Endoglycosidase-mediated Incorporation of ^{18}O into Glycans for Relative Glycan Quantitation

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Supporting Information

S-1. Unit definitions

Endo H and PNGase F: One unit is defined as the amount of enzyme required to remove > 95% of the carbohydrate from 10 μg of denatured RNase B in 1 hour at 37 °C in a total reaction volume of 10 μL (65 NEB units = 1 IUB milliunit).

Endo F_2 and Endo F_3: One unit will release *N*-linked oligosaccharides from 1 μmole of denatured porcine fibrinogen in 1 minute at 37 °C, pH 4.5.

S-2. Sample preparation of porous graphitized carbon (PGC) column

The sample preparation of glycans prior to MS using porous graphitized carbon (PGC) column was performed mainly according to the procedure previously reported:[1]

1) The column was washed with 6 column volumes of 80% (v/v) acetonitrile (ACN) containing 0.1% (v/v) trifluoroacetic acid (TFA), followed by 6 column volumes of water prior to use.
2) The sample (aqueous solution) to be desalted was loaded on the column and allowed to run into the adsorbent. The flow rate should be about 0.5–1.0 mL/min, and the flow through sample should be reloaded three times.
3) The column was washed with approximately ten column volumes of water to remove salts and detergent.
4) The adsorbed glycans were eluted with 3 column volumes of 25% (v/v) ACN containing 0.05% (v/v) TFA. The eluted solution was dried via vacuum centrifugation and redissolved in 50% (v/v) ACN containing 0.1% (v/v) TFA for MS analysis.

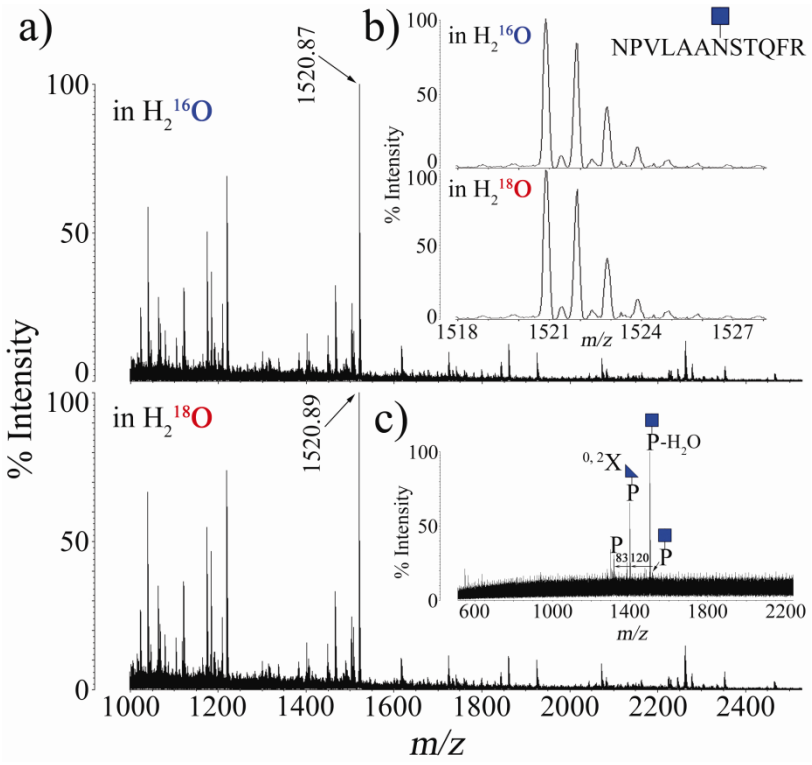


Figure S-1. MALDI-TOF mass spectra of invertase tryptic peptides treated by Endo H in $H_2{}^{16}O$ and $H_2{}^{18}O$. (a) Spectra of the tryptic peptides deglycosylated by Endo H in $H_2{}^{16}O$ (top) and $H_2{}^{18}O$ (bottom). (b) Enlarged spectrum of peptide NPVLAA**N**(GlcNAc)STQFR at *m/z* 1520. (c) MS^2 spectrum of peptide NPVLAA**N**(GlcNAc)STQFR at *m/z* 1520. Blue square: GlcNAc; P: peptide NPVLAANSTQFR.

No mass difference was observed between the ^{16}O- and ^{18}O-labeled species for the parent peptide. The $^{0,2}X$ ions of the GlcNAc cross-ring fragment on the peptide from the ^{18}O-labeled sample were distinctly observed in the MS^2 spectrum, displaying a characteristic unique pattern of triplets (P[-83], P-◣[-120], and P-■). These data suggest that, in contrast to PNGase, no ^{18}O is introduced to the glycosite in cleavage by endoglycosidase.

