Supplementary methods

Materials

Sodium dodecyl sulphate (SDS), urea, dithiothreitol (DTT), iodoacetamide (IAA), formic acid (FA), ammonium acetate (7.5M solution), sodium chloride, ammonium bicarbonate, L-fucose, methyl α-D-mammopyranoside and methyl α-D-glucopyranoside were from Sigma-Aldrich (Steinheim, Germany). LC grade water, methanol, acetonitrile (ACN), Tris (Trizma base) were purchased from Merck. Mass spectrometry grade trypsin was purchased from Promega (Madison, WI, USA). Neuraminidase A (α2-3, 6, 8, 9) was from New England Biolabs (NEB). Ammonium hydroxide (extra pure, 25% solution in water) was purchased from Acros organics. Amicon Ultra-0.5 mL centrifugal filters were from Merck Millipore.

Glycoprotein sample preparation for native MS

The asialo-AGP and asialo-Hp were obtained by digestion of 500 µg AGP and Hp with 400 unit neuraminidase A in 50 mM sodium acetate buffer (pH 5.5) at 37 °C overnight. Asialo-AGP and asialo-Hp were buffer exchanged into 200 mM ammonium acetate (pH 7.6) in 10 kDa MWCO centrifugal filters (Amicon Ultra-0.5 ml, Millipore) for native MS analysis.

Glycoprotein sample preparation for glycoproteomics and glycomics

Asialo-AGP and asialo-Hp were diluted with 100 mM Tris buffer (pH 8.0) containing 8 M Urea and 10 mM DTT to a concentration of 1 mg/ml for denaturing. The sample was incubated at 56 °C for 30 min for disulphide bond reduction and buffer exchanged to 50 mM NH_4HCO_3 with 10 mM IAA in a 10K Amicon centrifugal filter. The protein containing solution was then incubated at room temperature for 30 min in the dark and buffer exchanged to 50 mM NH_4HCO_3. The sample was then transferred to a new Eppendorf tube and digested with 2 µg trypsin at 37 °C overnight. For glycomics analysis, the denatured glycoprotein (about 50 µg) was incubated with 4 unit PNGase F at 37 °C overnight. The released N-glycans were desalted using a porous graphitic carbon StageTip and analyzed on a Q Exactive-Orbitrap mass spectrometer in positive mode.

Native MS data analysis

The raw native mass spectra were deconvoluted by UniDec to zero-charge spectra. Protein and glycan mass calculations were based on amino acid and monosaccharides average residue masses and led to a

calculated mass of 21560.12 Da for the AGP F1 variant peptide backbone. Pyro-Glu at the N-terminus (Gln to Pyro-Glu) results in a reduction of -17.03 Da. Hp (phenotype 1-1) α and β subunit peptide backbones are 9192.21 Da and 27265.07 Da, respectively. A disulfide bond reduces the mass by -2.01 Da. Hexose (Man and Gal) masses are 162.1424 Da. Fucose (Fuc) is 146.1430 Da, N-aceytlneuraminic acid (Neu5Ac) is 291.2579 Da and N-acetylglucosamine (GlcNAc) is 203.1950 Da.

Glycoproteomics data analysis

The monoisotopic masses of asialo-AGP and asialo-Hp tryptic glycopeptides without missed cleavages were manually calculated to 4 decimal places. Hexose (Man and Gal) monoisotopic masses are 162.0528 Da. GlcNAc is 203.0794 Da, Fuc is 146.0579 Da and Neu5Ac is 291.0954 Da. The extracted ion chromatogram (XIC) and its area under the curve (AUC) of each tryptic glycopeptide were processed and integrated using Xcalibur 2.2 with 50 ppm mass tolerance and 7 point Gaussian smoothing. Relative quantifications of the tryptic glycopeptides were based on their AUCs.

