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Common Antigenic Determinants in the Glycoproteins of Plants, Molluscs and Insects

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An antiserum raised against β-fructosidase isolated from the cell walls of suspension-cultured carrot cells cross-reacts with many plant proteins and hemocyanin of *Helix pomatia*. The shared epitope appears to be a small complex glycan with a β(1-2)-linked xylose residue attached to the β-linked mannose residue of the core of an asparagine-linked oligosaccharide. There is strong cross-reactivity with the proteins of many seed plants, molluscs and insects, and no cross-reactivity with the proteins of fungi, algae, mosses, ferns, or any of the vertebrates tested. Xylose-containing glycans appear to increase the immunogenicity of the proteins to which they are attached, and we suggest that they may be responsible for some allergic responses of people that are repeatedly exposed to plant or insect proteins.

Proteins of both plant and animal cells contain *N*-linked as well as *O*-linked glycans that modify the physicochemical properties of the polypeptides. It has become clear in recent years that glycans do not have a single function, and that their role depends on the functions of the proteins to which they are attached and on the manner in which they alter protein properties. Glycans often contribute to the maintenance of protein conformation: they may protect a protein against proteolytic degradation, modify the immunogenicity of proteins, or be involved in protein-protein recognition (lectins). It is well-established that the cell surface glycans of cancer cells differ significantly from those of untransformed cells, and these changes may be related to metastatis. With a few notable exceptions (e.g. the study of the cell wall glycoprotein, extensin), nearly all that we know about the structure of glycans and their biosynthesis is derived from studies of vertebrate glycoproteins (see [1-3] for reviews). Information about the glycans of algae, vascular plants, amoebae, and invertebrates is fragmentary, and an understanding of glycan function must await further studies on these organisms.

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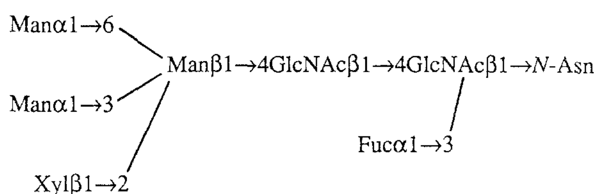

Figure 1. Structure of the xylose-containing glycan commonly found on plant glycoproteins and mollusc hemocyanin.

Asparagine-linked glycans are classified as high-mannose, with 5-9 mannose residues and two *N*-acetylglucosamine residues, or as complex. Complex glycans are derived from high-mannose glycans through a series of processing steps that occur in the Golgi apparatus, and involve glycosidases as well as glycosyltransferases. The complex glycans of mammalian glycoproteins are often large structures with two to six antennae, containing *N*-acetylglucosamine, mannose, galactose, fucose and sialic acid, but no xylose. The complex glycans of plant cells are quite different; the one most commonly found has the composition $Man_3XylFuc(GlcNAc)_2$. The structure of this glycan (Fig. 1) is characterized by a β(1-2)-linked xylose residue on the β-linked mannose of the core oligosaccharide, and an α(1-3)-linked fucose residue on the proximal GlcNAc [4-8]. There is, at present, almost no information about the complex glycans of invertebrates, with the exception of a few recent studies [9-12] on the glycans of hemocyanin of *Helix pomatia*, *Lymnaea stagnalis*, and *Panulirus interruptus*. These glycans are identical to the small complex glycans of plant glycoproteins.

Results obtained in many studies of glycoproteins using immunochemical techniques illustrate that certain glycans are immunogenic in mammals (see [13]). Immunogenic glycans have been found on the glycoproteins of amoebae [14, 15] and plants [16]. Antibodies prepared against a single glycoprotein carrying such a glycan reacted with many other proteins, indicating the presence of a shared epitope.

In the course of our work on secreted glycoproteins, we prepared a polyclonal rabbit antiserum against β-fructosidase purified from a cell wall extract of suspension-cultured carrot (*Daucus carota*) cells [17]. The antiserum against β-fructosidase, a glycoprotein with high-mannose and complex glycans, reacts strongly with endoglycosidase-H treated β-fructosidase, but not at all with chemically deglycosylated enzyme. In addition, we observed that the antiserum reacts with many proteins in a carrot cell extract [18]. These results indicate to us that the antibodies recognize an epitope common to many proteins; since only a few plant proteins are known to have *O*-linked glycans (extensin, potato lectin, and arabinogalactan protein), the common epitope is probably a complex asparagine-linked glycan.