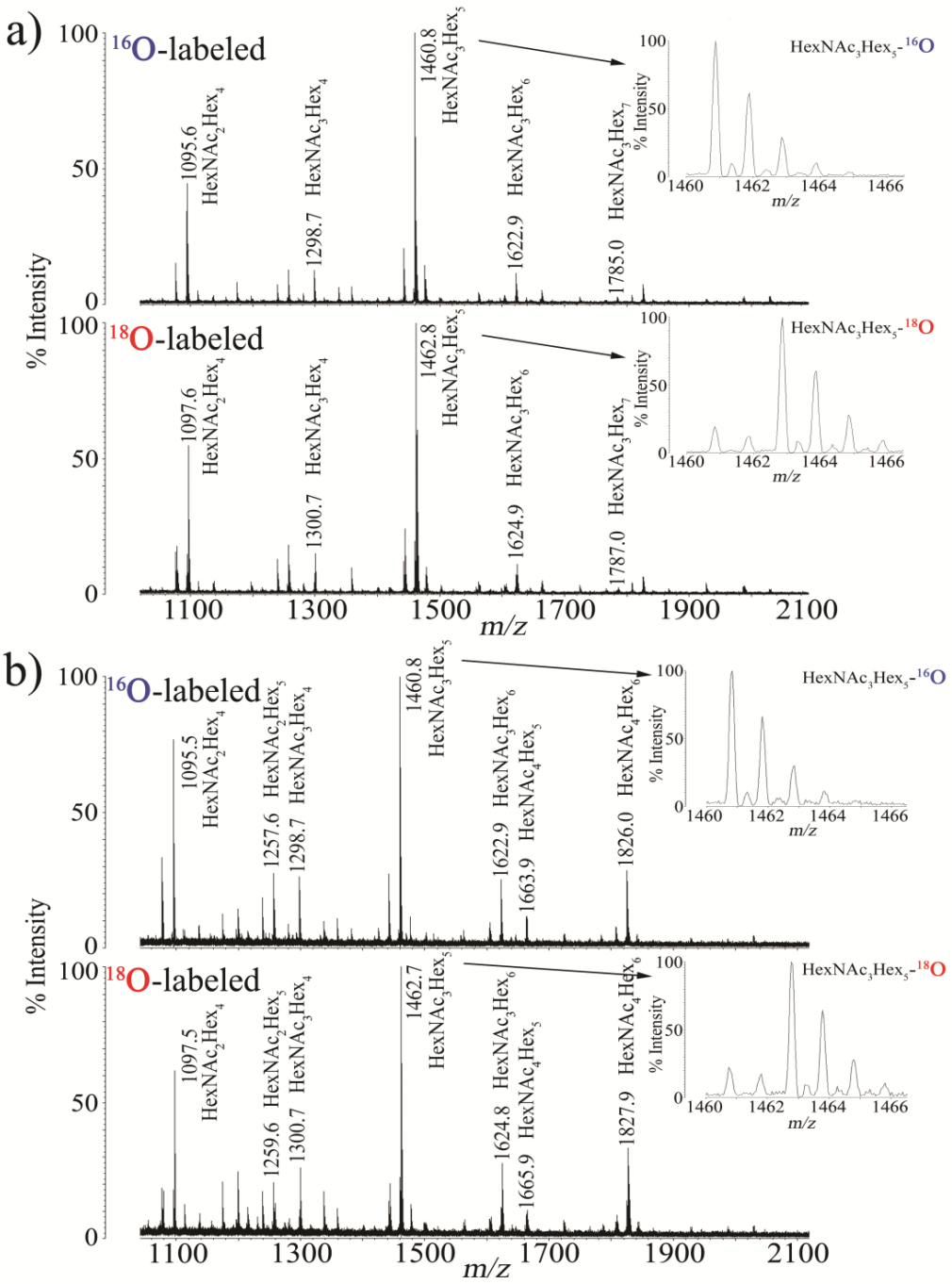


Figure S-2. MALDI-TOF mass spectra of ^{16}O- and ^{18}O-labeled glycans of asialofetuin treated by (a) Endo F_2 and (b) Endo F_3 in $H_2{}^{16}O$ and $H_2{}^{18}O$. All glycan ions are singly charged sodium adduct ions.

