Glycan Analysis of Murine IgG by Enzymatic Digestion with Endo S and PNGase F, Followed by Mass Spectrometric Analysis

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Introduction

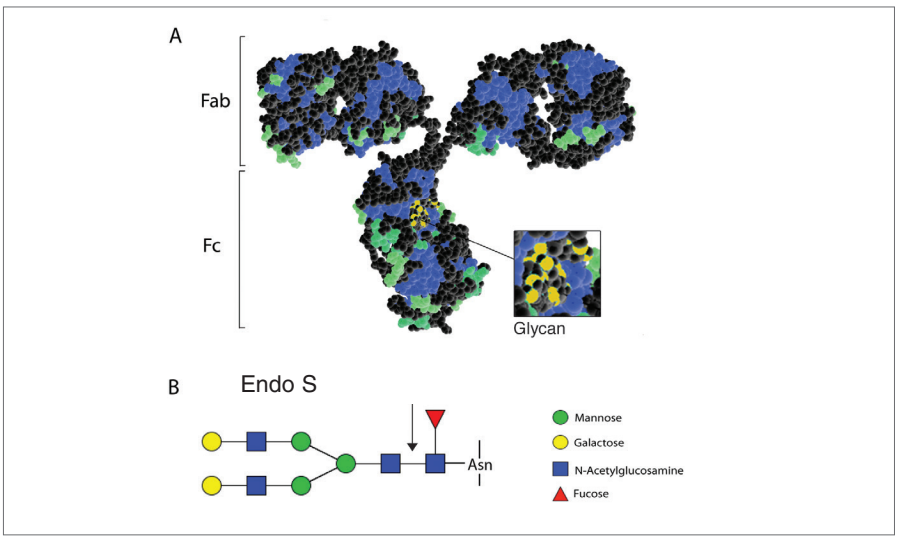
Immunoglobulin Gs (IgGs) are antibody molecules that are composed of four peptide chains — two heavy chains and two light chains. There are four IgG subclasses (IgG1, 2a, 2b, and 3) in mice. The heavy chains are known to be glycosylated, while the light chains are not. The *N*-glycan moiety attached to the asparagine 297 residue in the Fc domain of the antibody is critical for the structure and biological activity of the molecule (1). A growing number of monoclonal IgG antibodies are currently being developed and used as therapeutic agents and it is apparent that there are many variables in cell culture systems that can greatly influence the heterogeneity of the glycans on IgGs. Therefore, it has become increasingly important to monitor the glycosylation profiles of these molecules in the production process. Endoglycosidase S (Endo S) isolated from *Streptococcus pyogenes* has been shown to specifically and completely cleave the biantennary complex *N*-glycan at asparagine 297 of IgG under native conditions (2). This enzyme removes the *N*-glycan moiety after the first *N*-acetylglucosamine (GlcNAc) residue on the chitobiose core, leaving only a GlcNAc with or without a core fucose residue on the protein. In contrast, PNGase F cleaves between the innermost GlcNAc and asparagine residue of high mannose, hybrid and complex oligosaccharides from *N*-linked glycoproteins.

Endo S is cloned and expressed in *E. coli* as a fusion to the chitin binding domain (CBD). The specificity of Endo S is identical to Endo S with the added benefit of the CBD tag to remove the enzyme following a deglycosylation reaction if needed.

This application note compares the enzymatic removal of glycans on murine IgG using Endo S and PNGase F Glycerol free under native conditions. Endo S is a more robust enzyme for this purpose, completely removing the sugar residues from monoclonal mouse IgG. Conversely, the PNGase F digest does not result in a complete digestion under native conditions.

FIGURE 1:
Structure and glycosylation of a murine IgG

(A) Structural model of murine IgG. In the IgG, beta-sheets are colored blue, loops are colored black and the helices are colored green. The brackets indicate the antigen-binding Fab portion and the Fc effector portion of IgG. The inset highlights the two conserved glycans (yellow) attached to Asn-297 of the heavy chains. The model was generated using JMOL 12.2.23 from a model deposited in the Protein Data Bank by L. Harris (University of California, Riverside). (B) Schematic representation of the fully substituted IgG heavy-chain glycan and the location of the Endo S cleavage.



