

Affinity Purification of Antibodies Specific for Asn-Linked Glycans Containing $\alpha 1 \rightarrow 3$ Fucose or $\beta 1 \rightarrow 2$ Xylose

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Antisera raised against the plant glycoproteins β -fructosidase and horseradish peroxidase can be fractionated on an affinity column of honeybee venom phospholipase A₂ to produce serum fractions that are specific for either the $\alpha 1 \rightarrow 3$ fucose or $\beta 1 \rightarrow 2$ xylose epitopes commonly found on the Asn-linked glycans of plant glycoproteins. This affinity purification strategy relies on the absence of $\beta 1 \rightarrow 2$ xylose from the glycan of the venom protein. Such antibody preparations can be used for the detection of these sugar epitopes on glycoproteins. © 1993 Academic Press, Inc.

The characterization of Asn-linked complex glycans on mammalian glycoproteins has been aided substantially by the ready availability of plant lectins that recognize specific carbohydrate groups (1). Most of these well characterized lectins do not interact with the Asn-linked glycans found on plant glycoproteins. Plant glycoproteins have both typical high mannose glycans and complex Asn-linked glycans that differ substantially from mammalian complex glycans, and are characterized by the presence of an $\alpha 1 \rightarrow 3$ fucose residue on the proximal GlcNAc and a $\beta 1 \rightarrow 2$ xylose residue on the β -linked mannose of the core (for review see 2). Among the plant lectins, only concanavalin A (3) and *Ricinus communis* agglutinin (4) have proven useful for the analysis of plant glycans. The likely explanation for this lack of interaction between most seed lectins and the glycans on plant glycoproteins is that seed lectins are plant defense proteins that have evolved specifically to interact with the glycans and polysaccharides of other organisms. Because lectins have not proven to be particularly useful for the characterization of plant Asn-linked glycans, further progress in plant glycobiology depends on

the availability of other reagents that are specific for the glycans of plant glycoproteins.

When plant glycoproteins are used as immunogens in mammals, they elicit a mixture of antibodies directed at polypeptide epitopes, as well as the unusual glycan epitopes not found on mammalian glycoproteins (5–9). In this paper, we show that immobilized honeybee venom phospholipase A₂ (PA₂)² can be used as an affinity absorbent to further fractionate such sera to produce anti $\alpha 1 \rightarrow 3$ fucose and anti $\beta 1 \rightarrow 2$ xylose serum fractions. This purification strategy relies on the absence of $\beta 1 \rightarrow 2$ xylose residues from the glycan(s) present on honeybee venom PA₂ and the commercial availability of this protein.

MATERIALS AND METHODS

Materials

Phaseolus vulgaris cv. Greensleeves and *Arabidopsis thaliana* plants were grown in our greenhouse. Purified rice α -amylase was a gift from Dr. T. Mitsui (Nigata University, Japan). Chemicals and other purified glycoproteins used as reference were purchased from Sigma.

Homogenization

Seeds and seedlings used for protein analysis were homogenized directly in hot denaturing buffer (20 mM Tris-HCl, pH 6.8, 1% sodium dodecyl sulfate, 0.3% β -mercaptoethanol). The homogenate was boiled for 5 min and centrifuged at 10,000g for 15 min. Proteins were precipitated by the addition of 9 vol of cold acetone (2 h at -20°C). The sedimented proteins were dissolved

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² Abbreviations used: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PHA, phytohemagglutinin; ConA, concanavalin A; HRP, horseradish peroxidase; PA₂, honeybee phospholipase A₂; TBS, Tris-buffered saline; TTBS, Tween 20 containing TBS; PBS, phosphate-buffered saline.

in denaturing buffer (2 min at 100°C) and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