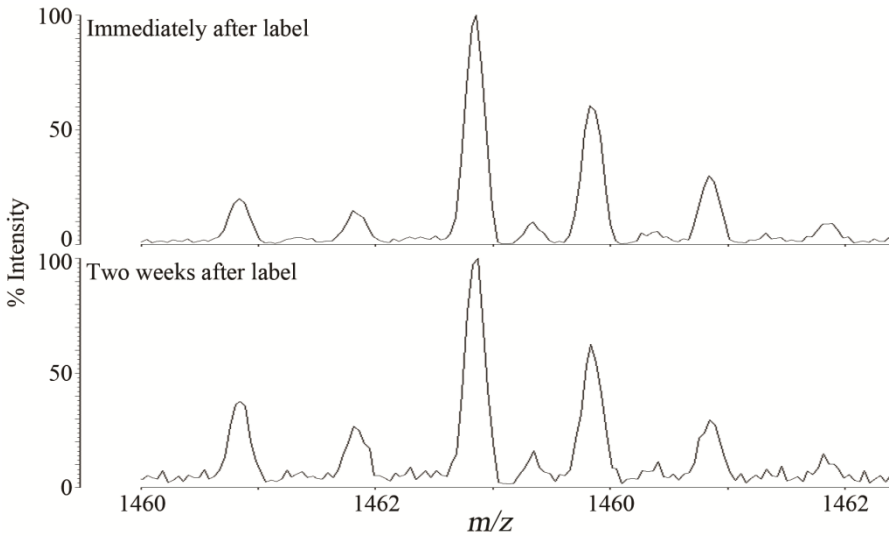


Figure S-3. Stability of the labeled ^{18}O in normal water (glycan *m/z* 1460 from ovalbumin as an example). Spectra of the glycan immediately after label (top) and two weeks later after label (bottom) stored in normal water at -20 ^{o}C show that the labeled ^{18}O is relatively stable in normal water. But stored as dried glycan or in ^{18}O-water is better. All glycan ions are singly charged sodium adduct ions.

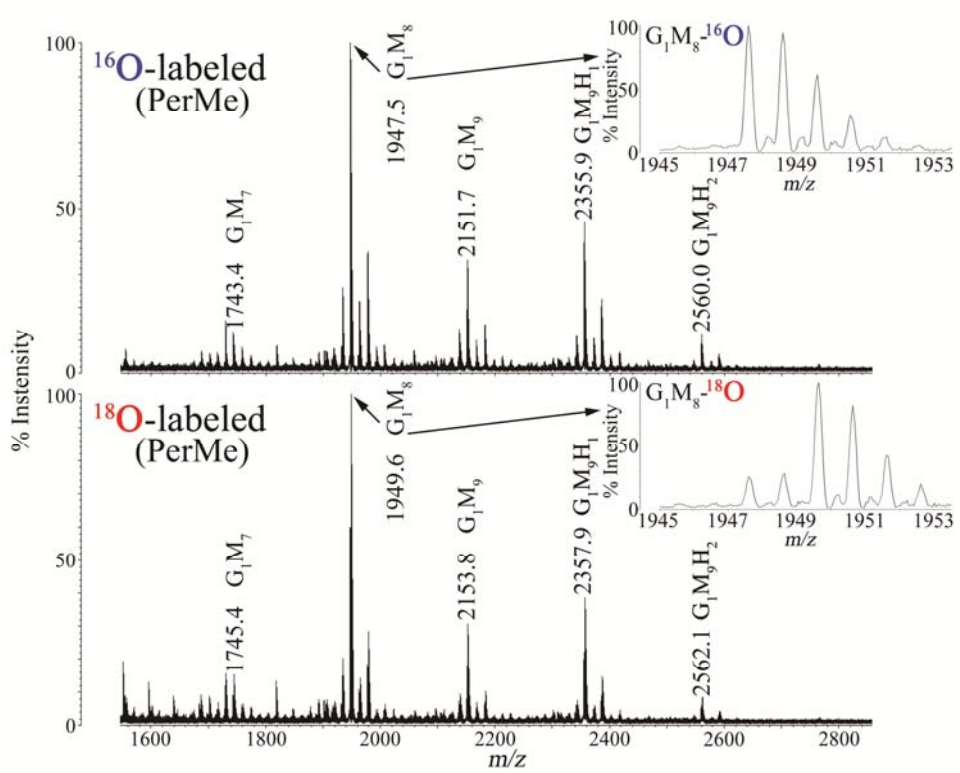


Figure S-4. MALDI-TOF mass spectra of the permethylated ^{16}O-labeled (top) and ^{18}O-labeled (bottom) glycans of invertase. M: Man; H: Hex; G: GlcNAc. All glycan ions are singly charged sodium adduct ions.

