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Distinguishing N-Acetylneuraminic Acid Linkage Isomers on Glycopeptides by Ion Mobility-Mass Spectrometry

Supporting Information

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Experimental Methods

If not noted otherwise, all chemicals were used as received and ultra-pure water was used for all experiments.

Synthesis and glycopeptide purification

All glycopeptides were synthesized by solid-phase peptide synthesis (SPPS) using previously reported fluorenylmethoxycarbonyl (Fmoc) protocols.[1, 2] Standard amino acids for SPPS were purchased from Iris Biotech GmbH (Marktredwitz, Germany), and NovaPEG resin was obtained from Novabiochem (Darmstadt, Germany). Fmoc-labelled asparagine (Asn) carrying mainly a disialylated, biantennary N-glycan were prepared from egg yolk as described previously.[3, 4] N-acetylneuraminic acid (NeuAc) residues were selectively protected by esterification with benzyl bromide prior use in SPPS.[1] The coupling of the glycosylated Asn building blocks was performed as described by Unverzagt and co-workers.[2]

GP1/GP2 purification. The crude glycopeptides GP1 and GP2 were purified using reversed-phase solid phase extraction (RP SPE; CHROMABOND® C18 ec, 6 mL, 1000 mg phase, MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany; preconditioned with 10 mL acetonitrile and equilibrated with 50 mL water prior to loading). Glycopeptides were dissolved in water, loaded onto the cartridge and subsequently treated with 5% acetonitrile followed by 20% acetonitrile (8 mL each). The glycopeptide eluting in the 5% fraction was further purified using preparative C18-RP HPLC on an Agilent 1200 series ELSD HPLC system (Agilent, Santa Clara, CA). The lyophilisate was dissolved in water and subjected to C18-RP chromatography (XBridge BEH C18 column, 130 Å, 3.5 µm, 4.6 mm x 150 mm, 1/pkg, Waters, Milford, MA) using a linear gradient (1 mL/min) from 8% to 13% acetonitrile within 25 min, followed by an increase to 100% in 10 min. Fractions of interest from several runs were combined and analysed using MS to confirm their composition.

GP3 purification. After synthesis crude GP3 was purified by reversed-phase solid phase extraction (RP SPE; 1 g, Sep-Pak Vac 6 cc cartridge, Waters, Milford, MA; preconditioned with 10 mL acetonitrile and equilibrated with 100 mL water prior to loading). The glycopeptide was dissolved in water, loaded onto the column and unbound material was subsequently washed out using 10 mL of water. The cartridge was sequentially treated with 5%, 10%, 15%, 20% and 30%

acetonitrile (10 mL each). The glycopeptide eluting in the 10% fraction was further purified using preparative C18 RP HPLC on an Agilent 1200 series ELSD HPLC system (Agilent, Santa Clara, CA). The lyophilisate was dissolved in water and subjected to C18-RP chromatography (XBridge BEH C18 column, 130 Å, 3.5 µm, 4.6 mm x 150 mm, 1/pkg, Waters, Milford, MA) using a linear gradient (1 mL/min) from 5% to 20% acetonitrile within 50 min, followed by an increase to 30% in 10 min. Fractions of interest from several runs were combined and analysed using MS.

Selective introduction of specific terminal monosaccharides on synthetic N-glycopeptides using specific recombinant glycosyltransferases

