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Structural analysis of N-glycans from allergenic grass, ragweed and tree pollens: Core α1,3-linked fucose and xylose present in all pollens examined

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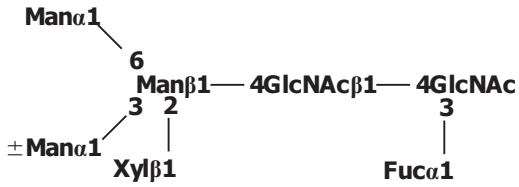
The N-glycans from soluble extracts of ten pollens were examined. The pyridylaminated oligosaccharides derived from these sources were subject to gel filtration and reverse-phase HPLC, in conjunction with exoglycosidase digests, and in some cases matrix-assisted laser desorption-ionisation mass spectrometry. In comparison to known structures, it was possible to determine the major structures of the N-glycans derived from Kentucky blue grass (*Poa pratensis*), rye (*Secale cerale*), ryegrass (*Lolium perenne*), short ragweed (*Ambrosia elatior*), giant ragweed (*Ambrosia trifida*), birch (*Betula alba*), hornbeam (*Carpinus betulus*), horse chestnut (*Aesculus hippocastanum*), olive (*Olea europaea*) and snake-skin pine (*Pinus leucodermis*) pollen extracts. For grass pollens the major glycans detected were identical in properties to:



Grass pollens also contained some minor structures with one or two non-reducing terminal *N*-acetylglucosamine residues. In the ragweed pollens, the major structures carried core α1,3-linked fucose with or without the presence of xylose. In tree pollen extracts, the major structures were either xylosylated, with or without fucose and terminal *N*-acetylglucosamine residues, with also significant amounts of oligomannose structures. These results are compatible with the hypothesis that the carbohydrate structures are another potential source of immunological cross-reaction between different plant allergens.

***Keywords*: N-glycans, pollens**

***Abbreviations:* MALDI-MS, matrix-assisted laser desorption-ionisation mass spectrometry; peptide: N-glycosidase A, peptide-*N*^{4}-(*N*-acetyl-β-glucosaminyl)asparagine amidase from almonds; peptide: N-glycosidase F, peptide-*N*^{4}-(*N*-acetyl-β-glucosaminyl)asparagine amidase from *Flavobacterium meningosepticum.* For an explanation of abbreviations for N-glycans, see Figure 5.**

Introduction

For many years, carbohydrate-dependent cross-reactions have been hypothesized to be responsible for some of the allergenic cross-reactions observed for IgE derived from patients allergic to plant pollen, food or arthropod materials [1]. The presence of carbohydrate IgE epitopes has been tested by examining loss of IgE binding after periodate oxidation or chemical or enzymatic deglycosylation or the retention of binding after protease digestion (e.g., refs. 2–4). In some recent studies, control glycoproteins with known N-glycan structures have been tested against patients' sera (e.g., refs. 3–5). It has also been recently reported that the carbohydrate of ryegrass (*Lolium perenne*) allergen *Lol p* XI is a cross-reactive epitope [4], that serum from a patient allergic to grass pollens and tomatoes has IgE cross-reacting with a bromelain glycopeptide cross-linked to bovine serum albumin [6], that Bermuda grass BG60 allergen has

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periodate-sensitive IgE epitopes [7] and that a glycopeptide from olive (*Olea europeae*) *Ole e* I is an IgE epitope [8]. Previous work from this laboratory has demonstrated that many patients allergic to honey bee venom have IgE that binds to the glycopeptide of bee venom phospholipase A_2(*Api m* I) and to the glycopeptide of bromelain [9,10]. There are also a number of reports of rabbit IgG polyclonal antibodies that recognise plant glycans [11–14], as well as one rat monoclonal YZ1/2.23 [15,16], thus indicating the immunogenicity of these glycans. Cross-reaction of anti-horseradish peroxidase with *Drosophila* neural tissue has been reported [11,17] and a report that anti-bee venom phospholipase A_2 cross-reacts with bromelain and horseradish peroxidase [18] added to indications that the presence of core α1,3-linked fucose, common to both plant and insect glycans, is the source of carbohydrate-based plant-insect cross-reactivity.

Grass, ragweed and tree pollens are major allergens and often are associated with cross-reactive food allergy [19], but no work has been published on any oligosaccharide structures from such sources, except for the recent data on the Japanese cedar (*Cryptomeria japonica*) *Cry j* I allergen and Bermuda grass BG60 antigen. *Cry j* I carries glycans with xylose and core α1,3-linked fucose, the smallest (and most abundant) being GnGnXF^3 and the largest having two terminal galactose and two outer arm α1,6-linked fucose residues [14], while BG60 contains mainly oligosaccharides with core α1,3-linked fucose, but no xylose [20]. Otherwise, only compositional analysis and studies on binding to concanavalin A have been performed on some grass pollen glycoproteins. For instance, 'Glycoprotein 2' from ryegrass (*Lolium perenne*) pollen was found to contain mannose, fucose, xylose and *N*-acetylglucosamine, consistent with the type of N-glycans described here for ryegrass, as well as arabinose and galactose whose presence is suggestive of plant-type O-glycans, although 'Glycoprotein 1' described in the same study did not contain xylose and fucose [21].

As part of a series of studies on the antibody binding activities and carbohydrate structures of pollen and food extracts, the reactivity of a number of pollens of allergological interest with anti-horseradish peroxidase and the 'anti-core α1,3-linked fucose' monoclonal YZ1/2.23 has been studied in this laboratory and it was concluded that all these pollens probably contained N-glycans with xylose and/or core α1,3-linked fucose [16]. Subsequently, as reported here, the structures of N-glycans from the complete soluble extracts of ten grass, ragweed and tree pollen samples were examined after fluorescent-labeling. The major pyridylaminated N-glycans from these extracts were compared with known glycans from horseradish peroxidase, bromelain, zucchini ascorbate oxidase and bee venom phospholipase. A 'two-dimensional' HPLC method in conjunction with exoglycosidase digestions was used in this comparison and allowed the determination of essentially all of the N-glycans present in the samples. Additionally, where sample quantity allowed, MALDI-MS analysis of various fractions corroborated the chromatographic data. The structural data verified that the previously determined antibody reactivity was indeed explicable by the presence of glycans containing xylose and fucose residues.

Materials and methods

Materials

Kentucky blue grass (smooth meadow grass, *Poa pratensis*), rye (*Secale cerale*), short ragweed (*Ambrosia elatior*), giant ragweed (*Ambrosia trifida*), birch (*Betula alba*) and olive (*Olea europaea*) pollens were purchased from Sigma and extracted, by the method of Ipsen and Løwenstein, overnight with 0.125 M ammonium bicarbonate pH 8.3 at 5 °C [22]. Pollen from snake-skin pine (*Pinus leucodermis*) was collected by Haralt Leiter and Thomas Dalik of this laboratory from a tree in a local park and subsequently extracted as above. Lyophilized extracts of pollens from ryegrass (*Lolium perenne*), horse chestnut (*Aesculus hippocastanum*), sweet chestnut (*Castanea sativa*) and hornbeam (*Carpinus betulus*) were the kind gift of Professor Christof Ebner (Allgemeines Krankenhaus der Stadt Wien) and had been extracted by the method of Hirschwehr *et al.* [23]. Pollen extracts were analyzed for amino sugars by an HPLC method [24]. Almond peptide:N-glycosidase A was obtained from Boehringer Mannheim. Pyridylaminated oligosaccharides of known structure from previous studies and derived from horseradish peroxidase, bromelain, *Aspergillus* α-amylase, soybean 7S glycoprotein, zucchini ascorbate oxidase (fraction 1) and bee venom phospholipase A_2 (fraction 5a) were used as standards [25–27]. The trimannosyl core MM structure had been previously generated from bovine asialofibrin pyridylamino-oligosaccharides by β-galactosidase and β-hexosaminidase digestion. Other materials were purchased from Merck or Sigma.

Preparation of fluorescently-labelled N-glycans

Pollen extracts (10–20 nmol in terms of GlcNAc) were digested overnight at 37 °C with pepsin (100 μg) in 5% (v/v) formic acid (200 μl). After drying and re-evaporation with methanol, the digests were taken up in 5% aqueous ammonia (50 μl) and reduced by addition of an equal volume of 1% (w/v) sodium borohydride. After addition of acetic acid, evaporation and re-evaporation, 20 μl of citrate-phosphate pH 5.0 buffer was added and the pepsin digest incubated at 95 °C for three minutes. The digests were then treated with peptide:N-glycosidase A (37 °C, two days) and subject to pyridylamination by the method of Hase *et al.* [28].