S-3. Glycan ^{18}O labeling by PNGase

Experiments using different buffers were conducted with ribonuclease B as a model glycoprotein. Four aliquots of ribonuclease B were separately treated by PNGase F in $H_2{}^{16}O$ or $H_2{}^{18}O$, of which one pair was digested in 50 mM ammonium bicarbonate buffer. The other pair was digested in 50 mM sodium phosphate buffer. The treated samples were then purified and analyzed by MS. The deamination reaction was almost completely inhibited in the buffer containing ammonium (Figure S-5b). Glycosylamine presented as the most intensive peak ([M-1]). The glycan labeled by ^{18}O ([M+2]) was highly inhibited. Labeling occurred in the buffer without ammonium (sodium phosphate buffer), but it was incomplete because of the coexisting glycosylamine (Figure S-5c).

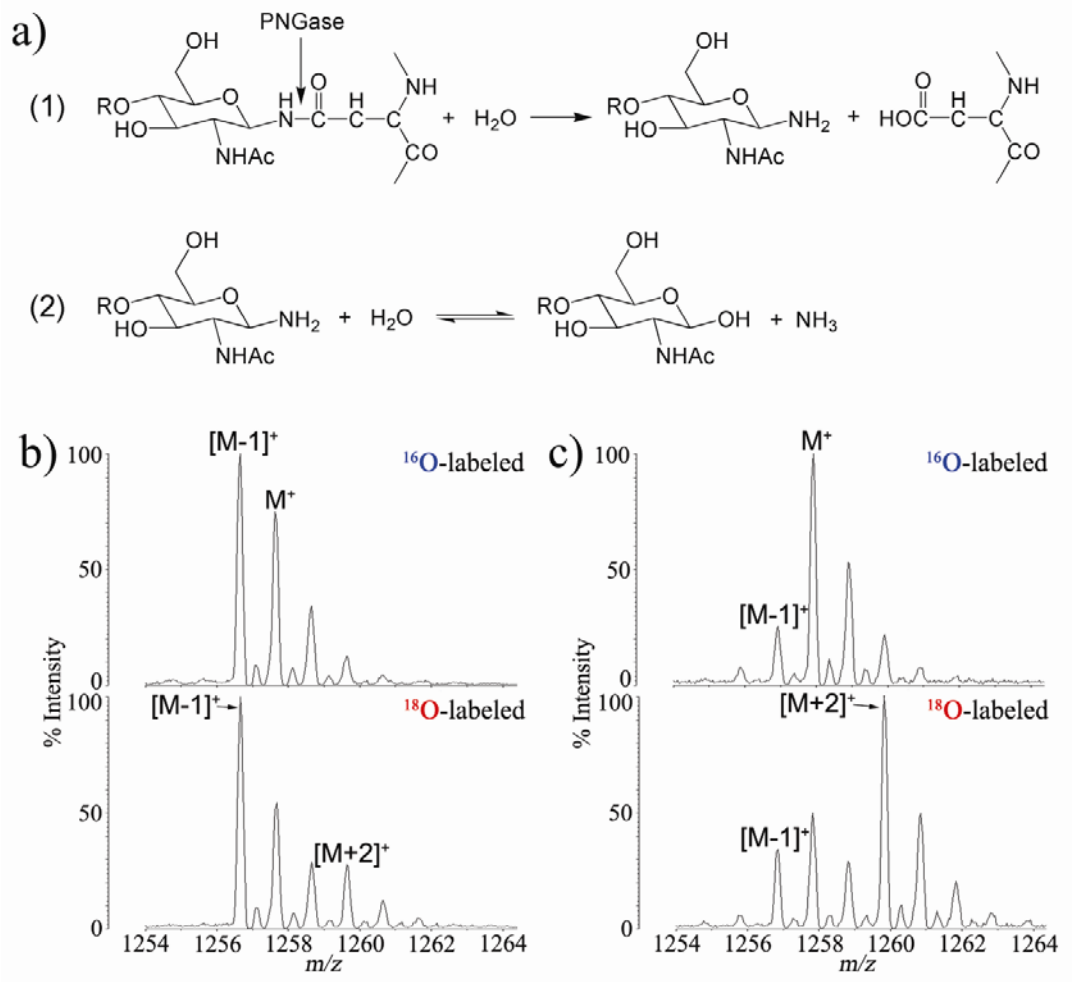


Figure S-5. Release of *N*-glycans from glycoproteins by PNGase. (a) Reaction mechanism of PNGase. (b) Enlarged MALDI-TOF mass spectra of an RNase B glycan (*m/z* 1257 as an example) treated by PNGase F in ammonium bicarbonate buffer prepared in $H_2{}^{16}O$ (top) and $H_2{}^{18}O$ (bottom). (c) Enlarged MALDI-TOF mass spectra of the same glycan digested in sodium phosphate buffer prepared in $H_2{}^{16}O$ (top) and $H_2{}^{18}O$ (bottom). M: glycan; [M+2]: ^{18}O-labeled glycan; [M-1]: glycosylamine. All glycan ions are singly charged sodium adduct ions.

