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Hydrophilic Interaction Anion Exchange for Separation of Multiply-Modified Neutral and Anionic *Dictyostelium* N-Glycans

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Abstract

The unusual nature of the N-glycans of the cellular slime mould *Dictyostelium discoideum* has been revealed by a number of studies, primarily based on examination of radiolabelled glycopeptides but more recently also by mass spectrometry. The complexity of the N-glycomes of even glycosylation mutants is compounded by the occurrence of anionic modifications, which also present an analytical challenge. In this study, we have employed hydrophilic interaction anion exchange (HIAx) HPLC in combination with MALDI-TOF MS/MS to explore the anionic N-glycome of the M31 (*modA*) strain, which lacks endoplasmic reticulum α -glucosidase II, an enzyme conserved in most eukaryotes including *Homo sapiens*. Pre-fractionation with HIAx chromatography enabled the identification of N-glycans with unusual oligo- α 1,2-mannose extensions as well as others with up to four anionic modifications. Due to the use of hydrofluoric acid treatment, we were able to discriminate isobaric glycans differing in the presence of sulphate or phosphate on intersected structures as opposed to those carrying GlcNAc-phosphodiester. The latter represent biosynthetic intermediates during the pathway leading to formation of the methylphosphorylated mannose epitope, which may have a similar function in intracellular targeting of hydrolases as the mannose-6-phosphate modification of lysosomal enzymes in mammals. In conclusion, HIAx in combination with MS is a highly-sensitive approach for both fine separation and definition of neutral and anionic N-glycan structures.

Keywords

mass spectrometry; HPLC; phosphate; sulphate; glycome

1 Introduction

The humble cellular slime mould (or social amoeba) *Dictyostelium discoideum* 1 has become one of the most used non-mammalian genetic model organisms alongside *Arabidopsis*, *Caenorhabditis*, *Drosophila*, sea urchin and zebrafish (with 8324 PubMed citations as compared to 60773, 26211, 97372, 10755 and 29710 respective citations until May 2017). Its genome sequence was first published in 2005 2, but predating this were a number of studies on its glycosylation (see, e.g., refs. 3–6). Basic features such as the presence of intersecting N-acetylglucosamine, methylphosphate and sulphate on

Conflict of interest

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oligomannose-type N-glycans of the slime mould were shown by a number of methods such as anion exchange, normal phase, lectin affinity and gel filtration chromatography of radiolabelled glycans, solvolysis, mass spectrometry and/or nuclear magnetic resonance (see, e.g., 5,7,8); nevertheless proof of the actual structures of many neutral and anionic N-glycans derived from either wild-type or mutant strains was only possible after more recent developments in mass spectrometry 9–11.

In our laboratory we have employed the strategy of off-line HPLC in combination with MALDI-TOF MS in order to examine the N-glycans of *Dictyostelium* 12,13. An important step in our protocol is to first employ non-porous graphitised carbon, with acetonitrile to elute neutral glycans and then acetonitrile supplemented with trifluoroacetic acid to enrich the anionic ones, in a way similar to that used with mammalian glycans 14,15. Subsequently, the neutral and anionic-enriched PA-labelled pools were subject to hydrophilic interaction anion exchange (HIA) HPLC, which separates glycans according to their size/charge, resulting in fractions containing less complex mixtures so facilitating the use of MALDI-TOF MS together with glycosidase or chemical treatments; the fractionation prior to mass spectrometry also reduces suppression effects. This type of procedure has been performed with the HL241/*alg9* and M31/*modA* strains deficient in two conserved enzymes of N-glycosylation in the endoplasmic reticulum 12,13: respectively, the ALG9 α 1,2-mannosyltransferase (EC 2.4.1.59) and the α 1,3-glucosidase II (EC 3.2.1.84; see also Figure 1A). Indeed, the initial stages of N-glycan biosynthesis in *Dictyostelium* (i.e., the formation of the Glc₃Man₉GlcNAc₂ precursor and the removal of the glucose residues in the endoplasmic reticulum) are conserved as compared to plants and animals, as is the process of mannose-6-phosphorylation known from mammals; thus, the slime mould can be used as a model for examining the glycomic repercussions of mutations affecting these processes. Pathological phenotypes resulting from mutations in the human glucosidase II α -subunit gene orthologous to *Dictyostelium modA* include some cases of autosomal-dominant polycystic kidney and liver diseases 16.