Size fractionation and reverse-phase HPLC

After crude gel filtration to remove excess 2-aminopyridine, pyridylaminated oligosaccharides were fractionated by

a 'two dimensional' mapping technique. Chromatography in the first dimension was according to size on a Micropak AX-5 column (0.4 x 30 cm), based on the method of Tomiya *et al.* [29] with a Shimadzu pump, system controller and fluorimeter at a flow rate of 1 ml/min. The starting buffer was 65 : 35 (v/v) acetonitrile/3% (w/v) acetic acid-triethylamine buffer pH 7.3 and a gradient increasing at 2% per min of 50:50 (v/v) acetonitrile/3% (w/v) acetic acid-triethylamine buffer pH 7.3 was applied. Peaks were collected and dried thoroughly prior to sub-fractionation in the second dimension by reverse-phase chromatography on a 5 μ Hypersil ODS column (0.4 x 25 cm, ÖFZ Seibersdorf), based on a previously published method [25], at a flow rate of 1.5 ml/min. The starting buffer was 0.1 M ammonium acetate, pH 4.0, and a gradient increasing at 1% per min 30% (v/v) methanol was applied. Columns were calibrated daily in terms of glucose units with a pyridylaminated partial dextran hydrolysate (3–10 glucose units). Peaks from either size fractionation or reverse-phase chromatographies were subject to exo- or endoglycosidase digestions at 37 °C as follows: *Canavalia ensiformis* (jack bean) α-mannosidase ('medium' dose; 25 mU in 20 μl 50 mM sodium acetate, 0.1 mM zinc chloride, pH 4.2); *Canavalia ensiformis* β-hexosaminidase (5 mU in 20 μl 0.1 M sodium citrate, pH 5.0); *Streptomyces plicatus* endoglycosidase H (2 mU in 20 μl, 0.1M citrate-phosphate, pH 5.0).

Matrix-assisted laser desorption-ionisation mass spectrometry (MALDI-TOF)

For mass spectrometry, collected fractions were lyophilized and reconstituted in 20 μl water yielding concentrations of pyridylaminoglycans of 0.2–0.8 pmol per μl for the major glycan species. Some of the samples (pine, hornbeam and olive pollens) contained considerable amounts of salt which were partially removed by membrane-dialysis using a 0.025 nm pore-size membrane (Millipore) floating on water. Generally, 2 μl of a sample was mixed with 1 μl of matrix (2% 2,5-dihydroxybenzoic acid in water containing 30% acetonitrile and 0.5 mM NaCl). Aliquots of 1 μl were applied to a flat sample platen and dried immediately under mild vacuum. MALDI-MS spectra were acquired on a DYNAMO (Thermo BioAnalysis, Hemel Hempstead, UK) linear time-of-flight mass spectrometer capable of 'dynamic extraction,' a synonym for delayed extraction. The instrument was operated with a dynamic extraction setting of 0.1. External mass calibration was performed with pyridylaminated N-glycans derived from bovine fibrin by sequential exoglycosidase digestions (see above), i.e., $Gal_2GlcNAc_2Man_3GlcNAc_2$ ($[M+Na]^+$ = 1742.6 Da), $GlcNAc_2Man_3GlcNAc_2$ (GnGn-PA, $[M+Na]^+$ = 1418.3 Da) and $Man_3GlcNAc_2$ (MM-PA, $[M+Na]^+$ = 1011.9 Da). Fifteen to twenty individual laser shots were summed.

Results

The size fractionation profiles for the pyridylaminated oligosaccharides derived from three grass, two ragweed and five tree pollens are given in Figures 1 and 2. The 'void' fluorescent peaks were not considered to be derived from N-glycans due to their small molecular mass and so were subsequently ignored. Each peak collected from size frac-

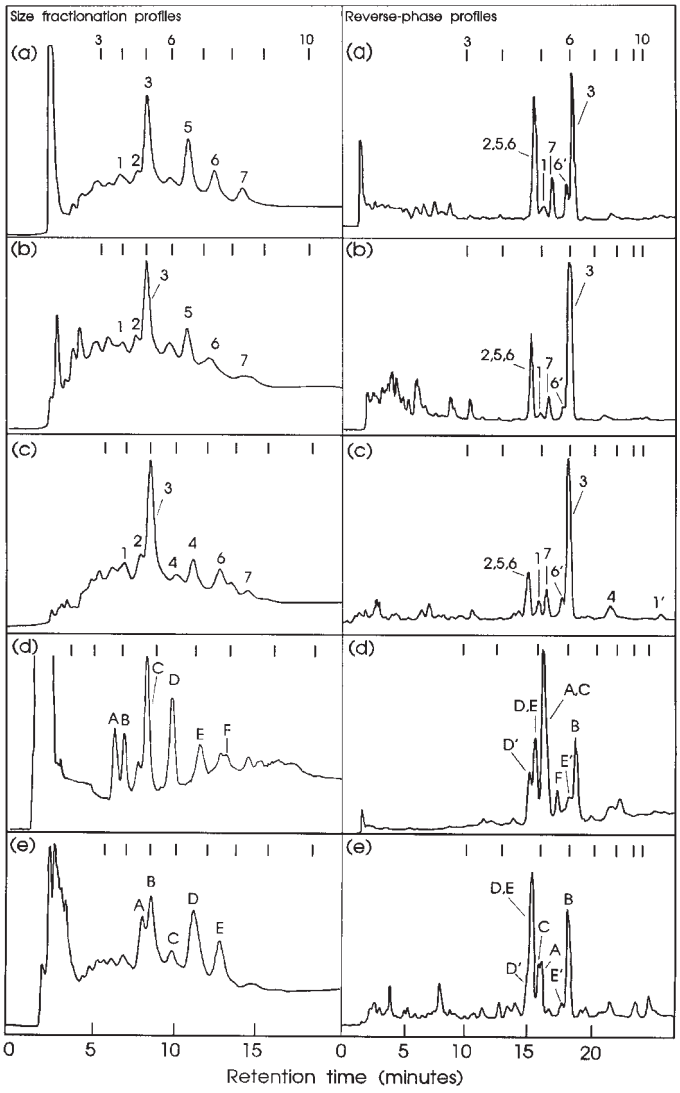


Figure 1. Complete chromatographic profiles of grass and ragweed pollen samples. For each sample, the complete pool of pyridylaminated oligosaccharides was injected onto either a size-fractionation column (Micropak) or a reverse-phase column (ODS) and the gradients applied were as described in Materials and Methods. External standard glucose units are indicated and retention times in minutes are also shown. Samples were as follows: kentucky blue grass pollen (row a), rye pollen (row b), ryegrass pollen (row c), giant ragweed pollen (row d) and short ragweed pollen (row e). Analysed Micropak peaks are marked **1**–**7** for grass pollen samples and **A**–**E** for ragweed pollen samples. Where the Micropak peak (for example) **E** is resolved into two peaks on reverse phase, the reverse phase peaks are designated (for example) **E** and **E′**.

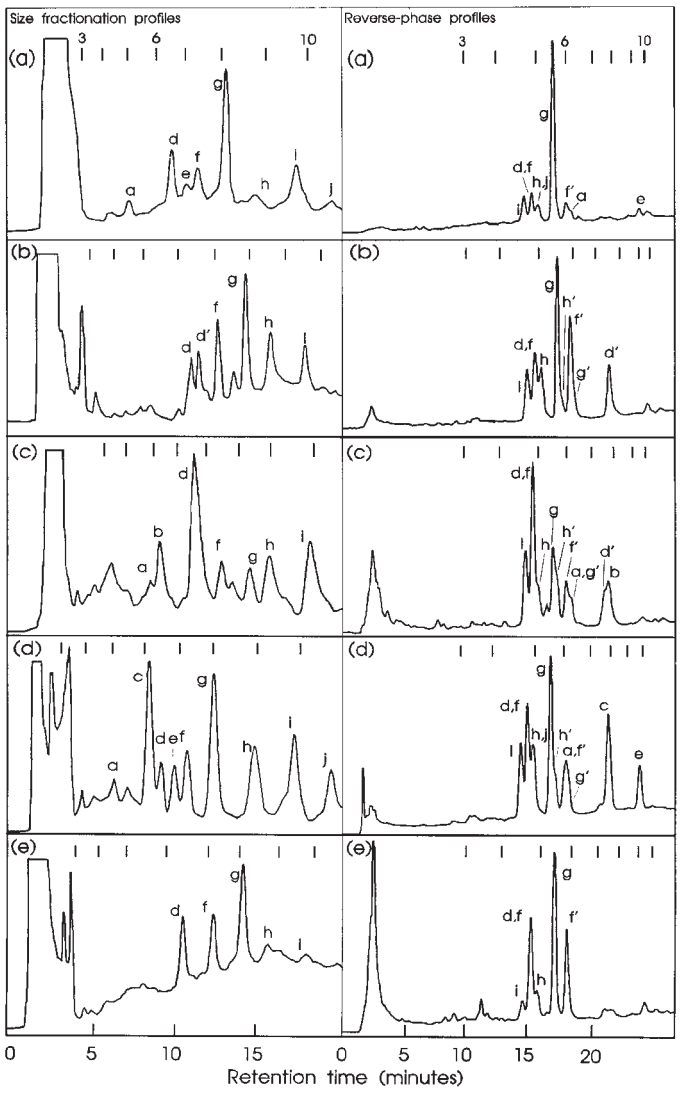


Figure 2. Complete chromatographic profiles of tree pollen samples. For each sample, the complete pool of pyridylaminated oligosaccharides was injected onto either a size-fractionation column (Micropak) or a reverse-phase column (ODS) and the gradients applied were as described in Materials and Methods. External standard glucose units are indicated and retention time in minutes are also shown. Samples were as follows: birch pollen (row a), hornbeam pollen (row b), horse chestnut pollen (row c), olive pollen (row d) and pine pollen (row e). Analysed Micropak peaks are marked **a**–**j.** Where a Micropak peak (for example) **f** is resolved into two peaks on reverse phase, the reverse phase peaks are designated (for example) **f** and **f′.**

