

N-Glycan Analysis by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry of Electrophoretically Separated Nonmammalian Proteins: Application to Peanut Allergen Ara h 1 and Olive Pollen Allergen Ole e 1

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A method has been developed which allows the analysis of glycoproteins separated by SDS-PAGE. The procedure, though applicable to N-glycosylated glycoproteins of any origin, is particularly devised for glycoproteins potentially containing fucose in α 1,3-linkage to the reducing GlcNAc as may be found in plants and invertebrates, e.g., insects and parasitic helminths. Starting with an established procedure for mass spectrometric peptide mapping, the analysis of N-glycans by matrix-assisted laser desorption/ionization mass spectrometry involved the use of peptide:N-glycosidase A, a triphasic microcolumn for sample cleanup, and a new matrix mixture consisting of 2,5-dihydroxybenzoic acid, 1-hydroxyisoquinoline, and arabinosazone. The method was tested on proteins with N-glycans of known structure, i.e., as horseradish peroxidase, zucchini ascorbate oxidase, soybean agglutinin, honeybee venom hyaluronidase, bovine ribonuclease B, and bovine fetuin. An electrophoretic band corresponding to 4 μ g of glycoprotein was generally sufficient to allow detection of the major N-glycan species. As an additional benefit, a peptide mass map is generated which serves to identify the analyzed protein. The method was applied to glycoprotein allergens whose glycan structures were unknown. Ara h 1 and Ole e 1, major allergens from peanut and olive pollen, respectively, contained mainly xylosylated N-glycans with the composition $\text{Man}_{3(-4)}\text{XylGlcNAc}_2$ in the case of Ara h 1 and $\text{GlcNAc}_{1-2}\text{-Man}_3\text{XylGlcNAc}_2$ in the case of Ole e 1 where also some $\text{GlcNAc}_{0-2}\text{Man}_3\text{XylFucGlcNAc}_2$ was found. © 2000

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Given the millions of species of plants, insects, and other invertebrate animals, relatively little research on the protein glycosylation of these organisms, as compared to mammals, especially humans, has been performed. Consequently, methods and knowledge in this area lag behind. However, there are at least two good reasons for a more extensive study of this area: (i) the obscuring influence of highly cross-reactive carbohydrate epitopes on *in vitro* allergen diagnosis (1, 2) and (ii) the increasing use of nonmammalian expression systems such as plants and insect cells for the production of recombinant glycoproteins for diagnosis (e.g., allergy diagnosis) or even therapy. These products, however, carry nonhuman, immunogenic oligosaccharides (3, 4).

In plants, glycoprotein N-glycans can either be of the oligomannosidic or of the complex type (5). The latter, in contrast to mammalian complex-type N-glycans are, according to the current knowledge, uncharged, non-sialylated oligosaccharides with up to 13 sugar residues. Most glycoproteins hitherto studied contain smaller, “truncated” structures terminating in mannose or GlcNAc (for examples, see Fig. 1) (6–9).

There is now ample evidence that plant glycoproteins with asparagine-linked oligosaccharides elicit antibodies which bind to xylosylated and/or core- α 1,3-fucosylated N-glycans structures of the so-called vacuolar type (2, 6, 10). In principle, also the Lewis A-containing antennae of “secreted-type” N-glycans may contribute to immunogenicity (11). Although being specific in terms of epitope structure, such antibodies are highly cross-reactive due the fact that numerous plant glycoproteins share a limited set of conserved glycan structures (2, 12, 13). In fact, recent studies on the N-glycans of 10 allergenic pollens (8) and of 27 foods (I. B. H. Wilson, R. Zeleny, D. Kolarich, E. Staudacher, C. Stroop, J. P. Kamerling, and F. Altmann, in

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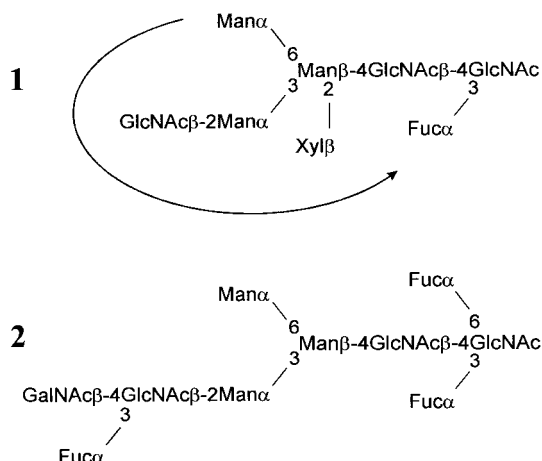


FIG. 1. Selected examples of *N*-glycans from plants and insects. Structure **1** shows the plant *N*-glycan structure MGnXF³. In this abbreviation the terminal residues are named starting with the residue on the 6-linked antenna and proceeding counterclockwise as indicated. The isomer with GlcNAc bound to the 6-linked mannose thus had the name GnMXF³. Where ambiguous, the linkage of the sugar is depicted as a superscript (for details, see (4, 8). Without any terminal GlcNAc residue the structure would be named MMXF³. Removal of the 3-linked mannose would lead to MUXF³ (U for unsubstituted; older name MOXF³ (8)). Structure **2** depicts the most complex *N*-glycan found in bee venom hyaluronidase (16).

preparation) have shown that immunogenic *N*-glycans such as MMXF³ or MUXF³ (see Fig. 1 for structures) are found, albeit in varying proportions, in all of these samples. The cross-reactivity of antibodies against plant *N*-glycans is, however, not confined to plant glycoproteins but, according to current knowledge, includes proteins from insects, snails, and parasitic helminths which all may contain core- α 1,3-fucose and/or xylose (2, 4, 12, 14–22).

The glycosylation of individual plant glycoproteins is currently a great unknown and this may well be caused by a lack of suitable methodology. In the present study we analyzed Ara h 1 and Ole e 1, the major allergens of peanut and olive pollen, respectively, which are both glycoproteins containing a single *N*-glycosylation site (23, 24). While for Ara h 1, an involvement of the carbohydrate in histamine release could not be found (25), the *N*-glycan of Ole e 1 was shown to be capable of eliciting histamine release (26). No structural analysis of *N*-glycans from these allergens has so far been reported.

The possibility of encountering core- α 1,3-fucose is, from the analytical biochemist's view, the common denominator of glycoproteins from plants, insects, and

other nonvertebrates and it has profound repercussions on the analytical strategies that might be employed, as will be exemplified later. Until a very few years ago, an analytical protocol for glycoprotein *N*-glycans started with the thorough purification of the protein of interest. Now procedures have been devised which allow the direct analysis of glycoproteins from SDS-PAGE gels thus obviating the need for complete purification of the protein. Post SDS-PAGE, one strategy is the fluorescent labeling of enzymatically released glycans followed by HPLC (27). Another approach makes use of mass spectrometry. Many aspects of glycan analysis by mass spectrometry are presented in the excellent introduction to a recent paper of Küster *et al.* (28) and need not to be repeated here. It may, however, be useful to remember that while carbohydrate analysis by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) suffers from being less sensitive than the analysis of peptides, it has the advantage that the ratios of peak intensities essentially reflect the abundance of the various glycans (29).

