

Enzymatic Removal of *N*- and *O*-glycans using PNGase F or the Protein Deglycosylation Mix

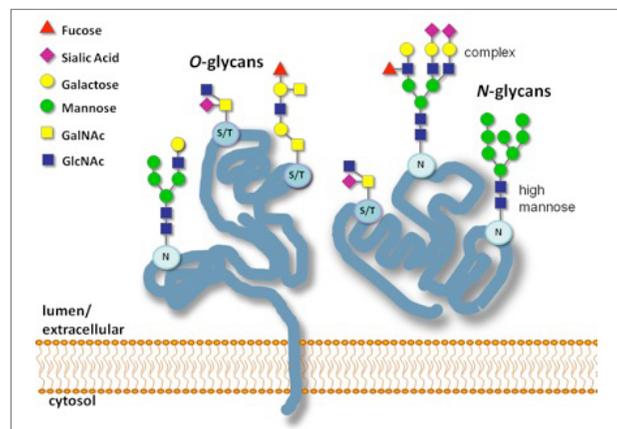
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Introduction

Glycosylation is a main post-translational modification in eukaryotic cells. There are various pathways for protein modification with sugars (glycans), the most common of which are the addition of glycans to an asparagine (*N*-linked) or to a serine or threonine (*O*-linked) in the secretory compartment (ER and Golgi).

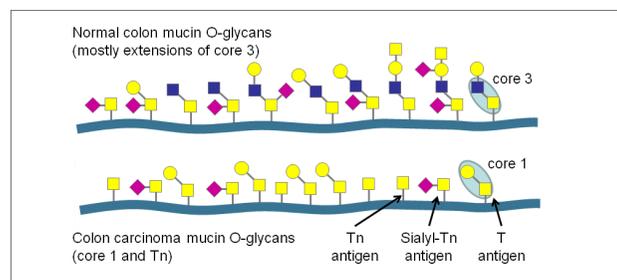
Some *O*-glycans are composed of a *N*-acetylgalactosamine (GalNAc) core of 1 to 3 sugars, while others are extensively modified by the addition of extra residues. The synthesis of *N*-glycans starts with the transfer of a common oligosaccharide to a nascent polypeptide in the ER. Some *N*-glycans remain unmodified ("high mannose"), while others are first trimmed and later extended as the glycoprotein matures in the Golgi ("complex") (1).

 FIGURE 1: *N*- and *O*- Glycosylation



The quantity and quality of glycosylation depends both on the protein itself and also on the cell type expressing the protein. Each tissue has a normal repertoire of glycans under physiological conditions, and changes in protein glycosylation are often indicators of a pathological state. For instance, the presence of certain *O*-glycans (Core1 or T, Tn, and sialyl-Tn) are known markers of malignancy in colon carcinoma (2).

 FIGURE 2: Colon Carcinoma



Materials

hCG β , recombinant, vial of 150 μ g (Sigma Aldrich #C6572)

PNGase F (NEB #P0704)

Protein Deglycosylation Mix (NEB #P6039)

GlycoBuffer 2 (10X), Glycoprotein Denaturing Buffer (10X) and NP-40 (10%) (supplied with PNGase F and the Protein Deglycosylation Mix)

α -*N*-Acetylgalactosaminidase (NEB #P0734)

α 1-2 Fucosidase (NEB #P0724)

β 1-3 Galactosidase (NEB #P0726)

α 1-3, 6 Galactosidase (NEB #P0731)

Blue Loading Buffer (NEB #B7703, supplied with 1.25 M DTT)

ColorPlus Prestained Protein Marker (NEB #P7709)