tionation HPLC was rechromatographed by reverse-phase HPLC, in addition to full reverse-phase profiles being made (see Figures 1 and 2). The 'two-dimensional' properties and the effects of exoglycosidase digestion on retention times of pollen glycans were compared with the properties of standard oligosaccharides MMXF^3, M0XF^3, MMF^3, MMX and MM (Table 1) in order to assign the structure. Example exoglycosidase digests of pollen extract glycans are shown in Figures 3 and 4, but a detailed examination of the data for each glycan type presented in the text below, with a summary giving percentages based on peak integrations given in Table 2. The structures referred to in the tables and in the text below are shown in Figure 5.

Manα1,6(Xylβ1,2)Manβ1,4GlcNAcβ1,4GlcNAc (M0X)

M0X was identified as a minor component of the ryegrass pollen peak **1** (Figure 1, row c) and its identify presumed from (i) size, (ii) having the same retention time as an intermediate during α-mannosidase digestion of MMX and (iii) being digestible with α-mannosidase to 8.5 glucose units, which corresponds to the retention time of the final digestion product of MMX (Table 1).

Manα1,6(Manα1,3)(Xylβ1,2)Manβ1,4GlcNAcβ1,4 GlcNAc (MMX)

MMX was identified as a significant component of horse chestnut pollen glycans (peak **b**; Figure 2, row c) due to the size and retention times before and after α-mannosidase digestion in comparison with the standard MMX from fraction 1 of ascorbate oxidase N-glycans [26].

Manα1,6(GlcNAcβ1,2Manα1,3)(Xylβ1,2)Manβ1,4Glc NAcβ1,4GlcNAc (MGnX) and GlcNAcβ1,2Manα1,6(GlcNAcβ1,2Manα1,3)(Xylβ1,2) Manβ1,4GlcNAcβ1,4GlcNAc (GnGnX)

Peaks **c** and **e** from olive pollen (Figure 2, row d) were both apparently resistant to α-mannosidase treatment as judged by their behavior on the reverse-phase column. While for peak **e** β-hexosaminidase treatment resulted in a shift to 7.7 glucose units (i.e., to apparently the same retention as MMX), for peak **c** even β-hexosaminidase treatment appeared to have no effect (Figure 3, panel a). However, for both peaks a combination of both hexosaminidase and mannosidase treatment resulted in peaks with reverse-phase retention of 8.5 glucose units, which is that expected for the final mannosidase digestion product of MMX. Thus, it was concluded that olive peak **e** corresponded to GnGnX, while the peak **c** could be MGnX due to its lower retention time on Micropak (consistent with the Micropak elution of GnMX from ascorbate oxidase, as reported by Altmann [26]), its apparent mannosidase insensitivity and apparent co-elution on reverse-phase with MMX. The reverse-phase retention and mannosidase insensitivity of this putative MGnX in comparison to MMX were thought to be possibly akin to the same parameters for MGnXF^3 in comparison to MMXF^3 (see below). In addition, in order to generate MGnX and GnMX *in vitro,* the effect of overnight acid defucosylation by 100% trifluoroacetic acid of a 4:1 mixture of MGnXF^3 and GnMXF^3 (peak **f** from olive) did result in major products of 7.7 and 10.0 glucose units on reverse-phase, compatible with the retention times for the

Table 1. Properties of standard oligosaccharides. N-glycans derived from horseradish peroxidase (HRP), pineapple stem bromelain (Brom), zucchini ascorbate oxidase (Asc. Ox.), bee venom phospholipase (PLA) and bovine fibrin were chromatographed as described in Materials and Methods and the retention times (RT) recorded in terms of glucose units. Retention times on reverse-phase after α-mannosidase digestion were also recorded.

Designation	*Source*	*Micropak RT*	*Reverse-phase RT*	*Post-mannosidase*
$MMXF^3$	HRP	6.5	4.8	6.0 then 5.2
$M0XF^3$	Brom	5.0	6.0	5.2
MMX	Asc. Ox.	5.6	7.7	11 then 8.5
MMF^3	PLA	5.8	5.0	4.3*
MM	fibrin	4.7	7.8	8.2 then 6.8

*Predicted intermediate $M0F^3$ (5.1 glucose units on reverse-phase) not detected.

putative MGnX and the known retention time for GnMX respectively, although an additional possibly artefactual peak of around 6 glucose units also appeared. As a final test, β-hexosaminidase-digested peak **c** from olive was chromatographed, in comparison to the undigested peak **c**, on the size-fractionation column. This indicated a shift from 6.2 glucose units to 5.6 (Figure 3, panel b), which is consistent with the removal of one *N*-acetylglucosamine residue to yield MMX.

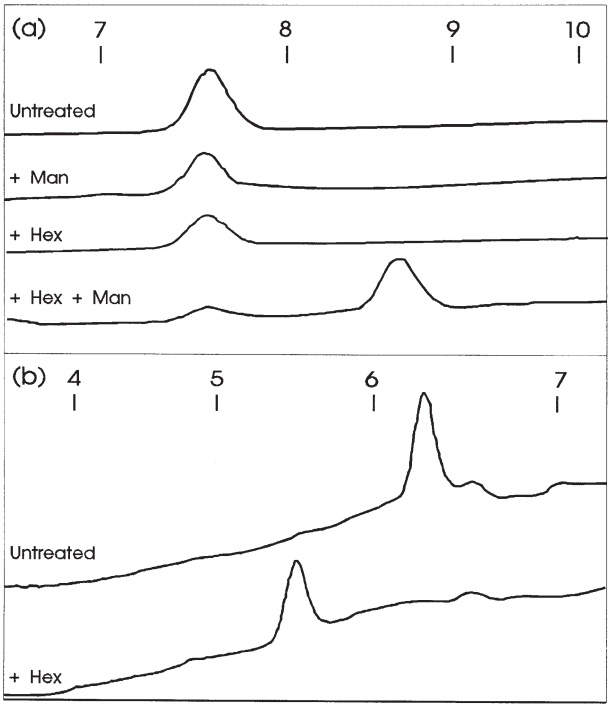


Figure 3. Effect of exoglycosidase digestions on olive peak c. The effect of exoglycosidase digestions on olive peak c are shown. In panel a the 'untreated' profile is the original peak from the size-fractionation column rechromatographed on the reverse-phase column. '+ Hex' indicates the profile after treatment with β-hexosaminidase. '+ Man' indicates the profile after α-mannosidase treatment. '+ Hex + Man' indicates the profile after first β-hexosaminidase, then mannosidase, treatment. Panel b shows the comparison of 'untreated' and '+Hex' on the size fractionation column as a final proof of the hypothesised MGnX structure.

