

Glycomic analysis of *Schistosoma mansoni* egg and cercarial secretions

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Abbreviations: 0-3h RP, zero-to-three hour released proteins; ESP, egg secreted proteins; CCA, circulating cathodic antigen; CAA, circulating anodic antigen; PNGase F, peptide *N*-glycosidase F; PNGase A, peptide *N*-glycosidase A; Deoxy-Hex, deoxyhexose; Hex, hexose; HexNAc, *N*-acetylhexosamine; Pent, pentose; Xyl, xylose; Fuc, fucose; Man, mannose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylglucosamine; Le^x, Lewis X; LacNAc, *N*-acetyllactosamine; LacdiNAc, *N,N'*-diacetyllactosamine; IL, interleukin; DC-SIGN, dendritic cell-specific intracellular adhesion molecule 3-grabbing nonintegrin; GC-MS, gas chromatography mass spectrometry; 2-DE, two-dimensional electrophoresis.

Summary

The parasitic helminth *Schistosoma mansoni* is a major public health concern in many developing countries. Glycoconjugates, and in particular the carbohydrate component of these products, represent the main immunogenic challenge to the host and could therefore represent one of the crucial determinants for successful parasite establishment. Here, we report a comparative glycomic analysis of the N- and O-glycans derived from glycoproteins present in *S. mansoni* egg (ESP) and cercarial (0-3h RP) secretions by a combination of mass spectrometric techniques. Our results show that *S. mansoni* secrete glycoproteins with glycosylation patterns that are complex and stage-specific. Cercarial stage secretions were dominated by N-glycans that were core xylosylated whereas N-glycans from egg secretions were predominantly core difucosylated. O-glycan core structures from cercarial secretions primarily consisted of the core sequence Gal β 1 $\rightarrow$ 3(Gal β 1 $\rightarrow$ 6)GalNAc, whereas egg-secreted O-glycans carried the mucin-type core 1 (Gal β 1 $\rightarrow$ 3GalNAc) and 2 (Gal β 1 $\rightarrow$ 3(GlcNAc β 1 $\rightarrow$ 6)GalNAc) structures. Additionally, we identified a novel O-glycan core in both secretions in which a Gal residue is linked to the protein. Terminal structures of N- and O-glycans contained high levels of fucose and include stage-specific structures. These glycan structures identified in *S. mansoni* secretions are potentially antigenic motifs and ligands for carbohydrate binding proteins of the host immune system.

Introduction

The blood fluke *Schistosoma mansoni* is a cause of significant human morbidity and mortality throughout Africa and parts of South America (1, 2). Infection is acquired when aquatic cercariae directly penetrate through the skin, transforming into schistosomula as they enter their host. Schistosomula migrate from the skin to the portal system, the ultimate site of parasitization, where maturation and pairing of adult worms occurs. Each female deposits approximately 300 eggs per day in the blood vessels of the gut wall; these either cross the tissues to reach the lumen and are voided in the feces, or are carried down the portal vein to embolise in the liver. Passage of both parasite stages across epithelial barriers involves the release of secretions; thus there is symmetry in the way that the parasite enters and leaves its mammalian host.

The cercarial secretions (termed 0-3hRP) originate from pre- and post-acetabular gland cells (4) whilst those of the eggs (termed ESP) are derived from the syncytial sub-shell envelope (3). Cercarial secretions appear weakly immunogenic and only with repeated exposure do they elicit marked immune responses. The function of cercarial secretions is to facilitate skin penetration and they are the first parasite components encountered by the mammalian host's immune surveillance mechanisms. In contrast, the eggs are highly immunogenic and are involved in interactions with the gut to facilitate the passage of eggs through these tissues and in the liver, driving granuloma formation that initiates the pathology of schistosomiasis (3, 5). However, there could be a dose effect at work since the total of 45,000 attenuated cercariae which are required to induce protective immunity in a baboon is only equivalent to the biomass of eggs deposited daily in the tissues of an animal with 500 worm pairs. Furthermore, much of the antibody response elicited by eggs is directed against glycan epitopes and is cross-reactive with glycans expressed on the cercarial surface and cercarial secretions (6-8). Recent papers have

described detailed proteomic characterization of cercarial secretions identifying novel proteases and immunomodulators (9, 10). When mammalian hosts are exposed to radiation-attenuated cercariae, delivering a very much larger antigenic load than the maximum feasible dose of normal cercariae, virtually the entire antibody response is directed against glycan epitopes common to both cercarial and egg secretions (6). These facts led us to suggest that highly immunogenic glycans could represent a smokescreen to subvert the immune system away from more vulnerable peptide epitopes (6). It was also shown that vaccination of mice with live schistosome eggs elicited high titers of antiglycan antibodies but afforded no protection against cercarial challenge (Kariuki T. *et al*, unpublished data).

It has long been recognized that schistosome glycoconjugates may play an important role in host-parasite interactions enhancing their survival within the host (11). Thus, a large number of studies have been carried out to characterize the schistosome glycome. Structural studies have demonstrated that *S. mansoni* parasites express a complex set of glycans throughout their lifecycle (12-18). However, most structural studies have focused on the glycans of whole stage extracts (12-14) and studies on schistosome secretions have been largely limited to the adult stage gut-associated antigens present in the host circulation, the circulating cathodic antigen (CCA) and circulating anodic antigen (CAA) (19-21), with the exception of a single purified glycoprotein, IPSE/alpha-1 (22). While the data generated from such studies on whole extracts have been informative, analysis of *S. mansoni* secretions will provide a more accurate representation of the molecules that directly interact with the host.

There is a wealth of evidence suggesting that glycans from parasitic helminths are involved in host immune evasion and modulation (see reviews (11, 23)). Therefore, we postulate that glycans of *S. mansoni* secreted glycoproteins are likely to be key players in the parasite-host

interactions associated with infection and onward transmission. We report here, in one of the most comprehensive studies of *S. mansoni* protein glycosylation that 0-3h RP and ESP contain a structurally diverse array of glycans. Glycan structures exhibited stage-specific as well as common structures with potential implications in terms of immunological recognition and host parasite interactions.

Experimental Procedures

Collection of cercarial secretions (0-3h RP)

The zero-to-three hour released proteins (0-3h RP) were prepared as described in (9).

Collection of egg secretions (ESP)

Egg secretions were prepared by extracting mature schistosome eggs from mouse livers seven weeks post-infection, purifying them on a Percoll gradient and culturing aseptically for 72h at 37°C in RPMI-1640 medium, as described in (3).

Glycoprotein analysis

Samples of either 50µg 0-3h RP or 25µg of ESP were separated by 2-DE on minigels as previously described (9). The distribution of glycans and proteins was determined by staining with ProQ Emerald (Invitrogen) and Sypro Ruby (BioRad, Hemel Hempstead, UK) respectively, according to manufacturers' instructions. Images were captured using a VersaDoc Imaging System Model 3000 (BioRad) and analysed with Phoretix 2D Evolution software (Nonlinear Dynamics, Newcastle, UK). ESP spots were identified by tandem mass spectrometry using a 4700 Proteomics Analyzer with ToF-ToF Optics (Applied Biosystems) as described in (9).

Glycomic analysis

Analysis was performed on the secretions of approximately 0.5 million cercariae, which produced approximately 330 µg 0-3hRP. Analysis was performed on the secretions of approximately 0.1 million eggs obtained from the livers of 8 mice, which produced approximately 250 µg ESP.

Digestion and reduction/carboxymethylation of parasite material

Parasite secretions were reduced, carboxymethylated and digested with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) bovine pancreas trypsin (EC 3.4.21.4) as previously described (24).

Release of N-glycans

N-glycans from approximately 250 µg of ESP and 330 µg 0-3h RP were enzymatically released from the peptide backbone by sequential digestion with PNGase F and PNGase A. PNGase F (Roche Molecular Biochemicals, EC 3.5.1.52) digestion was carried out in 50 mM ammonium hydrogen carbonate, pH 8.5 for 24 hours at 37°C with 3 U of enzyme. The reaction was terminated by lyophilization and the products were purified using a propanol /5% (v/v) acetic acid reverse-phase C₁₈ Sep-Pak (Waters Corp.) system as described (24). Glycopeptides remaining after the PNGase F digestion were further digested with 0.2mU of PNGase A (Roche Molecular Biochemicals, EC 3.5.1.52) for 24 hours at 37°C and products were purified on a C₁₈ Sep-Pak (Waters Corp.) as described previously (24).

Reductive elimination of O-glycans

O-glycans were chemically released by reductive elimination (400 μ l of 1 M KBH_4 in 0.05 M KOH at 45°C for 16 hours) from the glycopeptides remaining after the release of the N-glycan populations and desalted through a Dowex 50W-X8(H) column. Excess borates were removed by coevaporation under a stream of nitrogen with 10% (v/v) acetic acid in methanol.

Permethylation of glycans for mass spectrometric analysis

Prior to MS analyses, glycans were permethylated using the NaOH procedure as described (24). Permethylated glycans were then purified using an acetonitrile/water reverse-phase C_{18} Sep-Pak (Waters Corp.) system as described in (24). A third of each of the released N- and O-glycan fractions was permethylated as required for MS experiments.

MALDI-MS(/MS) analyses

The permethylated glycans were dissolved in 10 μ l of methanol and 1 μ l aliquot of dissolved sample were mixed with 1 μ l of matrix (2,5-dihydroxybenzoic acid, 20mg/ml). The sample/matrix mixture was subsequently spotted onto a MALDI target plate and dried under vacuum. A Voyager DE® STR MALDI-TOF (Applied Biosystems) mass spectrometer in the reflectron mode with Delayed Extraction was used to obtain MS spectra. Peaks observed in the MS spectra were selected for further MS/MS. MS/MS data were acquired using a 4800 MALDI TOF/TOF (Applied Biosystems) mass spectrometer. The collision energy was set to 1 kV and air was used as collision gas. All data expressed as monoisotopic masses.

CAD-ES-MS/MS analyses

The permethylated glycans were dissolved in 10 μ l methanol and 2-3 μ l of the samples were loaded into a NanoES spray capillary coated with a thin layer of gold/palladium (inner diameter 2 μ m (Proxeon, Odense, Denmark)). Glycans were sequenced by tandem mass spectrometry (MS/MS) using a hybrid quadrupole orthogonal acceleration time of flight (Q-TOF) mass spectrometer (Micromass, Manchester, UK) and/or a QSTAR Pulsar-i quadrupole TOF mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada). MS and MS/MS spectra were collected in the positive ion mode. For the Q-TOF instrument, a potential of 1.5 kV was applied to the nanoflow tip to produce a flow rate of 120-130 nl/min. The drying and collision gases used were nitrogen and argon respectively. Typical collision energies ranged from 30 to 80 eV depending on the size of the glycan and the collision gas pressure was maintained at 10^{-4} mbar. MS/MS spectra were acquired and processed using MassLynx software (Micromass, UK). For the QSTAR instrument, nitrogen was employed as the collision gas, with the pressure being maintained at 5.3×10^{-5} Torr and collision energies typically varied from 35 to 90 eV to analyse glycans. MS/MS spectra were acquired and processed using the Analyst QS data system (MDS Sciex, Toronto, Canada). Both instruments were pre-calibrated using [Glu¹]fibrinopeptide B in acetonitrile in 5% aqueous acetic acid (1:3 v/v). All data expressed as monoisotopic masses.

GC-MS

The permethylated glycans were dissolved in 10 μ l methanol and 5 μ l of the sample were hydrolyzed with 2 M trifluoroacetic acid for 2 h at 121 °C, reduced with 10 mg/ml sodium borodeuteride in 2 M aqueous ammonium hydroxide at room temperature for 2 h, and the reaction terminated by the addition of acetic acid. Samples were dried and then acetylated with acetic anhydride at 100 °C for 1 h. The reagent was removed under a stream of nitrogen. Samples

were then dissolved in chloroform and washed several times with water before drying under nitrogen. Samples were dissolved in 20 μ l of hexanes and 1 μ l aliquots were analyzed by GC-MS using a Perkin Elmer Clarus 500 instrument, fitted with a RTX-5 (30 m x 0.25-mm internal diameter, Restek Corp.). The sample was injected into the column at 60°C and the temperature was steadily increased at a rate of 8°C/min to a temperature of 300°C.

Results

Evaluation of glycosylation in ESP and 0-3h RP

ESP was separated by 2DE and subsequently stained with ProQ Emerald and Sypro Ruby (Figure 1 [A] and [B] respectively). A combined image was generated using Evolution software which revealed three spots reacting strongly to both protein and glycans (Figure 1 [C]). The spots were identified as hepatotoxic ribonuclease omega-1 (Omega-1; spot 1) and interleukin-4-inducing principle of *S. mansoni* eggs (IPSE; spots 2 and 3) (26). Supplemental Table 1 contains all protein identification data.

Similarly, 0-3h RP was separated by 2DE and stained with ProQ Emerald and Sypro Ruby (Figure 1 [D] and [E] respectively). Staining with Sypro Ruby produced a spot pattern similar to that described previously (9). All gel spot identities were assigned on the basis of their comparative position in the gel images presented in (9). Only a minority of constituents were glycoproteins, namely; the protease inhibitor serpin_c (Figure 1 [F], spot 3), dipeptidyl peptidase IV (Figure 1 [F], spot 4), two proteins with SCP domains (SmSCP_c and SmSCP_b, Figure 1 [F], spots 5 and 6) while the unnumbered spots correspond to glycoproteins whose identities are unknown. Notably, the most abundant constituents in 0-3h RP corresponded to the proteases,

metalloproteinase (SmPepM8) and cercarial elastase (SmCE) (Figure 1 [F], spots 1 and 2 respectively), which were not glycosylated.

Figure 1

General strategy for glycomic analysis of *S. mansoni* secretions

Collected secretions were reduced / carboxymethylated and digested with trypsin to facilitate the subsequent release of N- and O-glycans. N-glycans were released using two different enzymes, PNGase F and PNGase A. Digestion with PNGase F releases all N-glycans apart from those containing $\alpha 1 \rightarrow 3$ linked fucose to the reducing end GlcNAc of the core, such glycans are released by PNGase A. Sequential digestion with PNGase F and PNGase A allowed the analysis of two populations of N-glycans based on their core fucosylation (29). O-glycans were released by reductive elimination from the remaining glycopeptides after the removal of the two different N-glycan populations. Purified glycans were permethylated to increase the sensitivity of detection and to direct the subsequent MS/MS fragmentation. Consistent and reproducible data was obtained in multiple repeats of experiments.

Initially, MALDI-MS was used to obtain a profile of the molecular ions from each of the glycan pools. This gave singly charged sodiated molecular ions ($[M + Na]^+$) and allowed the assignment of the composition of molecular ions in terms of the number of Hex, Deoxy-Hex, HexNAc and Pent residues. Based on their molecular compositions, knowledge of the biosynthetic pathway and previously published structural data, putative structures could be proposed. To confirm putative structures, molecular ions observed in the MS spectrum were subjected to MS/MS analysis using two different ionization methods, ESI and MALDI, which

afforded sequence informative fragment ions that provided vital structural information such as the non-reducing end sequences i.e. antennae structures, branching patterns and sometimes linkage positions. GC-MS linkage analyses were performed in order to define monosaccharide compositions, glycosidic bond positions and relative abundance (RA) of each sugar component. Results from the various experiments were combined to assign detailed glycan structures.