In this paper, we demonstrate that the antiserum raised against β-fructosidase reacts specifically with the β(1-2)-xylose-containing glycans of several plant glycoproteins, and that it cross-reacts with proteins in many seed plants, molluscs, and insects, including hemocyanin of *Helix pomatia*, the glycan of which is known to have a β(1-2)-linked xylose. It appears, therefore, that these xylose-containing glycans which are absent from mammalian glycoproteins have a widespread occurrence in the plant and animal kingdoms.

Experimental Procedures

Materials

Phaseolus vulgaris cv. Greensleeves plants were grown in our greenhouse, and the seeds used as a source of phaseolin and phytohemagglutinin. Animals and plants were collected locally or purchased from a local supplier. All chemicals used were reagent grade or better.

Methods

Homogenization. All tissues were homogenized directly in hot denaturing buffer (20 mM Tris-HCl, pH 8.6, 1% sodium dodecylsulfate, 0.3% β-mercaptoethanol and 10% glycerol). The homogenate was boiled for 5 min and centrifuged at 10 000 × g 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 in denaturing buffer (2 min at 100°C) and separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis, electrotransfer and immunoblotting. The sodium dodecylsulfate-polyacrylamide gel electrophoresis was performed on 15% (w/v) acrylamide gels, and proteins were transferred to Trans-Blot nitrocellulose paper (Bio-Rad, Richmond, CA, USA) as described by Faye and Chrispeels [19]. Visualization of the antigens on the nitrocellulose sheets (immunoblots) was performed as described in the Bio-Rad technical information sheet, using a peroxidase-coupled goat anti-rabbit IgG as the second antibody. Affinoblotting using concanavalin A/peroxidase to detect polymannose glycans and treatment of the blots with jack-bean α-mannosidase was as described [19]. Protein standards (Bethesda Research Labs, Bethesda, MD, USA) used were myosin (M_r 200 000), phosphorylase (M_r 97 400), bovine serum albumin (M_r 68 000), ovalbumin (M_r 43 000), α-chymotrypsinogen (M_r, 25 700), β-lactoglobulin (M_r 18 400), and lysozyme (M_r 14 300).

Antiserum. Antibodies to purified carrot cell wall β-fructosidase were raised in a rabbit by injecting the protein without prior denaturation. The enzyme was purified to homogeneity using standard purification methods [17]. For the first injection, 300 μg of protein were mixed with complete Freund's adjuvant and injected in the lymph nodes. Subsequent injections (every two weeks) were with incomplete adjuvant. Blood samples were taken at two week intervals.

Results

The immune serum used in the present study was obtained by immunizing a rabbit with carrot cell wall β-fractosidase, a glycoprotein with polymannose and complex glycans [17]. When β-fructosidase was treated with endoglycosidase H, there was a decrease in its M_r of about 2 000, but no reduction in the reactivity with the antiserum. Chemical deglycosylation with trifluoromethylsulfonate resulted in a further reduction of the M_r by about 3 000, with complete loss of reactivity towards the antiserum [18].

To use this antiglycan serum as a tool for identification of defined glycan structures and processing events, we further characterized its specificity. Fig. 2 shows an immunoblot