Manα1,6Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc ($M0F^3$) and Manα1,6(Manα1,3)Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc (MMF^3)

Peaks **A** and **C** from both ragweed pollens contained glycans with 'two-dimensional' properties unlike $M0XF^3$ and $MMXF^3$ (Figure 1, rows d and e). Digestion with α-mannosidase resulted, for both peaks **A** and **C,** in a reduction of retention time to 4.3 glucose units (see Figure 4, panel a, for the data on ragweed peak **C**), which is identical to the effect of α-mannosidase on MMF^3 from bee venom phospholipase peak 5a [25]. Also, the retention time of the major component of ragweed peak **C** on size fractionation and reverse-phase chromatographies was verified to be identical to that of MMF^3 from insects, while the small difference in Micropak retention time between ragweed peak A and $M0XF^3$ is comparable to the difference between bromelain glycans treated with an almond xylosidase and untreated bromelain glycans (M. Lanner and F. Altmann, unpublished). That α-mannosidase digestion of ragweed peak **A** resulted in a lowering of retention time from 5.1 to 4.3 glucose units would suggest that peak **A** contained α-1,6-linked mannose rather than α-1,3-linked mannose and is comparable to the effect of α-mannosidase digestion on bromelain $M0XF^3$ which has an α 1,6-linked mannose [30]. In contrast, the structure A from Bermuda grass BG60 increases in retention time on reverse-phase after α-mannosidase digestion which led the authors to deduce that the structure contained an α 1,3-linked mannose (i.e., $0MF^3$) [20]. The $M0F^3$ structure, as found in ragweed peak **A,** was subsequently found to be present as a minor component of the grass pollen samples (labelled peak 2 in Figure 1, rows a–c) and in all cases was also digested with α-mannosidase to yield a structure of 4.3 glucose units on reverse-phase.

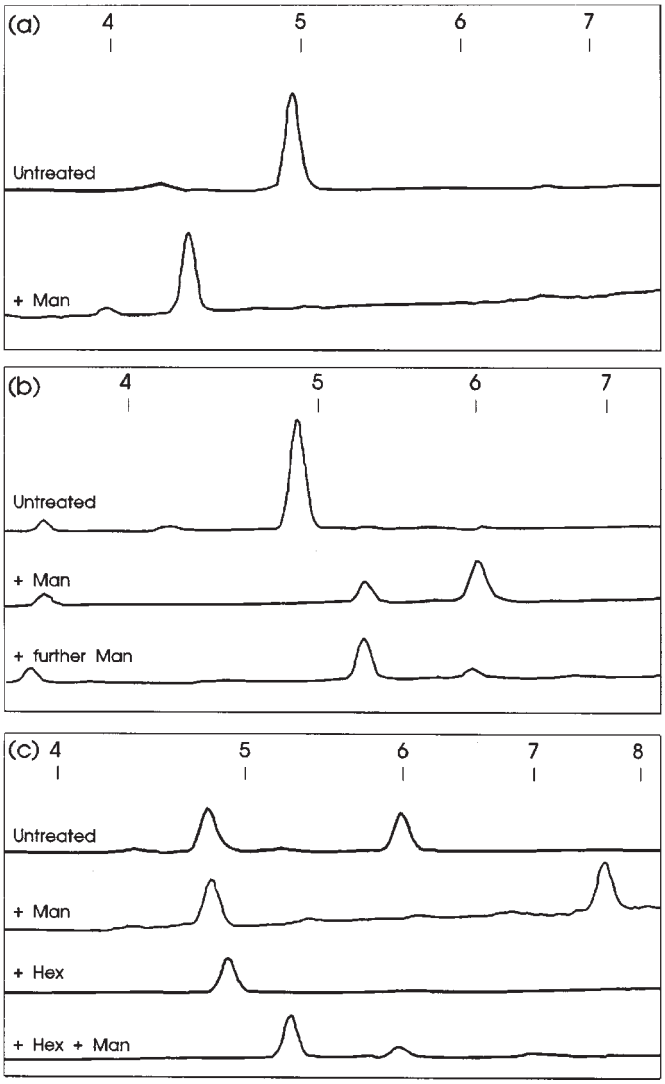


Figure 4. Examples of effect of exoglycosidase digestions on reverse-phase retention time. The effect of exoglycosidase digestions on reverse-phase retention times of peak **C** from giant ragweed (panel a), peak **5** from ryegrass (panel b) and peak **6** from ryegrass (panel c) are shown. 'Untreated' profiles are the original peaks from the size-fractionation column rechromatographed on the reverse-phase column. '+Hex' indicates a profile after treatment with β-hexosaminidase. '+ Man' indicates a profile after α-mannosidase treatment. '+ Hex + Man' indicates a profile after first β-hexosaminidase, then mannosidase, treatment. In panel b, '+ further Man' indicates a profile after re-incubation of peak **6** from ryegrass with additional mannosidase and shows that there remains a small residual amount of the M0XF^3 intermediate seen in the '+ Man' profile. The retention times of external standard glucose units are shown.

Manα1,6(GlcNAcβ1,2Manα1,3)Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc (MGnF^3)

A peak co-eluting on Micropak with the MMXF^3 structure was found in the giant ragweed sample (Figure 1, row d). However, by reverse-phase chromatography peak **D** from giant ragweed was split into two components in the range 4.7–4.9 glucose units designated **D** and **D′**. When **D** and **D′** were treated together with exoglycosidases, complicated digestion patterns were apparent, which suggested the presence of non-reducing terminal *N*-acetylglucosamine on a non-xylosylated structure as well as the MMXF^3 structure expected for that size. β-Hexosaminidase digestion products were also chromatographed on Micropak and about 50% of the peak had its retention time reduced. To define properly the structures, **D** and **D′** were separated by reverse-phase chromatography prior to further exoglycosidase treatments. While component **D** followed the exoglycosidase digestion pattern for MMXF^3 (see below), β-hexosaminidase digestion of **D′** resulted in a reproducible shift to 5.0 glucose units, which is the retention time expected for MMF^3. Subsequent α-mannosidase treatment confirmed that **D′** was indeed a glycan that required both β-hexosaminidase and mannosidase before digestion to a peak of retention time the same as 00F^3. Previous results have indicated that the presence of *N*-acetylglucosamine on the α1,3-linked mannose of the simple trimannosyl core MM (as well as on the corresponding α1,6-fucosylated core) causes a slight reduction in retention time on reverse phase, while there is a fairly large shift in the opposite direction if a *N*-acetylglucosamine residue is linked to the α1,6-linked mannose [29,31]. It is also known that the mannose residue α1,3-linked to the core β-linked mannose is highly susceptible to removal by α-mannosidase [32]. Thus since **D′** has an earlier retention time on reverse phase than **C** and since **D′** was apparently resistant to α-mannosidase, it was deduced that **D′** was MGnF^3 rather than GnMF^3.

ManαManα1,6(Manα1,3)Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc (M4F^3)

In short ragweed the Micropak peak **D** (containing MMXF^3 as the major component) also contained a small side peak (designated **D′**) of slightly lower retention time (i.e., 4.7 glucose units on reverse-phase). However, it did not appear to behave like the MGnF^3 from giant ragweed peak **D′** (see above) which had the same 'two-dimensional' chromatographic properties. Certainly this short ragweed side-peak did not require β-hexosaminidase in order for α-mannosidase to reduce its retention time to that of OOF^3. It was confirmed by co-injection on reverse-phase that **D′** was not a carry-over from peak **C** (the previous peak on Micropak) which contained MMF^3; however, the exact isomer of M4F^3 could not be determined due to the low amount of sample.

Xylβ1,2Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc (00XF^3)

This glycan was a very minor component of grass pollen samples (peak **1**) and its identify presumed from (i) size, (ii) resistance to α-mannosidase and (iii) having the same retention time (5.2 glucose units) on reverse-phase HPLC as

Table 2. Analysis of pyridylamino-N-glycan pools from grass and ragweed pollens. For each peak indicated in Figure 1, the structures identified are shown, together with the percentages. The structures corresponding to the abbreviations are shown in Figure 5. The percentages given are based on integration of all peaks of retention time greater than 5 minutes on the reverse-phase column, with reference to results of the glycosidase digestions and the size-fractionation column where necessary. Percentages for each pollen do not add up to 100% since not all fluorescent peaks could be identified.