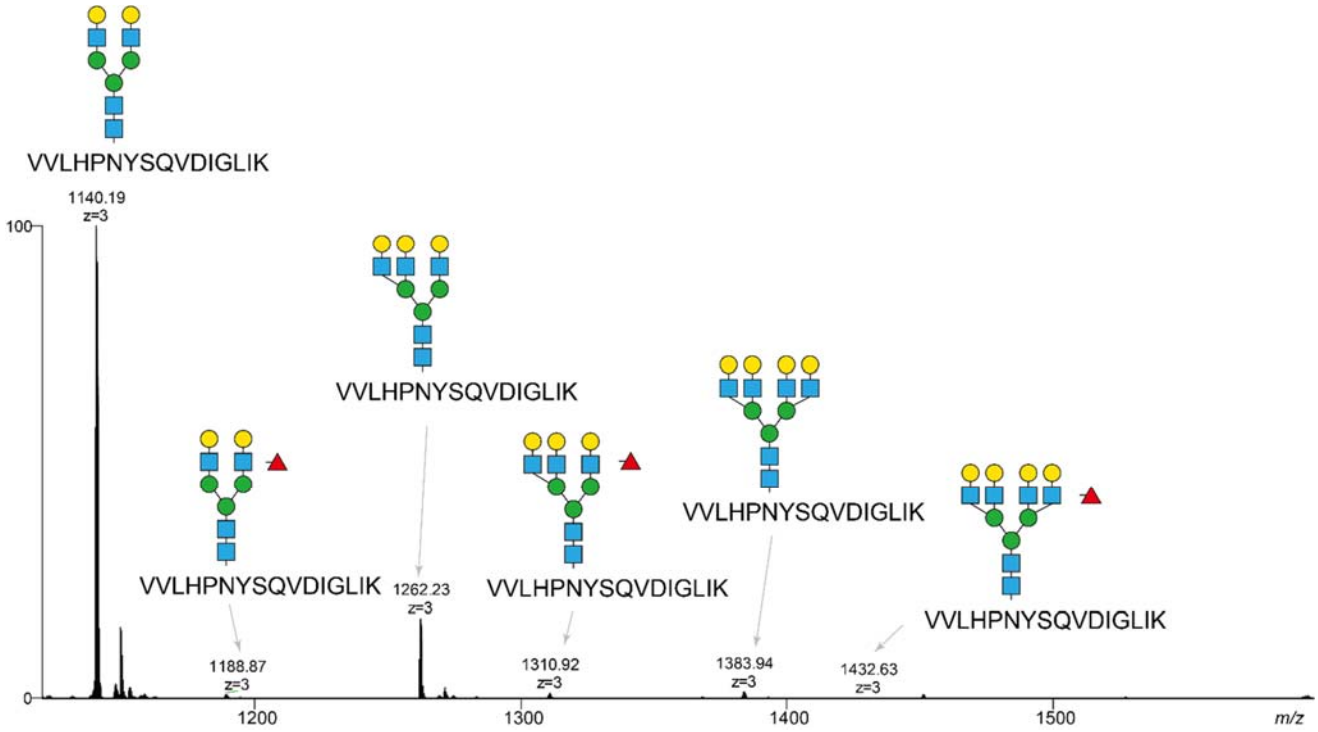

Figure S1. Glycoproteomics analysis of an asialo-Hp tryptic glycopeptide (Asn238). Intact glycopeptides with charge state +3 are shown. The glycan composition of intact glycopeptides are calculated based on peptide mass and monosaccharide residue monoisotopic masses. Bi-, tri- and tetra- antennary N-glycan without/with one fucose residue (red triangle, +146.0579 Da) are found at Asn238 of asialo-Hp. No sialylated (+291.0954 Da) N-glycan is observed. These suggest the complete removal of sialic acid residues. The monosaccharide residues are labeled according to the Consortium for Functional Glycomics guidance (blue square for GlcNAc, yellow circle for Gal, green circle for Man, and red triangle for Fuc).