Desialylation. The NeuAc residues of the synthetic N-glycopeptides were removed by chemical desialylation. A volume of 16 µL 10 mM trifluoroacetic acid (TFA) was added to the same volume of GP3 solution and the resulting mixture was incubated for 3 hours at 80°C. After addition of 50 µL methanol the glycopeptides were subjected to vacuum drying. The dried glycopeptides were dissolved in further 50 µL of methanol and once again dried under vacuum before they were dissolved in 100 µL HPLC grade water. Desialylation was confirmed by mass spectrometry. The total amount of desialylated glycopeptide was divided into three aliquots and the digalactosylated N-glycopeptides of each fraction were subjected to enzymatic α-2,3 sialylation with the help of a murine β-galactoside α-2,3-sialyltransferase 3 (ST3Gal3). The ST3GAL3 cDNA coding for this enzyme was purchased from Source Bioscience (IMAGE ID 3584323) and subcloned as a truncated version lacking the first 84 nucleotides into baculovirus transfer vector pVT-Bac-His1. Amplification of the nucleotide sequence coding for the truncated version of ST3Gal-III was done by PCR using forward primer 5'-GAGCTCAAGCTACACTTACTCCAATGGGAA-3' and reverse primer 5'-GAATTCTCAGATACCGCTGCTTAAGTCA-3'. PCR products containing terminal recognition sites for restriction endonucleases (underlined sequences) SacI and EcoRI were inserted into the multiple cloning site (MCS) of the cloning plasmid pGEM-T (Promega) and subjected to sequencing (Microsynth, Switzerland), in order to confirm the accuracy of DNA-amplification. Sequence- verified ST3GAL3 fragments were isolated from pGEM-T by treatment with SacI and EcoRI and ligated into baculovirus transfer vector pVT-Bac-His1 treated with the same enzymes. Recombinant expression of the protein missing the transmembrane domain was performed in *Spodoptera frugiperda* (Sf) 9 ovarian cells as described previously.[5]

Glycosyltransferase reaction. The donor substrate was mixed with the glycopeptide acceptor and subjected to vacuum drying before being dissolved in 37.5 µL of 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) reaction buffer adjusted to pH 7.0 supplemented with protease inhibitor (Roche, Vienna, AT). 2.5 µL of 40 mM $MnCl_2$ solution was added before addition of 10 µL enzyme concentrate, which was obtained by ultrafiltration of insect cell culture supernatant. Glycosyltransferase reactions were performed overnight at 37 °C.

Purification. Following enzymatic treatment glycopeptides were applied to reversed phase solid phase extraction (SPE) cartridges (Strata C18-E, Phenomenex) for purification. Prior to sample application the cartridges were equilibrated with 50% acetonitrile and rinsed twice with water. Bound glycopeptides were washed with water and eluted in 50% acetonitrile. Enzymatically modified glycopeptides were applied to a Thermo BioBasic C18 column (5 µm particle size, 150 x 0.360 mm) coupled to a maXis 4G ETD (Bruker, Bremen, Germany) mass spectrometer for subsequent LC-ESI-MS. LC was carried out using 80 mM ammonium formate (pH 3.0) as the aqueous phase and a gradient comprising an acetonitrile increase from 1-35% in 17 min.

Proteolytic digestion of α1-proteinase inhibitor (A1PI)

α1-proteinase inhibitor (A1PI), either isolated from human plasma (Sigma-Aldrich, St. Louis, MO, USA) or recombinantly produced in Chinese hamster ovary (CHO) cells (ProBioGen AG, Berlin, Germany), was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)[6] (Figure S1). Samples (~5 µg protein/lane) were dissolved in SDS-PAGE sample buffer consisting of 40% glycerin, 0.25 mM TRIS-HCl pH 6.8, 0.015% bromophenol blue (w/v), 4% SDS (w/v), 50 mM dithiothreitol (DTT) and incubated at 96°C for 5 min. After denaturation iodoacetamide (IAA) was added to a final concentration of 50 mM and samples were incubated at room temperature for 30 min in darkness. For electrophoretic separation a consort EV265 power supply (Consort bvba, Turnhout, Belgium) and a Mini-PROTEAN® Tetra Cell (Biorad, Munich, Germany) as well as self-cast polyacrylamide gels (0.75 mm thick) and Laemmli[7] system was used. Stacking gels (pH 6.8) consisted of 5%, separation gels (pH 8.8) of 10% polyacrylamide. The acrylamide/bisacrylamide ratio in both gels was 29:1. The running buffer contained 25 mM TRIS, 192 mM glycine, 0.1% SDS (w/v), pH 8.3. Electrophoresis was carried out with 160 V for 75 min. Gels were stained for 5 min with 50% (v/v) aqueous methanol containing 0.25% (w/v)

Coomassie blue R-250 and 7% (v/v) acetic acid. Excess dye was removed by 50% (v/v) methanol containing 10% (v/v) acetic acid for at least 3 h or until the background was clear.