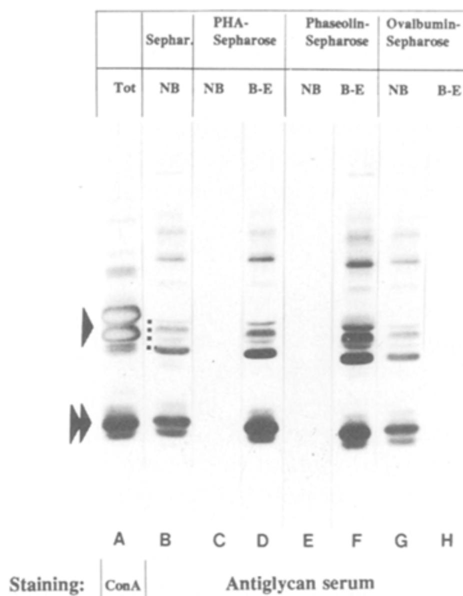

Figure 2. Immunoaffinity absorption of antiglycan antibodies on columns of Sepharose 4B covalently linked to glycoproteins with different glycans. Extracts of bean (*Phaseolus vulgaris*) seeds were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and fixed [24]. The proteins were visualized with the Con A/peroxidase method (high-mannose glycans) (lane A) or with the antiglycan serum (lane B). The seed extracts contain two abundant glycoproteins with different polypeptides closely related in M_r: phaseolin (single arrowhead) and phytohemagglutinin (PHA) (double arrowhead). With respect to phaseolin, the most abundant polypeptides have only high-mannose chains (compare A and B), while four less abundant polypeptides have complex glycans (dots). The antiglycan serum was chromatographed on Sepharose 4B with or without covalently linked glycoproteins into a fraction (NB) which did not bind to the column (lanes B, C, E, G) and a fraction (B-E) which bound and was eluted at low pH (lanes D, F, H). Fractionation on Sepharose 4B without covalently linked glycoproteins; lane B: on Sepharose 4B-phytohemagglutinin (PHA); lanes C and D: on Sepharose 4B-phaseolin; lanes E and F: on Sepharose 4B-ovalbumin; lanes G and H. Note that the antiglycan antibodies are retained by phaseolin or phytohemagglutinin, but not by ovalbumin.

obtained with an extract of developing seeds of the common bean, *Phaseolus vulgaris*. The predominant immunoreactive polypeptides recognized by the antiglycan serum are those of the lectin phytohemagglutinin (double arrowhead), and a group of polypeptides derived from the seed storage protein phaseolin (single arrowhead) (Fig. 2, lane B). Both proteins have high-mannose as well as xylose-containing complex oligosaccharide side-chains. Phytohemagglutinin contains both β(1-2)-linked xylose and α(1-3)-linked fucose [20], while phaseolin has no α(1-3)-linked fucose [21]. Staining of the same extract by the concanavalin A/peroxidase method is shown in Fig. 2, lane A. The two proteins have high-mannose glycans, although with respect to phaseolin, the two abundant polypeptides have only high-mannose glycans, while four non-abundant polypeptides have complex glycans. As illustrated in lanes C and E of Fig. 2, the anti-carbohydrate antibodies are completely absorbed when the serum is passed over a column of either phytohemagglutinin-Sepharose 4B or phaseolin-Sepharose 4B. The antibodies eluted at acidic pH from these immunoadsorbants gave the same pattern on

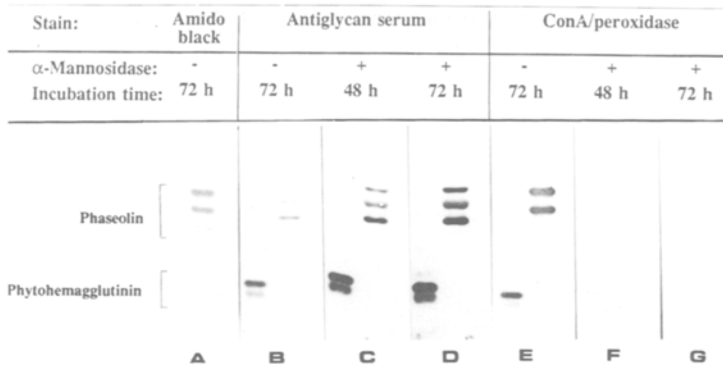

Figure 3. Detection of phytohemagglutinin and phaseolin after digestion of oligosaccharide sidechains with α-mannosidase. Purified phytohemagglutinin polypeptides (left lane on each nitrocellulose piece) and phaseolin polypeptides (right lane) were separated by sodium dodecylsufate-polyacrylamide gel electrophoresis, transferred to nitrocellulose and fixed [24]. The sheet was cut in strips and each strip was incubated for 48 h or 72 h at 37°C in a sealed plastic bag with buffer alone (lanes A, B, E) or in the presence of jack-bean α-mannosidase (10 units of activity) (lanes C, D, F, G) as described [24]. After this treatment, proteins were stained with amidoblack (lane A) and glycoproteins visualized with the Con A/peroxidase method (lanes E, F, G) or immunodetected with the antiglycan serum (and a second antibody coupled to peroxidase (lanes B, C, D). Lanes C and F were incubated 48 h and lanes A, B, D, E and G were incubated 72 h. Note that α-mannosidase incubation abolishes the Con A/peroxidase staining, but enhances the antiglycan staining.

immunoblots as an equivalent amount of the serum chromatographed on Sepharose 4B without covalently attached proteins (compare lane B to lanes D and F). As a control, when used under the same conditions, an ovalbumin-Sepharose column did not retain the antibodies (Fig. 2, lane G), and no antibodies could be eluted at acidic pH (lane H). These results demonstrate that the oligosaccharide structure, $Man_3(Xyl)(GlcNAc)_2$, on phaseolin is sufficient for binding of these antiglycan antibodies.