Dictyostelium glycomutants tend to modify their N-glycans with fewer anionic groups (methylphosphate and sulphate) on average as compared to the ‘wild-type’ AX3, due to the absence or blockage of acceptor sites on the glycans for these modifications 17. In the course of our previous study on the M31 glucosidase mutant 13, we were under the impression that more glycan structures were present than we could confidently assign and that we had reached the sensitivity limit with the mass spectrometers we were using, especially in negative mode MS/MS. However, by using instruments of increased sensitivity and performing further digests, we could extend our analysis of the neutral and anionic N-glycans of the M31 strain as fractionated by HIA chromatography. We thereby detected an extended range of glycans with one or two (methyl)phosphate and/or up to three sulphate groups and could also resolve a number of isobaric species; consequently, we can capture a number of metabolic intermediates of the mannose-6-phosphate glycan modification pathway in this organism, as well as identify examples of a novel ‘yeast-reminiscent’ α 1,2-mannose extension.

2 Experimental Procedures

2.1 Preparation of neutral and anionic N-glycans

The M31 (glucosidase II mutant) strain, obtained from the Dictyostelium Stock Centre, was grown in HL5 liquid medium according to standard techniques 18. Cellular material (2-3 g wet weight) was heat-treated in water, homogenised by sonication and proteolysed with pepsin in 5% formic acid, prior to purification of the (glyco)peptides and release of N-glycans with PNGase A and F (Roche) as previously published 13,19. After Dowex AG50 chromatography, the flow-through was subject to solid phase extraction using non-porous graphitised carbon (NPGC; $\approx 300 \mu\text{l}$ ENVITM Carb, Sigma-Aldrich), with 40% acetonitrile and then 40% acetonitrile containing 0.1% trifluoroacetic acid (each thrice 300 μl) to elute respectively the neutral and anionic N-glycans; the pools were then separately labelled with 2-aminopyridine at the reducing terminus prior to subsequent HPLC and MALDI TOF MS/MS analysis 19. According to fluorescence of the labelled pools (320/400 nm), the ratio of the neutral to anionic glycans was estimated to be 3:1; MALDI-TOF MS was used for quality control before and after labelling (Supplementary Figure 1).

2.2 Chromatography of N-glycans

Hydrophilic interaction anion exchange (HIAx) HPLC was performed with an IonPac AS11 column (Dionex, Sunnyvale, USA; $4 \times 250 \text{ mm}$, combined with a $4 \times 50 \text{ mm}$ guard column) on a Shimadzu Nexera UPLC system as described previously 12,13. A two solvent gradient with buffer A (0.8 M ammonium acetate, pH 3) and buffer B (80% acetonitrile; LC-MS grade) was applied at a flow rate of 1 ml/min: 0-5 min, 99% B; 5-50 min, 90% B; 50-65 min, 80% B; 65-85 min, 75% B. A pool of pyridylaminated (PA) oligomannosidic N-glycans from white beans (containing $\text{Man}_{3.9}\text{GlcNAc}_2$) was used to calibrate the column and test efficiency of separation. For RP-HPLC isomeric/isobaric comparison with wild-type glycans, an Ascentis Express RP-amide column ($150 \times 4.6 \text{ mm}$, $2.7 \mu\text{m}$; Sigma Aldrich, calibrated in terms of glucose units with an oligoglucose standard) was employed with a gradient of 30% (v/v) methanol (buffer B) in 100 mM ammonium acetate, pH 4 (buffer A) being applied at a flow rate of 0.8 ml/min as follows: 0-4 min, 0% B; 4-14 min, 0-5% B; 14-24 min, 5-15% B; 24-34 min, 15-35% B; 34-35 min, return to starting conditions 20. The fluorescence was measured at 320 nm (excitation) and 400 nm (emission) with a Shimadzu RF-20A XS detector.

2.3 MALDI-TOF MS/MS of N-glycans

HPLC fractions were dried and reconstituted in deionized water and analysed by MALDI-TOF MS using 6-aza-2-thiothymine as matrix, whereby the samples were dried under vacuum on a steel target prior to application of the matrix (dissolved in 50% ethanol) and drying once again 19. MS and MS/MS (laser-induced dissociation) spectra were generated using either an Autoflex Speed MALDI TOF/TOF or an UltrafleXtreme MALDI TOF/TOF (both equipped with a SmartbeamTM-II laser) in positive and negative reflectron ion modes. Spectra were processed with the manufacturer's software (Bruker Flexanalysis 3.3.80) using the SNAP algorithm with a signal/noise threshold of 6 for MS (unsmoothed) and 3 for MS/MS (smoothed four times) and thereafter analysed manually.