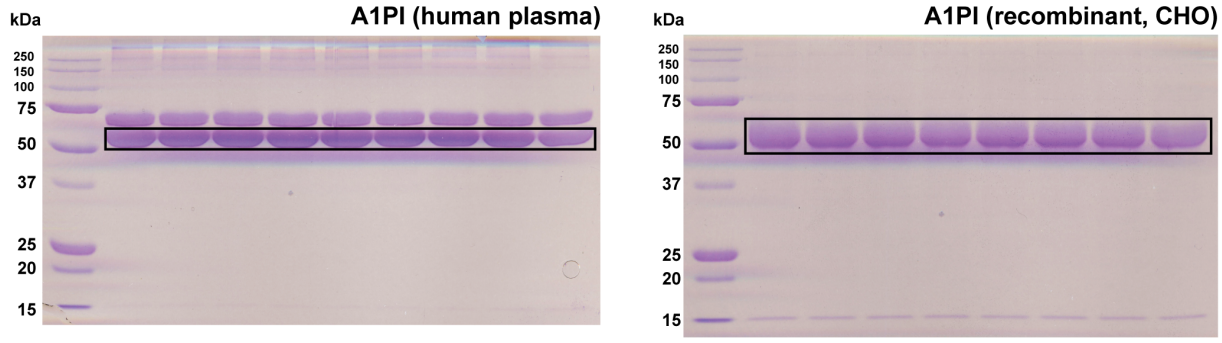

Figure S1. SDS-PAGE of A1PI obtained from human plasma and recombinantly produced in CHO cells. The box indicates the bands that were cut for later use.

Coomassie blue stained protein bands of interest were excised and cut into cubes, destained and trypsin (Roche, Indianapolis, IN, USA) was added in a ratio of 1:25-50 prior to incubation for at least 4 h or overnight at 37°C. Extracts were enriched for glycopeptides *via* an offline hydrophilic interaction chromatography (HILIC) step before using them for the IM-MS experiments to remove unglycosylated peptides, which can suppress the detection of glycopeptides.[8] The in-gel digested and extracted peptide/glycopeptide mixture was slowly dissolved in 50 µL of 80% acetonitrile/1% TFA and subjected to an in house made HILIC column/tip for purification. Briefly, 9 mg of PolyHYDROXYETHYL A (12 µm, 100 Å material; PolyLC Inc., Columbia, USA)[9] was packed onto ZipTip C18 (Merck KGaA, Darmstadt, Germany) and washed three times with 50 µL methanol, followed by 50 µL of ultra-pure water and was then equilibrated with three times 50 µL of 80% acetonitrile/1% trifluoroacetic acid. After loading, the column was washed four times with 50 µL of 80% acetonitrile/1% TFA before elution with 50 µL of 0.1% TFA, followed by 50 µL of 25 mM of ammonium bicarbonate and 50 µL of 50% ACN. Elution fractions were combined and dried.[10]

RP-LC ESI-IT-MS measurements of A1PI glycopeptides. Peptide mixtures from HILIC elution fractions (dissolved in 0.1% formic acid) were trapped on a C18 Acclaim™ PepMap™ RSLC Nano-Trap column (3 µm, 100 Å, 75 µm x 20 mm, Thermo Fisher Scientific, Waltham, MA, USA) and separated on a C18 Acclaim™ PepMap™ RSLC column (2 µm, 100 Å, 75 µm x 150 mm, Thermo Fisher Scientific, Waltham, MA, USA) using a linear gradient from 2% buffer B (acetonitrile with 0.1% formic acid) to 45% in 35 min. Buffer A was 0.1% formic acid. Flow rate was set to 300 nL/min and oven temperature to 45°C. Positive-ion-mode MS spectra were

acquired within an *m/z* range of 350–1800, tune was set to 1200, ICC to 200000 and max. acquisition time to 50 ms. Measurements were performed on an amaZon speed ETD ion trap mass spectrometer (IT-MS) equipped with CaptiveSpray nanoBooster™ (both Bruker, Bremen, Germany) coupled to an Ultimate 3000 UHPLC system (Dionex, Part of Thermo Fisher, Germany). The base peak chromatogram (BPC) of trypsin treated A1PI is shown in Figure S2.