SDS–PAGE, Affinodetection, and Immunodetection

Polypeptides were separated by 15% SDS–PAGE under reducing conditions according to Laemmli (10). The separated polypeptides were electrophoretically transferred onto a nitrocellulose membrane. Affinodetection of glycoproteins on the blot, using the ConA/ peroxidase method, was carried out as previously described (11). Before immunodetection, the free binding sites on nitrocellulose were blocked with 3% gelatin in a 15 mM Tris, pH 7.5, containing 500 mM NaCl buffer (TBS) by gently agitating the solution on a shaker platform for 1 h at room temperature. The sheet was next incubated in the first antibody solution (antiserum diluted 1/500 to 1/1000 in 1% gelatin in TBS) for 1–2 h and washed in 0.1% Tween 20 in TBS (four washes for 10 min each). The membrane was next incubated for 1 h in horseradish peroxidase-conjugated goat anti-rabbit IgG (GAR-HRP from Bio-Rad) diluted 1/1000 in 1% gelatin in TBS, and washed in Tween 20 in TBS as above, followed by a final wash in TBS alone. For both affino- and immunodetection, the horseradish peroxidase color reaction was carried out in a fresh solution of 30 mg of 4-chloro-1-naphthol (Bio-Rad) in 10 ml methanol mixed with 30 μ l H₂O₂ solution, 35% w/v in 50 ml TBS, and stopped by rising in water. After this staining step the blots were immediately photographed and kept dry for storage in darkness.

Antibody Production

The immunization schedule of the rabbits was basically as described in Harboe and Ingild (12). Phospholipase A₂ was dissolved at a concentration of 0.2 mg · ml⁻¹ in PBS. Each injection consisted of 250 μ l antigen solution mixed with the same volume of Freund's incomplete adjuvant except for the first injection in which Freund's complete adjuvant was used.

Antibodies specific for horseradish peroxidase (HRP) were prepared using HRP purified from preparative SDS–PAGE. HRP (500 μ g), from Sigma, was separated from other proteins by SDS–PAGE and then electrophoretically transferred onto a nitrocellulose membrane as described above. HRP was localized on the membrane after staining the blot 1 min in 0.2% Ponceau S in TCA, 3% (w/v). The band of interest was cut from the blot using a razor blade. The piece of nitrocellulose-containing HRP was then destained in TBS, frozen at -80°C, and ground with a mortar and pestle. Nitrocellulose powder containing HRP was then suspended in PBS and stored at -20°C in aliquots containing about 50 μ g of antigen. One aliquot was used for each injection as described above for the production of antibodies specific

for PA₂. Antibodies specific for carrot cell wall β -fructosidase were prepared as described in Laurière *et al.* (8).

Antibody Purification

Fucose- and xylose-specific antibodies were separated on a phospholipase A₂–Sepharose 4B column. The column was prepared by covalent attachment of 2.5 mg of phospholipase A₂ from honeybee venom (Sigma, P9279) to 0.5 g of cyanogen bromide-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. Rabbit serum (0.5 ml) diluted 1.5 ml with 0.1% Tween 20 in TBS (TTBS) was applied to the column and incubated by rotating gently with the gel at 5°C for 12 h. Unbound antibodies were eluted with 3 column vol of TTBS. Then the column was rinsed exhaustively (about 20 column vol) with TTBS until no more protein eluted. Bound antibodies were eluted using 0.2 M glycine, pH 2.2, and collected into 1 M Tris, pH 7.4, to neutralize the eluate as soon as possible.