	Kentucky blue grass		*Rye*		*Ryegrass*		*Giant ragweed*		*Short ragweed*		*Birch*		*Horse chestnut*		*Hornbeam*		*Pine*		*Olive*	
Structure	*peak*	*%*	*peak*	*%*	*peak*	*%*	*peak*	*%*	*peak*	*%*	*peak*	*%*	*peak*	*%*	*peak*	*%*	*peak*	*%*	*peak*	*%*
00XF[3]	1	2.1	1	2.2	1	3.4														
M0X					1′	2.1														
M0F[3]	2	2.0	2	1.2	2	4.8	A	10.5	A	10.8										
M0XF[3]	3	38.9	3	47.0	3	43.1	B	14.4	B	22.0	a	3.0	a	2.5					a	2.5
MMX													b	9.3						
MMF[3]							C	27.4	C	2.8										
Gn0XF[3]					4	1.2														
MGnX																			c	15.7
MMXF[3]	5	22.5	5	14.1	5	6.6	D	15.3	D	23.0	d	8.4	d	25.8	d	11.7	d	18.3	d	9.1
GnGnX											e	1.8							e	7.2
MGnF[3]							D′	7.6												
M4F[3]									D′	4.6										
M5													d′	7.5	d′	12.4				
MGnXF[3]	6	5.7	6	4.2	6	5.0	E	4.5	E	12.9	f	1.3	f	2.9	f	3.1	f	8.0	f	6.7
GnMXF[3]	6′	7.7	6′	3.9	6′	4.1	E′	3.1	E′	1.0	f′	4.4	f′	5.2	f’	15.2	f′	17.6	f′	4.4
GnGnXF[3]	7	8.9	7	4.2	7	1.7	F	3.4			g	59.4	g	12.1	g	26.8	g	39.6	g	22.7
M6													g′	4.4	g′	2.0			g′	4.6
M7.1											h	2.0	h	2.9	h	10.8	h	5.1	h	6.4
M7.2													h′	4.8	h′	1.0			h′	1.6
M8											i	10.4	i	10.3	i	10.2	i	4.6	i	10.3
M9											j	3.5							j	4.8

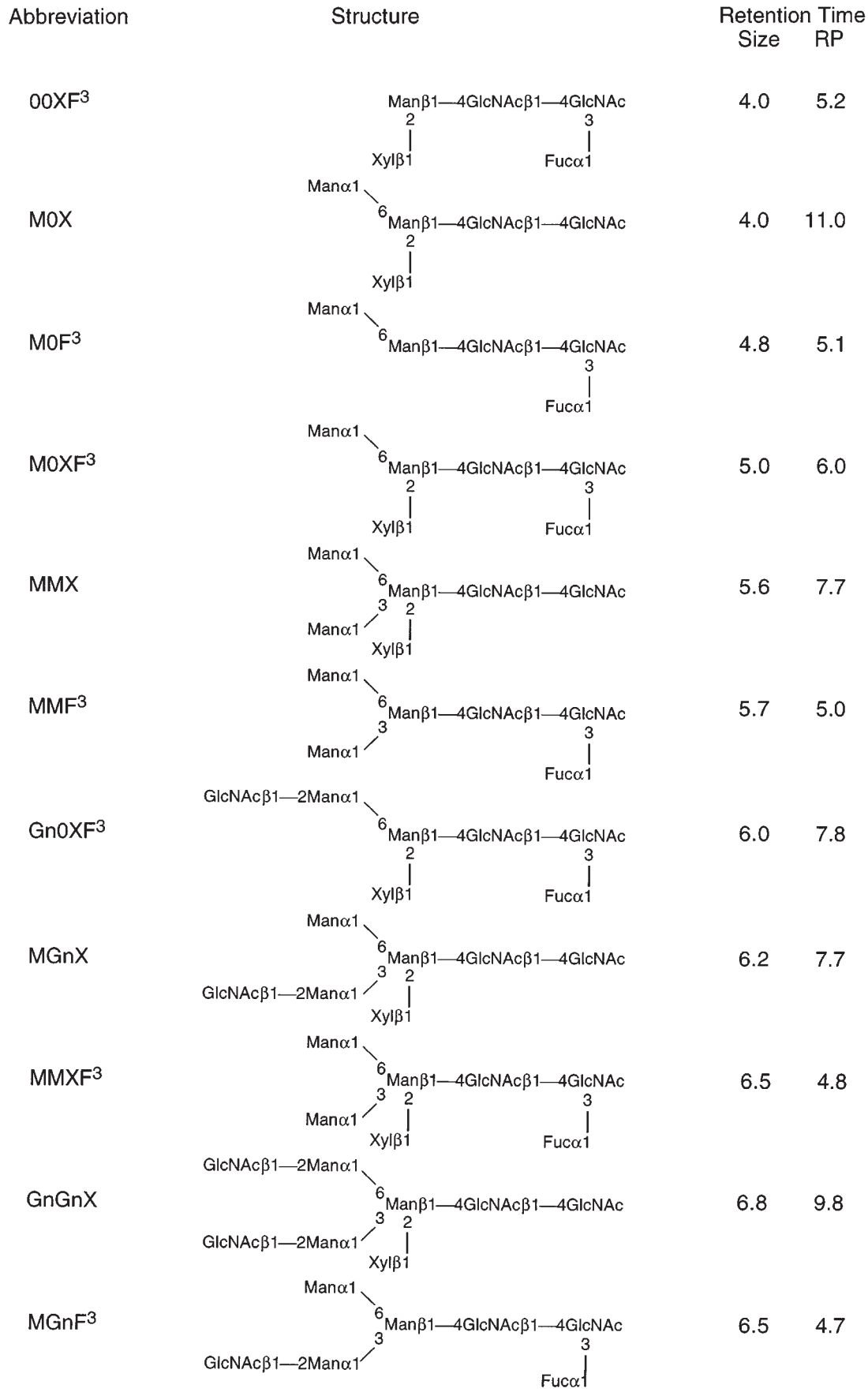


Figure 5. Structures of oligosaccharides. The structures found in grass, ragweed and tree pollens with the corresponding abbreviations used are shown, together with the size-fractionation and reverse-phase retention times in terms of glucose units.

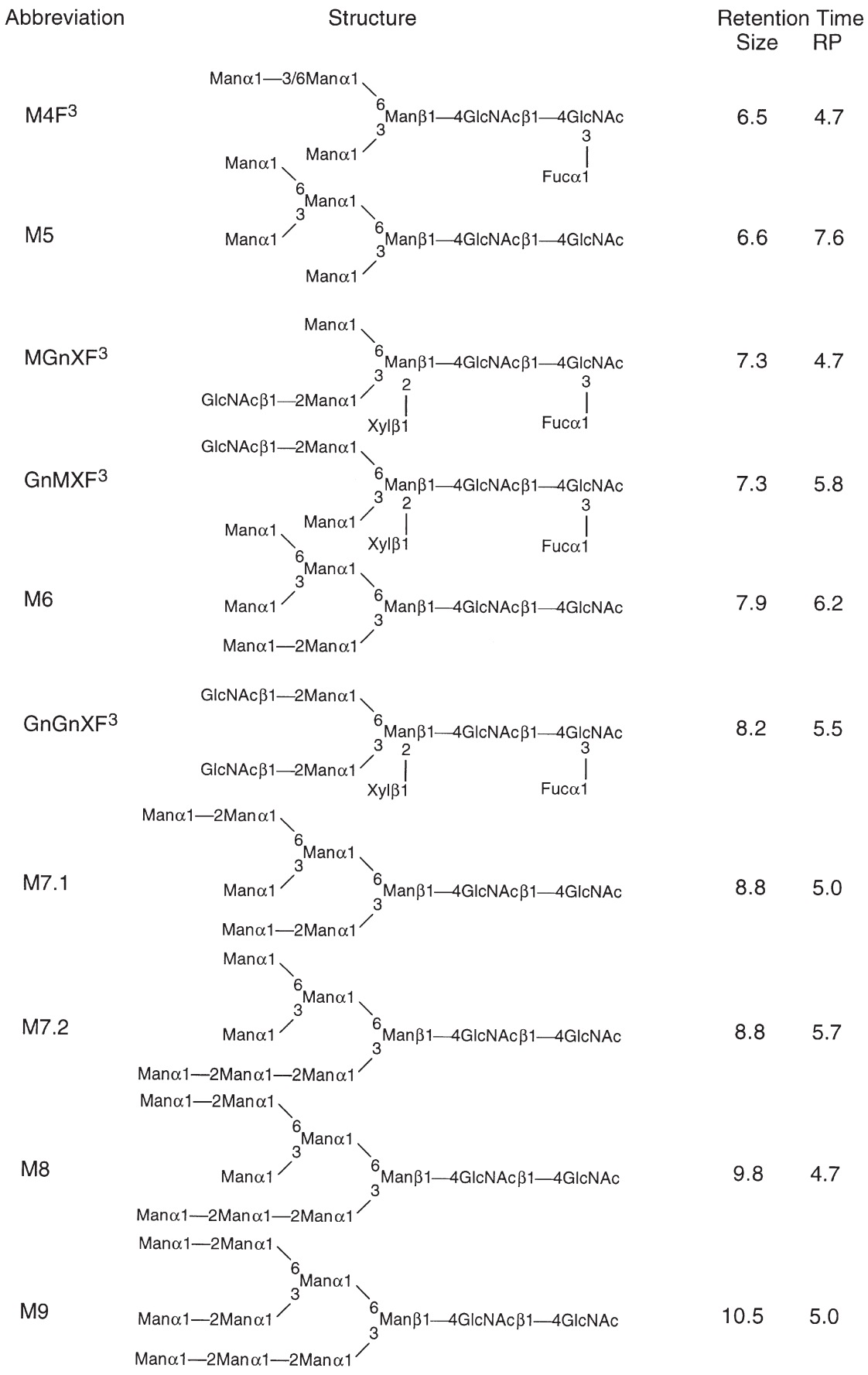


Figure 5. Structures of oligosaccharides. (continued)

the product of α-mannosidase digestions of the major horseradish peroxidase structure ($MMXF^3$).