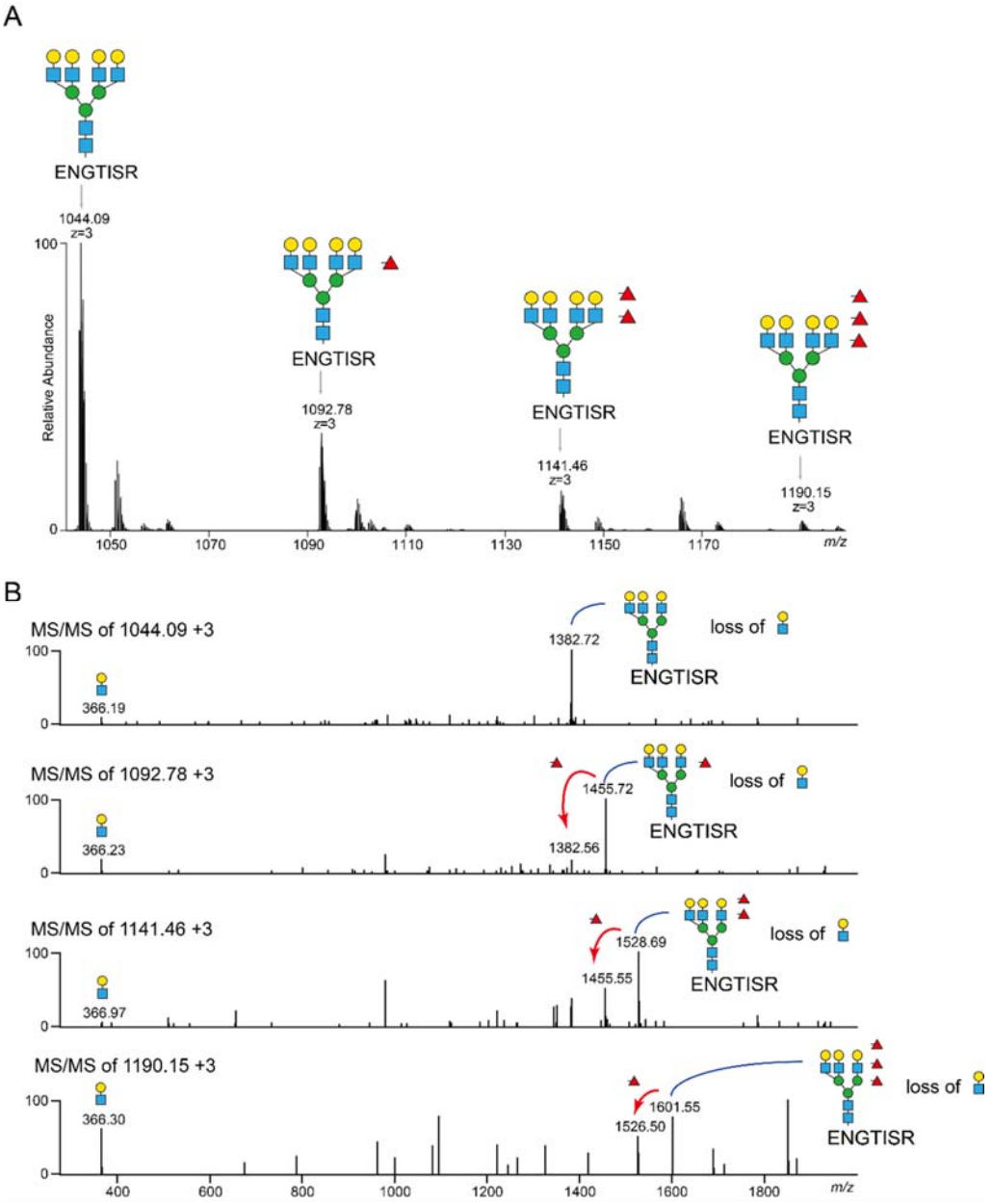

Figure S2. Glycoproteomics analysis of tetra-antennary asialo-AGP tryptic glycopeptides (Asn85). (A) MS of asialo-AGP tryptic glycopeptides (Asn85) carrying tetra-antennary N-glycans with zero to three additional fucose residues (red triangles, +146.0579 Da). Intact glycopeptides with charge state +3 are shown. No sialylated (+291.0954 Da) tetra-antennary N-glycopeptide is found. (B) MS/MS of asialo-AGP tryptic glycopeptides (Asn85). The neutral loss of fucose residue is indicated by red arrow. The high resolution mass spectrum of intact glycopeptides and tandem MS analysis confirm the complete removal of sialic acid residues and the presence of multiple fucose residues (red triangles).

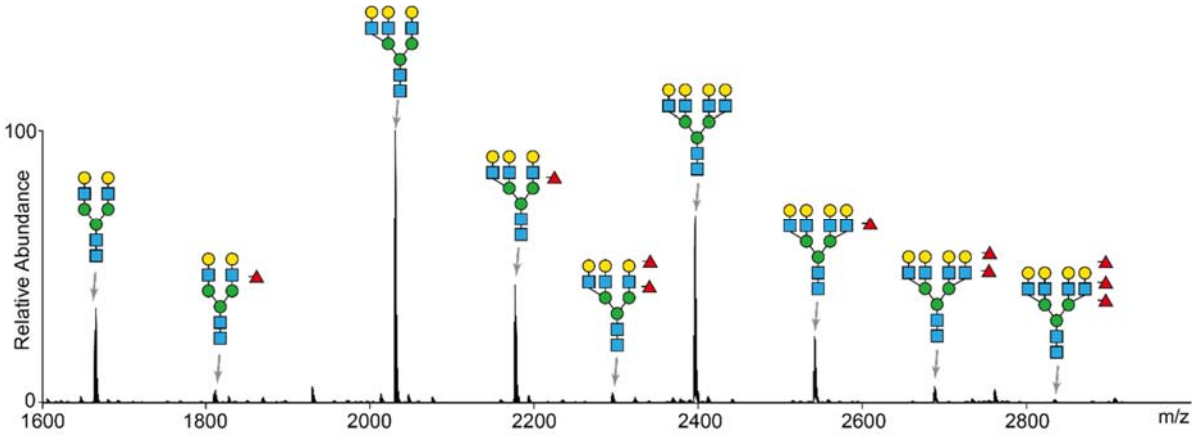

Figure S3. Glycomics analysis of the released N-glycans from asialo-AGP. N-glycans were enzymatically digested from denatured asialo-AGP using PNGase F treatment and analyzed by mass spectrometer. Bi-, tri- and tetra- antennary N-glycans are observed. We also identified mono-, bi- and tri- fucosylations (red triangles). No sialylated (+291.2579 Da) N-glycan is observed. This confirms the complete desialylation of asialo-AGP at the glycan level. All N-glycan ions in the spectrum are charge state +1.

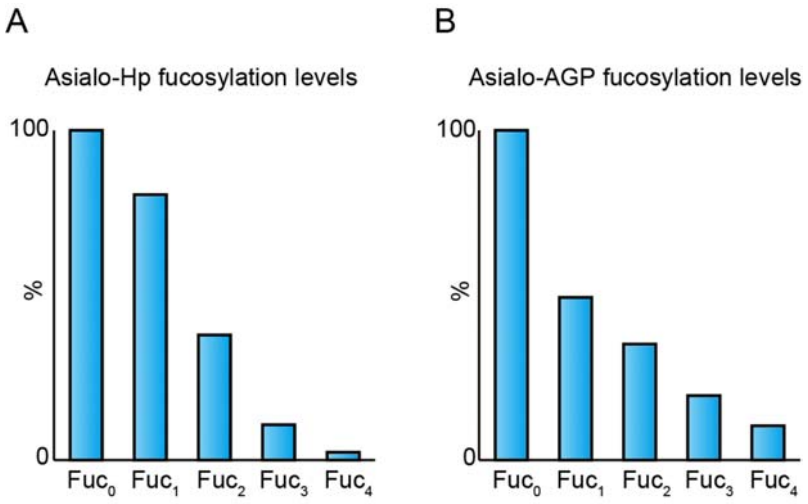

Figure S4. Fucosylation levels of asialo-Hp (A) and asialo-AGP (B). The relative abundances of Fuc_0, Fuc_1, Fuc_2, Fuc_3 and Fuc_4 peaks in asialo-Hp and asialo-AGP native mass spectra (Figure 1) are summed, normalized and plotted as bar graphs, respectively.