S-4. Overlapping-peak deconvolution method

Overlapping-peak deconvolution was realized by elimination of the 2 Da-higher species in the ^{16}O-labeled sample and the 2 Da-lower species in the ^{18}O-labeled sample, respectively.

First, the intensities of ^{16}O-labeled monoisotope peaks (I_a) and 2 Da higher peaks (I_{a+2}) in the ^{16}O-labeled sample (Figure 1b, top) as well as the intensities of ^{18}O-labeled monoisotope peaks (I_b) and 2 Da lower peaks (I_{b-2}) in the ^{18}O-labeled sample (Figure 1b, bottom) were obtained from mass spectra. The two samples were then mixed in proportion and analyzed by MS, after which the intensities of the monoisotope peaks (I_A) and 2 Da higher peaks (I_B) were obtained (Figure 1c). Subsequently, the ratios of ^{16}O-/^{18}O-labeled glycans were calculated using Eq. (1).

$$\mathrm{ratio}\left(\frac{^{16}\mathrm{O}}{^{18}\mathrm{O}}\right) = \frac{I_A - \frac{I_{b-2}}{I_b} I_B}{I_B - \frac{I_{a+2}}{I_a} I_A} \qquad (1)$$

An isotopic pair of glycans at *m/z* 1864 and 1866 ($GlcNAc_1Man_9Hex_1$) was selected in this study as example. The intensity ratio of *m/z* 1866/1864 in the ^{16}O-labeled sample (I_{a+2}/I_a) and the intensity ratio of *m/z* 1864/1866 in the ^{18}O-labeled sample (I_{b-2}/I_b) were obtained from MS spectra (before mixing), respectively (Table S-1). The intensity of *m/z* 1864 (I_A) and the intensity of *m/z* 1866 (I_B) were obtained from MS spectra after the two samples were mixed. The intensity of 2 Da-higher species in the ^{16}O-labeled sample was $I_A \times (I_{a+2}/I_a)$, and the intensity of 2 Da-lower species in the ^{18}O-labeled sample was $I_B \times (I_{b-2}/I_b)$. Thus, the ratio of ^{16}O-/^{18}O-labeled glycans after overlapping-peak deconvolution was $[I_A - I_B \times (I_{b-2}/I_b)]/[I_B - I_A \times (I_{a+2}/I_a)]$ (Table S-2).

Table S-1. I_{a+2}/I_a and I_{b-2}/I_b obtained from MS spectra before mixing ($GlcNAc_1Man_9Hex_1$)

glycan	I_{a+2}/I_a ± CV (n=6)	I_{b-2}/I_b ± CV (n=6)
$GlcNAc_1Man_9Hex_1$	0.4608±3.31%	0.0768±3.50%

Table S-2. Measured ratios before and after calculated by Eq. (1) ($GlcNAc_1Man_9Hex_1$)

$GlcNAc_1Man_9Hex_1$	Ratio ($^{16}O/^{18}O$) ± CV (n=6)						
Theoretical	10	5	2	1	0.5	0.2	0.1
Measured (I_A/I_B)	1.6701±3.94%	1.4446±1.36%	1.0607±1.79%	0.7116±3.35%	0.4273±2.12%	0.2526±1.68%	0.1703±3.83%
Measured [Eq. (1)]	7.0384±16.19%	4.0939±4.14%	1.9255±3.65%	0.9452±5.31%	0.4365±3.09%	0.1989±2.63%	0.1014±7.31%

