Glycoconj J (2006) 23:599–609
DOI 10.1007/s10719-006-8715-7

Structural characterization of the N-glycans of gpMuc from *Mucuna pruriens* seeds

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Received: 6 February 2006 / Revised: 6 April 2006 / Accepted: 7 April 2006

Abstract *Mucuna pruriens* seeds are used in some countries as a human prophylactic oral anti-snake remedy. Aqueous extracts of *M. pruriens* seeds possess *in vivo* activity against cobra and viper venoms, and protect mice against *Echis carinatus* venom. It was recently demonstrated that the seed immunogen generating the antibody that cross-reacts with the venom proteins is a multiform glycoprotein (gpMuc), and the immunogenic properties of gpMuc seemed to mainly reside in its glycan chains. In the present study, gpMuc was found to contain only N-glycans. Part of the N-glycans could be released with peptide-(N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGase F-sensitive N-glycans); the PNGase F-resistant N-glycans were PNGase A-sensitive. The oligosaccharides released were analyzed by a combination of MALDI-TOF mass spectrometry, HPLC profiling of 2-aminobenzamide-labelled derivatives and ^{1}H NMR spectroscopy. The PNGase F-sensitive N-glycans comprised a mixture of oligomannose-type structures ranging from $Man_5GlcNAc_2$ to $Man_9GlcNAc_2$, and two xylosylated structures, $Xyl_1Man_3GlcNAc_2$ and $Xyl_1Man_4GlcNAc_2$. The PNGase A-sensitive N-glycans, containing (α1-3)-linked fucose, were identified as $Fuc_1Xyl_1Man_2GlcNAc_2$ and $Fuc_1Xyl_1Man_3GlcNAc_2$. In view of the determined N-glycan ensemble, the immunoreactivity of gpMuc was ascribed to the presence of core (β1-2)-linked xylose- and core α(1-3)-linked fucose-modified N-glycan chains.

Keywords Allergens · Core-α1,3-linked fucose · Glycoprotein · *Mucuna pruriens* · N-glycans

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Introduction

It has long been known that seeds from the plant *Mucuna pruriens* act as an oral prophylactic against snake venom, and cross reactivity between components of the seeds and venom proteins has been suggested. By swallowing two seeds, individuals are protected from the toxic effects of snake venom for a whole year. Aqueous extracts of *M. pruriens* seeds (MPE) were shown to have activity against cobra and viper venoms *in vivo* [1]. It was found that MPE protected mice against *Echis carinatus* venom (EV) by an immunological mechanism [2]. More recently, it was demonstrated that the MPE immunogen generating the antibody that cross-reacts with venom proteins is a multiform glycoprotein (gpMuc) the immunogenic properties of which mainly reside in its glycan chains [3]. By Concanavalin A affinity chromatography, gpMuc was isolated from protein extract of *M. pruriens* seeds. It consisted of seven isoforms having molecular masses in the range from 20.3 to 28.7 kDa and pIs from 4.8 to 6.5. N-terminal amino acid sequencing of the isoforms revealed close similarity, since they shared at least 7/12 aa and all of them contained the consensus sequence DDREPV-DT found on the soybean Kunitz type trypsin inhibitor [3]. Preliminary studies on the glycan component of the protein suggested

that gpMuc contained both N- and O-glycans, and that the O-glycans were functional in its protective mechanism [3].

In the present study, detailed analysis of the glycosylation pattern of gpMuc yielded only N-glycans. Identification of core (β1-2)-xylosylated and α(1-3)-fucosylated N-glycans, identical to those found in food and pollen glycoproteins and insect venom, suggest an immunological cross reaction of allergologic significance.

Materials and methods

Plant material and purification of gpMuc from *M. pruriens* seeds

Mucuna pruriens (family: Fabaceae; subfamily: Papilionoideae; genus: Mucuna; species: pruriens) seeds were collected from the Rukuba area in Jos, Nigeria, with the aid of a traditional healer, and were authenticated by Prof. S.W.H. Hussini (Department of Botany, University of Jos, Nigeria). Voucher specimen Number A102 is deposited in the Pharmacy Herbarium of the University of Jos.

M. pruriens seed extract (MPE) was prepared as previously described [3]. Briefly, 50 g of grounded seeds were extracted in 100 ml Milli Q water for 24 h at 4°C, and the supernant, containing MPE proteins, was stocked after centrifugation (10000 g × 20 min). Four ml of Concanavalin A-Sepharose (Sigma, St. Louis, MO) packed in a column was pre-equilibrated at room temperature with 10 mM Tris-HCl (pH 8), 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.02% NaN_3 (binding buffer). MPE proteins were precipitated with $(NH_4)_2SO_4$ from the stock supernatant, and 20 μl of a 25 μg/μl solution of MPE proteins were diluted to 300 μl with binding buffer, then loaded onto the column and allowed to interact for 1 h. After elution of unbound material with 4-column volumes of binding buffer, gpMuc was eluted with 16 ml binding buffer containing 0.1 M methyl α-D-mannopyranoside, at a flow rate of 8 ml/h. The elution was monitored by UV absorbance at 206 nm. Further purification of gpMuc was carried out on 4 connected HiTrap columns containing Sephadex G-25 (5 ml, Pharmacia, Uppsala, Sweden; FPLC system), using 50 mM NH_4HCO_3 at a flow rate of 7 ml/min, and monitored by UV absorbance at 280 nm and conductivity measurements. GpMuc was stored at a concentration of 2 mg/ml for further analyses.

Reduction and carboxymethylation of gpMuc

Lyophilized gpMuc (10 mg) was dissolved in 10 ml 1 mM Tris-HCl (pH 8.25), containing 6 M guanidinium chloride and 1 mM EDTA. Then, 0.05 M DTT was added, and the mixture was incubated for 2 h at 37°C. After cooling to room temperature, 0.5 M iodoacetic acid in 0.5 M NaOH (v/v) was added to the mixture until a final concentration of 250 mM. The sample was immediately transferred into the dark for 30 min, then an excess of β-mercaptoethanol was added. Finally, the solution was dialyzed extensively against Milli Q water and lyophilized.