RESULTS AND DISCUSSION

A number of laboratories have raised antibodies against plant glycoproteins and found that such sera contain primarily antibodies directed against the Asn-linked glycans of these proteins (5–9). Such antisera invariably cross-react with most other plant glycoproteins because the unusual α 1 $\rightarrow$ 3 fucose and β 1 $\rightarrow$ 2 xylose residues are common determinants found on most complex Asn-linked glycans of plant glycoproteins (Fig. 1). Horseradish peroxidase and cell wall carrot β -fructosidase, two of the glycoproteins used to obtain sera, contain both types of residues. The specificity of such antisera has been determined by studying the inhibitory effect of glycans or glycoproteins of known structure on their reactivity with glycoprotein standards (7,13,14). Surprisingly, HRP sera have been found repeatedly to be primarily anti- α 1 $\rightarrow$ 3 fucose while the β -fructosidase serum was primarily anti- β 1 $\rightarrow$ 2 xylose (13,16). An α 1 $\rightarrow$ 3 fucose, specific antiserum was also obtained by Ramirez-Soto and Poretz (9) who injected a rabbit with *Wistaria floribunda* lectin. Although antibodies against one glycan epitope appear to predominate in such sera, antibodies to other glycan epitopes are likely to be present as well. Only a monoclonal antiserum against the complex glycans of HRP contained antibodies to a single glycan epitope (16).

Recent evidence indicates that Asn-linked glycans of insect glycoproteins share common features with both plant and mammalian complex glycans. In particular, PA₂ can have α 1 $\rightarrow$ 6 fucose (typically found on mammalian glycoproteins) and/or α 1 $\rightarrow$ 3 fucose (typically found on plant glycoproteins) on the proximal GlcNAc of its Asn-linked glycans (16). This finding explains our earlier observation that a serum specific for the complex glycans on plant glycoproteins cross-reacts with PA₂

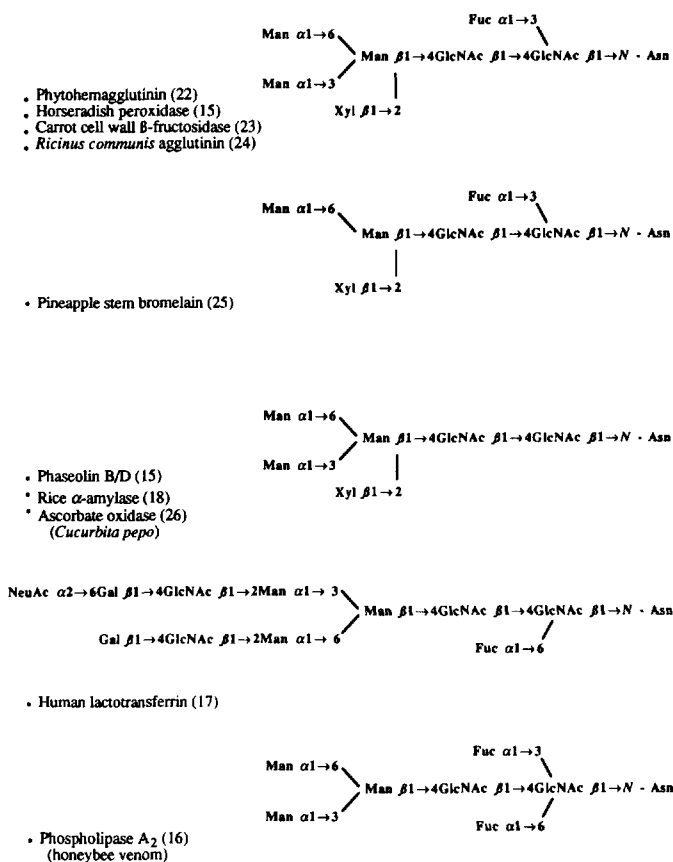


FIG. 1. Structure of the complex Asn-linked glycans of some glycoproteins used as standards in the present study.

(7), and more recent observations that an anti-HRP serum recognizes a specific neural glycan epitope in insects (13,16). The availability of PA₂ from a commercial source and the recent determination of the structure of its glycan (16) encouraged us to fractionate sera against plant glycoproteins on a PA₂ affinity column to produce serum fractions directed at specific sugar epitopes.