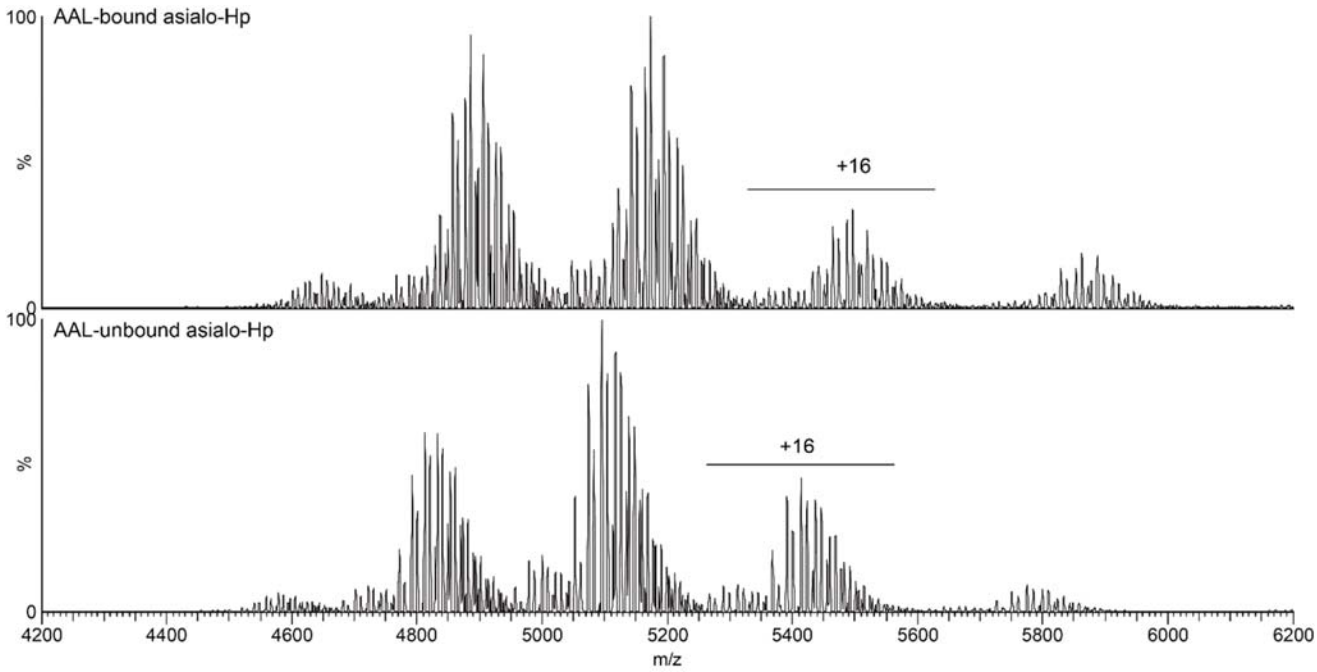

Figure S5. Raw native mass spectra of AAL-bound asialo-Hp and AAL-unbound asialo-Hp. The average molecular weight of AAL-bound asialo-Hp is significantly larger than AAL-unbound asialo-Hp suggesting AAL-bound asialo-Hp carries more fucose residues and/or fucose-containing glycan structures.

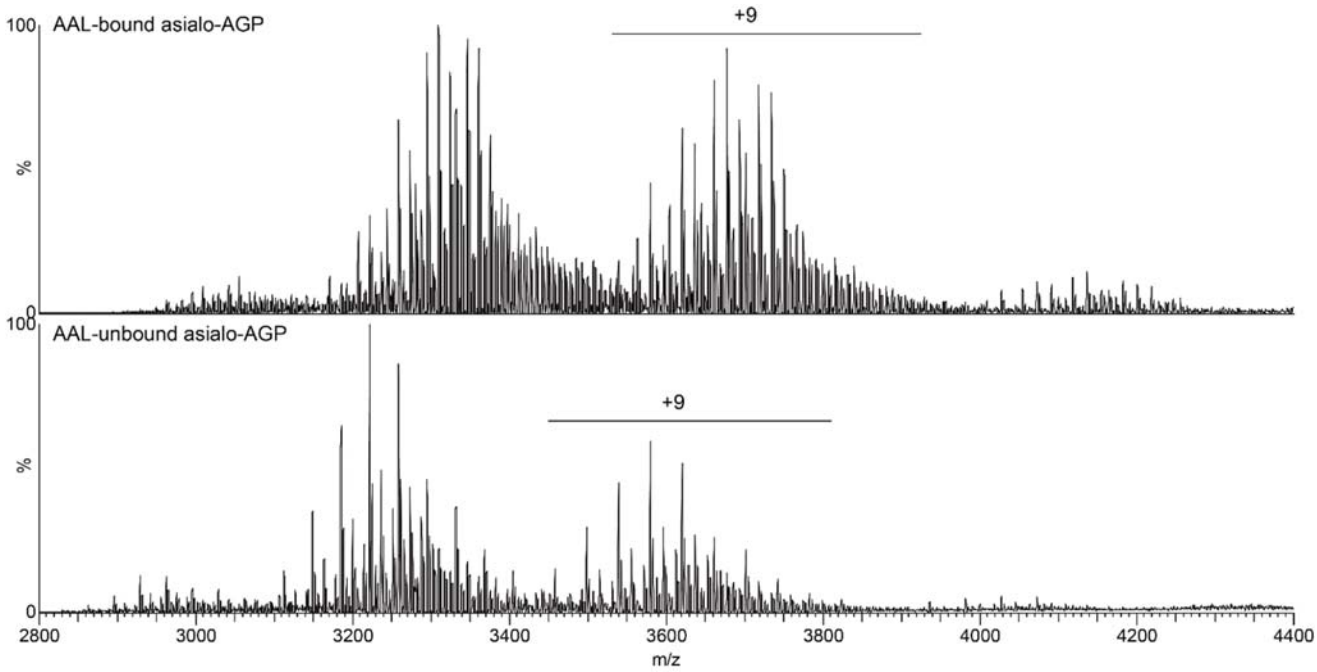

Figure S6. Raw native mass spectra of AAL-bound asialo-AGP and AAL-unbound asialo-AGP. The average molecular weight of AAL-bound asialo-AGP is also significantly larger than AAL-unbound asialo-AGP indicating the presence of additional fucose residues and/or fucose-containing glycan structures on AAL-bound asialo-AGP.