MALDI profiling of ESP N-glycans

Typical MALDI-MS spectra of PNGase F (Figure 2 [A]) and PNGase A (Figure 2 [B]) released ESP N-glycans are shown in Figure 2 and their assignments are compiled in Table 1. Based on the compositions of each molecular ion the types of N-glycans were deduced. In the PNGase F-released ESP N-glycan profile hybrid structures comprised the major N-glycan family, with dominant signals corresponding to compositions $\text{Fuc}_{0-1}\text{Hex}_{5-6}\text{HexNAc}_3$ (m/z 1999, 2030 and 2204). The next most abundant were complex type structures ($\text{Fuc}_{1-4}\text{Hex}_{4-6}\text{HexNAc}_{3-6}$) with dominant signals at m/z 2245 ($\text{Fuc}_1\text{Hex}_5\text{HexNAc}_4$) and 2420 ($\text{Fuc}_2\text{Hex}_5\text{HexNAc}_4$). Minor signals consistent with truncated type structures ($\text{Xyl}_{0-1}\text{Fuc}_{0-1}\text{Hex}_{1-3}\text{HexNAc}_{2-3}$) and high mannose structures (m/z 1580 and 1785, $\text{Hex}_{5-6}\text{HexNAc}_2$) were also present. The PNGase A released ESP N-glycan profile revealed new highly fucosylated signals (m/z 2143, 2184, 2347, 2551, 2768, 2807, 2837, 2982, 3012, 3257, 3390 and 3431, see Table 1).

Figure 2

Table 1

Characterization of ESP N-glycans by MS/MS analysis

To confirm the putative structures deduced from the MALDI-MS data, MS/MS analyses were performed on all ESP N-glycan molecular ions observed in Figure 2. Molecular ions observed in the PNGase F released N-glycan pool whose compositions indicated the presence of at least one fucose residue gave rise to a fragment ion at m/z 474, which is indicative of monofucosylated cores (Fuc α 1 $\rightarrow$ 6GlcNAc). Whilst molecular ions in the PNGase A released N-glycan pool containing two or more fucose residues produced a fragment ion at m/z 648, which implies the presence of difucosylated cores (Fuc α 1 $\rightarrow$ 6(Fuc α 1 $\rightarrow$ 3)GlcNAc). Both signals result from a glycosidic cleavage between the GlcNAc residues of the N-glycan core (see examples in Supplemental Figure 1-4).

Complex and hybrid type structures in both ESP N-glycan pools (m/z 1621, 1795, 1825, 1955, 1969, 1999, 2029, 2040, 2070, 2143, 2173 and 2203 to 3431 (see Figure 2) produced diagnostic fragment ions at m/z 660 (Fuc $_1$ Hex $_1$ HexNAc $_1$), with the concomitant elimination of Fuc giving rise to the signal at m/z 472, which is evidence for the Fuc residue being linked to the 3' position of the GlcNAc. Therefore, this indicates the presence of Le^x (Gal β 1 $\rightarrow$ 4(Fuc α 1 $\rightarrow$ 3)GlcNAc) structures. Additionally, the fragment ion at m/z 486 (Hex $_1$ HexNAc $_1$) provides evidence of LacNAc structures (see example in Supplemental Figure 1 and 2). Molecular ions above m/z 2693 also yielded fragment ions at m/z 935 (Hex $_2$ HexNAc $_2$), 1109 (Fuc $_1$ Hex $_2$ HexNAc $_2$) and 1283 (Fuc $_2$ Hex $_2$ HexNAc $_2$) which are consistent with tandem repeats of LacNAc and Le^x (see example in Supplemental Figure 3 and 4). ESP N-glycans whose molecular compositions indicated a higher content of HexNAc than Hex residues and contained at least one fucose residues (m/z 2010, 2184, 2633, 2807, 2909, 2982, 3082, 3257 and 3431) produced signals at m/z 701 (Fuc $_1$ HexNAc $_2$) and/or 875 (Fuc $_2$ HexNAc $_2$) and fragment ions

corresponding to the concomitant loss of these substituents from the molecular ion. These observations support mono-fucosylated and di-fucosylated LacdiNAc structures (see example in Supplemental Figure 1 and 2) in ESP N-glycans. In addition, when molecular ions contained at least two fucose residues and difucosylated LacdiNAc structures (e.g. m/z 2633) MS/MS spectra also produced a fragment ion at m/z 403 (Fuc₂) indicating that Fuc residues in difucosylated LacdiNAc structures can also be present in tandem (see example in Supplemental Figure 1 and 2).

MS/MS analyses on ESP complex N-glycan structures whose compositions implied the possibility of tri-antennary or tetra-antennary structures (m/z 2663, 2693, 2807, 2837, 2909, 3012, 3042, 3082, 3216, 3257, 3390 and 3431) exhibited fragmentation patterns showing that such structures were predominantly bi-antennary N-glycans. These contained an extended antennae comprising of tandem repeats of LacNAc and/or Le^x (see example in Supplemental Figure 3 and 4). Only a minority of the components were multiantennary N-glycans.

GC-MS linkage analysis of ESP N-glycans

The complexity of the glycan pools precluded the assignment of each detected component to an individual glycan. However, important conclusions could be drawn from the linkage data (Supplemental Table 2).

The PNGase F and PNGase A-released N-glycan pools comprised mostly similar components, with the exception of the presence of 3,4,6-linked GlcNAc in the PNGase A-released N-glycan pool, which is an indication of core difucosylation. This result together with the MS/MS data unambiguously confirmed the presence of core difucosylated N-glycans in ESP.

High levels of 3,6-linked mannose and 4-linked GlcNAc were in accordance with these residues being constituents of the core of the majority of N-glycans. Xylosylated cores were corroborated by terminal Xyl and 2,3,6-linked Man. However, these were present in relatively low amounts and were consistent with the MALDI-MS compositional assignments, indicating that xylosylation is not a dominant feature in ESP N-glycans. Monofucosylated cores in the PNGase F-released N-glycan pool were confirmed by the presence of 4,6-linked GlcNAc. The high abundance of 2-linked mannose indicated high levels of hybrid and complex structures. Minor quantities of 2,4- and 2,6-linked Man revealed the presence of tri- and possibly tetra-antennary complex N-glycans. This is consistent with observations made in MS/MS experiments where multiantennary complex N-glycans are minor components. Fuc, Man and Gal are the major terminal sugars with lesser amounts of terminal GlcNAc and GalNAc. In addition, GlcNAc and GalNAc residues are substituted at their 3- and 4- positions, which together with MS/MS data (see previous section), supports the presence of Le^x and fucosylated LacdiNAc ($\pm\text{Fuc}\alpha 1 \rightarrow 3\text{GalNAc}\beta 1 \rightarrow 4(\pm\text{Fuc}\alpha 1 \rightarrow 3)\text{GlcNAc}$) structures. The observation of 2-linked Fuc corroborates with the MS/MS data that some Fuc residues are further substituted.

Overall, these data indicates that hybrid and complex N-glycans are abundant structures in ESP N-glycan pools. Mono- and difucosylated cores are notable features in the majority of ESP N-glycans, whereas core xylosylation occurs at low levels. Major terminal structures are Le^x and LacNAc, which are also present as tandem repeats. Other terminal structures include LacdiNAc, mono- and di-fucosylated LacdiNAc, which are present in the less abundant high molecular weight structures. Complex N-glycans are predominantly bi-antennary structures with minor amounts of multiantennary structures. Taken together these data support the proposed structures summarized in Figure 4.

MALDI profiling of 0-3h RP N-glycans

Figure 3 shows typical MALDI-MS spectra of PNGase F (panel [A]) and PNGase A (panel [B]) released 0-3h RP N-glycans and assignments are shown in Table 2. Their compositions revealed that the majority of the N-glycans carry xylose. The PNGase F and PNGase A-released 0-3h RP N-glycans spectra exhibited very similar profiles. Truncated type structures ($\text{Xyl}_{0-1}\text{Fuc}_{0-1}\text{Hex}_{1-3}\text{HexNAc}_2$) and complex type structures ($\text{Xyl}_{0-1}\text{Fuc}_{1-4}\text{Hex}_{4-6}\text{HexNAc}_{3-6}$) were the two most abundant N-glycan families in both N-glycan pools. New minor high molecular weight peaks were present in the PNGase A pool (m/z 1988, 2070, 2489, 2839, 2868, 3358, 3532, 3706, 3880 and 4054) some of which could potentially contain difucosylated cores according to their compositions.

Figure 3

Table 2

Characterization of 0-3h RP N-glycans by MS/MS analysis

MS/MS analyses were performed on the majority of the most abundant molecular ions.

Molecular ions in the PNGase F released N-glycan pool containing at least one fucose residue resulted in a diagnostic fragment ion at m/z 474 (see example in Supplemental Figure 5), showing the presence of mono-fucosylated N-glycan cores. MS/MS analysis on molecular ions in the PNGase A released N-glycan pool with at least two fucose residues did not yield fragment ions at m/z 648, which suggested that difucosylated N-glycan cores are not present in 0-3h RP.

All signals in the MALDI-MS spectra (Figure 4) whose compositions indicated the presence of a

xylose (Table 2) consistently produced fragment ions corresponding to the loss of a xylose residue (see example in Supplemental Figure 5).

Major terminal structures in 0-3h RP were deduced from MS/MS experiments on hybrid and complex type components (m/z 1781 and above, see Figure 3). MS/MS spectra of such molecular ions revealed signals at m/z 486 and 660 supporting LacNAc and Le^x structures respectively. Complex and hybrid type molecular ions at m/z 2663, 2782, 2837, 2867, 3202, 3357, 3376, 3406, 3447, 3621 and 3651 gave rise to fragment ions at m/z 935 (Hex₂HexNAc₂), 1109 (Fuc₁Hex₂HexNAc₂) and 1283 (Fuc₂Hex₂HexNAc₂), revealing tandem repeats of LacNAc and Le^x. Moreover, MS/MS spectra of the molecular ions whose HexNAc content was higher than Hex e.g. m/z 2489, 2663, 2998, 3172, 3357, 3416, 3447, 3532 and 3591 produced fragment ions at m/z 731 (Hex₁HexNAc₂), 905 (Fuc₂Hex₁HexNAc₂) and 1079 (Fuc₂Hex₁HexNAc₂), which implicated the occurrence of the *S. mansoni*-specific terminal structure consisting of Galβ1→4(±Fucα1→3)GlcNAcβ1→4(±Fucα1→3)GlcNAc previously identified in whole cercarial extracts (14) (see example in Supplemental Figure 5).

In terms of branching patterns of 0-3h RP complex N-glycans, major species correspond to mono-antennary structures (e.g. m/z 1969 and 2129) with some bi-antennary structures (e.g. m/z 2244, 2418, 2578, 2592 and 2752) and minor amounts of multiantennary structures (molecular ions above m/z 2752).

GC-MS Linkage analysis of 0-3h RP N-glycans

The high relative abundance of 2,3,6-linked Man and terminal Xyl are indicative of xylosylated cores (Supplemental Table 3). This was in accordance to the MALDI-MS compositional data, which shows that many N-glycans carried a xylose residue. 2-linked Man

was present in high levels indicating that complex mono- and biantennary N-glycans were dominant structures and the lower abundance of 2,4 and 2,6-linked Man indicated that only a minority of the complex structures were multiantennary. The presence of LacNAc and Le^x antennae were supported by terminal Fuc, terminal Gal, 4-linked GlcNAc and 3,4-linked GlcNAc. Also, the detection of 3-linked Gal suggested that these terminal structures may be present in tandem repeats as in ESP N-glycans. Analysis of the PNGase A released N-glycans showed similar results to that obtained from PNGase F-released N-glycans (Supplemental Table 3). Moreover, 3,4,6-linked GlcNAc was absent and together with the MS/MS data, this strongly indicates that 0-3h RP N-glycans do not contain difucosylated cores.

Taken together, these results show that the 0-3h RP N-glycan profile contain abundant truncated and complex structures. The dominant groups of 0-3h RP N-glycans are core xylosylated and monofucosylated. Major terminal antennae structures include Le^x and LacNAc, which are also present in tandem repeats and the less abundant N-glycans carry the *S. mansoni*-specific terminal structure Gal β 1 $\rightarrow$ 4($\pm$ Fuc α 1 $\rightarrow$ 3)GlcNAc β 1 $\rightarrow$ 4($\pm$ Fuc α 1 $\rightarrow$ 3)GlcNAc. The proposed 0-3h RP N-glycan structures are shown in Figure 4.

Figure 4

ESP O-glycans

A representative MALDI-MS spectrum of the ESP O-glycans is shown in Figure 5 and compositional assignments are given in Table 3. The most intense molecular ions are *m/z* 738 and 983 which correspond to glycans with the compositions Hex₂HexNAc₁-itol and Hex₂HexNAc₂-itol respectively. O-glycan structures were further characterized by MS/MS

experiments, from which the core structures were confirmed. The MS/MS profile of the molecular ion m/z 738 is shown in Figure 6 with fragmentation patterns shown as insets.

Figure 5

Table 3

The fragment ions at m/z 486, 329 and 275 demonstrate the presence of a non-reducing end HexHexNAc unit implying the existence of a reducing end Hex-itol residue. In an attempt to identify this unknown Hex-itol residue, GC-MS linkage analysis on a range of sugar standards containing Hex-itols was carried out. A 4-linked Gal-itol sugar residue was demonstrated to co-elute and produced an EI-spectrum consistent with the unknown Hex-itol. These results are indicative of a novel core consisting of a Gal residue linked to the protein backbone (Figure 6 isomer [A]) rather than the more common GalNAc residue. In addition to this novel core, the molecular ion at m/z 738 also contained a second isomer, in which the non-reducing end consisted of two Hex units, supported by the fragment ions at m/z 259, 502 and 520 (Figure 6 isomer [B]). The HexNAc-itol residue is likely to be a GalNAc-itol residue, since a 3,6-linked GalNAc-itol residue is present in the linkage analysis (Supplemental Table 4). Therefore, these data supported the presence of two distinct isomers for this molecular ion: (i) isomer A (Figure 6 isomer [A]), that contains the novel Gal core and (ii) isomer B (Figure 6 isomer [B]) which contains the previously identified *S. mansoni*-specific core sequence Gal β 1 $\rightarrow$ 3(Gal β 1 $\rightarrow$ 6)GalNAc (12).

Figure 6

All major molecular ions observed in the ESP O-glycan MALDI-MS spectrum were subjected to MS/MS analyses. These MS/MS analyses together with GC-MS linkage analysis showed that the major ESP O-glycans (e.g. m/z 534, 779, 983, 1157, 1228, 1432, 1607, 1822) contained the more conventional mucin-type core 1 and 2 ($\text{Gal}\beta 1 \rightarrow 3(\pm \text{GlcNAc}\beta 1 \rightarrow 6)\text{GalNAc}$). Only a minor proportion also expressed the $\text{Gal}\beta 1 \rightarrow 3(\text{Gal}\beta 1 \rightarrow 6)\text{GalNAc}$ core (m/z 738 and 1607) (see example in Supplemental Figure 6 and 7).

Dominant terminal structures in ESP O-glycans were deduced from MS/MS spectra given by molecular ions such as e.g. m/z 983, 1157, 1228, 1433, 1607 and 1822. These produced fragment ions at 486 ($\text{Hex}_1\text{HexNAc}_1$) and 660 ($\text{Fuc}_1\text{Hex}_1\text{HexNAc}_1$) consistent with LacNAc and Le^x (see example in Supplemental Figure 6 and 7). Signals such as m/z 1228 and 1432 also produced fragment ions corresponding to the loss of $\text{Hex}_1\text{HexNAc}_2$ from the molecular ion which is consistent with $\text{Gal}\beta 1 \rightarrow 3\text{GalNAc}\beta 1 \rightarrow 4\text{GlcNAc}$. These observations were also in accordance with the presence of terminal Gal, 4-linked GlcNAc, 3-linked GalNAc and 3,4-linked GlcNAc in the GC-MS linkage analysis (Supplemental Table 4).