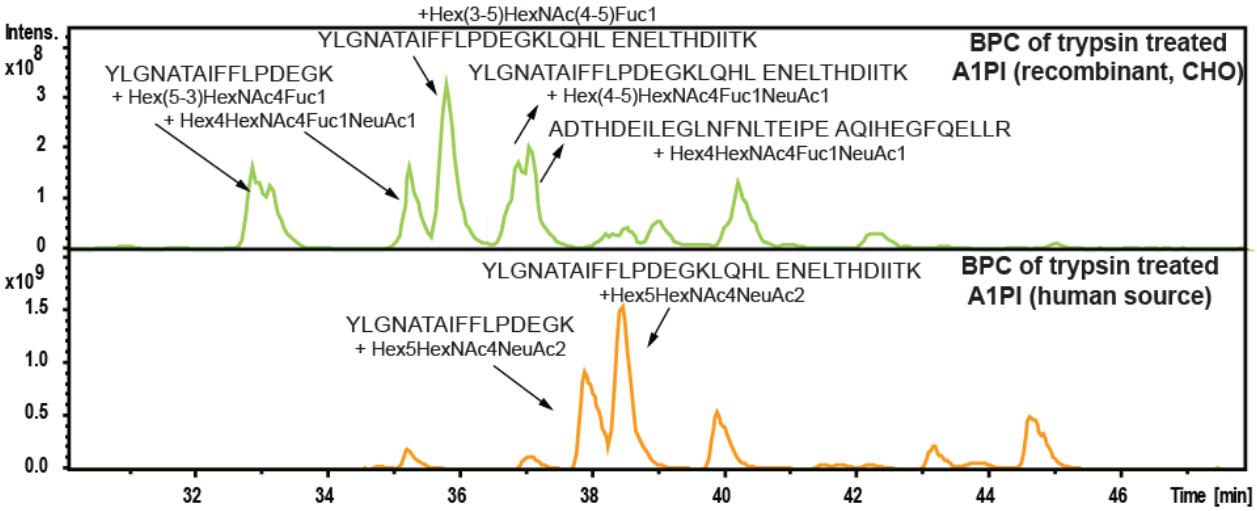

Figure S2. Reversed phase base peak chromatograms (BPCs) of tryptic glycopeptides ofA1PI from human plasma and recombinantly produced in CHO cells. Based on these results the HILIC enrichment was considered to be sufficient for later IM-MS experiments.

Confirming the NeuAc linkage from A1PI by PGC-LC ESI MS/MS of released N-glycans. To confirm that different NeuAc linkages are present on A1PI obtained from human plasma and CHO cells, glycans were enzymatically released and analysed using porous graphitized carbon (PGC) LC coupled to ESI-IT-MS detection (Figure S3) as described previously.[11, 12]