Monosaccharide analysis

An aliquot of lyophilized and dried gpMuc was subjected to methanolysis (1 M methanolic HCl, 24 h, 85°C), followed by re-*N*-acetylation and trimethylsilylation. The mixture of methyl glycosides was analyzed by gas-liquid chromatography (GLC) and GLC combined with electron impact mass spectrometry (GLC-EI-MS) [4]. GLC analyses were performed on a Chrompack CP 9002 instrument (Chrompack BV, Middelburg, The Netherlands) equipped with an EC-1 column (30 m × 0.32 mm, Alltech, Breda, The Netherlands), and using a temperature program of 140-240°C at 4°C/min. GLC-EI-MS analyses were carried out on a MD 800/8060 system (Fisons Instruments, Interscience, Breda, The Netherlands) equipped with an AT-1 column (30 m × 0.25 mm, Alltech, Breda, The Netherlands), using the same temperature program as for GLC.

Digestion of gpMuc with peptide-(N^4-(N-acetyl-β-glucosaminyl)asparagine amidase F and isolation of the released N-glycans

N-Glycans of gpMuc were enzymatically released with peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGase F; *Flavobacterium meningosepticum*, recombinant from *E. coli*; EC 3.5.1.52; Roche Applied Science, Indianapolis, IN) [5]. Briefly, 2 mg lyophilized sample were dissolved in 1 ml 20 mM sodium phosphate buffer, pH 7.5, containing 0.02% NaN_3. Subsequently, 10 U of fresh enzyme per mg glycoprotein were added, and the solution was incubated for 16 h at 37°C. After boiling the sample for 5 min, a fresh aliquot of enzyme (5 U) was added, and the incubation was continued for another 24 h, then boiled for 5 min.

The PNGase F digest was loaded on a column (47 × 1 cm) of Sephadex G-25 (Amersham Pharmacia Biotech, Piscataway, NJ), eluted with 50 mM NH_4HCO_3, pH 7.0, at a flow rate of 7 ml/min, and monitored by UV absorbance at 206 nm. The fractions containing the PNGase F-treated gpMuc and the PNGase F-released N-glycan pool, respectively, were lyophilized.

Digestion of PNGase F-treated gpMuc with pepsin

PNGase F-treated gpMuc (3 mg) were dissolved in 1 ml 5% (v/v) formic acid, and incubated with 100 μg pepsin (Sigma-Aldrich, St. Louis, MO) for 24 h at 37°C [6]. The digestion

was stopped by heating for 5 min at 100°C, and the mixture was lyophilized.

Digestion of PNGase F-treated, pepsin-treated gpMuc with peptide-(N^4-(N-acetyl-β-glucosaminyl)asparagine amidase A and isolation of the additionally released N-glycans

The mixture of peptides and glycopeptides, generated by pepsin digestion of PNGase F-treated gpMuc, was further digested with peptide-(N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase A (PNGase A; from sweet almonds, EC 3.5.1.52; Roche Applied Science) [7]. Briefly, about 3 mg of PNGase F-treated, pepsin-treated gpMuc mixture was dissolved in 0.5 ml 20 mM ammonium acetate, pH 5.5, and incubated with 0.5 mU PNGase A for 24 h at 37°C. After stopping the digestion by heating for 5 min at 100°C, 1/3 of the sample was used for HPLC profiling after 2-aminobenzamide (2AB) labelling (see below) and for MALDI-TOF-MS analyses. The remaining material was lyophilized, redissolved in Milli Q water, and passed over a 1-2 ml Dowex 50W × 8 column (Dowex Ion Exchange Resins, Dow Chemical Company, Michigan, USA) in order to separate additionally released N-glycans from peptide material. The column was eluted with Milli Q water, 400 mM ammonium acetate, pH 6.0, and 2% aqueous acetic acid and the N-glycan-containing fraction was used for HPLC profiling after 2AB labelling and MALDI-TOF-MS analyses.

2-Aminobenzamide derivatization of N-glycans for HPLC profiling

Oligosaccharides were fluorescently labelled with 2-aminobenzamide (2AB) essentially as described by Stroop *et al*., 2000 [8]. Briefly, 31.65 mg $NaCNBH_3$ were added to a solution of 23.6 mg 2AB in 500 μl 85% dimethylsulfoxide15% acetic acid, and the mixture was heated for 2 min at 65°C. An aliquot (8 μl) of the solution was added to about 160 μg dried oligosaccharide, and the mixture was incubated for 2 h at 65°C with intermediate mixing. After cooling to room temperature, the sample was transferred onto an acid-pretreated Whatman QM-A filter paper and dried. The residual reagents were eluted from the filter paper by washing extensively with acetonitrile. The labelled glycan mixture was eluted by using 2 ml water. The resulting solution was lyophilized, redissolved in 400 μl water, placed in a ultrafree MC centrifugal eppendorf filter (5000 NMWL) and the labelled glycans were recovered by centrifugation at 5000 g for 15 min. The sample obtained was used for HPLC profiling and MALDI-TOF-MS analysis.

HPLC profiling

HPLC was carried out using a Waters 2690 XE Alliance system equipped with an in-line degasser, a Waters temperature control unit (30°C) and a 474 fluorescence detector with excitation and emission wavelengths of 373 nm and 420 nm, respectively. The system was controlled via a LAC/E interface using Waters Millennium 32 software.

Normal phase HPLC was performed on a TSKgel Amide-80 column (4.6 × 250 mm; Tosoh BioSep, Tosoh Bioscience GmbH, Stuttgart, Germany), calibrated in glucose units (GU) with a standard mixture of glucose oligomers. The following gradient conditions were applied (solvent A, 50 mM ammonium formate, pH 4.4; solvent B, 20% solvent A in acetonitrile): following injection, a linear gradient of 6.5–43.8% A over 100 min, then a linear gradient of 43.8-100% A over 3 min, at a flow rate of 0.8 ml/min; using 100% A increase of flow rate to 1 ml/min over 1 min; isocratic elution with 100% A for 5 min; re-equilibration in 6.5% A over 31 min; total running time, 140 min.