MS/MS spectra of molecular ions such as m/z 1822, whose composition indicated the presence of at least one fucose residue and whose HexNAc content is higher than that of Hex yielded fragment ions at m/z 456 ($\text{Fuc}_1\text{HexNAc}_1$), 527 (HexNAc_2) and 701 ($\text{Fuc}_1\text{HexNAc}_2$) (data not shown). These signals together with the presence 3-linked GalNAc in the GC-MS linkage analyses (Supplemental Table 4) are consistent with minor components containing LacdiNAc and fucosylated LacdiNAc structures. In addition, the detection of 2-linked fucose in the linkage data (Supplemental Table 4) and the fragment ion at m/z 403 indicates that some fucose residues are further substituted in molecular ions with difucosylated LacdiNAc structures.

In summary, the major ESP O-glycans contain the more conventional mucin-type core 1 and 2 structures. Minor O-glycans carry the *S. mansoni*-specific Gal β 1 $\rightarrow$ 3(Gal β 1 $\rightarrow$ 6)GalNAc core and the novel type of core in which a Gal residue is attached to the protein backbone. The most abundant terminal structures are LacNAc and Le^x and fucosylated terminal LacdiNAc structures are found in minor components. Figure 8 illustrates the proposed ESP O-glycan structures.

0-3h RP O-glycans

A typical MALDI-MS O-glycan profile for the 0-3h RP O-glycans is shown in Figure 7 and compositional assignments are shown in Table 4. O-glycan sequences were confirmed by MS/MS analyses of molecular ions observed in the MALDI-MS spectrum.

Figure 7

Table 4

MS/MS experiments on the molecular ion at m/z 738 showed that 0-3h RP O-glycans also contained the novel core identified in ESP (Supplemental Figure 10) which is further supported by a 4-linked Gal-itol in the linkage data (Supplemental Table 4). Fragmentation patterns of molecular ions at m/z 534, 738, 779, 983, 1187, 1361, 1391, 1565, 1595, 1607, 1637, 1811, 1841, 1985, 2015, 2189, 2219, 2464 ions established that the predominant O-glycan core in 0-3h RP is Gal β 1 $\rightarrow$ 3($\pm$ Gal β 1 $\rightarrow$ 6)GalNAc (see example in Supplemental Figure 8 and 9). This is further corroborated by 3- and 3,6-linked GalNAcitol in the linkage analyses (Supplemental Table 4).

Minor 0-3h RP O-glycans (m/z 1157 and those above 2464), whose HexNAc residue content are higher than that of Hex residues contained the mucin-type 2 core structures (data not shown).

All molecular ions that were subjected to MS/MS analyses gave rise to fragment ions from which the terminal structures were also deduced. MS/MS spectra of the major molecular ions with relative intensities in the MALDI-MS (Figure 7) spectrum above 10% relative intensity e.g. m/z 1187, 1361, 1391, 1565, 1595, 1607, 1637, 1811, 1841, 1985, 2015, 2189 established that 0-3h RP O-glycan antennae mostly comprised of LacNAc (m/z 486) and Le^x (m/z 660 and 472) (see example in Supplemental Figure 8 and 9). Fragmentation patterns of higher molecular weight components present at below 5% relative intensity in the MALDI-MS spectrum (Figure 7) and whose masses were above m/z 2519 produced fragment ions at m/z 403 (Fuc₂), 456 (Fuc₁HexNAc₁), 577 (Fuc₃) and 630 (Fuc₂HexNAc₁) indicating multi-fucosylated LacdiNAc structures. These results are consistent with the detection of 3-linked GalNAc, 4-linked and 3,4-linked GlcNAc and 2-linked Fuc in the GC-MS linkage data.

Overall, the major 0-3h RP O-glycans are based on the Gal β 1 $\rightarrow$ 3(Gal β 1 $\rightarrow$ 6)GalNAc core. The lower abundant components carry mucin-type core 2 structures and an O-glycan with Gal as its reducing end residue is present in trace amounts. The main terminal structures found are LacNAc and Le^x whilst the less abundant O-glycans express highly fucosylated terminal epitopes. A summary of proposed O-glycan structures from 0-3h RP is shown in Figure 8.

Figure 8

Discussion

Here, using a combination of mass spectrometric techniques, we have identified the major glycoproteins from cercarial (0-3h RP) and egg (ESP) secretions and elucidated their N- and O-glycan structures. Our results show that ESP only contained two major glycoproteins, Omega-1 and IPSE. The former has recently been characterized and linked to a previously described egg protein, with hepatotoxic properties, consistent with its ribonuclease T2 domain (25). Its *in vivo* role remains to be established but possibilities include modulation of host cell-mediated immune responses. Extracellular ribonucleases have been implicated in inhibiting the proliferation of and inducing apoptosis of activated lymphocytes thus causing immunosuppressive activity (30-32). IPSE, also known as alpha-1, has been identified previously to be a major immunogenic secreted glycoprotein of *S. mansoni* eggs and was the subject of recent functional studies (26-28). The main glycoproteins present in 0-3h RP are inhibitor serpin_c, two glycoproteins with sperm coating protein (SCP) domains (SmSCP_b and SmSCP_c) that are related to venom allergens, a dipeptidyl peptidase IV and several unknown glycoproteins. The 0-3h RP have been demonstrated to drive Th2 responses (33) and stimulate macrophage cytokine production through TLR-4 dependent and independent pathways (34). The specific constituents responsible for these effects are unknown but glycan components are required for optimal cytokine production (34). Serpins are serine protease inhibitors and could therefore be active against proteins of the host immune system. The structural determination of a protein containing an SCP-domain in the human hookworm *Necator americanus* revealed similarities to chemokines, which could explain its immunomodulatory properties (35).

The comparative glycomic analyses in this study have clearly demonstrated that ESP and 0-3h RP express a complex and vast array of glycans. Detailed structural analyses revealed both common and unique stage-specific structures. The major terminal structures found in both

secretions are LacNAc, Le^x and tandem-Le^x/LacNAc in both the N- and O-glycans. Many functional roles have been attributed to such terminal structures and thus these epitopes could contribute to the immunomodulatory properties of the secreted glycoproteins and also explain the immunological cross-reactivity between the two secretions (Kariuki *et al* unpublished data).

Le^x has been demonstrated to induce the production of immune mediators such as IL-10 and PGE₂ that promote Th2 type responses; it can also act as a Th2 inducing adjuvant (36, 37). Le^x-containing glycoconjugates have also been shown to drive the maturation of naïve dendritic cells (DC) to a DC2 phenotype via the toll-like receptor 4 (TLR-4) dependent mechanisms, consequently leading to a Th-2 type response (38). The lectin dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN) binds Le^x and could therefore be involved in the glycan driven DC2 phenotype maturation (39). *In vitro*, it has been demonstrated that antibodies against Le^x mediate a complement dependent lysis of granulocytes (40), which may explain the mild neutropenia observed in patients suffering from schistosomiasis (41). Here, we show that Le^x can also be present in tandem repeats in both secretions. Studies investigating the effects of the multimericity of Le^x demonstrated that IgM antibody responses were higher for polyLe^x than monomeric Le^x and the different presentation of Le^x also evokes different classes of antibodies (42). Granulocytes are known to express polyLe^x on their surface (43), therefore anti-polyLe^x antibodies in infected individuals might bind more strongly to granulocytes subsequently leading to neutropenia. Also, the schistosome-specific terminal structure (Galβ1→4(±Fucα1→3)GlcNAcβ1→4(±Fucα1→3)GlcNAc) present in 0-3h RP N-glycans is likely to induce similar immune responses as those by Le^x.

ESP N- and O-glycans and 0-3h RP O-glycans express LacdiNAc and its fucosylated counterparts. Studies with schistosomes have shown that these epitopes stimulate the production

in monocytes of cytokines that induce antibody responses. This capacity is enhanced by the presence of fucosylated LacdiNAc structures (44). LacdiNAc binds to rat macrophage galectin-3 which plays a role in mediating recognition and phagocytosis of LacdiNAc-containing neoglycoconjugates by macrophages (45). The macrophage galactose-type lectin (MGL) expressed by dendritic cells binds both LacdiNAc and fucosylated LacdiNAc (46) and thus, potentially mediates the uptake of secretions by phagocytic cells. The multifucosylated LacdiNAc structures identified in ESP and 0-3h RP O-glycans resemble those found in the cercarial glycocalyx (CGX) (15). Studies have shown that immunization with the cercarial glycocalyx enhanced maturation of a secondary infection with cercariae (47).

A major difference between ESP and 0-3h RP N- and O-glycans is core modifications. Difucosylated cores are prominent in ESP N-glycans, whereas xylosylation is the major core modification in 0-3h RP. These types of modifications are not found in mammalian glycoproteins but are common in plants and invertebrates (48, 49). They induce a glycan-specific Th2 type cellular immune response to schistosomes in mice (50). The $\alpha 1 \rightarrow 3$ core fucose in honeybee venom phospholipase A₂ is a major allergenic determinant that induces substantial IgE responses in allergic individuals and in helminth infections (49-53). The core xylose residue is also largely responsible for IgE binding and allergenicity of plant and insect-derived glycoproteins (48). The majority of 0-3h RP O-glycans are based on the schistosome specific core (Gal β 1 $\rightarrow$ 3(Gal β 1 $\rightarrow$ 6)GalNAc) whereas ESP possess minor amounts of this core and higher levels of mammalian-like mucin-type core 1 and 2 structures. From a glycobiological perspective a significant finding is the presence of a novel O-glycan core where a Gal residue is attached to the protein backbone. The biological significance of this new form of protein O-glycosylation

remains to be elucidated but it implicates the presence of a novel polypeptide galactose transferase enzyme in schistosomes.

It is evident from our results that secretions contain stage-specific glycan epitopes which may play a role in the interplay with the immediate host environment for successful parasitism. There are several reasons as to why the egg may release potentially immunogenic glycoproteins. Eggs are deposited by females in the blood vessels of the gut wall in a completely undeveloped state and the miracidium larva takes 5-7 days to develop before the egg travels to the gut lumen for excretion in the feces (3). This process requires an intact host immune system since egg excretion via the feces is greatly reduced in immunocompromised mice and humans with HIV infection (54, 55). From this it can be inferred that the potent immunogenicity of the egg secretions is an integral part of their escape mechanism. Furthermore, since antibody responses are overwhelmingly directed to glycan epitopes in ESP (6), a proportion of the glycan structures we have defined must be the key epitopes. The mechanism of egg passage through the gut tissues thus represents an unusual example of a pathogen recruiting host immune responses for its own purposes. One further possibility, by analogy with glycosaminoglycans that serve to sequester chemokines at the surface of mammalian endothelial cells, is that the extensive glycosylation of the ESP also has a sequestering function (56). The mature egg releases ESP to assist its passage through the gut tissues. This process would be made more efficient if the secretions were concentrated in its vicinity instead of diffusing away; the glycans could provide such a mechanism by binding to host tissue constituents, or by assembling the ESP proteins into macromolecular complexes. Another property of the glycosylation of ESP proteins may be to confer resistance to digestion by the two proteases known to be present in the egg secretions (3). A likely explanation for the strong immunogenicity of the cercarial glycans is that they provide a

smokescreen to disguise more vulnerable peptide epitopes from immune attack. A more elaborate version of this hypothesis is that the cercarial epitopes are a “matador’s cloak”. The schistosomulum in transit from epidermis to blood vessel represents a moving target and it is easy to envisage how glycans released from the acetabular glands into the skin in the initial stages of penetration might decoy leucocytes away from the parasite during the brief period when it is acquiring its immune disguise (57).

Further glycoproteomic studies are being undertaken to determine the glycans expressed by the individual glycoproteins and their site specific occupancy. This information will allow issues of glycan clustering and presentation to be investigated which have been shown to be of critical importance for the binding of glycans to a whole range of lectins (39, 45, 46).

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Figure Legends

Figure 1. [A] 2DE gel stained with Sypro Ruby of ESP. [B] ProQ Emerald stained ESP 2DE gel. [C] Combined images of Sypro Ruby and ProQ Emerald stained ESP 2DE gel. Spot (1) Omega-1, Spots (2,3) IPSE [D] 0-3h RP 2DE gel stained with Sypro Ruby. [E] 0-3h RP 2DE gel stained with ProQ Emerald. [F] Combined images of Sypro Ruby and ProQ Emerald stained 0-3h RP 2DE gel Spot (1) SmPepM8; (2) SmCE; (3) SmSerpín_c; (4) SmDPP IV; (5) SmSCP_c; (6) SmSCP_b. The combined images were obtained using Evolution software.

Figure 2. MALDI-MS spectra of permethylated PNGase F [A] and PNGase A [B] released N-glycans from ESP. ESP N-glycans were sequentially released by PNGase F and PNGase A, permethylated and purified using an acetonitrile:water C₁₈ Sep-Pak system. Signals observed correspond to singly charged sodiated molecular ions [M+Na]⁺.

Figure 3. MALDI-MS spectra of permethylated PNGase F [A] and PNGase A [B] released N-glycans from 0-3h RP. 0-3h RP N-glycans were sequentially released by PNGase F and PNGase

A, permethylated and purified using an acetonitrile:water C₁₈ Sep-Pak system. Signals in the spectrum correspond to singly charged sodiated molecular ions $[M+Na]^+$.

Figure 4. Proposed structures of ESP and 0-3h RP N-glycans.

Figure 5. MALDI-MS spectrum of permethylated O-glycans from ESP. Signals observed correspond to singly charged sodiated molecular ions $[M+Na]^+$.

Figure 6. CAD-ES-MS/MS spectrum for the singly charged $[M + Na]^+$ molecular ion m/z 738 with the composition Hex₂HexNAc₁-itol. The spectrum was obtained using a Q-TOF instrument. Insets show fragmentation patterns from which two distinct isomers (**[A]** and **[B]**) can be assigned.

Figure 7. MALDI-MS spectrum of permethylated O-glycans from 0-3h RP. Signals observed correspond to singly charged sodiated molecular ions $[M + Na]^+$.

Figure 8. Proposed structures of O-glycans from ESP and 0-3h RP.

Tables

Table 1. Compositional assignments of singly charged sodiated molecular ions $[M+Na]^+$ observed in MALDI-MS spectra of permethylated PNGase F and PNGase A released ESP N-glycans.

^a indicates PNGase A released N-glycans.