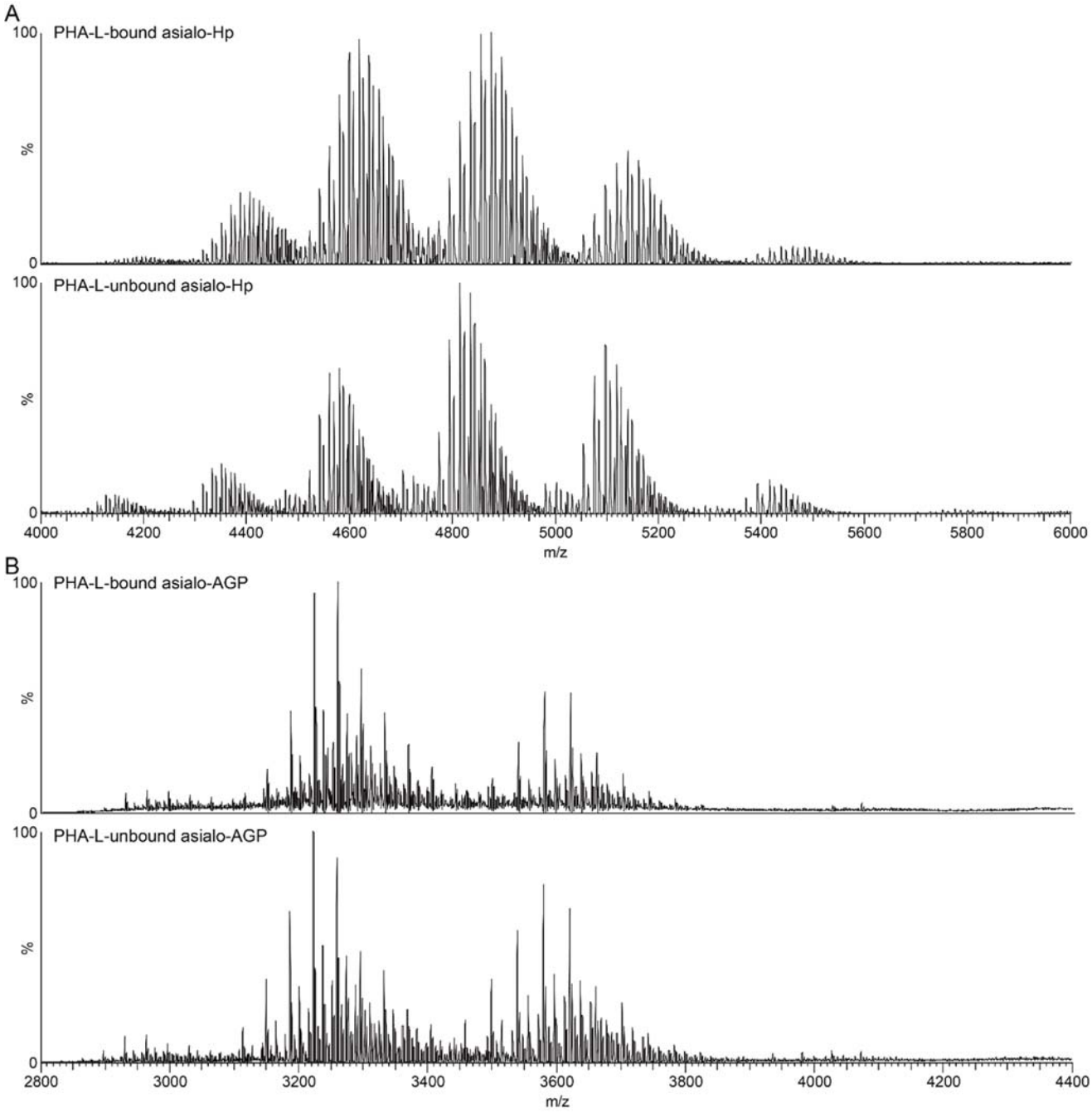

Figure S7. Raw native mass spectra of PHA-L fractionated asialo-Hp (A) and asialo-AGP (B). The PHA-L-bound and unbound asialo-AGP spectra are similar. PHA-L fractionation is more efficient to enrich highly branched asialo-Hp (with higher molecular weight), but less efficient to fractionate asialo-AGP.