Mass spectrometry

Prior to analyses by MALDI-TOF-MS, all analytes were desalted and concentrated by C18 MB Pipette tips (Omix pipette tips for micro extractions, Varian, Middelburg, The Netherlands). Before use, tips were conditioned with 50% aqueous acetonitrile and equilibrated with 1.0% aqueous TFA. After applying the sample, tips were firstly rinsed with 0.1% aqueous TFA, then the sample was eluted by using 0.1% aqueous TFA in 50–75% aqueous acetonitrile containing matrix (α-Cyano-4-Hydroxy-Cinnamic Acid 10mg/ml) and directly dispensed onto MALDI plate.

Positive-ion mode MALDI-TOF-MS analysis of N-glycan mixtures was performed on a Voyager-DE instrument (PerSeptive Biosystems), operating at an accelerating voltage of 23 kV (grid voltage, 91%; ion guide wire voltage, 0.1%), and equipped with a VSL-337ND-N_2 laser. An aliquot of the samples, dissolved in Milli Q water, was mixed in a 1:1 ratio with 2,5-dihydroxybenzoic acid (DHB, 20 mg/ml in 10% ethanol) in the sample well, and allowed to crystallize under mild vacuum. Linear mass scans were recorded over 3000 Da or 60000 Da, using a pulse delay time of 95 or 750 ns, depending on the sample analyzed. Recorded data were processed using Voyager software.

^{1}H NMR spectroscopy

Prior to NMR analysis, the oligosaccharide samples were repeatedly exchanged in D_2O (99.9 atom % D) with intermediate lyophilization, and finally dissolved in 500 μl D_2O (99.96 atom % D). 1D ^{1}H-NMR spectra were recorded at 500 MHz on a Bruker AMX-500 instrument (Bijvoet

Center, Department of NMR Spectroscopy, Utrecht University) at a probe temperature of 300 K. Chemical shifts (δ) are expressed in ppm by reference to internal acetone in D_2O ($\delta = 2.225$) [9].

Amino acid analysis

An aliquot of gpMuc (200 μg) was dissolved in 500 μl 6 M HCl, and hydrolyzed for 22 h at 110°C in a heating block. The solvent was evaporated using a flow of nitrogen, and the residue was dissolved in 500 μl water, filtered through a 0.22 μm filter, and analyzed on a LKB Alpha Plus Amino Acid Analyzer.

Results

General data for native gpMuc

Native gpMuc material, prepared from seeds of *M. pruriens* by extraction (MPE), protein precipitation (MPP) and Con A affinity chromatography, was checked for purity by SDS-PAGE. Figure 1 shows a typical pattern with a molecular species having an apparent molecular mass between 21-26 kDa and running as a broad band. Amino acid analysis of native gpMuc is shown in Table 1. Monosaccharide analysis [4] of native gpMuc revealed Fuc, Xyl, Man, and GlcNAc, and indicated a carbohydrate content of 12% (Table 2). Positive-ion mode MALDI-TOF-MS analysis of native gpMuc showed two patterns of broad peaks in the high-molecular-mass region, m/z 21724/23468 and m/z 10891/11777, assigned to mono-charged $[M + H]^+$ and double-charged $[M + 2H]^{2+}$ ions, respectively (Figure 2a). It should be noted that MALDI-TOF-MS of glycoproteins with a molecular mass above 20 kDa gives a very poor resolution, and glycoforms differing by the mass of one monosaccharide residue are often not resolved [10]. Multiple peaks, both in the single and double-charged pseudo-molecular ion region and broadening of peaks, may reflect the reported seven slightly different isoforms for gpMuc [3]. MALDI-TOF-MS analysis of protein material obtained after digestion with PNGase F (PNGase F-treated gpMuc) resulted in four well defined and sharper but still broad peaks at lower masses than those of the native glycoprotein: $[M + H]^+$, m/z 20755–22570; $[M + 2H]^{2+}$, m/z 10388–11283 (Figure 2b).

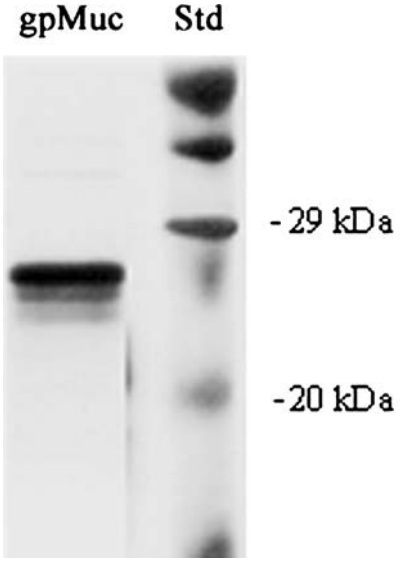


Fig. 1 SDS-PAGE pattern of native gpMuc. Samples were separated on a 12% gel. Left, native gpMuc; right, low-molecular standard mixture for calibration

Table 1 Aminoacid content of native gpMuc and percentage amount

Amino acid residue	Aminoacid content %
Alanine	1.14
α-Aminobutyric acid	0.30
Arginine	1.23
Aspartic acid	66.86
Glutamic acid	3.35
Glycine	3.55
Histidine	0.52
Isoleucine	2.23
Leucine	2.72
Lysine	3.08
Phenylalanine	2.04
Proline	3.00
Serine	3.03
Threonine	2.33
Tyrosine	1.93
Valine	2.68

Table 2 Monosaccharide composition and carbohydrate content of gpMuc and related fractions

		Molar ratio	
CHO	gpMuc	PNGase F-treated gpMuc	PNGase F-released N-glycans
Fuc	0.2	1.0	—
Xyl	0.4	1.0	0.6
Man	3.0	3.0	3.0
GlcNAc	1.7	2.4	1.8
CHO%	12%	5.5%	6.5%

Structural analysis of the PNGase F-released N-glycans of native gpMuc

The N-glycans released from native gpMuc by PNGase F were separated from PNGase F-treated gpMuc by size-exclusion chromatography on Sephadex G-25, and the pool of oligosaccharides was analyzed by positive-ion mode MALDI-TOF-MS before and after derivatization with