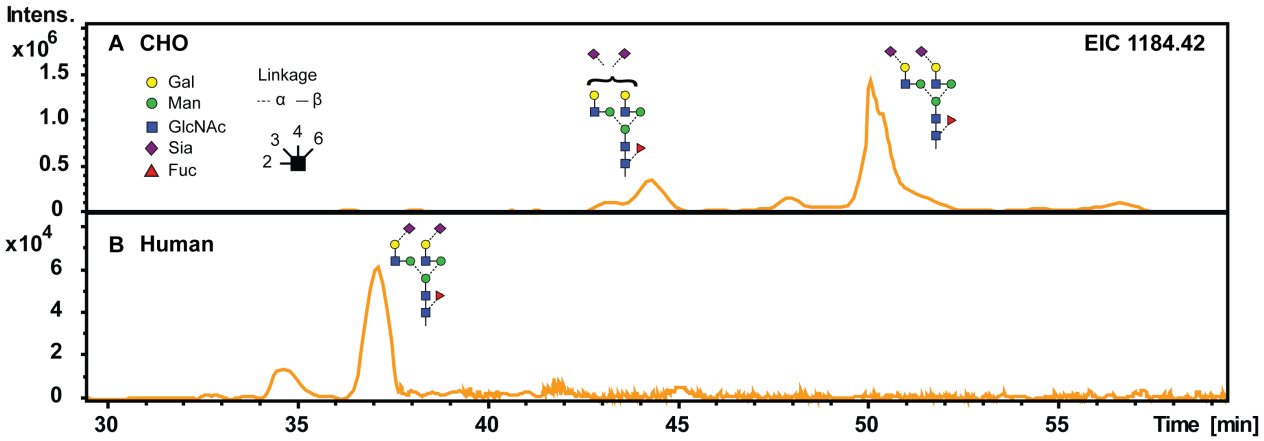

Figure S3. Extracted ion chromatograms (EICs) from PGC-LC of a representative N-glycan (Hex5HexNAc4NeuAc2Fuc, $[M-2H]^{2-}$ = 1184.43 Da) released from A1PI from two different origins as described above. (A) recombinantly produced A1PI from CHO cells and (B) purified from human serum. The difference in the retention time confirms that CHO derived A1PI contains largely α2,3 linked NeuAc residues, whereas the human derived A1PI carries just α2,6 linked NeuAc residues on the diantennary N-glycans, as shown previously.[13]

Ion mobility-mass spectrometry

Ion mobility experiments were performed on a travelling wave quadrupole/IMS/oa-ToF MS instrument, Synapt G2-S HDMS (Waters Corporation, Manchester, U.K.).[14, 15] All samples were dissolved in water/methanol (1:1, v/v) and ionized using a nano-electrospray source (nESI) from platinum-palladium-coated borosilicate capillaries prepared in-house. CCS estimations were performed using an established protocol with dextran as calibrant (Dextran M_n=1000 and Dextran M_n=5000, Sigma Aldrich).[16, 17] The calibration solution consisted of 0.1 mg/mL dextran1000, 0.5 mg/mL dextran5000, and 1 mM NaH_2PO_4 in water:methanol (1:1, v/v)

Positive ion mode: Source temperature, 25°C; needle voltage, 0.8 kV; sample cone voltage, 25 V; desolvation temperature, 150°C; cone gas, off; purge gas flow, off. Ion mobility parameters were: trap gas flow, 2 mL/min; helium cell gas flow, 180 mL/min; IM gas flow, 90 mL/min; drift time trimming, 5 bins; mobility delay after trap release, 0 µs; trap DC entrance, 3 V; trap DC bias, 35 V; trap DC exit, 0 V; IM wave velocity, 1000 m/s; IM wave height, 40 V; for MS/MS: trap collision energy, 15-25 V.

Negative ion mode: Source temperature, 25°C; needle voltage, 0.8 kV; sample cone voltage, 25 V; desolvation temperature, 150°C; cone gas, off; purge gas flow, off. Ion mobility parameters were: trap gas flow, 2 mL/min; helium cell gas flow, 180 mL/min; IM gas flow, 90 mL/min; mobility delay after trap release, 1000 µs; trap DC entrance, 3 V; trap DC bias, 45 V; trap DC exit 0 V; IM wave velocity, 800 m/s; IM wave height, 40 V.

IM-MS analysis of GP1 and GP2. GP1 and GP2 were analysed in positive and negative ion mode. For intact positively charged ions, no drift time differences were observed independent of the charge state (Figure S4A). However, a separation of GP1 and GP2 was possible by measuring the drift times of the quadruply deprotonated ions (Figure S4B).