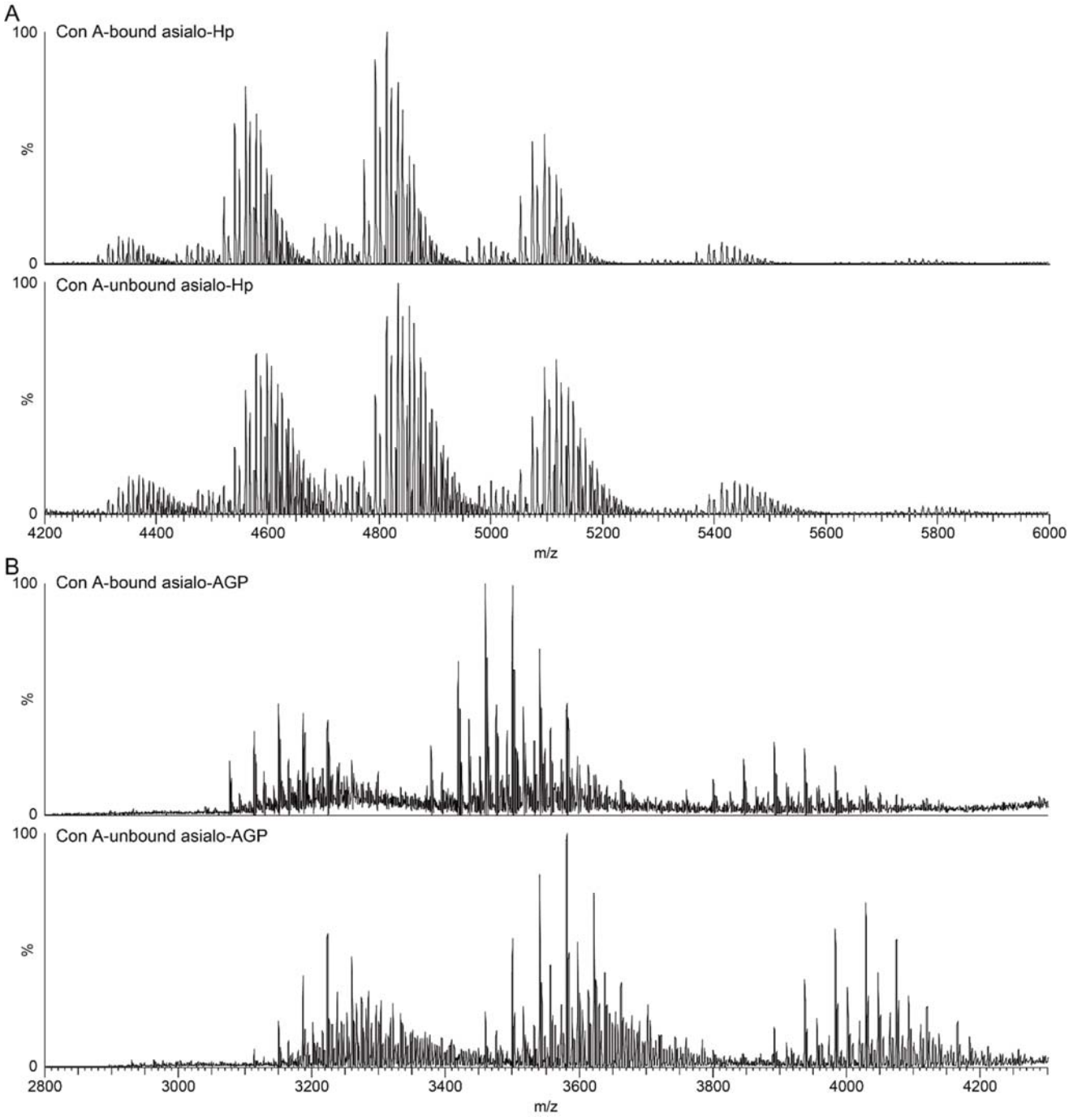

Figure S8. Raw native mass spectra of Con A fractionated asialo-Hp (A) and asialo-AGP (B). Con A- bound and unbound asialo-Hp spectra are almost identical. Comparing to PHA-L fractionation (Fig. S7), Con A lectin affinity purification is more practical to fractionate asialo-AGP.

Table S1. Annotation of asialo-Hp glycoproteoforms

Num.	Experimental Mass (Da)	Theoretical Mass (Da)	Mass Difference (Da)	Relative Abundance (%)	Hex	HexNAc	Fuc	Glycosylation status	Annotation
1	84260.1	84260.9	0.8	6.94	35	28	0	Partially	
2	84412.5	84407.1	5.4	4.57	35	28	1	Partially	
3	84625.8	84626.3	0.5	15.55	36	29	0	Partially	
4	84775.3	84772.4	2.9	10.84	36	29	1	Partially	
5	84924.2	84918.5	5.7	1.93	36	29	2	Partially	
6	84989.9	84991.6	1.7	16.42	37	30	0	Partially	
7	85138.4	85137.7	0.7	13.74	37	30	1	Partially	
8	85288.5	85283.9	4.6	5.74	37	30	2	Partially	
9	85356.4	85356.9	0.5	14.07	38	31	0	Partially	
10	85504.8	85503.1	1.7	12.88	38	31	1	Partially/Fully	P0F0
11	85654.9	85649.2	5.7	6.71	38	31	2	Partially/Fully	P0F1
12	85723.2	85722.3	0.9	10.06	39	32	0	Partially	
13	85881.4	85884.4	3	39	40	32	0	Fully	P1F0
14	86033.2	86030.5	2.7	15.84	40	32	1	Fully	P1F1
15	86091.3	86087.6	3.7	7.02	40	33	0	Partially	
16	86248.1	86249.7	1.6	79.12	41	33	0	Fully	P2F0
17	86398.7	86395.9	2.8	52.9	41	33	1	Fully	P2F1
18	86546.4	86542.0	4.4	6.89	41	33	2	Fully	P2F2
19	86615.3	86615.1	0.2	100	42	34	0	Fully	P3F0
20	86762.4	86761.2	1.2	82.42	42	34	1	Fully	P3F1
21	86913.0	86907.4	5.6	31.92	42	34	2	Fully	P3F2
22	86980.3	86980.4	0.1	95.69	43	35	0	Fully	P4F0
23	87127.6	87126.6	1	84.57	43	35	1	Fully	P4F1
24	87276.2	87272.7	3.5	44.4	43	35	2	Fully	P4F2
25	87346.7	87345.8	0.9	73.95	44	36	0	Fully	P5F0
26	87425.4	87418.8	6.6	7.02	43	35	3	Fully	P4F3
27	87493.7	87491.9	1.8	66.49	44	36	1	Fully	P5F1
28	87640.6	87638.0	2.6	41.21	44	36	2	Fully	P5F2
29	87711.0	87711.1	0.1	48.93	45	37	0	Fully	P6F0
30	87789.9	87784.2	5.7	15.45	44	36	3	Fully	P5F3
31	87859.6	87857.2	2.4	43.65	45	37	1	Fully	P6F1
32	88006.0	88003.4	2.6	29.27	45	37	2	Fully	P6F2
33	88077.4	88076.4	1	29.79	46	38	0	Fully	P7F0
34	88155.3	88149.5	5.8	14.74	45	37	3	Fully	P6F3
35	88223.8	88222.6	1.2	26.39	46	38	1	Fully	P7F1