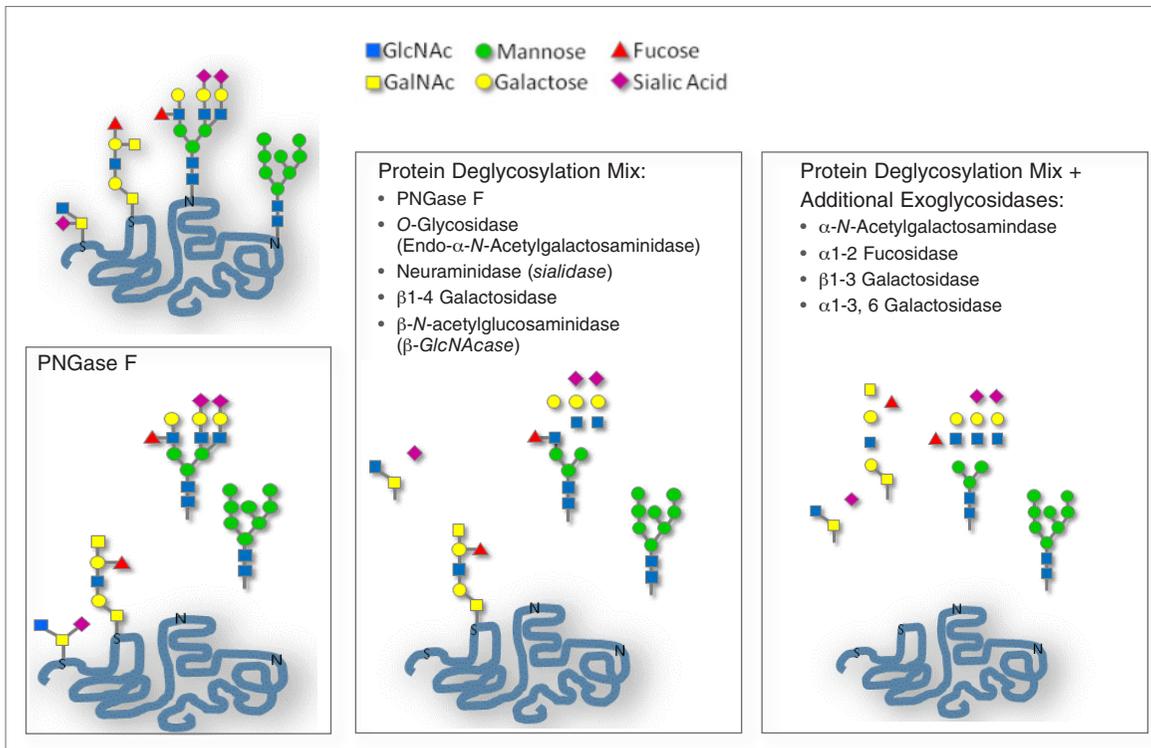
Pro-Q[®] Emerald 300 Glycoprotein Stain Kit (Invitrogen #P-21857)

Glycosylation is also important in the production of therapeutic proteins, since it can significantly affect the potency of a biological drug. Producing a homogeneously glycosylated protein is very difficult and often impractical. For this reason, development and manufacturing processes are highly monitored to minimize glycosylation variability as much as possible. The biology of cancer and the production of recombinant proteins are just two of the many subjects related to protein glycosylation. As interest in glycobiology keeps growing, novel analytical procedures are being developed. Mass spectroscopy of proteins and glycans is the current method of choice to study protein glycosylation, but it requires a high level of expertise and dedicated instrumentation.

Many researchers choose techniques that do not require special devices or training, such as the analysis of glycoproteins by glycosidase digestion, which can be easily executed in a common laboratory setting. Removing glycans with glycosidases often alters the gel migration of a protein, more or less dramatically depending on the size and number of sugar substitutions. Therefore, following the electrophoretic mobility before and after treatment with glycosidases provides a convenient way to understand the properties of a protein of interest, as illustrated in the following application.

This protocol describes the enzymatic removal of glycans from a protein using PNGase F or, alternatively, a mixture of glycosidases (e.g., Protein Deglycosylation Mix). PNGase F is able to remove most *N*-glycans, while the enzymes in the Protein Deglycosylation Mix [PNGase F, *O*-Glycosidase (Endo- α -*N*-Acetylglucosaminidase) and other enzymes] remove *N*-glycans, short *O*-glycans, and certain long chain *O*-glycans, as shown in Figure 3. We also demonstrate the use of additional exoglycosidases, which might release otherwise resistant sugars. An extensive characterization of these enzymes can be found in the technical reference section of our website.