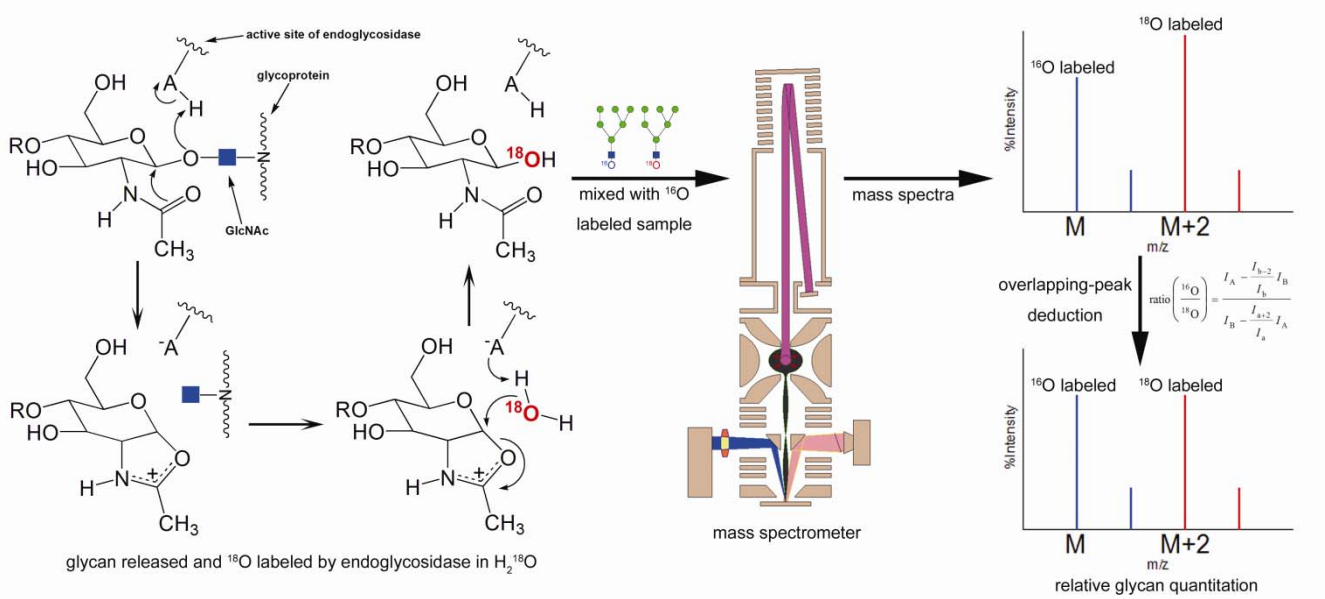
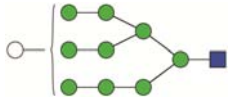
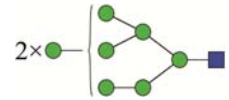
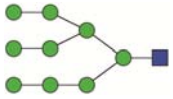
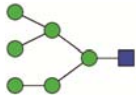
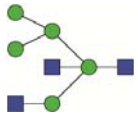
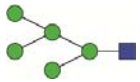


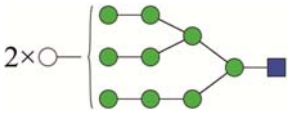
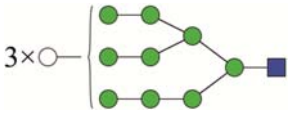
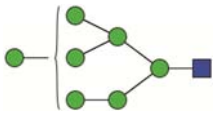
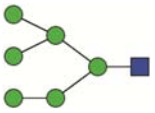
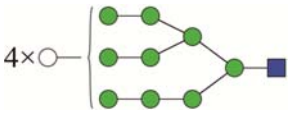
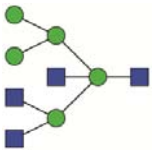
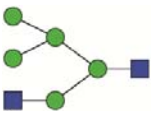
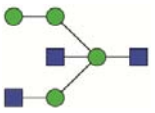
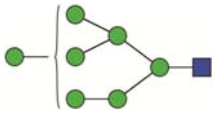
Figure S-6. Schematic illustration of GREOL

Table S-3. Coefficients of variation (CVs) and correlation coefficients (R^2) for the glycans used as examples