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and differences in the fragmentation pattern after these treatments was crucial for the prediction of the glycan structures.

In our previous study 13, we could confidently identify up to 36 N-glycans in 26 HIAx fractions of the M31 anionic glycan pool. Re-analysis enables us to detect 54 glycans in 30 anionic fractions (for chromatogram, see Figure 1C), all verified by MS/MS. The backbones of many of these glycans contained between eight and eleven hexose residues as well as up to four *N*-acetylhexosamines (Hex₈₋₁₁HexNAc₂₋₄); the anionic modifications were present in a number of combinations of one or two (methyl)phosphate and/or one to three sulphate residues. We did not detect any glycan with the unmethylated phosphate in addition to the isobaric sulphate. Furthermore, in the neutral pool, we can now identify five further glycans in addition to the 15 previously described 13.

3.2 Anionic N-glycans containing nine, ten or eleven hexose residues

Indicative of the possible combinations of anionic modifications are those glycans based on nine mannose residues (Figure 2; see also previously-published MS/MS 13) ranging from those with single (methyl)phosphate or sulphate modifications (Figure 2 A-F) through to those with up to one methylphosphate and three sulphates (Figure 2 G-L and Supplementary Figure 2). The presence of the anionic modifications renders the Man₉GlcNAc₂ structures partially resistant to jack bean α -mannosidase with maximally six residues being lost (Figure 2 B, D and F; see Supplementary Figure 3 for MS/MS of the digestion products), rather than eight for unmodified Man₉GlcNAc₂; thus, the positions of the sulphate or phosphate can be deduced since the A arm is not modified. The co-eluting larger sulphated and methylphosphorylated Hex₁₀₋₁₁-based glycans are, due to the glucose residues on the A antenna, losing maximally two mannose residues upon α -mannosidase digestion. The susceptibility towards α -mannosidase digestion is even more limited in the presence of two or more anionic modifications; the combined digestion and MS/MS data indicate that the phosphates are normally terminal on the C antennae (see also Supplementary Figure 4), as is commonly also the case in mammalian phosphorylated N-glycans 25 or the phosphoethanolamine-modified N-glycans of *Penicillium* species 26, whereas the sulphates tend to be subterminal; based on previous chemical desulphation data, it is assumed that the sulphates are 6-linked to mannose 27.

In the case of glycans with one sulphate and one methylphosphate, these moieties were on opposite antennae, unless the glycan also carried an intersecting *N*-acetylglucosamine on the N-glycan; in the latter case, an *m/z* 497 fragment is observed (Hex₂PMeS; Supplementary Figure 3F). Those glycans with three or four anionic modifications, though, could carry sulphate and methylphosphate on the same arm, but none of those detected possessed an intersecting GlcNAc. Nevertheless, analysis of glycans with multiple sulphate residues is complicated by in source fragmentation in MS mode as well as multiple sodium and potassium adducts; the charge-based separation as well as the digestion data facilitated the interpretation of complex spectra and the subsequent structural annotation (Figure 2 G, I and K and Supplementary Figure 2 I and J).

As previously hypothesised, although the glucosidase II is absent from the M31 strain, the anionic Hex₉HexNAc₂-based glycans lack glucose residues as these may be removed in a

post-Golgi compartment 13. Other common backbones of M31 glycans are those with ten or eleven hexose residues and in some cases, glycans with the same apparent mass were present across a number of fractions; without prior fractionation (either HPLC or RP-HPLC) it would not have been possible to distinguish these glycans by regular MALDI-TOF MS analysis.

