A Recombinant PNGase F for the Analysis of Glycoproteins

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May 21, 2012

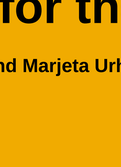
Introduction

PNGase F is an endoglycosidase first isolated from *Flavobacterium meningosepticum*. It is a 34 kDa enzyme with specificity for N-linked glycan removal. Given the difficulty of MS/MS-based sequencing of glycopeptides, upstream removal of glycan-chains by PNGase F is a powerful tool for proteomics research.

We have expressed PNGase F as a HaloTag™ fusion and purified it to homogeneity. Recombinant PNGase F is highly active and was shown to remove N-linked glycans from a wide panel of substrates including human serum glycoproteins. Furthermore, the enzyme is active under non-denaturing conditions and therefore compatible with MS workflows.

Methods

1. Purification of Recombinant PNGase F

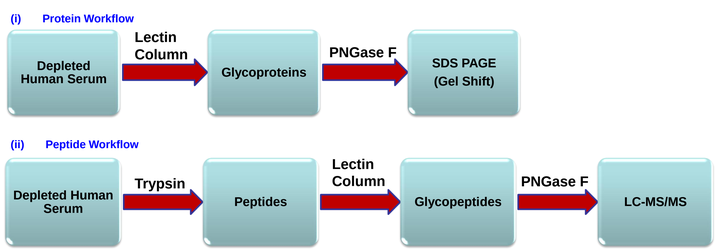


2. PNGase F Enzymatic Assays

A. Purified Glycoprotein Substrates

In a typical experiment, 50 µg of substrate was denatured by heating to 95°C for 10 minutes in 0.5% SDS + 40 mM DTT. Denatured substrate was diluted 2-fold into a final reaction buffer of 50 µL containing 1% NP-40 and 50 mM phosphate buffer, pH = 7.5. Reactions were initiated with PNGase F (500 – 2500 units) and deglycosylation was allowed to proceed for 4-18 hours at 37°C. Samples were analyzed by either SDS-PAGE (Gel-shift) or LC-MS/MS.

B. Human Serum



Human serum (1 mg) was depleted of albumin and IgG (Qiagen). Glycoproteins were enriched using a combined ConA/WGA lectin column. Glycopeptides were enriched by first digesting depleted human serum samples (0.2 mg) with trypsin followed by lectin enrichment. Glycopeptides were desalted with a 50 mg tC_{18} SEP-PAK cartridge (Waters), lyophilized and reconstituted in PNGase deglycosylation buffer. PNGase F (~1000 U) was added to the test samples while water was added to the controls.

PNGase F is Fully Active on Protein Substrates

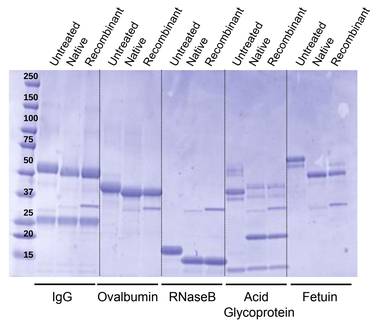


Figure 1. SDS-PAGE analysis of selected protein substrates treated with PNGase F, native and recombinant. Native and recombinant PNGase F effectively Deglycosylated all substrates tested.

Immobilization of PNGase F

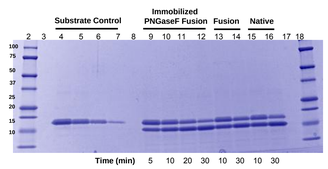


Figure 2. RNase B (100 µg) was denatured and treated with immobilized HaloTag-PNGase F, (soluble) HaloTag-PNGaseF or native PNGase F at 37°C for the indicated times. Samples (5 µg) were analyzed by SDS-PAGE and visualized with SimplyBlue stain.