Materials

Endo S (NEB #P0741)

PNGase F (Glycerol-free) (NEB #P0705)

Anti-MBP Monoclonal Antibody (Murine IgG2a) (NEB #E8032)

GlycoBuffer 2 [10X, supplied with PNGase F (Glycerol-Free) and Endo S]

3K Millipore Amicon Ultra Filter Unit (cat. #UFC500324)

Dilution Buffer: 20 mM Tris HCl pH 7.5, 50 mM NaCl, 1 mM EDTA

Protein Ladder (NEB #P7703)

Agilent 6210 TOF MS with both 1200 Series Capillary and Nano pumps with a ChipCube. Custom PLRP-S Chip (75 µm x 150 mm with 40 nl trap) or equivalent TOF or Q-TOF and nanoLC system.

General Protocols

Deglycosylation of IgG using Endo S occurs optimally under native conditions. Deglycosylation using PNGase F occurs optimally under denaturing conditions (using SDS, DTT and heat); however, this is not optimal for downstream mass spectrometry analysis and thus the reaction needs to be performed under native conditions without the addition of detergents.

Removal of Glycerol from Murine IgG Substrate

1. Dilute 50 µl of 1 mg/ml Anti-MBP Monoclonal Antibody (Murine IgG2a) with 450 µl of the Dilution Buffer: 20mM Tris HCl pH 7.5, 50 mM NaCl, 1mM EDTA.
2. Apply to a 0.5 ml 3K Millipore Amicon Ultra Filter Unit and spin in a microcentrifuge for 30 minutes at 12,000 rpm.
3. Discard flow-through and add an additional 450 µl of Dilution Buffer to the sample. Spin in a microcentrifuge for 30 minutes at 12,000 rpm.
4. Place the Amicon filter device upside-down in a clean microcentrifuge tube and spin for 2 minutes at 1,000 rpm to transfer glycerol-free murine IgG to the tube.

Deglycosylation of IgG with Endo S

1. Add 10 µl prepared glycerol free Anti-MBP Monoclonal Antibody (Murine IgG2a) at 1 mg/ml (10 µg total) to a 200 µl tube. Add 5 µl of 10X GlycoBuffer 2 (500 mM NaPhosphate pH 7.5) (see Note 1). Add 34 µl of water, and add 1 µl (200 units) of Endo S. Mix with pipette and incubate at 37°C for 1 hour.
2. Reserve 10 µl for SDS-PAGE gel analysis, if desired. Analyze remainder by nanoLC-TOF MS.

Deglycosylation of IgG with PNGase F (Glycerol-free)

1. Add 10 µl prepared glycerol free Anti-MBP Monoclonal Antibody (Murine IgG2a) at 1 mg/ml (10 µg total) to a 200 µl tube. Add 5 µl of 10X GlycoBuffer 2 (500 mM Sodium Phosphate pH 7.5) (see Note 1), 34 µl of water and 1 µl (500 units) of PNGase F (Glycerol-free).
2. Mix with pipette and incubate at 37°C for 1 hour. Reserve 10 µl for SDS-PAGE gel analysis if desired.
3. Analyze remainder by nanoLC-TOF MS.

Liquid Chromatography/Electrospray Ionization Time-of-Flight Mass Spectrometry (LC/ESI-TOF MS):

1. Protein was denatured using 10 mM DTT for 30 minutes at room temperature. Finally, formic acid was added to 0.1% v/v.
2. Samples were analyzed using a custom reverse-phase chip (see Note 2) on an Agilent 1200 series nano LC connected directly to an Agilent 6210 series ESI-TOF MS.
   a) The chip was equilibrated with 0.1% formic acid in 5% acetonitrile (ACN).
   b) Samples (1 µl) were injected, the chip trap column was loaded at 2 µl/min and the separation column developed at 500 nl/min with a 15 minute linear gradient from 5% to 95% ACN, followed by 5 minutes at 95% ACN. Protein was found to elute at approximately 10 minutes after injection.
   c) The spectra were extracted and deconvoluted (see Note 3).

