Reduction, Carboxyamidomethylation and Protease Digestion

The sample is placed in microcentrifuge tube, dissolved in 50 mM ammonium bicarbonate buffer (pH 8.4), reduced immediately with 25 mM dithiothreitol, and carboxyamidomethylated with 90 mM iodoacetamide at room temperature in the dark. The sample is digested with trypsin, incubating overnight at 37°C. The trypsin is deactivated by heating to 100 °C for 5 min, and the sample is dried down in a Speed Vac.
Release of N-linked glycans

After tryptic digestion, the dried sample is dissolved in 100 mM sodium phosphate buffer (pH 7.5), treated with peptide N-glycosidase F or A, and incubated at 37 °C to release the N-linked glycans. The sample is passed through a C18 reversed phase cartridge, eluting the N-linked glycans with 5% acetic acid. Finally, the sample is lyophilized.

Release of O-linked glycans

O-linked carbohydrate fractions are cleaved from the glycoprotein by the β-elimination procedure. Briefly, 500 µL of 50 mM NaOH containing 19 mg of sodium borohydride is added, and the sample is vortexed and incubated overnight. The incubated sample then is neutralized with 10% acetic acid, desalted by passing through a packed column of DOWEX\textsuperscript{TM} 50WX8-100 resin, and lyophilized. Finally, borate is removed from the sample by repeated co-evaporation with methanol:acetic acid (9:1) under a stream of nitrogen gas.

Preparation of the per-O-methylated carbohydrates

The lyophilized N- and O-linked fractions are dissolved in dimethylsulfoxide and then methylated with NaOH and methyl iodide (Ciucanu and Kerek, 1984). The reaction is quenched by addition of water, and per-O-methylated carbohydrates are extracted with dichloromethane. The organic phase is concentrated to dryness and then dissolved in methanol.

\textsuperscript{18}O-labeling of N-linked glycosylation sites

The sample is placed in microcentrifuge tube, dissolved in 50 mM ammonium bicarbonate buffer (pH 8.4), reduced immediately with 25 mM dithiothreitol, and carboxyamidomethylated with 90 mM iodoacetamide at room temperature in the dark. The sample is digested with trypsin, incubating overnight at 37°C. After trypsin digestion, the sample is treated with 1% formic acid to deactivate the enzyme and passed through a C\textsubscript{18} reversed phase cartridge. The sample then is completely dried to and rehydrated in \textsubscript{2}H\textsubscript{18}O. PNGase F or A is added and the mixture is incubated overnight at 37°C and dried in the Speed Vac. The sample is analyzed by liquid chromatography/mass spectrometry (LC-MS).

Matrix-assisted Laser-Desorption Time-of-flight Mass Spectrometry (MALDI)

MALDI-MS is performed in the positive ion mode using α-dihydroxybenzoic acid (DHBA) as a matrix. Full mass spectra of a sample are obtained initially using a MALDI TOF Mass Spectrometer (Applied Biosystems).

ElectroSpray Ionization – Linear Ion Trap Mass Spectrometry (ESI-LCQ/MSn)

The profile of glycan structures from sample is confirmed by electrospray ionization mass spectrometry (ESI-MS) using an LCQ-MS (Thermo Finnigan). The permethylated glycans dissolved in 1 mM NaOH in 50% methanol is infused directly into the instrument at a constant flow rate of 1 µL/min via a syringe pump (Harvard Apparatus) and sprayed. Mass spectra are obtained in the positive ion mode.
NanoelectroSpray Ionization – Linear Ion Trap Mass Spectrometry (ESI-LTQ/MSn)

Mass analysis by LTQ-MS (Thermo Finnigan) is performed by direct infusion of permethylated glycans dissolved in NaOH in 50% methanol into the LTQ instrument using a nanoelectrospray source at syringe flow rate of 0.40 \( \mu L/min \).