In a first experiment, we compared the specificity of an anti-HRP serum with that of an anti-PA₂ serum using a variety of glycoproteins as standards (Fig. 2). The anti-HRP serum (Fig. 2A) detected HRP (lane 3), phytohemagglutinin (PHA) (lane 2), RCA (lane 4), pineapple stem bromelain (lane 5), numerous proteins in a crude bean seed extract (lane 6), especially PHA (bracket), and a polypeptide of the seed storage protein phaseolin (asterisk), rice α -amylase (lane 7), proteins in crude PA₂, especially PA₂ itself (major band in lane 8) and the heavy (66 kDa) subunit of jack-bean α -mannosidase (lane 9). All these proteins, with the exception of α -mannosidase, are known to have $\alpha 1 \rightarrow 3$ fucose on the proximal GlcNAc and/or $\beta 1 \rightarrow 2$ xylose on the β -linked mannose. The serum did not recognize lactotransferrin (lane 1) which has $\alpha 1 \rightarrow 6$ fucose on the proximal GlcNAc (17). The anti-PA₂ serum (Fig. 2B) detected all

the same proteins except the rice α -amylase (lane 7). The phaseolin polypeptide is stained weakly (lane 6). These two proteins, rice α -amylase and the phaseolin polypeptide, both have $\beta 1 \rightarrow 2$ xylose on their Asn-linked glycans and either have no $\alpha 1 \rightarrow 3$ fucose (α -amylase) (18) or little $\alpha 1 \rightarrow 3$ fucose (phaseolin polypeptide) (19, and A. Sturm, personal communication). Anti-PA₂ failed to detect lactotransferrin (lane 1) indicating that the serum has no antibodies against $\alpha 1 \rightarrow 6$ fucose, although that epitope is present in PA₂. Thus, the HRP antiserum appears to contain a mixture of antibodies as it recognizes proteins that have $\alpha 1 \rightarrow 3$ fucose, as well as proteins that lack this epitope, but contain $\beta 1 \rightarrow 2$ xylose. The absence from the PA₂ serum of antibodies that recognize $\beta 1 \rightarrow 2$ xylose is consistent with previously published results (16) showing the absence of this epitope in PA₂ and encouraged us to use PA₂ as an immunoabsorbent to fractionate the HRP serum, as well as a previously prepared β -fructosidase antiserum. This antiserum was shown in earlier work to be largely anti- $\beta 1 \rightarrow 2$ xylose (14).

We fractionated these two sera on a PA₂ affinity column and obtained from the β -fructosidase serum the antibody fraction that passed through the column—this

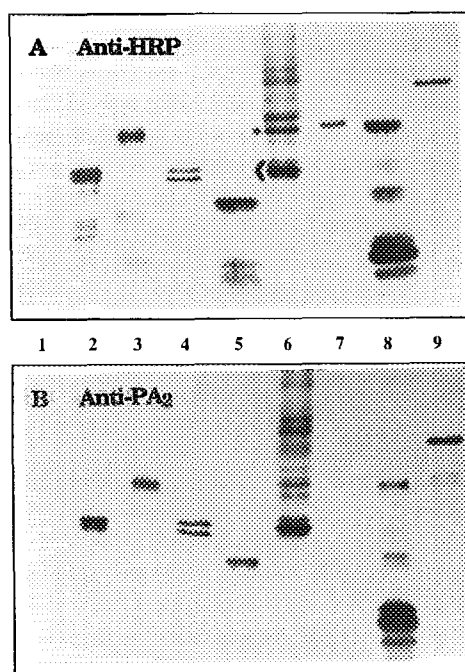


FIG. 2. Immunodetection of glycoproteins with crude antiglycan sera. Immune sera specific for horseradish peroxidase (anti-HRP, A) or honeybee phospholipase A₂ (anti-PA₂, B) were used for immunodetection of glycoproteins separated by SDS-PAGE and transferred onto nitrocellulose. Glycoproteins analyzed here are: lactotransferrin (lane 1), phytohemagglutinin (lane 2), horseradish peroxidase (lane 3), *Ricinus communis* agglutinin (lane 4), bromelain (lane 5), bean seed extract (lane 6), rice α -amylase (lane 7), honeybee venom phospholipase A₂ (lane 8), and α -mannosidase (lane 9).