Packer *et al.* (30) have devised an elegant approach for electrospray ionization-MS analysis which in principle should also be applicable to MALDI-MS. Herein, the gel is blotted to PVDF membranes and the glycoproteins are denatured and deglycosylated with *N*-glycosidase F. The *N*-glycans are then purified utilizing their affinity to graphitized carbon. The only method hitherto particularly dedicated to MALDI-MS was described by Küster *et al.* (28, 31). Here, the glycoproteins are digested by *N*-glycosidase F within the gel and the resulting oligosaccharides are eluted and purified by passage over a microcolumn containing cation and anion-exchange resin as well as reversed-phase matrix. This method appears superior as it omits the laborious blotting step which may result in substantial losses of glycoprotein. However, with regard to nonmammalian glycoproteins it suffers from a severe problem: *N*-glycosidase F cannot release glycans with a fucose in α 1,3-linkage to the reducing terminal GlcNAc (32, 33). This problem can be overcome by the use of *N*-glycosidase A, but with a mass of 75.5 kDa (34), this enzyme is much larger than *N*-glycosidase F having only 35 kDa (35). Thus penetration of *N*-glycosidase A into the gel might be hindered. Finally, but of utmost importance, *N*-glycosidase A acts efficiently only on glycopeptides but not on glycoproteins, denatured or not (33).

There is yet another problem associated with the analysis of a band from a gel and that is protein identity. Especially, if a crude preparation producing a multitude of bands is subjected to electrophoresis, identification of the protein in parallel with glycan analysis would be desirable. Therefore we describe herein a method that combines peptide and glycan mass mapping. First, tryptic peptides are generated, analyzed, and subsequently deglycosylated with *N*-gly-

² Abbreviations used: ACH, α -cyano-4-hydroxycinnamic acid; ARA, D-arabinosazone; DARCI, matrix mixture for MALDI-MS; DHB, 2,5-dihydroxybenzoic acid; HIC, 1-hydroxyisoquinoline; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; MMX, MMXF³, and others, *N*-glycan structures; see Tables 1 and 5 and Refs. (4, 8).

cosidase A. After cleaning of the oligosaccharides with a triphasic microcolumn they were analyzed by MALDI-MS using a new, powerful matrix mixture.

MATERIALS AND METHODS

Glycoproteins, Matrices, and Apparatus

Horseradish peroxidase (type VI-A), bovine fetuin, bovine ribonuclease B, soybean lectin, leukophytohemagglutinin, and olive pollen were purchased from Sigma. Zucchini (*Cucurbita* sp.) ascorbate oxidase was obtained from Roche and also from Sigma. Honeybee venom hyaluronidase was prepared from bulgarian bee venom as described (16). Unroasted peanuts were a kind gift of Dipl. Ing. Elisabeth Struppe (Farmgold, Wiener Neudorf).

2,5-Dihydroxybenzoic acid (DHB) was from Aldrich, whereas 1-hydroxyisoquinoline (HIC), α -cyano-4-hydroxycinnamic acid (ACH), and D-arabinose, used to prepare D-arabinosazone according to published procedures (36), were purchased from Fluka. Dowex 50 W $\times$ 8 (100–200 mesh) and AG3 $\times$ 4A (200–400 mesh) were from Bio-Rad. LiChroprep RP-18 (40–63 μ m) was from Merck. Gelloader tips from Molecular BioProducts (M β P) were used as microcolumns. Glycerol-free peptide:N-glycosidase A was prepared as described (34). Glycerol-containing peptide:N-glycosidase A, neuraminidase from *C. perfringens*, and sequencing grade trypsin (unmodified) were obtained from Roche.

Allergen Extracts

Peanuts were extracted with Tris/Tricine buffer as described (37). For electrophoresis, 10 μ l of a 1% (w/v) solution of the lyophilized extract was used.

Olive pollen extract was prepared by stirring 10 mg pollen in 0.4 ml 125 mM ammonium bicarbonate of pH 8.3 at 4°C overnight and subsequent centrifugation. To 80 μ l of this extract, 400 μ l cold methanol was added and the sample was kept at 4°C overnight to precipitate proteins. After centrifugation and drying under a stream of nitrogen, the pellet was dissolved in SDS sample buffer.

Electrophoresis and Peptide Mapping

Reducing SDS-PAGE was performed according to established procedures using a Bio-Rad Mini-Protein II cell and gels made of 12.5% (w/v) or, in the case of olive pollen, of 14% (w/v) total monomer with 1% cross-linker. Gels were fixed (50% (v/v) methanol, 7% (v/v) acetic acid) and stained with Coomassie blue G in perchloric acid (38). Bands of interest were excised with a scalpel and, after washing and S-carboxamidomethylation, subjected to tryptic digestion as described in detail elsewhere (39). The extracted peptides were typically taken up in 10 μ l (or more) of 5% (v/v) formic acid and analyzed by MALDI-MS. Deviating

from published protocols, the sample (0.8 μ l) was dried on the platen followed by addition of matrix solution (0.8 μ l of 1% (w/v) ACH in 70% (v/v) acetonitrile). Peptides were measured with a "dynamic extraction" setting of 0.1. Average masses of $[M+H]^+$ ions were determined using human bradykinin and human renin substrate tetradecapeptide for external calibration of the instrument. Theoretical peptide maps were constructed with the ExPASy "Peptide-Mass" program.

Preparation and MALDI-MS of N-Glycans

Following withdrawal of an aliquot of the tryptic digest for peptide mapping, the samples were dried in a speed-vac concentrator and dissolved in 25 mM ammonium acetate of pH 5.0. In order to inactivate any residual trypsin, the samples were heat-treated for 6 min at 95°C. After cooling and brief centrifugation, 30 μ U of N-glycosidase A (0.2 μ l of the glycerol-free enzyme preparation) was added and deglycosylation was performed overnight at 37°C. Where stated, the same amount of glycerol-containing N-glycosidase A (0.6 μ l) was employed. In the case of sialylated glycoproteins, 50 μ U neuraminidase was additionally included.

To remove peptides and salts, the digest was applied to a microcolumn prepared as follows. A 10- μ l portion of a slurry of Dowex 50W $\times$ 8 previously washed with 2% (v/v) acetic acid was transferred into a Gelloader tip. To compact the gel, two 20 μ l portions of 2% acetic acid were forced through it by gently applying pressure using a 1 ml pipette tip. Subsequently, 10 μ l of a suspension of LiChroprep RP18 in 96% (w/w) ethanol was layered on top of the cation exchanger and washed twice with 20 μ l 96% ethanol. Finally, 10 μ l of a slurry of AG 3 in 2% acetic acid was added. Finally the column was washed with several portions of 2% acetic acid. The entire sample was pipetted on top of the microcolumn, and the sample tube was rinsed with 10 μ l of 2% acetic acid which was likewise transferred to the microcolumn. It is crucial to prevent the column from running dry as then the sample will elute too quickly. The total eluate of approximately 30 μ l was collected in a new microcentrifuge tube and evaporated. If glycerol-containing N-glycosidase was used, a small drop of glycerol remained in the vial. The microcolumns could usually be used up to four times if thoroughly washed.