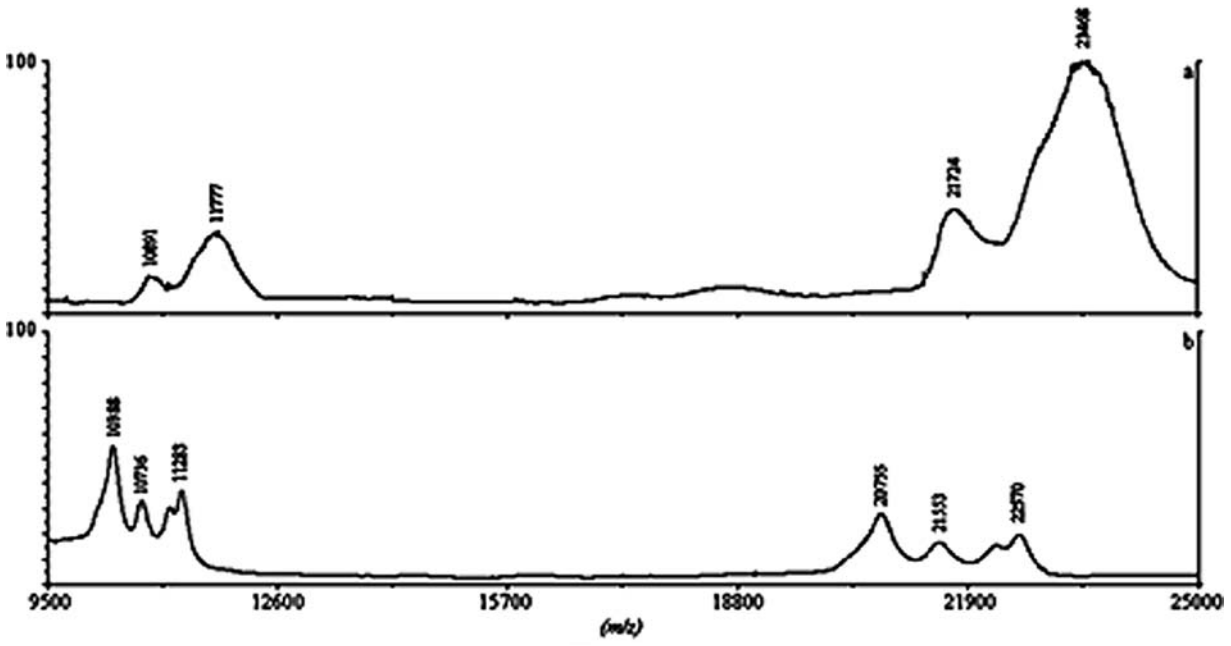


Fig. 2 Positive-ion mode MALDI-TOF mass spectra of (a) the molecular mass region of native gpMuc, and (b) the molecular mass region of PNGase F-treated gpMuc. The values indicated are average molecular masses measured in the linear mode

2-aminobenzamide (2AB), and by normal phase HPLC profiling of the 2AB derivatives. The various results are summarized in Table 3, assuming the usual N-glycan biosynthetic pathway. Monosaccharide analysis of the N-glycan pool revealed Xyl, Man, and GlcNAc, but Fuc was absent (Table 2).

After MALDI-TOF-MS analysis of the non-derivatized N-glycan pool, the peaks at m/z 1257.7, 1419.8, 1581.8, 1743.9, and 1906.0 were found to be correlated with the $[M+Na]^+$ adduct ions of oligomannose-type N-glycans ranging from $Man_5GlcNAc_2$ to $Man_9GlcNAc_2$ (Figure 3a and third column in Table 3). Considering the monosaccharide analysis data, the additional peaks at m/z 1065.6 and 1227.7 represent the $[M+Na]^+$ adduct ions of the xylosylated structures $Xyl_1Man_3GlcNAc_2$ and $Xyl_1Man_4GlcNAc_2$, respectively. Similar results were found for the 2AB-derivatized N-glycan pool (Figure 3b and fifth column in Table 3).

The HPLC profile of the 2AB-derivatized N-glycan pool on TSKgel Amide-80 is depicted in Figure 4b. The profile was compared with a 2AB-labelled $Man_5GlcNAc_2$-$Man_9GlcNAc_2$ mixture from RNAse B (Figure 4a), and with a dextran ladder to obtain peak values in terms of glucose units (GU). The peaks with RT values 42.575, 48.791, 54.581, 58.826, 59.614, and 63.261 min reflected the presence of $Man_5GlcNAc_2$-2AB (GU 5.19), $Man_6GlcNAc_2$-2AB (GU 6.09), $Man_7GlcNAc_2$-2AB (GU 7.00), $Man_8GlcNAc_2$-2AB (7.80), $Man_8GlcNAc_2$-2AB (GU 7.91), and $Man_9GlcNAc_2$-2AB (GU 8.70) (Figure 4b; Table 3, last column). Note that two isomers of $Man_8GlcNAc_2$ were detected, as in RNAse B. The very small shoulder in front of the $Man_7GlcNAc_2$ peak could stem from an isomer of that structure, but the amount is too small for structural assignment. The two early-eluting peaks with RT values of 32.927 and 38.868, corresponding to GU values of 3.98 and 4.75, respectively, were assigned to the two xylosylated structures, identified by MALDI-TOF-MS.

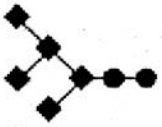
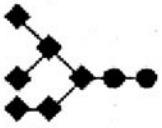
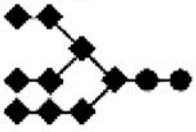
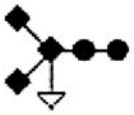
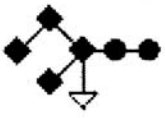
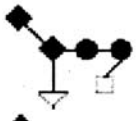
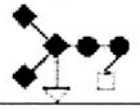
The N-glycan pool was analyzed by ^1H NMR spectroscopy. Figure 5 shows the structural-reporter-group region of the ^1H NMR spectrum, whereas Table 4 indicates the chemical shift values of the various structural-reporter-group signals. The ^1H NMR spectrum of the N-glycan pool showed the chemical shifts typical for either xylosylated or oligomannose-type structures. Assignments were made by comparison with ^1H NMR data of pure compounds [11]. The components identified in the N-glycan mixture sustained the results obtained by the above MS and HPLC analyses.