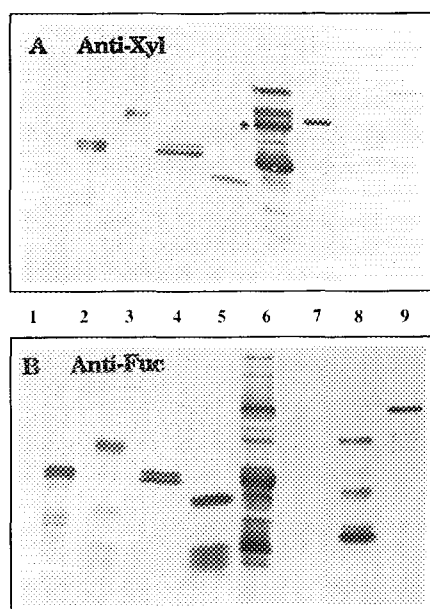


FIG. 3. Immunodetection of glycoproteins with purified anti $\alpha 1 \rightarrow 3$ fucose- and anti $\beta 1 \rightarrow 2$ xylose-antibody fractions. Lactotransferrin (lane 1), phytohemagglutinin (lane 2), horseradish peroxidase (lane 3), *Ricinus communis* agglutinin (lane 4), bromelain (lane 5), bean crude extract (lane 6), rice α -amylase (lane 7), honeybee phospholipase A_2 (lane 8), and α -mannosidase (lane 9) were separated with SDS-PAGE and analyzed on blots with xylose-specific (A) and fucose-specific (B) antibodies. The glycan specific antibodies used here were affinity purified on a PA_2 -Sepharose column from immunosera made against carrot cell wall β -fructosidase (anti-Xyl, A) or horseradish peroxidase (anti-Fuc, B). Antibodies specific for fucose bind, while antibodies specific for xylose do not bind immobilized PA_2 .

fraction is labeled "anti-xylose"—and from the HRP serum, the antibodies that bound to the column and could be eluted at low pH—this fraction is labeled "anti-fucose." The use of the terms anti-fucose and anti-xylose is not meant to imply that the immunoglobulin molecules interact only with the fucose or xylose residues. For example, the anti-fucose antibodies may also interact simultaneously with both the $\alpha 1 \rightarrow 3$ linked fucose and the $1 \rightarrow 6$ linked mannose residues located on the single glycan chain as proposed by Kurosaka *et al.* (13). The same set of proteins shown in Fig. 2 was probed with these two antibody preparations and the results are presented in Figs. 3A and 3B. The results clearly show that the anti-fucose fraction (Fig. 3B) recognized all proteins known to contain $\alpha 1 \rightarrow 3$ fucose on the proximal GlcNAc, while failing to recognize rice α -amylase (lane 7) which has no fucose. The phaseolin polypeptide which has very little fucose is weakly stained (lane 6). Thus, the PA_2 column effectively allowed the anti-xylose antibodies to be removed from the HRP serum, because the complete serum recognized rice α -amylase and phaseolin (Fig. 2A). The antibodies that bound to the PA_2 column and were eluted at low pH constituted the anti-fucose fraction of the HRP serum.

Fractionation of the β -fructosidase antiserum resulted in the retention of a small proportion of the antibodies (data not shown) while the majority passed straight through the column. The fraction that passed through the column recognized all the proteins containing $\beta 1 \rightarrow 2$ xylose, even those that lacked fucose (Fig. 3A). This leads to the conclusion that the fucose epitope does not have to be present for the antibodies to bind to the xylose epitope. The anti-xylose fraction does not recognize PA_2 on blots (Fig. 3A, lane 8) while PA_2 is recognized by the complete serum (7). This result indicates that the β -fructosidase antiserum contains some antibodies that were removed by the procedure. Whether these antibodies are directed at the fucose epitope or at another epitope that is common to the β -fructosidase and the PA_2 glycans remains to be determined.