For MALDI-MS analysis, the samples were redissolved in 10 μ l water. Generally, a 0.8- μ l sample was first spotted onto the target and dried, and then 0.8 μ l matrix was pipetted onto the dry sample. The platen was immediately transferred to a desiccator and vacuum was applied until all solvent had evaporated. Premixing of sample and matrix usually gave similar results. Several matrices were tested: (a) DHB alone was used as a 2% (w/v) solution in 30% (v/v) acetonitrile; (b) K-DHB was made adding 1% (v/v) of 1 M KCl; (c) arabinosazone (ARA) was dissolved to 1% (v/v) in ace-

TABLE 1
Summary of Identified Glycopeptides and Their Glycan Compositions^a

Peptide	Sugar residues				Average mass of [M+H] ⁺	
	Hex	HexNA	Pen	dHex	Calculated	Experimental
Horseradish peroxidase						
31–49 ^b	3	2	1	1	3379.5	3380.3
31–49 ^c	3	2	1	1	3393.6	3394.7
184–189	3	2	1	1	1843.8	1842.8
214–236	6	4	2	2	4986.1	4987.0
272–294	3	2	1	1	3673.9	3673.9
295–313	3	2	1	1	3355.5	3355.1
Ribonuclease B						
58–63	5	2	—	—	1935.9	1935.6
58–63	6	2	—	—	2098.1	2097.3
Hyaluronidase						
262–269	3	2		1	2002.0	2003.0
262–269	3	2		2	2148.1	2149.2
Soybean agglutinin						
95–117	9	2			4381.4	4383.2
Ole e 1						
105–111 ^d	3	2	1	1	2407.5 ^d	2407.1
105–111 ^d	5	2			2453.5	2454.0
105–111 ^d	3	3	1		2464.5	2464.5
105–111 ^d	3	3	1	1	2610.7	2610.9
105–111 ^d	6	2			2615.1	^e
105–111 ^d	4	3	1		2626.7	2626.4
105–111 ^d	3	4	1		2667.7	2667.8
105–111 ^d	7	2			2777.8	2778.0
105–111 ^d	3	4	1	1	2813.9	2814.1
105–111 ^d	8	2			2939.9	2940.3

^a The corresponding N-glycan structures are specified in Tables 2 and 3.

^b With pyroglutamic acid and acetamide-modified cystein (mass of protonated peptide = 2208.5).

^c With pyroglutamic acid and acrylamide-modified cystein (mass of protonated peptide = 2222.5).

^d Only for variants where residue 106 is Ile (mass of protonated peptide = 1236.4).

^e Unresolved shoulder of the peak at m/z = 2610.9.

tonitrile; (d) DHB-ARA was made by mixing the above matrix solutions in a ratio of 1:1; (e) HIC-DHB consisted of 0.2 M DHB and 0.06 M HIC in 50% (v/v) acetonitrile as described (40); and (f) DARCI was prepared by blending equal volumes of DHB, ARA, and HIC-DHB. It proved essential to mix the three solutions immediately before use. Oligosaccharides were analyzed with a “dynamic extraction” setting of 0.1. Spectra were compiled “manually” by addition of single shots rather than by automatic repetitions. Average masses of [M+Na]⁺ ions were determined using a partial dextran hydrolysate for external calibration of the instrument.

All spectra were recorded on a DYNAMO (Thermo-BioAnalysis), a linear time-of-flight MALDI-MS instrument capable of dynamic extraction, a synonym for delayed extraction.

RESULTS

Peptide Mapping and Identification of Glycopeptides

In principle, a MALDI peptide spectrum can also give information regarding N- and O-glycosylation of a

protein provided the glycopeptides can be identified. The N-glycans of purple acid phosphatase from red kidney beans have been studied in this way (41). However, suppression effects are a notorious problem in MALDI-MS (42). To overcome this problem, in one study the glycopeptides from interferon- γ have been isolated by HPLC prior to MALDI-MS, thus allowing the characterization of its N-glycosylation (43).

In the present study, glycopeptides could be identified in the digests from some of the glycoproteins studied but not from ascorbate oxidase, fetuin, and Ara h 1. Bee venom hyaluronidase contains two potential N-glycosylation sites (Swiss-Prot database). For glycosylation site 263 two N-glycopeptides could be identified, indicating that hyaluronidase contains at least two glycans species of different mass (Table 1). The oligomannosidic glycopeptides of soybean agglutinin and ribonuclease B could be detected, in the latter case, however, as a very small peak (Table 1). In horseradish peroxidase, no fewer than 6 of the 8 glycosylation sites could be identified which all contained MMXF³ glycans (Table 1). This screening of spectra for glycopeptide

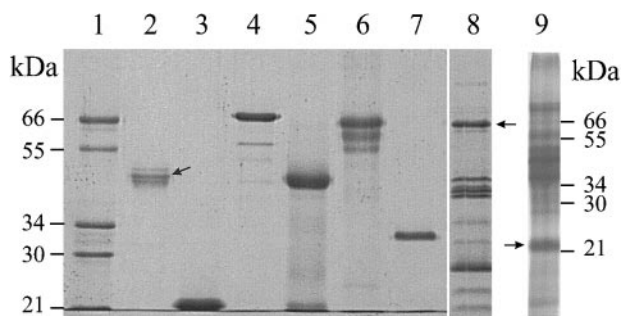


FIG. 2. SDS-PAGE of standard proteins and peanut extract. Four micrograms of the pure proteins, 100 μ g of peanut extract, and the precipitated proteins from 80 μ l of olive pollen extract (roughly corresponding to 2 mg of pollen) were applied on the gel. The lanes contained the following samples: 1, molecular mass standards; 2, horseradish peroxidase; 3, bovine ribonuclease B; 4, zucchini ascorbate oxidase; 5, bee venom hyaluronidase; 6, bovine fetuin; 7, soybean lectin; 8, peanut extract; and 9, olive pollen extract. The bands excised and analyzed either were the strongest band of the respective lane or were those identified by arrows.

peaks was performed with the *N*-glycan mass data already known—either from the literature or from this study. It remains doubtful whether it would have been possible to identify these sometimes multiply modified peptides (Table 1) without prior knowledge of the glycan types. Theoretically, a peptide-map performed after hydrolysis of glycopeptides by *N*-glycosidase A or F could yield information about the *N*-glycan. In practice however, the spectra were significantly poorer after this step, thus even some nonglycosylated peptides could no longer be found. Confirming a recent paper by Krause *et al.* (44), we observed that most of the detectable glycopeptides and most of the peptides giving high intensity peaks contained an Arg residue. For instance, when leuko-phytohemagglutinin was digested with trypsin in solution, only the Arg-terminated oligomannosidic glycopeptide but not the Lys-containing complex-type glycopeptide (45) could be identified (data not shown). The glycosylation itself does not seem to be a hindrance for effective laser desorption ionization as, e.g., the glycopeptide peaks from Ole e 1 were quite intense (Fig. 2, Table 1). Even though, MALDI-MS analysis of glycopeptides from digestion mixtures holds little promise as a generally applicable strategy.

N-Glycan Release and Purification

Owing to the above described problems, we redesigned the approach taken by Küster *et al.* (28, 31) for the analysis of nonvertebrate *N*-glycans. As outlined in the introduction, *N*-glycosidase A requires glycopeptides as substrates. Therefore, the protocol of Jensen *et al.* (39) was followed to isolate tryptic (glyco-)peptides from electrophoresis bands. As a highly valuable by-product, these tryptic peptides can be used for protein identification as was necessary in this work for the

allergens Ara h 1 and Ole e 1 (see below). Following enzymatic liberation of the *N*-glycans, peptides and other substances interfering with MALDI-MS of sugars had to be removed. The laser desorption/ionization of oligosaccharides is considerably less effective and more prone to interference by contaminations than that of peptides (29). Initial attempts with cation-exchange resin added directly to the sample gave very poor results. Therefore we tried to improve the ion yield of *N*-glycans by derivatization. Mo *et al.* (46) have claimed procaine derivatives to be highly useful compounds for MALDI analysis providing a 1000 times higher sensitivity than underivatized glycans. To this end, we derivatized maltohexaose with 4-aminobenzoic acid diethylaminoethyl ester and also with 2-aminopyridine and purified the products by HPLC. Comparative analysis of underivatized, procaine and aminopyridine-labeled hexaose did not reveal any significant gain in sensitivity on our instrument (data not shown). Since any derivatization procedure will inevitably lead to sample losses, we decided to analyze underivatized, reducing oligosaccharides. To this end, the cleanup strategy described by Küster *et al.* (28, 31) was adopted with minor changes: The reversed-phase matrix which tends to float in aqueous solvents was used as the middle layer, thereby being held in place by the anion-exchange resin. Dilute acetic acid was used as the solvent in order to promote binding of peptides to cation-exchange resin. Incidentally, this eluent allows at least monosialylated glycans to pass through the anion-exchange section of the column.