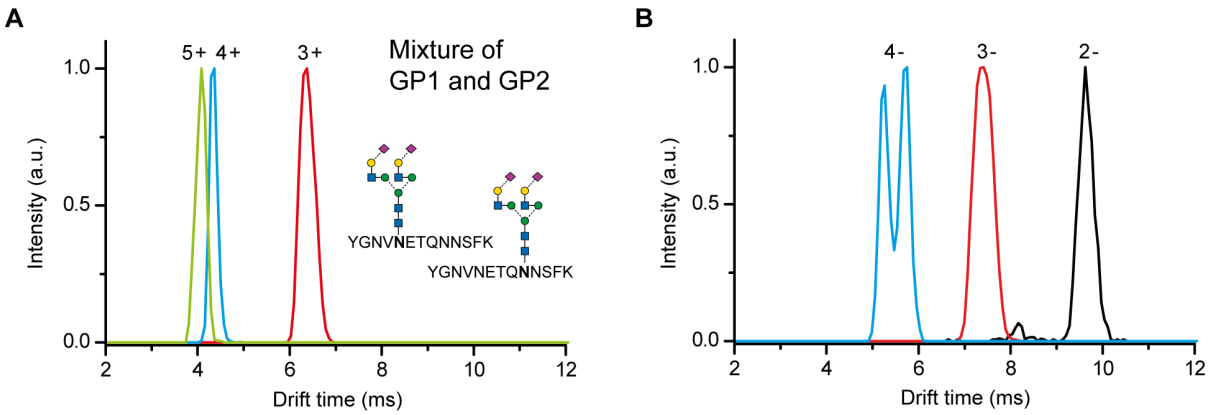

Figure S4. IM-MS analysis of a mixture of GP1 and GP2. (A) Arrival time distributions (ATDs) of differently charged intact ions in positive ion mode (green, $[M+5H]^{5+}$= 745; blue, $[M+4H]^{4+}$ = 931; red, $[M+3H]^{3+}$= 1240) did not show separation of the isomers GP1 and GP2. (B) For negatively charged ions a separation of GP1 and GP2 was observed for the quadruply charged intact ions (blue, $[M-4H]^{4-}$= 928). No drift time differences, however, were observed for other charge states (red, $[M-3H]^{3-}$ = 1238; black, $[M-2H]^{2-}$=1858).

IM-MS analysis of A1PI glycopeptides. The above described tryptic glycopeptides of A1PI, obtained from human plasma and recombinantly produced in CHO cells, were analysed using IM-MS. A variety of peptides and glycopeptides were observed as shown for Figure S5 and S6. Known glycopeptides containing sialylated N-glycans with the highest signal intensities were further analysed. For this, the species of interest were selected in the quadrupole and fragmented by collision-induced dissociation using argon as a collision gas. The resulting fragments are subsequently separated in the ion mobility cell and detected using a ToF mass analyser.