The relative amounts of the various PNGase F-released N-glycans chains, as reported in Table 5, were calculated by integration of the HPLC peaks.The most abundant glycan turned out to be $Xyl_1Man_3GlcNAc_2$, constituting about 24.1% of the mixture. The $Man_5GlcNAc_2$ to $Man_9GlcNAc_2$ series represented about 28.5% of the oligosaccharide mixture.

Structural analysis of the PNGase A-released N-glycans of PNGase F-treated gpMuc

Monosaccharide analysis of PNGase F-treated gpMuc revealed Fuc, Xyl, Man, and GlcNAc, indicating incomplete

Table 3 Molecular masses determined by MALDI-TOF MS and glucose units (GU) values for the 2AB-labelled N-glycan chains obtained from gpMuc after complete de-glycosylation

Structure	Mass $[M+Na]^+$		Mass 2-AB $[M+Na]^+$		GU value
	Theorical	Measured	Theorical	Measured	
Oligomannose-type structures					
	1257.4	1257.7	1377.6	1378.3	5.19
	1419.5	1419.8	1539.6	1540.2	6.09
	1581.5	1581.8	1701.7	1701.9	7.00
	1743.6	1743.9	1863.7	1863.8	7.80 7.91
	1905.6	1906.0	2025.8	2027.4	8.70
Structures with only Xyl					
	1065.4	1065.6	1185.5	1185.6	3.98
	1227.4	1227.7	1347.6	1347.6	4.75
Structures with Fuc and Xyl					
	1049.4	1048.3	1169.5	1167.5	4.53
	1211.4	1211.4	1331.6	1331.6	4.93

Symbols: *N*-Acetylglucosammine–●, Mannose–♦, Fucose–□, Xylose–▷.

de-N-glycosylation (Table 2). Carbohydrate content decreased from 12 to 5.5%. The finding of Fuc in PNGase F-treated gpMuc suggested the occurrence of structures containing (α1-3)-linked Fuc at the Asn-bound GlcNAc residue, making these glycan chains inaccessible for PNGase F digestion.

Since PNGase A removes N-glycans with (α1-3)-linked Fuc at the Asn-bound GlcNAc, PNGase F-digested gpMuc was treated with PNGase A. Analysis of the PNGase digest revealed that no glycan was released. According to the instructions for use, PNGase A is mainly used for cleavage of glycopeptides. We therefore decided to digest the

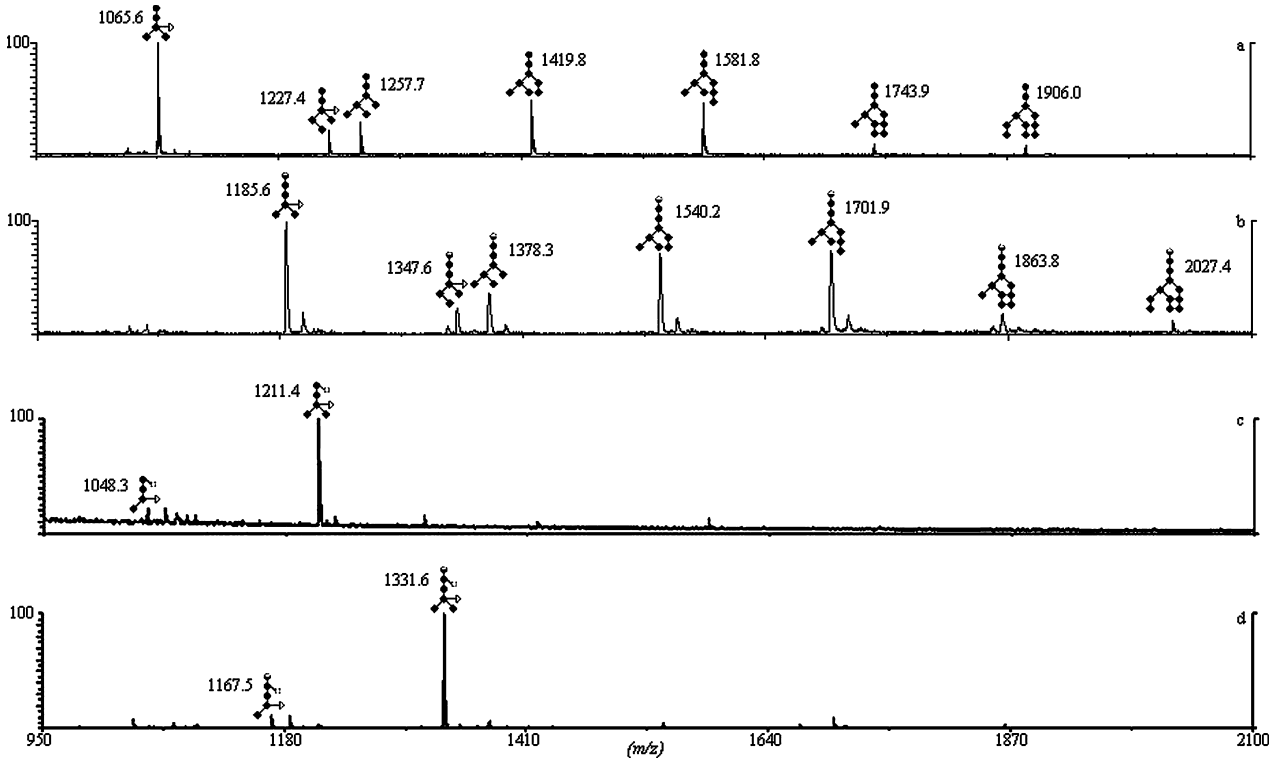


Fig. 3 Positive-ion mode MALDI-TOF mass spectra of PNGase F-released N-glycans from native gpMuc before (a) and after 2AB derivatization (b), and of PNGase A-released N-glycans from PNGase F/pepsin treated gpMuc before (c) and after 2AB derivatization (d). Explanation of the symbols: GlcNAc; Man; Fuc; Xyl; 2-AB For full structures, see Table 5