Matrices for *N*-Glycans

One obvious problem encountered when analyzing the glycans of electrophoretically separated glycoproteins is the low amount of sample. Therefore, strategies to optimize the sensitivity had to be developed. The first choice of matrix for MALDI analysis of carbohydrates is DHB (47). For the analysis of small amounts of sample, however, very small total sample volumes must be chosen, and thus (irremovable) contaminants appear in high concentration. In such samples, DHB required a few high-energy "preshots" before glycan peaks were detected. Unfortunately, this also may enhance the appearance of background peaks especially of mass 1135.4, 1209.1, and 1282.3 (± 0.3 Da). Various ways of forming the matrix layer were tried but none proved better than the technique of immediate drying of the matrix. Super-DHB (48) gave no improvement of signal on the DYNAMO. Arabinosazone, a promising new matrix for carbohydrates (36), when used alone, was too fluffy so that two or three shots often removed the entire matrix layer at the particular spot—even though the laser energy used was several percent below that required with DHB. Therefore we tried to stabilize the ARA by mixing it with DHB. Various

TABLE 2
Interpretation of MALDI-MS Data for Reference Glycoproteins

Molar composition				Structure name ^a	Calculated average mass of [M+Na] ^{±b} (Da)
Hex	HexNAc	Pen	dHex		
1	2	0	2	UUF ³ F ⁶	901.7
2	2	0	1	MUF ³ /MUF ⁶	917.8
3	2	0	0	MM (Man3)	933.8
2	2	1	1	MUXF ³	1049.9
2	2	0	2	MUF ³ F ⁶	1064.0
3	2	1	0	MMX	1065.9
3	2	0	1	MMF ³ /MMF ⁶	1080.0
4	2	0	0	Man4	1096.0
3	2	1	1	MMXF ³	1212.1
3	2	0	2	MMF ³ F ⁶	1226.1
4	2	1	0	Man4X	1228.1
5	2	0	0	Man5	1258.1
3	3	1	0	GnMX/MGnX	1269.1
4	2	1	1	Man4XF ³	1374.2
3	3	1	1	GnMXF ³ /MGnXF ³	1415.3
6	2	0	0	Man6	1420.3
4	4	0	0	GalGn/GnGal	1502.4
7	2	0	0	Man7	1582.4
3	4	0	2	^c	1632.5
5	4	0	0	GalGal	1664.5
8	2	0	0	Man8	1744.5
3	4	0	3	^d	1778.6
5	4	0	0	^e	1867.7
9	2	0	0	Man9	1906.7
5	5	0	0	^f	2029.8
5	5	+ Sialic acid		^g	2343.0

^a Abbreviated names for *N*-glycan structures are explained in detail in previous papers (4, 8) and are partially exemplified in Figs. 1 and 5. The structures explained in the following footnotes are not amenable to this nomenclature.

^b Experimental values agreed with these masses to within 0.3 Da. See also Fig. 3.

^c Similar to Structure 2 in Fig. 1 but lacking one of the fucose residues linked to the reducing GlcNAc.

^d Structure 2 in Fig. 1.

^e Triantennary *N*-glycan lacking one terminal Gal residue.

^f Triantennary asialo *N*-glycan.

^g Triantennary *N*-glycan with one *N*-acetylneuraminic acid residue present as the sodium salt (see also text).

ratios gave quite satisfactory results. Another highly useful matrix formulation proved to be HIC-DHB (40), which allowed desorption of glycans at lower laser energies than DHB.

For many samples, the best results were, however, obtained with the ternary mixture DARCI. The significantly reduced laser energy required as compared to DHB only matrices gave a better signal to noise ratio even in the mass range below 1000 Da. No, or just a minimum of, "burn-in" shots were required and the appearance of said background peaks was almost negligible. Thus the identification of oligosaccharide signals was in many cases easier and less equivocal than with the other matrices. Another advantage comes from the high sodium content of DARCI resulting in a strong dominance of the sodium adduct peak. The only minor drawback was the appearance of a peak at 972.2 Da which probably could be avoided by improved purification of the arabinosazone. The DARCI matrix mixture must be prepared fresh from the three solutions

before use. For unknown reasons, direct dissolution of the solid substances did not give satisfactory results. With some samples the best results were nevertheless obtained with plain DHB albeit with a higher operator effort. This may lead to equivocal results as often the potassium adduct ion of a glycan species has the same mass as another structure since the mass difference of hexose and deoxyhexose is 16 Da. In such cases, the potassium enriched matrix K-DHB can clarify ambiguities.

Use of Commercial, Glycerol-Containing Peptide: N-Glycosidase A

The observations described so far apply to samples treated with glycerol-free *N*-glycosidase A. However, the commercially available enzyme contains 50% glycerol. Thus, a small drop of glycerol will remain upon evaporation of the samples after purification on the microcolumn. In this case, DARCI was a less effective

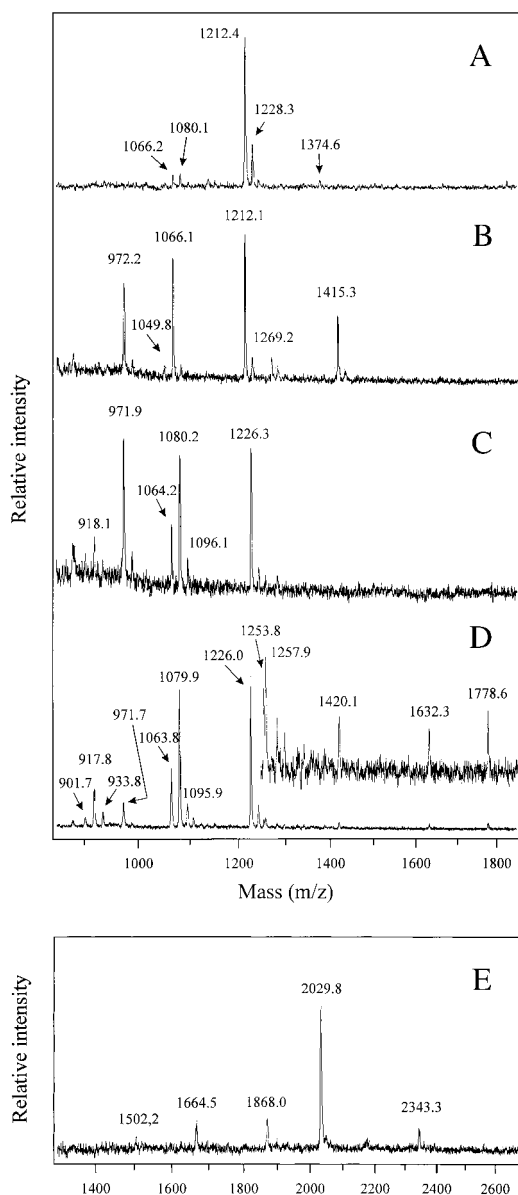


FIG. 3. MALDI mass spectra of *N*-glycans from reference glycoproteins. (A) *N*-Glycans from the major band of a sample of 4 μ g horseradish peroxidase; (B) oligosaccharides obtained from 4 μ g ascorbate oxidase (Roche); (C) *N*-glycans from a 4- μ g sample of bee venom hyaluronidase; (D) *N*-glycans from three combined 8- μ g samples of hyaluronidase; (E) "asialo"-oligosaccharides from 4 μ g of bovine fetuin. Interpretation of peak masses in terms of sugar composition and the calculated masses of the ions are summarized in Table 2. These spectra were acquired with DARCI as the UV-absorbing matrix. Peaks at m/z 972 derive from the matrix. Analysis of samples from peroxidase and ascorbate oxidase with K-DHB (data not shown) revealed the peaks at m/z = 1228 to be potassium adducts and not additional glycan species.

matrix than DHB which, however, crystallized very slowly even *in vacuo*. Instead of the uniform, microcrystalline layer obtained with glycerol-free samples, large irregular needles formed. While it was difficult to find suitable spots for MALDI, rather good spectra

were obtained if such spots could be found. MALDI analysis was considerably more awkward as compared to samples devoid of glycerol but the final results were sometimes only slightly inferior in terms of signal to noise ratio and the presence of impurity peaks. However, more often than not it will be advantageous to remove the glycerol by, e.g., microdialysis.