To test further the specificity of the antiserum towards glycan structures, we digested the glycans with jack-bean α-mannosidase after transfer of the proteins to nitrocellulose membranes [19]. The nitrocellulose membranes trated in this way were probed with the antiglycan antiserum (Fig. 3, lanes B, C, D) or with concanavalin A/peroxidase (Fig. 3, lanes E, F, G) to detect high-mannose glycans. Treatment of the blot with α-mannosidase for 72 h completely abolished the signal from phytohemagglutinin or phaseolin with Con A/peroxidase (Fig. 3, lane G, compared to the control in lane E). The same treatment enhanced the signal from the antiglycan serum (compare the control in lane B to lane D). These same antibodies did not bind to human transferrin which had been treated sequentially with sialidase, α-galactosidase, β-*N*-acetylglucosaminidase, and α-mannosidase (data not shown), suggesting that a $Man_1(GlcNAc)_2$ glycan is not sufficient for recognition. The antibodies do bind to pineapple stem bromelain which has a $XylMan_2GlcNAc_2$ glycan [7]. Together, these data indicate that the presence of a β(1-2)-linked xylose residue is crucial for the binding of the antibodies, and that binding increases when the terminal mannose residues are removed.

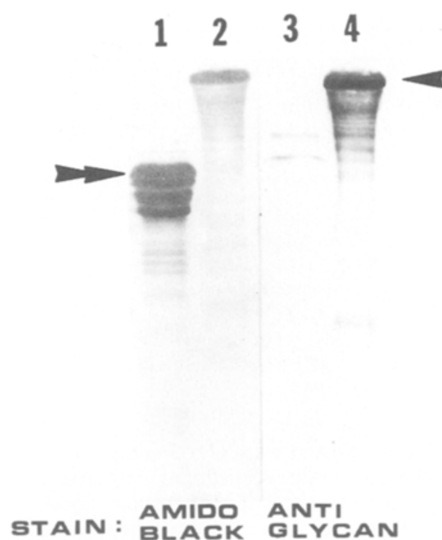

Figure 4. Immunodetection of *Helix pomatia* hemocyanin with the antiglycan serum. Hemolymph from *Panulirus interruptus* (lanes 1 and 3) and semi-purified hemocyanin from *H. pomatia* (lanes 2 and 4) were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Proteins were stained with amidoblack (lanes 1 and 2) or immunodetected with the antiglycan serum and a second antibody coupled to peroxidase (lanes 3 and 4). Arrowheads point to hemocyanins.

Recent studies [10, 11] on the glycans of *L. stagnalis* and *H. pomatia* hemocyanin have shown that the *N*-linked complex glycans of these mollusc glycoproteins are identical to those of plant glycoproteins. Hemocyanin of the arthroprod *P. interruptus* is also a glycoprotein, but its glycans do not have xylose [9, 12]. We used our antiglycan serum for an immunoblot of *P. interruptus* hemolymph (Fig. 4, lane 3) and semi-purified hemocyanin from *H. pomatia* (Fig. 4, lane 4). A comparison with the amido black stained lanes 1 and 2 shows that hemocyanin from *H. pomatia* (single arrowhead) gave a very strong signal, while hemocyanin from *P. interruptus* (double arrowhead) gave no signal. *P. interruptus* hemolymph contains a few other polypeptides that react with the antiglycan serum (faint bands in lane 3). The results show that the serum cross-reacts with animal glycoproteins that have glycans similar to those found on plant glycoproteins.

We tested for cross-reactivity with a wide variety of organisms (Table 1) and found cross-reactivity with many seed plants (Gymnosperms, Monocotyledons, and Dicotyledons) and invertebrates, but not with vertebrates, fungi, or algae (Table 1). Some of the positive results are shown in Fig. 5. Numerous bands were obtained with extracts of the honeybee (*Apis mellifera*) (lane 1) and the bumblebee (*Bombus sp.*) (lane 2), while a lesser number of bands was obtained with other arthropods. The specificity of this reaction was demonstrated by mild periodate oxidation prior to immunostaining [22] and with a pre-immune serum. We checked the cross-reactivity with two common sources of allergens: venom from the honeybee and *Dactylus glomerata* pollen (Fig. 6). In each case, we found very strong cross-reactivity. Phospholipase A, a glycoprotein from honeybee venom, gave a very strong reaction (arrowhead).