Signals (<i>m/z</i>)	Assignment $[M+Na]^+$	Signals <i>m/z</i>	Assignment $[M+Na]^+$
1506	Xyl ₁ Fuc ₁ Hex ₃ HexNAC ₂	2404	Xyl ₁ Fuc ₁ Hex ₅ HexNAC ₄
1580	Hex ₅ HexNAC ₂	2418	Fuc ₂ Hex ₅ HexNAC ₄
1621	Hex ₄ HexNAC ₃	2448	Fuc ₁ Hex ₆ HexNAC ₄
1751	Xyl ₁ Fuc ₁ Hex ₃ HexNAC ₃	2459	Fuc ₂ Hex ₄ HexNAC ₅
1784	Hex ₆ HexNAC ₂	2489	Fuc ₁ Hex ₅ HexNAC ₅
1795	Fuc ₁ Hex ₄ HexNAC ₃	2551 ^a	Fuc ₃ Hex ₆ HexNAC ₃
1825	Hex ₅ HexNAC ₃	2592	Fuc ₃ Hex ₅ HexNAC ₄
1836	Fuc ₁ Hex ₃ HexNAC ₄	2622	Fuc ₂ Hex ₆ HexNAC ₄
1955	Xyl ₁ Fuc ₁ Hex ₄ HexNAC ₃	2633	Fuc ₃ Hex ₄ HexNAC ₅
1969	Fuc ₂ Hex ₄ HexNAC ₃	2652	Fuc ₁ Hex ₇ HexNAC ₄
1999	Fuc ₁ Hex ₅ HexNAC ₃	2663	Fuc ₂ Hex ₅ HexNAC ₅
2010	Fuc ₂ Hex ₃ HexNAC ₄	2693	Fuc ₁ Hex ₆ HexNAC ₅
2029	Hex ₆ HexNAC ₃	2768 ^a	Fuc ₄ Hex ₅ HexNAC ₄
2040	Fuc ₁ Hex ₄ HexNAC ₄	2807 ^a	Fuc ₄ Hex ₄ HexNAC ₅
2070	Hex ₅ HexNAC ₄	2837 ^a	Fuc ₃ Hex ₅ HexNAC ₅
2143 ^a	Fuc ₃ Hex ₄ HexNAC ₃	2868	Fuc ₂ Hex ₆ HexNAC ₅
2159	Xyl ₁ Fuc ₁ Hex ₅ HexNAC ₃	2909	Fuc ₂ Hex ₅ HexNAC ₆
2173	Fuc ₂ Hex ₅ HexNAC ₃	2982 ^a	Fuc ₅ Hex ₄ HexNAC ₅
2184 ^a	Fuc ₃ Hex ₃ HexNAC ₄	3012 ^a	Fuc ₄ Hex ₅ HexNAC ₅
2203	Fuc ₁ Hex ₆ HexNAC ₃	3042	Fuc ₃ Hex ₆ HexNAC ₅
2214	Fuc ₂ Hex ₄ HexNAC ₄	3082	Fuc ₃ Hex ₅ HexNAC ₆
2244	Fuc ₁ Hex ₅ HexNAC ₄	3216	Fuc ₄ Hex ₆ HexNAC ₅
2285	Fuc ₁ Hex ₄ HexNAC ₅	3257 ^a	Fuc ₄ Hex ₅ HexNAC ₆
2347 ^a	Fuc ₃ Hex ₅ HexNAC ₃	3390 ^a	Fuc ₅ Hex ₆ HexNAC ₅
2377	Fuc ₂ Hex ₆ HexNAC ₃	3431 ^a	Fuc ₅ Hex ₅ HexNAC ₆

Table 2. Compositional assignments of singly charged sodiated molecular ions $[M+Na]^+$

observed in MALDI-MS spectra of permethylated PNGase F and PNGase A released 0-3h RP N-glycans.

^a indicates PNGase A released N-glycans.

Signals (<i>m/z</i>)	Assignment $[M+Na]^+$	Signals (<i>m/z</i>)	Assignment $[M+Na]^+$
1346	Fuc ₁ Hex ₃ HexNAC ₂	2578	Xyl ₁ Fuc ₂ Hex ₅ HexNAC ₄
1376	Hex ₄ HexNAC ₂	2592	Fuc ₃ Hex ₅ HexNAC ₄
1506	Xyl ₁ Fuc ₁ Hex ₃ HexNAC ₂	2664 ^a	Fuc ₂ Hex ₅ HexNAC ₅
1536	Xyl ₁ Hex ₄ HexNAC ₂	2752	Xyl ₁ Fuc ₃ Hex ₅ HexNAC ₄
1550	Fuc ₁ Hex ₄ HexNAC ₂	2782	Xyl ₁ Fuc ₂ Hex ₆ HexNAC ₄
1751	Xyl ₁ Fuc ₁ Hex ₃ HexNAC ₃	2839 ^a	Fuc ₃ Hex ₅ HexNAC ₅
1781	Xyl ₁ Hex ₄ HexNAC ₃	2868 ^a	Fuc ₂ Hex ₆ HexNAC ₅
1784	Hex ₆ HexNAC ₂	2927	Xyl ₁ Fuc ₄ Hex ₅ HexNAC ₄
1795	Fuc ₁ Hex ₄ HexNAC ₃	2998	Xyl ₁ Fuc ₃ Hex ₅ HexNAC ₅
1955	Xyl ₁ Fuc ₁ Hex ₄ HexNAC ₃	3172	Xyl ₁ Fuc ₄ Hex ₅ HexNAC ₅
1969	Fuc ₂ Hex ₄ HexNAC ₃	3202	Xyl ₁ Fuc ₃ Hex ₆ HexNAC ₅
1988 ^a	Hex ₇ HexNAC ₂	3346	Xyl ₁ Fuc ₅ Hex ₅ HexNAC ₅
1999	Fuc ₁ Hex ₅ HexNAC ₃	3358 ^a	Fuc ₂ Hex ₆ HexNAC ₇
2070 ^a	Hex ₅ HexNAC ₄	3376	Xyl ₁ Fuc ₄ Hex ₆ HexNAC ₅
2129	Xyl ₁ Fuc ₂ Hex ₄ HexNAC ₃	3407	Xyl ₁ Fuc ₃ Hex ₇ HexNAC ₅
2159	Xyl ₁ Fuc ₁ Hex ₅ HexNAC ₃	3417	Xyl ₁ Fuc ₄ Hex ₅ HexNAC ₆
2173	Fuc ₂ Hex ₅ HexNAC ₃	3447	Xyl ₁ Fuc ₃ Hex ₆ HexNAC ₆
2214	Fuc ₂ Hex ₄ HexNAC ₄	3532 ^a	Fuc ₃ Hex ₆ HexNAC ₇
2244	Fuc ₁ Hex ₅ HexNAC ₄	3591	Xyl ₁ Fuc ₅ Hex ₅ HexNAC ₆
2374	Xyl ₁ Fuc ₂ Hex ₄ HexNAC ₄	3621	Xyl ₁ Fuc ₄ Hex ₆ HexNAC ₆
2404	Xyl ₁ Fuc ₁ Hex ₅ HexNAC ₄	3651	Xyl ₁ Fuc ₃ Hex ₇ HexNAC ₆
2418	Fuc ₂ Hex ₅ HexNAC ₄	3706 ^a	Fuc ₄ Hex ₆ HexNAC ₇
2489 ^a	Fuc ₁ Hex ₅ HexNAC ₅	3880 ^a	Fuc ₅ Hex ₆ HexNAC ₇
2578	Xyl ₁ Fuc ₂ Hex ₅ HexNAC ₄	4054 ^a	Fuc ₆ Hex ₆ HexNAC ₇

Table 3. Compositions of singly charged sodiated molecular ions $[M+Na]^+$ observed in the MALDI-MS spectrum of permethylated ESP O-glycans. The reducing end of O-glycans were recovered as oligoglycosyl alditols and therefore compositions are denoted as “-itols.”

Signal (<i>m/z</i>)	Assignment $[M+Na]^+$	Signal (<i>m/z</i>)	Assignment $[M+Na]^+$
534	Hex ₁ HexNAC ₁ -itol	1852	Fuc ₁ Hex ₃ HexNAC ₄ -itol
738	Hex ₂ HexNAC ₁ -itol	1863	Fuc ₂ Hex ₁ HexNAC ₅ -itol
779	Hex ₁ HexNAC ₂ -itol	1882	Hex ₄ HexNAC ₄ -itol
983	Hex ₂ HexNAC ₂ -itol	1923	Hex ₃ HexNAC ₅ -itol
1024	Hex ₁ HexNAC ₃ -itol	1996	Fuc ₃ Hex ₂ HexNAC ₄ -itol
1157	Fuc ₁ Hex ₂ HexNAC ₂ -itol	2015	Fuc ₁ Hex ₅ HexNAC ₃ -itol
1198	Fuc ₁ Hex ₁ HexNAC ₃ -itol	2056	Fuc ₁ Hex ₄ HexNAC ₄ -itol
1228	Hex ₂ HexNAC ₃ -itol	2098	Fuc ₁ Hex ₃ HexNAC ₅ -itol
1270	Hex ₁ HexNAC ₄ -itol	2230	Fuc ₂ Hex ₄ HexNAC ₄ -itol
1372	Fuc ₂ Hex ₁ HexNAC ₃ -itol	2260	Fuc ₁ Hex ₅ HexNAC ₄ -itol
1402	Fuc ₁ Hex ₂ HexNAC ₃ -itol	2271	Fuc ₂ Hex ₃ HexNAC ₅ -itol
1432	Hex ₃ HexNAC ₃ -itol	2301	Fuc ₁ Hex ₄ HexNAC ₅ -itol
1474	Hex ₂ HexNAC ₄ -itol	2312	Fuc ₂ Hex ₂ HexNAC ₆ -itol
1515	Hex ₁ HexNAC ₅ -itol	2404	Fuc ₃ Hex ₄ HexNAC ₄ -itol
1607	Fuc ₁ Hex ₃ HexNAC ₃ -itol	2415	Fuc ₄ Hex ₂ HexNAC ₅ -itol
1648	Fuc ₁ Hex ₂ HexNAC ₄ -itol	2434	Fuc ₂ Hex ₅ HexNAC ₄ -itol
1678	Hex ₃ HexNAC ₄ -itol	2445	Fuc ₃ Hex ₃ HexNAC ₅ -itol
1689	Fuc ₁ Hex ₁ HexNAC ₅ -itol	2475	Fuc ₂ Hex ₄ HexNAC ₅ -itol
1719	Hex ₂ HexNAC ₅ -itol	2486	Fuc ₃ Hex ₂ HexNAC ₆ -itol
1781	Fuc ₂ Hex ₃ HexNAC ₃ -itol	2516	Fuc ₂ Hex ₃ HexNAC ₆ -itol
1822	Fuc ₂ Hex ₂ HexNAC ₄ -itol	2589	Fuc ₅ Hex ₂ HexNAC ₅ -itol
1841	Hex ₅ HexNAC ₃ -itol	2690	Fuc ₃ Hex ₃ HexNAC ₆ -itol

Table 4. Compositions of singly charged sodiated molecular ions $[M+Na]^+$ observed in MALDI spectrum of permethylated 0-3h RP O-glycans. The reducing end of O-glycans were recovered as oligoglycosyl alditols.

Signal (<i>m/z</i>)	Assignment $[M+Na]^+$	Signal (<i>m/z</i>)	Assignment $[M+Na]^+$
534	Hex ₁ HexNAC ₁ -itol	2219	Fuc ₂ Hex ₅ HexNAC ₃ -itol
738	Hex ₂ HexNAC ₁ -itol	2291	Fuc ₁ Hex ₆ HexNAC ₃ -itol
779	Hex ₁ HexNAC ₂ -itol	2385	Fuc ₅ Hex ₁ HexNAC ₅ -itol
983	Hex ₂ HexNAC ₂ -itol	2417	Fuc ₁ Hex ₆ HexNAC ₃ -itol
1157	Fuc ₁ Hex ₂ HexNAC ₂ -itol	2464	Fuc ₁ Hex ₆ HexNAC ₄ -itol
1187	Hex ₃ HexNAC ₂ -itol	2517	Fuc ₆ Hex ₂ HexNAC ₄ -itol
1361	Fuc ₁ Hex ₃ HexNAC ₂ -itol	2559	Fuc ₆ Hex ₁ HexNAC ₅ -itol
1391	Hex ₄ HexNAC ₂ -itol	2589	Fuc ₅ Hex ₂ HexNAC ₅ -itol
1433	Hex ₃ HexNAC ₃ -itol	2638	Fuc ₂ Hex ₆ HexNAC ₄ -itol
1565	Fuc ₁ Hex ₄ HexNAC ₂ -itol	2668	Fuc ₁ Hex ₇ HexNAC ₄ -itol
1595	Hex ₅ HexNAC ₂ -itol	2733	Fuc ₇ Hex ₁ HexNAC ₅ -itol
1607	Fuc ₁ Hex ₃ HexNAC ₃ -itol	2763	Fuc ₆ Hex ₂ HexNAC ₅ -itol
1637	Hex ₄ HexNAC ₃ -itol	2793	Fuc ₅ Hex ₃ HexNAC ₅ -itol
1811	Fuc ₁ Hex ₄ HexNAC ₃ -itol	2812	Fuc ₃ Hex ₆ HexNAC ₄ -itol
1841	Hex ₅ HexNAC ₃ -itol	2842	Fuc ₂ Hex ₇ HexNAC ₄ -itol
1985	Fuc ₂ Hex ₄ HexNAC ₃ -itol	2908	Fuc ₈ Hex ₁ HexNAC ₅ -itol
2015	Fuc ₁ Hex ₅ HexNAC ₃ -itol	2938	Fuc ₇ Hex ₂ HexNAC ₅ -itol
2189	Fuc ₂ Hex ₅ HexNAC ₃ -itol		

Figures

Figure 1.

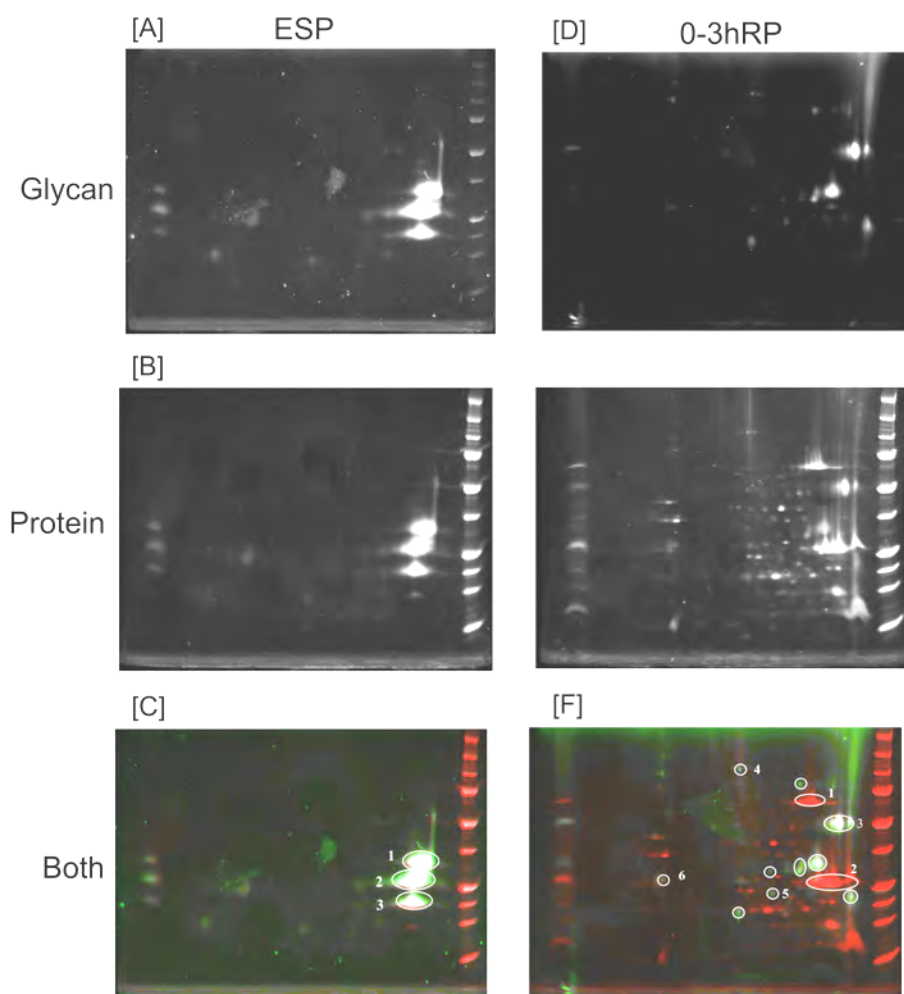


Figure 2.