Analysis of *N*-Glycans from Reference Glycoproteins

Horseradish peroxidase was a sample which proved easy to analyze as it contains per molecule eight *N*-glycans of almost uniform structure, i.e., MMXF³ (7, 49). Thus, even 1 μ g of peroxidase applied to the SDS-PAGE gel allowed identification of the major structure as a large peak. With 4 μ g (ca. 100 pmol) of protein, some minor *N*-glycans could be identified (see Table 2; Fig. 3A), which is in keeping with earlier reports (7, 49). As can be seen from Fig. 2 (SDS-PAGE of standard proteins and others), peroxidase divides into at least three bands, thus the actual amount of protein analyzed was in the range of 60 pmol. Given the eight glycosylation sites, the estimated amount of, e.g., MMX in the total sample amounted to 21 pmol and thus 1.5 pmol of glycan had been on the MALDI target, assuming no loss between electrophoresis and spotting.

Another plant glycoprotein with complex-type structures is ascorbate oxidase from zucchini, which previ-

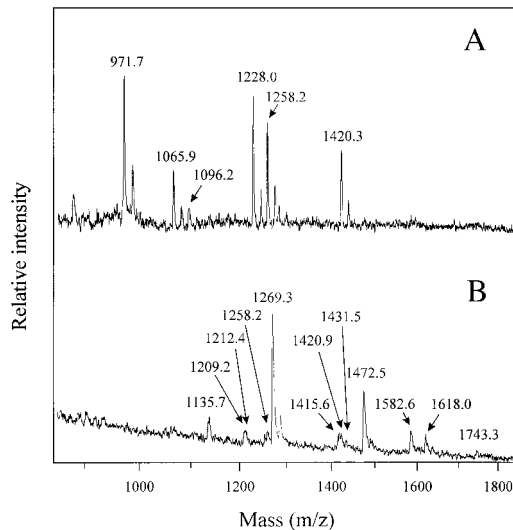


FIG. 4. MALDI mass spectra of *N*-glycans from peanut allergen Ara h 1 (A) and olive pollen allergen Ole e 1 (B). Interpretation of peak masses in terms of sugar composition and the calculated masses of the ions are summarized in Table 2. For the Ara h 1 spectrum, DARCI was used as the matrix. The spectrum of Ole e 1 glycans was made with plain DHB matrix. The peaks at 1135.7 and 1209.2 represent matrix impurities. The latter peak is poorly separated from a compound with 1212.4 Da, presumably MMXF³. The peaks identified by mass labels represent [M+Na]⁺ ions. Other peaks are potassium adducts of the same glycans or notorious impurities (see text).

TABLE 3
Asn-Linked Oligosaccharides from Allergens Ara h 1 and Ole e 1

Average mass of [M+Na] ⁺									
Experimental		Calculated	Molar composition				Structure name ^a	Peak area in %	
Ara h 1	Ole e 1		Hex	HexNAc	Pen	dHex		Ara h 1	Ole e 1
1065.9	—	1065.9	3	2	1	0	MMX	15.8	—
—	1212.4	1212.1	3	2	1	1	MMXF ³	—	3.0
1096.2	—	1096.0	4	2	0	0	Man4	4.9	—
1228.0	—	1228.1	4	2	1	0	Man4X	32.6	—
1258.2	1258.2	1258.1	5	2	0	0	Man5	27.5	1.0
—	1269.3	1269.1	3	3	1	0	GnMX/MGnX	—	54.0
—	1415.6	1415.3	3	3	1	1	GnMXF ³ /MGnXF ³	—	2.0
1420.3	1420.9	1420.3	6	2	0	0	Man6	19.1	2.0
—	1431.5	1431.3	4	3	1	0	Man4GnX	—	2.0
—	1472.5	1472.3	3	4	1	0	GnGnX	—	23.0
—	1582.6	1582.4	7	2	0	0	Man7	—	8.0
—	1618.0	1618.3	3	4	0	2	GnGnXF ³	—	4.0
—	1743.3	1744.5	8	2	0	0	Man8	—	1.0

^a Tentatively assigned *N*-glycan structures are depicted in Fig. 5.

ously was shown to contain four different *N*-glycan structures (9). All of these structures could be found by MALDI-MS analysis when 4 μ g protein was loaded on the gel (Fig. 3B). The theoretical amount of MUXF³ (m/z = 1049.8) on the MALDI target was calculated to be 65 fmol. The results shown in Fig. 3B and Table 2 were obtained with enzyme from Roche. The ascorbate oxidase from Sigma contained the same structures in a very similar proportion. Thus, in contrast to the ascorbate oxidase from *Cucurbita pepo medullosa* (50), these commercial products contain core α 1,3-fucose.

Two glycoproteins, one from a plant, one from a mammal, having oligomannosidic glycans were subjected to the procedure. In bovine ribonuclease B, four oligomannosidic *N*-glycans were found in a very similar ratio as described previously (64% Man5, 29% Man6, 4% each Man7 and Man8) (51); however, a trace of Man₉GlcNAc₂ found by other methods (31, 51) was not seen. Man₉GlcNAc₂ though was identified as expected as the major *N*-glycan in soybean agglutinin (52).

When the partially core- α 1,3-fucosylated insect glycoprotein hyaluronidase from honeybee venom was analyzed, essentially all structures previously identified where found in the spectra (Table 2). While 4 μ g of protein gave intense signals for all glycan species comprising at least 8% of the total (Fig. 3C) (16), the spectrum obtained from 25 μ g of protein—loaded on three separate lanes—allowed detection of even trace components (Fig. 3D). The complex-type *N*-glycan 2 (Fig. 1) which only represents 2.7% of the glycan pool according to HPLC could thus be found.

In the case of bovine fetuin which carries sialylated complex-type *N*-glycans, *N*-glycosidase A and neuraminidase were used simultaneously to allow

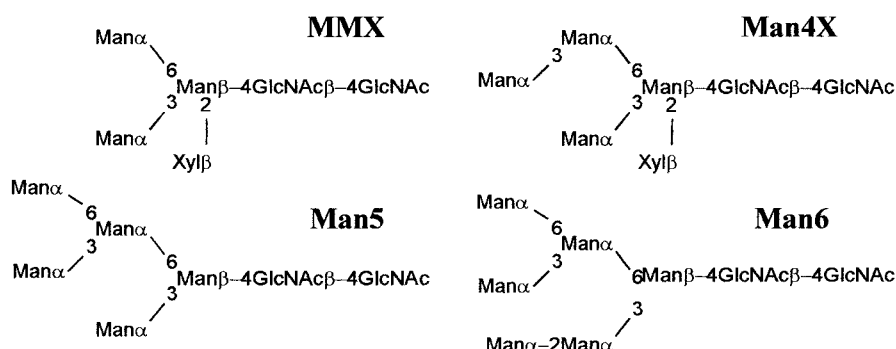
MALDI-MS using DHB or DARI as the matrix. Apart from the major compound possessing three complete antennae and three smaller variants, a peak (m/z = 2343.3) corresponding to a structure apparently containing *N*-acetylneuraminic acid as its sodium salt could also be observed. It may be speculated that this is a NeuNAc residue-linked α 2,6 to GlcNAc (53). This residue might well be in a configuration that makes it inaccessible to neuraminidase.