When plant glycoproteins are used as immunogens in mammals, they can elicit anti-polypeptide as well as anti-glycan antibodies. For the β -fructosidase serum, we showed earlier that it does not contain anti-protein antibodies because there was no reaction with deglycosylated β -fructosidase (8). The absence of anti-polypeptide antibodies from both the anti-fucose and the anti-xylose antibodies was also demonstrated by the sensitivity of the staining shown in Fig. 3 to periodate oxidation. When the blots were treated with periodate

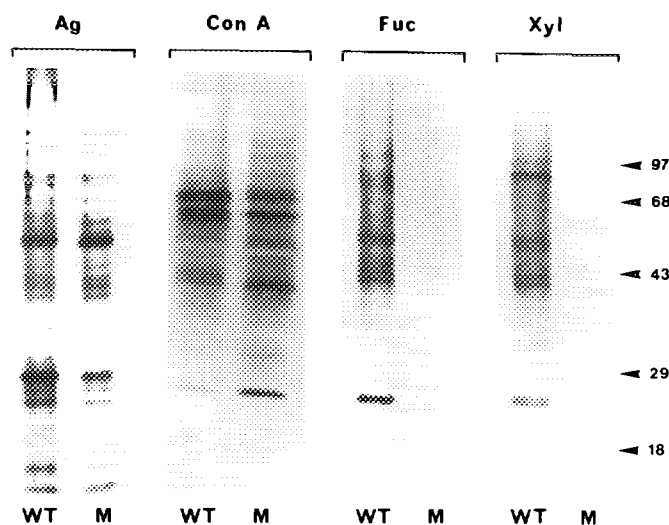


FIG. 4. Detection of glycoproteins with high mannose and complex-type glycans in the extracts from wild-type and mutant *A. thaliana* seedlings. The mutant analyzed here is unable to convert high mannose to complex glycans containing fucose or xylose residues. Proteins extracted from wild-type (WT) or mutant (M) *A. thaliana* seedling extracts were separated by SDS-PAGE and silver stained in the gel (Ag) or transferred to nitrocellulose paper. Glycoproteins with high mannose glycans were visualized by affinity detection with ConA and peroxidase on the blot (ConA). Glycoproteins containing either $\alpha 1 \rightarrow 3$ fucose (Fuc) or $\beta 1 \rightarrow 2$ xylose (Xyl) in their complex glycans were immunodetected on the blot with our fucose- and xylose-specific antibodies. Lanes are loaded for equal amount of protein.

before immunodetection, there was no reaction of the serum with the glycoproteins (data not shown).

Could the purified anti-fucose and anti-xylose sera contain antibodies to high mannose glycans or mannose residues of the plant complex glycan? That possibility was ruled out by testing the reactivity of the anti-fucose and the anti-xylose antibodies toward the glycoproteins of a mutant of *A. thaliana* that is blocked in the conversion of high mannose to complex glycans. Specifically, there is an accumulation of $\text{Man}_5(\text{GlcNAc})_2$ chains indicating the absence of GlcNAc-transferase I (21). The glycoproteins of this mutant contain the normal amount of high mannose (Man_9 , Man_8 , Man_7) glycans (21) and can be readily detected by affindetection with concanavalin A-peroxidase (Fig. 4). However, the glycoproteins do not react with the fucose-specific or the xylose-specific antibodies (Fig. 4). These results indicate the absence of antibodies directed at mannose epitopes.

We believe that the purified antibody fractions will be extremely useful for the further studies of plant glycans, such as the characterization of glycans on individual glycoproteins, the immunocytochemical localization of sugar addition reactions in the Golgi, the characterization of plant mutants that fail to complete the modification of complex glycans, and the effects of various inhibitors (such as monensin for example) on glycan modification.

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