36	88301.5	88295.7	5.8	4.39	45	37	4	Fully	P6F4
37	88371.7	88368.7	3	18.16	46	38	2	Fully	P7F2
38	88442.1	88441.8	0.3	16.61	47	39	0	Fully	P8F0
39	88518.9	88514.9	4	9.39	46	38	3	Fully	P7F3
40	88590.3	88587.9	2.4	15.2	47	39	1	Fully	P8F1
41	88666.6	88661.0	5.6	4.03	46	38	4	Fully	P7F4
42	88736.1	88734.1	2	10.12	47	39	2	Fully	P8F2
43	88808.4	88807.1	1.3	10.02	48	40	0	Fully	P9F0
44	88883.9	88880.2	3.7	4.31	47	39	3	Fully	P8F3
45	88955.4	88953.2	2.2	9.32	48	40	1	Fully	P9F1
46	89031.7	89026.3	5.4	1.88	47	39	4	Fully	P8F4
47	89102.7	89099.4	3.3	4.75	48	40	2	Fully	P9F2
48	89172.4	89172.4	0	3.75	49	41	0	Fully	P10F0
49	89254.6	89245.5	9.1	2.22	48	40	3	Fully	P9F3
50	89320.1	89318.6	1.5	3.2	49	41	1	Fully	P10F1

Table S2. Annotation of asialo-AGP glycoproteoforms

Num.	Experimental Mass (Da)	Theoretical Mass (Da)	Mass Difference (Da)	Relative Abundance (%)	Hex	HexNAc	Fuc	Genetic Variant	Annotation
1	30751.5	30752.5	1	4.75	28	23	0	F1	P0
2	30901.8	30898.7	3.1	2.37	28	23	1	F1	P0F1
3	30930.3	30926.7	3.6	1.17	28	23	1	S	P0F1
4	31042.9	31044.8	1.9	1.27	28	23	2	F1	P0F2
5	31115.7	31117.9	2.2	21.99	29	24	0	F1	P1F0
6	31145.4	31145.9	0.5	3.83	29	24	0	S	P1F0
7	31190.8	31191.0	0.2	1.91	28	23	3	F1	P0F3
8	31262.4	31264.0	1.6	10.55	29	24	1	F1	P1F1
9	31292.2	31292.1	0.1	4.63	29	24	1	S	P1F1
10	31324.2	31321.1	3.1	2.8	29	25	0	F1	
11	31408.4	31410.2	1.8	8.28	29	24	2	F1	P1F2
12	31437.5	31438.2	0.7	2.32	29	24	2	S	P1F2
13	31480.4	31483.2	2.8	47.91	30	25	0	F1	P2
14	31509.8	31511.3	1.5	18.35	30	25	0	S	P2
15	31556.6	31556.3	0.3	4.14	29	24	3	F1	P1F3
16	31584.8	31584.4	0.4	1.71	29	24	3	S	P1F3
17	31627.1	31629.4	2.3	21.62	30	25	1	F1	P2F1
18	31657.5	31657.4	0.1	9.7	30	25	1	S	P2F1
19	31689.7	31686.4	3.3	7.81	30	26	0	F1	
20	31706.0	31702.4	3.6	1.39	29	24	4	F1	P1F4
21	31774.1	31775.5	1.4	15.99	30	25	2	F1	P2F2
22	31803.7	31803.5	0.2	8.53	30	25	2	S	P2F2
23	31845.7	31848.5	2.8	71.79	31	26	0	F1	P3
24	31876.6	31876.6	0	32.08	31	26	0	S	P3
25	31921.5	31921.6	0.1	7.86	30	25	3	F1	P2F3
26	31950.3	31949.7	0.6	6.21	30	25	3	S	P2F3
27	31993.3	31994.7	1.4	34.55	31	26	1	F1	P3F1
28	32021.9	32022.7	0.8	18.81	31	26	1	S	P3F1
29	32054.6	32051.7	2.9	9.93	31	27	0	F1	
30	32070.1	32067.8	2.3	3.34	30	25	4	F1	P2F4
31	32094.6	32095.8	1.2	2.78	30	25	4	S	P2F4
32	32138.9	32140.8	1.9	25.96	31	26	2	F1	P3F2
33	32168.1	32168.9	0.8	15.58	31	26	2	S	P3F2
34	32212.5	32213.9	1.4	100	32	27	0	F1	P4
35	32243.1	32241.9	1.2	46.4	32	27	0	S	P4
36	32287.1	32287.0	0.1	13.18	31	26	3	F1	P3F3
37	32314.1	32315.0	0.9	9.41	31	26	3	S	P3F3
38	32360.0	32360.0	0	48.81	32	27	1	F1	P4F1
39	32387.7	32388.1	0.4	28.11	32	27	1	S	P4F1