Analysis of the Peanut Allergen Ara h 1 and Olive Pollen Allergen Ole e 1

As a realistic test of the new system two proteins with as yet unknown glycosylation were analyzed after SDS-PAGE of allergen extracts. Ara h 1 is the most significant peanut allergen (23). It is a glycoprotein of about 66 kDa with a single *N*-glycosylation site. From an SDS-PAGE of a peanut extract, the band of appropriate size was subject to the procedure herein described. The protein was identified by its peptide map as Ara h 1 (Swissprot P43237). *N*-glycan analysis revealed five glycan species (Fig. 4A; Table 3). From peak masses in conjunction with knowledge about structure and biosynthesis of plant *N*-glycans, structures were tentatively assigned as shown in Fig. 5. Remarkably, the single glycosylation site of Ara h 1 bears both oligomannosidic and xylosylated, complex-type *N*-glycans in a one to one ratio, while no fucosylated species could be found.

Ole e 1 exists in several isoforms (Swissprot P19963) (24). Peptide mapping revealed the isoform with Ile in position 106 as predominant in the pollen batch analyzed. Probably due to its terminal Arg residue, the signals corresponding to glycopeptides were very

Ara h 1



Ole e 1

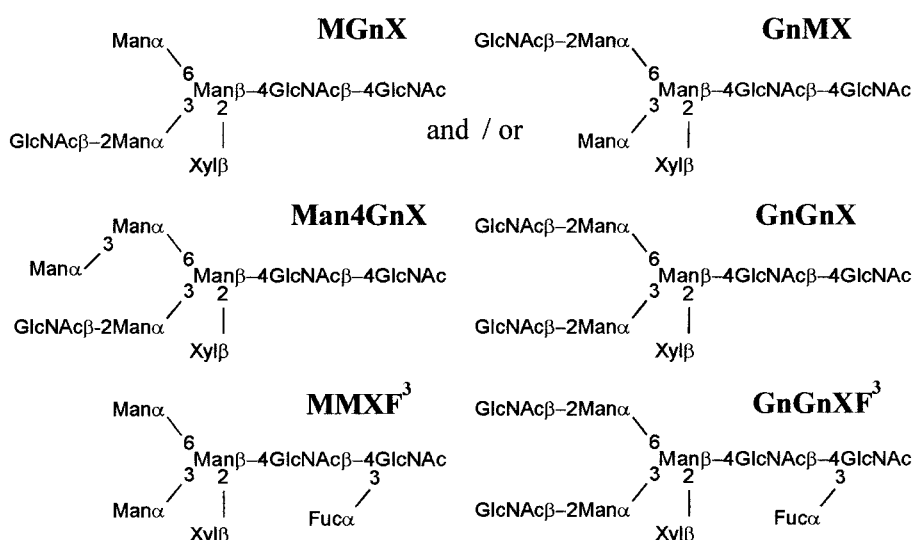


FIG. 5. Tentatively assigned structures of *N*-glycans from Ara h 1 and Ole e 1. The assignment of the Man4X structure is based on the specificity of a plant α -mannosidase II (56). Symbols for sugar residues are as follows: M, mannose; Gn, *N*-acetylglucosamine; F, fucose; X, xylose.

strong and allowed the compositions of at least nine *N*-glycan species to be assigned (Table 1). The glycopeptides were nonetheless digested with *N*-glycosidase A to yield the free oligosaccharides. Their analysis corroborated the identification of glycopeptides (Table 3). Xylosylated structures with one or two terminal GlcNAc residues were found to dominate and were accompanied by some additionally fucosylated glycans and oligomannosidic structures. These structures are a subset of those found during the previous analysis of entire olive pollen extracts (8).

DISCUSSION

The method described in this paper facilitates the analysis of glycoprotein *N*-glycans from electrophoretic

bands corresponding to a few micrograms of proteins from nonmammalian but also from mammalian origin. Depending on the amount of sample there is a certain threshold for minor species. This threshold can be lowered by combining two or three protein bands, e.g., in the case of hyaluronidase. Even then MALDI analysis of carbohydrates is in most cases less sensitive than that of peptides owing on one hand to the notoriously less efficient desorption/ionization mechanism of carbohydrates and, in addition, to the heterogeneity of *N*-glycans at a particular glycosylation site. However, with ascorbate oxidase, minor structures accounting for less than 100 fmol on the sample plate could still be identified. Labeling of glycans did not, in our hands, improve the detection limits. The use of a ternary ma-

trix mixture termed DARCI in many cases facilitated the mass spectrometric analysis but did not substantially lower the achievable detection limit.

Obviously, the basic concept of the method can be modified in several ways. For mammalian glycoproteins, the use of *N*-glycosidase F in a slightly basic, volatile buffer might be more economical. Glycans could be analyzed by electrospray ionization mass spectroscopy employing on-line desalting on graphitized carbon (30). This technique would obviate the need for the anion-exchange resin and thus permit the analysis of sialylated and sulfated *N*-glycans.

The new technique allowed determination of the *N*-glycan structures of two prominent allergens, Ara h 1 and Ole e 1. Carbohydrate specific antibodies toward Ara h 1 have recently been reported as having no clinical significance as judged by basophil histamine release assays (25). This was interpreted as a general inability of protein-oligosaccharides to elicit clinical symptoms. It could, however, also just reflect the notion that monovalent epitopes are unable to trigger a cellular response as they cannot crosslink cell-surface-bound IgE. A third explanation, however, comes from the results of this work. In several instances, core α 1,3-fucose was identified as the immunodominant sugar residue on complex plant carbohydrates both for IgG and IgE (2, 32, 54, 55). As the *N*-glycans of Ara h 1 contained no detectable amounts of core-fucosylated *N*-glycans, their affinity to IgE may be too low to elicit histamine release even though they bind IgE in an *in vitro* assay system. However, the free *N*-glycans of Ole e 1 released by *N*-glycosidase F were reported to elicit basophil histamine release (26). As both Ara h 1 and Ole e 1 contain only one glycosylation site and both bear mainly xylosylated structures these divergent results are not readily explained by the data presented here. However, we are confident that future applications of the method herein presented will assist in elucidating the role of Asn-linked carbohydrate in immunological processes.