Exemplary of these larger glycans were species with m/z 2566 in negative mode ($\text{Hex}_{11}\text{HexNAc}_3$ with an anionic modification of 80 Da). These isobaric glycans could be distinguished by treatment with hydrofluoric acid and re-analysis in positive and negative modes (Figure 3 and Supplementary Figure 5 A-G). The form which was resistant to hydrofluoric acid, as judged by the lack of a shift of the m/z 2566.7 $[\text{M-H}]^-$ ion, was concluded to carry one sulphate residue (Figure 3 A and C). The second isobaric glycan lost, however, 283 Da upon hydrofluoric acid treatment; this would be compatible with the combined loss of a phosphate and a *N*-acetylhexosamine (Figure 3 D and G). A third form, which lost 80 Da in positive mode but was no longer detectable in negative mode, was concluded to be modified by one phosphate (Figure 3 H and K). On the other hand, only the latter structure with a 'free' phosphomonoester was sensitive to phosphatase treatment, whereas sulphated and phosphodiester-modified (HexNAc-1-P and PMe) structures were resistant (Figure 3 B, F and I). The three m/z 2566 glycans were also differentially susceptible to jack bean α -mannosidase treatment (losses of one or two hexose residues; see also Figure 2 E-H). Considering also the differential ionisation in the positive mode (see also 28), as well as the fragmentation data (Supplementary Figure 5 H-L), we concluded that we detected isobaric/isomeric structures modified by sulphate, phosphate or $\text{HexNAc-1-phosphate}$ moieties. Thereby, the third *N*-acetylhexosamine in these structures are assumed to be either on the intersecting positions or linked as a phosphodiester; indeed, a fragment corresponding to $\text{Hex}_{0-1}\text{HexNAc}_1\text{P}_1$ (m/z 282 or 444) was found for a number of structures (as exemplified in Supplementary Figure 5 K).

Compared to the literature, the $\text{HexNAc-1-phosphate}$ is presumed to be a GlcNAc-P-Man phosphodiester; thus, the $\text{HexNAc-1-phosphate}$ modification found in the M31 strain is probably a biosynthetic intermediate of the mannose-6-phosphate pathway in which first $\text{GlcNAc-1-phosphate}$ is transferred by the *gpt1* gene product 29 and then, as in mammals 30, an 'uncovering enzyme' removes the GlcNAc 31, prior to the amoebal-specific methylation reaction 32; again, we assume that the uncovering enzyme operates less efficiently with the mutant glycans, which are in this mutant larger than in the wild-type and so resulting in steric hindrance. We conclude that for this reason, the biosynthetic intermediate is still detected on a few glycans (compositions of $\text{Hex}_{9-11}\text{HexNAc}_4\text{P}$ and $\text{Hex}_{11}\text{HexNAc}_3\text{P}$) in the final glycome. In the wild-type AX3, it would appear that all phosphate residues of the *N*-glycans are methylated, a biosynthetic end-point compatible with the presence of a specific methyltransferase detected in *Dictyostelium* extracts 32, whereas in this and another mutant (M31 and HL241) we have detected non-methylated phosphate; thus, one may conclude that the 'mutant' glycans are not the preferred substrates for the methyltransferase. Furthermore, as many structures with an intersecting GlcNAc also carry one methylphosphate, interesting questions are (i) whether first the $\text{GlcNAc-1-phosphate}$ or the intersecting GlcNAc is transferred and (ii) when the methylation reaction takes place relative to the other modifications.

3.3 Neutral N-glycans containing twelve or more hexose residues

Although Hex₈₋₁₁HexNAc₂₋₃ based-structures constitute the vast majority of backbones in M31 and apparently maximally two ‘unprocessed’ glucose residues were present, we were surprised to find neutral forms of Hex₁₂₋₁₄HexNAc₂₋₃; one question was whether they were glucosylated or not. Unexpectedly, these glycans lost up to six hexose residues when treated with endo- α -mannosidase (Figure 4) as compared to maximally three which would be lost from a diglucosylated structure including a single underlying mannose. The pattern of Y-fragments suggested an elongation on the A arm (Supplementary Figures 6 and 7), whereby the endo- α -mannosidase results indicate that the extension must be proximal to the glucose residues. Furthermore, only two glucose residues were removed using α -glucosidase II from Hex₁₁₋₁₃HexNAc₂₋₃ structures (Figure 4). Finally, the MS/MS data before and after α 1,2-mannosidase digestion led us to postulate that the mannose underlying the glucose extension can be branched and substituted with up to three α 1,2-mannose residues (Figure 4); the removal of the A-antennal extension by α 1,2-mannosidase was slower than that of the single B and C α 1,2-mannose residues, possibly for steric reasons as the additional elongation is predicted to be in close proximity to the diglucose cap on the A arm (Supplementary Figure 8). As compared to previously-analysed wild-type AX3 glycans, the MS/MS patterns as well as RP-HPLC elution after endo- α -mannosidase digestion were compatible with an intersecting position for the third GlcNAc when this was present (Figure 4 G-J). Due to low abundance, the presence of structures of higher than Hex₁₄HexNAc₂₋₃ cannot be confirmed, whereas potentially anionic versions of Hex₁₂₋₁₄HexNAc₂₋₃ could not be structurally verified by glycosidase digestions. Although, α 1,2-mannose modifications are known in yeasts and fungi, the position of such ‘outer chains’ is different and is also initiated with an α 1,6-linked residue transferred to the core α 1,3-mannose of the A antenna 26,33. Thus, the oligo- α 1,2-mannose extension in *Dictyostelium* represents a new eukaryotic glycan modification.