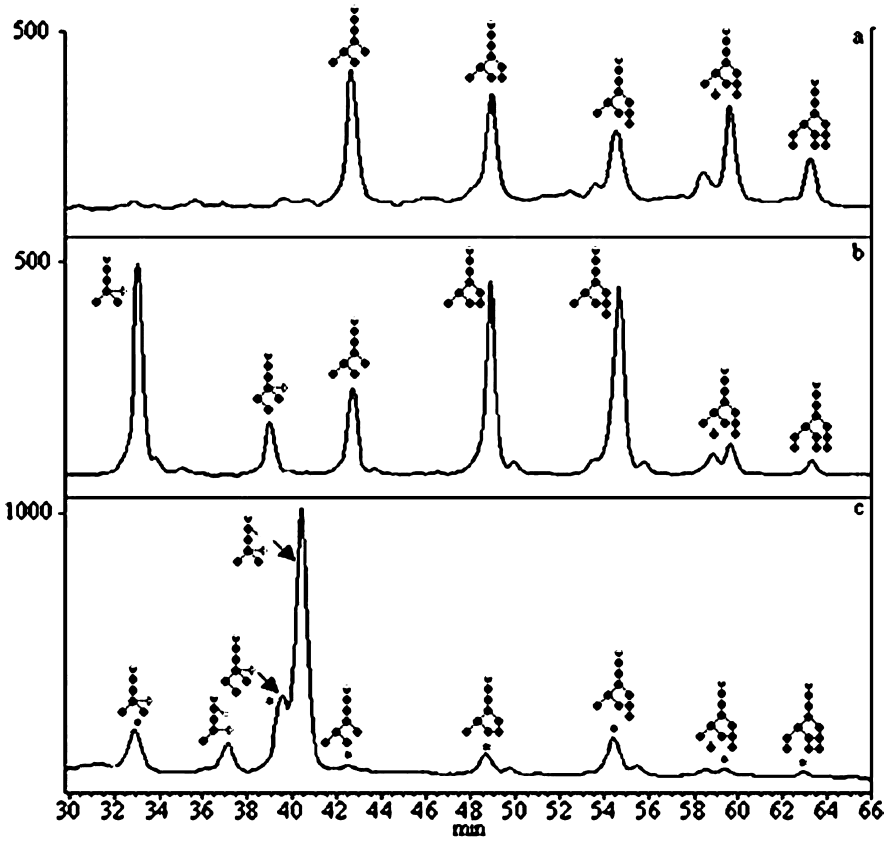


Fig. 4 HPLC profiling on TSKgel amide 80 of the 2AB-labelled derivatives of (a) oligomannose-type reference structures from RNAse B, (b) PNGase F-released N-glycans from native gpMuc, and (c) PNGase A-released N-glycans from PNGase F/pepsin treated gpMuc. Peaks marked with an asterisk (*) in (c) stem from an incomplete PNGase F digestion of native gpMuc. Explanation of the symbols: GlcNAc–●; Man–♦; Fuc–□; Xyl–△; 2-AB—○. For full structures, see Table 5

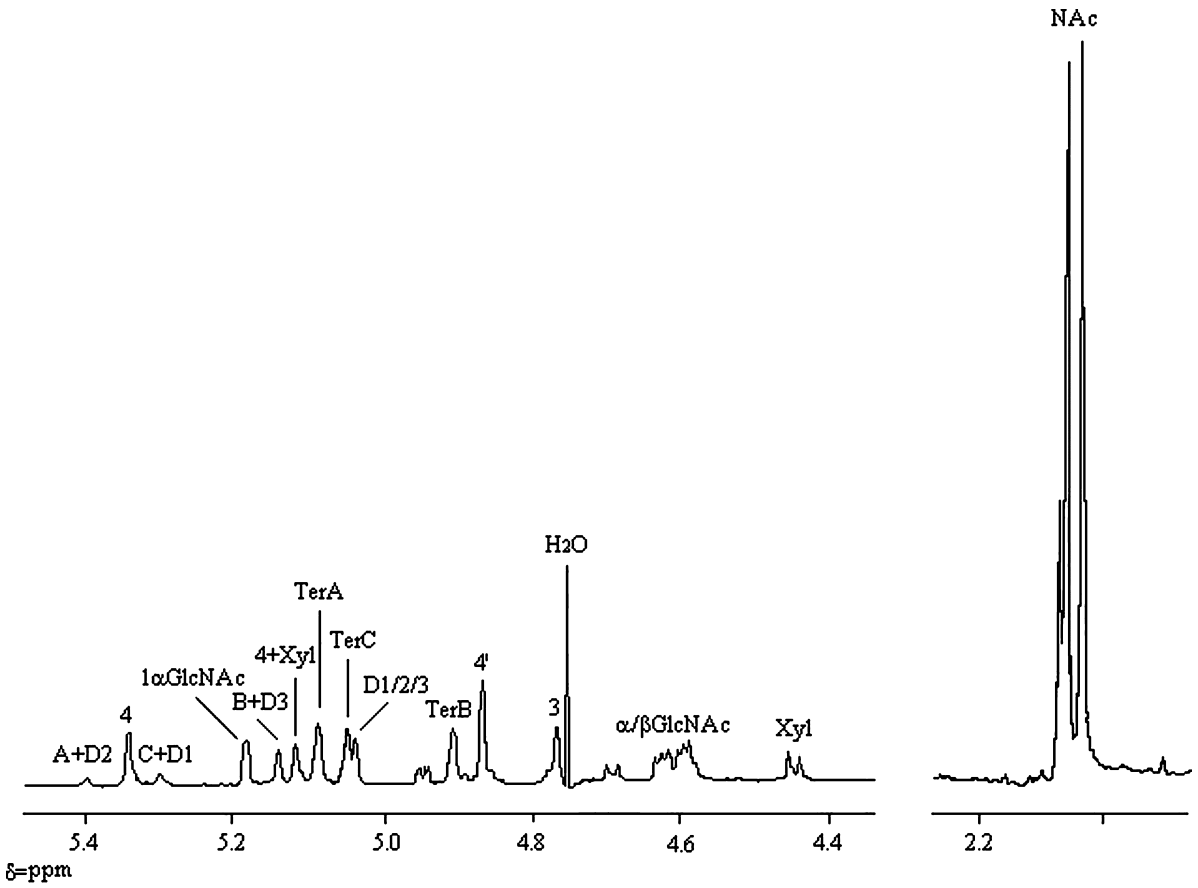


Fig. 5 Structural-reporter-group region of the 500 ^{1}H NMR spectrum of the N-glycan pool of PNGase F-treated native gpMuc, recorded in D_2O at 300 K

PNGase F-digested gpMuc with pepsin before PNGase A treatment. In this case glycans were successfully released. The cleaved glycans were directly 2AB-labelled and analyzed by MALDI-TOF-MS (Figure 3d) and normal phase HPLC profiling (Figure 4c).