Manα1,6(Xylβ1,2)Manβ1,4GlcNAcβ1,4(Fucα1,3) GlcNAc ($M0XF^3$)

Peak **3** from grass samples, peak **B** from ragweed samples and peak **a** from birch, horse chestnut and olive samples behaved comparably to $M0XF^3$ in terms of 'two-dimensional' retention times. α-Mannosidase digestions resulted in the expected shift to $00XF^3$ (5.2 glucose units) on reverse-phase. $M0XF^3$ is predicted to be the most abundant structure in rye, ryegrass and Kentucky blue grass pollen samples.

GlcNAcβ1,2Manα1,6(Xylβ1,2)Manβ1,4GlcNAcβ1,4 (Fucα1,3)GlcNAc ($Gn0XF^3$)

This structure is predicted to be present in small amounts in at least ryegrass pollen (peak **4**; see Figure 1, row c) on the basis that a structure co-eluting on reverse-phase with the α-mannosidase digestion product of $GnMXF^3$ (see below) and sensitive to β-hexosaminidase (yielding a peak of the same retention time as $M0XF^3$) was found to elute from the Micropak column in the region expected for its size.

Manα1,6(Manα1,3)(Xylβ1,2)Manβ1,4GlcNAcβ1,4 (Fucα1,3)GlcNAc ($MMXF^3$)

The glycan from peak **5** from all grass samples as well as the major peak **D** from both ragweed samples and peak **d** in all tree pollen samples behaved in terms of 'two-dimensional' coordinates like the $MMXF^3$ standard. The key diagnostic was that α-mannosidase digestion resulted in a time/concentration dependent conversion to the $M0XF^3$ (6.0 glucose unit on reverse-phase) structure and finally completely or almost completely to $00XF^3$ (5.2 glucose units on reverse-phase) in all cases (Figure 4, panel b). $M0XF^3$ is the sole intermediate in such digestions since the α1,6-linked mannose is more resistant to α-mannosidase digestion than the α1,3-linked mannose.

GlcNAcβ1,2Manα1,6(Manα1,3)(Xylβ1,2)Manβ1,4 GlcNAcβ1,4(Fucα1,3)GlcNAc ($GnMXF^3$) and Manα1,6(GlcNAcβ1,2Manα1,3)(Xylβ1,2)Manβ1,4 GlcNAcβ1,4(Fucα1,3)GlcNAc ($MGnXF^3$)

As shown in Figure 1 and Figure 2, peak **6** glycans from grass samples and peak **f** from tree samples eluted as one peak on Micropak, but were resolved into two peaks on reverse-phase of 4.7 and 5.8 glucose units which were designated **6** (or **f**) and **6′** (or **f′**) respectively. In all cases the grass peaks **6/6′** and tree peaks **f/f′** were treated with α-mannosidase and the 5.8 glucose unit peaks (i.e., grass peak **6′** and tree peak **f′**) were found to be sensitive, since they were all converted to a structure eluting at 7.8 glucose units on reverse-phase which is the same as the putative elution position for $Gn0XF^3$ (Figure 4, panel c). Therefore, the preliminary determinations of grass peak **6** (and tree peak **f**) as $MGnXF^3$ and grass peak **6′** (and tree peak **f′** as $GnMXF^3$ were based on the known sensitivity of α1,3-linked mannose residues to α-mannosidase [32], the documented effect of the addition of *N*-acetylglucosamine residues by GlcNAc transferases I and II on retention time [31] and on the similar pattern of α-mannosidase sensitivity reported for sycamore cell laccase glycans with a single non-reducing terminal *N*-acetylglucosamine residue [33]. Furthermore, β-hexosaminidase treatment of peak **6/6′** from grasses and peak **f/f′** from trees, as well as peak **E/E′** from ragweeds, resulted in a single peak of 4.8 glucose units on reverse-phase, which is the retention expected for $MMXF^3$. Subsequent α-mannosidase digestion of the β-hexosaminidase-treated fractions showed the same pattern of digestion indicative of the $MMXF^3$ structure.

GlcNAcβ1,2Manα1,6(GlcNAcβ1,2Manα1,3)(Xylβ1,2) Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc ($GnGnXF^3$)

An initial partial β-hexosaminidase digestion of peak **7** from Kentucky blue grass showed the appearance of structures of 4.7 and 5.8 glucose units akin to $MGnXF^3$ and $GnMXF^3$. Subsequently β-hexosaminidase digestion of peak **7** from all grass samples and peak **g** from all tree samples resulted in a reduction of retention time to 4.8 glucose units on reverse-phase. As for $MGnXF^3$ and $GnMXF^3$, subsequent α-mannosidase digestion of β-hexosaminidase-treated fractions showed the time-dependent pattern of digestion indicative of the $MMXF^3$ structure.

Oligomannosidic oligosaccharides ($Man_5GlcNAc_2$, $Man_6GlcNAc_2$, $Man_7GlcNAc_2$, $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$)

Endoglycosidase H treatment of complete N-glycan pools from pollen extracts suggested that tree pollens contain a number of oligomannosidic oligosaccharides. Percentages of endoglycosidase H sensitive glycans, as judged by the integration of the post-digestion *N*-acetylglucosamine peak of 3.5 glucose units on reverse-phase, were 25–30% in the cases of hornbeam, horse chestnut and olive and about 15% in the case of birch and pine. Verification of the nature of these presumed oligomannosidic oligosaccharides was by means of (i) size estimation on Micropak, (ii) comparison with retention times of the oligomannosidic series of soybean 7S glycoprotein ($Man_{6-8}GlcNAc_2$) [34], $Man_5GlcNAc_2$ from *Aspergillus* α-amylase [34], the putative $Man_{6-9}GlcNAc_2$ samples from soybean and pea that were examined in parallel (I.B.H. Wilson and F. Altmann, unpublished data) and retention times given in the literature [29,34] as well as (iii) demonstration that α-mannosidase treatment resulted in a final digestion product with

retention time of 6.8 glucose units. For $Man_{6-8}GlcNAc_2$, a number of isomers can exist. The isomeric designations given for peaks **d**′,g′,h,h′,i and **j** in Table 2 and Figure 5 are not definitive since they are based solely on the compatibility of the reverse-phase retention times with those given by Kubelka *et al.* [34] and Tomiya *et al.* [29] for these oligosaccharides. However, the retention times of Kubelka *et al.* and Tomiya *et al.* agree with eachother to within ±0.1 glucose units and so the isomeric designations in the present study can be given with some confidence.

Matrix-assisted laser-desorption-ionisation mass spectrometry

MALDI-MS was performed on a number of pyridylamino-N-glycan fractions. Due to the amount of glycan available, the presence of salts or losses during desalting it was possible to analyze only just over one-third of the fractions (i.e., 25 out of a total of 72) of the ten samples; however, since most of the structures are found in more than one sample, this translates into 13 of 20 structures found overall and all of the most abundant structures. In all cases where data could be gained (see Table 3 and Figure 6), the MALDI analyses yielded results which agreed with the theoretical masses calculated and so verified the structures that were proposed on the basis of the HPLC/exoglycosidase analyses.