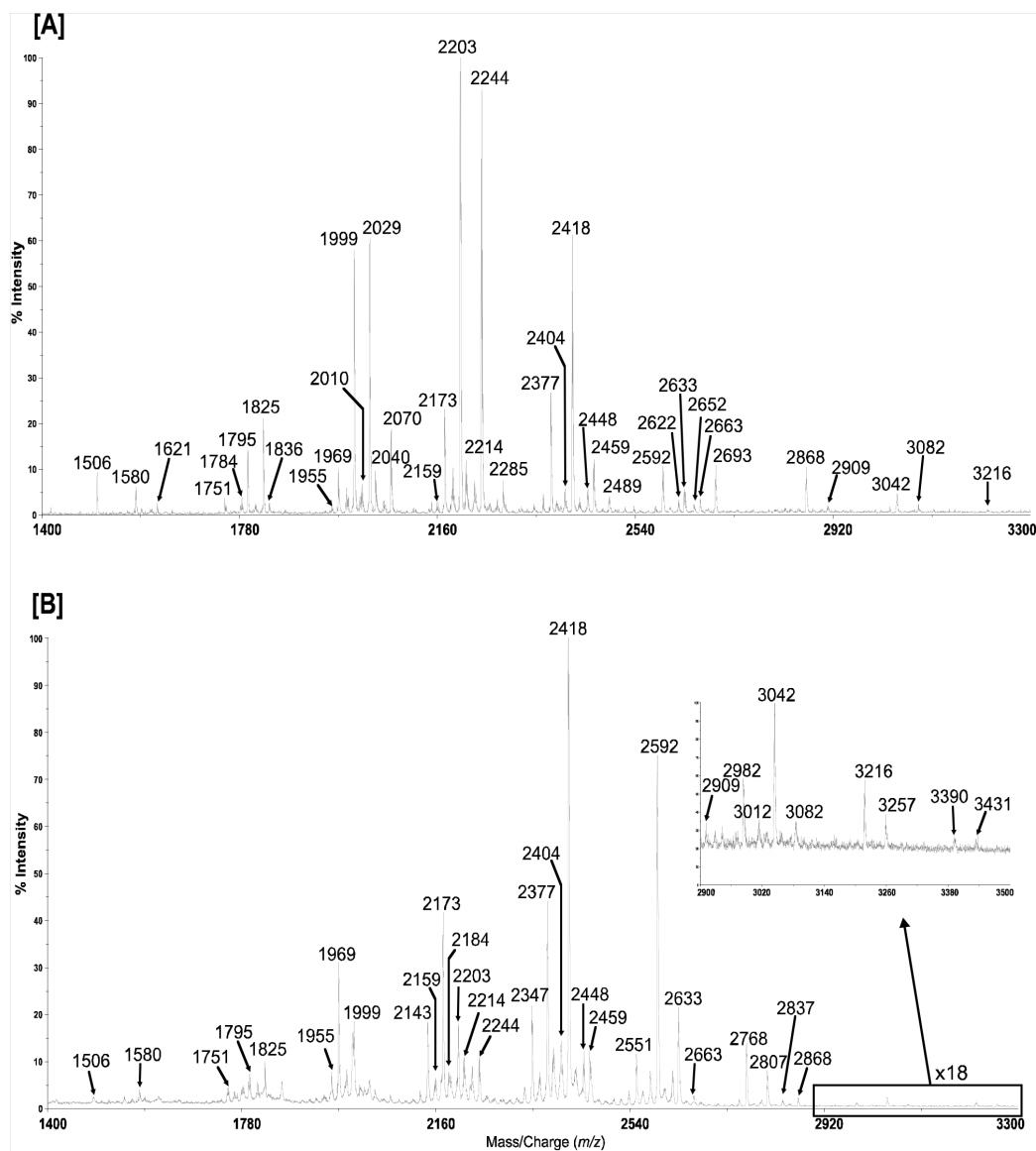


Figure 3.

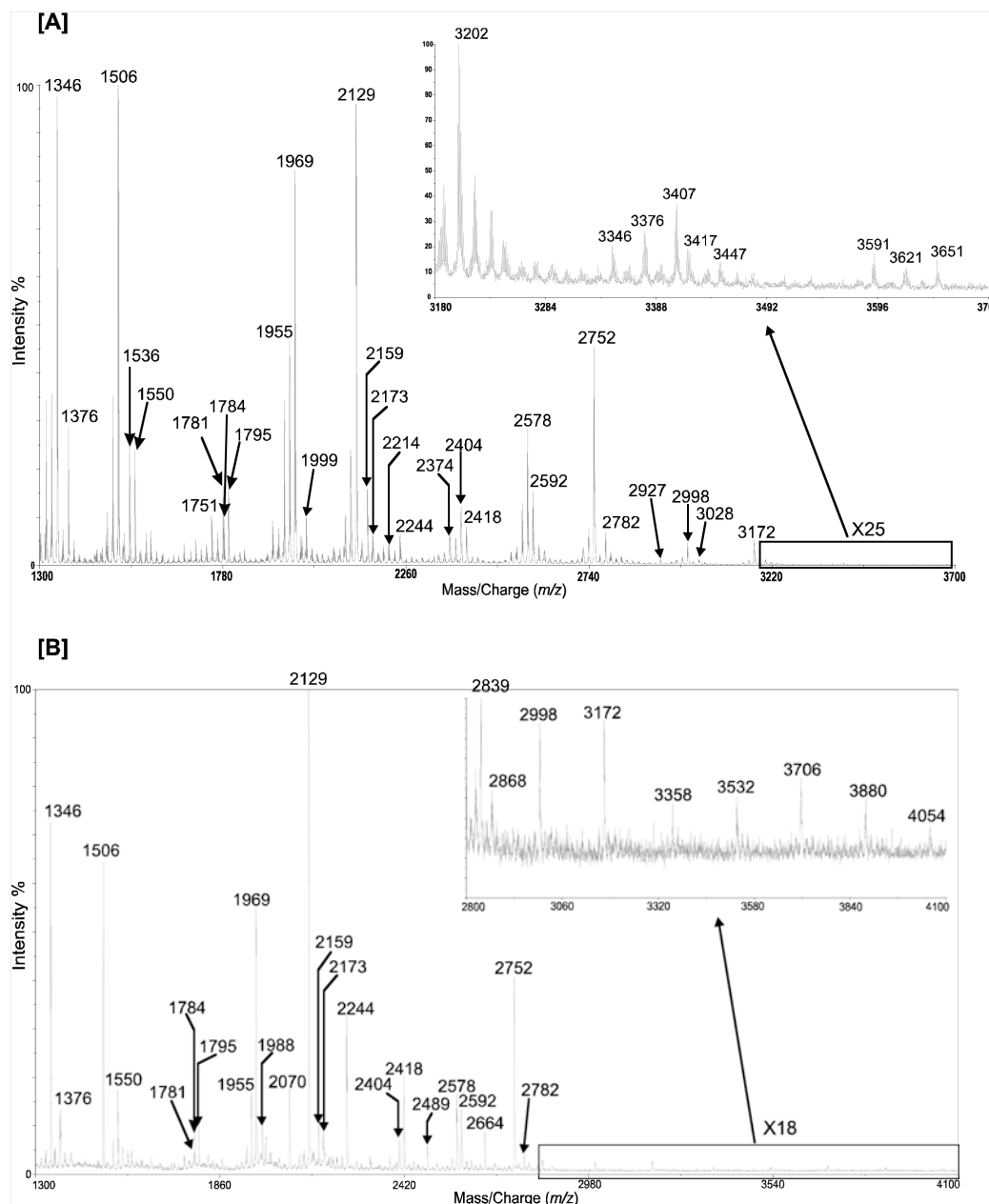


Figure 4.

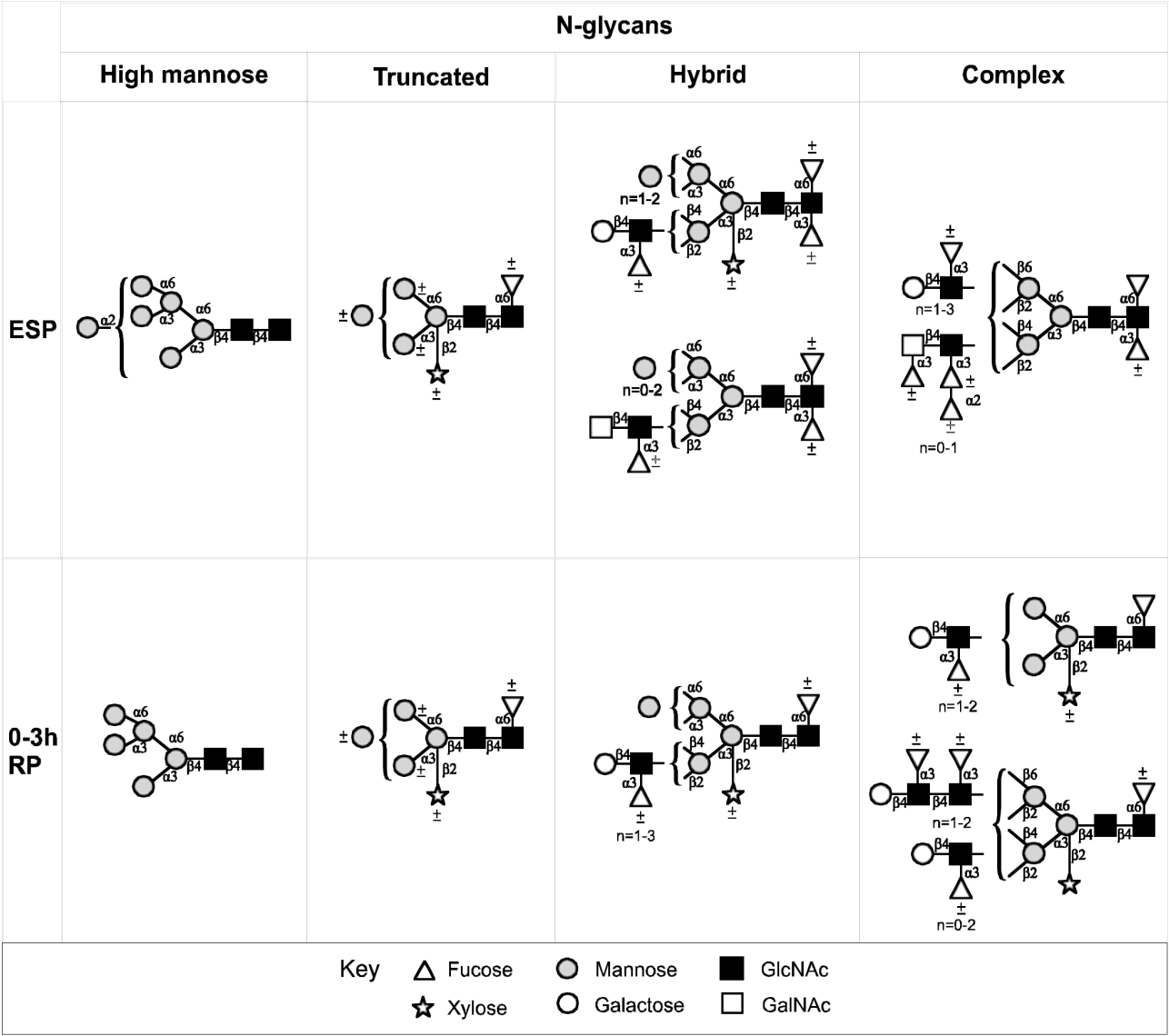


Figure 5.

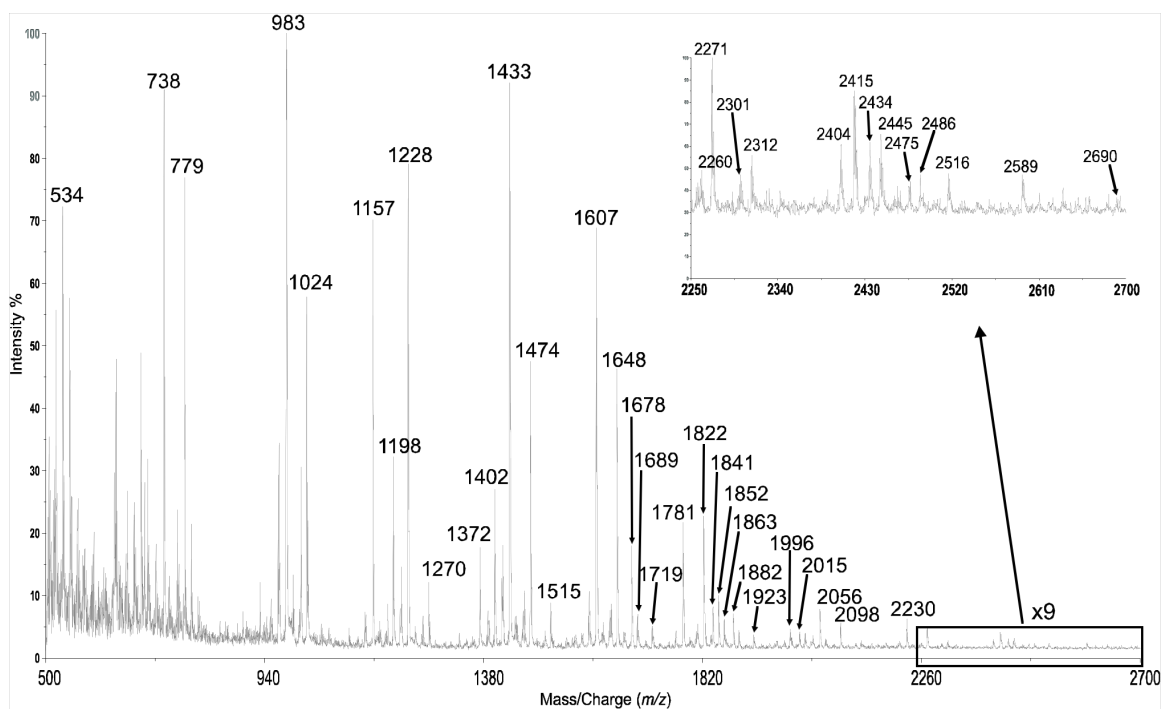


Figure 6.

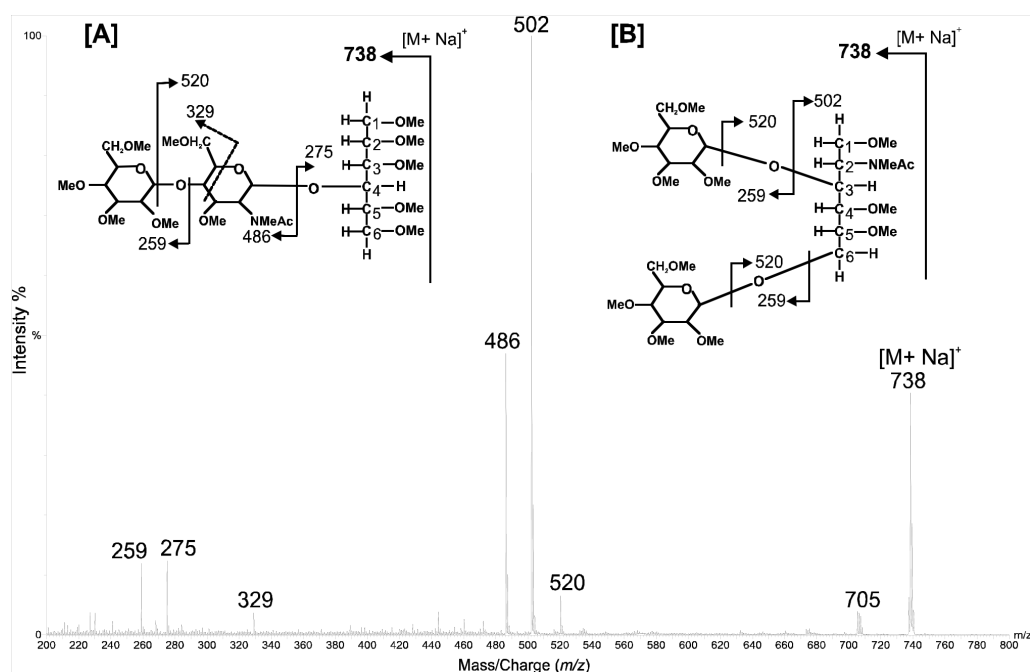


Figure 7.

