both contain xylose $\beta,1\rightarrow 2$ linked to the proximal core mannose residue, but differ by the presence in PHA of a fucose residue. Such a fucose residue is not present on the complex glycan of phaseolin (24). The cross-reactivity with phaseolin, therefore, indicates that fucose is not essential for immunoreactivity, but it does not exclude the possibility that the polyclonal serum contains antibodies directed against the fucose residues of the complex glycans of β-fructosidase (18).

Binding Requires Xylose, but Not Fucose

To determine whether anti-βF_1 contains antibodies that react with carbohydrate structures involving fucose, or with other antigenic determinants, the serum was adsorbed to phaseolin or PHA immobilized on Sepharose 4B. The assay on immunoblots of the bound and unbound fractions of the antiserum with a crude protein extract from carrot cells shows that all the reacting antibodies were adsorbed to the phaseolin-Sepharose (Fig. 3). The parallel experiment with PHA-Sepharose gave exactly the same result (data not shown). This means that the $\alpha,1\rightarrow 3$ linked fucose was not essential for retention of the antibodies on the column.

Further characterization of the anti-βF_1 serum was carried out in competition experiments utilizing haptens that were prepared by extensive pronase digestion of glycoproteins, followed by the purification of glycopeptides consisting of a glycan attached to an asparagine residue. We isolated the complex glycan of pineapple stem bromelain, $Man_3(Xyl)(GlcNAc)_2(Fuc)Asn$, and treated it exhaustively with jack-bean α-mannosidase to generate $Man(Xyl)(GlcNAc)_2(Fuc)Asn$. In addition, we prepared a glycopeptide from human transferrin that has the structure $Man_3(GlcNAc)_2$. These glycopeptides were included in the primary antibody solution during development of the blots. The results (Fig. 4) clearly show that the pineapple stem bromelain glycopeptide, before or after α-mannosidase digestion, competed well against the immobilized protein antigens. On the other hand,

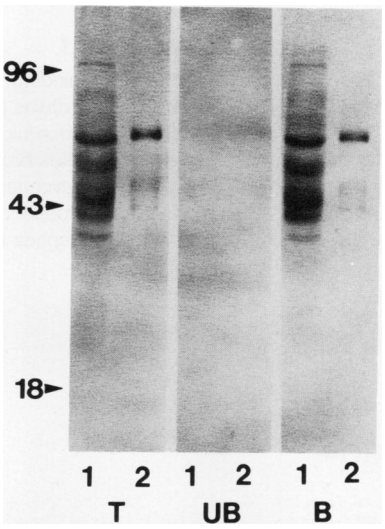

Figure 3. Comparison of the specificities of antibodies in the anti-βF_1 immune serum before and after affinity purification on a phaseolin-Sepharose column. Twenty μg of total protein from carrot cells per lane (1) or partially purified β-fructosidase (2) were separated by SDS-PAGE, transferred to nitrocellulose, then immunostained using antibodies that bound to the column (B), unbound antiserum (UB), or prepurified, total antiserum (T). Relative mol wt in kilodaltons are indicated by arrows.

$Man_3(GlcNAc)_2Asn$ did not compete. We thus conclude that the $\beta,1\rightarrow 2$ xylose residue found in the complex glycans of pineapple stem bromelain (27), phytohemagglutinin (31), and phaseolin (24) is critical for anti-βF_1 immunoreactivity. Inclusion of β-methylxyloside or xylose at 100 mM with the primary antiserum did not diminish the specific recognition of xylose-containing glycoproteins by this antiserum (data not shown).

Confirmation of this conclusion was obtained by incubation of a sample of PHA-L with α-mannosidase and β-xylosidase and analyzing the reactivity of the digested PHA-L with the antiserum on an immunoblot (Fig. 5). Treatment with α-mannosidase had little or no effect on the binding and subsequent staining (compare lane 2 with lane 1); treatment with β-xylosidase alone also had no effect (compare lane 3 with lane 1), while treatment with the two enzymes together

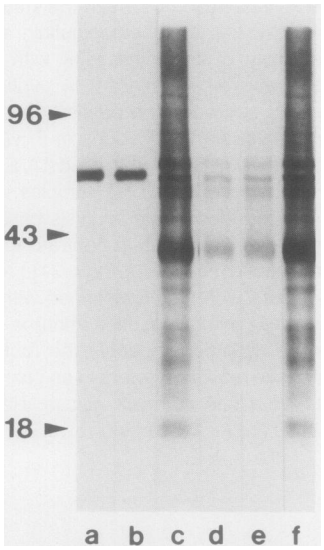

Figure 4. Glycopeptide inhibition of anti-βF_1 binding to carrot cell wall glycoproteins. Twenty μg carrot cell wall proteins per lane were separated by SDS-PAGE and transferred to nitrocellulose. The blots were immunostained with anti-βF_2 (a), anti-βF_2 plus bromelain glycopeptide (b), anti-βF_1 (c), anti-βF_1 plus bromelain glycopeptide (d), anti-βF_1 plus α-mannosidase-treated bromelain glycopeptide (e), and anti-βF_1 plus $Man_3(GlcNAc)_2Asn$. (f). The final glycopeptide concentration was 5 mM.