Notes

Note 1: Previous versions of this protocol called for other reaction buffers. As of 2015, a universal buffer system was introduced (10X GlycoBuffer 1 for exoglycosidases and 10X GlycoBuffer 2 for most endoglycosidases). You can find more information at: https://www.neb.com/faqs/2015/04/01/why-have-the-neb-glycosidase-enzymes-changed-reaction-buffers-what-are-the-new-reaction-buffers-a

Note 2: The reverse phase chip consisted of an integrated trapping column (40 nl), separation column and nano-ESI emitter (75 µm x 150 mm both packed with PLRP-S, 5 µm particles, 1000 A pore size).

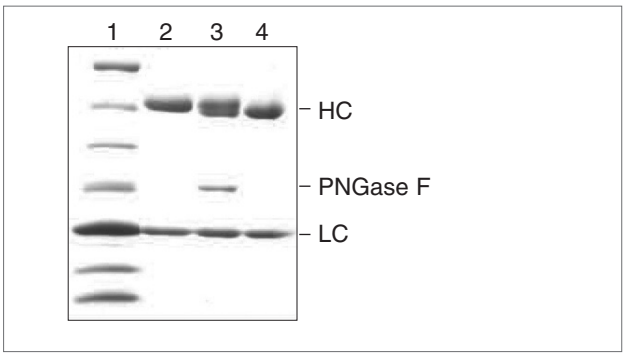
Note 3: The mass spectra were acquired from 150 to 3200 m/z, one cycle/sec and 10,000 transients per scan using an ionization energy of 1800 V, fragmentor of 215 V and drying gas of 325°C

Results

N-glycan removal under native conditions

Endo S has a high specificity for removing the N-glycan moiety of IgG under native conditions (Figure 3). Digestion of IgG with PNGase F (Glycerol-free) under native conditions (Lane 3) is not complete, as seen by the presence of a doublet band of the heavy chain (HC). Endo S (Lane 4) yields a complete deglycosylation of IgG under native conditions as shown by a complete shift of the band compared with the control (no enzyme, Lane 2).

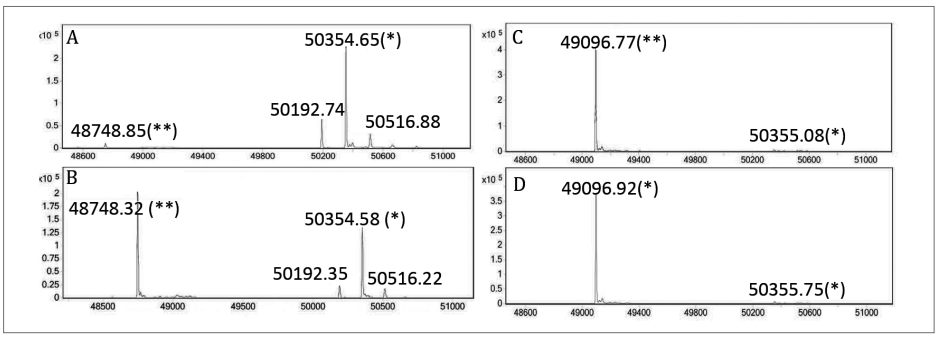
FIGURE 2:
IgG deglycosylation (native) with Endo S or PNGase F (Glycerol-free).



To confirm these results, samples were analyzed by ESI-TOF MS. Figure 3 shows the analysis of IgG digested under native conditions with PNGase F (Glycerol-free) (Figure 3A-B), or Endo S (Figure 3C-D).

FIGURE 3:
ESI-TOF analysis of deglycosylation.

(A,B) ESI-TOF MS of a PNGase F digested monoclonal mouse IgG incubated for 1 hour at 37°C (A) or 24 hrs at 37°C (B), where (*) indicates IgG with intact glycan at MW 50,354, and (**) indicates deglycosylated heavy chain of IgG at MW 48,748; (C,D). Endo S digested monoclonal mouse IgG incubated 1 hour (C) or 24 hrs (D), where (*) indicates IgG with intact glycan at MW 50,355, and (**) indicates deglycosylated heavy chain of IgG MW 49,096.



Conclusion

Endo S is a superior choice for glycobiology applications that demand rapid and reliable deglycosylation of IgG under native conditions.

References:
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2. Colin, M. and A. Olsén (2001) *EMBO Journal* 20, 3046–3056.
3. Vollmer, M and van de Goor (2009) *Methods in Molecular Biology* 544, 3–15.

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