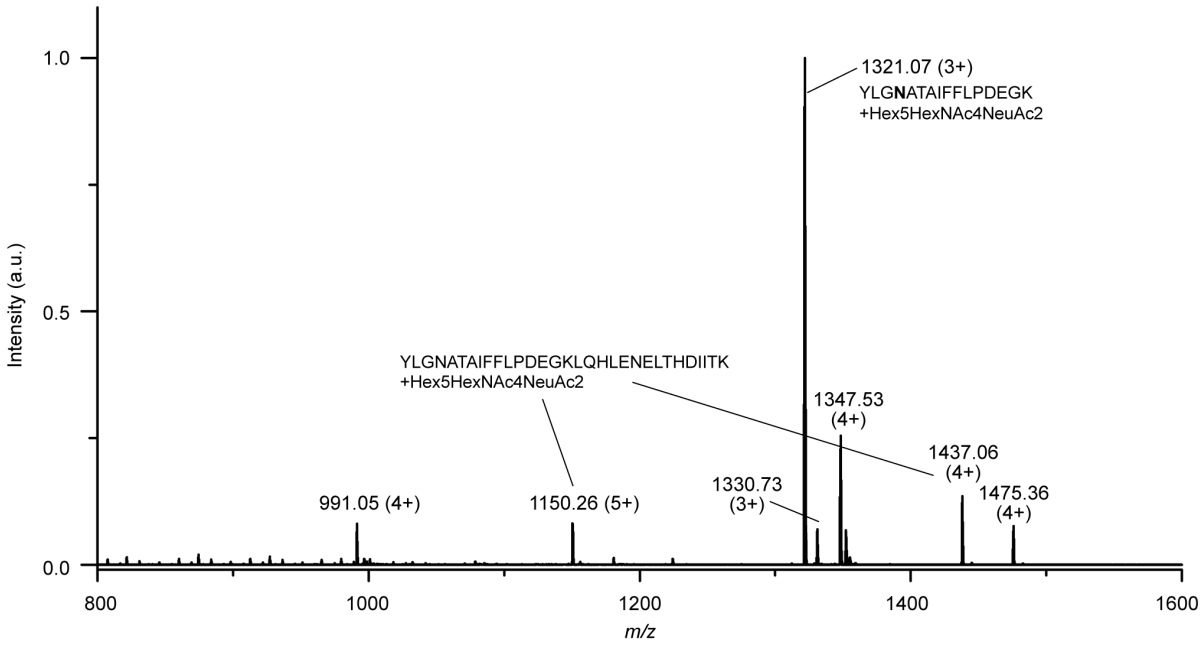

Figure S5. MS spectra of glycopeptides derived from human plasma A1PI in positive ion mode.

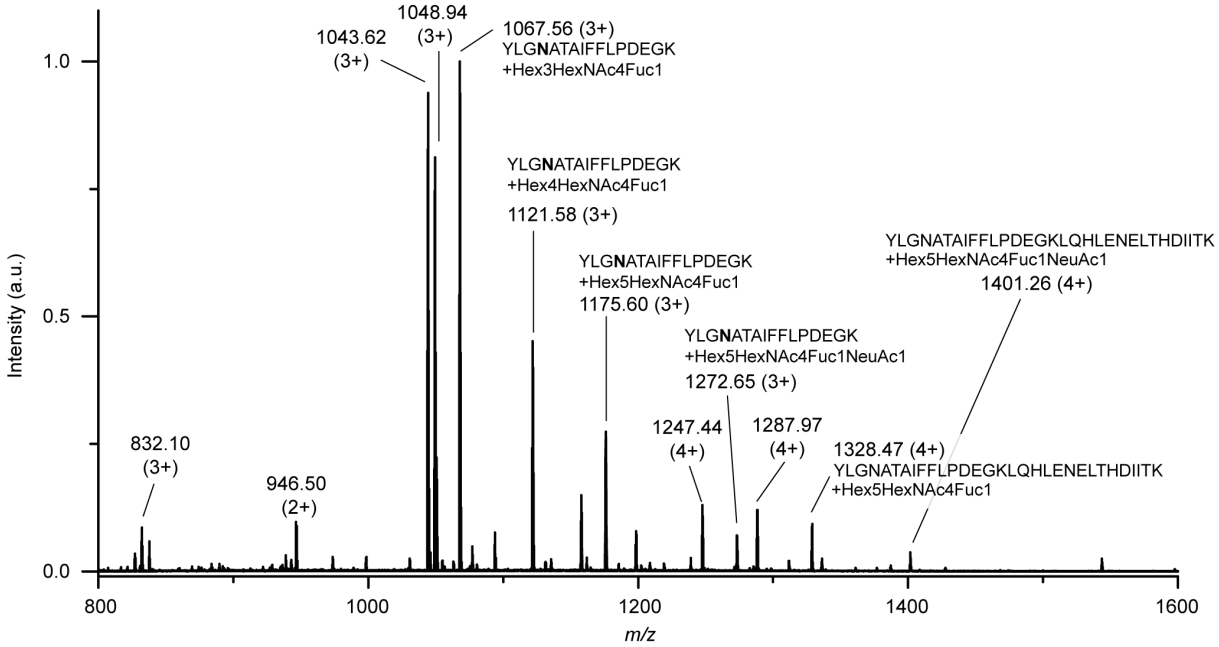

Figure S6. MS spectra of glycopeptides derived from recombinant A1PI (CHO) in positive ion mode.

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