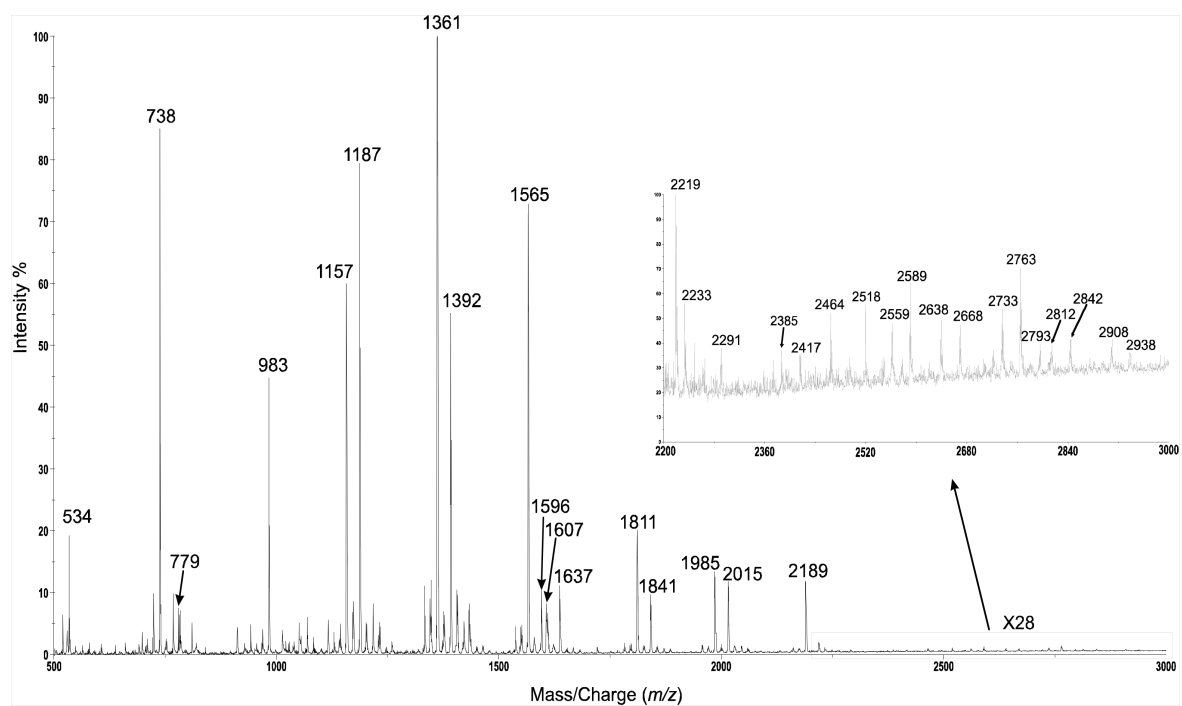
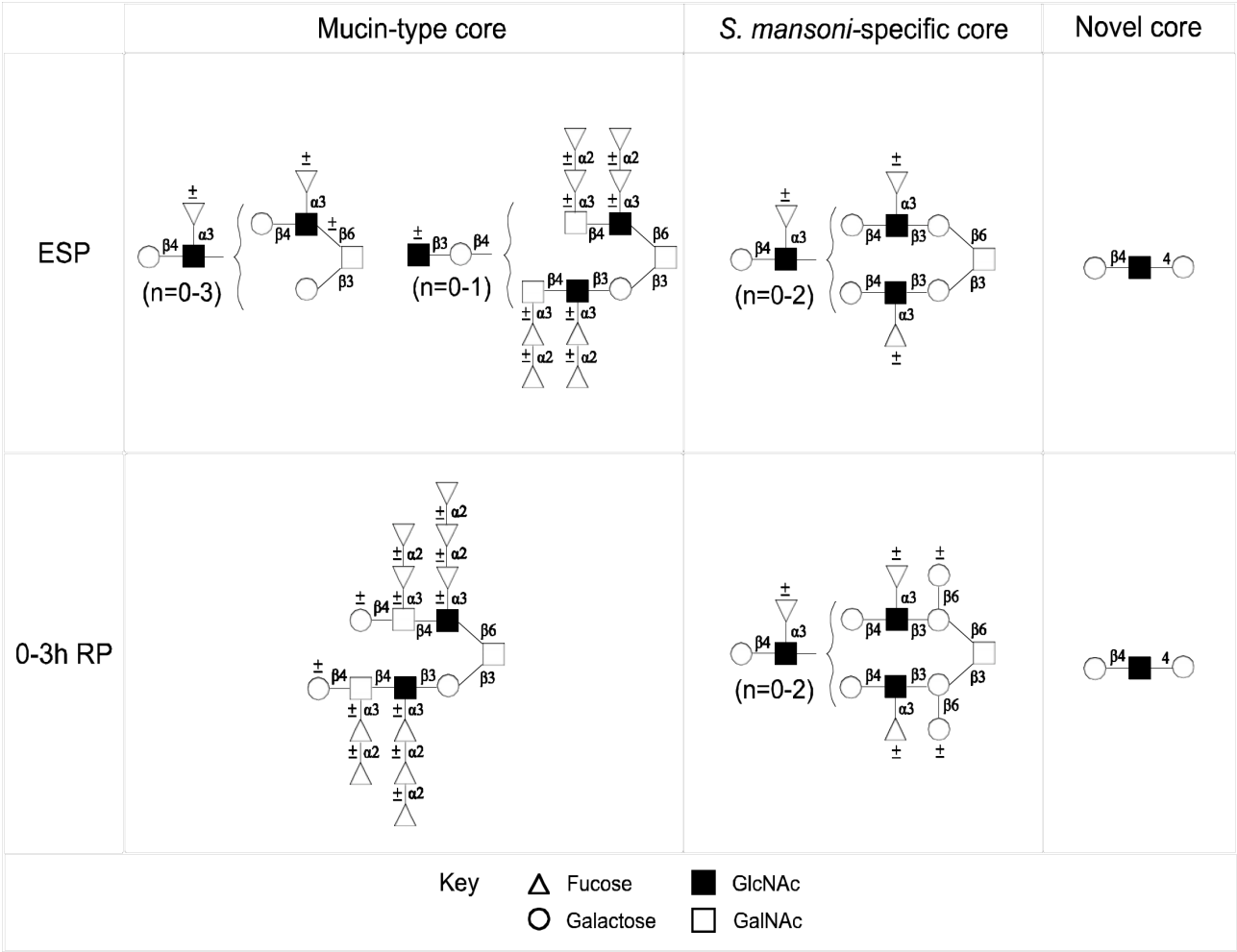


Figure 8.



Supplemental Data

Supplemental Table 1. Identification data for ESP spots 1-3, showing results of Mascot search and GPS Explorer (Applied Biosystems) analysis. A protein was considered identified if the total ion score confidence interval exceeded 99.9% and at least one individual ion score confidence interval also exceeded 99.9%.

Spot Number	Protein name	NCBI accession #	Sm genome #	Peptide count	Total ion score	Total ion score % CI
ESP1	Omega-1	ABB73003	Smp_193860	4	274	100
ESP2	IPSE	AAK26170	Smp_112110	4	318	100
ESP3	IPSE	AAK26170	Smp_112110	2	154	100
Spot Number	Sequence coverage (%)	Peptide calc. mass	Position in protein	Peptide obs. mass	Error (Da)	Error (ppm)
ESP1	22.2	1023.5662	218-225	1023.5657	-0.0005	0
		1608.8051	159-171	1608.7854	-0.0197	-12
		1756.8258	189-202	1756.7988	-0.027	-15
		1813.9385	54-68	1813.9174	-0.0211	-12
ESP2	40.3	1287.64	116-126	1287.63	-0.01	-7
		1634.78	85-99	1634.78	-0.01	-5
		1652.73	25-36	1652.72	-0.01	-7
		2092.99	69-84	2092.97	-0.02	-9
ESP3	20.1	1634.7843	85-99	1634.7725	-0.0118	-7
		1652.7261	25-36	1652.7076	-0.0185	-11
Spot Number	Sequence	Peptide ion score	Peptide ion score % CI	Modifications		
ESP1	KFIFPPFQ	64	99.832	Carbamidomethyl (C)[11]		
	QYDTINLMNVLER	85	99.998			
	GMYLEEVHVCLNR	44	81.684			
	DFTIHGLWPTIFPNR	81	99.996			
ESP2	DGKVECINQPK	46	93.998	Carbamidomethyl (C)[6]		
	ITGLGHGTCIDDFTK	68	99.956	Carbamidomethyl (C)[9]		
	YCLQLYDETYER	92	100	Carbamidomethyl (C)[2]		
	ERPYWYLFDNVNYTGR	112	100			
ESP3	ITGLGHGTCIDDFTK	81	99.997	Carbamidomethyl (C)[9]		
	YCLQLYDETYER	73	99.980	Carbamidomethyl (C)[2]		

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Supplemental Table 2. ESP N-glycan GC-MS linkage analysis of partially methylated alditol acetates obtained after PNGase F and PNGase A digestions. Permethylated N-glycans were hydrolyzed, reduced, acetylated and analyzed by GC-MS. (F) indicates PNGase F released N-glycans and (A) indicates PNGase A released N-glycans. Relative abundance is calculated relative to the most abundant sugar residue in both N-glycan pools.

Elution Time (min.) (F)	Elution Time (min.) (A)	Characteristic fragment ions	Assignment	Relative abundance (F)	Relative abundance (A)
17.133	17.162	101, 102, 117, 118, 161, 162	Terminal Xylose	0.01	0.01
17.601	17.599	102, 115, 118, 131, 162, 175	Terminal Fucose	0.95	0.41
18.838	18.845	88, 89, 130, 131, 190	2-linked Fucose	0.04	0.06
19.105	19.104	102, 118, 129, 145, 161, 205	Terminal Mannose	0.67	0.28
19.416	19.385	102, 118, 129, 145, 161, 205	Terminal Galactose	0.80	0.45
20.244	20.264	129, 130, 161, 190	2-linked Mannose	1	1
20.448	20.429	118, 129, 161, 202, 234	3-linked Mannose	0.23	0.06
20.538	20.540	118, 129, 161, 234	3-linked Galactose	0.06	0.03
21.048	21.05	99, 102, 129, 162, 189, 203, 233	6-linked Galactose	0.03	0.03
21.447	21.445	130, 190, 233	2,4-linked Mannose	0.02	0.15
21.838	21.836	129, 130, 189, 190	2,6-linked Mannose	0.05	0.05
22.029	22.019	118, 129, 189, 202, 234	3,6-linked Mannose	0.90	0.77
22.790	22.792	86, 87, 128, 129, 246, 262	2,3,6-linked Mannose	0.08	0.06
22.922	22.932	117, 143, 145, 159, 203, 205	Terminal GlcNAc	0.01	0.01
23.381	23.391	117, 159, 203, 205, 222	Terminal GalNAc	0.05	0.02
23.878	23.880	113, 117, 143, 159, 233	4-linked GlcNAc	0.95	0.06
24.082	24.097	117, 129, 159, 231	3-linked GlcNAc	0.01	0.02
24.358	24.356	117, 129, 159, 243, 273, 318	3-linked GalNAc	0.02	0.03
24.686	24.683	117, 142, 159, 301, 346	3,4-linked GlcNAc	0.49	0.47
25.111	25.117	117, 141, 143, 159, 261	4,6-linked GlcNAc	0.03	0.04
-	25.920	117, 159	3,4,6-linked GlcNAc	-	0.69

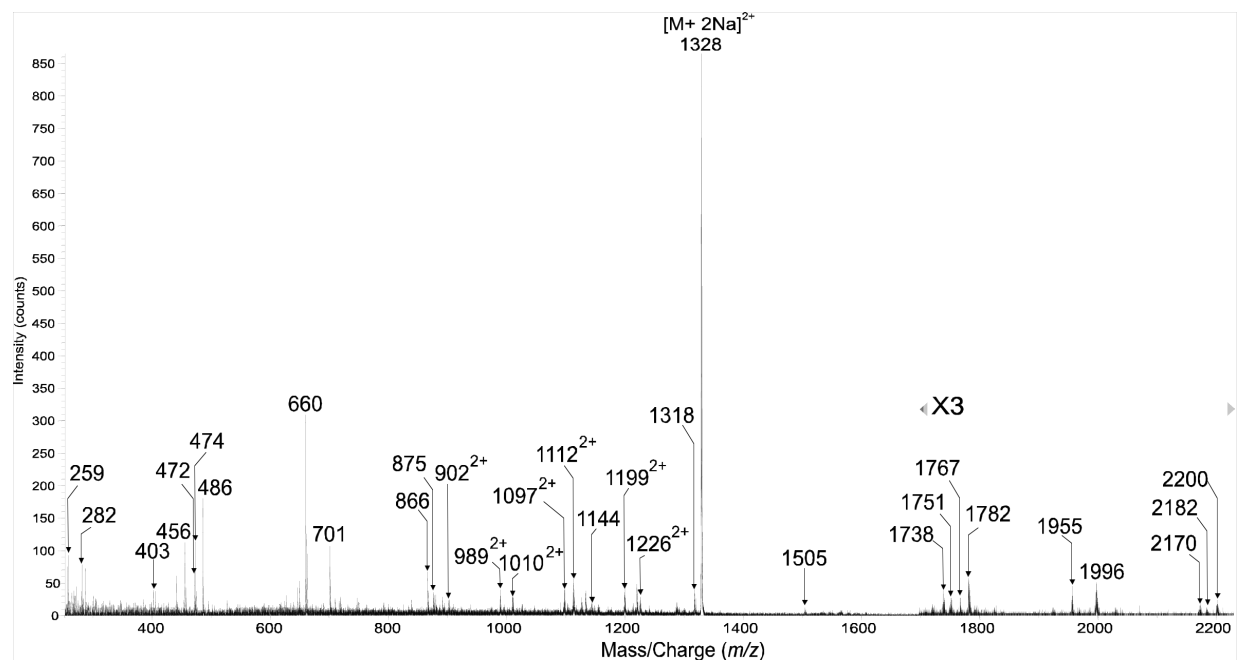
Supplemental Table 3. GC-MS linkage analysis 0-3h RP N-glycans. Released N-glycans were converted into partially methylated alditol acetates by hydrolyzing, reducing and acetylating permethylated N-glycans. The products were analyzed by GC-MS. The relative abundances of each sugar component were calculated relative to the most abundant sugar residue (terminal Galactose) in the 0-3h RP N-glycan pool.