40	32419.6	32417.1	2.5	17.22	32	28	0	F1	
41	32435.6	32433.1	2.5	5.48	31	26	4	F1	P3F4
42	32460.1	32461.2	1.1	6.09	31	26	4	S	P3F4
43	32505.8	32506.2	0.4	34.99	32	27	2	F1	P4F2
44	32533.4	32534.2	0.8	19.93	32	27	2	S	P4F2
45	32577.8	32579.2	1.4	81.83	33	28	0	F1	P5
46	32608.2	32607.3	0.9	43.91	33	28	0	S	P5
47	32651.8	32652.3	0.5	20.84	32	27	3	F1	P4F3
48	32680.4	32680.4	0	13.69	32	27	3	S	P4F3
49	32724.5	32725.4	0.9	40.48	33	28	1	F1	P5F1
50	32754.6	32753.4	1.2	26.24	33	28	1	S	P5F1
51	32785.2	32782.4	2.8	12.98	33	29	0	F1	
52	32799.5	32798.5	1	10.94	32	27	4	F1	P4F4
53	32827.5	32826.5	1	7.75	32	27	4	S	P4F4
54	32871.6	32871.5	0.1	28.89	33	28	2	F1	P5F2
55	32898.7	32899.6	0.9	18.68	33	28	2	S	P5F2
56	32944.2	32944.6	0.4	41.02	34	29	0	F1	P6
57	32972.9	32972.6	0.3	23.76	34	29	0	S	P6
58	33017.9	33017.6	0.3	18.41	33	28	3	F1	P5F3
59	33047.9	33045.7	2.2	13.48	33	28	3	S	P5F3
60	33090.3	33090.7	0.4	20.36	34	29	1	F1	P6F1
61	33119.0	33118.8	0.2	15.95	34	29	1	S	P6F1
62	33151.3	33147.8	3.5	4.59	34	30	0	F1	
63	33164.3	33163.8	0.5	12.85	33	28	4	F1	P5F4
64	33189.3	33191.8	2.5	8.38	33	28	4	S	P5F4
65	33236.8	33236.8	0	12.84	34	29	2	F1	P6F2
66	33265.8	33264.9	0.9	10.04	34	29	2	S	P6F2
67	33309.7	33309.9	0.2	29.56	35	30	0	F1	P7
68	33337.1	33338.0	0.9	18.01	35	30	0	S	P7
69	33383.1	33383.0	0.1	7.6	34	29	3	F1	P6F3
70	33411.8	33411.0	0.8	6.38	34	29	3	S	P6F3
71	33455.7	33456.0	0.3	17.38	35	30	1	F1	P7F1
72	33484.2	33484.1	0.1	11.52	35	30	1	S	P7F1
73	33516.1	33513.1	3	5.08	35	31	0	F1	
74	33529.7	33529.1	0.6	3.72	34	29	4	F1	P6F4
75	33557.4	33557.2	0.2	4.02	34	29	4	S	P6F4
76	33602.0	33602.2	0.2	11.03	35	30	2	F1	P7F2
77	33628.6	33630.2	1.6	7.47	35	30	2	S	P7F2
78	33674.2	33675.2	1	15.38	36	31	0	F1	P8F0
79	33703.6	33703.3	0.3	9.42	36	31	0	S	P8F0
80	33748.4	33748.3	0.1	6.52	35	30	3	F1	P7F3
81	33777.0	33776.4	0.6	4.56	35	30	3	S	P7F3
82	33822.1	33821.4	0.7	8.49	36	31	1	F1	P8F1

83	33850.0	33849.4	0.6	5.62	36	31	1	S	P8F1
84	33881.6	33878.4	3.2	1.91	36	32	0	F1	
85	33893.3	33894.5	1.2	3.26	35	30	4	F1	P7F4
86	33923.3	33922.5	0.8	3.11	35	30	4	S	P7F4
87	33967.8	33967.5	0.3	5.81	36	31	2	F1	P8F2
88	33996.7	33995.6	1.1	4.52	36	31	2	S	P8F2
89	34039.6	34040.6	1	9.76	37	32	0	F1	P9F0
90	34068.3	34068.6	0.3	6.1	37	32	0	S	P9F0
91	34111.0	34113.7	2.7	3.71	36	31	3	F1	P8F3
92	34141.5	34141.7	0.2	2.56	36	31	3	S	P8F3
93	34185.9	34186.7	0.8	4.43	37	32	1	F1	P9F1
94	34212.0	34214.8	2.8	3.25	37	32	1	S	P9F1
95	34261.5	34259.8	1.7	2.75	36	31	4	F1	P8F4
96	34288.3	34287.9	0.4	1.9	36	31	4	S	P8F4
97	34330.4	34332.9	2.5	4.67	37	32	2	F1	P9F2
98	34361.9	34360.9	1	2.77	37	32	2	S	P9F2
99	34404.9	34405.9	1	4.06	38	33	0	F1	P10F0
100	34433.2	34434.0	0.8	3.42	38	33	0	S	P10F0
101	34477.0	34479.0	2	1.69	37	32	3	F1	P9F3