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REFERENCES

- Mari, A., Iacovacci, P., Afferni, C., Barletta, B., Tinghino, R., Di Felice, G., and Pini, C. (1999) Specific IgE to cross-reactive carbohydrate determinants strongly affect the *in vitro* diagnosis of allergic diseases. *J. Allergy Clin. Immunol.* **103**, 1005–1011.
- Wilson, I. B. H., Harthill, J. E., Mullin, N., Ashford, D., and Altmann, F. (1998) Core α 1,3-fucose is a key part of the epitope recognised by antibodies reacting against plant *N*-linked oligosaccharides. *Glycobiology* **8**, 651–661.
- Cabanes-Macheteau, M., Fitchette-Laine, A. C., Loutelier-Bourhis, C., Lange, C., Vine, N. D., Ma, J. K., Lerouge, P., and Faye, L. (1999) *N*-Glycosylation of a mouse IgG expressed in transgenic tobacco plants. *Glycobiology* **9**, 365–72.
- Altmann, F., Staudacher, E., Wilson, I. B. H., and März, L. (1999) Insect cells as hosts for the expression of recombinant glycoproteins. *Glycoconj. J.* **16**, 109–123.
- Lerouge, P., Cabanes-Macheteau, M., Rayon, C., Fitchette-Laine, A. C., Gomord, V., and Faye, L. (1998) *N*-glycoprotein biosynthesis in plants: recent developments and future trends. *Plant Mol. Biol.* **38**, 31–48.
- Kurosaka, A., Yano, A., Itoh, N., Kuroda, Y., Nakagawa, T., and Kawasaki, T. (1991) The structure of a neural specific carbohydrate epitope of horseradish peroxidase recognized by anti-horseradish peroxidase antiserum. *J. Biol. Chem.* **266**, 4168–4172.
- Yang, B. Y., Gray, J. S., and Montgomery, R. (1996) The glycans of horseradish peroxidase. *Carbohydr. Res.* **287**, 203–212.
- Wilson, I. B. H., and Altmann, F. (1998) Structural analysis of *N*-glycans from allergenic grass, ragweed and tree pollens. Core α 1,3-fucose and xylose present in all pollens examined. *Glycoconj. J.* **15**, 1055–1070.
- Altmann, F. (1998) Structures of the *N*-linked carbohydrate of ascorbic acid oxidase from Zucchini. *Glycoconj. J.* **15**, 79–82.
- Faye, L., Gomord, V., Fitchette-Laine, A. C., and Chrispeels, M. J. (1993) Affinity purification of antibodies specific for Asn-linked glycans containing α 1-3 fucose or β 1-2 xylose. *Anal. Biochem.* **209**, 104–108.
- Fitchette, A. C., Cabanes-Macheteau, M., Marvin, L., Martin, B., Satiat-Jeunemaitre, B., Gomord, V., Crooks, K., Lerouge, P., Faye, L., and Hawes, C. (1999) Biosynthesis and immunolocalization of Lewis a-containing *N*-glycans in the plant cell. *Plant Physiol.* **121**, 333–344.
- Koshte, V. L., Kagen, S. L., and Aalberse, R. C. (1989) Cross-reactivity of IgE antibodies to caddis fly with arthropoda and mollusca. *J. Allergy Clin. Immunol.* **84**, 174–183.
- Faye, L., and Chrispeels, M. J. (1988) Common antigenic determinants in the glycoproteins of plants, molluscs and insects. *Glycoconj. J.* **5**, 245–256.
- Kubelka, V., Altmann, F., Staudacher, E., Tretter, V., März, L., Hård, K., Kamerling, J. P., and Vliegenthart, J. F. G. (1993) Primary structures of the *N*-linked carbohydrate chains from honeybee venom phospholipase A₂. *Eur. J. Biochem.* **213**, 1193–1204.
- Kubelka, V., Altmann, F., and März, L. (1994) Structures of the *N*-linked oligosaccharides of the membrane glycoproteins from three lepidopteran cell lines (SF-21, IZD-MB-0503, BM-N). *Arch. Biochem. Biophys.* **308**, 148–157.
- Kubelka, V., Altmann, F., and März, L. (1995) The asparagine-linked carbohydrate of Honeybee venom hyaluronidase. *Glycoconj. J.* **12**, 77–83.
- Hassani, O., Loew, D., Van Dorsselaer, A., Papandreou, M. J., Sorokine, O., Rochat, H., Sampieri, F., and Mansuelle, P. (1999) Aah VI, a novel, *N*-glycosylated anti-insect toxin from *Androctonus australis* hector scorpion venom: isolation, characterisation, and glycan structure determination. *FEBS Lett.* **443**, 175–180.
- Van Kuik, J. A., van Halbeek, H., Kamerling, J. P., and Vliegenthart, J. F. G. (1985) Primary structure of the low-molecular-weight carbohydrate chains of *Helix pomatia* alpha-hemocyanin. Xylose as a constituent of *N*-linked oligosaccharides in an animal glycoprotein. *J. Biol. Chem.* **260**, 13984–13988.
- van Tetering, A., Schiphorst, W. E., van den Eijnden, D. H., and van Die, I. (1999) Characterization of a core α 1,3-fucosyltransferase from the snail *Lymnaea stagnalis* that is involved in the synthesis of complex-type *N*-glycans. *FEBS Lett.* **461**, 311–314.
- van Die, I., Gomord, V., Kooyman, F. N., van den Berg, T. K., Cummings, R. D., and Vervelde, L. (1999) Core α 1-3-fucose is a