Table 1. Phylogenic distribution of glycoproteins with xylose-containing glycans. Extracts were made in the sodium dodecylsulfate-containing extraction buffer and assayed by the standard immunoblot technique after polyacrylamide gel electrophoresis. The presence of a strong signal from a single polypeptide resulted in a positive score.

			Reactivity
Bacteria		*Escherichia coli*	–
Fungi		*Aspergillus niger*	–
		Candida utilis	–
		Rhizopus	–
		Rhodotorulla	–
		Penicillium	–
		Saccharomyces cerevisiae	–
	Basidiomycota		
		Agaricus campestris	–
	Acresiales		
		Dictyostelium discoideum	–
Algae	*Phaeophyta*		
		Fucus serratus	–
		Fucus vesiculosus	–
	Rhodophyta		
		Chondrus crispus	–
		Polysiphonia violacea	–
	Chlorophyta		
		Enteromorpha intestinalis	–
		Ulva lactuca	–
Lichens		*Lobaria pulmonaria*	–
		Usnea ceratina	–
Bryophyta		*Atrichum undulatum*	–
		Isothecium myosuroides	–
		Polytrichum commune	–
		Equisetum sylvaticum	–
Pteridophyta		*Polystichum spinulosum*	–
		Polypodium vulgare	–
Gymnospermae		*Pinus torreyana*	+
Angiospermae		*Pinus laricio*	+
Monocotyledons		*Allium sativum*	+
		Dactylis glomerata	+
		Lolium perenne	+
		Triticum aestivum	+
Dicotyledons		*Acer pseudoplatanus*	+
		Beta vulgaris	+
		Brassica oleracea	+
		Daucus carota	+
		Fagus silvatica	+
		Lactuca sativa	+
		Malus domestica	+
		Nicotiana tabacum	+
		Phaseolus vulgaris	+
		Raphanus sativus	+
		Ribes nigrum	+

Porifera		*Halicondria panicea*	+
		Hymenicidon perleve	—
Cnidaria		*Actinia equina*	+
Annelida		*Nereis diversicolor*	—
		Lumbricus terrestris	+
Mollusca		*Arion rufus* (hemolymph)	+
		Helix pomatia (hemolymph)	+
		Lepidochitona cinereus	+
		Littorina littorea (hemolymph)	+
		Mytilus californicus	+/—
		Mytilus edulis	+
		Nucella lapillus	+
		Ocenelra erinacea	+
		Octopus vulgaris	+
		Patella vulgata (hemolymph)	+
Arthropoda	Arachnida		
		Araneus diadematus	+
		Aranea (grasshopper)	+
	Crustacea		
		Balanus balanoides	+
		Carcinus moenas (hemolymph)	+
		Eupagurus bernardus	+/—
		Panulirus interruptus (hemolymph)	+
	Insecta		
		Apis mellifera	+
		Bombus sp.	+
		Periplaneta americana	+
		Drosophila melanogaster	+
		Formica sp.	+
		Murgantia histrionica	+
		Phyllophaga sp.	+
		Lepisma saccharina	+
Echinodermata		*Asterias rubens*	—
Vertebrata	*Osteichthyes*		
		Conger conger	—
		Salmo gairdnerii irideus	—
		Solea solea	—
	Amphibia		
		Rana ridibunda (blood)	—
		Triturus alpestris	—
		Xenopus leavis	—
	Reptila		
		Pseudomys floridana	—
	Aves		
		Gallus domesticus (muscle)	—
		Columba livia domestica (muscle)	—
	Mammalia		
		Mus musculus (blood)	—
		Oryctolagus cuniculus (blood)	—
		Bos domesticus (muscle)	—