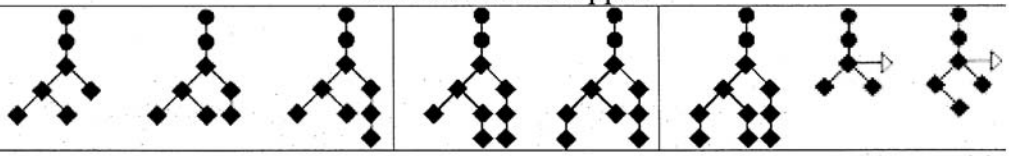
The main part of the pepsin/PNGase A digest was loaded onto a Dowex 50W × 8 column to separate released glycans from peptides and residual glycopeptides. Elution with milli Q water, 400 mM ammonium acetate, pH 6.0, and 2% aqueous acetic acid yielded three fractions, respectively, which were checked for N-glycans by MALDI-TOF-MS. The only fraction containing glycan chains turned out to be the water fraction. This fraction showed several peaks. Considering the monosaccharide analysis data, the two major peaks at m/z 1048.3 and m/z 1211.4 $[M+Na]^+$ (Figure 3c) were assigned as belonging to $Fuc_1Xyl_1Man_2GlcNAc_2$ and $Fuc_1Xyl_1Man_3GlcNAc_2$, respectively. The fraction also showed a series of very small peaks. Comparison of the molecular mass of these peaks with those of either xylosylated or oligomannose-structures already identified, indicated that they were due to incomplete digestion by PNGase F. These findings were confirmed by MALDI-TOF-MS analysis (Figure 3d) and HPLC profiling (Figure 4c) of the 2AB-derivatized N-glycan mixture. Here, the two additional peaks in the MALDI-TOF mass spectrum, correlated with $Fuc_1Xyl_1Man_2GlcNAc_2$-2AB and $Fuc_1Xyl_1Man_3GlcNAc_2$-2AB, had m/z values of 1167.5 and 1331.6 $[M + Na]^+$, respectively (for full structures see Table 3). Compared with the chromatogram in Figure 4b, the HPLC profile on TSKgel Amide-80 in Figure 4c showed two additional peaks (RT 37.089, GU 4.53; RT 40.382, GU 4.93), which were attributed to the (α1-3)-fucosylated chains.

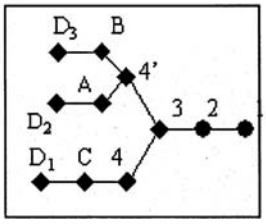
The relative amounts of the various PNGase A-released N-glycans of PNGase F-treated gpMuc, as reported in Table 5, were calculated by integration of the HPLC peaks. The $Fuc_1Xyl_1Man_2GlcNAc_2$ and $Fuc_1Xyl_1Man_3GlcNAc_2$ glycans occurred in relative amounts of about 2.3% and 32.0%, respectively, of the complete set of glycans present on gpMuc.

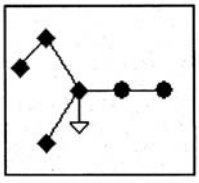
Discussion

We recently reported that the multiform glycoprotein gpMuc, isolated from the seeds of *M. pruriens*, plays a key role in generating immunological protection for individuals and

Table 4 500 MHz ^{1}H NMR chemical shifts of PnGase F-released N-glycans recorded at 300 K referenced to internal acetone $\delta = 2.225$

Reporter group	Residue	Chemical shift in ppm							
									
H-1	GlcNAc-1α	5.186	5.186	5.186	5.186	5.186	5.186	5.186	5.186
	Man-4	5.09	5.345	5.345	5.345	5.345	5.345	5.121	5.121
	Man-4'	4.869	4.869	4.869	4.869	4.869	4.869	4.91	4.91
	Man-A	5.09	5.09	5.09	5.40	5.09	5.40		5.09
	Man-B	4.91	4.91	4.91	4.91	5.14	5.14	–	–
	Man-C	–	5.05	5.30	5.30	5.30	5.30	–	–
		–	–	–				–	–
	Man-D_1	–	–	5.04	5.04	5.04	5.04	–	–
	Man-D_2	–	–	–	5.05	–	5.05	–	–
	Man-D_3	–	–	–	–	5.04	5.04	–	–
	Xyl(β1-2)	–	–	–	–	–	–	4.448	4.448
NAc	GlcNAc-1	2.036	2.036	2.036	2.036	2.036	2.036	2.036	2.036
	GlcNAc-2	2.060	2.060	2.060	2.060	2.060	2.060	2.071	2.071