- common modification of N-glycans in parasitic helminths and constitutes an important epitope for IgE from *Haemonchus contortus* infected sheep. *FEBS Lett.* **463**, 189–193.
21. Haslam, S. M., Coles, G. C., Munn, E. A., Smith, T. S., Smith, H. F., Morris, H. R., and Dell, A. (1996) *Haemonchus contortus* glycoproteins contain N-linked oligosaccharides with novel highly fucosylated core structures. *J. Biol. Chem.* **271**, 30561–30570.
 22. Khoo, K. H., Chatterjee, D., Caulfield, J. P., Morris, H. R., and Dell, A. (1997) Structural mapping of the glycans from the egg glycoproteins of *Schistosoma mansoni* and *Schistosoma japonicum*: Identification of novel core structures and terminal sequences. *Glycobiology* **7**, 663–677.
 23. Shin, D. S., Compadre, C. M., Maleki, S. J., Kopper, R. A., Sampson, H., Huang, S. K., Burks, A. W., and Bannon, G. A. (1998) Biochemical and structural analysis of the IgE binding sites on Ara h 1, an abundant and highly allergenic peanut protein. *J. Biol. Chem.* **273**, 13753–13759.
 24. Villalba, M., Batanero, E., Monsalve, R. I., Gonzalez de la Pena, M. A., Lahoz, C., and Rodriguez, R. (1994) Cloning and expression of Ole e I, the major allergen from olive tree pollen. Polymorphism analysis and tissue specificity. *J. Biol. Chem.* **269**, 15217–15222.
 25. Van der Veen, M. J., van Ree, R., Aalberse, R. C., Akkerdaas, J., Koppelman, S. J., Jansen, H. M., and van der Zee, J. S. (1997) Poor biologic activity of cross-reactive IgE directed to carbohydrate determinants of glycoproteins. *J. Allergy Clin. Immunol.* **100**, 327–334.
 26. Batanero, E., Crespo, J. F., Monsalve, R. I., Martin-Esteban, M., Villalba, M., and Rodriguez, R. (1999) IgE-binding and histamine-release capabilities of the main carbohydrate component isolated from the major allergen of olive tree pollen, Ole e 1. *J. Allergy Clin. Immunol.* **103**, 147–153.
 27. Anumula, K. R., and Du, P. (1999) Characterization of carbohydrates using highly fluorescent 2-aminobenzoic acid tag following gel electrophoresis of glycoproteins. *Anal. Biochem.* **275**, 236–242.
 28. Küster, B., Hunter, A. P., Wheeler, S. F., Dwek, R. A., and Harvey, D. J. (1998) Structural determination of N-linked carbohydrates by matrix-assisted laser desorption/ionization-mass spectrometry following enzymatic release within sodium dodecyl sulphate-polyacrylamide electrophoresis gels: Application to species-specific glycosylation of alpha1-acid glycoprotein. *Electrophoresis* **19**, 1950–1959.
 29. Naven, T. J., and Harvey, D. J. (1996) Effect of structure on the signal strength of oligosaccharides in matrix-assisted laser desorption/ionization mass spectrometry on time-of-flight and magnetic sector instruments. *Rapid Commun. Mass Spectrom.* **10**, 1361–1366.
 30. Packer, N. H., Lawson, M. A., Jardine, D. R., Sanchez, J. C., and Gooley, A. A. (1998) Analyzing glycoproteins separated by two-dimensional gel electrophoresis. *Electrophoresis* **19**, 981–988.
 31. Küster, B., Wheeler, S. F., Hunter, A. P., Dwek, R. A., and Harvey, D. J. (1997) Sequencing of N-linked oligosaccharides directly from protein gels: In-gel deglycosylation followed by matrix-assisted laser desorption/ionization mass spectrometry and normal-phase high-performance liquid chromatography. *Anal. Biochem.* **250**, 82–101.
 32. Tretter, V., Altmann, F., and März, L. (1991) Peptide- N^4 -(N -acetyl- β -glucosaminyl)asparagine amidase F cannot release glycans with fucose attached α 1-3 to the asparagine-linked N -acetyl-glucosamine residue. *Eur. J. Biochem.* **199**, 647–652.
 33. Altmann, F., Schweiszer, S., and Weber, C. (1995) Kinetic comparison of peptide:N-glycosidases F and A reveals several differences in substrate specificity. *Glycoconj. J.* **12**, 84–93.
 34. Altmann, F., Paschinger, K., Dalik, T., and Vorauer, K. (1998) Characterisation of peptide- N^4 -(N -acetyl- β -glucosaminyl) asparagine amidase A (PNGase A) and its N-glycans. *Eur. J. Biochem.* **252**, 118–123.
 35. Tarentino, A. L., Gomez, C. M., and Plummer, T. H., Jr. (1985) Deglycosylation of asparagine-linked glycans by peptide:N-glycosidase F. *Biochemistry* **24**, 4665–4671.
 36. Chen, P., Baker, A. G., and Novotny, M. V. (1997) The use of osazones as matrices for the matrix-assisted laser desorption/ionization mass spectrometry of carbohydrates. *Anal. Biochem.* **244**, 144–151.
 37. Vieths, S., Hoffmann, A., Holzhauser, T., Muller, U., Reindl, J., and Haustein, D. (1998) Factors influencing the quality of food extracts for in vitro and in vivo diagnosis. *Allergy* **53** (46 Suppl), 65–71.
 38. Reisner, A. H., Nemes, P., and Bucholtz, C. (1975) The use of Coomassie brilliant blue G250 perchloric acid solution for staining in electrophoresis and isoelectric focusing on polyacrylamide gels. *Anal. Biochem.* **64**, 509–516.
 39. Jensen, O. N., Shevchenko, A., and Mann, M. (1997) in *Protein Structure. A Practical Approach* (Creighton, T. E., Ed.), pp. 29–57, IRL Press, Oxford.
 40. Mohr, M. D., Bornsen, K. O., and Widmer, H. M. (1995) Matrix-assisted laser desorption/ionization mass spectrometry: Improved matrix for oligosaccharides. *Rapid Commun. Mass Spectrom.* **9**, 809–814.
 41. Stahl, B., Klabunde, T., Witzel, H., Krebs, B., Steup, M., Karas, M., and Hillenkamp, F. (1994) The oligosaccharides of the Fe(III)-Zn(II) purple acid phosphatase of the red kidney bean. Determination of the structure by a combination of matrix-assisted laser desorption/ionization mass spectrometry and selective enzymic degradation. *Eur. J. Biochem.* **220**, 321–330.
 42. Kratzer, R., Eckerskorn, C., Karas, M., and Lottspeich, F. (1998) Suppression effects in enzymatic peptide ladder sequencing using ultraviolet-matrix assisted laser desorption/ionization-mass spectrometry. *Electrophoresis* **19**, 1910–1919.
 43. Mortz, E., Sareneva, T., Haebel, S., Julkunen, I., and Roepstorff, P. (1996) Mass spectrometric characterization of glycosylated interferon-gamma variants separated by gel electrophoresis. *Electrophoresis* **17**, 925–931.
 44. Krause, E., Wenschuh, H., and Jungblut, P. R. (1999) The dominance of arginine-containing peptides in MALDI-derived tryptic mass fingerprints of proteins. *Anal. Chem.* **71**, 4160–4165.
 45. Faye, L., Sturm, A., Bollini, R., Vitale, A., and Chrispeels, M. J. (1986) The position of the oligosaccharide side-chains of phytohemagglutinin and their accessibility to glycosidases determines their subsequent processing in the Golgi. *Eur. J. Biochem.* **158**, 655–661.
 46. Mo, W., Takao, T., Sakamoto, H., and Shimonishi, Y. (1998) Structural analysis of oligosaccharides derivatized with 4-aminobenzoic acid 2-(diethylamino)ethyl ester by matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Chem.* **70**, 4520–4526.
 47. Stahl, B., Steup, M., Karas, M., and Hillenkamp, F. (1991) Analysis of neutral oligosaccharides by matrix-assisted laser/desorption mass spectrometry. *Anal. Chem.* **63**, 1463–1466.
 48. Karas, M., Ehring, H., Nordhoff, E., Stahl, B., Strupat, K., Hillenkamp, F., Grehl, M., and Krebs, B. (1993) Matrix-assisted laser desorption/ionization mass spectrometry with additives to 2,5-dihydroxybenzoic acid. *Org. Mass Spectrom.* **28**, 1476–1481.
 49. Harthill, J. E. (1991) N-Glycosylation of horseradish peroxidase. D. Phil. thesis, University of Oxford.
 50. D'Andrea, G., Bouwstra, J. B., Kamerling, J. P., and Vliegenterhart, J. F. G. (1988) Primary structure of the xylose-containing

- N-linked carbohydrate moiety from ascorbic acid of *Cucurbita pepo medullosa*. *Glycoconj. J.* **5**, 151–157.
51. Fu, D., Chen, L., and O'Neill, R. A. (1994) A detailed structural characterization of ribonuclease B oligosaccharides by ^1H NMR spectroscopy and mass spectrometry. *Carbohydr. Res.* **261**, 173–186.
52. Lis, H., and Sharon, N. (1978) Soybean agglutinin—A plant glycoprotein. Structure of the carbohydrate unit. *J. Biol. Chem.* **253**, 3468–3476.
53. Green, E. D., Adelt, G., Baenziger, J. U., Wilson, S., and Van Halbeek, H. (1988) The asparagine-linked oligosaccharides on bovine fetuin. Structural analysis of N-glycanase-released oligosaccharides by 500-megahertz ^1H NMR spectroscopy. *J. Biol. Chem.* **263**, 18253–18268.
54. Fötisch, K., Altmann, F., Haustein, D., and Vieths, S. (1999) Involvement of carbohydrate epitopes in the IgE response of celery-allergic patients. *Int. Arch. Allergy Immunol.* **120**, 30–42.
55. Petersen, A., Vieths, S., Aulepp, H., Schlaak, M., and Becker, W. M. (1996) Ubiquitous structures responsible for IgE cross-reactivity between tomato fruit and grass pollen allergens. *J. Allergy Clin. Immunol.* **98**, 805–815.
56. Altmann, F., and März, L. (1995) Processing of asparagine-linked oligosaccharides in insect cells. Evidence for α -mannosidase II. *Glycoconj. J.* **12**, 150–155.