3.4 Conclusion

In conclusion, not only is the neutral N-glycome of the M31 showing unusual features, the anionic N-glycome of the M31 strain is rather complex (see summary in Figure 5) due to the various possibilities and positions of the acidic modifications. On the HPLC column, the monosulphated glycans in any hexose series elute first, followed by the methylphosphorylated ones with or without an intersecting residue; the phosphorylated structures elute the latest. In general the more charged residues are present on a glycan, the greater the retention. The cumulative data indicate that phosphate residues, whether unsubstituted or as HexNAc- or methyl-diester, tend to be on terminal mannose residues, whereas sulphate is generally a subterminal substitution. The methyl residues are only associated with phosphate and not with hexose as is the case in the distantly-related social amoeba *Polysphondylium pallidum* 11.

As the phosphorylation and sulphation reactions are presumably late in the biosynthetic pathway, but the lower glucosylated A-antenna is blocked to such modifications in the M31 mutant, a maximum of four anionic moieties can be expected. The lack of endoplasmic reticulum glucosidase II in the mutant also results in a lack of core fucosylation and reduced amount of bisected GlcNAc (the bias towards intersected GlcNAc on M31 glycans may be

that the site of addition is less sterically hindered), but may reveal an acceptor site for the oligo- α 1,2-mannose extension as well as act as a brake on Golgi mannosidase processing. Indeed, the mannose substituted with this extension is absent from the Man8A-based glycans dominant in the wild-type 12; thus the lack of A-arm processing in the Golgi of the M31 mutant allows this otherwise 'unseen' modification to occur at a detectable level. The reduced complexity in the M31 mutant, as compared to the wild-type, makes the analytical challenge surmountable, both in terms of detection and interpretation of unusual glycans. Furthermore, the approach of using HIAX is applicable to analysis of other species, including insects 34, can be combined with RP-HPLC 26,35 opening up unprecedentedly highly-detailed glycomic studies capable of revealing new N-glycan modifications in wild-type and mutant model organisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Summary of biosynthesis of N-glycans in *Dictyostelium*, relevant diagnostic MALDI-TOF-MS/MS fragments and HPLC.

(A) Simplified 'wild-type' biosynthetic scheme starting with $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ showing the two-step action of glucosidase II (absent in the M31 strain) followed by the action of GlcNAc-1-phosphotransferase (GPT), removal of the GlcNAc and methylation of the 'uncovered' phosphate by a methyltransferase (MeT); the linkages, arms (A, B and C) and locations of intersecting and bisecting GlcNAc are also annotated. (B) Summary of important diagnostic ions of single and multiple anionic N-glycans obtained from MS/MS in

negative ion mode (positive ion mode m/z values of phosphate-containing fragment ions are also indicated with (+)); multiple sulphated ions were detected as sodium or potassium adducts (Na and/or K, shown in brackets). (C) HPLC chromatograms of the neutral and anionic pools highlighting the elution positions for three isobaric structures of m/z 2566.

Figure 2. Mannosidase digestion of selected glycans with single and multiple anionic modifications.

HIAx-fractionated singly-modified Hex₉HexNAc₂₋₃S₁ or Hex₉HexNAc₂₋₃PMe₀₋₁ N-glycan structures (A-F) and those with multiple sulphate and methylphosphate modifications (G-L) are shown before and after jack bean α-mannosidase digest in negative ion mode. Hex_{8,10-11}HexNAc₂₋₄-based sulphated or methylphosphorylated structures which co-elute with those based on Hex₉HexNAc₂ are shown in a greyscale; asterisks in panel A indicate contaminants of m/z 2154 and 2220. The parent structures, but not the in source fragment ions, are annotated with the composition (as H₈₋₁₁N₂₋₄P₀₋₁Me₀₋₁S₀₋₃; i.e.,