Composition	Major structure[a]	Theoretical *m/z* ($M+Na^+$)	Protein	Measured ratio ($^{16}O/^{18}O$) ± CV (n=6)							R^2
				10	5	2	1	0.5	0.2	0.1	
$GlcNAc_1Man_9Hex_1$		1864.61	Invertase	7.0384±16.19%	4.0939±4.14%	1.9255±3.65%	0.9452±5.31%	0.4365±3.09%	0.1989±2.63%	0.1014±7.31%	**0.9977**
$GlcNAc_1Man_8$		1540.50	Invertase	5.9334±6.92%	3.9275±9.34%	1.9098±4.78%	0.9499±4.59%	0.4369±4.14%	0.1996±4.00%	0.1049±4.53%	**0.9951**
$GlcNAc_1Man_9$		1702.55	Invertase	5.3527±12.86%	3.8406±6.60%	1.8466±6.45%	0.8849±3.37%	0.4020±4.48%	0.1812±7.64%	0.0927±10.34%	**0.9924**
$HexNAc_1Hex_6$		1216.40	Ovalbumin	11.2437±7.64%	6.0989±4.95%	2.1440±2.41%	1.1047±2.05%	0.5850±2.69%	0.2234±5.13%	0.1506±3.01%	**0.9962**
$HexNAc_3Hex_5$		1460.50	Ovalbumin	9.7179±5.92%	6.3229±7.19%	2.0073±4.92%	1.0316±3.49%	0.5566±4.60%	0.1981±2.07%	0.1347±9.32%	**0.9945**
$HexNAc_1Hex_5$		1054.34	Ovalbumin	8.1944±9.57%	5.7121±7.34%	2.1316±4.23%	1.0389±6.55%	0.5820±5.99%	0.1996±6.26%	0.1150±7.94%	**0.9957**

[a] The structures were partially proposed according to Glycan Mass Spectral DataBase (http://riodb.ibase.aist.go.jp/rcmg/glycodb/Top) and GlycoBase (Version 2, http://glycobase.nibrt.ie/glycobase.html). Green circle: Man; white circle: Hex; blue square: GlcNAc.

Table S-4. Coefficients of variation (CVs) and correlation coefficients (R^2) for the relative low-abundance glycans

Composition	Major structure[a]	Theoretical *m/z* ($M+Na^+$)	Protein	Min. CV (n=6)	Max. CV (n=6)	R^2
$GlcNAc_1Man_9Hex_2$		2026.66	Invertase	3.20%	8.16%	**0.9972**
$GlcNAc_1Man_9Hex_3$		2188.71	Invertase	5.19%	24.28%	**0.9968**
$GlcNAc_1Man_7$		1378.45	Invertase	4.53%	17.57%	**0.9842**
$GlcNAc_1Man_6$		1216.40	Invertase	5.39%	21.47%	**0.9924**
$GlcNAc_1Man_9Hex_4$		2350.77	Invertase	9.95%	20.59%	**0.9905**
$HexNAc_4Hex_5$		1663.58	Ovalbumin	6.14%	31.59%	**0.9980**
$HexNAc_2Hex_5$		1257.42	Ovalbumin	6.87%	17.22%	**0.9904**
$HexNAc_3Hex_4$		1298.45	Ovalbumin	3.65%	20.78%	**0.9972**
$HexNAc_1Hex_7$		1378.45	Ovalbumin	5.77%	13.34%	**0.9980**

[a] The structures were partially proposed according to Glycan Mass Spectral DataBase (http://riodb.ibase.aist.go.jp/rcmg/glycodb/Top) and GlycoBase (Version 2, http://glycobase.nibrt.ie/glycobase.html). Green circle: Man; white circle: Hex; blue square: GlcNAc.

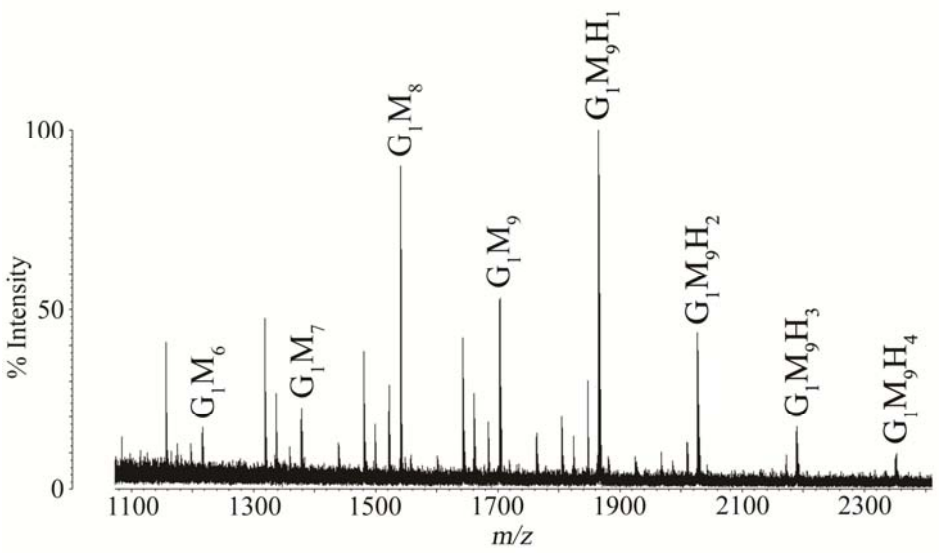
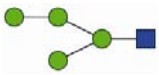
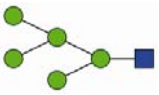
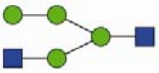
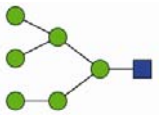
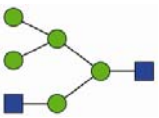
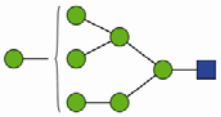
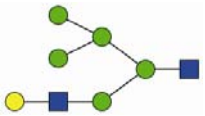
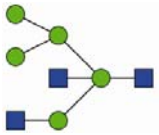
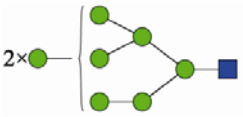
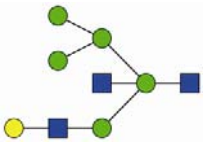
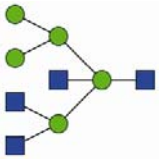
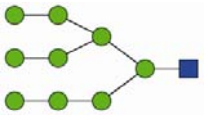
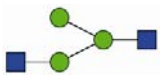
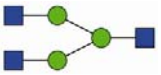
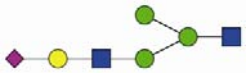
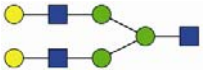
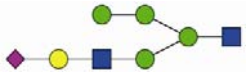
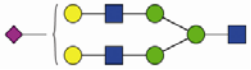