Elution Time (min.) (F)	Elution Time (min.) (A)	Characteristic fragment ions	Assignment	Relative abundance (F)	Relative abundance (A)
17.143	17.132	101, 102, 117, 118, 161, 162	terminal Xylose	0.28	0.07
17.571	17.561	102, 115, 118, 131, 162, 175	terminal Fucose	0.58	0.38
18.874	18.827	88, 89, 130, 131, 190	2-linked Fucose	0.04	0.29
19.103	19.087	102, 118, 129, 145, 161, 205	terminal Mannose	0.52	0.30
19.371	19.354	102, 118, 129, 145, 161, 205	terminal Galactose	1	1
20.241	20.242	129, 130, 161, 190	2-linked Mannose	0.74	0.6
20.420	20.408	118, 129, 161, 202, 234	3-linked Mannose	0.06	0.75
20.539	20.540	118, 129, 161, 234	3-linked Galactose	0.13	0.45
21.397	21.396	113, 129, 130, 190, 233	2,4-linked Mannose	0.01	trace
21.827	21.802	87, 100, 129, 130, 189, 190	2,6-linked mannose	0.02	0.06
21.988	21.985	118, 129, 189, 202, 233, 305	3,6-linked mannose	0.14	0.14
22.770	22.771	86, 87, 128, 129, 189, 246, 262	2,3,6-linked mannose	0.54	0.68
22.919	22.924	117, 129, 145, 159, 203, 234	terminal GlcNAc	0.01	0.05
23.824	23.816	113, 117, 143, 159, 233	4-linked GlcNAc	0.14	0.49
24.330	24.335	117, 129, 159, 243, 273, 318	3-linked GalNAc	0.01	0.28
24.670	24.654	117, 143, 145, 159, 203, 205	3,4-linked GlcNAc	0.09	0.51
25.099	25.100	117, 159, 233	4,6-linked GlcNAc	0.10	0.06

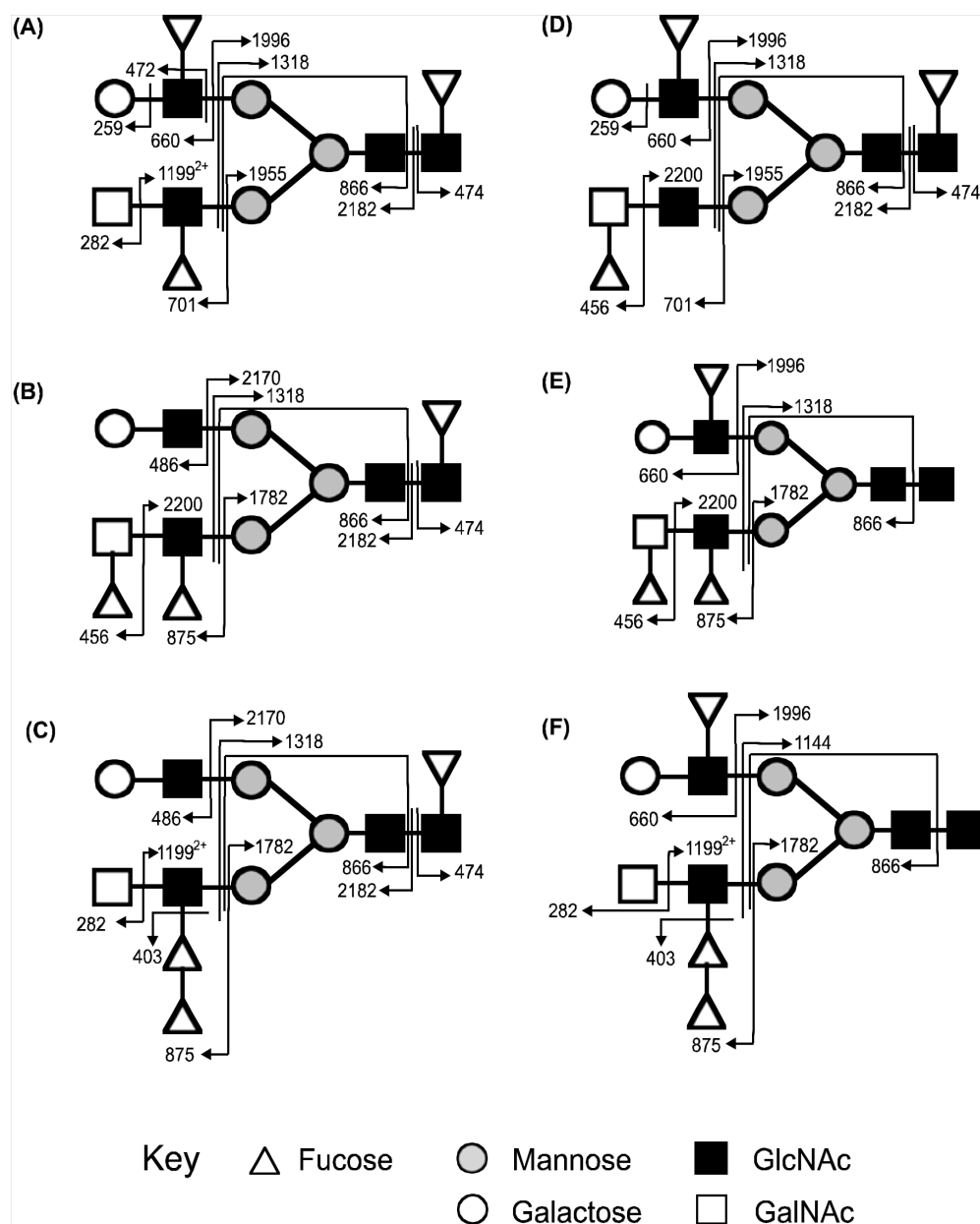
Supplemental Table 4. Linkage analysis reductively eliminated O-glycans from ESP and 0-3h RP. Permethylated 0-3h RP O-glycans were converted into partially methylated alditol acetates and analyzed by GC-MS.

Elution Time (min) (ESP)	Elution Time (min) (0-3h RP)	Fragment Ions	Assignment	Relative abundance (ESP)	Relative abundance (0-3h RP)
17.181	17.221	89, 101, 102, 205	4-linked Galitol	0.02	0.01
17.538	17.552	102, 115, 118, 131, 162, 175	terminal Fucose	0.86	0.68
18.800	18.820	89, 100, 115, 131, 190 234	2-linked Fucose	0.37	0.34
19.025	19.033	102, 118, 129, 145, 161, 205	terminal Mannose	0.41	0.08
19.318	19.364	102, 118, 129, 145, 161, 205	terminal Galactose	1	1
20.491	20.486	101, 118, 129, 161, 203, 234, 277	3-linked Galactose	0.48	0.29
-	20.983	102, 118, 129, 159, 162, 189, 233	6-linked Galactose	-	0.08
21.667	21.498	88, 101, 130, 142, 246, 258, 290	3-linked GalNAcitol	0.19	0.09
-	22.101	118, 129, 189, 234	3,6-linked Galactose	-	0.14
22.897	22.879	117, 143, 145, 159, 203, 205	terminal GlcNAc	0.04	0.07
23.33	23.291	88, 101, 130, 161, 246, 276, 318	3,6-GalNAcitol	0.06	0.12
23.339	23.334	117, 159, 203, 205	terminal GalNAc	0.12	0.01
23.772	23.763	117, 159, 203, 233	4-linked GlcNAc	0.82	0.13
24.291	24.281	117, 129, 159, 244, 273, 318	3-linked GalNAc	0.06	0.03
24.584	24.604	117, 142, 159, 171, 244, 301, 346	3,4-linked GlcNAc	0.35	0.19
25.851	25.828	117, 159, 346	3,4-linked GalNAc	0.03	0.01

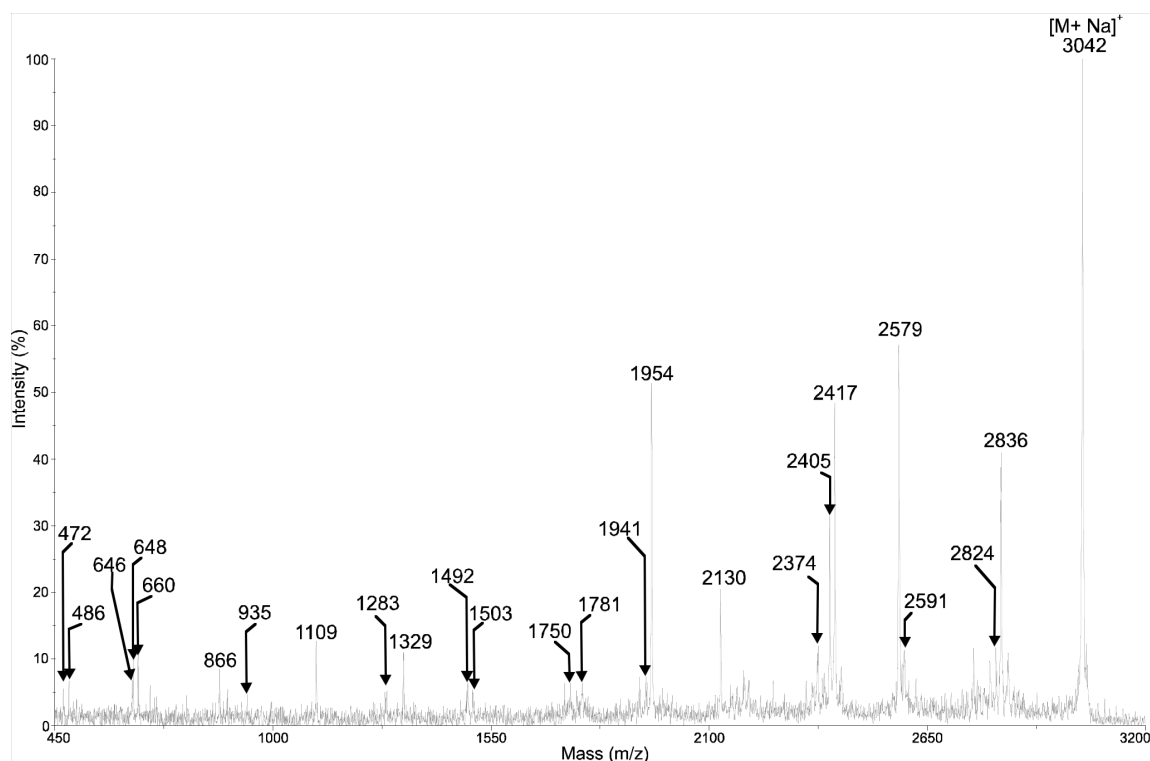
Supplemental Figure 1. CAD-ES-MS/MS spectrum of the doubly charged sodiated molecular ion $[M+2Na]^{2+}$ with the composition $Fuc_3Hex_4HexNAC_5$ (m/z 1328²⁺) observed in PNGase F released ESP N-glycan pool. Data was obtained using a Q-STAR instrument. The main fragment and molecular ions are indicated in the spectrum and proposed structures are shown in Supplemental Figure 2.



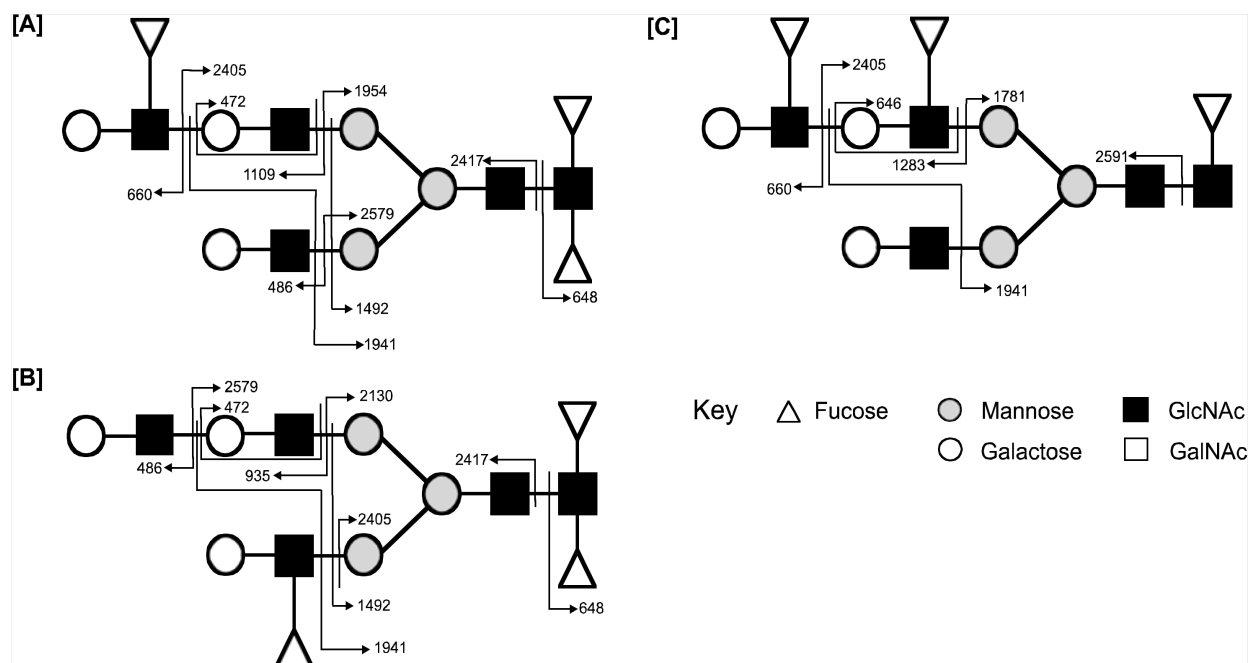
Supplemental Figure 2. The main structures for the molecular ion $\text{Fuc}_3\text{Hex}_4\text{HexNAc}_5$ present in the ESP PNGase F-released N-glycan pool. Five different isomers were deduced from the spectrum in Supplemental Figure 1 and characteristic fragmentation patterns of each isomer are indicated.



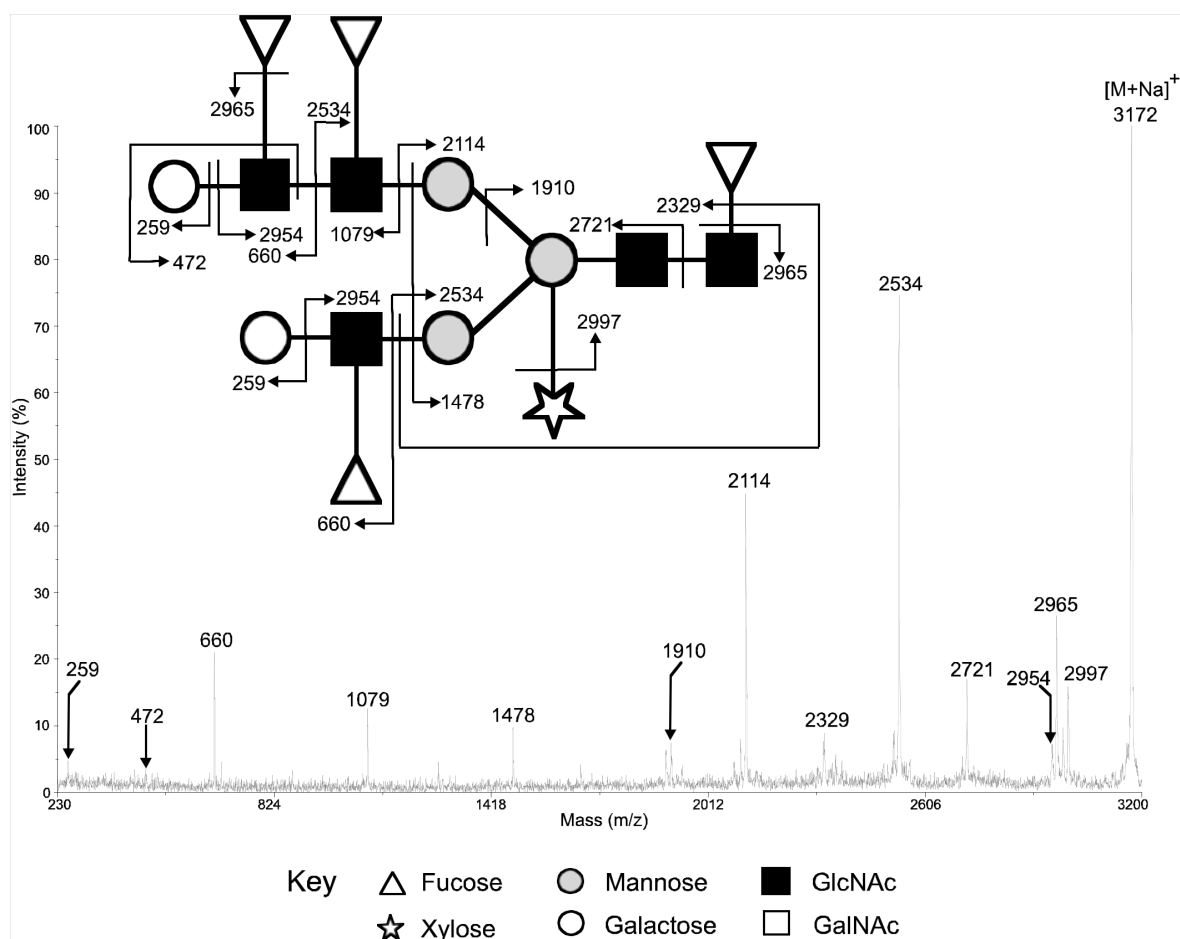
Supplemental Figure 3. MALDI-MS/MS spectrum of singly charged sodiated molecular ion $[M+Na]^+$ m/z 3042 with the composition $Fuc_3Hex_6HexNAC_5$ observed in the PNGase A-released ESP N-glycans. The spectrum was acquired using a MALDI TOF/TOF 4800 instrument. The main fragment and molecular ions are indicated in the spectrum and proposed structures are shown in Supplemental Figure 4.