Hex₈₋₁₁HexNAc₂₋₄P₀₋₁Me₀₋₁S₀₋₃); in source losses of sulphate are indicated by 102 or 118 (i.e., loss of sulphate and sodium or potassium counterions), whereas loss of mannose residues upon enzymatic digestion are indicated by red or blue dashed lines. Increasing numbers of anionic modifications as well as the presence of the intersecting GlcNAc residue result in decreased susceptibility to mannosidase digestion (a shift towards sodium adducts was observed due to the composition of the buffer in which the mannosidase is supplied). MS/MS for selected Hex₉-based structures have been previously published 13; further example digests as well as 'zoomed-in' regions of panels I and K (showing annotations of all adducts) are shown in Supplementary Figure 2 and selected MS/MS of mannosidase digestion products are in Supplementary Figure 3 A-D. Based on analyses of an RP-HPLC fraction containing Man₈₋₉GlcNAc₂PMe (panel C), it is concluded that PMe is on the C-arm (see also Supplementary Figure 4). Further data on the *m/z* 2566 glycans (panels E and G) are also presented in Supplementary Figure 5.

Figure 3. Analysis of isobaric anionic N-glycans found in the M31 glucosidase II mutant strain. N-glycan structures with the monoisotopic m/z 2566 as $[M-H]^-$ were identified in three different RP-HPLC fractions (**A**, **D** and **H**). In the case of the 7.8 g.u. structure (m/z 2566; **A**), resistance to both shrimp alkaline phosphatase (SAP; **B**) and hydrofluoric acid (HF; **C**) were compatible with it being sulphated (compare with Supplementary Figure 5 A and B). The 5.0 g.u. fraction contained two major glycans detected in both negative and positive modes (m/z 2256/2258 and 2566/2569; **D** and **E**), both of which were sensitive to hydrofluoric acid (**G**; loss of 94 [PMe] or 283 Da [HexNAcP], with products only detectable in positive mode) but resistant to phosphatase (**F**); thus the Hex₁HexNAc₃P glycan possesses a HexNAc-1-P phosphodiester (see also Supplementary Figure 5 E-G). The 5.6 g.u. fraction contained two major glycans (m/z 2457 and 2566; **H**), both of which are sensitive to hydrofluoric acid (**K** and **L**; loss of 94 [PMe] or 80 [P] Da); in this case, the Hex₁HexNAc₃P glycan possesses a phosphatase-sensitive phosphomonoester (see also Supplementary Figure 5 C and D), while the methylated phosphate on Hex₁HexNAc₂PMe is phosphatase-resistant. For MS/MS of the corresponding m/z 2566 glycans isolated from HIAx fractions as well as digestion products, see Supplementary Figure 5 H-L.

Figure 4. Identification of a novel oligo- α 1,2-mannose modification on neutral N-glycans. (A-C) MALDI-TOF MS of a late-eluting neutral HIAX fraction of M31 N-glycans (see also chromatogram in Figure 1C) before and after digestion with *Aspergillus* α 1,2-specific mannosidase alone or, after heat inactivation, subsequently with an insect cell α -glucosidase II; corresponding MS/MS are shown in Supplementary Figure 6. (D-F) MALDI-TOF MS of another late-eluting neutral HIAX fraction before and after digestion with *Aspergillus* α 1,2-specific mannosidase or bacterial endo- α -mannosidase; corresponding MS/MS are shown in Supplementary Figure 7. Losses of hexose residues are indicated by red or blue dashed lines, key Y-fragments by numbers in square brackets and contaminating ions with an asterisk. (G-J) The RP-HPLC elution time and MS/MS fragmentation pattern of the endo- α -mannosidase product (panel F and Supplementary Figure 7C) was compared to known bisected (isomer I) and intersected (isomer II) wild-type AX3 Hex₈HexNAc₃ glycans; on

this basis, it was concluded that the Hex₁₃HexNAc₃ glycan containing the oligo- α 1,2-mannose extension (panel D) contains an intersecting GlcNAc residue.

Figure 5. Summary of the anionic modifications found in the M31 glucosidase II mutant strain. Schematic overview of Glc₀₋₂Man₈₋₉GlcNAc₂₋₃P₀₋₁PMe₀₋₂S₀₋₃ structures based on their HPLC elution properties; the numbers of anionic modifications (one, two or more; i.e., R₁₋₄) are indicated by the grading of the shaded regions (with the lightest background for singly-modified glycans). The annotation of the structures was based on MALDI-TOF-MS/MS analysis in positive and negative ion modes combined with glycosidase and chemical treatments.