Figure S-7. The detection limit of GREOL. Glycans from about 2.68 pmol invertase are shown as examples. M: Man; H: Hex; G: GlcNAc. All glycan ions are singly charged sodium adduct ions.

Table S-5. Relative quantitation of glycans found in normal (^{16}O-labeled) and HCC (^{18}O-labeled) sera

Enzyme	*m/z* $(M+Na)^+$	Composition	Major structure[a]	Ratio (HCC/normal)	CV (n=6)	Change
Endo H	892.3	$HexNAc_1Hex_4$		**1.06**	**5.2%**	no
	1054.3	$HexNAc_1Hex_5$		**0.95**	**4.3%**	no
	1095.4	$HexNAc_2Hex_4$		**1.05**	**11.5%**	no
	1216.4	$HexNAc_1Hex_6$		**1.13**	**3.0%**	no
	1257.4	$HexNAc_2Hex_5$		**1.02**	**1.8%**	no
	1378.4	$HexNAc_1Hex_7$		**0.85**	**12.3%**	no
	1419.5	$HexNAc_2Hex_6$		**1.08**	**7.4%**	no
	1460.5	$HexNAc_3Hex_5$		**1.91**	**17.9%**	**up**
	1540.5	$HexNAc_1Hex_8$		**1.03**	**13.9%**	no
	1622.6	$HexNAc_3Hex_6$		HCC only	N/A	N/A
	1663.6	$HexNAc_4Hex_5$		HCC only	N/A	N/A
	1702.6	$HexNAc_1Hex_9$		**1.03**	**12.9%**	no

Endo F_2	933.3	$HexNAc_2Hex_3$		**1.67**	**3.8%**	**up**
	1095.4	$HexNAc_2Hex_4$		**1.37**	**1.8%**	**up**
	1136.4	$HexNAc_3Hex_3$		**2.09**	**2.6%**	**up**
	1298.4	$HexNAc_3Hex_4$		**1.86**	**3.8%**	**up**
	1386.5 $(M+H+Na)^+$	$NeuNAc_1HexNAc_2Hex_4$		**2.34**	**18.8%**	**up**
	1460.5	$HexNAc_3Hex_5$		**1.37**	**3.7%**	**up**
	1548.5 $(M+H+Na)^+$	$NeuNAc_1HexNAc_2Hex_5$		**2.09**	**8.6%**	**up**
	1773.6 $(M+2Na)^+$	$NeuNAc_1HexNAc_3Hex_5$		**2.03**	**12.0%**	**up**

[a] The structures were partially proposed according to Glycan Mass Spectral DataBase (http://riodb.ibase.aist.go.jp/rcmg/glycodb/Top) and GlycoBase (Version 2, http://glycobase.nibrt.ie/glycobase.html). Green circle: Man; yellow circle: Gal; blue square: GlcNAc; purple diamond: NeuNAc.

Reference of Supporting Information

(1) Packer, N. H.; Lawson, M. A.; Jardine, D. R.; Redmond, J. W. *Glycoconjugate J.* **1998**, *15*, 737–747.