Discussion

Methodological considerations

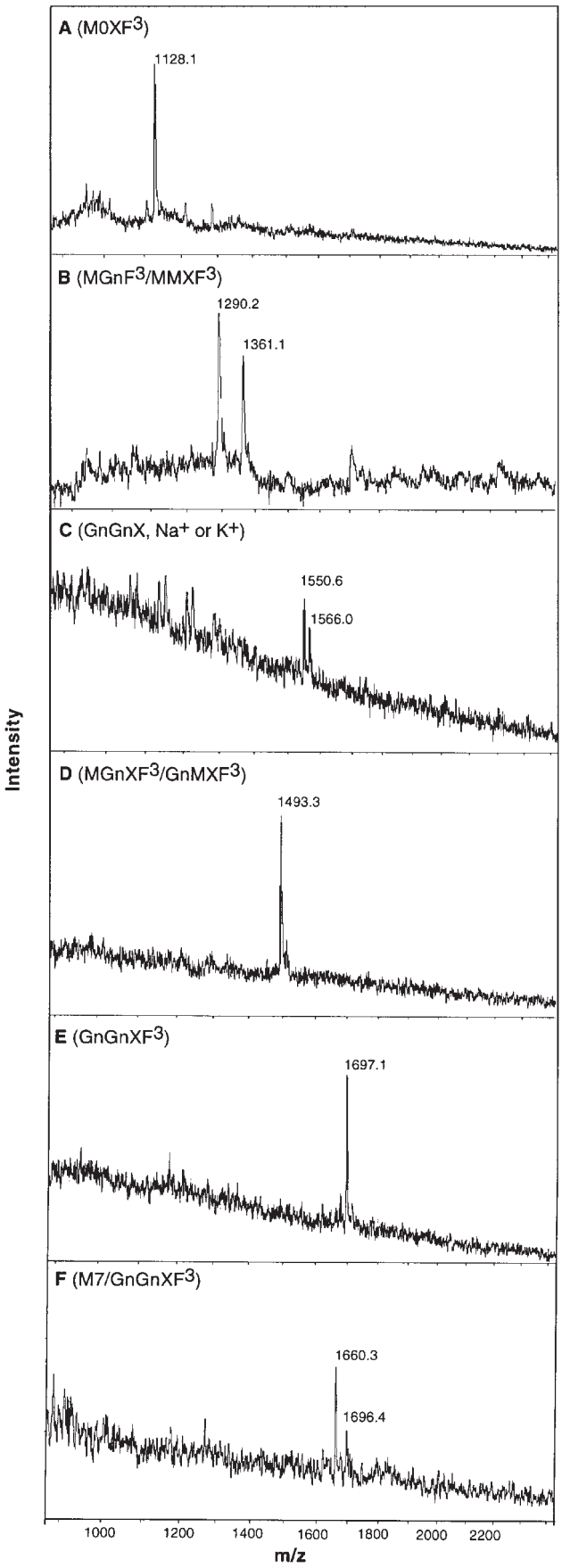
This study used a chromatographic methodology which has been employed on a wide variety of non-mammalian glycans, in this [25,26,34–36] and in other laboratories [20,33,37]; in addition, MALDI-MS was used as a corroborative method. In the ten pollens analyzed the vast majority of the fluorescent peaks with the two-dimensional chromatographic properties expected for N-glycans have been assigned. Addition of borohydride at alkaline pH to the pollen extracts after pepsin digestion and before deglycosylation was found to be important, since particularly tree pollen extracts that were not reduced contained a high percentage of mannosidase-sensitive peaks of low retention time on reverse-phase (but middling retention time on the size-fractionation column) which had similar properties to endoglycosidase products (data not shown). This suggested that free glycans with only one core *N*-acetylglucosamine residue were present. Reduction prior to digestion with peptide:N-glycosidase A ensured that these free glycans could not be labelled by the reductive amination procedure. Glycans with only one *N*-acetylglucosamine residue have been described before from preparations of *Erythrina cristagalli* lectin [38] and horseradish peroxidase [39], but these structures may be artefacts due to hydrazinolysis. However, in view of the presence of free glycans in crude pollen extracts, it is plausible to consider that small contaminating amounts of endoglycosidases in apparently pure plant glycoprotein preparations could lead to the detection of glycans with only one core *N*-acetylglucosamine residue. As regards enzymatic release, the use of peptide:N-glycosidase A from almonds was necessary, since peptide:N-glycosidase F from *Flavobacterium meningosepticum* cannot cleave glycans containing a fucose linked α1,3 to the reducing-terminal *N*-acetylglucosamine residue [27,40].

The employment of the 'two-dimensional' technique with pyridylaminated N-glycans has led to attempts to predict behavior of the labeled glycans on reverse-phase columns [41,42]. These estimation methods are not always compatible with the results from the present study. For instance, the similar retention times for $MMXF^3$ and MMF^3 are not predicted by the method of Lee *et al.* [41] (predicted 5.85 and 4.34 glucose units respectively based on their X series formula), although the formula of Lee *et al.* does predict that a structure lacking the fucose residue (*i.e.,* MMX) would display an increased retention time of 7.58 glucose units. In contrast the method of Hase and Ikenaka [42] suggests that $MMXF^3$ and MMF^3 will have similar retention times (a difference equivalent of 3% of the retention time for $Man_5GlcNAc_2$). Thus, it seems that the different brands of columns or different elution conditions used in different laboratories may lead to different estimations of retention times in terms of glucose units.

On occasion the behavior upon exoglycosidase digestion of apparently similar oligosaccharides is quite different. For instance, the difference in behavior of the $MMXF^3$ type of glycan compared to the MMF^3 type glycan is noticeable, since the final results of α-mannosidase treatment are completely opposite shifts of retention time. Fortunately, this behavior is useful to resolve $MMXF^3$ and MMF^3 which, as noted above, under the conditions used in the present study, migrate closely on reverse-phase. The role of xylose in affecting retention time is quite complicated, since $M0F^3$ and $M0XF^3$ have similar retention times on Micropak, while MMF^3 and $MMXF^3$ are much more resolved; the opposite retention time pattern occurs on reverse-phase. The absence of fucose though on MMX makes it behave on reverse-phase more like MM, although the pattern of α-mannosidase digestion allows MMX and MM to be discriminated on reverse-phase. The three dimensional structure of the oligosaccharides and the exact means of interaction with the chromatographic matrix may be responsible for these effects. There is not much information in the literature on the conformation of plant N-glycans, but for $M0XF^3$ from bromelain, the xylose residue is predicted to stick out from the linear Manα1,6Manβ1,4GlcNAcβ1,4GlcNAc sequence [43,44] and there is a crystallographic study of *Erythrina cristagalli* which shows a similar orientation for the xylose residue within an $MMXF^3$ structure [45]. From such limited spatial model information, though, it is not possible to

Table 3. MALDI-MS analyses of selected fractions of pyridylamino-N-glycans. Fractions of pyridylamino-N-glycans derived from nine of the ten pollen samples were analysed by MALDI-TOF as described in the text. ND indicates where determination was not possible.

		Kentucky blue grass	*Rye*	*Ryegrass*	*Giant ragweed*	*Birch*	*Horse chestnut*	*Hornbeam*	*Pine*	*Olive*
Structure	*Theoretical mass $[M+Na]^+$*	*Mass found*	*Mass found*	*Mass found*	*Mass found*	*Mass found*	*Mass found*	*Mass found*	*Mass found*	*Mass found*
00XF^3	965.9	ND	ND	966.6						
M0X	981.9			ND						
M0F^3	995.9	ND	ND	ND	995.6					
M0XF^3	1128.1	1127.8	1127.9	1128.1	1128.9	ND	ND			ND
MMX	1144.1						ND			
MMF^3	1158.1				1158.5					
Gn0XF^3	1331.2			ND						
MGnX	1347.2									ND
MMXF^3	1290.2	1290.6	ND	1289.7	1290.2	ND	1290.9	ND	1290.9	ND
GnGnX	1550.3					ND				1550.6
MGnF^3	1361.3				1361.1					
M4F^3	1320.2									
M5	1336.2						ND	1337.0		
MGnXF^3	1493.4	1493.3	ND	ND	ND	ND	ND	1492.7	ND	ND
GnMXF^3										
GnGnXF^3	1696.5	ND	ND	1698.6	ND	1696.8	ND	1696.3	1697.2	1696.4
M6	1498.4						ND	ND		ND
M7	1660.5					ND	ND	1660.0	ND	1660.3
M8	1822.6					ND	ND	1822.7	ND	ND
M9	1984.8					ND				ND



speculate on the reasons for the different chromatographic properties observed.

Taxonomic and other comparisons

Most of the structures found in the present study have been reported previously in plants by others. By far the most common type of structure found in the present study are N-glycans carrying both xylose and core α1,3-fucose residues regardless of subclass (mono- or dicotyledonous) or of taxonomic relationship. $MMXF^3$ was found in all samples; this glycan is very common in plants and is, for instance, the major structure of horseradish peroxidase [11,39,46]. Related structures with one or two non-reducing terminal *N*-acetylglucosamine residues were found also in the present study in all samples. Again, these structures are well known in plants: $GnMXF^3$ is a component of the glycans from zucchini ascorbate oxidase [26], while $GnMXF^3$, $MGnXF^3$ and $GnGnXF^3$ are all present on sycamore cell laccase [33] and red kidney bean Fe(III)-Zn(II) purple acid phosphatase [47]. With the $MGnXF^3$ and $GnMXF^3$ structures, the ratios of these isomers varies between samples (see Table 2 for the relevant percentages). For the grasses the ratio is around 1 : 1, while for short ragweed samples there is bias in favor of $MGnXF^3$ and for trees pollens the bias is normally towards $GnMXF^3$. To compare with other known cases, zucchini ascorbate oxidase has an isomeric composition almost 100% $GnMXF^3$ [26] while red kidney bean Fe(III)-Zn(II) purple acid phosphatase has a 4 : 1 ratio in favor of $GnMXF^3$ [47]. Presumably $MGnXF^3$ is a biosynthetic intermediate in all these cases, since GlcNAc transferase I is required prior to any development of complex N-glycans in plants [48], but subsequent actions of Golgi, vacuole or other β-hexosaminidases and GlcNAc transferase II to different degrees (such as have been proposed for insect cells [31,49]) are presumably the source of such different isomeric ratios. Some of the truncated structures could, of course, be due to degradation during extraction of the pollens, but various truncations are, as references herein show, very common amongst the oligosaccharides reported for purified plant glycoproteins.