 **FIGURE 3: Schematic representation of glycoprotein treated with PNGase F, the Protein Deglycosylation Mix or the Protein Deglycosylation Mix and additional exoglycosidases**



The model glycoprotein used in this study is human chorionic gonadotropin (hCG) recombinantly expressed in a mouse cell line. This heterodimeric glycoprotein consists of two subunits, alpha and beta, both glycosylated. The beta subunit of hCG is known to be *N*-glycosylated at N13 and N30, and *O*-glycosylated at S121, S127, S132 and S138 (4,5). After deglycosylation, hCG β samples were separated by SDS-PAGE and stained with Coomassie Blue. Alternatively, the sugar-specific stain ProQ[®] Emerald-300 (Invitrogen) can be used to determine the extent of glycan removal.

General Protocol

Deglycosylation is optimal under denaturing conditions (using SDS and heat), however it can be performed under native conditions without the addition of detergents (see notes 1,2).

1. Dissolve the contents of the hCG β vial (150 μ g) in 600 μ l of dH₂O. Dilute 0.5 μ l PNGase F in 25 μ l 1X GlycoBuffer 2 (see note 3). Prepare an exoglycosidase mix combining 2 μ l each of α -N-Acetylgalactosaminidase, α 1-2 Fucosidase, β 1-3 Galactosidase, and α 1-3, 6 Galactosidase. Dilute 100X BSA to 2.5 mg/ml (5 μ l BSA in 15 μ l dH₂O). Keep all solutions in ice.
2. Set up the reaction in small 200 μ l tubes as indicated:

TUBE	WITH SDS				WITHOUT SDS				CONTROLS		
	1	2	3	4	5	6	7	8	9	10	11
hCG β (0.25 mg/ml)	9 μ l (2.25 μ g)				9 μ l (2.25 μ g)				—		
10X Glycoprotein Denaturing Buffer	1 μ l				—				1 μ l		
H ₂ O	—				1 μ l				9 μ l		
Incubate for 10 minutes at 94°C											
10X NP-40(*)	2.5 μ l				—				2.5 μ l		
10X GlycoBuffer 2	2.5 μ l				2.5 μ l				2.5 μ l		
PNGase F	—	2 μ l	—	—	—	2 μ l	—	—	2 μ l	—	—
Protein Deglycosylation Mix	—	—	2 μ l	2 μ l	—	—	2 μ l	2 μ l	—	2 μ l	2 μ l
Additional Exoglycosidases Mix	—	—	—	2 μ l	—	—	—	2 μ l	—	—	2 μ l
25X BSA	1 μ l				1 μ l				1 μ l		
dH ₂ O	9 μ l	7 μ l	7 μ l	5 μ l	11.5 μ l	9.5 μ l	9.5 μ l	7.5 μ l	7 μ l	7 μ l	5 μ l
Total Volume	25 μ l				25 μ l				25 μ l		
Incubate for 4 hours at 37°C											

(*) NP-40 is added to prevent PNGase F inactivation by SDS.

3. Prepare fresh 3X reducing Blue Loading Buffer (4 μ l of 1.25M DTT, 130 μ l 3X Blue Loading Buffer). Add 17 μ l of 3X reducing Blue Loading Buffer to each sample mix and incubate at 94°C for 5 minutes. Load 30 μ l of each sample and the protein marker on a 10-20% Tris-glycine mini-gel. Save the remaining 10 μ l. Electrophorese at 130 volts, when the gel has finished running stain with Coomassie Blue solution, and destain. Record images.
4. Alternatively, used Pro-Q Emerald 300 for detection of glycosylation. This reagent gives better results with less total protein, loading only 10 μ l of each sample on a 10-20% Tris-glycine mini-gel is optimal. After electrophoresis, follow the Pro-Q Emerald 300 kit instructions to fix the gel, oxidize, and stain glycoproteins. Record images on an UV transilluminator.