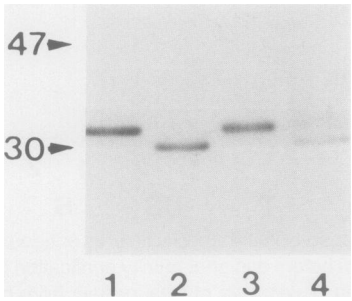

Figure 5. Effect of β-xylosidase treatment of PHA-L on its antigenicity toward anti-βF_1. Each lane contains 100 ng of PHA after preincubation in buffer alone (control; lane 1), after incubation with α-mannosidase (lane 2) or β-xylosidase (lane 3), or after sequential exposure to α-mannosidase and β-xylosidase (lane 4). PHA-L was denatured with SDS, treated with the enzymes, subjected to SDS-PAGE, and transferred to nitrocellulose. The blot was developed with anti-βF_1.

(lane 4) greatly diminished the antibody binding and staining of the antibodies to PHA-L. That the binding was not totally abolished may be due to the relatively ineffectiveness of this *Charonia lampas* β-xylosidase. Only by incubating small amounts of SDS-denatured PHA-L (1 μg) with a relatively large amount of the enzyme were we able to obtain a partial effect.

Antisera Against Other Glycoproteins

Comparison of anti-βF_1 with an immune serum raised against native PHA shows a similar pattern of reactivity on the immunoblot with glycoproteins from carrot and tobacco (Fig. 6). The same proteins reacted with the two immune sera. Differences in staining may reflect normal differences in affinity of the antibodies in the two sera for the same antigens. Similar results have been obtained with antisera raised against other glycoproteins with complex asparagine-linked glycans such as phaseolin and jack-bean α-mannosidase.

DISCUSSION

Immunization of rabbits with plant glycoproteins may lead to the production of anti-carbohydrate antibodies in addition to antibodies specific for the polypeptide moiety. In the case of the immunization with native carrot wall β-fructosidase, the complex glycans were apparently immunodominant, and antibodies that bind specifically to the polypeptide could not be detected. Results from our laboratory on the characterization of the β-fructosidase oligosaccharides (A Sturm, MJ Chrispeels, unpublished data) indicate that this secreted protein has one or two xylose-containing, complex sidechains per polypeptide. The structure of a typical complex glycan, found on many plant glycoproteins (see, for examples, 1, 9, 24, 25, 27), is shown in Figure 7. We suggest that the anti-βF_1 antibodies bind to the xyloseβ,1→2mannose portion of this

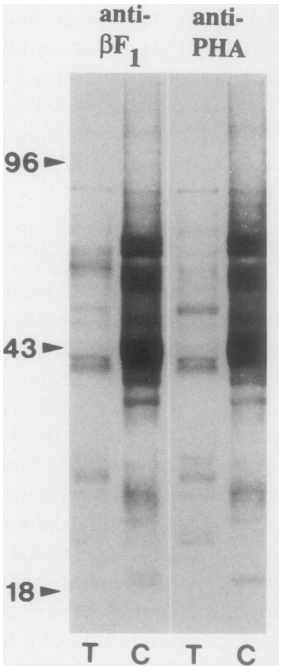

Figure 6. Comparison by immunoblotting of antiglycan antibodies raised against native β-fructosidase (anti-βF_1) and native phytohemagglutinin (anti-PHA) by immunoblotting. Twenty μg protein from tobacco seeds (T) and carrot cell walls (C) were loaded per lane.

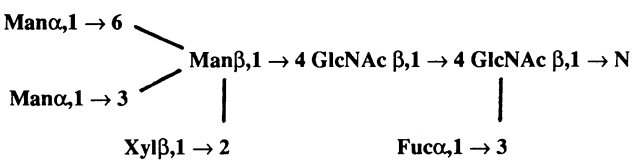

Figure 7. Structure of the typical asparagine-linked complex glycan found on a number of vacuolar glycoproteins.

glycan, and that the β,1→2 xylose residue found on the complex asparagine-linked glycans of plant glycoproteins is very antigenic. We reached this conclusion based on the following evidence. (a) The anti-βF_1 serum does not react with polypeptides that have only high-mannose glycans (phaseolin A and C), and its reactivity is not diminished by treatment of glycoproteins with endoglycosidase H (both PHA and β-fructosidase have at least one high-mannose glycan). (b) The antibodies are retained on affinity columns of phaseolin-Sepharose and PHA-Sepharose. These two proteins have complex glycans with β,1→2 xylose residues, but the glycan of phaseolin does not have an α,1→3 fucose residue on the proximal GlcNAc. (c) Hapten inhibition experiments indicate that the complex glycan shown in Figure 7 is an excellent competitor, while $Man_3GlcNAc_2$ does not compete. The incomplete inhibition of the antibody antigen reaction with 5 mM of complex glycopeptide may indicate that this polyclonal antiserum recognizes more than one epitope on the complex glycan, and that there is a differential affinity for these epitopes. (d) Treatment of the structure shown in Figure 7 with jack-bean α-mannosidase to remove the terminal mannose residues does not alter its activity as a hapten competitor. (e) Treatment of PHA with β-xylosidase plus α-mannosidase greatly diminished the antibody binding to PHA. Mannosidase alone had no effect on immunoreactivity. Incomplete removal of the xylose residues by β-xylosidase may be the cause of the residual antigenicity of PHA. (f) A serum made against native PHA also contains antibodies that react with the same spectrum of glycoproteins in bean and tobacco seed extracts.