For grass pollens, truncated xylosylated/fucosylated structures such as $M0XF^3$ and $MMXF^3$ predominate. This is in keeping with the knowledge of glycosylation of proteins

Figure 6. Examples of MALDI-MS spectra of pollen-derived pyridylamino- oligosaccharides. MALDI-MS on pyridylamino-oligosaccharides was performed as described in the text. The pyridylamino-oligosaccharides were generally detected as the $[M+Na]^+$ ion. The experimentally-determined m/z values were compared with the theoretical mass of the expected ions (Table 2). A, Fraction **3** from ryegrass pollen; B, Fraction **D** from giant ragweed pollen; C, Fraction **e** from olive pollen (note that a significant portion of this glycan was found as the $[M+K]^+$ ion; D, Fraction **6** from Kentucky blue grass pollen; E, Fraction **g** from birch pollen; F, Fraction **h** from olive pollen (note the detected $GnGnXF^3$ is a contaminant from the neighbouring fraction **g**).

from other monocotyledonous plants, such as pineapple bromelain ($M0XF^3$) [30], barley seed peroxidase [50] and β1,3/1,4-glucanase from germinating barley [51], although rice seedling α-amylase has structures such as MMX and oligomannose structures, but no structures with fucose [52]. Among the minor structures, $00XF^3$ has been previously reported as the major N-glycan of momordin-a [37], while $Gn0XF^3$ has been found as a free glycan from tomato [53].

Ragweed pollen glycans are noteworthy due to the significant presence of structures with fucose, but no xylose, which have been rarely detected as major structures in plants with the exception of Bermuda grass BG60 allergen [20]. $M0F^3$ is a minor component of glycans from bee venom phospholipase A_2 (allergen *Api m* I) and hyaluronidase (*Api m* II) [25,35], but its proposed presence in ragweed (and to a lesser extent in grasses) as opposed to the proposed $0MF^3$ isomer in Bermuda grass [20] would suggest that $M0F^3$ is a new plant glycan structure. MMF^3, though, is a structure previously reported in both insects [25,34,35] and plants; it has been found to constitute 1% of soybean peroxidase glycans [54], 4–6% of horseradish peroxidase glycans [39,47] and 80% of Bermuda grass antigen BG60 glycans [20]. An $M4F^3$ structure was also reported, in one study, to account for 2.6% of horseradish peroxidase glycans [39], but a solely α1,3-fucosylated structure with a non-reducing terminal *N*-acetylglucosamine residue (i.e., $MGnF^3$) has apparently not been found before, even in insects, although the xylosylated variant of this structure (*i.e.,* $MGnXF^3$) has been reported in plants in this and other studies [33,47]. The only previous information on glycan structures from a plant taxonomically related to ragweeds is for the *Art v* II allergen from mugwort (like ragweeds, a member of the Compositae): only oligomannose structures were found [55], although it must be noted that peptide:N-glycosidase F was used to release the glycans and not peptide:N-glycosidase A, so the presence of core α1,3-linked fucose on *Art v* II cannot be ruled out.

For tree pollens the glycosylation was more heterogenous than for grass and ragweed pollens. Regardless of taxonomic relationship, tree pollens contained not only xylosylated/fucosylated structures, but also oligomannose structures and, in the case of horse chestnut and olive, some solely xylosylated structures. Indeed, the presence of oligomannose structures in tree pollens sets them apart from the grass and ragweed pollens. Otherwise, though, oligomannose structures are well known in plants; for instance on phytohaemagglutinin [56] and soybean 7S glycoproteins [57], as well as the aforementioned *Art v* II allergen from mugwort pollen [55].

Additionally, in comparison to grasses and ragweeds, tree pollens display a bias towards larger structures carrying non-reducing terminal *N*-acetylglucosamine residues, although, unlike sycamore cell laccase [33,58] or Japanese cedar pollen *Cry j* I allergen [14], no N-glycans with galactose or outer arm fucose were detected. In addition to the full analyses of five tree pollens, preliminary analyses on the small quantities available of sweet chestnut (*Castanea sativa*) pollen also showed the presence of GlcNAc-terminated xylosylated/fucosylated type glycans. Taxonomic relationship also seemed no guide to glycosylation similarity, since, for instance, the glycan profile of a conifer pollen, from snake-skin pine, appears on first sight more similar to that of birch, than the profile of birch was to another member of the Betulaceae, hornbeam. Such differences though were merely quantitative than qualitative.

The finding of some solely xylosylated structures in olive pollen would correlate with peptide:N-glycosidase F being able to cause a diminution in IgE binding to a glycopeptide from the major olive allergen, *Ole e* I [3], since MGnX or GnGnX would be sensitive to peptide:N-glycosidase F. Xylosylated structures lacking fucose are known as major glycan components of zucchini ascorbate oxidase (MMX, GnMX) [26,59], rice α-amylase (MMX, MGnX, GnMX, GnGnX) [52] and S_3- and S_6-allele stylar self-compatibility ribonucleases of *Nicotiana alata* (MMX, M4GnX, M5GnX) [60] as well as, interestingly from an evolutionary perspective, being found on the sexuality-inducing glycoprotein from the alga *Volvox carteri* f. *nagariensis* [61]. Since *Volvox* has the capacity to xylosylate, it is not surprising that this modification is so widespread amongst the plant glycoproteins and extracts studied to date. The presence of fucose in algae cannot be ruled out, since peptide:N-glycosidase F was used to release the glycans from the *Volvox* protein and so the ancestry of core α1,3-linked fucosylation remains to be determined. Complicating this genealogy, structures with core α1,3-linked fucose and xylose have recently been found on egg glycoproteins of the parasitic worms *Schistosoma japonica* and *S. mansoni* [62], while xylosylation of snail glycoproteins, such as *Helix pomatia* haemocyanin, has been known for some time [63].

Immunological applications

The presence of xylose and α1,3-linked fucose in all these pollen samples is a possible source of immunological cross-reaction that may be of allergological importance. As reviewed in the Introduction, there are reports to suggest a role for glycans in allergenicity, but due to the absence of structural analyses and the lack of use of structurally-defined glycoconjugates in IgE binding studies it is not yet possible to ascertain whether plant N-glycans constitute a 'panallergen' such as profilins [19,64]. However, since plant N-glycans are known to be immunogenic and are conserved between species they are good candidates to account for some of the cross-reactions frequently observed. In comparison to the present study on pollens and of interest in regard to pollen-food cross-reactivity, preliminary results on extracts of soybean, pea, avocado, pear, almond, pistachio and coconut (I.B.H. Wilson, unpublished data) as well as tomato (R. Zeleny and F. Altmann, unpublished data) show

the presence of xylosylated and/or fucosylated glycans in all these food sources and so offer a structural explanation for reports such as a recent one suggesting the presence of carbohydrate IgE epitopes common to tomato fruit and grass pollen [6]. One issue is whether the xylose and α1,3-linked fucose residues of N-glycans are both important for immunogenicity. A recent study concluded that xylose was more significant as part of IgE epitopes than the fucose [5], but part of this conclusion rests on zucchini ascorbate oxidase of an unstated source giving a more positive response on a IgE dot-blot than bee venom and the claim that zucchini ascorbate oxidase contains no fucose. However, recent results from this laboratory show that, at least, a commercially available source of zucchini ascorbate oxidase has α1,3-fucosylated glycans released by peptide:N-glycosidase A [26], which is in contrast to results on peptide:N-glycosidase F released glycans from a non-commercial source of zucchini ascorbate oxidase [59]. This shows the importance of adequate structural determination of glycan structures before making conclusions as to whether xylose or α1,3-fucose is more important as an allergenic epitope.

In conclusion, xylosylated and/or α1,3-fucosylated N-glycans were found to account for all the detected N-glycan structures from soluble extracts of three grass family pollens and two ragweed pollens, while tree pollens contained xylosylated and xylosylated/α1,3-fucosylated glycans as well as some oligomannose structures. The finding of oligosaccharides of known immunogenicity in all these samples could account for some allergenic and immunogenic responses as well as immunological cross-reactions with foods. glycans as well as some oligomannose structures. The finding of oligosaccharides of known immunogenicity in all these samples could account for some allergenic and immunogenic responses as well as immunological cross-reactions with foods.

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