Analysis using LC-MS/MS

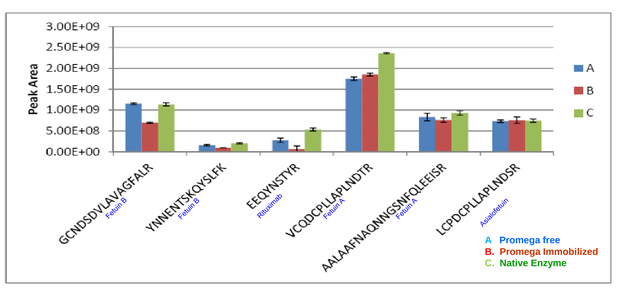


Figure 3. Specific examples of deglycosylated peptides prepared using (A) Soluble recombinant PNGase F, (B) immobilized PNGase F or (C) Native PNGase F.

PNGase F Deglycosylates Serum Proteins

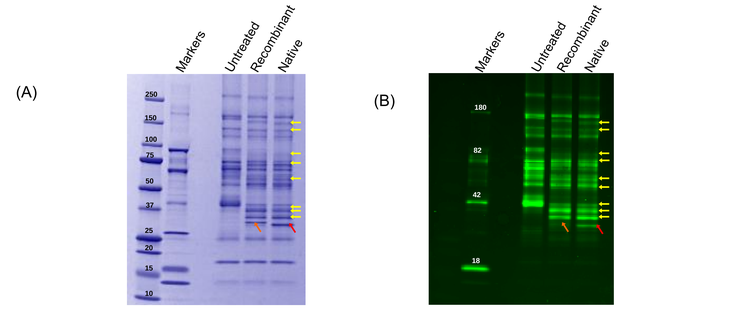


Figure 4. Enriched glycoproteins from human serum were treated with recombinant or native PNGase F at 37°C for 18 hours. Samples were analyzed by SDS-PAGE and visualized with SimplyBlue stain (A) or with Pro-Q Emerald™ glycoprotein stain (B). Arrows indicate deglycosylated proteins (yellow), recombinant (orange) and native (red) PNGase F. Deglycosylation patterns are virtually identical.

LC-MS/MS analysis of PNGase F treated Serum

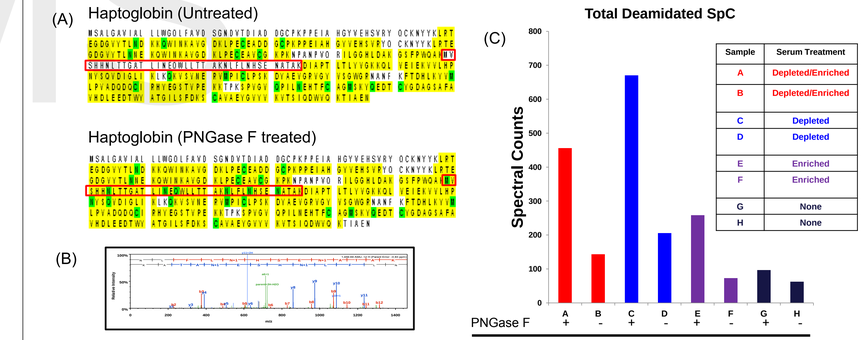


Figure 5. MS Analysis of Serum samples. (A) The sequence coverage of untreated Haptoglobin is unchanged relative to the PNGase F sample with the only exception being the identification of the glycopeptide whose spectrum is shown is panel (B). Panel (C) shows that in all cases PNGase F treatment significantly increases spectral counts for the deamidated peptides (2-5 fold)

Conclusions

- Recombinant PNGase F has been purified using HaloTag® technology to homogeneity.
- Recombinant PNGase F is fully active against both purified glycoprotein substrates and human serum glycoproteins as compared with native PNGase F.
- PNGase F improves sequence coverage is required to identify glycosylation sites.
- Deglycosylation of serum using PNGase F is compatible with MS workflows (i.e. can be used with non-denaturing buffers).
- Immobilization of PNGase F might be a useful tool for automation of sample prep workflows.