Notes

Note 1: Buffer formulations:

10X GlycoBuffer 2: 500 mM Sodium Phosphate pH 7.5
10X Glycoprotein Denaturing Buffer: 5% SDS, 400 mM DTT
10X NP-40: 10% NP40.
Final reaction concentrations (denaturing conditions): 50 mM Sodium Phosphate pH 7.5, 0.5% SDS, 40 mM DTT, 1% NP40.

Note 2: 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>

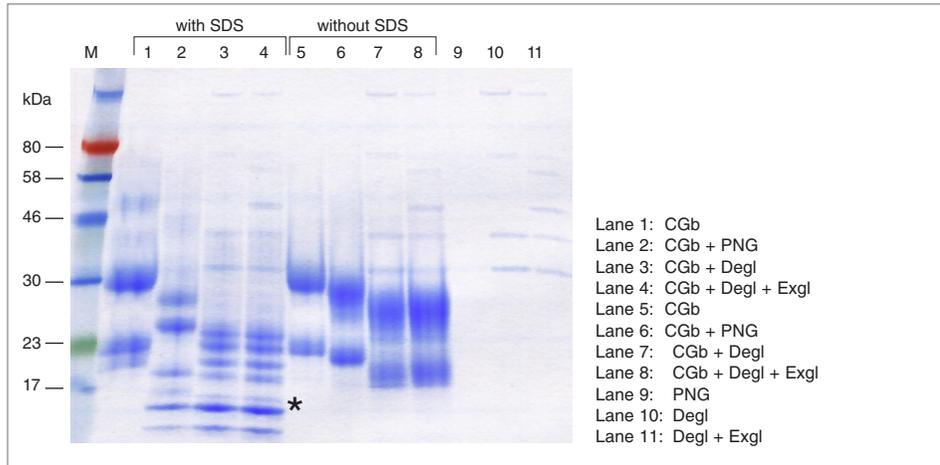
Note 3: Diluted PNGase F was sufficient for deglycosylation of the model protein used in this application. However, different samples might require higher PNGase F activity. In those cases, equivalent volumes of non-diluted PNGase F should be used instead. This is also important for native conditions, since PNGase F is often less active on folded proteins.

Results:



FIGURE 4: SDS-PAGE Analysis of Deglycosylation Reactions

After removal of *N*- and *O*-glycans, hCG β migrates faster. Bands become sharper (less polydisperse) and glycans are lost.
 ★ = Fully deglycosylated protein



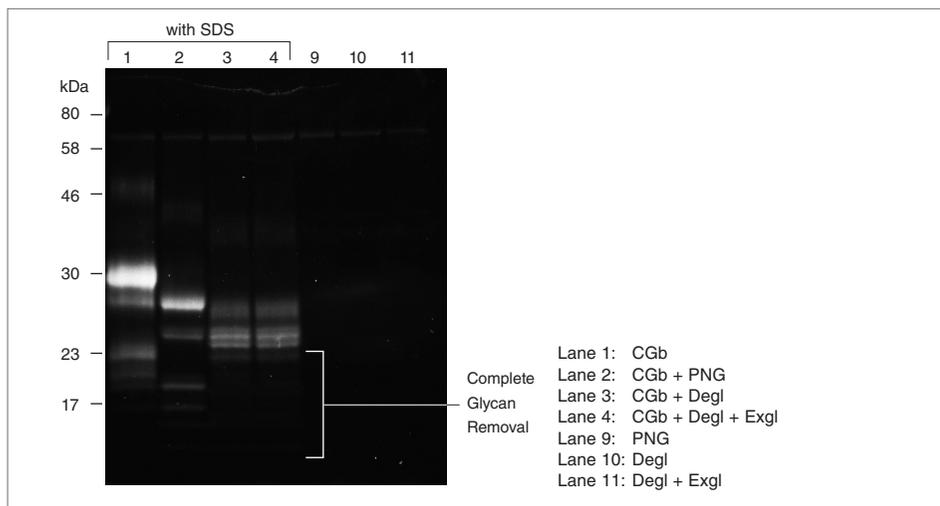
As previously reported (7,8), hCG β appears as an heterogeneous band near the 30 kDa mark (see lanes 1 and 5). A secondary band running close to 23 kDa might correspond to traces of alpha subunit in the hCG sample. Compare these lanes with the protein bands obtained after *N*-glycan removal (PNGase F, lanes 2 and 6), or with bands after *N*- and *O*-glycan removal (Protein Deglycosylation Mix, lanes 3 and 7). Digesting with additional glycosidases (lanes 4 and 8) does not further reduce the molecular weight. Deglycosylation is less efficient in native proteins (without treatment with SDS): under those conditions, overnight incubation might be necessary.