The xylose-containing glycans did not completely inhibit the antibody-recognition of the glycoproteins (Fig. 4) suggesting that there may be more than one epitope that is recognized by the antibodies. Some of the antibodies may not be directed at the mannoseβ,1→2xylose epitope. Xylose alone did not have any effect on the recognition reaction, indicating that the epitope is more complex than a single sugar residue.

Several years ago Kaladas *et al.* (14) described an antiserum elicited by the complex glycan of the lectin present in *Wistaria floribunda* seeds. This glycan is similar to the structure shown in Figure 7, except that it lacks the terminal α,1→3 mannose residue. The authors carried out binding experiments with pineapple stem bromelain glycopeptides and found a 57% inhibition of binding after treatment of the glycopeptides with jack bean α-mannosidase, and no inhibition after treatment with *Charonia lampas* β-xylosidase. They concluded that the antibodies were directed primarily against an epitope consisting of a linear array of three mannose residues. On the basis of new evidence, the structure of the glycan of pineapple stem bromelain has been revised (27), and there is no linear array of three mannose residues in this glycan. Their results are clearly different from ours, and it is not clear to which portion of the glycan their antibodies were directed. We have found that anti-complex glycan antibodies are elicited whenever plant glycoproteins with complex glycans are injected into rabbits. While this article was under review, McManus *et al.* (19) published evidence showing that a monoclonal mouse antibody is directed against these small fucose and xylose-containing complex glycans of plant glycoproteins. The β,1→2 xylose residue is absent from the Asn-linked glycans of mammalian cells (17), and the anti-βF_1 serum does not react with mammalian glycoproteins. Some mollusc glycoproteins contain complex asparagine-linked glycans with β,1→2 xylose residues (28, 29), and the anti-βFi1 serum reacts with numerous mollusc proteins (7). The absence of β,1→2 xylose from mammalian proteins may explain why this group is such a good immunogen. Other carbohydrate sidechains not found on mammalian proteins (*e.g.* arabinose sidechains) may be equally good immunogens.

To elicit antipolypeptide antibodies free of antiglycan antibodies, the glycoprotein can be chemically deglycosylated with TFMS prior to immunization. The procedure of Edge *et al.* (6) denatures the protein, and this may alter the immune response. A gentler procedure for chemical deglycosylation has recently become available (23). Antibodies against denatured protein may fail to recognize native protein. This is what we observed with β-fructosidase and has also recently been described for extensin (5). An alternative approach for obtaining antinative polypeptide antibodies free of antiglycan antibodies is to raise antibodies against the native glycoprotein and to absorb the antiglycan antibodies on an unrelated immobilized glycoprotein, such as PHA, phaseolin, or the commercially available pineapple stem bromelain, if the immunogenic glycan is N-linked and contains xylose. (When using stem bromelain as the immunoadsorbent, it is important to totally denature this protease before linking it to Sepharose 4B.) If antibodies specific for the polypeptide chain are elicited as well, this approach can yield a specific antipolypeptide serum fraction. We recently used this technique to purify anti-patatin antibodies. The antiserum, prepared in the laboratory of L. Wilmitzer (Berlin, FRG), contained a mixture of antipolypeptide and anti-complex glycan antibodies. Fractionation on a PHA-Sepharose column resulted in the separation of antiglycan from antipolypeptide antibodies (V Sonnewald, A Sturm, MJ Chrispeels, unpublished data).

Antiglycan sera, like anti-βF_1, are useful in identifying xylose-containing glycans on plant glycoproteins. This determination can be made even if very small amounts of protein are available or if the protein is impure. The antiglycan serum can also be useful in immunocytochemical studies, and to probe pathways of protein transport. For example, it is not known at present whether proteins of the plasma membrane, peroxisomal membrane, or tonoplast pass through the Golgi apparatus. Since the glycoprotein-modifying xylosyltransferase is located in the Golgi complex (13), a positive reaction with this antiserum would indicate that transport of these proteins to their target membranes occurred via the Golgi. The antiglycan serum might also be used to identify mutants in the glycan biosynthetic or glycan modification pathway, as was done with a different antiglycan antibody for the slime mold *Dictyostelium discoideum* (16). As the formation of xylose-containing glycans is dependent on the sequential action of Golgi glycosyltransferases and glycosidases (13), a null mutation in one of these enzymes would give rise to proteins that would not react with this antiserum.

Note Added in Proof

In the meanwhile, the linkage analysis on the complex glycan of PHA has been performed by ^{1}H-NMR. This glycan

has the structure shown in Figure 7 (G Strecker, A Sturm, MJ Chrispeels, unpublished results).

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