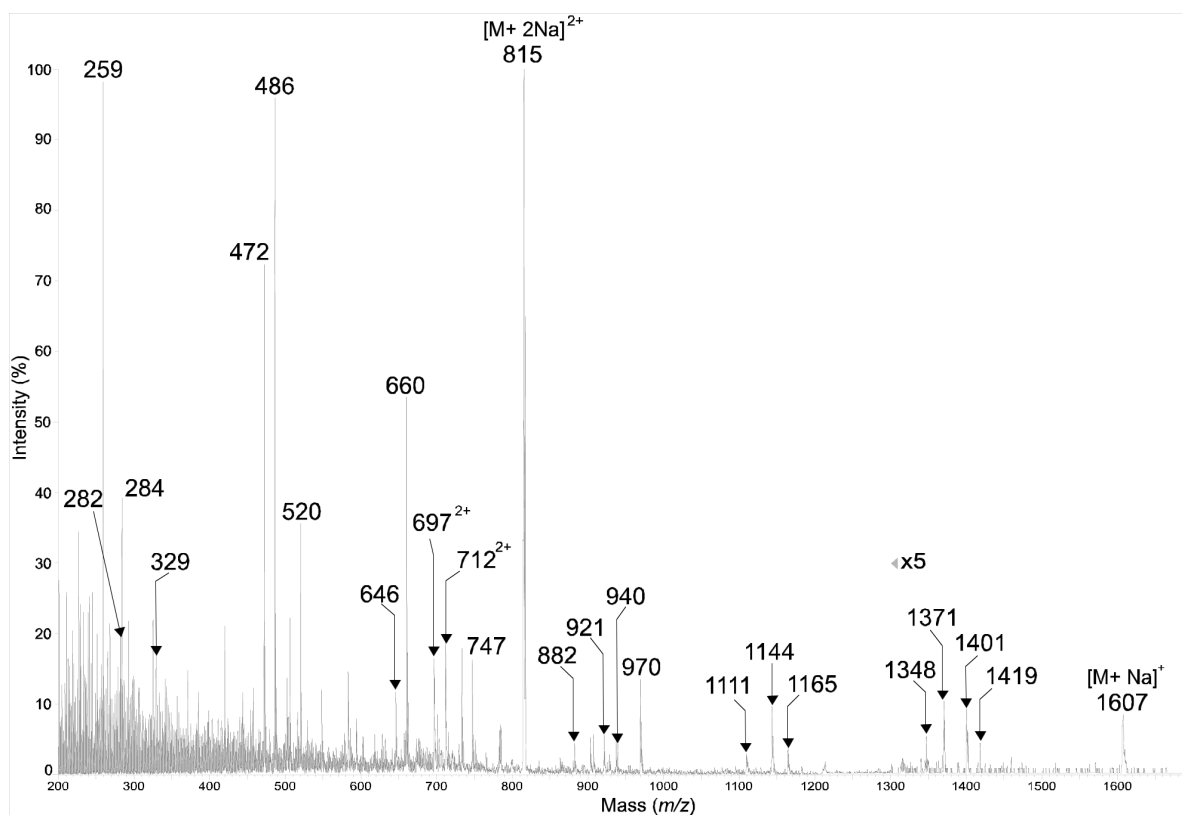
Supplemental Figure 4. The main structures for the molecular ion $\text{Fuc}_3\text{Hex}_6\text{HexNAc}_5$ present in the PNGase A-released ESP N-glycans. Three isomers were deduced from the spectrum shown in Supplemental Figure 3 and characteristic fragment ions for each isomer are indicated.



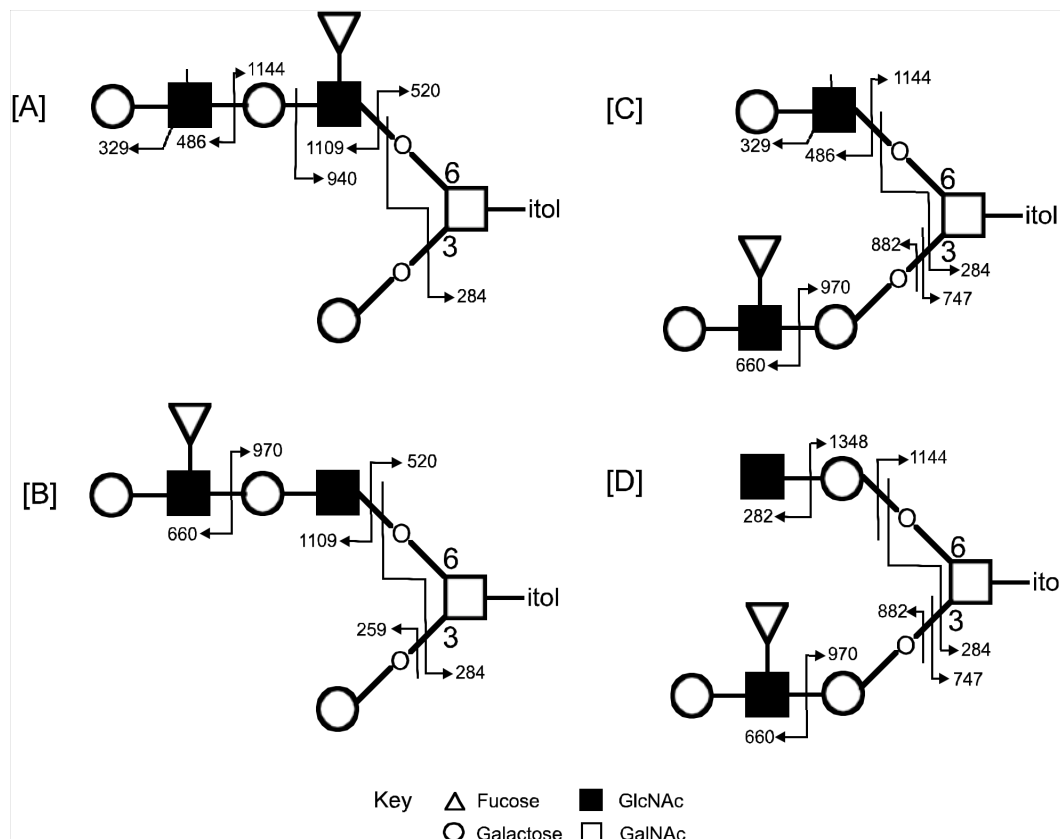
Supplemental Figure 5. MALDI-MS/MS spectrum of the singly charged sodiated molecular ion $[M+Na]^+$ m/z 3172 with the composition of $Xyl_1Fuc_4Hex_5HexNAc_5$ present in 0-3h RP N-glycan pool. The spectrum was obtained using a MALDI TOF/TOF 4800 instrument. The main fragment and molecular ions together with the proposed structure are illustrated.



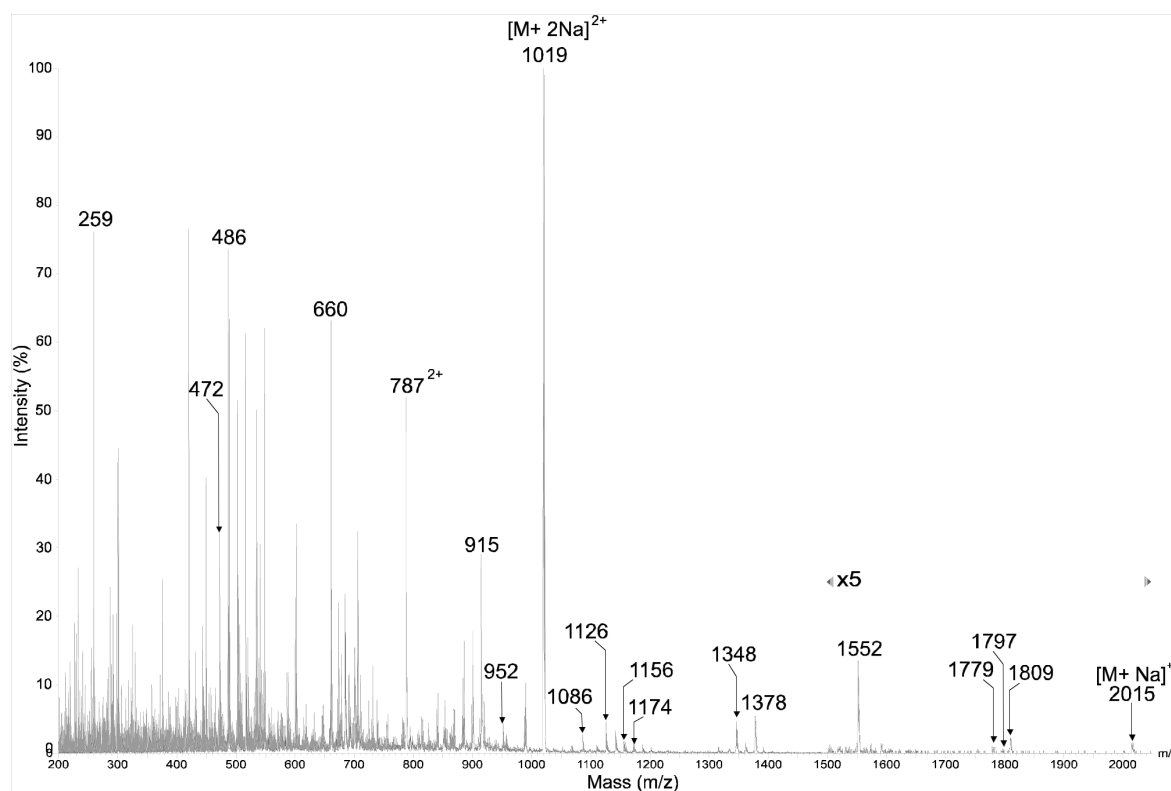
Supplemental Figure 6. CAD-ES-MS/MS spectrum of the doubly charged molecular ion $[M+2Na]^{2+}$ m/z 815²⁺ with the composition Fuc₁Hex₃HexNAc₃-itol observed in the ESP O-glycan fraction. The spectrum was obtained using a Q-TOF instrument. The main fragment and molecular ions are indicated in the spectrum and proposed structures are shown in Supplemental Figure 7.



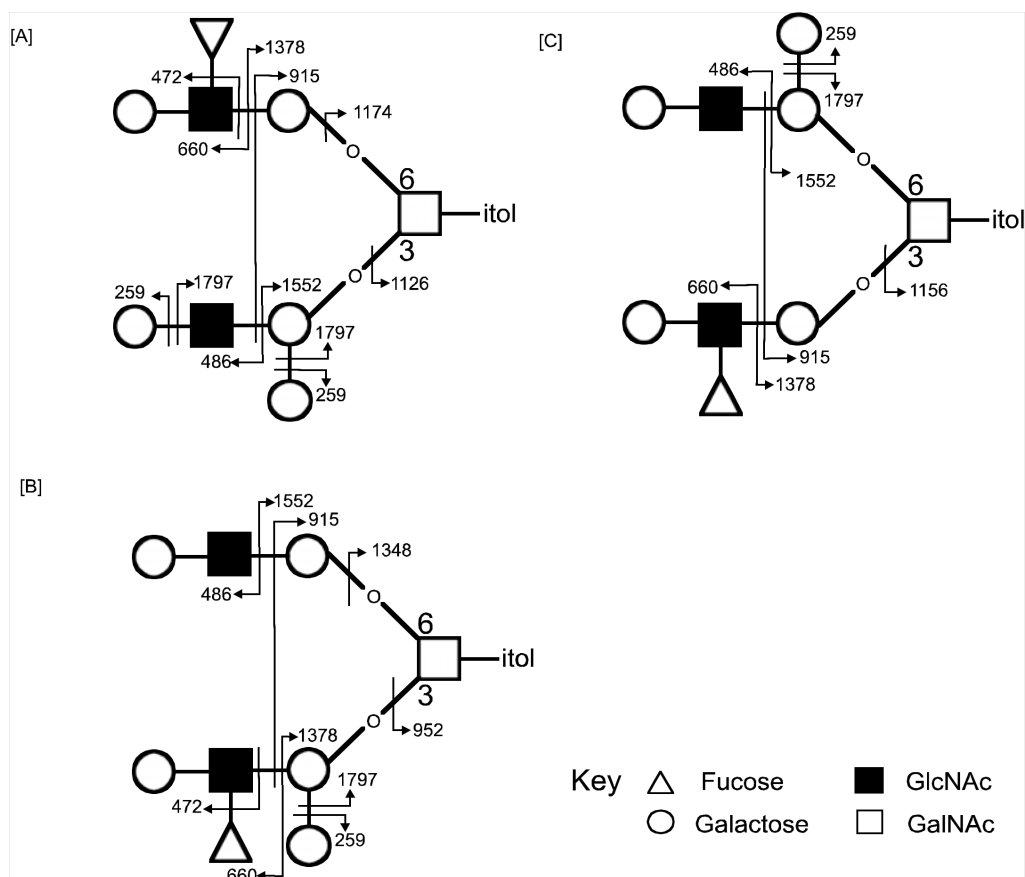
Supplemental Figure 7. The main structures of the molecular ion $\text{Fuc}_1\text{Hex}_3\text{HexNAc}_3\text{-itol}$ observed in the ESP O-glycan fraction. Four main isomers were deduced from the spectrum shown in Supplemental Figure 6 and characteristic fragment ions for each isomer are indicated.



Supplemental Figure 8. CAD-ES-MS/MS spectrum of the doubly charged sodiated molecular ion $[M+2Na]^{2+}$ m/z 1019 $^{2+}$ with the composition $Fuc_1Hex_5HexNAc_3$ -itol observed 0-3h RP O-glycans. The spectrum was obtained using a Q-TOF instrument. The main fragment ions and molecular ions are indicated and proposed structures are shown in Supplemental Figure 9.



Supplemental Figure 9. The main structures of the molecular ion $\text{Fuc}_1\text{Hex}_5\text{HexNAc}_3\text{-itol}$ observed in the ESP O-glycan fraction. Three main isomers were deduced from the spectrum shown in Supplemental Figure 8 and characteristic fragment ions for each isomer are indicated.



Supplemental Figure 10. MALDI-MS/MS spectrum of the singly charged sodiated molecular ion $[M+Na]^+$ m/z 738 with the composition Hex₂HexNAc-itol observed in the 0-3h RP O-glycan pool. The spectrum was obtained using a MALDI-TOF/TOF 4800 instrument. The schematic illustrates the two isomers for this O-glycan – isomer [A] with the novel Gal core and isomer [B] bearing a Gal β 1 $\rightarrow$ 3(Gal β 1 $\rightarrow$ 6)GalNAc core.