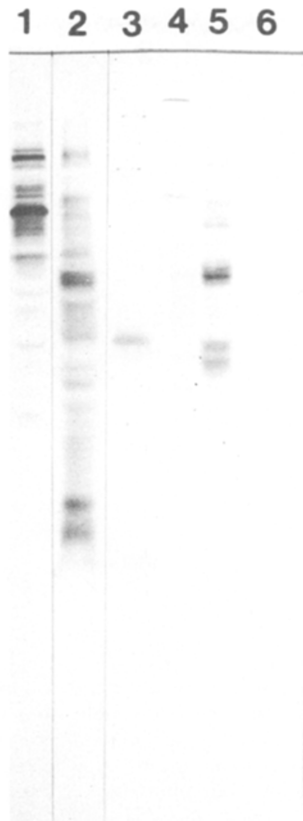

Figure 5. Visualization of insect glycoproteins with the antiglycan serum. Proteins extracted from insects and arachnida were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose sheet which was incubated with antiglycan serum for immunodetection. Each lane was loaded with approximately 100 μg of proteins from honeybee (*Apis mellifera*, Linn.) (lane 1), bumblebee (*Bombus sp.*) (lane 2), grasshopper (*Orthoptera*) (lane 3), June bug (*Phyllophaga sp.*) (lane 4), Harlequin Cabbage bug (*Murgantia histrionica*) (lane 5), and Spider (*Araneae*) (lane 6). No polypeptides were visible when pre-immune serum was used or when another nitrocellulose sheet was treated with periodate to oxidize the glycans [27] prior to immunodetection.

Discussion

The results presented in this paper show that a number of proteins present in many seed plants and invertebrates share a common epitope. This epitope consists of the small xylose-containing asparagine-linked glycan recently identified on a number of plant and a few mollusc glycoproteins (Fig. 1). This glycan is quite immunogenic, as shown by the fact that the antiserum against β-fructosidase contained no antibodies against the polypeptide. Injection of chemically deglycosylated β-fructosidase did result in a serum that was specific for the polypeptide moiety of this glycoprotein [17].

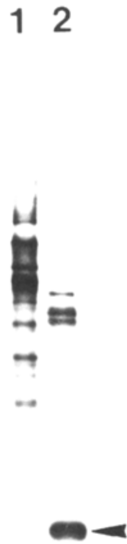

Figure 6. Visualization of *Dactylus glomerata* pollen extract and *Apis mellifera* venom glycoproteins with the antiglycan antibodies. Proteins from pollen and bee venom were separated by sodium dodecylsulfate-polyarylamide gel electrophoresis and transferred to a nitrocellulose sheet. Subsequent immunodetection on the sheet was performed using the antiglycan serum. Arrowhead indicates position of phospholipase A, a major allergen of honeybee venom. Controls for immunodetection specificity are the same as described for Fig. 5.

We have made antisera against phytohemagglutinin, phaseolin, and α-mannosidase; these are all glycoproteins that have xylose-containing glycans, and the antisera were always nonspecific (unpublished results). Similar results were obtained by Kaladas *et al.* [16] using *Wistaria floribunda* lectin as an antigen. This lectin also has a small xylose-containing complex glycan [4]. The immunogenicity of the complex glycan is further demonstrated by experiments in which monoclonal antibodies were made against another extracellular carrot glycoprotein (gP57). Of 40 hybridomas tested, 39 produced antibodies against the complex glycan of gP57 and only one hybridoma produced antibodies against a polypeptide epitope (S. Satoh, private communication).

The lack of cross-reactivity with vertebrate proteins may help explain why these glycans are so immunogenic in mammals. Many plant glycoproteins have both high-mannose and complex glycans, and results from our laboratory show that glycans which are modified in the Golgi are readily accessible to the modifying enzymes (glycosidases and glycosyltransferases), while glycans that remain in the high-mannose form are not

accessible [23, 24]. We assume that accessibility means that the glycans are displayed on the surface of the proteins, and are readily seen by the immunized mammals as foreign groups. Thus, these glycans increase the immunogenicity of the proteins to which they are attached.

The presence of xylose-containing glycans in invertebrates and their abundance in *Hymenoptera* shows that Golgi-based glycan modification systems must exist in these animals, contrary to earlier suggestions [25, 26]. The absence of such glycans from lower plants, algae, and fungi may indicate that these groups have only high-mannose glycans or that they have complex glycans without xylose.

Of particular interest is the strong reactivity with pollen protein and honeybee venom phospholipase A. The complex glycan of phospholipase A is specifically recognized as an epitope by IgE antibodies in the sera of allergic individuals [27, 28]. We suggest that these xylose-containing glycans may be a common determinant among glycoprotein allergens.

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