Before deglycosylation, the hCG β band is smeared due to the polydisperse nature of the glycan chains (not every protein chain is modified with the same set of glycans). Thus, bands become sharper as the glycans are removed. A ~ 15 kDa band (asterisk) shows the fully deglycosylated hCG β . The remaining bands represent incomplete deglycosylation or are unidentified glycoproteins present in the hCG β sample (which is not pure, see lanes 1 and 5). Lanes 9 to 11 correspond to the reagent controls.



FIGURE 5: SDS-PAGE Analysis of Deglycosylation Reactions

The results show that the intensity of the signal decreases as hCG β is deglycosylated.



The intensity of the signal decreases as hCG β is extensively deglycosylated under denaturing conditions (lane 1 to lane 4). Some bands in lanes 3 and 4 show residual signal, indicating the presence of sugars which cannot be removed under the conditions used in this experiment (see note 4). Adding extra glycosidases (lane 4) had a minor effect (only a slight reduction in intensity comparing with lane 3). Note that some bands (seen in Figure 4) are not detected by Emerald Green (bracket), indicating that they were fully deglycosylated. Our data is in accordance with previous reports indicating that hCG β contains multiple glycoforms (see note 5).

Conclusion

Endo- and exoglycosidases are useful tools to investigate the nature of protein glycosylation. The protocol presented here can be easily adapted for use in combination with other simple techniques.

The sample protein can be completely or partially purified. If the protein is tagged (or antibodies are available) the same protocol can be used on a cell extract, performing a Western Blot to analyze the mobility of a specific protein. Deglycosylation can use cell lysates prepared on a variety of buffers, including solutions containing SDS (like RIPA) (see note 5).

The protocol illustrates the simultaneous release of all glycans. However, other enzymes, enzyme combinations, or sequential treatments apply if one seeks to elucidate more details on the structure of the carbohydrate moieties.

After enzymatic treatment, proteins can be analyzed by a variety of methods. Many alternatives to Coomassie are available, which allow visualization of low abundance proteins. Proteins can be specifically detected by tag staining or immunoblots. Conjugated lectins (proteins that specifically bind glycans) can be used to detect specific sugar residues.

Finally, it should be noted that protein samples treated with enzymes remain intact, and therefore are ready for downstream proteomic analysis

References

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Notes

Note 4: The combination of enzymes used in this analysis should, in theory, suffice to remove the kind of modifications found in glycoproteins from mouse cells. However, the common incubation buffer (50 mM NaPhosphate pH 7.5) and/or the presence of detergent could be suboptimal for some of them, especially to remove difficult residues (branched substitutions for instance). The reader should remember that glycosidases are characterized on free glycans. When a carbohydrate is still attached to a protein, steric constraints can prevent the access to the glycosidase to certain residues.

Note 5: PNGase F is inhibited by SDS, so enough NP40 needs to be added after denaturation to prevent PNGase F inactivation. Some lysis buffers contain high NaCl concentrations as well. It is recommended to keep the total molarity of the reaction (from sample buffer and GlycoBuffer 2) below 200 mM, and the total SDS concentration not higher than 1%.

Note 6: Buffer formulations:
10X GlycoBuffer 2: 500 mM Sodium Phosphate pH 7.5
10X Glycoprotein Denaturing Buffer: 5% SDS, 400 mM DTT
10X NP-40: 10% NP40.
Final reaction concentrations (denaturing conditions): 50 mM Sodium Phosphate pH 7.5, 0.5% SDS, 40 mM DTT, 1% NP40.

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