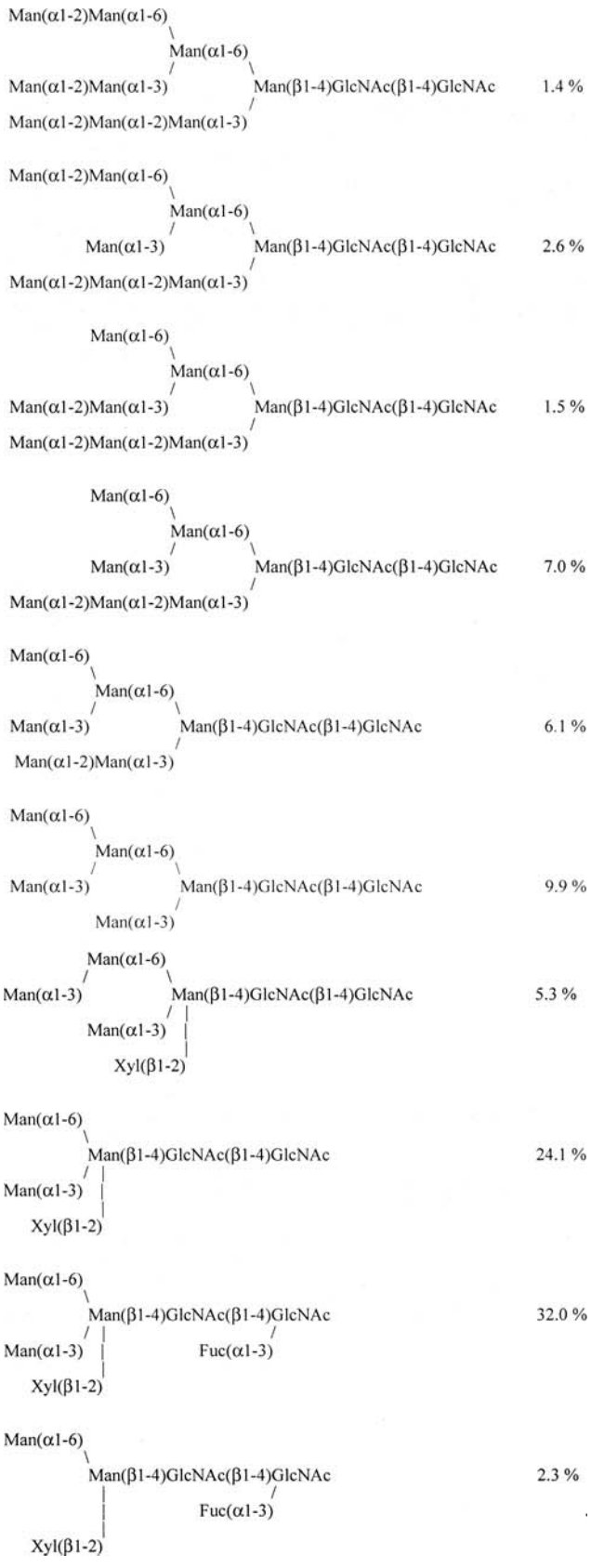
Compounds are represented by symbolic shorthand notation: N-Acetylglucosammine–●, Mannose–♦, Xylose –▷.

animals against snake venoms [3]. Based on the finding that gpMuc contained both PNGase F-sensitive N-glycans and alkaline borohydride-sensitive glycans, it was hypothesized that gpMuc should contain both N- and O-glycans. As the anti-gpMuc epitope did not include the peptide part or the N-glycans removed by PNGase F, it was considered that the anticipated O-glycans should be responsible.

In view of the results presented here, we suggest that the immunoreactivity detected for gpMuc is caused by core (β1-2)-linked Xyl and/or core (α1-3)-linked Fuc residues on part of the N-glycans. Indeed, the present results indicate that O-glycosylation [3] is absent, and that the N-glycan ensemble of gpMuc is built up of truncated oligomannose-type and [(α1-3)-fucosylated] (β1-2)-xylosylated structures, namely $Man_{5\text{-}9}GlcNAc_2$, $Xyl_1Man_{3\text{-}4}GlcNAc_2$, and $Fuc_1Xyl_1Man_{2\text{-}3}GlcNAc_2$. The major components are xylosylated and xylosylated/fucosylated pentasaccharide core structures $Xyl_1Man_3GlcNAc_2$ (24%) and $Fuc_1Xyl_1Man_3GlcNAc_2$ (32%). We also found that the molecular masses of gpMuc is in the range of 21–24 kDa, as determined by MALDI-TOF-MS, thereby redefining the molecular masses obtained earlier by SDS-PAGE [3]. Table 5 summarizes the N-glycans of gpMuc, having a carbohydrate content of 12%, together with their respective amounts, calculated from HPLC profiles, and checked by the ^{1}H NMR data in the case of PNGase F-treated gpMuc fraction.

Taken together, it is clear that the Fuc and/or Xyl core modifications of the N-glycans are the immunogenic component of gpMuc. This is in line with recent literature on cross-reactive carbohydrate determinants. The (α1-3)-fucosylated and (β1-2)-xylosylated N-glycans have been repeatedly reported to be immunogenic in mammals [12–17] and in fact (β1-2)-linked Xyl and (α1-3)-linked Fuc residues have been identified as the epitope structures for IgE binding [18,19] in patients allergic to tree and grass pollens. The vast potential for cross-reaction of antibodies with one or both of these carbohydrate determinants is further extended by the

Table 5 Survey of N-glycans obtained from gpMuc, together with their relative amounts



occurrence of the same epitope structures on glycoproteins of insects, molluscs and parasitic worms [20–22]. The suggestion of a contribution of (β1-2)-Xyl and (α1-3)-Fuc residues to cross-reaction mechanisms is further supported by the fact that anti-horseradish peroxidase and the anti-core (α1-3)-Fuc monoclonal YZ1/2.23 antibody bind to pollen [16].

In the context of our results it is noteworthy that core N-glycans modified with (α1-3)-Fuc and/or (β1-2)-Xyl residues in honeybee (HB) and vespid venoms [23] are proposed as immunogens responsible for cross-sensitisation to HB and yellow jacket (YJ). Whether protection of gpMuc against *Echis carinatus* venom depends upon the presence of the same (α1-3)-Fuc and/or (β1-2)-Xyl residues is now under study in our laboratories. The only fucosylation reported until now in snake venom is that of the motif Gal(α1-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-, present in N-glycans branches of cobra venom factor (CVF) [24]. On the other hand, the possibility that *Mucuna* seeds exert its protection against the snake venom with the cooperation of factors other than gpMuc cannot be excluded.

Acknowledgments This work was supported in part by a grant of Italian Ministery of Education and Scientific Research to F.R.

L.Di Patrizi has worked at Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University with a Marie Curie Fellowship, protocol number: HPMT-CT-2000-00045.

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